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Neural correlates of social behaviour in the honeybee brain

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Declaration

I hereby declare that the work presented in this thesis has been conducted independently and without inappropriate support. All sources of information are referenced. I 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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This dissertation includes the following manuscripts:

Chapter 2

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Contributions:

A.D. and B.P built the equipment, performed the recordings and analyzed the data. A.D. wrote the MathLab routines for analyzing the walking tracks. R.M. wrote the manuscript together with A.D. and B.P.

Contents

1 Summary

The honeybee (Apis mellifera) is an excellent model organism for social and navigational aspects of ethology. It is also heavily investigated with regard to neuronal circuits involved in vision, olfaction and memory. The aim of the current study is to correlate for the first time those peculiar and complex behaviours with the activity of neurons in certain brain areas. There is no knowledge yet on how social interactions are represented in the brain of an insect. To find answers in this regard, a mini colony of worker bees and one honeybee queen was situated in an arena. The behaviour within the artificial hive was monitored with a video camera by the aid of infra-red illumination. A custom written code was used to convert the video data into coordinates and head directions. For an experiment, a bee of interest was taken from the colony. The bee was then equipped with twisted one-meter-long two channel copper wire electrodes. The tips of the electrodes were plated with gold to lower the impedance by two orders of magnitude. Once the electrodes were situated at the beta exit of the mushroom body (MB), the electrodes were attached to the bee's head using non-toxic silicone. After this procedure, the immobilised bee was placed back into the hive. We recorded the bee's behaviour using a video and the neuronal activity with the extracellular setup. For the full duration of the experiment, the animal was free to move and behave as it does naturally. I could establish that the social behaviour of the whole colony is not restricted or modified in any way that might be of relevance in the current study. The bees exhibit, qualitatively, natural brood care, foraging waggle dancing, sleep and circadian rhythm. To some degree, the total amount or proportion does diverge due to the small number of initial worker bees of around 1 000. The extracellular recordings did not differ in any way from traditional recordings concerning the quality with regard to sortability and long-term stability for up to 24 hours. We found an overall low baseline activity when compared to experiments that were carried out with restrained honeybees. A multiverse analysis was created to search for any correlations between the neuronal activity of high-order MB output neurons and the behaviour extracted from the video recording. A result found over several of the successful experiments was an increase in spike rate variance for time windows in which social interactions occurred when compared to equal time windows in which the recorded bee was alone or random time windows. For the future I suggest to

iuntroduce a feeding mashine into the arena that may motivate the recorded bee to show more repeaded behaviour. Such devices can train bees in a classical or operant conditioning way.

2 Zusammenfassung

Zusammenfassung Die Honigbiene (Apis mellifera) ist ein ausgezeichneter Modellorganismus für soziale und navigatorische Aspekte der Ethologie. Es wird auch in Bezug auf neuronale Schaltkreise, die am She- und Geruchs-vermögen und am Gedächtnis beteiligt sind, intensiv untersucht. Das Ziel der vorliegenden Studie ist es, diese einzigartigen und komplexen Verhaltensweisen erstmals mit der Aktivität von Neuronen in bestimmten Hirnarealen zu korrelieren. Es gibt noch keine Erkenntnisse darüber, wie soziale Interaktionen im Gehirn eines Insekts representiert werden. Um diesbezüglich Antworten zu finden, befand sich in einer Arena eine Minikolonie von Arbeiterinnen und einer Honigbienenkönigin. Das Verhalten innerhalb des künstlichen Bienenstocks wurde mit Hilfe einer Videokamera und Infrarotbeleuchtung überwacht. Ein speziel hierfür geschriebener Code wurde verwendet, um die Videodaten in Koordinaten und Kopfrichtungen der Bienen umzuwandeln. Für ein Experiment wurde eine Biene von Interesse aus der Kolonie genommen. Die Biene wurde dann mit verdrillten, ein Meter langen, Zweikanal-Kupferdrahtelektroden ausgestattet. Die Spitzen der Elektroden wurden mit Gold plattiert, um die Impedanz um zwei Größenordnungen zu verringern. Sobald sich die Elektroden am Beta-Ausgang des Pilzkörpers (MB) befanden, wurden die Elektroden mit ungiftigem Silikon am Bienenkopf befestigt. Nach diesem Verfahren wurde die immobilisierte Biene in den Bienenstock zurückgebracht. Wir haben das Verhalten der Biene unter Verwendung eines Videos und die neuronalen Aktivität mit dem extrazellulären Aufbau aufgezeichnet. Während der gesamten Dauer des Experiments konnte sich das Tier frei bewegen und verhalten, wie es unter natürlichen Umständen der Fall ist. Ich konnte feststellen, dass das Sozialverhalten der gesamten Kolonie in keiner Weise eingeschränkt oder modifiziert wird, auf eine Weise die in der aktuellen Studie relevant sein könnte. Die Bienen zeigen qualitativ, natürliche Brutpflege, Schwänzeltanzen, Schlaf und zirkadianen Rhythmus. Bis zu einem gewissen Maß divergiert die Gesamtmenge

oder der Gesamtanteil aufgrund der geringen Zahl von etwa 1 000 Arbeiterbienen. Die extrazellulären Aufzeichnungen unterschieden sich in keiner Weise von traditionellen Aufzeichnungen hinsichtlich der Qualität in Bezug auf Sortierbarkeit und Langzeitstabilität für bis zu 24 Stunden. Im Vergleich zu solchen klassischen Experimenten, fanden wir eine insgesamt niedrige Grundaktivität. Eine Multiverse-Analyse wurde erstellt, um nach Korrelationen zwischen der neuronalen Aktivität von MB-Ausgangsneuronen höherer Ordnung und dem aus der Videoaufzeichnung extrahierten Verhalten zu suchen. Ein Ergebnis, das in mehreren der Experimente gefunden wurde, war eine Zunahme der Spikeratenvarianz für Zeitfenster, in denen soziale Interaktionen im Vergleich zu gleichen Zeitfenstern auftraten, in denen die abgeleitete Biene alleine war oder zufällige Zeitfenster. Für die Zukunft schlage ich vor, eine Futtermaschine in die Arena zu bringen, die die abgeleitete Biene dazu motivieren kann, mehr wiederholtes Verhalten zu zeigen. Solche Vorrichtungen können Bienen in einer klassischen oder operanten Konditionierungsweise trainieren.

3 General Introduction

The goal of this study was to acquire neuronal activity of high order interneurons in freely behaving honeybees (Apis mellifera L.) under near natural conditions. Therefore, I have constructed an experimental setup that mainly consists of a horizontally tilted (17) arena for a mini colony. The arena was connected to the outside world with a tube. The temperature and humidity in the arena was tightly controlled. The colonies consisted of 250 – 1000 worker bees and one queen. One bee of interest was picked and prepared with a one-meter-long two channel extracellular electrode. This bee was situated back into its hive to investigate its spontaneous behaviour. The behaviour was recorded by an infra-red camera and suitable illumination. Custom written scripts extracted the coordinates and head direction of the recorded bee and its twelve closest conspecifics. The neural activity of mushroom body output neurons was synchronously recorded and sorted based on their spike shapes.

These experimental conditions allowed me to investigate complex behaviour in the honey bee. The unhindered nature of the long recording electrodes enabled intrinsi-

cally motivated behaviour that cannot be seen in restrained bees. The bee of interest could follow a dance, care for brood or show any of the naturally occurring behaviours that makes the honey bee such an interesting to study model organism. This study was neuro ethologically motivated, correlating brain activity with free behaviour under natural conditions.

Chapter 1

Here I analyze the integrity of the social group. I discuss measures to check if the hive is showing natural behaviour in whole. This study is not meaningful as long the bees in the experimental arena do not show their full repertoire of behaviours. Therefore, I analyzed their group behaviour regarding the definitions of eusocial and sleeping behaviour. When the bees show cooperative brood care, overlapping generations and a division of labor I am confident to assume natural social behaviour. Sleeping behaviour can easily be disturbed by different factors and its circadian rhythm is passed on within the colony in a social manner. Therefore, I will use this measure as further evidence for natural behaviour.

Chapter 2

In this chapter we describe in great detail how we conducted the experiments and show our first iteration of analysis of the resulting data. To prepare the bee of interest with the recording electrodes as well as suspending the long electrodes some clever tricks were implemented. Precisely positioning the electrode at the investigated neuropil and attaching the wire at the bee's head was critical. For the bee to move within the arena freely the electrode wires were suspended by a spring that would move several centimeters when being actuated by just a few milligrams of pulling force. We discuss the quality of electrophysiological data and compare it with the well-established methods of having restrained animals.

Chapter 3

All data acquired within this study was analyzed here in search for any correlation of neuronal activity changes and the recorded behaviour. The unprecedented freedom in choice of behavior by the bee from its vast repertoire coupled with the complete

lack of control by the experimenter led me to an exploratory multiverse analysis. I hence searched for any correlation between any aspect of the neuronal domain versus any behavioral feature. All sensible combinations were checked systematically. I discuss spike frequency changes related to social interactions. Interestingly those were partially before, while or after the event. The neuronal response was surprisingly diverse as they varied from increase to decrease that stretched over seconds or in some animals several minutes.

The honey bee is a fascinating animal. It is impossible to sit in front of an observation hive and not be amazed. They run between each other eagerly, transporting foraged pollen and nectar. They care for brood and attend the queen. Quite frequently you will see bees communicate by symbolic dancing behaviour. This behaviour was first noted by Aristoteles (4. century) and later on investigated by Karl von Frisch (1946) who was then rewarded with the Nobel prize.

The bees are not just an important model organism in several sciences, they are of uttermost importance for the pollination of more than two thirds of the world's crop species (Roubik 1995). They are as important for the diversity in our ecosystem as well (Allen-Wardell et al. 1998). Insects inspire scientists across many disciplines (Beisel et al. 2013). The honey bee in particular is a well-established model in behavioral science and neuroscience.

In the behavioral science researchers are investigating the social construct of the colony (Michener 1974, Zayed and Robinson 2012), the supraorganism (SeeFseelly 1989, Rössler 2014, Lüttge et al. 2016).

In the neuroscientific field much research was carried out with respect to learning and memory in the honey bee (Menzel et al. 1974, Menzel 1983, Menzel 1993, Hammer and Menzel 1995, Okada et al. 2007, Denker et al. 2010, Menzel 2014, Strube-Bloss et al. 2011, 2016). The honey bee performs many behaviours under natural conditions in a social context. Bees, as eusocial animals, show division of labor (Lindauer 1952, Huang and Robinson 1996) and therefore a variety of interactions. They can inter-

act with each other by antennation (Rogers 2013) and trophylaxis (Korst and Velthuis 1982). When they forage they can navigate to food sources (Von Frisch 1967, Menzel et al. 1996, Menzel et al. 2005, Menzel and Greggers 2015). This very impressive behaviour is even overshadowed by their capability to communicate these places by the waggle dance behaviour to other forager bees (Von Frisch 1967, Grüter and Farina 2009, Seeley 2012).

None of the before mentioned behaviours is understood to a sufficient degree. They are not at all understood on the neuronal level since they are not investigated in such manner until now.

Honeybees show circadian rhythm (Bloch et al. 2001); they are more active at day time then at night. This is particularly true for the foraging worker bees (Bloch 2010); they need sunlight to navigate. Honeybees sleep. They have compound eyes so we cannot measure their rapid eye movement do detect REM sleep. But many other characteristics of sleep can be applied for the honeybee. They show specific sleep postures and specific sleep places (Kaiser, 1988), rapid reversibility of sleep, a reduced reaction threshold and sleep rebound after sleep deprivation (Siegel, 2008). Bees are rather picky concerning the physical properties of their nest; the volume of their nest as well as the hive entrance have to be within certain limits (Seeley 1976, 1977) so special attention will be paid towards stable colony behaviour.

The brain of the honeybee contains less than one million neurons in one cubic millimeter. It contains the mushroom bodies (MB), first identified in 1850 by Félix Dujardin. This paired structure consists of two calyces each that are connected with the alpha and beta lobe via peduncle. The input region, the calyxes are multimodal. They receive olfactory input from the antennal lobes and visual inputs from lobula and medulla and also mechanosensory and gustatory input (Mobbs 1982, Rybak and Menzel 1993, Gronenberg 2001, Schröter and Menzel 2003). The MB consists of Kenyon cells, an intrinsic neuron type. Not all Kenyon cells get input by projection neurons from other brain regions, some get inhibitory input from recurrent neurons of the protocerebral calyx tract (Grünewald 1999). The 14 000 Kenyon cells are connected to

roughly 100 MB output neurons. The connectivity in the MB is in parallel to the direct pathway, were the sensory input is connected to the central complex or the lateral protocerebrum to the premotor pathway (Menzel 2013). Functionally the MB is highly involved in learning and olfaction (Hammer and Menzel 1995, Grünewald 1999, Hussaini and Menzel 2013). More importantly for this study, it is also the candidate neuropil for attention (van Swinderen 2003 [Drosophila], Xi et al. 2008 [Drosophila]) and valence (Menzel 2012, Aso et al. 2014 [Drosophila]). MB output neurons can integrate context and cue so a learned context leads to an expectation of the learned cue (Filla and Menzel 2015).

We recorded at the beta exit of the alpha lobe, here we expect MB extrinsic neurons that most likely belong to the group of A1, A2 and A4 neurons (Rybak and Menzel 1993). They response to multiple sensory stimuli (Homberg and Erber 1979, Rybak and Menzel 1998). They adapt their response pattern during learning (Mauelshagen 1993, Okada et al. 2007, Strube-Bloss et al. 2011, Hussaini and Menzel 2013).

A growing number of neuroethological (Tinbergen 1963) experiments are taking place were the insects brain activity is recorded and the animal can behave freely (Mizunami et al. 1998 [cockroach], Takeuchi et al. 2004 [cockroach], Mu and Ritzmann 2005 [cockroach], Guo et al. 2014 [cockroach], Harrison et al. 2011 [dragonfly], Thomas et al. 2011 [dragonfly], Fischer et al. 1996 [locust]).

Furthermore, the honeybee is discussed as a model for cognition in insects (Menzel and Giurfa 2001, 2006, Srinivasan 2010, Menzel 2012, Giurfa 2013, Menzel 2017).

If the here proposed experimental approach can be demonstrated as successful concerning quality of neuronal data and naturality of behaviour, I will suggest a variety of cognitive neuroethological experiments.

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4 Chapter 1: Experimental Colony—Natural and Social Behaviour

4.1 Abstract

The social behaviour of honeybees (Apis mellifera) has been well investigated, but little is known about its neuronal correlates. Recently, it became possible to measure the activity of a small amount of neurons in the brain of a freely behaving bee inside a hive by means of extracellular recording. For this purpose, a mini colony of 1 000 honeybees was kept under near-natural conditions. One of these bees was equipped with an extracellular recording electrode that is long enough so the bee could move freely in the hive. The recorded animal behaves in the social context of its colony. The neuronal activity is correlated with that behaviour. Recorded data were further analysed using established methods of principle component analysis (PCA), cross-correlation and autocorrelation to evaluate the quality of neuronal signals. Evaluating the behaviour of the colony is more demanding and is the main aim of this chapter. The power of the experimental set-up employed in the current study relates to the opportunity to correlate neuronal activity with intrinsically motivated behaviour. The correlation of neuronal activity with performed behaviour requires the continuous monitoring of all exerted actions in the colony. Furthermore, the colony needs to be healthy to obtain reliable and meaningful data. It is, therefore, necessary to ensure the functioning of the eusocial group from an apiarist perspective by detailed observation of all known properties. Furthermore, the whole hive was continuously recorded on video for a period of two months and subsequently analysed by a custom-written computer program. To confirm that the honeybees behave naturally within the experimental hive, the animals were checked to see whether they exhibited typical changes in motor activity during the day (circadian rhythmicity). In addition, sleeping places and the circadian distribution of sleeping bouts were analysed to control the condition of the colony.

4.2 Introduction

The honeybee (Apis mellifera) is an established model organism for neuroscience (Menzel and Giurfa 2001, Srinivasan 2010, Galizia et al. 2011, Menzel 2012). Many

previous electrophysiological studies revealed neuronal pathways concerning olfaction (Mauelshagen 1993, Szyszka et al. 2005, Okada et al. 2007, Strube-Bloss 2011), colour vision (Menzel 1973, Menzel and Blakers 1976, Vorobyev et al. 2001) and learning (Hammer 1993, Hammer and Menzel 1995), to name a few (Review: Menzel and Giurfa 2006). Also well investigated are social behaviour (Michener 1974, Zayed 2012) and navigational capabilities (Von Frisch 1967, Menzel et al. 1996, Menzel et al. 2005, Menzel and Greggers 2015) of honeybees. Correlating neuronal activity in well-known neuropils such as the mushroom body (Rybak and Menzel 1993, Heisenberg 2003, Okada et al. 2007) with motivated behaviour would be desirable. Grooming, queen attending, food processing, and in particular waggle dancing (receiving or transmitting) would be such behaviours. The main goal of the current study is to prove the naturality of all behaviours exhibited by a group of bees in the study's experimental set-up. Essentially, the data consist of videos that capture the behaviour of the bees. Extracellular recordings from the brain region of interest capture the neuronal correlates of the behaviour. Data arising from extracellular recordings can be tested in well-established ways. For instance, principle component analysis (PCA) allows one to measure spike shape stability within a template and separability between templates. The inevitable refraction time can be used as an indicator for correctly separated spike units by computing their autocorrelations. Signals assigned to independent neuronal sources can be tested through cross-correlations. Such tools and their objectivity are not accessible for the investigation of natural and social behaviour. So, here I investigate different aspects of the behaviour to strengthen the argument that the behaviour, displayed by the bees in this setup, is natural. Certain hymenoptera (e.g., the honeybee, Apis mellifera) and termites are eusocial insects. A eusocial insect is characterised by cooperative brood care, overlapping generations and division of labour (Crespi and Yanega 1995). The division of labour in bees was described in detail by Rösch (1925) and Lindauer (1954). A healthy honeybee colony consists of 1 queen, 10 000 to 60 000 worker bees and, depending on the season, a few hundred drones. This group, often called a supraorganism (Lüttge et al. 2016), inhabits a nest called the hive. The natural nest volume of non-domesticated honeybees and bees that an apiarist takes care of varies between 20 and 100 litres (Seeley 1977). In nature, the nest's cavity and the outer end of its entrance can be as distant

as 74 cm (Seeley 1977). The entrance of the hive is typically located at a height below 2 m from the ground (Seeley 1977). Within the cavity, the bees build and live on wax combs, which are built from the top hanging down vertically. The wax combs consist of cells on which the bees walk, sleep and dance. Those cells can be empty or contain brood, pollen or honey. A worker bee has a life expectancy of around six weeks. However, in fall, when the outside temperature falls under 12°C, the bees become winter bees. Winter bees' lifespan is extended to roughly six months (Furgala 1975). The honeybee queen can survive for more than three years (Page and Peng 2001). A healthy queen can lay 2 000 eggs per day (Winston 1987). She lays fertilised eggs that become workers and unfertilised eggs that become drones (Dzierzon 1845). In a healthy hive, the queen is the only bee that reproduces, whereas young worker bees care for the brood. Queen-attending workers collect and distribute queen mandibular pheromone, which binds the social group together (Seeley 1996). Roughly a third of the workers are taking care of the brood (nurse bees), while another third takes care of the queen, cleans the hive, builds comb and processes food (house bees); the last third is foragers (Robinson 1992), which are of particular interest. They can forage for pollen, nectar and water. When a forager finds a food source, it can keep the distance and direction of the source relative to her hive in her memory. This vector is used by the bee to navigate to that particular food source again. This vector can also be communicated by a symbolic dance. This is called a waggle dance, and it allows other forager bees to also visit that food source (Von Frisch 1967). This remarkable information transfer would be extremely interesting to measure on the neuronal level. Honeybees actively regulate the temperature and humidity in the hive (Simpson 1961). They can lower the temperature inside the hive by both fanning and water evaporation (Lindauer 1954). The fanning behaviour also lowers the humidity. The development of the brood is temperature sensitive (Medrzycki et al. 2010). The bees can upregulate the comb temperature by both generating metabolic heat and forming a dense cluster (Seeley 2014). They keep the brood at 34.5–35.5 °C (Heran 1952). Under natural conditions, brood-free combs have a constant temperature of approximately 25°C. Accordingly, this characteristic property can be used to evaluate the state of the hive (Simpson 1961). Other voluntary behaviours of the bee that can be used as indicators of natural conditions are sleeping (Kaiser and Steiner-Kaiser 1983, Cirelli

and Tononi 2008) and motor activity changes based on the circadian rhythm (Moore et al. 1989). The circadian clock is socially regulated and important for synchronisation of worker activities (Bloch 2010). Bees that stay in the hive to take care of the brood, for example, work around the clock. They sleep independently of the time of day. Foraging bees sleep at night and are more active during day time (Bloch et al. 2001). Sleeping bees can be detected by immobility for a certain time frame. Since sleep is important for extinction learning (Hussaini et al. 2009) and precise waggle dances (Klein et al. 2010), sleep-deprived honeybees would behave unnaturally, possibly due to an environment that does not accommodate their needs. I expect cooperative brood care by nurse bees in the reproduction cycle of the honeybee. Overlapping generations are present when the queen produces eggs constantly. In addition, there should be adult bees for the whole experimental season, which is substantially longer than the lifespan of a worker bee. The division of labour is observable when bees take care of the brood, the nest and the foraging of resources.

4.3 Methods

4.3.1 The animals

In the experiments described in this chapter, 1 000 exclusively freshly hatched bees that were younger than one day were included in the colony. Additionally, a brood comb together with a fertilised queen was added to the arena.

4.3.2 The set-up

The freshly prepared arena consisted of a 15° tilted square board with a length of 55 cm per side. The board was surrounded by an acrylic glass frame of a 10 cm height. It was coated with a dry Polytetrafluorethylene (PTFE, also called Teflon) spray to prevent bees from walking over it as the barrier. The barrier had one opening where a silicone tube connected the arena to the outside of the building with a length of 50 cm. The channel did not consist of a continuous tube as described in Chapter 2. Instead, the middle third of the tube was cut out and replaced by a metal mesh bend in the form of a tube that was tightly connected to the two ends of the silicone tube. The whole part was surrounded by several layers of a mosquito net so bees could not escape by mistake. Under certain weather conditions, the air draft disturbed the bees,

especially at night when cold air was introduced into the arena. To further undock the air movement, three cardboard shields were introduced into the mesh part in a diagonal manner to push incoming air out. The arena was situated inside a wooden box that could be opened to interact with the bees but was generally closed to prevent air drafts as well as temperature shifts and to keep out light and odours. The inside of the wooden box was temperature regulated to ensure a constant air temperature of 30°C.

4.3.3 The observations

The health of the hives and the behaviour of the individual colony members were observed in person through the eye. Furthermore, a camera system collected video data of the whole colony for 2 months. To the roof of the set-up box, a camera, an infra-red light source and electrophysiology equipment were attached. Additionally, for this investigation, as well as for more comfortable day-to-day monitoring of the hive, a Raspberry Pi (Raspberry Pi 2, Raspberry Pi Foundation, Cambridge, UK) with a camera module (noIR cam, Raspberry Pi Foundation, Cambridge, UK) was introduced. The Pi was set up as a webcam server supplying the most recent frame of the cam as a picture tied to a fixed IP address and a port. Thus, any device connected to the internet can access this frame through a browser. The camera refreshed the picture every second by acquiring a new picture from the hive. The pictures were black and white. Because of the infrared illumination, there was no colour information. The resolution of the pictures was 1 200 by 1 600 pixels. For this experiment, the stream of pictures was grabbed consistently by a PC. The VLC media player (VideoLAN, Paris, France) handled the data stream and converted it to a highly compressed video. The videos were automatically named by date as well as time and compressed to one hour files. The used type of video compression, frame rate (1 fps) and grey-scale frames resulted in a data density of 250 megabytes per day. A custom MATLAB (MATLAB R2012a, The MathWorks, Natick, Massachusetts, USA) script was used to differentiate bees from the background of every frame to get coordinates of the bees over time. All of the analysis presented in the current study was done in MATLAB. A detailed description of the experimental set-up can be found in the methods section of Chapter 2. Relevant for this discourse are mostly the colonies' housing and climate conditions.

4.4 Results

Forming of the cluster A group of approximately 1 000 honeybees was introduced into the experimental arena. A tube of water and a feeder containing one molar sucrose solution were supplied. The bees explored the arena and covered the space evenly. None of them flew or made any quick movements. They started to form groups of 10 to 50 bees at several locations for short periods of time. Those groups were formed and disassembled within minutes. Over time, the groups grew in size and shrunk in number. After two hours, there were two to six groups at a time containing most of the bees. After four hours, the animals assembled to one group that was stable for four consecutive hours. Afterwards, the bees regrouped again within two hours below the feeder in the upper left corner of the arena. The position of the bee group changed a few more times since the position of the feeder was moved and a piece of brood comb as well as a fertilised queen were introduced. The queen was caged for the first two days. The piece of brood was introduced to bind the bees at a desired location. Young bees cared for the brood, and the queen stayed close to them. Some worker bees started building a comb near the brood. The bees aggregated in the upper left corner for the rest of the experiment. On the 12th day around noon, the empty piece of the brood comb was removed. A cluster of bees has formed around the queen and considered stable, since its centre had not moved anymore. When the cluster had formed, the bees behaved calmly.

Colony behaviour Daily inspections took place around noon, typically for 10 minutes. When light and air movement was introduced by opening the set-up hood, the animals did not display any aggressive or escapist behaviour. Within this time, one or no bee flew off the floor. Most cases of such behaviour seemed to be of exploratory nature. Only occasionally was a bee defensive. This was noticeable by a different tone in wing buzzing and a direct approach towards the experimenters face or hands. Additionally, there was no swarming behaviour. The queen laid eggs and stayed in the cluster (Fig. 4.1A) whenever she was inspected. She was constantly surrounded by bees and laid 20 to 50 eggs per day. The eggs were placed close to one another with only a few single gaps of empty cells. A healthy brood pattern was observed (Fig. 4.1B). The queen survived the whole experimental season, specifically from the

beginning of August to the middle of January 2016.

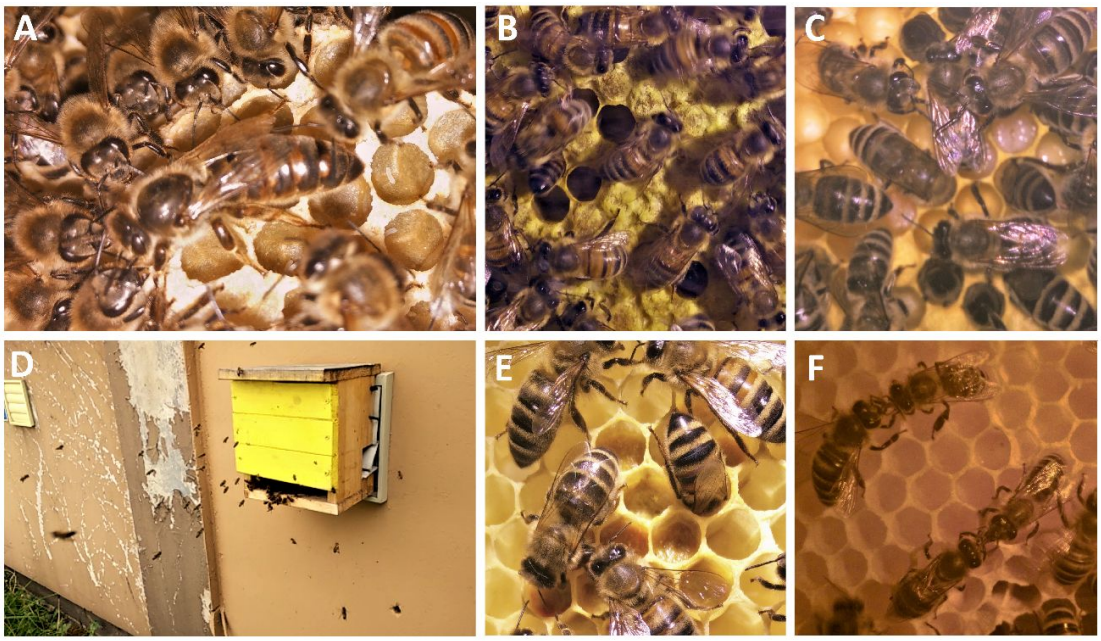

Figure 4.1: Observed examples of natural behaviour in the experimental hive. Photographs of different behaviours. **a** The honeybee queen attending other bees and freshly laid eggs (middle right). **b** Sealed brood with a regular brood pattern. **c** Open brood and nurse bees. **d** Orientation flights of young bees around the hive's entrance. **e** Cells with stored pollen the bees had gathered. **f** Trophallactic interactions near the hive's entrance on wax combs the bees had drawn.

The worker bees that were put into the arena lived for around six weeks. That was also true for bees that emerged from eggs the queen had laid. Only in November were there less dead bees carried out of the hive and fewer eggs laid. The animals seemingly had become winter bees; those bees stopped foraging outside and survived from November to January. The worker bees had behaviours for various labours. They built comb cells from the wax of the foundation on the ground. The area covered by wax combs quickly became larger than the size of the cluster of bees. Some workers cared for the eggs the queen had laid. This was noticeable from the occurrence of food surrounding larvae (Fig. 4.1C). Once the entrance of the arena was opened, bees were flying out to do orientation flights (Fig. 4.1D). The first few flights covered a small volume. They explored increasingly larger areas around the hive entrance and started

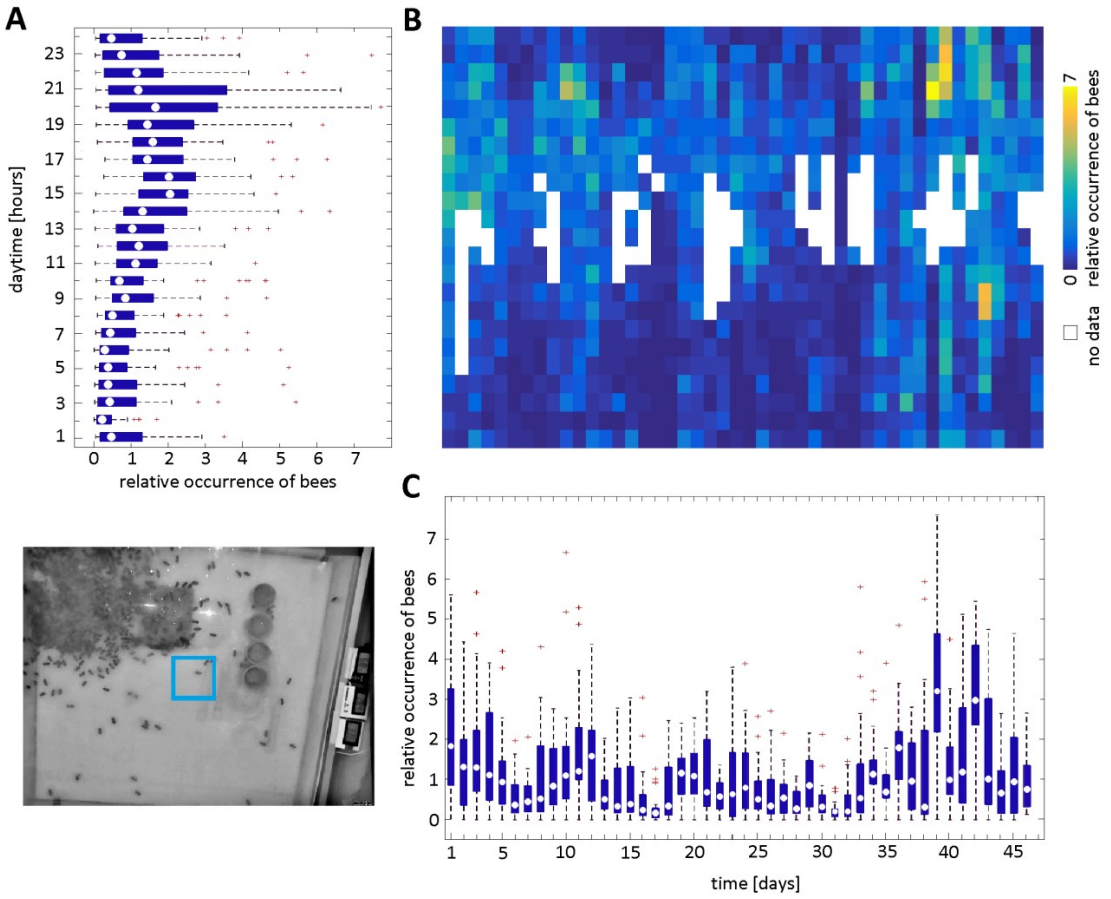

Figure 4.2: Temporal distribution of honeybees in the middle of the experimental arena. The area of 9 × 9 cm in the middle of the arena (blue box in the photograph) was taken to measure colony activity. This was achieved by counting bee tracks occurring in this field. This area was not occupied by the bee cluster or any obstacles. Forty-six days' worth of tracks were counted per hour and averaged according to the whole data set. **a** A boxplot of relative occurrence of bees per time of day in hours. Less bee occurrence was counted between 22:00 and 10:00 MEZ. **b** Heat map of relative bee occurrence in false colour. Experimental days are on the x axis, and times of day are on the y axis. White squares indicate missing data. **c** Boxplot of relative occurrence of bees per experimental day. High differences in distribution of bee attendance in the middle of the arena across a two-month period from August to September.

foraging within a day. This was observable by the presence of pollen on bees' legs and pollen in cells (Fig. 4.1E). The bees most likely foraged nectar. We only supplied a

sucrose solution within the hive, so we cannot know where the liquid in the cell came from. The bees had trophallactic interactions (Fig. 4.1F) and dancing behaviour (Subvideo 1). Most of the dancing bees had no pollen on them, so we conclude that they had been foraging for nectar. Each waggle phase per dance was indicating similar directions. The dances as a whole were evaluated as ordinary. The dancing bees had followers. We estimated that around 10% of the animals were foragers. The amount of dancing was varying, mainly depending on weather conditions. If the outside temperature was high enough and no rain occurred, one to three honeybees were constantly dancing. The portion of bees interacting with the queen and the brood was much higher. Other noticeable occupations were bees guarding the entrance by interacting with every bee coming in, seemingly guarding bees. Food-processing bees and, as mentioned earlier, bees cleaning out dead bees were also identified. The bees tried to regulate the hive temperature by fanning on very hot days. At night and in the winter time, they moved closer together in the cluster around the queen. This resulted in a much smaller diameter and higher density of the cluster.

Motor activity changes over time

To investigate undisturbed social behaviour and natural behaviour of honeybees in general, measuring circadian rhythm is appropriate. Since the video sequence that was analysed in the current study had a frame rate of 1 frame per second, the bee cluster could be tracked because of the good resolution, and individual animals could not be tracked because of the low frame rate. Bees walked faster between frames and were more densely distributed than it would be possible to correctly compute which individual bee belonged to each track from the preceding frame. Therefore, this analysis was focused on the positions of bees that could be captured. Unfortunately, the bee cluster surrounding the queen in the area of the brood nest could mostly not be analysed. The density of bees in the cluster was high enough that the used tracking algorithm was not suited to separate them. The tracking program was dependent on the condition that the object converted to dark pixels had to be mostly surrounded by a bright background. With the resolution of the used camera, nearly all bees were touching one another in the cluster. To acquire any information on the presence of the circadian rhythm, the attendance of bees in an empty area was used (Fig. 4.2C).

The area of 9 × 9 cm in the middle of the arena was taken to measure bee activity. This area was never occupied by any foreign objects or the cluster. The bees only walked through this area or rested there for a short time. The amounts of such bee tracks per time of day were compared. The bees exhibited greater occupation in this empty area during the afternoon (15:00–20:00) and particular low attendance during the night (22:00–10:00; Fig. 4.2A). The amount of bee tracks varied greatly throughout the stable 46 days of the two-month video sequence.

The distribution of bee occurrences was not linearly correlated with outside temperature, humidity or the hours of sunshine (the data are not shown). Nevertheless, this indicates a stable circadian rhythm in the sense of regular changes in behaviour over the course of 24 hours.

Another daytime behaviour is sleeping. The video resolution did not allow any analysis of the antennae position, so I defined sleep as immobility over a time window of five minutes to eight hours. The coordinates of bee tracks that were stable over such time periods were extracted from the videos and plotted on top of a single frame of a video recording from night time. The resulting plot (Fig. 4.3)) indicates a high density of sleeping places around the bee cluster. No bees slept outside the ring-shaped area. However, the area of the bee cluster, (red cycle Fig. 4.3) could not be analyzed due to the aforementioned high density of bees. We could neither by eye nor by video analysis account for the sleeping behaviour in this area.

The bees sleeping in the ring-shaped area surrounding the cluster were counted according to the time of day. The distribution of sleeping bees across all analysed days was the lowest around 11:00 (Fig. 4.4). It was the highest around midnight, thus confirming another indicator of natural bee behaviour.

To conclude, the queen laid eggs and stayed in the bee cluster. At no time did the queen swarm. The worker bees showed all labours, foraging, nurse care, comb building and the like. Different bees' motor activities were measured in an area in the middle of the arena. Those differences were repetitive and stable over a time of day. The bees' sleeping behaviour could be analysed, and it was found that the bees sleep

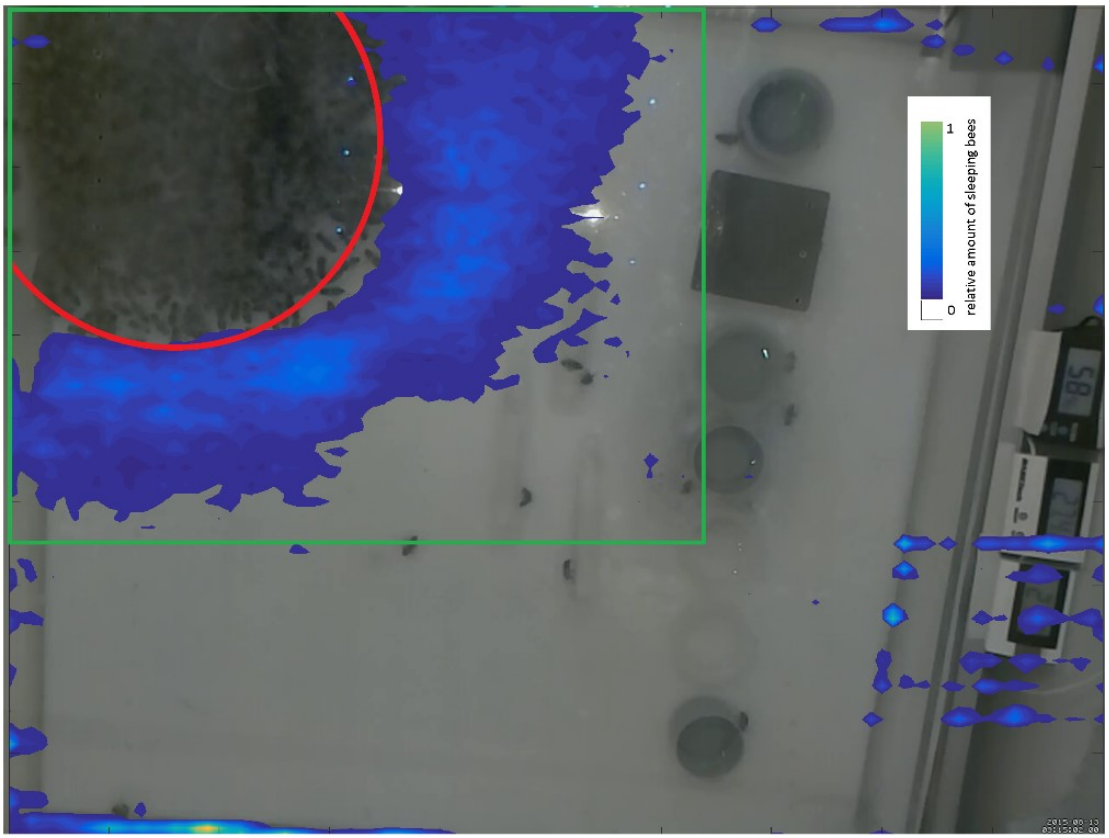

Figure 4.3: Qualitative representation of honeybee sleeping places in the experimental arena. Sleeping honeybees were characterised by a custom computer program using an immobile bee-sized blob for at least five minutes but no longer than 8 hours. Coordinates of all those sleeping bees are plotted as an overlay on a photograph of the arena. False colour of the overlay represents the amount of sleeping bees over a 46-day period. A continuous field of sleeping places is situated around the dense bee cluster. All other indicated places are outside the arena floor and are most likely due to video artefacts. For the following analysis, these data are excluded. The green box indicates the sub-area used for Fig. 4.4; the red circle indicates the area that was not analysed area because of high bee density.

around the bee cluster (or in the cluster; no measurements) primarily at night.

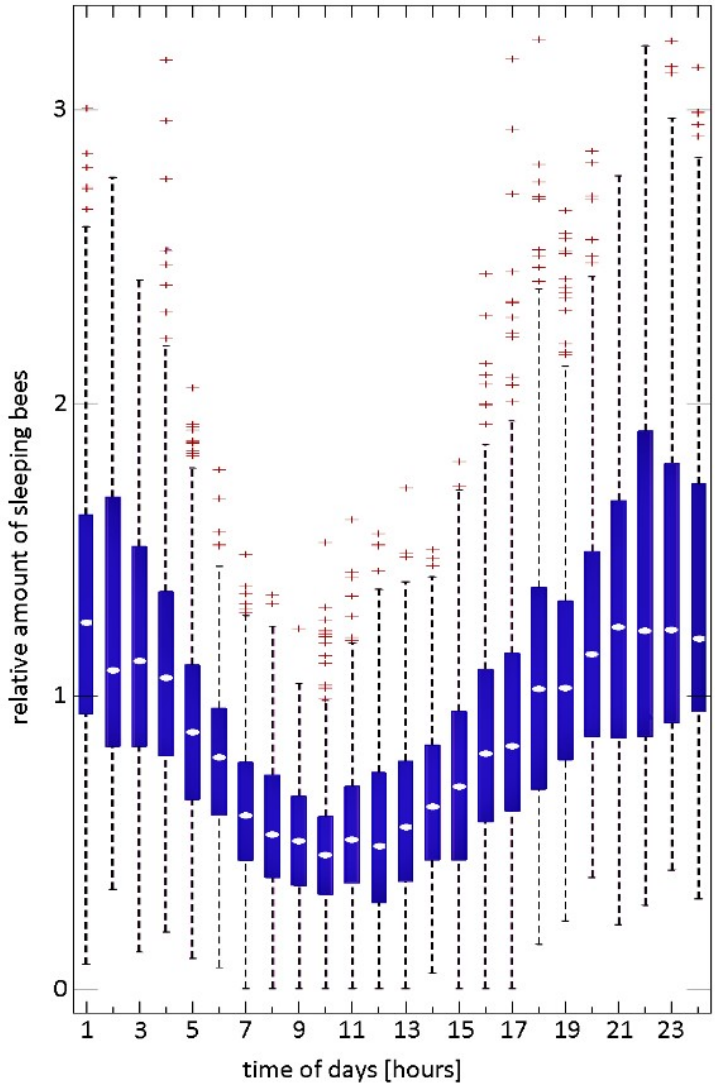

Figure 4.4: Daytime dependent amount of sleeping bees. A boxplot illustrating the distribution of amounts of bees sleeping per daytime hour. This experiment includes two months of data. The relative amount of sleeping bees is lower around noon and higher shortly before midnight. For further experimental and analytical information, refer to Fig. 4.3.

4.5 Discussion

Electrophysiological studies with freely moving and behaving insects are emerging and allow for a deeper look into neuronal representations of complex behaviour (Mizunami et al. 1998, Takeuchi and Shimoyama 2004, Mu and Ritzmann 2005, Guo et al. 2014). The opportunity to investigate social behaviour is even more compelling

(Paffhausen et al. 2015). The curse and blessing of such conditions are the necessity of the animals to freely form a social construct. The bees cannot be forced to develop a colony. The animals need to be in a situation close enough to their natural condition to do so. If that cannot be achieved, the bees will escape or die. The experimental setup discussed in the current study needs to supply an environment in which it is possible to do electrophysiological dissections and the bee colony can adequately live for a reasonable amount of time. The experimental animals were part of a mini-colony composed by around 1 000 worker bees and a queen. The main difference between this colony and any conventional colony is its size. The focus of the current study lies in the various interactions of one particular animal with a small group of other animals. If the colony consists of enough animals that represent all the different behaviours that are natural and can survive for a season, this will not pose a problem. The absence of drones is not worrying either. Drones exist only in spring and early summer, which outside the period investigated in the current study. The nest volume was chosen to be 50 litres, which is similar to conventional nests (Seeley 1977). The exit of the arena was connected to the outside by a 50 cm tube. The length of that tube was below the reported 74 cm that bees could accept (Seeley 1977). Moreover, another major difference to a conventional hive is the orientation of the wax combs and the geometry. Combs of undisturbed bee hives are built from top to bottom, i.e. vertically. A hive generally consists of several rows of wax sheets with combs on both sides. Our experimental arena had one layer of wax installed that was facing up and tilted vertically by only 17°. These major differences seem not to be a problem. The bees stopped building the comb once the area was around the size of the bee cluster. There were always empty combs available. The main concern with the low exposure to gravity on the almost horizontal combs was dancing. The bees, however, can dance on a comb that is tilted by more than 15° (Markl 1966) and did so in the arena (Sub. Video 1).

The bees exhibited a behaviour of all known labourers (Lindauer 1952): they took care of the nest as well as the brood and foraged. The nest was cleaned of dead bees, and cells were inspected. The amount of comb the bees drew was smaller than the amount produced by a regular colony. However, compared with a commercial

queen's mating box, the experimental colony had a similar bee-to-comb ratio. The bees foraged pollen as well as nectar and communicated those sites. Round and waggle dancing was observed. The waggle-phase length and direction were similar, as earlier studies of conventional beehive behaviour suggested (Von Frisch 1967). Contrary to the natural case, the bees were constantly fed in the arena by sucrose solution feeders. The proportion of foragers was too small to supply the needed energy.

The queen behaved as in natural conditions, despite laying only around 20 to 50 eggs per day instead of 2 000. She stayed constantly in the bee cluster. The queen's mandibular pheromone gave the colony a sense of being queenright, so the cluster stayed tightly together around the queen. The worker bees reached an average lifespan (6 weeks), and they transformed to winter bees when the outside conditions were accordingly. One generation of winter bees survives the whole period of winter, and it does not forage outside anymore.

The bees behaved similarly to a conventional colony as I had hoped for, despite the different geometry of the arena and the smaller size of animals. Temperature regulation as a predictor for a healthy colony was not possible, since the arena was too large for the bees to warm and cool the area by themselves. A smaller arena was not suitable for the recorded bee. Empty walking space and areas of low bee density were desirable. High bee density across the whole arena is likely to heighten chances of bees building the comb on other places then the ground. Everywhere else, the high density is of high disadvantage for the free movement of the long electrode. By keeping a large area of the arena free, maintenance was more easily performed. Thus, experiments concerning in-hive navigation and learning were still a possibility.

The interplay of the circadian clock and the social organisation of honeybees is of great importance (Bloch 2010). The activity of foraging bees entrains the circadian rhythm into the dark hive. Forager-dependent labours such honey processing pick up this entrainment. Bees of this experimental colony exhibited the circadian rhythm in the form of changes of motor activity and sleeping bouts. This is an important finding underlining the results of the apiaristic observations. The bees exhibited greater

activity in the afternoon than at night (Fig. 4.2). Furthermore, the amount of bees sleeping around the queen cluster was higher at midnight than at 11:00 hours. Nevertheless, the data also contain some daytime-independent activity. This also suggests the right functionality of the hive, since some labours such as brood care are independent of the daytime.

In summary, the bees showed co-operative brood care, overlapping generations and division of labour. Qualitatively, there were no differences from a wild or conventional honeybee hive. The amount and proportions of observed labours was different but not in ways that were relevant for the focus of the current study.

I conclude that the behaviour displayed by the experimental colony is equivalent to that in a conventional hive.

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5 Chapter 2: High order neural correlates of social behavior in the honeybee brain

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5.1 Highlights

Neural correlates of social interactions within the honeybee colony

Response properties of mushroom body extrinsic neurons

High order combinatorial coding of location, body direction, and social interactions

5.2 Abstract

5.2.1 Background

Honeybees are well established models of neural correlates of sensory function, learning and memory formation. Here we report a novel approach allowing to record high-order mushroom body-extrinsic interneurons in the brain of worker bees within a functional colony. New Method The use of a 100 cm long differential copper electrode allowed the recording of up to four units of mushroom body-extrinsic neurons simultaneously for up to 24 hours in animals moving freely between members of the colony. Every worker, including the recorded bee, hatched in the experimental environment. The group consisted of 200 animals in average.

5.2.2 Results

Animals explored different regions of the comb and interacted with other colony members. The activity of the units was not selective for location on the comb, body

[1]Equal contribution

direction with respect to gravity and olfactory signals on the comb, or the different social interactions. However, combinations of these parameters defined neural activity in a unit-specific way. In addition, units recorded from the same animal co-varied according to unknown factors.

5.2.3 Comparison with Existing Method(s)

All electrophysiological studies with honey bees were performed so far on constrained animals outside their natural behavioral contexts. Yet no neuronal correlates were measured in a social context. Free mobility of recoded insects over a range of a quarter square meter allows addressing questions concerning neural correlates of social communication, planning of tasks within the colony and attention-like processes.

5.2.4 Conclusions

The method makes it possible to study neural correlates of social behavior in a near-natural setting within the honeybee colony.

5.3 Keywords

Mushroom body extrinsic neurons, combinatorial coding, multi-unit extracellular recording, social behavior, insect

5.4 Introduction

Single-unit intracellular recordings and Ca2+ imaging of high-order interneurons in the brain of tethered honeybees have provided us with a wealth of data related to high-order integration of visual and olfactory information (Hammer (1993), Mauelshagen (1993), Faber (1999), Szyszka et al. (2005), Paulk et al. (2009), Mota et al. (2011)). Subsequent multi-unit extracellular recordings from mushroom body (MB) extrinsic neurons of tethered bees offered new insights into multimodal integration and learning-related plasticity in the insect brain (Okada et al. (2007), Denker et al. (2010), Strube-Bloss et al. (2011), Brill et al. (2013), Hussaini and Menzel (2013), review: Menzel (2012)). Recordings of local field potentials in bees walking on a treadmill have also been informative (Paulk et al. (2014)). However, so far it has not been possible

to record neurons in the brain of freely moving bees. Recording from multiple neurons in a social context, in particular, promises to provide further valuable insights. Honeybees are social insects that live in colonies of thousands of individuals. Social communication plays a major role in the life of a bee: it regulates the attendance of the queen, care of the brood, distribution and processing of incoming food, defense of the colony, and the allocation of foragers via the waggle dance (von Frisch (1967) Seeley (2011)). Social communication in the dark hive involves predominantly tactile and chemical signals between the queen and all members of the colony including the larvae. The waggle dance is a particular form of symbolic communication by which the foragers communicate about important sites (foraging places, new nest sites), and neurons related to the dorsal lobe are thought to provide a second and higher order code for the stimuli involved in dance communication (Ai et al. (2009)). Social interactions require across sensory integration, recognition of the self in relation to other group members, updating of conditions within the social environment and appropriate responses to these changes. High-order sensory integration and motor planning at the output side of the MB of this social insect may be particularly suited to the organization of social interactions. We developed a method that allows us to record from MB extrinsic neurons (ENs) in an animal freely moving within a miniature colony consisting of a queen, brood cells and about 250 workers (including foragers) living on a single wax comb. We ask whether the neural activity of ENs correlates with the behavior of the recorded animal including social interactions. Our method allows the animal to walk to any place on the comb, attend inbound and outbound foragers, join the queen group, visit the brood region and feed from the honey store. We focused on the recording of a subgroup of MB ENs, the A1, A2 and A4 neurons (Rybak and Menzel (1998)), because these neurons have already been well characterized by both intracellular and extracellular recordings in restrained animals, and were found to respond to multiple sensory stimuli (Homberg and Erber (1979), Rybak and Menzel (1998)), and to adapt their responses during learning (Mauelshagen (1993) Okada, Rybak, Manz, Menzel (2007) Strube-Bloss, Nawrot, Menzel (2011) Hussaini and Menzel (2013)).

5.5 Material and Methods

5.5.1 Setup

The miniature bee colony was housed in a wooden box next to an electrophysiology setup (Fig. 5.1). A tilted comb contained mostly empty cells and in one part cells filled with honey, pollen and water (each part 6 x 6 cells wide). An iron plate beneath the wooden plate on which the comb was lying was kept at a temperature of 32 – 34 ° C. The comb was surrounded by a vertical plastic frame (55 x 55 cm, height of the sides: 10 cm) whose inner sides were sprayed with Teflon in order to prevent bees escaping from the comb. Foragers left the colony at the exit via a plastic tube. The top housing (inner height 68 cm) made the space around the comb light-proof and acted as a Faraday cage. The ceiling of the top housing was fitted with a webcam (Logitech Pro 9000, Logitech international S.A., Apples, Schweiz, infrared filter removed) and infrared LEDs. These were used to monitor the movements of both the test animal and the animals of the colony. A support box (Fig. 5.1 B, C: 4) carried the head stages of the preamplifier (EXT, npi, Tamm, Germany) to which the electrode was connected via a DIP plug.

5.5.2 Behavior of the miniature bee colony and the recorded bee

Two normal sized combs were removed from a colony together with the queen and about 250 young workers. A piece of a comb of the same colony (14 x 14 cells) with closed cells containing pupae close to emergence was cut out and inserted into the combs in the experimental setup. Thus the bees in the miniature colony were close in age. Another section of cells containing honey and pollen was cut out of a comb of the same colony and inserted into the combs. Honey, pollen and water were replenished if needed. A few bees started foraging soon after the establishment of the colony. The queen attending group of bees usually settled at the furthest distance from the exit and moved around with the queen at a very low pace. Movement of all bees was continuously monitored with a webcam under infrared light.

The synchronously aligned data of the extracellular potentials and the location, orientation and behavior of the recorded bee could then be analyzed. The movement

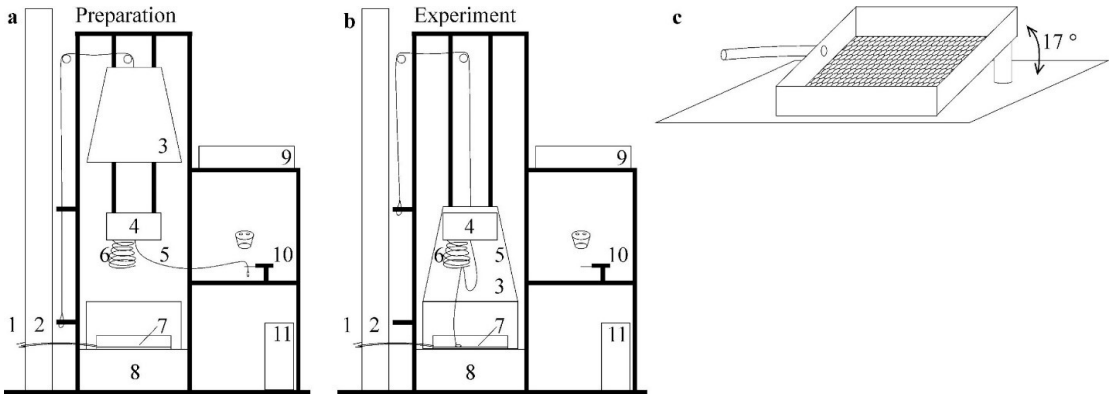

Figure 5.1: **Experimental setup**. a Setup during the preparation of the animal. b Setup during the recording from the animal inside the colony. c Side view of the tilted comb (# 7). Approximately 250 honeybees (Apis mellifera carnica) and a queen bee are situated on a 55 cm x 55 cm square of honeycomb (arena). The ground (#8) is tilted 17° and the whole area is heated. The walls of the arena (#7) are sprayed with Teflon. Foragers can leave the arena via a plastic tube (#2) through the wall of the building (#1). The electrode wire (#5) is connected to the head stages of preamplifiers (#4) and can either be moved to the dissection place (#10) or together with the experimental animal onto the wax comb inside the recording box (#7). When inside the recording box the electrode wire is attached to a coiled nylon threat acting as spring (#6) and thus prevents the wire touching the ground. The animal is prepared in the electrophysiology setup (# 9, 10, 11). When stable signals are found the animal is again immobilized with cold air (6° - 8° C) and transferred back onto the combs. The top housing (#3) is lowered toward the wax comb. The ceiling of the top housing carries a HD webcam and infrared LEDs. The top housing is surrounded by a metal mesh which acts as a Faraday cage. The head stages are connected to an analog/digital converter via an amplifier (#9). The numbers in the figure give the respective parts of the setup: 1: outside world, 2: plastic tubing connecting the colony to the outside world, 3: top housing which can be lowered and acts as a Faraday cage, 4: head stages of the preamplifier, HD web cam and infrared LED, 5: recording wire, 6: coiled nylon threat, 7: wax comb, 8: metal plate and heater, 9: preamplifier and analog-digital converter, 10: dissection place with micromanipulator and stereo microscope, 11: PC.

of the recorded animal was tracked with a custom made program which allowed not only the location of the animal but also the orientation of its body long axis, the speed of its movement and any close contacts to other animals. Using this program the following behavioral categories were distinguished: bee alone (no bee closer than the distance of a bee size), bee in close contact ("close") and bee touching another bee ("touch"), bee within the queen attending group ("group"), bee close to departing or arriving foragers at the exit ("exit"). As one can see from the supplementary video the recorded animal may experience these social interactions in different regions of the comb, may have come back to the same area from different directions, and may position its body in different directions relative to gravity in different areas.

5.5.3 Dissection

We found it essential that the animal was not too aroused or stressed during dissection. We blew cold air against the animal keeping it narcotized during dissection. Since the mechanoreceptors in the neck of the bee are very sensitive we avoided holding the head tightly by the neck; instead we caught the mandibles with tweezers that are very precisely manipulated by a micromanipulator very carefully avoiding any stretching of the neck. After a small hole was cut above one of the two alpha lobes we pushed the trachea sack above it to the site and immediately inserted the two Cu-recording wires, not the silver wire. The neural sheath of the brain is rather soft for a very short time (< 20 s) after the trachea sack has been pushed to the side, and thus the neural sheath was not needed to be cut. Promising neural activity was searched for when the animal had warmed up. A resting brain not covered with hemolymph was also very important for the stabilization with the two component silicon glue (KWIK-SIL Sarasota, FL, USA, mixture 1:1). We therefore sucked away the hemolymph (if there was any) from the brain surface, made sure that the cuticle was dry and put the mixed KWIK-SIL around the electrodes into the hole of the head capsule. Before we did this we checked that the grounding silver wire touched the brain surface without any pressure on it. After the KWIK-SIL had hardened the animal was again cooled with ice cold air, the squeezer of the abdomen removed, the mandibles released and then the animal carefully but quickly moved onto the wax comb of the colony.

5.5.4 Electrophysiology

Two polyurethane-coated copper wires (14 μm in diameter, Electrisola, Escholzmatt, Switzerland) were twisted over a length of 1 m. The carefully cut ends of these wires were plated with carbon nano fibers and gold using the method of Ferguson et al. (2009). This procedure reduced the impedance of the whole recording wires below 60 KΩ. We limited the number of the Cu wires to two because we observed that the animals were more likely to be restrained from freely moving with tetrodes. The other ends of the wires were de-insulated and attached to the amplifier input connectors by means of conducting silver glue. A silver wire (diameter 50 μm, Advent, Eynsham Oxon, UK) was twisted together with the two Cu wires. The weight of the three wires was counter balanced by a spring made of a thin fishing line. Such fishing lines come rolled up and when unrolled form a very flexible spring. The spring can counterbalance a weight of 8 mg (the weight of the twisted electrode wire). The mechanical stress of the recording wires was mostly determined by the ground electrode because it needed to be thicker than the active wires. The adjustment of the strength of this string and the location of fixing it to the electrode wires was critical and needed adjustment.

Each electrode wire was connected to the head stage of a preamplifier (npi electronic, Germany). Filters were set to high pass of 10 Hz and low pass of 2 kHz. Hum noise (50 Hz) was eliminated by an additional filter (Hum Bug; Digitimer, Hertfordshire, UK). Neural activity was sampled at a rate of 40 kHz through an analog-to-digital converter (1401 micro MKII; Cambridge Electronic Design, Cambridge, UK), and initial data analysis was performed by Spike2 software (Cambridge Electronic Design) including signal storage and pre-analysis of the data. The amplifier used a band pass filter with cut-off frequencies between 10 Hz and 2 kHz. Off-line analysis with Spike 2 software included calculation of the difference between the signals from the two electrodes which was then band pass filtered (300 Hz – 2000 Hz) with a digital FIR-filter (‚finite impulse response') and used for both multi-unit analysis (MUA) and sorting of single units. The semi-automated template matching algorithm of Spike2 was used for spike sorting. Besides careful visual inspection, sorting quality was controlled by means of a principal component analysis of the first three components of

each sorted unit. The dots of the single units (encoded in one particular color) needed to be clearly separated from each other in order to ensure good sorting quality and to avoid false positives and negatives in spike trains of a single unit. Muscle potentials could be identified by their broad spikes. These spikes were cut out of the recording (representative example in Fig. 5.2).

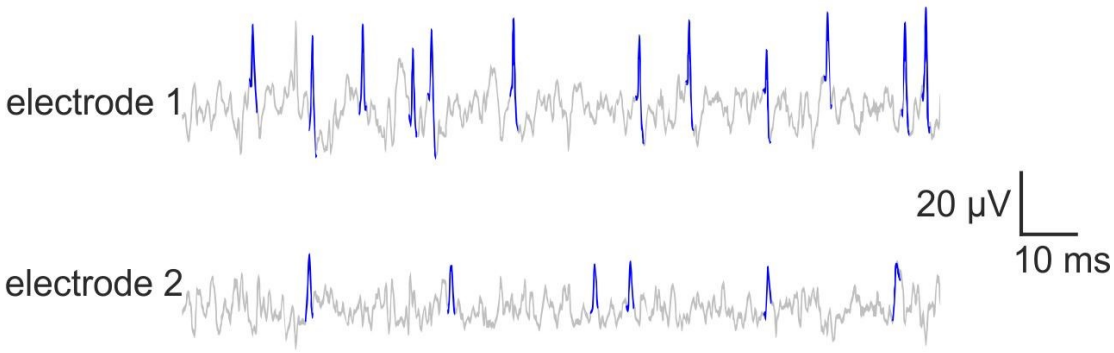

Figure 5.2: **Multi-unit extracellular recording** Here two units are recorded, one predominantly via electrode 1 and the other via electrode 2. Template sorting leads to the separation of these two units as marked in the two traces.

5.6 Results

Our recordings were performed in an experimental set-up consisting of a miniature honeybee colony and a nearby electrophysiological set-up. The colony (about 250 bees and the queen) lived on a tilted (17° to horizontal) layer of a wax comb (55 x 55 cm). The animals were prepared for recordings at the dissection place. After the electrode was inserted and stable recordings were established the animal was moved to the wax comb and watched by a webcam in order to record its behavior. The recorded animal was selected from the miniature colony and belonged either to the queen attending group (5 animals), the food processing bees (3 animals) or the foragers (1 animal). The recordings lasted up to 24 hours. One to four units were recorded simultaneously. Routine offline data analyses were performed in order to sort the spikes. Custom written programs in MatLab allowed extracting the coordinates of the recorded bee, its interaction with other bees and other categories of behavior. The analysis is based

on the results from 9 animals. The average recording time was 155 minutes, a total of 11 sorted units were evaluated (Fig. 5.2).

The tilted level of the combs together with other cues provided the bees with signals for orientation within the set-up. These additional cues were the signals from the exit (faint light, fresh air, temperature gradient), chemical signals from the queen group and the cells with honey and pollen. These signals formed an overlapping pattern of rather stationary gradients depicted schematically in Fig. 3. It can be expected, therefore, that bees walking on the tilted combs related these signals to the gravity cue and to each other possibly allowing localization on the combs and directed movements. Bees are able to perceive the gravity force on a tilted surface at an angle of $\geq$ 15° to horizontal and perform well orientated waggle dances (Markl, 1966).

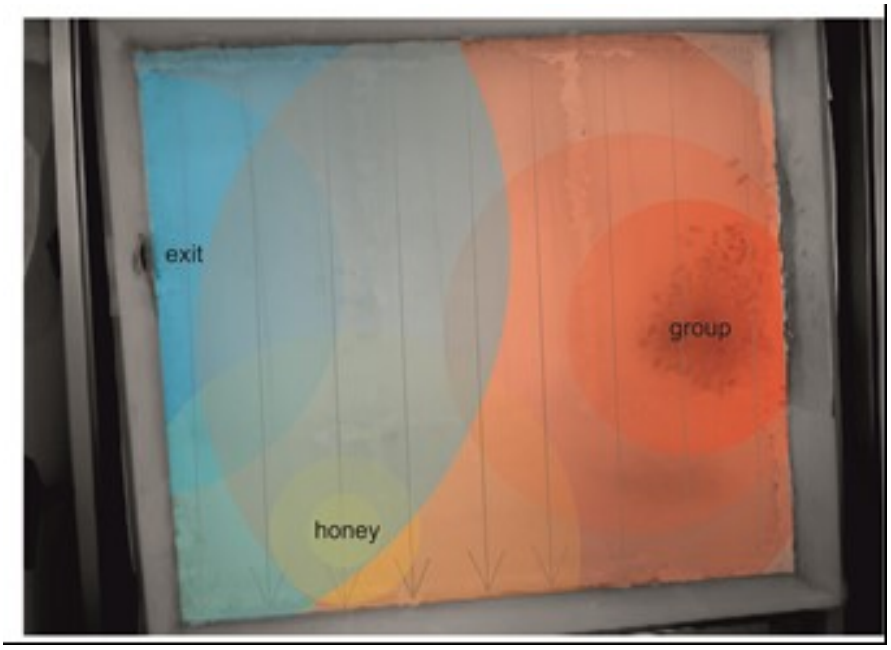

Figure 5.3: Schematic scheme of the effective gradients on the surface of the tilted comb. Gravity force (grey arrows pointing downwards) provided a compass in the dark, and several overlapping gradients added additional signals, e.g. faint light from the entrance together with fresh air and a temperature gradient marking the entrance (blue), humidity and specific odorant gradients emerging from the food area (honey, yellow), pheromones arising from the queen and chemical signals from the attending workers attending the queen (red).

The miniature colony showed normal behavior (Supplementary Video 1). The queen laid eggs and the young bees attended the queen. The group of bees moved around with her but preferred the upper part of the comb furthest away from the exit. The number of foraging bees was rather small, and under fortunate weather conditions a regular traffic of foragers via the exit was observed. Some of these bees performed round dances since the nectar and pollen sources were close to the colony during the test period. Unfortunately none of our recorded bees attended a dance, but other bees did. The queen related group and the foragers showed a circadian rhythm of movement with less dense grouping between 12am and 6pm. Such looser contacts to the queen were most obvious under perfect weather conditions and active foraging. The recorded animals traveled through most parts of the comb but were not particularly attracted by the queen group although some of them were collected from the queen group.

The recorded ENs belonged to the group of A1, A2 and A4 neurons (Rybak and Menzel, 1993) known to respond to multiple sensory inputs, to change their responses to learned odors and to develop different response properties for cue and context stimuli in the course of context dependent olfactory learning in harnessed bees (Mauelshagen, 1993, Okada and others, 2007, Strube-Bloss and others, 2011, Hussaini and Menzel, 2013). One characteristic of these ENs is their rather stable and high spontaneous activity in the range of 10 – 20 Hz in restrained bees. In contrast, ENs recorded in freely moving animals had a rather low spontaneous rate of spike discharge often well below 1 Hz (Supplementary Fig. 5.1). Fig. 5.4 shows an example in a time/space resolved plot giving spiking activity in false colors of the walking track. Bursts of spikes were observed without any obvious external stimuli or behavior of the animal. Prolonged periods of no spike activity may have occurred under certain conditions, e.g. when the animal was close to the exit (Fig. 5.4), and enhanced activity could be related to social contacts, to particular regions on the comb, to the direction of the body in the gravity field, or combinations of these conditions. Supplementary Video 2 shows a walking trajectory together with the unit´s activity in false colors. In some units higher activity correlated with faster walking speed.

We first asked whether neural activity of ENs depended on the location of the

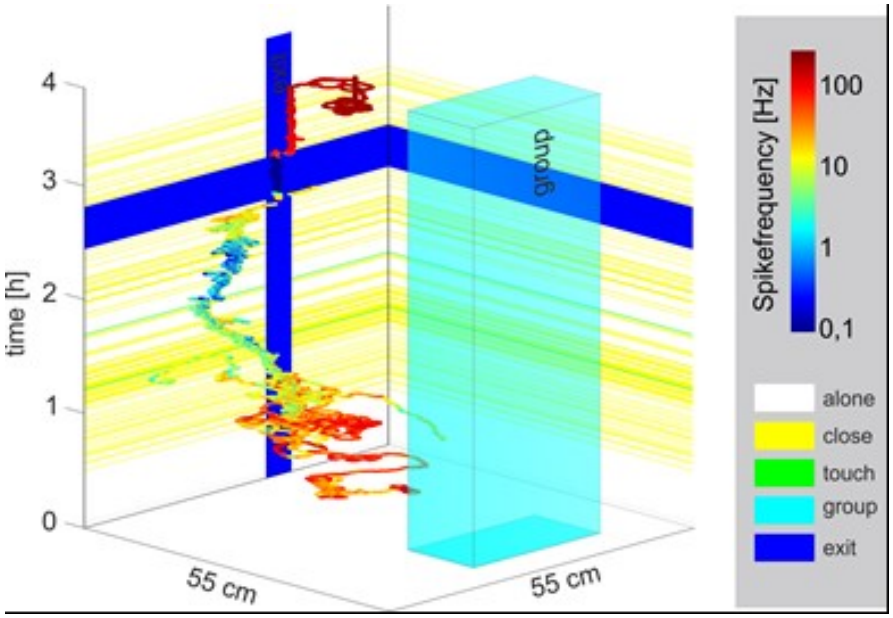

Figure 5.4: Time and space resolved activity of unit 2 of bee 4. Time is plotted vertically. Spike activity is given in false colors of the walking track (color code at the top of the insert at right). The location of the queen group is marked with the bluish vertical pillar and the exit by the dark blue vertical bar. The backgrounds mark 5 different behavioral categories of the animal in false colors (color code at the right side). The recording lasted for 3.5 hours.

animal on the comb. No such effect was found with the exception that a reduction of spiking activity appeared in some units when the animal was close to the exit (Fig. 5.4, Fig. 5.7). Next we analyzed the relationship between spiking activity, body direction and area on the comb. Supplementary Fig. 2 shows an example in which an animal performed many body rotations in the same area. One of the two units increased the firing rate when the body direction pointed at 120-130° and decreased it when the body was arranged at 30 -80°. The other unit was more active when the body pointed at 90-120°. All other animals walked around and had their body directions under different angles to gravity in different areas of the comb and at different times when they may have returned to the same area. Therefore, we included in the analysis, whether the animal was resting or walking and in which of 9 subareas the animal was on the comb (Fig. 5.5, Supplementary Fig. 5.2). Since the number of measurements taken in each of these subareas was rather different in different

animals we indicate in Fig. 5.5 and Supplementary Fig. 5.2 with black or red arrows how many measurements were evaluated for each of the 12 body directions. Body direction effects were seen only in few subregions, e.g. in bee 2 in the middle upper area of the comb in which the two recorded units coded for different body directions, an effect not seen in the resting animal (Supplementary Fig. 2). We conclude from these results that the recorded ENs may code body direction only in combination with other parameters (e.g. location on the comb, walking activity or resting.

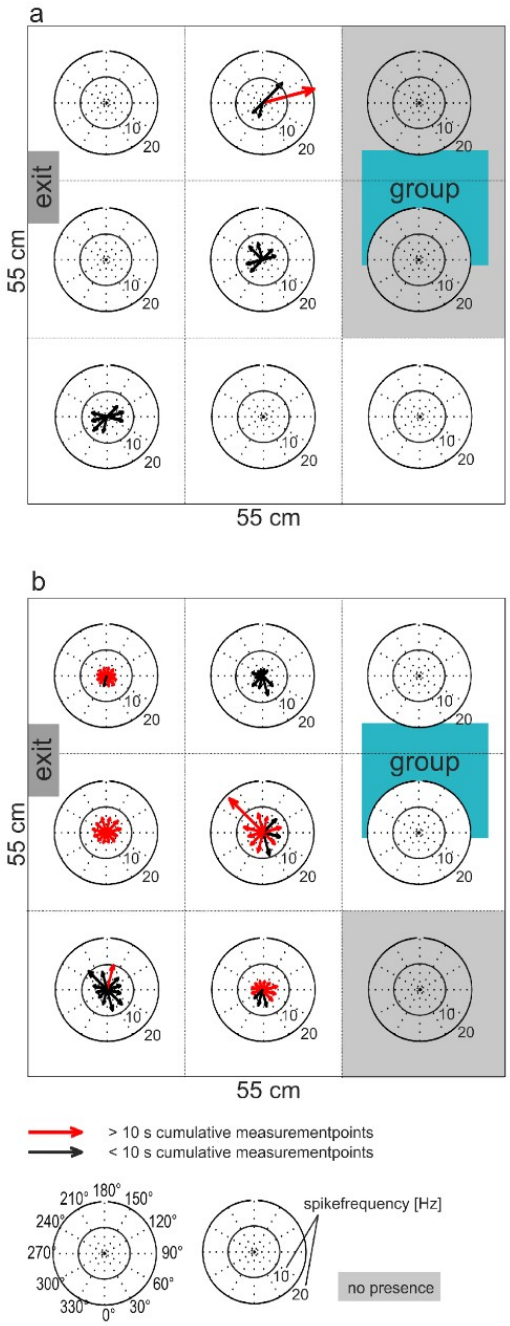

Figure 5.5: Activity of two units in relation to body direction and location in nine sub areas of the comb. Both bees were walking during the recording time and reached the respective areas from different directions. The upper graph shows unit 1 from bee 2, the lower graph unit 2 from bee 5. The two areas in the upper right (marked grey, overlapping partly with the location of the queen group marked blue) were not visited. The lower graphs and the arrows explain the design if the subfigures. The arrows give the body directions in 30° intervals relative to gravity. The length of the arrows relates to the relative frequency of spike in the particular direction. The black arrows indicate measuring times shorter than 10 s, and the red arrows measuring times longer than 10 s.

As shown in Fig. 4 and supplemental video 1 the recorded animals were well integrated in the social community of the miniature colony experiencing several kinds of social interactions (alone, close to another animal, touching another animal, being within the queen group or close to the exit and thus close to in and outbound foragers). Fig. 5.4 marks the 5 behavioral categories in false colors on the background of the 3D plot. The most frequent behaviors were alone and close to another animal. Fig. 5.6 gives a representative example of four units recorded in bee 9 over a period of about half an hour. Each category of social interactions (red dots in Fig. 5.6) was evaluated in intervals of 100 ms, therefore, the same interactions may have appeared in close temporal proximity, and the red dots fuse to a red line. Transitions between the various interactions may also happen in quick succession.

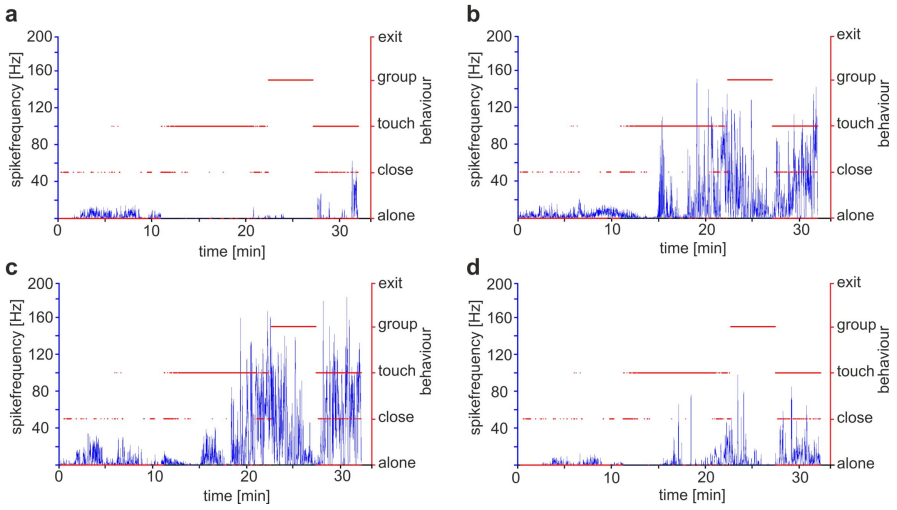

Figure 5.6: Time course of neural activity in four units of animal 9 in relation to five social interactions. The occurrence of the five social interactions (alone: no animal closer than the body length of a bee; close: another bee is closer than the body length of a bee; touch: the recorded bee touches another bee; group: recorded bee is within the queen group; exit: recorded animal is close to the exit and to foraging bees) are plotted with red dots (right ordinate). Spiking activity (left ordinate) is given in blue. Bee 9 was not close to the exit during the time period depicted here.

No units were found that were reliably and repeatedly active only when a particular social interaction occurred but preferential response patterns were seen. For example, unit 1 in bee 5 (supplementary Fig. 3) and unit 1 in bee 7 (Supplementary

Fig. 4) were rather specifically active during multiple encounters of close and touch, and were silent when the animal was close to in and out bound foragers at the exit. All 4 units given in Fig. 6 showed the lowest activity when the animal was within the queen group, an activity pattern which we also saw in a unit of bee 1 (not shown). Unit 1 in bee 8, on the contrary, was particularly active when the animal joined the queen group (not shown). The prevailing activity patterns were overlapping activities of the recorded units.

In order to test whether ENs may code for social interactions in a combinatorial way we analyzed two units of bee 4 which were recorded over 3.5 hours. Fig. 7 gives the joint activities of the two units for five behavioral categories (Supplementary Fig. 5 A – E shows the respective distributions separately for each of the five behavioral categories). The activities in both units were highly correlated indicating that a process not captured by the 5 behavioral categories drove activity in both units jointly. This unknown process was not related to walking speed. However, there were also selective effects in a combinatorial way. For example, high activity in both units was not related to any other social activity then the animal being alone. When the animal was within the queen group (pink) the activity was always very low in both units. This was also the

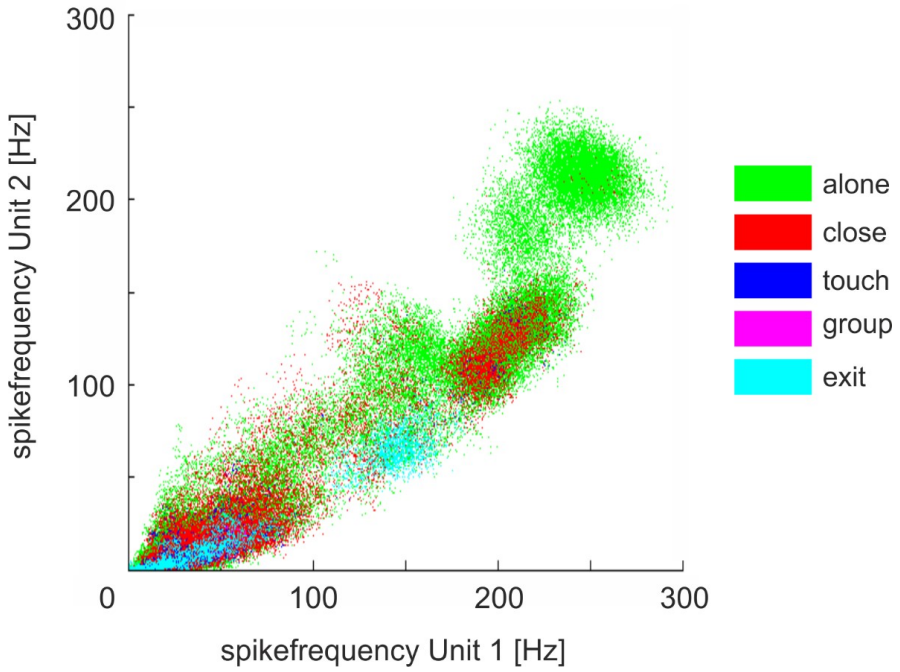

Figure 5.7: Neural activity of two units in animal 4 during 3.5 hours of recording. Five different social behaviors are color coded. The animal is alone (green), the animals is close to another animal (red), the animal touches another animal (deep blue), the animal is close to the exit (light blue), the animal is with the queen group (pink). Each point gives the number of spikes in unit 1 and unit 2 in Hz. See Supplementary Fig. 6 a- e for separate plots of the five behavioral categories.

case when the animal touched another animal (deep blue) but other than in the behavioral state of being within the queen group neural activity in both units reached also higher values. Neural activities during being close to the exit (light blue) and being close to another animal (red) appeared to be antagonistic. When activity of unit 1 was around 150 Hz and that of unit 2 around 50 Hz (cluster of light blue dots indicating "close to the exit") than other social behaviors were mostly lacking.

5.7 Discussion

Recording of high-order interneurons in freely ranging animals still poses a major challenge for neuroscientists. Large animals like primates can be fitted with a device that allows wireless monitoring of multiple units (Schwarz et al. 2014). Smaller animals like mice and birds are also able to carry miniature telemetric devices al-

lowing wireless connections within a small range and over rather short time periods (Schregardus et al. 2006).Only large insects can be fitted with such telemetric devices because the batteries required to operate them are too bulky and heavy, and wireless power transmission and energy harvesting technology with integrated circuits have yet been used only for dragonflies operating in a small volume of space Harrison, 2011 27306 /id). Thomas, 2012 27303 /id.. In cases where telemetric devices have been applied to insects so far they were not used to transmit recordings from interneurons but to stimulate the antennal nerves Holzer and Shimoyama (2014) or to record EMG Thomas, 2012 27303 /id.. Standard methods of recording via long, flexible cables connecting the electrodes with the amplifiers are used e.g. in rats (e.g. Moser et al. 2008) and in the large fruit bat (Rubin et al. 2014) in order to monitor many principle cells in the hippocampus. Such methods were found to be difficult to apply to insects because the wires were too heavy and too stiff. Mizunami et al. (1998a) applied such a method to the cockroach sampling recordings from mushroom body neurons but the recording wires were shorter and the animal was quite constrained in its movements. Bender et al. (2010) recorded units of the central complex of tethered cockroaches walking on a slippery surface and established correlations between firing rate and stationary walking speed. Here we solved the problem of the long recording wires by using only two twisted copper wires and a thin ground electrode. Furthermore, we counteracted the weight and mechanical stress of the wires with a loose spring made of thin fishing line. The fine adjustment of this loose spring is an essential part of our method. In addition, we substantially reduced the impedance of the recording wires by applying the gold plating method of Ferguson et al. (2009).

The aim of our study is to search for neural correlates of social interactions within the honeybee colony. It was therefore essential to establish a stable miniature colony with near-normal social interactions. Preliminary experiments showed that this requires a queen attended by young bees, brood cells, sealed cells, honey cells and pollen cells. The data reported here come from one colony that was established with 243 animals and contained 80 animals after 6 weeks at the end of the season. Initially young bees emerged from the sealed cells, and the larvae were cared for. Although the queen stopped laying eggs due to the late season of the year the queen court ap-

peared normal, foragers were flying in and out, and some of them performed round dances. Since such a small colony cannot control the temperature on such a large comb we kept the temperature of the surface below the comb at 32 - 34° C.

We recorded mushroom body extrinsic neurons (ENs) in the ventral aspect of the alpha lobe. These ENs belong to the A1, A2 and A4 neurons (Rybak and Menzel 1993), and one of the neurons is the single identified neuron PE1 (Mauelshagen 1993). Both PE1 and other ENs in this area respond to multiple sensory inputs (Homberg and Erber 1979); Rybak and Menzel 1993), Rybak and Menzel 1998) and their properties change during olfactory learning (Mauelshagen 1993); Okada, Rybak, Manz, Menzel 2007), Strube-Bloss, Nawrot, Menzel 2011); Hussaini and Menzel 2013). In fact olfactory cue stimuli and visual context stimuli were found to change the response properties of these ENs in bees categorically differently (decreased response to the olfactory cue, and increased response to the visual context stimuli) possibly indicating that they are involved in separating cue and context (Hussaini and Menzel 2013). No evidence has been found yet that these ENs in honeybees respond selectively to combinations of sensory modalities or context-dependent combinations of stimuli as was reported for ENs of the cockroach Li and Strausfeld (1999). The spontaneous response rate of these ENS in restrained bees ranged between 10 – 20 Hz but was found to be much lower in the freely moving animals in our experiments. It is thus likely that inhibitory input is lower when the animals are restrained and indeed spontaneous activity of putatively inhibitory neurons of the A3 ENs is rather high in restrained animals (Haehnel and Menzel 2010). The reduced response of units recorded in the central complex of the walking cockroach to antennal stimulation (Bender, Pollack, Ritzmann 2010) may also indicate higher inhibitory demand in actively ranging animals. Interestingly, putative pyramidal cells in the mammalian hippocampus of freely moving rats are characterized by low spontaneous activity (0.2 – 1 Hz) as compared to restrained animals (Kraus et al. 2013).

Multi-unit recordings in freely moving rats have revealed a large range of highly informative data on the multiple coding properties of principle cells in the hippocampus and prefrontal cortex (McNaughton et al. 2006), review: Moser, Kropff, Moser

2008). The activity of these neurons depends on place, body direction, viewing direction, and goal-seeking behavior as well as the integration of multiple state-dependent sensory inputs. A major insight concerns the multi-faceted properties of principle cells in the hippocampus of rats. Depending on the particular training and test conditions these neurons may respond selectively to additional stimuli, may "remap" their responses to place when the environment is changed (Colgin et al. 2008), may keep their place properties even in the dark, and change them when the task of the animal is altered (Manns and Eichenbaum 2009, review: Eichenbaum 2002). An indication for the possible involvement of mushroom body ENs in comparable tasks comes from extracellular recording in the cockroach (Mizunami et al. 1998b) Mizunami, Okada, Li, Strausfeld 1998a), who related neural activity of ENs to the place in a heat avoidance training task. However, molecular genetic manipulations in Drosophila do not support this conclusion and rather point to the ellipsoid body as a neural structure involved in place learning (Ofstad et al. 2011).

Although the animals in our experiment were in the dark they still should be able to localize themselves relative to the gravity field as a compass and to overlapping gradients of local cues like odors, substrate structure, temperature and light as well fresh air through the exit hole. No evidence was found that any of the recorded ENs coded a "place field" in the strict sense but some of them were more active in a particular area than in others. Some ENs also displayed a dependence on body direction, but this property was not independent of the area on the comb. The body direction effect in single units may also depend on whether the animal was walking or resting, but again this effect depended on the area. Obviously the recorded ENs did not code any one of these properties in isolation. The multiple coding properties of ENs of freely moving bees within the colony became particularly obvious when their activities were correlated with social behaviors. None of the ENs were exclusively activated by one of the five behavioral categories we distinguished. This finding is quite surprising since meeting with the queen group or intense contact to another animal could have been expected to lead to particularly strong effects. However, combinations of units´ activities could be rather specific.

Neural correlates of social behavior have not been studied so far in an insect model. The organization of social behavior requires integration of sensory information from group members, evaluation of these signals in the context of own needs and perception of specific signals for the coordination between group members. Olfactory social signals are often perceived by specialized receptors (e.g. the vomeronasal system in mammals, Dulac and Torello 2003), particular pheromone receptors, Christensen and Hildebrand 2002)). Specific social memory may be formed by filial imprinting (chick: Town 2011), mammals: Kendrick et al. 1992) and imprinting-like learning in honeybees (Masson and Arnold 1984) and ants (Bos et al. 2010).

Basic neural correlates of social behavior have already been found in the worm Coenorhabditis elegans (Garrison et al. 2012), Emmons 2012). The authors concluded that the nervous system of C. elegans houses a social circuitry which promotes positive social behavior. In Drosophila, formation and retrieval of a specific form of memory, ARM (amnesia resistant memory) is facilitated in social conditions (Chabaud et al. 2009). Flies trained for ARM interact within a group to improve their conditioned performance.

Multiple evidence exists for neuroanatomical correlates of social conditions in mammals (e.g. primates, Struble and Riesen 1978). Functional and structural neural correlates of social behaviors in birds and mammals depend on the context in which they are acquired or retrieved. For example, the pattern of singing-related neural activity in several high-level brain areas specialized for song learning in adult zebra finch depends on whether the bird sings by itself or to another bird (Hessler and Doupe 1999), and neurons of the intermediate and medial mesopallium of the chick respond differentially to familiar and unfamiliar conspecifics (Town 2011). Several aspects of such social circuits have meanwhile been characterized (Insel and Fernald 2004).

The methods established here will allow us to address similar questions in the honeybee. In addition, we shall search for the neural correlates of dance communication, a unique symbolic form of ritualized movement in the honeybee that transmits spatial information and requires the receiving bee to have explored the environment around

the hive. Therefore, we shall predominantly select experienced foragers as recorded animals and include units in the central complex in our analysis, since this neuropil in the insect brain is known to code sun compass-related cues (Homberg 2008) and to provide high-order motor commands to descending neurons (Bender, Pollack, Ritzmann 2010).

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6 Chapter 3: Increased Spike Rate Variance of MB Output Neurons due to Social Interactions in Honeybees (Apis mellifera)

6.1 Abstract

Honeybees (Apis mellifera) are a well-established model in the search for neural correlates of behaviour. Social behaviour is of particular interest, but due to its complexity, no neural correlates are known for social interactions. The aim of the current study is to correlate neuronal activity of multimodal high-order mushroom-body (MB) extrinsic neurons with social behaviour in a rather natural setting. Extracellular recording electrodes of a one-meter length were inserted in the beta exit of the MB of a freely moving bee within a small but functional colony. Its behaviour was video recorded. All meaningful parameters of the two data sets, behavioural and neurophysiological, were tested in an exploratory manner searching for neural correlations. Strong changes in the spike rate appeared before, during or after social interactions. The variance of the spike rate within a certain time window around such interactions was higher when compared with those within windows marked 'alone', in which the recorded animal did not interact. The window length ranged from 40 s to 12 min, depending on individual animals, for the strongest effect. Future experiments will involve indoor foraging as a repetitive task with components of social interaction and operant conditioning.

6.2 Introduction

The ecological and economical value of honeybees cannot be overestimated. Since the work of Von Frisch (1914, 1967), the honeybee has developed into a well-established model animal in several biological disciplines, particularly in behavioural science (Menzel 2005, Menzel and Giurfa 2006, Menzel 2017) and neuroscience (Homberg and Erber 1979, Rybak and Menzel 1993, Menzel 2012, Filla and Menzel 2015). The honeybee has a rich repertoire of behaviours, and it is a social insect with intriguing navigational skills. Extensive knowledge has been acquired about the bee's brain, neuropils and several types of neurons. How can we put these areas of interest closer

together? How does the brain of each individual bee code for social interactions?

Only an unrestrained bee can have an intrinsically motivated behaviour such as social behaviour and dancing. The bees in this study are, unlike any previous experiments, unhindered as well as free to move and behave. As already described in Chapters 1 and 2 of the current study, we investigate the neuronal correlates of behaviour that is observable in the presented experimental set-up.

What neurons are suitable candidates for coding for social interactions, dancing behaviour, brood care and interactions with the queen? Additionally, the experimental circumstances described here allow the animal to have signs of attention (Paulk et al. 2014), planning and learning. It would be fascinating to investigate the neuronal correlates of these behaviours (Menzel and Giurfa 2006, Menzel 2012).

There are two neuropils of interest: the mushroom body (MB) and the central complex (CC). The MB is associated with olfaction, learning and memory (Strube-Bloss et al. 2011, Menzel et al. 1996, Heisenberg 1998, 2003), and the CC is associated with functions around locomotor activity and orientation (Seelig and Jayaraman 2013, Strauss 2002, Neuser et al. 2008). The CC is situated deep in the brain (Homberg 1985) such that it is very difficult to record through with our technique. The MB extrinsic neurons (EN), by contrast, are situated shallow and right next to a clear landmark (Rybak and Menzel 1993, 1998). We decided to investigate the behaviour of social interactions in the context of olfaction (Cheal and Sprott 1971) and memory rather than location and orientation. At least in mammals, olfaction, memory and social behaviour seem to be tied closely together (Levine and McBurney 1986, Kirk-Smith and Booth 1987).

Here, I analyse the correlation of high-order output neurons of the MB with the behaviour of honeybees in their natural habitat. Extrinsic neurons of the alpha lobe project in many parts of the honeybees' brain (Rybak and Menzel 1993, 1998). They are multimodal (Homberg and Erber 1979, Erber 1978), and their responses are not related to motor activity (Huber 1960, Mauelshagen 1993). It was demonstrated that ENs change their activity in response to classical conditioning (Haehnel and Menzel

2010; Filla and Menzel 2015). They can code the value of a stimulus and distinguish between rewarded and non-rewarded odour (Okada et al. 2007, Strube-Bloss et al. 2011, 2016, Menzel 2014). Therefore, I expect to find correlates between the neuronal activity and social interactions.

The neuronal activity of high-order neurons in natural unhindered conditions compared to common laboratory conditions might have unforeseen insights into the emerging field of insect cognition (Menzel and Giurfa 2006, Webb 2012, Menzel 2012, Giurfa 2013, Perry et al. 2017).

The activity of these neurons is further analysed, building on the analysis already presented in Chapter 2. Here, I present an explorative analysis in search for correlations between any aspect of physiological data and behavioural data. The experiments in the current study are novel, and there are no data or literature on any closely related investigations. I present a multiverse analysis (Steegen et al.2016) to uncover any correlation between the two aforementioned types of data. This prevents any kind of confirmatory data analysis. A hypothesis-driven analysis would be inappropriate in these exploratory experiments. After exploring this new type of data and understanding, i.e. how spike rate changes are related with some of the behaviours, at least to some degree, one can think about a hypothesis (Tukey 1980, 1977). Here, an analytical toolbox is presented to investigate the data (Hoaglin 2003).

6.3 Materials and Methods

A detailed description of the experimental set-up can be found in the methods sections of Chapters 1 and 2. A wax-covered platform sized 55 × 55 cm was tilted 17° horizontally. This arena has PTFE-covered walls and an entrance tube to the outside. The arena serves as a hive for a mini-colony with around 1 000 worker bees and a queen. One animal was picked and prepared with a 1 m long electrode. The electrode consisted of two copper wires as mono-polar channels and one bare silver wire as the ground. The tip of the two copper wires was gold plated for lower impedance. Once the electrode was positioned in the area of interest, i.e. the ventral aspect of the alpha lobe of the alpha lobe, it was stabilised by applying two-component sil-

icone (Kwik-Sil, World Precision Instruments, Florida, USA). The prepared animal was situated back into the arena. Its behaviour was recorded by an infra-red camera synchronously with the extracellular recordings in Spike2 (Version 5.21, Cambridge Electronic Design Limited, Cambridge, England). For consistent curing of the two-component silicone in the honeybee's brain, a system to warm the whole bee shortly after applying the silicone was introduced. Plastic tubing was wrapped tightly around the metal bee holder. The tubing was attached on a hot-water reservoir on one end and a wastewater container on the other. The hot-water reservoir was elevated so the gravitational force moved the water through the system. The tubing could be gradually clamped to adjust the flow of water and thus the temperature of the bee holder and the bee. The temperature of the bee holder was adjusted to 35 °C. The analysis script of this chapter can be found in the appendix. It consists of a newly written custom MATLAB script MATLAB 2011, MathWorks Inc., Massachusetts, USA).

6.4 Results

Here, I analyse the behaviour extracted from the video recordings and the correlation with spike activity of extracellularly recorded neurons. These neurons are extrinsic high-order interneurons that output at the alpha lobe of the MB. The bees were unhindered in their behaviour, also meaning that their behaviour was as unpredictable and sparse as it is under natural conditions. To deal with the small number of known variables as well as the high dimensionality of the extracted behaviour and neuronal activity, the data were analysed in an exploratory fashion. The behavioural data are described and analysed concerning their quality in Chapter 1. Any behaviour that is analysed here was evaluated to be natural. The behaviour of the colony was not further analysed. However, some features of the experimental bee's behaviour were investigated to compare with bees that were not prepared and without an electrode. Of particular interest was the walking speed of the recorded bees. The walking speed distribution of random bees was compared with the distribution of any recorded bee. There were no differences between the two groups. It was also tested whether any of the recorded bees avoided certain areas of the arena. Plotting all recorded bees' trajectories together revealed that the bees did not avoid any place in the arena. The amount of rotations per direction was measured to see if any recorded animal was

turning mostly in one direction. Any experimental bees with most turns in one direction were completely excluded. There were few cases, and all of them exhibited poor quality of neuronal data. Those animals all died within two hours upon preparation. Interestingly, all recorded bees that did not indicate unidirectional turning behaviour accumulated less than a full turn in one direction but more than in the other direction, even though these bees turned 50° per minute on average. The analysis of the quality of extracellular recordings was discussed in great detail in Chapter 2. All those tests were also carried out with this data set. All recorded units that were included in this analysis obeyed the aforementioned controls regarding auto- and cross-correlation. The spike frequency of the units ranged from very low (0.1–1 Hz) to high (20 Hz). The baseline changes were of particular interest. Most units had a wide variety of frequencies over long periods of time. They could spike rather stably at 1 Hz for half an hour and then slowly increase their rate over 5 minutes to 10 Hz. At 10 Hz, they could be stable for an hour and then change to a different value. Within this stable baseline time window, there were changes in around 20% of the spike frequency in the order of seconds. However, there were greater changes over larger time windows. Overall, all tested units had a high dynamic range. It was not possible to assign any spike rate changes to any type of response category. However, as described later, some behavioural events were in sync with an increase and later with a decrease in the spike rate. Nevertheless, other features than the spike frequency of the spike data were tested against all of the behavioural data. The tested features included interspike intervals (ISI), high- and low-pass filtered spike rates and the combinatorial spike rates of units recorded in the same animal. The ISI was close to the reciprocal spike frequency but not the same, especially when they were different, for example, when a spike burst was followed by a decrease in the spike rate. These quick changes were not observed in the spike rate since it was always integrated over some time window, but this would be registered by the ISI. However, this approach did not result in any mentionable results. As described earlier, the great range of frequencies at which the units fired, the spike frequency changes were filtered. The cut-off frequency for both high pass and low pass was 0.1 Hz. The high-pass-filtered data resulted in data representing high frequency changes of the units with a baseline of 0 Hz. The low-pass-filtered data contained only the low-frequency components of the

spike-rate changes, i.e. the baselines. These processed neuronal data did not correlate in any way with the behaviour in any of the applied tests. The different units that were separated per experiment were tested independently as mentioned above and in combination to each other. The difference in spike rate and the synchrony of firing was investigated. As described later, their spiking properties were plotted in an X–Y manner, with different properties of the behaviour plotted as a false colour on top. To search for correlations between behaviour and neuronal activity, obvious misapprehension must be proven absent. Therefore, the data was tested for correlations between walking speed or turning behaviour and the neuronal activity. These correlations would be dangerous to interpret since they could possibly arouse muscle potentials or movement of the electrode in the bee brain. None of them correlations was found in the current study. The bee could move around in its hive freely. All positions (the queen group with the brood and the dancing area) have different importance since they are differently far located from the entrance. It was important to search for correlations between these places and the neuronal activities as well as place cells and grid cells. No correlations or trends could be found. To analyse the data in regard to place and grid cells, the problem was that none of the recorded bees explored the hive in multiple times. Observing random bees indicated that they do not explore the hive in a manner that would be sufficient to recognize those patterns. The recorded bees' trajectory in correlation with the spike activity as the false colour can be seen in Figure 5.4. The aforementioned importance of certain places can be investigated with these plots. None of the additional data analysed in this chapter exhibited any interesting results. In Figure 1 of this chapter, any locational importance of where a social interaction takes place can be investigated. The trajectory of the recorded bee is depicted in black. The closest bee at any given time point is plotted in the false colour of the spike activity of one unit of the recorded animal. Thus, a location dependence could be recognised. The social context of certain places could be revealed this way. However, this must be treated with caution as the bee in this example was visiting the upper area only once. The heightened spike rate in this area may not be correlated with the closer distance to the brood. None of the recorded bees made any repetitive visiting of places that were accompanied by different spiking behaviours.

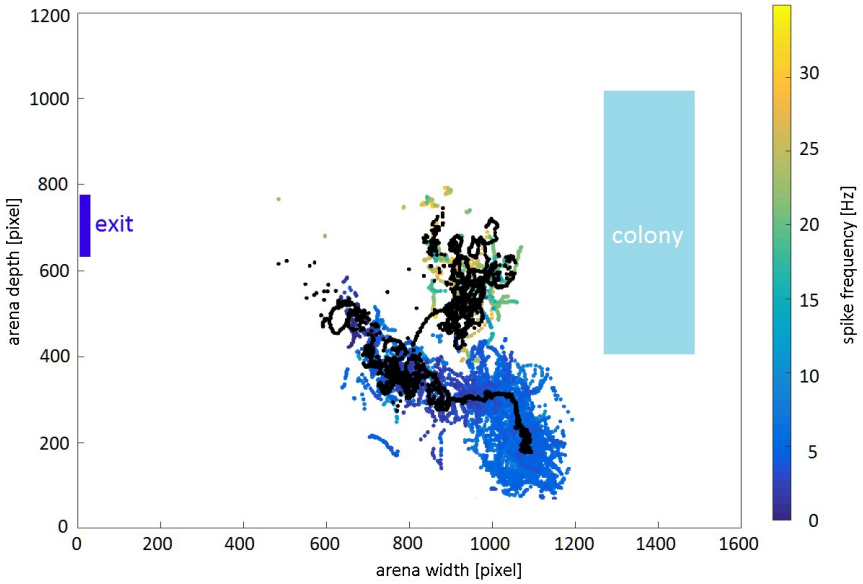

Figure 6.1: Neuronal activity in context of nearby bees. The trajectory of a free-moving bee in its hive is plotted in black. The corresponding spike activity of this bee is plotted in false colour (colour bar on the right) at the coordinates of the closest bee for each data point. In the upper right of the path, the activity is generally higher than in the rest of the experiment. Since this phenomenon has continuity, any assumption is speculative (dark blue represents the position of the hive exit; light blue represents the area of the queen group at the time of the experiment).

Nevertheless, the recorded bee and its locational in relation to any approaching bee may be of importance when one explores social correlates. Therefore, the relative distance and direction from the view of the recorded bee were analysed. The direction of the approaching bee was computed relative to the long axis of the recorded bee and the distance between the two bees. The result for one bee can be seen in Fig. 6.2. There seems to be no difference in spike activity when the recorded bee is approached from the back or front side. The distance indicates a trend of higher spike rates for smaller distances. The spike rate distribution and distance from the recorded bee to the closest bee was plotted separately, and it also confirmed this trend. In detail, low spike frequency was present at all distances, but high frequencies were more frequent at close distances (data not provided).

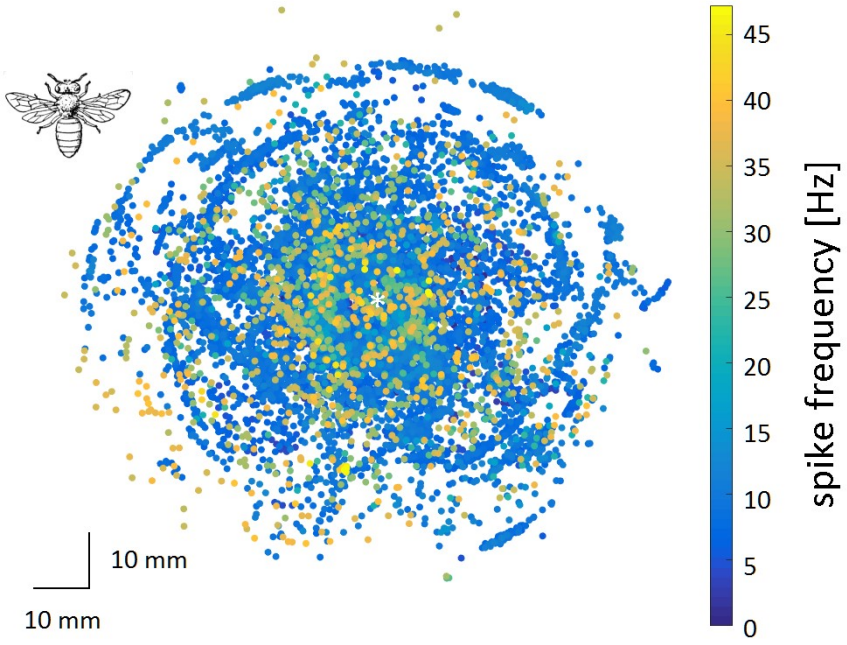

Figure 6.2: Neuronal activity in context of relative positions of the closest bee to the recorded bee. These coordinates are plotted for any given moment relative to the recorded bee positioned at the centre (white star) facing upwards (bee pictogram's upper left). The neuronal activity as spike frequency is plotted as the false colour on top of the associated coordinates of the bee with the closest distance to the recorded bee. No correlation was found concerning direction. A weak trend of higher neuronal activity for coordinates of bees particularly close to the recorded bees can be seen.

Angular properties of the bees' behaviour are analysed to some extent in Chapter 2. Here, I further investigate any possible correlations. Therefore, the occurrence of directions the recorded bee was facing were summed up (Fig. 6.3 a, b) to not falsely assume any relationships out of unequal angular distributions. With that in mind, one can appropriately evaluate the spike-rate distribution in relation to the head direction of the recorded bee (Fig. 6.3 c, d). Furthermore, the distribution of the spike rate of the recorded bee was analysed in relation with the angle of an approaching be relative to the long body axis of the recorded bee (Fig. 6.3 e, f). Neither the presented example nor the rest of the data that were analysed produced any trends in this regard. The head direction of the recorded bee was investigated in relation to the coordinates of

relevant places such as the entrance and the brood nest. This analysis was not fruitful. None of the bees changed their recorded spike activity towards any of those places in a meaningful way.

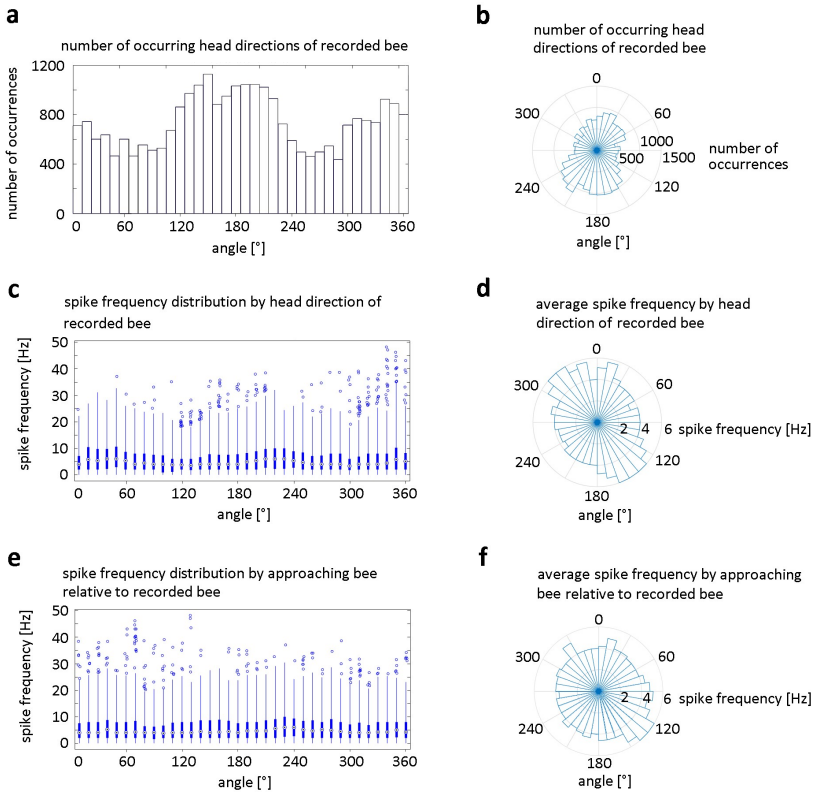

Figure 6.3: Circular analysis of orientation and approach direction of one exemplary animal. a, b Orientation of the recorded bee in relation to the arena. Distribution of occurrences per angle the bee was captured every 100 ms over the duration of the experiment. The plot on the right is the circular equivalent of the one on the left. A head direction of 0° corresponds to a bee facing up, and 270° corresponds to the wall that contains the exit tube. The bee in this example was facing downwards slightly more than any other direction. c, d Spike activity per orientation of the recorded bee in relation to the arena. Distribution of spike frequency for every angle the bee was captured each 100 ms over the duration of the experiment. The plot on the right is the circular equivalent of the average spike frequency of the plot on the left. There is no correlation between the spike activity and the orientation of the bee in the arena (Kuiper test of circular statistics, p = 0.47). e, f Distribution of spike frequency per approaching angle of the closest bee to the recorded bee. The angle and spike frequency were captured for each 100 ms over the duration of the experiment. The plots on the right (b, d, f) is the circular equivalent of the average spike frequency of the plot on the left. There is no correlation between the spike activity and the approaching angle.

The main goal of the current study was to investigate neuronal correlates of social interactions. The bees' social events in which we were most interested are as follows: following a dance, performing a dance, interacting with the queen or the brood and socially interacting with hive mates. Unfortunately, in all of our data, we could only observe the last behaviour. This behaviour was only observed in eight experiments that were frequent enough for us to analyse any correlation. Social interactions were defined as the moment when a bee approaches the recorded bee from far to the point where the distance between the two bees was close enough that they had to either touch or at least acknowledge each other. In particular, a social interaction happened when the recorded bee had no bee closer than 10 cm to it and only one bee approached the recorded bee closer than 1 cm. The moment these conditions were met are henceforth referred to as social interaction. The quality of the video prohibited any evaluation of antennation or proboscis extension. These events and a certain time window before and after were extracted from the behavioural and neuronal data. Initially. the time windows were chosen to be one second before and after the interaction. This did not have any effects. The windows were further enlarged gradually for all the experiments and started to be interesting when they were longer than 20 s. The longest period of time that was showing peak differences was 12 minutes. To evaluate them, these blocks of data were plotted one by one in order of occurrence. Plotted was the neuronal property of interest, but only spike-rate differences were found. So, the spike rate was plotted, and the moment of first interaction in the middle of the graph was marked by a vertical line. This exhibited many spike-rate changes, which were more when compared with time windows in which the bee was alone. "Alone" was defined as an equally sized time window in which the bee was always further than 10 cm apart from the next bee. In comparison, when the bee was alone, the spike rate was mostly stable. The interesting part of the spike rate changes in the time around an interaction is their unreliable nature. As can be seen in Figure 4 (blue graphs), the spike-rate changes were an increase, burst or decrease in the rate, before, during or after the interaction. Many examples of no spike rate change in the same animal worth mentioning. There is a spike rate difference in response to social interactions compared to the bee being alone. However, there are many different responses that needed to be categorised in a meaningful way. Therefore, behavioural features were

also plotted, especially the distance of the approaching bee and the walking speed of the recorded bee (Fig. 4). All other properties, for example, walking speed of an approaching bee or any kind of angular information, are not presented here but were tested. The behavioural data did not contribute to untangling different types of neuronal responses.

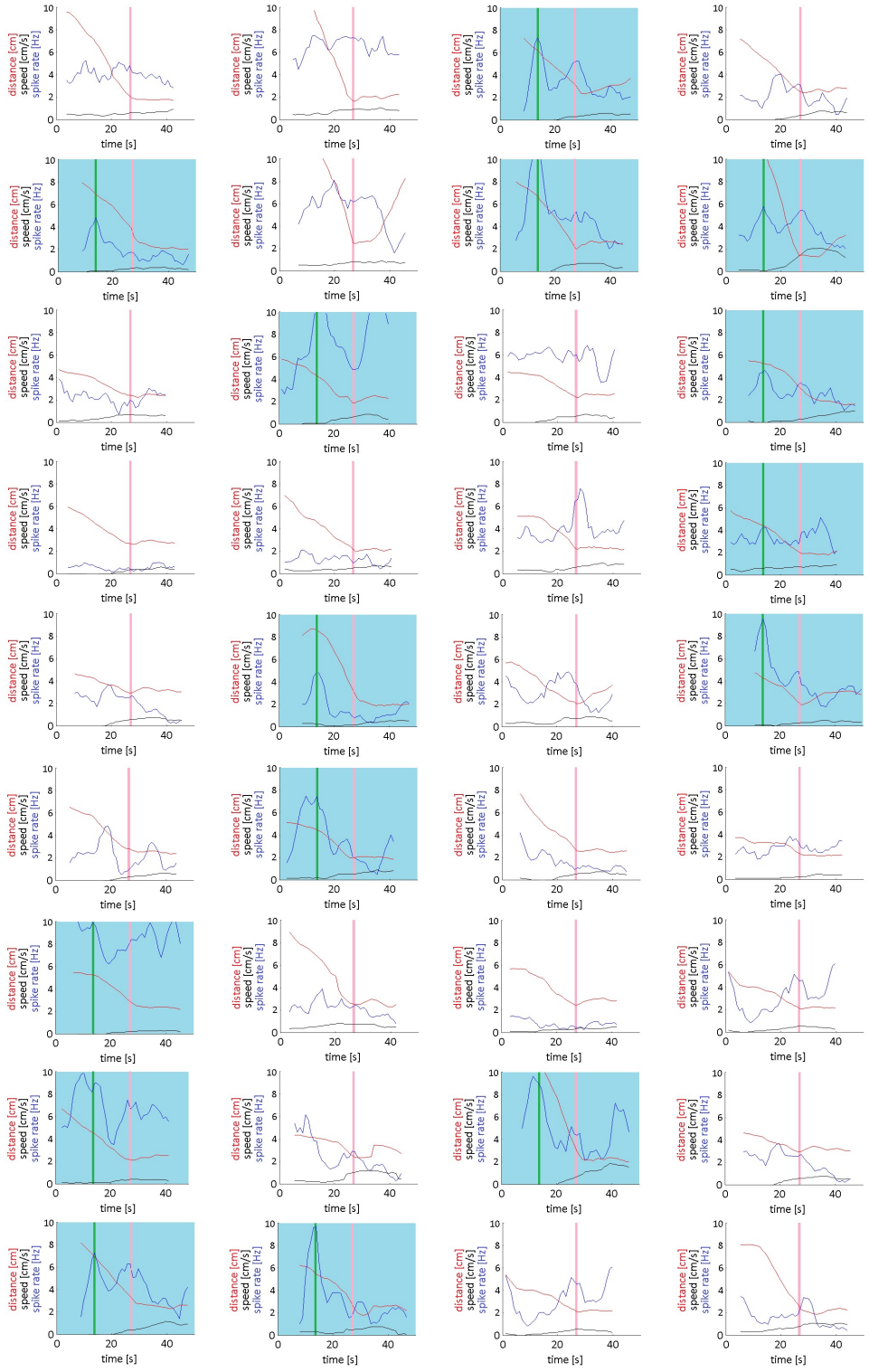

Figure 6.4: Temporal relationships between behavioural and other selected neuronal events. Each plot illustrates an encounter of a bee with the recorded bee of one experiment.

continue Fig. 6.4:The graphs are in order of occurrence. The red graph represents the distance from the closest bee to the recorded bee, and the pink vertical line depicts the first time point of the lowest distance. The black graph represents the walking speed of the recorded bee. There is no correlation between the walking speed or onset of walking and the neuronal activity. The blue graph illustrates the spike activity, and its first peak prior to the encounter was marked (the vertical green line on the light blue background). Events without any noticeable peak or a peak outside the green line do not have such green line. The focus of this figure is the temporal relation in a subset of events. The delay between the spike-rate peak and the bee's encounter is 13 to 14 seconds. This figure indicates 15 events out of 40 that did exhibit this phenomenon. Both events were marked by hand.

One particular case indicates regularity in their response pattern. In Figure 4, the social interactions of an animal are presented as mentioned earlier. Of interest are the plots with the light blue background. They illustrate occasions when the spike rate changes peaking 13.5 s before (green vertical line) the social interaction begins. This type of spike-rate change occurred 15 out of the 36 times in the recorded bee.

Another interesting case is illustrated in Figure 5. The spike rate increased and later decreased over a time period of 7 minutes when the bee had an interaction with another bee as well as when the recorded bee started to move. The firing rates were elevated 5 to 10 minutes.

The only behavioural events that were related with neuronal activity changes were social interactions and, to a less extent, the beginning of locomotion of the recorded bee.

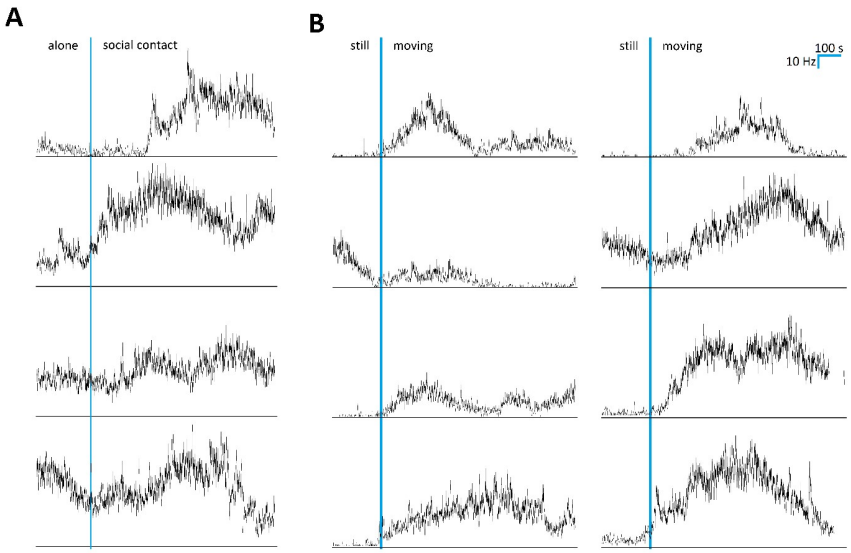

Figure 6.5: Temporal relationships between behavioural and neuronal events. **a** Each plot illustrates an encounter of a bee with the recorded bee (blue vertical line) of one experiment. **b** Each plot illustrates the onset of movement by the recorded bee independent of any other bee. The graphs are in order of occurrence. They show the spike activity over a time of 12 minutes. The events are distributed fairly evenly across a 10-hour experiment. The focus of this figure is the temporal relation in a subset of events. The data illustrate an overall trend of an increase and a following decrease of spike activity of the recorded units over a time frame of 5 to 10 minutes. Both events were marked by hand.

These spike-rate changes were not consistent in their timing, duration or polarity. To further investigate the varying spike rates, the variance of spike frequency was computed. The variance measures how far the spike frequency in the time window of a behavioural event spreads out from its average value. Therefore, the variance is independent of the spike rate baseline and does only incorporate the variety of spike frequencies within the time in question.

For the aforementioned interaction events, the variance of spike frequency was investigated with differently sized time windows to search for the greatest effect. It was found (Fig. 6) that a window of 400 seconds resulted in the greatest variance over the whole set of data that were eligible (>50 events/animal) for this type of analysis.

Notably, the effect was slightly weaker when the window started at the moment of the interaction and became stronger as time progressed. The variance of spike frequency of equally sized windows over which the bees were 'alone' and the variance for random events were computed. It can be seen that the distribution of all events per category of social interaction per animal was different in most cases. The variance distribution between 'alone' and 'random' was mostly similar, whereas the variance distribution of the spike frequency was elevated in the cases of social interaction, i.e. 'contact'.

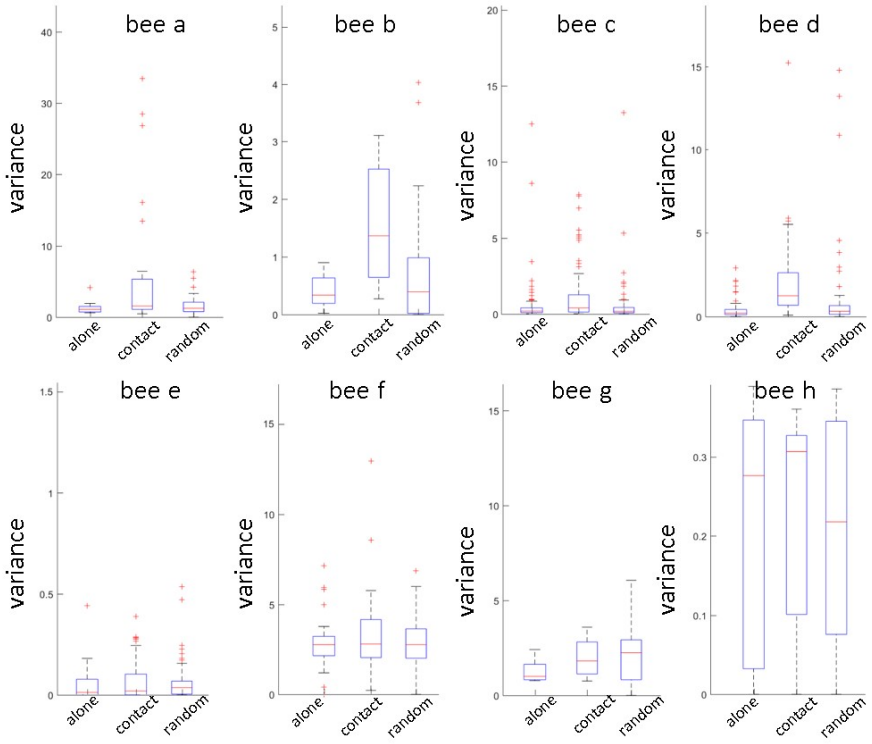

Figure 6.6: Spike frequency variance distribution in relation to social states. Eight independent experiments are presented. The variance of spike frequency changes in a 400 s window was computed for each social state and each experimental animal. The social state 'alone' was defined by a 400 s window in which the recorded bee had no contact with any other animal (e.g., no other bee was closer than 10 cm to the recorded bee). The state 'contact' was defined as a window in which the recorded bee was alone and one bee came closer than 1 cm to it. The moment of closest distance between the bees was marked as the 200th second. The resulting window of 400 s was thus 200 s both before and after the encounter. The 'random' states were 400 s windows randomly chosen by a script. The amount of windows per 'alone' and 'contact' state within one experiment were surprisingly similar. The amount for the 'random' state was chosen to be matched by the higher count of one of the naturally occurring states.

To summarise, the recorded bees behaved properly when compared to random bees. None of the neuronal properties, ISI, spike rate or their combination could be correlated with any behavioural feature related to location or direction. The only strong correlate was the variance of spike frequency in relation to social interaction.

6.5 Discussion

The aim of the current study is to find neuronal correlates of social behaviour. Due to the open exploratory nature of these experiments, the analysis of the resulting data was also exploratory. The experimental bees were prepared with a two-channel extracellular electrode to record extrinsic output neurons of the MB. The bees were situated into the social context of their natural habitat, i.e. the arena.

The challenge of the analysis of an open-world approach such as the one in the current study is the degrees of freedom. This obvious disadvantage of such an open experimental design was still outweighed by the quality of the resulting data. From a neuroethological point of view (Ewert 1980), it is exciting to investigate the neuronal activity of high-order neurons of an animal that is not restrained. The animals in the current study are neither in an unnatural position under strong illumination nor surrounded by strange doors as it is the case in many electrophysiological studies. The bee is surrounded by its peers. The light, odour and temperature in the hive are the same for the experiment as they were before the experiment.

The honeybee can do as it pleases, so unfortunately, there is a high chance of very little repetitions or the desired behaviour not occurring at all. My approach is to analyse the resulting data from any possible perspective. Such a multiverse analysis (Steegen et al. 2016) allows one to investigate any aspect that might prove meaningful afterwards. However, it is not allowed to introduce any confirmatory analysis using the data presented in the current study. Any hypothesis one could derive as a result of the data cannot be tested on the same data; this is known as HARKing (hypothesising after the results are known; Kerr 1998). When testing all dimensions of the data from extracellular recording of the brain versus all dimensions of the behavioural data extracted from the recorded videos, false negative and false positive are guaranteed.

Furthermore, the most compelling result, the increased variance of spike-rate changes when the experimental animal is interacting, was found as a result of changing the window sizes until they had a measurable difference (p-hacking; Head 2015).

In Chapter 1, I illustrated the naturality of the behaviour demonstrated by the bees, individually and as a social group. In Chapter 2, we demonstrated the stability and quality of the electrophysiological data. Additionally, the walking speed and the turning behaviour were compared with those of random bees in the arena. Included bees did not indicate any differences. Animals that behaved differently did so strongly; they walked extremely slowly, if at all, or they turned only in one direction. There were no cases that were not immediately clear while the experiment was still running. Therefore, I can rely on the acquired data and further analyse the correlation of behaviour and neuronal activity.

As presented in Chapter 2 of the current study, the spike-rate baseline of the investigated neurons is much lower than it is when the animal is restrained in a bee holder and experimented in laboratory conditions. This is also true for the data analysed here. This should be further investigated by untangling the social context and the restrained position of the bee. Therefore, I suggest that restrained bees and free moving bees be in their hive or in solitude under those two conditions. Preliminary experiments have indicated that bees in an arena such as the one presented here fly off when in solitude. When these bees have clipped wings, they explore the borders of the arena quickly and then stop moving until they die rather quickly. All of the 10 bees tested this way died within 12 hours. Restrained bees can survive up to several days when fed sufficiently.

The units spiked occasionally with 0.1 to 1 Hz. They also sporadically spiked with a frequency of up to 20 Hz. The frequency changed very slowly, over minutes, between somewhat stable values. There was no clear ON or OFF pattern. There was no pattern that could be observed without relating to the behaviour. Therefore, we included the analysis of frequency band-filtered spike rates, the interspike interval and combinatorial features of the spike frequency. The only correlation we found was based on changes in spike frequency. There was no additional information extractable by using the combinatorial spike pattern of two or more units recorded simultaneously. This is most likely due to the small number of experiments that resulted in a multiple of single unit activity. The intention of filtering the spike rate with a low-pass and

a high-pass filter was to separate time into different orders of magnitude. The low-pass filtered spike rate resulted in slow changes over minutes. I then investigated the difference in behaviour when the rate was stable for some time and at a different stable rate for a different period of time. The high-pass filtered data would reflect quick spike-rate changes that would more likely be connected to some behaviour in immediate temporal relation. The low-pass filtered data could be related to something such as a state (attention, planning, etc.), whereas the high-frequency component could code for direct responses to certain stimuli.

I investigated the special relation between the recorded bee's position and the neuronal activity in the sense of place cells and grid cells as they are known in mammals (Moser et al. 2008). No correlations were found. This is mostly due to the small space the bee covered in the period during the experiment. To further analyse such types of responses, one would need to design a walking arena that is significantly smaller. However, single bees in preliminary experiments have exhibited no walking behaviour when in solitude. It could be possible to encage recorded bees in small subsections within the arena to investigate place and grid cells. With such a motivation, recording from the central complex (CC) would also be worth considering (Pfeiffer and Homberg 2014). The orientation of the recorded bee relative to the arena as well as another bee that approached the recorded bee and thus the gravitational force were analysed. No correlation was found. One could further investigate indoor navigation by recording from the CC (Homberg 2004, Homberg et al. 2011, Seelig and Jayaraman 2013, Neuser et al. 2008). The social behaviour of the honeybee is of central interest in the current study. Therefore, I investigated spatial relations with the focus on social areas and events. I analysed the positions where social interactions occurred in search of context-dependent spike-rate changes (Gerber and Menzel 2000, Filla and Menzel 2015). I could not observe any correlations; this might be related to the small proportion of the arena the bees explored during the experiments. In different time windows of social interactions, the spike rate varied. It varied stronger then when the recorded bee was 'alone' in many cases. This unspecific correlation could not be tied to any behaviourally relevant condition. This might be of importance in determining which bee is in motion and approaching another one. There was no correlation

in such or any other way. This might be related to the small number of repetitions. Even though there were around 100 interactions per experiment, when divided based on approaching angle, walking speed, location or other factors, the number of occurrences per group is too small to give any insights.

The duration of the time windows that evoked the highest variance were surprising. The spike rate changed before, during and after interactions in seconds up to a few minutes. It was suggested that the MB is involved in the temporal integration of sensory signals (Schürmann 1987, Erber et al. 1987). There is evidence for plasticity at the input of ENs (Menzel and Manz 2005, Menzel 2012) and the coding of valency (Aso et al. 2014). These neurons can distinguish rewarded stimuli from non-rewarded stimuli (Strube-Bloss et al. 2011, 2016; Menzel 2014).

Interpretation of the results at this point is fairly speculative. The changed spike activity includes long, short, increased or decreased changes before, during or after social interactions. What I call social interaction here might include occasions on which the bees exhibited behaviour of antennation, feeding or only siting close to each other. There are too many possibilities to get to any conclusion. Therefore, I suggest getting more control of the experimental design. One feasible way would be to use cameras with a spatial and temporal resolution that would allow the identification of antennal and proboscis movement. It would be interesting to correlate each component of antennal movement with a high temporal resolution to correlate this important social behaviour with the neuronal activity. An effortless alternative would be to attach the recorded bee in some way at a location of interest. This would hinder the bee from free movement but allow for close recording, thus resulting in high spatial resolution. The position of interest would include the dance floor and the immediate proximity to the queen. This would enable one to correlate the different frequencies involved in the dancing behaviour of honeybees with high-order neurons. The movement of a dancing bee's abdomen and wings together as well as its distance from the recorded bee's head and the movement of its antenna would be very intruiging to correlate with the recorded bees neuronal activity. This should be repeated for many dances for different locations by different dancers.

A promising way of further controlling the experimental conditions without losing the existing natural conditions is to involve an automatic feeding machine. Such a device was preliminarily tested, and it worked just fine. It supplies a sucrose solution and an optic stimulus for a short period of time. This repeats in a loop with breaks of a few minutes. The first tests have indicated that the bees use the automatic feeder to gather the sucrose solution (data not shown). The bees interact with the device after a while only when the optic stimulus is present. In the context of our experiments, it would be useful to record from a bee with experience with the feeding device. It then would, most likely, continue to gather the sucrose solution when the stimulus is present and pass on the solution to a honey-processing bee. The recorded bee might move back and forth between the feeding device and honey-processing bees. It would plan, navigate, expect, be rewarded and interact socially in the process. If we can make that work, this series of behaviours could appear over and over again. The indoor foraging in general could be seen many times in this experimental set-up. The analysis of such an experiment would be straightforward. Every elemental behaviour such as the approach of the feeder can be pooled, and the neuronal activity changes can be investigated. Of special interest would be how the spike activity relates to unforeseen situations. We could easily change certain parameters such as the sucrose concentration and supply duration as well as the optic stimulus colour. How does the bee's brain respond to such changes?

By implementing the aforementioned adaptations, I am confident about exploring the neural correlates of natural behaviours further.

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7 General Discussion

The aim of the current study is the correlation of neuronal activity and social behaviour of honeybees. I demonstrate that the bees as a colony behaved as one would expect under natural conditions. The experimental bee displayed no difference in behaviour compared to any other bee. The electrophysiological data did not differ in signal-to-noise ratio or sortability when compared to traditional set-ups with tethered bees. The multiverse analysis in which I correlated any neuronal property with any meaningful aspect of behaviour revealed an increase in variance when the experimental bee interacted socially. Later on, I discuss the use of such signals as an indicator of cognitive abilities.

To investigate the social behaviour of honeybees on the level of neuronal activity, an experimental set-up must provide certain properties. The housing of a bee colony must be as close as possible to natural conditions. Additionally, the experimental bee must be accessible so that the probes to pick up neuronal data can be connected to a nearby data acquisition system. In this case, I used extracellular recording techniques. A 1m long two-channel electrode was utilised. One end of the electrode, the one connected to the amplifier of the data acquisition set-up, constituted a fixed point in space. The bee must be able to move around inside the artificial hive without any obstacles. Consequently, the colony has to be situated in a pseudo two-dimensional arena to ensure that the fixed end of the electrode and the recorded animal always have a line of sight. Therefore, I built a flat arena with one layer of wax on the ground. The surrounding border walls were covered in slippery Teflon (PTFE). To make sure the bees would not walk over each other, the number of bees per colony was considered. Furthermore, it is not possible to handle two or more recorded bees at the same time for reasons of entanglement. Generally, the wireless alternative does exist (Harrison et al. 2011) for bigger insects. The device Harrison use is roughly 1 cm2 in area and has two antennae attached that are around 8 cm in length. Between honeycomb alleys filled with other bees, this will not be suitable. Virtual reality approaches may have their applications elsewhere (Paulk 2014), but for the investigation of social behaviour, the temporal and spatial resolution of reality is unattainable for such machines. Especially here, where odours are most likely to play an extremely important

role (Michener 1974, Van Zenden 2010). Thus, it would be interesting to keep track of the flow of odours in the arena. The precise measurement of odours over time in a two-dimensional space is unreasonable at this time. Therefore, I conclude that long electrodes for one honeybee in a one-sided arena are for the short and medium terms the best approach to determining neuronal correlation of social interactions.

In Chapter 1, I dissect in great detail the social behaviour of the mini-colony. No relevant aspects of such eusocial group indicated any concerning deviation from natural conditions. The colonies used for experiments did not swarm even if they had the opportunity. The bees cared successfully for the eggs the queen had laid. The bees foraged nectar and pollen from nature as they would naturally. The amount of foraged resources did not suffice due to the small number of worker bees. Therefore, we added sucrose solution feeders. This somewhat unnatural condition did not pose any harm to the general aim of the current study. The bees do forage, and they communicate about profitable food sources by dancing. Under advantageous weather conditions, dances were more than every minute observable. Additionally, the indoor food gathering from the feeders substantiated the idea of introducing learning experiments within the arena, which is discussed later on. Even as the feeders were present, bees continued to forage outside. I investigated the circadian rhythm and found that after some time of group forming after the initial introduction of young bees into the arena, the bees quickly started to change their activity according to the time of day.

Although bees had dancing behaviour, the experimental bee did not attend one. Overall, the experimental bees did not do much. They did not explore the whole arena, and most of them were sitting some distance away from the queen group or the brood nest. This is not a surprise; bees rest much (Seeley 1989).

The total number of bees was considerably lower than in commercial or wild hives. This resulted in a small amount of available wax comb, eggs laid and brood taken care of. This is not natural but desirable; the amount of offspring matched the number of old dying bees. All colonies that were used for experiments were rather stable in count. The densely packed bees covered around a quarter of the area, and the loosely

distributed bees slightly more than a quarter. The rest of the arena was empty, thus leaving space for future experiments involving training devices.

We recorded extracellular at the beta exist of the alpha lobe; here, we expected MB extrinsic neurons that most likely belonged to the group of A1, A2 and A4 neurons (Rybak and Menzel 1993). We used some electrodes with a dye to check if we were at the correct region. Since this method did not work very often, and the dye spot was mostly larger that the region an experimenter can precisely aim for it, we rarely made this marking. The uncertainty of what cells we record from is the pitfall of extracellular recording techniques. Nevertheless, bees cannot be genetically engineered, yet as this is possible in fruit flies, and intracellular recordings are several orders of magnitude more sensitive to movement. The advantage of extracellular recordings, i.e. having some or even hundreds of electrodes (Spira and Hai 2013), is true for larger animals. Commercially available multi-electrode arrays are larger than a honeybee's whole body. We used custom-made electrodes with two electrode wires to get two channels; the more channels one records, the more details may be revealed about combinatorial differences in spike shape for a reliable spike-sorting process, even with many signals per channel. The number of wires in the current study is two; more wires seem to be damaging to the bee's brain. The signal-to-noise ratio of the electrophysiological recordings was a surprise. The 1 m long two-channel copper electrodes with a silver ground wire did not add any noise. When the recoded bee was situated in the experimental arena, the noise level was as low as in any other well-maintained electrophysiological set-up for restrained bees and close to the minimum noise the amplifier could perform. The subsequent spike sorting did not differ in any way from that of traditional experimental data from restrained animals in more controlled environments.

Nevertheless, the ratio of experiments involving bees prepared with electrodes to successful experiments was devastatingly low. It was most likely not qualitatively different from that in any other comparable experiment with restrained bees, and the percentage of electrodes delivering neuronal signals was as expected as long as the bee was still restrained. The difference derives from the bee showing behaviour af-

ter releasing into the arena for the duration of the experiments. Here, we could see the damage the preparation in some cases did to the animal's behaviour. Bees that did not behave like others in the arena behaved distinctly differently. They turned in small cycles for an hour or so and died. In those cases, we assume that the preparation of the electrode destroyed the bees' important neurons. There are experimental approaches carried out by other research groups involving free movement of extracellularly recorded insects such as the cockroach (Mizunami et al. 1998, Takeuchi et al. 2004, Mu and Ritzmann 2005, Guo et al. 2014, the dragonfly (Harrison et al. 2011, Thomas et al. 2011) and the locust (Fischer et al. 1996). However, those insects walk or fly in solitude. They do not feature a rich repertoire of behaviours as the honeybee does. These experiments are mostly aimed at walking behaviour and motor activity. The cockroach, the dragonfly and the desert locust are substantially larger than a bee, so they can carry much larger devices, and thicker or more electrodes can be used on them.

As described earlier, bees rest for a considerable amount of time, and even though they do a decent amount of patrolling (Seeley 1989), they did not explore the arena for several occasions as it would be necessary to investigate indoor navigation or concepts such as the mammalian place and grid cells. I invested the head direction with respect to gravitation, meaningful places such as the hive entrance as well as the brood nest and related the angle of approaching bees towards the recorded bee. None of those resulted in any correlation. The most interesting result with respect to the multiverse analysis was the decreased spike-rate variance for time windows around a social interaction when compared with equal time windows over which the experimental bee was alone or random time windows for the overwhelming majority of experiments. The spike frequency did not only increase or decrease before, during or after a social interaction. All these combinations occurred within one recorded animal. The spike frequency varied over a much larger time window as one would assume. Regarding the temporal context of neuronal activity and the related behaviour in an insect, an educated guess would aim for seconds. Notably, the strongest effect in difference in variance of the spike rate between animals in social contact and animals that are alone was between 40 seconds and 12 minutes.

I presume an increase in variance of spike rates as a neuronal correlate for social interaction. The known properties of MB output neurons are supporting that notion. The multimodality implies that olfaction, which is important in social behaviour, visual and mechanosensory stimuli are integrated (Mobbs 1982, Rybak and Menzel 1993, Gronenberg 2001, Schröter and Menzel 2003). This already abstract information is also coupled to some extent with valency (Aso et al. 2014 [Drosophila]). The odour–reward association can be coded by MB output neurons (Strube-Bloss 2011). Szyszka et al. (2008) demonstrated that Kenyon cells that give input to MB output neurons respond to a learned odour differently than before training. The possible responses include increase, decrease and no change in activity. Filla and Menzel (2015) found that a visual context and an olfactory cue change the firing rate of MB output neurons after learning.

To speculate about the possible meaning of these different response patterns, the amount of accumulated data is not sufficient. It might be that a certain combination of relevant stimuli leads to a stable spike-rate change, and a different combination leads to a different but stable response. To get reproducible results in this line of thoughts, the number of social events must be much higher, and the events must be differentiated much deeper on the behavioural level. The problem is that even though some bees were recorded with continuous signals for up to 24 hours, the number of recorded social events was too small. The behaviour is too multidimensional, and we lack knowledge of the relevant signals in the spike trains. The best chances of successfully analysing social interactions in the future are either to drastically increase the videos' time and spatial resolution to differentiate the behaviour much deeper or to introduce an incentive for much more repetition of a few behaviours. The first will happen in the medium term as consumer electronics drive developments in technology to higher bit rates, but one must not forget the additional overhead when handling massive amounts of data. I suggest the second approach, that is, to introduce an automatic feeding device (Paffhausen 2017) into the arena that can present a colour stimulus paired with provision of the sucrose solution. Stimulus and reward are only present for a short time; after some time, they appear again and cycle over the

whole time. Honeybees can associate the colour stimulus with the reward (Von Frisch 1914). In a preliminary test, I could see that the bees feed on such a device, and after some hours, they mostly approach the device when the colour stimulus is present. In future experiments, we would prepare with recording electrodes bees that have indicated beforehand that they have learned the association between colour stimulus and reward. When those bees are introduced back into the arena with the device, there is a reasonable chance that after some resting time the bee will continue to interact with the feeding device. This highly motivated bee could then collect sucrose and pass it on to a honey-processing bee. This would hopefully continue as the device repeats the reward and colour presentation. I have reason to believe that the experimental bee in such circumstances would socially interact with a honey-processing bee in a somewhat controlled and repetitive manner. Then, the bee would wait in anticipation for the next colour stimulus. The animal would then most likely navigate to the place where the device supplies the reward. Here, I expect some change in spike rate due to the valency of the rewarded stimulus. There will be several very interesting behaviours involved. Each behaviour within one category of behaviours will be rather stable, and the behaviour between experiments across animals will also be stable and comparable. Even more interesting is the opportunity to manipulate any parameter to the experimenters' needs. When investigating expectation, one can change the learned colour stimulus or the concentration of the sucrose solution. Additional dim light sources with the learned colour stimulus can be activated at different locations in the hive. When interested in the navigational aspect of such experiments, one can introduce a maze that gets increasingly more difficult for the bees to solve over time as walls are added. Since the experiments take place in the hive, many animals can learn without any supervision or interference. A maze also lowers the amount of bees interfering with the rewarding device (preliminary test, data not shown) what might be desirable in some cases. In the probable case in which those experiments can generally be carried out and the bees co-operate sufficiently, I propose cognitive neuroethological experiments. This topic is heavily debated (Menzel and Giurfa 2001, 2006, Srinivasan 2010, Menzel 2012, Giurfa 2013, Menzel 2017). I suggest use of the spike rate changes of the neurons investigated in the current study as indicators of complex behaviour. The neurons we most likely recoded from integrated multimodal

stimuli and meaning. They have mostly been input from neurons that are involved in learning and memory. In this case, I do not plan to tie a particular behaviour with a specific firing pattern and an identified neuron. I am rather interested in whether certain high-order behaviour exists in the honeybee. The recorded neuronal response may indicate attention or expectations.

This experimental set-up with the feeding device can easily be used to conduct experiments involving operant learning. A capacitance sensor can be integrated to measure the attendance of a bee at a particular position in the arena and then trigger the rewarding colour stimulus. Therefore, we could investigate the changes in spike rate of MB output neurons, as mentioned earlier, with the additional detour from the place the bee needs to go to activate the rewarding stimulus. In summary, I could illustrate that the experimental approach in the current study is suitable for recording high-order MB output neurons of freely behaving honeybees in their natural environment. However, the different spiking patterns that occur during social interactions could not be clearly assigned to particular subclasses of behaviour. Therefore, I suggest the use of higher-resolution behavioural monitoring to dissect behavioural differences more clearly. Alternatively, I suggest the introduction of a device that motivates repetitive indoor foraging to increase repetitive behaviour.

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B Appendix

Analysis of e-phys and behavioural data towards neuronal correlates of social behaviour

%%%
%%%%%%%%%%

spike2 and Tracker (Manu) - data

setup Ben for data from: Aron Isabella Inga

INPUT: 2 files that got exportet from spike2.smr to .mat unbinned

1 file from Manus Tracker (BeeTracker V1.2 (- instead of _)

->values.mat log.mat - if 0 new compute, if 1 take saved workspace.mat

OUTPUT: plots(not saved) and the workspacxeFULL.mat at the very end

```
clear all
close all
```

start

```
tic
figure_position1 = [2840 1080 1000 1080];   % upper window [2840 1080 1000 1080]
figure_position2 = [2840 0 1000 1080];      % lower window [2840 0 1000 1080]
figure_positiondouble = [2840 0 1000 2160];      % big window [2840 0 1000 1080]
```

loading

```
cd c1511241259_001_ALL
files=dir('*359_part_Ch1u2u3u4_log.mat');
files_amount=length(files);
if files_amount==0                                  % either import and format all the data
    % or if aleady done load the workspace
    load('1511241359_1_up_3.mat')
    times1 = V1511241359_002_Ch3.times;              % timepoint since recStart were is a spike
    codes1 = V1511241359_002_Ch3.codes;              % empty, templateNr
    values1 = V1511241359_002_Ch3.values;            % 1-30 show one analog template
    load('1511241359_1_down_4.mat')
    times2 = V1511241359_002_Ch4.times;              % timepoint since recStart were is a spike
    codes2 = V1511241359_002_Ch4.codes;              % empty, templateNr
    values2 = V1511241359_002_Ch4.values;            % 1-30 show one analog template
    load('1511241359_2_up_5.mat')
    times3 = V1511241359_002_Ch5.times;              % timepoint since recStart were is a spike
    codes3 = V1511241359_002_Ch5.codes;              % empty, templateNr
    values3 = V1511241359_002_Ch5.values;            % 1-30 show one analog template
    load('1511241359_2_down_6.mat')
    times4 = V1511241359_002_Ch6.times;              % timepoint since recStart were is a spike
    codes4 = V1511241359_002_Ch6.codes;              % empty, templateNr
    values4 = V1511241359_002_Ch6.values;            % 1-30 show one analog template
    clear V15*;
    cd c1511241359_002-1;
    files_track=dir('*values.mat');
    load(files_track.name)
```

data formating, time down, all end at 36006, cleaned from no tracking

```
    has_frame=has_frame';                           %time down
    has_frame=abs((has_frame*2)-1);
    object_count=object_count';                     %time down
    weite=size(x);                                  % 12...
    dauer=weite(1);
    weite=weite(2);
    temparray=zeros(sum(has_frame),weite);
```

other hiRes spikeformat, 10x res - new rAver + mean

```
    spikes1=zeros(10*(dauer),1);
    spikes2=zeros(10*(dauer),1);
    spikes3=zeros(10*(dauer),1);
    spikes4=zeros(10*(dauer),1);
    isi1=zeros((dauer),1);
    isi2=zeros((dauer),1);
    isi3=zeros((dauer),1);
    isi4=zeros((dauer),1);
    for i= 2:dauer*10
        spikes1(i)=length(times1(times1<(i/100) & times1>((i-1)/100)));
        spikes2(i)=length(times2(times2<(i/100) & times2>((i-1)/100)));
        spikes3(i)=length(times3(times3<(i/100) & times3>((i-1)/100)));
        spikes4(i)=length(times4(times4<(i/100) & times4>((i-1)/100)));
    end

    isiNOW=zeros(20,1);
```

```
k=1;
h=1;
for i=2:length(times1)
    if times1(i) >= k/10
        isi1(k)=max(isiNOW);
        k=k+1;
        h=1;
        isiNOW=zeros(20,1);
    end
    if k<=dauer
        isiNOW(h)=(times1(i)-times1(i-1));%+isi1(k);
        h=h+1;
    end
end

isiNOW=zeros(20,1);
k=1;
h=1;
for i=2:length(times2)
    if times2(i) >= k/10
        isi2(k)=max(isiNOW);
        k=k+1;
        h=1;
        isiNOW=zeros(20,1);
    end
    if k<=dauer
        isiNOW(h)=(times2(i)-times2(i-1));%+isi1(k);
        h=h+1;
    end
end

isiNOW=zeros(20,1);
k=1;
h=1;
for i=2:length(times3)
    if times3(i) >= k/10
        isi3(k)=max(isiNOW);
        k=k+1;
        h=1;
        isiNOW=zeros(20,1);
    end
    if k<=dauer
        isiNOW(h)=(times3(i)-times3(i-1));%+isi1(k);
        h=h+1;
    end
end

isiNOW=zeros(20,1);
k=1;
h=1;
for i=2:length(times4)
    if times4(i) >= k/10
        isi4(k)=max(isiNOW);
        k=k+1;
        h=1;
        isiNOW=zeros(20,1);
    end
    if k<=dauer
        isiNOW(h)=(times4(i)-times4(i-1));%+isi1(k);
        h=h+1;
    end
```

```
end

spikes1_smooth=smooth(spikes1,10);
spikes2_smooth=smooth(spikes2,10);
spikes3_smooth=smooth(spikes3,10);
spikes4_smooth=smooth(spikes4,10);
spikes1_down=zeros(dauer,1);
spikes2_down=zeros(dauer,1);
spikes3_down=zeros(dauer,1);
spikes4_down=zeros(dauer,1);
spikes1_down_neu=zeros(dauer,1);
spikes2_down_neu=zeros(dauer,1);
spikes3_down_neu=zeros(dauer,1);
spikes4_down_neu=zeros(dauer,1);

for i = 1:dauer
    spikes1_down_neu(i)=mean(spikes1(((i*10)-9):((i*10))))*10;
    spikes2_down_neu(i)=mean(spikes2(((i*10)-9):((i*10))))*10;
    spikes3_down_neu(i)=mean(spikes3(((i*10)-9):((i*10))))*10;
    spikes4_down_neu(i)=mean(spikes4(((i*10)-9):((i*10))))*10;
end
for i = 1:dauer
    spikes1_down(i)=mean(spikes1_smooth(((i*10)-9):((i*10))))*10;
    spikes2_down(i)=mean(spikes2_smooth(((i*10)-9):((i*10))))*10;
    spikes3_down(i)=mean(spikes3_smooth(((i*10)-9):((i*10))))*10;
    spikes4_down(i)=mean(spikes4_smooth(((i*10)-9):((i*10))))*10;
end
for j=1:weite                % only tracked parts stay
    temp=x(:,j);
    temparray(:,j)=temp(has_frame~=0);
end
x=temparray;
for j=1:weite
    temp=y(:,j);
    temparray(:,j)=temp(has_frame~=0);
end
y=temparray;
for j=1:weite
    temp=angle(:,j);
    temparray(:,j)=temp(has_frame~=0);
end
angle=temparray;
for j=1:weite
    temp=distance(:,j);
    temparray(:,j)=temp(has_frame~=0);
end
distance=temparray;
for j=1:weite
    temp=distance_to_main(:,j);
    temparray(:,j)=temp(has_frame~=0);
end
distance_to_main=temparray;
for j=1:weite
    temp=id(:,j);
    temparray(:,j)=temp(has_frame~=0);
end
id=temparray;
for j=1:weite
    temp=rel_angle(:,j);
    temparray(:,j)=temp(has_frame~=0);
end
```

```
    rel_angle=temparray;
    object_count=object_count(has_frame~=0);
    spikes1_down_neu=spikes1_down_neu(has_frame~=0);
    spikes2_down_neu=spikes2_down_neu(has_frame~=0);
    spikes3_down_neu=spikes3_down_neu(has_frame~=0);
    spikes4_down_neu=spikes4_down_neu(has_frame~=0);
    spikes1_down=spikes1_down(has_frame~=0);
    spikes2_down=spikes2_down(has_frame~=0);
    spikes3_down=spikes3_down(has_frame~=0);
    spikes4_down=spikes4_down(has_frame~=0);
    isi1=isi1(has_frame~=0);
    isi2=isi2(has_frame~=0);
    isi3=isi3(has_frame~=0);
    isi4=isi4(has_frame~=0);
    distance_to_main(distance_to_main==0)=NaN;
    x = double(x);
    y = double(y);
    angle=double(angle);
    distance_to_main=double(distance_to_main);
    id=double(id);
    object_count=double(object_count);
    rel_angle=double(rel_angle);
    x(x ==0) = nan;
    y(y ==0) = nan;
    y = 1200-y;         % mirror video upside down
    angle=angle-90;
    angle=mod(angle,360);
    for j=1:12
        for i=1:length(angle(:,j))-1
            if abs(angle(i,j)-angle(i+1,j))>130 && abs(angle(i,j)-angle(i+1,j))<200
                angle(i+1,j)=mod((angle(i+1,j)-180),360);
            end
        end
    end

    clear temp*;
    log=1;
    FileNameLog=[datestr(now, 'yyyy-mm-dd'),'_1511241359_part_Ch1u2u3u4_log.mat'];
    save(FileNameLog,'log')
    FileName=[datestr(now, 'yyyy-mm-dd'),'_1511241359_part_Ch1u2u3u4_workspace.mat'];
    save(FileName)
else
    workspace_file=dir('*359_part_Ch1u2u3u4_workspace.mat');
    workspace=workspace_file.name;
    load(workspace)
    cd ..
end
disp('data complete')
```

variables

```
distance_speed_threshold = 50;              % walkingspeed threshold [50]
rAverageSpeed_size = 50;                    % average' breite [50]
angle_resolution = 36;                      % polarPlot bin'ing [36]
turning_angle_smooth = 10;                  % bin'ing for direction/angle, 360-0° [10]
binning_dist=100;
binning_speed=100;
track_bins=50;
step_behavior=100;
```

```
density_plot_scaler=10;
rAverage1 = smooth(spikes1_down,5);          % smoothed beforehand by 10
rAverage2 = smooth(spikes2_down,5);
rAverage3 = smooth(spikes3_down,5);
rAverage4 = smooth(spikes4_down,5);

max1=max(rAverage1);
max2=max(rAverage2);
if max2<max1                                 % the bigger rAverage max stays for both colorbars
    max2=max1;
end
```

spikeshape overlay Unit 1 2 3 4 [1]

```
figure('OuterPosition',figure_position1)
subplot(2,2,1)
valuesHK1=values1';
plot(valuesHK1(:,1:100:end))
set(gca,'YDir', 'reverse')
str = {'spikes in Unit1: ',length(values1),'spikes displayed:',floor(length(values1)/100)};
annotation('textbox',[.2 .6 .3 .3],'String',str,'FitBoxToText','on');
subplot(2,2,2)
valuesHK2=values2';
plot(valuesHK2(:,1:100:end))
set(gca,'YDir', 'reverse')
str = {'spikes in Unit2: ',length(values2),'spikes displayed:',floor(length(values2)/100)};
annotation('textbox',[.7 .6 .3 .3],'String',str,'FitBoxToText','on');
subplot(2,2,3)
valuesHK3=values3';
plot(valuesHK3(:,1:100:end))
set(gca,'YDir', 'reverse')
str = {'spikes in Unit3: ',length(values3),'spikes displayed:',floor(length(values3)/100)};
annotation('textbox',[.2 0 .3 .3],'String',str,'FitBoxToText','on');
subplot(2,2,4)
valuesHK4=values4';
plot(valuesHK4(:,1:100:end))
set(gca,'YDir', 'reverse')
str = {'spikes in Unit4: ',length(values4),'spikes displayed:',floor(length(values4)/100)};
annotation('textbox',[.7 0 .3 .3],'String',str,'FitBoxToText','on');
```

rAverage combinatoric 4x [2]

```
figure('OuterPosition',figure_position1)
subplot(2,2,1)
scatter(rAverage2,rAverage3,[],rAverage1,'filled')
title('rAverage2,rAverage3,[],rAverage1')
subplot(2,2,2)
scatter(rAverage3,rAverage4,[],rAverage2,'filled')
title('rAverage3,rAverage4,[],rAverage2')
subplot(2,2,3)
scatter(rAverage4,rAverage1,[],rAverage3,'filled')
title('rAverage4,rAverage1,[],rAverage3')
subplot(2,2,4)
scatter(rAverage1,rAverage2,[],rAverage4,'filled')% spikerate over time rAverage1
title('rAverage1,rAverage2,[],rAverage4')
```

rAverage 1-4 timePlot [3]

```
figure('OuterPosition',figure_position1)
subplot(2,2,1)
plot(rAverage1*10)
title('spikerate over time rAverage1 in Hz')
subplot(2,2,2)
plot(rAverage2*10)
title('spikerate over time rAverage2 in Hz')
subplot(2,2,3)
plot(rAverage3*10)
title('spikerate over time rAverage3 in Hz')
subplot(2,2,4)
plot(rAverage4*10)
title('spikerate over time rAverage4 in Hz')
```

rAverage combinatoric 4x rAverage1_delta [4 & 5]

```
rAverage1_low=smooth(rAverage1,100);
rAverage2_low=smooth(rAverage2,100);
rAverage3_low=smooth(rAverage3,100);
rAverage4_low=smooth(rAverage4,100);
rAverage1_delta=rAverage1_low-rAverage1;
rAverage2_delta=rAverage2_low-rAverage2;
rAverage3_delta=rAverage3_low-rAverage3;
rAverage4_delta=rAverage4_low-rAverage4;
figure('OuterPosition',figure_position2)
subplot(2,2,1)
scatter(rAverage2_delta,rAverage3_delta,[],rAverage1_delta,'filled')
subplot(2,2,2)
scatter(rAverage3_delta,rAverage4_delta,[],rAverage2_delta,'filled')
subplot(2,2,3)
scatter(rAverage4_delta,rAverage1_delta,[],rAverage3_delta,'filled')
subplot(2,2,4)
scatter(rAverage1_delta,rAverage2_delta,[],rAverage4_delta,'filled')
figure('OuterPosition',figure_position1)
subplot(2,2,1)
plot(rAverage1_delta)
subplot(2,2,2)
plot(rAverage2_delta)
subplot(2,2,3)
plot(rAverage3_delta)
subplot(2,2,4)
plot(rAverage4_delta)
```

plot rAverage 1-4 LOW [6 & 7]

```
figure('OuterPosition',figure_position1)
plot([rAverage1_low rAverage2_low rAverage3_low rAverage4_low])
figure('OuterPosition',figure_position2)
plot([rAverage1 rAverage2 rAverage3 rAverage4])
```

spikerate over time rAverage2 [8]

```
figure('OuterPosition',figure_position2)
plot(rAverage2*10)
title('spikerate over time rAverage2')
```

trajectorie peripherie bienen / recBee [9]

```
figure('OuterPosition',figure_position1)
title('recBee trajectory over periferBees')
xlabel('video width in pixel')
ylabel('video hight in pixel')
hold on
plot(x(:,2:12),y(:,2:12),'c.')      % einfarbig
plot(x(:,1),y(:,1))                 % trajektorie Rec Bee
xlim([0 1600]);
ylim([0 1200]);
axis equal
hold off
```

neuro falsecolor over track rAverage1 [10]

```
figure('OuterPosition',figure_position1)
scatter(x(:,1),y(:,1),[],rAverage1,'filled')
title('trajectory of recBee, neuronal activity false color rAverage1')
colorbar
caxis([0 max1])
```

neuro falsecolor over track rAverage2 [11]

```
figure('OuterPosition',figure_position2)
scatter(x(:,1),y(:,1),[],rAverage2,'filled')
title('trajectory of recBee, neuronal activity false color rAverage2')
colorbar
caxis([0 max1])
```

unit1/unit2 TIMERESOLUTION [12]

```
figure('OuterPosition',figure_position2)
a = 10;
c = linspace(1,60,length(rAverage1));
scatter(rAverage1,rAverage2,a,c,'filled')
axis equal
axis square
title('Unit2 over Unit1, time cource color coded')
xlabel('sp.Frequency Unit1 in spikes/frame')
ylabel('sp.Frequency Unit2 in spikes/frame')
colorbar
clear a;
clear c;
```

ISI1 & ISI2 TIMERESOLUTION [13]

```
figure('OuterPosition',figure_position2)
c = linspace(1,60,length(rAverage1));
scatter(isi1,isi2,10,c,'filled')
title('ISI1 over ISI2, time cource color coded')
xlabel('ISI in sec')
ylabel('ISI in sec')
colorbar
clear c;
```

ISI1 VS ISI2 VS rAverage1 VS rAvewrage2 DIAGONAL HIST [14]

```
figure('OuterPosition',figure_positiondouble)
X=[isi1,isi2,isi3,isi4,rAverage1,rAverage2,rAverage3,rAverage4];
plotmatrix(X)
[S,AX,BigAx,H,HAx] = plotmatrix(X);
ylabel(AX(1),'ISI1')
ylabel(AX(2),'ISI2')
ylabel(AX(3),'ISI3')
ylabel(AX(4),'ISI4')
ylabel(AX(5),'rAverage1')
ylabel(AX(6),'rAverage2')
ylabel(AX(7),'rAverage3')
ylabel(AX(8),'rAverage4')
xlabel(AX(8),'ISI1')
xlabel(AX(16),'ISI2')
xlabel(AX(24),'ISI3')
xlabel(AX(32),'ISI4')
xlabel(AX(40),'rAverage1')
xlabel(AX(48),'rAverage2')
xlabel(AX(56),'rAverage3')
xlabel(AX(64),'rAverage4')
```

unit1/unit2 NOW IN 3D [15]

```
testrAverage1=ceil(density_plot_scaler*(rAverage1)/3);
testrAverage2=ceil(density_plot_scaler*(rAverage2)/3);
testrAverage1(testrAverage1==0)=1;
testrAverage2(testrAverage2==0)=1;
unit_hist=zeros(max(testrAverage1), max(testrAverage2));
for i=1:length(rAverage1)
    unit_hist(testrAverage1(i),testrAverage2(i))=unit_hist(testrAverage1(i),testrAverage2(i))+
end
unit_hist=sqrt(unit_hist);
figure('OuterPosition',figure_position1)
surface(unit_hist(2:end,2:end),'edgecolor','none')
%view(3)
title('Unit2 over Unit1 - density plot  WURZEL GEZOGEN!!!!!')
xlabel('sp.Frequency rAverage1 WURZEL GEZOGEN!!!!!')
ylabel('sp.Frequency rAverage2 WURZEL GEZOGEN!!!!!')
colorbar
```

unit1/unit2 NOW IN 3D TIMEBLOCKER (5min) 1/2 & 2/1 [16]

```
figure('OuterPosition',figure_position1)
for j=1:12
```

```
    subplot(2,12,j)
    unit_hist=zeros(max(testrAverage1), max(testrAverage2));
    for i=1+(floor(length(rAverage1)/12)*(j-1)):floor(length(rAverage1)/12)*j
        unit_hist(testrAverage1(i),testrAverage2(i))=unit_hist(testrAverage1(i),testrAverage2(
    end
    unit_hist=sqrt(unit_hist);
    surface(unit_hist)
    k=.0765*(j-1)+.01;
    set(gca,'Position',[k .52 .075 .45])
    %set(gca, 'ZScale', 'log')
end
str = {'Unit2 over Unit1'};
annotation('textbox',[.8 .6 .3 .3],'String',str,'FitBoxToText','on','EdgeColor','none');
for j=1:12
    subplot(2,12,j+12)
    unit_hist=zeros(max(testrAverage1), max(testrAverage2));
    for i=1+(floor(length(rAverage1)/12)*(j-1)):floor(length(rAverage1)/12)*j
        unit_hist(testrAverage1(i),testrAverage2(i))=unit_hist(testrAverage1(i),testrAverage2(
    end
    unit_hist=sqrt(unit_hist);
    surface(unit_hist')
    k=.0765*(j-1)+.01;
    set(gca,'Position',[k .02 .075 .45])
    %set(gca, 'ZScale', 'log')
end
str = {'Unit1 over Unit2'};
annotation('textbox',[.8 .1 .3 .3],'String',str,'FitBoxToText','on','EdgeColor','none');
```

unit1/unit2 spikeHistogramm - timeResolution [17]

```
figure('OuterPosition',figure_position1)
rAverage1_1000=smooth(rAverage1,1000);       % rolling average 5 werte gemittelt (standart)
rAverage2_1000=smooth(rAverage2,1000);
scatterhist(rAverage1_1000,rAverage2_1000,'MarkerSize',1)
axis square
axis equal
title('Unit2 over Unit1 rAverage 2min')
xlabel('sp.Frequency # Unit1 in spikes/frame')
ylabel('sp.Frequency # Unit2 in spikes/frame')
clear rAverage1_1000;
clear rAverage1_1000;
```

unit1/unit2 spikeHistogramm RolAverage [18]

```
figure('OuterPosition',figure_position2)
scatterhist(rAverage1,rAverage2,'MarkerSize',1)
axis square
axis equal
title('Unit2 over Unit1 rAverage')
xlabel('sp.Frequency # Unit1 in spikes/frame')
ylabel('sp.Frequency # Unit2 in spikes/frame')
```

isi1/isi2 spikeHistogramm interspikeIntervall [19]

```
figure('OuterPosition',figure_position2)
```

```
scatterhist(isi1,isi2,'MarkerSize',1)
axis square
axis equal
title('Unit2 over Unit1 ISI')
xlabel('interspikeInterval # Unit1 in sec')
ylabel('interspikeInterval # Unit2 in sec')
```

distance/speed periferBees

distance > 50 = sprünge - entfernen

```
speed=zeros(size(x));
for i=1:length(x)
    for j=1:12
        if distance(i,j)<distance_speed_threshold
            speed(i,j)=distance(i,j);
        end
    end
end
% rolling average speed zu rAverageSpeed
rAverageSpeed=zeros(size(x));
for i=1:12
    rAverageSpeed(:,i)=smooth(speed(:,i),rAverageSpeed_size);
end
```

Histogramm RecBee SPEED [20]

```
figure('OuterPosition',figure_position1)
hist(rAverageSpeed(:,1),1000)
title('walking speed, averaded, histo of RecBee')
xlabel('speed in pixel/frame')
ylabel('#')
xlim([0 10])
```

RecBee SPEED vs spiking [21]

```
rAverageSpeed_rAverage1 = [rAverageSpeed(:,1), rAverage1];
rAverageSpeed_rAverage1_sort=sortrows(rAverageSpeed_rAverage1);
speed_hist_rAverage1=zeros(floor(10*length(x)/binning_speed),binning_speed);
speed_hist_rAverage1(speed_hist_rAverage1==0)=nan;
max_speed=max(rAverageSpeed_rAverage1_sort(:,1));
step_speed=max_speed/binning_speed;
j=1;
k=1;
for i=1:length(x)
    if  rAverageSpeed_rAverage1_sort(i,1)<step_speed*j && rAverageSpeed_rAverage1_sort(i,1)...
            >= step_speed*(j-1)
        speed_hist_rAverage1(k,j)=rAverageSpeed_rAverage1_sort(i,2);
        k=k+1;
    else
        speed_hist_rAverage1(1,j+1)=rAverageSpeed_rAverage1_sort(i,2);
        j=j+1;
        k=2;
    end
end
```

```
speed_hist_rAverage1=speed_hist_rAverage1(:,1:end-1);
figure('OuterPosition',figure_position1)
subplot(3,1,1)
boxplot(speed_hist_rAverage1)
title('walking speed, averaded vs rAverage1 spike activity')
xlabel('speed from lowest to highest')
ylabel('spikes per frame')

speed_hist_rAverage1_amount=NaN(1,binning_speed);
for i=1:size(speed_hist_rAverage1,2)
    speed_hist_rAverage1_amount(i)=sum(isnan(speed_hist_rAverage1(:,i)));
end
subplot(3,1,2)
plot((size(speed_hist_rAverage1,1))-speed_hist_rAverage1_amount);
title('walking speed, ocurance')
xlabel('speed from lowest to highest')
ylabel('#')

rAverageSpeed_rAverage2 = [rAverageSpeed(:,1), rAverage2];
rAverageSpeed_rAverage2_sort=sortrows(rAverageSpeed_rAverage2);
speed_hist_rAverage2=zeros(floor(10*length(x)/binning_speed),binning_speed);
speed_hist_rAverage2(speed_hist_rAverage2==0)=nan;
j=1;
k=1;
for i=1:length(x)
    if  rAverageSpeed_rAverage2_sort(i,1)<step_speed*j && rAverageSpeed_rAverage2_sort(i,1)...
            >= step_speed*(j-1)
        speed_hist_rAverage2(k,j)=rAverageSpeed_rAverage2_sort(i,2);
        k=k+1;
    else
        speed_hist_rAverage2(1,j+1)=rAverageSpeed_rAverage2_sort(i,2);
        j=j+1;
        k=2;
    end
end
speed_hist_rAverage2=speed_hist_rAverage2(:,1:end-1);
subplot(3,1,3)
boxplot(speed_hist_rAverage2)
title('walking speed, averaded vs rAverage2 spike activity')
xlabel('speed from lowest to highest')
ylabel('spikes per frame')
```

Histogramm periferBees SPEED [22]

```
rAverageSpeedrow=zeros(length(x)*11,1);
for j=2:12                          % all in one row for histogramm
    for i=1:size(x)
        rAverageSpeedrow(i+(size(x)*(j-2)))=rAverageSpeed(i,j);
    end
end
figure('OuterPosition',figure_position2)
hist(rAverageSpeedrow,1000)
xlim([0 10])
ylim([0 20000])
title('walking speed, averaded, histo of periferBees')
xlabel('speed in pixel/frame')
ylabel('#')
```

angle distro of rec bee PolarPlot [23]

```
figure('OuterPosition',figure_position2)
rad_angle=degtorad(angle(:,1));
rose(rad_angle,180)
title('angular distribution of RecBee')
```

turning directions [24]

```
turn=zeros(length(x),1);
smooth_angle=smooth(angle(:,1),turning_angle_smooth);
for i=1:length(x)-60
    turn(i)=angle(i,1)-angle(i+1,1);
end
direction_hist=hist(turn);      % 2 and 9 turn oder 360; 5-decrease, 6 increase
disp('left turns:')
disp(direction_hist(5))
disp('right turns:')
disp(direction_hist(6))
disp('left turnsovers:')
disp(sum(direction_hist(1:2)))
disp('right turnsovers:')
disp(sum(direction_hist(9:10)))


% angle over time RecBee
figure('OuterPosition',figure_position1)
subplot(2,1,1);
plot(angle(:,1));
xlabel('time in frames')
ylabel('angle of recBee in degree')
title('angular orientation over time of RecBee')
testtext=0;
angle_full=angle(:,1);
for i=1:length(x)-1
    if angle_full(i)-angle_full(i+1)>200
        angle_full(i+1:end)=angle_full(i+1:end)+360;
        testtext=testtext+1;
    elseif angle_full(i+1)-angle_full(i)>200
        angle_full(i+1:end)=angle_full(i+1:end)-360;
    end
end
subplot(2,1,2);
str = {'overall turns: ',floor((angle_full(end)-angle_full(1))/360)};
annotation('textbox',[.2 0 .3 .3],'String',str,'FitBoxToText','on','EdgeColor','none');
plot(angle_full)
xlabel('time in frames')
ylabel('angle of recBee in degree')
title('angular orientation over time of RecBee cumulative (360° = 0°)')
```

Activity1 per angel [25 & 26]

```
figure('OuterPosition',figure_position1)
angle_resolution_corr=360/angle_resolution;
angleUnits=zeros(floor((length(x)/100)*angle_resolution_corr),angle_resolution);
angleUnits(angleUnits == 0) = NaN;
```

```
for j=1:angle_resolution
    h=1;
    for i=1:length(x)
        if angle(i,1) > (j-1)*angle_resolution_corr && angle(i,1) <= j*angle_resolution_corr
            angleUnits(h,j)=rAverage1(i);
            h=h+1;
        end
    end
end
boxplot(angleUnits,'plotstyle','compact','whisker',3)
ylabel('distro of spike Activity Unit1')
xlabel('angle of recBee in degree')
title('amound of spikes per frame of Unit 1 per angle RecBee')

% Activity2 per angel
figure('OuterPosition',figure_position2)
angle_resolution_corr=360/angle_resolution;
angleUnits=zeros(floor((length(x)/100)*angle_resolution_corr),angle_resolution);
angleUnits(angleUnits == 0) = NaN;
for j=1:angle_resolution
    h=1;
    for i=1:length(x)
        if angle(i,1) > (j-1)*angle_resolution_corr && angle(i,1) <= j*angle_resolution_corr
            angleUnits(h,j)=rAverage2(i);
            h=h+1;
        end
    end
end
boxplot(angleUnits,'plotstyle','compact','whisker',3)
ylabel('distro of spike Activity Unit1')
xlabel('angle of recBee in degree')
title('amound of spikes per frame of Unit 2 per angle RecBee')
```

activity1 over angle in timeColor [27 & 28]

```
figure('OuterPosition',figure_position1)
a = 10;
c = linspace(1,60,length(rAverage1));
scatter(rAverage1,angle(:,1),a,c,'filled')
ylim([0 360])
xlabel('spiks per frame of Unit1')                          %Label the horizontal axis
ylabel('angle of recBee')                                   %Label the vertical axis
title('angular distr. of spikeActivity of Unit1, color-timecourse')
colorbar
clear a;

% activity2 over angle in timeColor
figure('OuterPosition',figure_position2)
a = 10;
c = linspace(1,60,length(rAverage2));
scatter(rAverage2,angle(:,1),a,c,'filled')
ylim([0 360])
xlabel('spiks per frame of Unit1')                           %Label the horizontal axis
ylabel('angle of recBee')                                    %Label the vertical axis
title('angular distr. of spikeActivity of Unit2, color-timecourse')
colorbar
clear a;
```

distance to the closest periferBee over spike Activity [29]

```
distance_to_main_sort=distance_to_main;
distance_to_main_sort(distance_to_main == 0) = nan;           % get rid of 0's by jumps/bee ch
for i=1:length(x)
    distance_to_main_sort(i,2:12) = sort(distance_to_main_sort(i,2:12));
                                                              % all other then the recBee sort
end
distance_to_main_sort_rAverage = [distance_to_main_sort(:,2),rAverage1,rAverage2];
                                                              % closest distance, unit1, unit2
distance_to_main_sort_rAverage = sortrows(distance_to_main_sort_rAverage);
dist_hist_rAverage1=zeros(floor(10*length(x)/binning_dist),binning_dist);
dist_hist_rAverage1(dist_hist_rAverage1==0)=nan;
dist_hist_rAverage2=zeros(floor(10*length(x)/binning_dist),binning_dist);
dist_hist_rAverage2(dist_hist_rAverage2==0)=nan;
max_dist=max(distance_to_main_sort_rAverage(:,1));
step_dist=max_dist/binning_dist;
j=1;
k=1;
for i=1:length(x)
    if  distance_to_main_sort_rAverage(i,1)<step_dist*j && distance_to_main_sort_rAverage(i,1)
            >=step_dist*(j-1)
        dist_hist_rAverage1(k,j)=distance_to_main_sort_rAverage(i,2);
        dist_hist_rAverage2(k,j)=distance_to_main_sort_rAverage(i,3);
        k=k+1;
    else
        dist_hist_rAverage1(1,j+1)=distance_to_main_sort_rAverage(i,2);
        dist_hist_rAverage2(1,j+1)=distance_to_main_sort_rAverage(i,3);
        j=j+1;
        k=2;
    end
end
figure('OuterPosition',figure_positiondouble)
subplot(3,1,1)
boxplot(dist_hist_rAverage1)
ylabel('spiks per frame of Unit1')
xlabel('distance of closest periferBee')
title('distance to closest periferBee vs spikes per frame rollAverage, unit1')
dist_hist_rAverage1_amount=NaN(1,binning_dist);
for i=1:binning_dist
    dist_hist_rAverage1_amount(i)=sum(isnan(dist_hist_rAverage1(:,i)));
end
subplot(3,1,2)
plot((size(dist_hist_rAverage1,1))-dist_hist_rAverage1_amount);
ylabel('#')
xlabel('distance of closest periferBee')
title('distance to closest periferBee vs #')
subplot(3,1,3)
boxplot(dist_hist_rAverage2)
ylabel('spiks per frame of Unit1')
xlabel('distance of closest periferBee')
title('distance to closest periferBee vs spikes per frame rollAverage, unit2')
```

plotting the closest bees with neuronal activity as color on tracks [30 & 31]

```
combiTest=cat(3,distance_to_main,x,y,angle);
for i=1:length(x)
```

```
    temp(:,:)=combiTest(i,2:end,:);
    temp= sortrows(temp); % all other then the recBee sorted
    combiTest(i,2:end,:)=temp;
    clear temp;
end

figure('OuterPosition',figure_positiondouble)
subplot(2,1,1)
hold on
scatter(combiTest(:,2,2),combiTest(:,2,3),[],rAverage1,'filled')
scatter(combiTest(:,1,2),combiTest(:,1,3),[],'k','filled')
title('trajectory of periferBee CLOSE, neuronal activity false color rAverage1')
colorbar
caxis([0 max1])
hold off
subplot(2,1,2)
hold on
%scatter(combiTest(:,3,2),combiTest(:,3,3),[],rAverage2,'filled')
scatter(combiTest(:,2,2),combiTest(:,2,3),[],rAverage2,'filled')
scatter(combiTest(:,1,2),combiTest(:,1,3),[],'k','filled')
title('trajectory of periferBee CLOSE, neuronal activity false color rAverage2')
colorbar
caxis([0 max1])
hold off

figure('OuterPosition',figure_positiondouble)
subplot(2,1,1)
hold on
scatter(combiTest(:,7,2),combiTest(:,7,3),[],rAverage1,'filled')
scatter(combiTest(:,6,2),combiTest(:,6,3),[],rAverage1,'filled')
scatter(combiTest(:,5,2),combiTest(:,5,3),[],rAverage1,'filled')
scatter(combiTest(:,4,2),combiTest(:,4,3),[],rAverage1,'filled')
scatter(combiTest(:,3,2),combiTest(:,3,3),[],rAverage1,'filled')
scatter(combiTest(:,2,2),combiTest(:,2,3),[],rAverage1,'filled')
scatter(combiTest(:,1,2),combiTest(:,1,3),[],'k','filled')
title('trajectory of periferBees, neuronal activity false color rAverage1')
colorbar
caxis([0 max1])
hold off
subplot(2,1,2)
hold on
scatter(combiTest(:,7,2),combiTest(:,7,3),[],rAverage2,'filled')
scatter(combiTest(:,6,2),combiTest(:,6,3),[],rAverage2,'filled')
scatter(combiTest(:,5,2),combiTest(:,5,3),[],rAverage2,'filled')
scatter(combiTest(:,4,2),combiTest(:,4,3),[],rAverage2,'filled')
scatter(combiTest(:,3,2),combiTest(:,3,3),[],rAverage2,'filled')
scatter(combiTest(:,2,2),combiTest(:,2,3),[],rAverage2,'filled')
scatter(combiTest(:,1,2),combiTest(:,1,3),[],'k','filled')
title('trajectory of periferBees, neuronal activity false color rAverage2')
colorbar
caxis([0 max1])
hold off
```

plotting the closest bees with neuronal activity as color on tracks relative to recBee [32 & 33]

angle and position of periferBee RELATIV to egocentric recBee

```
x_rel=NaN(size(x));
```

```
y_rel=NaN(size(x));
angle_rel=NaN(size(x));
for i=1:length(x)
    if isnan(angle(i,1))
    else
        x_rel(i,:)=x(i,:)-x(i,1);
        y_rel(i,:)=y(i,:)-y(i,1);
        angle_rel(i,:)=mod(angle(i,:)-angle(i,1),360);
        R=rotx(angle(i,1));
        for j=2:12
            temp_a=[1;x_rel(i,j);y_rel(i,j)];
            temp_b=R*temp_a;
            y_rel(i,j)=temp_b(3);
            x_rel(i,j)=temp_b(2);
        end
    end
end

combiTest_rel=cat(3,distance_to_main,x_rel,y_rel);
for i=1:length(x)
    temp(:,:)=combiTest_rel(i,2:12,:);
    temp= sortrows(temp); % all other then the recBee sorted
    combiTest_rel(i,2:12,:)=temp;
    clear temp;
end

figure('OuterPosition',figure_positiondouble)
subplot(2,1,1)
hold on
scatter(combiTest_rel(:,5,2),combiTest_rel(:,5,3),[],rAverage1,'filled')
scatter(combiTest_rel(:,4,2),combiTest_rel(:,4,3),[],rAverage1,'filled')
scatter(combiTest_rel(:,3,2),combiTest_rel(:,3,3),[],rAverage1,'filled')
scatter(combiTest_rel(:,2,2),combiTest_rel(:,2,3),[],rAverage1,'filled')
hold off
title('trajectory of periferBees, neuronal activity false color rAverage1')
subplot(2,1,2)
hold on
scatter(combiTest_rel(:,5,2),combiTest_rel(:,5,3),[],rAverage2,'filled')
scatter(combiTest_rel(:,4,2),combiTest_rel(:,4,3),[],rAverage2,'filled')
scatter(combiTest_rel(:,3,2),combiTest_rel(:,3,3),[],rAverage2,'filled')
scatter(combiTest_rel(:,2,2),combiTest_rel(:,2,3),[],rAverage2,'filled')
hold off
title('trajectory of periferBees, neuronal activity false color rAverage2')

figure('OuterPosition',figure_positiondouble)
subplot(2,1,1)
hold on
scatter(combiTest_rel(:,2,2),combiTest_rel(:,2,3),[],rAverage1,'filled')
hold off
title('trajectory of periferBee CLOSEST, neuronal activity false color rAverage1')
subplot(2,1,2)
hold on
scatter(combiTest_rel(:,2,2),combiTest_rel(:,2,3),[],rAverage2,'filled')
hold off
title('trajectory of periferBee CLOSEST, neuronal activity false color rAverage2')
```

PSTH closest contact - peak delta > 10pxls && closness < 50pxls [34 & 35]

```
[closest_peak,closest_peak_i] = findpeaks(-1*smooth(distance_to_main_sort(:,2),50));
```

```
                                                                % find peaks, negativ so close
contact=zeros(length(x),1);
PSTH_contact_unit1=zeros(floor(length(closest_peak_i)/10),50);
PSTH_contact_unit1(PSTH_contact_unit1 == 0) = nan;     % get rid of 0's
PSTH_contact_unit2=PSTH_contact_unit1;
PSTH_contact_rAverage1=PSTH_contact_unit1;
PSTH_contact_rAverage2=PSTH_contact_unit1;
j=1;
for i=5:length(closest_peak_i)-10
    if    (closest_peak(i)+distance_for_contact)>0     % if a peak is closer then last its rea
        contact(closest_peak_i(i)) = closest_peak(i);  % all 0's but max
        PSTH_contact_rAverage1(j,:)=rAverage1(((closest_peak_i(i)-25):(closest_peak_i(i)+24)))
        PSTH_contact_rAverage1(j,:)=PSTH_contact_rAverage1(j,:)-PSTH_contact_rAverage1(j,25);
        PSTH_contact_rAverage2(j,:)=rAverage2(((closest_peak_i(i)-25):(closest_peak_i(i)+24)))
        PSTH_contact_rAverage2(j,:)=PSTH_contact_rAverage2(j,:)-PSTH_contact_rAverage2(j,25);
        j=j+1;
    end
end

figure('OuterPosition',figure_positiondouble)          % plot closness over time, cycle contac
hold on
plot(-1*(distance_to_main_sort(:,2)))
plot(contact,'o','MarkerSize',12)
ylim([-50 0])
title('plot closness over time, cycle contact(isch)')
hold off

figure('OuterPosition',figure_positiondouble)
subplot(2,1,1)
boxplot(PSTH_contact_rAverage1)                        % PSTH of rAverage1 @ closeness
str = {'contacts: ',size(PSTH_contact_rAverage1,2)};
annotation('textbox',[.5 .3 .2 .2],'String',str,'FitBoxToText','on','EdgeColor','none');
title('PSTH of rAverage1 @ closeness +/- 25 bin')
%set(gca,'Position',[.53 .53 .44 .44])
subplot(2,1,2)
boxplot(PSTH_contact_rAverage2)                        % PSTH of rAverage2 @ closeness
title('PSTH of rAverage2 @ closeness +/- 25 bin')
%set(gca,'Position',[.53 .03 .44 .44])
```

neuro activity before and after contact [36]

```
figure('OuterPosition',figure_positiondouble)
j=0;
PSTH_contact_rAverage1_bin=zeros(size(PSTH_contact_rAverage1,1),2);
for i=1:size(PSTH_contact_rAverage1,1)
    PSTH_contact_rAverage1_bin(i,1)=0.05*sum(PSTH_contact_rAverage1(i,1:20));
    PSTH_contact_rAverage1_bin(i,2)=0.05*sum(PSTH_contact_rAverage1(i,31:50));
end
subplot(2,1,2)
boxplot(PSTH_contact_rAverage1_bin)
title('20 bins befor and 20 bins after contact summed boxplot rAverage1')

for i=1:size(PSTH_contact_rAverage1,2)
    if PSTH_contact_rAverage1_bin(i,1)<PSTH_contact_rAverage1_bin(i,2)
        PSTH_contact_rAverage1_bin(i,:)=PSTH_contact_rAverage1_bin(i,:)+1;
        j=j+1;
    end
end
subplot(2,1,1)
```

```
plot(PSTH_contact_rAverage1_bin')
str = {'up: ',j,'down: ',length(PSTH_contact_rAverage1_bin(~isnan(PSTH_contact_rAverage1_bin(:
annotation('textbox',[.5 .4 .3 .3],'String',str,'FitBoxToText','on','EdgeColor','none');
title('20 bins befor and 20 bins after contact summed, sorted & counted ascending/descending r
```

behavior analysis, contact+-step

```
j=0;
for i=1:length(x)
    if contact(i) ~= 0
        j=j+1;
    end
end
contact_counter=j;
behavior_contact=zeros(contact_counter,step_behavior,10);
behavior_contact_norm=behavior_contact;
j=1;
for i=1+(step_behavior/2):length(x)
    if contact(i) ~= 0
        behavior_contact(j,:,1)=x(i-(step_behavior/2):i+(step_behavior/2)-1,1);
        behavior_contact(j,:,2)=y(i-(step_behavior/2):i+(step_behavior/2)-1,1);
        behavior_contact(j,:,3)=angle(i-(step_behavior/2):i+(step_behavior/2)-1,1);
        behavior_contact(j,:,4)=rAverageSpeed(i-(step_behavior/2):i+(step_behavior/2)-1,1);
        behavior_contact(j,:,5)=x(i-(step_behavior/2):i+(step_behavior/2)-1,2);
        behavior_contact(j,:,6)=y(i-(step_behavior/2):i+(step_behavior/2)-1,2);
        behavior_contact(j,:,7)=angle(i-(step_behavior/2):i+(step_behavior/2)-1,2);
        behavior_contact(j,:,8)=rAverageSpeed(i-(step_behavior/2):i+(step_behavior/2)-1,2);
        behavior_contact(j,:,9)=rAverage1(i-(step_behavior/2):i+(step_behavior/2)-1);
        behavior_contact(j,:,10)=rAverage2(i-(step_behavior/2):i+(step_behavior/2)-1);
        j=j+1;
    end
end
for i=1:contact_counter
    for j=1:10
        behavior_contact_norm(i,:,j)=behavior_contact(i,:,j)-behavior_contact(i,(step_behavior
    end
end

behavior_contact_mean=zeros(length(behavior_contact(:,1,1)),2,length(behavior_contact(1,1,:)))
behavior_contact_mean_norm=behavior_contact_mean;
for i=1:length(behavior_contact(:,1,1))
    for j=1:10
        behavior_contact_mean(i,1,j)=mean(behavior_contact(i,1:(step_behavior/2),j));
        behavior_contact_mean(i,2,j)=mean(behavior_contact(i,(step_behavior/2)-1:step_behavior
    end
end
for i=1:length(behavior_contact(:,1,1))
    for j=1:10
        behavior_contact_mean_norm(i,1,j)=mean(behavior_contact_norm(i,1:(step_behavior/2),j))
        behavior_contact_mean_norm(i,2,j)=...
            mean(behavior_contact_norm(i,(step_behavior/2)-1:step_behavior,j));
    end
end
```

behavior analysis, contact+-step [37]

```
figure('OuterPosition',figure_positiondouble)
```

```
str = {'each contact(close) +- step, x y angle speed REC, x y angle speed perifer close,'...
    'rAverage1 & rAverage2'};
annotation('textbox',[.1 0 .9 .99],'String',str,'FitBoxToText','on','EdgeColor','none');
j=1;
for i=1:10
    subplot(5,4,j)
    plot(behavior_contact(:,:,i)')
    subplot(5,4,j+1)
    boxplot(behavior_contact_mean(:,:,i))
    j=j+2;
end
```

behavior analysis, contact+-step NORMALIZED [38]

```
figure('OuterPosition',figure_positiondouble)
str = {'each contact(close) +- step, x y angle speed REC, x y angle speed perifer close,'...
    'rAverage1 & rAverage2 NORM'};
annotation('textbox',[.1 0 .9 .99],'String',str,'FitBoxToText','on','EdgeColor','none');
j=1;
for i=1:10
    subplot(5,4,j)
    plot(behavior_contact_norm(:,:,i)')
    subplot(5,4,j+1)
    boxplot(behavior_contact_mean_norm(:,:,i))
    j=j+2;
end
```

behavior analysis, contact+-step NORMALIZED UP & DOWN rAverage1 [39]

```
behavior_contact_mean_norm_rauf1=zeros(contact_counter,step_behavior,10);
behavior_contact_mean_norm_rauf1(behavior_contact_mean_norm_rauf1==0)=nan;
behavior_contact_mean_norm_runter1=zeros(contact_counter,step_behavior,10);
behavior_contact_mean_norm_runter1(behavior_contact_mean_norm_runter1==0)=nan;
j=1;
k=1;
for i=1:length(behavior_contact(:,1,1))
    if behavior_contact_mean_norm(i,1,9) < behavior_contact_mean_norm(i,2,9)
        behavior_contact_mean_norm_rauf1(j,:,:)=behavior_contact_norm(i,:,:);
        j=j+1;
    else
        behavior_contact_mean_norm_runter1(k,:,:)=behavior_contact_norm(i,:,:);
        k=k+1;
    end
end
figure('OuterPosition',figure_positiondouble)
str = {'each contact(close) +- step, x y angle speed REC, x y angle speed perifer close,'...
    'rAverage1 & rAverage2 sortet to rAverage1 left down right up'};
annotation('textbox',[.1 0 .9 .99],'String',str,'FitBoxToText','on','EdgeColor','none');
rauf_counter1=length(behavior_contact_mean_norm_rauf1(~isnan(behavior_contact_mean_norm_rauf1.
    (:,1,10))));
runter_counter1=length(behavior_contact_mean_norm_runter1(~isnan(behavior_contact_mean_norm_ru
    (:,1,10))));
str = {'rAverage1 up:',rauf_counter1,'down:',runter_counter1};
annotation('textbox',[.1 .05 .9 .92],'String',str,'FitBoxToText','on','EdgeColor','none');
j=1;
for i=1:10
    subplot(5,4,j)
```

```
    plot(behavior_contact_mean_norm_rauf1(:,:,i)')
    subplot(5,4,j+1)
    plot(behavior_contact_mean_norm_runter1(:,:,i)')
    j=j+2;
end
```

behavior analysis, contact+-step NORMALIZED UP & DOWN rAverage2 [40]

```
behavior_contact_mean_norm_rauf2=zeros(contact_counter,step_behavior,10);
behavior_contact_mean_norm_rauf2(behavior_contact_mean_norm_rauf2==0)=nan;
behavior_contact_mean_norm_runter2=zeros(contact_counter,step_behavior,10);
behavior_contact_mean_norm_runter2(behavior_contact_mean_norm_runter2==0)=nan;
j=1;
k=1;
for i=1:length(behavior_contact(:,1,1))
    if behavior_contact_mean_norm(i,1,10) < behavior_contact_mean_norm(i,2,10)
        behavior_contact_mean_norm_rauf2(j,:,:)=behavior_contact_norm(i,:,:);
        j=j+1;
    else
        behavior_contact_mean_norm_runter2(k,:,:)=behavior_contact_norm(i,:,:);
        k=k+1;
    end
end
figure('OuterPosition',figure_positiondouble)
str = {'each contact(close) +- step, x y angle speed REC, x y angle speed perifer close,'...
    'rAverage1 & rAverage2 sortet to rAverage2 left down right up'};
annotation('textbox',[.1 0 .9 .99],'String',str,'FitBoxToText','on','EdgeColor','none');
rauf_counter2=length(behavior_contact_mean_norm_rauf2(~isnan(behavior_contact_mean_norm_rauf2.
    (:,1,10))));
runter_counter2=length(behavior_contact_mean_norm_runter2(~isnan(behavior_contact_mean_norm_ru
    (:,1,10))));
str = {'rAverage2 up:',rauf_counter2,'down:',runter_counter2};
annotation('textbox',[.1 .05 .9 .92],'String',str,'FitBoxToText','on','EdgeColor','none');
j=1;
for i=1:10
    subplot(5,4,j)
    plot(behavior_contact_mean_norm_rauf2(:,:,i)')
    subplot(5,4,j+1)
    plot(behavior_contact_mean_norm_runter2(:,:,i)')
    j=j+2;
end
%min(property)
```

histo rAverage1 vs distance closest - density plot [41] distance perifer rA1

```
figure('OuterPosition',figure_position1)
property=distance_to_main_sort(:,2);
property_step=0.5;
fullrAverage1=ceil(density_plot_scaler*(rAverage1));
fullrAverage1(fullrAverage1==0)=1;
fullproperty=ceil(property_step*(property));
fullproperty(fullproperty==0)=1;
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(rAverage1)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
```

```
end
hist_block(1,1)=0;
subplot(2,5,1)
surface(sqrt(hist_block(2:end,2:end)'))
title('rAverage1 vs distance closest - density plot')
xlabel('sp.Frequency rAverage1')
ylabel('property')
set(gca,'Position',[.02 .51 .19 .44])
```

histo rAverage2 vs distance closest - density plot distance perifer rA2

```
fullrAverage1=ceil(density_plot_scaler*(rAverage2));
fullrAverage1(fullrAverage1==0)=1;
fullproperty=ceil(property_step*(property));
fullproperty(fullproperty==0)=1;
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(rAverage1)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,6)
surface(sqrt(hist_block(2:end,2:end)'))
title('rAverage2 vs distance closest - density plot')
xlabel('sp.Frequency rAverage1')
ylabel('property')
set(gca,'Position',[.02 .02 .19 .44])
```

histo rAverage1 vs x - density plot x coordinate recBee rA1

```
property=x(:,1);
property_step=0.5;
fullrAverage1=ceil(density_plot_scaler*(rAverage1));
fullrAverage1(fullrAverage1==0)=1;
fullproperty=ceil(property_step*(property));
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(rAverage1)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,2)
surface(sqrt(hist_block(2:end,2:end)'))
title('rAverage1 vs x coordinat - density plot')
xlabel('sp.Frequency rAverage1')
ylabel('property')
set(gca,'Position',[.22 .51 .19 .44])
```

histo rAverage2 vs x - density plot x coordinate recBee rA2

```
fullrAverage1=ceil(density_plot_scaler*(rAverage2));
fullrAverage1(fullrAverage1==0)=1;
fullproperty=ceil(property_step*(property));
```

```
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(rAverage1)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,7)
surface(sqrt(hist_block(2:end,2:end)'))
title('rAverage2 vs x coordinat - density plot')
xlabel('sp.Frequency rAverage2')
ylabel('property')
set(gca,'Position',[.22 .02 .19 .44])
```

histo rAverage1 vs y - density plot y coordinate recBee rA1

```
property=y(:,1);
property_step=0.5;
fullrAverage1=ceil(density_plot_scaler*(rAverage1));
fullrAverage1(fullrAverage1==0)=1;
fullproperty=ceil(property_step*(property));
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(rAverage1)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,3)
surface(sqrt(hist_block(2:end,2:end)'))
title('rAverage1 vs y coordinat - density plot')
xlabel('sp.Frequency rAverage1')
ylabel('property')
set(gca,'Position',[.42 .51 .19 .44])
```

histo rAverage2 vs y - density plot y coordinate recBee rA2

```
fullrAverage1=ceil(density_plot_scaler*(rAverage2));
fullrAverage1(fullrAverage1==0)=1;
fullproperty=ceil(property_step*(property));
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(rAverage1)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,8)
surface(sqrt(hist_block(2:end,2:end)'))
title('rAverage2 vs y coordinat - density plot')
xlabel('sp.Frequency rAverage2')
ylabel('property')
set(gca,'Position',[.42 .02 .19 .44])
```

histo rAverage1 vs angle - density plot angle recBee rA1

```
property=angle(:,1);
property_step=0.5;
fullrAverage1=ceil(density_plot_scaler*(rAverage1));
fullrAverage1(fullrAverage1==0)=1;
fullproperty=ceil(property_step*(property));
fullproperty(fullproperty<0)=0;
fullproperty=fullproperty+1;
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(rAverage1)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,4)
surface(sqrt(hist_block(2:end,2:end)'))
title('rAverage1 vs angle recBee - density plot')
xlabel('sp.Frequency rAverage1')
ylabel('property')
set(gca,'Position',[.62 .51 .19 .44])
```

histo rAverage2 vs angle - density plot angle recBee rA2

```
fullrAverage1=ceil(density_plot_scaler*(rAverage2));
fullrAverage1(fullrAverage1==0)=1;
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(rAverage1)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,9)
surface(sqrt(hist_block(2:end,2:end)'))
title('rAverage2 vs angle RecBee - density plot')
xlabel('sp.Frequency rAverage1')
ylabel('property')
set(gca,'Position',[.62 .02 .19 .44])
```

histo rAverage1 vs distance closest - density plot [42] distance perifer isi1

```
figure('OuterPosition',figure_position2)
property=distance_to_main_sort(:,2);
property_step=0.5;
fullrAverage1=ceil(density_plot_scaler*(isi1)*100);
fullrAverage1(fullrAverage1==0)=1;
%fullrAverage1(fullrAverage1>100)=100;

fullproperty=ceil(property_step*(property));
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(isi1)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,1)
```

```
surface(sqrt(hist_block(2:end,2:end)'))
title('isi1 vs distance closest - density plot')
%xlabel('isi in sec')
ylabel('property')
set(gca,'Position',[.02 .51 .19 .44])
```

histo rAverage2 vs distance closest - density plot distance perifer isi2

```
fullrAverage1=ceil(density_plot_scaler*(isi2)*100);
fullrAverage1(fullrAverage1==0)=1;
fullproperty=ceil(property_step*(property));
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(isi2)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,6)
surface(sqrt(hist_block(2:end,2:end)'))
title('isi2 vs distance closest - density plot')
%xlabel('sp.Frequency rAverage1')
ylabel('property')
set(gca,'Position',[.02 .02 .19 .44])
```

histo rAverage1 vs x - density plot x coordinate recBee isi1

```
property=x(:,1);
property_step=0.5;
fullrAverage1=ceil(density_plot_scaler*(isi1)*100);
fullrAverage1(fullrAverage1==0)=1;
fullproperty=ceil(property_step*(property));
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(isi1)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,2)
surface(sqrt(hist_block(2:end,2:end)'))
title('isi1 vs x coordinat - density plot')
%xlabel('sp.Frequency rAverage1')
ylabel('property')
set(gca,'Position',[.22 .51 .19 .44])
```

histo rAverage2 vs x - density plot x coordinate recBee isi2

```
fullrAverage1=ceil(density_plot_scaler*(isi2)*100);
fullrAverage1(fullrAverage1==0)=1;
fullproperty=ceil(property_step*(property));
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(isi2)
```

```
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,7)
surface(sqrt(hist_block(2:end,2:end)'))
title('isi2 vs x coordinat - density plot')
%xlabel('sp.Frequency rAverage1')
ylabel('property')
set(gca,'Position',[.22 .02 .19 .44])
```

histo rAverage1 vs y - density plot y coordinate recBee isi1

```
property=y(:,1);
property_step=0.5;
fullrAverage1=ceil(density_plot_scaler*(isi1)*100);
fullrAverage1(fullrAverage1==0)=1;
fullproperty=ceil(property_step*(property));
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(isi1)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,3)
surface(sqrt(hist_block(2:end,2:end)'))
title('isi1 vs y coordinat - density plot')
%xlabel('sp.Frequency rAverage1')
ylabel('property')
set(gca,'Position',[.42 .51 .19 .44])
```

histo rAverage2 vs y - density plot y coordinate recBee isi2

```
fullrAverage1=ceil(density_plot_scaler*(isi2)*100);
fullrAverage1(fullrAverage1==0)=1;
fullproperty=ceil(property_step*(property));
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(isi2)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,8)
surface(sqrt(hist_block(2:end,2:end)'))
title('isi2 vs y coordinat - density plot')
%xlabel('sp.Frequency rAverage1')
ylabel('property')
set(gca,'Position',[.42 .02 .19 .44])
```

histo rAverage1 vs angle - density plot 000rAverage1 VS isi2

```
property=rAverage1;
property_step=10;
fullrAverage1=ceil(density_plot_scaler*(isi1)*100);
```

```
fullrAverage1(fullrAverage1==0)=1;
fullproperty=ceil(property_step*(property));
fullproperty(fullproperty<0)=0;
fullproperty=fullproperty+1;
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(isi1)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,5)
surface(sqrt(hist_block(2:end,2:end)'))
title('isi1 vs rAverage1 - density plot')
%xlabel('sp.Frequency rAverage1')
ylabel('property')
set(gca,'Position',[.82 .51 .16 .44])
```

histo rAverage2 vs angle - density plot 000rAverage1 VS isi2

```
fullrAverage1=ceil(density_plot_scaler*(isi2)*100);
fullrAverage1(fullrAverage1==0)=1;
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(isi2)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,10)
surface(sqrt(hist_block(2:end,2:end)'))
title('isi2 vs rAverage1 - density plot')
%xlabel('sp.Frequency rAverage1')
ylabel('property')
set(gca,'Position',[.82 .02 .16 .44])
```

histo rAverage1 vs angle - density plot angle recBee isi1

```
property=angle(:,1);
property_step=0.5;
fullrAverage1=ceil(density_plot_scaler*(isi1)*100);
fullrAverage1(fullrAverage1==0)=1;
fullproperty=ceil(property_step*(property));
fullproperty(fullproperty<0)=0;
fullproperty=fullproperty+1;
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(isi1)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,4)
surface(sqrt(hist_block(2:end,2:end)'))
title('isi1 vs angle recBee - density plot')
%xlabel('sp.Frequency rAverage1')
ylabel('property')
```

```
set(gca,'Position',[.62 .51 .19 .44])
```

histo rAverage2 vs angle - density plot angle recBee isi2

```
fullrAverage1=ceil(density_plot_scaler*(isi2)*100);
fullrAverage1(fullrAverage1==0)=1;
fullproperty(isnan(fullproperty))=1;
fullrAverage1(isnan(fullrAverage1))=1;
hist_block=zeros(max(fullrAverage1), max(fullproperty));
for i=1:length(isi2)
    hist_block(fullrAverage1(i),fullproperty(i))=hist_block(fullrAverage1(i),fullproperty(i))+
end
hist_block(1,1)=0;
subplot(2,5,9)
surface(sqrt(hist_block(2:end,2:end)'))
title('isi2 vs angle RecBee - density plot')
%xlabel('sp.Frequency rAverage1')
ylabel('property')
set(gca,'Position',[.62 .02 .19 .44])
```

rAverage change up or down quickly! plots [43 & 44 & 45 & 46]

```
rAverage1_up=rAverage1;
for i=10:length(rAverage1)
    if rAverage1(i)-rAverage1(i-1) > .2    % if spikes go up
        rAverage1_up(i)=rAverage1_up(i);
    else
        rAverage1_up(i)=nan;
    end
end
rAverage1_down=rAverage1;
for i=10:length(rAverage1)
    if rAverage1(i-1)-rAverage1(i) > .2    % if spikes go down
        rAverage1_down(i)=rAverage1_down(i);
    else
        rAverage1_down(i)=nan;
    end
end

dist_test_up=distance_to_main_sort(:,2);
dist_test_down=distance_to_main_sort(:,2);
dist_test_neither=zeros(length(x),1);
for i=1:length(x)
    if isnan(rAverage1_up(i))
        dist_test_up(i)=0;
    end
end
for i=1:length(x)
    if isnan(rAverage1_down(i))
        dist_test_down(i)=0;
    end
end
for i=1:length(x)
    if dist_test_down(i) == 0 && dist_test_up(i) == 0
        dist_test_neither(i)=distance_to_main_sort(i,2);
    end
end
```

```
figure('OuterPosition',figure_positiondouble)
subplot(2,1,1)
bin_distance_to_main_thisplot=30;
zeroline=zeros(bin_distance_to_main_thisplot,1);
hist([dist_test_up dist_test_down],bin_distance_to_main_thisplot)
upper_lim=(mean(hist([dist_test_up dist_test_down],bin_distance_to_main_thisplot)));
ylim([0 upper_lim(2)])
title('spike rate rise (blue) or falls(red) over distence to main SORT histo')
subplot(2,1,2)
hold on
spike_change_hist1=hist([dist_test_up dist_test_down],bin_distance_to_main_thisplot);
plot((spike_change_hist1(:,1)-spike_change_hist1(:,2)));
plot(zeroline)
hold off
title('spike rate rise (positiv) or falls (negativ) over distence to main SORT histo')


dist_test_up=x(:,1);
dist_test_down=x(:,1);
dist_test_neither=zeros(length(x),1);
for i=1:length(x)
    if isnan(rAverage1_up(i))
        dist_test_up(i)=0;
    end
end
for i=1:length(x)
    if isnan(rAverage1_down(i))
        dist_test_down(i)=0;
    end
end
for i=1:length(x)
    if dist_test_down(i) == 0 && dist_test_up(i) == 0
        dist_test_neither(i)=x(i,1);
    end
end
figure('OuterPosition',figure_positiondouble)
subplot(2,1,1)
bin_x_thisplot=300;
zeroline=zeros(bin_x_thisplot,1);
hist([dist_test_up dist_test_down],bin_x_thisplot)
upper_lim=(mean(hist([dist_test_up dist_test_down],bin_x_thisplot)));
ylim([0 upper_lim(1)])
title('spike rate rise (blue) or falls(red) over x-position recBee histo')
subplot(2,1,2)
hold on
spike_change_hist1=hist([dist_test_up dist_test_down],bin_x_thisplot);
plot((spike_change_hist1(:,1)-spike_change_hist1(:,2)));
plot(zeroline)
hold off
title('spike rate rise (positiv) or falls (negativ) over x-position recBee histo')


dist_test_up=x_rel(:,2);
dist_test_down=x_rel(:,2);
dist_test_neither=zeros(length(x),1);
for i=1:length(x)
    if isnan(rAverage1_up(i))
        dist_test_up(i)=0;
    end
end
for i=1:length(x)
```

```
    if isnan(rAverage1_down(i))
        dist_test_down(i)=0;
    end
end
for i=1:length(x)
    if dist_test_down(i) == 0 && dist_test_up(i) == 0
        dist_test_neither(i)=x_rel(i,2);
    end
end
figure('OuterPosition',figure_positiondouble)
subplot(2,1,1)
bin_x_rel_thisplot=30;
zeroline=zeros(bin_x_rel_thisplot,1);
hist([dist_test_up dist_test_down],bin_x_rel_thisplot)
upper_lim=(mean(hist([dist_test_up dist_test_down],bin_x_rel_thisplot)));
ylim([0 upper_lim(2)])
title('spike rate rise (blue) or falls(red) over x-position perifer RELATIVE histo')
subplot(2,1,2)
hold on
spike_change_hist1=hist([dist_test_up dist_test_down],bin_x_rel_thisplot);
plot((spike_change_hist1(:,1)-spike_change_hist1(:,2)));
plot(zeroline)
hold off
title('spike rate rise (positiv) or falls (negativ) over x-position perifer RELATIVE histo')


dist_test_up=angle(:,1);
dist_test_down=angle(:,1);
dist_test_neither=zeros(length(x),1);
for i=1:length(x)
    if isnan(rAverage1_up(i))
        dist_test_up(i)=0;
    end
end
for i=1:length(x)
    if isnan(rAverage1_down(i))
        dist_test_down(i)=0;
    end
end
for i=1:length(x)
    if dist_test_down(i) == 0 && dist_test_up(i) == 0
        dist_test_neither(i)=angle(i,2);
    end
end
figure('OuterPosition',figure_positiondouble)
subplot(2,1,1)
bin_angle_thisplot=30;
zeroline=zeros(bin_angle_thisplot,1);
hist([dist_test_up dist_test_down],bin_angle_thisplot);
upper_lim=(mean(hist([dist_test_up dist_test_down],bin_angle_thisplot)));
ylim([0 upper_lim(2)])
title('spike rate rise (blue) or falls(red) over angle of RecBee histo')
subplot(2,1,2)
hold on
spike_change_hist1=hist([dist_test_up dist_test_down],bin_angle_thisplot);
plot((spike_change_hist1(:,1)-spike_change_hist1(:,2)));
plot(zeroline)
hold off
title('spike rate rise (positiv) or falls (negativ) over angle of RecBee histo')
```

Activity1 per angel_rel [47 & 48]

```
figure('OuterPosition',figure_position1)
angle_resolution_corr=360/angle_resolution;
angleUnits_rel=NaN(floor((length(x)/100)*angle_resolution_corr),angle_resolution);
for j=1:angle_resolution
    h=1;
    for i=1:length(x)
        if angle_rel(i,2) > (j-1)*angle_resolution_corr && angle_rel(i,2) <= j*angle_resolutio
            angleUnits_rel(h,j)=rAverage1(i);
            h=h+1;
        end
    end
end
boxplot(angleUnits_rel,'plotstyle','compact','whisker',3)
ylabel('distro of spike Activity Unit1')
xlabel('angle of closest periferBee in degree')
title('amound of spikes per frame of Unit 1 per angle_REL periferBee')

% Activity2 per angel
figure('OuterPosition',figure_position2)
angle_resolution_corr=360/angle_resolution;
angleUnits_rel=NaN(floor((length(x)/100)*angle_resolution_corr),angle_resolution);
for j=1:angle_resolution
    h=1;
    for i=1:length(x)
        if angle_rel(i,2) > (j-1)*angle_resolution_corr && angle_rel(i,2) <= j*angle_resolutio
            angleUnits_rel(h,j)=rAverage2(i);
            h=h+1;
        end
    end
end
boxplot(angleUnits_rel,'plotstyle','compact','whisker',3)
ylabel('distro of spike Activity Unit1')
xlabel('angle of closest periferBee in degree')
title('amound of spikes per frame of Unit 2 per angle_REL periferBee')
```

headdircetion implementation | Track of recBee with headdirection as blue line [49]

```
figure('OuterPosition',figure_positiondouble)
arrows=nan(length(x),5);    % x recbee y recbee trash(z-axis) y arrowhead x arrowhead
arrows(:,1)=x(:,1);
arrows(:,2)=y(:,1);
hold on
%arrows(:,4)=y(:,1)+1;       % one up so 0°, gets turned by angle(i,1) later

for i=1:length(x)-1
    if isnan(angle(i,1))
    else
        R=rotx(angle(i,1));
        arrows(i,3:5)=R*[0;10;0];      % building the arrow starting from [0 0 0]
        arrows(i,4)=arrows(i,4)+arrows(i,2); % adding the arrow to the current pos of RecBee
        arrows(i,5)=arrows(i,5)+arrows(i,1); % y and x are twisted
        arrows(i,3)=arrows(i,2);
        arrows(i,2)=arrows(i,5);
        plot(arrows(i,1:2),arrows(i,3:4),'Color',[((rAverage1(i)/max2)),0,(1-(rAverage1(i)/max
    end
```

```
end
hold off
title('headdirection on /as track of Recbee')
```

headdircetion implementation | Track of periferBees with headdirection as blue line [50]

```
figure('OuterPosition',figure_positiondouble)
arrows=nan(length(x),5);    % x recbee y recbee trash(z-axis) y arrowhead x arrowhead
arrows(:,1)=combiTest(:,2,2);
arrows(:,2)=combiTest(:,2,3);
hold on
%arrows(:,4)=y(:,1)+1;       % one up so 0°, gets turned by angle(i,1) later
for i=1:length(x)
    if isnan(combiTest(i,2,4))
    else
        R=rotx(combiTest(i,2,4));
        arrows(i,3:5)=R*[0;10;0];              % building the arrow starting from [0 0 0]
        arrows(i,4)=arrows(i,4)+arrows(i,2);   % adding the arrow to the current pos of RecBee
        arrows(i,5)=arrows(i,5)+arrows(i,1);   % y and x are twisted
        arrows(i,3)=arrows(i,2);
        arrows(i,2)=arrows(i,5);
        plot(arrows(i,1:2),arrows(i,3:4))
    end
end
scatter(combiTest(:,2,2),combiTest(:,2,3),[],log10(rAverage1),'filled')
hold off
title('headdirection on track of periferBees with sp.Activity rAverage1Recbee')
```

rAverage1 hist over time [51]

```
figure('OuterPosition',figure_positiondouble)
subplot(2,2,1)
clear histoarray;
k=1;
bins=100;
under_even=floor(-1+length(isi1)/300)*300;
histoarray=nan(under_even/300,bins);
for i=1:300:under_even
    histoarray(k,:)=hist(rAverage1(i:i+499),bins);
    k=k+1;
end
histoarray=log10(histoarray);
histoarray(histoarray==-Inf)=0;
contourf(histoarray,200,'EdgeColor','none');
title('rAverage1 hist over time ROOTED')
```

rAverage2 hist over time

```
subplot(2,2,2)
clear histoarray;
k=1;
bins=100;
under_even=floor(-1+length(isi1)/300)*300;
histoarray=nan(under_even/300,bins);
for i=1:300:under_even
```

```
    histoarray(k,:)=hist(rAverage2(i:i+499),bins);
    k=k+1;
end
histoarray=log10(histoarray);
histoarray(histoarray==-Inf)=0;
contourf(histoarray,200,'EdgeColor','none');
title('rAverage2 hist over time ROOTED')
```

isi1 hist over time

```
subplot(2,2,3)
clear histoarray;
k=1;
bins=100;
under_even=floor(-1+length(isi1)/300)*300;
histoarray=nan(under_even/300,bins);
for i=1:300:under_even
    histoarray(k,:)=hist(isi1(i:i+499),bins);
    k=k+1;
end
histoarray=log10(histoarray);
histoarray(histoarray==-Inf)=0;
contourf(histoarray,20,'EdgeColor','none');
title('isi1 hist over time ROOTED')
```

isi2 hist over time

```
subplot(2,2,4)
clear histoarray;
k=1;
bins=100;
under_even=floor(-1+length(isi1)/300)*300;
histoarray=nan(under_even/300,bins);
for i=1:300:under_even
    histoarray(k,:)=hist(isi2(i:i+499),bins);
    k=k+1;
end
histoarray=log10(histoarray);
histoarray(histoarray==-Inf)=0;
contourf(histoarray,20,'EdgeColor','none');
title('isi2 hist over time ROOTED')
```

video Overlays & plots to show syncrony data alignment and use the 4Th Dimension when nessesary

needs workspace & video

video with real background red periferBees, greenRecBee rAverage1&2 box-size

```
tic
clear video
clear speicherbar
videoFReader = vision.VideoFileReader('cutMGPEG1408191255_020-1.avi');
```

```
info = mmfileinfo('cutMGPEG1408191255_020-1.avi');
vid_delta=int16(abs(10*info.Duration-length(x(:,1))));
overall_time=abs(10*info.Duration-length(x(:,1)));

overall_time= 200;%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%200 frames fit the memory, NO MORE!!

video(1,1:int16(overall_time)) = struct('cdata',zeros(1200,1600,3,'uint8'),...
    'colormap',[]);
for frame=1:overall_time
    video(1,frame).cdata = step(videoFReader);

    video(1,frame).cdata(-floor(rAverage1(frame+vid_delta))...
        +1195-(y(frame+vid_delta,1))...
        :floor(rAverage1(frame+vid_delta))...
        +1205-y(frame+vid_delta,1),...
        -floor(rAverage2(frame+vid_delta))...
        +x(frame+vid_delta,1)-5 ...
        :floor(rAverage2(frame+vid_delta))...
        +x(frame+vid_delta,1)+5,2)=1;

    for i=2:12
        video(1,frame).cdata(1195-(y(frame+vid_delta,i))...
            :1205-y(frame+vid_delta,i),...
            x(frame+vid_delta,i)-5:x(frame+vid_delta,i)+5,1)=1;
    end
end
vid = VideoWriter(['vid1_' num2str(rand()) '.avi']);
vid.FrameRate = 10;
vid.Quality = 100;
open(vid)
writeVideo(vid, video);
close(vid);
toc
```

trajectory of blue-recBee; red-closesd bee; green-rest of bees; pink-contact

```
figure('OuterPosition',[100 10 800 500])      % windwos size more important for memory then i=
clear M;
k=1;
for i=1:3000
    plot(combiTest(i*10:i*10+100,3:12,2),combiTest(i*10:i*10+100,3:12,3),'.g')
    xlim([0 1600])
    ylim([0 1200])
    hold on
    plot(combiTest(i*10:i*10+100,2,2),combiTest(i*10:i*10+100,2,3),'.r')

    if sum(abs(contact(i*10:i*10+100,1)))>0
        plot(x(i*10:i*10+100,1),y(i*10:i*10+100,1),'ms','MarkerFaceColor','m')
    else
        plot(x(i*10:i*10+100,1),y(i*10:i*10+100,1),'.b')
    end

    hold off
    M(k) = getframe;
    k=k+1
end
close
vid = VideoWriter(['vid_2_' num2str(rand()) '.avi']);
vid.FrameRate = 10;
```

```
vid.Quality = 100;
open(vid)
writeVideo(vid, M);
close(vid);

figure('OuterPosition',[100 10 800 500])         % windwos size more important for memory ther
arrows=nan(length(x),5);                         % x recbee y recbee trash(z-axis)
arrows(:,1)=combiTest(:,1,2);
arrows(:,2)=combiTest(:,1,3);                    % 1.bee for tests
clear M;
k=1;
videoFReader = vision.VideoFileReader('cutMGPEG1408191255_020-1.avi');
info = mmfileinfo('cutMGPEG1408191255_020-1.avi');
vid_delta=int16(abs(10*info.Duration-length(x(:,1))));
for i= vid_delta:10:vid_delta+6000                % in steps of 10 //secoundwise
    fresh_pic.cdata = step(videoFReader);
    R=rotx(combiTest(i,1,4));
    arrows(i,3:5)=R*[0;50;0];                    % building the arrow starting from [0 0 0]
    arrows(i,4)=arrows(i,4)+arrows(i,2);         % adding the arrow to the current pos of Rec
    arrows(i,5)=arrows(i,5)+arrows(i,1);         % y and x are twisted
    arrows(i,3)=arrows(i,2);
    arrows(i,2)=arrows(i,5);
    imshow(fresh_pic.cdata)
    hold on

    plot(arrows(i,1),1200-arrows(i,3),'or')
    plot(arrows(i,1:2),1200-arrows(i,3:4))
    xlim([0 1600])
    ylim([0 1200])

    M(k) = getframe;
    k=k+1;
    hold off

    step(videoFReader);
    step(videoFReader);
    step(videoFReader);
    step(videoFReader);
    step(videoFReader);
    step(videoFReader);
    step(videoFReader);
    step(videoFReader);
    step(videoFReader);
end
close
vid = VideoWriter(['vid_2_' num2str(rand()) '.avi']);
vid.FrameRate = 10;
vid.Quality = 100;
open(vid)
writeVideo(vid, M);
close(vid);
```

save the workspace

```
save('workspaceFULL_5rA.mat');
analyse_time=toc;
disp(['    ',num2str(floor(analyse_time)), ' sec'])
```

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