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# **Individual olfactory learning in the ant *Ooceraea biroi***

**Master Thesis**

to gain the academic grade as a

*Master of Science* in the Study Program *Evolution, Ecology, and Systematics* (EES)

(M. Sc.)

submitted by

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## Declaration of Self-Dependence

# 1. Introduction

## 1.1 Definition and history of learning experiments

How do we define when an individual has learned something? In science, there exists a common definition of learning. While the exact wording varies across sources (Hall et al., 1998b; Marler et al., 1984a; Papaj & Lewis, 1993b; Pearce, 2013; Thorpe, 1956), the underlying meaning remains consistent. Learning signifies the process through which an individual alters their behavior in response to an experience..

Learning experiments have a history spanning almost 140 years (Ebbinghaus, 1885; Seel, 2012). During this time, both animal and human memory underwent various testing methodologies (Luriā, 1987; Pavlov, 1927; Thorndike, 1898). Initially, learning experiments predominantly involved vertebrates, such as birds (Quicke, 2017), with insects gaining popularity in learning experiments only in the last few decades (Giurfa & Sandoz, 2012; Marler et al., 1984b; Papaj & Lewis, 1993a). Discussions regarding the cognitive abilities of ants date back to the late 19th century (Bethe, 1898; Forel, 1874; Thorndike, 1898; Wasmann, 1899). Wasmann's work, in particular, contributed to a lively debate questioning the comparability of intelligence between ants and humans, especially concerning the capacity to associate two stimuli (Wasmann, 1899). Although there was no general affirmation about individual learning, scientists like Rudolf Brun (Brun, 1914) or Charles Turner (Turner, 1907) actively started to write about the capability of ants to memorize.

Recent papers have increasingly centered on classical (individual) associative learning (see chapter 1.2) in insects, building on the groundwork laid by researchers like Brun, who conducted individual learning experiments, primarily in eusocial Hymenopteran species such as the honey bee, etc. (Behmer, 2008). Ants, in particular, have been shown to be capable of learning (Aron et al., 1993; Johnson, 1991; Wehner & Raber, 1979). Although, bees were and are very promising in invertebrate learning experiments (Giurfa & Sandoz, 2012), in some experiments, ants even showed learning after a single trial making them therefore highly interesting for further research (Czaczkes & Kumar, 2020; Huber & Knaden, 2018; Piqueret et al., 2019). In general experiments, classical associative learning has been conducted (Bos et al., 2012; Desmedt et al., 2017; Guerrieri & d'Ettorre, 2010) as well as investigations into the extinction time of their memory (Piqueret et al., 2019). Moreover, various experiments have highlighted specific factors influencing learning performance and memory in ants, including

the species (Aron et al., 1993; Dupuy et al., 2006; Johnson, 1991), the number of trials conducted (Dupuy et al., 2006; Fernandes et al., 2018; Piqueret et al., 2019), the stimulus (Huber & Knaden, 2018), or even the age of the ants (Cammaerts, 2013; Franklin et al., 2012). The learning performance, as I will write about in the upcoming chapters, refers to the overall trend observed wherein a specific group of ants exhibits successful completion of learning experiments compared to others within the same group.

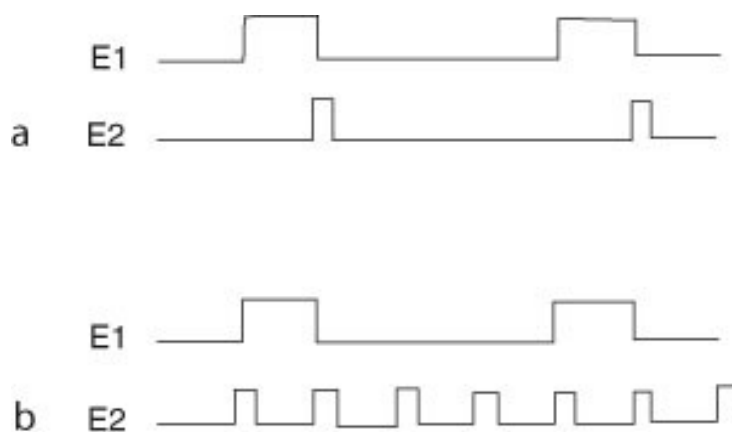
## 1.2 Principles of conditioned learning experiments

All of these experiments are based on the principles of Pavlovian conditioning, or to be precise classical associative learning. In this paradigm, two stimuli are needed: a neutral stimulus (conditioned stimulus, CS) that does not elicit a specific response from the individual, and an unconditioned stimulus (US), that has a given value (either positive or negative) for the individual. During the 'learning phase,' both stimuli are presented together to the trained individual. Then, only the CS is presented to the individual during the 'test phase/memory phase'. Successfully trained individuals should produce a response to the CS known as the conditioned response (Hall et al., 1998a; Pavlov, 1927). Consequently, the tested individual either avoids the conditioned stimulus (aversive learning) (Litvin et al., 2009) or is attracted to it (appetitive learning) (Martin-Soelch et al., 2007) once it has successfully learned the CS. In experiments involving ants, olfactory components are commonly used as stimuli for various types of learning experiments (Behmer, 2008; Dupuy et al., 2006; Quinn et al., 1974; Roces, 1994).

Moreover, in conditioned odor experiments, a second odor, previously unfamiliar to the individual, can be introduced. This unfamiliar odor serves as a control during the memory test, allowing the individual to choose between this new odor and the conditioned odor (CS). Both odors are alternated as the conditioned stimulus (CS) and the additional unfamiliar odor across trials. Adding a second odor is done to exclude any olfactory pre-bias towards an odor (CS) (Thiagarajan et al., 2022; Tully & Quinn, 1985). The precise manner in which these two stimuli are presented is crucial. The unconditioned stimulus (US) must be presented in a way that correlates with the conditioned stimulus (CS) (see Figure 1a) and should generally be presented simultaneously or shortly after the CS, but not continuously and without contextual information (see Figure 1b) (Behmer, 2008). However, there can be exceptions in some

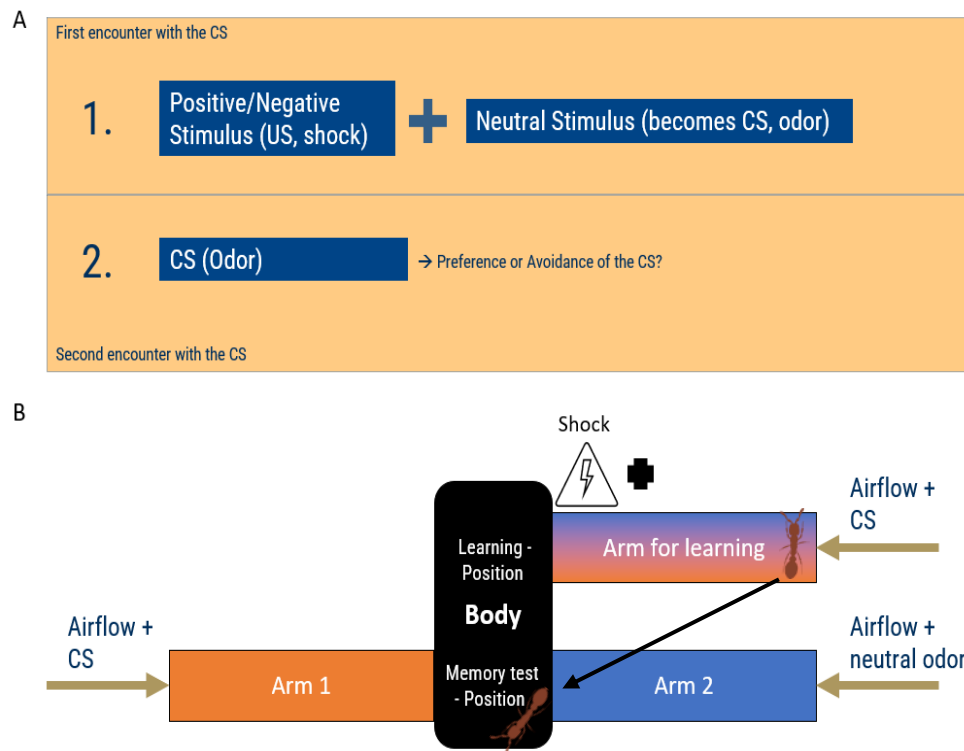
animals. For example, mice can learn even when there were hours between the US (drinking poison) and the CS (saccharin, sucrose solution, ...) (Welzl et al., 2001).

One reason for the prominent role of olfaction in learning experiments is that the olfactory system is relatively similar across many species (convergent features for similar tasks) (Ache & Young, 2005; Eisthen, 2002), even between mice and ants (Duan & Volkan, 2020). Ache and Young (2005) conducted a comparative analysis of learning and memory development across different taxa, highlighting intriguing similarities. For instance, they noted a resemblance in the spatial pattern of oscillatory dynamics used to store and develop odor memory among mammals, bees, and slugs. Moreover, species such as *Drosophila spp.*, commonly used in laboratory experiments (Yamaguchi & Yoshida, 2018) are highly responsive to odors and are therefore used in olfactory learning experiments (Davis, 2004). For graphical visualization, Figure 2a shows the general procedure of a learning experiment and 2b how it is implemented in this thesis.



**Figure 1: Different patterns of correlation between two stimuli.**

Possible patterns of how to correlate the two stimuli are illustrated in the upper version (a) and the lower version (b). In the upper version (a), E2 (US) is activated after or during the activation of E1 (CS), with no E2 activations occurring between the interval of E1. This pattern allows for the formation of an association between the two stimuli. In contrast, in the lower version (b), E2 is continually present and not correlated with E1. Consequently, no association can develop, and learning would not be possible in (b). Source: Behmer (2008).



**Figure 2: Principles of learning experiments**

A: The fundamental procedure for conducting olfactory learning experiments following the Pavlovian model. In the first phase, the US is presented along with the CS. In the second phase, only the CS is presented to the individual in a manner that allows for preference or avoidance to become apparent (Pavlov, 1927).

B: Schematic representation of the T-maze used in thesis. The first phase of the learning experiment occurs in the 'learning position,' located in one arm of the T-maze, where the electric shocks (negative stimuli) are induced simultaneously with the CS odor. In the lower part of the T-maze, the second phase assesses whether the ant has successfully learned the CS. In this phase, the ant must choose between two arms, which are identical to the arm used in the first phase. One arm provides the CS odor through an airflow and is expected to induce an avoidance response in the ant, while the other arm contains a neutral odor that the ant has not previously encountered. .

### 1.3 Learning in ants and ecological reasons

The procedure for conducting learning experiments in ants is similar across many publications but can vary in a few aspects. In the case of appetitive learning, ants are provided with a reward, such as sugar water (US), combined with various olfactory stimuli (CS) like plant volatile odors (Duymaz & Aksoy, 2023; Guerrieri & d'Ettorre, 2010; Huber & Knaden, 2018; Piqueret et al., 2019) or cuticular hydrocarbons (Bos et al., 2012). In some studies, the US is visual (Aron et al., 1993; Fernandes et al., 2018). To assess whether the ants have learned the odor, two common indicators are used. The first is the active approach which was also done



in this thesis. The ants walk actively towards the stimulus (Aron et al., 1993; Bos et al., 2012; Huber & Knaden, 2018; Piqueret et al., 2019). A second possible indicator is the Maxillary-Labium extension response (MaLER), where ants extend their mouthparts to receive food, which is a behavior positively associated with successful learning. This simple yet effective indicator has been successfully applied over the years with *Camponotus aethiops* (Guerrieri & d'Ettorre, 2010), *Camponotus fellah* (Guerrieri et al., 2011), *Formica clara* (Duymaz & Aksoy, 2023) and *Formica rufa* (Fernandes et al., 2018).

In contrast, in aversive learning experiments with ants, different types of US can be used to form an association with the CS, like quinine (bitter taste) (Dupuy et al., 2006; Guerrieri & d'Ettorre, 2010), heat (Desmedt et al., 2017) or electric shocks (Wenig et al., 2021).

In the second phase of aversive learning experiments ('test phase/memory phase', see 1.2), ants for example, avoid the CS in free-walking experiments like open arenas or mazes (Bar et al., 2022; Wenig et al., 2021) or show aggressive response (e.g., mandible opening response - MOR) (Desmedt et al., 2017) For example, in Desmedt et al. (2017), the ants were fixed and exposed to high temperature (US), resulting in the induction of the MOR. After learning and exposure to the CS, the ants showed significantly more frequently the MOR compared to the control group.

Henaut et al. (2014) incorporated an ecologically relevant context - a inter-species interaction - into a learning experiment involving ants, revealing individual learning within a defensive prey/predator-predator interaction. They observed that both species, the ant species *Ectatomma tuberculatum*, characterized by a stinger and strong mandibles, and the golden silk spider (*Trichonephila clavipes*), for which the ant is a highly-defended and unsuitable prey. In nature, both species can be found in the same habitat and probably it comes to encounters between both species. In the experiment, ant workers were exposed to the golden silk spider's web on purpose and became ensnared. Due to the ant's defensive characteristics, attempts by the spider to prey upon the ant often resulted in the spider struggling or being harmed. This led to a rapid and enduring 24-hour memory in the spider, causing it to avoid attacking the trapped ant in its web in subsequent encounters. Concurrently, the ants altered their behavior after such incidents, demonstrating a swifter escape from the spider's web due to learned experience. This behavior carries ecological principles, as the learned behavior conserves energy resources of both sides that would otherwise be invested in conflict.

In ants, both types of learning are relatively rapid, albeit with variation in the number of trials required. For instance, in appetitive learning, successful learning may be observed after just one trial (Huber & Knaden, 2018; Piqueret et al., 2019). However, it typically necessitates a range of 3 to 15 trials to show successful learning (Duymaz & Aksoy, 2023; Fernandes et al., 2018; Guerrieri & d'Ettorre, 2010; Rossi et al., 2020; Steck et al., 2011). This variation may be attributed to differences in the stimuli used (reward and conditioned stimulus), as well as factors related to species and experimental setup.

Learning can have a significant ecological impact on the success of an ant colony and, by extension, the evolutionary success of a population or even a species. Two examples highlighting the importance of learning in this context are drawn from the studies by Hollis et al. (2017) and Bar et al. (2022). The first study demonstrated the success in foraging of *Tetramorium sp.* workers by avoiding pitfall traps of ant lions, underscoring the contribution of learning to predator-avoidance strategies in ants. The second study, conducted with *Cataglyphis niger* workers, revealed a similar pattern when exposed to similar pitfall traps. In their quest to reach a food resource situated behind these traps, the ants exhibited a reduced likelihood of falling into the traps and became more efficient at obtaining the reward with increasing experience. In the case of ant lions, which can exist in large numbers (Gatti & Farji-Brener, 2002), the ability of a significant proportion of foragers in an ant colony to avoid these predators can affect the colony's overall success. This is further supported by observations that ant communities actively avoid ant lion-infested areas in their vicinity (Gotelli, 1996).

In the symbiotic relationship between fungus and leaf-cutter ants, learning and long-lasting memory play crucial roles in the colony's survival. For the ecological success of leaf-cutter ants, the ability to detect the most suitable food for their fungus is of paramount importance. Research has shown that ants can memorize odors from leaf fragments brought back to the colony by foragers, which facilitates further foraging (Roces, 1994). Equally critical is the ability to determine which plants they should avoid collecting and providing to their symbiotic partner, the fungus. Saverschek et al. (2010) illustrated this process in detail, showcasing how leaf-cutter ant colonies learn to avoid harmful food. In their study, plant fragments treated with a fungicide (harming the symbiotic fungus) were offered to the ants. The ants, unable to detect the fungicide, initially accepted the plants. However, upon recognizing the harm done to their fungus, they rejected the plant species that had been exposed to the fungicide. Remarkably, foragers avoided this plant species even after 18 weeks. While the precise

mechanisms for the ants' ability to detect the negative impact are likely linked to olfactory, tactile, or gustatory communication between the fungus, the ants, and detection of the stimuli, the exact process remains unclear. Despite different contact intensities with the harmful plants inside the colony, the entire colony retains the knowledge of avoiding harmful plants, confirming the collective learning capacity of the colony (Arenas & Roces, 2016).

Learning abilities that have colony-survival related are also observed in harvester ants, as they tend to remain on specific available seeds and develop preferences for them. Learning can last several months and likely provides an advantage in foraging by allowing them to exploit temporarily available resources more efficiently (Johnson, 1991; Rissing, 1981).

Olfaction in ants is notably well-developed, and several factors contribute to this feature. It is linked to their social lifestyle, enabling them to discern members of their colony from others by detecting unique cuticular hydrocarbon (CHC) profiles (Morel et al., 1988; Sprenger & Menzel, 2020), detection of the alarm pheromone to react on threats (Lopes et al., 2023) or to detect trail pheromone(s) of colony members to follow them to a food resource (Klowden, 2008). This strong reliance on scent is further emphasized by their underground habitat, common among many species, often associated with limited eye development (Aksoy & Camlitepe, 2018; Andersen & Brault, 2010), particularly in smaller ant species (Palavalli-Nettimi et al., 2019). However, there are exceptions, with some ant species possessing relatively well-developed vision (Yilmaz & Spaethe, 2022).

Ants exhibit a substantial olfactory apparatus, e.g. housing around 470 (Babu et al., 2011) to 1080 olfactory sensilla (Nakanishi et al., 2009) across all flagella of their antennae in workers. In comparison, *Drosophila*, for instance, possesses approximately 410 olfactory sensilla across its entire antenna (Laissue & Vosshall, 2008). Ants also possess around 390 to 500 glomeruli in their antennal lobes, along with a similar number of odorant genes, enabling them to detect various chemical compounds (Ferguson et al., 2021). This starkly contrasts with solitary insects like *Drosophila spp.*, which typically have fewer glomeruli (Grabe et al., 2015). Additionally, the olfactory processing pathway in ants is more complex, featuring a dual-olfactory processing pathway as opposed to the single pathway in *Drosophila*. This dual pathway connects to higher brain structures, such as the mushroom bodies and the lateral horn, enabling ants to process information more intricately (Zube et al., 2008). In particular, the mushroom bodies play an important role in learning (Erber et al., 1980) and the lateral horn is associated with innate behavioral responses (Schultzhaus et al., 2017). Given these

characteristics, ants hold significant promise as subjects for olfactory learning experiments in insects (from a neurobiological view) - even for complex tasks.

#### 1.4 The clonal raider ant: potential in learning experiments

Ants are one of the few insect families capable of forming colonies up to millions of individuals, characterized by a eusocial structure with distinct castes comprising non-reproductive workers and reproductive queens. The evolution of their altruistic eusocial lifestyle is probably also linked to the relatedness among individuals within these colonies. While theoretical calculations based on an ideal scenario within an ant colony (only one father and one mother/queen) can explain the altruistic behaviors observed in ant workers, several observations in nature present a different picture. Many ant colonies, though not all species, feature individuals exhibiting significant genetic variations among workers and even queens. Several factors contribute to the observed variations in relatedness among ant workers within colonies. These include the variability in the number of queens present and their genetic relatedness, the number of males the queen(s) have mated with, and the proportion of males born from unfertilized workers (phenomenon known as the competition between workers and queens). These dynamics result in a spectrum of relatedness among workers ranging from nearly zero to approximately 0.75, presenting challenges in determining the genetic background of workers in many ant species (Hölldobler & Wilson, 1990b).

The age structure within an ant colony is normally heterogeneous. Queens exhibit significantly longer lifespans, while males have shorter life expectancies compared to the workers, leading to an absence of uniformity in age distribution. But of particular significance is that the workers, commonly used in experiments, are typically present in various ages and coexist with brood in different developmental stages within the colony simultaneously. This diverse age structure within colonies not only impacts population dynamics but also influences the colony's functioning, thereby representing a critical factor in their biology (Hölldobler & Wilson, 1990a).

With regard to the previous factors of genotype and age-structure in ant colonies, the clonal raider ant (*Ooceraea biroi*) stands out as an exceptionally promising subject for standardized behavioral research for several reasons. First, this species reproduces clonally (Tsuji & Yamauchi, 1995) and second, follows a strictly matched brood cycle (see figure 5) inside the

colony (Ravary & Jaisson, 2002). The clonal reproduction results in genetically identical individuals within colonies and the brood cycle facilitates age standardization by marking newly born individuals.

Age and/or experience (Cammaerts, 2013; Franklin et al., 2012; Stieb et al., 2010) as well as the genotype (Walsh et al., 2022) can influence behavior, and learning in insects. In ants, behaviors are often age-dependent, with tasks shifting as individuals age (age-polyethism) and younger individuals start to do nursing tasks and then with age shift to the task of foraging outside the nest. It makes sense from the colony perspective, that old workers forage which is a dangerous task. If they die, they supported the reproductive female(s) during life at least as nursers (Gordon, 2010b). Therefore, the behavior of *O. biroi* could alter during life which is easier traceable due to its matched brood cycle.

A study focusing on pupae molting fluid emphasized the essential role of the fluid excreted by late-stage pupae within ant colonies. This fluid serves as a crucial source of nutrients for larvae and workers. Notably, workers are responsible for its removal, ensuring the maintenance of hygiene of the pupa. The presence of this fluid across various ant species suggests a potential influence on the evolution of sociality among ants. (Snir et al., 2022). Due to the importance of the fluid for the ants, it may also offer the opportunity to use it in an appetitive learning setup (as US).

### 1.5 Aversive stimulus: Electric shocks

Electric shocks are a common method employed to induce aversive learning in conditioning experiments with invertebrates (Peckmezian & Taylor, 2015). This strong stimulus has been extensively used in *Drosophila sp.* (Pauls et al., 2010; Preat, 1998; Quinn et al., 1974; Thiagarajan et al., 2022). To date, there is only one study using electric shocks as aversive US in ants (Wenig et al., 2021). Researchers examined in this study whether *Lasius niger* workers would avoid pheromone trails when exposed to the negative stimulus of electric shocks or in combination with quinine (bitter solution). The ants were offered a choice within a Y-maze: one arm contained a pheromone trail paired with punishment (shock/quinine), while the other arm provided a positive reward (sucrose solution) without the pheromone trail. Interestingly, the electric shock affected the ants, causing them not to avoid the pheromone trail but to ignore it and proceed to one arm of the maze during the memory test without

rewards/punishments but with the presence of the CS. However, a classical learning experiment with ants with a neutral stimulus for conditioning and an electric shock has not been done so far.

### 1.6 Ant's consistency in learning

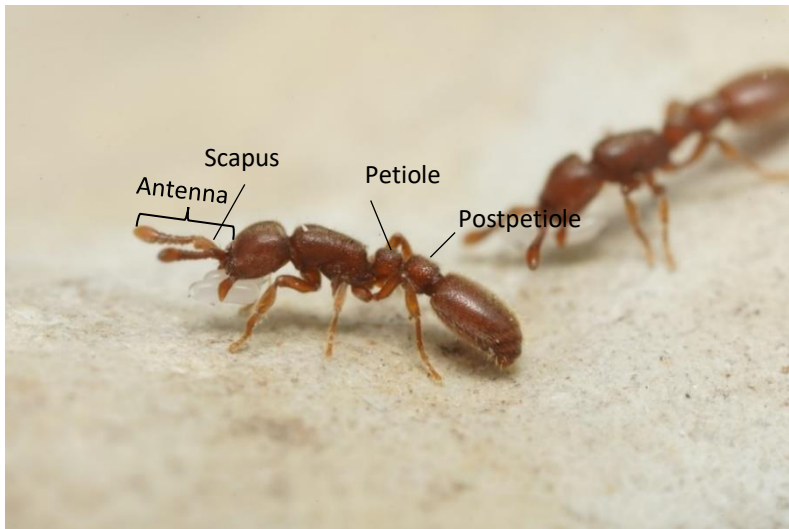
Few studies have examined individual consistency in learning among insects (e.g. in bees by Finke et al. (2023), ants by Udino et al. (2016), and *Drosophila* by (Smith et al., 2022)). The existence of 'good learners' in insects remains relatively underexplored. Finke et al. (2023) conducted a comprehensive study with honey bees. They tested different learning paradigms including the classical learning (see 1.2) and reversal learning in which bees learned a negatively associated odor A and positively associated odor B in a first phase and then reciprocally in a second phase. After these 2 conditioning phases, they did a choice test. As a third paradigm, they did also a negative patterning experiment in which bees were punished when the CS (two odors) are presented together and they were rewarded when odors were presented separately. Then two choice tests without the US in an identical array test for learning. The study revealed a positive and significant correlation between individuality and learning performance including the three different learning paradigms. While not all comparisons between these learning paradigms to find individual consistency were significant (performance in reversal learning correlates without significance with negative patterning), their research encompassed a broad range of setups, involving olfactory and visual components, as well as Pavlovian and operant learning paradigms, yet it could not definitively answer the role of individual variability in learning. On the other hand, Udino et al. (2016) found a clear relationship between personality traits (Exploratory activity, sociability, and aggression) and the time needed to learn in *Camponotus aethiops* which were taught to associate two odors (CS) with a positive US (sucrose) and tested via MaLER. Li et al. (2017) showed optimistic results as well which indicated not only the existence of individual consistency in bumblebee's (visual) learning abilities but also that there is a morphological reason/correlation between learning performance and brain structure. Individuals which were "good learners" – making fewer errors – had more microglomeruli in the mushroom body compared to the "bad learners". These more optimistic findings, indicate not only the presence of individual consistency in bumblebees' visual learning abilities but also suggesting a morphological basis in the brain structure. Further support for this observation comes from

a remarkably similar study conducted on leaf-cutter ants trained on plants that were detrimental to their symbiotic fungus due to fungicide treatment. Throughout the aversive learning experiment and the establishment of memory, the ants' heads were dissected to assess brain morphology and potential changes. The study revealed a notable increase in the number of microglomeruli in the ants' brains as a result of the experiment. Intriguingly, this change in microglomeruli was a short-term phenomenon and returned to its original state after four days. Despite this reversion, the ants remained capable of remembering the harmful plant (Falibene et al., 2015).

In this thesis, I conducted an aversive olfactory learning study with *O. biroi* using electric shocks. I investigated age and genotype as factors that might influence learning performance. Furthermore, I explored consistency in individual learning by testing individuals after a period of 2 months for a second time. The hypotheses arising from this study are as follows: 1) Clonal raider ants are capable of aversive olfactory learning, 2) there are significant differences in learning performance between different genotypes, 3) different ages have an impact on learning performance and (4) how consistent are the ants in making the right choice.

## 2. Species description

The species *O. biroi* (formerly *Cerapachys biroi* (Borowiec, 2016)) is around 2 mm long, red-brownish in color and has a very strong cuticula, short and thick antennae. Additionally, the species has strongly reduced eyes and distinct postpetiole (Forel, 1907) (Fig. 3).



**Figure 3:**

Two workers of *O. biroi* carrying eggs in their mandibles.

*O. biroi* is a globally distributed species (Fig. 4), primarily found in tropical regions, where it has established itself as an invasive species. Its origins can be traced back to central Asia, specifically Bangladesh (Trible et al., 2020; Wetterer et al., 2012). The asexually reproduction

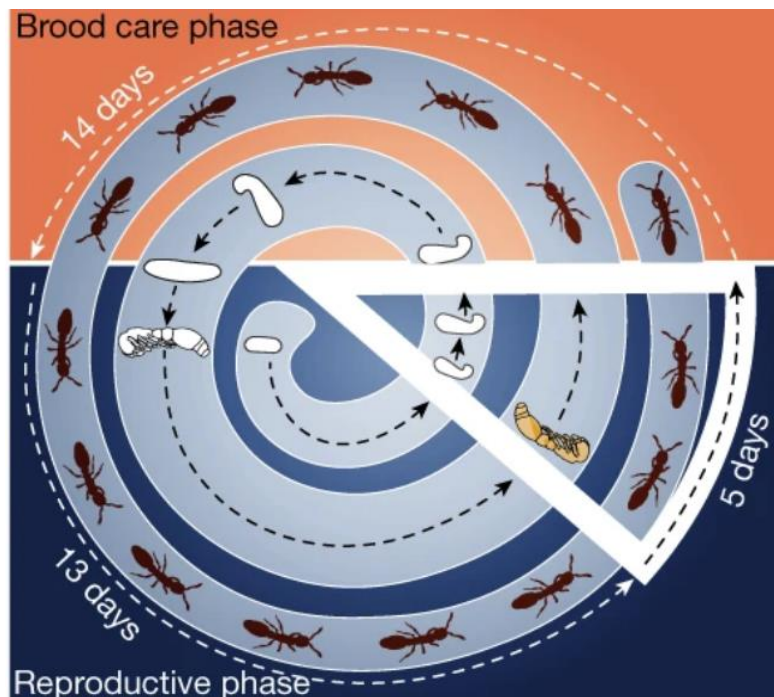


**Figure 4: Global distribution of *O. biroi***

The species can be found world-wide, close around the equator and therefore in tropical regions. The origin is Asia, specifically Bangladesh from which the species was introduced to Indonesia, Madagascar and the Caribbean. Source: antweb.com



through parthenogenesis in workers (Tsuji & Yamauchi, 1995), results in a low genetical variability whereby researchers have in total identified 13 distinct genetic lineages (labeled A to M), (Trible et al., 2020). The colony cycle of *O. biroi* comprises a static phase, characterized as the reproductive phase (workers lay eggs), and another phase in which the workers shift their focus to foraging for food, known as the brood care phase. These cycles are profoundly influenced by the developmental stage of the brood and typically span a duration of over two weeks each (Ravary & Jaisson, 2002; Fig. 5).



**Figure 5: Colony (reproductive) cycle of the clonal raider ant**

The colony has two cycles, restricted to the stage of the brood. The presence of eggs and pupae serves as an indicator for the reproductive phase, during which all workers within the colony are acting very passively and do not forage. With the development from eggs to larvae and simultaneously hatching of pupae to new workers, the colony transitions to the brood care phase. In this phase, the workers actively forage to provide food for the larvae. The phase ends with the larvae turning into pre-pupae which do not need any further food and the workers stop foraging. Both phases last roughly around two weeks, the reproductive phase's overlap close before the pupae are hatching and undergo melanization. Modified from Snir et al. (2022).

### 3. Methodology

#### 3.1 Ant keeping

The ants were kept in a climate chamber (Dry Oven, Binder Serie FP Classic line), at 28 °C constantly, daily fed with frozen flies (Calliphoridae) and ant brood from *Tetramorium bicarinatum* (Nylander, 1846) if the colony was in the brood care-phase (Snir et al., 2022). Ants were kept in a plastic box with plaster as substrate, with humidity around 90-99 %. To ensure

age-controlled colonies, new colonies were established using approximately 90-300 callows (freshly hatched individuals) from another colony. Subsequent identification (in case more than one generation was present in the colony) was possible by color-marking the ants (of a given generation) on the gaster (S. Fig. 10, using paint markers (UNI-ball)). Unfortunately, I faced considerable mortality among ants that dug into the plaster substrate and subsequently died or others that drowned in the condensation that formed on the lids of the nest boxes. This unfortunate circumstance significantly affected the available number of replicates, especially among the older individuals. As a result, it became unfeasible to perform a comparison between different genotypes among the oldest individuals. Initially, all colonies were free from infection by the nematodes of the genus *Diposcapter* (Li et al., 2023). However, after a few months, the colony of genotype B became infected (in dissected heads of 4/5 workers nematodes could be found). Subsequently, the colony of genotype A, which was 6 months old during the age-dependence experiments, also experienced contamination (3/5 dissected workers). Finally, the last colony, genotype M, was likewise affected by nematode contamination (no nematodes in the head but on the plaster of the nest). These nematodes were likely introduced through infected brood from *T. bicarinatum*, and the discovery of the contamination occurred too late to prevent an overall infection. Consequently, a consistent infection status of the colonies could not be maintained.

### 3.2 The setup and procedure of the experiment

#### Experimental setup

The experimental setup for this study was modified from Thiagarajan et al. (2022). Detailed technical specifications for these modifications are provided in the supplementary materials (S. Fig. 4)

To ensure the odors were strictly neutral for the ants (no innate preference), a series of pre-experiments were performed. I performed choice tests (without conditioning) between the two odors, and the ants did not show any preference for one odor over the other ( $n = 50$  Chi-Square test,  $X^2 = 1.0588$ ,  $df = 1$ ,  $p\text{-value} = 0.3035$ ; S. Fig. 1). I also conducted tests to determine whether ants exhibited a preference or avoidance for any of these odors over the absence of an odor (attractivity test). However, the ants did not demonstrate a significant

preference or avoidance for any odor compared to its absence. (n = 100, Chi-Square test, X-squared = 1.2462, df = 2, p-value = 0.5363; S. Fig. 3).

The neutral odors used as conditioned stimuli were hexanal (CAS: 66-25-1) and 1-octanol (CAS: 111-87-5) (Sigma Aldrich®, 99% purity). To ensure a consistent release of these odors into the experimental arms, a specific method was employed:

Each odor was placed in a glass vial with a volume of 5ml, containing 60µl of undiluted odor. These vials were then positioned inside larger glass bottles (Schott, 250ml; S. Fig. 11a) to establish an equilibrium within the larger bottle with similar odor in- and outflow. The rate of odor released from the small vial matched the rate of odor removal from the larger bottle due to the same diameter of the opening of the small glass vial and the diameter of the Teflon tubes which lead to the arms of the maze. Furthermore, to provide a stable concentration of odor in the maze, odor was accumulated in the bottle and then released for 45-60 minutes before testing. The velocity of the flow (0.25 – 0.3 litres per minute (LPM)) was controlled by analogous flowmeters (Key instruments, FR2000). The water bottles (250ml, Schott) were integrated in both - in the airflow without odor and with odor (see S. Fig. 4) and additionally heated by a water bath up to 29°C (Memmert WNB7) to provide a constant high relative humidity of around 90% +/- 2%. This was essential to ensure contact between the ant and the copper circuit board and therefore an electric shock of the ant – and a proper aversive learning. The results of the odor measuring showed only significant changes in the concentration of 1-octanol over 4.5 hours not in hexanal or between the time steps. Details about the dynamics of odor outflow over time are provided in the supplements (see Supplemental part, page VI-VIII; S. Fig. 3). Nevertheless, the differences did not impact the behavior of the ants (S. Fig. 7).

The odor flow was constantly present for 10 seconds after pressing a pedal to release the odors (Marquardt, MAR2410.0401) and then absent during a break of 30 seconds (only humidified air from both paths). Whether the odor was released or not was controlled by valves connected before and behind the odor bottle. The 3/2-way magnetic valve (Pneumatikwelt, M3M5ES24V) channeled the air to the odor bottle if activated. The 2/2-way magnetic valve (Pneumatikwelt, M2M5ES24V) blocked or released the air coming from the odor bottle and therefore opening if activated (S. Fig. 4).

Simultaneously when pressing the pedal the controller produced an electric shock, provided by a generator (Joy it) set to 40 V and 5 mA, DC. The shock was delivered by a copper circuit board (constructed at the Max-Planck-Institute For Chemical Ecology) with a contact distance of 0.2mm, between 0.2 mm - wide circuits inside the arms. Similar T-maze arms were used for the learning and for the memory tests. The only difference was the presence (learning) or absence (memory tests) of the electric shocks. The arms themselves were 3D printed out of black Polyoxymethylene (POM) and had an acrylic glass cover to make it possible to check the ant's behavior and possible problems during the test.

The synchronization between shock and odor was provided by a custom-made controller with the circuit board Arduino Nano and software Arduino (version 2.04) responsible for controlling the valves and generator after the pedal was pressed (code provided in S. fig. 9). After the pedal had been pressed (beginning of the trial) the odor was 2.5 s released before the shock started - ensuring association with the unconditioned stimulus as in fig. 1. This means, the valves are activated and release the odor flow out of the larger bottles into the arms to the ant (permanently for 10 seconds). Subsequently an electric shock starts for 0.5s, then 0.5s break, and this for 8 repetitions (7.5 seconds). Then the shocks stop and the valves close to prevent any further release of odor into the arms. In sum, the ant encountered the odor permanently for 10 seconds and the shock shortly after for 7.5 seconds in intervals.

### 3.2.1 Procedure of the experiment

#### Learning

The learning phase, where the ant forms an association between the odor and the negative stimulus, began with a single ant placed within one of the arms, which is connected to the electrical circuit (S. Fig. 11a). Activating the pedal initiated a conditioning trial by opening the valves, thereby exposing the ant to the odor, followed by the electric shock. Between one trial and the following one (Inter-trial interval), ants had a 30-second break. Then, the pedal is pressed again to carry out another trial. This cycle was repeated six times, amounting to a total duration of 3 minutes and 20 seconds. At the end of the sixth (last) trial, the ant was relocated to a petri dish for 5 minutes.

## Memory

The second phase, referred to as the memory phase, is designed to test the association previously formed by the ant. It begins with the ant placed in a chamber in the body of the T-maze (made of polyoxymethylene; Fig. 2b - upper part of the body). This chamber is mobile, allowing the ant within to be placed between the two arms connected to the body, located in the lower section of the body, between arm 1 and arm 2. Following a brief 10-second acclimatization, the chamber opens, allowing the ant the choice to walk to either of the two arms. Simultaneously, the pedal is pressed to initiate the flow of odor. However, in this phase, the ant will not receive an electric shock if it walks on the copper circuit board. Similar to the "learning phase" the odors were continuously present for 10 seconds, then only fresh air for 30 seconds, followed by again 10 seconds of odors. This cycle is repeated for a duration of three minutes, marking the conclusion of the experiment. The position of the ant was noted at the end of the memory test with three possible outcomes: (1) the ant stayed between the two arms (no decision ND), (2) the ant went to the arm with the aversively conditioned odor (wrong choice), or (3) the ant went to the arm with the novel odor not associated with a shock (right choice). The individuals which pass the test, were promptly frozen at -21°C and not returned to the colony (except in the consistency experiment) to prevent any influence on other individuals within the colony, which were tested later on. Individuals which escaped or were injured during the experiment were removed from the analysis (less than 1%).

### 3.2.2 Structure of the experiment

The order of testing individuals was as randomized. I included alternating positions of the odor, either on the left or right side of the T-maze. This was done in a pseudo-random order (Sequence: RLRLRLRL...LRLRLRRL..., L = left/R = right) similar to Desmedt et al. (2017). The odor on which the ants were trained was also alternating - one pair of ants was conditioned on hexanal the next pair on 1-octanol. Between all ants, the arms and the T-maze body were cleaned with cotton-tube cleaners (Vauen) plus 70% ethanol and subsequently with demineralized water. Additionally, each week, the body and all arms of the maze were cleaned with odorless soap (Degent Powder, Alconox) to remove all remaining scents.

To gain a comprehensive overview about the experiments and which colonies with which status were used for it, additional details can be found in Supplementary Table 1.

### 1) General learning experiments

The results of the general learning experiments were divided into two groups, one in which the experiment was conducted without age standardization and different genotypes. This experiment was referred to as tests with “mixed-age” colonies (1a).

The ants from colonies that were tested in the other learning experiments will be referred to as from “pure-age” colonies (1b) in which a comparison between age cohorts and genotypes was possible/aimed. To test how the genotype affects the learning ability in *O. biroi*, colonies from 3 different genotypes were used (called “A”, “B”, and “M” (Trible et al., 2020)). For each genotype (from A, B and M), three age cohorts were tested: 1 month old, 3 month-old, and 9 month-old, to analyze the impact of age on learning. Except the general learning experiment, pure-age colonies were also used for subsequent experimental designs (effect of older individuals by a cohort of 4-month-old individuals, 1-month old individuals of genotype A and M for the consistency experiment)

### 2) Consistency in learning across time

In an experiment, to test whether individuals are consistent in learning and therefore may be good learners and others not, I did an identical procedure as explained before. The sole variation in this experiment was that individuals were marked with two dots, establishing a specific color combination. This distinction allowed for the recognition and differentiation of their decision-making patterns based on the first learning experiment. For this, I used the individuals in the experiment 1b) of 1-month-age (Individuals from genotype A and M) and then tested them a second time with an age of 3 months. Additionally, a colony from genotype B was used to have in total three different genotypes in this experiment. The results from the second testing were not included in 1b) in 3-month-old individuals, because these ants would have been biased after they already passed the experiment once. Efforts were made to ensure that the experimental groups were similar in size.

### 3) Impact of older individuals inside a colony

After the pure-age memory experiment I tested for an effect by older on younger individuals in the learning experiment. I will here refer to it as “effect of older individuals”. Almost all

pure-age cohorts were the oldest adult individuals within their colony (except 1- and 3-month-old individuals of genotype B). To test for a significant impact of the presence of older workers on the learning ability of younger workers, I additionally (beside the two age-cohorts of genotype B) tested one generation of 4-month old ants from genotype A, which were reared in the presence of older individuals. These individuals were tested after the pure-age cohorts and were raised by workers from the 9- and 3-month-old cohort.

### 3.2.3 Statistical analysis

Data were analyzed in RStudio 2023.6.0.421 (posit team, 2023). Statistical significance was fixed at  $\alpha = 5\%$ . The behavioral data which do not include ND-results were binomial (wrong decision : 0, right decision: 1).

First, Chi-Square tests were applied to test for deviation from random choice (50% right and 50% wrong) in all learning experiments tests. The same test was applied to check for differences between for example age cohorts and the proportion of ND results but also for the individuals with the effect of older individuals compared to a random choice distribution. To examine the effect of older individuals (3), I applied the Chi-Square test on the first two age cohorts of genotype B from the pure-age colonies and the results from the 4-month-old cohort of genotype A which grew up with older individuals.

The data of the general learning experiments of pure-age colonies (1b) were analyzed using a binomial GLMM (generalized linear mixed model) to check for effects of age and genotype on learning (glmer-function from the package lme4 version 1.1-33). The focus was on the response of individuals either choosing right or wrong. Therefore, the third outcome, that individuals did no decision (ND) was not included in the GLMM. We analyzed the effect of age (3 levels: 1 month-old, 3 month-old, and 9 month-old) genotype (3 levels: A, B or M) on decision, with day (32 levels: date), position of the odors (2 levels: left or right), and cleaning order of the arms (4 levels: 1-4, a reference to how many ants had been tested after the arms were cleaned with 70% ethanol and water which repeats after 4 individuals) as random factors. The model was finally tested for different pre-assumptions like within-group deviation from uniformity and homogeneity of variance via the DHARMA-package (version 0.4.6) which showed no significance. Thus, the GLMM can be used to predict significant influences in the learning performance in *O. biroi* derived from different variables.

In the beginning, the model was set up with the additional variables: used odor for conditioning and larvae day appearance (to check if the life cycle of the ants had an impact on the activity in the maze) until simplification led to the exclusion of them due to non-significance ( $p > 0.05$ , see R-code).

Subsequent to the GLMM, a post hoc Tukey HSD test by using the emmeans-function of the package 'emmeans' (version 1.8.7) was applied to test for specific significance inside the variables/between groups (between the 3 genotypes and 3 age-cohorts).

A similar approach (compared to the memory test data) has been done in the consistency experiment (2), in which a GLMM was used as well to test whether the previous decision (2 levels: right/wrong, fixed factor) was linked to the decision the ant made in the second experiment. The GLMM had also random factors, identical to the GLMM from the general learning experiment with day (5 levels: date), position of the odors (2 levels: left or right), and cleaning order of the arms (4 levels: 1-4).

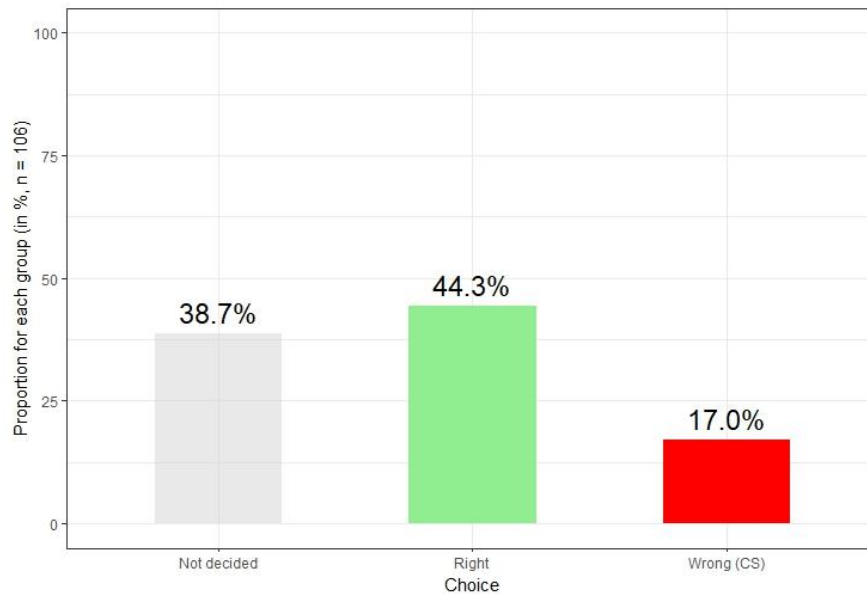
## 4. Results

In total, 623 individuals were tested after being conditioned. Of these, 58.91% (218 ants, without ND-results) made a decision by walking in one of the arms of the maze. The results can be separated into (1a) Learning experiment in mixed-age colonies, (1b) Learning experiment in pure-age colonies, (2) individual consistency experiment and (3) learning experiment with the effect of older individuals. The results from the variables odor and larvae appearance were not significant and therefore not included in the final GLMMs. However, graphical distributions of these excluded variables are shown in Supplemental Fig. 5-7.

### 4.1 Learning experiment in mixed-Age colonies

(1a): 106 individuals were tested. Ants showed learning and avoided the aversive CS more often than expected by chance (Chi-Square test, X-squared = 12.938, df = 1, p-value < 0.001\*\*\*; Fig. 6). Testing for a preference for one side in the T-maze showed no significance (Chi-Square test, X-squared = 7.2188e-31, df = 1, p-value = 1) and also the position of the odor offered in the maze did not play a role in the experiment (Chi-Square-test, X-squared = 0.36231, df = 1, p-value = 0.5472). This means that ants learned to associate the CS (odors) and the US (electric shock).





**Figure 6: Memory test in mixed-age colonies**

Results of the memory test using colonies with a mixed-age structure of genotype A. The graph shows the proportional distribution of how the ants chose in the memory test (counts ND: 41, R: 47, W:18). The light grey bar shows how high the proportion of individuals is which did no decision and neither walked to the arm with the CS (red, “wrong”) or the arm without the CS and with the second odor (green, “right”). The three possible choices are shown on the x-axis.

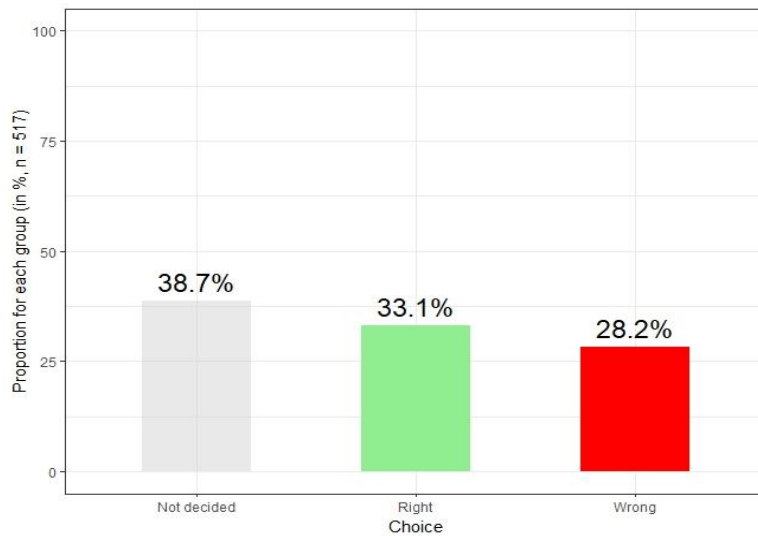
#### 4.2 Pure-Age colonies

(1b): 517 individuals were tested. Out of these individuals, 216 were 1-month, 167 3-month, and 134 9-month old. In terms of the genotypic distribution, I tested 258 individuals of genotype A, 99 of genotype B, and 160 of genotype M. Amongst the 517 ants, 317 made a decision: 53.94% made the right decision, 46.06% the wrong decision.

Interestingly, the first cohort of 1-month-old individuals in contrast to the 3-month-old individuals was showing a higher proportion of ants making no decision (Chi-Square test, X-squared = 23.915, df = 2, p-value < 0.001\*\*\*; S. Fig. 8). However, this trend changed with the 9-month-old individuals, which showed a very high proportion of ND compared to 3-month-old individuals (Chi-Square test, X-squared = 55.328, df = 2, p-value < 0.001\*\*\*; S. Fig. 8).

Testing for the variable age, there was a tendency for the last age cohort to perform better and show learning (GLMM ~ Age + Genotype + random variables, 1 - 9 month-old, p-value = 0.0706) but not for the other combination (GLMM, 1 - 3 month-old, p-value = 0.7886; Fig. 8).

The post hoc Tukey HSD test which checked for any significance between groups, showed no significance as well, even in the last age cohort (Table 1).

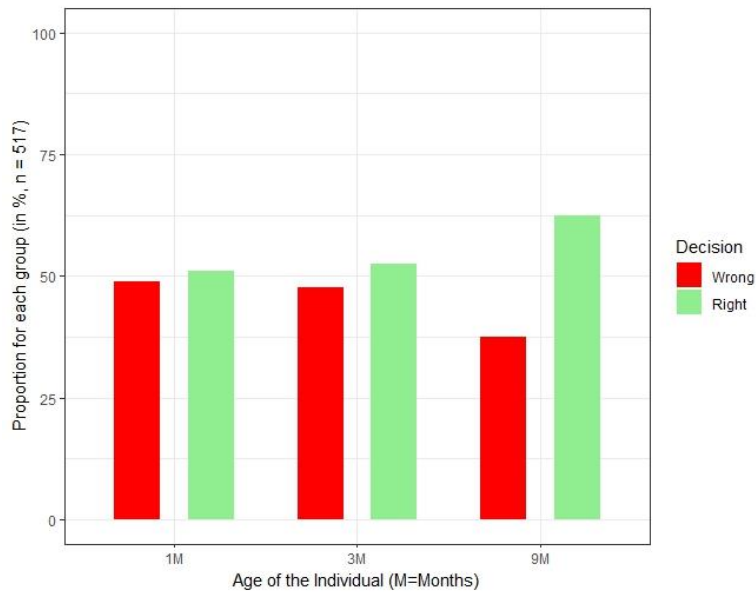


**Figure 7: Memory test of the pure-age colonies**

Results of the memory test of the general learning experiment in colonies with pure-age cohorts of 1-, 3-, and 9 months age from 3 different genetic lineages. The three bars show the proportional distribution of all individuals across the three possible choices without reference to their age or genotype (counts ND: 200, R: 171, W:146). For instance, individuals that avoided the CS after conditioning (and learned) are referred to by the bar “Right” on the x-axis (possible choices).

Using the GLMM, there was no effect of the genotype on learning visible (GLMM, ~genotype + age, A-B: p-value = 0.3477, A-M: p-value = 0.3405). The afterwards applied post hoc Tukey HSD test showed also no significant differences between genetic lineages on the learning performance (genotype-combinations: A - M | A - B | B - M, Table 1). The relationship between genotype and learning performance is shown in fig. 9.

(3): I found no significant evidence for learning in ants that grew up with older individuals in pure-age colonies of genotype A and B, but a tendency that they perform better in learning compared to a random distribution (Chi-Square test, X-squared = 3.722, df = 1, p-value = 0.054, n = 97; Fig. 10).



**Figure 8: Learning as a function of age**

Results of the memory test of the general learning experiment in colonies with pure-age cohorts with reference to their age (x-axis, 1M: 1-month-old). For each age cohort, the decisions (binomial: right and wrong) are shown in bars next to the corresponding age. For instance, individuals that avoided the CS after conditioning (and learned) are represented in the green bars at 1-, 3- or 9 month-age. The results are proportional and grouped for each age cohort to show learning at each age (counts: 1M, ND: 87 | R:66 | W:63; 3M, ND: 43 | R: 65 | W:59; 9M, ND: 70 | R: 40 | W:24).

**Table 1: Results from the Tukey test of the GLMM**

Results of the post hoc (Tukey) test from the GLMM in pure-age colonies. The two main variables age and genotype were tested for significant differences between treatments.

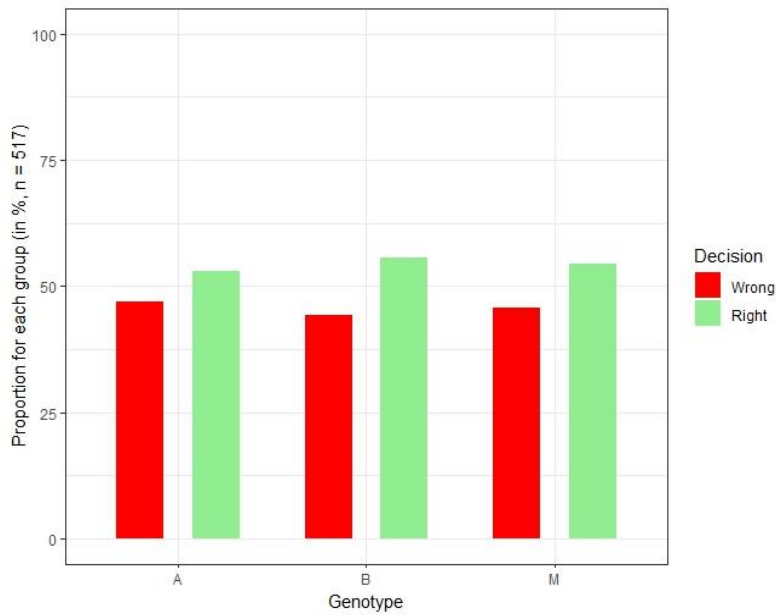
Table 1: Memory test standardized colonies. Influences of variables.

Combinations	Age			Genotype <sup>c</sup>		
	1 M - 3 M <sup>b</sup>	1 M - 9 M	3 M - 9 M	A - B	A - M	B - M
p-values <sup>a</sup>	0.9611	0.1668	0.2352	0.6155	0.6065	0.9933

<sup>a</sup> P-values were calculated by a Tukey post hoc test of the GLMM. Random effects are mentioned in the methodology.

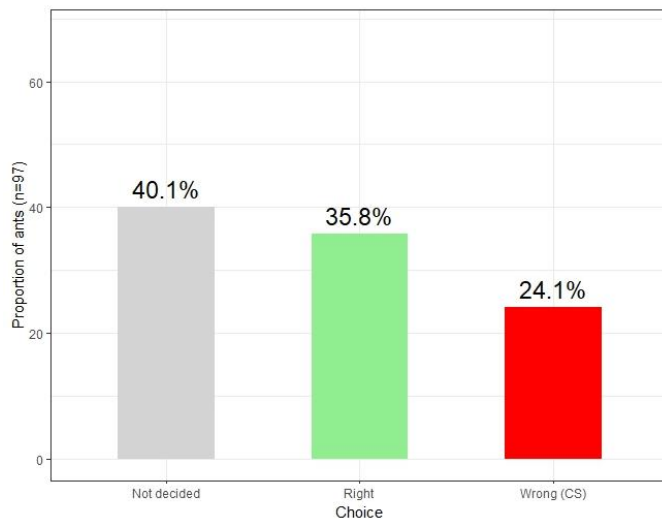
<sup>b</sup> M (except for the genotype) = Month(s)

<sup>c</sup> Genotype lines after Tribble (2020)



**Figure 9: Learning as a function of the genotype**

Results of the memory test of the general learning experiment in colonies with pure-age cohorts with reference to their genotype (x-axis, A: genetic lineage). For each genotype, the decisions (binomial: right and wrong) are shown in bars next to the corresponding genotype. For instance, individuals that avoided the CS after conditioning (and learned) are represented in the green bars of genotype A, B and M. The results are proportional and grouped for each genotype to show learning in each (counts: A, ND: 105 | R:81 | W:72; B, ND: 38 | R:34 | W:27; M, ND: 57 | R:56 | W:47).

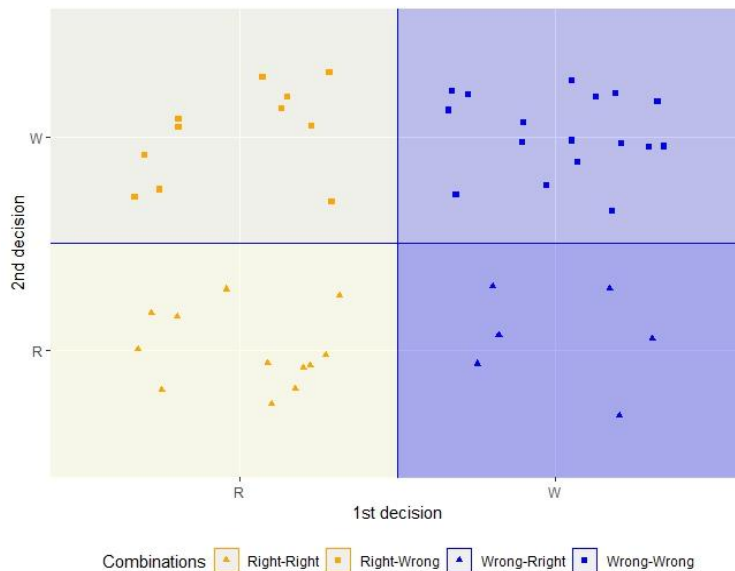


**Figure 10: Memory test in ants with older individuals**

The outcome of the memory tests in ants which grew up with individuals inside the colony which were older than themselves. The three bars show the proportional distribution of the individuals across the three possible outcomes in the maze (counts: ND: 65 | R:58 | W:39). For instance, individuals that avoided the CS after conditioning (and learned) are referred to by the green bar “Right” on the x-axis (possible choices).

### 4.3 Consistency experiment

(2): 81 individuals were tested two times. The distribution of how ants performed in the second memory test in regard to the first one is shown in fig. 11. Individuals which successfully passed the first test decided very equally in the second test with a distribution of almost 50/50 in making the right decision ( $n = 12/23$  (52%)). Individuals that decided wrong in the first test, had a higher proportion in making the wrong decision in the second test as well ( $n = 17/23$  (74%)). A GLMM that checked for a connection between the first and second memory test's decision showed a tendency for ants to make the same choice between both memory tests (GLMM,  $p$ -value = 0.0743). Additional results are found in the Annex (S. Fig. 12b) which shows a bar plot of the data (dependence of the decision of the first memory test on the outcome of the second memory test).



**Figure 11: Learning consistency in *O. biroi***

The results of the consistency experiment and about the distribution of the decision-making in individuals doing the learning experiment twice. Every dot means one individual, the blue color relates to a previously wrongly made memory test where the ant was preferring the CS. The orange color refers to the individuals which made the right decision in the first test. The shape on the other hand is related to their second decision (triangle = right, square = wrong). The dots are dispersed in their corresponding square which refers to the combination of decisions from both tests one individual made.

## 5. Discussion

The results revealed an ambiguous learning ability in *O. biroi*. A few groups with a different background (with effect of older individuals inside the nest and the 9-month-old individuals) yielded results with a trend for learning, only individuals of the mixed-age showed significant learning.

Age and genotype, the two primary variables of interest, exhibited no general effects on the learning ability - neither the genotype nor the age showed significant results. In a third factor - the effect of older individuals, ants show no significant learning abilities, only a tendency to make the right decision if they have experienced it. Here, I will discuss how to interpret these findings and draw comparisons with previous research. I will also suggest potential improvements, and outline future experiments involving *O. biroi* in a learning context.

### 5.1 Factor: The genotype

Genotype, at least for workers up to 3 months of age, appears to have no discernible impact on learning ability. However, the inability to compare genotypes for the last age cohort, primarily composed of genotype A individuals, due to the loss of several ants (as discussed in section 2.2), highlights the need for a larger sample size in similar long-term experiments with this species. A repetition of testing the last age cohort with the two missing genotypes would be promising, to observe possible differences between genotypes at this very old life stage and to check for the ability of 9-month-old individuals to learn. The influence of genotype on learning in ants has not been extensively explored in prior research, to my knowledge. As such, making direct comparisons with previous studies to discuss the results in a broader context is challenging. Furthermore, achieving prior genetic control over experimental groups in (ant) species that reproduce sexually, as opposed to the clonal reproduction observed in *O. biroi*, can be logistically complex. While some studies have investigated genetic influences on behavior in ants, such as foraging behavior in the pharaoh ant (Walsh et al., 2022) or the lifecycle of whole colonies in the clonal raider ant (Jud et al., 2022), examinations of genotype-based comparisons and their effects on ant behavior remain relatively scarce. Instead, many studies have focused on comparing gene expression patterns between groups in different ant species (Alleman et al., 2019; Friedman et al., 2020; Ingram et al., 2011). The lack of a significant influence of genotypes on learning performance in *O. biroi*, as suggested by my

results, could be related to a phenomenon in science where negative results are less likely to be published. This phenomenon has been observed in various fields (Fanelli, 2012), indicating that other research groups might have encountered similar findings but chose not to publish them.

Nonetheless, this experiment provides an initial insight that genotype may simply not play a significant role in determining learning outcomes in ants, at least in the case of the clonal raider ant. This suggests that learning could be similarly important among different populations of ants with varying genotypes and that they may not experience strong contrast in selection from both biotic and abiotic factors to be good in learning or not.

## 5.2 Factor: The age

The trend in my results indicates limited changes in ant behavior that may arise with age. However, there is a lack of sources discussing the impact of age on learning in ants. Only the two studies by Franklin et al. (2012) and Cammaerts (2013) have explored this aspect in ants. Including polyethism and resulting task specialization, studies observing differences in learning between castes may indirectly include the influence of age.

Franklin et al. (2012) did not observe a significant influence of age on learning to do tandem runs in ants - a behavior used to recruit other colony members. They rather suggested that this behavior was more closely related to experience and learning than to age as well. In contrast, Cammaerts (2013) demonstrated that the spatial-temporal memory of *Myrmica sabuleti* ants was better in mid- or old-age workers compared to young workers, which aligns with the results of my experiment. Additionally, other studies have shown that the brain structure of ants changes with experience and age, particularly in the early stages of a worker's life (around 1 month), affecting brain regions like the mushroom body, which plays a crucial role in ant learning and memory formation (Groh et al., 2014). A similar study conducted with the species *Acromyrmex ambiguus* revealed a significant increase in boutons in the mushroom body immediately after learning. Although this increase declined after a few days, the ants retained their ability to remember the learned conditioned stimulus (CS). The study also included different age-groups which showed no significant morphological differences (Falibene et al., 2015). This structural change in the brain was not exclusive to leafcutter ants but was also observed in the carpenter ant *Camponotus floridanus*. In this species, the

morphological change in the mushroom body volume was not only age-related but also caste-dependent. Foragers exhibited a substantial increase in mushroom body volume, which may play a role in learning and memory (Gronenberg et al., 1996). In contrast, in *Temnothorax albipennis*, the efficiency in foraging was not associated with specialization, which suggests limited differences in memorization abilities (Dornhaus, 2008). These different outcomes could be related to differences between species in this specific ability. In conclusion, it would be intriguing to conduct a similar study with clonal raider ants and investigate morphological differences in their brain structure before, immediately after, and several days after memory tests. This could provide insights into how effectively these ants respond to learning experiments and whether they exhibit brain structural changes similar to those observed in other ant species. Furthermore, it would be worthwhile to explore potential variations in the learning abilities of individuals more inclined to forage as opposed to nurse, as there is a possibility that foragers might excel in learning (Perez et al., 2013). An examination of their brain structure in comparison could reveal valuable insights into these potential differences. Especially with the background, that there can be further methods to explore that in *O. biroi*. The experiment by Hart et al. (2023) with transgenic clonal raider ants that expressed the genetically encoded calcium indicator GCaMP6s to examine their brain activity while exposing them to the alarm pheromone - an olfactory stimulus - could here be applied on activity patterns/morphology changes when working on learning experiments.

### 5.3 The learning consistency of ants

The results from the consistency experiment also yielded mixed findings and did not exhibit a distinct pattern. Notably, a higher proportion of individuals consistently made incorrect choices in both trials, in contrast to the anticipated random distribution where individuals would choose the conditioned stimulus (CS) or the alternative odor in roughly equal numbers. Ants that made the right decision in the first memory test on the other hand choose equally both odors.

It is hard to imagine that ants possess personalities but it has been observed in many studies that some kind of personality (on colony and/or individual level) in ants exists (Carere et al., 2018; Maák et al., 2020; Mailleux et al., 2005; Scharf et al., 2012). Pinter-Wollman (2012) even discussed a possible future to scientifically explore worker and colony personalities in ants. A few of these studies are also linked to learning



The ability of ants to remember odors after a prolonged period is relatively improbable and has only been demonstrated in a limited number of experiments e.g. in Saverschek et al. (2010). Consequently, these results in this thesis about consistency appear to be indicative of individual consistency in the ants' behavior when making the wrong choice. Nevertheless, the reason why this consistency was not observed in individuals that successfully passed the first memory test remains elusive. For example, Finke et al. (2023) observed very similar results of (restrained) olfactory learning in their study with bees. In their reversal learning experiments, individuals which did not learn in the first test significantly tended to choose identically wrong in the second test (91%, n = 47 | here: 74%). The same pattern as in this thesis was observed for individuals which did pass the first test successfully. Individuals then were not consistent and chose right and wrong in more or less equal proportions (Finke et. al: only 52% right | here: 52% as well). In contrary to their experiments, the ants in this thesis were conditioned on the same odor as they were in the first memory test. Although in their study with significantly correlating combinations to test consistency in different paradigms, they could not find any significance between the second reversal learning and a negative patterning experiment for example. Test in which the bees were punished when the odors were presented together and rewarded when presented separately). Signs of consistency or even personality can therefore vary depending on the combination used in the study. Udino et al. (2016) found a significant correlation between consistent exploratory activity and learning performance in *C. aethiops*, but not for other factors like sociability and aggression. Consistency was observable in my experiment, but only for individuals which could not learn.

One hypothesis to consider in the experiment of this thesis (in which the proportion of ants only making the wrong decision in both tests (1<sup>st</sup> and 2<sup>nd</sup> memory test) was high) is that ants may encounter the aversive stimulus differently. As a result, they exhibited a preference for the odor they had previously encountered. The differences in the ants' behavior could be attributed to variations in a specific structure related to their actual contact with the electrical circuit. The way ants make contact with a surface is a complex process and may involve tarsal hairs and their orientation on the surface (Endlein & Federle, 2015). It is well-known that ants within a colony can exhibit variation in characteristics such as hairiness (Seifert, 2018). Furthermore, in other insects like bumblebees, it is known that hairs can become electrically charged (Koh & Robert, 2020). In general, the distance between two electrically conductive bodies plays a crucial role in allowing current to flow (Kovacs, 2001). These factors could

potentially contribute to the differences observed in the ants' response to the aversive stimulus. Maybe simply, because the electric shocks reduced the hairiness (singed hair) of the ants after the first memory test (Schulze et al., 2016). Indeed, investigating the morphological characteristics of the ants, particularly their feet that are in contact with the circuit, could provide valuable insights into the observed variations in behavior. Such an examination was not conducted in this thesis but could be a valuable direction for future research. It would be intriguing to determine whether the ants that initially chose the conditioned stimulus (CS) and showed no further learning in subsequent experiments consistently maintained this behavior. Especially, with the knowledge that ants are able to maintain their learning performance over several extinction trials by what it would be interesting to examine a consistent pattern in a similar consistency experiment (as in this thesis) but with more repetitions (Guerrieri & d'Ettorre, 2010; Piqueret et al., 2019). While it is unlikely that *O. biroi* consistently makes the wrong decision, understanding the factors that influence their response to aversive stimuli could shed light on the observed behavior.

#### 5.4 Potential factors influencing learning in *O. biroi*

##### 5.4.1 Task specialization

The influence of task specialization on learning and behavior is an interesting aspect of ant biology and may have influenced the results in this thesis as well. Task specialization has already been combined with learning experiments in other ant species - e.g. in subsequent passages to show possibilities on how to implement task specialization in learning experiments with the clonal raider ant.

Iakovlev and Reznikova (2019) experiment on wood ants, which examined learning in workers with a different task specialization, is an example of how task-specific learning could occur. In their study, they observed distinct behaviors between workers in response to a particular stimulus, in this case, a hoverfly larva that can glue the ant with a viscous secretion, if attacked. However, despite the task-specific differences in behavioral response, neither nurses nor foragers (task specializations) learned to avoid attacking the larva. More general was the study from Perez et al. (2013) who challenged (sucrose learning with MaLER) task specialized workers (nurses and foragers) of *C. aethiops* in an appetitive learning experiment. The workers were monitored for a period of two weeks to assign them to a specific specialization. They showed that foragers are better at learning than nurses. This highlights the importance of

considering various factors, including task specialization and the specific nature of the stimulus. Differently specialized members within an ant colony may respond similarly different to certain stimuli. Exploring the role of task specialization in learning and behavior in ants can provide valuable insights into their social structure and division of labor, especially because the clonal raider ant displays task specialization (Ravary et al., 2007). Further research in this area could help clarify the extent to which task-specific learning occurs and under what circumstances in the clonal raider ant. Perez et al. (2013) results suggest differences between specialized *O. biroi* workers in the learning performance (for example foragers, that show a stronger learning performance than nurses). The factor of different task specialized workers could have influenced the results in the learning experiment in this thesis, by what adding this factor could shed light on this question.

#### 5.4.2 Age structure in the colony

The age structure of the colony is another factor that might have influenced the learning abilities of the ants. The experiments tested individuals from the oldest cohort within the colony in the pure-age colonies (not in the mixed-age colonies, two small cohorts of genotype B and a cohort of genotype A with the effect of older workers (than themselves). This implies that learning could potentially be more evident in colonies where older ants are already present, as they might have an important impact on younger workers to memorize food sources. This could potentially come from teaching as in *T. albipennis*, which shows the ability to teach knowledge about efficient tandem runs from experienced to naïve workers (Franks & Richardson, 2006). To explore this hypothesis, an additional experiment was conducted with a cohort of ants that were younger than the other individuals within the colony (see methods and results section). The outcome of this experiment showed a tendency for ants to learn. This tendency points out a possible interaction between two factors: colony structure/individuals inside the colony older than the challenged individuals, along with the age factor. This interaction might result in an increased and noticeable learning ability, as observed in the mixed-age colony, because these colonies had individuals with probably the oldest age of all colonies used in this thesis (~9 months - 2 years), were large (around 1000 - 3000 individuals) and possessed most cohorts inside the colony (various age). For a more robust evaluation of these assumptions, conducting comparisons with larger groups that incorporate would be insightful.

#### 5.4.3 Colony size

The colony's size may potentially have impacted the experimental outcomes. Studies have revealed that ant colonies can exhibit reduced activity in the short term when workers outside the nest are removed. This decrease in activity could be attributed to colonies becoming more cautious (O'Shea-Wheller et al., 2015) or due to the removal of the most active individuals, typically found outside the nest, within a colony (Ishii & Hasgeawa, 2013). Conversely, observations on the clonal raider ant's activity within a group have shown a relationship between individual activity and group size, suggesting slightly decreased activity (per individual) and increased variation with larger group sizes (Ulrich et al., 2018). The group sizes were quite low (1-16), but maybe this also applies for individual workers, that do a learning experiment and speak against an influence by the colony size. In the data collection for this thesis, colony size varied among genotypes and within genotypes (S. Fig. 8). For instance, the 1-month-old individuals were essentially daughter colonies of the 3-month-old individuals in genotypes A and B, and thus smaller. It would have been valuable to control for this factor, especially considering that larger colonies tended to exhibit more pronounced learning in this thesis. Specifically, the mixed-age colonies and the pure-age colonies with 9-month-old workers were larger than the pure-age colonies at the time the first two age cohorts had been tested. Indeed, larger colonies possess the advantage of being more resilient to the loss of individual members compared to smaller colonies (Shorter & Rueppell, 2012). As a result, the willingness to take risks may be higher in larger colonies, potentially affecting the proportion of ants making a choice in the T-maze. Furthermore, ants in larger colonies are exposed to a greater amount of social interactions with their fellow colony members throughout their lives, which may influence their learning (Gordon, 2010a).

#### 5.4.4 Type of US and CS

Several factors may contribute to less individuals exhibiting learning than potentially possible, including the nature of the CS and of the US. Ants can exhibit significant variation in their behavior when forming associations with different USs. For example, they tend to learn odors associated with food items more readily than those linked to the nest entrance (US), and the latter associations are often less robust and slower to form (Huber & Knaden, 2018). It remains a challenge to determine whether the electric shock serves as the optimal unconditioned stimulus (US) for use in experiments with clonal raider ants. Wenig et al. (2021) were successful in using electric shocks as US (see section 1.5), but the ants could not avoid the

pheromone trail after the shocks had been applied which maybe indicates, that a shock is not the most optimal US in a learning experiment. However, a comparison between species and different setups might be inappropriate. For instance, Wenig et al. (2021) used a much lower voltage (7.5 V) whereas I applied 48 V to shock the ants (the ants were not visually affected with a lower voltage). Although Wenig et al. (2021) did not specify the amperage used, it presumably differed from the 5mA I applied. An alternative to electric shocks could involve conditioning ants to associate an olfactory conditioned stimulus (CS) with an aversive heat source, as demonstrated by Desmedt et al. (2017). Additionally, the quality of the unconditioned stimulus (US) holds the potential to influence learning performance, such as in appetitive learning. For instance, the concentration of sugar in sugar water can impact the speed of learning in maze experiments (Oberhauser et al., 2018). This principle can be relevant to aversive learning, as the effectiveness of applying the US to the tested individual would then depend on the quality. In conclusion, there are several ways to enhance the experiment, making the clonal raider ant's learning more evident. One approach is to increase the quality of the US, ensuring it is sufficiently aversive to facilitate the formation of associations. Minor adjustments, such as increasing the number of trials (as in some studies, (Guerrieri & d'Ettorre, 2010; Wenig et al., 2021)), and longer sessions with extended breaks, could be beneficial for ants to memorize odors in the T-maze. This improved association formation is critical, as a lack of it can lead to imperceptible learning in ants (Fernandes et al., 2018). On the other hand, it could also be expected, that after a certain number of trials, there is no improvement in learning (after ca. 5 conditioning trials) as observed in bees (Giurfa & Sandoz, 2012). Furthermore, the proper application of the shocks can be more critical than one might anticipate. If the ant is not sufficiently shocked in some trials, this absence of the US results in trials that can act as extinction trials (unrewarded trials with the CS), making the formation of associations even more challenging (Sandoz & Pham-Delegue, 2004). However, it is difficult to determine the exact threshold for these non-aversive/punished trials in aversive experiments. For instance in appetitive learning, in Piqueret et al. (2019), there was no evidence of extinction even after nine unrewarded trials in *Formica fusca*. This suggests that a similar number of non-aversive trials may not result in a significant extinction response, assuming the shocks in my experiment were not consistently 100% accurate.

Another significant aspect to consider is the specific odor used as the CS, as ants may respond differently to various olfactory components (e.g. in CHC's) (Bos et al., 2012). While 1-octanol

and hexanal have been employed in previous experiments (Huber & Knaden, 2018; Piqueret et al., 2019), exploring different odors could be valuable for understanding the performance of *O. biroi*. However, like the odors used here, any new compounds should ideally be relatively simple/volatile. When multiple odors are used, their difference in chain length could be important for the ants to discriminate between them, to avoid generalization in the memory phase (Bos et al., 2012). This consideration can be crucial in designing future experiments to optimize learning in clonal raider ants.

#### 5.4.5 The ant species

To assess the general learning ability of *O. biroi*, it could be worthwhile to use the same experimental setup with another ant species and compare the results with the findings of this study - taking the limited ability to include the same standardization of variables as in *O. biroi* (genotype, age) into account. Research has shown that different ant species vary in learning strategies in experiments. Two such species, *L. niger* and *Linepithema humile*, demonstrated different orientation strategies in an experiment involving the association of a visual cue with a food reward. The setup comprised a Y-maze which was connected to the nest at one end and obtained the food rewards (sugar water) behind the fork, at each end of the Y. One of the arms was coupled with a visual cue (light bulb). The ants then foraged and were marked while consuming the reward. While both species learned to associate the visual cue with a reward and significantly favoring the path to the food with the visual clue (in contrast to inexperienced individuals choosing both), experienced workers of *L. niger* displayed more individual and cue-guided behavior driven by the visual stimulus in a subsequent choice test. In this test, workers chose to take the arm of the Y with the visual cue (pheromone trails had been removed) instead of the other branch which was marked with a pheromone trail from the previous experiments. In contrast, experienced workers of *L. humile* (priorly conditioned on the visual clue) were primarily focused on the chemical cue of the pheromone trail and relied heavily on it, leading to a preference for the pheromone trail over the associated visual stimulus (Aron et al., 1993). Applied on the experiment in this thesis, a reduced individuality within the clonal raider ants to overcome pheromone trails may have a more negative impact on their learning abilities. Even though, I tried to remove any pheromone trails, the influence of that could have been present during the experiment. On the other hand, species that place less emphasis on visual cues and rely more on olfactory signals may simply exhibit higher levels of success in

olfactory learning. Comparing (in an olfactory context) *O. biroi* with a species such as *L. niger* could offer valuable insights into interspecies variations in learning behavior. Infection by nematodes

One problematic change that occurred during the experiment was the increasing infection by nematodes of the genus *Diploscapter*. This nematode is known to infect the clonal raider ant and can alter the host's behavior to spend more time in the nest or can decrease the expression of genes responsible for aggressive behavior (Li et al., 2023). Although it is unclear whether this nematode influences the ants' learning ability, parasites can affect the outcomes of learning in bees negatively (Gómez-Moracho et al., 2022) and positively (Kralj et al., 2007). In this experiment, there seems to be a slight, albeit trend for older individuals to perform better in learning, which might be linked to the infection with *Diploscapter* but cannot be confirmed due to missing data about the actual infection load. The groups which performed here the best were the oldest cohort of the pure-age colonies, the cohort which was tested for effects of older individuals inside a colony and the colony of the mixed-age - and all of them encompassed the presence of nematodes since a minimum of 3 months. Longer than in the others. In retrospect, it is possible that the ants in the colonies with higher infection rates performed better in learning. To investigate this hypothesis, incorporating a uninfected group and perhaps a highly infected pure-age group in this experiment could provide valuable insights.

#### 5.4.6 Ecological role

It's also possible that the ecological role of learning in *O. biroi* is not as pronounced as in other ant species. For instance, *O. biroi*'s morphological characteristics (see 2.1) may not require them to be aware of certain ecological threats, such as ant lions creating their funnels above the surface, as other ant species. In contrast, species like *Tetramorium sp.* are more frequently exposed to this threat (Taylor et al., 2013) and can learn to avoid such traps (Hollis et al., 2017). Applied on the US in this thesis, the clonal raider ant is not confronted with electric shocks or any similar stimuli, their behavior in response to this stimulus may not align with initial expectations and therefore, the US is not as effective as required to yield high rates of learning in the experiment.

#### 5.4.7 Painting of the ants

Painting ants could influence their decision-making in the T-maze and therefore the outcome in the memory test. However, when comparing the performance of workers from the pure-age colonies and workers with the effect of older individuals (the ones which were painted), and workers of the mixed-age colonies as well as the first cohort of genotype A and M of the pure-age colonies (which were not painted), the results did not support this theory (Chi-squared = 1.4056, df = 1, p-value = 0.8409). As a result, the idea was discarded.

#### 5.5 Outlook

Here, I focused on individual learning, not social learning (with at least two individuals, learning from other individuals) or learning in groups (several individuals are doing the learning experiment simultaneously). However, exploring the effect of colony size and the influence of social behavior/effects in ants on their learning ability is an interesting avenue of research. Previous observations have suggested that ants tend to prefer individual learning in smaller colonies and social learning in larger colonies. Environmental factors also play a role in determining whether social or individual learning is more efficient, potentially impacting the ant's ability to memorize stimuli (Glaser & Gruter, 2023). One simple modification to the setup could involve adding more than one worker in the "learning phase" and challenging groups of workers, perhaps 5 or 10 individuals, simultaneously to investigate social learning in *O. biroi*.

It would also be promising to refine the experiment by incorporating improvements such as introducing different sets of learning trials and inter-trial interval periods, then specifying the time points at which memory tests are conducted and enhancing the design of extinction trials. Although the memory test was done close after the learning phase and the learning performance is expected to be lower when the memory test starts later, it would be worth it to verify or maybe find non-linear relationships with the already mentioned different number of learning trials. That means for example, to find a peak in learning performance with a specific number of trials and a decrease/stagnation in performance with less or more trials. These modifications have been implemented in several previous studies in various species (Desmedt et al., 2017; Dupuy et al., 2006; Piqueret et al., 2019; Sandoz & Pham-Delegue, 2004). This enables also the investigation of a long-term memory in *O. biroi* (prior with an



optimal number of learning trials) similar to studies conducted by Arenas and Roces (2018); Piqueret et al. (2019), and Iakovlev and Reznikova (2019).

Finally, it's worth considering the intriguing possibility of introducing a component to enhance the learning and memorization abilities of ants. In a handful of studies, ants changed their behavior after they were fed with drugs which are known to have a (cognitive) neurobiological impact (Entler et al., 2016; Friedman et al., 2018; Kostowski & Tarchalska, 1972). For example, dopamine can increase the foraging activity of harvester ants which was also supported by higher expression levels of transcripts in the brain tissue of treated workers which speaks for a higher brain activity (Friedman et al., 2018). One study even worked on the effect of drugs on learning in ants which showed that *L. niger*'s learning performance is affected by blocking dopamine (with flupentixol) and octopamine (with epinastine) receptors. The workers were tested in an appetitive learning experiment on two linear hydrocarbons (CS) on which one of them they were conditioned using a sucrose reward. One group had previously been treated with the drugs, the other group not. Although blocking the dopamine receptor only reduced the memory (long-term) to associate an odor with a reward, blocking the octopamine receptor resulted in a complete inhibition of appetitive learning in the ants (Wissink & Nehring, 2021). Additionally, Carlesso et al. (2021) demonstrated that at least one secondary plant compound,  $\gamma$ -Aminobutyric acid (GABA), has the potential to improve both appetitive and aversive learning performance in bees. It remains a question whether the clonal raider ant may be similarly influenced by GABA and dopamine and octopamine. This avenue of investigation opens up exciting possibilities for future experiments.

## 6. Summary

Ants have established their reputation as excellent learners, with the ability to remember conditioned stimuli over extended periods (from days to weeks). The clonal raider ant, being an intriguing species for laboratory studies due to the control it offers over variables like age and genotype, was chosen for an aversive olfactory learning experiment. The study aimed to explore the general aversive learning capacity of clonal raider ants and the possible influence of age and genotype. The results indicate that clonal raider ants, in general, can exhibit abilities in olfactory learning, but this capability appears to be influenced by several factors and was not present in most of the experimental groups. Among these factors, age and the

potential effect by older ants showed most impact. Conversely, the influence of genotype on learning appeared to be negligible. Other factors that could potentially, such as the experimental setup or social learning, may influence learning performance. In summary, this experiment represents an initial step in conducting controlled aversive learning experiments in the clonal raider ant but also shows possible limitations in the learning ability of this species.

## 7. Zusammenfassung

Es ist mehrfach bewiesen worden, dass Ameisen ein sehr gutes Lernvermögen besitzen und sich an konditionierte Stimuli über mehrere Tage oder sogar Wochen erinnern können. Die klonale Raubameise ermöglicht aufgrund ihrer Biologie die Standardisierung von Genetik und Alter, das Durchführen sehr interessanter Laborexperimente. Mit diesem Hintergrund wurde ein aversives, olfaktorisches Lernexperiment durchgeführt, in der die allgemeine Fähigkeit der klonalen Raubameise (*O. biroi*) zu lernen, getestet worden ist. Zusätzlich sind die Faktoren Genotyp and das Alter der Individuen in das Experiment eingeflossen, um ihren Einfluss auf das Lernverhalten zu untersuchen. Die Ergebnisse zeigen, dass die klonale Raubameise im Allgemeinen zu olfaktorischen lernen fähig sein kann, jedoch viele Ergebnisse keine Signifikanz aufwiesen. Hierbei erscheinen das Alter und wie die Ameisen aufgewachsen sind die hauptsächlichen Faktoren zu sein. Vor allem die ältesten Individuen zeigten einen Trend zu Lernverhalten, sowie Individuen aus Kolonien mit gemischtem Alter. Hingegen hat der Genotyp keine große Rolle gespielt gegenüber der Lernfähigkeit. Andere Faktoren, welche Einfluss auf die Ergebnisse des Verhaltensexperiments haben könnten, sind angesprochen worden - Beispielsweise Verbesserungen im Aufbau des Experiments oder das Hinzuziehen des sozialen Lernens in welchem *O. biroi* womöglich noch besser sein könnte. Insgesamt ist das Experiment ein erster Schritt in (genetisch und altersbedingt) kontrollierte Lernexperimente in Ameisen und das erste mit der klonalen Raubameise, zeigte jedoch auch die Grenzen der Lernfähigkeit dieser Art auf.

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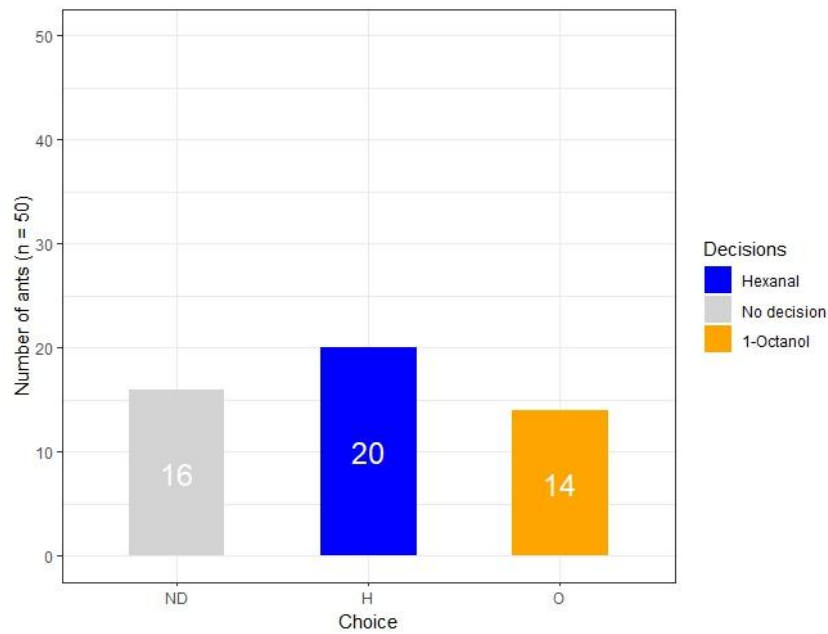
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## Supplemental part

Before conducting learning experiments, preliminary tests were performed to evaluate any bias towards one of CS odors. These preliminary tests aimed to assess a preference for one of the two odors, the general aversive or attractive influence of each odor, and the learning

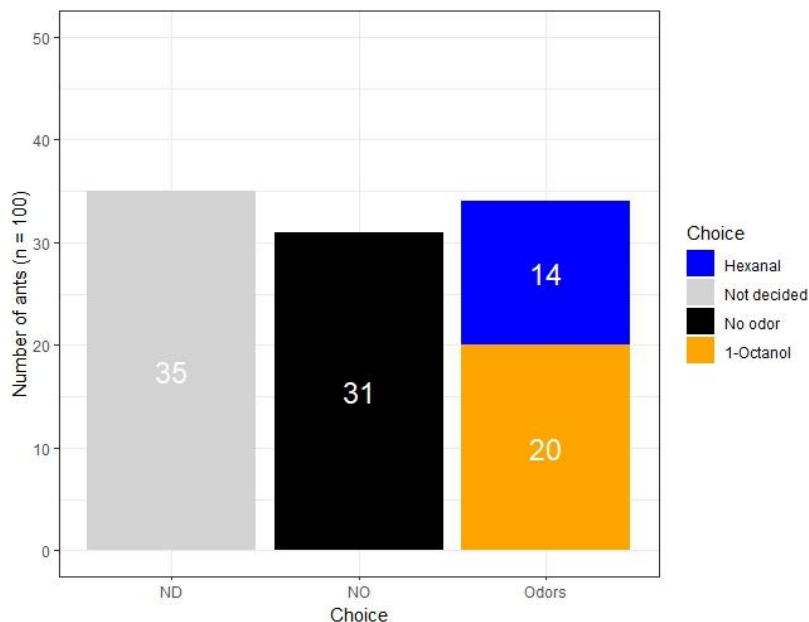


**S. Fig. 1: Preference test**

The odor preference test as one of the three main pre-tests. Overall, 50 naïve individuals have been tested in a similar way as in the 2<sup>nd</sup> phase of the learning experiment. The graph shows how many individuals decided for which odor and how many did no decision (ND). The ants showed no clear significance nor a tendency to prefer hexanal or 1-octanol (p-value > 0.05).

performance within mixed-age colonies of a single genotype. Odor preference was assessed by placing naïve ants in the central chamber of the T-maze. The arms, delivering the odors were attached to the maze, and the chamber containing the ant was moved to the position of the attached arms (memory test; Fig. 2b). The ants were given two minutes to choose between the odors. To determine whether there was a significant preference for one odor, I generated expected values assuming an even distribution and compared them to the actual outcomes. The results showed no preference between the two odors (Chi-Square test, X-squared = 1.0588, df = 1, p-value = 0.3035; S. Fig. 1).

Another test was conducted to assess the aversive or attractive influence of each odor when presented in one arm of the T-maze, with no odor (only humidified air) in the other arm. Results showed no significant attraction or aversion towards both odors compared to no odor (Chi-Square test, X-squared = 1.2462, df = 2, p-value = 0.5363; S. Fig. 3).

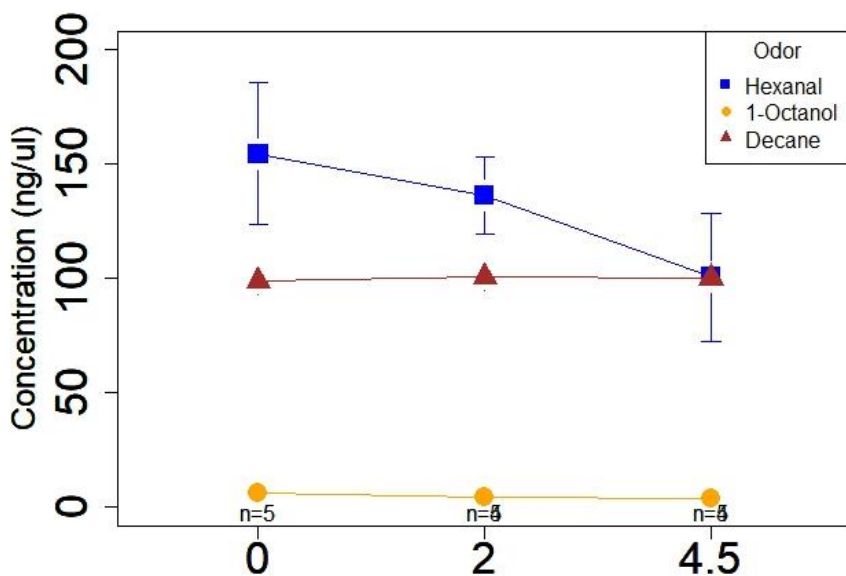


**S. Fig. 2: Attractivity test**

The attractivity test, which was quite similar to the preference test, but the odors were offered separately and as a second option, an empty arm was offered without any odor. The results showed no significant tendencies for any option. Either for an odor, no odor or to stay in the chamber with a distribution of around 1/3 for each of the possibilities.

I measured the concentration of odor that the ants were exposed to during the experiment (S. Fig. 3) using a volatile compound collection system (Super-Q filter; Porapak™, Sigma Scientific LLC, USA). Similar to the experiment setup, the odors were allowed to accumulate inside the bottles for one hour and then released for one hour before the collection process began. Collection spanned 5 hours, similar to the duration of experiments. Collection times were at 0, 2, and 4.5 hours, during which the filter collected odors for 30 minutes each time. Following this, the filters were washed with 200µl of Dichloromethane (DCM), which also

contained a standard (100ng/μl n-Decane) to ensure accurate concentration calculations. The collected samples were analyzed using GC-FID (Gas Chromatography with Flame Ionization Detection), and the results revealed significant differences between the absolute concentration of the odors hexanal and 1-octanol (linear-mixed model, concentration~time, lmer()-function from the lme4-package, p-value < 0.001\*\*\*). On average, the concentration of hexanal was more than 18 times higher than that of 1-octanol, with concentrations of 4.53 ng/μl for hexanal and 83.46 ng/μl for 1-octanol. Further tests to assess the significance of changes in concentration over time showed that the concentration of 1-octanol exhibited a more pronounced change. In the case of hexanal, the concentration remained relatively stable over the entire 5-hour time period, and no significant changes were observed at any time point (p-adjusted values (Bonferroni corr.): 0 - 2 h: p = 0.4062; 0 - 4.5 h: p = 0.0627; 2 - 4.5 h: p = 0.0743 for hexanal). Conversely, for 1-octanol, the concentration showed a statistically significant change over the entire 5-hour period, though not at all time points (p-adjusted values (Bonferroni corr.): 0 - 2 h: p = 0.0743; 0 - 4.5 h: p = 0.0498\*; 2 - 4.5 h: p = 1.0000 for 1-octanol, order: 0 - 2 h, 0 - 4.5 h, 2 - 4.5 h). The concentration trends in both hexanal and 1-octanol were both decreasing, with different time points showing varying significance.



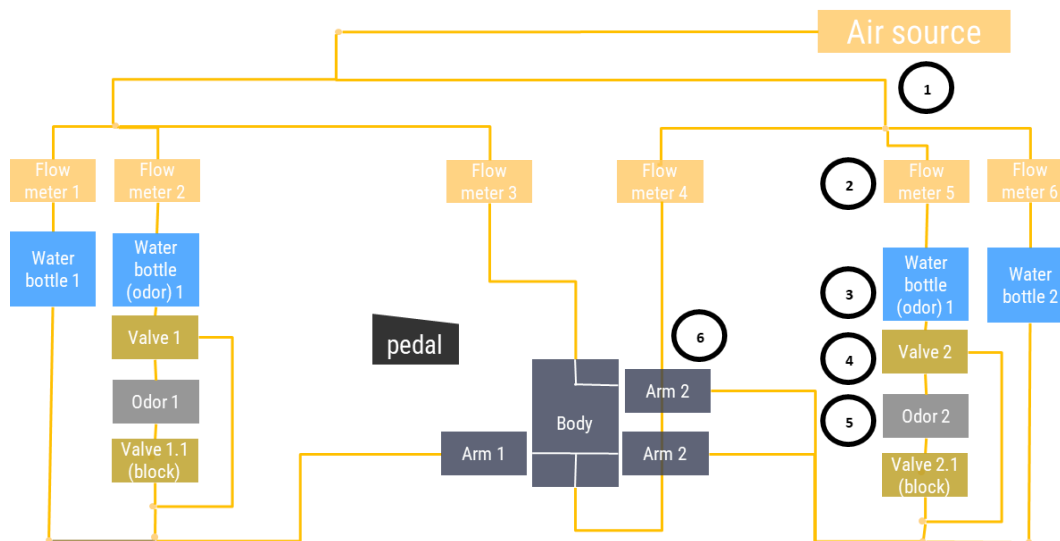
S. Fig. 3: Dynamics of the odor concentration in the setup

The odor measuring experiment which shows the decrease of odor in the setup over time. The small experiment was done over 5 hours which is related to a usual day doing the learning experiment. The odor was collected at three different time steps (0,2 and 4,5 hours) for 30 minutes with a SuperQ filter. Both odors at the same time.

Hexanal experienced a more rapid decrease in concentration during the second half of the experiment, while 1-octanol exhibited a faster decline in the first half.

In addition to the main results and analysis of the two variables, genotypes, and age, I considered other variables that could have potentially introduced noise into the results of the learning experiment. These additional variables included larvae appearance, odor, position of the arms, and day order. "Larvae appearance" refers to the developmental stage of the ants and indicates the day when the first larvae became visible, providing insight into the status of the colony's lifecycle (Fig. 5). The ants which were tested started at this time a behavioral switch with the beginning to the brood-care phase in which they forage and are usually less clustered and inactive as in the previous stage (Reproductive phase). Based on my own observations and test trials conducted during the reproductive phase, the ants exhibited significantly different behavior, generally appearing more passive when in the reproductive phase compared to the brood care phase, during which they engage in foraging (Fig. 5 and 2.1 in the methodology). The aim was then to test whether the behavior during the brood-care phase significantly differs in the motivation of the ants to walk in one of the arms during the learning experiment. Subjecting the data to a Chi-Square test to identify any potential trends for different behavior related to day differences in the lifecycle, no significant correlation with the ND results was observed (Chi-Square test,  $X^2 = 34.367$ ,  $df = 28$ ,  $p\text{-value} = 0.1891$ ), and there was no discernible impact on decision-making (right/wrong) related to colony cycle (Chi-Square test,  $X^2 = 5.7727$ ,  $df = 14$ ,  $p\text{-value} = 0.9719$ ; S. Fig. 5). Consequently, these



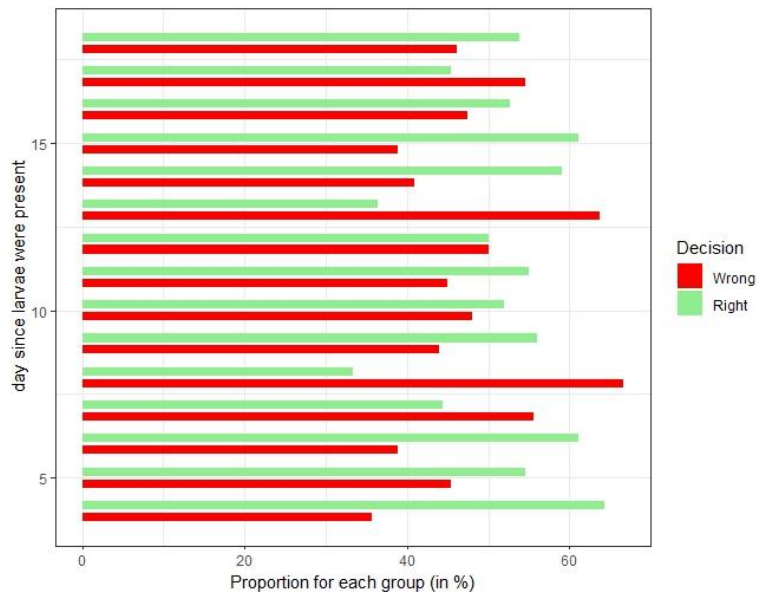


**S. Fig. 4: Scheme of the airflow of the entire setup**

Schematic display of the set up respectively airflow(s). (1) The starting point is the air source which provides air for both arms which are offered to the ants in the T-Maze. (2) First, the air passes the flowmeters which allows setting the flow rate. For flowmeters 3 and 4, the soaking rate is set with which air (with odor) is removed from the maze to avoid accumulation. (3) The water bottles - to provide sufficient humidity for the ants which is in the case of *O. biroi* very high with almost 99% RH in the nest. (4) After the water bottles the air passes valves which ensure the presence or absence of the odor in the arms. (5) If they are activated and opened, the odor is passed through the tubes, out of the bottle into the arms and the ant encounters the odor. (6) In this scheme, arm 2 is present twice and refers to the possibility of positioning the arm at the upper part of the body to train the ant or at the lower position, to check the learning ability when offering the other odor opposite, at the lower position of the body.

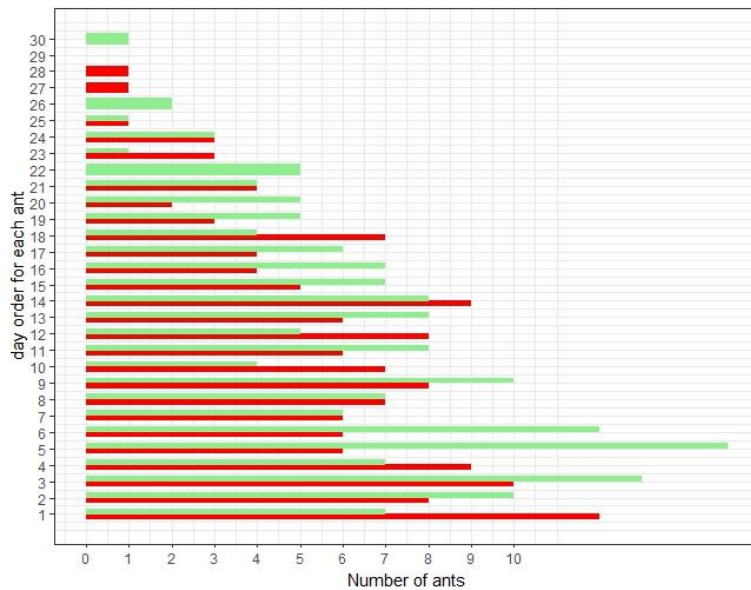
findings indicate that the colony cycle does not influence learning performance, and it is irrelevant on which day of the brood care phase the experiment is conducted.

The “day order” respectively the order in which the ants had been tested each day was another factor considered for its potential influence on the results. It aimed to rule out any effects caused by, for example, the accumulation of pheromones, changes in airflow, or declining odor concentration. To investigate the significance of the day order, Chi-Square tests were used by looking for a significant relationship between right decisions in the memory test and the day order of the ants. However, the results indicated no significant correlation, regardless of whether the ND results were included (Chi-Square test, X-Squared = 60.603, df = 58, p-value = 0.3821) or not (Chi-Square test, X-squared = 23.731, df = 28, p-value = 0.6957; S. Fig. 16).



**S. Fig. 5: Influence of the colony cycle on the memory test**

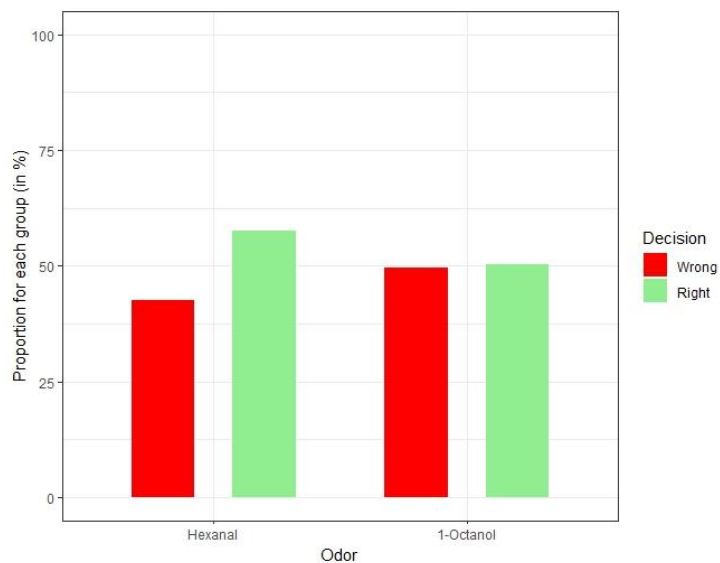
This figure shows the dependence of the colony cycle on the memory experiment and the learning ability of *O. biroi*. This is evaluated by the presence of larvae, which is directly connected to the beginning of the brood-care phase in which the clonal raider ant starts to forage. On the Y-axis, the stage of the lifecycle of the ant colonies is shown (by days since larvae appearance) and on the x-axis the scale from 0-100% for the results. The bars are visualizing the proportion for each day of right and wrong decisions.



**S. Fig. 6: Influence of the day order on the memory test**

Strength of the factor “day order” to test whether individuals from the first trials are different from individuals which were tested later during the day respectively one session of experiments. The y-axis shows the ant’s position of the day order. The x-axis the number of ants which were tested with this position. The red bars indicate the number of wrong decided individuals (chose the CS) and the green bars show the individuals which did the right decision in the memory test.

Another variable considered was the type of odor used as the conditioned stimulus (CS), specifically whether the ants exhibited different behaviors when conditioned with hexanal or 1-octanol. A previous study has demonstrated that ants can react differently in learning experiments based on the odor used as the CS (Huber & Knaden, 2018). Here, we found no significant effect (Chi-Square-test, X-squared = 1.6917, df = 2, p-value = 0.242; S. Fig. 7) of odor on learning performance.



**S. Fig. 7: Differences between odors in the memory test**

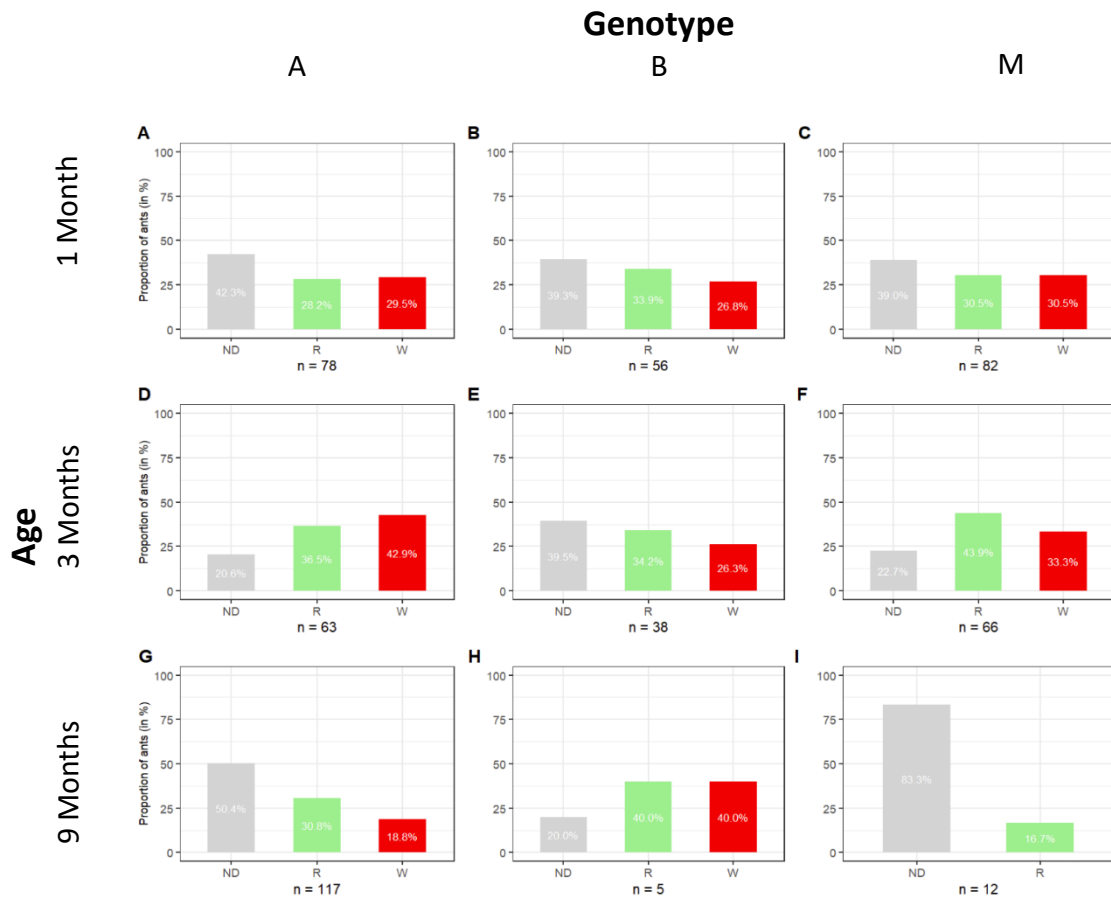
How strong is the influence of using either hexanal or 1-octanol in the memory test to look for (aversive olfactory) learning abilities in the clonal raider ant. The bars show no significant trend for both odors. Especially for 1-octanol, the distribution is almost totally even (49/51 %). For Hexanal, there is a small tendency for making the right decision (57.6 %) and avoiding the CS, but it is not significant (Chi-Square-test, X-squared = 1.6917, df = 2, p-value > 0.1). Therefore, the choice of odor has no significant impact on the learning in the clonal raider ant. Results are from pure-age colonies.

Several changes and improvements to the experimental setup were implemented during the thesis to ensure successful shock application, proper handling of the ants, and unbiased results. The initial version of the setup used was the same as the one employed by Thiagarajan et al. (2022) for *Drosophila*. Apart from the light source, I started with the same generator, arms, T-maze body, shock controller, and tube system in the learning experiment. However, issues arose with the generator's strength, which was too high and had the unintended consequence of nearly incapacitating or killing the ants when delivering shocks. Low humidity also posed a problem, resulting in a lower proportion of successful shock applications. The arms of the setup also presented issues, primarily due to the copper circuit board's coarseness, which was not suitable for the small size of the ants. This coarseness allowed ants to find "safe spots" where they would not receive a shock, and it prevented visual tracking of the ants. To address these problems, I transitioned to using a less powerful generator with a maximum of 48 V (Joy it). This voltage setting was similar to the one used by Peckmezian and

Taylor (2015) but higher than the 7 V used by Wenig et al. (2021). The optimal voltage and current strength were determined by monitoring the ants during the experiment. The ants were considered to have received the appropriate shock when they exhibited minor muscle spasms without being severely affected by the previous shock, either during the breaks or in the second phase of the experiment. The issue of humidity was resolved by incorporating a water bottle into the setup, before the odor bottle. Additionally, the water temperature, initially at around 22°C, was adjusted to 29°C using a water bath (Memmert, WNB7). This adjustment significantly increased the relative humidity from approximately 70% to a stable 90% (+/- 2). Moreover, the arms underwent a complete redesign. Notably, the arm shape changed from circular to cuboid, and a fine copper circuit board was introduced, with contact points situated just 0.2 mm apart from each other, in contrast to the previous setup, where the distance was approximately 1 mm. The transparent acrylic cover allowed for close observation of the ants and immediate response to any issues that might arise during the experiment, such as ant escapes or the presence of water on the contacts leading to a short circuit. Furthermore, changes were implemented in the odor presentation method. Rather than offering the odor in a small vial on filter paper, I decided to use a small, volume of pure odor (60µl), which was more aligned with the methods employed by Thiagarajan et al. (2022). This adjustment was made due to the rapid evaporation of the odor from the filter paper in the new setup, which could not ensure detection by the ant after several hours. The final setup which was then used and how it worked in all tests after the pre-tests is presented in s. fig. 4 which shows a scheme of how the (improved) parts are interconnected.

Another aspect of this thesis involved exploring the potential for incorporating typical appetitive learning. However, given that *O. biroi* primarily feeds on ant brood and not on sugar water, the conventional approach of using sugar water and odor as the conditioned stimuli was not feasible. Although using prey items like flies or *T. bicarinatum* brood was theoretically possible, it would have required a significant amount of time for each individual. The process would involve the food item being brought back to the colony, and then motivating the ant to engage in a second foraging activity, which would take several hours for the six required trials for each individual (Ravary et al., 2007) and therefore not feasible in my thesis. A recent study uncovered several intriguing aspects of the pupal fluid (see section 1.4) in *O. biroi* and other ants (Snir et al., 2022). This discovery presents an opportunity to use pupal fluid as an equivalent to sugar water in experiments involving the clonal raider ant. A recent study

(Chandak et al., 2023) confirms the feasibility of using pupae as a positive reward in learning experiments. The study focused on *Diacamma indicum* and challenged the workers in a Y-maze to associate pupae with either a visual or tactile stimulus. The workers would navigate from the connected nest to the maze to retrieve a pupa located in one of the arms. The experiment involved training ants to associate cues in the form of white dots (visual cue) and variations in floor texture (coarse or fine, tactile cue) with the presence of pupae. The results demonstrated the ability of the workers to learn both tactile and visual cues when motivated by pupae. Here, I collected Pupae shortly before hatching, when they exhibited the highest level of fluid secretion. The pupae were then affixed to a small lid with glue (see Annex; S. Fig. 13) that could be place within the arms of the T-maze. Then the pupae were kept them for 24 hours in a small petri dish with moistened filter paper. Despite making adjustments to various factors such as the contact area with the glue, the size of the area where the ant and pupae were located, and the age of the pupae, there was no evidence of worker ants showing sufficient interest in the fluid or brood. While signs of interaction were visible in approximately 25% of the individuals, this was insufficient for further learning experiments. Additionally, it is worth noting that contacts were primarily associated with attempts to pick up the pupae for transportation rather than displaying any licking behavior towards the pupal fluid. The idea of transporting the pupae, similar to the study by Chandak et al. (2023), would be too time-consuming, given the observations of *O. biroi's* relatively slow pace in exploring and retrieving the pupa to bring it back to the nest. The study by Chandak et al. (2023) did not test the ants in an olfactory context, but the notion of using a tactile cue for learning would be particularly intriguing in *O. biroi* due to their blindness and potential reliance on such stimuli.



**S. Fig. 8: Interplay of genotype and age in the memory test**

Overview of different combinations between genotype and age in the pure age colonies and how they are performing in the memory test. In the youngest individuals (one month age (A, B, C)) the constant high proportion of ants making no decision is very striking with around 40 %. This was the same in genotype B of 3-month-old individuals, but much lower in the other genotypes of this age (around 21 %). Contrary to expectations, the last age cohort together seems to act very reluctant with a high proportion of ND individuals. Variation between the genotypes seems to be very low (additionally see results). A general trend of older individuals being better in learning and memorizing is possible to observe in these graphs as well as in fig. 4 and the statistical results. Apart from that, there are no special outlier-groups.

```

1  /*
2  --> Frequenzgenerator für Ameisen
3  Fußpedal
4  Ventile
5  DC Netzteil
6  */
7
8  // constants won't change. They're used here to set pin numbers:
9  const int schock = 11;
10 const int ventil = 3;
11 const int pedal = 13;
12
13 // variables will change:
14 int buttonState = 0;      // variable for reading the pushbutton status
15
16 void setup() {
17   // initialize the LED pin as an output:
18   pinMode(ventil, OUTPUT);
19   pinMode(schock, OUTPUT);
20   // initialize the pushbutton pin as an input:
21   pinMode(pedal, INPUT);
22 }
23
24 void loop() {
25   // read the state of the pushbutton value:
26   buttonState = digitalRead(pedal);
27
28   // check if the pushbutton is pressed. If it is, the buttonState is HIGH:
29   if (buttonState == HIGH) {
30     digitalWrite(ventil, HIGH);
31     delay (2500);
32     digitalWrite(schock, HIGH);
33     delay (500);
34     digitalWrite(schock, LOW);
35     delay (500);
36     digitalWrite(schock, HIGH);
37     delay (500);
38     digitalWrite(schock, LOW);
39     delay (500);
40     digitalWrite(schock, HIGH);
41     delay (500);
42     digitalWrite(schock, LOW);
43     delay (500);
44     digitalWrite(schock, HIGH);
45     delay (500);
46     digitalWrite(schock, LOW);
47     delay (500);
48     digitalWrite(schock, HIGH);
49     delay (500);
50     digitalWrite(schock, LOW);
51     delay (500);
52     digitalWrite(schock, HIGH);
53     delay (500);
54     digitalWrite(schock, LOW);
55     delay (500);
56     digitalWrite(schock, HIGH);
57     delay (500);
58     digitalWrite(schock, LOW);
59     delay (500);
60     digitalWrite(schock, HIGH);
61     delay (500);
62     digitalWrite(schock, LOW);
63     digitalWrite(ventil, LOW);
64     delay (1000);
65
66   }
67   else {
68     digitalWrite(ventil, LOW);
69     digitalWrite(schock, LOW);
70   }
71 }

```

**S. Fig. 9: Code to control the generator and valves**

Code from Arduino (Vers. 2.04) to use the controller for starting the shock-program after the pedal has been pressed. The code works with the pedal as being the indicator for starting the code. The valves and the shock from the generator are then activated. In detail, after pressing, there is a 2.5-second lasting break before the first shock is implemented (line 31) but the odor is already released. This is due to a possible delay and the unwanted possibility of having the shock first before the odor. Then, there is an alternating break and shock phase of overall 7.5 seconds (lines 32 - 62). After this, the valves and shock are deactivated until the pedal is pressed another time. The code is additionally provided as INO-file for Arduino (version 2.0.4)





**S. Fig. 10: Painted ant workers**

Picture of the painted workers of the clonal raider ant. Here, they are painted on the gaster, but also painting on the thorax is possible. This offers a huge variety of color coding to recognize individuals and in my case, can associate them to their specific age or previous decisions. Previous experiments with these ants in our lab have not noticed any severe impact from the paint on the behavior. Picture taken by Frederic Braun.

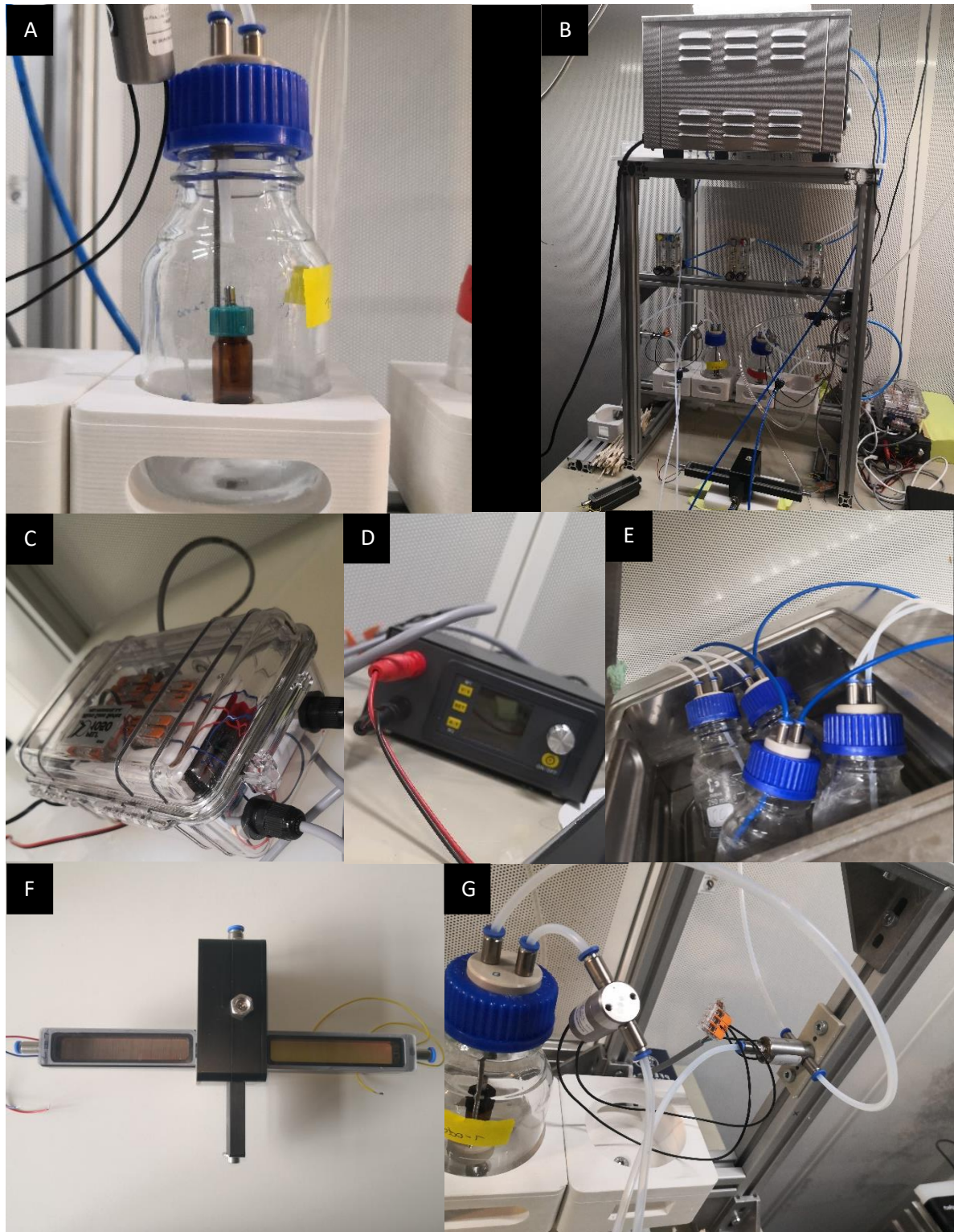
**S. Table 1:**

Table about the experiments and which colonies belonged to them with information about the age, genotype and whether the ants were painted.

Experiment	Colony <sup>1</sup>	Genotype	Age	Painted
general learning experiment/memory test with mixed-age colonies	OIST1.1;OIST1.1.2;OIST1	A	unknown	No
general learning experiment/memory test with pure-age colonies	OIST1.2;OIST1.2.1;STC1.2.1.2.1.1.1; BG14.1.1;BG14.1.1.1	A, B and M	1 month, 3 months and 9 months	Yes, except genotype A and M 1-month-old
Consistency experiment	OIST1.2.1;STC1.2.1.2.1.2.2; BG14.1.1.1	A, B and M	1 month and tested twice at 3 months	yes
Effect by older individuals	OIST1.2; STC1.2.1.2.1.1 (two cohorts)	A and B	1 month and 3 months for STC and 4 months for OIST	yes

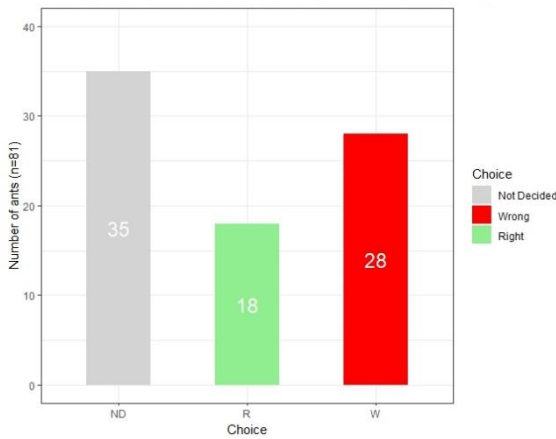
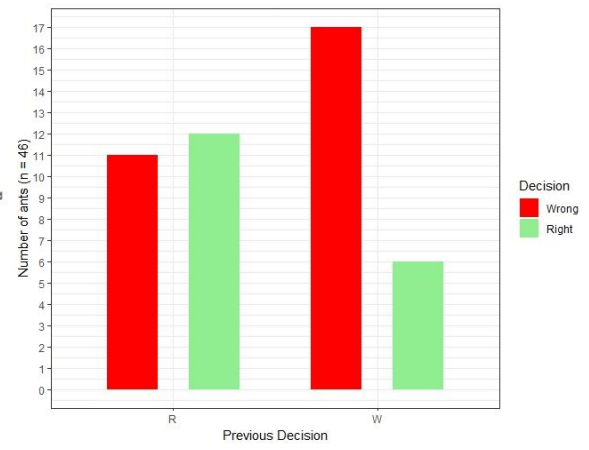
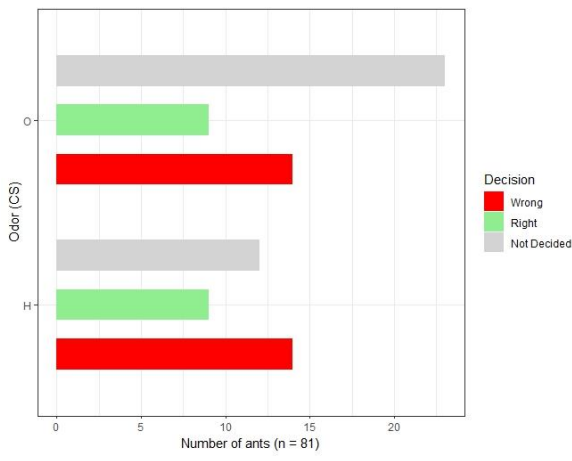
<sup>1</sup>The colony codes are having a reference to a specific genotype. Here the codes belong to following genotypes:

Genotype A: OIST  
 Genotype B: STC  
 Genotype M: BG14



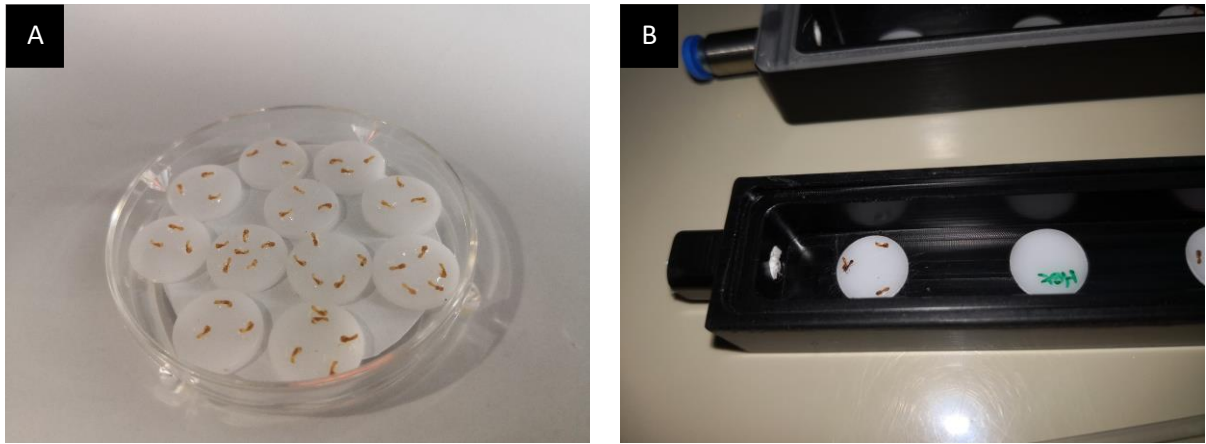
**S. Fig. 11: Pictures of the most important parts of the setup**

Pictures of the electrical part of the setup to ensure a shock and association with an odor. (A): Shows how the odor was implemented in the setup. The pure odor was in a smaller vial with a small opening. Around it was a larger bottle in which the air with the accumulated and then released in the maze. (B): Pictures of the whole setup and the most important parts of the airflow. (C): The controller to time shock and odor. (D): The generator provided the exact amount of strength of electric current and voltage to shock the ants. (E): The water bottles were inside this water bath and provided very important, warm, and humidified air. (F): Shows the T-maze with arms and body in which the ant was tested. (G): Two valves were controlling the input of odor in the airflow. They simultaneously opened and then closed the air channel to the odor-bottles.

**A****B****C**

**S. Fig. 12: Graphs from the second memory test in the consistency experiment**

Additional graphs from the consistency experiment. (A): Outcome of the memory test when the individuals were tested the 2<sup>nd</sup> time. (B): Graph, which shows the distribution of right and wrong decisions, depending on their first decision. Or in other words, how consistent the ants were. (C): Any separation of the decision making depending on the CS which was used, showed no significant differences.



**S. Fig. 13: Pictures of parts of the planned experiment with pupae as CS**

Insight into the planned experiment with pupae as a positive US (reward). The pupae were collected from a colony and then glued onto small circular lids. To prevent them from drying out, I put them in a petri dish on moistened filter paper (A). After 24 hours, I tested the pupae which had accumulated pupa fluid during this period. They were then positioned into the arms of the T-maze, which had no copper underground but notches to put in the lids on which the pupae had been placed (B). However, due to insufficient interest of the workers and a potentially long test period for each individual with other conditions, I could not continue this idea.

## Declaration of Self-Dependence

Herewith I declare that I prepared this thesis on my own, that I did not use any other sources and resources than those that are specified, that all arguments and ideas that were literally or analogously taken from other sources are sufficiently identified, and that the thesis in identical or similar form has not been use as part of an earlier course achievement or examination procedure.

Place/Date: \_\_\_\_\_

Signature: \_\_\_\_\_