Economic decision making in ants

A comparative approach to investigating individual decision making in ants

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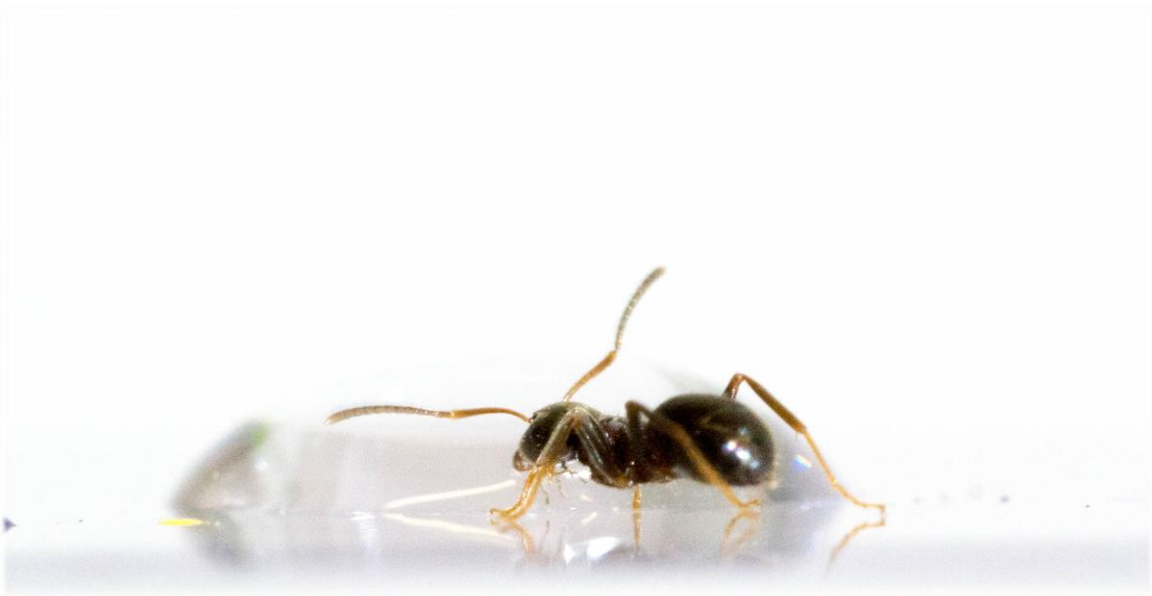aus Kappeln (an der Schlei)

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A single Lasius niger *forager feeding at a sucrose droplet. Once it filled its crop, it returns to the nest. On its way back home, it deposits pheromone to recruit other nestmates. Upon reaching the nest, it shares the food with its nestmates, and then leaves it for another foraging bout.*

Summary

Social insects have the striking ability to collectively choose the most profitable among all available options, even without a central control. This extremely successful system is built on the subjective decisions of many individuals. By following a comparative approach, integrating theories from behavioural economics and consumer psychology, this thesis provides deep insights into individual decision making in ants and how it is affected by factors apparently independent of an option's absolute value.

In chapter 2, we demonstrated that expectations of upcoming reward qualities affect value perception in individual ant foragers and provide, to our knowledge, the first relative value curve for an invertebrate, covering a wide range of reward expectations. Specifically, we show that medium quality food is rated as higher quality by ants which expected to find poor quality food based on previous experience (positive incentive contrast) compared to ants which expected good food and were thus disappointed (negative incentive contrast).

Through association formation, ants can learn to predict reward qualities based on odour cues. By confronting ants with medium food along with odours previously associated to good or poor food, in chapter 3, we show that odour labels can affect perceived value, and thus diminish contrast effects, by adding a value assigned to them in the past.

The presence of other nestmates often leads to compensatory behaviour aimed at preventing crowding on trails and at food sources. Chapter 4 reveals that experienced foragers not only downregulate their recruitment effort to prevent crowding, but also prefer unoccupied over occupied food sources, allowing uninformed recruits to focus on already established food sources.

Ants, as central-place foragers, strongly discount time to maximize their individual food intake rate. We demonstrate in chapter 5 that experienced foragers can forego low quality food close to the nest in favour of good food far-away, showing self-control. If the close food is of similar or identical quality, however, they ignore far-away food, displaying impulsivity.

Finally, despite many factors affecting value perception being described, the genetic and neuronal mechanisms underlying relative value perception are widely unknown. Thus in chapter 6 presents multiple attempts to train *Drosophila* fruit flies to expect different reward qualities

depending on previously associated odours, a prerequisite for revealing these mechanisms. However, flies did not show a preference for one of the associated odour cues.

Taking an interdisciplinary approach, and thus benefiting from the work of behavioural economists and comparative and consumer psychologists, allowed us to gain fresh insights into the behaviour and cognition of individual ant foragers. This work reveals a broad spectrum of factors affecting value perception in ants. These factors in turn allow ants to adapt their foraging decisions to a changing environment and thus maximize colony-level food intake.

Manuscripts arising from this thesis

This thesis is composed of the following manuscripts, of which two are published, one is under review, one submitted and the last one in preparation for publication:

Chapter 2

Wendt S, Strunk KS, Heinze J, Roider A, Czaczkes TJ (2019) Positive and negative incentive contrasts lead to relative value perception in ants. *eLife* 8: e45450. doi: 10.7554/eLife.45450.

Author contributions: Stephanie Wendt (SW) performed the experiments and analysed the data. SW and Tomer J. Czaczkes (TJC) designed and coordinated the study. SW, Kim S. Strunk (KS), Jürgen Heinze (JH), Andreas Roider (AR) and TJC wrote the manuscript and interpreted the data.

Chapter 3

Wendt S, Czaczkes TJ (revision submitted) Labelling effect in insects: cue associations influence perceived food value in ants (*Lasius niger*). *Submitted to the Journal of Comparative Psychology.*

Author contributions: SW performed the experiments, analysed and visualised the data, and designed the study. SW and TJC interpreted the data and wrote the manuscript.

Chapter 4

Wendt S, Kleinhoelting N, Czaczkes TJ (submitted) Negative feedback: Ants choose unoccupied over occupied food sources and lay more pheromone to them. *Submitted to The Journal of The Royal Society Interface.*

Author contributions: Nico Kleinhoelting (NK) collected the data. SW analysed and visualised the data. SW and TJC designed the study, interpreted the data and wrote the manuscript.

Chapter 5

Wendt S, Czaczkes TJ (2017) Individual ant workers show self-control. *Biology Letters* 13 (10): 20170450. doi: 10.1098/rsbl.2017.0450.

Author contributions: SW performed the experiments and analysed and visualised the data. TJC designed and coordinated the study. SW and TJC wrote the manuscript and interpreted the data. SW and TJC revised the manuscript.

Chapter 6

Wendt S, Seeholzer L, Czaczkes TJ (in preparation) Odour conditioning in *Drosophila melanogaster*.

Author contributions: Lea Seeholzer (LS) and SW performed the experiments. SW analysed and visualised the data. TJC and SW designed and coordinated the study. SW and TJC wrote the manuscript and interpreted the data.

I also contributed to the following manuscripts which are not included in this thesis:

Oberhauser FB, Schlemm A, **Wendt S**, Czaczkes TJ (2019) Private information conflict: *Lasius niger* ants prefer olfactory cues to route memory. *Animal Cognition*. doi: 10.1007/s10071-019-01248-3.

Oberhauser FB, **Wendt S**, Czaczkes TJ (in preparation) Social information in the form of pheromone trails does not distort perceived value in ants.

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Chapter 1
General Introduction

1.1   Overview

Insects represent about half of all described animal species, but only 2% are social (Hölldobler and Wilson 2011). However, social insects can represent up to 75% of a habitat's animal biomass, with ants being a major part of that. Ants alone constitute up to 30% of the terrestrial animal biomass (Wilson 1990). More than 13,000 ant species have been classified (Bolton 2012). They can be found in nearly all terrestrial ecological niches (Hölldobler and Wilson 1990) and, apart from their extraordinary social organization (Wilson and Hölldobler 2005), have developed numerous strategies which have ensured their survival for around 130 million years (Agosti et al. 1998).

Their extraordinary ecological success is due at least partly to their collective approach towards tackling the challenge of a constantly changing environment and their ability to solve complex tasks without a central control (Detrain et al. 1999). Working as a collective allows for division of labor, in which individuals can specialize on distinct tasks. Ant colonies are characterized by a differentiation between reproductive and non-reproductive individuals. The non-reproductives – the workers – are mostly sterile, and are responsible for all non-reproductive tasks (Hölldobler and Wilson 1990; Wilson and Hölldobler 2005) such as brood care, food collection and nest defense. These tasks are again handled collectively by groups of often specialized individuals (Hölldobler and Wilson 2011). While collective decisions are less susceptible to individual errors and incomplete information about available options (Feinerman

and Korman 2017), they still strongly depend on the subjective decisions of hundreds to millions of single individuals. A colony's success thus mainly relies on the efficiency of individuals' choices and their collective performance (Feinerman and Korman 2017).

There are several possible strategies for decision making. Random choice likely represents the least information and processing intensive decision making system. Alternatively, various heuristics ("rules of thumb") can be used, like choosing the option which was chosen in the last decision (Angner 2012), or copying choices of conspecifics (Caldwell et al. 2012; Toelch et al. 2010; Worden and Papaj 2005). A very common, and very powerful, system for decision making is comparing the value of available options, and choosing the option with the greatest value (von Neumann and Morgenstern 1944). In this system, the way in which value is perceived has huge repercussions on the outcome of a decision. It furthermore requires information about, ideally, all available options.

Over the course of this thesis, various types of both social and private information which individual ant foragers acquire and how they can influence decision making by affecting the perceived value of food options are investigated. Moreover, it is discussed how distorted value perception can affect collective decision making. As previous research on decision making has had a strong focus on foraging, probably due to the ecological importance of these decisions, and since foraging behaviour is well investigated and can be easily studied in ants (Detrain and Deneubourg 2008; 2009; Leadbeater and Chittka 2009), this thesis focuses on gaining deeper insights into decision making in a foraging context.

1.2   Foraging and signaling in ants

Because ants act as a collective, all individuals of a colony benefit from communicating information, increasing the inclusive fitness of individuals which feed information into the system as well. Dishonesty in communication, at least in the context of foraging, is thus believed to be minimal in social insects (Heinze and d'Ettorre 2009). While honeybees have developed the waggle-dance for communicating the quality and location of available food sources (von Frisch 1965), many ant species, as well as termites and some stingless bees, use pheromone trails to recruit other nestmates (Czaczkes et al. 2015c; Jackson and Ratnieks 2006; Schmidt et al. 2006; Traniello and Leuthold 2000). Both systems allow colonies to collectively exploit the best available food sources (Beckers et al. 1990; de Bisaeu et al. 1991; Detrain and Deneubourg 2008; Seeley et al. 1991). At its core, collective decision making via pheromone trails strongly relies on

the quality assessments of single individuals and is thus likely to be affected by factors distorting the perceived value of options.

Upon finding a food source of sufficient quality and quantity, foragers deposit pheromone on their way back to the nest (Beckers et al. 1993; Mailleux et al. 2000; 2003). Individuals can adapt the strength of a pheromone trail by a) either depositing pheromone or not and b) increasing or decreasing the intensity of pheromone depositions (Beckers et al. 1993). To apply pheromone to the ground, Formicine ants, such as the main study species of this thesis, *Lasius niger*, stop walking for a fraction of a second and bend the tip of their gaster to the ground, from which a droplet of pheromone is secreted from a gland and applied to the substrate (Beckers et al. 1992a; Hölldobler and Wilson 1990). Ants are more likely to follow stronger trails (Hangartner 1969; Sumpter and Beekman 2003) and deposit stronger trails when returning from higher quality food (Beckers et al. 1993; Hangartner 1970). This stereotypic behaviour can be easily quantified and, along with the assumption of signals being honest in social insects, provides a powerful mechanism for investigating how individuals evaluate food. If the food source found at the end of a pheromone trail is of sufficient quality and quantity (Detrain and Deneubourg 2008; Mailleux et al. 2000), recruited nestmates also deposit pheromone when returning to the nest (Beckers et al. 1992a). This leads to an accumulation of pheromone depositions via a positive feedback loop (Sumpter and Beekman 2003), and the development of a collective pheromone trail whose strength reflects the quality of the food found at its end (Czaczkes et al. 2015c). Recruiting too many nestmates to a food source may lead to queuing at the food source and slower travel speed due to crowded trails (Burd 1996; 2000; Burd and Aranwela 2003), ultimately leading to a decreased colony-level food intake, or reduced efficiency. Thus, upon walking on a crowded trail, returning foragers tune down their pheromone depositions (Grüter et al. 2012; Czaczkes 2014; Czaczkes et al. 2013b), leading to a passive evaporation of accumulated pheromone on the trail. Such negative feedback systems maintain flexibility in otherwise rigid collective choice mechanisms (Grüter et al. 2012; Czaczkes 2014; Czaczkes et al. 2013b). However, even though crowded food sources are strongly discounted through negative feedback systems, unexperienced recruits of *Lasius niger* appear to preferentially move on crowded trails (Czaczkes et al. 2015b), presumably increasing the potential crowding at an already overexploited food source. Experienced individuals can, however, integrate both private information, such as memories, and social information like pheromone trails (Grüter et al. 2011; Middleton et al. 2018; Oberhauser et al. 2019) into their decisions, allowing them to make more flexible choices. Individual ant workers can distinguish specific reward qualities (Josens et al. 1998), associate them to different odours in a relatively

short amount of time (Dupuy et al. 2006; Josens et al. 2009; Oberhauser et al. 2019) and integrate this information into later foraging decisions (Beckers et al. 1994; Josens et al. 2016; Oberhauser and Czaczkes 2018; Provecho and Josens 2009; Roces 1990; 1994; Saverschek and Roces 2011). Individuals can also learn the location of food sources using a combination of different strategies such as patch integration and visual landmarks (Collett et al. 2013; Salo and Rosengren 2001). Given that experienced foragers have a larger pool of private information which they can incorporate into decision making in addition to social information, it can be assumed that they are less likely to mistakenly follow outdated or suboptimal pheromone trails. Indeed, while naïve bumblebees prefer to feed at food patches at which other nestmates are present, experienced bees rather avoid them (Kawaguchi et al. 2007), likely due to associative learning processes the experienced workers have undergone (Leadbeater and Chittka 2011). The question whether experienced ant foragers also prefer unoccupied over occupied food sources is addressed in chapter 4.

Unexperienced recruits may also be in danger of exploiting low quality food sources which are located on the trail leading to a better food source. As time costs are a major factor in central-place foraging, many animals strongly discount time in order to maximize their food collection rate (Hayden 2016; 2019), and ant workers are less likely to recruit to far-away food sources (Devigne and Detrain 2006; Fewell et al. 1992). The ability to resisting the impulse of choosing an immediate reward in favour of a better one later is defined as self-control (Logue 1988). A lack of self-control, also called impulsivity, is said to be a central factor in many human problems, such as failures at school, depression and criminal tendencies (Moffitt et al. 2011). However, in animals which strongly discount time, impulsivity may yield greater overall profits in some situations (Hayden 2019). In chapter 5, experienced foragers which are informed about all available food sources (one far-away and one closer to the nest) are tested for their ability to show self-control. Unexperienced recruits, due to their limited access of private information, may end up trapped in exploiting a close, but low quality feeder located on the trail to a much better food source far-away.

To solve this problem, ant colonies have developed further behavioural patterns allowing naïve ants to receive information about available food sources before leaving the nest. In addition to depositing pheromone trails to guide unexperienced workers to valuable food sources, returning foragers unload collected food inside the nest by sharing it with other nestmates via trophallaxis, a direct mouth-to-mouth food transfer (Hölldobler and Wilson 1990). These food transfers provide samples of food sources available outside the nest, which may contain valuable

information about available sucrose concentrations or odours of resources. Ants can demonstrably receive odour information through trophallaxis (Josens et al. 2016; Provecho and Josens 2009), and can distinguish and memorize familiar odours (Dupuy et al. 2006; Josens et al. 2009). Odour information acquired through trophallaxis can also be used on future foraging trips, and drives choices towards familiar odours (Josens et al. 2016; Provecho and Josens 2009). The question of whether sucrose concentration information can also be shared with nestmates, and whether this information can be integrated into future foraging decisions, is addressed in chapter 2. As experienced foragers rely on private information more strongly, especially when it is conflicted with social information such as pheromone trails (Grüter et al. 2011), social transfer of additional information may contribute to maintaining flexibility in an already efficient collective decision making system (Letendre and Moses 2013).

1.3   Value perception and how it can be distorted

The study of decision making is not limited to animal behaviour. Indeed, the study of human decision making has likely been studied for much longer, and comparative approaches may offer deeper insights into both animal and human behaviour. In humans too, the first step towards making a rational decision is often a comparison of available options, often followed by a choice for the option yielding the greatest profit (von Neumann and Morgenstern 1944). The value of an option and how value is perceived thus strongly influences which option is ultimately chosen.

Kahneman and Tversky (1979) suggested with their Prospect Theory that an option's value is not always perceived based on its absolute value, but relative to a reference point, such as the *status quo* or former experience (Kahneman and Tversky 1979; Parducci 1984; Tversky and Kahneman 1992; Ungemach et al. 2011; Vlaev et al. 2011). A high reference point can thus lead to an option being perceived as more negative, and a low reference point can make the same option more positive (Kahneman and Tversky 1979). Following this theory, for example, subjects expecting to receive €100, but ultimately getting only €50 may perceive the reward as less rewarding compared to subjects expecting €1 and ultimately receiving €50.

Humans are not unique in having a relative perception of value. In animals, value perception relative to expectations has been studied for decades using the successive contrasts paradigm. In this experimental procedure, animals are successively trained to one quality or quantity of reward which is then suddenly either increased (positive incentive contrast) or decreased (negative incentive contrast) (Flaherty 1999). Animals react to successive negative contrasts by

disrupting their behaviour compared to control animals which had not experienced a change in reward (Bower 1961; Tinklepaugh 1928; Vogel et al. 1968). For example, in a classic study, Crespi (1942) showed monkeys a piece of banana and placed it under a cup, eliciting expectations of a high quality reward. Afterwards, hidden from the monkey's view, the piece of banana was replaced with a piece of lettuce, producing a successive negative contrast. Due to their high expectations of receiving a piece of banana, the monkey quickly moved towards the cup, but after raising it and finding a piece of lettuce beneath it, dropped its hand and did not touch it, displaying a negative incentive contrast effect. There are good theoretical reasons for expecting both positive and negative contrast effects to evolve (McNamara et al. 2013). According to McNamara et al.'s (2013) theory based on an optimality model, contrast effects could evolve as an adaptive response to environmental instability and unpredictability. Individuals living in environments usually offering good food benefit from showing negative incentive contrasts towards the worse option, because they are very likely to find good food again in the near future. In contrast, individuals used to low quality options strongly benefit from showing positive incentive contrasts towards a better option appearing, and should focus on exploiting it as long as it's available, because chances are high that following options will again yield low quality (McNamara et al. 2013). However, while negative successive contrasts have been demonstrated in a broad array of animals, positive successive contrasts have often proved elusive (Black 1968; Bower 1961; Capaldi and Lynch 1967; Dunham 1968; Papini et al. 2001).

For an animal to show successive contrasts, it must be able to compare a remembered option with the one currently on offer. This might be broadly expected for vertebrates, but insects, specifically honeybees, have also been shown to be able to do this (Bitterman 1976; Couvillon and Bitterman 1984; Richter and Waddington 1993). For example, honeybees rejected otherwise acceptable lower quality food when they expected high quality food due to previous experience (Bitterman 1976; Couvillon and Bitterman 1984). However, a detailed description of value perception relative to a wide range of food qualities – a relative value perception curve – has so far not been demonstrated. Furthermore, even though ants can learn the location of, and quality cues assigned to food sources very rapidly (Oberhauser et al. 2019), and are a common study object for both collective and individual decision making (Detrain and Deneubourg 2008), the effect of expectations on their perceived value of food sources has not yet been described. Thus in chapter 2, relative value perception for a wide range of sucrose concentrations in individual ant foragers is investigated and a detailed relative value curve described. Because successive contrast designs suffer from some physiological and conceptual limitations (Bitterman 1976), we

further controlled for these effects and ultimately demonstrate that the revealed contrast effects are based on a cognitive process rather than being of purely physiological origin. These results are also presented in chapter 2.

Even though relative value perception has been investigated for decades in humans and animals, the underlying genetic and neuronal mechanisms are still widely unknown. *Drosophila melanogaster,* with its well investigated genetic background and short generation time (Hadler 1964) along with its rich behavioural repertoire suggesting that it can use memories to make decisions (Kahsai and Zars 2011), may allow us to gain deeper insights into these mechanisms. Unfortunately, expectation driven value perception has not yet been shown in *Drosophila* fruit flies. Chapter 6 thus presents multiple attempts to condition *Drosophila* fruit flies to associate different odours to high and low quality food, as a precursor to demonstrating cognition-based relative value perception. Unfortunately, after training flies did not show a preference for one of the offered odours, regardless of the experimental approach. The approaches presented in this thesis are thus not sufficient for investigating cognition-based relative value perception in *Drosophila melanogaster.*

A closer look at the field of human behavioural economics reveals another way in which expectations can be formed and used as a reference point: Humans can attend to factors they believe (consciously or unconsciously) to be associated with predicting value, such as brand labels (French and Smith 2013). Naturally value-neutral brand labels can be associated to socially-driven quality statements (French and Smith 2013; Macklin 1996). A brand label is usually linked to an accumulation of associations, each affecting perceived value either positively (Fornerino and d'Hauteville 2010; McClure et al. 2004; Woodside and Taylor 1978) or negatively (Breneiser and Allen 2011; Fornerino and d'Hauteville 2010). Given that the discrepancy between a label-driven expectation and an option's objective value is small, subjects are likely to align the perceived value to the expectation specified by the value assigned to the label in a mechanism called assimilation (Cardello and Sawyer 1992). For example, drinks presented along with strong brands such as "Coca Cola" (which has strong positive associations due to successful marketing campaigns) tend to be rated as being tastier or more attractive compared to identical drinks which were presented with weaker brand labels or without any labels, even though there is rarely a preference found in blind tests (Breneiser and Allen 2011; Fornerino and d'Hauteville 2010; Kühn and Gallinat 2013; McClure et al. 2004; Yamada et al. 2014). In the case of a large discrepancy between the label and an option's value, however, contrast effects, as described above and shown in chapter 2, are likely to occur. In ants, learned odour cues can significantly affect future food

choices, with ants showing a strong preference for familiar odours (Oberhauser and Czaczkes 2018; Provecho and Josens 2009), even when they are presented in deterrent food (Josens et al. 2016), suggesting that ants form an expectation based on learned odour-labels as well. In chapter 3, it is investigated whether a) individual ant workers, following the theory of Rescorla and Wagner (Rescorla and Wagner 1972), can assign different values to odour cues presented with different food qualities, and b) whether odour-labels present during food consumption can diminish contrast effects by decreasing the discrepancy between a label-driven expectation and an option's objective value, ultimately leading to assimilation in ants.

1.4   Learning in social insects

As central-place foragers, social insect foragers steadily commute between food sources (to gather food) and the nest (to unload collected food and recruit other nestmates). Studies on navigation and route learning in social insects have revealed various mechanisms allowing individuals to accurately localize nest or food sites (Collett et al. 2013; Knaden and Graham 2016). As learning and memory play important roles in many of these mechanisms, umpteen studies investigating these abilities exist in social insects (Collett and Collett 2002; Josens et al. 2009; Giurfa 2007; Giurfa and Sandoz 2012; Menzel 1990; Narendra et al. 2007; Salo and Rosengren 2001).

The impressive learning abilities of social insects make them an ideal model for studies requiring previous conditioning. Through associative learning, naturally value-neutral odours are assigned a value dependent on the reward quality (Rescorla and Wagner 1972), presumably leading to rewards presented with familiar odours being preferred over novel odours regardless of the option's objective quality (Josens et al. 2016; Oberhauser and Czaczkes 2018). As social insects return to food sources multiple times, they can easily be trained to different food sources, allowing scientists to ask specific questions incorporating individuals' memory and expectations.

1.5   *Lasius niger* as a model organism for comparative cognition

While there are many studies following comparative approaches for investigating psychology and behavioural economics, these arerarely done on insects, despite their easy maintenance and availability of flexible experimental designs. However, while experiments in humans are often bound to pitfalls, such as cultural and educational differences (Carter and Irons 1991; Guiso et al. 2006), second-guessing of experimenters, and non-relevant reward sizes (Levitt and List 2007), signaling in ants is thought to be honest(Heinze and d'Ettorre 2009) which can –

assuming a well designed and balanced experiment – be investigated in an ecologically meaningful and mostly unbiased manner. Furthermore, reward sizes offered are biologically meaningful in experiments with ants, unlike the rewards used in most psychological or economic studies in humans. This in addition to their behavioural flexibility and integration of both social and private information into decision making make ants an ideal model for investigating economic decision making and cognitive processes.

All studies presented in this thesis, apart from the last one, were conducted on the common black garden ant *Lasius niger* (Hymenoptera: Formicinae) (Linnaeus, 1758), which is a widespread European pioneer species (Seifert 2007). It is a flexible commensal species often found in cities as well as in parks, gardens and grasslands (Seifert 2007), which makes them easily acquirable both in nature and for keeping them in the lab. Colonies form robust trails to extrafloral nectaries or aphid colonies, which serve as a main food source of this species (Detrain et al. 2017; Völkl and Mackauer 1993). Individual workers can incorporate expectations of upcoming reward qualities (chapter 2) and other private information such as learned odours (chapter 3) into future foraging decisions. Moreover, individuals can use social information such as nestmate presence (chapter 4) or other information acquired socially like food samples shared through trophallaxis (chapter 5) to guide their decisions.

1.6   Aims of this thesis

The aim of this thesis was two-fold. The first aim was to gain deeper insights into individual decision making and how it is affected by reward expectations and further shaped by socially acquirable information. The second aim was to pioneer *Lasius niger* as a model economic agent by following a comparative approach towards investigating economic decision making. Using ants as models in pilot studies would allow behavioural economists to further pin down factors affecting value perception independent of an option's absolute value while simultaneously avoiding common pitfalls associated with experimental studies in humans.

Ants, like humans, live in huge societies and can solve complex coordination tasks without central control (Detrain and Deneubourg 2008; Hölldobler and Wilson 1990; 2011). No matter how complex or flexible a collective choice is, it is always composed of an accumulation of many individual choices, making a deep understanding of strategies used by individuals to choose between options extraordinary important for understanding collective choices. Individuals often make decisions based on a comparison of available options. However, identical options are not

always perceived alike, but can be perceived relative to sometimes arbitrary reference points (Flaherty 1999; Kahneman and Tversky 1979; Tversky and Kahneman 1992; Vlaev et al. 2011).

This thesis addresses the question whether an option's perceived value can change relative to reference points such as expectations of upcoming reward qualities (chapter 2) and associated odour labels (chapter 3) in individual ant workers, and provide evidence that this is due to cognitive processes rather than simple physiological mechanisms. Additionally, multiple approaches for odour conditioning in *Drosophila melanogaster* were deployed in order to investigate whether flies can associate different food qualities to odours in a similar way than ants do (chapter 6). A successful association formation is a prerequisite for investigating relative value perception and its underlying genetic and neuronal mechanisms in this species.

Social cues such as nestmate presence can drastically affect food choices in naïve bumblebees (Kawaguchi et al. 2007; Worden and Papaj 2005) and path choices in unexperienced recruited ant workers (Czaczkes et al. 2015b). Chapter 4 thus investigates to what extent the presence of other nestmates drives choices in experienced foraging ants.

Furthermore, odour information of available food sources can also be shared socially inside the nest, and significantly influences future foraging decisions outside the nest. Chapter 2 thus addresses the question whether the nest can serve as an information hub, where information about available food qualities can be collected, synthesized, and fed back to outgoing foragers, and how this information affects value perception outside the nest.

Finally, as central-place foragers, individual foragers presumably strictly discount time during food collecting. This raised the question of whether experienced foragers can show self-control by foregoing a close low quality food source in favour of a better one far away. This question is answered in chapter 5). It is also investigated whether this behaviour is plastic, and dependent on the relative value of the options.

Chapter 2

Positive and negative incentive contrasts lead to relative value perception in ants

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2.1   Abstract

Humans usually assess things not according to their absolute value, but relative to reference points – a main tenant of Prospect Theory. For example, people rate a new salary relative to previous salaries and salaries of their peers, rather than absolute income. We demonstrate a similar effect in an insect: ants expecting to find low quality food showed higher acceptance of medium quality food than ants expecting medium quality, and vice versa for high expectations. Further experiments demonstrate that these contrast effects arise from cognitive rather than mere sensory or pre-cognitive perceptual causes. Social information gained inside the nest can also serve as a reference point: the quality of food received from other ants affected the perceived value of food found later. Value judgement is a key element in decision making, and thus relative value perception strongly influences which option is chosen and ultimately how all animals make decisions.

2.2   eLife Digest

What did you expect?

We make many decisions every day, often by comparing options and choosing the one with the greatest profit. But how much we value something often does not depend solely on our needs. Instead, this value may depend on our expectations or other arbitrary reference points. For example, how satisfied you are with your income might depend on how much your colleagues or friends earn.

Animals, including insects, also make decisions when feeding, choosing a partner, or finding a nesting site. Sometimes animals behave in ways that look like disappointment. For example, monkeys may reject a cucumber as a reward if they have seen another monkey get a grape for completing the same task. But it is hard to tell if this behavior reflects a value judgment.

To investigate whether insects evaluate their options against their expectations, Wendt et al. offered black garden ants sugar water over multiple trials. Some ants grew to expect low quality sugar water (containing little sugar); some expected medium quality; and others expected high quality sugar water (containing a high concentration of sugar). Ants that expected to find low quality sugar water were more likely to accept medium quality options than ants that expected the medium quality sugar water. Similarly, ants that expected high quality sugar water were less likely to accept lower quality sugar water. Further experiments confirmed that the ants were not using physical cues such as satiation to guide their behavior.

Furthermore, Wendt et al. found that ants that returned to the nest after foraging passed on information that altered the expectations of the next group of foragers about nearby food. This suggests that the value that ants place on food sources depends both on individual experiences and on information gained from others.

Studies of decision making in humans can be difficult to perform and interpret, because volunteers may try to second-guess what the experimenters want to find, and culture and education may also influence choices. Studying ants instead could help to avoid these pitfalls, as the results presented by Wendt et al. suggest they make decisions in similar ways to humans. Future work building on these findings could also help researchers to predict how insects behave, particularly in rapidly changing environments.

2.3   Introduction

We all compare options when making both large and small decisions, ranging from career choices to the choices of an evening's entertainment. Understanding how options are compared has thus been central to the study of behavioural economics. Theories explaining the mechanisms by which options are compared and decisions are made have a long tradition (Vlaev et al. 2011), with Expected Utility Theory (EUT) being the most widely used theory in economic models (Mankiw 2011; von Neumann and Morgenstern 1944). EUT suggests that decisions are made by evaluating and comparing the expected utility from each option. A rational decision maker then chooses the option resulting in the best end state: the option providing the greatest utility (von Neumann and Morgenstern 1944).

However, over the past decades economic research on human decision making has started to shift away from a view of (absolute) utility maximization towards more nuanced notions of relative utility, such as reference-dependent evaluations. Kahneman and Tversky (Kahneman and Tversky 1979) made a major contribution to this shift by introducing Prospect Theory, suggesting that decision making is not based on absolute outcomes, but rather on relative perceptions of gains and losses. In contrast to EUT, the utility attributed to options being evaluated is determined relative to a reference point, such as the *status quo* or former experience (Kahneman and Tversky 1979; Parducci 1984; Tversky and Kahneman 1992; Ungemach et al. 2011; Vlaev et al. 2011). Various examples of relative value perception have been described. For example, satisfaction gained from income is perceived not absolutely, but relative to the income of others in the social reference group – such as one's colleagues (Boyce et al. 2010). Overall, Prospect Theory has enriched our understanding of human decision making by conceptualizing it as more nuanced and less rational than previously assumed (Tversky and Kahneman 1974; 1981).

The concept of malleable value perception is not just relevant to humans. Value judgments in animals are also influenced by factors apparently independent of the absolute value of options. For example, capuchin monkeys refuse otherwise acceptable pay (cucumber) in exchanges with a human experimenter if they had witnessed a conspecific obtain a more attractive reward (grape) for equal effort (Brosnan and de Waal 2003). Rats, starlings, and ants, like humans, place greater value on things they work harder for (Aw et al. 2011; Czaczkes et al. 2018a; Lydall et al. 2010), and starlings, fish and locusts demonstrate state-dependent learning, wherein they show a preference for options experienced when they were in a poor condition (Aw et al. 2009; Pompilio et al. 2006; Schuck-Paim et al. 2004). Roces and Núñez (1993; 1993) aimed to show that in leaf

cutting ants perceived value can be influenced by other ants. Ants recruited to higher quality food sources ran faster, deposited more pheromone, but cut smaller leaf fragments, even if the food source the recruits find is replaced by a standardised food source (Roces 1993; Roces and Núñez 1993). However, in these experiments the absolute value and nature of the reference remains unclear, and indeed pheromone presence may have caused the observed behaviours without influencing the ants' expectations or value perception at all.

Healey and Pratt (Healey and Pratt 2008) showed that colonies of the house-hunting ant species *Temnothorax curvispinosus* move into a nest of mediocre quality faster when they were previously housed in a high-quality nest compared to colonies which were previously housed in a poor-quality nest (Healey and Pratt 2008). In contrast, Stroeymeyt et al (Stroeymeyt et al. 2011) showed that colonies of *Temnothorax albipennis* developed an aversion towards mediocre-quality nests available in their environment when they were housed in a high-quality nest, whereas colonies housed in a low-quality nest did not, and thus show an experience-dependent flexibility in nest choice (Stroeymeyt et al. 2011). However, critically missing from the existing works is a systematic description of value judgment relative to a reference point.

'Value distortion by comparison' effects have been studied for decades using the successive contrasts paradigm, in which animals are trained to a quality or quantity of reward which is then suddenly increased (positive incentive contrast) or decreased (negative incentive contrast) (Bentosela et al. 2009; Bitterman 1976; Couvillon and Bitterman 1984; Crespi 1942; Flaherty 1982; 1999; Mustaca et al. 2000; Weinstein 1970b). Many mammals, including apes, monkeys, rats, and dogs (Bentosela et al. 2009; Brosnan and de Waal 2003; Crespi 1942; Flaherty 1999; Mustaca et al. 2000; Pellegrini and Mustaca 2000; Weinstein 1970a) have been shown to respond to successive negative contrast by disrupting their behaviour compared to control animals which had not experienced a change in reward. The animals display behaviour akin to disappointment – slower running speeds to a reward (Bower 1961), depressed licking behaviour (Flaherty et al. 1985; Vogel et al. 1968), or reward rejection (Tinklepaugh 1928).

Contrast effects were also successfully described in invertebrates (Bitterman 1976; Couvillon and Bitterman 1984; Richter and Waddington 1993). Bitterman (Bitterman 1976) found negative incentive contrast effects in honeybees which were trained to a high quality feeder and then received a downshift to a lower quality feeder. In contrast, bees which experienced an upshift in feeder quality did not show any feeding interruptions (Bitterman 1976; Couvillon and Bitterman 1984). While negative successive contrast effects – akin to disappointment – have been well

described in animals, positive successive contrast effects – akin to elation – have often proved elusive (Black 1968; Capaldi and Lynch 1967; Bower 1961; Dunham 1968; Papini et al. 2001). There are several factors which may prevent positive contrast effects from being detected. Firstly, ceiling effects may occur when the performance of animals receiving a large reward is at or near a physical limit. The absence of positive contrast effects may then not be due to the absence of perceived positive contrast, but rather due to an artefact of experimental design (Bower 1961; Campbell et al. 1970). Secondly, neophobia counteracts positive contrast effects: animals may be reluctant to eat a novel food – even if the food is of higher quality than normal (Flaherty 1999; Oberhauser and Czaczkes 2018). Finally, generalisation decrement may prevent stronger responses to positive contrast. Generalisation decrement occurs when animals are trained under one set of stimuli and then tested under another. The strength of the tested response may decrease with increasing differences between the training and testing stimuli (Kimble 1961), which may then result in weaker positive contrast effects following a reward shift. Thus, the reward change itself may lead to a decrease in responding just as would any other change in context, such as a change in the brightness of the runway or scent of the food (Oberhauser and Czaczkes 2018; Capaldi 1978; Premack and Hillix 1962).

Even though positive contrast effects proved to be hard to demonstrate in laboratory experiments, there are good theoretical reasons for expecting both positive and negative contrast effects to evolve (McNamara et al. 2013). Incentive contrasts have also been demonstrated for rewards other than food. Females become more (or less) likely to accept a mate of given quality if they have prior experience of better (or worse) mates. Such mate quality contrast effects are reported in both vertebrates (Collins 1995) and invertebrates (Dukas 2005; Reid and Stamps 1997; Wagner et al. 2001).

In this study, we investigate positive and negative contrast effects using the successive contrasts paradigm, and, in addition to demonstrating positive and negative contrast effects, define the first relative value curve in an invertebrate; the ant *Lasius niger*. We conduct a critical control experiment to rule out physiological or psychophysical effects which may lead to the same pattern (see experiment 2) and thus provide strong evidence for a purely cognitive relative value effect in a non-human animal. Furthermore, we demonstrate that information flowing into the nest can influence value perception in outgoing foragers. This suggests that food sources are not only valued based on individual experiences, but also based on social information gained inside the nest. The perceived value of a food source influences social information dissemination, by affecting the strength of pheromone trails which then lead further ants to the food source.

Thus, the way in which value is judged is likely to strongly affect the foraging mechanics of a whole colony.

2.4   Methods

2.4.1   Study animals

Eight stock colonies of the black garden ant *Lasius niger* were collected on the University of Regensburg campus. The colonies were kept in 30 x 30 x 10cm foraging boxes with a layer of plaster covering the bottom. Each box contained a circular plaster nest box (14 cm diameter, 2 cm height). The colonies were queenless with around 1000-2000 workers and small amounts of brood. Queenless colonies still forage and lay pheromone trails, and are frequently used in foraging experiments (Devigne and Detrain 2002; Dussutour et al. 2004). The colonies were fed with *ad libitum* 0.5M sucrose solution and received *Drosophila* fruit flies once a week. Water was available *ad libitum.*

One sub-colony of 500 individuals was formed from each stock colony, and these eight fixed-size sub-colonies were used for our experiments. Sub-colonies were maintained identically to the stock colonies, but did not receive any *Drosophila* fruit flies to prevent brood production, and were starved four days prior to the experiments in order to achieve a uniform and high motivation for foraging (Mailleux et al. 2006; Josens and Roces 2000). During starvation, water was available *ad libitum.* Any ants which died or were removed from the sub-colonies were replaced with ants from the original stock colonies.

2.4.2   General setup, ant selection, and monitoring

The general setup used for all of our three experiments was identical and consisted of a 20 x 1 cm long paper-covered runway which was connected to the sub-colony's nest box via a 40 cm long drawbridge (figure 2-1A). A 5mm diameter drop of sucrose solution (Sigma-Aldrich) was placed on an acetate feeder at the end of the runway (60cm from the nest). The molarity of the sucrose droplet depended on the experiment, treatment and on the ants' number of visit to the food source.

To begin an experiment, 2-4 ants were allowed onto the runway, and the first ant to reach the feeder was marked with a dot of acrylic paint on its gaster. This procedure may select for the

more active foragers, but does not introduce any selection bias between treatments. The marked ant was allowed to drink to repletion at the food source, while all other ants were returned to the nest.
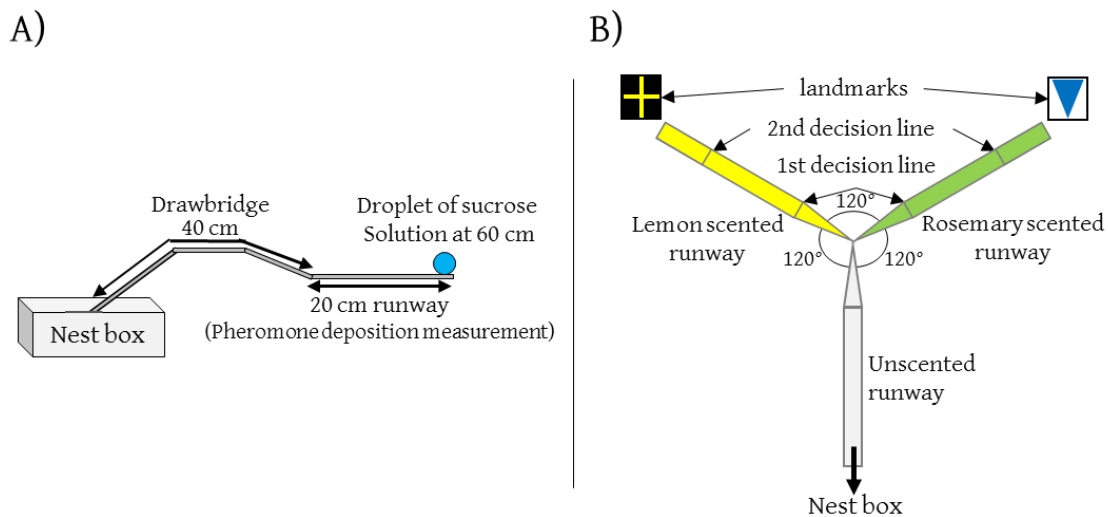
A)

B)

Figure 2-1: A) General setup used for all presented experiments. The 20 cm long runway is connected to the nest box via a 40 cm long drawbridge. The droplet of sucrose solution is placed at the end of the runway (60 cm distance to the nest). B) Y-maze used on the 10th visit of experiment 2. All arms were 10 cm long. The arm connected to the nest box was covered with unscented paper overlays while the other two arms were covered with lemon and rosemary scented paper overlays (one scent on each side). Visual cues (landmarks) were placed directly behind the two scented arms. The first decision line was located 2 cm from the Y-maze centre and marked the initial decision of an ant while the second decision line was located 8 cm from the centre and marked the final decision.

Food acceptance scores as a measure of perceived value were noted for each ant and visit as follows: Full acceptance (1) was scored when the ant remained in contact with the drop from the moment of contact and did not interrupt drinking within 3 seconds of initial contact (see ESM: video 2-1). Partial acceptance (0.5) was scored if feeding was interrupted within 3 seconds after the first contact with the food source, but the ant still filled its crop within 10 minutes (as can be seen by the distention of the abdominal tergites). Ants which interrupt feeding within the first seconds after contacting the food usually show successive feeding interruptions and generally show a rather 'impatient' behaviour compared to ants which show a food acceptance score of 1 (see ESM: video 2-2). Lastly, rejection (0) was scored if the ant refused to feed at the sucrose solution and either returned to the nest immediately or failed to fill its crop within 10 minutes.

When the ant had filled its crop or decided not to feed at the sucrose droplet, it was allowed to return to the nest. Inside the nest, the ant unloaded its crop to its nestmates and was then

allowed back onto the runway for another visit. The drawbridge was now used to selectively allow only the marked ant onto the runway.

In addition to measuring food acceptance, we also measured pheromone deposition. Individual pheromone deposition behaviour correlates with the (perceived) quality of a food source (Beckers et al. 1993; Hangartner 1970; Czaczkes et al. 2015c). Individual ants can adapt the strength of a pheromone trail by either depositing pheromone or not, or varying the intensity of a pheromone trail through number of pheromone depositions (Hangartner 1970; Beckers et al. 1993). Pheromone deposition behaviour in *L. niger* is highly stereotypic. To deposit pheromone, an ant briefly interrupts running to bend its gaster and press the tip of the gaster onto the substrate (Beckers et al. 1992a). This allows the strength of a pheromone trail to be quantified by counting the number of pheromone depositions over the 20 cm runway leading to the feeder. Pheromone depositions were measured each time the ant moved from the food source back to the nest (inward trip), and each time the ant moved from the nest towards the food source (outward trip). Because *L. niger* foragers almost never lay pheromone when they are not aware of a food source (Beckers et al. 1992a), we did not measure pheromone depositions for the very first outward trip (visit 1). The presence of trail pheromone on a path depresses further pheromone deposition (Czaczkes et al. 2013a). Thus, each time an ant had passed the 20 cm runway, the paper overlay covering the runway was replaced by a fresh one every time the ant left the runway to feed at the feeder or returned to the nest.

All experimental runs were recorded with a Panasonic DMC-FZ1000 camera to allow for later video analysis. Each tested ant was observed until all experimental runs were finished and then discarded from the colony before switching to the next ant. If an ant did not return before finishing all experimental runs, we waited for 15 minutes, then discarded it from the colony and moved to the next ant.

2.4.3   Statistical Analysis

Statistical analyses were carried out in R v. 3.4.1 (R Core Team 2016) using Generalized Linear Mixed Models (GLMMs) in the LME4 package (Bates et al. 2014) to analyse pheromone depositions data and Cumulative Link Mixed Models (CLMMs) in the ordinal package (Christensen 2015) to analyse food acceptance scores. CLMMs were used to analyse the acceptance data since we used an ordered factor with three levels (1 = full acceptance, 0.5 = partial acceptance, 0 = rejection).

As multiple ants were tested per colony, colony identity was added as a random effect to each model. GLMMs were tested for fit, dispersion and zero inflation using the DHARMa package (Hartig 2017). The model predictors and interactions were defined *a priori*, following Forstmeier and Schielzeth (2011). All p-values presented were corrected for multiple testing using the Benjamini–Hochberg method (Benjamini and Hochberg 1995). A total of 1070 ants were tested, with 829 in experiment 1, 73 in experiment 2 and 168 in experiment 3 (see table S1-5 in supplement S1.5). Sample sizes were set ahead of time by deciding how much time we will invest in data collection (1 day per treatment per colony).

2.4.3.1   Food acceptance data

Depending on the experiment, we either used treatment (experiment 1 & 3 = Reference Molarity; experiment 2 = expected molarity triggered by a scented runway and the odours presented on the runway) or an interaction between treatment and visit number, and the odours presented on the runway (training visits of experiment 2) or trophallaxis time (experiment 3) as fixed factors. The interaction between expected molarity and visit number in the training runs of experiment 2 was added, because experience with a food source is likely to affect the behaviour at a food source. The odours presented on the runway were added as fixed factors to test for odour preferences regardless of sucrose molarity. The interaction between trophallaxis time and reference molarity in experiment 3 was added because trophallaxis time may affect food acceptance through crop load and information gained through trophallaxis (for the effects of trophallaxis time on food acceptance see supplement S1.4 figure S1-4 and table S1-4). Because individual ants were tested multiple times in experiments 1 and 2, we included AntID nested in colony as a random factor for statistical analyses of the training visits.

We used the following general model formula (this formula varied depending on experiment as described above):

$$FoodAcceptance \sim treatment + (random\ factor: colony)$$

2.4.3.2   Pheromone Deposition Data

As the pheromone deposition data is count data, they were analysed using a GLMM with a Poisson distribution.

Depending on the experiment, we either used treatment (experiment 1 = Reference Molarity; experiment 2 = expected molarity triggered by a scented runway and the odours presented on the runway) or an interaction between treatment and visit number (training visits of experiment

2) as fixed factors. The interaction between expected molarity and visit number in the training runs of experiment 2 was added, because experience with a food source is likely to affect the behaviour at a food source. The odours presented on the runway were added as fixed factors to test for odour preferences regardless of sucrose molarity. Because individual ants were tested multiple times in experiment 2, we included AntID nested in colony as a random factor for statistical analyses of the training visits.

For statistical analysis of experiment 1, we also added a variable indicating if ants deposited more or less pheromone compared to the average to correct for individual strength of pheromone depositions and overdispersion. The variable was calculated as follows:

Difference to average ~

((Number Pheromone Depositions 1st visit - mean number Pheromone Depositions 1st visit) +

(Number Pheromone Depositions 2nd visit - mean number Pheromone Depositions 2nd visit)) / 2

We used the following model formulae in the model:

Experiment 1:

NumberPheromoneDepositions ~

treatment + Difference to average + (Difference to average)^2 + (random effects: colony/ AntID)

Experiment 2:

NumberPheromoneDepositions ~ scent associated to molarity + (random effects: colony)

2.4.3.3   Other analyses

The number of drinking interruptions was quantified via video analysis in experiment 2 (see below). This was analysed statistically in a manner identical to number of pheromone depositions.

Trophallaxis time in seconds in experiment 3 were used in full seconds and treated as count data. We performed a GLMM with Poisson distribution and Reference Molarity as a fixed effect, while colony identity was added as a random factor:

TrophallaxisTimeseconds ~ ReferenceMolarity + (random effects: colony)

2.5   Experiment 1 – Defining a relative value perception curve

The aim of this experiment was to test whether *Lasius niger* ants value a given absolute sucrose solution concentration relative to a reference point or based on its absolute value. We used a range of twelve molarities as reference points in order to describe a value curve. To exclude effects of the researcher's expectations on the data, the data for this experiment were collected blind to treatment (Holman et al. 2015).

2.5.1   Experiment 1 - Methods

Ants made two initial training visits to a feeder at the end of a runway in order to set their reference point (figure 2-1A). The quality of the sucrose solution was varied between ants, with each ant receiving the same quality twice successively. Twelve different molarities were used: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5 or 2M (also referred to as pre-shift solution or reference point). *Lasius niger* workers learn the quality of a feeder within 2 visits (Wendt and Czaczkes 2017). On the third visit (test visit), the food source was replaced by a 0.5M sucrose solution droplet for all ants (also referred to as post-shift solution). Thus, ants trained to qualities <0.5M experienced a positive successive contrast, ants trained to >0.5M experienced a negative successive contrast, and the ants trained to 0.5M constituted the control (no contrast). 97% of ants successfully finished the training procedure and participated in the test visit (third visit).

2.5.2   Experiment 1 - Results

Ants seemed to value sucrose solution droplets relative to their reference point (figure 2-2, supplement S1.5 table S1-5). In the training visits, acceptance scores increased significantly with increasing molarity of the reference quality (*CLMM*: estimate= 1.97, z= 9.65, p< 0.001, figure 2-2). However, in the test (contrast) visit, acceptance scores decreased significantly with increasing molarity of the reference quality (*CLMM*: estimate= -2.59, z= -13.57, p<0.001, figure 2-2). Ants which were trained to the lowest molarity (0.1M: p<0.001) showed significantly higher acceptance of 0.5M sucrose than control ants, while ants trained to high molarities (1.5M: p<0.001, 2M: p<0.001) showed lower acceptance of 0.5M than the control group (see table S1-1 in supplement S1.1).
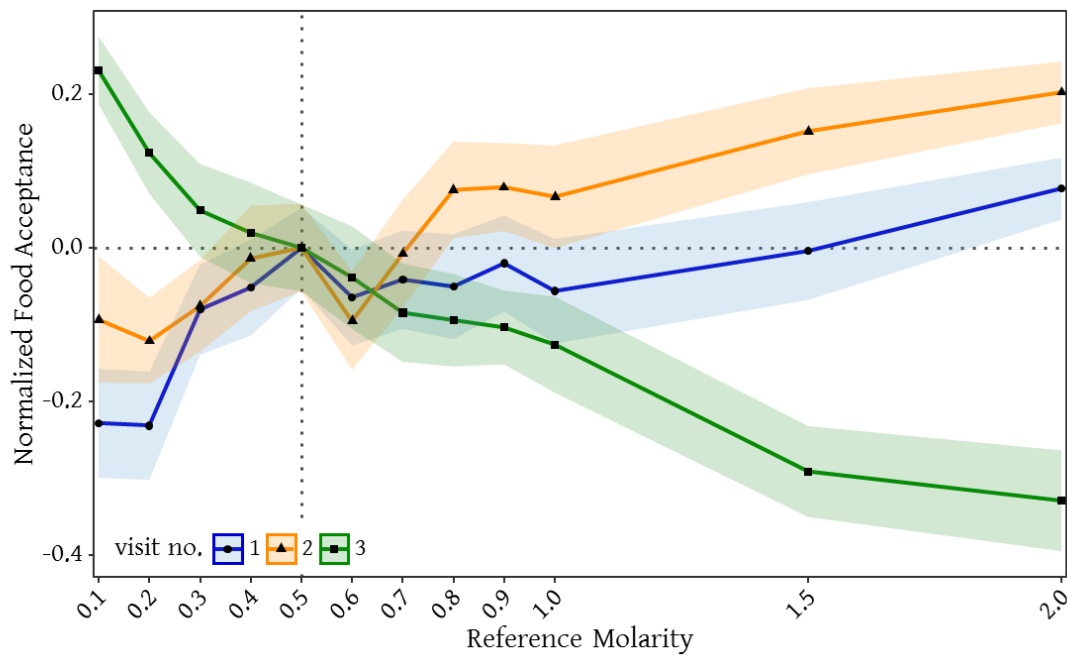
Figure 2-2: Food acceptance shown in experiment 1 for the two training visits (visit 1 & 2) in which ants received one of 12 molarities and the test visit (3) in which all ants received 0.5M (sample sizes: 0.1M: 57; 0.2M: 80; 0.3M: 76; 0.4M: 66; 0.5M: 77; 0.6M: 65; 0.7M: 73; 0.8M: 66; 0.9M: 72; 1M: 55; 1.5M: 72; 2M: 70). Shown are the mean food acceptance (points) and the 95% confidence intervals (coloured ribbons) for each reference molarity and visit. Data was normalized to show the mean food acceptance of the control group (received 0.5M on each visit) at 0 for all three visits. For a non-normalized graph of the data see figure S1-1 in supplement S1.1.

A similar pattern was found for pheromone deposition behaviour on the way back to the nest (figure 2-3). In the training visits, number of pheromone depositions increased significantly with increasing molarity of the reference solution (*GLMM*: estimate = 0.86, z = 13.87, p < 0.001). By contrast, on the test visit pheromone depositions decreased significantly with increasing molarity of the reference solution (*GLMM*: estimate = -0.82, z = -9.75, p < 0.001, figure 2-3). Ants which deposited more pheromone during the training visits generally deposited more pheromone on the test visit compared to ants which deposited less pheromone during the training visits (*GLMM*: estimate = 0.16, z = 15.99, p < 0.001). Ants which were trained to a low molarity (0.2M: p < 0.01) deposited significantly more pheromone in the test visit than control ants, while ants trained to high molarities (1M: p < 0.001, 1.5M: p < 0.001, 2M: p < 0.001) deposited less pheromone than the control group (see table S1-2 in supplement S1.2 for pairwise comparisons).
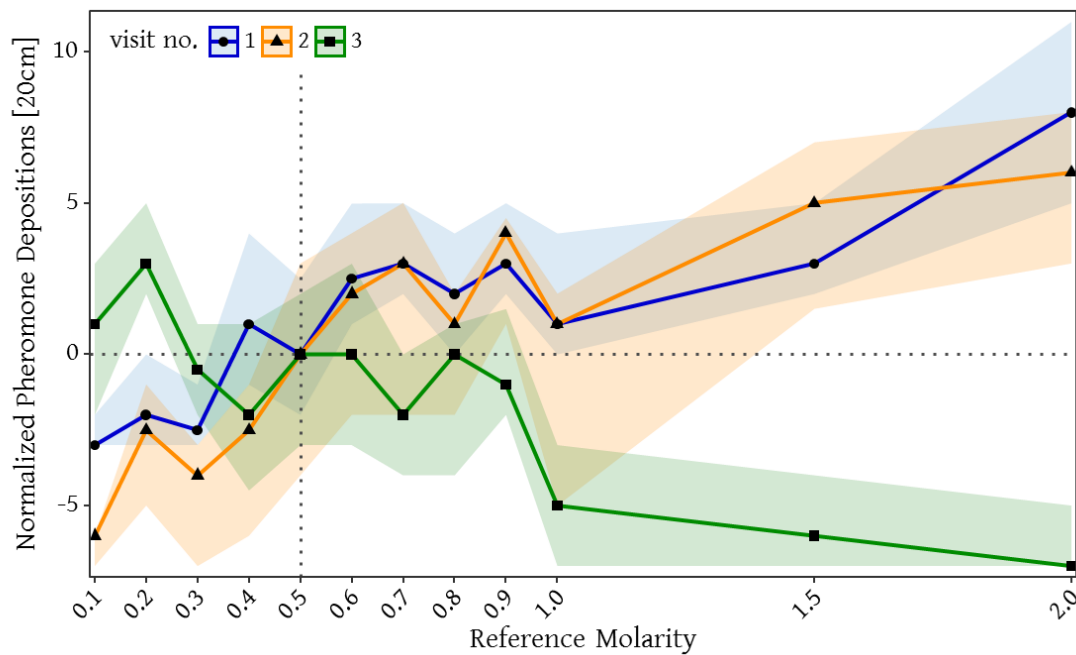
Figure 2-3: Pheromone depositions on the way back to the nest shown in experiment 1 for the two training visits (visit 1 & 2) in which ants received one of 12 molarities and the test visit (3) in which all ants received 0.5M (sample sizes: 0.1M: 57; 0.2M: 80; 0.3M: 76; 0.4M: 66; 0.5M: 77; 0.6M: 65; 0.7M: 73; 0.8M: 66; 0.9M: 72; 1M: 55; 1.5M: 72; 2M: 70). Shown are the median number of pheromone depositions (points) and the 95% confidence intervals (coloured ribbons) measured on a 20 cm track right behind the food source for each reference molarity and visit. Data was normalized to show the median number of pheromone depositions of the control group (received 0.5M on each visit) at 0 for all three visits. For a non-normalized graph of the data see figure S1-2 in supplement S1.2.

These results are consistent with relative value perception stemming from the psychological effects of successive contrasts. We could further define a relative value perception curve similar to that described in Prospect Theory, as well as showing positive contrast effects for both food acceptance and number of pheromone depositions.

However, there is another possible explanation for these results: non-random selection of individuals with different acceptance thresholds. Different individuals from the same colony may have different acceptance thresholds. Animals with lower acceptance thresholds may readily exploit low-quality food sources while animals with higher thresholds may not. When training to lower-molarity sucrose, ants with high thresholds may not have completed training, leaving only a non-random subset of ants with low acceptance thresholds at the test phase (Robinson et al. 2009). Thresholds may also be influenced by experience, by which animals use the best experienced option as a threshold for accepting a new option or not (Stroeymeyt et al. 2011; Robinson et al. 2011). However, we can exclude this possibility, as the proportion of ants not

completing training was uniformly low and did not vary with treatment (table S1-5 in supplement S1.5).

2.6   Experiment 2 – ruling out alternative explanations using scent training

Alternative hypotheses could also explain the results from experiment 1 and lead to the same behavioural patterns observed. Five possible 'lower-level' mechanisms must be excluded: sensory satiation, ingested sucrose changing haemolymph-sugar levels, psychophysical sensory contrast effects, the fact that ants may expect pre-shift solutions to return in later visits, and non-random selection of individuals with different food acceptance thresholds in different treatments.

Sensory satiation

This may occur in ants which were trained to higher molarity food due to the blocking of more sweetness receptors compared to low molarity sucrose. The more sweetness receptors are blocked by a sweet reference solution, the fewer receptors will fire when confronted with a post-shift reward, thus making solutions taste less sweet for ants trained to high-molarity solutions, and sweeter for ants which were trained to low molarities (Bitterman 1976).

Haemolymph-sugar levels

Ants may not only have stored sucrose solutions in their crop while foraging, but may also have ingested small amounts of it, leading to an increase of haemolymph-sugar levels. Higher blood-sugar levels negatively affect sweetness perception in humans (Mayer-Gross and Walker 1946; Melanson et al. 1999), and a similar effect could cause a post-shift solution to taste less sweet to animals trained on high sucrose concentrations.

Psychophysical sensory contrasts

The contrast effects shown in experiment 1 could also derive from simple psychophysical mechanisms (Fechner 1860; Zwislocki 2009), and thus arise from sensory perceptual mechanisms rather than higher-level cognitive processing of value. Sensory judgements are usually made relative to reference points and through constant comparisons with former stimuli (Helson 1964; Vlaev et al. 2011). Thus, identical stimuli may be perceived differently depending on the context they are presented within. The

position of the reference point in the range of stimuli may thus bias how the stimulus, and thus the value, of a post-shift reward is perceived (Zwislocki 2009). For example, the sweetness of a sucrose solution may be perceived as much stronger when the reference point to which it is compared is low. Psychophysical sensory contrasts are physiological or low-level cognitive phenomena, found in all animal taxa studied, and even potentially in bacteria (Akre and Johnsen 2014; Kojadinovic et al. 2013; Mesibov et al. 1973).

Future expectations

Animals may rationally expect the pre-shift reward to be available in the future again and therefore rationally show lower acceptance towards the post-shift reward, because they are waiting for the pre-shift reward to reoccur.

All of these alternative factors would lead to the same behavioural patterns found in experiment 1 without relative value perception necessarily being present. Experiment 2 was designed to rule out these alternative explanations.

2.6.1   Experiment 2 - Methods

To rule out the alternative non-psychological explanations for the contrast effects we described above, we needed to change the expectations of the ants while exposing all ants to identical training regimes. This would provide a reference point for testing relative value perception while keeping sensory saturation, haemolymph-sugar levels, psychophysical effects, future expectations, and ant subsets the same until the testing phase.

Ants were allowed to make 8 training visits. The quality of the sucrose solution offered at the end of the runway alternated each visit, always beginning with the low quality solution. The solutions were scented using either rosemary or lemon essential oils (0.05µl essential oil per ml sucrose solution, rosemary: *Rosmarinus officinalis*; Lemon: *Citrus limon*, Markl GbR, Grünwald). In half the trials the 1.5M solution was scented with lemon and the 0.25M with rosemary, and vice versa for the other trials. In addition, to support learning and to allow solution quality anticipation, we also scented the paper overlays covering the runway leading to the feeder. Paper overlays were scented by storing them for at least one day in an airtight box containing a droplet of essential oil on filter paper in a petridish. Finally, in addition to odours cuing sucrose molarity, visual cues were also provided. These consisted of printed and laminated pieces of paper (22 x

16.5 cm, displayed in figure 2-1B) displayed at the end of the runway, directly behind the sucrose droplet.

On the 9th (test) visit, the odour of the runway and the visual cue signified either 1.5M or 0.25M, while the sucrose solution provided was unscented and of intermediate (0.5M) quality. Runway scents in the test visit were varied systematically between ants, but each ant was confronted with only one of the two runway scents coupled with unscented 0.5M sucrose. While the ant fed at the sucrose droplet, the scented runway overlay was replaced with an unscented overlay in order to eliminate possible effects of scent association on pheromone deposition behaviour. Previous work has shown that *L. niger* foragers can form robust expectations of upcoming reward quality based on runway odour after 4 visits to each odour/quality combination (Czaczkes et al. 2018b). Nonetheless, to ensure that learning had taken place, on the 10th visit, we carried out a memory probe. The linear runway was replaced with a Y-maze (figure 2-1B), with two 10cm long arms and a 10cm long stem. The Y-maze stem was covered with an unscented paper overlay while one arm was covered with the 1.5M-associated odour overlay, and the other with the 0.25M-associated odour overlay. The matching visual cues were placed directly behind the relevant Y-maze arms. Trained ants were allowed to walk onto the Y-maze and their arm choice was noted. We used two decision lines to define arm choice – an initial decision line (figure 2-1B, 2 cm after the bifurcation) and a final decision line (8 cm after the bifurcation). After testing on the Y-maze, the ants were permanently removed from the colony.

97.2% of ants successfully finished the training procedure and participated in the last test visit.

Additionally to the other measures, on the 9th (test) visit of this experiment we counted the number of food interruptions made by an ant from the moment of first hitting the food source until it had finished feeding at the sucrose droplet. The number of food interruptions are likely to reflect and support the behaviour encoded in food acceptance scores and was thus investigated to give stronger support for the results of this experiment.

2.6.2   Experiment 2 - Results

During training, ants behaved as expected, showing higher acceptance and pheromone deposition for 1.5M compared to 0.25M on all but the very first visit to 0.25M (Food acceptance: *CLMM*: estimate = -7.34, z = -8.9, $p < 0.001$; pheromone depositions outward journey: *GLMM*: estimate = 0.23, z = 2.89, $p < 0.01$; pheromone depositions inward journey: *GLMM*: estimate= -2.49,

$z = -19.46$, $p < 0.001$, figure 2-4A, C & E). Furthermore, food acceptance and pheromone depositions both on the outward and inward journeys decreased with increasing experience with the 0.25M feeder and increased with increasing experience with the 1.5M feeder (Food acceptance: *CLMM*: estimate = -2.84, $z = -3.63$, $p < 0.001$; pheromone depositions outward journey: *GLMM*: estimate = -0.94, $z = -10.00$, $p < 0.001$; pheromone depositions inward journey: *GLMM*: estimate = -0.53, $z = -4.41$, $p < 0.001$).

On the outward journey of the 9th (test) visit, ants walking towards the feeder while exposed to 1.5M sucrose-associated cues deposited more pheromone (median=15, figure 2-4D) compared to ants exposed to 0.25M-associated cues (median = 2, *GLMM*: estimate = -1.31, $z = -12.94$, $p < 0.001$). Moreover, in the learning probe, 87% of ants chose the 1.5M associated arm. This demonstrates that ants formed a robust expectation of food molarity based on the cues learned during training.

Ants exposed to 1.5M-associated cues during the 9th visit showed significantly lower food acceptance towards the unscented 0.5M feeder than ants exposed to 0.25M-associated cues (*CLMM*: estimate = 1.04, $z = 2.049$, $p < 0.05$, figure 2-4B, table S1-5 in supplement S1.5). Although ants exposed to high molarity associated cues – presented through scented runways on the way to the food – showed a significantly higher number of pheromone depositions on their return journey than ants confronted with low molarity scent (*GLMM*: estimate= -1.65, $z = -3.03$, $p < 0.01$, figure 2-4E & F), the number of pheromone depositions decreased drastically for both treatments compared to training visits (median 1.5M = 0, median 0.25M = 0, figure 2-4E & F, table S1-5 in supplement S1.5).
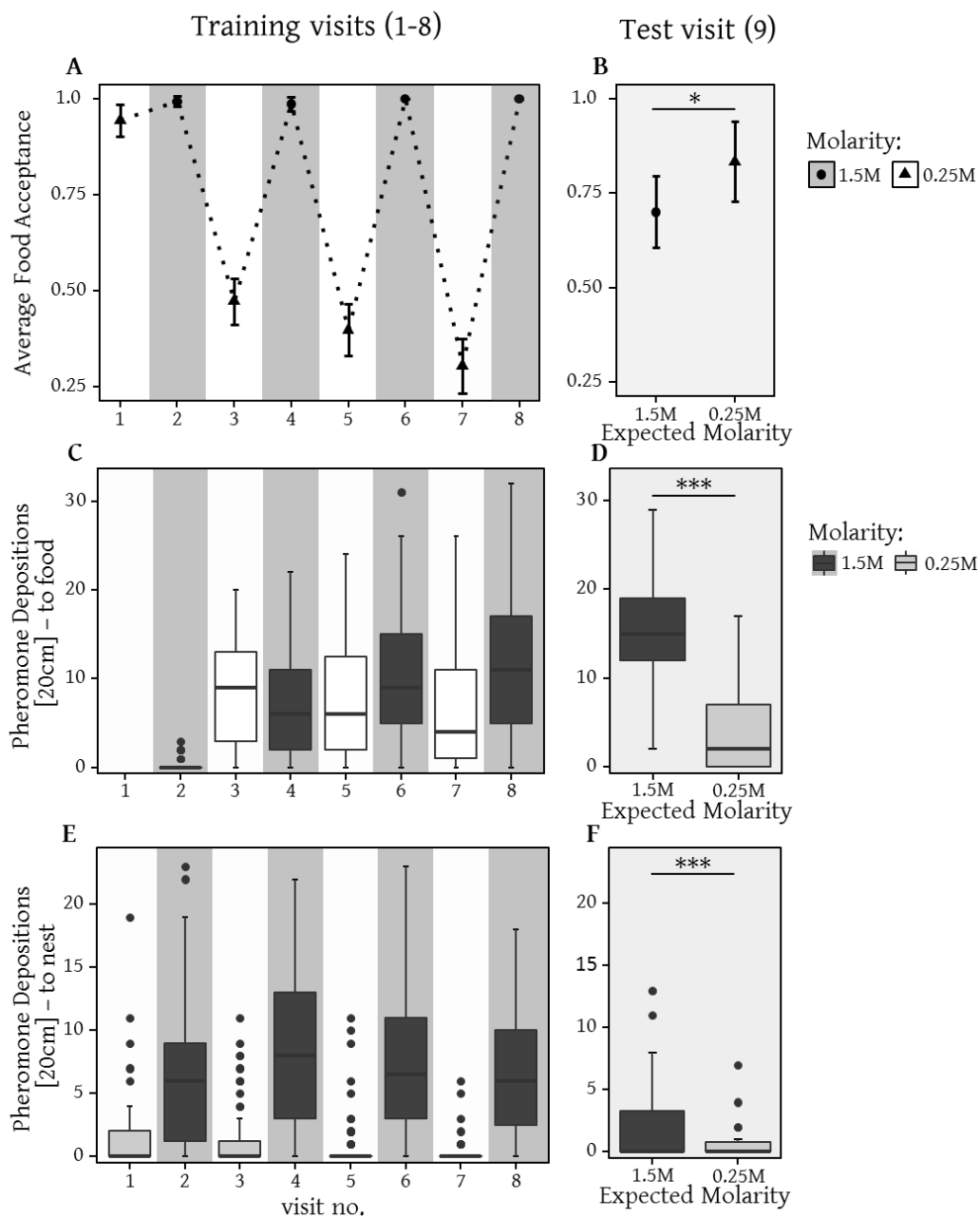
Figure 2-4: Food acceptance (A & B) and number of pheromone depositions towards the food source (C & D) and towards the nest (E & F) in experiment 2. The left panels (A, C, E) show behaviour over the 8 training trials, in which ants received 0.25M coupled with one scent and 1.5M coupled with another scent on alternating visits. The right panels (B, D, F) show behaviour on the test visit, in which ants always received unscented 0.5M sucrose solution, but the runway leading towards the food source was impregnated with one of the learned scents, causing ants to expect either a high or low reward. 40 ants were induced to expect a high reward, and 32 to expect a low reward. A & B show the mean food acceptance (points) and the 95% confidence intervals (error bars) for each visit; C – F show the median number of pheromone depositions on a 20 cm track leading to the food source and the 75%/25% quantiles for each visit.

Even after controlling for alternative explanations, ants still show contrast effects depending on the quality of the post shift solution. This is in spite of all ants undergoing identical training experiences. The only difference between the groups was the odour of the runway on the 9th (test) visit. It is thus unlikely that sensory saturation, increased haemolymph-sugar levels, simple psychophysical effects or ants expecting pre-shift solutions to return can fully explain the behaviour of the ants in our experiments. All videos were re-analysed by a naïve scientific assistant and this blind analysis of the ants behaviour confirmed the stated results (*CLMM*: estimate= 1.42, z = 2.35, p = 0.019), and also found that ants interrupted drinking significantly more often when expecting high rather than low food qualities (*GLMM*, estimate = 0.36, z = 2.74, p = 0.006, see figure S1-3 in supplement S1.3 and ESM: Figure 2-4 - Figure supplement 1 - Source Data File 1).

Non-random selection of individuals with different acceptance thresholds can also be excluded for the results of this experiment as the proportion of ants not completing training was again uniformly low (see table S1-5 in supplement S1.5) and all ants had to taste both low and high molarities in order to complete training.

2.7   Experiment 3 – expectation setting via trophallaxis: the nest as an information hub

Ants receive information about available food sources, such as food odour and palatability, through food exchanges (trophallaxis) inside the nest (Provecho and Josens 2009; Josens et al. 2016). An ant beginning a food scouting bout may not have direct information about the quality of the food sources available in the environment, but nonetheless must make a value judgement on their first visit to a food source. The aim of this experiment was to ascertain whether information about sucrose concentrations gained through trophallaxis in the nest affected the perceived value of food sources found outside the nest.

2.7.1   Experiment 3 - Methods

An ant was allowed to feed at an unscented sucrose solution droplet of either 0.16, 0.5 or 1.5M (also referred to as pre-shift solution or reference point) and return to the nest to unload its crop via trophallaxis. When trophallaxis began, we noted the time spent in trophallaxis with the first trophallactic partner. When trophallaxis stopped, the receiving trophallactic partner (receiver) was gently moved from the nest and placed onto the start of a runway offering

unscented 0.5M sucrose solution at the end (also referred to as post-shift solution). As the receiver fed, we noted its food acceptance.

2.7.2   Experiment 3 - Results

Acceptance scores of receivers towards 0.5M decreased with increasing molarity of the sucrose solution received through food exchanges inside the nest (*CLMM*: estimate= -0.57, z= -3.07, p <0.01). The interaction of reference molarity and trophallaxis time significantly predicted acceptance (*CLMM*: estimate=-0.48, z= -2.33, p= 0.02, figure 2-5) and longer trophallaxis times led to lower food acceptance in ants as well (*CLMM*: estimate= -0.70, z= -3.62, p<0.001). Ants which received 0.16M inside the nest showed significantly higher acceptance of 0.5M sucrose than ants which received 1.5M (p<0.01, see table S1-3 in supplement S1.4 for pairwise comparisons). The time spent in trophallaxis with the receiver increased significantly with increasing molarity (*GLMM*: estimate= 0.13, z= 4.79, p<0.001, see ESM: Figure 2-5 - Source Data File 1).
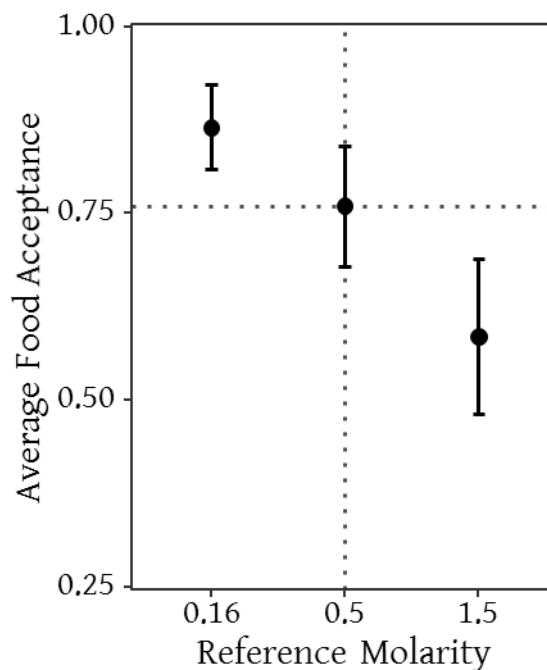
Figure 2-5: Food acceptance shown in experiment 3 for the receivers which received either 0.16, 0.5 or 1.5M through trophallaxis in the nest and then found 0.5M at the end of the runway (sample sizes: 0.16M 63; 0.5M: 52; 1.5M: 53). Shown are the mean food acceptance (points) and the 95% confidence intervals (error bars) for each reference molarity.

Ants valued a standard quality food source relative to the molarity which they received from a returning forager inside the nest. This suggests that information about the quality of a food

source received through trophallactic interactions inside the nest can be used by naïve foragers when evaluating new food sources outside the nest. Thus, the nest serves as an information hub in which information about available food sources can be gathered, processed, and disseminated.

2.8   Discussion

The introduction of Prospect Theory (Kahneman and Tversky 1979) contributed to a major shift in economic research by suggesting that humans do not perceive value in absolute terms, but relative to reference points. Here, we demonstrate parallel findings in an insect. To the best of our knowledge, we provide the first detailed description of relative value perception in an invertebrate based on individual experience, but also induced by social information. Furthermore, we demonstrate the elusive positive contrast effects in ants which were trained to low molarities (figure 2-2and figure 2-3).

Similar results in house-hunting ants were explained by a simple threshold rule (Stroeymeyt et al. 2011; Robinson et al. 2009; 2011) which suggests that individuals have different acceptance thresholds and ants with lower thresholds accept lower quality options. The higher the quality of the option, the more often it exceeds the acceptance threshold of individual ants, and thus the option is accepted more readily. This could have potentially affected our results in experiment 1, as we would expect fewer individuals to accept very low reference points. Ants which did not accept the low quality sucrose would thus not be tested. Therefore, at low reference points, we would only select individuals with very low acceptance thresholds, while no threshold selection would occur at high reference points. When confronting ants with medium quality food after training, the differently selected acceptance thresholds may lead to the same pattern as we observed. However, 97% of all ants finished both the training and the test phases and no higher proportion of cancelled training can be seen at lower reference molarities (see table S1-5 in supplement S1.5). It is thus unlikely that a simple threshold rule leads to the results shown in experiment 1 (figure 2-2and figure 2-3).

While a second major prediction of Prospect Theory, that "losses loom larger than gains" (Tversky and Kahneman 1992), is not supported by the data of our main experiment, it is also not ruled out. We believe ants do overemphasise losses, but, due to limitations in the experimental design and physiological limitations of the animals, we cannot make strong claims about this (47–49). The lack of strong evidence for losses being overemphasised may stem from the psychophysics of our study system: a basic tenant of psychophysics is that the Just Noticeable

Difference (JNDs) between two stimuli is a function of the relative difference between the stimuli (Fechner 1860; Stevens 1957; Zwislocki 2009). Thus, ants shifted from 0.1M to medium (0.5M) quality experience a 5-fold increase in molarity, while those down-shifted from 0.9M to 0.5M experience less than a two-fold decrease, although the absolute change was of the same magnitude. This would predict larger shift-changes, in terms of absolute molarity change, for gains than for losses. Indeed, the fact that this is also not seen may imply that losses are indeed – relatively speaking – looming larger than gains for the ants. Finally, it must be kept in mind that acceptance scores are unlikely to be linear, and that pheromone deposition behaviour shows large variation (Beckers et al. 1992a), making it difficult to use either of these factors to quantitatively test for over- and undervaluation of gains and losses.

The results of experiment 2 allow us to exclude all but a cognitive relative value effect (figure 2-4). This cognitive effect is subjectively very familiar to humans, and its presence in an invertebrate is at first glance surprising. However, insects have been shown to display many cognitive traits in parallel with humans (Cheng et al. 2002; Czaczkes et al. 2018a, 2018b; Pompilio et al. 2006; Wendt and Czaczkes 2017), and contrast effects are likely selected for (McNamara et al. 2013).

The smaller effect size in experiment 2 is presumably driven either by the exclusion of the additional driving factors (see chapter 2, section 2.6), or the additional complications involved in an extensive training regime, or both. Specifically, the expectations leading to contrast effects in experiment 2 were driven by differential learning of odour-quality associations, rather than a simple one-component memory of food quality as may have been the case in experiment 1. This may have weakened the observed effect.

Another possible explanation for smaller effect sizes may be that in experiment 2 ants had access to two reference points (0.25M and 1.5M) to use for value judgement of the medium quality food in the control experiment, while in experiment 1 they only had one reference. Thus, while the odour cue may have overemphasized the role of the associated quality as reference, the competing reference quality may have been acting as a second reference. Additional reference points are likely to affect the scale post-shift rewards are compared to (Zwislocki 2009). This possibility is supported by the acceptance data collected during training in experiment 2. On the first training visit, all ants encountered low quality food and showed a high food acceptance towards the feeder (figure 2-4A). However, as soon as ants had experienced a high-quality sucrose solution, the previously acceptable low quality food became unattractive, and food acceptance

scores decreased from a mean of 0.99 to 0.39. This strongly suggests that the ants were valuing the training solutions in relation to each other, and may therefore have used both reference points to judge the value of an unscented medium quality food source. It is possible that the ants may have calculated an average from both reference points, and used the average as comparison to judge the value of the post-shift reward (Flaherty 1999), as shown in rats (Peters and McHose 1974). However, the fact that medium quality elicited different food acceptance scores depending on the runway scent makes it unlikely that this would be the only factor affecting acceptance scores.

Lastly, masking effects may also explain the smaller contrast effects of experiment 2 compared to experiment 1: learning theory suggests that neutral cues associated to positive stimuli will elicit positive responses even when no reward is given and vice versa (Rescorla and Wagner 1972). Therefore, since ants were confronted with the scent associated to high quality food, food acceptance may have been affected by the scent itself, leading to an elevated food acceptance compared to ants tested in experiment 1 which did not receive a positive cue, but only medium quality food.

The reduced pheromone deposition seen in the final return in experiment 2 may be due to the change in environment (scented runways to unscented runways) causing a disruption in recruitment behaviour, perhaps due to generalization decrement (Capaldi 1978; Kimble 1961) or neophobia (Barnett 1958; Johnson 2000; Mitchell 1976; Pliner and Loewen 1997). Furthermore, since only the scented paper overlays were replaced by unscented ones, but not the runways themselves, it is possible that small portions of the odours were still present, driving the ants to deposit pheromone according to the remaining odours, with higher deposition rates for the high-quality associated odour. In a separate experiment, such pheromone deposition directly related to quality-associated odours on runways was clearly demonstrated (Wendt and Czaczkes 2019). This would explain why pheromone depositions were higher for ants returning to the nest from a high molarity scent than in ants returning from a low molarity scent.

Information about sucrose concentrations gained through trophallactic interactions inside the nest can affect the way newly discovered food sources are valued outside the nest (figure 2-5), as well as providing other information (Provecho and Josens 2009; Josens et al. 2016; LeBoeuf et al. 2016). By taking into account information gained inside the nest, recruited workers are able to evaluate newly discovered food sources in relation to other food sources available in the environment. Ants could thus forego food sources which are of lower quality than the average

available food sources (Wendt and Czaczkes 2017). Even though higher trophallaxis times led to lower acceptance scores and trophallaxis times were higher at high reference molarities, this does not necessarily imply that ants ingested more sucrose at higher references and were thus less hungry or motivated. Higher sucrose solutions are more viscose and thus ants take longer to ingest the same amount of sucrose compared to low molarities (Josens et al. 1998). If, however, more sucrose solution was transferred between the returning forager and the recruit at longer trophallaxis times, it is likely that information input increases and food acceptance decreases. The longer the trophallaxis time, the more the recruit can fill its crop through trophallaxis and therefore the food acceptance may decrease, because the recruit is less starved than an ant which showed a short trophallaxis time. However, even if more food was transferred, the food acceptance scores are a measure of the first assessment of ants at a food source, not the ingested volume. Thus, while some ants may have had less space in their crop left, this may not necessarily affect the food acceptance score, while it is very likely to affect ingested volume after trophallaxis. Additionally, if longer trophallaxis times lead to more ingested sucrose solution, it is also more likely that a higher amount of information about the past food quality is transferred. Thus, more transferred food during trophallaxis may have led to better informed ants reaching the post-shift solution and thus stronger contrast effects. Since the data shows clear effects of both trophallaxis time and reference solution on the food acceptance of 0.5M sucrose, longer trophallaxis times cannot be the only factor driving the contrast effects found in this experiment (see figure S1-4 in supplement S1.4 and ESM: Figure 2-5 - Source Data File 1). Even at high trophallaxis times, ants with a 0.16M reference showed no low food acceptance scores, unlike ants with high reference solution after long trophallaxis times.

Ultimately, we see the nest serving as an information hub, in which information about currently available food sources can be collected, synthesised, and fed back to outgoing foragers. Relative value perception can therefore be expected to have strong effects not only on the individual behaviour of animals, but also on the collective behaviour of insect colonies. For example, colonies of house-hunting ants developed an aversion towards mediocre nests when housed in high-quality nests, but not when they were housed in low-quality nests. Such mediocre nests are then avoided when colonies have to find a new nest site while newly discovered mediocre nests are readily accepted (Stroeymeyt et al. 2011; Robinson et al. 2011). However, while in house-hunting the reference resource is directly experienced by scouts only, we demonstrate that information brought back to the nest can set a reference point for ants which have not directly experienced the resource *in situ.*

A broad range of behaviours relevant to behavioural economics have been described in invertebrates (Wendt and Czaczkes 2017; Czaczkes et al. 2018a; Cheng et al. 2002; Pompilio et al. 2006; Czaczkes et al. 2018b). We propose that invertebrates make attractive models for a broader understanding of behavioural economics in humans. The benefits of an interdisciplinary approach will likely flow both ways. Using animal models allows researchers to avoid pitfalls associated with studies on humans, such as cultural and educational differences (Carter and Irons 1991; Guiso et al. 2006), second-guessing of experimenters, and non-relevant reward sizes (Levitt and List 2007) as well as relaxing ethical concerns. The game-like designs of many economic experiments are highly artificial and the incentive magnitudes that can be provided are limited (Kahneman and Tversky 1979; Levitt and List 2007). While there has been much progress in field studies on humans to clearly measure causal relationships (Harrison and List 2004), the usefulness of these new techniques is constrained by the range of questions and settings to which they can be applied. Hence, while behavioural studies on invertebrates also have their limitations (for example, in that inducing expectations is more of a challenge), they can be easily designed to be ecologically meaningful, and offer rewards which are in line with the real-life budgets under which the animals operate. Finally, due to human complexity, building economic models which accurately predict human behaviour is challenging. Insect economic behaviours are demonstrably similar to that of humans, but likely simpler. We therefore propose that economic models to predict invertebrate decision making may be a complementary step on the way to predicting human behaviour.

There is a well-developed tradition of integrating economics and biology (Aw et al. 2009; 2011; Cheng et al. 2002; Czaczkes et al. 2018a; Evans and Westergaard 2006; Lydall et al. 2010; Wendt and Czaczkes 2017). Here we provide a systematic description of value judgment relative to a reference point in ants, define a relative value curve as described in Prospect Theory, and provide some of the first strong evidence for a purely cognitive element to relative value judgement. Reference points can not only be set by individual experiences but also through social information such as pheromone trails or through trophallactic contacts inside the nest. We feel a critical mass of evidence is now available to consider comparative behavioural economics as a relevant discipline for both biologists and economists.

Acknowledgements

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All animal treatment guidelines applicable to ants under German law have been followed.

Chapter 3

Labelling effect in insects: cue associations influence perceived food value in ants *(Lasius niger)*

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3.1   Abstract

Humans usually assess options not in terms of absolute value, but relative to reference points. The framing of alternatives can strongly affect human decision making, leading to different choices depending on the context within which options are presented. Similar reference-point effects have been recently reported in ants, in which foragers show contrast effects: ants overvalue a medium-quality food source if they were expecting a poor one, and vice versa for expectations of good food. However, studies of human consumer psychology have demonstrated that expectations, for instance from product labels, can drive value perception in the opposite direction, via a process of assimilation. For example, an expensive bottle of wine is perceived as more enjoyable compared to a cheaper bottle, even if the wine is the same. In this study, we demonstrate a similar labelling-association effect in an insect: ants showed assimilation effects by spending twice as long drinking at medium quality food if it was scented with an odour previously associated with high quality than if it was scented with a poor-quality label. The presence of odour cues in the food during consumption and evaluation is critical, as without them, odour-driven expectations of quality result in contrast, not assimilation effects. The addition of a quality label in the food thus reverses contrast effects and causes value to be aligned with expectations, rather than being contrasted against them. As value judgement is a key element in decision making, relative value perception strongly influences which option is chosen, and ultimately how choices are made.

3.2   Introduction

A decision is often made by evaluating and comparing available options. This comparison usually leads to a choice for the option promising the greatest profit (von Neumann and Morgenstern 1944). The way in which options are evaluated may, however, strongly influence which option is ultimately chosen. Thus, understanding the factors influencing the perceived value of available options helps us understand human behaviour and decision making (Slovic 1995; Thaler and Sunstein 2008; Tversky and Kahneman 1981). Understanding the drivers of option evaluation and comparison are thus central to the study of behavioural economics and consumer psychology.

Although early economic theories described humans as rational decision makers who always choose the option with the greatest utility regardless of other factors (von Neumann and Morgenstern 1944; Vlaev et al. 2011), a large body of evidence has accumulated demonstrating that this is not always the case. Kahneman and Tversky (1979) suggested that decision making is not based on absolute outcomes, but rather on the relative perceptions of gains and losses. According to Prospect Theory, which incorporates these assumptions, the value of options being evaluated is determined relative to a reference point, such as the *status quo* or former experience (Kahneman and Tversky 1979; Parducci 1984; Tversky and Kahneman 1992; Ungemach et al. 2011; Vlaev et al. 2011). Thus, the same option can be perceived more negatively if a reference point is high, and more positively given a lower reference point (Kahneman and Tversky 1979). For example, satisfaction gained from income is perceived not absolutely, but relative to the income of one's colleagues (Boyce et al. 2010). Therefore, human decision making tends to be relative rather than rational.

The concept of malleable value perception is not just relevant to humans. Value judgments in non-human animals are also influenced by factors apparently independent of the absolute value of options, such as the state an animal is in during learning in birds (Aw et al. 2011), rats (Lydall et al. 2010), fish (Aw et al. 2009) and insects (Czaczkes et al. 2018a; Pompilio et al. 2006), and expectations about upcoming rewards in dogs (Bentosela et al. 2009), rats (Annicchiarico et al. 2016; Crespi 1942; Flaherty 1982; 1999; Papini et al. 2001; Webber et al. 2015; Weinstein 1970a), mice (Mustaca et al. 2000) and insects (Bitterman 1976; Couvillon and Bitterman 1984; Oberhauser and Czaczkes 2018; Roces and Núñez 1993; Roces 1993; Wendt et al. 2019). Banded tetra fish and desert locusts, for example, prefer cues associated with food presented when they were very hungry compared to identical food received when they were less hungry (Aw et al. 2009; Pompilio

et al. 2006). Expectations can make animals perceive identical options differently depending on whether a better or worse option was expected instead of the presented one. For example, capuchin monkeys refuse otherwise acceptable pay (cucumber) in exchanges with a human experimenter if they had witnessed a conspecific obtain a more attractive reward (grape) for equal effort (Brosnan & de Waal, 2003; van Wolkenten, Brosnan, & de Waal, 2007). Similarly, *Lasius niger* ants showed lower food acceptance towards medium quality food when they expected high quality food (negative contrast) and higher acceptance of medium food when expecting poor food (positive contrast) (Wendt et al. 2019). Honeybees too rejected otherwise acceptable lower quality food when they expected high quality food due to previous experience (Bitterman 1976; Couvillon and Bitterman 1984). Such incentive contrast effects (Flaherty 1999) represent one of the main influences on subjective value. We see incentive contrasts as a subset of relative value perception.

Another factor altering perceived value in humans are product and brand labels, which can also directly affect purchasing decisions. Such labels convey expectations and thus act as reference points for judging an option (French and Smith 2013). Depending on previous associations with the label, perceived option value can increase (Breneiser and Allen 2011; Fornerino and d'Hauteville 2010; Kühn and Gallinat 2013; Lee et al. 2013; Nevid 1981; McClure et al. 2004; Wansink 2000; Woodside and Taylor 1978; Yamada et al. 2014) or decrease (Lee et al. 2006; Wansink 2000). For example, drinks presented along with strong brands such as "Coca Cola" (which has strong positive associations due to successful marketing campaigns) tend to be rated as being tastier or more attractive compared to identical drinks which were presented with weaker brand labels or without any labels, even though there is rarely a preference found in blind tests (Breneiser and Allen 2011; Fornerino and d'Hauteville 2010; Kühn and Gallinat 2013; McClure et al. 2004; Yamada et al. 2014). Compared to these strong international brands, store brands are often perceived as offering lower product quality and nutritional value (Cunningham et al. 1982; Dick et al. 1995). If the difference between a label-driven expectation and the products' objective value is small, the perceived value aligns with the expectation in a process called assimilation (Cardello and Sawyer 1992; Hovland et al. 1957; Schnurr et al. 2017). For example, a soft drink, which previously received a low rating, may receive a significantly better rating when subjects were told that it is of a favourable brand (Cardello and Sawyer 1992). In humans, labels are an accumulation of various associative cues which evoke a positive or negative response once the label is seen. Such associated attributes may affect value perception in animals as well.

Associative learning, through which cues or actions are learned to predict a positive or negative experience, is almost ubiquitous in the animal kingdom as well, including insects (Couvillon and Bitterman 1980; Giurfa 2007; Menzel 1993; Siwicki and Ladewski 2003; Spatz et al. 1974) and other invertebrates (Hawkins and Byrne 2015; Rankin 2004; Sahley et al. 1981). Like associative labelling in humans, perceived option value varies for animals as well. Naïve *Camponotus mus* ants, for example, prefer food presented alongside an odour which had already been received through food exchanges inside the nest over food presented with a novel odour, because the familiar odour was previously associated with a positive event (Provecho and Josens 2009). An example of negative associations was shown in the leaf cutter ants *Acromyrmex ambiguous* and *Acromyrmex lundi*: odour cues associated with damage to the ants' cultivated fungus drive aversion to otherwise acceptable fungal substrate, with the odour cue acting as a negative food label (Roces 1994; Saverschek and Roces 2011).

The aim of this study was to investigate whether labelling effects as shown in humans can be demonstrated in insects, whether they could act against contrast effects and whether this is affected by the timing of cue presentation. Specifically, we ask whether ants align their perception of a food sources' value with value-associated odour cues presented in the food during consumption. We previously demonstrated a contrast effect in ants, whereby ants undervalue or overvalue food if they were expecting a better or worse food quality, respectively (Wendt et al. 2019). In the previous study, expectations generated *before* perception of the objective food quality drove value perception. Here, we ask how value-related labels experienced *during* consumption affect perceived value and whether the time of label-presentation changes the perceived value of an option in ants. In order to counteract contrast effects, one first has to elicit them – hence we first aimed to form a cue-based expectation of different molarities, which would normally result in contrast effects during the test, and added assimilation-driving cues within the food (flavour cues previously associated to either high or low quality food), in order to counteract this assimilation. We hypothesized that incentive contrast effects could be counteracted by the mere presence of associative odour cues during consumption.

3.3   Methods

3.3.1   Study animals

Eight colonies of the black garden ant *Lasius niger* were collected on the University of Regensburg campus. The colonies were housed in 30 x 30 x 10 cm foraging boxes with a layer of plaster covering the bottom. Each box contained a circular plaster nest box (14 cm diameter, 2 cm height). The colonies were queenless with around 1000-2000 workers and small amounts of brood. Workers from queenless colonies forage and lay pheromone trails, and are frequently used in foraging experiments (Detrain et al. 2019; Dussutour et al. 2004). The colonies were fed with 0.5M sucrose solution and received *Drosophila* fruit flies once a week. Colonies were deprived of sucrose solution four days prior to the experiments in order to achieve a uniform and high motivation for foraging (Mailleux, Detrain, and Deneubourg 2006; Josens and Roces 2000). Water was always available *ad libitum*.

3.3.2   General setup

The setup consisted of a 20 cm x 1 cm long paper-covered runway which was connected to the colony's nest box via a 40 cm long drawbridge (figure 3-1A). A 5 mm diameter drop of sucrose solution (Sigma-Aldrich) was placed on an acetate feeder (2 x 1.5 cm) at the end of the runway (60 cm from the nest).
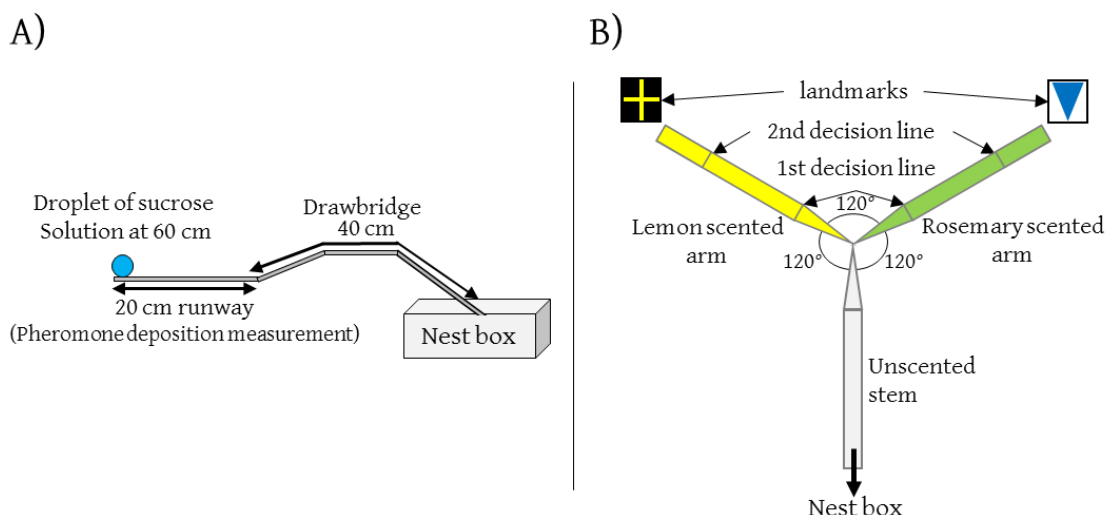

Figure 3-1: A) Setup used during training (visits 1 to 8) and on the 9th (test) visit of ants. The 20 cm long runway was connected to the nestbox via a 40 cm long drawbridge. The droplet of sucrose solution was

placed at the end of the runway (60 cm distance to the nest). All runways were covered with paper overlays while the overlay on the last 20 cm was replaced by pheromone-free ones each time an ant had walked over it. B) Y-maze used on the 10th visit of the ants to conduct a memory probe. All Y-maze arms were 10 cm long. The arm connected to the nest box was covered with unscented paper overlays while the other two arms were covered with lemon and rosemary scented paper overlays (one odour on each side). Visual cues (landmarks) were placed right behind the two scented Y-maze arms (at a distance of about 5 cm from the Y-maze arm end, so that ants could not walk onto it). The first decision line was located 2 cm from the y-maze centre and marked the initial decision of an ant while the second decision line was placed 8 cm from the centre and marked the final decision.

To begin an experiment, the colony was connected to the runway via the drawbridge. Two to four ants were allowed onto the runway, and the first ant to reach the feeder was marked with a dot of acrylic paint on its abdomen. The marked ant was allowed to drink to repletion at the food source, while all other ants were returned to the nest. As the ant drank at the droplet it was given one of three food acceptance scores, following Wendt et al. (2019). Full acceptance (1) was scored when the ant remained in contact with the drop from the moment of contact and did not interrupt drinking within 3 seconds of initial contact (see ESM: Appendix B video B1). Partial acceptance (0.5) was scored if feeding was interrupted within 3 seconds after the first contact with the food source, but the ant still filled its crop within 10 minutes (as can be seen by the distention of the abdominal tergites). Ants that interrupt feeding within the first seconds after contacting the food usually show successive feeding interruptions and generally show a rather 'impatient' behaviour compared to ants that show a food acceptance score of 1 (see ESM: Appendix B video B2). Lastly, rejection (0) was scored if the ant refused to feed at the sucrose solution and either returned to the nest before filling its crop or failed to fill its crop within 10 minutes. Regardless of whether the ant had drunk to satiety or failed to feed to repletion within 10 minutes, it was allowed to freely return to the nest at any time. Inside the nest, the ant unloaded the collected sugar load to its nestmates and was then allowed back onto the runway for another visit. The drawbridge connecting the nest with the runway leading to the food source was now used to selectively allow only the marked ant onto the runway by lowering it only for the marked ant and quickly raising it once the marked ant had moved onto the bridge. As an additional measure of perceived value, we counted the pheromone depositions the ant performed on the way to and from the feeder. Individual pheromone deposition behaviour correlates with the (perceived) quality of a food source (Beckers et al. 1993; Czaczkes et al. 2018b; Hangartner 1970; Wendt et al. 2019). Individual ants can adapt the strength of a pheromone trail by either depositing pheromone or not, or varying the intensity of pheromone depositions (Hangartner 1970; Beckers et al. 1993). Pheromone deposition behaviour in *Lasius niger* is highly stereotypic. To deposit pheromone, an ant briefly interrupts running to bend its abdomen and press the tip onto the ground (Beckers et al. 1992a). This allows the strength of a pheromone trail to be

quantified by counting the number of pheromone depositions over the 20 cm runway leading to the feeder. Pheromone depositions were measured each time the ant moved from the food source back to the nest (inward trip), and each time the ant moved from the nest towards the food source (outward trip). Because *Lasius niger* foragers almost never lay pheromone when they are not aware of a food source (Beckers et al. 1992a), we did not measure pheromone depositions for the very first outward trip (visit 1). The presence of trail pheromone on a path depresses further pheromone deposition (Czaczkes et al. 2013a). Thus, each time an ant had passed the 20 cm runway, the paper overlay covering the runway was replaced by a fresh one. All experimental runs were recorded with a Panasonic DMC-FZ1000 camera to allow for later video analysis.

3.3.3   Experimental Procedure

3.3.3.1   Overview

Ants were trained to associate a high sucrose molarity (1.5M) with one odour, and a low molarity (0.1M) with a different odour. Then, in the testing phase, odours were placed on the runway to trigger an expectation of either high or low molarity, which was then contrasted with a medium (0.387M) solution containing one of the learned odour cues. The molarity of the medium quality solution (0.387M) was chosen because we wanted to present the ants with identical relative increases in sucrose molarity. 0.1M was chosen as the low food quality as it is suggested to be the minimum sucrose concentration which *L. niger* ants reliably detect, distinguish, and accept (Detrain and Prieur 2014). 1.5M was chosen as the high food quality as acceptance scores plateau after 1.5M (Wendt et al. 2019).

3.3.3.2   Detailed methods

Training to associate food quality with odour cues took place over eight visits. The quality of the sucrose solution offered at the end of the runway alternated each visit, always beginning with the low quality solution. The solutions were scented using either rosemary or lemon essential oils (0.5 µl essential oil per ml sucrose solution, rosemary: *Rosmarinus officinalis*; lemon: *Citrus limon*, Markl GbR, Grünwald). Molarities were presented along with the same odours throughout a whole training run (i.e. 1.5M presented with rosemary and 0.1M presented with lemon). For half of the trained ants the 1.5M solution was scented with lemon and the 0.1M with rosemary, while for the other half 1.5M was scented with rosemary and 0.1M with lemon. In addition, to support learning and to allow solution quality anticipation (Czaczkes et al. 2018b), the paper overlays covering the runway leading to the feeder were also scented. Paper overlays

were scented by storing them for at least one day in an airtight box containing a drop of essential oil on filter paper in a Petri dish. Finally, in addition to odours cueing sucrose molarity, visual cues were also provided at the end of the runway. These consisted of printed and laminated pieces of paper (22 x 16.5 cm, figure 3-1B) displayed at the end of the runway, directly behind the sucrose droplet. Each odour was presented along with another visual cue (a yellow cross on black background when lemon odour was presented and a blue triangle on white background when rosemary odour was presented, figure 3-1B). Runways were scented on both outbound and inbound visits of the ant.

On the 9th (test) visit, the odour and visual cue associated with either 1.5M or 0.1M were presented, while the sucrose solution provided was of intermediate (0.387M) quality, but also scented according to the runway odour. Runway odours in the test visit were varied systematically between ants, but each ant was confronted with only one of the two runway odours coupled with scented 0.387M sucrose. This resulted in a balanced experimental design in which half of the ants were confronted with the odour associated to 1.5M (for half of the ants, the odour was lemon, for the other half rosemary) and the same flavour in a 0.387M sucrose drop on the 9th visit, and the other half was confronted with the odour associated to 0.1M sucrose and the same flavour in 0.387M sucrose. These methods are nearly identical to those used in Wendt et al. (2019) in which contrast effects in ants are reported. The key difference is that in Wendt et al. (2019) odours were presented only on the runway on the 9th (test) visit, but not in the food. In contrast, in the current study the medium quality food (0.387M) was scented in addition to presenting the associated odours on the runway leading to the food source.

Previous work has shown that *L. niger* foragers can form robust expectations of upcoming reward quality based on runway odour after four visits to each odour/quality combination (Czaczkes et al. 2018b; Wendt et al. 2019). Nonetheless, to ensure that learning had taken place, we carried out a memory probe at the end of each training and test run, i.e. on the 10th visit. The linear runway was thus replaced with a Y-maze (figure 3-1B), with two 10 cm long arms and a 10 cm long stem. The Y-maze stem was covered with an unscented paper overlay while one arm was covered with the 1.5M-associated odour overlay, and the other with the 0.1M-associated odour overlay. The matching visual cues were placed directly behind the relevant Y-maze runways (ca. 5 cm from the end of the Y-maze arms, so that ants could not walk onto it and escape from the setup). Trained ants were allowed to walk onto the Y-maze on their 10th visit (after 8 training visits and the 9th test visit) and their Y-maze arm choice was noted. We used two decision lines to define Y-maze arm choice – an initial decision line (figure 3-1B, 2 cm after the bifurcation) and a

final decision line (8 cm after the bifurcation). 98.5% of ants chose the side in the Y-maze which was covered in an odour previously associated to high molarity food and thus made a correct decision, suggesting that ants had successfully formed an association between both given sucrose/odour combinations (1.5M with one odour and 0.1M with another odour) and showed a strong preference for the odour previously associated to high molarity. Furthermore, on the 9th (test) visit, ants deposited significantly more pheromone when presented with a high quality associated odour on the runway on their way to the food source compared to when the runway was impregnated with a low quality associated odour (see figure S2-1B in supplement S2.1). Pheromone depositions towards the high quality odour increased with increasing experience with the food source during training, while they decreased for the low quality odour (see figure S2-1A in supplement S2.1). This shows that ants were able to associate a given odour to a food quality and formed a robust expectation of upcoming food qualities based on the odour. After testing on the Y-maze, the ants were permanently removed from the colony.

3.3.4   Statistical Analysis

Statistical analyses were carried out in R v. 3.5.0 (R Core Team 2016) using Generalized Linear Mixed Models (GLMMs) in the LME4 package (Bates et al. 2014) to analyse first interruption times, total drinking times and pheromone depositions data and Cumulative Link Mixed Models (CLMMs) in the ordinal package (Christensen 2015) to analyse food acceptance scores. CLMMs were used to analyse the acceptance data since we used an ordered factor with three levels (1 = full acceptance, 0.5 = partial acceptance, 0 = rejection).

As multiple ants were tested per colony, colony identity was added as a random effect to each model. GLMMs were tested for fit, dispersion and zero inflation using the DHARMa package (Hartig 2017). The model predictors and interactions were defined *a priori*, following Forstmeier and Schielzeth (2011). All p-values presented were corrected for multiple testing using the Benjamini–Hochberg method (Benjamini and Hochberg 1995). A total of 70 ants were tested – 34 with low quality associated cues and 36 with high quality associated cues.

3.3.4.1   Food acceptance data

Model formula slightly differed depending on the experimental phase (training = visits 1 to 8, test = visit 9). Fixed factors used for statistical analysis of the training phase were "presented molarity" (1.5M or 0.1M) interacting with the "visit number" (1 to 8). Visit number was brought into the model as an interaction with presented molarity, because molarities were presented in

an alternating order, always starting with low molarity on the first visit. Because individual ants were tested multiple times, we included AntID nested in colony as a random factor for statistical analyses of the training visits.

We used the following model formula for statistical analysis of the training visits:

FoodAcceptance ~ Molarity * scale(visit) + (random factor: colony/AntID)

The fixed factor used for statistical analysis of the test visit was "high or low molarity associated odour cues" (odours were associated to 1.5M and 0.1M during the training phase).

This resulted in the following model formula for the test visit:

FoodAcceptance ~ AssociatedMolaritytoOdour + (random factor: colony)

3.3.4.2   First Interruption Times, Total Drinking Times & Pheromone Deposition Data

The total drinking times and pheromone deposition data were analysed using a GLMM with a Poisson distribution for: total drinking time during the test visit, and first interruption times and pheromone depositions for the training phase and the test visit. Total drinking times of the training phase were tested with a negative binomial distribution to receive a better model fit.

Model formula again slightly differed depending on the experimental phase (training = visits 1 to 8, test = visit 9). Fixed factors used for statistical analysis of the training phase were "presented molarity" (1.5M or 0.1M) interacting with the "visit number" (1 to 8). Visit number was brought into the model as an interaction with presented molarity, because molarities were presented in an alternating order, always starting with low molarity on the first visit. Because individual ants were tested multiple times, we included AntID nested in colony as a random factor for statistical analyses of the training visits.

We used the following model formulas for statistical analysis of the training visits:

FirstInterruptionTime ~ Molarity * scale(visit) + (random factor: colony/AntID), distribution = poisson

TotalDrinkingTime ~

Molarity * scale(visit) + (random factor: colony/AntID), distribution = negative binomial

NumberPheromoneDepositions ~

Molarity * scale(visit) + (random factor: colony/AntID), distribution = poisson

The fixed factors used for statistical analysis of the test visit were "high or low molarity associated odour cues" (odours were associated to 1.5M and 0.1M during the training phase) and the used odours (rosemary or lemon).

This resulted in the following model formulas for the test visit:

FirstInterruptionTime ~

AssociatedMolaritytoOdour + Odour + (random factor: colony), distribution = poisson

TotalDrinkingTime ~ AssociatedMolaritytoOdour + Odour + (random factor: colony), distribution = poisson

NumberPheromoneDepositions ~

AssociatedMolaritytoOdour + Odour + (random factor: colony), distribution = poisson

3.4   Results
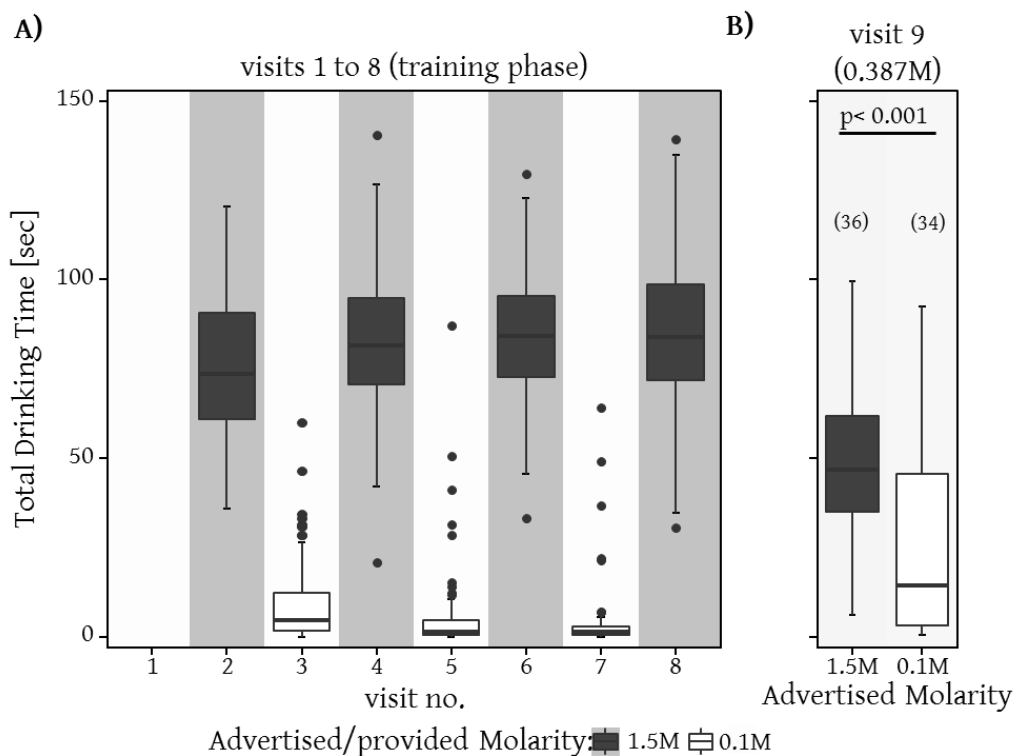
Figure 3-2: A) & B) Total Drinking Time in seconds for A) the eight training visits (visits 1-8) in which ants received 0.1M coupled with one odour and 1.5M coupled with another odour in an alternating order, always starting with 0.1M, B) the test visit (visit 9) in which ants always received 0.387M sucrose solution. Both the sucrose solution and the runway leading towards the food source were impregnated with one of the learned odours, triggering an expectation towards receiving either high or low molarities at the end of the runway. There is no data for total drinking time of the first visit displayed, because ants were sometimes

disturbed when marking them, occasionally resulting in unclear feeding patterns. Shown are the median, the 75%/25% quantiles, and the range of total drinking time for each visit. Sample sizes for the 9th visits of both experiments are displayed in parentheses of B).

During training (visit 1 to 8), ants showed a higher total drinking time in seconds when confronted with the high molarity than when confronted with the low molarity (*GLMM:* estimate = -3.02, z = -41.81, p < 0.001, *OR* = -3.02, 95% C.I. [-3.16, -2.88], figure 3-2A). On the 9th (test) visit, the quality indicated on the runway and in the medium quality (0.387M) food strongly affected total drinking times. Drinking times were significantly higher when high-quality associated odours were present than when low-quality odours were present (median drinking time with high molarity cues: 44.63 seconds, low molarity cues 21.38 seconds, *GLMM:* estimate = -1.18, z = -4.47, p < 0.001, *OR* = -1.18, 95% C.I. [-1.7, -0.66], figure 3-2B).

Because we believe that a direct comparison of the current study to those of Wendt et al (2019) is important in order to fully explain the experimental differences included to counteract contrast effects, we reproduce the key results of Wendt et al (2019) in figure 3-2C and figure 3-5C.
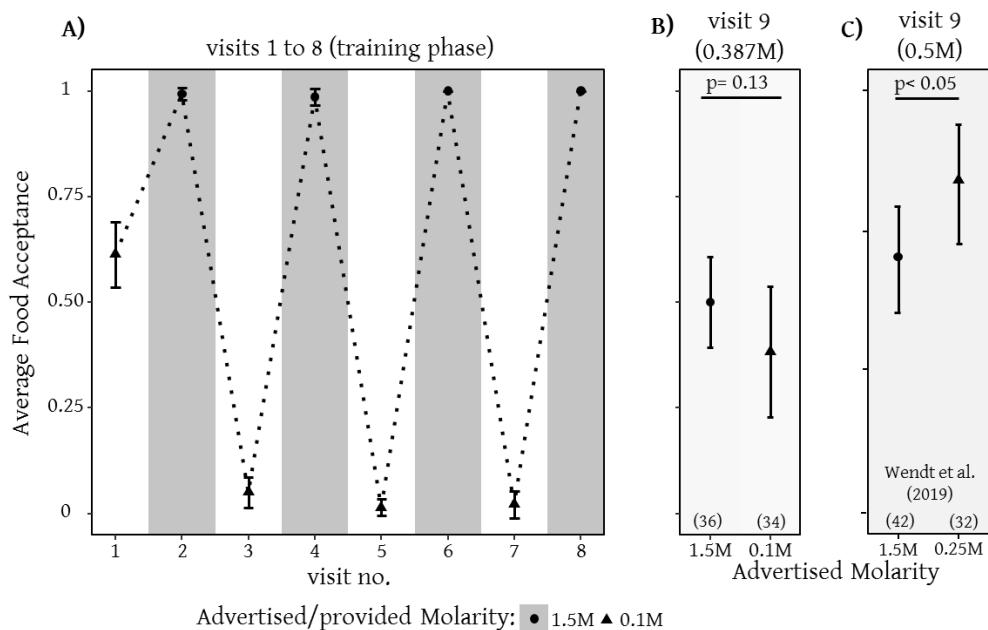
Figure 3-3: Average food acceptance for A) the eight training visits (visits 1-8) B) the 9th (test) visit and C) the 9th (test) visit of ants tested in Wendt et al. (2019) in which only the runway, but not the medium quality food (0.5M) was impregnated with learned odours. Shown are the mean food acceptance (points) and the 95% confidence intervals (error bars) for each visit. Sample sizes for the 9th (test) visits of both experiments are displayed in parentheses of B) and C).

Acceptance scores during training mirrored total drinking times, with ants showing a significantly higher food acceptance when confronted with the high molarity than when

confronted with the low molarity (*CLMM:* estimate = -4.54, z = -4.97, p < 0.001, *OR:* 95% C.I. [-12.99, -9.11], figure 3-3A). However, even though food acceptance scores were higher on average when high quality food was advertised, they did not differ significantly in the 9th (test) visit between the two advertised qualities (*CLMM:* estimate = -0.69, z = -1.51, p = 0.13, *OR:* 95% C.I. [-1.62, 0.18], figure 3-3B). This is in contrast to the pattern found in Wendt et al. (2019), in which ants exposed to 1.5M-associated cues during the 9th (test) visit showed significantly lower food acceptance towards the unscented 0.5M feeder than ants exposed to 0.25M-associated cues (*CLMM:* estimate= 1.07, z= 2.15, p= 0.03, *OR:* 95% C.I. [0.08, 2.08], figure 3-3C).
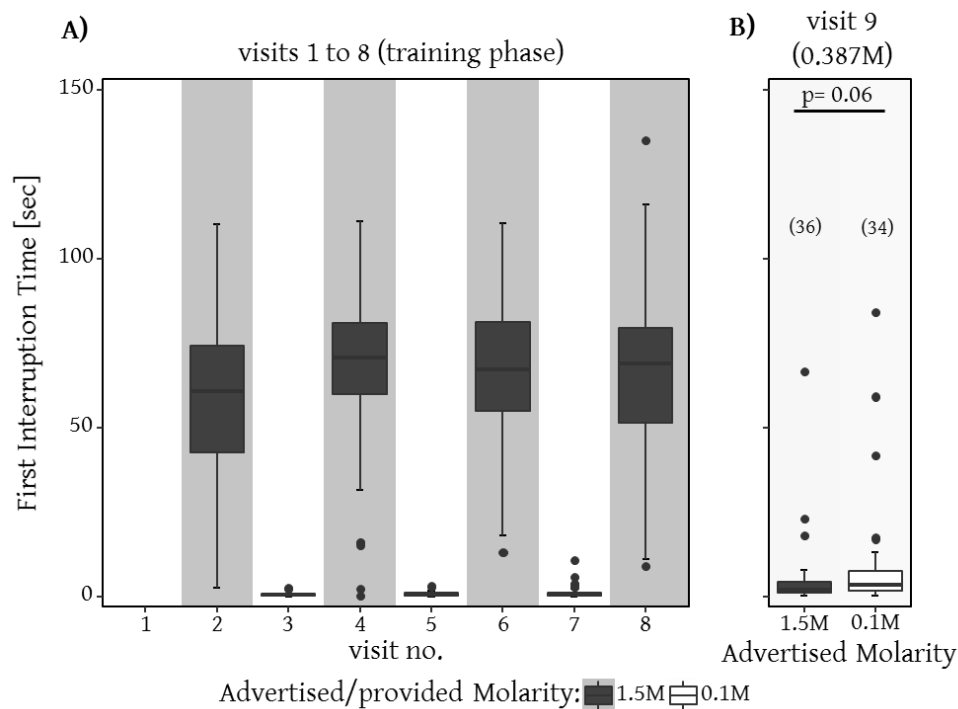
Figure 3-4: A) & B) First interruption time in seconds for A) the eight training visits and B) the 9th (test) visit. See figure 3-2 legend for details.

First interruption times also mirrored acceptance scores and total drinking times during training, with higher first interruption times for the high quality food (*GLMM:* estimate = 0.53, z = 3.60, p < 0.001, *OR* = -5.21, 95% C.I. [-5.45, -4.97], figure 3-4A). In the 9th (test) visit, there was a strong tendency towards ants showing lower first interruptions times for medium quality food advertised as high quality (*GLMM:* estimate = 0.71, z = 1.88, p = 0.06, *OR* = 0.71, 95% C.I. [-0.03, 1.46], figure 3-4B).
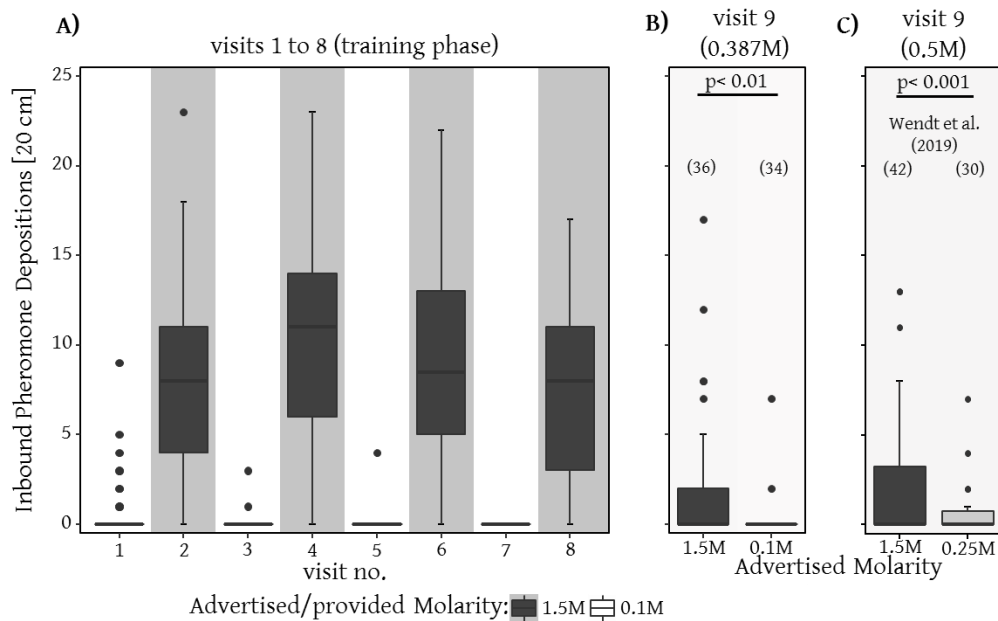
Figure 3-5: Pheromone depositions towards the nest [20 cm] (inbound pheromone depositions) for A) the eight training visits (visits 1-8), B) the 9th (test) visit and C) the 9th (test) visit of ants tested in Wendt et al. (2019) in which only the runway, but not the medium quality food (0.5M) was impregnated with learned odours. Shown are the median number of pheromone depositions on the measured 20 cm track on the way back to the nest and the 75%/25% quantiles for each visit.

Finally, pheromone depositions when returning from the feeder to the nest also mirrored the other measured variables, with higher pheromone deposition for higher quality (*GLMM: estimate = -1.58, z = -5.28, p <0.001, OR = -5.46, 95% C.I. [-6.27, -4.65], figure 3-5A*). On the 9th (test) visit, advertised food quality affected pheromone deposition, with ants depositing more pheromone having consumed medium food advertised as high quality (*GLMM: estimate = -3.19, z = -3.24, p < 0.01, OR = -3.19, 95% C.I. [-5.12, -1.26], figure 3-5B*). However, note that ants deposited much less pheromone on the return from the 9th (test) visit than on training visits (*GLMM: estimate = 1.15, z = 9.37, p < 0.001*). The data reported are similar to Wendt et al. (2019). Ants experiencing 1.5M-associated cues during the 9th visit – provided only through runway odours towards the food, but not in the food – showed a significantly higher number of pheromone depositions on their return than ants exposed to 0.25M-associated cues (*GLMM: estimate= -1.36, z= -5.50, p<0.001, OR = -1.65, 95% C.I. [-2.72, -0.58], figure 3-5C*).

3.5   Discussion

Ants spent more time feeding at medium quality (0.387M) sucrose solution when it was accompanied by a flavour previously associated to high molarity food compared to a flavour

associated to low molarity food (figure 3-2B). The number of pheromone depositions performed after feeding on medium quality food was also significantly higher when ants returned to the nest from 0.387M accompanied with high quality flavour compared to low quality odour, suggesting assimilation effects both in the feeding times and inbound pheromone depositions. Food acceptance scores, although not significant, displayed a similar pattern (figure 3-3B): Ants showed higher acceptance of the medium quality food when it was presented along with a high molarity odour. Thus, ants reacted differently to food sources of identical sucrose solution, depending on the associative cue presented before, during, and after consumption.

However, ants also showed some evidence of contrast effects in the first few seconds after finding the food. The time until first feeding interruption at medium quality food was almost twice as long when ants expected low quality compared to expecting high quality food (p=0.06), suggesting that the medium quality food was initially perceived as better when ants expected to find poor food, and vice versa (figure 3-4B).

Finally, also as in Wendt et al. (2019), there was a significant difference in pheromone deposition depending on the ants' expectations (figure 3-5B & C), with ants experiencing good food-associated cues depositing on average more pheromone than those experiencing poor food cues. However, our experimental manipulation seems to interfere with pheromone laying, and the number of pheromone depositions is generally so low on the 9th (test) visit that the difference does not seem biologically meaningful.

Wendt et al. (2019) showed clear contrast effects in terms of food acceptance, where expectations caused an inversion in perception, so that high expectations caused an undervaluing of medium quality, and vice versa for low expectations. Here, with the minor addition of odour in the food, we eliminated the acceptance contrast effects reported in Wendt et al. (2019), and even found the reverse pattern in terms of drinking times (figure 3-2B), indicating an assimilation effect: if a label was present in the food indicating high quality during consumption, the perceived quality of the food increased. The assimilation effect can be very clearly seen in the total drinking time data (figure 3-2B), and also, though to a lesser extent, in the pheromone deposition data (figure 3-5B). Hovland et al. (1957) argued that assimilation effects are likely to occur in humans when the expectation is not very different from reality, whilst contrast effects are more likely to occur when the expectation is very different from reality. The results of this study together with those of Wendt et al. (2019) support this assumption in ants as well. The presence of an associated odour during consumption leads to a higher similarity

between expectation and experience in the current experiment, in turn leading to assimilation rather than contrast effects, which were shown in the previous study (Hovland et al. 1957).

We argue that this is directly analogous to the labelling effect described in humans. There, brand labels are based upon an accumulation of associative cues, which have been linked to a label affecting perceived value (Levin et al. 1998; Macklin 1996; Mao et al. 2013; van Osselaer and Janiszewski 2001). For example, our social environment has led us to assign a negative value to fat and a partly negative value to soy. Humans tend to assign greater value to meat advertised as 75% lean compared to when it was advertised as containing 25% fat (Levin et al. 1998). Soy as a product label decreases the perceived quality of taste while at the same time increasing the perceived healthiness of a product (Wansink 2000). In this case, the label forms an expectation about taste and healthiness mostly based on cultural biases and advertisements. Products can thus be paired with already positively or negatively associated labels to influence costumers' perception of product value. Just as humans prefer to purchase a brand with which they have previously had positive experiences over a novel brand (Russo et al. 1996), ants may also be affected by a familiar food label (associated odour), which previously offered positive (or negative) experiences, and may thus be more (or less) likely to "buy" a novel medium-quality food source if it is presented with the familiar odour cue.

Our findings extend those of Oberhauser & Czaczkes (2018), who trained *Lasius niger* workers to a 1M food source presented along with either lemon or rosemary odour. After training, ants received a food source of identical quality, but presented with an unfamiliar odour. Ants showed significantly lower food acceptance towards the unfamiliar odour. There, as in this study, it is likely that the naturally value-neutral odour cue gained an associated value, which affected value perception. Once the associated cue was missing, the reward lost part of its assigned value, leading to contrast effects, as also shown in Wendt et al. (2019). We propose that in the current experiment the odour on the runway and the taste of the food are playing different roles in the ant's evaluation process: the odour is signalling what to expect, setting a reference point against which the measure of value obtained during feeding is contrasted. The taste is adding an associated value (positive or negative) during feeding, which is added to the objective sensory measure of food quality to form the complete measure of value obtained during feeding. This is then contrasted against the ant's expectation. The results of this study support the prediction that the presence of an associative cue during food consumption affects value perception, and that it can counteract expectations – even if the expectations and the associations are triggered by the same cue.

However, in some ways the odour cues used in this study may not be directly analogous to brand labels affecting perceived value in humans. Odours often have important innate biological meanings, whereas brand labels in humans are naturally value neutral and biologically meaningless. Indeed, ants strongly rely on odour cues both in navigation (Czaczkes et al. 2014b; Josens et al. 2009; Oberhauser et al. 2019; Provecho and Josens 2009; Roces 1994) and nestmate recognition (Akino et al. 2004; Brandstaetter et al. 2008; Sturgis and Gordon 2012). However, since *Lasius niger* ants mainly feed on honeydew (Detrain et al. 2017; Devigne and Detrain 2002; Völkl and Mackauer 1993; Völkl et al. 1999) it is unlikely that a positive value is assigned to the odours used in this study (rosemary and lemon), even though they may be naturally available to ants. Previous studies show no innate preference for either of the odours (Oberhauser and Czaczkes 2018). In contrast to odours, the value assigned to brand labels in humans comes from social knowledge and marketing, not solely from direct experience. The cues used in this study may thus not be the completely equivalent to brands and food labels used in human studies, but seem to be the best alternative for investigating value distortion effects in ants. We demonstrated an assimilation effect driven by labels in feeding time and recruiting behaviour of ants (and a tendency in food acceptance scores), mirroring effects found in humans. In contrast, ants showed weak contrast effects in first interruption times. This study shows that the time of cue presentation strongly influences its effect on perceived value in ants. When odours where presented *before* food consumption, ants showed clear contrast effects (Wendt et al. 2019). Presenting odours *during* food consumption, however, reversed the perceived value of a medium quality food source, resulting in assimilation (this study). The fact that we see weak contrast effects during the first reactions of ants (first interruption times), but not later during or after feeding (total drinking time and inbound pheromone depositions) furthermore suggests that contrast effects can be acted against by presenting a label during consumption and that this change in value perception happens while the label is presented, and not through expectations induced before food consumption or through other factors. Because contrast effects can be seen in the first interruption times, but not in the acceptance scores which measure the first three seconds of an ant's response, we argue that a food label gradually counteract and overwhelm contrast effects over the course of several seconds. To further pin down contrast and assimilation effects, future studies may wish to investigate consummatory effects without first inducing contrast effects. This would give a clear picture of ant responses to food labels alone, and allow for a clearer interpretation of both contrast and assimilation effects, because assimilation effects will likely be stronger when odour labels do not have to overcome contrast effects.

The evidence presented in this study adds to prior studies showing parallel value-distorting effects in humans and insects, including decoy effects (Sasaki and Pratt 2011; Tan et al. 2014; 2015), risk aversion (De Agrò et al. 2019; Shafir et al. 1999; Shapiro 2000; Waddington et al. 1981), discounting (Cheng et al. 2002; Wendt and Czaczkes 2017) and expectation-driven valuation (Bitterman 1976; Couvillon and Bitterman 1984), suggesting that insights into human behaviour can, in part, be transferred to insects. Insect-based comparative psychology studies allow much tighter control over experimental subjects and conditions, offering stringent tests of basic insights from human psychology, and the experimental flexibility to test hypotheses untestable on human subjects. We hope that our work inspires consumer psychologists and behavioural economists to consider insects as a viable model system in which to test their underlying assumptions and thinking, in order to gain deeper insights into both human and animal behaviour.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All animal treatment guidelines applicable to ants under German law have been followed.

Chapter 4

Negative feedback: Ants choose unoccupied over occupied food sources and lay more pheromone to them

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4.1   Abstract

In order to make effective collective decisions, ants lay pheromone trails to lead nestmates to acceptable food sources. The strength of a trail informs other ants about the quality of a food source, allowing colonies to exploit the most profitable resources. However, recruiting too many ants to a single food source can lead to over-exploitation, queuing, and thus decreased food intake of the colony. The non-linear nature of pheromonal recruitment can also lead colonies to become trapped in suboptimal decisions, if the environment changes. Negative feedback systems can ameliorate these problems. We investigated a potential source of negative feedback: whether the presence of nestmates makes food sources more or less attractive. *Lasius niger* workers were trained to food sources of identical quality, scented with different odours. Ants fed alone at one odour. At the other odour ants fed either with other feeding nestmates, or with dummy ants (black cuticular hydrocarbon-coated glass beads). Ants avoided food sources at which other nestmates were present. They also deposited less pheromone to occupied food sources, suggesting an active avoidance behaviour, and potentiating negative feedback. However, ants did not avoid the food associated with dummy ants, suggesting that cuticular hydrocarbons and static visual cues alone may not be sufficient for nestmate recognition in this context. This effect may prevent crowding at a single food source when other profitable food sources are available elsewhere, leading to a higher collective food intake. It could also potentially protect colonies from becoming trapped in local feeding optima.

4.2   Introduction

Distributing labour and coordinating collective tasks is a challenge faced by both social insects and human societies. A critical task is to allocate effort to where it will be most productive, while avoiding crowding consequences and queuing costs. This must be achieved without centralised control in both social insect societies, and in many human endeavours (e.g. distributed computation, telecommunication networks). Social insects have developed numerous strategies to inform nestmates about valuable food sources, allowing collective exploitation of resources in the surrounding environment. For example, honeybees can share information about the direction and distance of food sources with other nestmates via the waggle dance (von Frisch 1965). Both the duration and the number of waggle runs increase when bees dance for higher food qualities, thus increasing the likelihood that foragers are recruited to better resources (Seeley et al. 1991; 2000). In ants, information about distance and direction is not directly shared with nestmates. Instead, many ant species deposit pheromone when returning from a resource, such as a food source or new nest site. These pheromone trails lead other nestmates to newly discovered food sources. The more pheromone ants lay when returning from a food source, the stronger the trail will be. Ants can modulate both their decision to deposit pheromone, and the intensity of pheromone deposition (Beckers et al. 1993; Hangartner 1970). Both trail lay rates and pheromone deposition intensity increase as the perceived value of the resources increases (Beckers et al. 1992a; Detrain and Deneubourg 2008; Wendt et al. 2019). Stronger trails result in more ants being recruited from the nest and a higher proportion of ants following the trail at a bifurcation (Hangartner 1970; von Thienen et al. 2015; Wilson 1962). This simple system results in a positive feedback mechanism, leading to colonies often collectively focusing their foraging effort on the most valuable resources (Beckers et al. 1990; 1993; Czaczkes et al. 2016; Frizzi et al. 2018; Latty and Beekman 2013).

However, recruiting too many nestmates to a food source can lead to crowding and an overexploitation of food sources, and thus decrease colony food intake (Burd 1996; 2000). For example, although crowding increases foraging efficiency in leaf-cutter ants (Dussutour et al. 2007), it decreases walking speed of ants affected by head-on collisions (Burd and Aranwela 2003) and can have negative effects in other species as well. Many natural food sources are limited by quantity and replenishment time. For example, honeydew-producing aphids or extrafloral nectaries slowly produce a variable amount of food over the course of a day (Fischer et al. 2005; Völkl et al. 1999). If a resource is fully exploited, recruiting more individuals will lead to increased waiting times and foragers returning to the nest without food. An optimal distribution of foragers

would allocate foragers to a resource until the efforts of any additional forager would be better focused on a different resource.

Decentralised decision making in non-limited situations also poses challenges for positive-feedback based coordination systems. If positive feedback is non-linear, as is the case in mass-recruiting ants which deposit pheromone trails (Detrain and Deneubourg 2008; Sumpter and Beekman 2003), recruitment can rapidly become extremely strong to one option. If the environment then changes, colonies may not be able to break out of their previous decision and become trapped in exploiting a temporal local optimum (Beckers et al. 1990; 1992b; Czaczkes et al. 2016; Goss et al. 1989), but also see (Dussutour et al. 2009).

The problems of overexploitation and crowding, and of trapping in local optima, can be ameliorated or overcome by building negative feedback into the collective decision making system. Social insects have developed a number of negative feedback systems which decrease the number of recruited nestmates as recruitment progresses. These systems include both active and passive processes (Kietzman and Visscher 2015; Czaczkes et al. 2013b). Honeybees, for example, use an acoustic signal as an active inhibitory stop signal, stopping returning foragers from recruiting (Kietzman and Visscher 2015; Kirchner 1993; Nieh 1993; 2010; Pastor and Seeley 2005; Thom et al. 2003). Ants reduce pheromone deposition when walking on a pheromone laden path and when encountering other nestmates on the trail (Czaczkes et al. 2013b; 2013a).

In addition to active recruitment signals, social insects also rely on cues when deciding where to forage. A very important cue for a wide variety of animals is the presence of fellow foragers, both con- and heterospecific (Clark and Mangel 1984; Krebs et al. 1972). The presence of conspecifics provides information about the safety and productivity of a foraging patch. Naïve bumblebees, for example, prefer to visit food sources at which other bees are present (Avarguès-Weber and Chittka 2014; Kawaguchi et al. 2007; Leadbeater and Chittka 2007; Worden and Papaj 2005). Ants show a similar behavioural pattern, preferentially choosing to follow paths on which other nestmates are present (Czaczkes et al. 2015b). This is somewhat at odds with the finding that ants reduce recruitment in the presence of others ants (Czaczkes et al. 2013b; 2013a). This highlights a trade-off foragers have to make: well-used patches imply productivity and safety, but also competition for resources and potential over-exploitation.

The aim of this study was twofold: Firstly, we ask whether the presence of nestmates at a food source (as opposed to on a trail, Czaczkes, Grüter, and Ratnieks 2013) triggers a negative-feedback effect by reducing recruitment. Secondly, we ask whether unoccupied food sources are

more attractive than otherwise equally profitable occupied food sources. We trained individual ants to two alternating food sources associated with different odours. At one food source, ants fed alone. At the other, either live nestmates or black glass beads coated with nestmate cuticular hydrocarbons (CHCs) were present at the food source. After training, odour preference was tested. If nestmate presence has an inhibitory effect in this context, the ants should follow the odour associated with feeding alone, and deposit less pheromone when returning from occupied food sources. In contrast, if nestmate presence enhances the attractiveness of a food source, ants should prefer the odour associated with the presence of nestmates or CHC-coated glass beads, and deposit more pheromone when returning from occupied feeders.

4.3   Methods

4.3.1   Study animals

Eight stock colonies of the black garden ant *Lasius niger* were collected on the University of Regensburg campus. The colonies were housed in 30x30x10cm foraging boxes with a layer of plaster covering the bottom. Each box contained a circular plaster nest box (14 cm diameter, 2 cm height). The colonies were queenless with around 1000-2000 workers and small amounts of brood. Queenless colonies forage and lay pheromone trails, and are frequently used in foraging experiments (Detrain et al. 2019; Dussutour et al. 2004). The colonies were fed with 0.5M sucrose solution and received *Drosophila* fruit flies once a week. Water was available *ad libitum.* Colonies were starved for four days prior to the experiments in order to achieve a uniform and high motivation for foraging (Mailleux et al. 2006; Josens and Roces 2000). During starvation, water was available *ad libitum.*

4.3.2   Setup & Experimental Procedure

4.3.2.1   Training

Two to four ants were given access to a 20 x 1 cm long plastic runway overlaid with scented paper via a 40 cm long drawbridge. A 5mm diameter drop of 1M sucrose solution (Sigma-Aldrich) was placed in the centre of a feeding platform (4 x 4 cm;) surrounded by a water barrier (figure 4-1, 1.75 cm wide and 1.3 cm deep, platform size including the surrounding water barrier: 7.5 x 7.5 cm) at the end of the runway (60cm from the nest). The first ant to reach the feeder was marked with a dot of acrylic paint on its abdomen. The marked ant was allowed to drink to

repletion at the food source, while all other ants were returned to the nest. When the ant had filled its crop, it was allowed to walk back into the nest. Inside the nest, the ant unloaded its crop to its nestmates and was then allowed back onto the runway for another visit. The drawbridge was now used to selectively allow only the marked ant onto the runway.

The ant was allowed to make 8 return visits to the feeder, with alternating odour cues on each subsequent visit: in half of the visits, ants were allowed to feed alone in the presence of one odour. In the other visits, ants fed together with either i) 5 other nestmates or ii) 5 black CHC-coated glass beads (dummy ants which were placed in a semicircle around the sucrose droplet at a distance of 5 mm) in the presence of a second odour (see figure 4-1, see below for dummy ant creation details). Companion nestmates were gently placed onto the feeding platform shortly before the test ant arrived by allowing them to walk onto a piece of paper, and walk off the paper onto the platform. They displayed no signs of alarm behaviour and fed calmly at the food source when they discovered it. It is thus unlikely that they emitted alarm pheromones which may have led to the test ant avoiding this food source (Nonacs 1990). Mimicking nestmates with dummy ants allowed us to control for tactile stimuli and pheromone which may be deposited on the runway or the feeding platform by foraging or returning nestmates. Previous work has shown that *Lasius niger* foragers can form robust expectations of upcoming reward quality based on runway odour after just 1 visit to each odour/quality combination (Wendt et al. 2019; Czaczkes et al. 2018b; Oberhauser et al. 2019). Sucrose solutions and the runway overlays were scented, with the 'nestmate' and 'alone' treatments each having a fixed odour. The solutions were scented using either rosemary or lemon essential oils (0.5μl essential oil per ml sucrose solution, rosemary: *Rosmarinus officinalis*; Lemon: *Citrus limon*, Markl GbR, Grünwald). For half of the ants, lemon was associated with the 'nestmate' treatment and rosemary with the 'alone' treatment, and vice versa for the other ants. Paper overlays were scented by storing them for at least one day in an airtight box containing a droplet of essential oil on filter paper in a petridish.

As the ant returned to the nest from the food source, we counted the number of pheromone depositions performed. Individual pheromone deposition behaviour correlates with the (perceived) quality of a food source (Beckers et al. 1993; Czaczkes et al. 2018b; Hangartner 1970; Wendt et al. 2019). Individual ants can adapt the strength of a pheromone trail by either depositing pheromone or not, or varying the intensity of pheromone depositions (Hangartner 1970; Beckers et al. 1993). Pheromone deposition behaviour in *Lasius niger* is highly stereotypic. To deposit pheromone, an ant briefly interrupts running to bend its gaster and press the tip onto the ground (Beckers et al. 1992a). Pheromone depositions were measured each time the ant

moved from the food source back to the nest (inward trip), and each time the ant moved from the nest towards the food source (outward trip). Because *Lasius niger* foragers almost never lay pheromone when they are not aware of a food source (Beckers et al. 1992a), we did not measure pheromone depositions for the very first outward trip (visit 1). The presence of trail pheromone on a path depresses further pheromone deposition (Czaczkes et al. 2013a). Thus, each time an ant had passed the 20 cm runway, the paper overlay covering the runway was replaced by a fresh one.
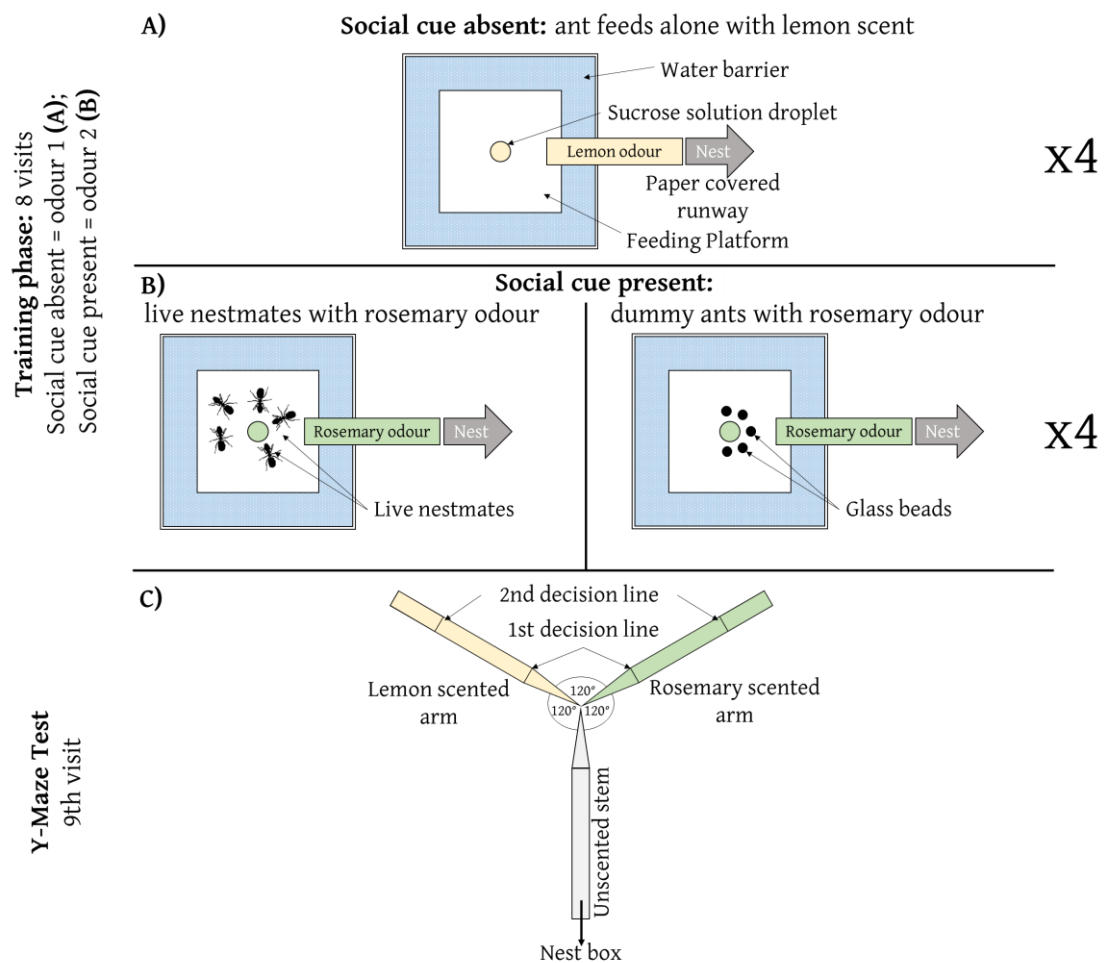
Figure 4-1:A) & B) Experimental Setup used during training visits 1 to 8. A 1M sucrose solution droplet was placed in the centre of a platform surrounded by a water barrier. The platform was connected to the nest via a paper-covered 20 cm long runway and a 40 cm drawbridge. A) Shows the platform for visits on which the social cue was absent. In this case, sucrose solution was presented with one odour (e.g. lemon) on the runway and in the food. B) Shows the platform for visits on which the social cue was present. In this case, sucrose solution was presented with another odour on the runway and in the food (e.g. rosemary). Half of the tested ants were confronted with live nestmates as a social cue, the other half was confronted with dummy ants (black CHC-coated glass beads). Social cue presence (and the associated odours) alternated each visit. C) Y-maze used on the 9th (test) visit. All arms were 10 cm long. The arm connected to the nest box was covered with unscented paper overlays while the other two arms were covered with lemon and

rosemary scented paper overlays (one scent on each side). The first decision line was located 2 cm from the Y-maze centre and marked the initial decision of an ant while the second decision line was placed 8 cm from the centre and marked the final decision.

4.3.2.2   Choice tests

On the 9th visit (the testing phase), the linear runway was replaced with a Y-maze (figure 4-1C), with two 10cm long arms and a 10cm long stem. The Y-maze stem was covered with an unscented paper overlay while one arm was covered with the odour overlay associated to the social cue present (e.g. rosemary), and the other with the odour overlay associated to the social cue absent (e.g. lemon). The trained ant was allowed to choose between the two arms, and its decision was recorded. We used two decision lines to define arm choice – an initial decision line (figure 4-1C, 2 cm after the bifurcation) and a final decision line (8 cm after the bifurcation). After an ant had made a choice, it was allowed to walk onto a piece of paper at the end of the Y-maze arm and moved to the beginning of the Y-maze in order to allow it to make another choice. This was repeated until an ant had made 3-10 choices in the Y-maze. The number of choices made by an ant depended on its motivation to make another choice, but was limited to a maximum of 10 choices.

After each experimental run the ant was permanently removed from the colony.

4.3.2.3   Preparation of Dummy Ants

To simulate the presence of other nestmates, we used black glass-beads (dummy ants) coated in nestmate cuticular hydrocarbons (CHCs). CHC profiles differ between colonies and allow ants to identify nestmates and distinguish them from non-nestmates (Sturgis and Gordon 2012). CHC-coated glass beads are regularly used to mimic nestmates (Czaczkes et al. 2013b; 2014a; Greene and Gordon 2003) and non-nestmates (Akino et al. 2004; Guerrieri et al. 2009; Ozaki et al. 2005) in ants, including *Lasius niger*. Bead preparation followed Czaczkes et al. (2013b): Clean black glass beads were first washed with pentane multiple times, then baked for 1-2 hours at 300 °C and again washed with pentane after baking to remove any substances or odours which may interfere with nestmate identification. To coat the beads in ant CHC-profiles, twenty workers out of the colony to be tested were refrigerated at -20 °C for about 10 minutes. The ants were then placed in a 2 ml extraction vial (Sigma Aldrich) and covered in pentane. To dissolve the cuticular hydrocarbons from the ants' cuticle, the vial was agitated for 5 minutes at 30 °C. After this, the ants were removed from the pentane solution containing ant CHCs and 8 black glass beads (diameter 2.3 mm, height 1.5 mm) were placed into the solution instead. The solution and beads were then

again agitated at 30 °C until all the pentane had evaporated. This procedure left the beads coated in CHCs.

A pilot aggression test revealed that CHC-coated beads elicited aggressive behaviour such as mandible opening (Guerrieri and d'Ettorre 2008) when they were coated with non-nestmate CHCs, while no aggressive behaviour was shown when beads were coated with nestmate CHCs. This suggests that beads were sufficiently coated to allow ants to recognize them as other ants and differentiate between nestmates and non-nestmates.

4.3.3   Statistical Analysis

Statistical analyses were carried out in R v. 3.5.0 (R Core Team 2016) using Generalized Linear Mixed Models (GLMMs) in the LME4 package (Bates et al. 2014). As multiple ants were tested per colony, and we took multiple measurements from each ant, colony identity and individual ant identity were added as random effects to each model. GLMMs were tested for fit, dispersion and zero inflation using the DHARMa package (Hartig 2017). The model predictors and interactions were defined *a priori*, following Forstmeier and Schielzeth (2011). All p-values presented were corrected for multiple testing using the Benjamini–Hochberg method (Benjamini and Hochberg 1995). A total of 49 ants was confronted with real nestmates as a social cue, making a total of 278 choices. In the bead treatment, a total of 43 ants was tested, of which 248 choices were made.

4.3.3.1   Choice tests

The initial and final choice of the ants matched in 92.4% of choices, so for simplicity we only considered final choices in the statistical analysis. Choice preference was tested using a GLMM with a binomial distribution. We included the fixed factors social cue type (nestmates or CHC-coated glass beads), the odour associated to the social cue (lemon or rosemary, to test for innate odour preferences), the side of the social cue odour in the Y-maze (right or left, to test for a side bias) and a binomial factor indicating whether ants were confronted with a social cue or fed alone on the first training visit in order to test for primacy and recency effects (Lipatova et al. 2006). This resulted in the following model formula:

FinalDecision ~

SocialCueType + SocialCueOdour + SocialCueSide + SocialCueFirst + (random factor: Colony/AntID)

Additionally, we conducted an exact binomial test of choice behaviour against a null hypothesis of 0.5. This test was run for both social cue types (nestmates and CHC-coated glass beads) separately to test whether Y-maze choices were different from a random choice.

4.3.3.2   Inbound pheromone depositions during training

Inbound pheromone deposition behaviour was analysed using GLMMs with a poisson distribution. First, we attempted to predict pheromone deposition using the fixed factor social cue type in interaction with whether social cues were present that visit and a scaled visit variable included to model changes in pheromone deposition over subsequent visits. This resulted in the following model formula:

Complete Model:

InboundPheromoneDepositions ~

SocialCueType * SocialCuePresence + VisitNumber + (random factor: Colony/AntID)

As the interaction was not significant (see results), but social cue presence had a significant effect on ant choices and visual inspection of the data showed a clear difference between ants confronted with live nestmates and those confronted with dummy ants, we ran two further models in order to explore the data in more detail. We thus subsetted the data according to social cue type This resulted in the following model formula:

Split by SocialCueType:

InboundPheromoneDepositions ~ SocialCuePresence + VisitNumber + (random factor: Colony/AntID)

4.4   Results

4.4.1   Choice tests
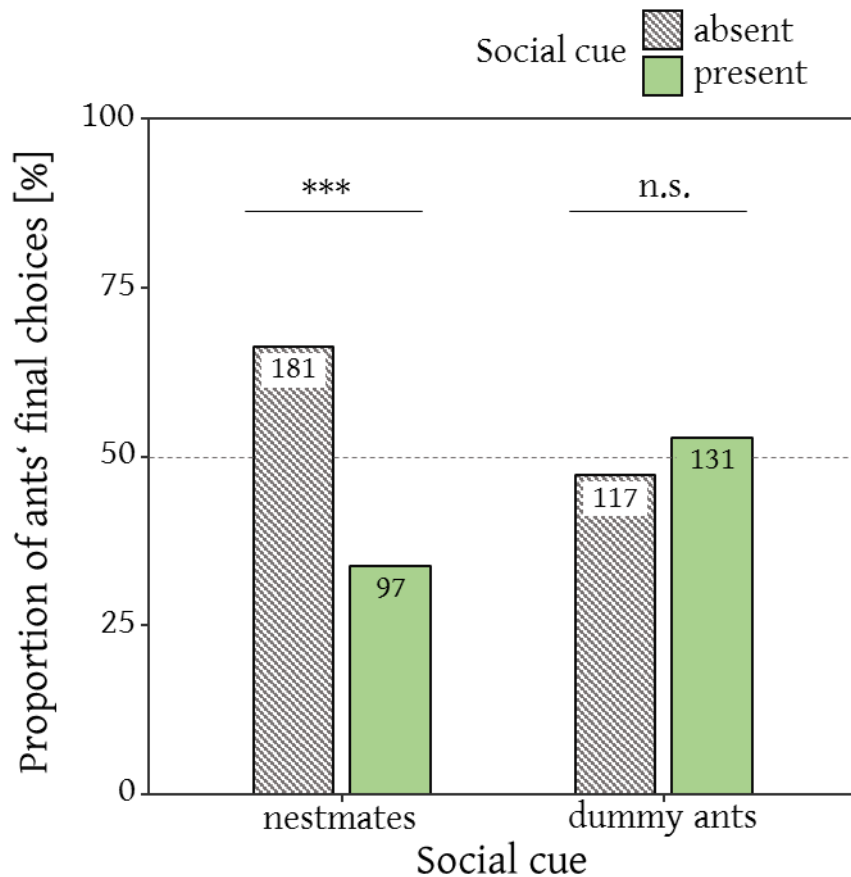
Figure 4-2: Proportions of all final choices made by ants in the Y-maze. Ants fed with either real nestmates or with dummy ants (black CHC-coated glass beads) when the social cue was present and fed alone when the social cue was absent. 49 ants were trained with real nestmates and 43 with dummy ants. Numbers in the bars represent sample sizes (individual choices). *** = p < 0.001, n.s. = p > 0.05.

Only 34.8% (97 of 278) of choices in the Y-maze were made for the odour previously associated with the presence of other nestmates, which is significantly different from chance (figure 4-2, *exact binomial test* testing vs $H_0 = 0.5$: p< 0.001). By contrast, when the social cue was dummy ants, 52.8% (131 of 248) of choices were made for the arm containing the social-associated cue, which does not differ from chance (p = 0.41). Social cue type had a significant effect on ant choices, with ants being more likely to choose the social cue side in the bead treatment compared to the nestmate treatment (*GLMM*: estimate= 0.84, z= 3.13, p< 0.01). A significant effect of the first

presentation of the social cue during training was also found. Ants were significantly more likely to choose the social cue side in the Y-maze when the social cue was first presented on the first training visit compared to the second training visit (*GLMM*: estimate = 0.89, z = 4.11, p < 0.001). Furthermore, ants showed side and odour biases with significant preferences for lemon odour and the left side in the Y-maze (odour preference *GLMM*: estimate = -0.61, z = -2.71, p < 0.01; side preference *GLMM*: estimate = -0.69, z = 3.19, p < 0.01).

4.4.2   Inbound Pheromone Depositions during training
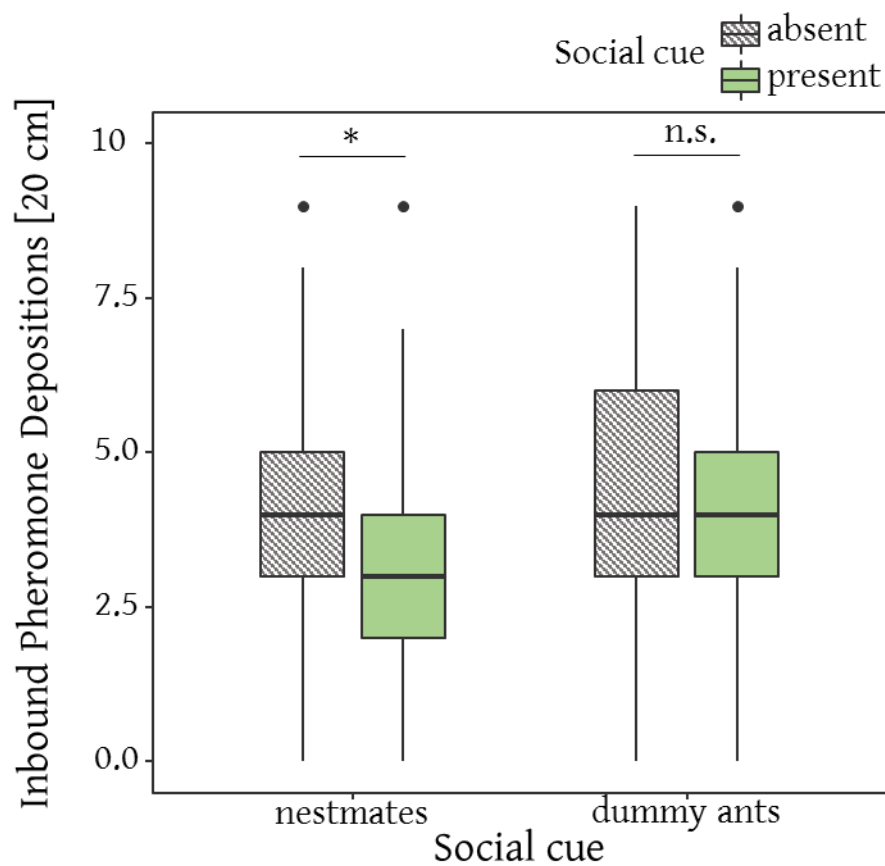
Figure 4-3: Number of pheromone depositions deposited during the way back to the nest on a 20 cm track right behind the feeding platform. Horizontal lines are medians, boxes are along with interquartile ranges, whiskers are 5%/95% ranges and dots are outliers. * = p < 0.01, n.s. = p > 0.05.

The GLMM analyzing the complete dataset indicated a significant effect of the social cue presence on the number of pheromone depositions: ants deposited significantly less pheromone when returning from a food source at which a social cue (live nestmates or dummy ants) was

present (*GLMM*: estimate = -0.13, z = -2.39, p < 0.05). Ants further deposited significantly more pheromone on later training visits (*GLMM*: estimate = 0.05, z = 2.76, p < 0.05).

We did not find a significant effect of social cue type (*GLMM*: estimate = 0.1, z = 1.76, p = 0.10) or the interaction between social cue type and social cue presence (*GLMM*: estimate = 0.11, z = 1.53, p = 0.13) on choice. However, ants deposited significantly less pheromone when a social cue was present (*GLMM*: estimate = -0.13, z = -2.38, p < 0.05) and visual inspection of the data clearly showed a difference between ants confronted with live nestmates and those confronted with dummy ants. We thus examined the data more closely by splitting it by social cue type and ran another GLMM to check for the effect of social cue presence for each of the two social cue types respectively. Ants deposited significantly less pheromone when returning from a food source at which other nestmates were present compared to when they fed alone (*GLMM*: estimate= -0.13, z= -2.4, p< 0.05, figure 4-3). However, when beads were used as a social cue, there was no significant effect of social cue presence on the number of pheromone depositions during the ants' return to the nest (*GLMM*: estimate= -0.01, z= -0.2, p= 0.84, figure 4-3). The order of social cue presentation did not have a significant effect on number of pheromone depositions in both treatments (*GLMM* for live nestmates treatment: estimate = 0.03, z = 0.52, p = 0.8; *GLMM* for dummy ant treatment: estimate = 0.08, z = 1.23, p = 0.26).

4.5   Discussion

Ants showed a significant preference for food sources at which they fed alone over food sources at which other ants were feeding, and also deposited more pheromone when returning from solitary feeding (figure 4-2 and figure 4-3) However, cuticular-hydrocarbon-coated beads failed to elicit this effect.

These results demonstrate that ants actively avoided feeding at already occupied food sources and recruited more heavily to unoccupied food sources. The results further suggest that the attractiveness of a food source is not solely based on direct traits such as sugar concentration, flow rate or distance to the nest (Beckers et al. 1993; Fewell et al. 1992; Josens et al. 1998; Schilman and Roces 2003), but can also be affected by the status of occupancy and most likely also by other indirect traits. The reduction of pheromone depositions on crowded trails has already been described in *Lasius niger* ants (Czaczkes et al. 2013b). In addition to a similar effect from occupancy at the food source, here we report an apparent aversion to occupied food sources. Both behaviours may combine and lead to the exploitation of numerous valuable food sources in the

environment rather than overexploiting only one good food source. This may counteract the tendency of the positive-feedback component of the ant recruitment system to result in only choosing one option, a phenomenon termed symmetry breaking (Beckers et al. 1990; Czaczkes 2014; de Bisaeu et al. 1991; Grüter et al. 2012; Sumpter and Beekman 2003). This may be beneficial for two reasons: Firstly, overexploitation can lead to queuing at the food source and slower travel speed due to crowded trails (Burd 1996; 2000; Burd and Aranwela 2003). A reduction of pheromone strength on already occupied trails and preference for unoccupied food sources may lead to a more evenly distributed food exploitation and thus a higher colony-level food intake. Secondly, an aversion to occupied food sources may act as a negative feedback system, preventing colonies from becoming trapped in local foraging optima. Non-linear positive feedback systems in general, and pheromone-mediated recruitment, particularly in ants, can result in such a strong recruitment that the system cannot react to changing environments. Thus, if an ant colony is allowed to forage extensively at a good food source, and then the quality of the food is reduced, colonies often fail to refocus their foraging effort to newly available, better food sources (Beckers et al. 1990; Czaczkes et al. 2016; de Bisaeu et al. 1991; Sumpter and Beekman 2003). The negative feedback system we describe may be an effective method of mitigating these effects, especially in combination with other such effects (Czaczkes 2014; Grüter et al. 2012).

However, the presence of only 5 nestmates at a relatively large ($\varnothing$ 5mm) food source might not be reasonably considered as a crowded food source. Furthermore, the presence of nestmates at a food patch may serve as an indicator for a safe and productive food source which is worth exploiting and should be concentrated on while it is not yet completely crowded (Czaczkes et al. 2015b). Why then do the ants reduce exploitation of such food sources? In Czaczkes et al.'s study (2015b), even though ants downregulated pheromone depositions on crowded trails, colonies showed a clear preference for paths on which dummy ants were present compared to control paths. The authors argue that the presence of nestmates and a simultaneous absence of alarm pheromones on a path inform foragers that the path is safe and productive and is thus preferred over one at which nestmates are absent (Czaczkes et al. 2015b). Furthermore, colonies may benefit from an increased information transfer and recruitment potential upon usage of paths at which nestmates are present (Dussutour et al. 2007; Czaczkes et al. 2015b; Farji-Brener et al. 2010; Roces 1990; 1994). Why then do ants reduce their pheromone deposition and preference for a food source which is occupied by just a few nestmates? Nestmate density at a food source may be an indicator for how many foragers are already potentially available to exploit a food source and may also inform ants about whether additional nestmates should be recruited (Jarau and Hrncir

2009). Given the positive-feedback nature of recruitment in these ants, even a few present nestmates suggest that this food source will soon be well occupied. Foragers with experience of other, unoccupied, food sources could thus concentrate on recruiting to other food, or on scouting for new food sources. These foragers may thus accept the risk of feeding alone at a newly discovered food source until more nestmates have been recruited. Such scouting ants have been described in various ant species (Breed et al. 1987; Chadab and Rettenmeyer 1975; Jaffe and Howse 1979; Mailleux et al. 2006). A similar pattern was reported in foraging bumblebees: Bees which were experienced with a food source avoided occupied food sources, but naïve bees preferred them (Kawaguchi et al. 2007). The behavioural pattern reported here and in Czaczkes et al. (2015b) can also be seen in this light: In the current study, we trained individual ants to food sources over the course of 8 visits, allowing them to become familiar with the food, odour and nestmate presence or absence. In contrast, Czaczkes et al. (2015b) investigated path preference of complete colonies in which a number of recruited ants unfamiliar with available food sources followed a pheromone trail. These naïve ants would be more likely to visit occupied food sources, while informed ants would rather avoid them.

The fact that dummy ants (black CHC-coated glass beads) did not elicit a decrease in recruitment strength and food attractiveness suggests that the mere presence of a nestmate odour may not be sufficient for nestmate recognition in this context. Although CHC-coated glass beads have successfully been used in previous studies on recruitment behaviour (Greene and Gordon 2007), including in *Lasius niger* (Czaczkes et al. 2013b; 2014a), the lack of other stimuli such as movement, home-range markings (Depickère et al. 2004; Detrain and Deneubourg 2009; Devigne and Detrain 2002), or feeding signals (Bouchebti et al. 2015; Hölldobler and Wilson 1990; Roces 1994; Roces and Hölldobler 1996) may have caused the ants to underestimate the local density of ants, or have prevented them from being perceived as nestmates.

Ants showed a stronger preference for the odour associated to a no social cues when it was first experienced on the first training visit, with only 34.1% of choices for the the odour at which a social cue was present. In contrast, if the social cue was first presented on the first training visit, 51.8% of choices were for the Y-maze side covered in social cue odour. This strongly suggests a primacy effect, in which memory of the first-exposed cue is stronger than memory of a cue experienced later (Pineño and Miller 2005; Wright et al. 1985; Wright and Roediger 2003). Ants also chose the social cue odour more often when it was associated to lemon odour and placed on the left side of the Y-maze, suggesting an innate odour preference for lemon over rosemary odour and a side bias. Side biases especially have been widely reported in ants and other animals

(Buchanan et al. 2015; Cooper et al. 2011; Frasnelli 2013; Glick and Ross 1981; Guo et al. 2009; Hunt et al. 2014; Kight et al. 2008; Stancher et al. 2006). However, as our treatments were fully balanced by treatment presentation order, neither the primacy effect nor the innate biases should interfere with our interpretation.

We demonstrate that negative feedback is not only elicited by crowded paths in *Lasius niger,* but also through nestmate presence during food consumption. Moreover, ants also prefer food sources without fellow foragers. The avoidance of already occupied food sources allows ants to distribute their foraging effort and exploit multiple food sources simultaneously. This can result in a more efficient exploitation of the environment (Czaczkes et al. 2015a). In addition, such behaviour may increase the amount of information about available food sources and may thus increase the colony-level food intake rate. Moreover, such negative-feedback systems may play a critical role in maintaining collective flexibility and preventing trapping in local optima. Taken together, the combination of passive negative feedback from reduced pheromone deposition, and active negative feedback via occupied food avoidance, may be a powerful mechanism for increasing collective foraging efficacy.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All animal treatment guidelines applicable to ants under German law have been followed.

Chapter 5

Individual ant workers show self-control

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5.1   Abstract

Often, the first option is not the best. Self-control can allow humans and animals to improve resource intake under such conditions. Self-control in animals is often investigated using intertemporal choice tasks – choosing a smaller reward immediately, or a larger reward after a delay. However, intertemporal choice tasks may underestimate self-control, as test subjects may not fully understand the task. Vertebrates show much greater apparent self-control in more natural foraging contexts and spatial discounting tasks than in intertemporal choice tasks. However, little is still known about self-control in invertebrates. Here, we investigate self-control in the black garden ant *Lasius niger*. We confront individual workers with a spatial discounting task, offering high quality reward far from the nest and poor quality reward closer to the nest. Most ants (69%) successfully ignored the closer, poorer reward in favour of the further, better one. However, when both the far and the close rewards were of the same quality, most ants (83%) chose the closer feeder, indicating that the ants were indeed exercising self-control, as opposed to a fixation on an already known food source.

5.2   Introduction

Self-control – the ability to choose a large delayed reward over a small immediate one – is an important feature of human behaviour (Logue 1988). A lack of self-control, also called impulsivity, is said to be a central factor in many human problems, such as failures at school, depression and criminal tendencies (Moffitt et al. 2011).

Research on apes, monkeys and ravens suggests that they, like humans, can show good self-control (Osvath and Osvath 2008; Evans and Westergaard 2006; Kabadayi and Osvath 2017). However, many other animal species, such as pigeons and rats, were shown to have poor self-control unless they had received extensive training before testing (Ainslie 1974; Tobin and Logue 1994). Many of these experiments were carried out using laboratory protocols which may have had little ecological meaning for the animals tested. In pigeons, for example, self-control was often examined using intertemporal choice tasks (Grosch and Neuringer 1981) in which subjects must choose between an immediate small and a larger reward later. Hayden (2016) suggests that animals which were shown to behave impulsively in such intertemporal choice tasks did not understand the task of waiting for a better reward, and may have been attempting to maximise food intake per unit time (Kagel et al. 1986). To prevent animals from choosing the smaller reward in order to proceed to the next trial faster, a post-reward delay, equalizing the length of both small and large reward trials, is often added after small rewards. Nonetheless, animals may still not understand the task unaided. Pearson et al. (Pearson et al. 2010) tested monkeys in an intertemporal choice task, adding visual cues to show the length of delays. Monkeys with access to such cues showed less impulsivity than monkeys which had no information about delay times. These results suggest that many experiments testing self-control in animals may overestimate impulsivity (Hayden 2016).

Spatial discounting tasks may be a more ecologically appropriate test of self-control in animals. Here, an animal must choose between a small reward nearby and a larger reward further away (Stevens et al. 2005). For example, Cheng et al. (Cheng et al. 2002) trained honeybees to find a small reward (10 µl syrup) at the entrance of a box, and a larger (*ad libitum*) reward 15 cm further away. After extensive training on both feeders, the bees were offered both simultaneously. Most bees preferred the larger reward and thus showed self-control in a foraging context. A similar temporal discounting task, where bees had to wait 5 seconds for sweeter food, showed similar results. However, this study of self-control in invertebrates is not without drawbacks. The sample

sizes were as low as 5 individuals for some treatments, and data were expressly excluded to emphasize self-control, making it difficult to draw firm conclusions.

Another study about self-control in insects investigated collective rather than individual behaviour (Franks et al. 2008). *Temnothorax albipennis* colonies were induced to abandon their nest and choose a new one. Colonies were given the choice between a far good nest and a closer poor one. Ant colonies collectively chose the far, good nest in almost all trials, even when it was more than nine times further away, showing 'collective' self-control. However, the behaviour of individuals cannot be inferred from behaviour of colonies. In the same species, colonies chose bad nests when tandem running is used, but predominantly chose the best option without tandem running (Burns et al. 2016). Effects like these make a comparison of individual and colony behaviour difficult. More broadly, the average behaviour of animals is a poor representation of the behaviour of individuals (Pamir et al. 2011).

Here, we investigate whether hungry *Lasius niger* foragers can ignore a food source they would normally exploit, if they are aware of a higher quality food source elsewhere. *L. niger* learns the quality and location of food sources relatively quickly (Grüter et al. 2011), which makes it easy to conduct spatial discounting experiments without excessive training.

5.3   Material and Methods

5.3.1   General Methods

An ant foraged at a feeder (1M sucrose) located either at the end of a 120cm runway or at 70cm distance from the nest (see figure 5-1 and treatment description). On the ants' second visit to the food source, an additional, closer sucrose droplet of varying molarity (1M, 0.75M or 0.25M depending on treatment) was introduced at 60cm from the nest. We then observed whether the ant drank at the near feeder (scored 0), the original feeder (scored 1), or at both feeders (scored 0.5).
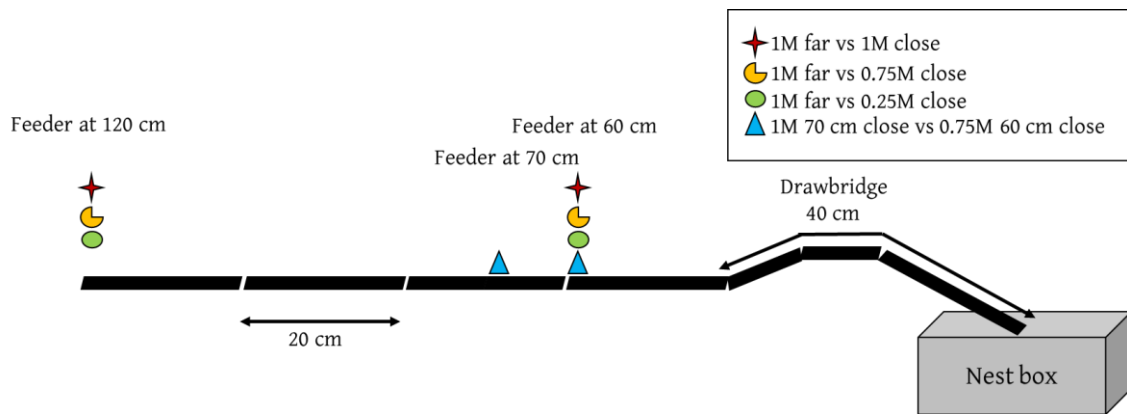
Figure 5-1: Experimental setup for the 4 possible treatments. Each unbroken segment is 20cm long and covered with paper stripes which can be replaced with pheromone-free paper stripes. The sucrose droplets were located at 120 or 70cm (1M) and 60cm (1M, 0.75M or 0.25M) from the nest, depending on treatment.

5.3.2   Treatments

We conducted 4 treatments, which varied in the location of the first feeder (1M at 120 or 70cm) and the molarity of the second feeder (1M, 0.75M or 0.25M at 60cm).

In the first treatment, the initial feeder (1M) was placed at 120cm from the nest while the second feeder (0.25M), which was introduced on the ants' second visit, was located at half the distance of the original feeder (60cm).

The second treatment was identical to the first treatment, except that the new feeder offered 1M sucrose. In the third treatment, it provided 0.75M sucrose.

In treatment 4, which served as a last control treatment, the first (1M) feeder was placed only 70cm from the nest and the second feeder (0.75M) was placed 60cm from the nest.

Finally, we repeated the '1M far vs 0.25M close' and '1M far vs 1M close' treatments, but allowed the ants to visit the far feeder twice before presenting the near feeder.

For detailed methods, see supplement S3.1.

5.4   Results
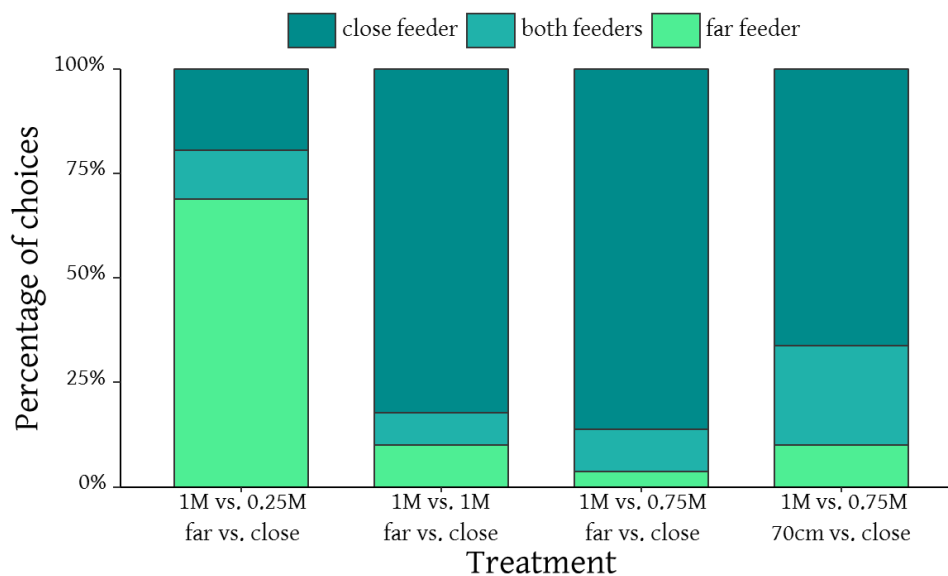
Figure 5-2: Proportions of decisions made for the different feeders for all treatments. The close feeder was located 60cm from the nest, the far-away feeder was located either 120cm or 70cm (for treatment 1M at 70cm vs. 0.75M) from the nest. Treatment is named as molarity of the far-away feeder vs. molarity of the close feeder. Sample sizes are as follows: 1M vs. 0.25M: n= 77; 1M vs. 1M: n= 79; 1M vs. 0.75M: n= 80; 1M at 70cm vs. 0.75M: n= 80. Decisions in 1M vs. 0.25M significantly differed from the other treatments. Decisions in 0.75M vs. 1M close significantly differed from 0.75M vs. 1M. The other treatments were not significantly different from each other.

69% of ants successfully rejected a close, low quality (0.25M) food source at 60cm when a higher quality (1M) food source was available at 120cm (see figure 5-2). By contrast, when the food sources were of equal quality (1M), only 10% of ants rejected the closer food source. However, most ants (96%) failed to reject the closer food source when it was of only marginally lower quality (0.75M) than the 1M food source at 120cm. Even when the farther, better food source was only 10cm further than the close food source most ants (66%) failed to reject the slightly poorer, slightly closer food source. More ants rejected the closer feeder in the 1M vs 0.25M than in the other three treatments (Ordered Linear Regression, $z < 7.21$, $p < 0.001$, see Table S1 in ESM: supplement ESM3.1 for pairwise comparisons). Additionally, ants in the 0.75M vs. 1M at 70cm treatment were significantly more likely to reject the closer feeder than ants in the 0.75M vs. 1M treatment ($z = -2.777$, $p < 0.01$). Finally, there were no significant differences in the rate of close feeder rejection between the 1M vs. 1M & 1M vs 0.75M ($z = 0.3777$, $p > 0.05$), and 1M vs. 1M & 1M vs. 0.75M at 70cm treatments ($z = -0.7464$, $p > 0.05$).

An additional training visit strengthened the reaction of the ants which had to choose between a closer poor feeder (0.25M) and a far-away better feeder (1M). With two training visits before testing, 97% of tested ants successfully rejected the poorer feeder (see figure S3-1). These differences are significant (z = 2.399, P = 0.16, see supplement S3.3). With an additional training visit, 83% of ants which were confronted with 1M at 120cm and 1M at 60cm chose the closer feeder. There is no significant difference between one and two training visits for this treatment (for statistics please refer to ESM: supplement ESM3.1). All data are provided in the ESM.

5.5   Discussion

Self-control is the ability to choose a large delayed reward over a small immediate (Logue 1988). Here we showed that individual *Lasius niger* workers can avoid consuming a low-quality reward earlier in order to exploit a known higher-quality food source later, but can successfully choose a closer food source if its quality is identical to the farther food source. This demonstrates that individual ants exhibit good self-control.

Ants failed to reject slightly poorer food even if the higher quality food source was very close. This strongly suggests that the foragers could not tell these food sources apart. This may be due to an inability to sense the molarity difference, a failure in distinguishing both food sources as different locations, or, since they only made one visit to the good food source, due to a poor representation of the good food source in their memory.

It seems counterintuitive that individual ants should show good self-control, while many vertebrates have been found to be impulsive. However, the conclusion of impulsivity from many vertebrate studies based on inter-temporal choice may be spurious. Vertebrates show a higher degree of self-control when tested in more natural foraging contexts (Blanchard and Hayden 2015).

Most animals try to maximize the food intake per unit time. For this reason, it may be misleading to describe animals as impulsive when they do not show self-control in an intertemporal choice task. Self-control experiments in animals should be performed with regard to the ecology of the studied animals. In unstable environments or environments which suffer from high predation risks, it may be advantageous to show impulsivity. Animals exploiting predictable environments may show good self-control. By testing ants in an ecologically sensible spatial discounting test, we demonstrated impressive self-control abilities in individual ant workers.

Conflict of interest

The authors declare no competing interests.

Ethical approval

All animal treatment guidelines applicable to ants under German law have been followed.

Chapter 6

Attempts at multiple cue conditioning in *Drosophila melanogaster*

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In preparation

Note: this chapter is a summary. For a detailed report, see supplement S4.

6.1   Abstract

Comparing multiple options and choosing the most profitable one is a challenge constantly faced by humans and animals. However, an option's profitability may change depending on the decision maker's current state. An options' value is thus not only rated based on its absolute value, but relative to the *status quo*, expectations, or former experience. Such relative perception of value was recently demonstrated in ants, which display higher acceptance of medium quality food when expecting low quality food and vice versa for high expectations. However, the genetic and neuronal mechanisms underlying relative value perception are still largely unknown. *Drosophila melanogaster,* with their short generation time and the broad array of available genetic tools, may be an ideal model for investigating the molecular basis of relative value perception. Here, we present multiple attempts to condition flies to expect either high or low quality food based on associated odour cues, a prerequisite for investigating relative value perception and its underlying mechanisms. However, none of the conditioning methods used led to a preference of one odour, suggesting that flies either cannot associate different food qualities to different odours, or that the approaches were not suitable for reliable olfactory conditioning in this system. For that reason, we suggest potential directions for future attempts at investigating relative value perception in *Drosophila.*

6.2   Introduction

Almost all organisms are constantly faced with multiple options, the challenge of comparing them to each other, and finally choosing among them. Understanding how options are compared has thus been central to the study of behavioural economics. In humans, decisions are not necessarily made based on the absolute values of options, but can be relative to reference points such as the *status quo*, expectations or former experience (Kahneman and Tversky 1979; Parducci 1984; Tversky and Kahneman 1992; Ungemach et al. 2011; Vlaev et al. 2011).

Value judgments in animals are also influenced by factors apparently independent of the absolute value of options (Aw et al. 2009; 2011; Bitterman 1976; Couvillon and Bitterman 1984; Czaczkes et al. 2018a; Oberhauser and Czaczkes 2018; Pompilio et al. 2006; Richter and Waddington 1993; Roces 1993; Roces and Núñez 1993; Schuck-Paim et al. 2004). Studies investigating reference-dependent perception of value have often been conducted using the successive contrasts paradigm in which animals are trained to a quality or quantity of reward which is then suddenly increased (positive incentive contrast) or decreased (negative incentive contrast) (Bentosela et al. 2009; Bitterman 1976; Couvillon and Bitterman 1984; Crespi 1942; Flaherty 1982; 1999; Mustaca et al. 2000; Weinstein 1970b). Thus, the reference point upon which a newly given reward is valued in these studies is their expectation of an upcoming reward, often based on former experience with a food source. These lead to either negative incentive contrasts, often shown in form of a disruption in behaviour or behaviour akin to disappointment, if expectations were higher than the received reward (Bower 1961; Flaherty et al. 1985; Tinklepaugh 1928; Vogel et al. 1968), or positive incentive contrasts if expectations were low (Eisenberger et al. 1975; Flaherty 1999). Apart from mammals, including apes, monkeys, rats, and dogs (Bentosela et al. 2009; Brosnan and de Waal 2003; Crespi 1942; Flaherty 1999; Mustaca et al. 2000; Pellegrini and Mustaca 2000; Weinstein 1970a), contrast effects were also successfully described in bees and ants (Bitterman 1976; Couvillon and Bitterman 1984; Richter and Waddington 1993), also see chapter 2. For example, Bitterman (1976) found negative incentive contrast effects in honeybees which were trained to a high quality feeder and then received a downshift to a lower quality feeder. In contrast, bees which experienced an upshift in feeder quality did not show any feeding interruptions (Bitterman 1976; Couvillon and Bitterman 1984). However, care must be taken to ensure that the reported effects truly arise from psychological processes, and are not merely physiological or psychophysical in nature (for an overview of these alternative factors, please refer to chapter 2 (section 2.6). One possibility to control for these alternative factors is by training the study organism to food sources of high and low molarities accompanied with

different odours, so that they form robust expectations of upcoming rewards based on the odour cues, but undergo the same training procedure. The ability to remember past qualities, and compare the current options on offer to options stored in the memory, is a pre-requisite for incentive contrasts.

However, even though relative value perception and incentive contrast effects have been demonstrated in a broad variety of species, including a few insects, the underlying genetic and neuronal mechanisms of this behaviour are yet not fully understood. The fruit fly *Drosophila melanogaster,* with its well investigated genetic background and short generation time (Hadler 1964) may help to gain deeper insights into the mechanisms underlying relative value perception. It also offers a large pool of genetic mutants, some of them showing impairments in various different behaviours, including learning and memory (Dudai et al. 1976; Folkers et al. 1993; Kim et al. 2007; Sehgal et al. 1994; Siegel and Hall 1979; Tempel et al. 1983; Tully 1987). Over the last 30 years, about 80 genes that are potentially involved in olfactory memory have been identified (Keene and Waddell 2007), and using such genetically modified flies may help investigate the genetic and neuronal basis for relative value perception in insects (Kahsai and Zars 2011).

Learning and memory in *Drosophila* is usually investigated by classical as well as operant appetitive and aversive conditioning to, for example, different odours, shapes or colours. Adult flies can learn to approach rewarded odours (Kim et al. 2007; Tempel et al. 1983) and avoid punished stimuli such as odours (Quinn et al. 1974; Tully and Quinn 1985), colours (Brembs and Heisenberg 2001; Wolf and Heisenberg 1997) or shapes (Brembs and Heisenberg 2001). Various approaches are available for investigating decision making, learning, and memory in adult flies, including classical PER approaches (Shiraiwa and Carlson 2007), conditioning of freely moving flies (Tempel et al. 1983; Wustmann et al. 1996), navigation in flight simulators (Brembs and Heisenberg 2001) and egg-deposition (Yang et al. 2008). Freely moving flies, for example, can be conditioned to avoid one side of a small test chamber if it is heated whenever the fly enters this side (Wustmann et al. 1996).

Moreover, learning and value perception in *Drosophila* is demonstrably affected by factors apparently independent of an option's value. For example, flies display state-dependent judgement biases when allowed to choose between air and an ambiguous odour (a 1:1 blend of previously positively and negatively reinforced odours). Control flies which had not undergone any additional treatment after training approached the ambiguous odour more often than flies which were shaken to induce a purported negative state, suggesting that the 1:1 odour blend was

perceived more negative in disturbed flies compared to the undisturbed control (Deakin et al. 2018). Even more relevant to relative value perception, female flies display incentive contrasts in mate choice depending on their prior experience with males. Females become more (or less) likely to accept a mate of given quality if they have prior experience of better (or worse) mates (Dukas 2005), suggesting that incentive contrasts appear in foraging contexts as well. However, in these experiments a change in physiological state due to different mating experiences cannot be ruled out.

In order to investigate the underlying mechanisms of relative value perception in a foraging context, an experimental approach eliciting contrast effects while simultaneously avoiding alternative explanations for the observed effects must be found. This will require training flies to expect different reward levels depending on the conditional stimulus provided. Here, we present multiple approaches for training *Drosophila melanogaster* to predict the quality of upcoming rewards based on learned odour cues. Approaches presented here are either similar to previous studies in which flies successfully learned combinations of reward/punishment and odour (Chabaud et al. 2006; Fujita and Tanimura 2011; Kim et al. 2007; Quinn et al. 1974; Tempel et al. 1983), modified versions of these experiments, or similar to the training setup used in chapter 2. Finding a working paradigm would allow an investigation of whether *Drosophila melanogaster* shows relative value perception behaviour in the context of feeding which is a prerequisite for investigating the genetic and neuronal mechanisms underlying relative value perception.

6.3   Methods

In this study, multiple approaches to appetitive olfactory conditioning following both individual and group conditioning paradigms were used, and these will be presented in the following section.

6.3.1   Study animals

We used *Drosophila melanogaster* fruit flies (Wild Type Berlin; WTB) for all presented experiments. Flies were kept in 12 vials (7cm height, 2.5cm ⌀) and always bred from the same vial number to allow statistical control for possible mutations which may affect learning abilities (Dudai et al. 1976; Tempel et al. 1983). The vials were filled to one third with standard fly medium and closed with sponge plugs. The flies were kept at constant 25°C and a 12/12 hours light-dark-cycle (8am – 8pm). Vials were renewed once a week and hatched flies transferred to new vials

marked with the same number to continue breeding. Thus, the flies used for experiments were one to two weeks old, but were not selectively chosen for experiments by age nor sex. Flies were starved for 18 to 20 hours prior to experiments in order to maximize the learning and feeding motivation (Chabaud et al. 2006; Tempel et al. 1983; Fujita and Tanimura 2011). For starvation, flies were anesthetized with CO_2 for around 30 seconds and ca. 50 flies moved from the breeding vials into clean vials covered with common kitchen paper soaked in water.

6.3.2   Grouped Training in Vial and Block Slide Setups

Flies were trained to associate two sucrose molarities (0.1 and 1M) to different odours (rosemary and lemon or lemon and orange). One molarity was presented along with one of the given odours (e.g. rosemary with 0.1M) throughout a whole training procedure while the other molarity was presented along with the other odour (e.g. lemon with 1M). Odour-molarity pairs were presented in an alternating order, so that each pair was presented three times throughout training. Previous work has shown that *Drosophila melanogaster* flies can form robust expectations of upcoming rewards or punishments based on odours present in the air after 1-4 presentations of each odour/quality combination (Fujita and Tanimura 2011; Quinn et al. 1974; Tempel et al. 1983). Sucrose rewards were presented via filter paper which had been soaked in sucrose (0.1 or 1M) and then dried for 2 hours at 50°C until all water had evaporated. Sweetened filter papers were scented using either rosemary and lemon essential oils (rosemary: *Rosmarinus officinalis*; Lemon: *Citrus limon*, Markl GbR, Grünwald) or lemon and orange food flavours ("Mit allen 5 Sinnen", Grünwald). They were scented by either storing them for at least one day in an airtight box containing a droplet of essential oil (lavender, rosemary and lemon) on filter paper in a petridish or by directly dropping either undiluted or 1:50 diluted food flavours (blackberry, lemon and orange) on the sweetened filter papers.

To find an optimal training method, group training occurred in one of two setups: a vial and a block slide setup (figure 6-1 and figure 6-2A). Similar versions of both setups have previously been reported to be sufficient for olfactory conditioning in fruit flies (Fujita and Tanimura 2011; Quinn et al. 1974; Tempel et al. 1983). For different treatments using the same setups, feeding time (10 or 30 minutes), pause time (10 or 30 minutes), odours (essential oils (lavender, rosemary and lemon) or food flavours (blackberry, lemon and orange, undiluted or 1:50 diluted solution) and the method of fly movement into the setup (with or without CO_2) were changed (for a detailed methods overview, please refer to supplement S4.1, for further details on treatment differences refer to tables S4-2 and S4-7 in supplement S4.4).

6.3.2.1   Vial training setup

Flies were trained in cylindric plastic vials (figure 6-1; 10cm height and 5cm Ø). The vials were closed with a foam plug on one side and fine mesh wire on the other side through which odours used during feeding intervals could escape during pause intervals, thus preventing an accumulation of training odours inside the training vial (figure 6-1). Odour-sucrose combinations were presented via previously prepared pieces of filter paper which were pinned on the inside of the foam plug. After the vials had been closed with the foam plug, they were turned around so that the scented sucrose filter papers were presented on the vial's bottom. Training was switched into pause intervals or another feeding interval (with a different odour/sucrose combination) by simply changing the foam plugs of the vials with plugs to which differently prepared filter papers were pinned.
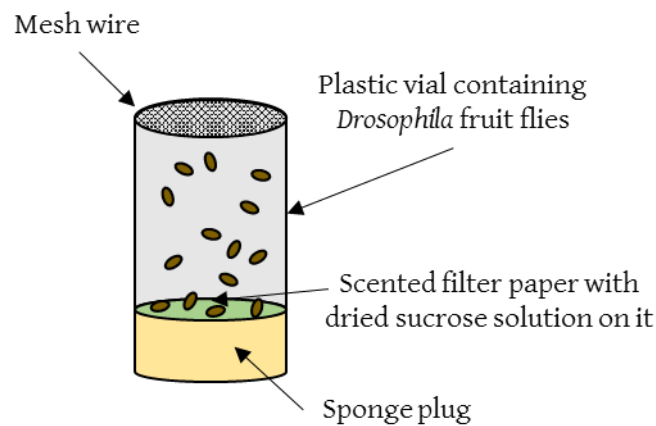
Figure 6-1: Vial training setup consisting of a plastic vial (10cm height and 5cm Ø) closed with a sponge plug on the lower side and fine mesh wire on the upper side. The mesh wire allowed a constant air flow into the vial and an evaporation of training odours during pause intervals. Sucrose/odour pairs were presented in form of filter papers with dried sucrose solutions on it which were pinned on the sponge plugs. Odours were additionally directly applied to the sponge plugs (below the filter papers). Sponge plugs were changed according to training intervals. Two sucrose/odour combinations were presented in an alternating order for a given feeding time. After each feeding interval, a pause interval followed in which the sponge plug was replaced by an unscented one without any sucrose filter papers pinned on top of them. Each sucrose/odour pair was presented three times, resulting in a total of 6 training intervals. After training, flies were transferred into a T-maze for a final choice test.

6.3.2.2   Block slide training setup

Other flies were trained in a block slide setup similar to the setup used in Quinn et al.'s (1974) odour and visual discriminative learning experiments. The setup was 3D printed in PLA and consisted of 1 plastic block and 3 tube holders which could independently be slid over the large block (figure 6-2A). Each tube block contained a hole into which a falcon tube (11.4cm height,

3cm ∅) was plugged. The large block also contained 3 holes and tube holders were slid over them to present odour-sucrose pairs. Odour/sucrose filter papers were placed on the bottom of the large block. The central block contained no odour/sucrose combination and served as the "pause block" to which the flies were moved in between two feeding intervals. The two remaining blocks each contained one of the odour/sucrose combinations (i.e. either rosemary + 1M or lemon + 0.1M). Water was available via a piece of filter paper soaked in water and pinned into the training tube throughout the whole training procedure.
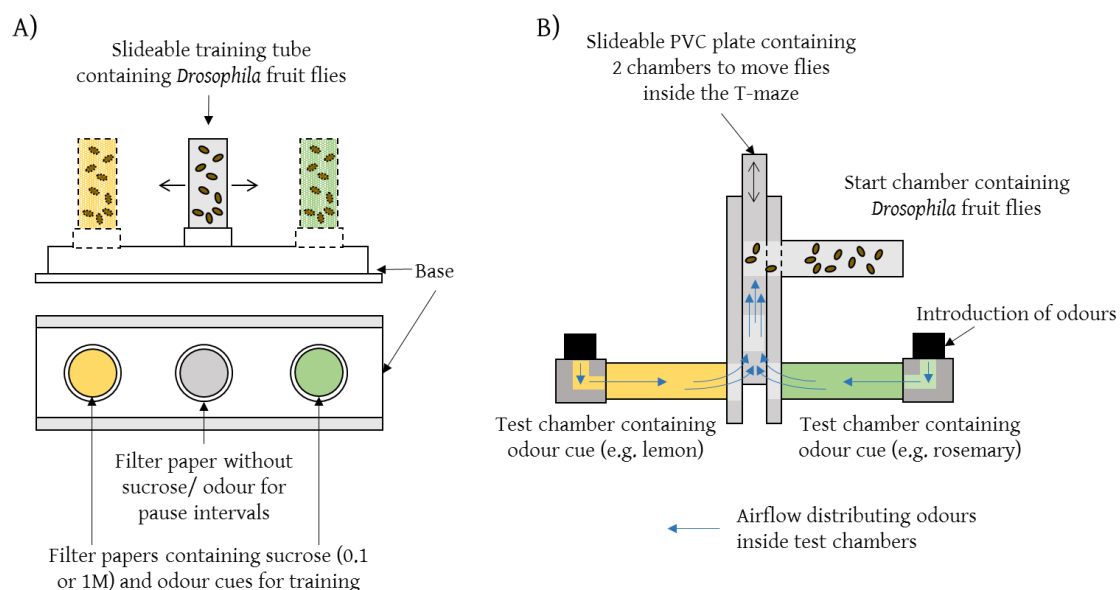
Figure 6-2: Block slide setup and T-maze used after training in the vial and block slide training setups. A) Overview of the block slide training setup. Flies were introduced via one of three falcon tubes mounted on a 3D printed base. These could be moved to both sides (left or right). Odours and dried sucrose filter papers were presented on the floor of the base and falcon tubes placed on top of them. One tube contained the flies to be trained. Two other tubes (one for each odour) were used to prevent odours from evaporating while flies were in a pause interval. The pause position was in the middle of the setup and flies were then moved from the mid position to the left (where sucrose/odour pair 1 was presented for a given feeding time) and then again moved back to the mid position for a given pause time. After the pause, the tube was moved to the right position (where sucrose/odour pair 2 was presented) and then again back to mid position. This was repeated until each sucrose/odour pair had been presented 3 times (making a total of 6 feeding intervals. After training, flies were transferred into a T-maze for a final choice test. B) Common T-maze setup consisting of three PVC plates and 3 tubes. Flies were introduced in the upper tubes from where they could move into a chamber in the mid PVC plate. This plate was then moved down, aligning the chamber in between both test tubes. The test tubes were filled with two associated odour cues (e.g. rosemary and lemon) which were introduced via vessels at the end of the tube and were spread inside the test tubes via a constant air flow (see blue arrows).

6.3.2.3   T-Maze test

After training, a choice test was conducted in a T-maze (figure 6-2B). The T-maze consisted of three PVC plates, of which the middle plate was vertically moveable. It held a chamber through

which flies were transferred from the odour-neutral start chamber into the T-maze (figure 6-2B). Flies were introduced via a vial connected to the upper end of the setup. They were knocked into the chamber inside the mid slice and stayed there for 3 minutes to become familiar with the new environment. Afterwards, flies were moved down into the T-maze. Here, two other vials were connected to each side of the setup with each chamber containing one of the associated odours, but no sucrose (i.e. rosemary odour left and lemon odour right). Odours were introduced to the T-maze via small reservoirs connected to the end of each T-maze vial and circulated through the setup via a constant air flow. Odours used in the reservoirs were either undiluted essential oils or undiluted food flavours depending on the training procedure flies had gone through (for further details, please refer to supplement S4.4). Flies were allowed to make a choice for 1 minute and then the T-maze was closed. Number of flies present on the right and left sides of the T-maze (flies which made a decision) and in the middle chamber (flies which had not made a decision) were counted.

6.3.3   Individual training via Proboscis Extension Reflex (PER) response

Drosophila were shown to successfully discriminate a reward and non-reward through a classical PER paradigm (Chabaud et al. 2006) and PER conditioning proved to be a successful tool in honeybee conditioning as well (Giurfa and Sandoz 2012). Therefore, in other approaches, we conducted individual training via the PER response. Odours were presented through puffing scented air through Pasteur pipettes containing a piece of kitchen paper soaked with either essential oils or food flavours. Training was conducted in a similar manner to the PER paradigm described in Shiraiwa and Carlson (2007). Training molarities (0.5M, 0.1/1.5M, 0.01/1M or 0.1/1M pairs), odour types (rosemary and lemon essential oils or undiluted food flavours with lemon and orange flavor/odour) and fixation methods (yellow panel stickers, pipette tips, sponge or PVC slides) differed depending on the training treatment. For detailed methods overviews or further details on fixation differences, please refer to supplement S4.2. Sucrose solutions were presented via small paper wicks. These were prepared beforehand by twisting a ca 6mm wide piece of thin paper tissues (e.g. Kimwipes®) into a thread. The thread was then torn into multiple 5mm long pieces (also see Shiraiwa & Carlson (2007) for a video and step by step protocol on wick preparation and PER assay procedure). The wicks were soaked in water or sucrose solution and then presented to the fly's proboscis.

For individual training via PER, we used four different fixation methods: pipette tip fixation following Shiraiwa and Carlson (2007), yellow panel stickers (figure 6-3A), PVC slides (figure 6-3B)

and sponges (figure 6-3C). Flies were fixed in pipette tips by flipping them towards the slim end of a pipette tip, freeing their head and forelegs and preventing them from escaping by plugging the tip with a small piece of cotton. Flies fixed on the yellow panel stickers (figure 6-3A) were glued to a piece of a yellow panel sticker with their wings, leaving their head and legs free to use. The yellow panel stickers were glued to a piece of a cardboard box with their free side, and flies placed in a vertical position. Flies fixed via PVC slides were fixed in V-shaped holes into which their neck was placed (figure 6-3B). They were then fixed with a piece of modelling clay, preventing them from escaping, and then brought into a vertical position. Flies fixed on a sponge were placed on the sponge and fixed with a small PVC slide, leaving their head and forelegs free for use (figure 6-3C). They were then also brought into a vertical position before training started. Flies which were trained while fixed via PVC slides or sponges were transferred into a Y-maze (figure 6-3D) after training to make a final choice for one of the conditioned odours.

For a more detailed description of the fixation methods, please refer to supplement S4.2.
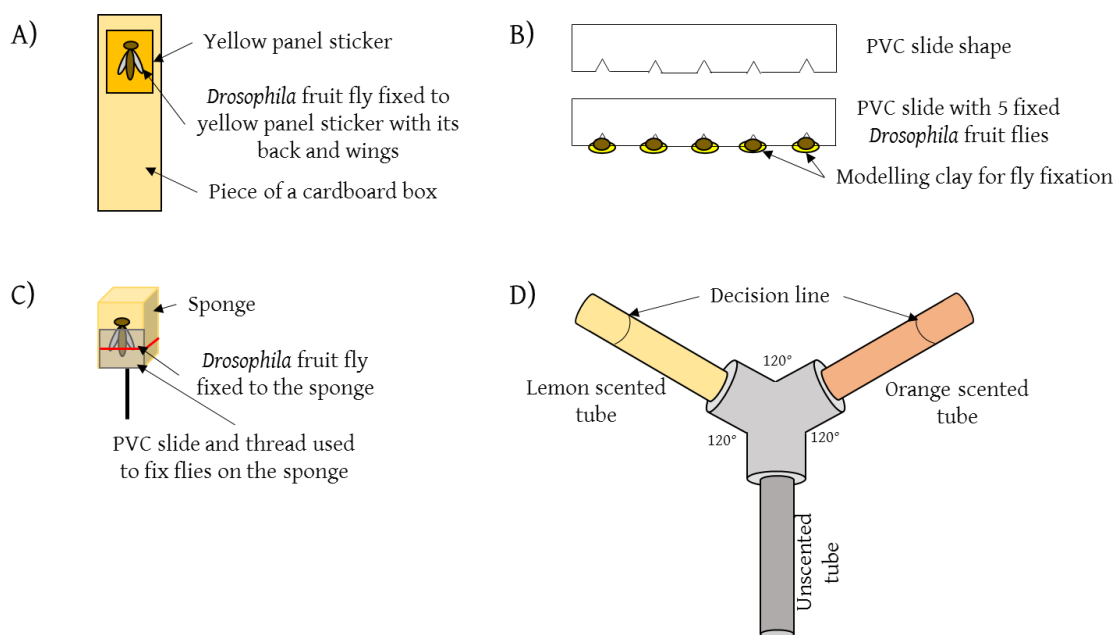
Figure 6-3: Fixation methods of the individual trainings via PER assay (pipette tip method not shown) and Y-maze training. A) Yellow panel sticker fixation: Flies were glued to a yellow panel sticker with their backs and wings, leaving the proboscis and legs free of use. The other side of the yellow panel sticker was glued to a piece of a cardboard box which allowed the flies to be positioned in a vertical position. B) PVC slide fixation: 5 V-shaped holes were cut into a piece of a PVC slide. Flies were placed individually into of the holes and the holes closed with modelling clay in front of them. This left the flies fixed between their heads and thorax, leaving the proboscis and legs free of use. Flies were then arranged in a vertical position for PER training. C) Sponge fixation: Flies were individually placed on top of a sponge wrapped with soft kitchen paper to prevent flies from being injured due to fixation. A piece of a PVC slide was then placed on top of flies and fixed with a thin thread, leaving flies fixed with their proboscis (and most of the times forelegs) free of use. Flies were arranged in a vertical position for PER training. D) Y-maze training: Flies

were introduced to the Y-maze via an unscented falcon tube (12cm height, 1.7cm ⌀). A 3D-printed Y-shaped tube holder connected 2 more falcon tubes to the unscented one. The tubes were arranged in a 120° angle to each other. In one tube, sucrose/odour pair 1 (e.g. 0.1M and lemon odour) and in the other tube, sucrose/odour pair 2 (e.g. 1M and orange odour) was presented. Flies were allowed an acclimatization time of 5 minutes. Then, flies choices were recorded for up to 8 times. A choice was made when a fly had crossed the decision line.

6.3.4   Individual Training in the Y-Maze

The last approach used for olfactory conditioning in *Drosophila melanogaster* was similar to an experimental procedure commonly used for conditioning in ants: the Y-maze (Czaczkes 2018; Dupuy et al. 2006; Oberhauser et al. 2019). In contrast to Y-mazes used for conditioning in ants which consist of open runways, the Y-maze used for olfactory conditioning here consisted of tubes (figure 6-3D).

Individual flies were directly trained in the Y-maze which consisted of 3 falcon tubes (12cm height, 1.7cm ⌀) connected by a 3D-printed Y-shaped tube holder (figure 6-3D). Sweetened and odoured pieces of filter paper were placed in two Y-maze tubes (i.e. 0.1M with undiluted orange food flavor and 1M with undiluted lemon food flavor on it), one odour on each side, while one tube remained empty. To begin a training, an individual fly was placed in the empty tube of the Y-maze and allowed to get familiar with the setup for 5 minutes. Afterwards, the fly's choice was recorded for up to 8 times in between which it was knocked back into the starting tube to begin another choice trial. The Y-maze was placed on a fixed position inside the room to keep visual and light cues constant.

6.4   Results

None of the presented approaches motivated flies to choose the odour which had been presented with higher molarity food in the T- and Y-mazes after training. Flies conditioned via the PER assay did not show a higher proportion of PER responses towards high molarity compared to low molarity during training, suggesting that there was no preference for one of the presented odour/sucrose combinations. In fact, the majority of flies transferred into the Y-maze after PER conditioning did not make a choice for one of the presented odours at all. Flies trained directly in the Y-maze were also either not motivated to explore the Y-maze or did not show a preference for the higher molarity odour.

For further details on the results depending on setups and treatments, as well as visualized results and statistical analysis, please refer to supplement S4.

6.5   Discussion

None of the training setups and training procedures trialed led a robust preference for an odour previously presented along with high quality food compared to an odour presented with low quality food. This may have been caused by the flies' inability to associate different odour/sucrose pairs and integrating memory of associated odours into future decisions or by inappropriate methods.

Odour conditioning in *Lasius niger* ants was successfully performed using lemon and rosemary essential oils as odour cues on the runway and in the food (Oberhauser and Czaczkes 2018; Oberhauser et al. 2019; Wendt et al. 2019; Wendt and Czaczkes 2019). We thus initially used identical odours for odour conditioning in *Drosophila melanogaster*, but switched to food flavours due to high mortality of flies in later treatments. None of the odours induced association formation in flies. However, previous studies showing a successful association formation between rewards or punishment presentations and odours in *Drosophila* used different substances as odour cues (Fujita and Tanimura 2011; Kim et al. 2007; Quinn et al. 1974; Tempel et al. 1983). Using odours such as 4-methylcychohexanol (MCH) and DL-3-octylalcohol (OCT), which were shown to be effective for conditioning in *Drosophila* fruit flies, may have led to a higher proportion of flies preferring the odour presented along with higher molarity sucrose.

In contrast to ants which collect food for the whole colony over multiple visits and share it with their nestmates, *Drosophila melanogaster* fruit flies forage only for their own needs. Because of that, flies have to collect much lower amounts of food in order to become satiated. A training paradigm via a feeding response may thus not be an optimal choice for odour conditioning to different qualities in this case. Indeed, *Drosophila* offers other behavioural patterns, such as mate choice, which may be more effective for investigating relative value perception. Females which had previously been in contact with small males are more likely to accept both small and large males as mating partners compared to flies which had been in contact with large males only (Dukas 2005), suggesting an evaluation of available options relative to former experience. Mate choice behaviour may thus be more suitable for investigating the genetic and neuronal mechanisms underlying relative value perception.

After mating, females are in need to find sites for egg deposition which offer a sufficient quality, quantity, and composition of food to ensure a fast and successful growth of the larvae. Flies avoid a sucrose enriched medium if they can choose a sucrose-poor medium for egg deposition (Yang et al. 2008; 2015). On the other hand, *Drosophila* females trade off individual diets

with substrate suitable for their offspring by feeding at sites enriched with nutrient contents depending on their own nutritional state, but moving to carbohydrate enriched sites for egg-deposition (Lihoreau et al. 2016). Even though larvae showed faster development on high protein foods, both survival and learning performance were increased on balanced food, and thus egg-deposition in high carbohydrate ripened fruits which gradually enrich in proteinaceous yeast as they start rotting may prove beneficial for the offspring (Lihoreau et al. 2016). These studies suggest egg-laying behaviour as an alternative approach for investigating relative value perception.

Drosophila larvae can also be conditioned relatively easy in petri dishes, yielding sufficient odour-taste learning (Gerber and Stocker 2007; Gerber et al. 2013). Larvae can not only learn to approach previously rewarded odours (Neuser et al. 2005; Scherer et al. 2003) or light-dark cues (Gerber et al. 2004) and avoid previously punished odours (Eschbach et al. 2011), but also show concentration-dependent odor-taste memories for only-sweet sugars such as xylose and arabinose (Burke and Waddell 2011; Rohwedder et al. 2012), possibly making them a more suitable model for the approaches presented in this chapter.
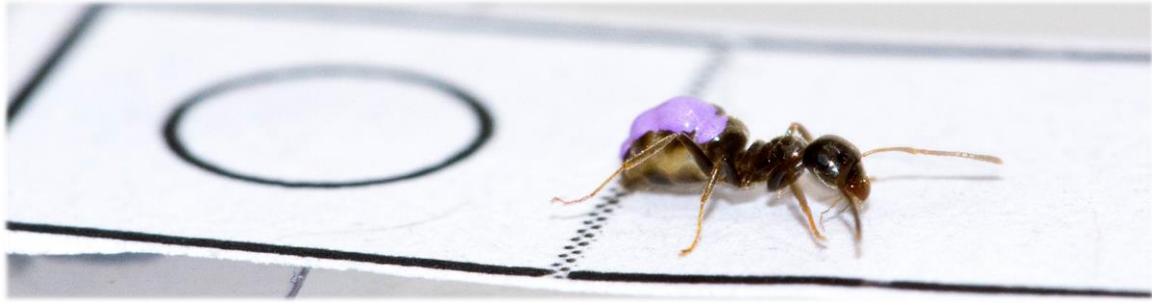
Chapter 7

Discussion and conclusion

7.1   Discussion

The ability of social insects to successfully solve complex tasks without a central control fundamentally relies on the decisions of independent individuals. Understanding the factors influencing the perceived value of options is key to understanding decision making. Apart from the direct traits characterizing an option's quality, such as sucrose concentration (Josens et al. 1998) and quantity (Mailleux et al. 2000) (in the case of a food source), an option's value can also be perceived relative to sometimes arbitrary reference points (Bitterman 1976; Couvillon and Bitterman 1984; Czaczkes et al. 2018a; Oberhauser and Czaczkes 2018; Richter and Waddington 1993; Roces 1993; Roces and Núñez 1993), and see chapter 2 and chapter 3. In this thesis, various factors serving as potential reference points during food evaluation were investigated in the ant *Lasius niger*. The work presented here demonstrates that value perception in ants is, like in humans, rather relative than absolute, and also affected by traits apparently independent of an option's absolute quality.

Colonies are constantly faced with the challenge of optimizing their food intake. Intriguingly, a direct comparison of options by single individuals is not required for a colony to make a collective choice for the best option (Beckers et al. 1990; Mallon et al. 2001; Robinson et al. 2009; 2014; Seeley et al. 1991). A collective choice is composed of numerous single individuals independently assessing the quality of options and adapting their recruitment effort to an option's perceived value (Beckers et al. 1993; Robinson et al. 2009). The way in which value is

perceived by single individuals is thus an extraordinary important part of collective decision making. Value attribution relative to reference points, such as expectations or former experience, affects value perception in humans (Kahneman and Tversky 1979; Tversky and Kahneman 1992; Vlaev et al. 2011), vertebrates (Annicchiarico et al. 2016; Bentosela et al. 2009; Crespi 1942; Flaherty 1982; 1999; Mustaca et al. 2000; Papini et al. 2001; Webber et al. 2015; Weinstein 1970a) and invertebrates (Bitterman 1976; Couvillon and Bitterman 1984; Richter and Waddington 1993), and see also chapter 2 and chapter 3. However, although successive negative contrasts have been frequently demonstrated in animals (Bower 1961; Tinklepaugh 1928; Vogel et al. 1968), positive incentive contrasts have often proved elusive (Black 1968; Bower 1961; Capaldi and Lynch 1967; Dunham 1968; Papini et al. 2001).

In chapter 2, we demonstrate both positive and negative incentive contrasts in ants and provide, for the first time of our knowledge, a detailed curve for value perception relative to a wide range of food quality expectations in an invertebrate. If ants valued a given food source only based on its absolute value, we would expect a similar acceptance of medium quality food for all ants regardless of the quality they received on earlier visits to the same location. In reality, ants which – based on former experience with a food source – expected to find high quality food displayed lower acceptance upon finding medium quality food than ants expecting to find low quality food. Inbound pheromone depositions mirrored this pattern, suggesting value perception relative to food qualities found on earlier visits.

However, the contrast effects demonstrated in chapter 2 (section 2.5) are not solely explainable by higher-level cognitive processing of value, but could have been driven by simple sensory perceptual mechanisms affecting value. For example, ants trained to higher molarity food may be affected by sensory saturation due to more sweetness receptors being blocked from previous visits, causing sucrose solutions to taste less sweet for subjects trained to high molarity solutions, and sweeter for subjects trained to low molarities (Bitterman 1976). The revealed contrast effects are also explainable by several other explanations of which a detailed description is provided in chapter 2 (section 2.6). In order to further pin down the mechanisms underlying the demonstrated contrast effects, ants had to be given the opportunity to form robust expectations of upcoming rewards while exposing all ants to the same stimuli. Ants can associate odour cues to different food qualities in a relatively short amount of time (Dupuy et al. 2006; Josens et al. 2009; Oberhauser et al. 2019; Provecho and Josens 2009) and integrate learned odour cues into later food choices outside the nest (Beckers et al. 1994; Czaczkes et al. 2014b; Oberhauser and Czaczkes 2018; Oberhauser et al. 2019; Provecho and Josens 2009; Roces 1990; Saverschek and

Roces 2011; Steck et al. 2011; Wolf and Wehner 2005). We thus trained ants to two different food qualities, offering different odours along with each quality. Ants could form robust expectations of upcoming rewards by associating the given molarities to their assigned odours. After training, ants received medium quality food, even though they were made to expect either high or low molarity food based on the runway odour. Even after carefully controlling for alternative factors through this experimental procedure, ants showed contrast effects by displaying higher acceptance towards the medium quality food when they expected to get low quality compared to when they expected high quality food. These results strongly suggest that the contrast effects arising from different reference solutions as shown in chapter 2 (section 2.5) are based on higher-level cognitive processing rather than on basic psychophysical mechanisms.

While we are developing good understanding of the psychological and behavioural aspects of expectation-based value perception, the genetic and neuronal mechanisms underlying it are still widely unknown. The fruit fly *Drosophila melanogaster* offers a well investigated genetic background and short generation time (Hadler 1964). This, along with its rich behavioural repertoire which suggests that it can use memories to make decisions (Kahsai and Zars 2011), may help gain deeper insights into these mechanisms. As expectation-driven value perception has not yet been shown in *Drosophila* fruit flies, we attempted to train flies similar to high and low quality food assigned with different odours (see chapter 6), in order to pursue a similar approach to that taken with ants in chapter 2. Flies can associate odours to a given reward (or punishment) and prefer odours associated to rewards (or avoid if the odour was associated to a punishment) over odours previously presented without a reward (mostly water) in a T-maze (Fujita and Tanimura 2011; Kim et al. 2007; Quinn et al. 1974; Tempel et al. 1983). However, none of our attempts resulted in sufficient evidence of learning to choose higher quality food based on its assigned odour. Flies were thus either incapable of associating odour cues to multiple different food qualities, or were not motivated to choose the better quality. In contrast to ants which collect food for the whole colony over multiple visits and share it with their nestmates, *D. melanogaster* feed for their own need, but have a higher responsibility concerning their own reproductive success. It is thus not surprising that females have preferences regarding substrate composition, and trade-off substrates composed of valuable food with substrates suitable for a quick and a high survivability and quick growth of offspring larvae, preferentially depositing eggs into substrates suitable for the offspring (Lihoreau et al. 2016; Yang et al. 2008; 2015). Females also show incentive contrasts in mate choice by incorporating experience with previously available males (Dukas 2005). Investigating relative value perception using an egg-deposition or mate choice assay

rather than through food preferences in this species may thus be a more successful research direction. As all our attempts to condition flies to expect a reward based on odour cues failed, we do not yet have a suitable approach for investigating the genetic and neuronal mechanisms underlying relative value perception in this model system.

In ants, valuable food sources are quickly connected to the nest via pheromone trails deposited by returning foragers, leading naïve recruits to them (Beckers et al. 1992a; Czaczkes et al. 2015c). However, even though ants are more likely to follow stronger trails, the strength of a trail does not appear to influence the perceived quality of a food source. In a study which could not be included into this thesis, we investigated the effect of a trail's strength on food acceptance and drinking time by applying pheromone extracted from *Lasius niger* workers (following von Thienen et al. (2014)) to the runway leading to a food source. Apparently, pheromones neither affected food acceptance, nor drinking time, and ants also showed no preference for odours presented along with food sources connected to the nest via pheromone (Oberhauser et al. in preparation). If a pheromone trail does not inform ants about the quality of a food source, colonies are in danger of becoming trapped in suboptimal choices if a previously high-quality food source with a strong pheromone trail drops in quality. Experienced foragers can, in addition to relying on social information, incorporate private information into decisions and often prefer them when both types are conflicted (Czaczkes et al. 2016; Grüter et al. 2011; Harrison et al. 1989; Oberhauser et al. 2019). This allows them to make more flexible choices and may reduce the chance of exploiting worse food sources when individuals have information about better ones elsewhere.

In chapter 5, we show that individual ant workers which are informed about all available options can also forego a poor food source close to the nest in a favour of a better one far away. This ability to take the time to move to a better reward without giving in to the impulse of choosing an immediately available worse reward is often referred to as self-control (Logue 1988). In humans, success in self-control tasks is believed to be correlated with success in other life situations, with a lack of self-control arguably increasing the probability of failing at school, or developing depression and criminal tendencies (Moffitt et al. 2011). However, waiting for a better reward is risky, because food sources may be exploited in the meantime or not become available at all. Whether showing self-control by foregoing immediate rewards is beneficial may strongly depend on specific environmental characteristics. Species living in stable habitats where food is easily acquirable, or species living in larger groups which mitigate the risk of individual failures, may benefit from showing self-control and are 'insured' in the case that waiting for a better

reward leads to no reward at all. In contrast, a starved animal which is in need of food, or species living in unstable environments where food is not available, may be more successful with impulsive choices, because waiting for a later reward risks death (Mayack and Naug 2015; McNamara et al. 2013). Indeed, animals strongly discount time when choosing between options and this is often ignored in experiments investigating self-control in animals (Hayden 2016; 2019), even though a choice for immediate rewards can be an ecologically meaningful decision. A small reward may still yield higher profit if the time needed to collect it is very low. In contrast, a high reward may become unattractive if it takes too long to reach. Many ant species preferentially forage close to their nest site in the presence of passively laid home-range markings and are less likely to continue laying trails on their way home in the absence of home-range markings (Devigne and Detrain 2006). In our study, ants showed robust self-control abilities when high quality food was available far away and low quality food closer to the nest. However, an increase of the close feeder's quality to either the same quality as offered by the far feeder or only slightly lower led to impulsive choices, with ants preferentially choosing the close feeder. While poor self-control is often linked to lower cognitive abilities (Hayden 2019), this behaviour seems perfectly ecologically reasonable considering that ants likely discount time to increase food intake per unit time. This study, however, investigated experienced foragers which had access to information about all available food sources, and could thus make direct comparisons. The low quality food source was only rejected by workers which were aware of a better quality further away. Naïve foragers of a starved colony would, however, readily accept the low quality food source (see chapter 2) and may end up trapped with a suboptimal choice that is available on the way to a better food source. This could be overcome by providing naïve workers information about the food available in the environment.

One way for naïve foragers in the nest to obtain such information would be to attend to the food brought in by returning foragers. In chapter 2 (section 2.7), we investigate whether the nest can serve as an information hub, where information about available food sources is collected, processed and fed back to outgoing foragers, thus providing an information pool which may allow ants to learn about available food sources outside the nest. We demonstrate that recruited workers can use the quality of food samples received through trophallactic interactions inside the nest as a reference point for future food evaluations outside the nest. Recruited ants which had not recently been in contact with other food sources displayed similar contrast effects to those demonstrated in the previous experiments of chapter 2 (section 2.5), suggesting that ants are not required to directly sample all available options, and can use the information gained from

returning foragers as reference points for later food evaluations. This system may decrease the risk of individuals being trapped in suboptimal choices if food sources of different qualities are available on the same pheromone trail and allows them to either accept a valuable close food source or reject it if a better one is available elsewhere. Czaczkes et al. (2019) support this assertion by demonstrating that foragers are more likely to follow pheromone trails upon discovering a highly concentrated small sucrose droplet when leaving to forage, indicating that high quality food is available in the environment. Using fluorescence microscopy, they also reveal that food is frequently shared between fed workers and outgoing foragers. Returning ants contacting outgoing foragers on the trail may thus share valuable information about available food qualities (Czaczkes et al. 2019), promoting trail following when the returning foragers have found good food, and leading to a reliance on private information of previous good food when other nestmates share worse food.

Sucrose concentration is not the only information demonstrably shareable inside the nest. Ants and bees show a preference for odours previously found rewarding on their own foraging trips (Grüter et al. 2008; Oberhauser and Czaczkes 2018) as well as odours brought back by successful returning foragers (Arenas et al. 2007; Josens et al. 2016; Provecho and Josens 2009). The use of odour cues allows ants to reject harmful food sources (Saverschek and Roces 2011), but can also lead to suboptimal choices such as exploiting a deterrent and harmful food source instead of a valuable one (Josens et al. 2016). During association formation, value-neutral cues can be assigned a value which may affect future value perception of options holding learned cues (Rescorla and Wagner 1972). Confirming this theory, ants prefer food sources offering already learned odour cues over identical food sources which offer novel odours (Oberhauser and Czaczkes 2018). In addition, a bee's experience with scented food decreases its responsiveness to sucrose, making it more likely to accept lower concentrations as long as they contain a previously rewarding odour (Finkelstein et al. 2019). This suggests that the value assigned to previously associated odour cues affects value perception of novel food sources. A similar effect can be seen in humans, where brand labels carry associations and social information (French and Smith 2013; Macklin 1996). The associations linked to a label can serve as an expectation-based reference point and affect how the quality of a product advertised with such label is perceived. Depending on the values assigned to the linked associations, perceived quality can either increase (Breneiser and Allen 2011; Fornerino and d'Hauteville 2010; Kühn and Gallinat 2013; Lee et al. 2013; Nevid 1981; McClure et al. 2004; Wansink 2000; Woodside and Taylor 1978; Yamada et al. 2014) or decrease (Lee et al. 2006; Wansink 2000). Whether perceived value of an option is aligned with

the value of its assigned label or is contrasted against it strongly depends on the discrepancy between a label-driven expectation and the products' objective value. If the discrepancy is small, an option's perceived value will be aligned with the value of a label, a process called assimilation (Cardello and Sawyer 1992; Hovland et al. 1957; Schnurr et al. 2017). In the case that the discrepancy is large, contrast effects are likely to appear (Cardello and Sawyer 1992). Such contrast effects due to expectations elicited by previously learned odour labels were shown in chapter 2. Increasing the similarity between a food source's objective quality and the label-driven expectation by simply adding corresponding flavours to the food changed ants' behaviour from displaying contrast effects to aligning the value of the odour label to the perceived value of a new food source (see chapter 3). These results suggest that naturally value-neutral food labels can influence decision making in a foraging context and may even counteract contrast effects.

In addition to social information shared actively through pheromone trails or through direct mouth-to-mouth food transfers, the mere presence of other nestmates affects foraging choices in social insects as well. For example, naïve bumblebees copy food choices of experienced foragers (Leadbeater and Chittka 2011; Worden and Papaj 2005) and are more likely to visit food sources at which other nestmates are present (Kawaguchi et al. 2007). Indeed, the presence of other nestmates may serve as an indicator for a safe and productive food source which is worth exploiting and should be concentrated on while it is not yet completely crowded (Czaczkes et al. 2015b). This, however, must be weighed against recruiting too many nestmates to a food source, which may lead to queuing at the food source and slower travel speed due to crowded trails (Burd 1996; 2000; Burd and Aranwela 2003), ultimately leading to a decreased colony-level food intake. Ants and bees have developed different negative feedback systems preventing them from recruiting too many nestmates to a food source. These negative feedback systems are deployed upon visiting crowded feeders (Kietzman and Visscher 2015), walking on a crowded trail (Czaczkes 2014; Czaczkes et al. 2013b) or experiencing long waiting times when unloading the food inside the nest (Nieh 1993; Seeley 1992). Honeybees produce an acoustic stop-signal which is often delivered while bumping into a dancing bee, to prevent it from recruiting more nestmates (Kietzman and Visscher 2015; Pastor and Seeley 2005). Individual ant workers, on the other hand, tune down their pheromone depositions (Grüter et al. 2012; Czaczkes 2014; Czaczkes et al. 2013b), leading to a passive evaporation of accumulated pheromone on the trail. Although crowded food sources are strongly discounted through negative feedback systems, unexperienced recruits of *Lasius niger* appear to preferentially move on occupied trails (Czaczkes et al. 2015b), thus increasing the potential crowding at an already overexploited food source. However, bumblebee

foragers seem to shift from preferring unfamiliar food sources occupied by a conspecific to rather avoiding them when they are familiar (Kawaguchi et al. 2007) and a similar behaviour is demonstrated for ants in chapter 4: Ants which had been trained to food sources only differing in the presence or absence of other nestmates deposited less pheromone when returning from occupied food sources and preferred unoccupied food sources in a Y-maze choice test. These results, in conjunction with the finding that experienced individuals are more likely to rely on private information when it is conflicted with social information (Grüter et al. 2011), suggest that experienced foragers make up for the higher amount of naïve workers moving towards occupied food sources by actively avoiding them. This in turn allows them to increase recruitment strength to identical unoccupied food sources, ultimately leading to a more even distribution of the available work force.

7.2   Conclusion

Even though collective decision making processes appear to be robust to differences in individual access to information (Robinson et al. 2014), they are fundamentally dependent on the subjective decisions of single individuals. Understanding the factors influencing how information is integrated into the comparison of different options is thus key to a better understanding of both individual and collective decision making. The studies presented in this thesis provide new insights into factors affecting individual value perception, such as expectations based on former experience (chapter 2) or induced by learned odour labels (chapter 2 and chapter 3) and nestmate presence (chapter 4). Moreover, they provide evidence about mechanisms preventing individuals from being trapped in suboptimal choices, such as information sharing through trophallaxis (chapter 2) and crowding avoidance (chapter 4), and making flexible choices based on private information incorporating time-discounting (chapter 5) and crowding (chapter 4) into food choices.

All presented behavioural patterns, even though not always strictly economically rational, appear to lead to ecologically sensible collective decisions, very likely leading to improved food intake by colonies. While the private information of individual foragers can demonstrably trap colonies in local feeding optima (Czaczkes et al. 2016), social information can lead to similar problems (Josens et al. 2016). Maintaining a flexible quality assessment based on private memory of other qualities currently available or remembered from the past while at the same time incorporating information received from other nestmates and pheromone trails may allow social

insect colonies to effectively exploit resources in a changing environment (Czaczkes et al. 2011; 2019).

With the many parallels between ants' and vertebrate (including human) behaviour, in addition to their easy maintenance, ants offer a tractable and flexible model system for examining perceived value. Experiments can easily be designed in an ecologically meaningful and well-balanced way. Moreover, pitfalls associated with experiments in humans, such as non-relevant reward sizes (Levitt and List 2007) can be avoided in experiments investigating foraging behaviour in ants, where sufficient reward sizes can be offered in form of a simple sucrose droplet (Mailleux et al. 2000). The cross-disciplinary nature of this thesis, comprising behavioural ecology, behavioural economics, cognitive psychology and consumer psychology, has proven a productive approach to investigating decision making in ants. By referring to already existing theories from the fields of behavioural economics and consumer psychology, we were able to reveal further factors influencing value perception and decision making in ants. Likewise, other fields may gain further insights from considering findings from behavioural ecology as well. All these disciplines may benefit from bringing tools and concepts from one field into another, thereby increasing flexibility in experimental designs and expanding the pool of knowledge to draw from.

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Supplementary Material

All electronic supplementary material (ESM) referred to in this thesis, including all raw data used in the presented studies, Source Data Files and Model Outputs can be found on the attached compact disc. All files are sorted by the chapters they are presented in within this thesis.

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S1 Supplement to Chapter 2

Positive and negative incentive contrasts lead to relative value perception in ants

S1.1   Supplement to Figure 2-2

Figure S1-1: Food acceptance shown in experiment 1 for the two training visits (visit 1 & 2) in which ants received one of 12 molarities (Reference Molarity) and the test visit (3) in which all ants received 0.5M. Shown are the mean food acceptance (points) and the 95% confidence intervals (coloured ribbons) for each reference molarity and visit. Coloured dashed lines mark the mean food acceptance for ants which received 0.5M (control). This figure can also be found in the ESM (Figure 2-2 – Figure supplement 1).

Table S1-1: Pairwise Comparisons Table for the Food Acceptance Scores of Experiment 1. Estimates, z-values and p-values for the pairwise comparisons of all twelve treatments on the third visit for food acceptance of Experiment 1. Pairwise comparisons were calculated in R with the lsmeans function and a Benjamini-Hochberg correction. This table can also be found in the ESM (Figure 2-2 – Source Data File 1).

treatment	value	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1	1.5	2
0.1	estim.		1.35	1.98	2.11	-2.39	2.69	2.98	3.18	3.34	3.43	5.19	5.63
	z-value		0.48	0.47	0.48	-5.05	0.49	0.49	0.50	0.49	0.52	0.53	0.53
	p-value		0.31	<0.01	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001
0.2	estim.	1.35		0.63	0.75	-1.04	1.34	1.63	1.83	1.99	2.08	3.84	4.27
	z-value	0.48		1.87	2.17	0.33	3.73	4.61	4.98	5.55	5.24	9.32	10.30
	p-value	0.31		1	1	0.13	<0.05	<.001	<.001	<.001	<.001	<.001	<.001
0.3	estim.	1.98	0.63		0.12	-0.41	0.70	1.00	1.20	1.36	1.45	3.21	3.64
	z-value	0.47	1.87		0.36	0.33	1.99	2.87	3.32	3.85	3.69	7.90	8.91
	p-value	<0.01	1		1	1	1	0.27	0.059	<0.01	<0.05	<.001	<.001
0.4	estim.	2.11	0.75	0.12		-0.28	0.58	0.88	1.08	1.23	1.33	3.09	3.52
	z-value	0.48	2.17	0.36		0.34	1.59	2.43	2.87	3.37	3.30	7.40	8.38
	p-value	<.001	1	1		1	1	0.99	0.27	<0.05	0.06	<.001	<.001
0.5	estim.	-2.39	-1.04	-0.41	-0.28		0.30	0.59	0.80	0.95	1.04	2.80	3.24
	z-value	-5.05	0.33	0.33	0.34		0.35	0.35	0.36	0.35	0.39	0.40	0.41
	p-value	<.001	0.13	1	1		1	1	1	0.44	0.5	<.001	<.001
0.6	estim.	2.69	1.34	0.70	0.58	0.30		0.30	0.50	0.65	0.74	2.50	2.94
	z-value	0.49	3.73	1.99	1.59	0.35		0.80	1.31	1.76	1.82	5.97	6.98
	p-value	<.001	<0.05	1	1	1		1	1	1	1	<.001	<.001
0.7	estim.	2.98	1.63	1.00	0.88	0.59	0.30		0.20	0.35	0.45	2.21	2.64
	z-value	0.49	4.61	2.87	2.43	0.35	0.80		0.53	0.97	1.10	5.36	6.38
	p-value	<.001	<.001	0.27	0.99	1	1		1	1	1	<.001	<.001
0.8	estim.	3.18	1.83	1.2	1.08	0.80	0.50	0.20		0.15	0.25	2.00	2.44
	z-value	0.50	4.98	3.32	2.87	0.36	1.31	0.53		0.41	0.59	4.77	5.79
	p-value	<.001	<.001	0.059	0.27	1	1	1		1	1	<.001	<.001
0.9	estim.	3.34	1.99	1.36	1.23	0.95	0.65	0.35	0.15		0.09	1.85	2.28
	z-value	0.49	5.55	3.85	3.37	0.35	1.76	0.97	0.41		0.23	4.53	5.58
	p-value	<.001	<.001	<0.01	<0.05	0.44	1	1	1		1	<.001	<.001
1	estim.	3.43	2.08	1.45	1.33	1.04	0.74	0.45	0.25	0.09		1.76	2.19
	z-value	0.52	5.24	3.69	3.30	0.39	1.82	1.10	0.59	0.23		3.99	4.97
	p-value	<.001	<.001	<0.05	0.06	0.5	1	1	1	1		<0.05	<.001
1.5	estim.	5.19	3.84	3.21	3.09	2.8	2.50	2.21	2.00	1.85	1.76		0.43
	z-value	0.53	9.32	7.90	7.40	0.40	5.97	5.36	4.77	4.53	3.99		1.14
	p-value	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<0.05		1
2	estim.	5.63	4.27	3.64	3.52	3.24	2.94	2.64	2.44	2.28	2.19	0.43	
	z-value	0.53	10.30	8.91	8.38	0.41	6.98	6.38	5.79	5.58	4.97	1.14	
	p-value	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	1	

S1.2   Supplement to Figure 2-3

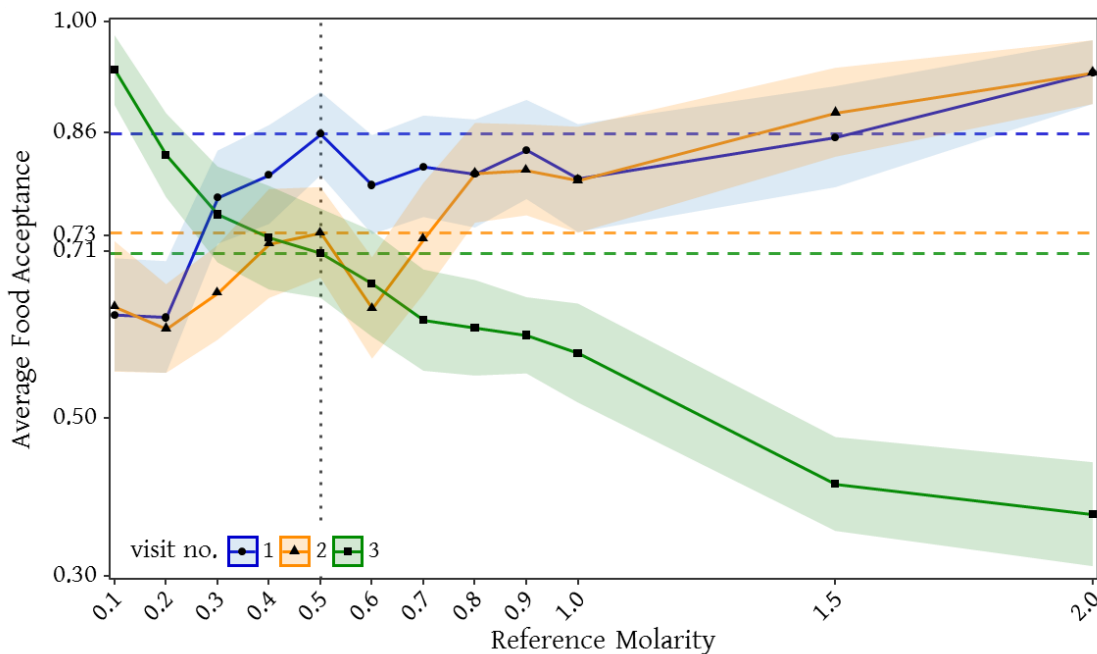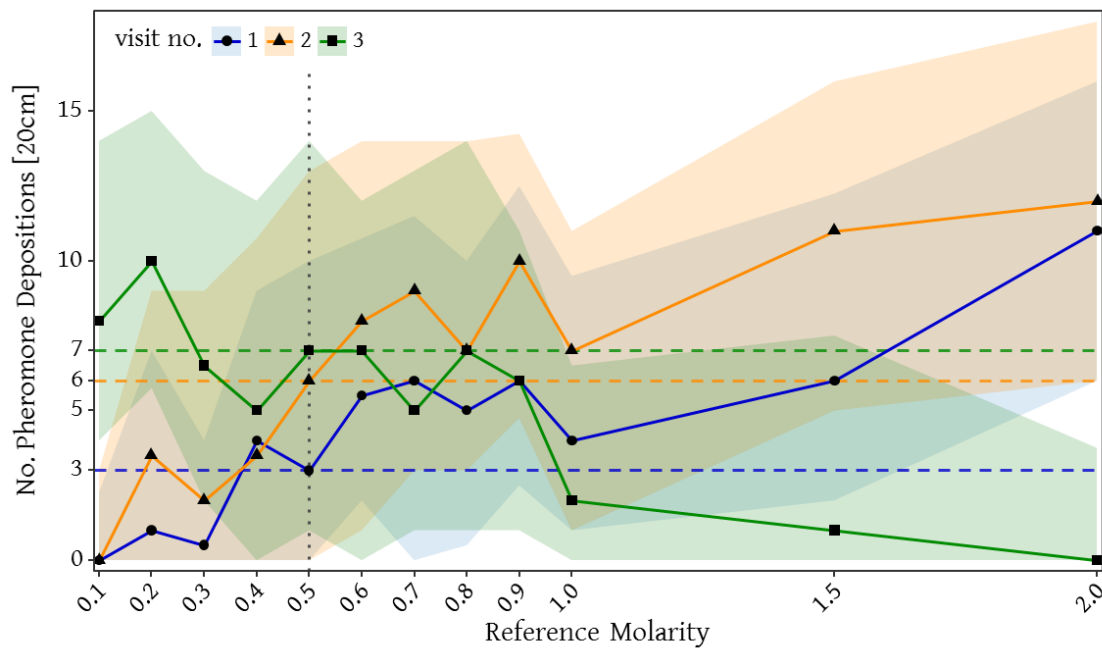Figure S1-2: Pheromone depositions on the way back to the nest shown in experiment 1 for the two training visits (visit 1 & 2) in which ants received one of 12 molarities (Reference Molarity) and the test visit (3) in which all ants received 0.5M. Shown is the median number of pheromone depositions (points) and the 25/75% quartiles (coloured ribbons) measured on a 20 cm track right behind the food source for each reference molarity and visit. Coloured dashed lines mark the median pheromone depositions for ants which received 0.5M (control). This figure can also be found in the ESM (Figure 2-3 – Figure supplement 1).

Table S1-2: Pairwise Comparisons Table for the Number of Pheromone Depositions on the way back to the nest of Experiment 1. Estimates, z-values and p-values for the pairwise comparisons of all twelve treatments on the third visit for inbound pheromone depositions on a 20 cm track right behind the food source of Experiment 1. Pairwise comparisons were calculated in R with the lsmeans function and a Benjamini-Hochberg correction. This table can also be found in in the ESM (Figure 2-3 – Source Data File 1).

treatment	value	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1	1.5	2
0.1	estim.		-0.42	0.04	0.30	-0.08	0.05	0.12	0.18	0.14	0.94	0.94	1.34
	z-value		-2.23	0.21	1.44	-0.41	0.25	0.60	0.87	0.70	4.11	4.41	6.07
	p-value		1	1	1	1	1	1	1	1	<0.01	<.001	<.001
0.2	estim.	-0.42		0.47	0.72	-0.51	0.48	0.55	0.60	0.57	1.37	1.36	1.77
	z-value	-2.23		2.63	3.83	-2.86	2.56	3.00	3.22	3.11	6.44	6.98	8.62
	p-value	1		0.56	<0.01	0.28	0.70	0.18	0.08	0.12	<.001	<.001	<.001
0.3	estim.	0.04	0.47		0.26	-0.04	0.01	0.08	0.14	0.10	0.64	0.64	1.04
	z-value	0.21	2.63		1.33	-0.22	0.06	0.43	0.71	0.54	2.86	3.03	4.75
	p-value	1	0.56		1	1	1	1	1	1	0.28	0.16	<.001
0.4	estim.	0.30	0.72	0.26		0.22	-0.25	-0.18	-0.12	-0.16	0.64	0.64	1.04
	z-value	1.44	3.83	1.33		1.12	-1.23	-0.89	-0.59	-0.79	2.86	3.03	4.75
	p-value	1	<0.01	1		1	1	1	1	1	0.28	0.16	<.001
0.5	estim.	-0.08	-0.51	-0.04	0.22		-0.03	0.04	0.10	0.06	0.86	0.86	1.26
	z-value	-0.41	-2.86	-0.22	1.12		-0.15	0.22	0.50	0.32	3.97	4.28	6.03
	p-value	1	0.28	1	1		1	1	1	1	<0.01	<0.01	<.001
0.6	estim.	0.05	0.48	0.01	-0.25	-0.03		0.07	0.12	0.09	0.89	0.88	1.29
	z-value	0.25	2.56	0.06	-1.23	-0.15		0.36	0.63	0.45	3.97	4.25	5.94
	p-value	1	0.70	1	1	1		1	1	1	<0.01	<0.01	<.001
0.7	estim.	0.12	0.55	0.08	-0.18	0.04	0.07		0.05	0.02	0.82	0.81	1.22
	z-value	0.60	3.00	0.43	-0.89	0.22	0.36		0.28	0.10	3.71	3.98	5.70
	p-value	1	0.18	1	1	1	1		1	1	<0.05	<0.01	<.001
0.8	estim.	0.18	0.60	0.14	-0.12	0.10	0.12	0.05		-0.04	0.76	0.76	1.16
	z-value	0.87	3.22	0.71	-0.59	0.50	0.63	0.28		-0.18	3.39	3.64	5.35
	p-value	1	0.08	1	1	1	1	1		1	<0.05	<0.05	<.001
0.9	estim.	0.14	0.57	0.10	-0.16	0.06	0.09	0.02	-0.04		0.80	0.80	1.20
	z-value	0.70	3.11	0.54	-0.79	0.32	0.45	0.10	-0.18		3.62	3.89	5.62
	p-value	1	0.12	1	1	1	1	1	1		<0.05	<0.01	<.001
1	estim.	0.94	1.37	0.64	0.64	0.86	0.89	0.82	0.76	0.80		-0.01	0.40
	z-value	4.11	6.44	2.86	2.86	3.97	3.97	3.71	3.39	3.62		-0.02	1.67
	p-value	<0.01	<.001	0.28	0.28	<0.01	<0.01	<0.05	<0.05	<0.05		1	1
1.5	estim.	0.94	1.36	0.64	0.64	0.86	0.88	0.81	0.76	0.80	-0.01		0.40
	z-value	4.41	6.98	3.03	3.03	4.28	4.25	3.98	3.64	3.89	-0.02		1.81
	p-value	<.001	<.001	0.16	0.16	<0.01	<0.01	<0.01	<0.05	<0.01	1		1
2	estim.	1.34	1.77	1.04	1.04	1.26	1.29	1.22	1.16	1.20	0.40	0.40	
	z-value	6.07	8.62	4.75	4.75	6.03	5.94	5.70	5.35	5.62	1.67	1.81	
	p-value	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	1	1	

S1.3   Supplement to Figure 2-4

For CLMM and GLMM outputs for figure 2-4 please refer to the ESM (Figure 2-4 – Source Data Files 1-6).
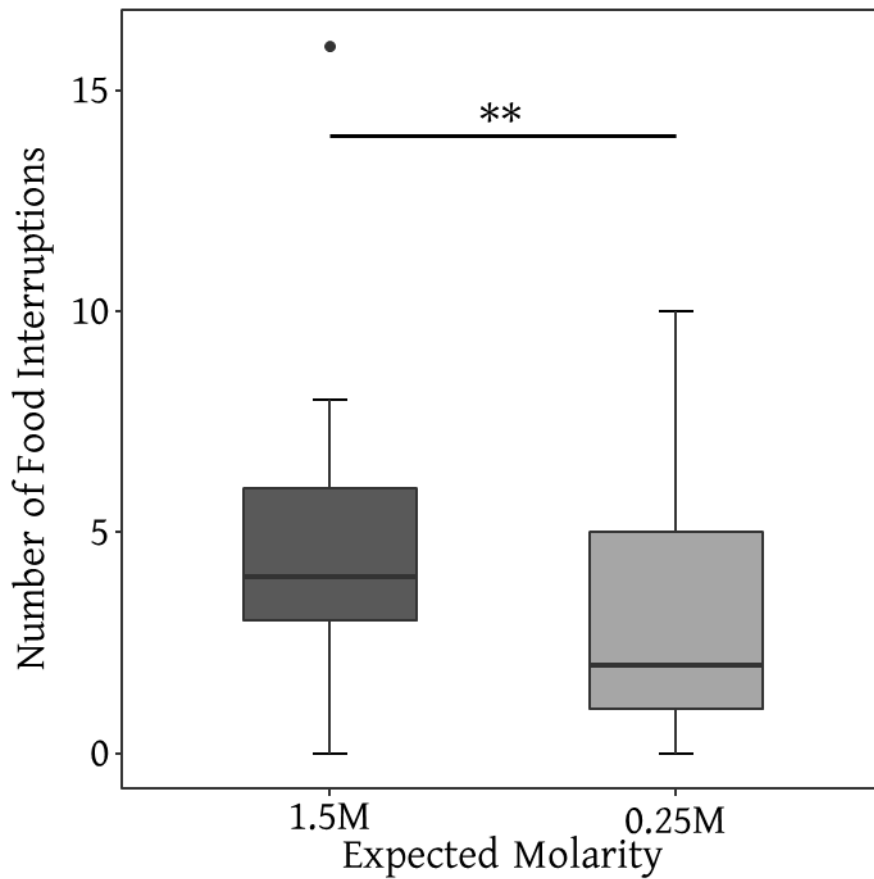

Figure S1-3: Number of food interruptions on the last (9th) visit depending on the ant's expectations until the crop was filled. This figure can also be found in the ESM (Figure 2-4 – Figure supplement 1).

S1.4 Supplement to Figure 2-5

Table S1-3: Pairwise Comparisons Table for the Food Acceptance Scores of Experiment 3. Estimates, z-values and p-values for the pairwise comparisons of all three treatments for the recruit for food acceptance of Experiment 3. Pairwise comparisons were calculated in R with the lsmeans function and a Benjamini-Hochberg correction. This table can also be found in the ESM (Figure 2-5 – Source Data File 1).

treatment	value	0.166	0.5	1.5
0.166	Estimate		0.92	1.53
	z-value		2.00	3.33
	p-value		0.13	**<0.01**
0.5	Estimate	0.92		0.61
	z-value	2.00		1.29
	p-value	0.13		0.59
1.5	Estimate	1.53	0.61	
	z-value	3.33	1.29	
	p-value	**<0.01**	0.59	

Table S1-4: Pairwise Comparisons Table for Trophallaxis Time in seconds of Experiment 3. Estimates, z-values and p-values for the pairwise comparisons of all three treatments for the recruit for trophallaxis time in seconds of Experiment 3. Pairwise comparisons were calculated in R with the lsmeans function and a Benjamini-Hochberg correction. This table can also be found in the ESM (Figure 2-5 – Source Data File 1).

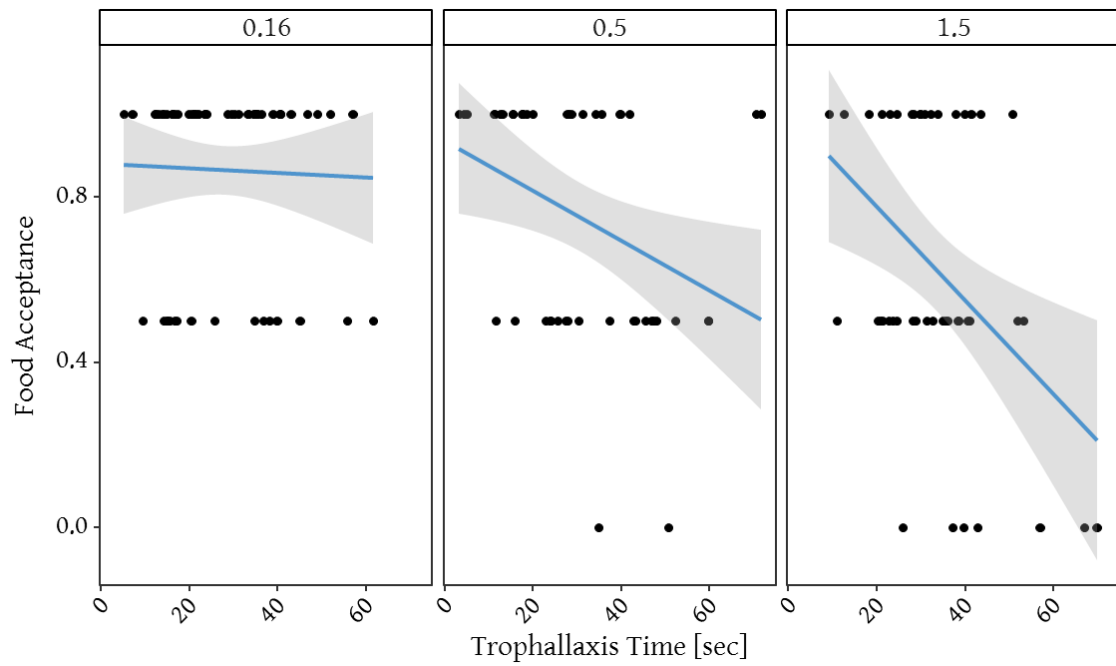| treatment	value	0.166	0.5	1.5
0.166	Estimate		-0.12	-0.19
	z-value		-3.08	-5.08
	p-value		**<0.01**	**<.001**
0.5	Estimate	-0.12		-0.07
	z-value	-3.08		-1.76
	p-value	**<0.01**		0.23
1.5	Estimate	-0.19	-0.07	
	z-value	-5.08	-1.76	
	p-value	**<.001**	0.23	

Figure S1-4: Food acceptance scores dependent on the trophallaxis time [sec] of receiving foragers for all 3 reference molarities (each plot represents data for 1 of 3 reference molarities). This figure can also be found in the ESM (Figure 2-5 – Figure supplement 1).

S1.5 Further Supplements

Table S1-5: Sample sizes, mean food acceptance and median pheromone depositions (inward and outward journeys) for the test visits of each experiment and treatment. This table can also be found in the ESM (supplementary file 1).

Experiment	Reference /Expected Molarity	Sample Size	Nr. of Ants excluded	proportion finished [%]	Mean Food Acceptance	Median Pheromone Depositions to Nest [20cm]	Median Pheromone Depositions to Food [20cm]
Relative Value Perception (Experiment 1)	0.1M	57	0	100	0.94	8	2
	0.2M	80	0	100	0.83	10	8
	0.3M	76	2	97.43	0.76	6.5	6
	0.4M	66	3	95.65	0.73	5	6
	0.5M	77	2	97.47	0.71	7	10
	0.6M	65	1	98.48	0.67	7	9
	0.7M	73	2	97.33	0.62	5	11
	0.8M	66	2	97.06	0.61	7	10
	0.9M	72	1	98.63	0.60	6	12
	1M	55	5	91.66	0.58	2	10
	1.5M	72	7	91.14	0.42	1	13
	2M	70	0	100	0.39	0	14
Scent Training Control (Experiment 2)	1.5M	38	2	95	0.70	0	15
	0.25M	32	0	100	0.83	0	2
Trophallaxis (Experiment 3)	0.16M	63	0	100	0.86		
	0.5M	52	0	100	0.76		
	1.5M	53	0	100	0.58		

S1.5.1 Video Supplements

The video supplements referred to in chapter 2 can be found on the accompanying disc (ESM).

Video 2-1: ant displaying food acceptance score 1. It shows no food interruptions within the first seconds of feeding.

Video 2-2: ant displaying food acceptance score 0.5. It interrupts feeding within the first seconds of feeding and repeatedly interrupts feeding, but still feeds at the food source (an ant displaying food acceptance score 0 would refuse to feed at the sucrose solution and either returned to the nest immediately or fail to fill its crop within 10 minutes).

S2 Supplement to Chapter 3

Labelling effects in insects: cue associations influence perceived food value in ants *(Lasius niger)*

S2.1 Appendix A
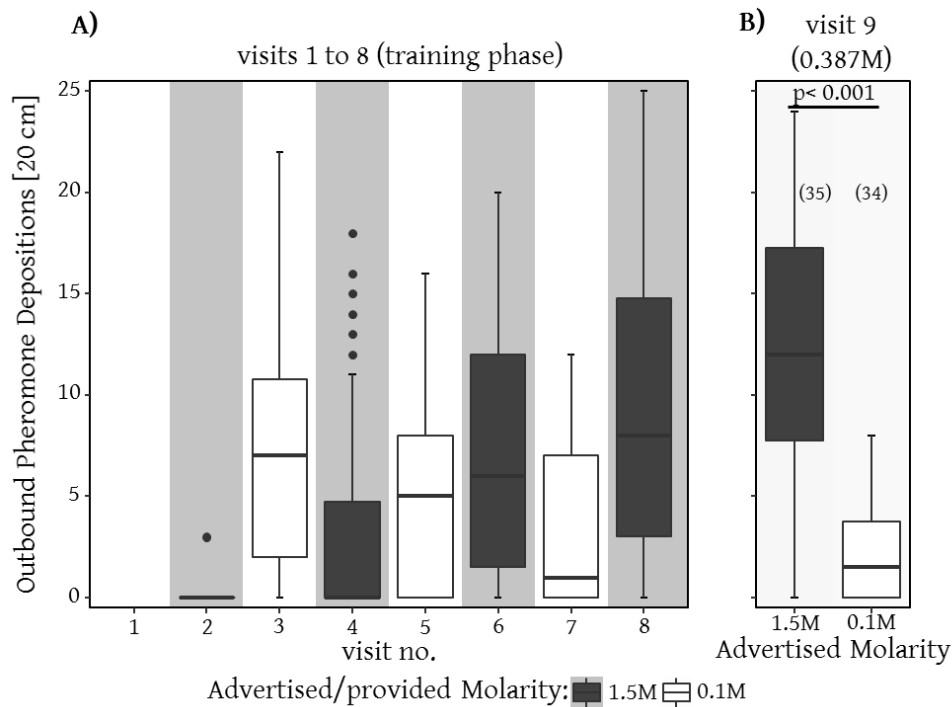
Figure S2-1: A) & B) Outbound Pheromone Depositions [20 cm] (to the Food Source) for A) the eight training visits (visits 1-8) in which ants received 0.1M coupled with one scent and 1.5M coupled with another scent in an alternating order, always starting with 0.1M, B) the test visit (visit 9) in which ants always received 0.387M sucrose solution. Both the sucrose solution and the runway leading towards the food source were impregnated with one of the learned scents, triggering an expectation towards receiving either high or low molarities at the end of the runway. Shown are the median number of pheromone depositions on the measured 20cm track on the way to the food source and the 75%/25% quantiles for each visit.

During training (visit 1 to 8), ants deposited significantly more pheromone on the measured 20 cm track on the way to the food source when confronted with the high molarity than when confronted with the low molarity (*GLMM:* estimate = 0.52, z = 4.65, p < 0.001, *OR* = 0.52, 95% C.I. [0.3,

0.73], figure S2-1A). The number of visit also had a significant effect on the number of deposited pheromone with pheromone depositions towards high molarity scent generally increasing and pheromone depositions towards low molarity scents generally decreasing over time (*GLMM: estimate = 1.14, z = 13.64, p < 0.001, OR = 1.14, 95% C.I. [0.97, 1.3]*). Ants which expected high molarity on the 9th visit (test visit) deposited significantly more pheromone than ants which expected to find low molarity food at the end of the runway (*GLMM: estimate = -1.80, z = -8.85, p < 0.001, OR = -1.81, 95% C.I. [-2.21, -1.41]*, figure S2-1B). Number of outbound pheromone depositions were also significantly higher for ants confronted with the high molarity scent on the 9th visit compared to the training phase (visit 2 vs 9: estimate = 0.46, z = 11.14, p < 0.001, visit 4 vs 9: estimate = 0.57, z = 13.96, p < 0.001, visit 6 vs 9: estimate = 0.59, z = 14.5, p < 0.001, visit 8 vs 9: estimate = 0.58, z = 14.36, p < 0.001).

S2.2 Appendix B

Captions for Supplementary Videos B1 and B2

The video supplements referred to in chapter 3 can be found on the accompanying disc.

Video B1: ant displaying food acceptance score 1. It shows no food interruptions within the first seconds of feeding.

Video B2: ant displaying food acceptance score 0.5. It interrupts feeding within the first seconds of feeding and repeatedly interrupts feeding, but still feeds at the food source until the crop is filled (an ant displaying food acceptance score 0 would refuse to feed at the sucrose solution and either returned to the nest immediately or fail to fill its crop within 10 minutes).

S3 Supplement to Chapter 5
Individual ant workers show self-control

S3.1 Materials and Methods

S3.1.1 Study species

Eight stock colonies of the black garden ant *Lasius niger* were collected on the University of Regensburg campus. The colonies were kept in 30x30x10cm foraging boxes with a layer of plaster covering the bottom. Each box contained a circular plaster nest box (14 cm diameter, 2 cm height). The colonies were queenless with around 1000-2000 workers and small amounts of brood. The colonies were fed three times a week with Bhatkar mix (Bhatkar and Whitcomb 1970) and *Drosophila* fruit flies.

One sub-colony of 500 individuals was formed from each stock colony, and these eight fixed-size sub-colonies were used for our experiments. Sub-colonies were maintained identically to the stock colonies. The sub-colonies were starved four days prior to the experiments in order to achieve a uniform and high motivation for foraging. Any ants which died or were removed from the sub-colonies were replaced from the original stock colonies. Water was available *ad libitum.*

S3.1.2 General Methods

The aim of this series of experiments was to test whether hungry *Lasius niger* foragers can ignore a food source they would normally exploit, if they are aware of a higher quality food source elsewhere. To begin the experiment, a sub-colony was connected to an 80 x 1 cm long paper-covered runway via a 40 cm drawbridge (figure 5-1). A 5mm diameter drop of 1M sucrose (Sigma-Aldrich) was placed on an acetate feeder either at the end of the runway (120cm from the nest), or at 70cm (see below for treatment description). 2-4 ants were allowed onto the runway, and the first ant to reach the feeder was marked with a dot of acrylic paint on its gaster. The other ants were then returned to the nest. The marked ant was allowed to fill its crop and return to the nest (training run). In the nest, the ant unloaded her crop to her nestmates and was then allowed back onto the runway for a second visit. On the second visit, an additional 5mm sucrose droplet of

varying molarity (1M, 0.75M or 0.25M depending on treatment) was placed in the middle of the runway at 60cm from the nest. The ant could not pass the new droplet without contacting it as the drop filled almost the entire width of the runway (about 0.5cm in diameter). However, the ant could bypass the droplet with ease if it chose to. Both food sources were marked with landmarks located beside the runway which the ants could use to aid location memorisation. Since *Camponotus rufipes* workers can travel from 0.5 to 9 km with the energy gained in a single foraging trip only, the costs to move between 120 and 60 cm are considered to be very minimal (Schilman and Roces 2006). We then observed whether the ant drank at the near feeder, chose to skirt around it to reach the original feeder, or drank at both feeders. The ant was considered to have fed at a feeder if her mandibles were dipped into a drop for more than 1 second. Ants which fed on both feeders usually fed on the close feeder first and then moved on to the far feeder. Some ants again returned to the close feeder after that, but this proportion was very minimal. Again, the ant was allowed to drink and return to the nest when it had finished foraging. Shortly before the ant reached the nest, it was removed, to prevent pseudo replication. The average time between the training and testing visits was about three to seven minutes.

S3.1.2.1 Treatments

Each ant experienced one of 4 possible treatments, which varied in the location of the first feeder and the molarity of the second feeder.

1M far vs 0.25M close

In this treatment the initial feeder (1M) was placed at the end of the runway (120 cm from the nest). On its second visit, the ant encountered a 0.25M feeder 60 cm from the nest; that is, 60 cm before the location of the original feeder. The aim of this treatment was to test whether ants can forego foraging on a relatively poor quality food source when they are aware of a higher quality food source further away. *L. niger* workers from colonies deprived of food for 4 days will readily drink and deposit pheromone to 0.25M sucrose (Detrain and Prieur 2014), Oberhauser, Koch and Czaczkes *in prep*).

1M far vs 1M close

This treatment was identical to the first treatment, except that the new food source had an identical quality (1M) to the original food source. The aim of this treatment was to test whether ants that forego a close feeder in favour of a farther one do so because they are comparing the values of the two feeders, or whether ants, once they memorise a food location, ignore other food sources, for example due to behavioural momentum (Podlesnik and Jimenez-Gomez 2016).

1M far vs 0.75M close

This treatment was identical to the first treatment, except that the second feeder provided 0.75M sucrose. The aim of this treatment was to test whether ants would forego a lower quality feeder if the quality difference was not very large.

1M 70cm close vs 0.75M 60cm close

After collecting data on treatments 1-3, we found that ants mostly chose the far, 1M quality feeder when the new feeder offered 0.25M, but mostly chose the new feeder when it offered 0.75M. To ascertain whether this was due to the ants not being able to reliably distinguish 0.75M from 1M, or whether it was due to an explicit choice of the nearer feeder to save energy and walking time, we carried out treatment 4. Here, the first (1M) feeder was placed only 70cm from the nest and the second feeder, offering 0.75M, was placed 60cm from the nest, as in treatment 3. This maintained the quality relationships used in treatment 3, but greatly reduced the distance difference.

We tested 10 individuals from each colony on each treatment, for a total of 80 individuals.

In an additional test, we repeated the '1M far vs 0.25M close' and '1M far vs 1M close' treatments, but allowed the ants to visit the far feeder twice before presenting the near feeder. Sample sizes in these experiments were lower (29 and 28 ants for the two treatments, respectively).

S3.1.2.2 Statistical Analysis

Statistical analyses were carried out in R v. 3.3.2 (R Core Team 2016) using Cumulative Link Mixed Models (CLMMs) in the "Ordinal" package (Christensen 2015). We used CLMMs, because the response variable contains three ordered factors. Thus, an ordered linear regression was necessary. As multiple ants were tested per colony, colony identity was added as a random effect. The three decision codes (1 = far-away feeder, 0.5 = both feeders, 0 = close feeders) were brought into the model as ordered factors.

We used the following model formulae:

Choice ~ DecisionCode ~ treatment + (random effects: colony)

With treatment as a fixed effect.

In addition to that, to explicitly disentangle the effects of food quality and distance to the nest, we performed *post hoc* pairwise comparisons between the four different treatments.

S3.2 Data for two training visits before testing self-control

Methods for this data were the same as those provided in the main text. The difference here is that ants were allowed to visit the far feeder twice before they were confronted with a second, closer feeder. In the data shown in figure 5-2 in the main text, ants were allowed to visit the far-away feeder only once.
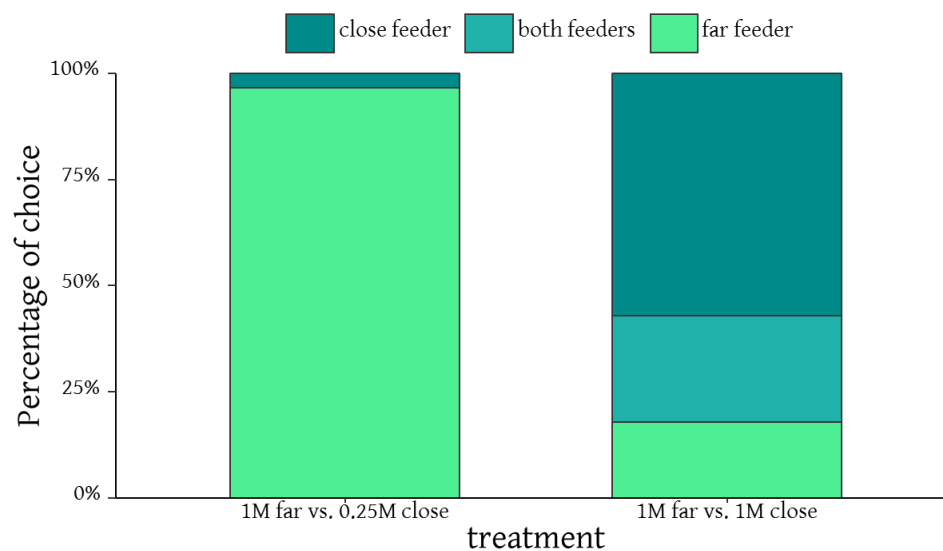
Figure S3-1: The proportions of decisions made for the different feeders for all treatments with two training visits before testing self-control instead of one training visit. The close feeder was located at a distance of 60 cm from the nest while the far-away feeder was located at 120 cm distance to the nest. Treatment is named as molarity of the far-away feeder vs. molarity of the close feeder. Sample sizes are as follows: 1M vs. 0.25M: n= 29; 1M vs. 1M: n= 28. Decisions in 1M vs. 0.25M were significantly different from the other treatment.

S3.3   Comparison between one training visit and two training visits

This model compares the decisions made by ants which were allowed to visit the far feeder once (1M vs. 1M = treatment newgood1 and 0.25M vs. 1M = treatment test1-0.25/1) and ants which were allowed to visit the far feeder twice (1M vs. 1M = treatment newgood 2 and 0.25M vs. 1M = treatment test 2) before confronting them with a second, closer feeder.

```
Coefficients:
            Estimate Std. Error z value Pr(>|z|)
treatmentnewgood1     -0.8563    0.5184 -1.652  0.0986 .
treatmenttest 2        4.8248    1.1419  4.225 2.39e-05 ***
treatmenttest1-0.25/1  2.2756    0.5542  4.106 4.03e-05 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
Coefficients:
            Estimate Std. Error z value Pr(>|z|)
treatmenttest1-0.25/1 -2.549     1.062 -2.399  0.0164 *
treatmentnewgood1     -5.681     1.086 -5.231 1.68e-07 ***
treatmentnewgood 2    -4.825     1.142 -4.225 2.39e-05 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

When ants received two training visits before testing self-control, there are significantly more choices for the far feeder in the 0.25M vs. 1M treatment than in the 1M vs. 1M treatment (z = 4.225, p < 0.001).

There is no significant difference between the 1M vs. 1M treatment with one visit before testing and with two visits before testing. However, in treatment 0.25M vs. 1M, there are significantly more choices for the far feeder when ants were allowed to feed on the far feeder twice before testing self-control (z = 2.399, p < 0.05). This shows that ants show even better self-control when they are more experienced with a food source due to more visits before testing.

S4 Supplement to Chapter 6

Attempts at multiple cue conditioning in *Drosophila melanogaster*

Detailed methods and results overview for all olfactory conditioning approaches presented in chapter 6.

S4.1 Grouped Training in Vial and Block Slide Setups

S4.1.1 Methods

S4.1.1.1 General training procedure

Training occurred on scented pieces of filter paper (1/3 of a 5cm ⌀ filter paper) impregnated with 0.1 or 1M sucrose solution. Pieces of filter paper were soaked in 0.1M or 1M sucrose solution until everything had just been covered in sucrose solution. Afterwards, the filter paper was dried for 2 hours at 50°C until all water had evaporated, leaving just the sugar on the filter paper.

To begin a training, starved flies were transferred into the training setup and provided with filter paper soaked in water which was accessible throughout the whole training procedure. 3 different setups were used: vials, block slide and individual training (figure 6-1, figure 6-2A and figure 6-3A, B, C & D). Within the different setups, feeding time (10 or 30 minutes), pause time (10 or 30 minutes), odours (essential oils (lavender, rosemary and lemon) or food flavours (blackberry, lemon and orange) undiluted or 1:50 diluted solution) and the method of fly movement into the setup (with or without CO_2) were changed (for an overview for the vial and block slide training setups please see tables 2 & 7). Training started with the presentation of a neutral odour (lavender or blackberry) and without reward for the given feeding time to allow flies to form a rewardless association to the first presented odour and thus prevent primacy effects in the following used odours which were presented along with different rewards (Pineño and Miller 2005; Wright et al.

1985; Wright and Roediger 2003). After each feeding interval followed a pause of a given amount of time (see tables 2 & 7 for feeding and pause times of each treatment of the vial and block slide training setups) during which no rewards or odours were present. Flies passed through 6 feeding intervals with a pause in between each 2 feeding intervals. During feeding intervals, flies were trained to associate two sucrose molarities (0.1 and 1M) to different odours (rosemary and lemon or lemon and orange). One molarity was presented along with one of the given odours (e.g. rosemary with 0.1M) throughout a whole training procedure while the other molarity was presented along with the other odour (e.g. lemon with 1M). Odour-molarity pairs were presented in an alternating order, so that each pair was presented three times throughout training. Previous work has shown that *Drosophila melanogaster* flies can form robust expectations of upcoming rewards or punishments based on odours present in the air after 1-4 presentations of each odour/quality combination (Fujita and Tanimura 2011; Quinn, Harris, and Benzer 1974; Tempel et al. 1983). Sweetened filter papers were scented using either rosemary or lemon essential oils (rosemary: *Rosmarinus officinalis*; Lemon: *Citrus limon*, Markl GbR, Grünwald) or lemon and orange food flavours ("Mit allen 5 Sinnen", Grünwald). They were scented by either storing them for at least one day in an airtight box containing a droplet of essential oil (lavender, rosemary and lemon) on filter paper in a petridish or by directly dropping either undiluted or 1:50 diluted food flavours (blackberry, lemon and orange) on the sweetened filter papers.

To find an optimal training method, group training occurred in one of two setups, both of which have previously been reported to be sufficient for odour association in fruit flies (Fujita and Tanimura 2011; Quinn, Harris, and Benzer 1974; Tempel et al. 1983). Furthermore, both feeding and pause times, the nature of the odour cues (either essential oils or food flavours) and the procedure of moving flies into the training setup (either by anesthesia via CO_2 or by knocking flies into the training vials and thus avoiding CO_2 usage). Feeding and pause intervals were first (treatments X & Y) chosen based on previous studies used for fly conditioning (Fujita and Tanimura 2011) and later changed independently. Longer feeding intervals were argued to allow for longer odour/sucrose presentation and may thus improve learning in flies. However, the longer flies are allowed to feed during one interval, the more likely they are to be satiated before training has ended, thus decreasing learning motivation. Longer pause intervals provide more time for previously used odours to escape from the training vials, thus decreasing the probability of odour mixing during feeding intervals and increasing association strength. Flies may also be more motivated to feed on the sweetened filter papers when they are deprived of food for longer times during pause intervals. However, longer pause intervals may also decrease learning due to

longer times without cue presentation during which flies have to remember the previous odour/sucrose combination and the one presented before that. Flies were, however, shown to form robust memory during odour conditioning tasks when the combinations to remember were a reward (or punishment such as electric shock) coupled to an odour and no reward (or punishment such as electric shock) coupled to another odour (Quinn, Harris, and Benzer 1974; Tempel et al. 1983). Essential oils used in these experiments (rosemary and lemon with lavender as neutral odour to prevent primacy effects) have led to robust memory formation in *Lasius niger* ants (Oberhauser and Czaczkes 2018; Oberhauser et al. 2019; Wendt et al. 2019; Wendt and Czaczkes 2019) and were first used in order to keep the methods as comparable as possible. Food flavours were used as another odour cue because essential oils can carry an unattractive taste onto the sweetened filter papers and thus lead to flies disliking one odour over the other which may prevent learning. Flies were previously tested to show no preference for any of the used food flavours in small pilot experiments in order to keep the odour cues neutral. CO_2 has been shown to significantly impair learning, memory and other behavioural traits in *Drosophila melanogaster* (Barron 2000; Bartholomew et al. 2015). Because of that, we trained some flies after moving them via a CO_2 anesthesia into the training vials and others after moving them via knocking (without CO_2 anesthesia).

S4.1.1.2 Vial training setup

Flies trained in treatments A, B, C, D, X, Y and Z were trained in cylindric plastic vials (figure 6-1; 10cm height and 5cm Ø). The vials were closed with a foam plug on one side and fine mesh wire on the other side through which odours used during feeding intervals could escape during pause intervals, thus preventing an accumulation of training odours inside the training vial (figure 6-1). Odour-sucrose combinations were presented via previously prepared pieces of filter paper (see general training procedure for details) which were pinned on the inside of the foam plug. After the vials had been closed with the foam plug, they were turned around so that the scented sucrose filter papers were presented on the bottom of the vial. Training was switched into pause intervals or another feeding interval (with a different odour/sucrose combination) by simply changing the foam plugs of the vials with ones pinned with differently prepared filter papers. Treatments in the vial training setup (treatments A, B, C, D, X, Y & Z) differed in feeding and pause times, whether essential oils or food flavours were used as associative cues and whether flies were anesthetized with CO_2 or not in order to move them into the training vials. If flies were not anesthetized via CO_2, they were moved by simply knocking the open end of the

starvation vial on top of the open end of the empty training vial. For a detailed list of the treatment differences please see table S4-2 in supplement S4.4.

S4.1.1.3 Block slide training setup

Flies trained in treatment E, F, G and H were trained in a block slide setup similar to the setup used in Quinn et al.'s odour and visual discriminative learning experiments (Quinn, Harris, and Benzer 1974). The setup was printed in a 3D printer and consisted of 1 plastic block and 3 tube holders which could independently be slid over the large block (figure 6-2A). Each tube block contained a hole into which a falcon tube (11.4cm height, 3cm ⌀) was plugged. The large block also contained 3 holes onto which the tube holders could be slid to allow for cue and reward presentation. Odour/sucrose filter papers were placed on the bottom of the large block. One block contained no odour/sucrose combination and served as the "pause block". The two remaining blocks each contained one of the used odour/sucrose combination (i.e. either rosemary + 1M or lemon + 0.1M). Two tubes remained empty and were slid on top of the odour/sucrose filter papers to act against a decrease of odour concentration during training. One tube contained the flies which had to be trained and this tube was successively slid over the pause block and both feeding blocks. Water was available via a piece of filter paper soaked in water and pinned into the training tube throughout the whole training procedure.

Training routine used in the block slide training setup also slightly differed depending on treatment (different feeding/pause times, odour types and CO_2 anesthesia during vial movement). For a detailed list of the treatment differences please see table S4-7 in supplement S4.4.

S4.1.1.4 T-Maze test

After training, a choice test was conducted in a T-maze (figure 6-2B). The T-maze choice test was conducted in a dark room under red light with constant temperature at room temperature level to prevent phototaxis and possible effects of different temperatures. The T-maze consisted of three PVC slices. The middle slice was vertically moveable and held a small chamber through which the flies could be transferred from the odour neutral rest chamber into the T-maze. Flies were introduced via a vial connected to the upper end of the setup and stayed in this chamber for 3 minutes under red light to become familiar with the new environment. Afterwards, flies were knocked into the chamber inside the mid slice and slid down into the T-maze. Here, two other vials were connected to each side of the setup with each chamber containing one of the associated odours, but no sucrose (i.e. rosemary odour left and lemon odour right). Flies were now allowed to choose a side for 1 minute until the T-maze was closed and flies present on the

right and left sides of the T-maze (flies which made a decision) and in the middle chamber (flies which had not made a decision) were counted. Odours were introduced to the T-maze via small reservoirs connected to the end of each T-maze vial and circulated through the setup via a constant air flow. Odours used in the reservoirs were either undiluted essential oils or undiluted food flavours depending on the training procedure flies had gone through.

S4.1.1.5 Statistical Analysis

Statistical analyses were carried out in R v. 3.5.3 (R Core Team 2016) using Generalized Linear Mixed Models (GLMMs) in the LME4 package (Bates et al. 2014) to test whether choices differed depending on treatments inside the same training procedures and exact binomial tests to test whether the obtained choices differed from random choices. Number of the breeding vials were included as a random factor in the GLMMs, because flies of different vials may show differences in learning success due to breeding and possible mutations which may have occurred over time and impair learning and memory (Dudai et al. 1976; Quinn, Sziber, and Booker 1979; Tempel et al. 1983). GLMMs were tested for fit, dispersion and zero inflation using the DHARMa package (Hartig 2017). The model predictors and interactions were defined *a priori*, following Forstmeier and Schielzeth (2011). All p-values presented were corrected for multiple testing using the Benjamini–Hochberg method (Benjamini and Hochberg 1995). Exact binomial tests were performed for each treatment and setup in which choices for the high odours and choices for the first presented odour/reward combination were tested against a random distribution of 50% of choices for each odour respectively. For individual training, an exact binomial test was performed for PER responses towards water + blackberry odour to test whether flies showed a preference for water significantly different from random choice. This test was done to confirm that flies included in the statistics were not biased due to thirst.

GLMMs used to analyse differences between the vial and block slide setups were run with a binomial T-maze choice variable (proportion of flies at the high/first odour side in the T-maze) as response variable. Fixed factors included were Setup (vial or block slid), feeding time, odour presented along with high molarity food, whether the first odour introduced in the training was presented along with 0.1 or 1M (1 if it was presented with 1M, 0 if it was presented with 0.1M), odour type (essential oils, food flavours either undiluted or as a 1:50 diluted solution and whether CO_2 for fly movement from the starvation to the training vials. This resulted in the following model formula for the vial and block slide setups:

Proportion of flies at high/first odour ~

Setup + FeedinTime + HighScent + FirstScentHigh + OdourType + CO$_2$Pushing + (random factor: VialNr)

S4.1.1.6 Results

We conducted a total of 52 complete trainings in the vial training setup, in which a total of 2166 flies was trained and allowed to make a choice in the T-maze. In the block slide training setup, a total of 18 complete trainings was run. In this setup, a total of 732 flies was trained and allowed to make a choice in the T-maze. For a more detailed description of sample sizes tested in the different treatments, please refer to tables S4-3, S4-5, S4-8 and S4-10.
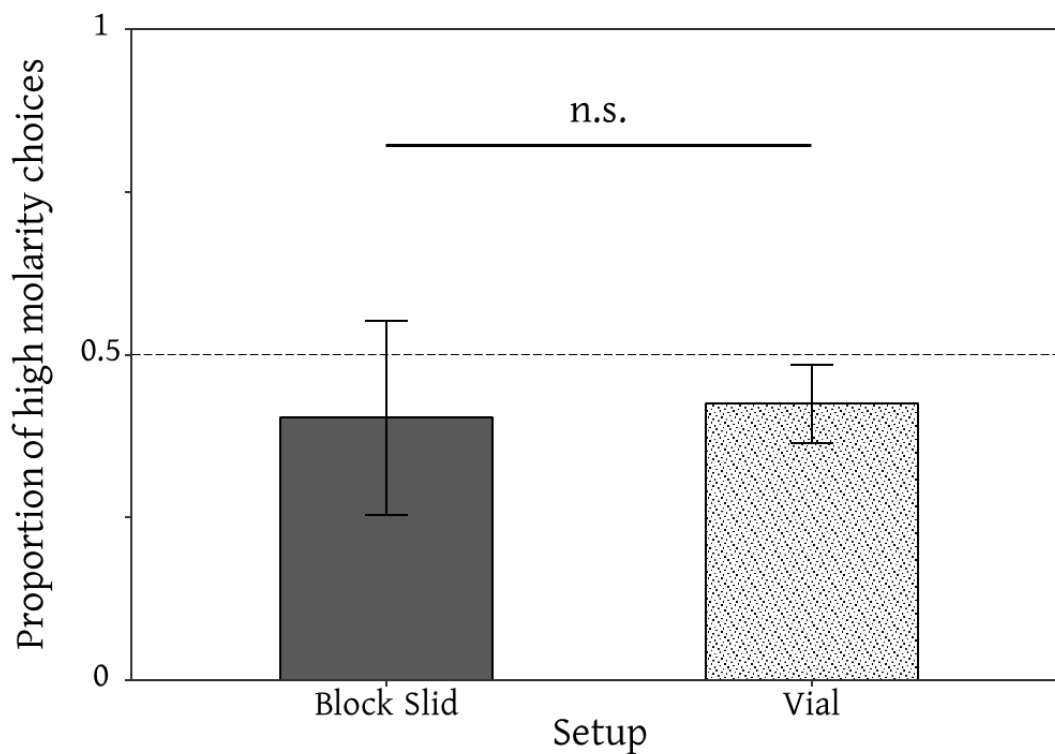
Figure S4-1: Proportion of choices for the high molarity odour cue side in the T-maze for flies which were trained in the vial and block slide training setups, respectively. Flies had been presented a combination of low sucrose with one odour (e.g. rosemary or lemon) and high sucrose and another odour (e.g. lemon or orange) during training. Afterwards, flies were allowed to make a choice in a T-maze. In one arm of the T-maze, the odour previously presented along with high sucrose was offered. In the other arm, the odour presented with low sucrose was offered. The dotted line represents a hypothetical choice of 50% for each odour side.

The proportion of choices for the odour which had been presented along with high molarity sucrose during training did not differ between the vial and block slide training setups (*GLMM*: estimate = 0.12, z = 1.2, p = 0.26). 42.5% of flies which had been trained in the vial training setup

chose the T-maze side in which a high molarity odour cue was presented (figure S4-1). This proportion was significantly different from random choice (*exact binomial test* p < 0.001), suggesting that flies avoided the T-maze side in which a high molarity odour was offered. Only 36.2% of flies trained in the block slide setup chose the T-maze side in which a high molarity odour was offered (see figure S4-1, table S4-1). This proportion was also significantly different from chance level (*exact binomial test* p < 0.001). Feeding times did not affect proportion of choices in the T-maze (*GLMM*: estimate = 0.01, z = 1.38, p = 0.22). The usage of CO_2 before fly movement did also not affect choice behaviour in the T-maze (*GLMM*: estimate = 0.19, z = 1.67, p = 0.14). Proportion of choices for the high molarity odour in the T-maze were significantly higher when the odour presented first during training was offered along with high molarity food (*GLMM*: estimate = -0.46, z = -6.1, p < 0.001). Flies trained with essential oils were were significantly more likely to choose the high molarity odour in the T-maze compared to flies trained with undiluted food flavours (*GLMM*: estimate = -0.99, z = -2.69, p < 0.05) and a 1:50 diluted solution of food flavours (*GLMM*: estimate = -0.49, z = -2.71, p < 0.05) as odour cues. Furthermore, proportion of choices for the high molarity odour in the T-maze were higher when the offered odour cue was orange (*GLMM*: estimate = 1.08, z = 5.58, p < 0.001).

Flies trained in the vial training setup showed a similar proportion of choices for the first rewarded odour which was presented during training (47.8%, table S4-1) than flies trained in the block slide setup (57%, *GLMM*: estimate = 0.02, z = 0.22, p = 0.8). The molarity presented along with the odour offered first during training also did not affect T-maze choice in the vial and block slide training setups (*GLMM*: estimate = -0.05, z = -0.7, p = 0.73). The proportion of choices for the first odour was not different from a random choice in flies trained in the vial training setup (*exact binomial test* p = 0.17), suggesting that flies did not show a preference for the first rewarded odour cue when they were trained in the vial training setup. In the block slide training setup, the proportion of choices for the first rewarded odour cue was significantly different from chance (*exact binomial test* p < 0.001) with 57% of flies being found in the side of the T-maze in which the odour which had first been presented during training was offered (table S4-1).

Table S4-1: Detailed overview of fly's choices made in the T-maze after training in the vial and block slide training setups. The table includes choices of flies trained in all treatments of the vial and block slide training setups (treatments A, B, C, D, X, Y and Z for the vial training setup and treatments E, F, G and H for the block slide training setups). Given are the sum of choices for both odours presented in the T-maze and flies which didn't make a choice (stayed in the middle of the T-maze), the number of choices for the odour presented along with high molarity sucrose, the number of choices for the odour presented first during training, the percentage for both the odour presented with high molarity and odour presented first during training and the p-values of exact binomial tests against the null hypothesis of random choices for high molarity odour and odour presented first during training.

		Vial Training Setup	Block Slide Setup
	Sum of choices	2166	732
High odour choices	No. choices for high molarity odour	932	265
	% for high odour	42.5	36.2
	Exact binomial test p-value	**< 0.001**	**< 0.001**
First odour choices	No. choices for first presented odour	1115	417
	% for first presented odour	47.8	57.0
	Exact binomial test p-value	0.17	**< 0.001**

For a detailed overview of fly choices in different vial and block slide treatments please see figures S4-7 and S4-8, and tables S4-3, S4-5, S4-8 and S4-10.

S4.2   Individual training via Proboscis Extension Reflex (PER) response

Drosophila were shown to successfully discriminate a reward and non-reward through a classical PER paradigm (Chabaud et al. 2006) and PER conditioning proved to be a successful tool in honeybee conditioning as well (Giurfa and Sandoz 2012). Furthermore, *Lasius niger* workers investigated in our previous studies (Oberhauser and Czaczkes 2018; Oberhauser et al. 2019; Wendt et al. 2019; Wendt and Czaczkes 2019) were also trained individually and successfully preferred an odour presented along with higher quality food over a low food quality odour pair

after training. Therefore, we conducted individual training via the PER response in addition to the above presented grouped training setups. Odours were presented through puffing scented air out of commonly used glas pipettes containing a piece of kitchen paper soaked with either essential oils or undiluted orange, lemon or blackberry food flavours. Training was conducted similar to the PER paradigm described in Shiraiwa and Carlson's work (2007). Training molarities (0.5M, 0.1/1.5M, 0.01/1M or 0.1/1M pairs), odour types (rosemary and lemon essential oils or undiluted food flavours with lemon and orange flavor/odour) and fixation methods (yellow panel stickers, pipette tips, sponge or PVC slides) differed depending on the training treatment.

Sucrose solutions were presented via small paper wigs. These were prepared beforehand by twisting a ca 6mm wide piece of thin paper tissues (e.g. Kimwipes®) into a thread. The thread was then torn into multiple 5mm long pieces (also see Shiraiwa & Carlson (2007) for a video and step by step protocol on wick preparation and PER assay procedure). The wigs were soaked in water or sucrose solution and then presented to the fly's labellum.

S4.2.1 Pipette Tip fixation

S4.2.1.1 Methods

Flies trained with this fixation method were fixed inside a pipette tip following Shiraiwa & Carlson's (2007) PER assay protocol. Starved flies were individually trapped in an aspirator and a pipette tip placed over the opening. Now, the open end (with the pipette tip on it) was flipped once while blowing air into the aspirator. This resulted in the fly being trapped in the pipette tip. The fly was gently blown further into the pipette tip, to a point at which it could not move. A mark was made at the position of the fly's eye and the fly slightly soaked out of the tip. Now, using a sharp razor blade, the end of the tip was removed at the marked position and the fly again blown back into the tip. The tip was removed from the aspirator and small pieces of cotton pushed inside it, up to the point at which the fly was trapped. This procedure left the fly trapped in the pipette tip with only its head (and mostly forelegs) being free for use and no way out on the open end.

Flies fixed in pipette tips were trained only to 0.5M sucrose solution paired with lemon or rosemary odour (essential oil). The sucrose/odour pair was presented four times with an intertrial interval of 10 minutes. Each training procedure started with the presentation of water, as suggested by Shiraiwa & Carlson (2007) and was followed by the presentation of a low molarity sucrose solution (0.1M). These two pre tests allowed us to discard flies which i) were thirsty and

would thus have been likely to show a PER response towards any offered solution and ii) flies, which were not hungry or motivated to react to any offered reward. PER responses were recorded for each presentation trial. After 4 training trials in which flies were presented 0.5M sucrose solution along with rosemary or lemon odour, flies were puffed with just the odour and the PER response recorded. To end a trial, we conducted a second test, offering the flies just water and on another trial 0.1M sucrose solution in order to test whether flies got thirsty during training or conditioned to something being poked into their faces (e.g. the paper wick soaked in sucrose solution).

S4.2.1.2 Statistical Analysis

Data for flies trained while fixed with pipette tips was analysed with a GLMM using binomial distribution and PER response (binomial; did flies show PER or not?) as response variable. Phase (training or test) and odour (lemon or rosemary) were included as fixed factors. Because flies had been tested multiple times, but vial number had not been recorded for this data, we only included FlyID as a random factor. This resulted in the following model formula:

PER Response ~ Phase + Odour + (random factor: Fly ID)

S4.2.1.3 Results

A total of 7 flies was fixed in pipette tips and trained to only 0.5M sucrose solution associated to one odour. We thus recorded a total of 7 PER responses in the test phase and 28 during training.
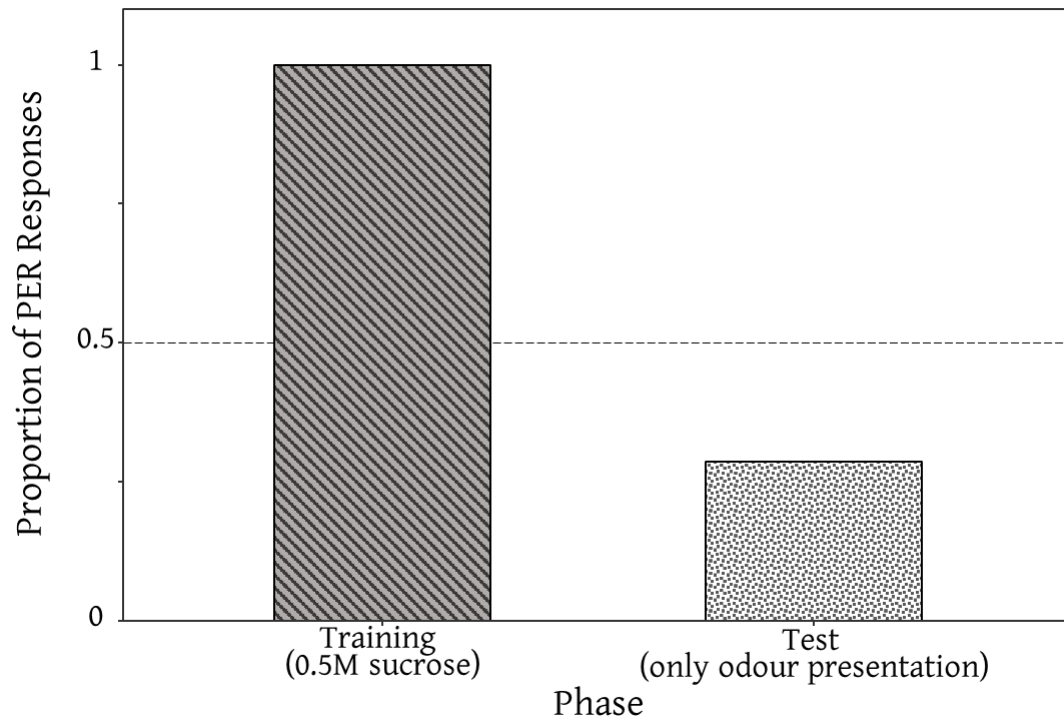
Figure S4-2: Proportion of PER responses for flies trained individually in a PER assay when they were fixed in pipette tips. Flies were offered a 0.5M sucrose solution via a paper wick soaked in the solution accompanied with one odour (lemon or rosemary) four times and then tested by offering them only the odour, but no reward. The dotted line represents a hypothetical choice of 50% for each odour side.

Figure S4-2 shows that the rate of positive PER responses was uniformly high during training (when flies were presented and odour and a 0.5M sucrose solution simultaneously). However, due to a low sample size, a stable model could not be run over this data and thus significant differences cannot be stated. During training, flies showed a PER response in 100% of sucrose/odour presentations which was significantly different from random choice (*exact binomial test* p < 0.001). In contrast, only 28.5% of flies showed a PER response when only the odour was presented after 4 training presentations. Due to a low sample size (only 7 flies tested), a significant difference from random choice could now be shown (*exact binomial test* p = 0.45).

S4.2.2 Yellow Panel Sticker fixation

S4.2.2.1 Methods

Because a majority of flies did not remain in a fixed position inside the pipette tips, we moved to yellow panel stickers for fly fixation. Furthermore, sucrose concentration was decreased for

training and a second sucrose/odour pair added to the training procedure to allow flies to form associations based on different sucrose concentrations and develop a preference for one sucrose/odour pair.

Flies trained in the "Yellow Panel Sticker fixation" treatment were glued to sticky yellow panels (frequently used for control of fruit flies and sciarids in households) with their wings and back. The panels were glued to a piece of cardboard box with one side, allowing us to fix the flies in a vertical position (also see figure 6-3A). Flies in this treatment were trained to a high sucrose molarity (1M or 1.5M) paired with one odour (rosemary or lemon) and a low sucrose molarity (0.01M or 0.1M) paired with the other odour. Training always started with the low molarity sucrose/odour pair. After an intertrial interval of 2 or 5 minutes, the high molarity sucrose/odour pair was presented. Sucrose/odour pairs were then presented in an alternating order with each pair being presented 5 times during training. After 5 presentations of each sucrose/odour pair, flies were puffed with only the associated odour. Either flies were first puffed with the odour associated to high molarity sucrose and then, after an intertrial interval of 2 or 5 minutes (the interval was kept constant for each fly, but differed on different training days in order to find possible positive effects of intertrial intervals on association formation), were presented the odour associated to low molarity sucrose, or the other way around. To finalize and experimental run, each fly was presented a paper wick soaked in water, but no odour to control for flies which had gotten thirsty throughout the training assay.

The PER response was recorded for each fly and each presentation throughout the PER assay.

S4.2.2.2 Statistical Analysis

Data for flies trained while fixed with yellow panel stickers was analysed with a GLMM using binomial distribution and PER response (binomial; did flies show PER or not?) as response variable. Phase (training or test), whether high (1 or 1.5M) or low (0.01 or 0.1M) molarity sucrose was presented, odour (lemon or rosemary) and trial number (as a measure for experience with the sucrose/odour pairs) were included as fixed factors. Because flies had been tested multiple times, but vial number had not been recorded for this data, we only included FlyID as a random factor. This resulted in the following model formula:

PER Response ~ Phase + HighLowMolarity + Odour + scale(TrialNumber) + (random factor: Fly ID)

S4.2.2.3 Results

A total of 39 flies was trained to a high and low molarity odour pair, resulting in 39 PER responses for the high molarity and low molarity association tests, respectively (16 for the 0.01/1M and 23 for the 0.1/1.5M combinations).
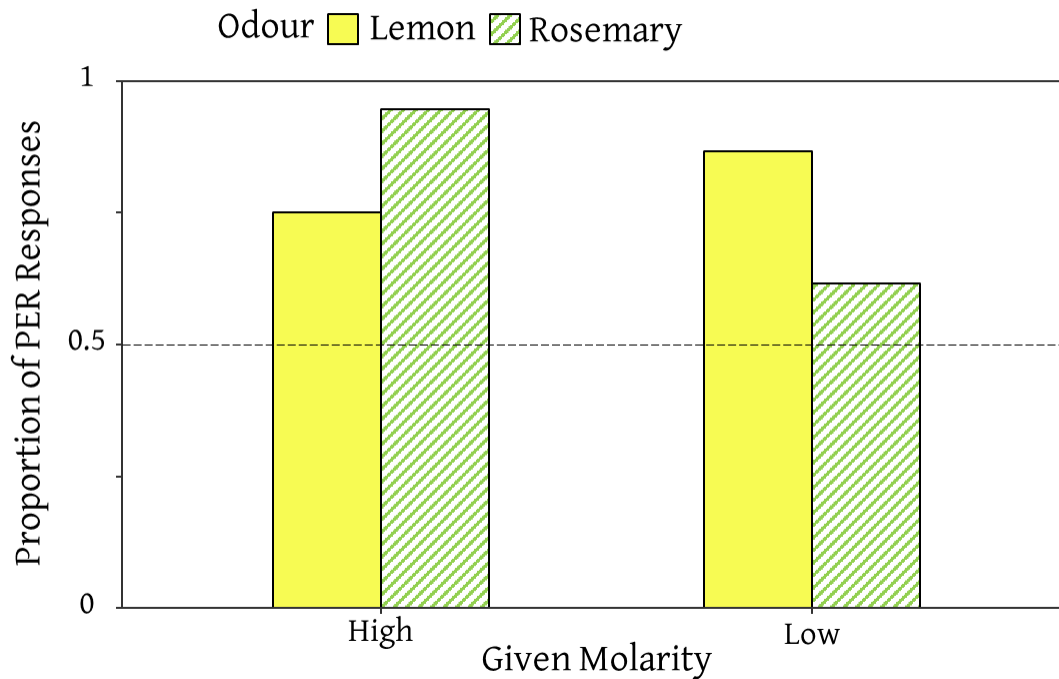

Figure S4-3: Proportions of positive PER responses during training in the PER assay with yellow panel sticker fixation. Responses are split by high (1 or 1.5M) and low (0.01 or 0.1M) sucrose reward and by odour (lemon or rosemary), respectively, to display possible differences in odour preferences. The dotted line represents a hypothetical choice of 50% for each odour side.

The proportion of positive PER responses was significantly higher when flies were presented a paper wick soaked in sucrose (during training) compared to the test presentations on which flies were only puffed with the odour which had previously been presented along with the rewards (see figure S4-3 and figure S4-4; Training compared to test at which odour cue for low molarity was offered alone: *GLMM pairwise comparison* estimate = -6.08, z = -7.36, p < 0.001; Training compared to test at which odour cue for high molarity was offered alone: *GLMM pairwise comparison* estimate = -7.11, z = -7.37, p < 0.001). Additionally, flies were more likely to show a PER response when high molarity sucrose (1 or 1.5M, 82.5% positive PER responses) was offered compared to low molarity sucrose (0.01 or 0.1M, 71.3% of positive PER responses, *GLMM*: estimate = -1.3, z = -3.5, p < 0.001). The trial number (can be interpreted as a measure of experience with

the sucrose/odour combinations) did not significantly affect PER responses (*GLMM*: estimate = -0.15, z = -0.86, p = 0.39). Proportions of positive PER responses during training were 62.5% when 0.01M, 77.4% when 0.1M, 83.7% when 1M and 81.7% when 1.5M sucrose was presented (see figure S4-4 for data split by high (1 and 1.5M merged) and low (0.01 and 0.1M merged) molarities and odour). All of these proportions were significantly different from chance level (0.01M: *exact binomial test* p < 0.05; 0.1M: *exact binomial test* p < 0.001; 1M: *exact binomial test* p < 0.001; 1.5M: *exact binomial test* p < 0.001). In contrast, when flies were only puffed with an odour but not presented a reward after training in the PER assay, the proportion of positive PER responses was very low with only 10.2% when flies were puffed with the odour which had previously been presented along with the high molarity (1M or 1.5M) sucrose solutions and 17.9% when the odour previously presented along with low molarity was presented (see figure S4-4 for a display of PER responses split by high or low molarity odour cue and odours). PER responses were significantly different from random choice in both unrewarded tests (odour cue for high molarity presented: *exact binomial test* p < 0.001; odour cue for low molarity presented: *exact binomial test* p < 0.001). Even though figure S4-4 strongly suggests it, the presented odour cue (rosemary or lemon) did not significantly affect PER responses (*GLMM:* estimate = 0.08, z = 0.22, p = 0.83).
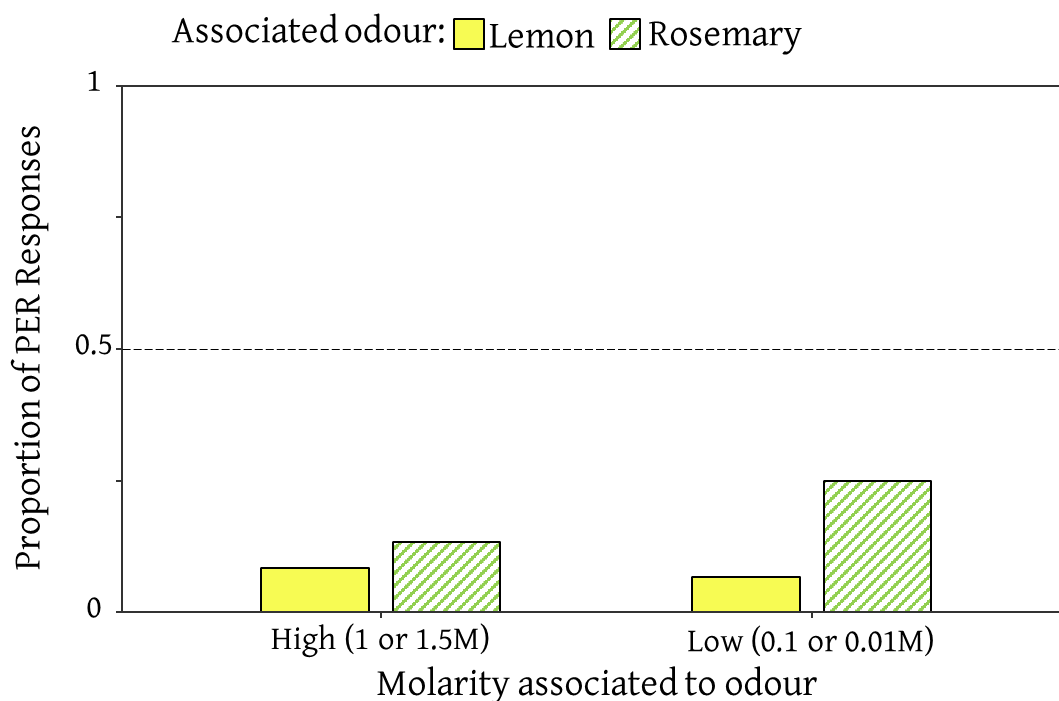

Figure S4-4: Proportions of positive PER responses after training in the PER assay with yellow panel sticker fixation. Odours which had been presented along with either low (0.01 or 0.1M) or high (1 or 1.5M) molarity

odours were now presented without a reward via puffing out of a glass pipette. Responses are split by odour (lemon or rosemary) to show a slightly stronger response rate when presented with rosemary odour. The dotted line represents a hypothetical choice of 50% for each odour side.

S4.2.3 Sponge and PVC slide fixation

These training procedures were conducted as a continuation of the PER assays mentioned above. Because flies did not seem to form an expectation based on a given odour in the PER assay, we continued to train them individually, but then transferred them to a Y-maze for a final choice test. However, because flies had to be transferred into the Y-maze alive and without injuries, we had to refrain from the previously used fixation methods (pipette tips and yellow panel stickers) and find a method which allowed flies to be freed and transferred to the Y-maze after individual training via a PER assay.

S4.2.3.1 Methods

Flies were fixed in two different ways: they were either fixed to i) a soft PVC stripe with V-shaped cavities leaving space for the flies' necks (treatment I). The opening was then closed with a piece of modeling dough, leaving 5 flies fixed with 2 cm distance to each other (figure 6-3B) or to ii) a piece of a sponge (2 x 1cm) wrapped with a piece of kitchen paper providing a smooth surface (treatment K). A piece of a PVC sheet was then bound on top of the flies, leaving them fixed with their back to the sponge-side, but leaving their heads and forelegs free of fixation (figure 6-3C). Flies had to be anesthetized with CO_2 prior to fixing.

In these treatments, flies were presented food flavours instead of essential oils (as used in the other two PER assays) as associative cues. To begin a training, flies were presented water to control for thirsty flies and exclude these beforehand. In contrast to the treatments described above, flies were puffed with blackberry odour simultaneously to water presentation in this treatment. With this presentation of a first odour without reward presentation, the likelihood of a formation of primacy effects – in which the first associative cue has a stronger associative strength than any cue associated later during training – could be decreased. Training consisted of 8 trials (4 to each sucrose/odour pair) in which flies were first puffed with odour and then offered a paper wick soaked in sucrose solution of either low (0.1M) or high molarity (1M). Each molarity was presented along with one odour (lemon or orange undiluted food flavours) and each sucrose/odour combination was presented 4 times in an alternating order. PER responses were noted for each sucrose/odour presentation. The intertrial interval was kept constant at 2 minutes for each fly. After training, flies were again confronted with blackberry odour and water to

exclude flies which had gotten thirsty throughout the training procedure and may thus have reacted to all offered sucrose solutions in order to receive the water from it. Flies were anesthetized with CO_2 before fixing them for PER training. After training, flies were transferred into a Y-maze for a final choice test in which one odour was presented in one Y-maze arm and the other odour in the other arm (see figure 6-3D).

S4.2.3.2 Statistical Analysis

Data for flies trained while fixed with sponges and PVC slides were merged and analysed in one model, because the setup did not affect learning ability and the training procedure was identical. The model was run with a binomial distribution and PER response (binomial; did flies show PER or not?) as response variable. Molarity, odour and trial number (as a measure of experience with the sucrose/odour pairs) were included as fixed factors. This resulted in the following model formula:

PER Response ~ Molarity + Odour + TrialNumber + (random factor: Vial Nr)

S4.2.3.3 Results

A total of 21 flies was trained in the PER assays with sponge (10 flies) and PVC slide (11 flies) fixation. However, only 3 of the trained flies were successfully transferred into the Y-maze to note their odour/side choice after training. Of these 3 flies, only 1 made a choice in the Y-maze. Other flies transferred into the Y-maze did not show any sign of movement and some died after removement from the PER assay fixation. Because of that, this data is not further included in the interpretation of the learning success.
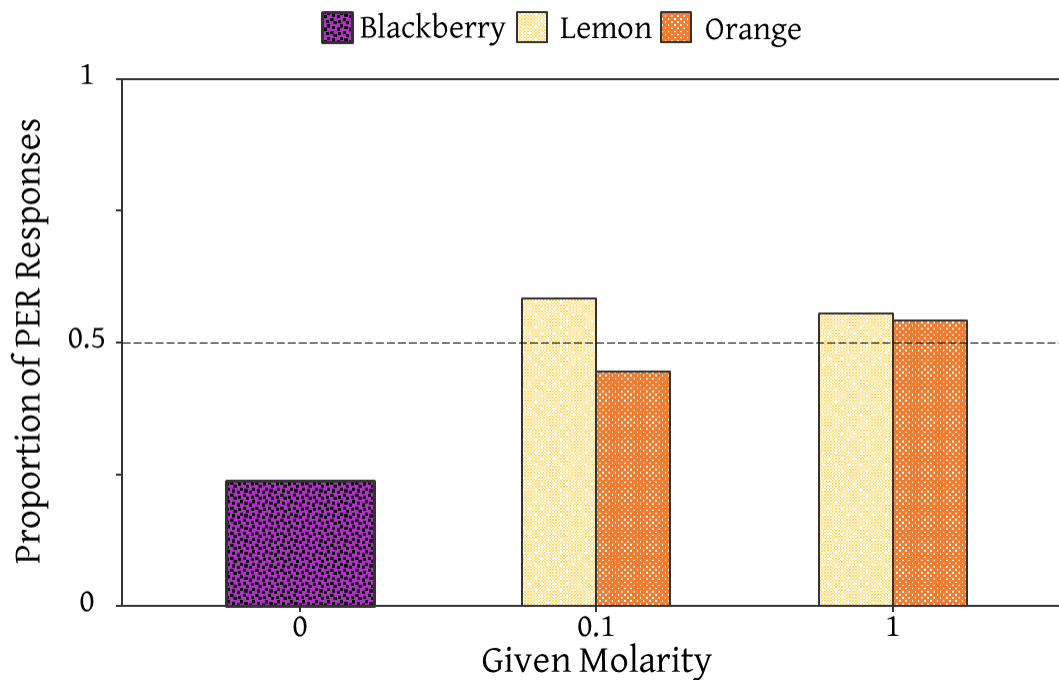
Figure S4-5: Proportion of positive PER responses of flies trained individually in a PER assay (fixed either to a sponge or via PVC slides) for all possible food/water - odour combinations (water + blackberry odour, 0.1M + lemon or orange odour and 1M + lemon or orange odour). The dotted line represents a hypothetical choice of 50% for each odour side.

Flies trained individually via PER assay with fixation on a sponge or through PVC slides did show an increased PER response when 1M sucrose was presented via a paper wick soaked in sucrose solution compared to when 0.1M was presented (figure S4-5; *GLMM*: estimate = 0.17, z = 0.52, p = 0.6). Odours and trial numbers did also not significantly affect PER response in flies (Odour: *GLMM*: estimate = -0.28, z = -0.87, p = 0.51; Trial Nr: *GLMM*: estimate = -0.3, z = -1.8, p = 0.28). Furthermore, as can also be seen in figure S4-5, the proportion of PER responses did not differ from chance level for both offered molarities (1M: *exact binomial test* p = 0.38; 0.1M: *exact binomial test* p = 0.66). However, the proportion of PER responses upon water presentation along with black berry odour was significantly lower than that expected from a random choice (*exact binomial test* p < 0.001), suggesting that flies were not thirsty and were more likely to react when a reward was offered. Because flies could not be motivated to make a choice in the Y-maze after training, this data is not shown here.

S4.3   Individual Training in the Y-Maze

S4.3.1 Methods

Flies did not seem to learn odours offered along with a high or low molarity sucrose solution in the individual training procedures using PER assays and grouped training in the vial and block slide training setups. Furthermore, flies did not show any movement or motivation to make a choice in the Y-maze after being transferred from the PER fixation. Therefore, instead of transferring them to the Y-maze after training through a PER assay, we wanted to train and test them directly in the Y-maze. This procedure is similar to odour conditioning in individual *Lasius niger* ants (Czaczkes 2018; Oberhauser and Czaczkes 2018; Oberhauser et al. 2019; Wendt et al. 2019; Wendt and Czaczkes 2019) with the Y-maze consisting of tubes rather than open runways in this case. This experimental procedure is commonly used in odour conditioning in ants and other invertebrates and and was shown to lead to high learning rates (Czaczkes 2018; Dupuy et al. 2006; Josens, Eschbach, and Giurfa 2009; Oberhauser et al. 2019).

In this treatment, individual flies were directly trained in a Y-maze consisting of 3 falcon tubes (12cm height, 1.7cm ⌀) connected by a 3D-printed Y-shaped tube holder (figure 6-3D). Sweetened and odoured pieces of filter paper were placed in two Y-maze tubes (i.e. 0.1M with undiluted orange food flavor and 1M with undiluted lemon food flavor on it), one odour on each side. To begin a training, an individual fly was placed in the unscented tube of the Y-maze and allowed to get familiar with the setup for 5 minutes. Afterwards, the fly's choice was recorded for up to 8 times in between which it was knocked back into the starting tube to begin another choice trial. The Y-maze was placed on a fixed position inside the room to keep visual and light cues constant.

S4.3.2 Statistical Analysis

Choices made in the Y-maze after flies had been individually trained via a PER assay and choices made by flies trained directly in the Y-maze were analysed in a binomial GLMM with a binomial variable of whether flies chose the high odour side in the Y-maze as response variable. Fixed factors included in the model for were the phase in which the flies were in (training choices or the last test choice), odour presented along with high molarity sucrose (orange or lemon) and trial number (as a measure of experience with the sucrose/odour pairs). Because flies were tested

multiple times, we also included Fly ID nested in Vial number in the model. This resulted in the following model formulas:

Y-maze choice ~ Phase + Odour + TrialNr + (random factor: VialNr and Fly ID nested in Vial Nr)

S4.3.3 Results

In the individual Y-maze training, a total of 15 flies was investigated, of which 97 choices were recorded (43 for the high molarity odour side and 55 for the low molarity odour side). Final and initial choices matched in 93.8%. Thus, only final choices were further considered for data interpretation.
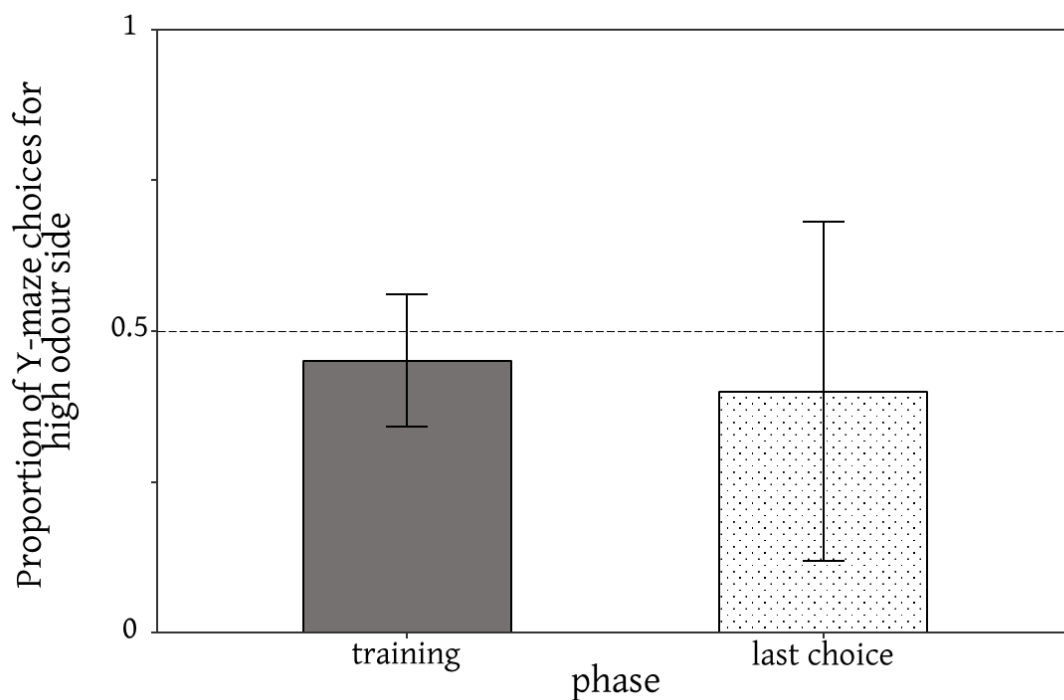
Figure S4-6: Proportion of Y-maze choices of flies trained individually in the Y-maze. Shown is the proportion of choices made during the training runs and during the last choice of trained flies. Flies could either choose the high molarity side on which one odour (e.g. lemon) was presented along with high molarity (1M) sucrose filter papers or the low molarity side on which another odour (e.g. orange) was presented along with low molarity (0.1M) sucrose. Shown are only the final choices. The dotted line represents a hypothetical choice of 50% for each odour side.

Y-maze choices did not significantly differ between phases (training or last choice (see figure S4-6), *GLMM*: estimate = 0.42, z = 0.6, p = 0.8). Trial number and and odour cues associated to the molarities (orange or lemon) did also not have a significant effect on Y-maze choices (trial number: *GLMM*: estimate = 0.13, z = 0.51, p = 0.8; odour: *GLMM*: estimate = 0.002, z = 0.006, p = 0.99).

The proportion of flies choosing the odour side presented along with high molarity (1M) odour did not significantly differ from a random choice (*exact binomial test* p = 0.31) when they were trained directly in the Y-maze. The choices made for the odour presented with low molarity (0.1M) odour was also not significantly different from a random choice (*exact binomial test* p = 0.31). Including the "training" choices, flies made 44 choices for the high molarity odour side and 55 choices for the low molarity odour side. The pattern of choices does not change when looking at only the last choices of trained flies in which flies were supposed to have learned to predict the food quality based on the accompanied odour. Here, 7 flies made a choice for the high molarity odour side and 10 flies chose the side on which low molarity odour was presented (see figure S4-6). The last choices were also not significantly different from chance level (high molarity odour: *exact binomial test* p = 0.6; low molarity odour: *exact binomial test* p = 0.6).

S4.4 Vial and Block Slide Training Setups – Treatment differences

Treatments in the vial (treatments A, B, C, D, X, Y & Z; see figure 6-1 for the training setup) and block slide (treatments E, F, G & H; see figure 6-2A for the training setup) training setups differed in feeding and pause times, the odour types used as associative cues (essential oils and food flavours undiluted or 1:50 diluted solution) and whether flies were anesthetized with CO_2 or not in order to move them into the training vials. If flies were not anesthetized via CO_2, they were moved by simply knocking the open end of the starvation vial on top of the open end of the empty training vial. The above changes were made based on learning success of flies and general observations during training and the T-maze test. For example, the amount of essential oils used was decreased due to flies showing a higher mortality when confronted with rosemary odour. This may also have caused an avoidance and acted against association learning in flies. Because of that, essential oils were replaced by food flavours in later treatments. Food flavours may taste and smell better than the previously used essential oils and thus increase fly's motivation to learn sucrose/odour combinations.

Furthermore, feeding times were changed frequently throughout treatments in order to find a feeding/pause time combination at which flies showed robust odour preference. In addition to that, we resigned from using CO_2 anesthesia for moving flies from the starvation to the training vials, because CO_2 has been shown to have negative effects on various traits such as fecundity and climbing abilities (Barron 2000; Bartholomew et al. 2015; de Crespigny and Wedell 2008; Nicolas and Sillans 1989; Perron et al. 1972). For a detailed list of all treatment differences please see tables S4-2 and S4-7.

S4.4.1 Statistical Analysis

GLMMs used to analyse differences between treatments of the vial and block slide setups were run with a binomial T-maze choice variable indicating the proportion of fly choices for either the high molarity odour or the odour presented first during training as response variable. Fixed factors included were treatment, HighScent (the odour cue presented along with high molarity sucrose) and FirstScentHigh (whether the first odour introduced in the training was presented along with 0.1 or 1M; 1 if it was presented with 1M, 0 if it was presented with 0.1M). This resulted in the following model formula for the vial and block slide setups:

Proportion of flies at high/first odour ~ treatment + HighScent + FirstScentHigh + (random factor: VialNr)

S4.4.2 Vial training setup

S4.4.2.1 Methods

Table S4-2: Detailed overview of all differences between all treatments conducted in the vial training setup.

Treatment	Feeding Time [min]	Pause Time [min]	CO_2 Anestetization	Odour Type	Odours used	Filter Paper
A	10	30	yes	essential oils	(lavender, rosemary, lemon)	dried
B	30 10	10 30	no	essential oils	(lavender, rosemary, lemon)	dried
C	30 10 10	10 30 10	no	food flavours (1:50 dilution)	(blackberry, lemon, orange)	dried
D	30 10	10 10	no	food flavours (undiluted)	(blackberry, lemon, orange)	dried
X	10	60	yes	essential oils	(rosemary, lemon)	dried
Y	10	60	yes	essential oils	(rosemary, lemon)	solution
Z	10	30	yes	essential oils	(rosemary, lemon)	dried

S4.4.2.1.1   Treatment A

Flies were confronted with each sucrose/odour pair 3 times, adding up to a total of 6 training trials. Feeding times were always 10 minutes, while pause time was always 30 minutes. Flies were anesthetized with CO_2 in order to move them from the starvation into the training vials. Odour types used were essential oils. The used odours were lemon and

rosemary as associative cues for sucrose solutions and lavender as the first presented odour to prevent the formation of primacy effects. The used filter papers had been soaked in sucrose solution and dried thereafter. Filter papers were scented by storing them for at least one day in an airtight box containing a piece of cotton soaked in the essential oil.

S4.4.2.1.2 Treatment B

In this treatment, flies were not anesthetized with CO_2 in order to prevent memory impairments which may occur after CO2 anesthesia (Colinet and Renault 2012). Furthermore, feeding and pause times were changed from 10 to 30 minutes in later trainings of this treatment. This was done in order to test whether longer feeding times improved association formation in *Drosophila* fruit flies. Odour types used were essential oils. The used odours were lemon and rosemary as associative cues for sucrose solutions and lavender as the first presented odour to prevent the formation of primacy effects. The used filter papers had been soaked in sucrose solution and dried thereafter. Filter papers were scented by storing them for at least one day in an airtight box containing a piece of cotton soaked in the essential oil.

S4.4.2.1.3 Treatment C

Treatment C differed from treatment B in the used odour type. Instead of essential oils, odours were introduced via 3 droplets of a 1:50 diluted solution of food flavours which was placed directly on the dried sucrose filter papers. We thus also used lemon and orange odours as associative cues and blackberry odour as a first unrewarded odour to prevent primacy effects. Feeding and pause times were changed between trainings, so that the combinations 30 min feeding/10 min pause, 10 min feeding/30 min pause and 10 min feeding/ 10 min pause were tested. In this treatment, flies were again not anesthetized via CO_2.

S4.4.2.1.4 Treatment D

This treatment differed from treatment C just in the usage of undiluted food flavours instead of a 1:50 diluted solution of food flavours. The dilution was changed because odours of the 1:50 diluted solution appeared to evaporate before training had ended. We imagined that stronger food flavours would take longer to evaporate and thus stay available for cue association until training had ended. Feeding times in this treatment were 30 and 10 minutes, while pause times were kept constant at 10 minutes. Again, no CO_2 was used for fly movement.

S4.4.2.1.5   Treatments X, Y and Z

Treatments X, Y and Z were there first treatments conducted within the vial setup and conducted following the methods used in Fujita & Tanimura's study (2011). In these treatments, we did not yet control for primacy effects by presenting a first unrewarded odour. Thus, the used odours were only lemon and rosemary essential oils. Flies were anesthetized via CO_2 in all of these three treatments. In treatments X and Y the feeding time was 10 minutes for each sucrose/odour pair. However, flies were not switched into a pause time after each pair, but were presented the high and low molarity odour pairs without a break in between. After both pairs had been presented, flies were switched to a pause that lasted 60 minutes. Each pair was presented four times, resulting in a total of 8 "training runs". The difference between treatments X and Y was the presentation type of the sucrose reward. In treatment X, flies were presented normally prepared dried pieces of filter paper which had been impregnated with sucrose solution prior to drying. In contrast, pieces of filter were not dried in treatment Y, but just soaked in sucrose solution. In treatment Z, flies again received dried pieces of filter paper as reward. Flies in this treatment were allowed to feed for 10 minutes and were then switched to a pause of 30 minutes after each pair.
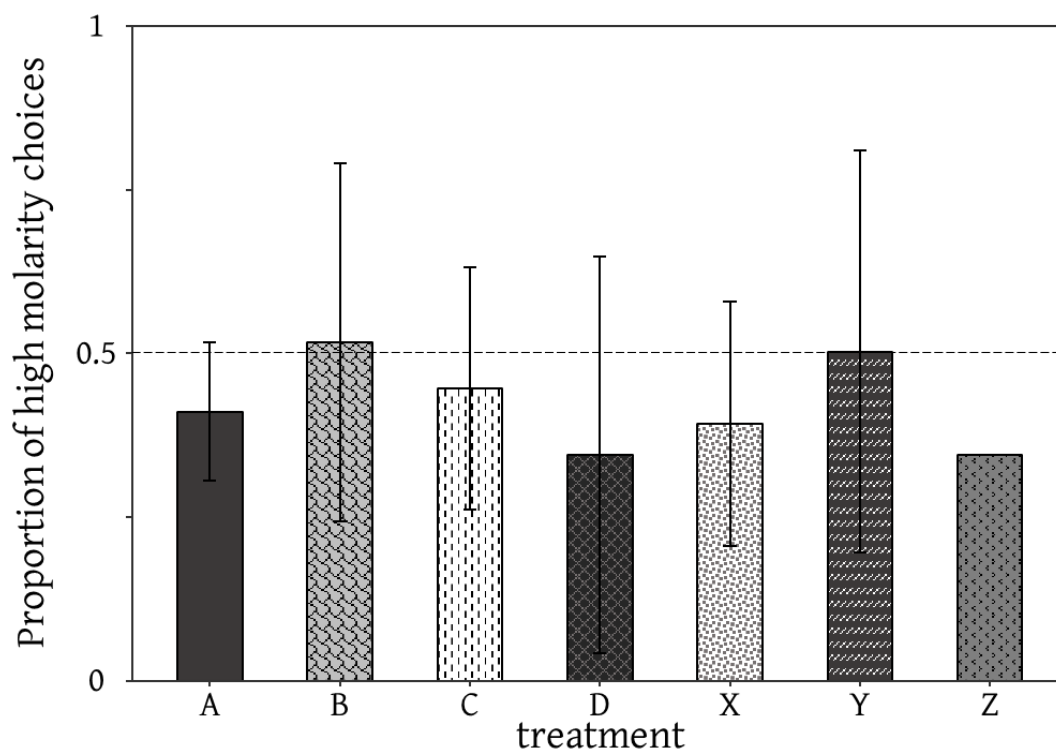
S4.4.2.2 Results

Figure S4-7: Proportion of choices for the T-maze side which offered the odour previously presented along with high molarity sucrose during training for flies of all treatments (treatment A, B, C, D, X, Y and Z) trained in the vial training setup. Treatments differed in feeding and pause times, used odour types, whether a control for primacy effects was included, the usage of CO_2 during fly movement from the starvation into the test vials and whether dried filter papers with sucrose applied or filter papers soaked in sucrose solution were used. For a detailed overview of the treatment differences, please refer to table S4-2. The dotted line represents a hypothetical choice of 50% for each odour side.

Apart from between treatments X and Y, the proportion of choices made for the high molarity odour side in the T-maze did not differ significantly between treatments of the vial training setup (for a detailed list of GLMM pairwise comparison estimates, z- and p-values please refer to table S4-4). Choices made in the treatments B and C were not significantly different from random choice (figure S4-7; treatment B: 48.7% of choices for high molarity odour cue, *exact binomial test* $p = 0.6$; treatment C: 45.7% for high molarity odour cue, *exact binomial test* $p = 0.17$), suggesting that flies either did not learn to associate the given sucrose molarities to their odours or did not prefer any of the combinations. However, choices of treatments A and D were significantly different from a random choice (figure S4-7; treatment A: 38% of choices for high molarity odour, *exact binomial test* $p < 0.001$; treatment D: 33.3% for high molarity odour, *exact binomial test* < 0.05), suggesting an avoidance for the high molarity sucrose/odour combination in

the T-maze. Furthermore, flies were significantly less likely to choose the high odour side in the T-maze when the first odour was presented along with low molarity food (*GLMM*: estimate = -0.44, z = -4.78, p < 0.001), but significantly more likely to choose the high molarity odour side when it was presented with orange odour (*GLMM*: estimate = 0.79, z = 3.45, p < 0.01).

Table S4-3: Detailed overview providing information on fly's choices made in the T-maze after training for all treatments of the vial training setup (treatments A, B, C, D, X, Y and Z). Given are the number of choices for the odour presented along with high molarity sucrose, the sum of all choices made (sum of both odours and fly's staying in the mid of the T-maze and thus not making a choice), the percentage of choices for the odour presented with high molarity sucrose and the p-value of the exact binomial test (testing against the null hypothesis of random choice).

Treatment	No. choices for high molarity odour	Sum of all choices made	% choices for high odour	Exact binomial test p-value
A	320	840	38.1	**< 0.001**
B	264	541	48.8	0.6
C	128	280	45.7	0.17
D	17	51	33.3	**< 0.05**
X	79	208	39.2	**< 0.001**
Y	92	161	50.2	0.08
Z	32	85	34.5	**< 0.05**

Table S4-4: Pairwise Comparisons Table for T-maze choices flies trained in the different treatments (treatments A, B, C, D, X, Y and Z) of the vial training setup for the high odour side.

Treatment	A	B	C	D	X	Y	Z
A		estim.=-0.13 z = -1.1 p = 1	estim.=0.02 z = 0.08 p = 1	estim.=0.6 z = 1.66 p = 1	estim.=-0.02 z = -0.05 p = 1	estim.=-0.63 z = -1.66 p = 1	estim.=-0.07 z = -0.16 p = 1
B	estim.=-0.13 z = -1.1 p = 1		estim.=0.12 z = 0.69 p = 1	estim.=0.73 z = 1.95 p = 1	estim.=0.11 z = 0.28 p = 1	estim.=-0.5 z = -1.3 p = 1	estim.=0.06 z = 0.14 p = 1
C	estim.=0.02 z = 0.08 p = 1	estim.=0.12 z = 0.69 p = 1		estim.=0.59 z = 1.71 p = 1	estim.=-0.03 z = -0.08 p = 1	estim.=-0.64 z = -1.55 p = 1	estim.=-0.08 z = -0.19 p = 1
D	estim.=0.6 z = 1.66 p = 1	estim.=0.73 z = 1.95 p = 1	estim.=0.59 z = 1.71 p = 1		estim.=-0.62 z = -1.21 p = 1	estim.=-1.23 z = -2.4 p = 0.34	estim.=-0.67 z = -1.25 p = 1
X	estim.=-0.02 z = -0.05 p = 1	estim.=0.11 z = 0.28 p = 1	estim.=-0.03 z = -0.08 p = 1	estim.=-0.62 z = -1.21 p = 1		estim.=-0.61 z = -3.2 **p < 0.05**	estim.=-0.05 z = -0.2 p = 1
Y	estim.=-0.63 z = -1.66 p = 1	estim.=-0.5 z = -1.3 p = 1	estim.=-0.64 z = -1.55 p = 1	estim.=-1.23 z = -2.4 p = 0.34	estim.=-0.61 z = -3.2 **p < 0.05**		estim.=0.56 z = 2.25 p = 0.51
Z	estim.=-0.07 z = -0.16 p = 1	estim.=0.06 z = 0.14 p = 1	estim.=-0.08 z = -0.19 p = 1	estim.=-0.67 z = -1.25 p = 1	estim.=-0.05 z = -0.2 p = 1	estim.=0.56 z = 2.25 p = 0.51	

The proportion of flies choosing the odour which had been presented first during training was also not significantly different for most treatments. However, significant differences were found between treatments B&C, A&Y, B&Y, X&Y and Y&Z (for a detailed list of GLMM pairwise comparison estimates, z- and p-values please refer to table S4-6). The molarity presented along with the first offered odour and the odour presented with the high molarity sucrose did not affect T-maze choice after training (first odour: *GLMM*: estimate = -0.06, z = -0.78, p = 0.51; high odour: *GLMM*: estimate = -0.21, z = 0.91, p = 0.51). Flies of treatments A and B showed a significant preference for the odour offered first during training, regardless of the food quality (treatment A: 55.5% of choices for the first offered odour, *exact binomial test* p < 0.01; treatment B: 64.1% for first odour, *exact binomial test* p < 0.001). In contrast, flies of treatments C and D chose the first offered odour significantly less often than a random choice would explain (treatment C: 35.7% of choices for the first offered odour, *exact binomial test* p < 0.001; treatment D: 7.8% for first odour, *exact binomial test* < 0.001). For a detailed overview of fly choices, proportions of choices and p-values of exact binomial tests please refer to table S4-3 for high molarity choices and table S4-5 for choices for the odour offered first during training (because first offered odour was not recorded in treatments X, Y and Z, there is no data included for these treatments).

Table S4-5: Detailed overview providing information on fly's choices made in the T-maze after training for treatments A, B, C and D of the vial training setup (data for treatments X, Y and Z was not recorded and it thus not included in the table). Given are the number of choices for the odour first presented during training, the sum of all choices made (sum of both odours and fly's staying in the mid of the T-maze and thus not making a choice), the percentage of choices for the first presented odour and the p-value of the exact binomial test (testing against the null hypothesis of random choice).

Treatment	No. choices for first presented odour	Sum of all choices made	% choices for first odour	Exact binomial test p-value
A	320	840	38.1	**< 0.001**
B	264	541	48.8	0.6
C	128	280	45.7	0.17
D	17	51	33.3	**< 0.05**

Table S4-6: Pairwise Comparisons Table for T-maze choices of flies trained in the different treatments (treatments A, B, C, D, X, Y and Z) of the vial training setup for the side in which the odour which was first presented during training was offered.

Treatment	A	B	C	D	X	Y	Z
A		estim.=-0.25 z = -2.32 p = 0.43	estim.=0.36 z = 2.02 p = 0.9	estim.=0. z = 1.28 p = 1	estim.=0.18 z = 0.74 p = 1	estim.=0.88 z = 3.14 **p < 0.05**	estim.=-0.14 z = -0.52 p = 1
B	estim.=-0.25 z = -2.32 p = 0.43		estim.=0.61 z = 3.34 **p < 0.05**	estim.=0.65 z = 1.2 p = 0.98	estim.=0.43 z = 1.72 p = 1	estim.=1.13 z = 3.94 **p < 0.01**	estim.=0.11 z = 0.38 p = 1
C	estim.=0.36 z = 2.02 p = 0.9	estim.=0.61 z = 3.34 **p < 0.05**		estim.=0.04 z = 0.14 p = 1	estim.=-0.17 z = -0.61 p = 1	estim.=0.52 z = 1.64 p = 1	estim.=-0.50 z = -1.6 p = 1
D	estim.=0. z = 1.28 p = 1	estim.=0.65 z = 1.2 p = 0.98	estim.=0.04 z = 0.14 p = 1		estim.=-0.22 z = -0.57 p = 1	estim.=0.48 z = 1.17 p = 1	estim.=-0.54 z = -1.34 p = 1
X	estim.=0.18 z = 0.74 p = 1	estim.=0.43 z = 1.72 p = 1	estim.=-0.17 z = -0.61 p = 1	estim.=-0.22 z = -0.57 p = 1		estim.=0.7 z = 3.11 **p < 0.05**	estim.=-0.32 z = -1.5 p = 1
Y	estim.=0.88 z = 3.14 **p < 0.05**	estim.=1.13 z = 3.94 **p < 0.01**	estim.=0.52 z = 1.64 p = 1	estim.=0.48 z = 1.17 p = 1	estim.=0.7 z = 3.11 **p < 0.05**		estim.=-1.02 z = -4.01 **p < 0.01**
Z	estim.=-0.14 z = -0.52 p = 1	estim.=0.11 z = 0.38 p = 1	estim.=-0.50 z = -1.6 p = 1	estim.=-0.54 z = -1.34 p = 1	estim.=-0.32 z = -1.5 p = 1	estim.=-1.02 z = -4.01 **p < 0.01**	

S4.4.3 Block slide training setup

S4.4.3.1 Methods

S4.4.3.1.1   Treatment E

In treatment E, flies were anesthetized via CO2 in order to move them into the block slide training setup. Odour types used were essential oils, including an unrewarded control for primacy effects (lavender odour). Flies were first offered a sucrose/odour pair for 10 minutes and then switched to a cueless pause of 30 minutes.

S4.4.3.1.2   Treatment F

This treatment was basically identical to treatment E. It only differed in the procedure of odour presentation. In contrast to only presenting odour cues via scented filter paper, in this treatment, a piece of kitchen paper soaked in 0.1ml (rosemary) or 0.2ml (lemon) essential oils was placed inside the training tube additionally to scented filter papers. The amount of rosemary essential oils was reduced to 0.1ml due to high mortality rates. However, kitchen paper was soaked in 0.2ml lemon because its smell appeared to be weaker (personal observation) and we aimed for an even odour distribution during training.

S4.4.3.1.3   Treatment G

This treatment was very similar to treatment F, but differed in feeding and pause times and CO_2 anesthesia method. In contrast to treatments E and F, flies were not anesthetized via CO_2 in this treatment. Furthermore, feeding times were increased to 30 minutes, and pause times decreased to 10 minutes.

S4.4.3.1.4   Treatment H

This treatment was identical to treatment G, apart from the used odour types. Instead of essential oils, we here poured a 1:50 diluted solution of food flavours directly onto the dried filter papers instead of storing them in an airtight box containing essential oils for a day. This change was done, because rosemary essential oils had led to a higher mortality in earlier treatments. We thus imagined that food flavours may lead to increased learning due to higher attractivity of odour and taste.

Table S4-7: Detailed overview of all differences between all treatments conducted in the block slide training setup.

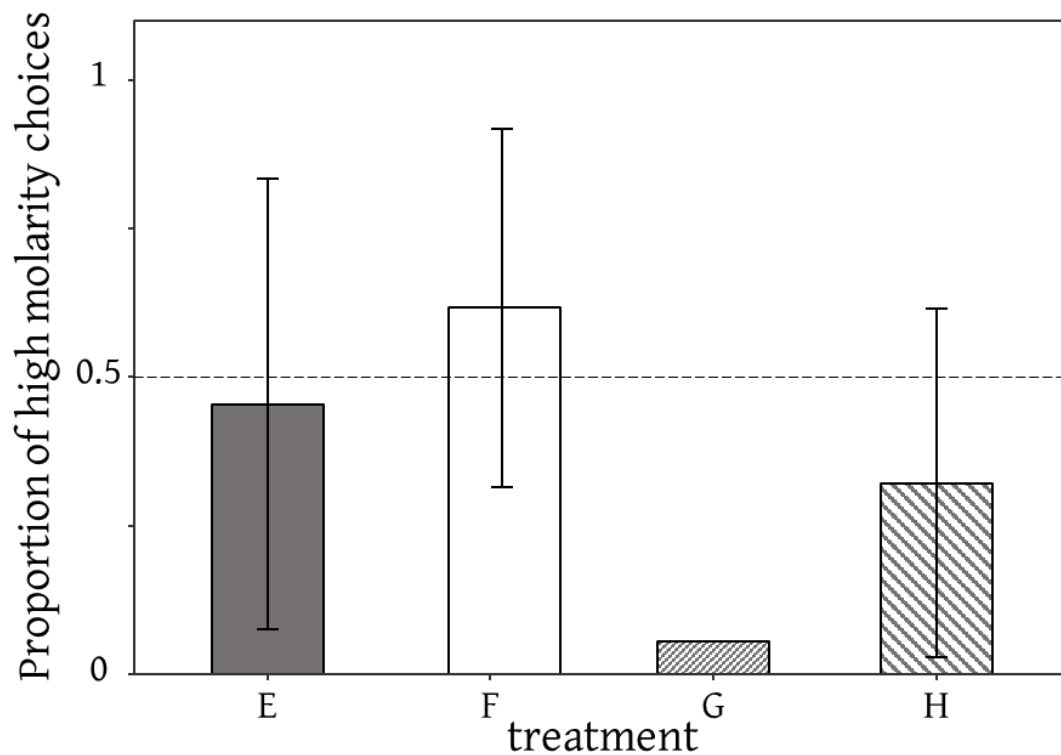| Treatment	Feeding Time [min]	Pause Time [min]	CO_2 Anesthesia	Odour Type	Odours used	Filter Paper
E	10	30	yes	essential oils	(lavender, rosemary, lemon)	dried
F	10	30	yes	essential oils	(lavender, rosemary, lemon)	dried
G	30 10	10 30	no	essential oils	(lavender, rosemary, lemon)	dried
H	10 30 10	30 10 10	no	food flavours (1:50 dilution)	(blackberry, lemon, orange)	dried

S4.4.3.2 Results

Figure S4-8: Proportion of choices for the T-maze side which offered the odour previously presented along with high molarity sucrose during training for flies of all treatments (treatments E, F, G and H) trained in the block slide training setup. Treatments differed in feeding and pause times, used odour types and the usage of CO_2 during fly movement from the starvation into the test. For a detailed overview of the treatment differences, please refer to table S4-7. The dotted line represents a hypothetical choice of 50% for each odour side.

Flies trained in treatments F and E in the block slide training setup chose the high molarity odour side in the T-maze significantly more often than flies trained in treatments G and H (figure S4-8, for a detailed overview of GLMM estimates, z- and p-values, please refer to the pairwise comparisons displayed in table S4-9). Furthermore, flies trained in treatment F chose the high molarity odour side in the T-maze significantly more often than flies trained in treatment E (*GLMM pairwise comparison*: estimate = -0.73, z = -2.93, p < 0.05). Proportions of choices did not significantly differ between treatments G and H (*GLMM pairwise comparison*: estimate = -0.26, z = -0.57, p = 1). Proportion of choices for the high molarity odour were not significantly different from random choice in treatment E (45.1% of choices for high molarity odour, *exact binomial test* p = 0.32). Flies of treatment F showed a significant preference with 63.5% of choices for the high molarity odour (figure S4-8, *exact binomial test* p < 0.001). In contrast, flies trained in treatments G

and H chose the high molarity odour side significantly less often in the T-maze than a random choice would explain (treatment G: 6.7% of choices for high molarity odour, *exact binomial test* p < 0.001; treatment H: 27.5% for high molarity odour, *exact binomial test* p < 0.001). Furthermore, flies were significantly more likely to choose the high molarity odour in the T-maze when the odour offered first during training was presented along with high molarity sucrose (*GLMM*: estimate = -0.58, z = -2.69, p < 0.01). Flies were also more likely to choose the high molarity odour when the odour was orange (*GLMM*: estimate = 1.52, z = 4.87, p < 0.001).

Table S4-8: Detailed overview providing information on fly's choices made in the T-maze after training for all treatments of the block slide training setup (treatments E, F, G and H). Given are the number of choices for the odour presented along with high molarity sucrose, the sum of all choices made (sum of both odours and fly's staying in the mid of the T-maze and thus not making a choice), the percentage of choices for the odour presented with high molarity sucrose and the p-value of the exact binomial test (testing against the null hypothesis of random choice).

Treatment	No. choices for high molarity odour	Sum of all choices made	% choices for high odour	Exact binomial test p-value
E	56	124	45.2	0.32
F	127	200	63.5	0.72
G	8	139	5.8	**< 0.001**
H	74	269	27.5	0.22

Table S4-9: Pairwise Comparisons Table for T-maze choices flies trained in the different treatments (treatments A, B, C, D, X, Y and Z) of the vial training setup for the high odour side.

Treatment	E	F	G	H
E		estimate=-0.73 z = -2.93 **p < 0.05**	estimate=1.5 z = 3.26 **p < 0.01**	estimate=1.21 z = 3.85 **p < 0.001**
F	estimate=-0.73 z = -2.93 **p < 0.05**		estimate=2.2 z = 5.67 **p < 0.001**	estimate=1.94 z = 6.44 **p < 0.001**
G	estimate=1.5 z = 3.26 **p < 0.01**	estimate=2.2 z = 5.67 **p < 0.001**		estimate=-0.26 z = -0.57 p = 1
H	estimate=1.21 z = 3.85 **p < 0.001**	estimate=1.94 z = 6.44 **p < 0.001**	estimate=-0.26 z = -0.57 p = 1	

Looking at the proportion of choices made for the odour offered first during training, flies trained in treatment E chose the first odour significantly less often in the T-maze compared to flies trained in treatments F and G (for a detailed overview of GLMM pairwise comparison

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estimates, z- and p-values, please refer to table S4-11). Flies trained in treatment G also chose the first odour significantly more often than flies trained in treatment H (*GLMM pairwise comparison*: estimate = 0.94, z = 3.15, p < 0.01), with 85.6% of choices made for the odour offered first during training in treatment G. This proportion was significantly different from chance, suggesting a preference for the first odour in this treatment (*exact binomial test* p < 0.001). Choices of treatments E, F and H were not significantly different from a random choice (treatment E: 45.1% of choices for the first odour, *exact binomial test* p = 0.32; treatment F: 48.5% for the first odour, *exact binomial test* p = 0.72; treatment H: 53.9% for first odour, *exact binomial test* p = 0.22). Even though there was a strong tendency, the sucrose molarity presented along with the first odour offer during training did not significantly affect odour choice in the T-maze test after training (*GLMM*: estimate = -0.55, z = -1.94, p = 0.05). For a detailed overview of fly choices, proportions of choices and p-values of exact binomial tests please refer to table S4-8 for high molarity choices and table S4-10 for choices for the odour offered first during training.

Table S4-10: Detailed overview providing information on fly's choices made in the T-maze after training for all treatments of the block slide training setup (treatments E, F, G and H). Given are the number of choices for the odour first presented during training, the sum of all choices made (sum of both odours and fly's staying in the mid of the T-maze and thus not making a choice), the percentage of choices for the first presented odour and the p-value of the exact binomial test (testing against the null hypothesis of random choice).

Treatment	No. choices for first presented odour	Sum of all choices made	% choices for first odour	Exact binomial test p-value
E	56	124	45.2	0.32
F	97	200	48.5	0.72
G	119	139	85.6	**< 0.001**
H	145	269	53.9	0.22

Table S4-11: Pairwise Comparisons Table for T-maze choices of flies trained in the different treatments (treatments A, B, C, D, X, Y and Z) of the vial training setup for the side in which the odour which was first presented during training was offered.

Treatment	E	F	G	H
E		estimate=-1.12 z = -2.85 **p < 0.05**	estimate=-1.74 z = -4.03 **p < 0.001**	estimate=-0.8 z = -2.45 p = 0.08
F	estimate=-1.12 z = -2.85 **p < 0.05**		estimate=-0.62 z = -2.41 p = 0.09	estimate=0.31 z = 1.18 p = 1
G	estimate=-1.74 z = -4.03 **p < 0.001**	estimate=-0.62 z = -2.41 p = 0.09		estimate=0.94 z = 3.15 **p < 0.01**
H	estimate=-0.8 z = -2.45 p = 0.08	estimate=0.31 z = 1.18 p = 1	estimate=0.94 z = 3.15 **p < 0.01**	

A group of Lasius niger *foragers feeding at a drop of sucrose.*