Laboratory tests of navigation in bumblebees: pesticide effects and neural correlates

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Declaration of Independence

Herewith I certify that I have prepared and written my thesis independently and that I have not used any sources and aids other than those indicated by me.

Hereby declare that this thesis has not been submitted either in the same or a different form to this or any other university for a degree.

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Contents

1 Summary

The utilization of bumblebees in the laboratory setting is increasing primarily for the purpose of behavioral analysis and neural recordings. Their remarkable physiological resilience and their willingness to adapt to diverse environmental conditions render them exceptionally suitable for a wide range of experimental investigations. Moreover, they represent the vast group of wild bees, which are also struggling with the current problems arising from agricultural mismanagement.

To find a suitable setup to perform behavioral tests accompanied by electrophysiological recordings for bumblebees, a novel experimental apparatus was designed and constructed. The bumblebee could autonomously navigate between the colony and the test/feeding arena. Initially, the focus was set on the normal behavior and development of the colonies within this controlled artificial environment. Through a series of iterative refinements, a training protocol was established. The bumblebees had to learn a specific feeding place marked by a local cue and matching panorama. Notably, extinction tests revealed an enhanced behavior towards the learned goals. This change in behavior is proving, that the artificial arena with the connection to the colony and animals foraging on their own motivation adequately fulfills the requisite criteria for conducting such experiments.

Furthermore, tests were performed treating animals with Calypso®, an insecticide formulation with neonicotinoid Thiacloprid. The extinction tests resulted in a shift in behavioral patterns towards the border area compared to the controls, displaying a change in exploratory behavior. These findings serve as evidence, that the experimental procedure is suitable for laboratory based navigational experiments researching sub-lethal effects of treatments.

Electrophysiological recordings were performed on trained animals using an established setup, yielding stable and sortable data. However, no discernible pattern indicative of memory retrieval was observed. Instead, subtle indications of involvement in exploratory behavior or exploratory learning were detected. The limitations of comparing simultaneously recorded neurons prevented the identification of a network pattern for learned object identification.

Nevertheless, the study emphasizes the suitability of the setup for conducting electrophysiological recordings in trained bumblebees. The recommendation to upgrade the outdated recording devices is made to enable long-lasting multi-unit recordings and facilitate further intriguing analyses.

2 Zusammenfassung

Hummeln werden im Labor zunehmend für Verhaltensanalysen und neuronale Aufzeichnungen eingesetzt. Aufgrund ihrer bemerkenswerten physiologischen Widerstandsfähigkeit und ihrer Bereitschaft, sich an unterschiedliche Umweltbedingungen anzupassen, eignen sie sich hervorragend für ein breites Spektrum an experimentellen Untersuchungen. Außerdem stellen sie die größte Gruppe der Wildbienen dar, welche ebenfalls mit den aktuellen Problemen zu kämpfen haben, die sich aus der Misswirtschaft in der Landwirtschaft ergeben.

Um einen geeigneten Aufbau für die Durchführung von Verhaltenstests in Verbindung mit elektrophysiologischen Aufzeichnungen für Hummeln zu finden, wurde ein neuartiger Versuchsapparat entworfen und gebaut. Die Hummeln konnten autonom zwischen der Kolonie und der Test-/Fütterungsarena navigieren. Anfänglich lag der Schwerpunkt auf dem normalen Verhalten und der Entwicklung der Kolonien in dieser kontrollierten künstlichen Umgebung. Durch eine Reihe von iterativen Verfeinerungen wurde ein Trainingsprotokoll erstellt. Die Hummeln mussten einen bestimmten Futterplatz erlernen, der durch einen lokalen Hinweis und ein passendes Panorama gekennzeichnet war. Die Extinktionstests zeigten ein verbessertes Verhalten gegenüber den erlernten Zielen. Diese Verhaltensänderung beweist, dass die künstliche Arena mit der Verbindung zur Kolonie und den Tieren, die aus eigener Motivation auf Futtersuche gehen, die erforderlichen Kriterien für die Durchführung solcher Experimente ausreichend erfüllt.

Außerdem wurden Tests durchgeführt, bei denen die Tiere mit Calypso® behandelt wurden, einer Insektizid-Formulierung mit dem Neonicotinoid Thiacloprid. Die Extinktionstests führten zu einer Verschiebung der Verhaltensmuster in Richtung des Randbereichs im Vergleich zu den Kontrollen, was eine Änderung des Erkundungsverhaltens darstellt. Diese Ergebnisse zeigen, dass das Versuchsverfahren für Navigationsexperimente im Labor zur Erforschung subletaler Effekte von Behandlungen geeignet ist.

Die elektrophysiologischen Aufzeichnungen wurden an trainierten Tieren mit einem etablierten Aufbau durchgeführt und ergaben stabile und verwertbare Daten. Es wurde jedoch kein erkennbares Muster beobachtet, das auf das Abrufen von

Gedächtnisinhalten hinweist. Stattdessen wurden schwache Hinweise auf eine Beteiligung am Erkundungsverhalten oder am Erkundungslernen festgestellt. Die Beschränkungen des Vergleichs gleichzeitig aufgezeichneter Neuronen verhinderten die Identifizierung eines Netzwerkmusters für die erlernte Objektidentifikation.

Dennoch unterstreicht die Studie die Eignung des Versuchsaufbaus für die Durchführung elektrophysiologischer Ableitungen bei trainierten Hummeln. Es wird empfohlen, die veralteten Aufzeichnungsgeräte aufzurüsten, um langanhaltende Multi-Unit-Aufzeichnungen zu ermöglichen und weitere interessante Analysen zu erleichtern.

3 Introduction

Social bees and polylectic solitary bees are efficient pollinators that adapt their foraging strategies to the highly dynamic and competitive flower market. They achieve this by acquiring knowledge of flower cues and optimizing their flights between the nest and the nectar or pollen producing flowers (Faegri & van der Pijl, 1978; Feinsinger, 1983). The sensory processes involved in flower discrimination have been studied particularly precisely for the honeybee (Karl von Frisch, 1967), employing training protocols with artificial feeders. The honeybee exhibits color discrimination through a trichromatic color vision system (Peitsch et al., 1988), while also demonstrating the ability to discern a wide array of odors through combinatorial neural activation patterns of approximately 160 chemo-sensory inputs (Galizia & Menzel, 2000; Laska et al., 1999). Additionally, the honeybee distinguishes between visual shapes based of their geometric patterns (Srinivasan, 2010; Wehner, 1967). The learning processes were systematically examined within the ecological framework (Heinrich, 1983; Pyke, 1979), and considered within the behavioral context (Couvillon & Bitterman, 1985; Greggers & Menzel, 1993). Notably, distinct adaptations were observed between the floral cues, spatial distribution of flowers and the resultant choice behavior of bees (Menzel, 1985). More recently, the flight paths between nest sites and the food sources were studied using harmonic radar for tracking bees across natural foraging distances (Riley et al., 2005). During their initial flights from the hive, bees engage in landscape exploration, wherein they acquire knowledge about numerous landmarks and their respective spatial relationships (Capaldi et al., 2000; Degen et al., 2015). This acquired information enables them to navigate back to the hive safely from any location within the explored area (Degen et al., 2016; Menzel et al., 2011).

Navigation in honeybees has been shown to be based on multiple cues and their associations. For example, the direction of an elongated landmark like a forest border is intricately linked to the direction determined relative to the sun compass (K. v. Frisch & Lindauer, 1954). Additionally, when honeybees embark on homing flights subsequent to displacement, their ability to return home depends on the exploration of the area into which the bees were displaced (Degen et al., 2016). It is assumed, that bees also associate the panorama with local landmarks, but evidence is lacking for the dimensions of natural navigation. Empirical support for this claim comes from

experimental trials, in which the bees were tested in the close range, showing that they searched for the food or the hive entrance by the best fit between panorama and local cues (Collett, 1992; Philippides et al., 2013). These associations can be considered as necessary and fundamental components of navigation in general and in particular for flying insects. The panorama, as a parallax and optic flow independent cue, can act as a compass and in natural navigation it will be associated with the sun compass (K. v. Frisch & Lindauer, 1954). Thus, the association of the panorama and the local landmarks may not only play a major role in proximate orientation tasks at the hive entrance and the food sources, but possibly also in long-range navigation as well.

We have focused our search for neural substrates of navigation in the bumblebee. Specifically, we have concentrated our efforts on the beforementioned essential and elementary component of navigation. This approach is motivated by the fundamental work in rodents, in which navigation in a laboratory setting was studied by mazes. In these studies, a location was characterized in relation to a panorama alone or a panorama and a local marker (Biegler & Morris, 1993; Morris, 1982). The implementation of this experimental paradigm has proven crucial in facilitating significant discoveries, concerning hippocampal neurons implicated in navigation (Moser et al., 2008; O'Keefe & Nadel, 1978). To the best of our knowledge, investigation of neural correlates of navigation, specifically with regards to panorama-object relations, have not been studied so far in insects. The numerous and highly informative data on the central complex as a neural hub of compass and vector based forms of processing (Homberg et al., 2011) do not speak to the questions raised here, because localized objects appear not to impact on the properties of the central complex neurons or those of the closely attached neuropil (e.g. protocerebral bridge). We selected mushroom body extrinsic neurons because we expect neuronal processes to be involved in solving this spatial task, that requires segmentation of background and object, as well as object identification. These neurons were already examined in walking bumblebees in the context of exploratory behavior. However, no discernible patterns of neural activity were discovered, that could be directly linked to spatial locations within a confined arena or the direction of the observed field of view (Jin et al., 2020). The behavior of the bees in a latter study was dominated by escape, and thus the predominant neural activity correlated most strongly with walking speed and walking directions between the peripheral escape area and a central crossing area.

Consequently, we established experimental parameters that effectively facilitated the voluntary entry of the test bees into the designated environment at any given time. They lived with their colony, including a queen, in an attached box and were trained to navigate between the colony and the color marked feeding site on their own motivation. These conditions ensured a stress-free testing environment. The bees had to learn a stable association of an object (a blue trapezoid target) with the panorama regardless of the angle from which it was perceived upon entry into the maze. Other than honeybees (personal observations) bumblebees are highly cooperative and display competent learning capabilities under the specific experimental conditions described. Furthermore, it has been observed that walking bumblebees respond also very well to simulated sky patterns and show components of path integration and searching behavior (Patel et al., 2022). These studies taken together, emphasize the successful transfer of basic navigational components to laboratory conditions in bumblebees, and, as it is shown here, the simultaneous recording from mushroom body extrinsic neurons.

3.1 The Bumblebee

Bumblebees are fascinating creatures which live in an annual colony and are divided into over 250 species worldwide. They are part of the hymenoptera order and further they belong to the family of Apidae. Most of the species can be found in the higher parts of the northern hemisphere; only few are identified in South America. In Germany we have around 30 species and 10 species of the so-called cuckoo bumblebees, which belong to the subgenus Psithyrus (von Hagen & Aichhorn, 2014). Bumblebees live in a primitive eusocial colony, meaning that they live in a social colony with one queen and worker bees which take care of each other. However, their social structure is less complex compared to that of honeybees, lacking for example communication methods such as waggle dances. Notably, they are endotherm implying that they can regulate their body core temperature independently from their surroundings. Bumblebees are experts in maintaining a high enough body temperature to fly even when it is almost below 0°C outside. To do this, probably two mechanisms are combined. Firstly, bumblebees can generate muscle heat by shivering with their flight muscles without

engaging in flight (Heinrich, 2004). Secondly, enzymatic heat production via substrate cycling has also been proposed (Newsholme et al., 1972). Furthermore, bumblebees can keep their body temperature high because of some intelligent mechanism. Given their anatomy, it is very important to keep the thorax warm, were the muscles for the legs and the wings are located. The abdomen remains typically approximately 10°C cooler than the thorax. Besides the furry surface, the combination of an isolating air sac in front of the petiole (waist) and the utilization of the countercurrent principle in pumping the blood through the waist, reduces the heat loss of the thorax to a minimum (Alford, 1975; Goulson, 2010; Heinrich, 1972, 2004). Bumblebees can be categorized as either pocket maker or pollen storer based on their pollen storage strategies for the brood. Pocket makers store the pollen in clumps between the larvae. The larvae feed from the pollen and stay in one cluster together. Pollen storer larvae only remain clustered for a few days before each larva receives an individual pot to stay in. Workers will feed them with regurgitated pollen and nectar. Bumblebees which are pocket makers are broadly the long-faced bumblebees which also tend to have smaller colonies (up to max. 100) and the pollen storer are the short-faced bumblebees possessing shorter tongues (Alford, 1975; Free & Butler, 1959; Goulson, 2010; Prys-Jones & Corbet, 2014; von Hagen & Aichhorn, 2014). In this work the bumblebee *Bombus terrestris* is used for the experiments. The main reason is the availability to obtain such colonies easily and repeatedly most of the time. This species belongs to the short-faced bumblebees, nests underground and produces colonies with up to 600 workers. Furthermore we receive *Bombus terrestris dalmatinus*, which are mainly reared commercially (Hart et al., 2021; Ings et al., 2009)

3.1.1 Structure and function of a colony

The colony of a bumblebee consists of three different types of animals. The queen is the foundress of the colony. After hibernating in an adequate hole, which she found in late summer the previous year, she arrives around march and searches for a right place to start her colony. In that time, she forages on spring blooming flowers and has a high protein intake to maintain a good maturation of her eggs. When she was successful in finding the correct spot (e.g., an empty rodent hole), she starts building a pot for nectar and her first egg lump containing 8-11 eggs. Unlike other bees she

shows avian-like incubation behavior to keep the eggs warm. Periodically she leaves the nest to refill her nectar reserves. The hatched larvae will feed on the pollen which is located in their former egg lump. After the pupating of the larvae, the queen lays her second batch of eggs and is keeping them warm. The hatched individuals represent the second caste, referred to as workers. As the number of emerging workers increases, the queen ceases foraging activity. She will stay in the nest to lay eggs, feed larvae, keep the nest temperature constant and clean the emptied pupae pots for the incoming nectar (Alford, 2009; Free & Butler, 1959; Goulson, 2010; Heinrich, 2004; Sladen, 1912; von Hagen & Aichhorn, 2014). A bumblebee queen is the only known bee, who does everything by itself spanning from finding a hibernation site to finding a nest site to start a colony and executing maintenance work. Within the colony all workers are female, and their division of labor follows a system based on size (alloethism) (Bloch, 2019; Kapustjanskij et al., 2007; Shpigler et al., 2013; Spaethe & Weidenmüller, 2002). Smaller individuals predominantly perform nest-related tasks, and it might be that they never leave the hive. Larger workers will become forager almost right after hatching. If the weather is especially bad tempered or the nest resources are abundant, the larger workers may also perform maintenance jobs in the colony, but they tend to be slower than the smaller nest workers (Cartar, 1992; O'Donnell & Jeanne, 1992; Sakagami & Zucchi, 1965). If the resources are plenty and the colony attains sufficient size the so-called turning point occurs, and the queen commences egg laying to produce reproductives. Worker brood production stops at this state. Here the third caste comes in place. Together with new queens the haploid drones will be raised. These bees stay in the colony only for self-feeding purpose and depart the hive during mating period, a few days after emergence. Mating behavior varies among bumblebee species. True for almost all species (exception for example *Bombus hypnorum*) is a single mating event typically resulting in the deposition of a gelatinous plug by drones to prevent further mating. After the turning point, the workers and the old queen will die mostly naturally. Occasionally, conflicts arise between the workers and the old queen, as her ability to suppress worker egg laying weakens. Eggs from workers are not favorable for the queen, resulting in rising aggression potential in the colony (Alford, 1975; Goulson, 2010).

Because of the haploid drones and the very common behavior to mate only once, all workers within a colony are full siblings (~75% related to each other) (Goulson, 2010).

This promotes collaboration between the workers but is simultaneously raising conflicts between the queen and the workers. Moreover, the reduction of genetic variability in the colony increases the vulnerability to parasites and diseases (Baer & Schmid-Hempel, 1999; Liersch & Schmid-Hempel, 1998). So why do bumblebees mate only once? The mating process in bumblebees has a duration of 10 to 80 minutes (Brown & Baer, 2005). In that time the animals cannot defend themselves and are exposed to predators. The colonies are also smaller and last for a single summer, so the decreased fitness is negligible. In experiments it is shown that the artificially insemination of bumblebee queens with sperm from multiple males reduced the hibernation survival rate (Baer & Schmid-Hempel, 2005). Similarly, the fitness levels decrease when mated twice or three times. From the fourth mating on the fitness raises again (Baer & Schmid-Hempel, 2001). However, due to the duration of the mating process, mating multiple times holds significant risks. The gelatinous plug, remaining in place for up to 3 days, prevents other sperm to enter the spermatheca. Even in bumblebees which mate multiple times (e.g., *Bombus hypnorum*) research demonstrates a fatherhood of one dominant male in 69% of the offspring (Paxton et al., 2001). So, the preference for a single mating event arises from minimizing predation rates and foster worker collaboration.

Because of the ability of the bumblebee to fly at very low temperatures and their robustness against inclement weather conditions they are well-suited pollinators in the northern hemisphere even in early springtime. Together with other wild bee species, they play a vital role in pollinating plants that are less favored by honeybees. Furthermore, their ability to perform buzz pollination makes bumblebees remarkably effective for Solanaceae plants, including tomatoes.

3.1.2 Communication and interaction

The communication between bumblebees and other insect can be divided into communication inside and outside the hive. Outside the hive, olfactory cues play an important role, either to find a suitable mating partner or to avoid recently exploited flowers. Due to variations in mating behavior across species, divers methods of scent placement are employed (Goulson, 2010).

To avoid recently visited flowers requiring time for nectar replenishment, bumblebees scent a mixture of cuticular hydrocarbons which are deposited unintentionally by other insects through their tarsi. They manage to determine different thresholds for different flowers, indicating optimal foraging times. It is not quite known how the scent changes over time, whether it evaporates (though the long chain hydrocarbons do not easily evaporate) or if the hydrocarbons are absorbed by the flowers. Notably, bumblebees not only detect the presence of conspecifics that have exploited the flower, but also keep distance from flowers which were visited by other bumblebee species, other bees, and even other insects (Goulson et al., 1998; Stout & Goulson, 2001; Witjes & Eltz, 2009). The components are widespread in the world of insects and so it is possible that it is a rather universal system to conserve energy by avoiding already depleted flowers when searching for food.

Inside the bumblebee hive the communication is not as sophisticated as in a honeybee hive. Bumblebees do not engage in waggle dance behavior. The dance of the honeybees has evolved as a means of communication to locate a new nesting site. Given the annual life cycle of bumblebees, this evolutional step did not occur. They use a pheromone mixture of eucalyptol, farnesol and ocimene together with running around vividly to persuade workers in the hive to fly out and forage. The incoming forager carries the scent of visited flowers, providing cues to the other foragers (Dornhaus & Chittka, 1999; 2001). Moreover, the fact that bumblebee colonies are annual the urgent need to forage for a winter depot is not present. Once the colony reservoir is sufficiently stocked, the incoming forager start her persuading ceremony but stops rather soon. Further there are pheromones emitted by the queen, to suppress the workers juvenile hormone, preventing them from laying eggs. The composites of this pheromone are not totally understood yet. As long as the workers do not lay their own eggs, they will take care of the queens' brood with self-sacrifice. An aged queen will not produce enough of that pheromone and workers will start laying unfertilized eggs. These they will nurse and defend against other workers, who will try to remove those non-queen eggs. The aggression in the colony rises by then.

3.1.3 Advantages and disadvantages compared to a honeybee hive

Honeybees are widely used in neuroscience because of their splendid intra-hive communication, their learning and navigating skills and their capacity to form perennial hives. The presence of bees throughout the year allows continuous experiments without the need of a winter break. So why the study with bumblebees? Bumblebees are part of the extensive family of wild bees containing around 20.000 species worldwide, with over 570 species found in Germany. It is important to not only comprehend the learning and navigation in honeybees but also investigate how wild bees cover different tasks, identifying potential vulnerabilities. Bumblebee hives are considerably smaller than honeybee hives. A reduction of workers because of navigational errors would have a more severe impact compared to honeybees. Therefor it is crucial to understand the behaviors of wild bees, such as bumblebees, and explore their memory retrieval and which factors disturb it.

Working with bumblebees one must take care about different factors. Bumblebee colonies typically remain in the lab for only 6-12 weeks before new colonies must be procured. On one hand that makes the work with the colonies more intense since implementing a new colony in the setup and marking the animals consumes additional time. On the other hand, it is useful for toxicology studies because every 3 months it provides a new “clean” hive. Moreover, laboratory experiments are facilitated, because cutting the wings to make them walk in an arena is well accepted in bumblebees and not at all in honeybees. They explore their surrounding by walking. After the dissection, the bumblebees recover rather fast, resuming normal behavior within 1 to 5 minutes. Additionally, the full illumination of the setup does not pose any problem. Precautions must be taken to master potential escape rates but after some trial-and-error, oiling the walls keep them in the setup. Their natural peacefulness simplifies working with them, except during later stages when aggression arises within the colony. Working with honeybees in that setup by clipping their wings would impair their normal behavior and the full illumination for 12 hours would induce excessive stress.

In order to uphold bumblebees’ foraging and exploration behaviors within the arena, it is essential to deprive the food in the colony. Bumblebees show an increased motivation to forage and respond more readily to the recruitment pheromone with a low nutritional status in the colony (Molet et al., 2008). As long as there is little or no

nectar in the colony, foragers motivate other bees to also start foraging and the number of available bumblebees for training and testing rises. However, one must take care to supply the colony with sufficient pollen to sustain the colony's overall well-being, aiming to preserve a normal colony life as much as feasible.

3.1.4 Bumblebees and the neonicotinoid insecticide Thiacloprid

Neonicotinoids are chemical substances invented in the 1980s as an effective agent against insect pests. They are extensively used and abundant everywhere (Simon-Delso et al., 2015; Van der Sluijs et al., 2013). With the used method of seed coating and spraying, it is very effective on sucking pest insects such as whiteflies, thrips and aphids (Elbert et al., 2008). Though Thiacloprid has only been recently banned in the EU (Commision, 2020), it is worth looking at the agent ahead of its possible admission in the near future. Also, it is known, that private persons can still buy it from different websites in Poland (personal observations).

Thiacloprid is known as less toxic than other neonicotinoids, its LD 50 being much higher than other neonicotinoids (Iwasa et al., 2004). It is still worth looking at it as the sublethal effects might be similarly severe. It was sold by the Bayer AG in the formulation Calypso® and Biscaya® and could be bought by every farmer and private person.

Neonicotinoids bind on the post-synaptic nicotinic acetylcholine receptor (nAChR). Their function is based on the permanently docking of the active agent on the nAChR, disturbing the normal transit of nerve impulses by continuous stimulating it, causing severe muscular impairment (Brown et al., 2006; Tomizawa & Casida, 2005). An effect which was not foreseen was, apart from being very effective to so called pest insects, the disruption in behavior of pollinating insects. Several studies found that the memory of the animals and their natural behavior is disrupted (Gill et al., 2012; Rundlöf et al., 2015; Smith et al., 2020; Tison et al., 2016). In Bumblebees Ellis et al., 2017 found a strongly striking effect on the efficiency in colonies in producing reproductive, producing 46% fewer reproductives than colonies in the control environment. Affected colonies were also much lighter in weight, suggesting a disturbed foraging work. The nAChR are mainly expressed in the tissue of the central nervous system in insects

(Jones et al., 2006). Therefore, it is necessary to take a deeper look at the structure of the brain.

3.1.5 The layout of a bumblebee brain

The structure of the bumblebee brain does not differ much from a honeybee brain.

Insects' nervous systems contain a brain in the head and multiple ventral chord ganglia in a segmental arrangement within the thorax and abdomen. The brain encompasses various regions responsible for the optic processing: the lamina, the medulla and the lobula, each present bilaterally. They take up about half of the brain volume indicating the great role of vision in their natural behavior, e.g., swift reactions during flight and visual precision in locating food and home. Visual pathways include the anterior optic tubercle, a gateway to the mushroom body and other brain regions (e.g., the central complex). The pharyngeal opening is located centrally and can be used for guidance of recording electrodes in the brain. Adjacent to this opening are the antennal lobes, processing the olfactory signals coming from the antennae. Comprised of roughly 165 glomeruli, they transmit the processed information via projection neurons to the mushroom body and the lateral horn. Underneath the pharyngeal opening, the subesophageal ganglion is located. It is the gateway to the ventral ganglia and the motor control of the mouth parts as well as the sensory input from the proboscis. It also houses the modulatory neurons of the VUM neurons (ventral unpaired median neurons). The central body can be found above the pharyngeal opening. It is crucial for the adjustments of the optical signals to navigate and orientate and for the basic locomotor patterns. The lateral horn is also part of the integration process. It receives inputs from multiple parts of the brain including mushroom body extrinsic neurons and the second order interneurons from the antennal lobes. In general, the higher order integration centers in the insect brain are the mushroom bodies, which will be discussed in the subsequent section.

3.1.6 Mushroom body: the center of high order integration

The mushroom bodies are a paired neuropil formed by a large number of Kenyon cells (360.000 in the honeybee brain) and consists of three major elements: the calyx, the peduncle and the lobes. It lays in the dorsal part of the protocerebrum and is the main integrative center. The dominant input part is the calyx, and the dominant outputs are the lobes. Each mushroom body has two calices. Both are formed out of a lip, a collar, and a basal ring. The axons of the Kenyon cells form the peduncle. The outputs of the mushroom body are formed by extrinsic neurons at the lobes. There the information is signaled to many other parts of the brain including the lateral horn in the case of descending neurons.

The recordings were conducted at the beta exit of the alpha lobe. The selected site consists most likely of mushroom body extrinsic neurons (EN) belonging to the group A1, A2 and A4 (J Rybak & Menzel, 1993), which are extensively studied through intracellular and extracellular recordings in restrained animals. (Homberg & Erber, 1979) and (Rybak & Menzel, 1998) revealed their responsiveness to multiple sensory stimuli. Notably, these neurons exhibit a remarkable capability to modify and adjust their response patterns during the process of learning (Hussaini & Menzel, 2013; Okada et al., 2007; Strube-Bloss et al., 2011, Mauelshagen, 1993).

3.2 Electrophysiological studies: State of the Art

Recording neural activity in freely ranging animals still poses inherent challenges. While larger animals can be equipped with wireless telemetric devices to record multiple units (Schregardus et al., 2006; Schwarz et al., 2014), this method is not feasible for insects due to their small size and limited carrying capacity. Standard methods using long flexible cables as used in rats or bats (Moser et al., 2008; Rubin et al., 2014) are also not applicable to the usage in insects since they are too heavy and rigid to conclude in normal movement.

To record from a tethered insect has proven to be a popular technique, yielding valuable insights into the integration of visual and olfactory information (Mauelshagen,

1993; Mota, Yamagata et al., 2011; Szyszka et al., 2005). Additionally, extensive multi-unit extracellular recordings of mushroom body extrinsic neurons in tethered bees have provided insights into multimodal integration and learning plasticity (Denker et al., 2010; Hussaini & Menzel, 2013; Okada et al., 2007; Strube-Bloss et al., 2011; Zwaka et al., 2019). To advance the research, experiments were conducted with freely moving insects, preferably within a social context. This was achieved by using honeybees inhabiting in a miniature hive (Duer et al., 2015; Paffhausen et al., 2020) and in individually trained bumblebees (Jin et al., 2020). Here, a new method was established. The test animals were part of a full functioning colony in the lab. They underwent training using beforehand verified protocols. Following successful training, the animals were equipped with an electrode and released back into the familiar pathway to the test arena. However, this method is not allowing any conclusion about the exact identification of neurons, complicating the comparison of the recordings with each other. Consequently, separate analyses were performed for each individual animal.

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4 Material and Methods

4.1 Developing a working setup

A working setup had to allow the bumblebees to walk around freely while also have them easily accessible for electrophysiological recordings. A modular configuration of plastic boxes proved to be efficient because the boxes were simple to install for varying arrangements and for cleaning. The setup of the boxes changed over time and handling experience with the animals (Figure 1).

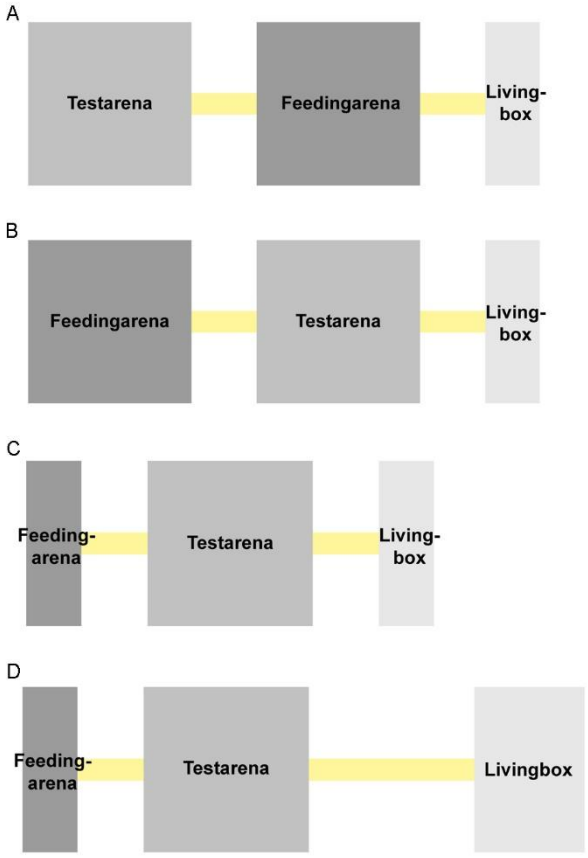

Figure 1: Adaptations of the experimental configuration. The changes of the setup over time. **A**: Initial configuration: compact living box, short tubes, expansive feeding- and test arena, first feeding arena when leaving living box. **B**: Reorder of the feeding- and test box because of decreasing visitation of test arena in setup A. **C**: implementation of a reduced size feeding box to make it less attractive. **D**: Elongated tube after leaving bigger living box for improved separation of the animals.

Also, the type of passages underwent a series of changes from bigger tubes over flexible tubes to the stable small tubes used at the end. A main concern was the escape rate. The walls on the tubes changed in form and borders. Additionally self-adhesive foil and a thin layer of sunflower oil was applied on the walls to mitigate escape occurrences. The panorama and the local cue were also changed during the first period of experiments. Originally, the local cue was just a colored ring around the feeding pot. Because of visibility limitations upon entering the arena, a three-dimensional object was used instead. Initially the panorama was located only at the corners but was then fitted around the arena to enhance visibility. A feeding machine with tubes and a motor was built to automatically replenish the food in the pot when the bumblebee drank from it. It turned out that food ad libitum did not elicit increased foraging efforts, rather the bumblebees tended to stay in the colony and foraging activity was relatively low. Moreover, the initial approach of introducing multiple bumblebees into the arena simultaneously turned out to be inefficient because the bumblebees distracted each other, and no real searching behavior resulted. The doors were also a significant challenge. An automatic door would have been preferable as no interference should take place. Since a single door selecting one bee could not exclude harming the animal, a double door system was constructed. As the animals got used to the experiment, the automatic door was abandoned, and a manual two-door system was installed. Efforts to maintain the humidity at medium to high levels included different methods like dripping water on towels or an electric humidifier. Ultimately, manual refillable water pots with cloth in it were used as it was the most hygienic (easy to change and wash) and most effective method.

Regarding the electrophysiological setup, an old amplifier was exchanged by a new custom build one. Cables were changed to proper quality and plugs were fitted as this also needed to be modular. The optimal shielding took huge amount of efforts, and the amount of shielding was increased to the maximum degree as it is used in the final version. The Raspberry Pi boards were exchanged by their latest version for faster computing.

After this road of trial and errors the following version was used as the final setup.

4.2 The behavioral setup

The setup consisted of 3 plastic boxes: the training- and test arena (30 x 30 cm), the feeding box (17 x 30 cm), and the colony box (at the beginning 17 x 30 cm, later changed to 25 x 30 cm for handling reasons, Figure 3A). The boxes were joined by 15 and 30 cm long plastic tubes with the upper sections removed. Further the plastic tubes were equipped with plastic walls in V-shape to prevent the escape of the animals (Figure 3C). For the same reason the walls of the boxes and tubes were coated with a thin layer of sunflower oil. The setup was designed for straightforward and cost-effective maintenance (weekly cleaning, changing of parts). The cleaning was done with a neutral cleaner (Frosch® neutral cleanser, Werner & Mertz GmbH, Mainz, Germany). When the colony changed, all cleanable parts were also sterilized with a fragrance-free disinfectant (SOS® Disinfection spray, Districon GmbH, Franfurt am Main, Germany). Components such as the tubes and the mesh under the combs (beekeeping ventilation grille, Imgut® Aluminium Lüftungsgitter, Heinrich Holtermann KG, Brockel, Germany) were replaced by new ones. After assembling the boxes and tubes, white cat sand (Dein Bestes Hygienestreu, dm-drogerie markt GmbH + Co. KG, Karlsruhe, Germany) was filled in the boxes to absorb the excrements of the bumblebees. The light-colored sand as background was also favorable for video recording. The entire setup was placed in a wooden box to eliminate external light sources. Furthermore, a curtain was installed inside the box in order to avoid startle responses of the bumblebees when opening the wooden doors of the box. Both the curtain and the surrounding walls had the same black color. Tubes, filled with water and a cloth sticking out of it, were positioned adjacent to the colony box to maintain the high humidity during the heating period. The temperature and the humidity were monitored via a thermo-hygrometer (Aplusdeal Digitales Thermo-Hygrometer) next to the colony box. Additionally, the brood temperature was checked at various locations in the colony with an infrared thermometer (infrared thermometer 1001, FERM®, Zwolle, The Netherlands) on a daily basis, and the mean temperature was determined.

The video recordings were run with a framerate of 30 frames per second from above the training and test arena with a camera module of the microcontroller Raspberry Pi Camera Module v2 and a Raspberry Pi 3 (later Raspberry Pi 4, Raspberry Pi Ltd,

Cambridge, UK). Illumination was provided by LED modules wired together with a power adapter creating a 12/12-hour light-dark cycle. For the behavioral experiments, the Raspberry Pi was connected to a display and a software with a GUI (Graphical User Interface) was installed to facilitate the operation of the camera. The software can be accessed on GitHub (https://github.com/Billwilliams1952/PiCameraApp).

4.3 The electrophysiological setup

The electrophysiological setup contained the identical parts as found in the behavioral setup with addition of the electrical devices for recording purposes and a preparation setup located next to the boxes. The electrophysiological setup consisted of a moveable shielded box including the new amplifier, which was designed by Dr. Benjamin Paffhausen and Thorsten Thiele (Figure 2 A) and was connected to an audio amplifier (TDA 7297 dual channel audio amplifier board, STMicroelectronics International N.V., Amsterdam, the Netherlands). The mobility was necessary because it was first connected to the input at the preparation setup and was then relocated to the recording setup within the arena. The audio amplifier was also connected to a pair of speakers to facilitate the location of the electrode during the search for neurons. The amplifier was linked via a car connector to a STM32-F4 microcontroller (STMicroelectronics International N.V., Amsterdam, the Netherlands) (Figure 2 B), which stored the data on a SD card. The video recording was run by a Raspberry Pi 4 and a fitting camera module (Raspberry Pi Camera Module v2, Raspberry Pi Ltd, Cambridge, UK). Both were shielded, and the connecting cable was fitted in a cable insulation (material from Schirmung 2000, Freiberg, Germany). The light signals, which were crucial for stitching the neural signals with the video recordings, were provided by an Arduino Uno. All components had an external power supply via a car battery to limit the electrical interference within the arrangement. Apart from having 2 Faraday cages (one outer around the setup, one surrounding the arenas) all parts were individually shielded and grounded. The preparation setup consisted of a micromanipulator for handling the electrode, a binocular (Olympus SZ-CTV, OLYMPUS EUROPA SE & CO. KG, Hamburg, Germany), a lighting system and a

mandible clamp. This clamp was used to stabilize the bumblebee's head without disrupting the fine mechanosensory hairs in the neck.

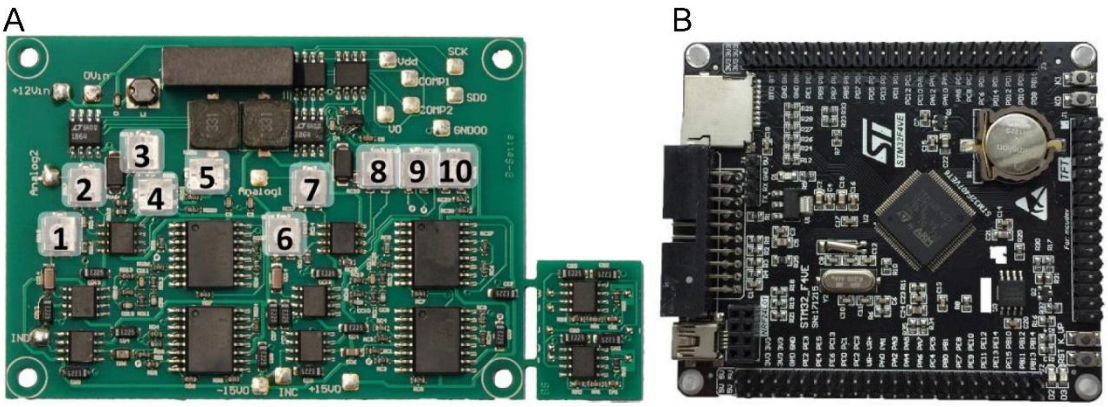

Figure 2: The main electronical parts for the recording. A: The amplifier board with numbered potentiometers. B: STM32 for saving the data.

4.4 Laboratory procedures for maintenance of bumblebee colonies

The test animals were procured from Katz Biotech AG (Baruth, Germany) to a fixed date and were shipped from Belgium (Biobest Group NV, Westerlo, Belgium) within 24 hours to ensure a low stress level for the bumblebees. Two-weeks younger colonies than usually supplied colonies were bought because personal experience showed that the queen survived longer, leading to healthier colonies in the setup. The colonies were approximately 10 weeks old (info of Biobest Group NV) and consisted of 60-90 worker bees plus one queen. After arriving in the lab, the box was placed on a table for at least 20 minutes to establish a state of reduced stress. Sugar solution was used to keep the animals quiet while moving them from the delivery box to a pyramidal box under red light (Figure 3 B). The queen was taken out separately for clipping her wings with nail scissors and marking her. This helped to separate her from new developing queens within the laboratory setup. The original combs were cautiously placed on a metal mesh within the prepared boxes. Pollen dough (powdered pollen mixed with water, pollen ordered at Heinrich Holtermann KG, Brockel, Germany) was placed around the

combs and the prepared queen was put on top of the combs. Then the worker bumblebees were taken out of the pyramid one by one, and their wings were also clipped with nail scissors. By cutting all wings, it was ensured that they did not try flying (with only one half cut they still tried to fly and became stressed). The bumblebees became used to this non-flying state quite fast and showed no impaired behavior corroborating the observations by Sladen, 1912. These prepared workers were also placed on the combs afterwards. Sugar solution (1/1 sugar/water) was positioned in a feeder in the feeding arena. The colonies were kept under a 12 h light/dark cycle. The animals were allowed to explore their surrounding for 20 hours before individual marking started. Wings of newly hatched bees were cut every morning and the death and birth of animals were recorded. Also, temperature and humidity of the surrounding and the temperature of the brood together with some general information about the behavior of the animals were documented in the laboratory journal. For the weekends, the feeder was replenished and placed in the feeding arena and new pollen dough balls were provided. During the training sessions, the feeding arena was closed to ensure the bumblebees were hungry and motivated to forage following the experience of Cartar, 1989 and Free, 1955. Throughout the week, only pollen was offered to keep the queen busy producing eggs and to ensure a normal larval development. The colony was dismissed when the foragers exhibited aggressive behavior and ceased foraging completely. If (under any circumstance) the queen died, the colony was also dismissed because the normal foraging behavior disappeared.

4.5 Individual marking of bumblebees in a laboratory setting

The tags for individual marking (Figure 3 F) were printed on photo paper to ensure stability and were subsequently punched out using a hole puncher. They consisted of 5 distinct colors with alphanumeric characters (33 each color) at a size of 5 mm. Foragers were selected from the feeding site and were placed in a marking tube (Imgut® Königinnen Zeichenrohr ordered at Heinrich Holtermann KG, Brockel, Germany). Fixed there, the thoracic hairs were shaved, and the tags were affixed on to the thorax with a minimal amount of super glue gel (UHU® Sekundenkleber

Supergel, UHU GmbH & Co KG, Bühl / Baden, Germany). The animals were put back into the arena after keeping them in tubes for 5 minutes to allow the glue to adequately dry. This procedure was repeated every Monday morning to create enough marked foragers for subsequent training sessions until the first appearance of drones. At this stage, the marking discontinued to ensure only foragers were marked.

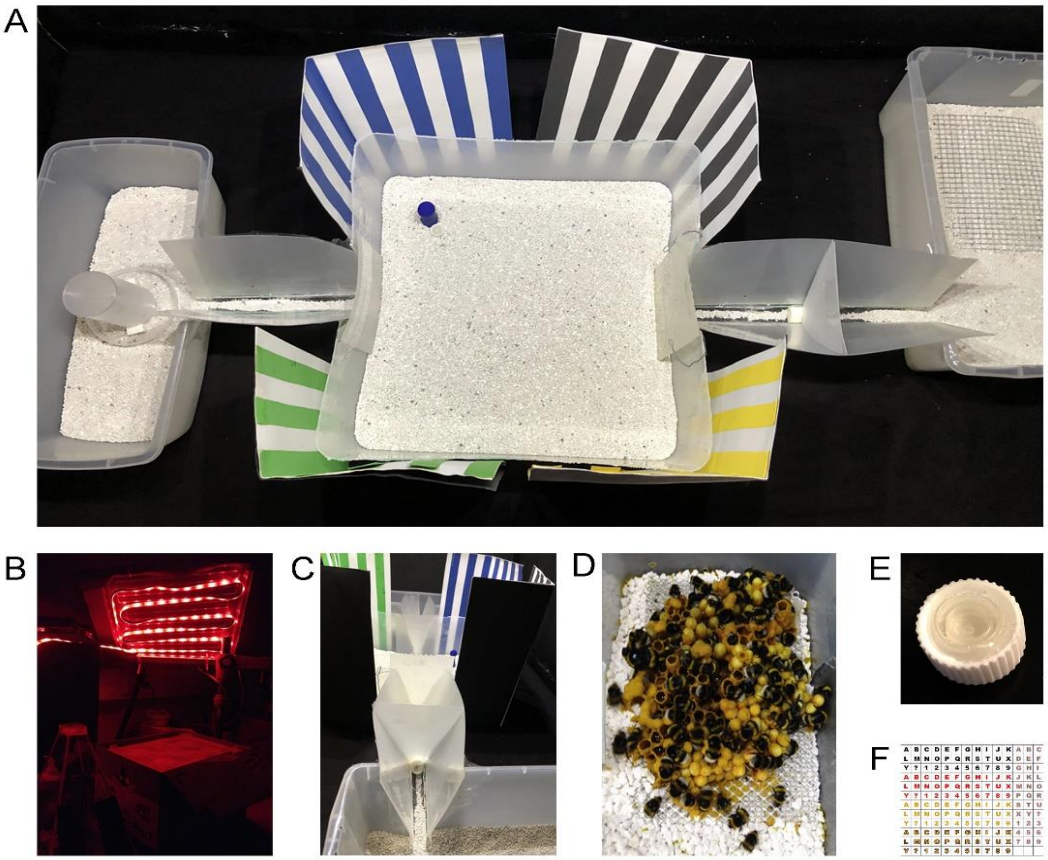

Figure 3: Configuration and components of the behavioral experimental setup. A: The test arena in the middle during the training situation. The feeding box on the left and the colony box with the prepared mesh on the right side. B: The red-light device for taking the bumblebees out of their delivery box while minimizing stress levels. C: The V-shaped transitions with the prepared doors. D: A bumblebee colony on the mesh. E: A cup which is utilized in the training to offer the sugar solution. F: The marking tags for one colony.

4.6 How to build electrodes

The method of building the recording electrodes is explained in Duer et al., 2015 and is here only described shortly. Two polyurethane-coated copper wires (14 µm in

diameter, Electrisola, Escholzmatt, Switzerland) and a silver wire (50 µm in diameter. Advent, Eynsham, Oxon, UK) were twisted over a length of approximately 1,2 meters. The loose end of the twisted wires was de-insulated and soldered to a dpi-plug for connecting the electrode with the amplifier. The other end was cut at an angle, and the silver wire was bent in an 90° angle. After finishing the preparation, the silver wire was just touching the brain for grounding. The two copper wires were formed to an inverted 'V' shape and glued together such that they stayed in place (Figure 6 D). This end was then gold plated, using the method of Ferguson et al., 2009 to reduce the impedance of the recording wires below 60 kΩ. During the procedure of dissection and preparation the electrode was fixed on a micromanipulator with a droplet of low heat wax. While recording an animal in the test arena, the electrode was counterbalanced with a spring made from a fish line.

4.7 The behavioral training

The training started always at the beginning of the week. The entrance to the arena was closed with two doors. The two-barrier system ensured that only one bumblebee at a time entered the arena. Only individually marked bumblebees were selected for training purposes. Sugar solution (1.5/1 sugar/water ratio) was used for the training process. Two pieces of filter paper soaked with farnesol were put in the arena to enhance foraging behavior (Granero et al., 2005; Strube-Bloss et al., 2015). It was ensured that no sugar solution was in the combs, so the bumblebees were hungry and motivated to forage (Cartar, 1989; Free, 1955). The Raspberry Pi with the recording software was started and the cups (Figure 3 E) were filled with 100 µl sugar solution. For the panorama, cardboard walls with colored stripes were installed. The area covered by the color on the white background was the same on each wall. The colors (in human terms) were yellow, green, black, and blue. A painted game piece in royal blue served as the local cue. The panorama was placed around the arena and the local cue was placed at the selected spot. During training the local cue was always placed in front of the same panorama color (blue). The cat sand was freshly mixed, so no odor cues were present (Goulson et al., 1998; 2000; Schmitt & Bertsch, 1990). Then the

first bumblebee entered the arena. The video recording was initiated, and the test bee started to explore the arena ad libitum. When she returned, the doors were opened, and she could exit the arena. The cup and the position were then changed, and the cat sand was thoroughly mixed to distribute any odor marks possibly left by the former forager. The next bumblebee could enter the arena. Such training sessions usually ran for 3 hours during 3 - 4 consecutive days. Tests without sucrose reward were conducted with trained bumblebees after each training session to examine the training progress. At the end of the week, 3 different test procedures were performed as explained next. Only 2 - 4 bumblebees could be tested per week due to their lower motivation to forage. Though it was taken care that the colony had no food supply, and the combs were empty, the motivation consistently stayed at that level.

The videos from the years 2017 and 2018 were omitted from the in-depth analysis due to significant structural, technical, and methodical changes over time. The former camera lacked a steady framerate while recording, the boxes altered, and the test setups differed. Hence, these data cannot be compared to the ones used for the analysis. For the analysis only data from the revised and improved setup was used.

4.8 The testing procedures

4.8.1 Testing procedure without former contamination with the insecticide Calypso®

The test situations were the following: a) panorama only, b) mismatch (the panorama and the local cue were located at different positions), c) match (same conditions as during training) (Figure 4). A fourth test was performed, which was the same mismatch situation as explained before. While testing, no sugar solution was provided (extinction test). All runs were video recorded, tracked and analyzed. Similar to the training runs, the bees were allowed to enter the arena whenever they wanted except if the arena was occupied by another bee. The animals were not touched or moved by the experimenter.

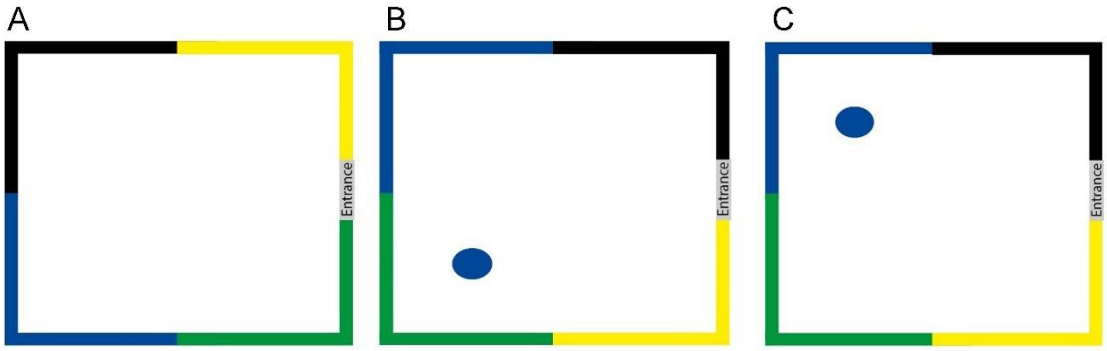

Figure 4: Test situations. A: Panorama only. B: Mismatch (panorama and local cue located at different positions). C: Match (panorama and local cue coupled to each other, like training). Fourth test not shown here was the same as B.

Also, for different analysis steps the arena was divided into 5 areas which were occupying the same area size (Figure 5).

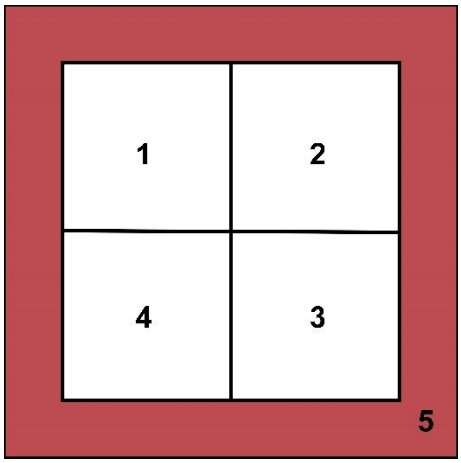

Figure 5 Division in areas.

4.8.2 Test with Calypso®

Two groups of bees (control and calypso group) were run in parallel tests. The animals of these two groups were handled individually by moving them in and out of separate tubes. The control group received 4µl of sugar solution (1/1 sugar /water ration). The calypso group was fed with 4µl calypso in sugar solution. Different concentrations were used as well (400 ng/animal and 800 ng/animal; end sugar concentration maintained at 1/1 sugar/water ratio). Each bee was placed in a separate tube under red light, and

they were video recorded for approximately one hour. This procedure allowed me to ensure that the animals digested the food and were not able to distribute it into the colony afterwards. It also allowed me to detect any misbehavior (e.g., cramps or other unnatural behavior). The animals were then put into the colony again and the testing followed the aforementioned protocol.

As stated before, after each training session a mismatch test was performed. The mismatch test after the last training was later used in the analysis to compare the performance of the bees which received a treatment with their own performance without any treatment.

4.8.3 Electrophysiological Recordings

The trained animals were prepared one at a time. The animal was removed from the colony and was put on ice for a short period of time to calm it down. Then it was fixed in a special bee holder, which was designed by Dr. Benjamin Paffhausen on demand, improved by my own experience and 3D printed by Ralph Schön. Conventional bee holders designed for honeybees were too small for normal sized bumblebees. Because of their natural size variation, 7 different sizes of bee holders were prepared (Figure 6 A+B). After fixing the bumblebee in the appropriate bee holder, her mandibles were secured, to ensure no movement of the head while inserting the electrode. The antennae were glued with a two-component silicon glue (Kwik-Sil™, World Precision Instruments, Sarasota, USA) that was removed later without causing harm. A hole was cut into the caput of the bumblebee and after shifting the mandibular glands, which lay right under the chitinous cuticle, the brain became visible, and the electrode could be placed at the selected site (the alpha exit of the mushroom body, Figure 6 C). The electrode was first fixed with a drop of tissue glue (Surgibond Tissue Glue, SMI AG, St. Vith, Belgium) to the surface of the brain and then the hole was filled with Kwik-Sil preventing it from drying and to keep the smell of the tissue glue at a low level. The animal was then kept in the dark to allow the glue to harden. The box with the amplifier and audio amplifier was put in the arena. They were connected to the STM, and the Arduino was placed next to the arena to emit the light signal for offline synchronization between video and electrophysiological recordings. The camera of the Raspberry Pi was focused and everything got connected to the external power supply (car battery).

After 20-30 minutes the bumblebee was placed in the passage between the colony and the arena. The electrode was connected to the amplifier. The electronic parts were turned on and the Arduino generated the light signals. Then the door to the arena opened and the bumblebee was allowed to enter. The aim of the experiments was to test both situations (match and mismatch) while recording. If the animal did not move for 30 minutes or the neural signals were lost, the animal was removed, and the procedure was repeated with the next trained animal.

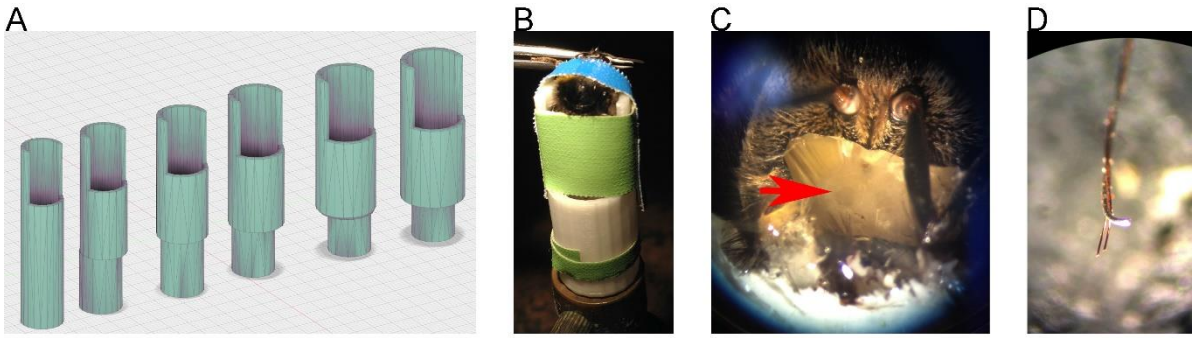

Figure 6: Parts and procedure of the preparation for electrophysiological recordings. A: The 3D prints of the bee holders in different sizes. B: A bumblebee fixed in a bee holder with her mandibles secured with forceps. C: The opened head capsule with perspective of the brain. The red arrow marks the vertical lobe. D: The end of the electrode with the two copper wires slightly afar from each other and the silver wire bend to be laid on the brain after the insertion.

4.9 Video recording

All videos were recorded with a Raspberry Pi 4 and a camera module (Raspberry Pi Camera module v2). The videos of the behavioral tasks were recorded via a software obtained from GitHub. These videos had to be converted into the AVI (Audio Video Interleave) format. All videos were then tracked with the Biotracker of the Landgraf research group (FU Berlin, Robotics group). Further information about this tracker can be found here: https://github.com/BiorboticsLab/biotracker_core/wiki. The tracks were stored as CSV (Comma-separated values) files and used for further analysis.

4.10 Electrophysiological recordings

For efficiency the STM saved the data as binary files. The files had to be converted by a binary converter written by Julian Petrasch. The resulting time series were imported into the software Spike2 (v7.1, CED, Cambridge, UK). The original data contained sample rate irregularities that were compensated.

4.10.1 Filtering and sorting

The data in Spike2 (v7.1, CED, Cambridge, UK) were filtered by a bandpass filter between 156 and 1500 Hz. Semi-automated spike sorting was run with a time window of 0,8 to 1 ms and the threshold was set between 80 and 200 mV. After spike sorting the spikes were clustered into one group and manually clustered into units by shape. This clustering was controlled with a PCA (Principal Component Analysis) and a PSTH (Peristimulus time histogram) for quality check. Then each functional unit was exported as a Matlab file for the stitching process.

4.10.2 Light signal

The light signal for synchronizing the spike data with the tracking data was obtained by an Arduino UNO (Adafruit Industries, LLC, New York City, USA) with a blinking LED and a timing code programmed on it. The output of the light was led around the connection between electrode and amplifier, so the signal was visible in the video and recorded by the amplifier synchronously. The light signal in the recording was filtered separately in Spike2 and exported as a Matlab file. The light signal in the videos was manually reported in the tracking CSV file by running the videos frame by frame.

4.10.3 Synchronization of video and electrophysiological recording

The stitching was done in MATLAB (MATLAB by MathWorks®, Natick, USA) by implementing a stitching code originally developed by Dr. Benjamin Paffhausen. The

code was refactored and customized to suit the specific dataset. The tracking CSV file with the light signals was imported as well as the Matlab files of the units and the Matlab file of the light. Double light signals were filtered out and the data was merged. The spikes were binned according to the 30-fps rate of the video and distributed between the corresponding light signals. After stitching and putting the data together, it remained one CSV file with the tracking data and the associated spikes for each recording.

4.10.4 Definition of behavior

To define phases of distinct behavioral patterns, the most promising recordings were visually inspected and then analyzed according to the following behavioral categories:

- Movement from entrance gate to position 1
- Movement from entrance gate to position 4
- Movement from position 1 to entrance gate
- Movement from position 4 to entrance gate
- Movement from position 1 to position 4
- Movement from position 4 to position 1
- Movement from position 2 to position 1
- Movement from position 2 to position 4
- Movement from position 3 to position 1
- Movement from position 3 to position 4
- Border walking
- Walking around (position 2/position 3; not between the goals)
- Movement from position 2 or 3 to entrance gate
- sitting
- Occupying position 1
- Occupying position 4
- Moving away from position 1 (excluding movement towards position 4 or entrance gate)
- Moving away from position 4 (excluding movement towards position 1 or entrance gate)

It should be noted that not every behavior was observed in every recording.

4.11 Statistical analysis of behavioral and electrophysiological data

The analysis was mainly done in RStudio (Posit Software PBC, Boston, USA) using the R programming language. Scripts of the used code can be found in the appendix. The corresponding statistics were done in GraphPad Prism (GraphPad Software, Inc., Boston, USA).

For the heatmaps shown later, the density of the video fixes is the basis.

All groups, that were used for the statistics were checked for normality with a Kolmogorov-Smirnov Test and fitted tests for further investigation were used based on these results.

Various parameters were examined in the analysis of the behavior. The head direction could not be determined because the tracker did not recognize the head of the bumblebee separately from its body. Similarly, the angle she faced while sitting could not be calculated. Not shown in this work are multiple analyses of different parameters e.g., related to walking angle towards the learned locations, because there was no hint of any correlation between these behavioral parameters and the spike rate.

For the electrophysiological analysis all recordings were sorted and merged. Following a first intense evaluation, the recordings were filtered with the following exclusion criteria:

- Duration of recording too short (less than 5 minutes)
- No natural behavior:
    - sitting too long (more than 2 minutes)
    - frequent circling
    - attempts to remove the electrode
    - staggering locomotion
- Neural signal:
    - diminishing signal over time

- muscles contractions interfering with recording (can be audibly recognized by their stereotypical pattern, a high amplitude and a low frequency)

All electrophysiological recordings with both testing situations were filtered through a mask (1 x 1 cm) to use only the data points which occur in both situations. For this filtering a matrix was built for the 30 x 30 cm arena. The data points from one test condition were then superimposed onto the matrix. Whenever there was occurrence of data in one bin, the second test condition was laid over and checked whether there is also an occurrence. If in both test situations data points could be found, these datapoints where used.

4.12 Residue analysis of the insecticide Calypso®

Preparing the bees for biochemical measurements for pesticide residue, animals were carefully selected from a functional colony. These animals were then fed with the sugar calypso solution (400 ng and 800 ng calypso per animal; end sugar concentration 1/1 sugar/water ratio). Following the ingestion, the animals were kept under red light for the different time slots (1,5 h, 3 h, 4,5 h). After the time had elapsed, the animals were promptly frozen in -80 °C. After completely frozen, the body parts were separated, packed, appropriately marked and dispatched to the laboratory technician. The Labor Friedle GmbH (Tegernheim, Germany), a specialized laboratory, for conducting the analysis, then provided the results via email for further interpretation.

4.13 References

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5 Results

5.1 Behavioral experiments

Throughout the entire duration of all experiments, around 30 colonies, approximately 450 animals, underwent training, out of which 230 of them were tested. The majority of the video recordings of the tested bumblebees were subjected to semi-automated tracking and underwent partial analysis. Due to modifications and adjustments made to the experimental setup and training procedure, I will not combine all data obtained over the time. Consequently, only data from the most recent experiments with the optimized procedure and the final setup were selected for further analysis.

5.1.1 Development of the colonies in the setup

Looking at the plain number of animals in the colony, I observed that the number of animals first increased, then remained stable for a short amount of time before experiencing a decline (Figure 7 A). After I fitted the curves with a nonlinear regression analysis for growth (Yin et al., 2003) a standardized model was obtained (Figure 7 B).

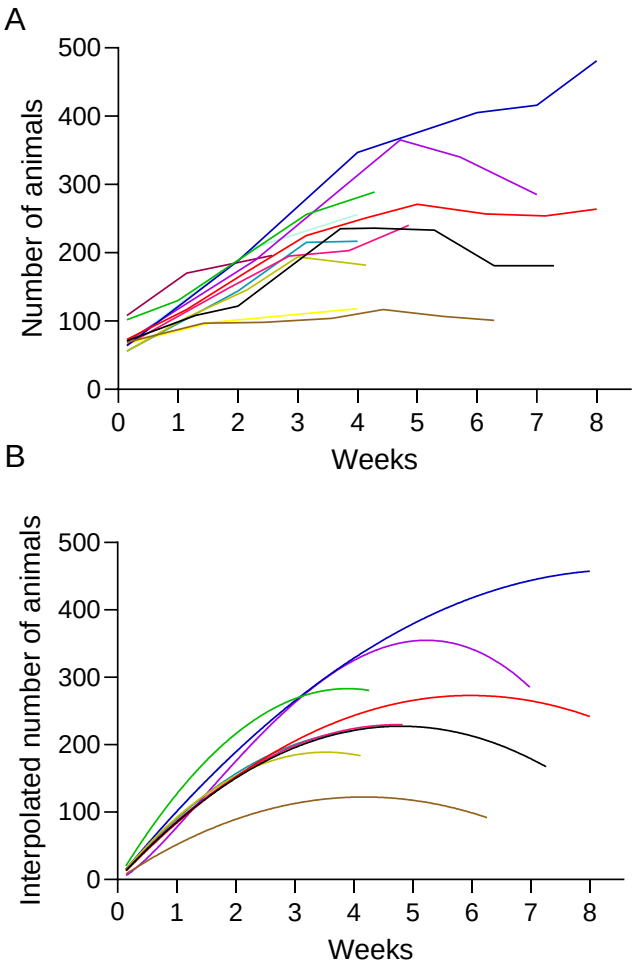


Figure 7: Development of the colonies in the setup. A: The development of the population of the colonies in the setup here shown from 12 example colonies. B: Interpolation of colony development of 9 example colonies.

Most colonies were removed of the setup upon the emergence of new queens, which was either at their maximum peak or shortly after.

Figure 8 shows the development of single colonies within the setup over time. Notably, peaks in the number of animals occurred at the beginning of each week. These were indicated by the number of wings cut and dead animals, which were the counting after the weekend, when no wings were removed. Moreover, the humidity dropped over the course of the week. The peaks here are not at the first day but second or third day of the week after the wet clothes were renewed on the first day of the week. The temperature was maintained constant throughout the experiments (Figure 8).

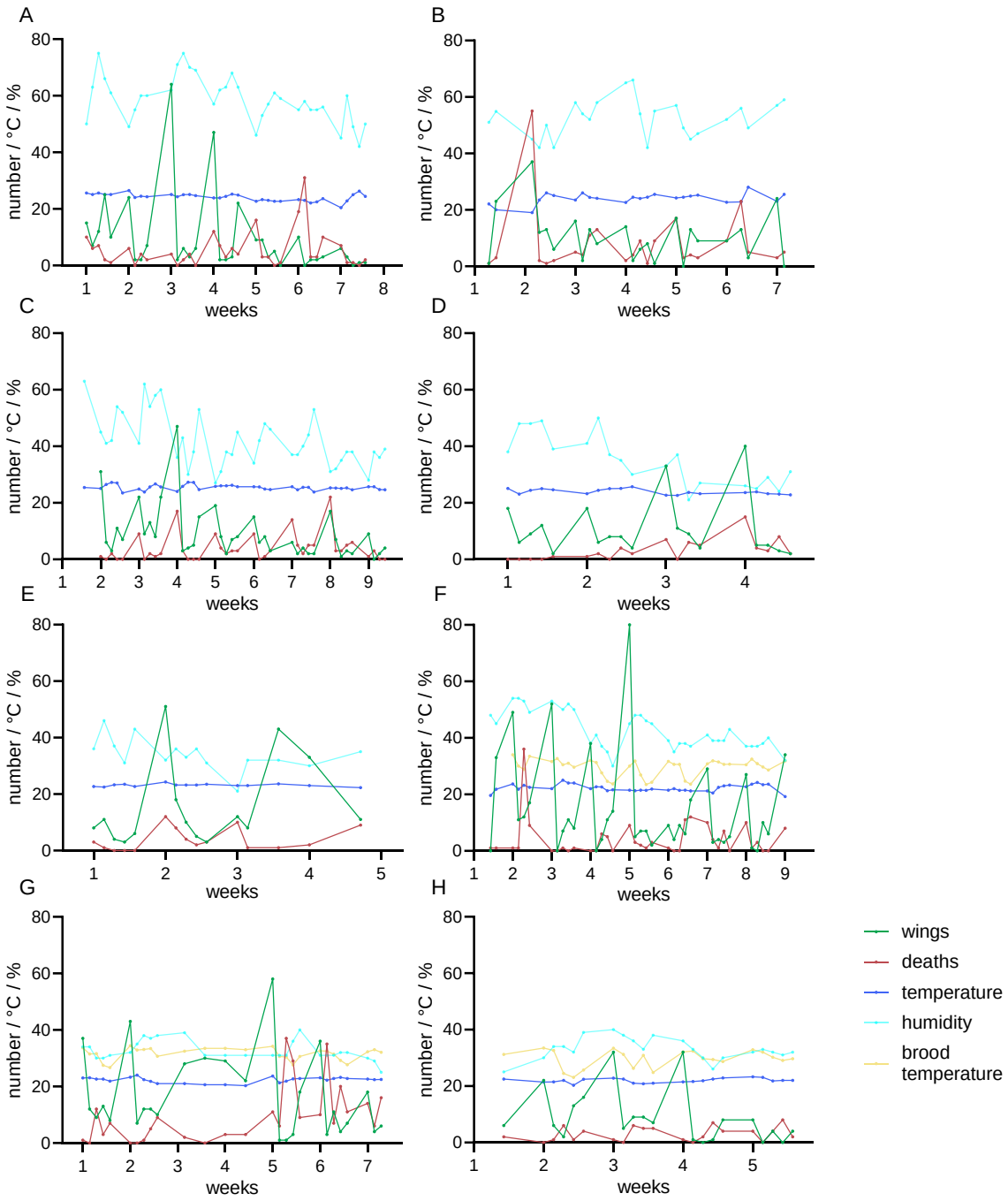


Figure 8: Development of single colonies throughout their time in the setup. 8 of the colonies used in the setup. Shown are the number of wings cut (newly hatched bees), the number of dead animals, the surrounding temperature and humidity. Additionally for F, G, H the brood temperature is displayed.

In the later colonies the brood temperature was additionally recorded. This variable, unlike the room temperature, shows fluctuations in correspondence with the population size of the colony (Figure 8). The duration of the time in the setup differs with each

colony and depends on different parameter which will be explained in the discussion part.

5.1.2 Spontaneous Color preference

All animals used in the experiments underwent an initial assessment of color preference while they were still completely untrained and did not know the arena yet. Solely the panorama boards were used as stimuli, not the local cue. To determine the significance of color preferences, a statistical analysis was performed comparing the percentage of time spent in each area relative to the others (ANOVA with Bonferroni post hoc test). No significant spontaneous color preference was found between the four areas marked with different panorama cues (Figure 9 A). The bees stayed significantly longer (by about 20% longer) in the border area, that stretches all panorama cues, then in any other area ($p < 0{,}001$) (Figure 9 B).

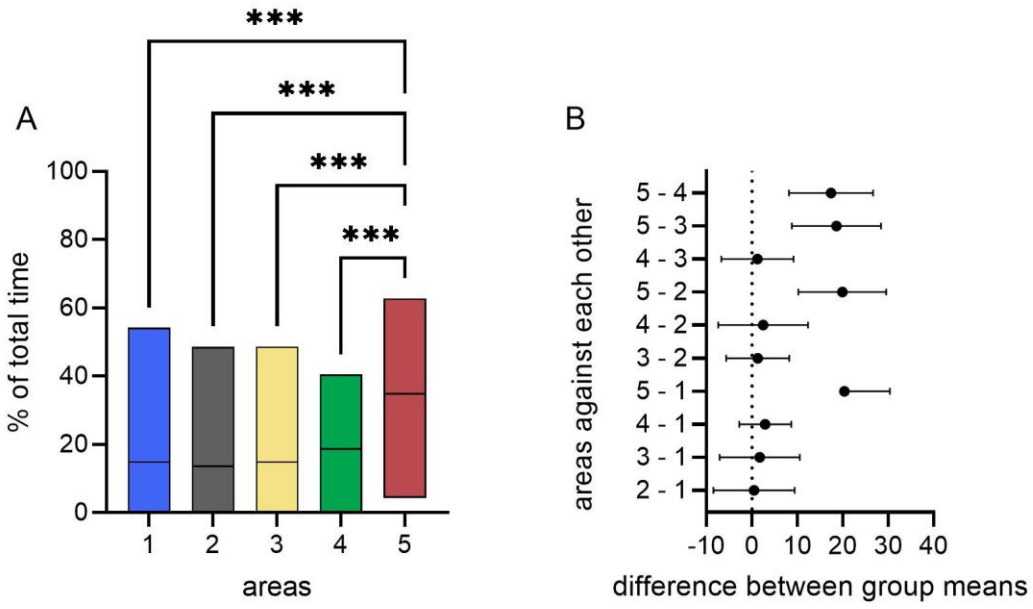


Figure 9: Test of spontaneous color preference. Differences of untrained bees in their time distribution. A: Boxes from the minimum and maximum of the values and the mean as hyphen. Asterisks indicate statistical difference (n = 35, $p < 0{,}001$, ANOVA with Bonferroni post hoc test). The colors of the plots represent the colors of the panorama at that specific area (area 1-4). The 5th (red) area depicts the border area. B: The delta between the groups means with standard error.

Examining the average walking speed within the different areas (Figure 10), one finds a significant differences between area 1 and area 5 (p = 0,02, paired t test) and between area 3 and area 5 (p = 0,04, paired t test). The differences between area 2 and area 5 do not reach statistical significance (p = 0,07, paired t test), however a tendency can be seen. Importantly, no significant differences were found between the 4 areas characterized by different panorama cues.

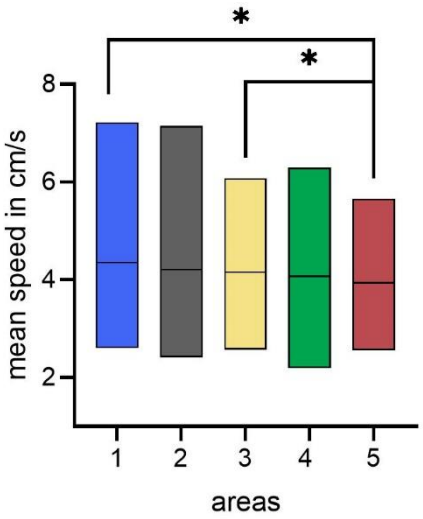


Figure 10: Mean speed (cm/s) in areas. The boxes show the mean speed of the animals (n=35) from minimum to maximum with the mean as hyphen. Asterisks mark significance ($p < 0.05$, paired t tests). The colors of the plots represent the colors of the panorama at that specific area. The 5th (red) area depicts the border area.

5.1.3 Behavior of bumblebees during testing after training

Animals were tested in the setup in the four distinct test situations within a controlled experimental setup (1. Panorama only; 2. Mismatch; 3. Match; 4. Mismatch). To ensure comparability, data from the final experiments was exclusively utilized, while the data from earlier stages of the setup was disregarded.

The value of the straightness of the animals is significantly higher comparing the 1st training with test 3 (match situation like training, $p < 0,001$ Wilcoxon test). The tested animals showed a significantly shorter and therefor more direct trajectory to the goal in the extinction test 3 then the naïve untrained animals, thereby suggesting a heightened efficiency in their navigational abilities (Figure 11).

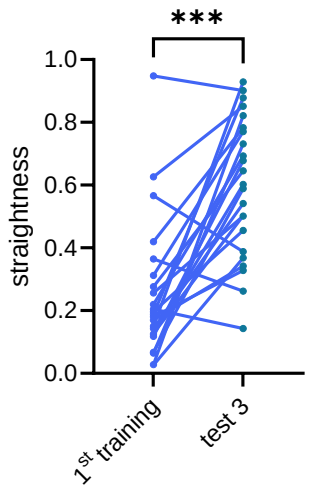


Figure 11 Comparison between the straightness of the walking trajectory during the first successful training run and the extinction test 3. Statistical significance is shown by asterisk (n = 23; p < 0,001; Wilcoxon test).

Next the data of the percentage of the total length of walking trajectory was analyzed with an ANOVA with Bonferroni post hoc test (Figure 12). Significant differences were found in the first test (panorama alone) between area 1 and area 4 (p = 0,04), area 2 and area 3 (p < 0,001), area 2 and area 4 (p = 0,02) and area 4 and area 5 (p = 0,03). In test 2 (mismatch: panorama around area 1, local cue located in area 4) significant differences were found between area 1 and area 4 (p < 0,001), area 2 compared to area 3 (p = 0,007) and area 5 (p = 0.001), area 4 compared to area 3 (p < 0,001) and area 5 (p < 0,001). Test 3 (match: panorama and local cue in area 1) shows high significant differences between area 1 and all others (p < 0,001 for all areas). A smaller number of animals participated in test 4, as their motivation had waned by that point. Notably, the results of test 4 (again mismatch) exhibited a statistically distinction solely between area 1 and area 4 (p = 0,02).

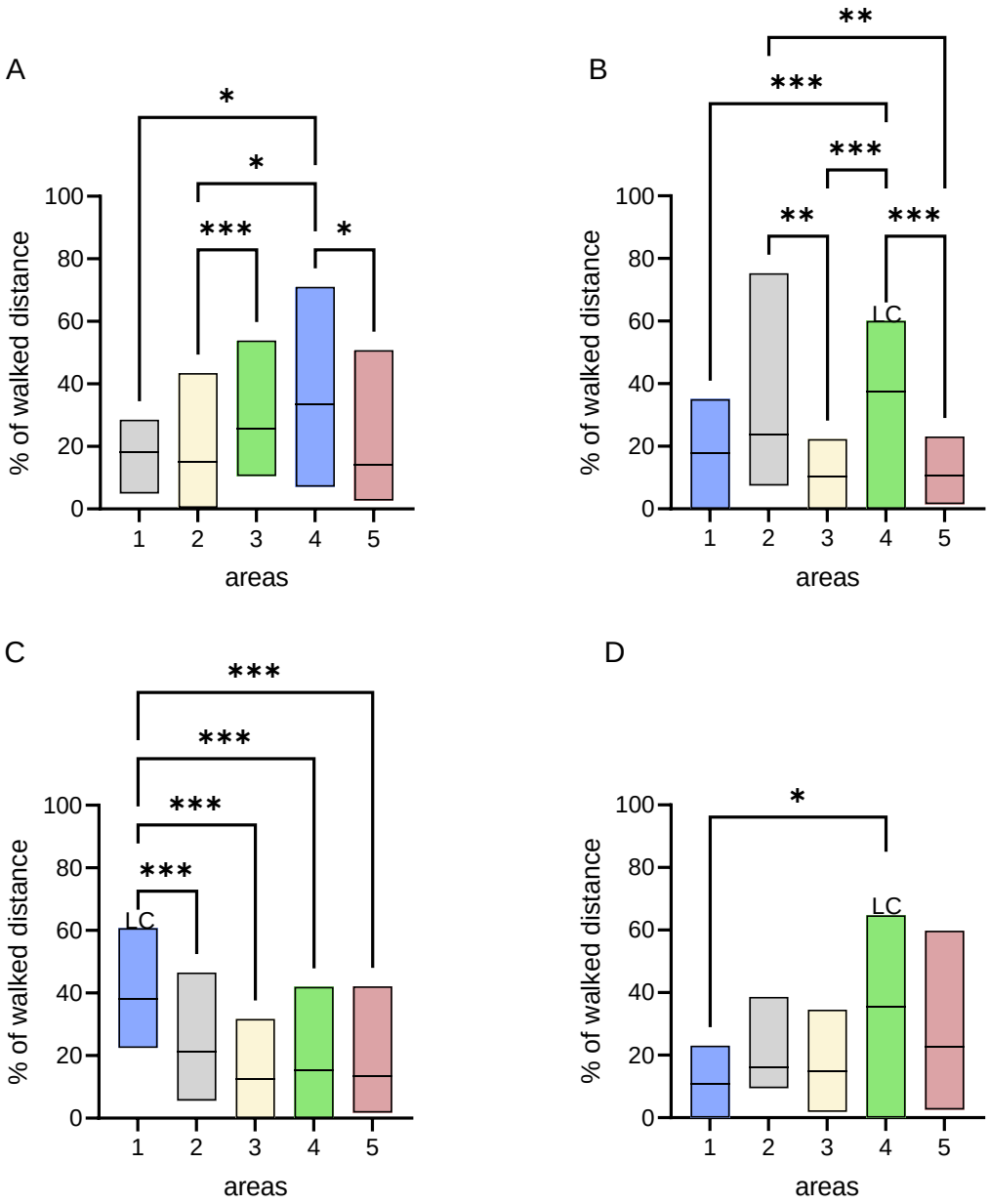


Figure 12: The percentage of the total length of walking trajectory in the areas of the 4 extinction tests. The boxes show the percentage of the total length of the walking trajectory in the test run per area (n = 23; in test 4 n = 14) from minimum to maximum with the mean as hyphen. Asterisks mark three level of significant differences (* p < 0,05; ** p < 0,01; *** p < 0,001). The colors of the plots represent the colors of the panorama at that specific area. The position of the local cue is labeled (LC). The 5th (red) area depicts the border area. A: Test 1 only panorama. B: Test 2 mismatch (different position of the panorama and local cue). C: Test 3 match (coupled position of panorama and local cue). D: Test 4 again mismatch

A next attempt to analyze the training effect was to compare the walking lengths in the spontaneous preference test with the first extinction test. The color preference test and the extinction test 1 (panorama only) are in principle the same situation in the arena: only the blue panorama is shown, not the local cue. The data from both of the tests

(same animals) show significant changes in area 4 (p = 0,009; Wilcoxon test) and area 5 (p < 0.001; Wilcoxon test) (Figure 13). Area 4 is the area that is marked with the trained panorama (blue), and area 5 is the border area. The values are significantly higher for area 4 and lower for area 5, indicating the training effect. The reduced walking in area 5 may indicate in addition that the animals were more adapted to the training/test situation after training since walking in area 5 is indicative for escape behavior. Notice that in this test no local cue was shown. Thus, the combined effect of training to the panorama and the local cue cannot be derived from these data.

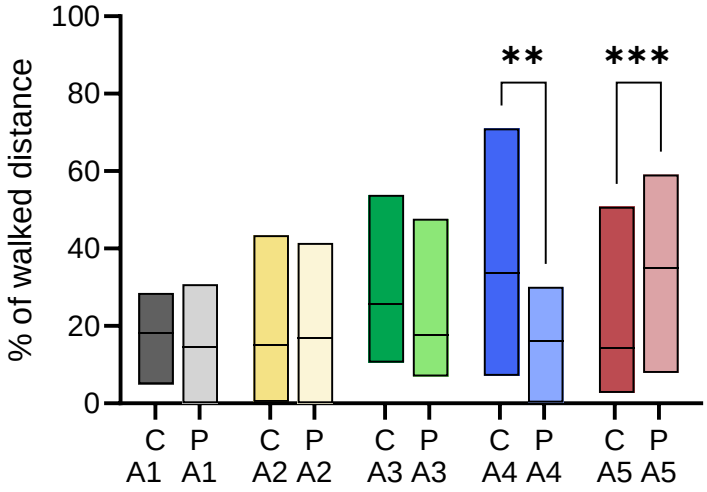


Figure 13 Comparison of the percentage of the length of walking trajectory during extinction test 1 with the results of the color preference test. The boxes show the percentage of the length of the walking trajectory of the test run per area of the groups (C: control n = 23, P: color preference test, n = 23) from minimum to maximum with the mean as hyphen. Asterisks mark significance (Wilcoxon test). The colors of the plots represent the colors of the panorama at that specific area. The 5th (red) area depicts the border area. Significance in area 4(p = 0,009) and area 5 (p < 0,001).

5.1.4 The effects of uptake of the neonicotinoid Thiacloprid, an agonist of the nicotinic receptor

The extensive use of neonicotinoids poses a threat to pollinating insects (Brandt et al., 2016; Pisa et al., 2014; Stanley & Raine, 2016; Tsvetkov et al., 2017; Van der Sluijs et al., 2013; Wood & Goulson, 2017) and led to the ban of 3 highly toxic neonicotinoids used in Europe (EFSA, 2018) and meanwhile also to the ban of the less toxic neonicotinoid Thiacloprid. Neonicotinoids act as agonists on nAChRs opening cation channels (Casida & Durkin, 2013; Jones et al., 2006; Lu et al., 2022) located in the central nervous system of insects. Their agonistic action induces continuous excitation

of the post-synaptic membrane, producing discharges leading to cell energy exhaustion, paralysis and death (Tomizawa & Casida, 2011). The behavioral test procedures developed by me in the course of recording neural activity of high order interneurons during free walking in a semi navigational tasked allowed me to address in the nutshell the action of Thiacloprid in the laboratory

5.1.4.1 400 ng of Thiacloprid per animal

In the following tests bumblebees were tested after an intake of 400 ng/animal Thiacloprid in the formulation Calypso®. The concentration was reached by diluting Calypso® by an equal amount of sugar water. A volume of 4 µl was provided to each animal. It was carefully controlled that the full dose was ingested.

First, walking tracks of the experimental and control animals were compared. Heat maps were calculated on the basis of the density of videos fixes in the arena. The results are given in Figure 14 for test 1 (panorama only), in Figure 15 for test 2 (mismatch), in Figure 16 for test 3 (match) and in Figure 17 for test 4 (again mismatch).

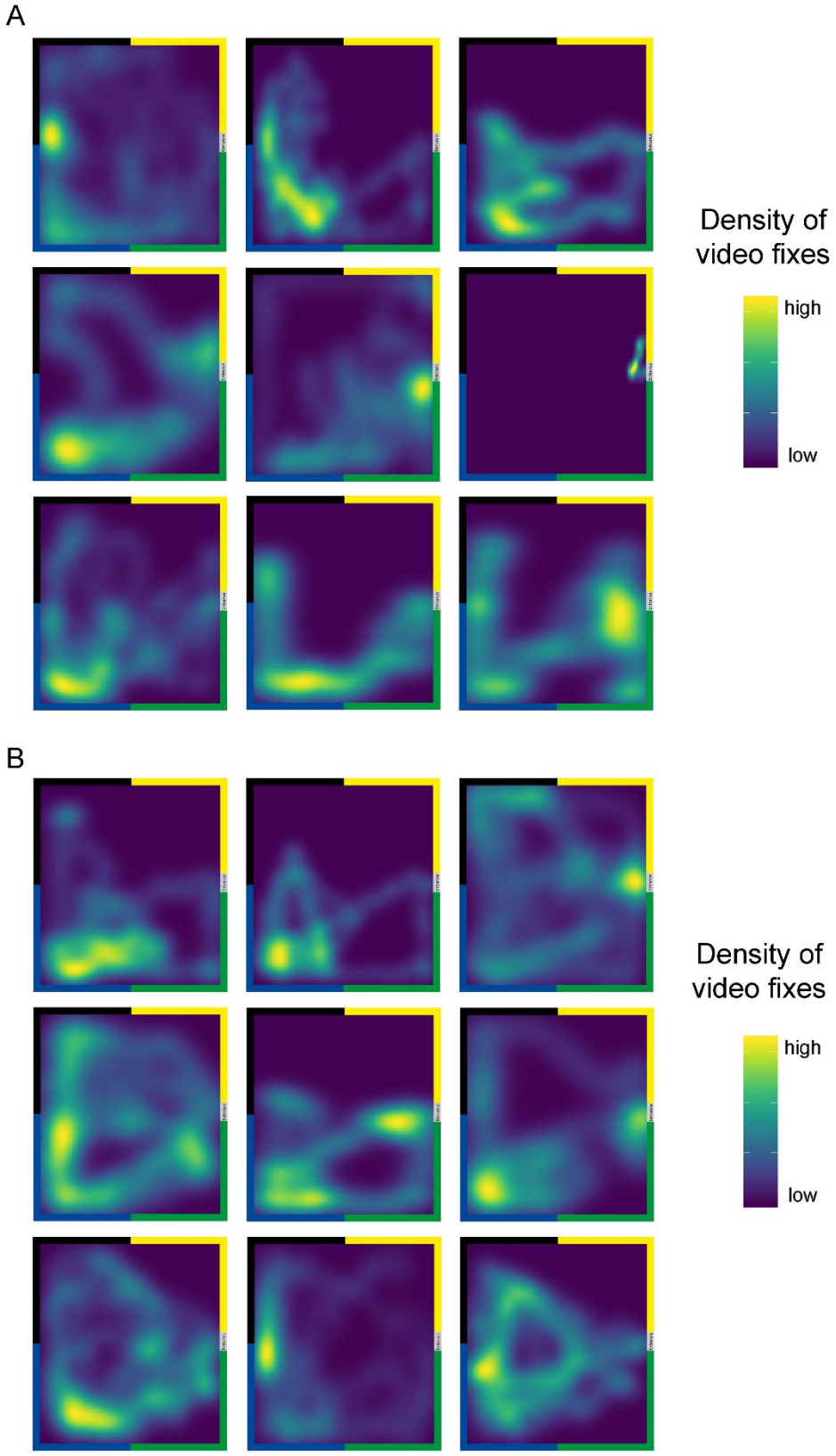


Figure 14: Heat maps of the density of video fixes plotted onto the arena of test 1 (panorama only). A: treated group (n = 9), B: control group (n = 9). Panorama is depicted around the arena.

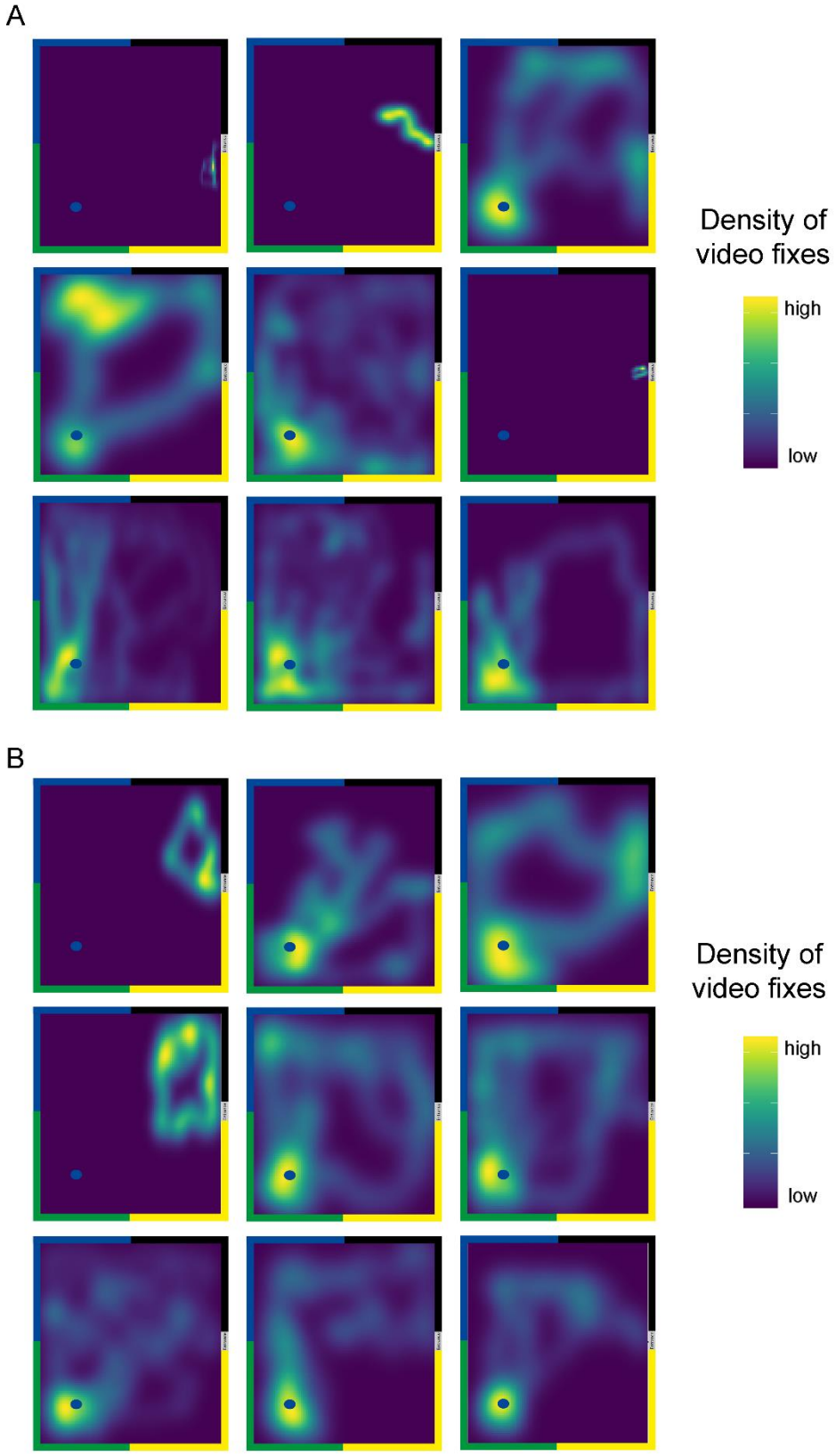


Figure 15: Heat maps of the density of video fixes plotted onto the arena of test 2 (mismatch). A: treated group (n = 9), B: control group (n = 9). Panorama and local cue (dark blue dot) are depicted at mismatching locations.

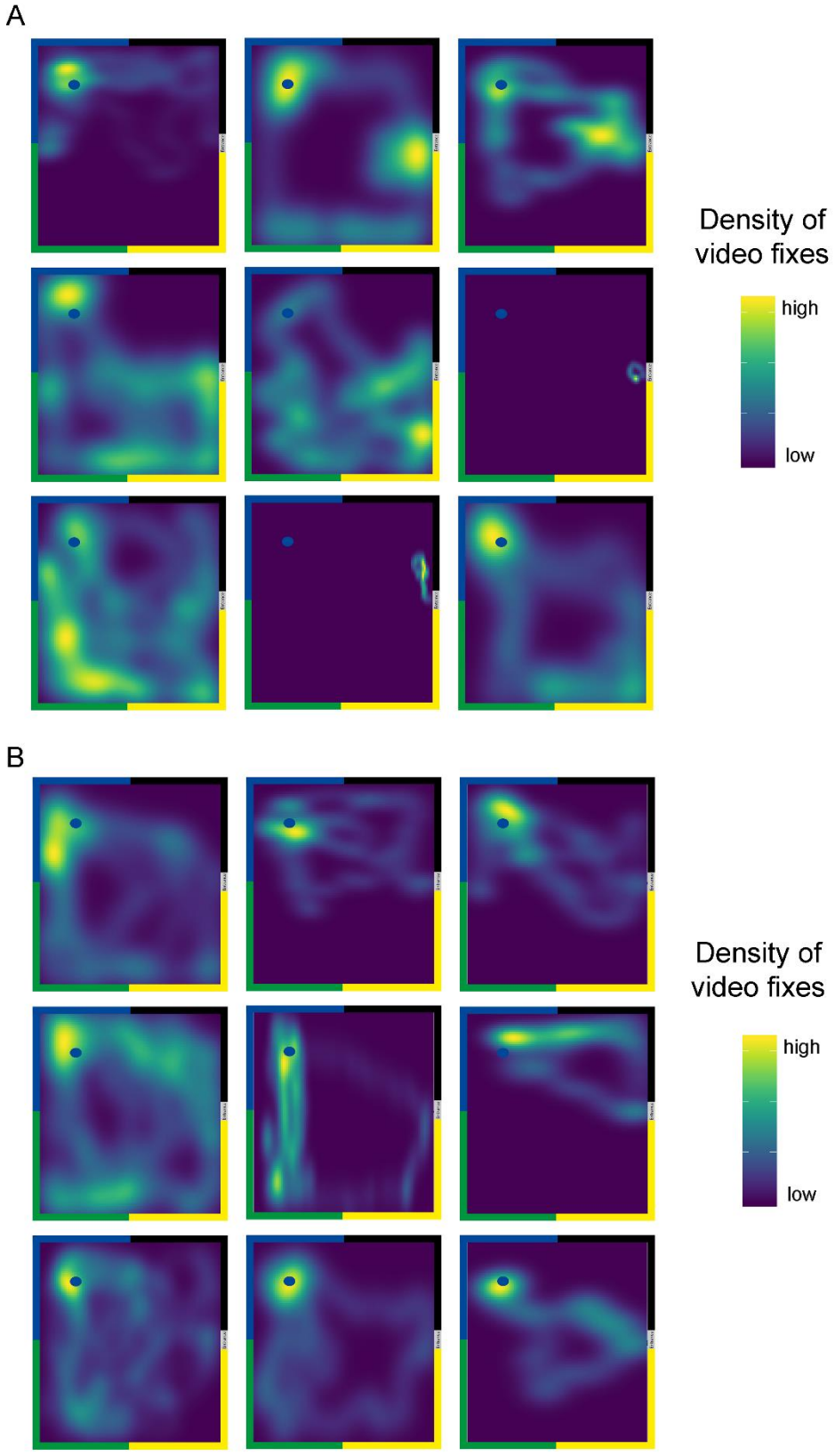


Figure 16: Heat maps of the density of video fixes plotted onto the arena of test 3 (match*).* A: treated group (n = 9), B: control group (n = 9). Panorama and local cue (dark blue spot) are depicted at matching locations.

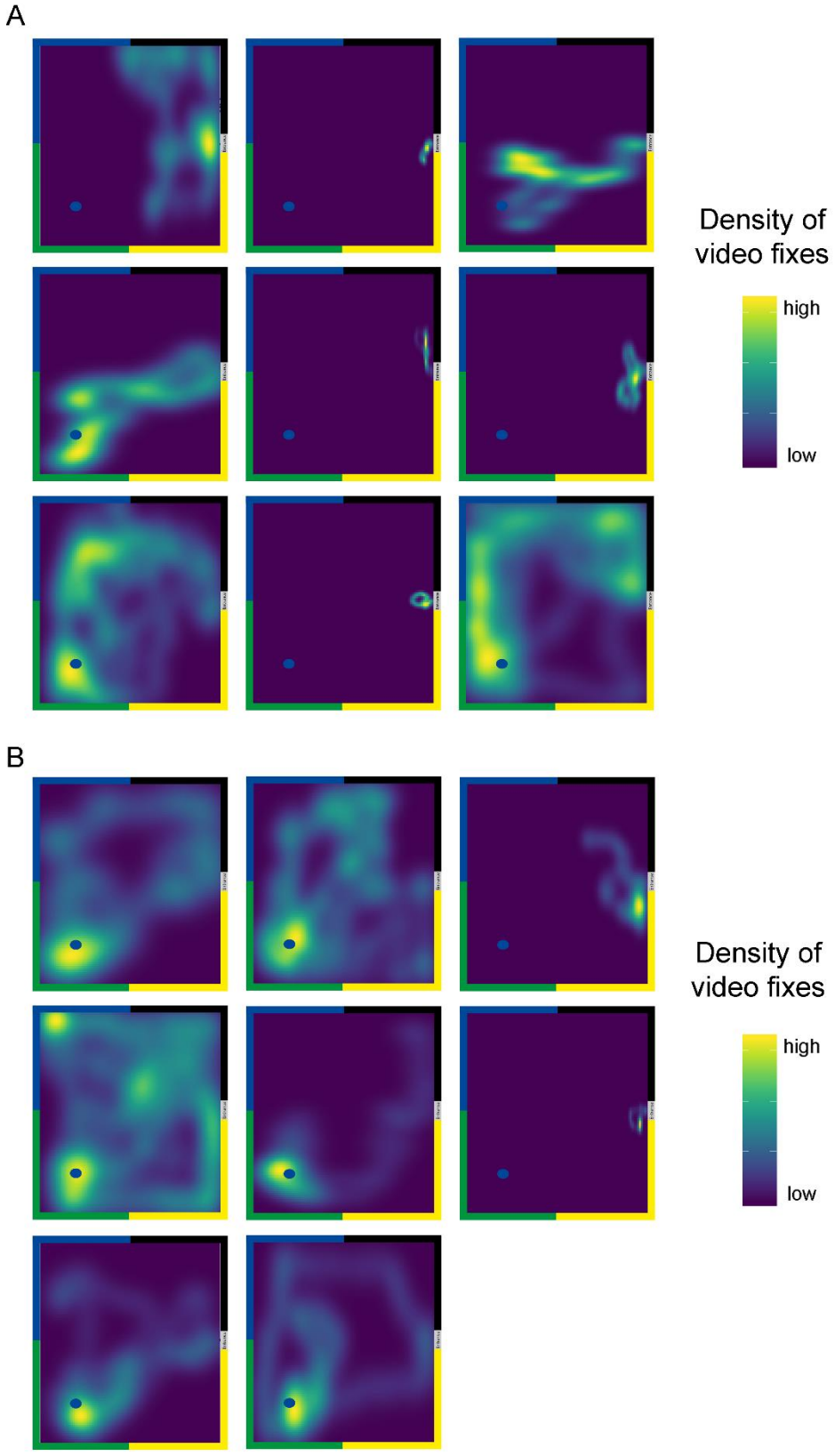


Figure 17: Heat maps of the density of video fixes plotted onto the arena of test 4 (mismatch*).* A: treated group (n = 9), B: control group (n = 8). Panorama and local cue (dark blue spot) are depicted at mismatching locations.

Next, several parameters of the walking trajectory were analyzed. Analyzing the total length of walking trajectory, no significant difference can be seen. (Figure 18 A). The straightness of the trajectory towards a goal was calculated by the formula:

$$\text{straightness} = \frac{\text{direct route length}}{\text{actual route length}}$$

The optimal straightness is 1. The closer to zero the more imprecise the walk to the goal is. Only in test 3 the straightness between the treated and control group differed significantly (p = 0,04; t-test with Welch correction) (Figure 18 B). The total length of the walking trajectory and straightness did not change significantly before and after the treatment (comparing the data from test 2 with the data of the corresponding animals of the mismatch test after the 3rd training session) (Figure 18 C+D). Taken all the straightness values of all tests together, there was also no significant difference (Figure 18 E).

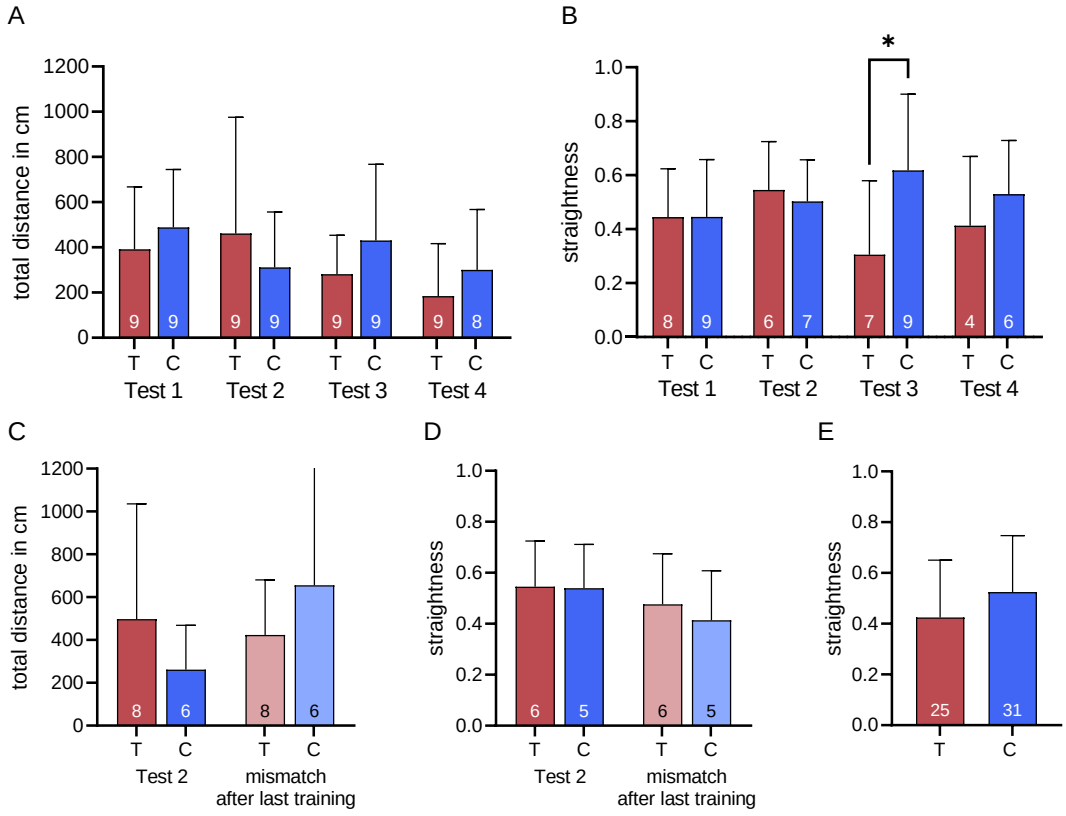


Figure 18: The total length and straightness of the walking trajectory compared between the control and treated group. The plots represent the mean of the total length of the walking trajectory and the straightness with the standard deviation for the tests of the 2 groups (treated (T, 400 ng, red) and control (C, blue) group). The number of animals is marked in the bars. Asterisks mark significance. A: the total length of the walking trajectory in the different tests. B: straightness of the two groups in the different test. Significance in test 3 (p = 0,04; t-test with Welch correction). C: total length of the walking trajectory in test 2 compared to the mismatch test after training. D:

straightness in test 2 compared to the mismatch test after training. E: the straightness of all tests taken together for the two groups.

Next, the success of the training was tested by analyzing the difference between the straightness of the first successful training (naïve bees finding the food in the training situations) and test 3, which represents the training situation. The treated animals showed no significant difference between the first successful training and test 3 whereas the control group significantly improved in test 3 (after being trained) (p = 0,02, Wilcoxon test) (Figure 19).

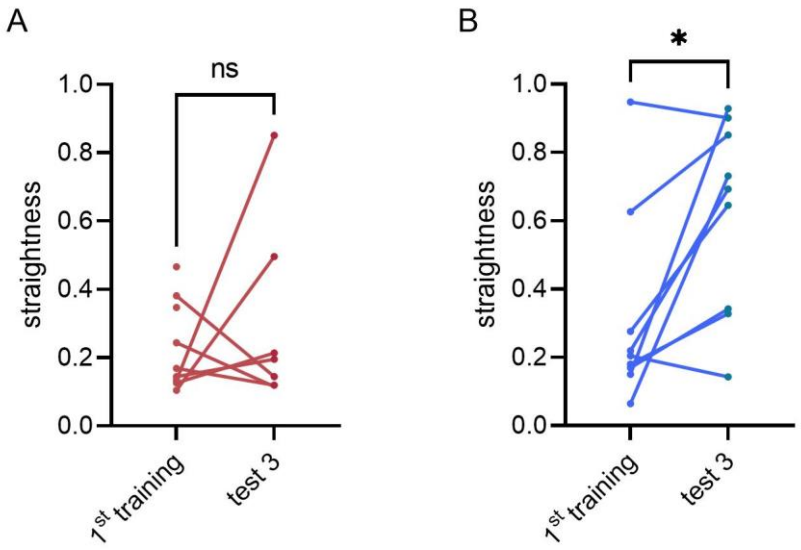


Figure 19: Comparison between the straightness of the walking trajectory during the first successful training run and the extinction test 3 of the treated (A) and control group (B). Statistical significance is shown by asterisk (p = 0,02; Wilcoxon test).

A further comparison between control and treated animals focused on the time spent in each of the 5 areas. No significant differences were found in test 1 (only panorama). In test 2 and test 3 the time the treated group stayed in the border area is significantly longer than the control group (p = 0,02, t-test with Welch correction). In test 3 the time around the local cue was significantly lower in the treated group then of the control group (p = 0,02, t-test with Welch correction) (Figure 20).

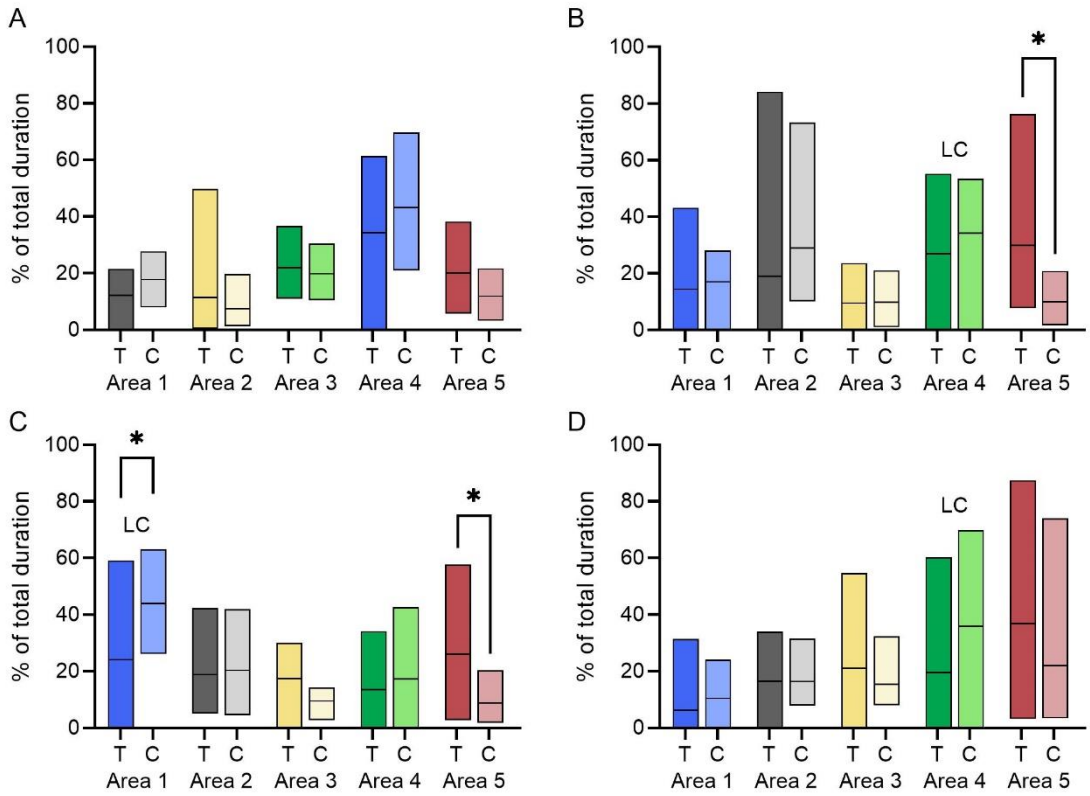


Figure 20: The percentage of the total duration in the areas for the 2 groups (treated and control) in the 4 test situations. The boxes show the percentage of the total duration of the test run per area and test group (T: Thiacloprid intake 400 ng, n = 9; C: control n = 9) from minimum to maximum with the mean as hyphen. Asterisks mark significance. The colors of the plots represent the colors of the panorama at that specific area. The position of the local cue is labeled (LC). The 5th (red) area depicts the border area. A: Test 1 only panorama. B: Test 2 mismatch (with different position of the panorama and local cue). Significance in area 5 (p = 0,04, t-test with Welch correction) C: Test 3 match (with coupled position of panorama and local cue). Significance in area 1 (p = 0,02, t-test with Welch correction) and area 5 (p = 0,02, t-test with Welch correction) D: Test 4 mismatch. Without significance.

The duration of being in an arena could result from slower walking or no walking at all.

Therefore, I analyzed the walking time excluding periods of not walking.

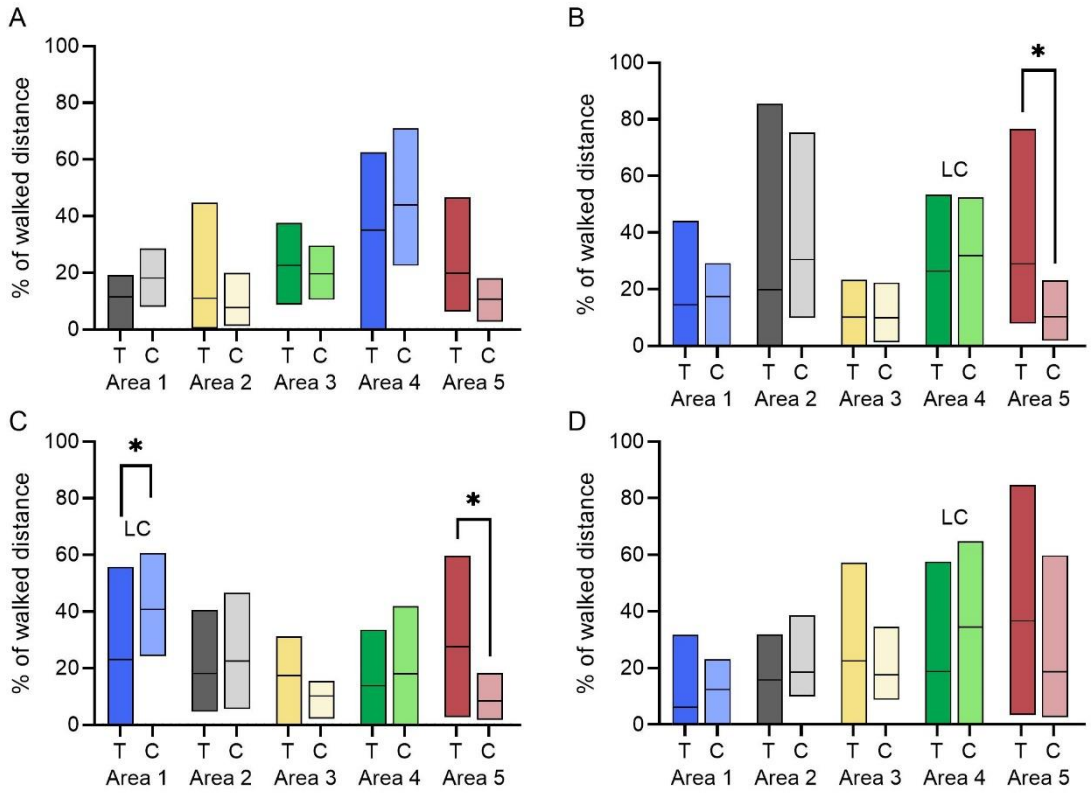


Figure 21: The percentage of the total length of walking trajectory in the areas for the 2 groups (treated and control) in the 4 test situations. The boxes show the percentage of the total length of walking trajectory of the test run per area and test group (T: Thiacloprid intake 400 ng, n = 9; C: control n = 9) from minimum to maximum with the mean as hyphen. Asterisks mark significance. The colors of the plots represent the colors of the panorama at that specific area. The position of the local cue is labeled (LC). The 5th (red) area depicts the border area. A: Test 1 only panorama. B: Test 2 mismatch (with different position of the panorama and local cue). Significance in area 5 (p = 0,05, t-test with Welch correction) C: Test 3 match (with coupled position of panorama and local cue). Significance in area 1 (p = 0,02, t-test with Welch correction) and area 5 (p = 0,02, t-test with Welch correction) D: Test 4 mismatch. Without significance.

To check whether the intake of Thiacloprid changes the behavior of the animal the mismatch test after the last training was compared to the extinction test 2 (mismatch) (Figure 22 A+B). Here the treated group shows a significant increase in the length of the walking trajectory compared in area 5 (p = 0,05; Wilcoxon test). No significant difference was seen to the control group. To confirm that the prerequisite is the same the mismatch test after the training was analyzed between the two groups (control and treatment) and no difference can be measured (Figure 22 E). Another test was performed whether there is change between test 2 and test 4 (Figure 22 C+D). No significant difference was revealed.

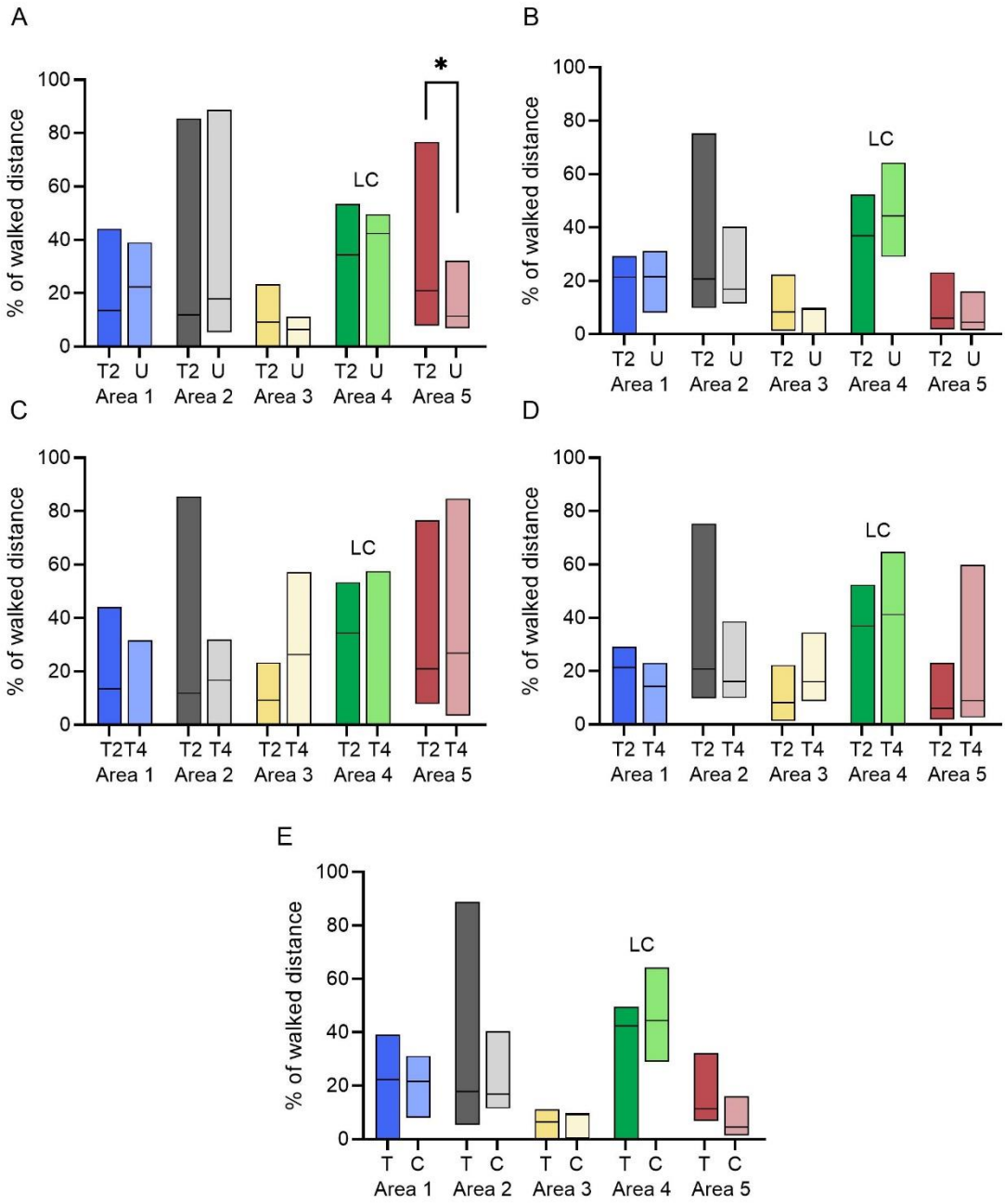


Figure 22: The percentage of the total length of walking trajectory in the different areas in the treated and control group. The boxes show the percentage of the total length of walking trajectory of the test run per area and test group (T: Thiacloprid intake 400 ng, n = 9; C: control n = 9) from minimum to maximum with the mean as hyphen. Asterisks mark significance. The colors of the plots represent the colors of the panorama at that specific area. The position of the local cue is labeled (LC). The 5th (red) area depicts the border area. A: Comparing the data from test 2 with the mismatch test after the last training (U) of the treated group. Significance in area 5 (p = 0.05, Wilcoxon test) B: Comparing the data from test 2 with the mismatch test after the last training (U) of the control group. C: comparing the data from test 2 with test 4 of the treated group. D: comparing the data from test 2 with test 4 of the control group. E: Comparing the mismatch test after the last training between the treated and control group.

Also interesting is the analysis of the mean walking speed (cm/s) in the different areas (Figure 23). In test 1 one does already see a tendency but only area 1 is significant

(p = 0,03). In test 2 all areas show a significant difference between the treated and the control group (area1: p = 0,01; area 2: p < 0,001; area 3: p= 0,02; area 4: p = 0,02; area 5: p < 0,001). In test 3 the difference is significant only in area 2 (p = 0,004), and in test 4 two areas show significant differences, area 2: p = 0,01 and area 3: p = 0,05.

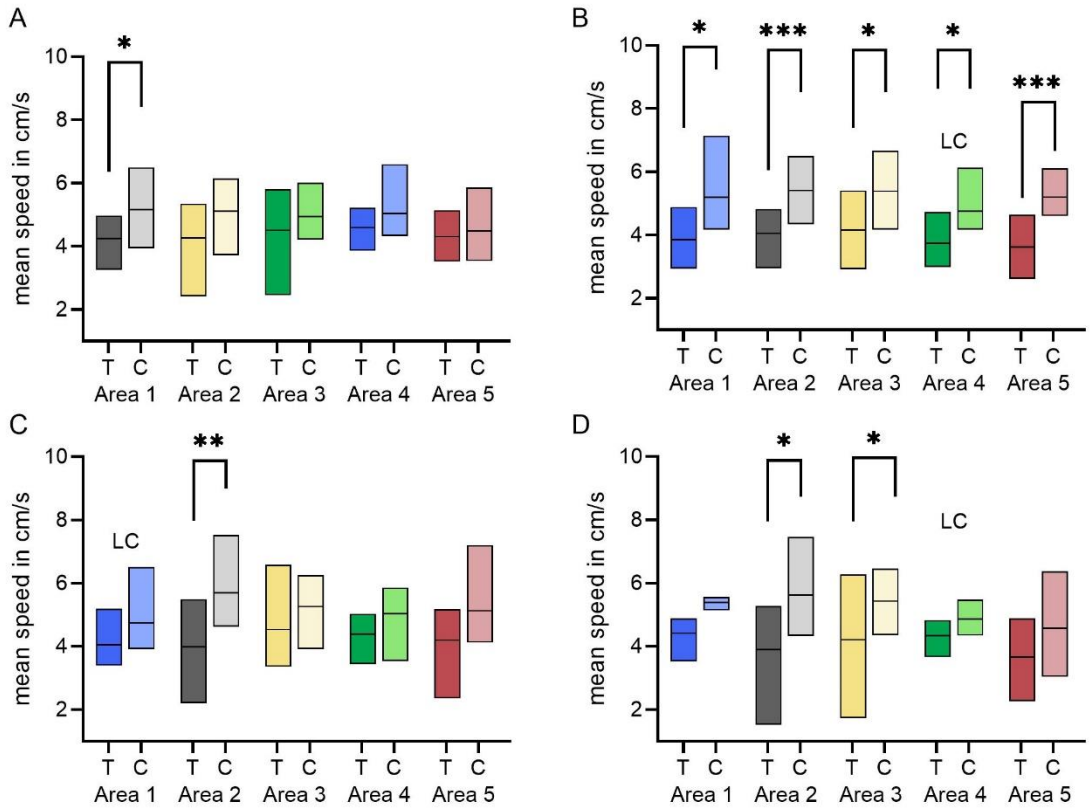


Figure 23: Mean walking speed of both groups in each test situation divided per area. The boxes show the mean speed of the test run per area and test group (T: Thiacloprid intake 400 ng, n = 9; C: control n = 9) from minimum to maximum with the mean as hyphen. Asterisks mark significance (t-test with Welch correction). The colors of the plots represent the colors of the panorama at that specific area. The position of the local cue is labeled (LC). The 5th (red) area depicts the border area. A: Test 1. Significance in area 1 (p = 0.03) B: Test 2, mismatch. Significance in all areas (area 1: p = 0,01; area 2: p < 0,001; area 3: p = 0,02; area 4: p = 0,02; area 5: p < 0,001). C: Test 3, match Significance in area 2 (p = 0,004). D: Test 4 mismatch. Significance in area 2 (p = 0,01) and in area 3 (p = 0,05).

The mismatch test after the last training was analyzed and compared with test 2 on the test day (Figure 24 A+B). While the mean speed in the control group only differs in one area (area 3: p = 0,02) the treated group showed a significant decrease in mean speed in the two areas they must walk through to get to the goal (area 2: p = 0,03; area 3: p = 0,03) and the border area (p = 0,02). No significant differences were found comparing test 2 and test 4 (both mismatch) for the treated and the control group

(Figure 24 C+D). Also, there is no difference between the two groups (treated and control) in the prior mismatch test after the last training (Figure 24 E).

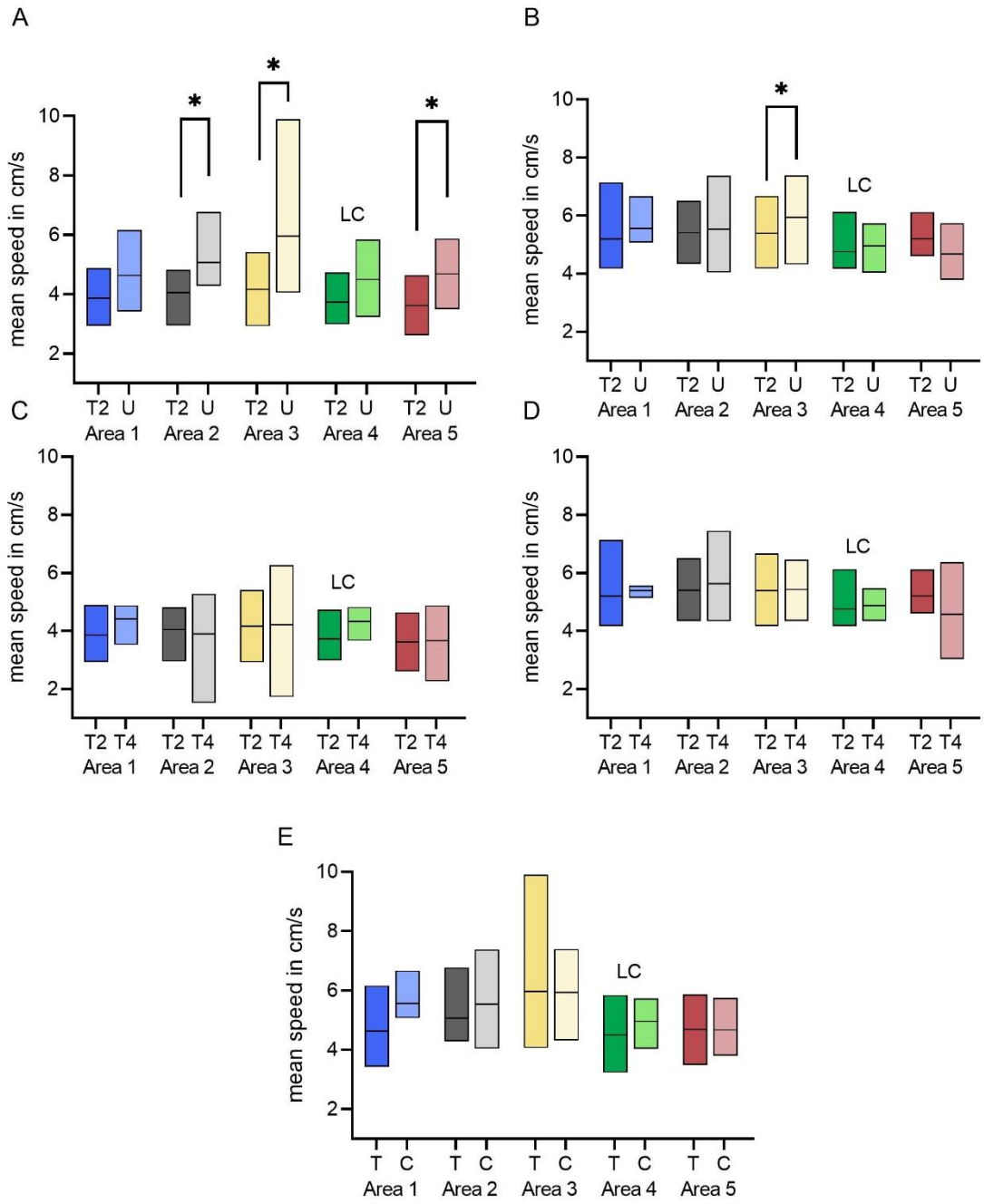


Figure 24 The mean walking speed in the areas of the 2 groups (treated and control). The boxes show the mean walking speed in cm/s of the test run per area and test group (T: Thiacloprid intake 400 ng, n = 9; C: control n = 9) from minimum to maximum with the mean as hyphen. Asterisks mark significance. The colors of the plots represent the colors of the panorama at that specific area. The position of the local cue is labeled (LC). The 5th (red) area depicts the border area. A: Comparing the data from test 2 with the mismatch test after the last training (U) of the treated group. Significance in three areas (area 2: p = 0.03; area 3: p = 0,03; area 5: p = 0,02; Wilcoxon test) B: Comparing the data from test 2 with the mismatch test after the last training (U) of the control group Significance in area 3 (p = 0,02; Wilcoxon test). C: comparing the data from test 2 with test 4 of the treated group. D: comparing the data from test 2 with test 4 of the control group. E: Comparing the mismatch test after the last training between the treated and control group.

The results of test 1 of the treated and control group were also compared with the respective color preference group (t test with Welch correction). The treated group showed a higher relative length of walking trajectory in area 4 to the preference test group ($p = 0,03$) whereas there was no difference comparing the mean speed of these two groups (Figure 25 A+C). The control group showed a significantly higher relative length of walking trajectory in area 4 ($p < 0,001$) whereas the relative length of trajectory of the preference test group was increased in area 5 ($p < 0,001$). The mean speed of the control is significantly higher in 3 areas than the mean speed of the preference test group (area 2: $p = 0,03$; area 3: $p = 0,02$; area 4: $p = 0,01$) (Figure 25 B+D).

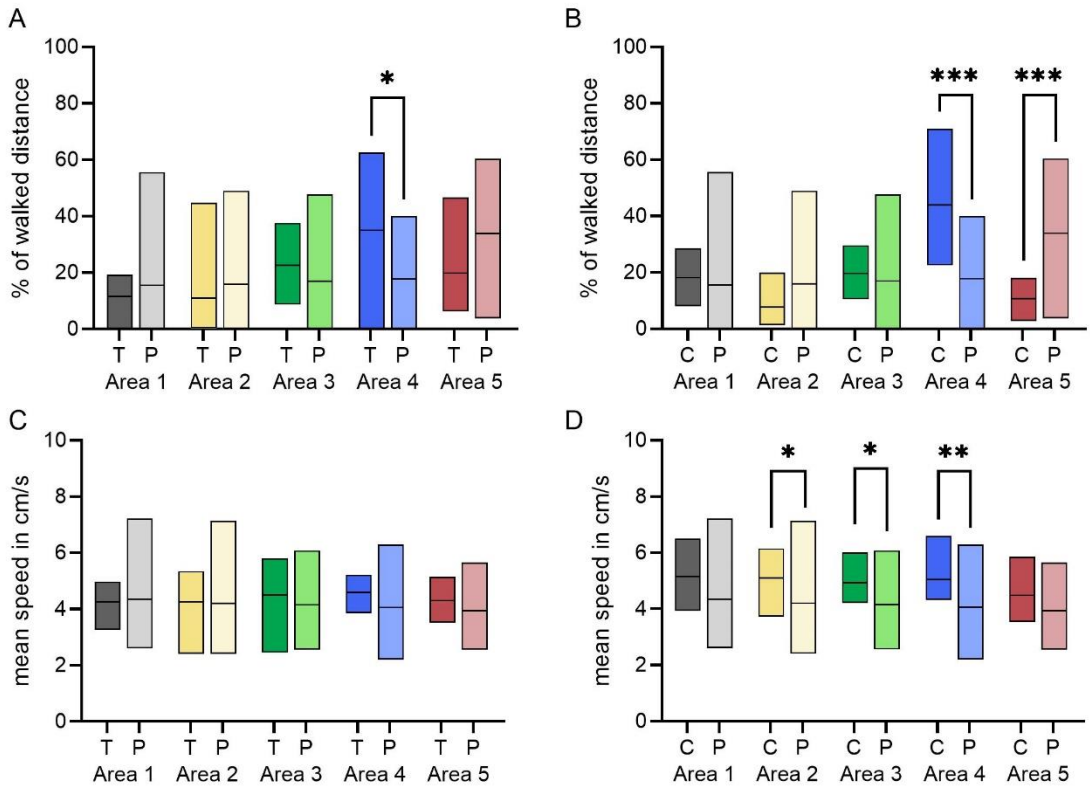


Figure 25: Comparison of the percentage of the length of walking trajectory and mean speed of the treated group and control group (test 1) compared with the results of the color preference test. The boxes show the percentage of the length of the walking trajectory of the test run per area (A+B) and the mean speed per area (C+D) of the groups (T: Thiacloprid intake 400 ng, n = 9; C: control n = 9, P: color preference test, n = 9) from minimum to maximum with the mean as hyphen. Asterisks mark significance (t test with Welch correction). The colors of the plots represent the colors of the panorama at that specific area. The position of the local cue is labeled (LC). The 5th (red) area depicts the border area. A: significance in area 4 ($p = 0,03$). B: Significance in area 4($p < 0,001$) and area 5 ($p < 0,001$). C: no significance. D: significance in area 2 ($p = 0,03$), area 3 ($p = 0,02$) and area 4 ($p = 0,01$).

5.1.4.2 800 ng of Thiacloprid per animal

In these tests, control animals were compared with animals that consumed 800 ng of Thiacloprid per animal. These data have to be handled with care because of the modest number of animals per group.

For an overview, the total length of walking trajectory in the different test situations and the straightness of walking trajectories were calculated. There is no statistical difference between the two groups in the total length of walking trajectories in the different test conditions or comparing the test 2 (mismatch) with the mismatch test after the last training. (Figure 26 A+C). The straightness of the treated group decreased in test 2 compared to the control (Figure 26 B), and also decreased compared to the performance in the mismatch test after the last training (Figure 26 D). Taken together the straightness values from all tests the treated animals walked significantly less straight (Figure 26 E).

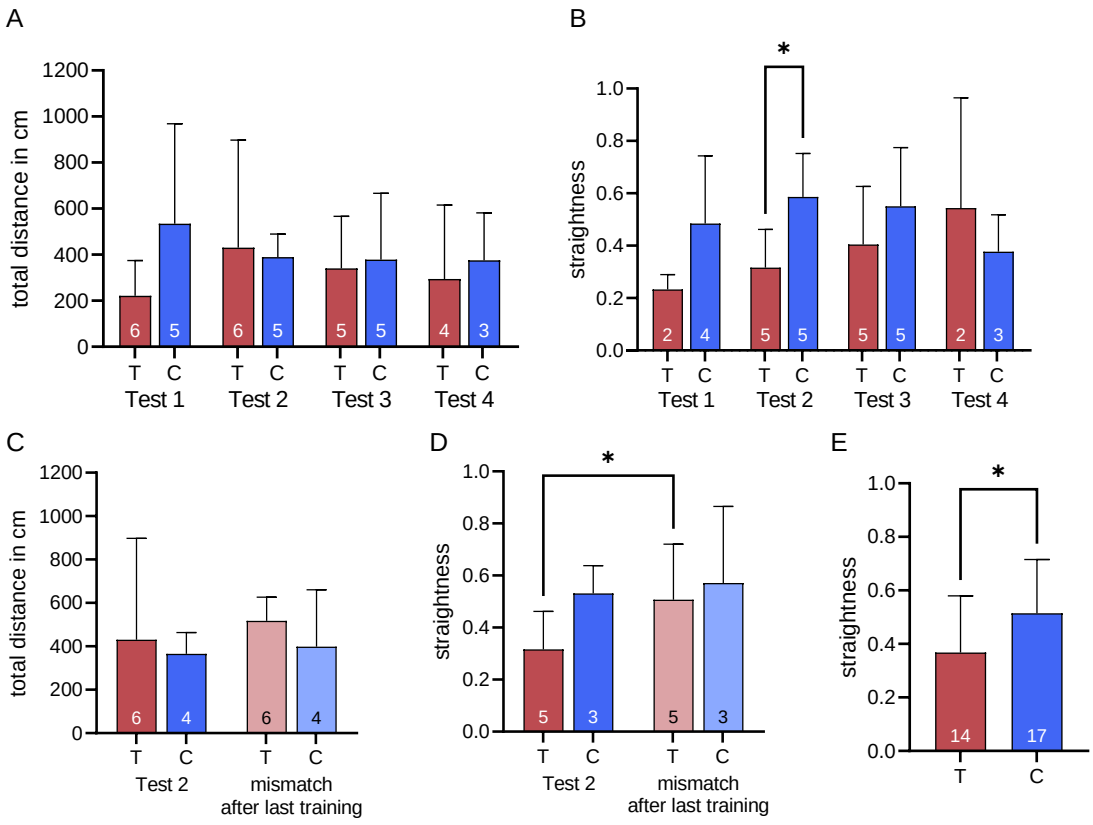


Figure 26: The total length of walking trajectories and straightness compared between the control and treated group. The plots represent the mean of the total length of walking trajectories and the straightness with the standard deviation for the tests of the 2 groups (treated (T, 800 ng, red) and control (C, blue) group). The number of animals is marked in the bars. Asterisks mark significance. A: the total length of walking trajectory in the different tests. B: straightness of the two groups in the different test. Significance in test 2 ($p = 0,03$; t-test with Welch

correction). C: total length of walking trajectory in test 2 compared to the mismatch test after training. D: straightness in test 2 compared to the mismatch test after training. Significance between test 2 and mismatch test of the test group (p = 0,03; paired t-test). E: the straightness of all tests taken together for the two groups (p = 0,03; Mann-Whitney test).

To analyze the success of the training, the first successful training (naïve animals finding the food source in the training conditions) and test 3 were compared. Neither the treated group nor the control group showed a significant change (Figure 27).

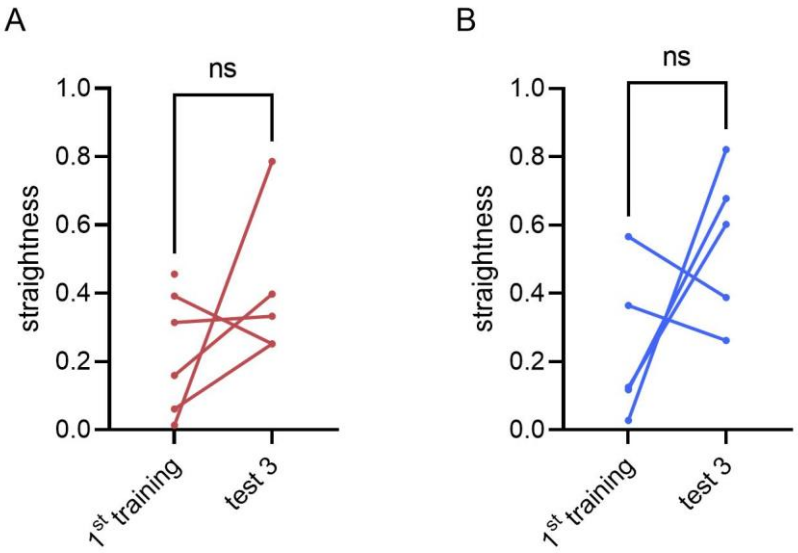


Figure 27: Comparison between the straightness of the walking trajectory during the first successful training run and the extinction test 3 of the treated (A) and control group (B). No statistical significance.

No significant differences were found for the 4 test situations analyzed separately (Figure 28). In test 4 the number of tested animals is further reduced because a lack of motivation.

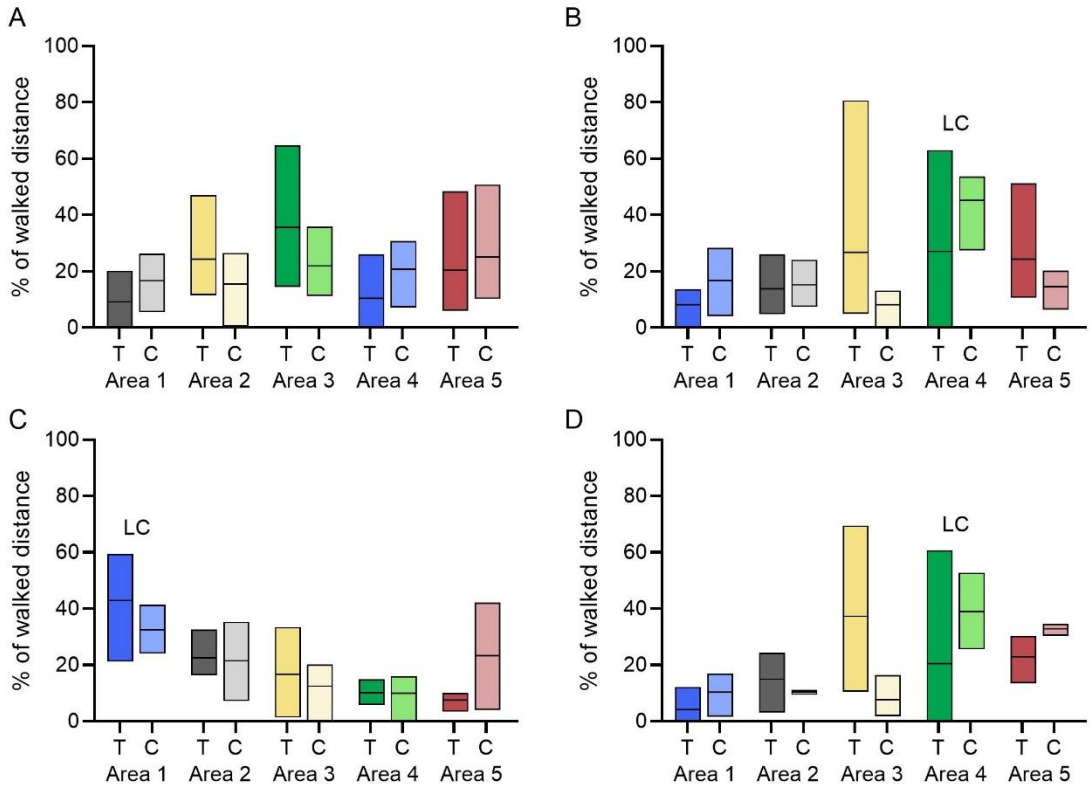


Figure 28: The percentage of the total length of walking trajectory in the areas for the 2 groups (treated and control) in the 4 test situations. The boxes show the percentage of the total length of walking trajectory of the test run per area and test group (T: Thiacloprid intake 800 ng, n = 6; C: control n = 5) from minimum to maximum with the mean as hyphen. Asterisks mark significance. The colors of the plots represent the colors of the panorama at that specific area. The position of the local cue is labeled (LC). The 5th (red) area depicts the border area. A: Test 1 without the local cue. B: Test 2 with different position of the panorama and local cue. C: Test 3 with coupled position of panorama and local cue. D: Test4 same as test 2 (here T: n = 4; C: n = 3).

To find out a direct effect, the mismatch test after the last training was analyzed and compared with test 2 on the test day. Here one can see a significant increase in the length of walking trajectory to the total in area 5 of the treated group ($p = 0,02$; paired t test). The control group showed no significant change (Figure 29 A+B). Comparing the performance of the two groups in the mismatch test after the last training no difference was found (Figure 29 C). Test 2 versus test 4 were not analyzed because of missing motivation in test 4 and therefor even less animals in test 4.

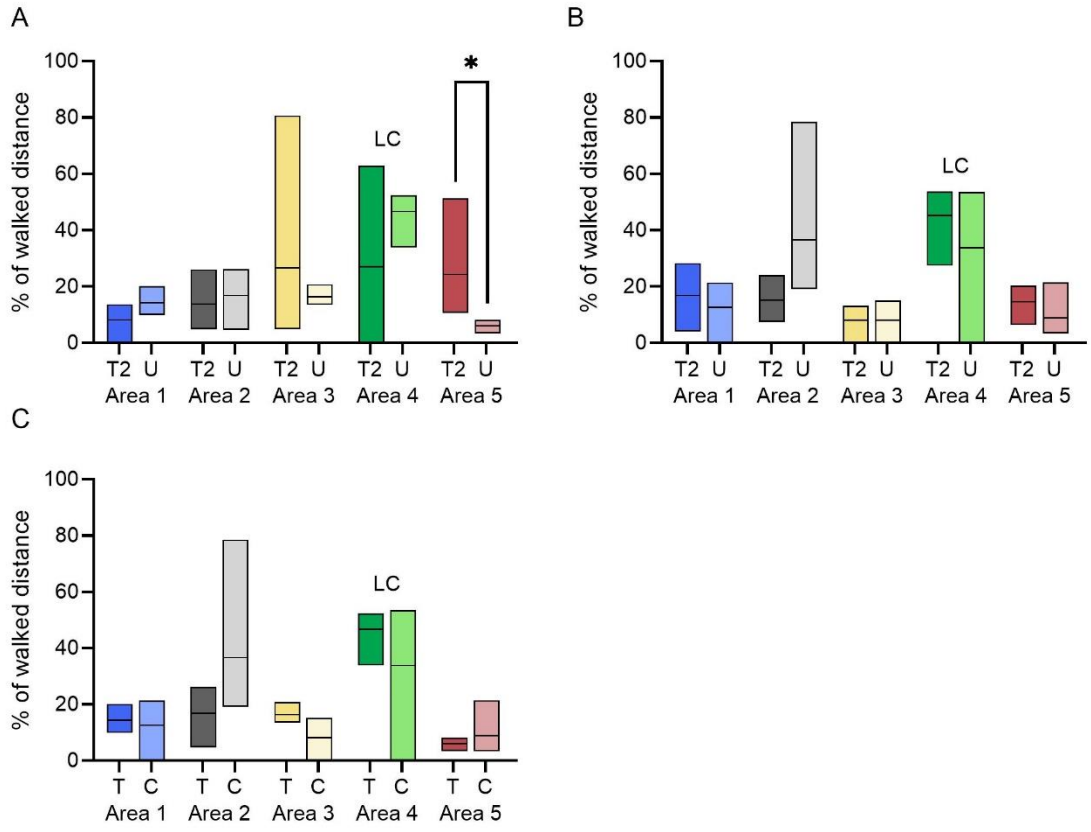


Figure 29: The percentage of the total length of walking trajectory in the areas for the 2 groups (treated and control). The boxes show the percentage of the total length of the walking trajectory of the test run per area and test group (T: Thiacloprid intake 800 ng, n = 6; C: control n = 5) from minimum to maximum with the mean as hyphen. Asterisks mark significance. The colors of the plots represent the colors of the panorama at that specific area. The position of the local cue is labeled (LC). The 5th (red) area depicts the border area. A: Comparing the data from test 2 with the mismatch test after the last training (U) of the treated group. Significance in area 5 (p = 0.02, paired t test) B: Comparing the data from test 2 with the mismatch test after the last training (U) of the control group. C: Comparing the mismatch test after the last training between the treated and control group.

When I analyzed the walking speed of the animals only test 1 showed a significant change in area 3 (p = 0,04; t test with Welch correction). In all other tests no changes were seen (Figure 30).

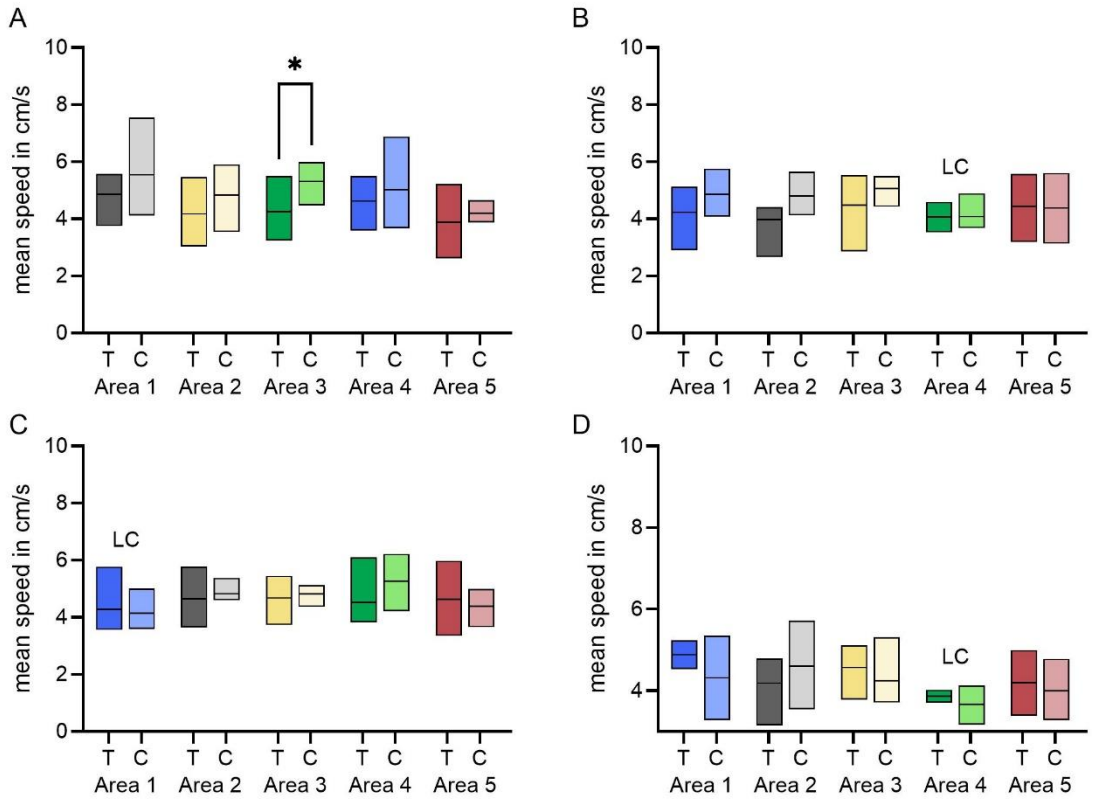


Figure 30: Mean walking speed of both groups in each test situation divided per area. The boxes show the mean speed of the test run per area and test group (T: Thiacloprid intake 800 ng, n = 6; C: control n = 5) from minimum to maximum with the mean as hyphen. Asterisks mark significance (t-test with Welch correction). The colors of the plots represent the colors of the panorama at that specific area. The position of the local cue is labeled (LC). The 5th (red) area depicts the border area. A: Test 1. Significance in area 3 (p = 0.04) B: Test 2, the mismatch test. C: Test 3, training situation. D: Test 4 same as test 2 (here T: n = 4; C: n = 3).

Again, test 1 of the treated and control group were also analyzed in comparison with the color preference group (t test with Welch correction). The test group showed no significant difference in either of these tests (Figure 31 A+C). The control group showed a significantly higher mean speed in area 3 (p = 0,01) but no difference in the relative distance (Figure 31 B+D).

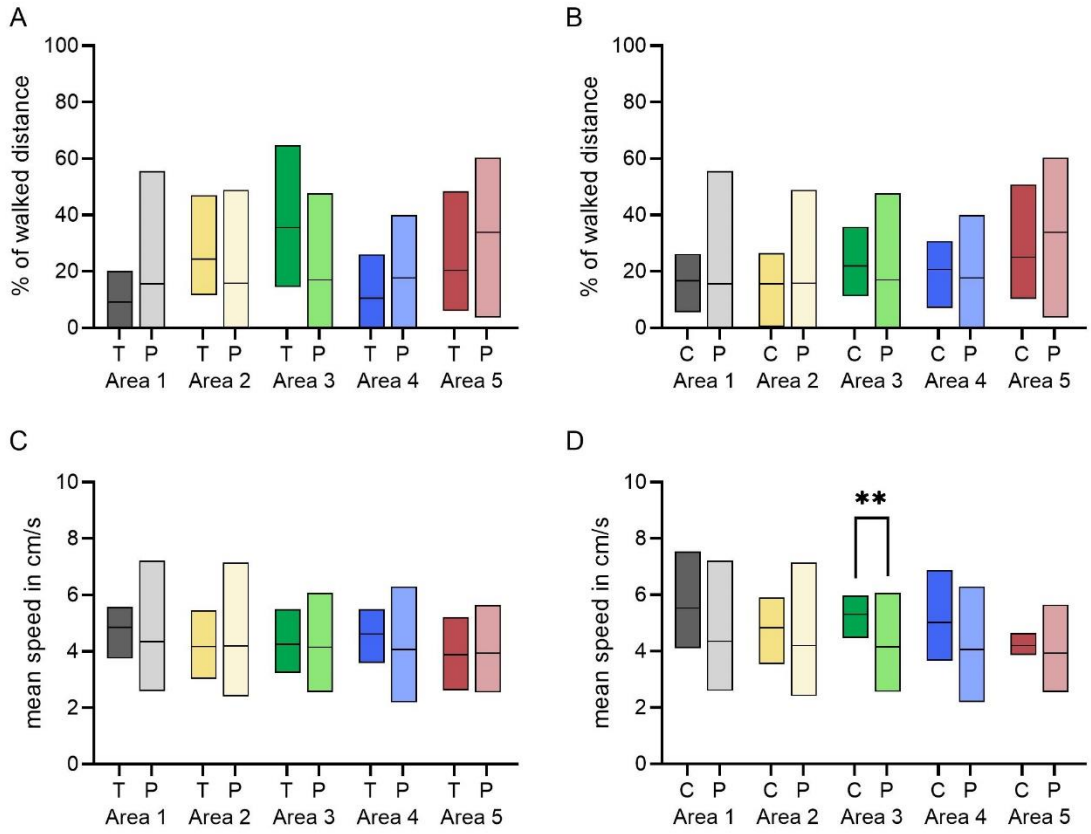


Figure 31 Comparison of the percentage of length of walking trajectory and mean speed of the treated group and control group (test 1) compared with the results of the color preference test. The boxes show the percentage of the total length of walking trajectory of the test run per area (A+B) and the mean speed per area (C+D) of the groups (T: Thiacloprid intake 800 ng, n = 6; C: control n = 5, P: color preference test, n = 6/5) from minimum to maximum with the mean as hyphen. Asterisks mark significance (t test with Welch correction). The colors of the plots represent the colors of the panorama at that specific area. The position of the local cue is labeled (LC). The 5th (red) area depicts the border area. A: no significance. B: no significance. C: no significance. D: significance in area 3 (p = 0,01).

5.1.4.3 400 ng versus 800 ng of Thiacloprid per animal

Since two Thiacloprid concentrations were tested one can ask whether there is a dose effect.

Firstly, I analyzed the overall length of walking trajectories per test and the straightness to get an overview. The total length of walking trajectory is not different between the groups, whereas the straightness differs in test 2 (p = 0,04; t test with Welch correction) (Figure 32).

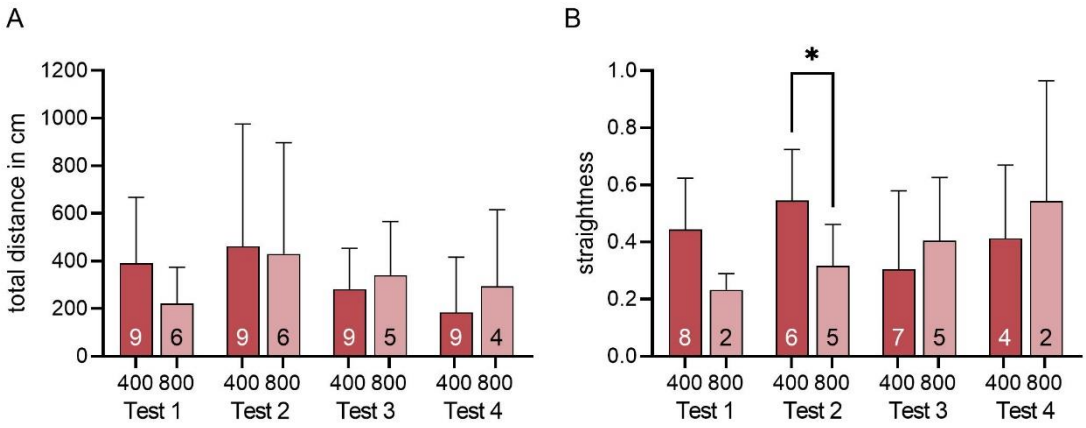


Figure 32: The total length of walking trajectory and straightness compared between the two treated groups. The plots represent the mean of the total length of walking trajectory and the straightness with the standard deviation for the tests of the 2 groups. The number of animals is marked in the bars. Asterisks mark significance. A: the total length of walking trajectory in the different tests. B: straightness of the two groups in the different test. Significance in test 2 (p = 0,04; t-test with Welch correction).

Next, for the first three test situations the percentage of the length of the walking trajectory was analyzed. The controls were checked against each other and the two treated groups (400 ng and 800 ng) with a t test with Welch correction. The controls differed only in test 1 in area 4 significantly, showing less relative length of trajectory of the control group recorded with the 800 ng tests (p = 0,003) (Figure 33 A-C). The treated groups did not differ in test 2 but in test 1 the animals that ingested 800 ng covered less percent of their total walking in area 4 (p = 0,01). In test 3 they differed in 2 areas. In area 1 the 400 ng test group made less way of their total then the 800 ng treated group (p = 0,05) whereas in area 5 their relative length of walking trajectory was significantly higher (p = 0,01) (Figure 33 D-F).

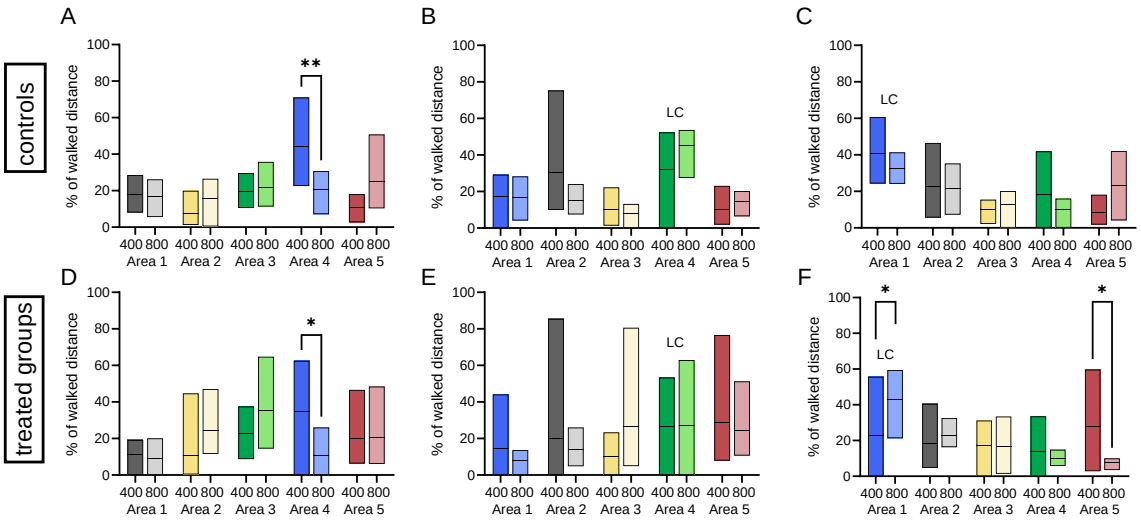


Figure 33: Comparison of percentage of the length of walking trajectory per area of the controls and the two treated groups. The boxes show the percentage of length of walking trajectory of the test run per area and group (upper row: controls of the two test situations, lower row: the two treated groups against each other; 400 ng: n = 9; 800 ng: n = 5 (control) and 6 (treated)) from minimum to maximum with the mean as hyphen. Asterisks mark significance (t-test with Welch correction). The colors of the plots represent the colors of the panorama at that specific area. The position of the local cue is labeled (LC). The 5th (red) area depicts the border area. A: Test 1 of the two control groups. Significance in area 4 (p = 0,003). B: Test 2 of the two control groups. C: Test 3 of the two control groups D: Test 1 of the two treated groups. Significance in area 4 (p = 0,01). E: Test 2 of the two treated groups. F: Test 3 of the two treated groups. Significance in areas 1 (p = 0,05) and area 5 (p = 0,01).

Additionally, the mean walking speed was analyzed in the same combinations with the same statistical tests. The controls showed a low statistical significance only in test 3 area 2 (p = 0,05) (Figure 34 A-C). The treated groups show no significant differences in either test situation (Figure 34 D-F).

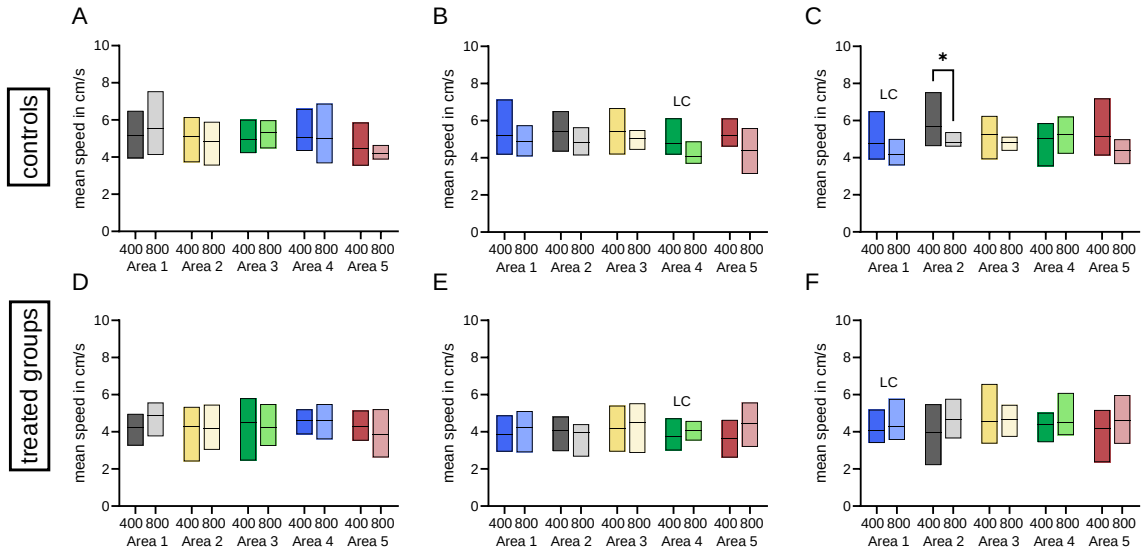


Figure 34: Comparison of mean walking speed per area of the controls and the two treated groups. The boxes show the mean walking speed of the test run per area and group (upper row: controls of the two test situations, lower row: the two treated groups against each other; 400 ng: n = 9; 800 ng: n = 5 (control) and 6 (treated)) from minimum to maximum with the mean as hyphen. Asterisks mark significance (t-test with Welch correction). The colors of the plots represent the colors of the panorama at that specific area. The position of the local cue is labeled (LC). The 5th (red) area depicts the border area. A: Test 1 of the two control groups. B: Test 2 of the two control groups. C: Test 3 of the two control groups. Significance in area 2 (p = 0,05) D: Test 1 of the two treated groups. E: Test 2 of the two treated groups. F: Test 3 of the two treated groups.

Although I knew which animals were exposed to the sucrose/Thiacloprid mixture, I had no control over how much the respective animal had taken up. I, therefore collected samples of three body parts from the treated animals for which I knew to how much concentration of Thiacloprid they were exposed to over which ingestion times. The analysis of the residue of Thiacloprid was performed by the Labor Friedle. An increase of the dose (concentration over uptake time) leads to an increase of dose in each body part measured 1,5 h after ingestion (Figure 35 A). The dose in the abdomen decreases over time between ingestion and measurement. The dose in the head and thorax show no clear tendency for the ingestion time (Figure 35 B). The dose dependence in the head is the relevant finding documenting that Thiacloprid reaches the head already after 1,5 hours, most likely predominantly the brain, judging from the volumes of tissue in the head.

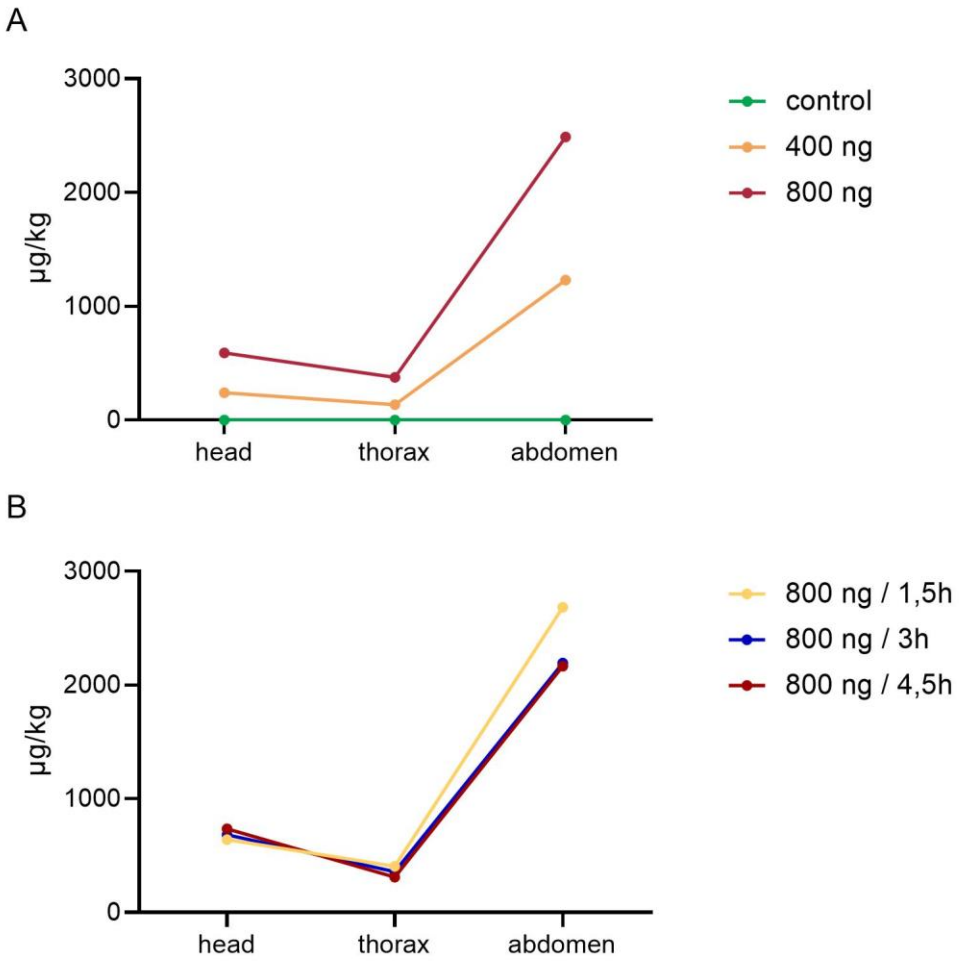


Figure 35: Analysis of the concentration of Thiacloprid in different body parts after the intake. A: The concentration (µg/kg) of Thiacloprid in the 3 body parts (head, thorax, abdomen) of control (no intake), 400 ng and 800 ng intake of Thiacloprid. Animals (n = 10 per group) frozen after 1,5 h of ingestion under red light. B: The concentration (µg/kg) of Thiacloprid in the 3 body parts (head, thorax, abdomen) of 800 ng intake of Thiacloprid. Animals (n = 10 per group) frozen after 1,5 h (yellow), 3 h (blue) and 4,5 h (red) of ingestion under red light.

5.2 The search for neural correlates of learned maze navigation

The central aim of my study was to establish a laboratory-based test procedure for a learned goal directed navigational task that includes two key components of navigation, panorama matching and goal localization with respect to the panorama. The data presented so far document that bumblebees are highly cooperative in learning this task in the context of their natural behavior to search for food when leaving the colony on a self-motivated level (hungry state). Learning through searching leads to an optimized performance in the sense that the association between target and panorama guides their walking trajectories in extinction tests. Given these ideal situations, the first step in the electrophysiological experiments was to test whether the dissections, necessary to expose the brain for inserting the recording electrodes, did not alter the behavior significantly.

They are 23 recorded animals from which 12 recordings (recordings of 12 animals) were sorted in Spike2 and overall, 86 units were filtered. Eight of the recordings were particular informative because of their recording duration and animal activity. Four of them were used for the behavior categorization. These animals showed the most interesting behavioral patterns. One of these recordings was later excluded, because it was only presented to one of the 2 test situations.

All animals performed well in all 3 consecutive training days before the test day. All of them showed goal directed behavior before and after the insertion of the electrodes. Since the recorded animals were not able to leave the arena and go back into their colony, their walking trajectory encompasses the whole recording time. The evaluation of straightness in the initial goal-directed walks shows no significant difference between the mismatch test, following the final training session, and the recorded mismatch situation. The distance traveled towards the goal exhibits no significant increase compared to the route length observed during the test conducted after the last training session, which took place one day prior to the recording session. This indicates that the dissection procedure did not alter their behavior (Figure 36).

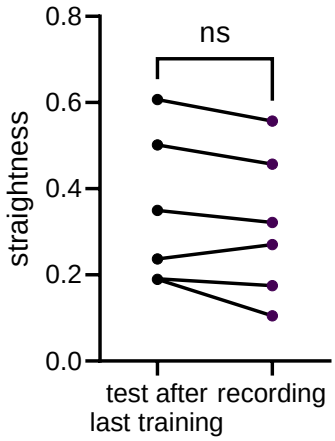


Figure 36 Test of the effect of the dissection procedure. Straightness of the animals in the test after the last training session compared to the values of the same animals while being recorded. No significant difference.

5.2.1 Evaluating the quality of the sorting

The quality check of the sorting was done (as mentioned before) with PCAs, PSTHs and a shape analysis.

To further ensure the quality of the sorting, correlation plots of each recording were analyzed. Correlation plots show the Pearson correlation coefficient as color code. It explains the relationship between the spike patterns of the units. If they show the same behavior at the same time, they are positively correlated. If their show an antagonistic effect at the same time, they are negatively correlated. If they show no common pattern at all, they are not correlated. In Figure 37 one can see all bumblebees after a rough sorting of all recordings. Clear differences between the units can be seen. In some recordings (e.g.: rU, g2, rL) one can even see a positive and negative correlation of particular units (bumblebee rU with unit 5, bumblebee g2 with unit 2, bumblebee rL with unit 6).

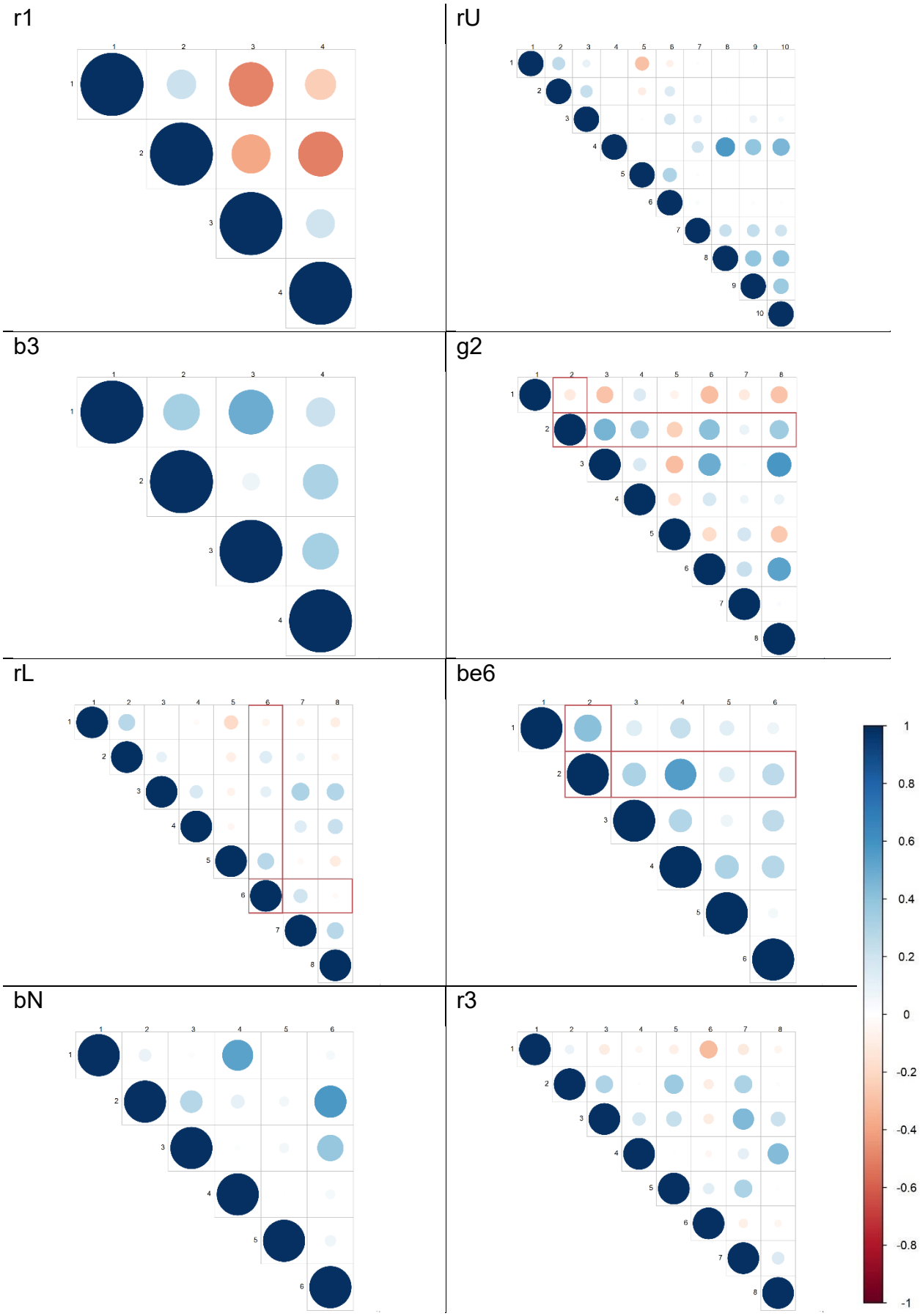


Figure 37 Corplots of units of the 8 animals. Correlation plots per animal of their units. Positive correlation shown in blue and negative correlation in red. Marked units in red rectangles are the units used for further analysis.

5.2.2 The testing situations

The bumblebees were trained over 3 days in the same setup, which was used for the recording. In the training, the panorama rotated randomly with the blue local cue always being in front of the blue panorama (match). The sequence of the panorama colors stayed the same. Each bumblebee was trained to this situation, so each bumblebee experienced the local cue with the panorama coupled on all 4 possible positions at least once. This experimental design should uncouple any tendency to learn a relationship between an angular run after leaving the gateway from the colony to the arena and emphasize the spatial relationship between the local cue and the panorama.

The recorded bumblebees were confronted with two different situations (Figure 38). The match situation displayed the learned situation: the blue local cue in front of the same-colored panorama in area 1. The mismatch situation displayed the same lineup of the panorama background like in the match situation, but the local cue was shifted to area 4 to uncouple the learned goals from each other.

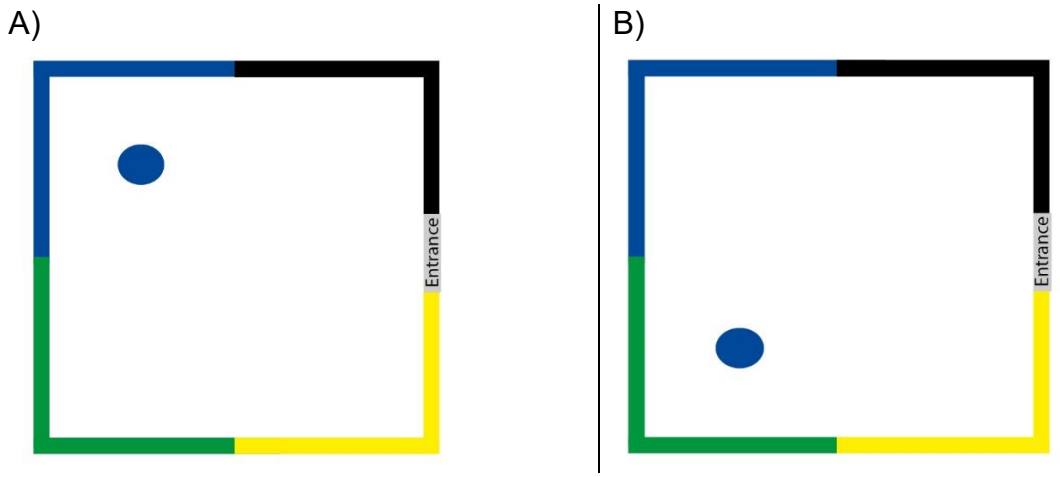


Figure 38 Different test setups. A: Match (panorama and local cue are located at the same position, like training). B: Mismatch (panorama and local cue are located at different positions)

Also, for different analysis steps the arena was divided into 5 areas which were occupying the same area size (Figure 39).

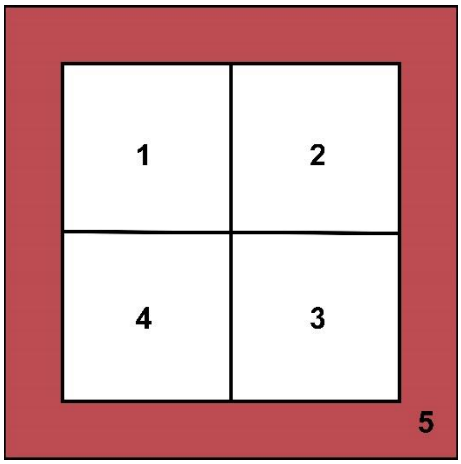
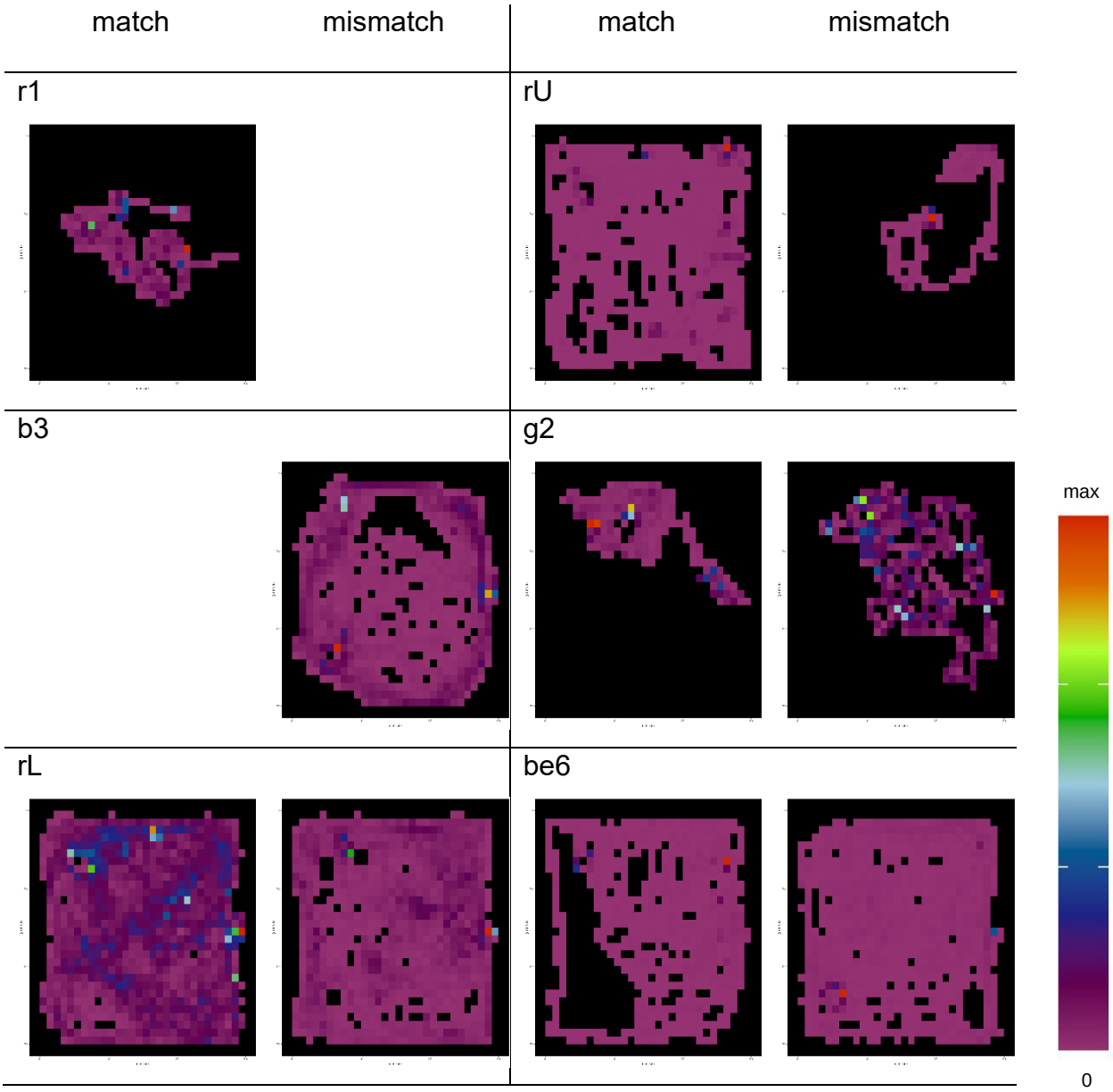


Figure 39 Division in areas.

The heatmaps of the pure tracking data of the bumblebees show a rather evenly distributed pattern of search throughout the arena (Figure 40). Single points with higher frame count show places of rest.

For further analysis, the most interesting bumblebees were sorted (criteria see Material and Methods).



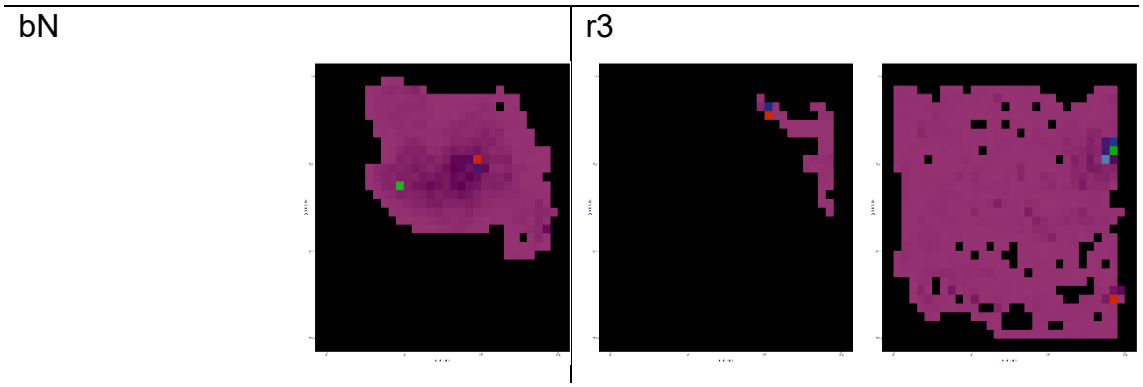


Figure 40 Heatmap of the trajectory of the 8 different bumblebees. The heatmaps display the density of the video fixes and show possible hot spots (locations with more visits than others). The color code bar to the right shows the count of frames and depends on the length of the recording. Overall, the 8 animals explored the arena rather uniformly.

5.2.3 Search for fundamental connections

5.2.3.1 Walking speed

The first step in the search for correlations between neural activity and behavior was to ask, whether walking speed drives neural activity irrespective of where the animal is in the arena. This is an important issue because of two arguments, units may be contaminated or completely determined by muscle activity, units may simply code a premotor output of the mushroom bodies. Figure 41 shows the correlation analysis for the animals g2, rL, be6 with binned walking speeds in the 5 areas. Walking speed 0 marks sitting animals. No consistent correlation is found but a tendency can be seen with low spike rates when the animal is sitting. Similar results were found for the other 5 animals.

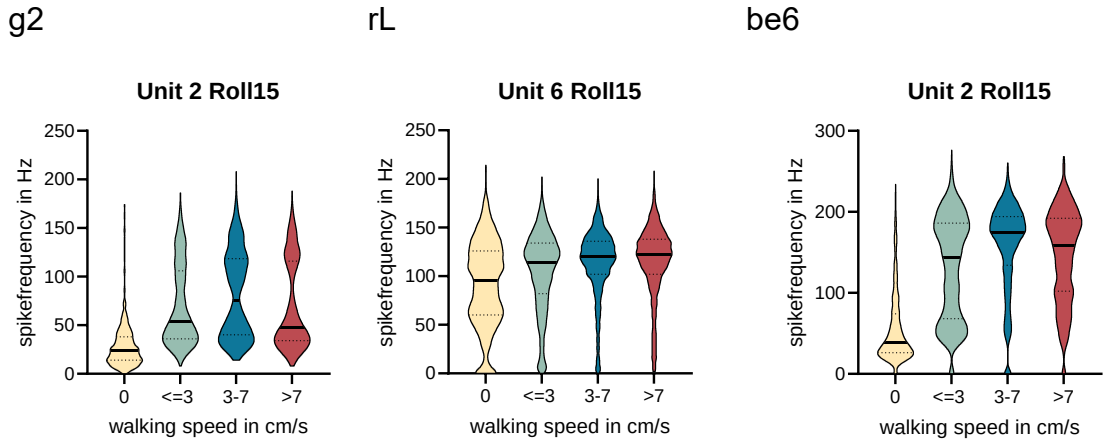


Figure 41 Violin plots of binned speed against spike frequency. The speed is binned: yellow: no walking; green: under 3 m/s; blue: 3-7 m/s; red: above 7 m/s. The spike frequency on the y axis depends on the activity of the specific unit.

For each animal and experimental unit, a set of images was generated, in order to assess the correlation between the behavioral data and the occurrence of local maxima (positive peak) or minima (negative peak) in the spike rate. A local maximum (or local minimum) is defined as the highest (lowest) peak in a distinct series of frames. The behavioral criteria were: angle towards the goal, distance to the goal, angle towards the entrance, distance to the entrance, and walking speed. A meticulous manual examination and analysis of over 84.000 images were conducted, but no pattern was revealed. Figure 42 presents illustrative examples of unit 6 in animal rL. The subfigures depict the central representation of local maxima for the unit, indicated by a red vertical line. The angle to the goal is represented in green, the distance to the goal in blue, and the walking speed is displayed in beige.

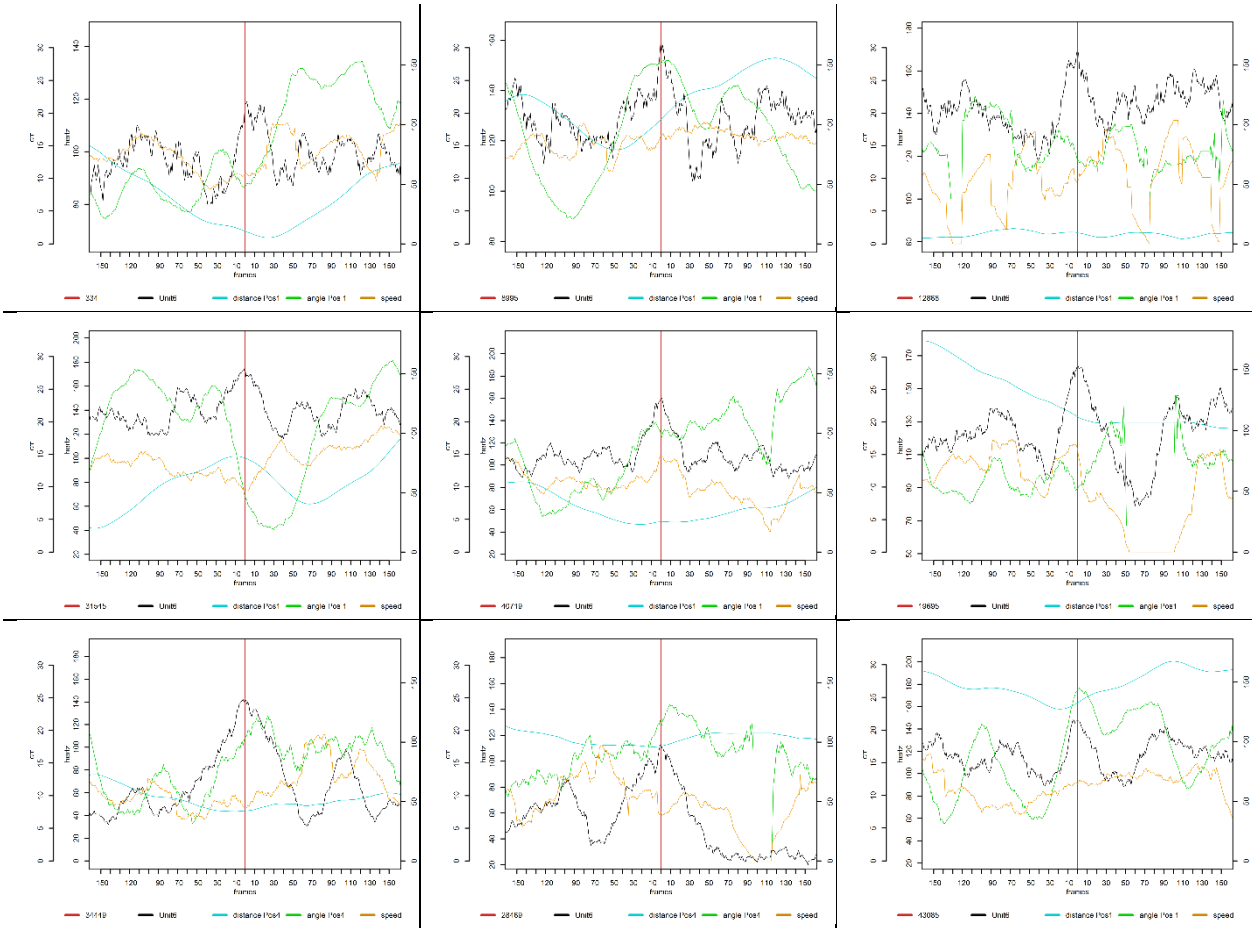


Figure 42 Temporal relationship between local maxima of the spike rate and behavioral parameter of unit 6 of bumblebee rL in the matched situation. Displayed are the local maxima of the spike rate in black in the middle of each subfigure with 5 seconds before and after. The event is marked with the red vertical line. The angle in which the animal is walking towards the position 1 (panorama and local cue located here) is depicted in green, the distance to position 1 in light blue and the walking speed of the animal in beige.

5.2.4 Single Unit analysis

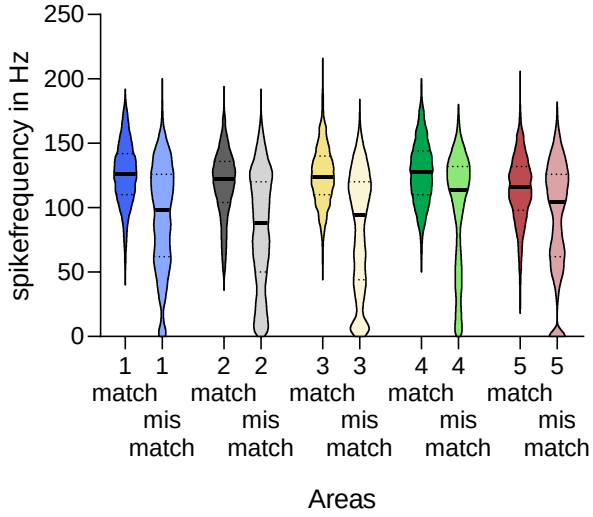
5.2.4.1 Unit 6 in bumblebee rL

The bumblebee rL used the whole arena in test conditions (match and mismatch) (Figure 40). Several quite active units were recorded. First to be analyzed will be unit 6.

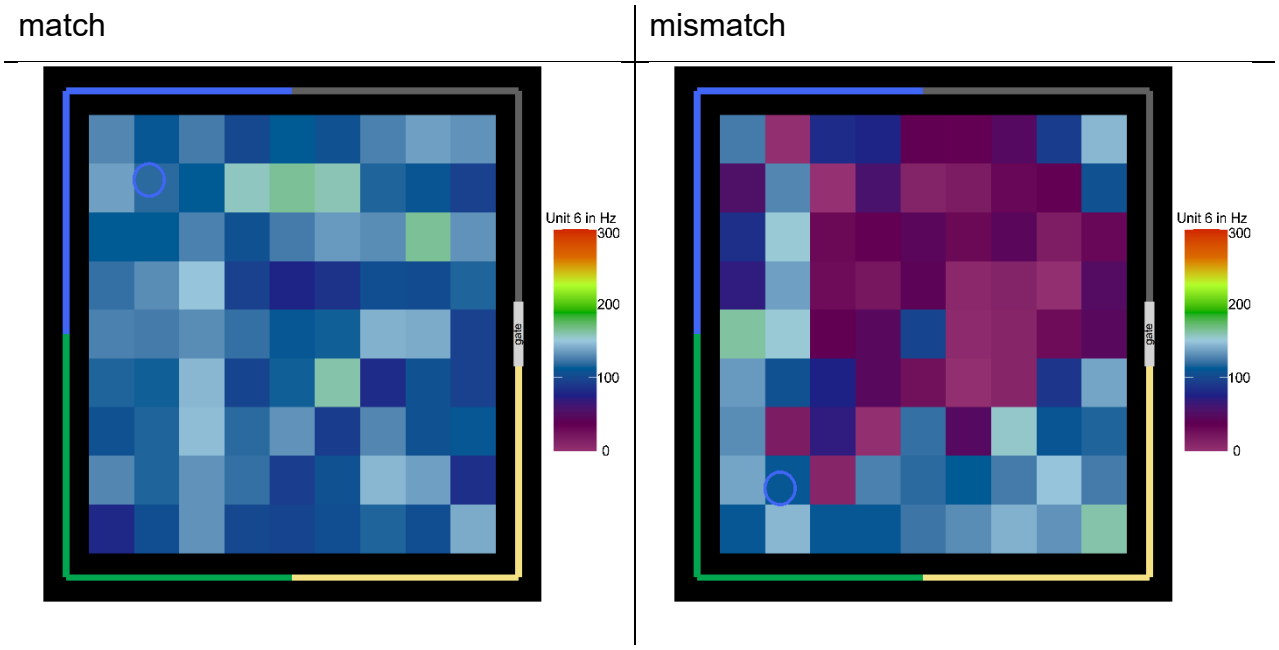
Most interesting significantly lower spikes were found for the mismatch situation in all areas ($p < 0{,}001$, Wilcoxon test for each area) (Figure 43 a) indicating that the unit´s activity correlates with a deviation from the learned situation possibly coding a discrepancy of the expected conditions in the arena. This result is particularly relevant since it can be excluded that the animal explored different areas differently (Figure 43 b).

The animal's trajectory covers all areas in both test situations. In the match situation the spike rate shows no difference between any part of the arena making it unlikely that the neuron´s activity is related to a specific selection of the current localization of the local cue and the panorama. It needs to be remembered that different areas housed the local cue together with the panorama during training, and thus the match/mismatch effect can well be a property of the whole arena. Further, the two different spatial resolutions of the spike rate analysis indicates that there is no hidden fine structure of spike rate distribution, and the effects appears to be fully captured by the overall analysis across areas.

a)


spikefrequency in Hz
250
200
150
100
50
0
1 1 2 2 3 3 4 4 5 5
match mis match match mis match match mis match match mis match match mis match
Areas

b)


match
mismatch
Unit 6 in Hz
300
200
100
0
gate
Unit 6 in Hz
300
200
100
0
gate

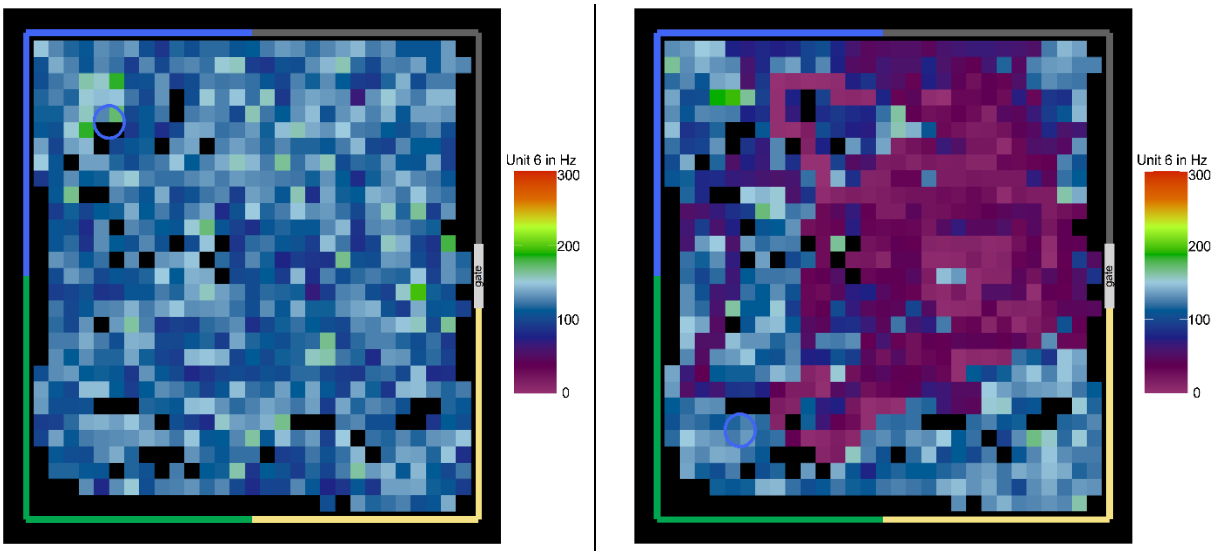


Figure 43 Distribution of spike rates of unit 6 in animal rL. The distribution of the firing rate in Hz. a) The spike frequency in Hz in the different areas in the matched/mismatched situation. The color coding of the bars displays the color of the panorama at the different areas. In the mismatched situation the blue local cue was located in area 4. In the match situation the blue local cue was located in area 1. Area 5 is the border area. b) heatmaps of the spike rate in Hz in the matched (left panel) and the mismatched (right panel) situation, with two different spatial resolutions. The colored rectangles around the arena display the panorama colors. The light blue circle indicates the position of the local cue. The light grey rectangle in the mid right shows the entrance gate.

The evaluation of the correlation between spike rate and distances to the goal (the local cue together with the panorama in the match situation and the separate locations of local cue and panorama in the mismatch situation) shows a rather uniformly distribution of spike frequencies in the matched situation, whereas in the mismatched situation there is a significant drop of frequencies ($p < 0{,}001$, Wilcoxon test) at a distance between 10 cm and 20 cm (distance to panorama; distance to local cue even up to 30 cm). (Figure 44). This result corroborates the data shown in Figure 43 b supporting the conclusion that the unit 6 appears not to be involved in a localization of the two aspects of the goal, local cue and panorama.

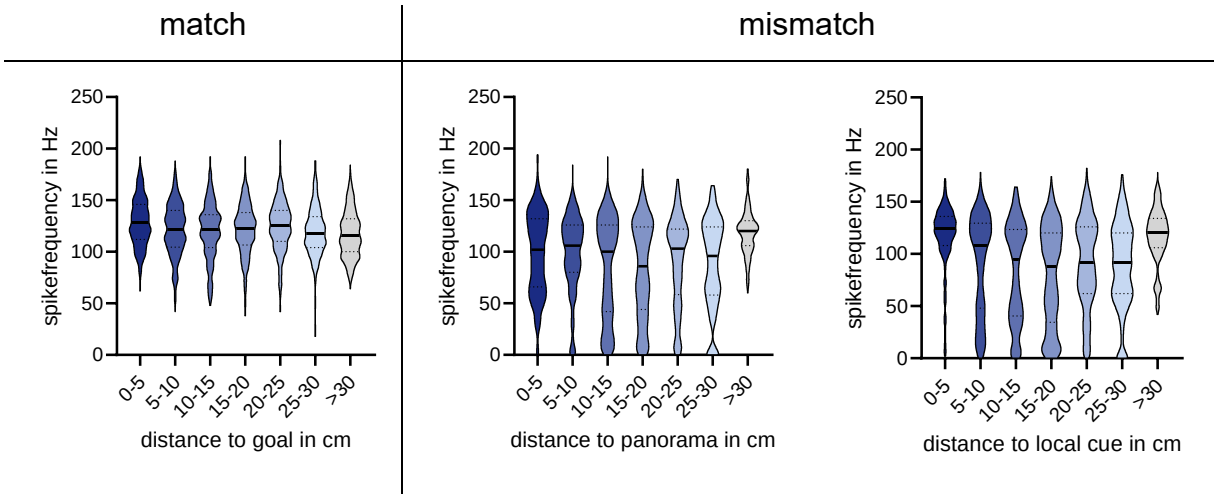


Figure 44 Spike frequency at different distances to the goal in matched/mismatched situation of unit 6 in animal rL. Abscissa: Distances in cm to the goal (match: local cue in front of panorama; mismatch: panorama vs. local cue). Ordinate: the spike activity in Hz. Violin plots are shown, with their mean indicated as line.

For the analysis of the spike rate in different behavioral patterns, unit 6 will be compared to the simultaneously recorded unit 1. Thus, one can address the question if a different unit may behave differently during the same tests. I address this question in an exemplatory way for the different behavioral states.

I investigated the question whether the unit´s spike rate differences between match and mismatch requires the active walking of the animal and found that indeed the spike rate is lowest when the animal does not walk. For this analysis, I pooled all locations of sitting in both, the match and mismatch situation and compared it with walking around either in all 4 areas pooled or in area 5 (the border area). Interestingly, highest spike rates of unit 6 are found in area 5, an effect hard to reconcile with the result of the analyses so far (Figure 45). Unit 1 shows no significant differences comparing the 3 different states.

Since the border of the arena is the least goal directed area, I next analyzed repeated walks towards the goal in the matched situation (unit 6: Figure 46; unit 1: Figure 47). For unit 6, consecutive walks towards the goal and panorama in the match situation led initially to a stepwise increase of spike rate and then to a plateau. One may argue that detecting the local cue and the panorama in the area 1 although the combined location of both was experienced in any of the 4 areas during training led to some learning via exploration rather than reward (since it was an extinction test). In such a case one would expect that consecutive walks to the separated goals in the mismatch situation may not lead to stepwise increase (Figure 48 a). This is indeed the case. An

extremely slight increase can be seen but it is not significant. Therefore, one can safely conclude that unit 6 is involved in coding the learned local cue/panorama situation in the arena.

The consecutive walks towards the goal in the matched situation led to no change in spike rate in unit 1. The same appears for the goal directed walks in the mismatch situation. There is a slight tendence in Unit 1 to decrease spike rates over repeated goal walks, but it is not significantly (Figure 48 b).

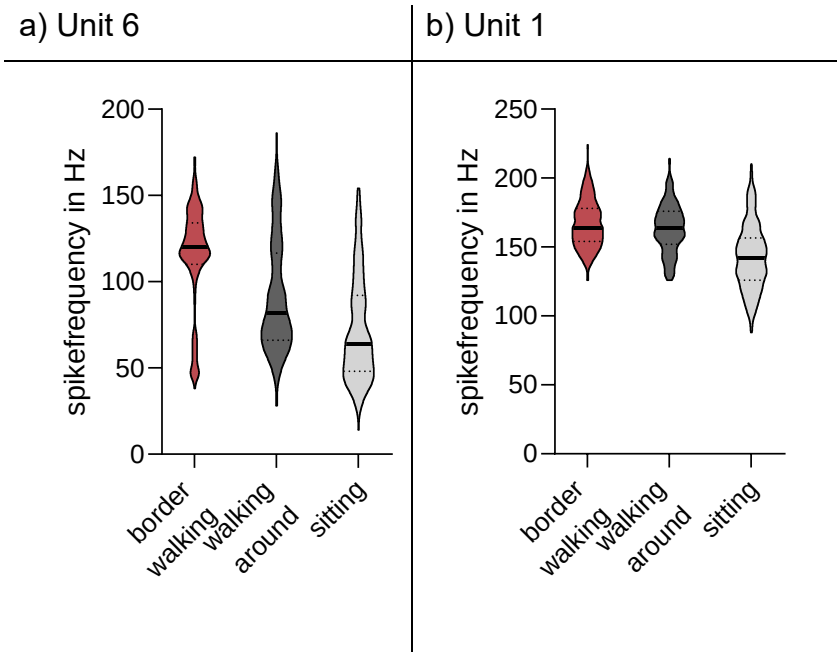


Figure 45 Spike frequency at two different states of walking of unit 6 and unit 1 in animal rL. Displayed in violin plots is the distribution of spike frequency in Hz against the different behavior states. The mean is indicated as bold line. a) Unit 6: Significance each against each other $p < 0{,}001$ (Wilcoxon test) b) Unit 1

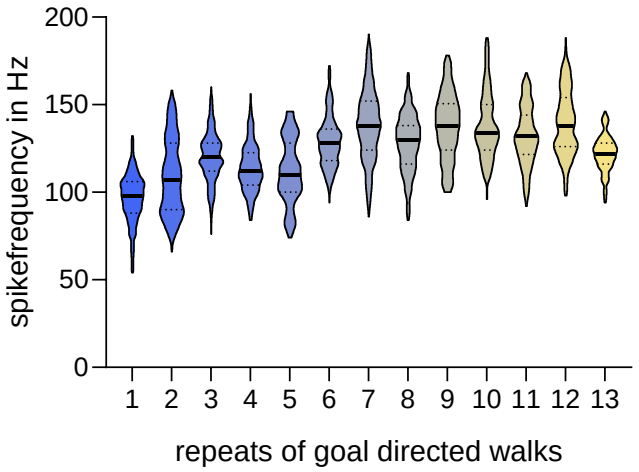


Figure 46 Repeated walks to the goal in matched setup and their respective occurrence of spike frequencies of unit 6 in animal rL. In violin plots displayed, is the distribution of spike frequency in Hz with the mean indicated as bold line. The x axis is the number of repeated walks to the goal.

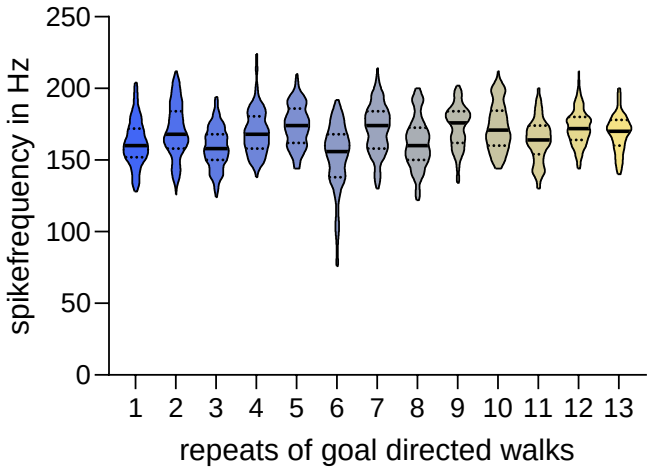


Figure 47 Repeated walks to the goal in matched setup and their respective occurrence of spike frequencies of unit 1 in animal rL. In violin plots displayed, is the distribution of spike frequency in Hz with the mean indicated as bold line. The x axis is the number of repeated walks to the goal.

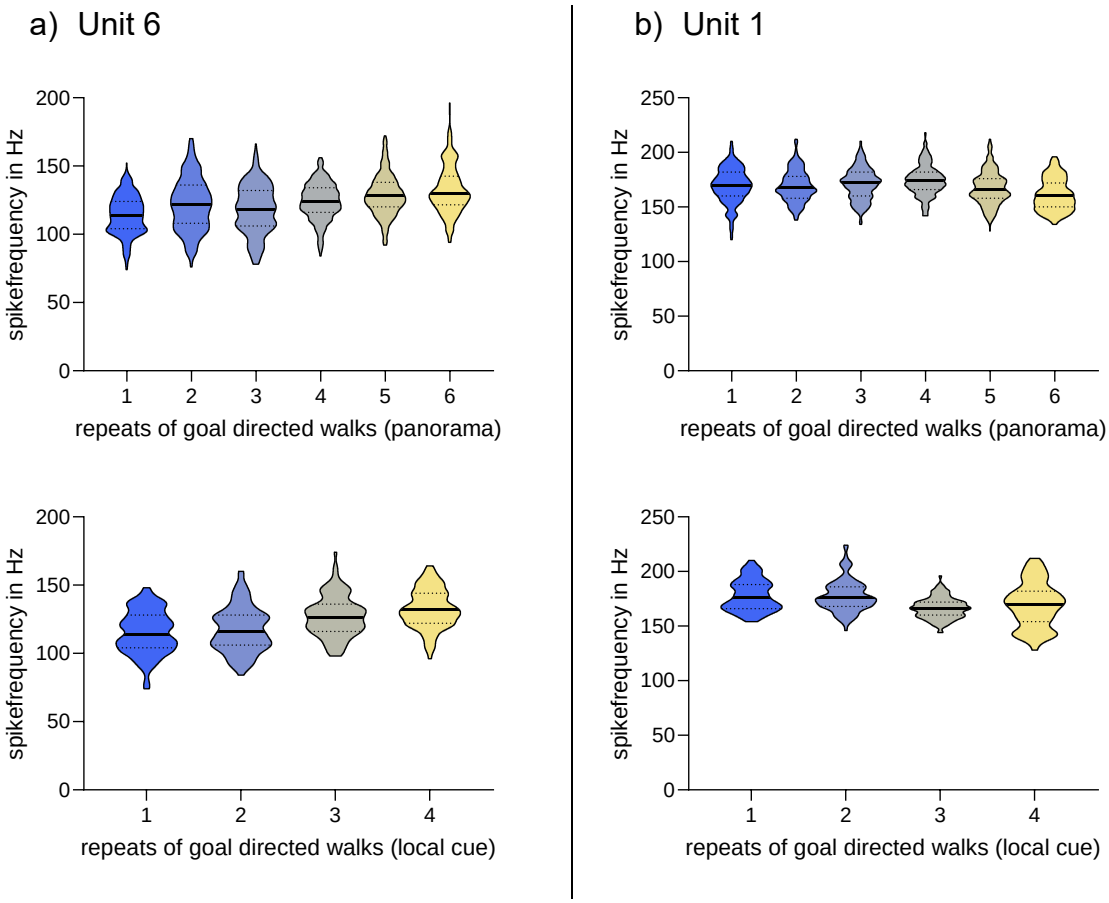


Figure 48 Comparison of spike rate distribution in repeats of goal directed walks to the local cue and panorama of unit 6 and unit 1 of animal rL. Displayed in violin plots is the distribution of spike frequency in Hz with the mean indicated as bold line. The x axis is the number of repeated goal directed walks to the panorama (above graphs) and the local cue (lower graphs) from a) unit 6 and b) unit 1 of animal rL in comparison.

The mismatch test provides us with the opportunity to study a component of the search behavior that might uncover the retrieval of memory related to the local cue and the panorama. Animals perform repeated walks between the two goals, the area which houses the local cue and the area that is attached to the panorama. I analyzed spike rates for these trajectories for both unit 6 and unit 1 (Figure 49 a and b). No salient effect is seen, though it is interesting that the 11th repetition of the walk from the local cue to the panorama led to a significant drop of spike rate in unit 6 but only a tendency of lower spike rate in unit 1. Although, these findings on unit 6 and unit 1 appear to be rather unspectacular, I want to stress again the unique conditions. The repeated spike rate measurements for the same behavioral component (repeated goal directed walks, Figure 46 -Figure **49**) document the stability of the recordings and render small deviations relevant.

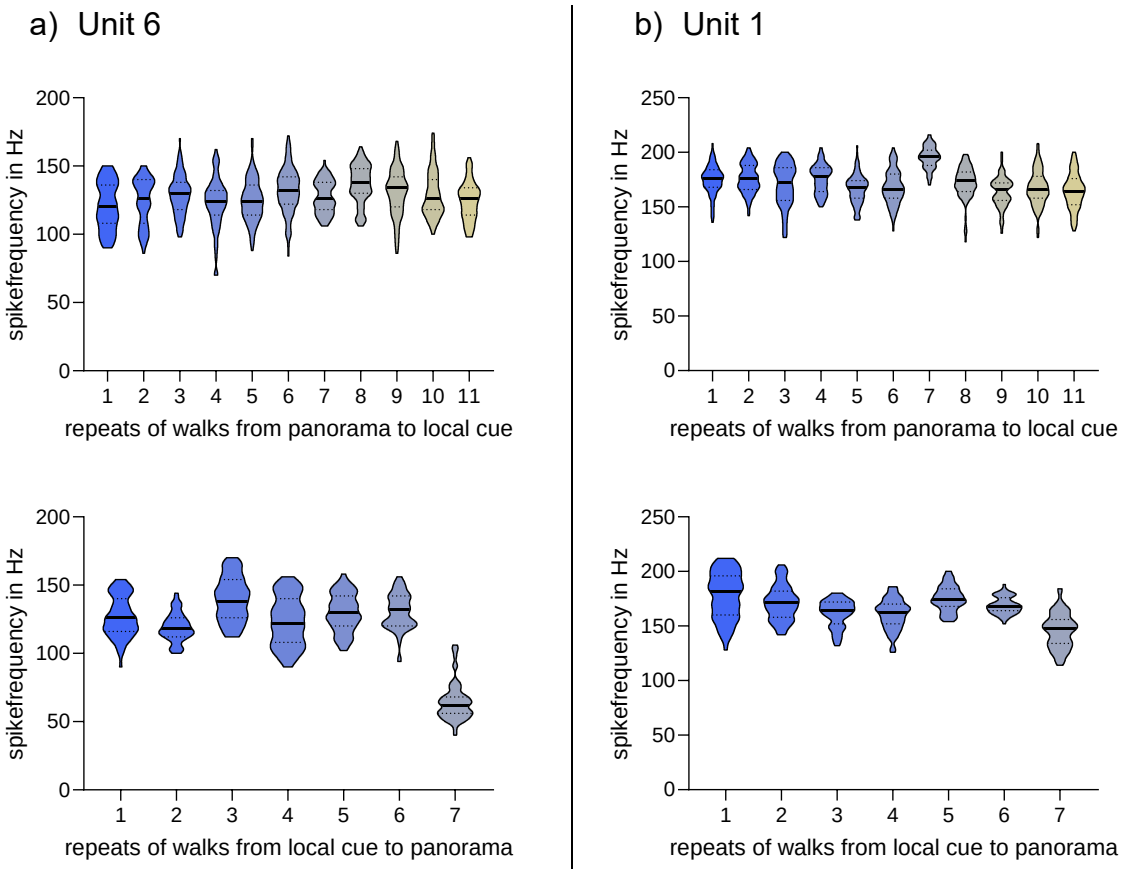


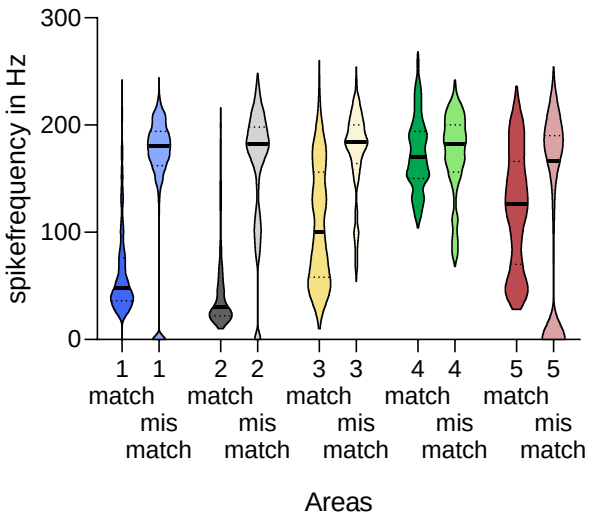
Figure 49 Comparison of spike rate distribution in repeats of walks between the goals (panorama to the local cue and local cue to the panorama) during the mismatch test of unit 6 and unit 1 of animal rL. Displayed in violin plots is the distribution of spike frequency in Hz with the mean indicated as bold line. The x axis is the number of repeated walks from the panorama to the local cue (above graphs) and from the local cue to the panorama (lower graphs) from a) unit 6 and b) unit 1 of animal rL in comparison.

5.2.4.2 Unit 2 in bumblebee be6

The bumblebee be6 used the arena quite uniformly in both test conditions. Several active units were recorded. The firing rate of the unit 2 was used for further analysis. For comparing different behavior patterns, additionally unit 1 and unit 5 of this animal were included.

The unit shows an overall higher spike frequency in the mismatch situation ($p < 0{,}001$, Wilcoxon test for each area; except area 4). In the matched situation the spike frequency is highest in area 4 and in the border area (Figure 50 a). The walking trajectory of the animal covered all areas. However, area 4 was not fully visited. In the mismatch situation the spike rate distribution shows no specific pattern according to a spatial place. In the match situation the lowest spike rates can be found in the middle of the arena and right at the located local cue/panorama combination (Figure 50 b). Thus, this unit appears to code the trained location in the matched situation by reduced spike rates and contrasts in a characteristic way unit 6 of animal rL. Figure 50 b for the mismatch test indicates clearly, that this reduced spike rate accounts for the local cue and not for the panorama. However, this conclusion is weakened by the fact that the animal did not explore area 4 equally well as the other areas.

a)



b)

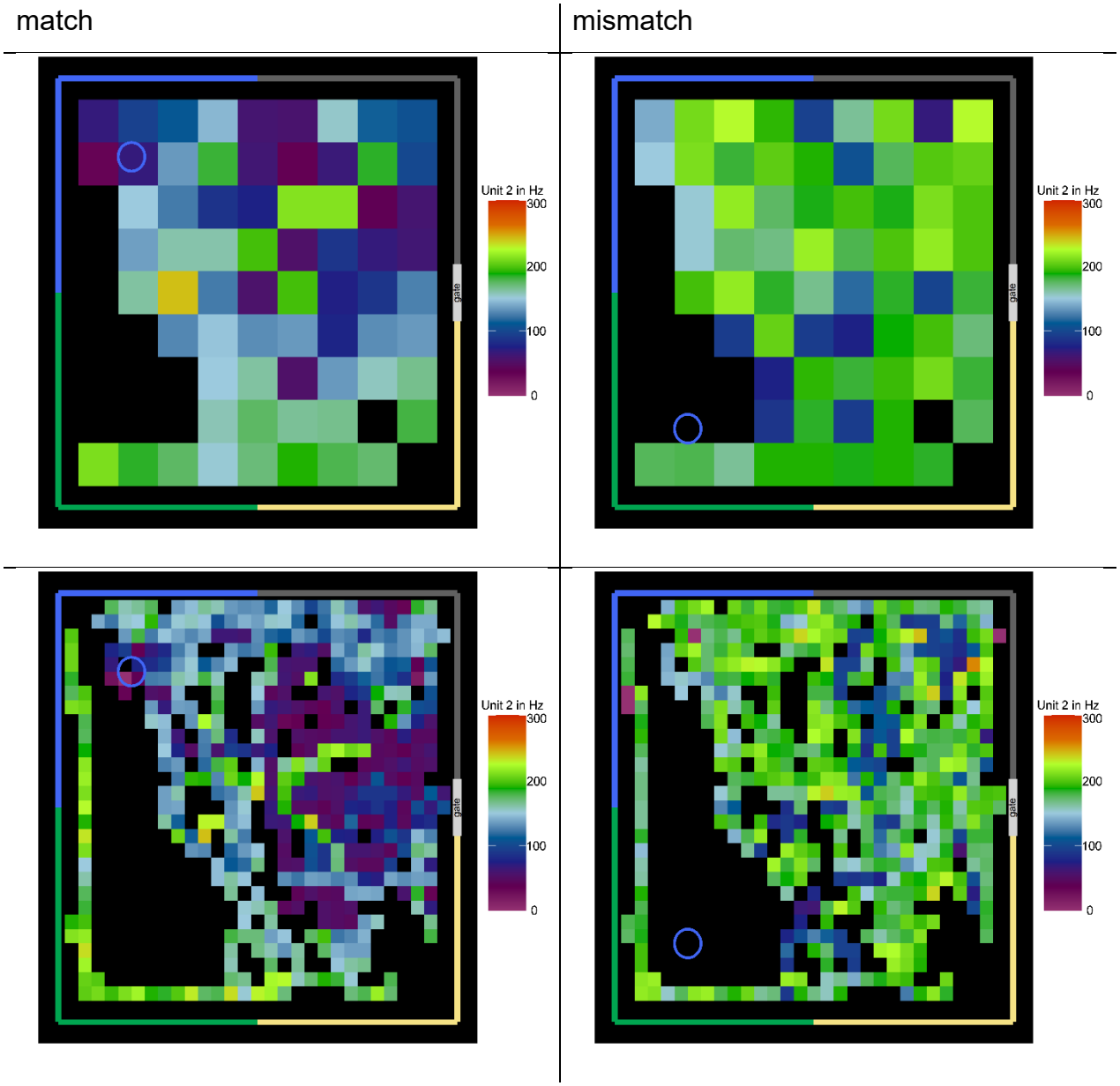


Figure 50 Distribution of spike rates of unit 2 in animal be6. The distribution of the firing rate in Hz. a) The spike frequency in Hz in the different areas in the matched/mismatched condition. The color coding of the bars displays the color of the panorama at the different areas. In the mismatched condition the blue local cue was located in area 4. In the match condition the blue local cue was located in area 1. Area 5 is the border area. b) heatmaps of the spike rate in Hz in the matched (left panel) and the mismatched (right panel) situation in different resolutions. The colored rectangles around the arena display the panorama colors. The light blue circle indicates the position of the local cue. The light grey rectangle in the mid right shows the entrance gate.

In the analysis of distances for the match condition, the spike rate was low, close and further away from the local cue and significantly higher for middle distances ($p < 0{,}001$, Wilcoxon test). The high spike frequency at the furthest distance resulted most likely from the low spike rate in area 5 (see Figure 50 a). In the mismatched condition, being

close but not near to the local cue (5 - 10 cm) also resulted in a drop of spike frequencies ($p < 0{,}001$, Wilcoxon test).

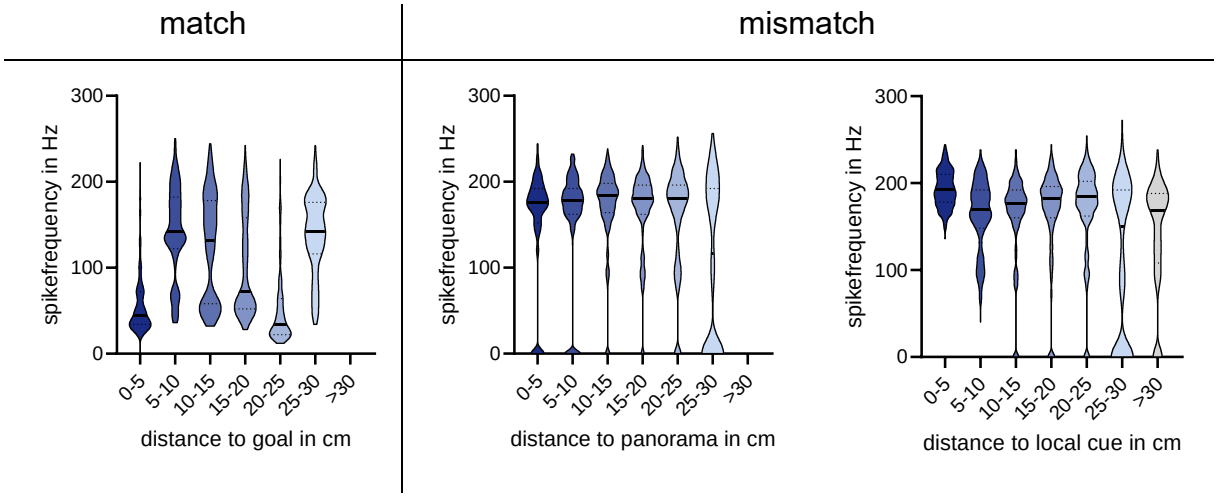


Figure 51 Spike frequency at different distances to the goal in matched/mismatched setup of unit 2 in animal be6. Distances in cm to the goal (match: local cue in front of panorama; mismatch: panorama vs. local cue) on the x axis. The y axis displays the spike activity in Hz. Violin plots are shown, with their mean indicated as line.

In the mismatch situation the animal is confronted with two potential goals, the location of the local cue (at the wrong panorama) and the correct panorama (now without the local cue). Therefore, one can ask whether unit 2 in animal be6 codes these two walking directions differently, when it moves from one to the other partly matching goal. Figure 52 shows a significant increase of firing rate of unit 2 when walking away from the local cue rather than to the local cue, supporting the interpretation of the data above, that the spike rate reduction is specific for the local cue.

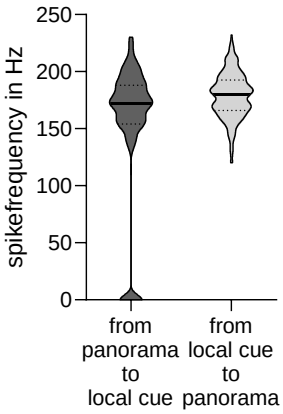


Figure 52 Spike rate of unit 2 of animal be6 walking between local cue and panorama in the mismatch situation. The spike rate is significantly lower when the animal walks from the panorama to the local cue (p-value < 0,001; Wilcoxon test). Displayed in violin plots is the distribution of spike frequency in Hz against the two different states (dark gray: walking from panorama to local cue; light gray: walking from local cue to panorama).

Animal be6 performed repetitive walks between the two goals in the mismatch situation. Fortunately, it was possible to separate 3 units in this animal (unit 2, unit 1 and unit 5) that were analyzed for the spike rates during repetitive walks between the two goals (Figure 53). The patterns of spike rates arising from these walks are complex with little similarities for the repetition effect and the directional effect (away from the local cue or towards the local cue). For example, there is a drop in the spike rate at the 6th attempt walking from the panorama to the local cue in unit 1. This drop is less in unit 2 than in unit 5 and opposite in unit 2 (from local cue to panorama). Overall, it appears that units 2 and 1 are more similar and unit 5 is more different from these two units. Taken together the units code the directionality of walks between the two goals in a combinatorial way.

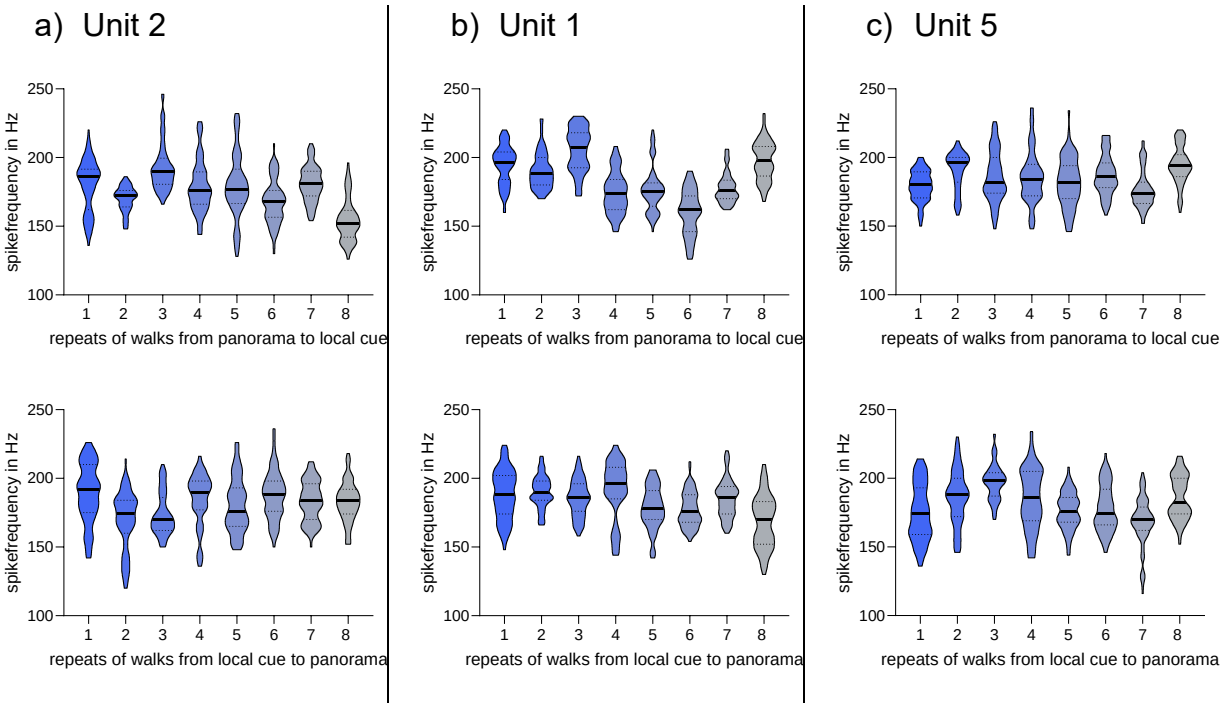


Figure 53 Comparison of spike rate distribution in repeats of walks between the goals (panorama to the local cue and local cue to the panorama) of unit 2, unit 1 and unit 5 of animal be6. Displayed in violin plots is the distribution of spike frequency in Hz with the mean indicated as bold line. The x axis is the number of repeated walks from the panorama to the local cue (above graphs) and from the local cue to the panorama (lower graphs) from a) unit 2, b) unit 1 and c) unit 5 of animal be6 in comparison.

This animal was active in almost the whole arena and performed 3 clear repetitions of goal directed walks towards the local cue from the entrance gate in the mismatched situations. A distinct differentiation of the units is revealed (Figure 54). Unit 2 exhibits a rise in spike frequency with each repetitive walk to the goal while unit 5 shows an

opposite pattern and unit 1 shows no change. The spike frequency in unit 1 remains consistent, displaying no tendency. This result is particularly interesting for unit 2 on the background of the results in Figure 50. Being within the area of the goal led to a reduced spike rate (Figure 50) but walking towards the goal to an increase over repeated walks. This finding is a hint for complex response patterns of these units that are not well captured by the behavioral paradigm applied in this study.

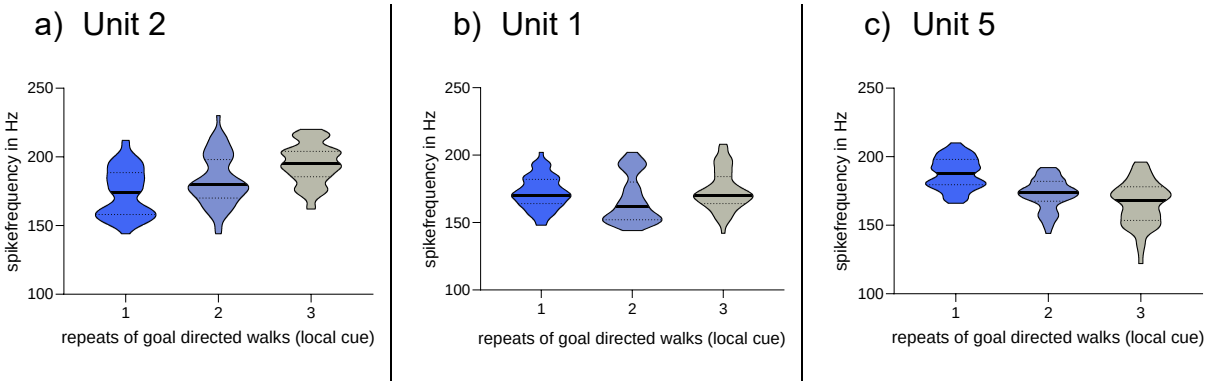


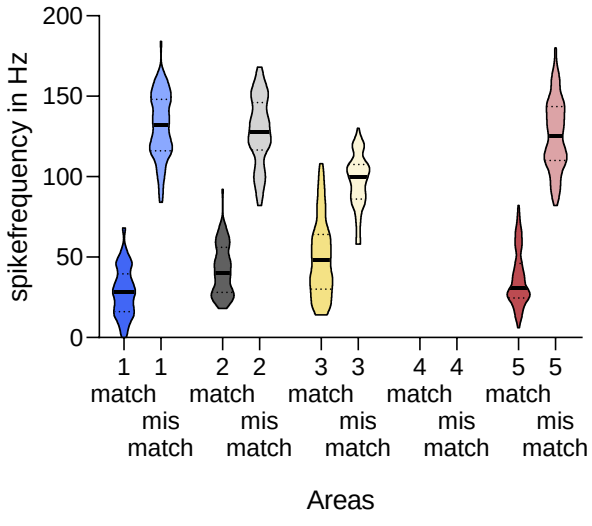
Figure 54 Comparison of spike rate distribution in repeats of goal directed walks towards the local cue of unit 2, unit 1 and unit 5 of animal be6. Displayed in violin plots is the distribution of spike frequency in Hz with the mean indicated as bold line. The x axis is the number of repeated walks from the entrance to the local cue in a) unit 2, b) unit 1 and c) unit 5 of animal be6 in comparison.

5.2.4.3 Unit 2 in bumblebee g2

Bumblebee g2 did not explore the whole arena evenly and explored only a similar part of the arena in the matched and mismatched situation (Figure 55 b). The trajectory of this animal is limited because of a shorter exploratory phase in both test condition then in the other animals. However, the spike rate distributions showed an interesting pattern (Figure 55 a, b)

Similar to unit 2 of animal bee6 (Figure 50 a, b), the spike rate unit 2 of animal g2 is significantly higher in all visited areas during the mismatch situation (area 4 was not visited) (respective areas against each other: p-value < 0,001, Wilcoxon test) (Figure 55 a). In the matched situation, the spike rate difference is highest for area 1, the area that contained both local cue and panorama. Here the spike rate was higher in the areas closer to the entrance (areas 2 and 3). In the mismatch situation, however, the highest spike rates occur in the area 1 (only panorama located here) and at the border (Figure 55 a). The results for area 5 have to be taken with care because the animal visited only those parts of area 5 that were closest to area 1 and 2. In the mismatch situation, the area housing the local cue (area 4) was not visited. Thus, there is no information what the spike rate would be for the local cue separated from the panorama. Based on the limited exploratory activity in animal g2 the reliable result accounts for the difference between area 1 and 2 in the matched situation.

a)



b)

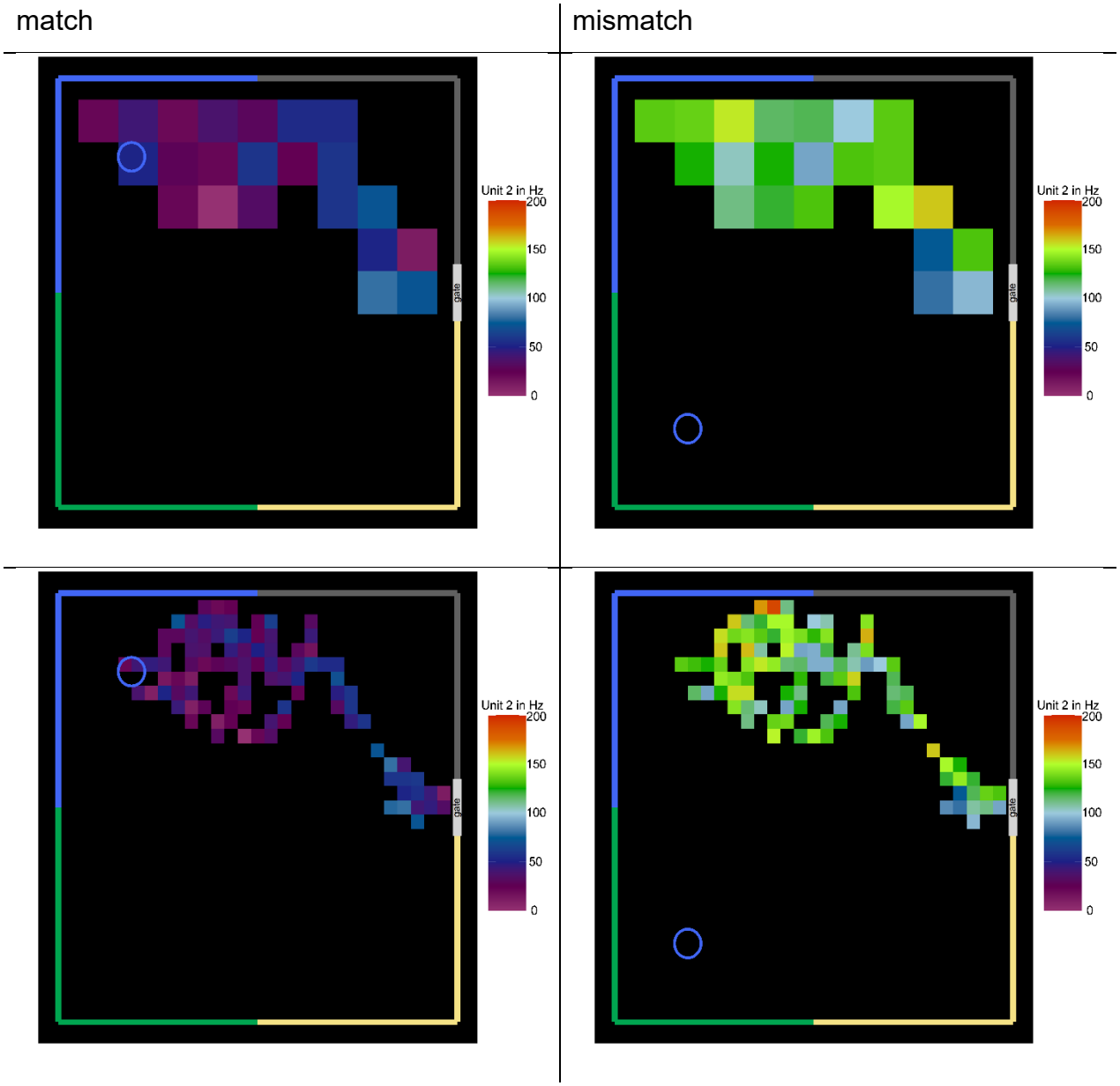


Figure 55 Distribution of spike rates of unit 2 in animal g2. The distribution of the firing rate in Hz. a) The spike frequency in Hz in the different areas in the matched/mismatched condition. The color coding of the bars displays the color of the panorama at the different areas. In the mismatched setup the blue local cue was located in area 4. In the match setup the blue local cue was located in area 1. Area 5 is the border area. Significance comparing the respective areas: $p < 0{,}001$ (Wilcoxon test) b) heatmaps of the spike rate in Hz in the matched (left panel) and the mismatched (right panel) situation in different resolutions. The colored rectangles around the arena display the panorama colors. The light blue circle indicates the position of the local cue. The light grey rectangle in the mid right shows the entrance gate.

Analyzing the effects of distance to the goals in the matched and mismatched situations, it is also important to consider the uneven distribution of walking trajectories. Therefore, far distances in the matched situation occur only from area 2 (not from area 3 and 4), and in the mismatch situation for the local cue (located in area 4) from area 1 and 2. Still the results given in Figure 56 are very interesting, showing that the spike

rates are mostly independent of the distance to the respective goals. Besides this finding, the results confirm the data reported in Figure 55 a with low spike rates for the matched condition and high spike rates for the mismatched conditions.

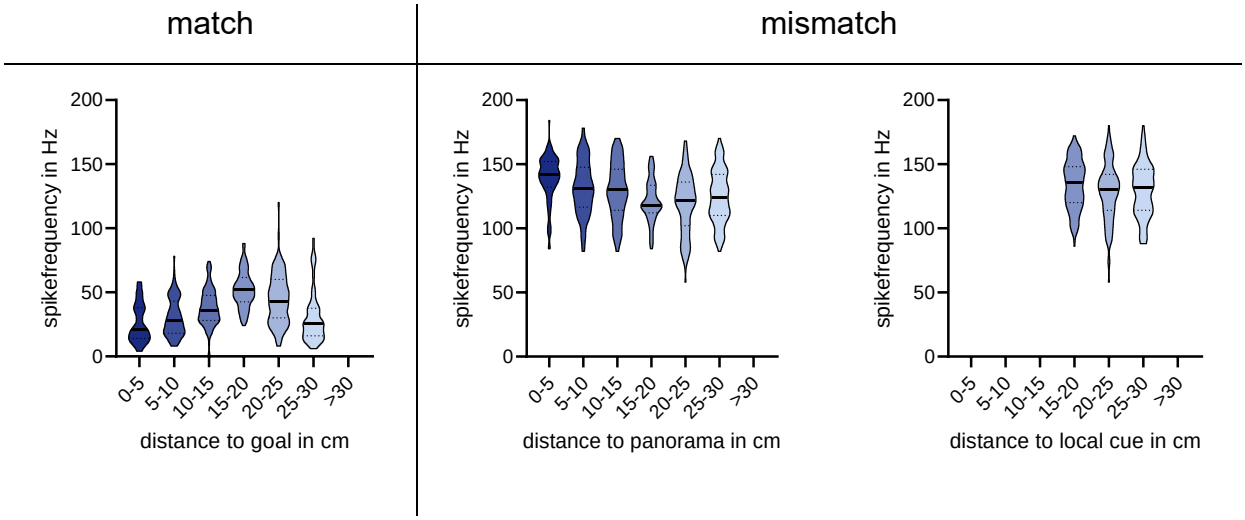


Figure 56 Spike frequency at different distances to the goal in matched/mismatched setup of unit 2 in animal g2. Distances in cm to the goal (match: local cue in front of panorama; mismatch: panorama vs. local cue) on the x axis. The y axis displays the spike activity in Hz. Violin plots are shown, with their mean indicated as line.

The findings reported above are also confirmed by an analysis, in which only data points were analyzed when the animal was walking, and the walks were directed towards area 1 from the entrance gate. In the matched situation area 1 contained both local cue and panorama, but only the panorama in the mismatch situation. Significantly higher spike rates were found in the mismatch situation (p-value < 0,001, Wilcoxon test) (Figure 57) indicating that the low spike rate codes for the local cue and not for the panorama.

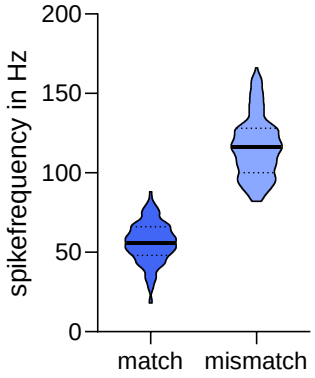


Figure 57 Spike frequency of unit 2 in animal g2 when walking towards area 1 from entrance gate. Displayed in violin plots is the distribution of spike frequency in Hz. In match condition panorama and local cue located in area 1 (shown in dark blue). In mismatch condition only the learned panorama without local cue located in area 1 (shown in light blue). Significance: p < 0,001 (Wilcoxon test).

5.3 References

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6 Discussion

6.1 Behavioral experiments

6.1.1 Bumblebee colonies under laboratory conditions

Bumblebee colony development is an intensively studied subject (Alford, 1975, 2009; Free & Butler, 1959; D. Goulson et al., 2002; Dave Goulson, 2010; Heinrich, 2004; Prys-Jones & Corbet, 2014). Already Frederic Sladen (Sladen, 1912) was fascinated by their life and behavior and reared them in artificial boxes with transparent covers. He was the first, who described that clipping the wings is a good precaution against escape. He also described that regular feeding of the bumblebees makes them inactive foragers. The setup for this work was designed and optimized for foraging experiments without interaction. Every effort was made to ensure stable and adequate conditions for the bumblebees.

The colonies undergo distinct phases, starting with the nest establishment by the queen, followed by an exponential growth phase, characterized by workers production and rearing. When sufficient resources are available in the colony (pollen, nectar), it transitions to the reproduction phase, during which males and new queens are reared. The number of animals remains stable or even decreases during this phase (Alford, 1975; Dave Goulson, 2010; Heinrich, 2004). The overall colony development exhibits a natural progression with the steady growth and a subsequent turning point marked by a decline in the number of individuals. After the nonlinear regression performed based on (Yin et al., 2003) the standardized evolvement of a colony is visible. In some cases of my colonies, they had to be removed from the setup prior to the decline due to the death of the foundress queen accompanied with a raise of aggression levels. Furthermore, the inability of evolved drones to exit the colony (no wings) increased the aggression potential. Consequently, the handling procedure had to be adjusted, and the foragers were not willing to work as before. Due to the limited size of the boxes, the colonies rarely reached the maximal number of individuals observed in the wild (Alford, 1975, 2009; von Hagen & Aichhorn, 2014). Looking at the individually

developments, it became evident, that a higher mortality occurred at the end of the stay in the setup. This resulted from increased aggression within the colonies. The higher mortality at the beginning, as seen in colony B and F, was the consequence of stress during the preparation of the colonies for the setup.

While the temperature could be held stable, the humidity control was challenging especially during the autumn/winter heating seasons. In the heated rooms, the humidity dropped below 30% and it was nearly impossible to maintain the optimal humidity within the setup. However, higher humidity is crucial for stimulating bumblebee queens to lay eggs and to ensure the foraging of workers (Dave Goulson, 2010; Peat & Goulson, 2005). Monitoring the brood temperature proved to be essential, as it provided insight into the availability of sufficient food levels in the colony for optimal breeding conditions. Too much food discourages the foraging behavior, while not enough food lowered brood temperatures (Heinrich, 1972, 2004; Sladen, 1912). If the brood temperature dropped below 30 °C, the workers tended to stay on the brood for heating, reducing the availability of foraging animals. Consequently, the brood temperature was recorded from early 2019 onwards to detect and respond to temperature decline promptly.

Overall, the development of the colonies and the behavior the animals displayed closely resemble those observed in natural colonies. Performing experiments with freely moving single animals from social insects was done before and was found to pose risks of interfering too much with the animals (Duer et al., 2015; Jin et al., 2014; Paffhausen et al., 2020; Patel et al., 2022; Weidenmüller et al., 2002). Here I applied a novel combination of a laboratory-based setup with freely moving animals, employing a learning paradigm, including electrophysiological recordings. Ensuring that each colony displayed normal behavior was of utmost importance. Every colony was under observation for the time in the setup. I confirm, that the behavior the animals displayed, was consistent with the natural behavior described in the literature and which can be found in naturally occurring colonies (personal observation, Alford, 2009; Free & Butler, 1959; Dave Goulson, 2010; Heinrich, 2004; Sladen, 1912)

6.1.2 The absence of spontaneous color preferences

Any training experiment requires knowledge about the response of the animals to the stimuli involved before training. In the case of my training paradigm, I had to determine the spontaneous responses to the colored local cue and the panorama. No color preference was found indicating that any change of response to the two-colored signals resulted from training.

It is known that commercially reared bumblebees belong predominantly to the subspecies *Bombus terrestris dalmatinus.* (Ings et al., 2009) demonstrated an innate preference for the color blue but at a lower level in this particular subspecies as compared to the other tested subspecies *Bombus terrestris audax* (Rohde et al., 2013; Spaethe et al., 2001). In general, this validates my observations made across all bumblebee colonies purchased for my experiments, but not even a low level of spontaneous color preference was detected. However, a distinct preference for the border region of the setup was observed. This thigmotactic behavior (Jin et al., 2020) is characteristic for stressed and confined animals. This behavior mostly diminished as the animals experienced the arena by exploratory runs and in the course of reward training. The analysis of the mean walking speed indicates that the animals walked at their normal pace in the subareas 1 - 4 of the arena. On the contrary, they walked slower in the border area 5, possibly indicating a sort of uncertainty, an interesting phenomenon because one would expect escape behavior to be more erratic and faster.

Bumblebees explore new environments with high motivation, as indicated by their far-ranging walks usually covering the whole area. Once acclimated to the initially unfamiliar surroundings, their walking trajectories continued to encompass the entire arena, indicating a lack of fear or discomfort. I genuinely took care to ensure that, despite the placement of the colony in an artificial arena and limited sugar supply for motivational reasons, all other aspects were carefully designed to imitate a natural setting. Unnecessary disturbances were minimized, and the surrounding parameters were maintained as stable as possible. These efforts are reflected in the typical exploration patterns and behavior within the setup.

6.1.3 The learning paradigm: the bumblebees display a distinct goal directed behavior after the training sessions

Utilizing this novel experimental setup, it was important to demonstrate that the training paradigm results in training specific behavioral changes according to the training conditions and verified by the different test situations. Training consisted of different phases including different forms of learning. Under natural conditions, learning in the navigation context is separated in two distinct phases both at the colony entrance and at the feeding place: associative reward learning when approaching the target (entrance, feeding location), exploratory learning when leaving these places (Degen et al., 2015; Lehrer, 1997; Opfinger, 1931). The former can also be characterized as the inbound phase and the latter as the outbound phase. In the laboratory conditions as applied here, the bees were exposed to an elongated gateway that led them through the test arena to the feeding arena with a reward placed in the middle of the latter. At that point, no marking with the local cue or with the panorama took place. In this situation, bees explored the maze and became motivated to search for food outside the nest at a further distant location. Both exploratory and reward learning are involved in this initial phase. In addition to become familiar with the unusual situation, foraging for food outside the nest by walking, a form of motivational learning is involved preparing the selected bees to explore the arena. The second phase included again an exploratory phase in which the bees were exposed to the novel conditions with the panorama pattern and the blue local cue for food now placed in the test arena marked by the blue parts of the panorama. After the local cue was found and the reward was taken up, the bees underwent reward learning. In the third step of training the bees had to learn the stable relationship between panorama and local cue and the unreliable relationship with the angle at which they leave the gateway on the way to the reward. Importantly, navigational learning as simulated under these conditions is composed of two different phases of a foraging trip, the outbound search for food and the inbound return to the colony and the related forms of learning. The analysis of the walking trajectories focused on the outbound component both with respect to the panorama and the local cue simulating natural foraging conditions. The separation of panorama and local cue in the mismatch situation has been chosen to simulate a central property of navigational learning, the association between localized parameters for food and the embedding of those cues in the further distant landmarks by exploratory learning.

Furthermore, the extinction tests, as applied here, simulate the traditional forms of tests with bees under natural conditions in which bees experience the various signals, both local and peripheral, but do not receive a reward (extinction tests, (Menzel & Giurfa, 2006)). Under natural conditions, it is practically impossible to separate local cues and peripheral landmarks like the patterns of the panorama. This was possible in my laboratory setup, providing a unique situation. That allowed me to search for neural correlates, that might reflect characteristic navigational memory which stores local cues in relation to far distant cues (see below).

Successful learning, as shown in Figure 11, documents the suitability of the training paradigm for studying navigation under laboratory conditions. Highest learned performance was found for matching conditions, and separate learning of cue and panorama in the mismatch condition. The results on the walking trajectories in area 5 (the border area) allows insight into a switch of search behavior not accessible under natural test conditions, here most likely the switch to escape behavior. This is again of high value during the attempts to unravel neural correlates of navigation because any specific neural events for area 5 characterize a deviation from navigation related neural events (see below).

In the extinction test 2 (mismatch), it was observed that the animals exhibited a clear preference for the region characterized by the local cue over the area with the panorama stimulus. The significance of area 2, which encompasses the path leading to area 1, can be succinctly explained. As the animals traversed this path towards the learned panorama, they encountered an absence of the local cue, indicating a different spatial location. Consequently, they tended to deviate from their intended trajectory before entering area 1.

I found that the straightness toward the learned targets was significantly increased after the training compared to the performance in the first training session. Furthermore, comparing the performance of the animals in test 1 (panorama only) with their behavior in the color preference test showed an increase of walked distance in area 4 and decrease in area 5 documenting the successful learning also of the panorama during the training sessions.

Thus, the sequential extinction tests 1 – 4 uncovered the results of the sequential learning steps including both exploratory and reward learning mimicking navigational

learning under natural conditions. Trajectories in area 5 are indicative for the switch to innate escape behavior with unknown relationship to the outbound and inbound motivation.

6.1.4 Application of the training paradigm to a pharmacological manipulation of central brain function: Effect of the nicotinic agonist Thiacloprid

Neonicotinoids play an important role in the agricultural business. Their usage rapidly increased over the last decades. They are very effective against the target pest insects which include sucking insects such as aphids or whiteflies, but they are also taken up by none target insects like pollinating insects. The abundancy of these pesticides is rather alarming as they can be found everywhere and not just in the treated plants. Thus, other insects get in contact with these chemicals via consumption of contaminated pollen or nectar or even via direct acute contact. Chronic exposure and sublethal effects are already the subject of various studies for various neonicotinoids (Goulson, 2013; Morrissey et al., 2015; Sánchez-Bayo, 2014; Wood & Goulson, 2017). Though the 4 ‘known as the most toxic‘ neonicotinoids (Thiametoxam, Imidacloprid, Acetamibrid, Clothianidin) are already banned in the EU, it is still worth further evaluating their impact, because of the possibility of future re-admission. Thiacloprid is played down because of its lower LD 50 value compared to other neonicotinoids (Manjon et al., 2018), though the sublethal effects are known to interfere with navigation, learning and communication (see below).

The action of the neonicotinoid Thiacloprid is well understood both at the molecular and cellular level (Jones & Sattelle, 2010; Lu et al., 2022; Tomizawa & Casida, 2005; Tomizawa et al., 2008). Here, the sublethal effect of Thiacloprid in the formulation Calypso® was tested. In the study of Tison et al., 2017 it was found that Thiacloprid in the formulation Calypso® impaired natural navigation performance, motivation to forage and performance of waggle dances in honeybees. I, therefore selected Calypso® as a pharmacological treatment of central nACh receptors to study the effect in a laboratory setting using my training and test procedure. The goals were twofold, to collect additional behavioral data from the control group (untreated animals) to strengthen the behavioral data set and to explore the usefulness of my setup for laboratory tests on the effect of neonicotinoids. The latter goal is particularly important

in the context of the search for laboratory test conditions of sub-lethal effects. So far, the standard tests on the effect of insecticides are the LD 50 tests, percentage of dead animals after 24 hours. The European Commission is actively searching for standardized tests of sub-lethal effects that include the bumblebee as a test animal.

There was one hour digestion time under red light between the ingestion and start of the tests. Since the bumblebees were foraging on their own motivation, they were put back onto their colony after the digestion pause. Additional time elapsed until they entered the test arena ensuring that the drug was completely absorbed in their body.

The treated animals showed no difference to the controls when analyzing the total walking distance during all different test situations. Thus, no hint was found for motor impairments after the treatment. Also, goal directedness overall did not change. The straightness of the walks however decreased in the matched test situation in the treated animals whereas it increased in the control animals, indicating an effect to retrieve the memory for the trained condition. This finding is in line with the results of Tison et al., 2017, who found longer and less precise trajectories of homing flights. In addition, an increase of the length of the walking trajectories in the border area (area 5) was found after Calypso® treatment both respect to their own trajectories in area 5 after the last training session as well as after treatment in the match as well as in the mismatch test. A similar effect was detected in naïve bees after they entered the arena for the color preference test. Furthermore, walking speed decreased during all 4 test situations in comparison both with the control animals as well as with their own walking speed during the mismatch test after the last training session.

Since the animals showed no motor defects and covered the whole arena, I conclude that the changed behavior resulted from compromised retrieval of memory of the location of the local cue and the panorama. These results are in line with tests under natural conditions not only in the case of quantified flight trajectories but also with respect to the weight of colonies (treated colonies are lighter) (Ellis et al., 2017).

The changes of behavior after the intake of 400 ng/animal Thiacloprid were not enhanced by a higher dose (800 ng/animal). However, the sample size may have been too low to propose a clear statement. Furthermore, I observed but did not quantify behaviors inside the colony and the probability of entering the test arena. Observations

indicated an overall reduction of activity and prolonged periods of returning to the colony.

Taken together I conclude that my laboratory setting is well suited to test and quantify the sub-lethal effects on navigation under laboratory conditions. These detrimental effects can be accounted to compromised retrieval of navigational memory corroborating the results under natural conditions. It is also a tool to access memory retrieval phenomena by pharmacological methods. The results found here confirm the importance of nicotinic transmission in the context of memory formation and retrieval.

6.1.5 Analysis of residuals

It appeared to me to be necessary to know the amount of Thiacloprid in the treated animals. Fortunately, I found a laboratory which did these residue measurements for me (Labor Friedle GmbH, Von-Heyden-Straße 11, 93105 Tegernheim, Germany). The analyses revealed an increase of Thiacloprid in all body parts of the animals 1.5 h following ingestion. The abdomen showed the highest increase, while the thorax the least. The amount in the abdomen suggests the presence of unabsorbed residue, which may be degraded by two enzymes and then excreted (Manjon et al., 2018). Given that Thiacloprid primarily binds to receptors expressed in the central nervous system, the increase detected in the head is most relevant and proves that after 1.5 h Thiacloprid has reached the brain. The doses found there were well below the LD50 of 10.000 ng/animal in line with the finding that no obvious dead fall was observed and corroborating the conclusion that doses far below LD50 have substantial effects on memory retrieval. Additionally, investigations were conducted to assess the residual concentrations at various time intervals after the ingestion of 800 ng per animal. The thorax and head exhibited negligible variations whereas the abdomen manifested the highest concentration 1.5 h after ingestion. The consistent concentration observed in the head after different time periods supports the appropriateness of the experimental timeframe selected: the main absorption occurred within the initial 1.5 h interval following ingestion.

As pointed out above the pharmacological experiments with the neonicotinoid Thiacloprid were performed by two motivations, to add additional behavioral data

(control animals) and to test the suitability of a laboratory setting of navigation in a nutshell. The latter is well supported and will hopefully contribute to the search for standardizable and easy to perform laboratory tests for sub-lethal effects of neonicotinoids.

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6.2 The search for the neuronal correlates of navigation in a laboratory setting

6.2.1 Mushroom body extrinsic neurons as a target of search

Navigation is an important faculty of highly integrated brain function in a large range of animal species. A comparative approach appears as a promising strategy, aiming to find corresponding neural processes when animals solve similar problems, e.g., navigating to an important location. Indeed, some fundamental navigational strategies, e.g. path integration (Collett & Collett, 2000; Mittelstaedt & Mittelstaedt, 1982), mapping to compass values (Kramer, 1952; Valkova & Vacha, 2012), panorama matching (Cartwright & Collett, 1982; Collett, 1992; Morris, 1984), vector integration (Wehner, 1984) and cognitive mapping (Tolman, 1948; Wang et al., 2023) are documented for many species including invertebrates (Shettleworth, 2010; Wiener et al., 2011). Invertebrates solve these tasks with less neural resources than most vertebrate species, and thus may be suitable for uncovering the essential components of navigation (Collett & Collett, 2002).

Navigation in social insects is particularly impressive since they need to return safely to their hive, forage at unreliable resources and may communicate about locations in the environment (Seeley, 2011; von Frisch, 1967). Given their small brains and the complexity of their homing strategies, it can be expected that across sensory integration, learning, motivational switch and appropriate memory retrieval may be accessible to neural analysis (Menzel & Giurfa, 2001; Srinivasan, 2010; Wehner, 2003). An essential step in this endeavor will be laboratory-based test conditions comparable to those used in laboratory mammals, e.g., the Morris water maze (Morris, 1984).

Although studies under natural conditions provide us with multiple and highly interesting data about the behavioral strategies, they allowed only in a preliminary way to combine in-flight natural navigation with neural studies (Paffhausen et al., 2021). Simplified test conditions for neural studies are thus required that try to keep essential components of natural navigation. Laboratory studies of navigation use mazes of different design to address the question which environmental stimuli are used and how

they are integrated in order to enable goal directed behavior (Morris, 1984), and ground breaking results were found on the neural basis of navigation in the mammalian hippocampus (place cells, grid cells, border cells, head direction cells, (Moser et al., 2008)). Laboratory based methods have been applied for walking insects (e.g., ants (Lent, Graham, & Collett, 2013; Vandersal, 2008), cockroaches (Durier & Rivault, 1999; Moore et al., 1997), *Drosophila* (Kuntz et al., 2012; Ofstad et al., 2011; Seelig & Jayaraman, 2013, 2015)). Bees were studied only recently in two different laboratory settings although they are known to perform visually guided decisions when walking in confined arrangements (e.g., in a T-maze (Menzel, 1981; Menzel, 1985) or in galleries (Bisetzki, 1957; Lindauer, 1963)). The two settings combined with extracellular neural recordings were performed in bumblebees walking in a small round arena with a local cue (Jin et al., 2020), and in honeybees walking on an air supported floating ball in a virtual reality (Zwaka et al., 2019). In both cases mushroom body extrinsic neurons (MB EN) exiting the mushroom body at the ventral aspect of the alpha lobe were recorded based on the argument that the mushroom body is the center of high order integration and an essential neural hub for learning and memory processing (Menzel, 2014). The test conditions, as described here, allow simultaneous extracellular recordings from these MB ENs under much more favorable conditions since the recorded bumblebees are housed in a colony, enter a two-part maze by self-motivation and learn an essential component of navigation, the local cue-panorama association, very well.

MB ENs are located at the output of the mushroom body, they are likely to represent high order integration, and in this sense may be comparable to prefrontal cortex neurons in mammals. MB ENs neural correlates of differential classical odour conditioning were found for two different groups of neurons (Okada et al., 2007; Strube-bloss et al., 2021, 2011). They exhibit learning related plasticity mostly as an increased response (in the case of the identified PE1 neurons a decrease) towards the rewarded odour, and through recruitment of additional responding neurons that have not responded to the rewarded odour before. However, multiple other forms of associative plasticity were observed including enhanced or reduced responses to specifically not rewarded stimulus (CS-), drop-out of initially responding neurons (to both CS+ or CS-) and network effects as e.g., enhanced ensemble activity after learning (Menzel, 2014). Furthermore, MB ENs may code the value or meaning of highly integrated

sensory input with respect to the expected outcome of an action as prefrontal neurons do (Filla & Menzel, 2015). So, probably, MB ENs are not solely responsible for learning induced plasticity, but might also mediate goal-oriented behaviour and expectation in more complex behavioural conditions as in the navigational task studied here.

I also focused on these neurons because they are multisensory and are known to change their response properties during olfactory stimulation and learning (Grünewald, 1999; Haehnel & Menzel, 2010; Zwaka et al., 2019), and they were successfully recorded under conditions in which the animal ran freely in a small experimental colony (Paffhausen et al., 2020). These neurons were also recorded in honeybees attached to a model helicopter that flew along the trained flight path of the recorded animal (Paffhausen et al., 2021). In this latter case, clear evidence was found for turning related flight behaviour in a close to natural setting, but no correlates were found for navigational components during their tethered flights.

6.2.2 The quality of the recording

In this study, I employed the method outlined in Duer et al., 2015. Due to the larger setup and the greater robustness of bumblebees, the electrodes used were required to be longer. To mitigate any adverse effects on the mobility of the bumblebee, the electrodes were properly counterbalanced with a spring mechanism as introduced by Duer et al., 2015.

In all setups used so far, it was possible to isolate distinct units with principal component analyses (PCAs), peri-stimulus time histograms (PSTHs) and manual verification of patterns and shapes, as it was done here. The confirmation of the sorting process was done through correlation plots. The presence of both negative and positive correlation within one unit, exhibiting distinct expression patterns, validates the sorting in these units is sensible (Figure 37).

To ensure that the observed variations in spike frequencies were not influenced by potentials arising from muscles, I assessed the walking speeds of bumblebees (Figure 41). Due to the scarcity of available data on the walking speeds of bumblebees, particularly since most experiments involving these insects are conducted within flight arenas, I relied on personal experience to bin the different walking speeds. Also, any

bumblebees displaying abnormal behavior were excluded from the sorting process. Such behaviors included: circling, no walking activity, attempts to remove the electrode, stumbling and any other indications of motor impairment following the insertion of the electrode. These excluding criteria reduced the success rate to less than 12 preparations (animals) with a total of 83 units recorded, but only 3 of them could be used for complete analysis of behavioral tests and unit activity due to the strict excluding criteria. Furthermore, two third of the time invested in the course of the doctoral work was spent on establishing this novel experimental set-up, verifying the successful learning, building, and testing the preamplifier and fighting the electrical noise and instability of electrodes of a length of over 1 m. I also want to mention here loss of time and the investment of time due to the move of the lab from the old building to the Zoology building. All these factors combined lead to the extremely low ration of successful recordings per invested time and effort.

6.2.3 Stable electrophysiological recordings from three trained animals with six distinct units

Approximately 180 MB ENs leave the mushroom body at the alpha exit (Rybak & Menzel, 1993). It is very likely that different units were recorded in different animals. Consequently, it is not surprising that distinct activity patterns are observed in the recorded region among different animals.

All units showed a significant lower spike frequency while sitting then during walking. Rother et al., 2023 shows that walking bumblebees and the consequential higher temperature of the head results in a faster processing of the visual system. The rise of spike frequency when walking compared to the low frequency when sitting might be taken as a hint for this temperature effect. Most importantly, no specific relationship between the activity of the units and the position of the recorded bee in the arena was revealed. Thus, no hint was found for place cell–like or grid cell-like activity patterns as it is known in mammals (Moser et al., 2008). Furthermore, the analysis of key behavioral components of the walking trajectory (angle, distance and speed) and time windows of sharp increases or decreases of spike frequency showed no relationship, making it unlikely that MB ENs code parameters of animal´s body in relation to the arena cues. The spike rate changes found in all units for the border area are unlikely

a border cell effect as in the maze studies of hippocampal neurons but a signature of a motivational change from exploration to escape. It thus rather appears that the MB ENs are concerned about the complex coding of learned object properties in a combinatorial way more like the hippocampus/prefrontal network in mammals (Eichenbaum, 2002; Manns & Eichenbaum, 2009).

In the following I will substantiate this conclusion by looking into the activity patterns of the recorded units comparing the match/mismatch test situations and the goal directed phenomena.

In the case of the bumblebee rL (unit 6, Figure 43), a difference is observed between the two different test conditions (mismatch/match) in all areas. In the mismatch condition, the spike rates in these areas are significantly lower compared to the matched condition and higher in the match condition. Thus, higher spike rates are indicative for a test situation that satisfies the expected conditions. This conclusion is supported by the higher spike rates while the animal is close to the learned goals, even in the mismatch situation (Figure 44). Most interestingly an increment in spike rate is observed with each repetition of walking to the learned cues (local cue and panorama) in the matched condition (Figure 46). Since a higher spike rate occurs when the learned situation is detected it appears likely that this unit may be involved in exploratory learning rather reward learning because during these tests no reward was available (extinction test).

In animal rL two active units were recorded simultaneously (units 6 and 1), allowing a comparison across units for the same behavioral components. Unit 1 did not show any particular pattern of spike rates changes that I could correlate with a behavioral component. This finding might suggest that unit 1 is neither part of the memory retrieving process nor the exploratory learning process. To quantify and illustrate the difference between the responses of unit 6 and unit 1 I selected the repeated walks toward the goals (local cue, panorama) in the mismatch situation and found different spiking properties (Figure 48). The walks to the goal in the matched situation show no effect in unit 1.

Unit 2 in bumblebee be6 responds to the experience of mismatch with a higher spike frequency than to that of the matching situation in all areas (Figure 50), conditions that contrast with the results of unit 6 in animal rL. This effect is independent of the distance

to the goals in both match/mismatch situations (Figure 51). Since the mismatch situation displays a discrepancy between expectation and reality, the spike rate differences could be indicative for the exploratory behavior of the animal. There is one exception from the lower spike rate in the matching situation, namely a higher spike rate for long distances (> 30 cm) in the matching situation (Figure 51 left panel). Under these conditions the animal may not have expected to see the local goal, and thus a discrepancy is not detected.

The mismatch test provides me with the opportunity to unravel the effects of local cue and panorama separately. To take advantage of this unique conditions, I analyzed spike rates when the animal was walking between the two areas housing the local cue or the panorama. Unit 2 showed a slight but significantly lower spike rate during walkers from the panorama area to the local cue area (Figure 52). Since the specific coding property of this unit is a spike rate reduction, I conclude that the local cue plays a more important role in its response properties.

Animal be6 allowed me to compare three simultaneously recorded units. The analysis focused on multiple repeated walks between the goals in the mismatch situation (Figure 53) and 3 consecutive walks toward the local cue in the mismatch situation (Figure 54) asking whether and how the spike rates differ. Spike rates rose and fell in the course of the multiple repeated walks from the local cue to the panorama and those from the panorama to the local cue. No obvious pattern across units and repeated walks is apparent, suggesting that the complex pattern codes the responses to the cues in a combinatorial way. The picture is clearer for the three walks from further distances toward the area that houses the local cue. Spike rates increased for unit 2, stayed constant for unit 1 and decreased for unit 5. These antagonistic effects corroborate the interpretation of the effects for repeated walks between the separated cues namely the combinatorics in neuronal coding during memory retrieval processes.

The analysis of unit 2 in animal g2 has been included although the walking trajectories cover only part of the arena, a criterion that usually led to the exclusion of units. Unit 2 is active very differently during mismatch and match situations with low spike rates during matching situation and higher spike rates during mismatch situation (Figure 55). This pattern resembles the activity patterns seen in unit 2 in animal bee6 (Figure 50 a, b).

The neural activity of MB ENs of the alpha exit in bees (both honeybees and bumblebees) have been studied in multiple test conditions both under confined and freely walking conditions (see Introduction). All data collected so far allow to reject the proposal that these neurons are involved in coding specific sensory stimuli or are involved in specific premotor control. They are multimodally responding to single and combined sensory stimuli, and their activities cannot be related to motor output in experiments with freely walking bees or in bees walking in a virtual reality setup. Since they are the output of the mushroom body it has been proposed already early on (Mauelshagen, 1993; Okada et al., 1999) that their key properties are related to multimodal object identification, context dependent evaluation, learning and memory retrieval (Menzel, 2014). It is thus not surprising that the spike rates recorded in test conditions examined here do not correlate with elementary forms of sensory input.

Neural correlates of self-localization and decision making in the navigational context are unknown for insects. Directional information in relation to the sun azimuth is known to be coded in the central complex (Pfeiffer & Homberg, 2007; Seelig & Jayaraman, 2015), but these properties address only one of several components of navigation. The most important components in navigation, learned object recognition and multimodal combinations of directional, distance and spatial object relationships are not addressed in these studies. Such aspects are coded in hippocampal place cells and grid cells (Moser et al., 2008). Given the knowledge we have about the coding properties of MB Ens, one wonders why we did not find any place cell-related properties. The mushroom body lies in parallel pathways of the visual, olfactory and mechanosensory high order interneurons and has little neural connectivity to the central complex. Since all signals relevant for far distance navigation are visual, it is likely that pathways via the visual ganglia, the outputs of the central complex and the mushroom body converge downstream to form a hub for combined navigational coding. However, the location of such a hub is unknown and may not even be localized in a structurally identifiable brain part. Our ignorance about the central coding of the navigational capacities is also not resolved by my recordings.

Neural codes of learned object identification are most likely a network property in which the contribution of single neurons would be best uncovered if several to many neurons would be recorded simultaneously, and their joint coding properties identified. I have aimed for such experimental conditions embedded in a behavioral paradigm that

captures important aspects of memory retrieval under natural foraging conditions and self-motivated decision making. The behavioral paradigm was successfully established but the recording under these demanding conditions was much less successful. Earlier attempts with a similar goal suffered either from too restricted training and test conditions (e.g., proboscis extension conditioning to odors in restrained animals (Filla & Menzel, 2015; Okada et al., 1999)), too complex behavioral paradigms (e.g. animals attached to a flying helicopter or running stationary in an artificial reality (Paffhausen et al., 2021; Zwaka et al., 2019)), or lacked controllable stimulus conditions (e.g. animals in a semi-natural setting of the social environment (Paffhausen et al., 2020)). Furthermore, none of these studies managed to record from more than one MB EN simultaneously. Given this background and the extremely demanding methodological problems of multi-unit recording even under restrained conditions, the data collected here promise a fruitful avenue into more effective experimental conditions. Together with new recording devices (electrodes, amplifiers) as they exist meanwhile, and the successful behavioral paradigm will certainly transcend what I have reached.

6.3 Conclusion and future possibilities

A setup used for behavioral training and associated electrophysiological recordings was established. Freely moving bumblebees foraged on their own motivation and showed learned behavior. Stable recordings were conducted, and single units could be sorted and analyzed. The technical aspects of the setup were refined, and the artificial environment adjusted to the highly flexible behavior of the bumblebee colonies. Experiments revealing sub-lethal effects of treatments can be conducted and shown in such setups. The recordings revealed small deviations between units and their response patterns.

Future behavioral experiments with different treatments are possible. Working with bumblebees can be quite challenging. The low number of test animals per week must be considered for future time planning. Also, the breakout rate and the brood temperature are crucial aspects to monitor.

Performing electrophysiological recordings in bumblebees pose the risk of losing the electrode due to the robustness and strength of the animals. The used tissue glue minimized the chance of losing a recording due to dismantling by the bumblebee. This was also the main reason, the recorded animals were not allowed back into their colony and no recordings could be performed during training. Bumblebee workers are very strong insects and an intruder with unnatural attachments will be attacked rather fast and ruthless.

Apart from these struggles, future experiments could include further recordings with trained bumblebees with an additional change in the training paradigm. Also, treated animals could be used for recordings and could reveal the impact they have on the mushroom body. The few trials (not shown here) proved that these animals may need a different dissection procedure. All too often animals showed only stressed, circling behavior or no walking at all. Also, longer lasting recordings should be aimed for, including 3 - 4 training trials and an additional test run, to compare the effect of training.

Though it took me a long time refining the setup and adjusting the method to the nature of bumblebees, the outcome was promising and will hopefully be used in future studies.

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A) Appendix

I) R code for Analysis

```
library(tidyverse)
library(zoo)
library(RColorBrewer)
library(colorspace)
library(ggplot2)
library(viridis)
library(colorRamps)
library(Hmisc)
library(corrplot)
library(beanplot)
library(vioplot)
library(mltools)
library(pracma)
library(plyr)
library(jcolors)
library(plot3D)
library(plotly)
library(stringr)
library(ggforce)
library(readr)
library(LearnGeom)

####Data import
path <- 'D:/path'
results_path <- 'D:/resultsPath'
fname<-"name"
food<-"food4"
Unit<-""
dataUnit<-
data.frame(read.csv(paste0(path,sprintf("/%s_%s_%s_data.csv",fname,food,Uni
t)),sep=";",skip=1,header=FALSE))
food1Werte<-filter(dataUnit,dataUnit$Food=="food1")
food4Werte<-filter(dataUnit,dataUnit$Food=="food4")
Food1filtered<-c()
Food4filtered<-c()
xmin <-c()
xmax <-c()
ymin <-c()
ymax <-c()

####mask overlay
for (i in 1:30) {
  ymin=i-1
  ymax=i
  for (m in 1:30) {
    xmin=m-1
    xmax=m
    Food1Temp<-c()
    Food4Temp<-c()
    for (j in 1:nrow(food1Werte)) {
      currentX <-food1Werte[j,]$x
      currentY <- food1Werte[j,]$y
      if (currentX >= xmin && currentX < xmax && currentY >= ymin && currentY
< ymax){
```

```
        Food1Temp<-rbind(Food1Temp,food1Werte[j,])
      }
    }
    if (!all(is.null(Food1Temp))) {
      for (k in 1:nrow(food4Werte)) {
        currentX <-food4Werte[k,]$x
        currentY <- food4Werte[k,]$y
        if (currentX >= xmin && currentX < xmax && currentY >= ymin &&
currentY < ymax){
          Food4Temp<-rbind(Food4Temp,food4Werte[k,])
        }
      }
      if (!all(is.null(Food4Temp))) {
        Food1filtered<-rbind(Food1filtered,Food1Temp)
        Food4filtered<-rbind(Food4filtered,Food4Temp)
      }
    }
  }
}
write.table(Food1filtered,file=paste0(results_path,sprintf("%s_Food1Filtere
d_2.csv",fname)),sep=';',row.names = F)
write.table(Food4filtered,file=paste0(results_path,sprintf("%s_Food4Filtere
d_2.csv",fname)),sep=';',row.names = F)

####starting analysis
data <- dataUnit
dx <- data$x
dy <- data$y
data$dist                    <-                    c(0,sqrt((dx[1:(length(dx)-1)]-
dx[2:length(dx)])^2+(dy[1:(length(dy)-1)]-dy[2:length(dy)])^2))
data$speed                   <-                   c(0,(sqrt((dx[1:(length(dx)-1)]-
dx[2:length(dx)])^2+(dy[1:(length(dy)-1)]-dy[2:length(dy)])^2))*30)
data$keep <- TRUE
data<-filter(data,data$dist<15) ####filter strays
data$Q1 <- as.numeric((data$x>=1.58)&(data$x<15)&(data$y>=1.58)&(data$y<15))
data$Q2                                                                    <-
as.numeric((data$x>=15)&(data$x<28.42)&(data$y>=1.58)&(data$y<15))
data$Q3                                                                    <-
as.numeric((data$x>=15)&(data$x<28.42)&(data$y>=15)&(data$y<28.42))
data$Q4                                                                    <-
as.numeric((data$x>=1.58)&(data$x<15)&(data$y>=15)&(data$y<28.42))
data$Q5                                                                    <-
as.numeric((data$x<1.58)|(data$x>=28.42)|(data$y<1.58)|(data$y>=28.42))

# calculate angle
for (i in 1:nrow(beePosition)-1 ){
  di<- as.numeric(beePosition[i,])
  dj<- as.numeric(beePosition[i+1,])
  anglei<- Angle(dj, di, food1, label = FALSE)
  angle1P[length(angle1P)+1]<-anglei
}
write.csv(angle1P,paste0(results_path,sprintf("%s_Angle1.csv",fname)),row.n
ames = FALSE)

####distances per area
data$Q <- data$Q1+data$Q2*2+data$Q3*3+data$Q4*4+data$Q5*5
d5Q <- aggregate(data$dist,by=list(paste0(fname,'-Q',data$Q)),sum)
labels <- matrix(unlist(strsplit(d5Q$Group.1,'-')),ncol=2,byrow=TRUE)
d5Q$name <- labels[,1]
d5Q$Q <- labels[,2]
d5Q1 <- spread(d5Q[2:4],key="Q",value="x")
```

```
write.table(d5Q1,file=paste0(results_path,sprintf('/%s_distanzen_proQuadran
t.csv',fname)),sep=';',row.names = F)
d5Q1P<-d5Q1
d5Q1P <- ((d5Q1P[,2:5]*100)/sum(data$dist))
write.table(d5Q1P,file=paste0(results_path,sprintf('/%s_distanzen_Prozent_p
roQuadrant.csv',fname)),sep=';')

####Speed mean per area
data$Q <- data$Q1+data$Q2*2+data$Q3*3+data$Q4*4+data$Q5*5
d6Q <- aggregate(data$speed,by=list(paste0(fname,'-Q',data$Q)),mean)
labels <- matrix(unlist(strsplit(d6Q$Group.1,'-')),ncol=2,byrow=TRUE)
d6Q$name <- labels[,1]
d6Q$Q <- labels[,2]
d6Q1 <- spread(d6Q[2:4],key="Q",value="x")
write.table(d6Q1,file=paste0(results_path,sprintf('/%s_speed_mean_proQuadra
nt.csv',fname)),sep=';',row.names = F)

write.table(data,file=paste0(results_path,sprintf('/%s_data.csv',fname)),se
p=';',row.names = F)

####correlation plots
unitsTogether<-
cbind(dataUnit$Unit1.FPS,dataUnit$Unit2.FPS,dataUnit$Unit3.FPS,dataUnit$Uni
t4.FPS,dataUnit$Unit5.FPS,dataUnit$Unit6.FPS,dataUnit$Unit7.FPS,dataUnit$Un
it8.FPS)
res<-cor(unitsTogether)
png(paste0(results_path,sprintf('/%s_CorPlotUnits_final.png',fname)),width=
3060,height=2036,res=300,type = "cairo-png")
par(mfrow=c(1,1))
corrplot(res,type="upper",tl.col = "black",tl.srt = 0.01)
dev.off()

####Heatmaps
MaxUnit1=max(round_any(max(food1Werte$roll15Unit1Hz,na.rm=TRUE),100,f=ceili
ng),round_any(max(food4Werte$roll15Unit1Hz,na.rm = TRUE),100,f=ceiling))
MaxUnit2=max(round_any(max(food1Werte$roll15Unit2Hz,na.rm                  =
TRUE),100,f=ceiling),round_any(max(food4Werte$roll15Unit2Hz,na.rm          =
TRUE),100,f=ceiling))
MaxUnit3=max(round_any(max(food1Werte$roll15Unit3Hz,na.rm                  =
TRUE),100,f=ceiling),round_any(max(food4Werte$roll15Unit3Hz,na.rm          =
TRUE),100,f=ceiling))
MaxUnit4=max(round_any(max(food1Werte$roll15Unit4Hz,na.rm                  =
TRUE),100,f=ceiling),round_any(max(food4Werte$roll15Unit4Hz,na.rm          =
TRUE),100,f=ceiling))
MaxUnit5=max(round_any(max(food1Werte$roll15Unit5Hz,na.rm                  =
TRUE),100,f=ceiling),round_any(max(food4Werte$roll15Unit5Hz,na.rm          =
TRUE),100,f=ceiling))
MaxUnit6=max(round_any(max(food1Werte$roll15Unit6Hz,na.rm                  =
TRUE),100,f=ceiling),round_any(max(food4Werte$roll15Unit6Hz,na.rm          =
TRUE),100,f=ceiling))
######plot single unit####
##food 1
if (length(food1Werte$X)!=0){

ggplot(food1Werte,aes(x=floor(x),y=floor(y)))+geom_tile(aes(fill=roll15Unit
1Hz))+scale_fill_gradientn(colours=rev(jcolors("rainbow")),limits=c(0,MaxUn
it1))+     theme(legend.key.size     =unit(2,"cm")     ,panel.background     =
element_rect(fill="#000000"),panel.grid.major                              =
element_blank(),panel.grid.minor = element_blank())+

geom_segment(aes(x=0,y=0,xend=0,yend=15),colour="#4166F5",size=4)+geom_segm
```

```
ent(aes(x=0,y=0,xend=15,yend=0),colour="#4166F5",size=4)+geom_segment(aes(x
=15,y=0,xend=30,yend=0),colour="#606060",size=4)+geom_segment(aes(x=30,y=0,
xend=30,yend=13),colour="#606060",size=4)+

geom_segment(aes(x=30,y=17,xend=30,yend=30),colour="#F4E285",size=4)+geom_s
egment(aes(x=15,y=30,xend=30,yend=30),colour="#F4E285",size=4)+geom_segment
(aes(x=0,y=30,xend=15,yend=30),colour="#00A550",size=4)+geom_segment(aes(x=
0,y=15,xend=0,yend=30),colour="#00A550",size=4)+

geom_segment(aes(x=30,y=13,xend=30,yend=17),colour="#D4D4D4",size=6)+xlim(0
,30) + ylim(30,0)+xlab("x in cm")+ylab("y in cm")+labs(fill="Unit1 in
Hz")+annotate("text",x=29.9,y=15,label="gate",color="black",size=5,angle=90
)+
    geom_circle(aes(x0=5.5,     y0=5.5,     r=1),     color='#4166F5',lwd=2,
inherit.aes=FALSE)

ggsave(paste0(results_path,sprintf('/%s_F1_1_HeatmapUnit.png',fname)),heigh
t = 10, width=11)


ggplot(food1Werte,aes(x=floor(x/3)*3,y=floor(y/3)*3))+geom_tile(aes(fill=ro
ll15Unit1Hz))+scale_fill_gradientn(colours=rev(jcolors("rainbow")),limits=c
(0,MaxUnit1))+  theme(legend.key.size  =unit(2,"cm")  ,panel.background  =
element_rect(fill="#000000"),panel.grid.major                              =
element_blank(),panel.grid.minor = element_blank())+

geom_segment(aes(x=0,y=0,xend=0,yend=15),colour="#4166F5",size=4)+geom_segm
ent(aes(x=0,y=0,xend=15,yend=0),colour="#4166F5",size=4)+geom_segment(aes(x
=15,y=0,xend=30,yend=0),colour="#606060",size=4)+geom_segment(aes(x=30,y=0,
xend=30,yend=13),colour="#606060",size=4)+

geom_segment(aes(x=30,y=17,xend=30,yend=30),colour="#F4E285",size=4)+geom_s
egment(aes(x=15,y=30,xend=30,yend=30),colour="#F4E285",size=4)+geom_segment
(aes(x=0,y=30,xend=15,yend=30),colour="#00A550",size=4)+geom_segment(aes(x=
0,y=15,xend=0,yend=30),colour="#00A550",size=4)+

geom_segment(aes(x=30,y=13,xend=30,yend=17),colour="#D4D4D4",size=6)+xlim(0
,30) + ylim(30,0)+xlab("x in cm")+ylab("y in cm")+labs(fill="Unit1 in
Hz")+annotate("text",x=29.9,y=15,label="gate",color="black",size=5,angle=90
)+
    geom_circle(aes(x0=5.5,     y0=5.5,     r=1),     color='#4166F5',lwd=2,
inherit.aes=FALSE)

ggsave(paste0(results_path,sprintf('/%s_F1_3_HeatmapUnit.png',fname)),heigh
t = 10, width=11)
}
###food4
if (length(food4Werte$X)!=0){

ggplot(food4Werte,aes(x=floor(x),y=floor(y)))+geom_tile(aes(fill=roll15Unit
1Hz))+scale_fill_gradientn(colours=rev(jcolors("rainbow")),limits=c(0,MaxUn
it1))+    theme(legend.key.size    =unit(2,"cm")    ,panel.background    =
element_rect(fill="#000000"),panel.grid.major                              =
element_blank(),panel.grid.minor = element_blank())+

geom_segment(aes(x=0,y=0,xend=0,yend=15),colour="#4166F5",size=4)+geom_segm
ent(aes(x=0,y=0,xend=15,yend=0),colour="#4166F5",size=4)+geom_segment(aes(x
=15,y=0,xend=30,yend=0),colour="#606060",size=4)+geom_segment(aes(x=30,y=0,
xend=30,yend=13),colour="#606060",size=4)+

geom_segment(aes(x=30,y=17,xend=30,yend=30),colour="#F4E285",size=4)+geom_s
```

```
egment(aes(x=15,y=30,xend=30,yend=30),colour="#F4E285",size=4)+geom_segment
(aes(x=0,y=30,xend=15,yend=30),colour="#00A550",size=4)+geom_segment(aes(x=
0,y=15,xend=0,yend=30),colour="#00A550",size=4)+

geom_segment(aes(x=30,y=13,xend=30,yend=17),colour="#D4D4D4",size=6)+xlim(0
,30)  +  ylim(30,0)+xlab("x  in  cm")+ylab("y  in  cm")+labs(fill="Unit1  in
Hz")+annotate("text",x=29.9,y=15,label="gate",color="black",size=5,angle=90
)+
    geom_circle(aes(x0=5.5,     y0=24.5,     r=1),     color='#4166F5',lwd=2,
inherit.aes=FALSE)

ggsave(paste0(results_path,sprintf('/%s_F4_1_tileUnit1HzRoll15.png',fname))
,height = 10, width=11)


ggplot(food4Werte,aes(x=floor(x/3)*3,y=floor(y/3)*3))+geom_tile(aes(fill=ro
ll15Unit1Hz))+scale_fill_gradientn(colours=rev(jcolors("rainbow")),limits=c
(0,MaxUnit1))+  theme(legend.key.size  =unit(2,"cm")  ,panel.background  =
element_rect(fill="#000000"),panel.grid.major                              =
element_blank(),panel.grid.minor = element_blank())+

geom_segment(aes(x=0,y=0,xend=0,yend=15),colour="#4166F5",size=4)+geom_segm
ent(aes(x=0,y=0,xend=15,yend=0),colour="#4166F5",size=4)+geom_segment(aes(x
=15,y=0,xend=30,yend=0),colour="#606060",size=4)+geom_segment(aes(x=30,y=0,
xend=30,yend=13),colour="#606060",size=4)+

geom_segment(aes(x=30,y=17,xend=30,yend=30),colour="#F4E285",size=4)+geom_s
egment(aes(x=15,y=30,xend=30,yend=30),colour="#F4E285",size=4)+geom_segment
(aes(x=0,y=30,xend=15,yend=30),colour="#00A550",size=4)+geom_segment(aes(x=
0,y=15,xend=0,yend=30),colour="#00A550",size=4)+

geom_segment(aes(x=30,y=13,xend=30,yend=17),colour="#D4D4D4",size=6)+xlim(0
,30)  +  ylim(30,0)+xlab("x  in  cm")+ylab("y  in  cm")+labs(fill="Unit1  in
Hz")+annotate("text",x=29.9,y=15,label="gate",color="black",size=5,angle=90
)+
    geom_circle(aes(x0=5.5,     y0=24.5,     r=1),     color='#4166F5',lwd=2,
inherit.aes=FALSE)

ggsave(paste0(results_path,sprintf('/%s_F4_3_tileUnit1HzRoll15.png',fname))
,height = 10, width=11)
}
####plotLocalMax
plotLocalMaxAlles                                                          <-
function(idxLocalMax,timeRange,Unit,behavior1,labelBehav1,titleBehav1,behav
ior2,labelBehav2,titleBehav2,behavior3,labelBehav3,titleBehav3,titleUnit,po
sition){
  xMaxDefault=150
  xMinDefault=-150
  for(i in idxLocalMax) {
    xMin=timeRange[i]+xMinDefault
    xMax=timeRange[i]+xMaxDefault
    xLimits=c(xMin,xMax)

png(paste0(results_path,sprintf('/All_%s_%s_%s_%s_%s_LocalMaxima.png',fname
,position,i,titleUnit,titleBehav1)),width=3060,height=2036,res=300,type    =
"cairo-png")
    par(mar = c(6,8,2,8)+0.1)
    plot(timeRange,Unit,xlim=xLimits,ylim=c(min(Unit[i-
2700:i+2700],na.rm=TRUE),max(Unit[i-
2700:i+2700],na.rm=TRUE)),type="l",xaxt="none",yaxt="none",xlab="",ylab="")
```

```
axis(1,at=seq(xMin,xMax,by=10),labels=seq.int(xMinDefault,xMaxDefault,by=10
),lwd=2)
    mtext(("frames"),side=1,line=2)
    axis(side=2,lwd=2,at=seq(round_any(min(Unit[i-
2700:i+2700],na.rm=TRUE),10),round_any(max(Unit[i-
2700:i+2700],na.rm=TRUE),10),by=20))
    mtext(("hertz"), side = 2, line = 2)
    abline(v=timeRange[i],col="firebrick3",lwd=2)
    par(new=TRUE)

plot(timeRange,behavior1,xlim=xLimits,ylim=if(titleBehav1=="speed"){c(0,10)
}else{c(0,max(behavior1,na.rm=TRUE))},type="l",col="cyan3",xaxt="none",yaxt
="none",xlab="",ylab="")
    axis(side=2,lwd=2,line=4)
    mtext((labelBehav1), side = 2, line = 6)
    par(new=TRUE)

plot(timeRange,behavior2,xlim=xLimits,ylim=if(titleBehav2=="speed"){c(0,10)
}else{c(0,max(behavior2,na.rm=TRUE))},type="l",col="green3",xaxt="none",yax
t="none",xlab="",ylab="")
    axis(side=4,lwd=2)
    mtext((labelBehav2), side = 4, line = 2)
    par(new=TRUE)

plot(timeRange,behavior3,xlim=xLimits,ylim=if(titleBehav3=="speed"){c(0,10)
}else{c(0,max(behavior3,na.rm=TRUE))},type="l",col="orange2",xaxt="none",ya
xt="none",xlab="",ylab="")
    axis(side=4,lwd=2,line=4)
    mtext((labelBehav3), side = 4, line = 6)
    par(fig = c(0,1,0,1),oma = c(0,0,0,0) ,mar = c(0,0,0,0),new=TRUE)
    plot(1, type = "n", axes=FALSE, xlab="", ylab="")
    plot_colors <- c("firebrick3","black", "cyan3","green3","orange3")
    legend(x = "bottom",inset = 0,
           legend = c(i,titleUnit,titleBehav1,titleBehav2,titleBehav3),
           col=plot_colors, lwd=5, cex=1, horiz = TRUE,bty="n")
    dev.off()
  }
}
```