An innovative computer-based method for continuous behavioural detection in honeybee colonies (*Apis mellifera*) and the social organization of moving behaviour on the comb.

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Christina Blut Düsseldorf, den 28.05.2018

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Chapter I

General Introduction

Honeybees (*Apis mellifera*) are one of the most intensively studied insects since their social structure and various individual behaviours provide a large range of studying fields. The most striking and intensively studied social pattern found in honeybee colonies is the division of labour exhibited by the thousands of female worker bees (Rösch 1925, Lindauer 1952, Johnson 2010a). The various tasks essential for colony maintenance, including brood rearing, foraging for and accumulating food are performed by and coordinated among the colony's many worker bees (Winston 1987, Seeley 1995). The history of research on division of labour in honeybees goes back to the 17th century. Since, studies have focused on investigating (i) the temporal basis of worker bees' task performances (Butler 1609, Dönhoff 1855a, b, Gerstung 1891 - 1926) including the order and nature, and the flexible performance of these (Rösch 1925, 1930, Lindauer 1952, Seeley 1982, 1995, Johnson 2003, 2010a, Johnson and Frost 2012), (ii) and the colony-level factors which determine why worker bees perform tasks at different times (Winston 1987, Seeley 1989, Fewell and Winston 1992, 1996, Pankiw et al. 1998, Fewell and Bertram 1999). Division of labour enables honeybee colonies to maximize their growth and food accumulation (Johnson 2010a). The collective efforts of the colony's worker bees lead to social outcomes far exceeding the abilities of an individual bee (Theraulaz et al. 2003). By working together the worker bees construct hives of well-structured wax combs in which they rear brood and store large amounts of honey and pollen (Winston 1987). For example, every year a honeybee colony collects an average of 20 kg of pollen, which can only be achieved through the collaborative efforts of the many forager bees (Seeley et al. 1991). A single bee would not be able to collect that amount of pollen.

Organization of the honeybee colony

Honeybees live in complexly structured social societies. These societies consist of up to 100 000 essentially sterile female workers (Bourke 1999), a single fertile female, the queen, and several hundred males (drones) (Winston 1987). Despite this vast number of individuals, the honeybee colony is a highly organized system which is maintained by its individuals' performances and their coordinated efforts (Lampeitl 1995). The worker bees perform altruistic, social behaviours that are generally related to colony growth and development, while the queen and drone behaviours are related to reproduction (Winston 1987).

Within a day and throughout their life, worker bees perform a number of tasks, while task performances are most often related to age and physiological condition of the worker bee (Rösch 1925, Seeley 1982). Collectively, worker bees store food and rear brood in well-structured wax combs. A typical honeybee wax comb contains a brood area with wax cells holding the honeybee larvae, a pollen and a honey area in which the collected pollen and processed nectar is stored. The majority of tasks is bound to these three major task areas, for example the task of brood feeding can only be performed in the brood area while packing pollen is bound to the pollen area (Seeley 1982, Johnson 2008a). While engaging in different tasks, worker bees move about and switch between the different task areas of the wax combs (Seeley 1982). Within the first three weeks of their lives, honeybee workers engage in a variety of in-hive tasks, for example, cell cleaning, brood feeding, pollen and nectar storing, while foraging is performed later (Lindauer 1952, Seeley 1982). Typically, worker bees perform the task of cell cleaning in the first three days of their life, followed by brood rearing tasks, like feeding brood and capping brood cells which they perform up to an age of 12 days (Seeley 1982, Johnson 2008b). Between the age of 13 to 20 days, worker bees typically perform a variety of tasks that include nest construction, food storage and processing (Seeley 1982, Johnson 2008b). Finally, they become foragers collecting pollen and nectar at an age of about 21 days (Seeley 1982).

Although tasks are most often performed by bees of certain ages, an overlap and variability in task performance of different age groups is recognizable (Seeley 1982, Winston 1987). Young and old bees tend to perform fewer tasks than middle-aged bees (Seeley 1982, Winston 1987, Seeley 1995). The latter, thus, most often have a greater overlap in tasks than younger or older bees. Some bees tend to specialize in certain tasks, like undertaking, guarding or social grooming (Moore et al. 1995). However, despite their specialization these ‘specialists’ also perform other tasks, but in a reduced manner compared to unspecialized worker bees (Trumbo et al. 1997). If colony conditions require it, worker bees can flexibly and to some degree regardless of age, adapt their task performances according to the colony’s needs (Rösch 1930, Page and Erber 2002, Gordon 2016). For example, worker bees may begin foraging at an early age while active foragers may return to in-hive task performance if required (Rösch 1930, Robinson 1985, 1987).

Taken together, the thousands of worker bees in a honeybee colony take upon the many tasks required for colony maintenance, while individual task performances are most often related to certain stages of age, but nevertheless flexible in order to meet the colony's shifting needs.

Division of labour in honeybees

Division of labour can take place between the queen and workers, which is referred to as reproductive division of labour (Oster 1978, Robinson 1992), and between the workers of a colony (Oster 1978, Hölldobler and Wilson 2008). The division of labour between a colony's workers can emerge from physical or physiological, age-related differences between the workers (Wilson 1976, Seeley 1982, Wilson and Holldobler 1988, Calderone and Page 1991, Page and Robinson 1991, Robinson 1992, Calderone and Page 1996, Huang and Robinson 1996, Johnson 2003). Latter, is referred to as temporal polyethism which is prominent in honeybees and implies a correlation between the worker bee's task performances and her age (Lindauer 1952, Ribbands 1953, Winston 1987, Robinson 1992). Specific tasks, for example, feeding brood or comb construction, require a specific physiological development of the worker bee, which is typically met at a specific age (Winston 1987, Page and Peng 2001). Hypopharyngeal and wax glands for brood food and wax production are needed for these tasks and are typically active at an age of 3 to 11 and 10 to 15 days (Page and Peng 2001). Worker bees meeting the required developmental state of such specific physiological-dependent tasks will perform these tasks (Johnson 2003, 2008b). However, physiological-dependent tasks only make up a small portion of the total task repertoire in a honeybee colony (Johnson 2003). The larger portion of tasks is independent of the physiological condition of the worker bee and can be performed by almost any worker bee at any time (Seeley 1982, Robinson 2002, Johnson 2003, 2008b, 2010a).

To understand how division of labour emerges, we need to understand how tasks are allocated among the individual worker bees of a honeybee colony. The allocation of worker bees into specific tasks, is based on the decision of an individual bee to perform a certain task (Beshers and Fewell 2001). This decision, which partly is known and partly is hypothesized, is influenced by different factors. These factors may be internal or external factors, whereas internal factors are those originating in the internal state of the individual, such as genetic, neuronal and hormonal factors (Robinson 1985, 1987, Robinson et al. 1989, Robinson 1995, Sullivan et al. 2003). As implied by temporal polyethism, physiological and thus internal conditions of worker bees can initiate specific task engagements (Robinson 1992, Beshers et al. 2001b).

External factors are those generated by interactions with the colony's environment, which include stimuli triggering task performances and worker-worker interactions (Beshers and Fewell 2001). In honeybees, worker-worker interactions are characterized by two worker bees facing each other and the repeated contact of their moving antennae. Four worker-worker interactions displaying these behavioural features can be recognized: (i) antennation, which is used to initialize and maintain a contact; (ii) offering, which is used by worker bees to offer food to other nestmates (De Marco and Farina 2003); (iii) begging, which a worker bee uses to beg for food from another nestmate (Free 1956, De Marco and Farina 2003); and, (iv) trophallaxis, in which nectar from the crop is exchanged between two bees (Korst and Velthuis 1982, Goyret and Farina 2005). It has been proposed that worker-worker interactions are ways for worker bees to gather information about their colony's state (Lindauer 1948, Ribbands 1953, Seeley 1986, Farina 1996). For example, it is known that interactions between foragers and hive-bees enable the access of information about the quality and source of nectar and the honey stores of the colony. To gain information about the quality and source of nectar offered by an incoming forager bee, begging behaviour is performed by other worker bees (Bozic and Valentincic 1991, Goyret and Farina 2003, Gil and De Marco 2005, Goyret and Farina 2005). The colony's state and the amount of stored honey, hereby, affect the rate of begging behaviour (Schulz et al. 2002). Additionally, the quality of the incoming nectar affects worker-worker interactions and establishes a control mechanism for the workers' foraging engagement, performance and the influx of high-quality nectar (Seeley et al. 1991). Foragers returning with high-quality nectar will more often find a recipient bee to unload their crop load to because they show increased offering behaviour as well as increased dancing behaviour (De Marco and Farina 2001). Therefore, these foragers more often return with nectar to the colony. In addition to the regulating effect on workers' foraging engagement and performance (Frisch 1965, Farina and Nunez 1991), Smith et al. (2017) found that worker-worker interactions are most likely a way for hive-bees to assess when their colony is large enough to invest in reproduction by building drone combs. A honeybee colony will not construct drone combs before it has not reached a number of individuals of at least 4000 bees (Smith et al. 2014). Most likely, worker bees sense that their colony has reached this critical number through interactions with nestmates since the rate of worker-worker interactions increases with increased worker density (Smith et al. 2017). Despite the role of worker-worker interactions in the regulation of foraging behaviour (Frisch 1965, Farina and Nunez 1991) and the onset of reproduction in honeybee colonies (Smith et al. 2014, Smith et al. 2017), little is known about other possible effects of worker-worker interactions on task engagements and colony organization.

Stimuli triggering worker bees’ task performances are represented by signals and cues. Both are sources of information that worker bees can use to assess the need for task performance (Seeley 1998). In honeybees, signals are primarily of chemical and mechanical nature and present evolved sources of information that convey distinct information between the sender and the receiver of the signal (Seeley 1998, Johnson 2010b). Pheromones and dances present such signals. For example, a pheromone released by honeybee larvae, called brood pheromone, triggers pollen foraging behaviour (Pankiw et al. 1998, Pankiw 2004). The tremble dance is a signal used by incoming nectar foragers that experience long waiting times for a recipient bee to accept their nectar load (Seeley 1992). This dance conveys the need for more recipient bees (Seeley 1992).

Alternative to signals, worker bees may use cues when deciding to perform a certain task. Cues may be any information from the social or physical environment perceived by worker bees that were not shaped under natural selection to convey distinct information as signals do (Seeley 1998, Johnson 2010b). It is thought that cues are more important than signals when it comes to how a worker bee decides which task to engage in (Seeley 1998, Beshers and Fewell 2001). Cues received by bees may be chemical cues, such as the floral odor on a waggle dancer, tactile cues, such as unpacked pollen loads in a cell as indication for the need of packing pollen, temporal cues, such as the time spent searching for a nectar receiver bee, visual and temperature cues, the later as an indication for heating or cooling the nest (Seeley 1998). Worker bees may perceive the cues’ information by passing through different comb areas and encountering the cues present in the specific areas (Johnson 2009, 2010b).

Task allocation is based on the decision of a worker bee to perform a certain task. This decision may be dependent upon the worker bee’s physiological state and the information she gains through direct interactions with nestmates, signals and cues.

Models on emergence of individual task engagements

Different models predicting how individual task engagements emerge have been proposed (Beshers and Fewell 2001), but still the mechanisms leading to these are not fully understood. The models address the question of how an individual decides which task to perform, combining the internal and external factors thought to influence task engagement.

The response threshold model proposes that individual task engagements emerge from differences in individuals’ response thresholds for task specific stimuli (Page et al. 1995, Bonabeau et al. 1998, Beshers and Fewell 2001). The different task areas of a honeybee comb provide different task-specific stimuli. A starving larvae in the brood area, for example, presents stimuli that may trigger a worker bee’s feeding behaviour (Huang and Otis 1991). A worker bee performs a particular task when the stimulus for this task exceeds the worker's internal task-specific stimulus threshold. Each worker bee may respond to task-specific stimuli, such as a starving larvae, differently because their individual response threshold for these specific stimuli vary due to genetic differences between the worker bees (Page & Mitchel 1998, Robinson & Page 1989). The model assumes that all workers can perform a task with the same probability. It also assumes that there is a negative feedback loop for task-specific stimuli that results in a reduction of the task-specific stimulus when this task is performed. So, if a worker bee performs one task, she reduces the stimulus for that task for other worker bees, thereby reducing the likelihood that other workers with a higher stimulus threshold will perform this task (Page & Mitchell 1991, Page & Mitchel 1998).

The foraging-for-work model proposes that a worker bee actively seeks for work (Tofts and Franks 1992, Franks and Tofts 1994, Beshers and Fewell 2001). In doing so, she switches between the different task areas of the comb until she finds a task to perform (Franks and Tofts 1994). The worker bee will continue performing a task until there is no need for its performance and will move on to a different comb area to find another task to perform (Tofts 1993, Franks and Tofts 1994). Hereby, tasks are located in different areas of the comb while specific tasks can only be performed in specific task areas. For example, the task of brood feeding can only be performed in the brood area. The foraging-for-work model presumes that bees begin their life and task performance in the centre of the nest and move into the periphery of the nest with increasing age (Tofts 1993, Franks and Tofts 1994). The model therefore can be used to simulate the emergence of temporal polyethism.

Self-reinforcement models propose that experience in performing a task and self-reinforcement play a role in the decision of worker bees to perform specific tasks (Theraulaz et al. 1998, Beshers and Fewell 2001). Once engaged in a task, a worker bee may repeatedly perform the task when being successful at it. Hereby, the successful completion of a task increases the likelihood of reperforming that task, whereas failure to perform a task or the lack of opportunities to do so reduce the likelihood of performing that task (Plowright 1988, Theraulaz et al. 1998). These conditions can potentially lead to specialists for specific tasks.

Social inhibition and information transfer models propose that direct interactions with nestmates may cause a worker bee to engage in a specific task (Beshers and Fewell 2001, Beshers et al. 2001b). The decision to engage in a task is based on the information gained through encounters with other workers (Beshers and Fewell 2001) and the physiological state of the worker bee (Huang 1999, Beshers et al. 2001b). Experimental evidence has shown that worker-worker interactions can regulate the physiological development of a worker bee and thus influence individual task engagements (Huang and Robinson 1992, 1996).

The different models are all based on the assumption that individual workers operate independently and rely on information obtained from their immediate vicinity when deciding which task to perform. Strikingly, honeybee workers spend a large percentage of their time either patrolling, walking around the nest, (Lindauer 1952, Johnson 2008a) or resting, standing and seemingly doing nothing (Winston 1987). Patrolling may be very important for a bee's 'decision' of task performance since tasks and possibly certain cues triggering task performance are spatially distributed within the bee's hive (Seeley 1982, Johnson 2008a). Middle-aged bees have been found to randomly quit their current task independent of the demand for it, and patrol throughout the nest (Johnson 2008a). This behaviour is not displayed by nurse bees whose task repertoire and the area in which the nursing tasks can be performed are much more specific than for middle-aged bees (Seeley 1982, Johnson 2008a).

Based on the behavioural observations mentioned above, Johnson (2009) formulated a new model to explain how task allocation emerges in honeybees. This model focuses on the middle-aged bees who perform many different tasks dispersed throughout the entire nest (Johnson 2008b). The model presumes that bees can be in three behavioural states: inactive, working, or patrolling. Worker bees in the working state perform the first task they encounter while they perform a search for work if they fail to find a task in their current location. Bees in the patrolling state conduct a random walk across the nest while they do not react to task specific stimuli during the patrol (Johnson 2009). They may decide to switch from patrolling to working and perform a task in any given area. Searching for work relocates bees to area with the highest task demand while patrolling randomly displaces bees to different areas independent of task demand. Patrolling therefore opposes the effect of searching for work (Johnson 2009).

It is most likely that individual task engagements emerge from a combination of the proposed models. However, experimental evidence is rare up to date.

Methods for behavioural studies in honeybees

Throughout the centuries, different methods for studying honeybee behaviour have been established, most of which are based on manually following the actions of an individual bee or a small group of bees. To study the behaviour of honeybee workers within their natural environment of the hive, observation hives have been used since the beginning of the 20th century (Rösch 1925, 1930). Such hives hold up to several wax combs that are encased by transparent walls allowing the observer to watch the bees with minimal disturbance (Frisch 1923, Lindauer 1952, Seeley 1982).

To manually track bees during behavioural studies, individual bees are marked with a paint dot on the thorax or abdomen, or with a numbered, coloured plastic disc called Opalithplättchen that is glued to the bees' thorax (Fig. 1) (Frisch 1923, Lindauer 1952, Frisch 1965, Scheiner et al. 2013). The observer may choose to follow an individual bee for a period of time making frequent observations of the focal bee's behaviour, or he may choose to observe the behaviour of a random subset of bees in less frequent time intervals (Lindauer 1952, Seeley 1982, Johnson 2008a). Video recordings of bees can also be used to analyse the behaviour under natural or experimental conditions (Seeley 1992, Gempe et al. 2012). Bees' task performances, interactions with nestmates and the area on the comb in which the bee was detected can be noted by the observer.

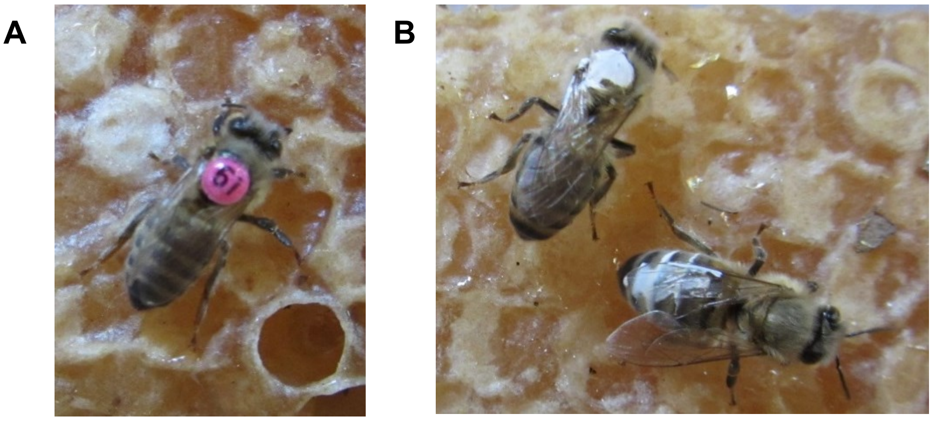

Figure 1: Examples of labelling worker bees for behavioural studies. Worker bees may be individually marked with numbered, coloured plastic discs (**A**) or paint dots on the thorax or abdomen (**B**) in order to manually track their behaviour within the colony (Source: Personal collection).

Manually performed behavioural observations have brought insight into the organization of the honeybee colony and the task repertoire of its worker bees (Rösch 1925, Lindauer 1952, Seeley 1982). However, the question of how tasks are allocated among the thousands of worker bees has so far not been answered, mainly because the current methods do not provide the necessary information to answer this question. To understand how task allocation is coordinated in honeybee colonies, continuous information on task engagements and interactions of several hundred bees at once is needed. Manual observation methods can only provide fractional information on bees' behaviours.

The 21st century has brought forth computer-based methods that can be used to automatically obtain behavioural information of many different model organisms (Noldus et al. 2001, Spink et al. 2001, Branson et al. 2009, Dankert et al. 2009, Gautrais et al. 2009). Many of these methods rely on tracking individuals' movements within an artificial environment and function by automatically detecting individuals based on contrast differences between the environmental background and the individual (Noldus et al. 2001, Spink et al. 2001, Branson et al. 2009, Dankert et al. 2009). The disadvantage of such tracking methods is that only individual animals or small groups can be tracked, whereas the reliable identification of individuals in a group is difficult or not possible.

Honeybees have so far not been subject to computer-based observation methods and have never been automatically tracked within the colony. A computer-based system which is promising for the application in honeybees, was introduced by Mersch et al. (2013). They established an automated tracking system for ants that can generate information on trajectories of more than 2000 individually marked ants. Ants are marked with small tags bearing 2D barcodes that each encode an individual identification number (Fig. 2). Thus, via tracking software, marked individuals can be reliably identified within a group and continuous information on trajectories of these can automatically be obtained for any time period. Adapting the automated tracking system for the use in honeybees would enable researchers to gather large amounts of continuous positional information of hundreds of worker bees at once within the hive.

Based on the automatically obtained information on individuals' trajectories, further computer-based methods can be used to automatically detect behaviours (Kabra et al. 2013). Automatic detection of behaviours based on trajectories has been introduced for the model organism *Drosophila melanogaster* and is based on machine learning (Kabra et al. 2013). Machine learning builds on an algorithm that first learns and subsequently identifies behaviours based on behavioural features that are specific for a certain behaviour. For example, the head to head orientation of two individuals and their distance to each other may be used as behavioural features to describe an interaction. Behavioural features specific for a certain behaviour are generated from the trajectories and associated with a specific behaviour during the machine learning process (Kabra et al. 2013). The specific behaviour is learned based on manually labelled examples for this behaviour (Kabra et al. 2013).

Applied to trajectories of honeybee workers obtained by an automated tracking system such as the one introduced by Mersch et al. (2013), automated detection of behaviours via machine learning would facilitate the gathering of behavioural information on honeybees. Most importantly, the combination of automated tracking and subsequent, automated behaviour detection would enable the collection of large amounts of behavioural information of bees. Such information is needed to understand the emergence of individual task engagements in honeybees.

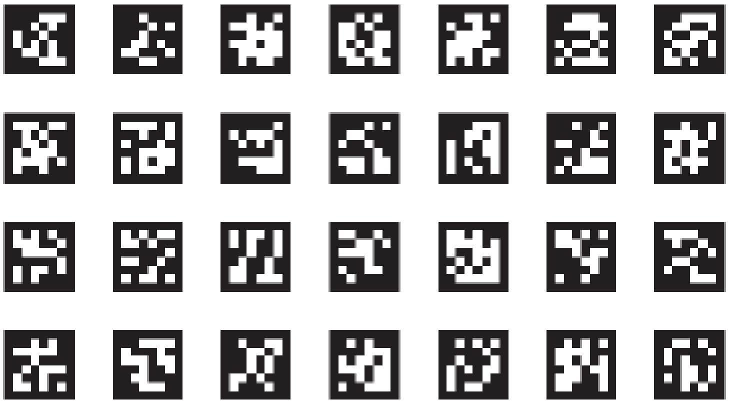

Figure 2: Examples for 2D barcodes. Each 2D barcode encodes for an individual identification number and can be identified by tracking software. Glued to the thorax of ants or bees, the marked individuals can thus be reliably and distinctively identified (Source 2D barcodes (AprilTags): Olson (2011); source picture: Personal collection).

Questions and goals

Although studies have brought insight into the organization of the honeybee colony and the task repertoire of its worker bees (Rösch 1925, Lindauer 1952, Seeley 1982), the mechanisms underlying individual task engagements are still not fully understood. The functionality of the colony relies on the actions of thousands of individual bees which seem to perform exactly those tasks needed for the given colony state. The task engagements of worker bees are most likely dependent on the different information perceived by the individual bees. Social information is constantly exchanged via inadvertent cues and intentional signals (Wray et al. 2012). Patrolling, trophallaxis, pheromones, the rate of incoming resources, and dances are mechanisms suggested to be involved in the control of task performance (Frisch 1923, Lindauer 1952, Frisch 1965, Lindauer 1986, Farina and Nunez 1991, 1993, Crailsheim 1998, Pankiw et al. 1998, Pankiw and Page 2003, Pankiw 2004, Farina and Wainselboim 2005, Johnson 2008a, 2009, Muenz et al. 2012). Still, it is mainly unknown how the individual worker bees receive the information about the colony's needs and thus perform the required tasks at a given colony state. The mechanisms underlying the transition from individual behaviours to colony-level outcomes can only be assumed for most of the behaviours found in a honeybee colony up to date. For pollen and nectar foraging mechanisms are largely known. Pollen foraging activities depend on the pollen stores in the hive (Fewell and Winston 1996), the number of larvae (brood), the foragers' genotype and the availability of the resource in the environment (Pankiw et al. 1998). For nectar, on the other hand, this is not the case. Nectar foraging is independent of nectar stores in the hive and rather depends on the quality and availability of nectar in the environment (Fewell and Winston 1996). Also, brood pheromones influence the pollen foraging activity (Pankiw et al. 1998), whereas for nectar foraging brood pheromones have no effect on the number of nectar foragers (Pankiw 2004). For other tasks, the mechanisms leading to the specific task engagement are largely unknown.

Experimental evidence for mechanisms underlying individual task engagements is largely missing, mainly because current research techniques lack to provide the data necessary for the understanding of these key mechanisms. The current research techniques, in which focal bees or small groups of bees are observed for short periods, offer only fractional behavioural observations (Frisch

1923, Lindauer 1952, Seeley 1982). However, solving the question of the underlying mechanisms of individual task engagements requires continuous observations as only these can reveal possible links between individual behaviour and collective level outcomes that have been missed so far.

The goal of this project is to establish an innovative method for the collection of continuous behavioural information on several hundred bees at once within the hive. The continuous behavioural information obtained with this method will be used to investigate the mechanisms underlying the emergence of individual task engagements in the context of division of labour. The first part of my thesis will deal with the establishment of an automated system for the collection of continuous positional information and subsequent automated detection of behaviours of several hundred honeybees at once. My goal is to adapt two computer-based systems for the use in honeybees that were originally introduced for ants (Mersch et al. 2013) and fruit flies (*Drosophila melanogaster*) (Kabra et al. 2013). I will combine these two systems to one system that will enable the automated detection of worker bee behaviours within the natural environment of the hive. The second part of my thesis is based on the successful establishment of the automated system for honeybees. It will deal with 1) the quantification and automated detection of encounter behaviours occurring in small groups of honeybees and 2) the investigation of the regulation of task allocation through the analysis of task area visiting behaviour performed by worker bees in different sized groups.

Theses

The following theses can be formulated in the context of this work:

1) Unlike current methods for behavioural studies in honeybees *Apis mellifera*, the computer-based and automated Bee Behavioral Annotation System (BBAS) can provide continuous behavioural data of up to 1000 honeybee workers at once. Such continuous behavioural data are necessary to understand the mechanisms underlying the regulation of task allocation in honeybees.
2) BBAS can automatically and reliably detect encounter behaviours of worker bees in small groups. It can thus provide the information necessary to understand the role of encounter behaviours in the exchange of information between honeybee workers and their individual task engagements.
3) BBAS can reliably detect and distinguish trophallaxis behaviour from the other encounter behaviours, antennation, begging and offering, based on the characteristic duration of this encounter behaviour.
4) Independent of group size, all worker bees repeatedly visit the different task areas, namely the brood, pollen and honey area. This suggests that each worker bee can individually and continuously access information on task demand by visiting the different task areas.
5) Mobility constraints on the comb reduce individual task area visiting behaviour whereas task demand and per capita workload marginally affect this behaviour. This suggests that worker bees only marginally adjust their task area visiting behaviour according to task demand and per capita workload.
6) Worker bees' individual task area visiting behaviour creates a stable group-level pattern of task area visits throughout different group sizes. This suggests that honeybees collectively gain task information through repeated individual task area visits.

Chapter II: Manuscripts

Manuscript I

Automated computer-based detection of encounter behaviours in groups of honeybees

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Abstract

Honeybees form societies in which thousands of members integrate their behaviours to act as a single functional unit. We have little knowledge on how the collaborative features are regulated by workers' activities because we lack methods that enable collection of simultaneous and continuous behavioural information for each worker bee. In this study, we introduce the Bee Behavioral Annotation System (BBAS), which enables the automated detection of bees' behaviours in small observation hives. Continuous information on position and orientation were obtained by marking worker bees with 2D barcodes in a small observation hive. We computed behavioural and social features from the tracking information to train a behaviour classifier for encounter behaviours (interaction of workers via antennation) using a machine learning-based system. The classifier correctly detected 93% of the encounter behaviours in a group of bees, whereas 13% of the falsely classified behaviours were unrelated to encounter behaviours. The possibility of building accurate classifiers for automatically annotating behaviours may allow for the examination of individual behaviours of worker bees in the social environments of small observation hives. We envisage that BBAS will be a powerful tool for detecting the effects of experimental manipulation of social attributes and sub-lethal effects of pesticides on behaviour.

Introduction

Honeybees, like other eusocial insects, form societies in which their members integrate their behaviours to form a single functional unit (often described as 'superorganisms') (Hölldobler and Wilson 2008). In honeybee colonies, for example, the brood is collectively reared by the worker bees under constant temperature conditions in worker-made and well-structured wax combs (Winston 1987). We still have little knowledge on how the collaborative features are regulated within the colony by single workers' task engagements, worker-worker interactions and environmental cues.

A honeybee may engage in many behavioural tasks, for example, cell cleaning, brood feeding, comb building, pollen and nectar storing, and foraging (Seeley 1982). The many in-hive tasks are usually performed within the first three weeks of their life, whereas foraging tasks are performed later (Seeley 1982). Individual task engagements are flexible and are adapted according to the colony's needs (Page and Erber 2002, Gordon 2016). Differences in individuals' internal response thresholds for task-specific stimuli (response threshold model) (Page et al. 1995, Bonabeau et al. 1998, Beshers and Fewell 2001), actively seeking for tasks (foraging for work model) (Tofts 1993), repeatedly performing the same task when being successful at it (self-reinforcement models) (Theraulaz et al. 1998, Beshers and Fewell 2001) and information transferred by social partners through direct contact (Beshers et al. 2001a) may play an important role in the organisation of task engagements within the colony.

Gaining continuous behavioural information on each single worker, their direct contacts (encounters) to other worker bees and their interactions with the local environment would facilitate the further characterization of the underlying mechanisms of colony organization. However, we currently lack methods that enable the collection of simultaneous and continuous behavioural information for each individual worker bee in the environment of a colony (Scheiner et al. 2013). In current methods, behaviours are manually detected by an observer either from video recordings of small observation hives or from direct observations (Frisch 1923, Lindauer 1952, Seeley 1982, Gempe et al. 2012). These manually detected behaviours represent only a fraction of the behaviours that the many worker bees can display in a colony, especially when the behaviour is frequently performed, for example, in the case of encounter behaviours.

In honeybees, encounter behaviours between workers are characterized by the following: the two worker bees face each other head to head and their moving antennae are repeatedly in contact. Encounter behaviours summarize different worker-worker interaction behaviours that display constant antennal contact and can be further grouped into the following behaviours: (i) antennation behaviour, which is required to initialize and maintain a contact (Free 1956), whereby the antennae of two worker bees are in constant contact but no other features of the following behaviours are displayed; (ii) begging behaviour, in which a worker bee begs for food from another nestmate (Free 1956, De Marco and Farina 2003); (iii) offering behaviour, in which a worker bee offers food to another nestmate (De Marco and Farina 2003); and, (iv) trophallaxis behaviour, in which nectar from the crop is exchanged between two bees (Korst and Velthuis 1982, Goyret and Farina 2005).

Worker bees may perform begging behaviour to gain information about the quality and source of nectar offered by the incoming forager bees (Bozic and Valentincic 1991, Goyret and Farina 2003, Gil and De Marco 2005, Goyret and Farina 2005). Incoming forager bees perform offering behaviour to unload the collected nectar to a recipient in-hive worker bee via trophallaxis (Rösch 1925, Nixon and Ribbands 1952, Frisch 1965, Goyret and Farina 2003). Returning foragers presenting high-quality nectar show increased offering behaviour as well as increased dancing behaviour (De Marco and Farina 2001). They more often find a recipient bee and will more often return with nectar to the colony. Effects of different nectar qualities on worker-worker interaction establish a control mechanism for the workers' foraging engagement, performance and the influx of high-quality nectar (Seeley et al. 1991). Despite their role in regulating workers' foraging engagement and performance (Frisch 1965, Farina and Nunez 1991), we have little knowledge on other possible roles that these encounter behaviours may have in task engagements and colony organization.

In this study, we introduce the Bee Behavioral Annotation System (BBAS), which enables the automated classification of worker-worker encounters within a group of honeybees. We obtained continuous information on workers' positions and orientations over time by simultaneously tracking 100 bees tagged with a 2D barcode by adapting a tracking device that was developed for ants (Mersch et al. 2013). From this tracking information, behavioural and social features were computed, and a behaviour classifier was trained based on machine learning using the Janelia Automatic Animal Behavior Annotator (JAABA) program (Kabra et al. 2013). Our study demonstrates that we can automatically and accurately classify encounter behaviours within a group of bees. This system has the prospect of automatically obtaining quantitative and continuous behavioural information on hundreds of bees at once in small colonies.

Results

Automatic classification of encounter behaviours in a group of worker bees

To automatically classify worker behaviours in a small observation hive, we developed the BBAS. We obtained tracking information from individual worker bees in a small group and computed behavioural features (per-frame features), which were utilized to classify behaviours. Per-frame features represented parameters calculated from the tracking information that provided information on the bees' behavioural properties in each frame. Such properties included, for example, a bee's speed or orientation towards a nestmate (see Kabra et al. (2013) for a detailed listing of per-frame features). We applied the per-frame features to manually labelled behaviour classes to train a machine learning-based system and thus generate an automatic behaviour classifier.

First, we adapted a tracking device developed for ants (Mersch et al. 2013) to obtain information on the position and orientation of individually tagged bees at a rate of four frames per second. In our setting, we tracked 100 newly emerged worker bees for two days. Bees were individually tagged with 2D barcodes from the AprilTags library (Olson 2011) printed on 2x2 mm tags and housed in a small observation hive on a single comb providing food (Fig. 1a-c). We chose a rate of four frames per second to ensure that we obtained sufficient information on the bees' position and orientation for subsequent use in automatic behaviour classification. To test whether the chosen rate captured sufficient information we determined the average change in position and orientation of bees (see Supplementary information online). On average, bees' positions changed by 0.9 mm (SD ± 0.9 mm) from one frame to another, which corresponds to ~ 0.06% of an *Apis mellifera* worker size. Bees' average change in orientation from one frame to another was 6° (SD ± 4°). These small changes in position and orientation suggest that we can capture sufficiently detailed information on the bees' movements with the chosen rate. The AprilTag system was chosen because it is an actively maintained open source project and provides a robust system to minimize inter-tag confusion. It also has better performance on images taken under non-uniform lighting conditions as compared to several other similar systems (Olson 2011).

The results of the detection rate and positional accuracy of the tracking device of immobile tags glued to a comb and tags attached to moving and resting worker bees are summarized in Table 1. On average, resting bees were detected in

98.2% of the frames, whereas moving bees were detected in 90.8% of the frames. The orientation accuracy of immobile tags glued to a comb was 1.5° and the positional accuracy was 0.04 mm. The high detection rate and positional accuracy suggest that we can obtain a considerable amount of detailed information on the movement of each single worker in a group of bees.

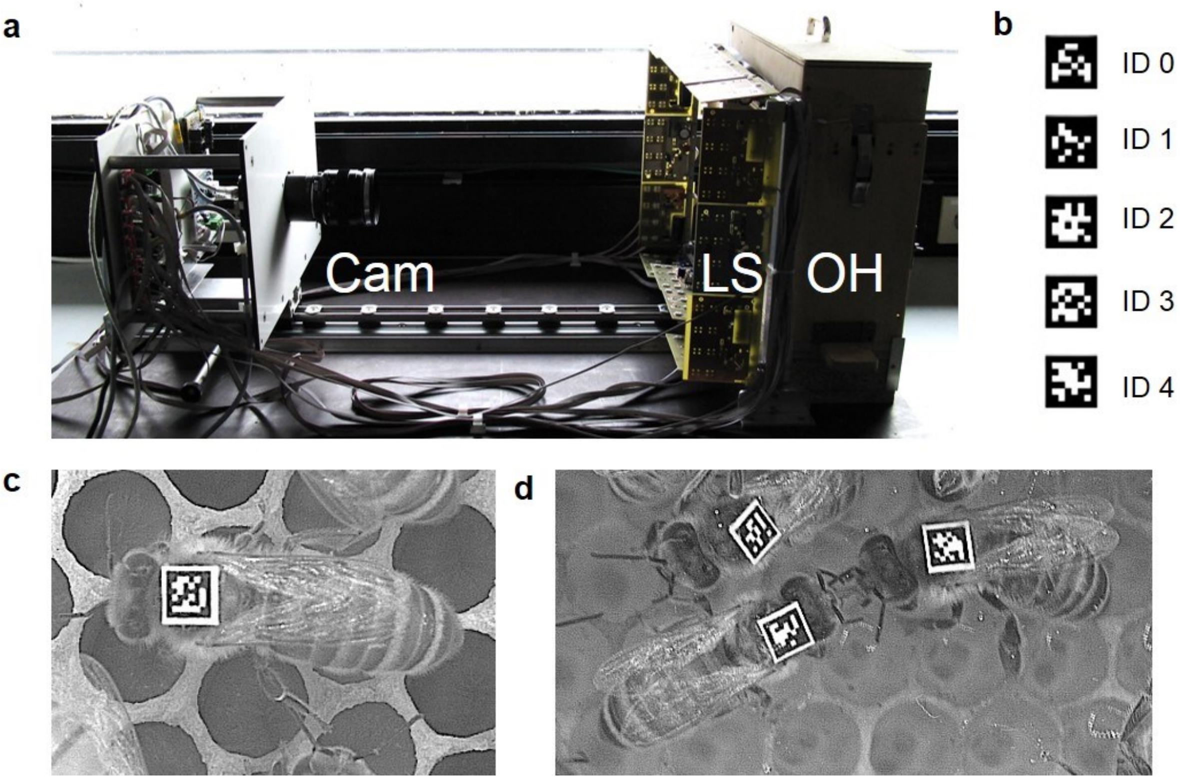

Figure 1: Setup of the tracking device. (**a**) The tracking device consisted of a high-resolution camera (Cam), an infrared lighting system (LS) and the observation hive holding one “Deutsch Normal” comb (OH). The entire device was placed under a cardboard box in a laboratory. (**b**) Examples of 2D barcodes from the AprilTags library. (**c**) Bee marked with a tag bearing a 2D barcode. (**d**) Encounter behaviour between two worker bees defined by the head to head orientation and the antennal contact of the interacting bees. This specific encounter shown is trophallaxis.

Table 1: Detection rate and positional accuracy of the tracking device.

		No. of tracked tags	**No. of frames analysed (sequence duration) (3)**	**Detection rate (4) (%)**	**x/y position accuracy (5) (mm ± SD)**	**Orientation accuracy (5) (degrees ± SD)**
Tags glued to a comb	immobile	100	1200 (5 min)	99.9	0.04 (6) ± 0.03	1.5 ± 0.8
Tags glued to a bee	resting (1)	10	240 (1 min)	98.2	n.d (7)	n.d (7)
	moving (2)	30	240 (1 min)	90.8	n.d (7)	n.d 7)

(1) Bee sits in one position without moving for ≥ 5 seconds.
(2) Bee walks across the comb without interacting with other bees, inspecting cells or performing any other task.
(3) Duration of the tracking.
(4) The percentage of frames in which tags were detected.
(5) Accuracy of the tracking device for the detected x/y centre position and the orientation.
(6) i.e., ~0.003% of an *Apis mellifera* worker size.
(7) Not determined (n.d.) because changes could result from the bees' behaviours.

Second, to generate an automatic behaviour classifier, we computed per-frame features from the tracking information using the JAABADetect program (Kabra et al. 2013). Computing the per-frame features for the tracking information on 100 worker bees required a high-performance computing cluster. We used the social per-frame features to train a classifier for honeybee encounter behaviours (Kabra et al. 2013). The social per-frame features are a set of per-frame features providing information on an individual's state in each frame in relation to its nearest nestmates. For example, the distance, orientation and speed towards another worker may be described by these features (see Kabra et al.(2013) for a detailed listing of social per-frame features).

Third, we determined whether we could automatically classify encounter behaviours between workers using an automatic behaviour classifier generated with the JAABA program. The automatic behaviour classifier was expected to classify the four different behaviours - antennation, begging, offering and trophallaxis - as a single class, which have the behavioural features of head to head orientation and antennal contact of two worker bees in common (Fig. 1d). To train the automatic behaviour classifier, we manually labelled 76 encounter behaviours and 77 non-encounter behaviours from 105 minutes of video recording and corresponding tracking information of the 100 tracked bees. We only labelled encounter behaviours of which we were highly confident that encounter behaviour was truly displayed. The 76 encounter behaviours (EBs) comprised a sample of 28 antennation, 8 begging, 6 offering and 34 trophallaxis behaviours (see Supplementary Videos V1-V4 online for examples of the four encounter behaviours). The non-encounter behaviours (NEBs) represented a sample of 46 sitting, 20 walking, 7 self-grooming, 1 social grooming and 3 sitting with subsequent walking behaviours. We trained the classifier by entering the 76 EBs and 77 NEBs (training set) bit by bit into the JAABA program in five training rounds until we observed no further improvement in the cross-validation estimates (see Supplementary information online for details on cross-validation). Cross-validation estimates were obtained by randomly splitting the EBs and NEBs from the training set into testing and training subsets. The training subset was used to train the classifier while the testing subset was used to subsequently estimate the classifier’s error rate on classifications (Kabra et al. 2013). Table 2 presents the final cross-validation estimates from 10 cross-validation rounds for our trained ‘encounter classifier’. The estimates represent the percentage of frames automatically classified as EB* and NEB* by the ‘encounter classifier’ (asterisks indicate automatically classified behaviours; see Supplementary information online for details on calculation of estimates). Of the EB frames, 77.3% were correctly classified by our ‘encounter classifier’ (SD ± 1.3%, Table 2), whereas 73.7% of the NEB frames were correctly classified (SD ± 1.2%, Table 2). The false positive rate was 26.3% (NEB frames falsely classified as EBs*), whereas the false negative rate was 22.7% (EB frames falsely classified as NEBs*; Table 2).

Table 2: The accuracy of the trained ‘encounter classifier’ estimated through cross-validation on the labelled frames for EBs and NEBs

		Automatically detected by the ‘encounter classifier’	
		Encounter (EB*) (6) **(± SD) (%)** (2)	**Non-encounter (NEB*)** (6) **(± SD) (%)** (2)
Manually annotated (1)	Encounter (EB)	77.3 (± 1.3) (3)	22.7 (± 1.3) (5)
	Non-encounter (NEB)	26.3 (± 1.3) (4)	73.7 (± 1.2) (3)

(1) The manually labelled high-confidence behaviours (EBs and NEBs) used to train the classifier.
(2) Mean estimates with standard deviation (SD) of the 10 rounds of cross-validation. Estimate values are given as percentage of frames correctly or falsely classified as EBs or NEBs using the classifier.
(3) Frames correctly classified as EB or NEB (true positives).
(4) NEB frames falsely classified as EB* (false positives).
(5) EB frames falsely classified as NEB* (false negatives).
(6) Asterisks indicate automatically classified behaviours.

Next, we examined whether our classifier was able to correctly classify all 76 manually labelled EBs from our training set. Since the training set included the different behaviour classes - antennation, begging, offering and trophallaxis - we examined whether the classifier could correctly classify these four different behaviours as encounter behaviour. We determined the classification rate and observed that all manually labelled encounter behaviours of the training set were correctly detected by our classifier (training set in Table 3; Supplementary Table S1 online).

We then determined the accuracy of our ‘encounter classifier’ by comparing manual annotations and automatic classifications of behaviours that were not included in our initial training set. We manually annotated 43 encounter behaviours comprising 4 trophallaxis, 8 begging, 12 offering and 19 antennation behaviours (testing set; see Supplementary Table S1 online). Our ‘encounter classifier’ detected 93% of the manually annotated encounter behaviours in this testing set. The false negative rate was 7%, whereas 28% of the automatically detected behaviours were falsely classified as EBs* (testing set in Table 3; Supplementary Table S1 online).

We re-examined the falsely classified EBs* and found that 15% of the 28% falsely classified EBs* displayed similar features to those of encounter behaviours, i.e. head to head orientation and proximity of two bees. However, these falsely classified EBs* collectively lacked antennal contact. Of the behaviours falsely classified as encounters, 13% were unrelated to encounter behaviour, i.e. displayed no features characterizing encounter behaviours. The results on the high classification rates suggest that the BBAS can be used to automatically and accurately annotate encounter behaviours in groups of honeybees.

Table 3: Comparison of manually annotated behaviours (EBs and NEBs) and automatically classified behaviours (EBs* and NEBs*)

		Automatically detected by the 'encounter classifier'	
		Encounter (EB*) (%)	**Non-encounter (NEB*) (%)**
Training set (1)	Encounter (EB)	100	0
	Non-encounter (NEB)	0	100
Testing set (2)	Encounter (EB)	93	7
	Non-encounter (NEB)	28 (3)	n.d. (4)

(1) The manually labelled high-confidence behaviours (EBs and NEBs) used to train the classifier.
(2) Manually annotated behaviours not used to train the classifier.
(3) Automatically detected behaviours falsely classified as EB* by the 'encounter classifier'.
(4) Not determined (n.d.) because we did not manually annotate NEBs for the testing set and thus could not determine the automatic classification rate.

Classification of trophallaxis behaviour based on the duration of the encounter behaviour

We demonstrated that we could automatically classify the different encounter behaviours, antennation, begging, offering and trophallaxis, as a single behavioural class with our 'encounter classifier'. Next, we considered whether we could use the duration of the different encounter behaviours to distinguish these from each other. In 105 minutes of the 22 hours of video recording, we measured the frequency and duration of antennation, begging, offering and trophallaxis behaviours in the group of 100 worker bees.

We manually detected 658 encounter behaviours from which 57% were antennation behaviours, 26% were offering behaviours, 9% were begging behaviours and 8% trophallaxis behaviours (Table 4; Supplementary Videos V1-V4 online). The median duration of the trophallaxis behaviours was 8 seconds (75% percentile: 13 seconds; range of duration: 5 - 30.5 seconds; Table 4; Fig. 2). The median duration of antennation, offering and begging behaviours was much shorter, ranging from 1 to 2 seconds with a considerable overlap in the 75% percentile (range of durations: antennation: 0.25 - 9.25 seconds, offering: 0.25 - 4.5 seconds, begging: 0.75 - 6.75 seconds; Table 4; Fig. 2). There was a significant difference between the duration of the four different encounter behaviours (One Way ANOVA on Ranks: $N = 658$, $\alpha = 0.05$, $H = 175$, d.f. = 3, $P = < 0.001$). Post hoc tests showed that pairwise comparisons were significantly different except for begging vs. antennation behaviours (Dunn’s Method, $\alpha = 0.05$: trophallaxis vs. offering: $N = 222$, $Q = 13$, $P < 0.001$; trophallaxis vs. antennation: $N = 427$, $Q = 10.7$, $P < 0.001$; trophallaxis vs. begging: $N = 109$, $Q = 6.7$, $P < 0.001$; begging vs. offering: $N = 231$, $Q = 5.3$, $P < 0.001$; antennation vs. offering: $N = 549$, $Q = 5.2$, $P < 0.001$; begging vs. antennation: $N = 436$, $Q = 2.3$, $P = 0.138$). This result suggests that the duration of encounter behaviours could be utilized to distinguish the different encounter behaviours from each other.

Next, we tested whether encounter behaviours could be accurately classified as antennation, begging, offering or trophallaxis based solely on their duration. Therefore, we analysed the ranges of duration of the 658 encounters from the four behaviour classes to determine whether duration thresholds could be used as classifier for the different encounter behaviours. Hereby, we attempted to find thresholds above which behaviours could be reliably classified as one of the four behaviour classes. We observed that duration thresholds could not be utilized as classifiers for begging, offering and antennation behaviours since their ranges of duration overlapped too strongly (Table 4; Fig. 2). When considering only behaviours with duration of 5 and more seconds, we observed that all trophallaxis behaviours could be correctly classified (100%; Table 5). Non-trophallaxis behaviours (i.e. begging and antennation behaviours), however, were falsely classified as trophallaxis behaviours with a false positive rate of 8% (Table 5).

We then tested whether trophallaxis behaviours could be automatically classified based on their duration. We applied the duration threshold of ≥ 5 seconds to the automatically classified EBs* from the testing set comprising 43 encounter behaviours. We observed that 100% of the trophallaxis behaviours were automatically classified (Table 5). However, 28% of the detected behaviours were falsely classified as trophallaxis (false positive rate; Table 5). These classification rates suggest that we can automatically classify the vast majority of trophallaxis behaviours in a group of worker honeybees using our 'encounter classifier' together with the duration threshold of ≥ 5 seconds.

Table 4: Frequency and duration of the different manually detected encounter behaviours.

Encounter behaviour	No. of encounters	Relative proportion (%)	Min. duration (sec)	Max. duration (sec)	Median (sec)	75% percentile (sec)
Antennation	377	57	0.25	9.25	1.8	2.5
Offering	172	26	0.25	4.5	1	1.9
Begging	59	9	0.75	6.75	2	3
Trophallaxis	50	8	5	30.5	8.4	12.9

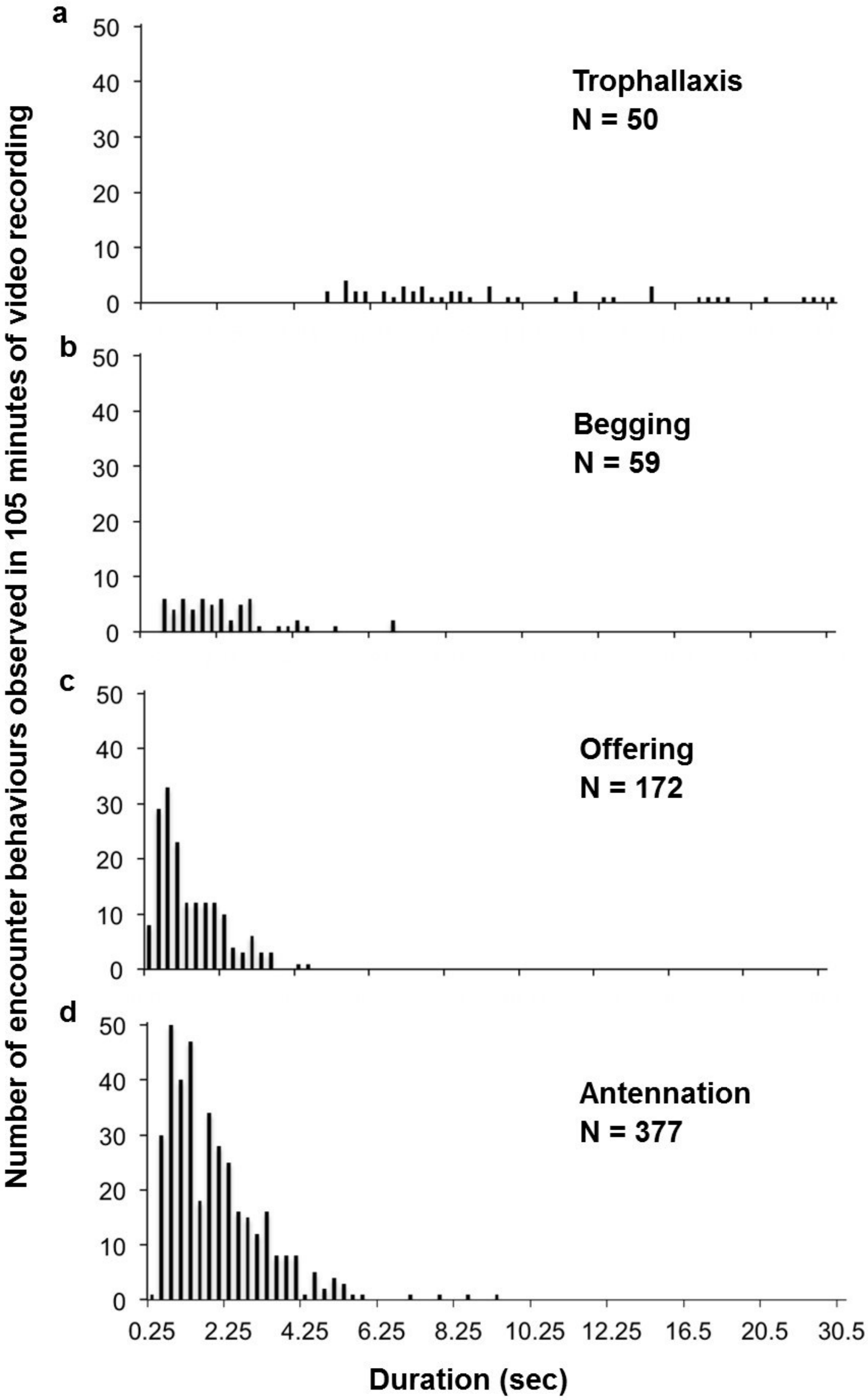

Figure 2: Number of encounter behaviours observed for the different duration of encounter behaviours from the four behaviour classes. (**a**) Trophallaxis, (**b**) Begging, (**c**) Offering, (**d**) Antennation.

Table 5: The classification of trophallaxis behaviours of manually detected and automatically detected encounter behaviours using the duration threshold of ≥ 5 seconds.

	Manually classified by duration among the 658 manually detected behaviours (1)		**Automatically classified by duration among the EBs* from the testing set** (2)	
	Trophallaxis (%) (3)	**Non-trophallaxis (%)** (3)	**Trophallaxis* (%)** (4)	**Non-trophallaxis* (%)** (4)
Trophallaxis (5)	100	0	100	0
Non-trophallaxis (5)	8	92	28	72

(1) We manually classified trophallaxis behaviours from the 658 manually detected encounter behaviours using the duration threshold of ≥ 5 seconds.

(2) We applied the ‘encounter classifier’ with the duration threshold of ≥ 5 seconds to the 43 manually annotated encounter behaviours not used for training.

(3) Percentage of the manually detected trophallaxis and non-trophallaxis behaviours that were manually classified as trophallaxis using the duration threshold of ≥ 5 seconds.

(4) Percentage of the manually annotated trophallaxis and non-trophallaxis behaviours from the testing set that were automatically classified as trophallaxis* and non-trophallaxis* (asterisks indicate automatic classification) using the duration threshold of ≥ 5 seconds.

(5) Trophallaxis and non-trophallaxis behaviours that were manually annotated by the observer.

Discussion

We introduced the BBAS, a system that can automatically classify stereotypical behaviours of individual workers in a group of honeybees. Our results show that the BBAS can be reliably used to automatically detect encounter behaviours.

Current behavioural observation methods usually require the manual detection of behaviours by an observer (Scheiner et al. 2013). Manual detection limits the number of observable behaviours, especially when the behaviour is frequently displayed by the many worker bees in a colony, as is the case for encounter behaviours. In this study, we accurately classified encounter behaviours between worker bees using automatic classification. Of the encounter behaviours that were manually annotated, 93% were accurately detected. Even though our classifier may not detect 7% of the encounter behaviours, the large number of behaviours of the many worker bees that can be detect over multiple days of observation produces a reliable test sensitivity. This statistical power will support the identification of even tiny differences between internal physiological states or the effects of experimental manipulation. According to the manual annotations, our classifier falsely classified other behaviours as encounter behaviours. Of these false detections, 13% had no similarity to encounter behaviours, whereas 15% had a close similarity to encounter behaviours, possibly suggesting that our classifier can detect a broader spectrum of encounter and encounter-related behaviours than can be manually annotated. These borderline cases may have a similar biological function and require further investigation.

In this study, the duration of the four different classes of encounter behaviours - trophallaxis, begging, offering and antennation - was obtained from 100 same-aged bees kept in a one-frame observation hive without a queen and brood. Our results showed that trophallaxis behaviours lasted between 5 and 30.5 seconds. The duration of offering and begging behaviours ranged from 0.25 to 6.75 seconds while antennation lasted 0.25 to 9.25 seconds. These measurements correspond to previous reports on the duration of trophallaxis, begging and offering behaviour that were obtained under more natural conditions (queenright colonies in one- or two-frame observation hives (Korst and Velthuis 1982, De Marco and Farina 2001, 2003)). Trophallaxis behaviours of different aged worker bees in these small queenright colonies lasted 4 to 30 seconds while begging and offering lasted less than 0.5 to 10 seconds (Korst and Velthuis 1982, De Marco and Farina 2001, 2003).

This constancy under different conditions suggests that duration can possibly be used as a predictive parameter to distinguish among the behavioural classes of encounters.

Our survey of manually annotated encounter behaviours suggests that a duration threshold of ≥ 5 seconds for an encounter behaviour can be used to accurately separate trophallaxis behaviour from the other encounter behaviours (begging, offering and antennation). When we applied our ‘encounter classifier’ together with the duration threshold, we were able to classify 100% of the manually annotated trophallaxis behaviours. However, the false positive rate was relatively high (28%), suggesting that we may need further adjustments of the behaviour duration parameter to reduce false classifications.

It has been proposed that encounter behaviours and the transmission of food are ways for worker bees to gather information about their colony’s state and thus can adjust their behaviours according to the colony’s need (Lindauer 1948, Ribbands 1953, Seeley 1986, Farina 1996). So far, we have detailed knowledge on the role of trophallaxis, begging and offering behaviours between incoming foragers and worker bees inside the colony in accessing information about the quality and source of nectar and the honey stores of the colony. Foraging worker bees usually unload the nectar from the honey crop to the in-hive worker bees via trophallaxis (Frisch 1965, Seeley 1995, Goyret and Farina 2005). The recipient worker bees store the nectar within the wax cells or further reduce the water content. Offering behaviour is performed by the returning nectar foragers, which are willing to unload their crop contents to a recipient worker bee (De Marco and Farina 2003). Inside the nest, worker bees beg incoming forager bees to receive nectar (Frisch 1965, Bozic and Valentincic 1991, De Marco and Farina 2003, Farina and Wainselboim 2005). The rate of begging behaviour is affected by the colony’s state and the amount of stored honey in the colony (Schulz et al. 2002). Antennation behaviour is essential in making and maintaining the contact between encountering bees (Free 1956, Goyret and Farina 2003). We envisage that with more classifiers trained for other behaviours, we can further examine the possible effects of encounter behaviours on subsequent task engagement.

For training the classifier and for measuring the accuracy of detection, we used 100 tagged worker bees in this study. However, with the current setup the BBAS can track up to 1000 worker bees on a brood comb in a small observation hive (preliminary data). It can be further scaled up to over 2000 worker bees by adding an additional camera, lighting system and cluster of five computers. Hence, we suggest that the BBAS will enhance our ability to gather knowledge on worker bees' individual and collective behaviours. With more classifiers trained to detect different behaviours in honeybees, the BBAS can be used to examine single-worker behavioural phenotypes and worker-worker interactions within small observation hives. We envisage that the BBAS will be a powerful tool to detect the experimental effects of genetic and physiological manipulations on single workers (Liang et al. 2012, Schulte et al. 2014). Additionally, we propose that the BBAS can be an accurate method for measuring the sub-lethal effects of pesticides on behaviour (Charreton et al. 2015). The key to understanding the effects of pesticides on honeybee colonies is gaining knowledge on how these influence individual behaviour. With the BBAS we will be able to analyse the effects of pesticides on individual behaviour because we can continuously and simultaneously quantify the in-hive behaviours of hundreds of worker bees under standardized conditions with computer-based classifiers. For encounter behaviours, for example, we can analyse the effects of pesticides on the duration of encounters or their quantity.

In conclusion, we foresee that the BBAS will be beneficial in various research areas for honeybee researchers who need to obtain detailed behavioural information of hundreds of individual bees.

Methods

Tracking device and procedure

Video recordings of worker bees on a comb and tracking information were obtained with a tracking device that was developed for ants by Mersch et al. (2013) and modified for tracking honeybees (see Supplementary information online). The honeybee tracking device consisted of a monochrome high-resolution camera, a cluster of five desktop computers, an infrared lighting system and an observation hive holding a single “Deutsch Normal” comb (Fig. 1a). The infrared light was provided in flashes synchronized with the images taken every quarter second (4 frames per second) by the camera. To omit daylight exposure, both the observation hive and the camera stood in a laboratory covered by a cardboard box that was lined with infrared-reflecting foil, which intensified the infrared illumination of the comb area. The cardboard box was equipped with a ventilation device that kept the temperature at approximately 29°C (± 1°C).

We used 2x2 mm tags bearing 2D barcodes from the AprilTags library (Fig. 1b) (Olson 2011) to tag and track honeybee workers. These 2D barcodes consisted of a square outline with a 36-bit code word encoded in the interior, which could generate up to 2320 unique identification (ID) numbers. An experiment on mortality and behavioural observations of tagged bees showed that bees bearing tags survived and behaved as untagged bees did (see Introductory experiments and observations in Supplementary information online). The tracking information obtained by the tracking software (Mersch et al. 2013) contained (after postprocessing) the tag’s ID number, the x- and y-coordinates of its centre and its orientation with the corresponding frame number and timestamp in UNIX time (with a precision of 1/100 seconds).

Automatic behaviour classification using the tracking information

From the tracking information, we used the JAABADetect program (Kabra et al. 2013) to compute social per-frame features to provide information on the bees’ properties in relation to their nearest nestmate in each frame (for example, the distance, speed, and orientation to the closest bee; see Kabra et al. (2013) for a detailed listing of social per-frame features).

To produce the ‘encounter classifier’, we labelled examples of encounter and non-encounter behaviour in 105 minutes of tracking information and video material using the graphical user interface of the JAABA program (Kabra et al. 2013). We only labelled encounter and non-encounter behaviours for which we had high confidence in classification. Thus, for encounter behaviours we only labelled those for which we could confidently identify that behavioural features characterizing encounter behaviours were displayed. Information about the social per-frame features that were computed from the tracking information was used to train the 'encounter classifier' via machine learning implemented in the JAABA program (Kabra et al. 2013).

The classifier's accuracy was determined using the cross-validation method implemented in the JAABA program (Kabra et al. 2013). We used JAABA's default settings for the cross-validation and performed 10 cross-validation rounds to obtain an average estimate on the classifier's accuracy (see Supplementary information online for more details on calculation of accuracy and cross-validation).

Manual annotation of encounter behaviours and further analysis

We manually examined the video recordings to detect all encounter behaviours. We determined the duration in seconds and the type of encounter behaviour: i) antennation behaviour, ii) begging behaviour, iii) offering behaviour, iv) trophallaxis behaviour.

Statistical analyses were performed using the SigmaPlot 13 software.

Bee handling

We used newly emerged honeybees that originated from a colony of western honeybee *Apis mellifera* from our bee yard at the Heinrich-Heine University of Düsseldorf, Germany. A sealed brood comb was taken from the source colony and incubated at 34°C. Emerging worker bees were collected when they were 0-24 hours old. One hundred bees were marked with hand-cut tags by gluing these centrally on the thorax of the bees with glue ("Opalith Zeichenleim", Heinrich Holtermann KG, Brockel, Germany).

The bees were tracked from May 3rd to May 4th, 2016 on a comb comprising 40 capped cells filled with honey. Bees were restricted to one side of the comb without a queen. As worker-worker encounters were the interest of this study, neither a queen nor drones were included in the group. The comb did not contain brood because we used newly emerged worker bees for tracking, and it is known that brood rearing first begins at an age of two to three days (Rösch 1925, Seeley 1982).

To ensure that sufficient encounter behaviours occurred during the tracking process, a proportion of the bees were either fed ad libitum with a sugar solution (Ambrosia Bienenfutter-Sirup, Nordzucker AG, Braunschweig, Germany) or starved before tracking was started. On the first day of tracking, 16 bees were fed with the sugar solution before starting the tracking experiment, whereas the remaining bees were starved for approximately an hour. For sustenance, we provided the bees with a sugar pastry (Apifonda Futterteig, Südzucker AG, Mannheim, Germany) two hours after tracking was started. On the second day of tracking, we removed the sugar pastry and fed 15 of the 100 bees again with the sugar solution. The other 85 bees were starved for three hours. The 15 bees were reintroduced into the observation hive before tracking began. In total, information from 22 hours of tracking was generated for 96 bees. Four bees lost their tags during tracking.

Data availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request. Programs developed for this study will be shared and can be requested from the corresponding author.

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Author contributions

CB designed and performed the experiments and conducted the behaviour analyses. CB and MB conceived the study, supervised its design and coordination, and wrote the manuscript. AC, DM and LK developed the tracking software. LZ and MK worked on programming required for use of JAABA with our tracking data. CF and BS developed and manufactured the electronics for the tracking device. All authors reviewed the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Supplementary Information

Supplementary Table S1: Comparison of manual annotations (EBs) and automatic classifications (EBs*) among the different classes of encounter behaviours

	No. of behaviours from the training set		**No. of behaviours from the testing set**			
			Manually annotated EBs ⁽³⁾	**Automatically classified EBs* ⁽⁴⁾**		
Classes of encounter behaviours	**Manually annotated EBs ⁽¹⁾**	**Automatically classified EBs* ⁽²⁾**		**Correctly classified**	**False negatives ⁽⁵⁾**	**False positives ⁽⁶⁾**
Trophallaxis	34	34	4	4	0	17
Begging	8	8	8	8	0	
Offering	6	6	12	12	0	
Antennation	28	28	19	16	3	

(1) The manually labelled high-confidence behaviours (EBs) used to train the classifier (training set).

(2) EBs* correctly classified by the trained ‘encounter classifier’. A confidence threshold of > 0 was set for automatic classification; thus, only EBs with frames displaying confidence values above 0 were classified as EBs*.

(3) Manually annotated EBs not used to train the ‘encounter classifier’.

(4) EBs* correctly classified by the trained ‘encounter classifier’. A confidence threshold of > 0.2 was set for automatic classification; thus, only EBs with frames displaying confidence values above 0.2 were classified as EBs*.

(5) EBs falsely classified as NEBs* by the ‘encounter classifier’.

(6) NEBs falsely classified as EBs* by the ‘encounter classifier’.

Supplementary Methods

Tracking device

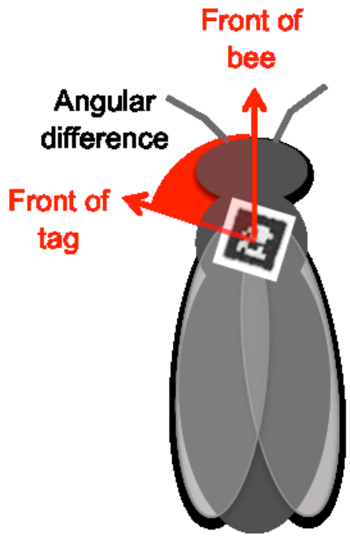
Video recordings of worker bees on a comb and tracking information were obtained with a tracking device that was developed for ants by Mersch et al. (2013). For honeybee tracking, the following modifications were made to the tracking device: We used a monochrome high-resolution camera (hr29050MFLGEA GigE Compact CCD-camera, 6576x4384 pixels, SVS-VISTEK GmbH, Seefeld, Germany) equipped with an 50 mm F/2.0 camera lens (ZEISS Objektiv Makro-Planar T* 2.0/50 ZF, Carl Zeiss AG, Oberkochen, Germany) and an infrared-transmitting filter that was transparent to wavelengths starting from 780 nm (Heliopan RG780 Infrared Filter, Heliopan Lichtfilter-Technik Summer GmbH & Co KG, Gräfelfing, Germany). The tracking software was run on a cluster of five desktop computers (2x Intel Xeon E5-1620, 3.70 GHz, 16GB RAM, Rombus, Büren, Germany; 1x Intel Xeon E5-1630, 3.70 GHz, 16GB RAM, 1x Intel Core i5-3330, 3.00 GHz, 12GB RAM, 1x Intel Core i7-4790, 3.60 GHz, 8GB RAM, 1x Intel Xeon E5-1630, 3.70 GHz, 16GB RAM, Wortmann AG, Hüllhorst, Germany) that were connected through a Gigabit Ethernet switch (D-Link DGS-1016D Gigabit 16-Port Switch, D-Link GmbH, Eschborn, Germany). The camera was connected to the cluster via two Ethernet cables and took images of the surface of a single “Deutsch Normal” comb that was placed in an observation hive between two Plexiglas panels. The distance between the Plexiglas panels and the comb was about 12 mm (for more information see Introductory experiments and observations). To omit daylight exposure, both the observation hive and the camera were covered by a cardboard box in the laboratory. The box was lined with infrared-reflecting foil (LEE Farbfolie 273, Eckert Bühnenlicht, Wuppertal-Langerfeld, Germany). The infrared-reflecting foil intensified the infrared illumination of the comb area. The cardboard box was equipped with a ventilation device that kept the temperature at approximately 29°C (± 1°C). The lighting system consisted of fourteen custom-made electronic boards each equipped with six infrared light emitting diodes. The boards were arranged directly around the observation hive to avoid light reflections on the Plexiglas (Fig. 1a). The infrared light was provided in 5 ms flashes with a peak wavelength at 850 nm (outside of the bees’ visible range (Backhaus 1991)) that were synchronized with the images taken every quarter second (4 frames per second). We used infrared light flashes rather than continuous infrared light since flashes had the advantage of producing less heat and thus facilitated temperature control under the cardboard box. Also, motion blur of fast moving bees was decreased because light flashes permitted us to use a higher light intensity and thus a reduced exposure time.

Tracking procedure

The tracking software and image processing were as described in Mersch et al. (2013), except for the following modifications: Among the various 2D barcode sets provided by the AprilTags library, we used the 36h10 set (Fig. 1b), which provides up to 2320 unique 2D barcodes. Each barcode is encoded as a unique 36-bit code word having a minimum Hamming distance (i.e. number of differences when comparing bit-by-bit with another code) of 10 bits to all the other barcodes (Olson 2011). Tags bearing the 2D barcodes were 2x2 mm in size, printed on waterproof white polyester foil (laserFOL.135 a4, matte surface, 135 µm, Papier & mehr, Neuenhaus, Germany) with a laser printer and cut out by hand. The tags were filmed by the camera at a resolution of 17 pixels/mm. During processing, the images were divided into 96 segments by the tracking software because it can only handle small images. Image segments overlapped by 100 pixels to ensure that tags located on segment borders were detected. Images were saved as video (AVI files) after they were resized to 1644x1096 pixels and compressed with the Xvid codec. The tracking software output the ID number, the x- and y-coordinates of the four corners of each tagged bee's tag with the corresponding timestamp in UNIX time (with a precision of 1/100 seconds) and the image number, and this information was stored in a comma-separated values (CSV) data file. This CSV data file was postprocessed to obtain the tag's centre and its orientation and to generate a binary data file containing the tag's ID number, its x- and y-coordinates of its centre and its orientation with the corresponding frame number and timestamp in UNIX time (with a precision of 1/100 seconds). Finally, to obtain the final binary data file, we processed the angle difference between the front of the tag and the front of the bee using a program from Mersch et al. (2013) to ensure that the orientation given in the final data file represents the front of each bee (see Supplementary Fig. S2).

To estimate whether we can capture sufficient information on bees' position and orientation with a tracking rate of four frames per second, we measured the average change in x/y position and orientation of bees in four consecutive frames. We randomly chose 10 bees moving across the comb, calculated the average change in x/y position and orientation for each bee and then calculated the average change in x/y position and orientation with its standard deviation for all bees.

We examined the accuracy of our tracking device by determining the average detection rate of immobile tags (glued to a comb) and tags glued to bees. To measure the detection rate of the immobile tags, we distributed 100 immobile tags on a wax foundation positioned in a “Deutsch Normal” frame and tracked the tags for five minutes. To measure the detection rate of the tags on bees, we randomly selected 30 moving and 10 resting worker bees from three different video sequences and tracked them for one minute. For each tag, we calculated the percent of frames in which the tag was detected, and from this we calculated the average percent of detected frames for immobile tags and tags glued to bees. For the immobile tags, we also determined the accuracy for the detection of the x/y position and orientation. For both the position and orientation, we calculated the average difference between the tracking information of consecutive frames with its standard deviation.



Supplementary Figure S2: Angle difference between the front of the tag and the front of the bee. If the front of the tag did not align with the front of the bee, we corrected the angle difference to ensure that orientations given in the tracking information corresponded to the bee’s front.

Automatic behaviour classification using the tracking information

From the tracking information, we computed social per-frame features that provide information on the bees' properties in each frame in relation to its nearest neighbour (for example, the distance, speed, and orientation to the closest bee). We used the JAABADetect program (Kabra et al. 2013) that was run on a high performance-computing cluster at the Heinrich-Heine University Düsseldorf, Germany. The JAABADetect and JAABA program were run in MATLAB version 8.3.0.532 (R2014a; The MathWorks GmbH, Ismaning, Germany). To obtain tracking data for the classifier's training procedure, we extracted three 15-minute and two 30-minute sequences of the total tracking data and video recordings using a program from Mersch et al. (2013) and the free video tool VirtualDub (VirtualDub-1.9.11, virtualdub.org). From these data, we generated MATLAB-compatible .mat files using an in-house MATLAB application. This application also performed the following steps: first, if the tracking information in a single frame of a worker's trajectory was missing, the information was extrapolated from the mean of data points before and after the gap; second, trajectory segments representing missing information from more than 6 frames were excluded from the further analysis; third, trajectories shorter than 10 frames (for 15-minute sequences) and shorter than 100 frames (for 30-minute sequences) were not inherited by the MATLAB .mat file.

To train the 'encounter classifier', we first labelled examples of encounter and non-encounter behaviours using the video recordings and the graphical user interface of the JAABA program (Kabra et al. 2013). We only labelled encounter behaviours and non-encounter behaviours for which we had high confidence in classification. For encounter behaviours, we thus only labelled encounters for which we could confidently identify that behavioural features characterizing encounter behaviours were displayed. For automatic classification of encounter behaviours, we applied the hysteresis method which is a postprocessing tool implemented in the JAABA program (http://jaaba.sourceforge.net/Training.html#PostProcessing). This tool allows for reduction of falsely classified behaviours by setting a confidence threshold. Automatically classified behaviours are discarded if their confidence values lie below the set confidence threshold. Most falsely classified behaviours had low confidence values, thus by setting confidence thresholds these falsely classified behaviours could be reduced significantly. To determine confidence thresholds for the different data sets analyses in this study, we examined the classifier's positive and false positive classification rates for each data set with different confidence thresholds. We chose the confidence threshold providing the lowest rate of false classifications. We applied a confidence threshold of > 0 (i.e. no threshold) for the training set (EB) and a confidence threshold of > 0.2 for the behaviours not used for training. For the automatic classification of trophallaxis behaviours using our 'encounter classifier' with the duration threshold of ≥ 5 seconds, we applied a confidence threshold of > 0.4.

Training was performed with the social per-frame features computed from the tracking information (see the detailed list of social per-frame features in the Supplementary information of Kabra et al. (2013)). We performed cross-validation to measure the classifier's accuracy using JAABA's default settings (Kabra et al. 2013). For cross-validation, the training set consisting of the labelled EBs and NEBs was spilt into subsets. By default JAABA does 7-fold cross-validation, thus the training set was split into 7 subsets. Hereby, 1/7 of the EBs and NEBs were assigned to a testing subset while 6/7 were assigned to a training subset. Later was used to train the classifier whereas the classifier's error rate was estimated by testing its accuracy on the testing subset (Kabra et al. 2013). The accuracy is tested by quantifying how many frames of the manually labelled EBs and NEBs were predicted on correctly or incorrectly by the 'encounter classifier'. Therefore, the number of frames manually labelled as EBs and NEBs and automatically predicted on as EB* and NEB* by the 'encounter classifier' are compared (asterisks indicate automatically classified behaviours). This results in a percentage value for frames correctly and falsely classified as EB* and NEB* by the 'encounter classifier'. The assignment of EBs and NEBs to either the testing or training subset is done randomly. Thus, the EBs and NEBs maybe part of a different subset for each cross-validation round performed. The estimated accuracy of the classifier may therefore slightly vary for each cross-validation round. We performed 10 cross-validation rounds to obtain an average estimate of the classifier's classification accuracy.

Manual annotation of encounter behaviours and classification of trophallaxis

We manually examined the video recordings to detect encounter behaviours using the video tool VirtualDub (VirtualDub-1.9.11, virtualdub.org) with a software component from Mersch et al. (2013) that enables the visualization of the bee’s ID number in the video recording. We noted all encounters with the corresponding behaviour duration in seconds and determined the type of encounter behaviours: i) begging behaviour, ii) offering behaviour, iii) trophallaxis behaviour and iv) antennation behaviour. The different encounter behaviours are characterized by the following behavioural features: i) Begging bees tilted their head upwards, opened their mandibles and outstretched their proboscis (Korst and Velthuis 1982) towards the mouthparts of the other bee (Free 1956). The begging bee moved its antennae more or less intensely and oriented them towards the other bee. Additionally, the begging bee could show a grasping movement with its front legs (Korst and Velthuis 1982). ii) A bee displaying offering behaviour opened its mandibles, but it did not show tilting of its head or the outstretching of its proboscis (as observed for begging bees). The antennae were held low and close to the head (Korst and Velthuis 1982). iii) During trophallaxis behaviour the antennae of the bees were in contact, whereas the receiving bee outstretched its proboscis towards the mouthparts of the donating bee (Korst and Velthuis 1982). In addition to the behavioural features, we used the duration (> 4 seconds) of encounter behaviour to characterize trophallaxis behaviours and to differentiate them from the three other encounter behaviours because different studies have shown that an effective food transfer requires a contact of at least 4 seconds (Kühnholz 1997, Farina and Wainselboim 2001). iv) Antennation behaviour was noted if two bees stood facing each other with their moving antennae in contact and no other behavioural features as described for the other three classes were shown (Bell 1979, Lenoir 1982, Montagner 1982, Gorton et al. 1983).

Statistical analyses were performed using the SigmaPlot 13 program.

Introductory experiments and observations

Experiments and observations were performed prior to the tracking experiments in this study. We used newly emerged honeybees that originated from colonies of western honeybee *Apis mellifera* from our bee yard at the Heinrich-Heine University of Düsseldorf, Germany. Bees from the same experiment were of the same age. Sealed brood combs were taken from the source colonies and incubated at 34°C. Emerging worker bees were collected when they were 0–24 hours old and marked either with tags bearing 2D barcodes (2D barcodes were printed on waterproof foil) or a coloured dot on the thorax using a marker (POSCA Zeichenstift “Europa”, Heinrich Holtermann KG, Brockel, Germany).

First, behavioural observations of bees marked with tags were conducted to determine if the tags affect their behaviour. We introduced 150 tagged bees into a queenright colony of a two-frame observation hive. Observations were conducted from June 14th to July 26st, 2013 at the Heinrich-Heine University of Düsseldorf, Germany. We observed the bees’ behaviour every second day for two hours. Tagged bees performed all in-hive tasks equally than their non-tagged nestmates. They entered comb cells and also left the hive for foraging trips returning with pollen loads.

Second, from July 8th to August 8th, 2013 we tested the mortality of bees marked with tags. We introduced 100 bees tagged with 2D barcodes and 100 bees marked with a coloured dot on their thorax into a queenright colony housed in a one-story beehive box. The hive stood in a flight cage. Bees were provided with pollen (Echter Deutscher Spezial Blütenpollen, Werner Seip – Biozentrum GmbH & Co. KG, Butzbach, Germany), sugar solution (Ambrosia Bienenfutter-Sirup, Nordzucker AG, Braunschweig, Germany) and water that they could forage for ad libitum. Over the period of the experiment, we counted the dead bees each day by searching the floor of the flight cage for marked bees. There was no difference in the survival of bees marked with tags and coloured dots (Log-rank test: $N = 200$, $\chi^2 = 2.7$, d.f. = 1, $P = 0.1$).

Third, when constructing our observation hive for the tracking experiments we started off with a distance of about 8 mm between the Plexiglas panels and the comb. This distance corresponds to the common distance of 8 mm between combs in a beehive box. We ascertained, however, that this distance was too narrow. First, this distance affected drone behaviour because they got stuck between the two surfaces. Second, the narrow space caused water to condense on the Plexiglas, which affected visibility. We thus increased the distance between the glass and the comb by 4 mm resulting in a distance of 12 mm.

Supplementary Video V1: Antennation behaviour. The highlighted bees with the ID numbers 2296 and 2302 (green) show antennation behaviour. The bees face each other with their moving antennae in contact.

Supplementary Video V2: Begging behaviour. The bee highlighted in red with the ID number 2276 (green) shows begging behaviour towards the bee highlighted in blue with the ID number 2261 (green). The begging bee (red) orientates its antennae towards the other bee, tilts its head upwards and grasps the other bee with its front legs.

Supplementary Video V3: Offering behaviour. The bee highlighted in red with the ID number 2302 (green) shows offering behaviour towards the bee highlighted in blue with the ID number 2045 (green). The offering bee (red) holds its antennae low and close to its head and does not tilt its head upwards as a begging bee would.

Supplementary Video V4: Trophallaxis behaviour. The highlighted bees with the ID numbers 2134 (green) and 2279 (green) show trophallaxis behaviour. The antennae of both bees are in contact and the bee highlighted in red outstretches its proboscis towards the other bee.

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Automated computer-based detection of encounter behaviours in groups of honeybees

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Author’s contribution: 80%

- Concept of the study
- Design and setup of the tracking system
- Experimental design
- Implementation of tracking experiments
- Video analyses and manual detection of encounter behaviours
- Implementation of training procedure in JAABA
- Authoring the manuscript

Manuscript II

Honeybees (*Apis mellifera*) collectively gain information about task demand through repeated individual visits to different task areas.

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Abstract

Inside a honeybee hive, thousands of worker bees perform various tasks, which are located in different task areas distributed throughout the comb. How worker bees obtain information on the need for task performances appropriate for their colony's current task demand is uncertain. It has been proposed that worker bees access information on task demand by moving throughout the task areas and gathering local information. Worker bees therefore would not simply rely on local information when deciding which task to perform, but could access information on a much larger scale. In this study, we experimentally tested whether worker bees may gain information on task demand by visiting task areas and whether individual task area visiting behaviour can generate stable group-level patterns of task area visits. We measured task area visits and moving behaviour of individual worker bees in different sized groups with varied task demand using the Bee Behavioral Annotation System (BBAS). Our results demonstrate that all worker bees visit task areas independent of group size suggesting that worker bees may individually and continuously access information on their group's task demand. Individual task area visiting behaviour created a stable group-level pattern of task area visits throughout the different group sizes, suggesting that honeybees collectively gain task information through repeated individual task area visits.

Introduction

The functionality of a honeybee colony (*Apis mellifera*) relies on the actions of thousands of individual worker bees who seem to perform exactly those tasks needed for the given colony state. How worker bees assess information on the need for task performances appropriate for the current task demand within the hive is a central question.

Inside the hive, worker bees have a large task repertoire. Tasks associated with brood rearing, nest construction, food storage and processing are performed by the many hive-bees (Lindauer 1952, Winston 1987, Seeley 1995, Johnson 2008b, 2010a). Two age cohorts of worker bees perform the majority of in-hive tasks (Seeley 1982, Johnson 2008b). Nurse bees, typically aged 4 to 12 days primarily perform tasks associated with brood rearing (Seeley 1982, Winston 1987, Johnson 2008b). Such tasks include feeding larvae and other nestmates which involves pollen consumption (Crailsheim et al. 1992, Camazine 1993), capping and inspecting brood cells (Seeley 1982, Winston 1987, Johnson 2003, 2008b, 2010a). Middle-aged bees, typically aged 13 to 20 days perform 15 different tasks associated with nest construction, food storage and processing (Seeley 1982, Johnson 2008b). Such tasks are, for example working wax, packing pollen, receiving, storing and processing nectar (Seeley 1982, Seeley et al. 1996, Johnson 2003, 2008b). The majority of tasks performed by nurse bees and middle-aged bees is bound to three major task areas on the comb, namely the brood, pollen and honey area. While brood rearing tasks of nurse bees, such as feeding larvae are mainly restricted to the brood area, the various tasks performed by middle-aged bees are dispersed throughout the entire comb (Seeley 1982). For example, packing pollen and processing nectar are performed in the pollen and honey area while working wax can be performed throughout the entire comb. Since the comb is quite large compared to the size of worker bees and distances separate the different task areas, it is unlikely that worker bees are able to sense changes in task demand in areas other than where they are present (Seeley 1995, Johnson 2008a). Unlike forager bees who produce signals through dances, vibration and sound in order to convey information about resource location and quality, and the need for additional receiver bees and foragers (Frisch 1923, Frisch 1965, Michelsen et al. 1986, Seeley et al. 1991, Seeley 1992, Kirchner 1993, Seeley et al. 1996, Nieh and Tautz 2000, Schneider and Lewis 2004), hive-bees have so far not been found to produce signals in order to communicate about task demand (Johnson 2002, 2003, 2008a, b).

Group-level patterns emerge from worker bees' individual decisions which are thought to be solely based on local information such as a hungry larva indicating the need for feeding or a partially capped cell as indication for the need of cell capping (Deneubourg and Goss 1989, Bonabeau et al. 1997, Seeley 1998, Deneubourg et al. 2002). If individual worker bees base their decision of task performances solely on local information then the question arises how worker bees determine changes in the demand for tasks that are spread out over the task areas. Based on behavioural observations, Johnson (2008a, 2009) proposed that worker bees assess their group's task demand by moving throughout the nest and gathering local information in the different task areas (global information sampling). Middle-aged bees spend a large percentage of their time patrolling, walking around the comb and inspecting random cells (Lindauer 1952, Johnson 2002, 2008a). Additionally, they have been found to randomly quit their current task independent of the demand for it (Johnson 2008a). Studies using cohorts of 100 middle-aged bees reported that these visited different task areas during an observation period of 30 minutes (Johnson 2008a) and frequently quit the task of wax working within an hour suggesting that task quitting and patrolling are regular behaviours of middle-aged bees (Johnson 2002). Strikingly, patrolling and task quitting have not been found to be exhibited by nurse bees whose task repertoire and the area in which the nursing tasks can be performed are much more specific than for middle-aged bees (Seeley 1982, Johnson 2008a). However, it is unknown whether patrolling and task quitting behaviour of middle-aged bees are related to changes in task demand. A critical component of Johnson's model (2009) is that long patrols are performed throughout the nest without reference to variation in task demand. Despite the constant task switching and movement of worker bees at the individual level a stable task allocation pattern at the collective level is generated (self-organizing properties) (Johnson 2009).

In this study, we experimentally tested whether worker bees may gain information on their group's task demand by visiting task areas and whether individual task area visiting behaviour can generate stable group-level patterns of task area visits despite changes in task demand. Task area visits and moving behaviour of individual worker bees in small queen-right groups were measured using the Bee Behavioral Annotation System (BBAS) (Blut et al. 2017). We changed the task demand by altering group size while keeping the task areas and workload in the varying group sizes constant. Our results demonstrate that all worker bees visit the different task areas independent of group size suggesting that worker bees may individually and continuously access information on their group's task demand. The individual task area visiting behaviour created a stable group-level pattern of task area visits throughout the different group sizes, suggesting that honeybees collectively gain task information through repeated individual task area visits.

Results

To understand how individual worker bees obtain information about colony needs we examined the visits to the brood, pollen and honey area, path length and change in direction of worker bees from different group sizes kept on standardized combs with equal workload. The combs were standardized for areas in which cells contained an equal amount of brood (151 third to fourth instar honeybee larvae), pollen and honey (sugar syrup) (Fig. 1B). Queen-right groups in three different sizes (250, 500 and 750 worker bees) with three biological replicates for each group size were produced with same-aged worker bees. Over 48 hours, continuous information on the number of visits to the brood, pollen and honey area per hour (see Supporting Information S1 Table for quality of tracked group sizes), the path length per hour and the change in direction per second was obtained for each bee when she was seven days old. For comparison and as control group, the same information was obtained over 20 minutes for worker bees individually kept on standardized combs (14 biological replicates). Values were calculated from the tracking data obtained with the Bee Behavioral Annotation System (BAAS) introduced by Blut et al. (2017).

Figure 1: Worker bee tagged with 2D barcode and standardized comb. A) For automated tracking with the Bee Behavioral Annotation System (BBAS), bees were marked with tags bearing 2D barcodes. Each bee can be individually identified by the identification number encoded in the 2D barcode. **B**) For tracking, we used standardized combs with an equal workload. Each comb contained a brood area with 151 third to fourth instar honeybee larvae (centre) flanked by areas containing the same amount of pollen (yellow areas) and sugar syrup (areas surrounded by green lines).

All worker bees have access to task and resource information by visiting task areas

To learn whether all bees or only a group of bees obtains task information, we examined the frequency distributions of the visits to all task areas (brood, pollen and honey area visits totalled) and the visits to the brood, pollen and honey area for the bees under the condition of different group sizes. If there was a group of specialist bees that gained more task information than non-specialist bees (more visits to task areas) we would expect bimodal frequency distributions.

The biological replicates of the three group sizes displayed a single, unimodal frequency distribution for all task area visits and the visits to the three different task areas. All bees visited at least one area per hour (Fig. 2). At least 60% of the bees visited the brood area at least once per hour. Both, the pollen and honey area were visited by at least 20% of the bees at least once per hour (Supporting Information S2 – S4 Fig.). These frequency values suggest that each bee may individually and continuously access task information by visiting the different task areas.

The frequency distributions differed from various probability distributions (Normal, Lognormal, Gamma, and Poisson distribution: $P < 5 \times 10^{-4}$, Supporting Information S5 Table). All unimodal distributions were leptokurtic while the kurtosis increased with a tripled amount of bees ($\text{Kurtosis}_{(250\ \text{bees})} = 5$, $\text{Kurtosis}_{(750\ \text{bees})} = 121$; Supporting Information S6 Table). This suggests that with more bees on the comb and low per capita workload the difference in task area visits between individual bees is lower in large groups.

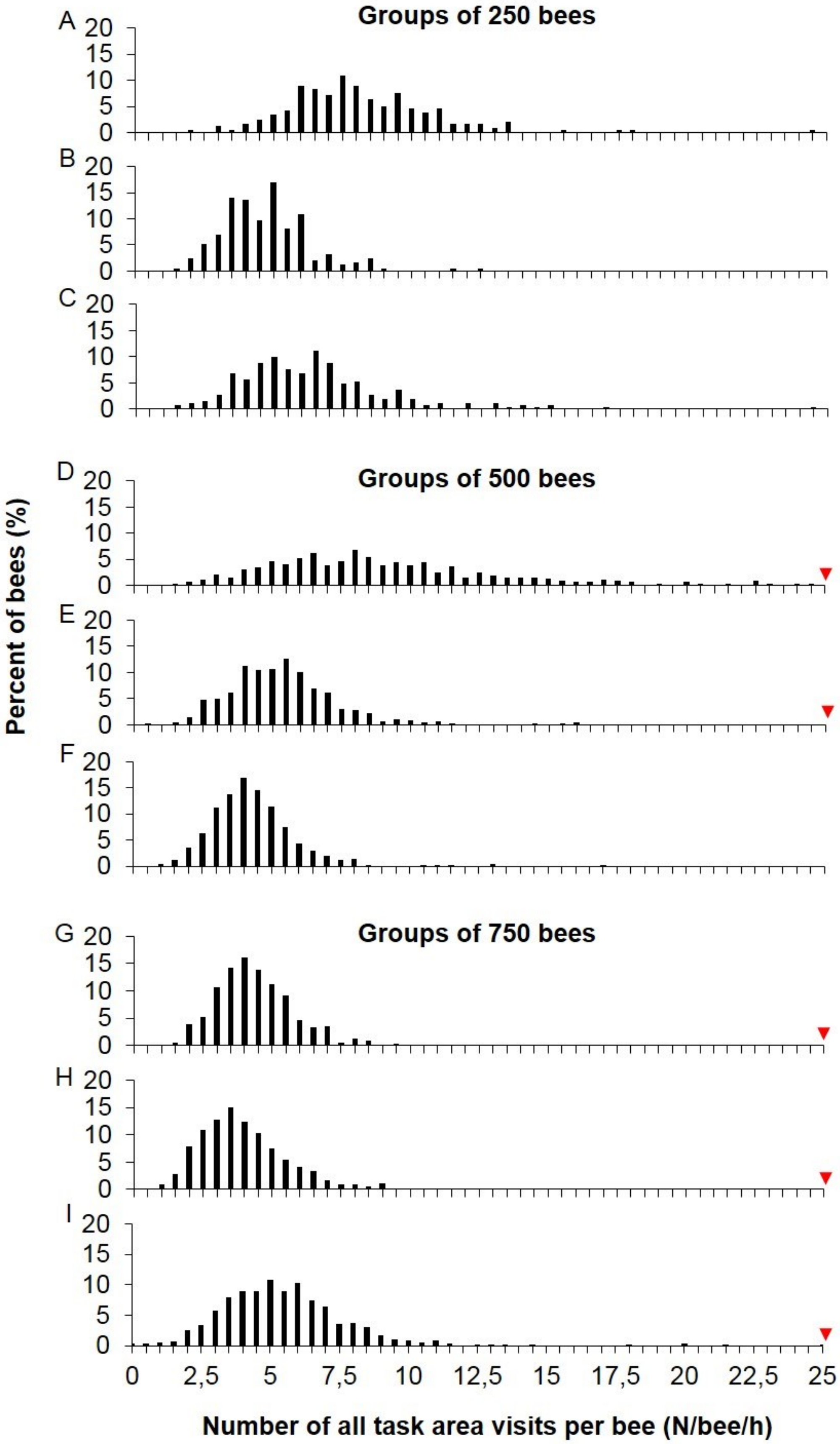

Figure 2: Frequency distributions for the visits to all task areas for the biological replicates. A) – **C**) Frequency distributions for the three biological

replicates of the group size of 250 bees. **D**) – **F**) Frequency distributions for the three biological replicates of the group size of 500 bees; red triangles represent outliers (x/y): D) (0.2/25.5); (0.2/26.5); (0.4/28); (0.2/28.5); (0.2/29.5); (0.2/30); (0.2/33); (0.4/35); (0.2/38); (0.2/39.5); (0.2/40.5); (0.2/53.5); (0.2/80), E) (0.2/25.5); (0.2/27.5); (0.2/29); (0.2/31). **G**) – **I**) Frequency distributions for the three biological replicates of the group size of 750 bees; red triangles represent outliers (x/y): G) (0.1/52), H) (0.2/39.5), I) (0.1/57.5).

Effect of increased number of worker bees on the access to the different task areas

Next, we examined how all worker bees gain task information if the amount of worker bees is tripled while the group-level workload is kept constant. More bees on the comb may constrain the access to different task areas in which tasks are performed. Alternatively, a lower workload per bee may influence the number of task area visits: worker bees may be more active in order to find a task or they may show lower activity due to the reduced workload per bee.

First, we tested whether group size influenced the visits to the different task areas by comparing the visits to all task areas between the group sizes. Visits to all task areas of worker bees differed significantly between the groups of 250 (median: 5.7 visits/bee/h), 500 (median: 5.1 visits/bee/h) and 750 bees (median: 4.1 visits/bee/h; ANOVA on ranked and bootstrapped datasets: $F_{(2,\ 4324\text{-}4326)} = 198.6$, $P < 5 \times 10^{-4}$; Fig. 3A, S7 Table). Worker bees kept singularly on a comb had a median number of 102 visits per hour, which are about 25 fold more visits compared to the group of 750 bees. Post hoc tests revealed that the number of task area visits consistently decreased with increasing group size (Dunnett’s T3 Test: 250 vs. 500 bees: D = 236; 250 vs. 750 bees: D = 862; 500 vs. 750 bees: D = 627; S7 Table). When the number of worker bees was tripled the task area visits decreased by 28%. This suggests that factors raised through increased group size such as mobility constraints and lower workload per bee affect a bees’ task area visits.

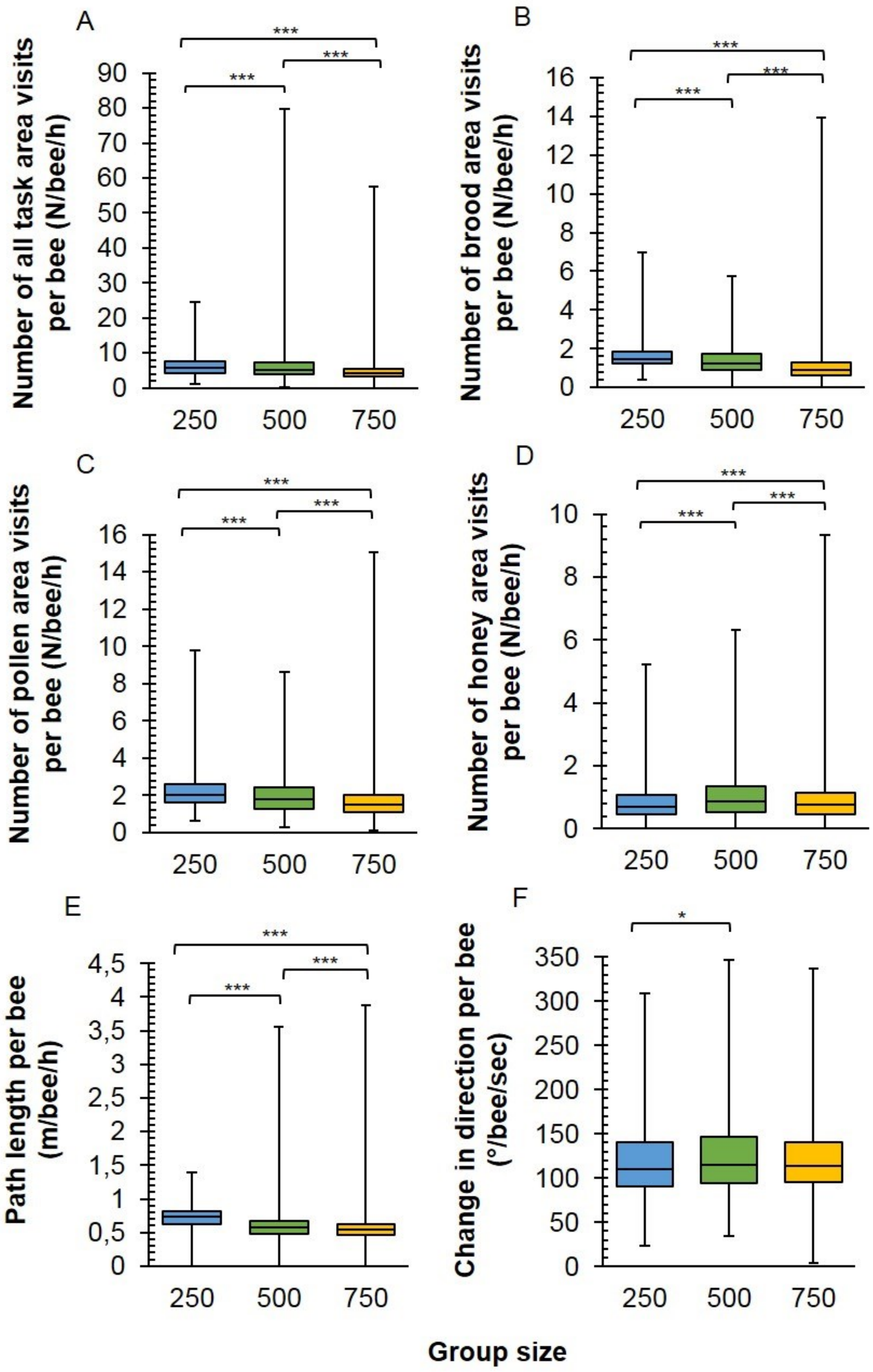

Figure 3: Boxplots for the number of all task, brood, pollen and honey area visits, the path length and change in direction of worker bees from three different group sizes. The median, 75% and 25% percentile and minimum and maximum are presented. **A**) Number of all task area visits (brood, pollen and honey

area visits totalled). **B**) Number of brood area visits. **C**) Number of pollen area visits. **D**) Number of honey area visits. **E**) Path length. **F**) Change in direction.

In addition, we examined whether the found relationship between group size and all task area visits held true for the visits to the brood, pollen and honey area. Worker bees' tasks within the brood area include feeding and heating the brood. Pollen and honey areas are, among others, visited to eat pollen and honey, pack pollen, unload and process nectar. For all three task areas, we found that the visits differed significantly between the groups of 250, 500 and 750 bees (ANOVA on ranked and bootstrapped datasets: brood area: $F_{(2,\ 4324\text{-}4326)} = 422.2$, $P < 5 \times 10^{-4}$; Fig. 3B, S7 Table; pollen area: $F_{(2,\ 4324\text{-}4326)} = 146.2$, $P < 5 \times 10^{-4}$; Fig. 3C, S7 Table; honey area: $F_{(2,\ 4324\text{-}4326)} = 29.5$, $P < 5 \times 10^{-4}$; Fig. 3D, S7 Table). However, post hoc tests revealed that the found relationship between group size and task area visits held true only for the brood and pollen area (Fig. 3B and 3C; S7 Table). Tripling the number of worker bees on the comb decreased both the brood and pollen area visits per bee, suggesting that more bees on the comb and a lower per capita workload reduce the number of visits to these two areas. For the honey area visits, on the other hand, we found no such relationship. Although honey area visits differed significantly between group sizes, these did not consistently decrease with increasing group size (Dunnett's T3 Test: all pairwise: $P < 5 \times 10^{-4}$; Fig. 3D, S7 Table). This suggests that an increased number of bees on the comb does not consistently affect the number of honey area visits. For bees kept singularly on the comb we found the visits to the three areas to be notably higher compared to the bees' visits in the large groups (S7 Table). For example, worker bees kept singularly on a comb visited the brood area 21 times per hour which is around 20 times more than the bees' brood area visits in the large groups (S7 Table).

The previous results demonstrated that the task area visits of individual worker bees decreased due to increased group size. This suggests that the individual worker bees obtain less local information when more worker bees are on the comb. Therefore, we analysed how this reduction on the individual level relates to the information gained on the collective level. We estimated the number of all task area visits performed by the group as a whole (Table 1). We found that tripling the amount of workers on the comb doubled the number of task area visits. This indicates that the reduction of task area visits per bee caused by the increased number of bees is compensated solely by a higher number of bees involved.

Table 1: The task area visits at the collective level

Group size	Biological replicates	Number of all task area visits per bee per hour [a]	Total number of all task area visits per hour
250	3	5.7	1430
500	3	5.1	2535
750	3	4.1	3050

[a] All task area visits represent the total sum of brood, pollen and honey area visits. The values represent the medians that were obtained from the cumulated data of the three biological replicates per group size.

Increased group size constrains access to different task areas

To learn whether the decrease in task area visits is caused by a reduction of mobility on the comb due to the higher density of worker bees, we analysed the path length and change in direction of individual worker bees from the different group sizes. If worker bees' mobility is affected by a higher density of bees on the comb we would expect bees' path length to decrease with increasing group size while we would expect bees' change in direction to increase. With higher worker density on the comb we expect bees' paths to be occluded more often resulting in bees having to change their direction more frequently.

Path lengths of worker bees differed significantly between the groups of 250 (median: 0.73 m/bee/h), 500 (median: 0.57 m/bee/h) and 750 bees (median: 0.55 m/bee/h; ANOVA on ranked and bootstrapped datasets: $F_{(2,\ 4318\text{-}4329)} = 416.3$, $P < 5 \times 10^{-4}$; Fig. 3E, S8 Table). Worker bees kept singularly on a comb had a median path length of 1.2 m per hour which is a 2.2 fold increase compared to the median path length of bees from the largest group. Post hoc tests revealed that bees' path length consistently decreased with increasing group size (Dunnett's T3 Test: 250 vs. 500 bees: D = 1141, 250 vs. 750 bees: D = 1401, 500 vs.

750 bees: D = 260; Fig. 3E, S8 Table), suggesting that bees' mobility is influenced by worker density on the comb.

The change in direction of worker bees differed significantly between the groups of 250 (median: 109.7 °/bee/sec), 500 (median: 115.2 °/bee/sec) and 750 bees (median: 113.6 °/bee/sec; ANOVA on ranked and bootstrapped datasets: $F_{(2,\ 4318\text{-}4329)} = 3.5$, $P = 2 \times 10^{-2}$; Fig. 3F, S8 Table). However, post hoc tests revealed that bees' change in direction did not consistently increase with increasing group size (Dunnett's T3 Test: 250 vs. 500 bees: P = 0.04; 250 vs. 750 bees: P = 0.22; 500 vs. 750 bees: P = 0.52; Fig. 3F; S8 Table). Worker bees kept singularly on a comb showed a median change in direction of 90.8 degrees per second. This is 1.2 fold less degrees per second compared to the median change in direction from the group of 500 bees. These results suggest that bees' change in direction is not consistently affected by an increase in worker bees on the comb.

The previous results indicate that the higher density of bees on the comb induce a constraint on the distance a worker bee can walk within an hour. The bees' reduced path length may in turn affect the number of task areas a worker bee can visit. To learn whether the reduced number of task area visits may directly result from the reduced path length and thus, from the mobility constraints imposed by the higher density of worker bees on the comb, we examined correlations between task area visits and path lengths. We found correlations between path length and task area visits for all group sizes ($P < 5 \times 10^{-4}$; Table 2). While we found path length to weakly correlate with brood, pollen and honey area visits in the group size of 500 bees (R = 0.2 – 0.3, $P < 5 \times 10^{-4}$, Table 2), we consistently found strong correlations for the other two group sizes (250 and 750 bees: R = 0.4 – 0.5, $P < 5 \times 10^{-4}$; Table 2). These results indicate that part of the reduction of task area visits is caused by the shorter path lengths of worker bees resulting from the higher density of bees on the comb and the imposed mobility constraints. Additionally, differences in workload per bee in the three group sizes may affect the distance a worker bee walks within an hour. We propose that the higher workload per bee in smaller group sizes may weaken the correlation. The higher demand for tasks to be performed (task demand) should direct the visits to specific areas which would itself not necessarily result in a difference in path length. However, correlation coefficients were not consistently affected by group size, suggesting that the higher task demand and workload do not strongly affect the relationship of path length and task area visits.

Table 2: Correlations between path length and task area visits for the three group sizes.

		Correlation with path length			
Group size	**Biological replicates**	**Number of all task area visits (R(df))**	**Number of brood area visits (R(df))**	**Number of pollen area visits (R(df))**	**Number of honey area visits (R(df))**
250	3	R(732) = 0.6	R(732) = 0.5	R(732) = 0.5	R(732) = 0.4
500	3	R(1434) = 0.4	R(1434) = 0.2	R(1434) = 0.3	R(1434) = 0.2
750	3	R(2142) = 0.6	R(2142) = 0.4	R(2142) = 0.4	R(2142) = 0.4
				All $P < 5 \times 10^{-4}$	

Finally, we explored whether factors imposed through group size independent of mobility constraints, such as task demand and per capita workload can explain the fewer visits found for all task areas as well as the brood and pollen area in the larger groups. Therefore, we examined the effects of group size on task area visits while controlling for the covariate path length using ANCOVA on ranked and bootstrapped datasets.

When controlling for path length we found that visits to task areas in total (brood, pollen and honey summed up) did not consistently decrease with increasing group size (Dunnett's T3 Test: 250 vs. 500 bees: D = -391; Table 3). This suggests that bees' task area visits are not consistently influenced by factors imposed through group size independent of mobility constraints that may arise from higher worker density on the comb.

We found the same results for the brood and pollen area visits when controlling for path length. Visits to both, the brood and pollen area did not consistently decrease with increasing group size (Dunnett's T3 Test: 250 vs. 500 bees: brood: D = -163, pollen: D = -66; Table 3). This indicates that factors influenced by group size other than mobility constraints, such as task demand and per capita workload do not consistently affect visits to these areas.

The pattern of honey area visits throughout the group sizes did not change after controlling for path length. As found previously, honey area visits did not consistently decrease with increasing group size validating that honey area visits are not consistently affected by factors imposed through group size (Dunnett's T3 Test: 250 vs. 500 bees: P < 5 x 10^{-4}; 250 vs. 750 bees: P = 0.2; 500 vs. 750 bees: P < 5 x 10^{-4}; Table 3).

Table 3: ANCOVA results on the influence of group size on task area visits when controlling for path length.

		Path length as covariate [a] **Median [b]**			
	Group size	**Number of all task area visits (N/bee/h)**	**Number of brood area visits (N/bee/h)**	**Number of pollen area visits (N/bee/h)**	**Number of honey area visits (N/bee/h)**
F-value (2, 4324-4326)		102.4 P < 5 x 10^{-4}	225.6 P < 5 x 10^{-4}	34.3 P < 5 x 10^{-4}	99.9 P < 5 x 10^{-4}
Multiple R^2		0.34	0.26	0.21	0.13
Post hoc test [b] D-value (2, 4324-4326)	250 vs. 500	-391 P < 5 x 10^{-4}	-163 P < 5 x 10^{-4}	-66 P < 5 x 10^{-4}	-788 P < 5 x 10^{-4}
	250 vs. 750	94 P < 5 x 10^{-4}	830 P < 5 x 10^{-4}	239 P < 5 x 10^{-4}	-612 P = 0.2
	500 vs. 750	484 P < 5 x 10^{-4}	667 P < 5 x 10^{-4}	305 P < 5 x 10^{-4}	176 P < 5 x 10^{-4}

[a] F-values were obtained by performing One Way ANCOVA on the ranked and bootstrapped data of the cumulated datasets for each tested parameter (all task, brood, pollen and honey area visits). Path length was set as covariate while group size (250, 500, 750 bees) was the independent factor.

[b] The F-values and associated P-values represent the median of the values obtained from 1000 bootstrap iterations.

[c] Pairwise comparisons were performed with the Dunnett's T3 Test.

Discussion

We investigated how in-hive worker bees access information about their group's task demand and how group size affects the information access. Our results demonstrate that all worker bees repeatedly visit the different task areas and thus may individually and continuously access task information. We found that tripling group size while keeping the collective workload constant only marginally affected the individual task area visiting behaviour, suggesting that worker bees do not considerably adjust this behaviour according to group size and task demand.

The unimodal frequency distributions of task area visits in this study showed that all worker bees in all three group sizes visited at least one task area per hour. First, this suggests that each worker bee can individually and continuously access information on task demand by visiting the different task areas. Task information is thus not restricted to a group of worker bees. Secondly, the unimodal frequency distributions suggest that task area visiting behaviour is an inherent property of worker bees. Despite the difference in task demand and per capita workload between the three group sizes all worker bees visited the different task areas. Additionally, the number of task area visits per bee between the different group sizes differed only slightly suggesting that individual task area visiting behaviour is only marginally adjusted according to task demand and per capita workload.

Behavioural observations have shown that middle-aged bees regularly visit the different task areas while performing intervals of cell inspections without subsequent task performance (Lindauer 1952, Johnson 2008a, b). Additionally, pollen foragers have been found to perform a similar behaviour within the nest (Calderone and Johnson 2002, Weidenmuller and Tautz 2002).The hypothesized function of this patrolling behaviour is the individual collection of group-level information in order to assess task demand and pollen stores (Johnson 2008a, 2009). Nurse bees have not been observed patrolling (Johnson 2008b) indicating that this particular behaviour is specific for middle-aged bees and pollen foragers. We did not perform manual behaviour observations and can thus not determine whether patrolling behaviour was performed by our bees. Our results however show that bees of the age of nurse bees perform repeated task area visits. Similarly to patrolling behaviour of middle-aged bees, nurse bees may thus gather task information by visiting the different task areas.

Correlations between bees' path lengths and their task area visits as well as the continuous decrease of task area visits with increasing group size showed that mobility constraints on the comb affected individual task area visiting behaviour. Therefore, individual information access is dependent on group size and the associated mobility constraints caused by higher numbers of worker bees on the comb. Task demand and per capita workload, on the other hand, did not consistently affect individual information access. Our results showed that independent of mobility constraints, task area visits in total and visits to the brood and pollen area decreased with tripled but not doubled amount of worker bees on the comb. This suggests that task demand and per capita workload may only affect task area visiting behaviour when groups have reached a certain size. Studies from Smith et al. (2014, 2017) indicated that worker density influences in-hive behaviour of worker bees. Worker bees from different sized colonies only constructed drone comb if their colony had reached a critical number of individuals (Smith et al. 2014). These results and our findings on task area visiting behaviour show that worker density influences individual worker behaviour in honeybee colonies.

The reduced number of task area visits per bee found in large groups of this study suggests that worker bees may individually gain less information on task demand if worker density is high. Although worker bees in large groups may individually be less informed about their group's task demand than worker bees from small groups, our results suggest that group-level information access is kept constant regardless of group size. Worker bees' individual task area visiting behaviour created a stable group-level pattern of task area visits throughout the different group sizes. We found that the reduced information access on the individual level is most likely compensated by the higher number of worker bees involved in task area visiting behaviour in larger groups. Tripling the amount of worker bees doubled the amount of task area visits on the group level suggesting that honeybees collectively gain task information through repeated individual task area visits.

Finding the links between group-level patterns and individual behaviour requires large amounts of continuous behavioural data of a colony's entire members. A honeybee colony can consist of up to 100 000 individuals (Bourke 1999). However, it is unnecessary to obtain the required data on 100 000 individual worker bees. Small groups of worker bees kept under artificial conditions closely simulating natural conditions of honeybee colonies can be used to gather the required data. In this study, we used small queen-right groups of same-aged worker bees that we provided with a brood comb containing larvae, pollen and honey as is found in natural honeybee colonies. We used brood rearing success and the consumption of pollen and honey as an indicator for the quality of our groups. Worker bees typically consume pollen and honey for their own nutrition or to produce brood food (Haydak 1970, Brodschneider and Crailsheim 2010, Frias et al. 2016). Pollen and honey consumption is thus a good indicator for worker bee health. Brood rearing success is typically used as an indicator for colony health and productivity (Mattila and Seeley 2007, DeGrandi-Hoffman et al. 2008, Smart et al. 2016). We found our groups to rear up to 60% of the brood, eat approximately 20% of the provided pollen and up to 79% of the sugar syrup. We especially took the amount of reared brood as an indicator for a good quality of the tracked groups and thus the gathered behavioural data.

Our groups consisted of same-aged bees that were seven to nine days old during tracking. Typically bees of this age primarily perform tasks associated with brood rearing (Seeley 1982, Johnson 2008b). Same-aged worker bees were chosen in order to exclude possible variability in moving behaviour and task area visits caused by age differences. We chose the age typical for nurse bees to ensure that brood rearing would be performed. Natural honeybee colonies typically consist of worker bees of mixed ages (Rösch 1925, Lindauer 1952, Winston 1987). Natural occurrences such as swarming, predation, nest damage and diseases, however, may alter the typical demographic structure of a honeybee colony (Winston and Fergusson 1985, Morse 1990). When honeybees swarm, for example, most of the nurse bees leave the colony with the prime swarm (Winston 1978, 1987). Thus the newly founded colony predominantly consists of nurse bees who will undertake various tasks unusual for their age, for example building comb or foraging (Winston 1987, Johnson 2008b). Honeybee colonies consisting of similarly or same-aged bees therefore naturally occur and are able to function as usual demographically structured colonies (Rösch 1930, Robinson 1985, 1987).

In this study, we demonstrated that all worker bees repeatedly performed task area visits which generated a stable group-level pattern of task area visits with unimodal frequency distributions irrespective of group size. This suggests that repeated task area visits are a way for worker bees to individually and continuously access information on their group’s task demand. Additionally, repeated individual task area visits may constitute a mechanism for collectively receiving task information in honeybees.

Materials and Methods

Brood combs with defined brood, pollen and honey areas were used for tracking

Across experiments, worker bees were tracked on brood combs that were standardized for the same amount of brood, pollen and honey located in specific areas on the comb. To prepare these "standardized" combs we took "Deutsch Normal" combs with empty cells. In defined regions nearby the later brood area, one side of these combs was filled with 30 g ground pollen (Echter Deutscher Spezial Blütenpollen, Werner Seip – Biozentrum GmbH & Co. KG, Butzbach, Germany) (Fig. 1B). To keep the pollen in the cells, we added a 25 µl drop of sugar syrup to each pollen cell (Ambrosia Bienenfutter-Sirup, Nordzucker AG, Braunschweig, Germany). Located in defined regions of the combs' periphery, we filled 550 cells each with 200 µl sugar syrup (70% sugar solution) using a Multipette (Eppendorf Multipette Plus pipette, Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany) (Fig. 1B). Immediately before tracking, we inserted a piece of brood comb containing 151 honeybee larvae of the third to fourth instar into the centre of the combs (Fig. 1B). The brood originated from the same colonies as our worker bees used for tracking. As measurement for the quality of the tracked groups, brood survival was determined at the end of each tracking experiment by determining the percent of reared larvae (capped brood cells) and emptied pollen and honey cells (Supporting Information S1 Table).

Experimental setup and bee handling

With each three biological replicates, the different group sizes of 250, 500 and 750 same-aged worker bees were tracked together with a queen in a laboratory at the Heinrich-Heine University of Düsseldorf, Germany. The biological replicates were performed from June 28th to July 21st 2016, July 28th to August 20th 2016, and August 30th to September 29th 2016. The groups were consistently tracked for 48 hours. For the first biological replicate of the group of 250 bees we only obtained tracking information for 31 hours. Worker bees originated from several *Apis mellifera* source colonies that were located at our bee yard at the Heinrich-Heine University, Germany. Newly emerged worker bees (0-24 hours old) were collected from sealed brood combs incubated at 34°C and marked with tags bearing 2D barcodes (Fig. 1A; for more information see Blut et al. (2017)). Together with a tagged queen, the tagged worker bees were kept on a pollen and honey containing comb in a one-frame observation hive (Blut et al. 2017) at 34°C for the first 24 hours. The next five days the hive was kept in a dark laboratory at room temperature. On the sixth day, to replace the pollen and honey containing comb with the standardized brood comb, bees were kept at 4°C for 30 to 45 minutes and transferred to a one-frame observation hive holding the standardized brood comb. This observation hive was incubated at 34°C over night before starting the tracking experiment the next day. During tracking, 50 g of sugar pastry (Apifonda Futterteig, Südzucker AG, Mannheim, Germany) was provided above the comb.

For individual tracking of bees, five bees from the group of 500 bees and nine bees from the group of 250 bees (second biological replicates) were tracked on standardized brood combs. Individual tracking of worker bees was restricted to only 20 minutes because the brood was not provided for otherwise. Bees were 10 days old at the time of tracking.

Data generation from tracking information

With the Bee Behavioral Annotation System (BBAS) (Blut et al. 2017), we obtained information on each tagged bee's trajectory (see Supporting Information S12 Text and S13 Table for detection performance of BBAS). Trajectories resulted from the tracking information on position and orientation gained every quarter second (4 frames per second). Due to gaps in the detection, bees' trajectories were not continuous, but consisted of several smaller trajectories. For all trajectories, gaps of a single frame were filled with tracking information estimated through linear interpolation from the data points before and after the gap. From the resulting trajectories, we calculated the bees' task area visits per hour, path length per hour and change in direction per second. The detection rate per bee (number of frames in which bees were detected) and thus the amount of trajectory information varied during tracking. Therefore, bees' trajectory information was only used for calculation if it represented at least 10% of an hour's total tracking information (see Supporting Information S12 Text and S13 Table for detection performance after application of the 10% threshold).

Calculation of path length was performed with the high-performance computing system of the Heinrich-Heine University Düsseldorf and was based on the information on bees' position in each frame. The change in direction was computed based on the information on bees' orientation per frame.

Worker bees’ visits to task areas were determined by the first detection of each bee in the brood, pollen or honey areas. Therefore, for each standardized comb we determined the x- and y-coordinates of the brood, pollen and honey areas that were used to define when the bee was detected in the different areas (Mersch et al. 2013). From this information, the number of visits to the brood, pollen and honey area per hour were determined for each bee. In addition, for each bee we determined the average number of all task area visits per hour (brood, pollen and honey area totalled).

Visits to the different task areas were counted as demonstrated with following examples: a) a bee is in the undefined region of the comb (not brood, pollen or honey area) and walks into the brood area. This is counted as a brood area visit; b) a bee is in the brood area, crosses the undefined region (not counted as visit) and walks into the pollen area. This is counted as a pollen area visit; c) a bee is in the pollen area, exits into the undefined region and re-enters the pollen area. This is not counted as a pollen area visit.

Statistical analyses

All statistical analyses were performed using the Systat 13 software (Systat Softarwe GmbH, Erkrath, Germany).

First, in frequency distributions we displayed the data on all task area visits and the visits to the brood, pollen and honey area per bee for each biological replicate. For statistical analyses, we cumulated the data on all task area visits, visits to the brood, pollen and honey area, path length and change in direction for the three biological replicates of each group size (see Supporting Information S10 Fig. and S11 Table for data on each biological replicate). For the cumulated data we determined the kurtosis values and performed Kolmogorov-Smirnov One-Sample Tests to test for Normal, Lognormal, Gamma and Poisson distribution. We used the default settings of Systat for the Kolmogorov-Smirnov One-Sample Test (Normal(mean = 0, STD = 1), Lognormal(mean = 0, STD = 1), Gamma(mean = 5, STD = 1), Poisson(mean = 5)).

Second, for the cumulated data we determined the median values per group size. Before performing further statistical analyses, we tested the cumulated data for variance of homogeneity and normality by performing the Leven’s Test and Shapiro-Wilk Test (Supporting Information S9 Table). The data failed both tests, therefore we performed the following statistical tests on the ranks of the cumulated data and bootstrapped the ranked data for the Analysis of Variance (ANOVA) and the Analysis of Covariance (ANCOVA). For the bootstrapping process, we chose 1000 bootstrap iterations for both, the ANOVA and ANCOVA and retained all other default settings of Systat (Type III Sum of Squares, 95% confidence interval). From the output of the 1000 bootstrap iterations, we determined the median values for the F-value, P-value and the degrees of freedom (between and within group). We performed the ANCOVA for the task area visits and the visits to the brood, pollen and honey area with the path length as covariate. Further, we performed pairwise comparisons on the ranks of the cumulated data by performing the Dunnett's T3 Test with a 95% confidence interval.

We tested the ranks of the cumulated data for correlations by performing hypothesis tests for correlations that assume a correlation coefficient of zero (null-hypothesis) while testing for non-equality (alternative hypothesis) with a confidence interval of 95%.

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Author contributions

CB designed and performed the experiments and conducted the data analyses. CB and MB conceived the study, supervised its design and its coordination, and wrote the manuscript. SR and SC performed and supervised programming required for analyses of the tracking data. All authors reviewed the manuscript.

Competing financial interests

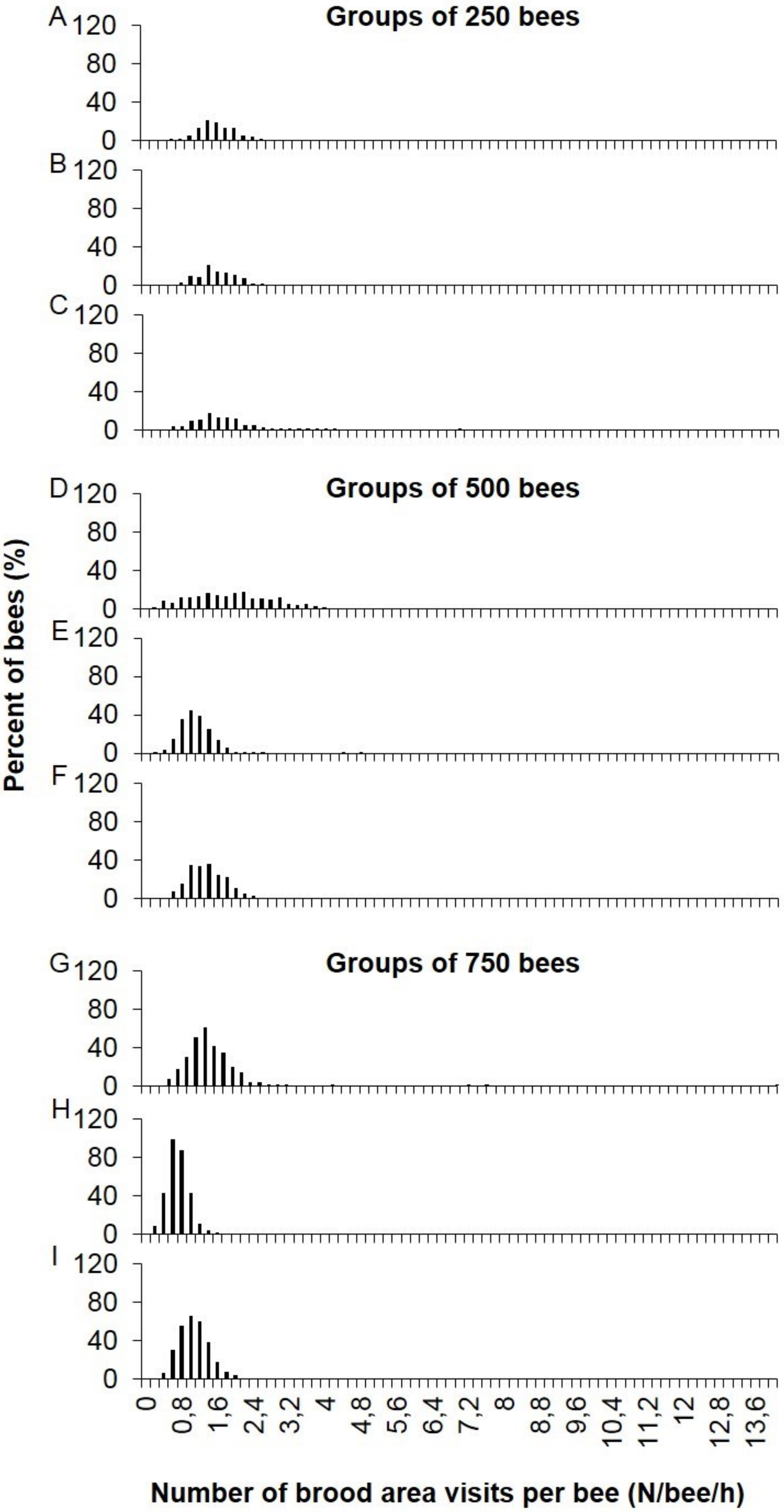
The authors declare no competing financial interests.

Supporting Information

S1 Table: Larvae rearing success and the percent of emptied pollen and honey cells for the different group sizes

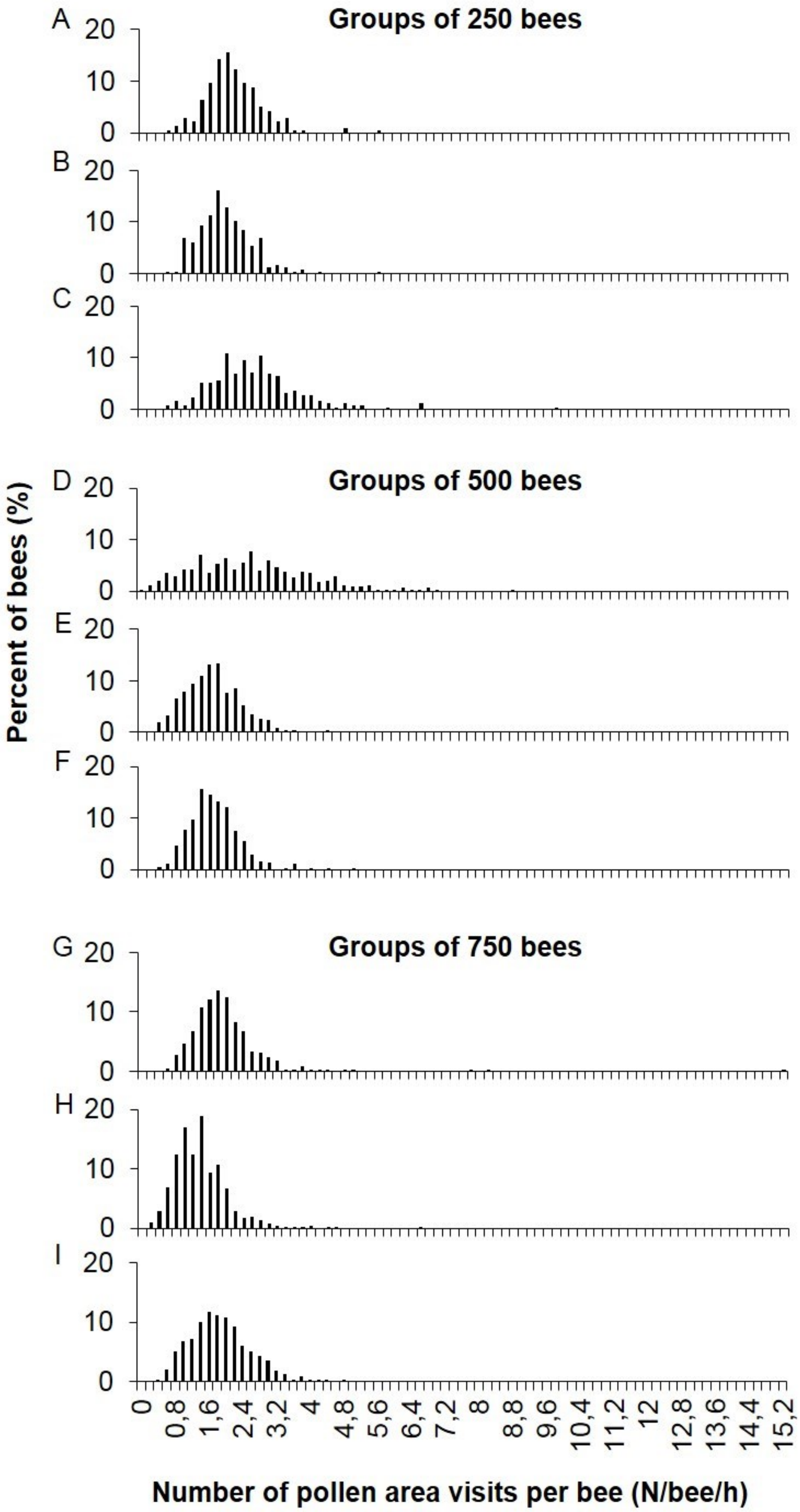
Group size	Biological replicates	Percent of reared larvae [a] (%)(± STD)	Percent of emptied pollen cells (%)(± STD)	Percent of emptied honey cells (%)(± STD)
250	3	44 (± 9)	15 (± 12)	48 (± 5)
500	3	46 (± 16)	16 (± 4)	41 (± 14)
750	3	60 (± 32)	21 (± 20)	79 (± 17)

[a] Honeybee larvae were counted as reared when the brood cell was fully capped. We determined the average percent of reared larvae for each group size from the three biological replicates.



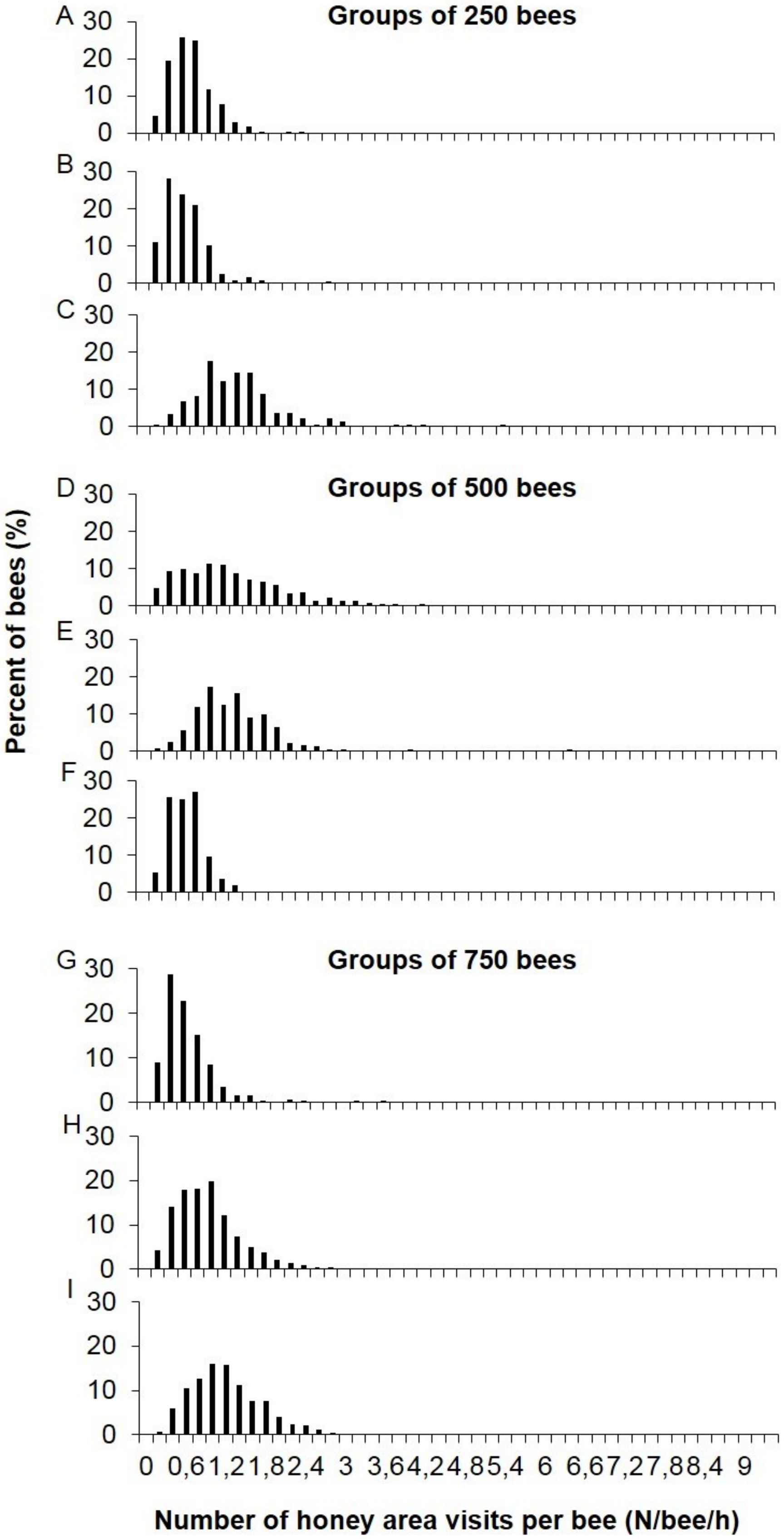
S2 Figure: Brood area visit frequency distributions for the biological replicates. **A**) – **C**) Frequency distributions for the three biological replicates of the

group size of 250 bees. **D**) – **F**) Frequency distributions for the three biological replicates of the group size of 500 bees. **G**) – **I**) Frequency distributions for the three biological replicates of the group size of 750 bees.



S3 Figure: Pollen area visit frequency distributions for the biological replicates. **A**) – **C**) Frequency distributions for the three biological replicates of the

group size of 250 bees. **D**) – **F**) Frequency distributions for the three biological replicates of the group size of 500 bees. **G**) – **I**) Frequency distributions for the three biological replicates of the group size of 750 bees.



S4 Figure: Honey area visit frequency distributions for the biological replicates. **A**) – **C**) Frequency distributions for the three biological replicates of the

group size of 250 bees. **D**) – **F**) Frequency distributions for the three biological replicates of the group size of 500 bees. **G**) – **I**) Frequency distributions for the three biological replicates of the group size of 750 bees.

S5 Table: Tests for different types of distributions of the cumulated datasets.

		Kolmogorov-Smirnov One-Sample Test			
Type of distribution	**Group size**	**Number of all task area visits (D(df))**	**Number of brood area visits (D(df))**	**Number of pollen area visits (D(df))**	**Number of honey area visits (D(df))**
Normal	250	D(734) = 1	D(734) = 0.8	D(734) = 0.8	D(734) = 0.5
	500	D(1438) = 1	D(1438) = 0.7	D(1438) = 0.7	D(1438) = 0.6
	750	D(2152) = 0.9	D(2152) = 0.6	D(2152) = 0.7	D(2152) = 0.5
		All P = 5 x 10^{-4}			
Lognormal	250	D(734) = 0.8	D(734) = 0.4	D(734) = 0.5	D(734) = 0.2
	500	D(1438) = 0.8	D(1438) = 0.3	D(1438) = 0.4	D(1438) = 0.2
	750	D(2152) = 0.7	D(2152) = 0.2	D(2152) = 0.3	D(2152) = 0.2
		All P = 5 x 10^{-4}			
Gamma	250	D(734) = 1	D(734) = 0.8	D(734) = 0.8	D(734) = 0.5
	500	D(1438) = 1	D(1438) = 0.7	D(1438) = 0.7	D(1438) = 0.6
	750	D(2152) = 0.9	D(2152) = 0.6	D(2152) = 0.7	D(2152) = 0.5
		All P = 5 x 10^{-4}			
Poisson	250	D(734) = 1	D(734) = 0.8	D(734) = 0.8	D(734) = 0.5
	500	D(1438) = 1	D(1438) = 0.7	D(1438) = 0.7	D(1438) = 0.6
	750	D(2152) = 0.9	D(2152) = 0.6	D(2152) = 0.7	D(2152) = 0.5
		All P = 5 x 10^{-4}			

S6 Table: Kurtosis values for the number of all task, brood, pollen and honey area visits (cumulated data) and group sizes.

	Kurtosis			
Group size	**Number of all task area visits**	**Number of brood area visits**	**Number of pollen area visits**	**Number of honey area visits**
250	5	14	10	8
500	42	3	4	7
750	121	117	42	27

S7 Table: ANOVA results on the task area visits of bees from the three group sizes.

			Median[a]			
	Group size	**Biological replicates**	**Number of all task area visits (N/bee/h)**	**Number of brood area visits (N/bee/h)**	**Number of pollen area visits (N/bee/h)**	**Number of honey area visits (N/bee/h)**
	250	3	5.7	1.5	2	0.7
	500	3	5.1	1.2	1.8	0.9
	750	3	4.1	0.9	1.5	0.8
	1	14	102	22.5	46.5	30
F-value (2, 4324-4326)			198.6 $P < 5 \times 10^{-4}$	422.2 $P < 5 \times 10^{-4}$	146.2 $P < 5 \times 10^{-4}$	29.5 $P < 5 \times 10^{-4}$
Post hoc test [b] D-value (2, 4324-4326)	250 vs. 500		236	545	423	-363
	250 vs. 750		862	1300	839	-92
	500 vs. 750		627	755	417	271
			All pairwise $P < 5 \times 10^{-4}$			

[a] For each measured parameter (all task, brood, pollen and honey area visits), median values were obtained from the cumulated data of the three biological replicates per group size. F-values were obtained by performing One Way ANOVA on the ranked and bootstrapped data of the cumulated datasets. The F-values and associated P-values represent the median of the values obtained from 1000 bootstrap iterations.

[b] Pairwise comparisons were performed with the Dunnett's T3 Test.

S8 Table: Comparison of the path length and change in direction of bees from the three group sizes.

			Median [a]	
	Group size	**Biological replicates**	**Path length (m/bee/h)**	**Change in direction (°/bee/sec)**
	250	3	0.73	109.74
	500	3	0.57	115.24
	750	3	0.55	113.58
	1	14	1.2	90.8
F-value (2, 4318-4329)			416.3 $P < 5 \times 10^{-4}$	3.5 $P < 2 \times 10^{-3}$
Post hoc test [b] D-value (2, 4318-4329)	250 vs. 500		1141 $P < 5 \times 10^{-4}$	-149 $P = 0.04$
	250 vs. 750		1401 $P < 5 \times 10^{-4}$	-96 $P = 0.22$
	500 vs. 750		260 $P < 5 \times 10^{-4}$	-53 $P = 0.52$

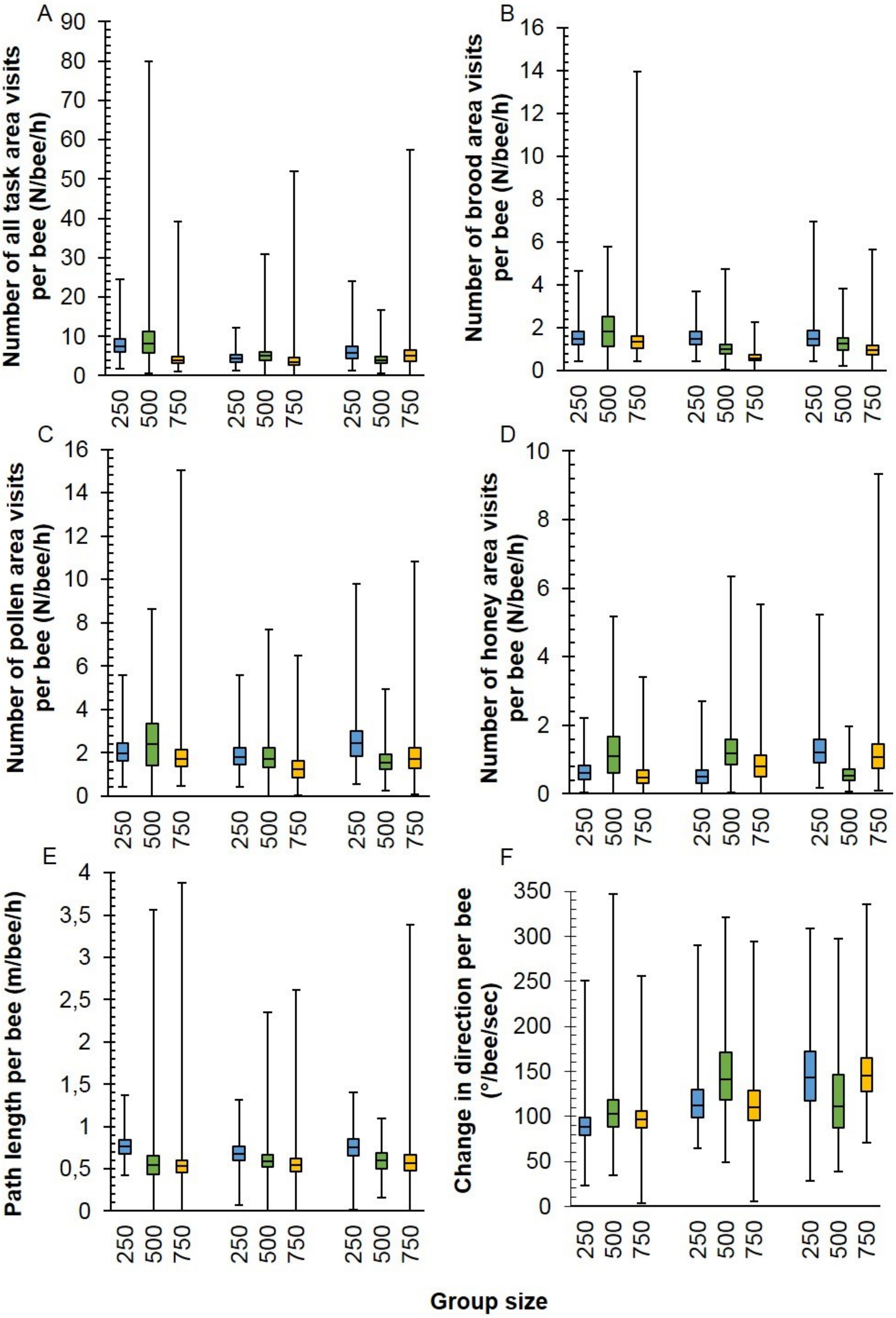
[a] For the path length and change in direction, median values were obtained from the cumulated data of the three biological replicates per group size. F-values were obtained by performing One Way ANOVA on the ranked and bootstrapped data of the cumulated datasets. The F-values and associated P-values represent the median of the values obtained from 1000 bootstrap iterations.

[b] Pairwise comparisons were performed with the Dunnett's T3 Test.

S9 Table: Test for homogeneity of variance and normality for the cumulated datasets.

	Number of all task area visits	Number of brood area visits	Number of pollen area visits	Number of pollen area visits	Path length	Change in direction
Test for homogeneity of variance (Levene's Test) [a]	F(2, 4324) = 17	F(2, 4324) = 72.9	F(2, 4324) = 31.9	F(2, 4324) = 2.9	F(2, 4318) = 84.8	F(2, 4329) = 15.7
	All $P < 5 \times 10^{-4}$					
Normality Test (Shapiro-Wilk Test) [a]	W(2, 4318) = 0.8	W(2, 4329) = 0.9	W(2, 4324) = 0.7	W(2, 4324 = 0.8	W(2, 4324) = 0.9	W(2, 4324) = 0.8
	All $P < 5 \times 10^{-4}$					

[a] Tests were performed on the cumulated data of the three biological replicates for the group sizes of 250, 500 and 750 bees.



S10 Figure: Boxplots for the number of all task, brood, pollen and honey area visits, the path length and change in direction for worker bees from the three biological replicates. The median, the 75% and 25% percentile and the minimum and maximum are presented. **A**) Number of all task area visits (brood, pollen and honey area visits totalled). **B**) Number of brood area visits. **C**) Number of pollen area visits. **D**) Number of honey area visits. **E**) Path length. **F**) Change in direction.

S11 Table: Group medians of the biological replicates.

		Median					
Group size	**Biological replicate**	**Number of all task area visits (N/bee/h)**	**Number of brood area visits (N/bee/h)**	**Number of pollen area visits (N/bee/h)**	**Number of honey area visits (N/bee/h)**	**Path length (m/bee/h)**	**Change in direction (°/bee/sec)**
250	1	7.6	1.5	2	0.6	0.76	88.5
	2	4.4	1.5	1.8	0.5	0.58	112.4
	3	5.8	1.5	2.4	1.2	0.75	142.6
500	1	8.1	1.8	2.4	1.1	0.54	102.3
	2	5	1	1.7	1.2	0.59	141.3
	3	3.9	1.3	1.5	0.5	0.6	110.8
750	1	4	1.3	1.7	0.5	0.53	96.8
	2	3.5	0.6	1.2	0.8	0.54	109.8
	3	5	1	1.7	1.1	0.57	145.1

S12 Text: Detection performance of the BBAS

For each biological replicate and group size we determined the percent of detected bees per hour (S13 Table). On average, we detected approximately 90% of the bees (S13 Table). We applied a 10% threshold to discard information from bees that were detected in less than 10% of the frames in each hour. As a result, we detected between 60 to 80% of the bees (S13 Table).

S13 Table: Detection performance of the BBAS with and without the 10 % threshold

		Average % of detected bees per hour (± STD)	
Group size	Biological replicates	Without 10% threshold [a]	With 10% threshold [a]
250	3	96 (± 0.01)	79 (± 0.05)
500	3	86 (± 0.06)	65 (± 0.14)
750	3	90 (± 0.07)	59 (± 0.15)

[a] We applied a 10 % threshold to the tracking information to discard all bees that were detected in less than 10 % of the frames in each hour.

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Author's contribution: Manuscript II

Honeybees (*Apis mellifera*) collectively gain information about task demand through repeated individual visits to different task areas.

Journal: Manuscript submitted to Scientific Reports

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1st author

Author's contribution: 90%

- Concept of the study
- Experimental design
- Implementation of tracking experiments
- Design of analysis programs
- Implementation of analysis programs
- Statistical analyses
- Authoring the manuscript

Chapter III

Summary

Honeybees (*Apis mellifera*) collectively construct well-structured wax combs, rear brood and gather large amounts of pollen and honey. Little is known on how such collaborative features are regulated by individual worker bees' task performances. Patrolling, trophallaxis, pheromones, the rate of incoming resources, and dances are mechanisms suggested to be involved in the control of individual task performance. However, experimental evidence for mechanisms underlying individual task engagements, especially within the hive is largely missing because current research techniques lack to provide the data necessary for the understanding of these key mechanisms. In this work, I introduced the computer-based and automated Bee Behavioral Annotation System (BBAS). Unlike current methods for behavioural studies in honeybees, BBAS can provide continuous behavioural data of up to 1000 honeybee workers at once. I demonstrated that BBAS can automatically and reliably detect encounter behaviours of worker bees in small groups based on these continuous behavioural data. Encounter behaviours comprise antennation, begging, offering and trophallaxis behaviour and are suggested to play a role in the exchange of information between worker bees and their individual task engagements. BBAS can reliably detect and distinguish trophallaxis from the other encounter behaviours, antennation, begging and offering, based on the characteristic duration of this encounter behaviour. In addition to demonstrating the functionality of BBAS, I revealed a possible mechanism underlying individual information access on task demand in small groups of worker bees. I showed that independent of group size, all worker bees repeatedly visit the different task areas, namely the brood, pollen and honey area of a honeybee comb. Hereby, mobility constraints on the comb reduce individual task area visiting behaviour whereas task demand and per capita workload marginally affect this behaviour. These findings suggest that each worker bee can individually and continuously access information on task demand by visiting the different task areas whereas this behaviour is only marginally adjusted according to task demand. Worker bees' individual task area visiting behaviour created a stable group-level pattern of task area visits throughout different group sizes. This suggests that honeybees collectively gain task information through repeated individual task area visits.

Zusammenfassung

Gemeinsam konstruieren Honigbienen (*Apis mellifera*) strukturierte Waben, ziehen Brut auf und sammeln große Mengen an Pollen und Honig. Die Regulierung solcher kollaborativen Merkmale durch individuelle Ausübung von Tätigkeiten der Arbeitsbienen ist vorwiegend unbekannt. Patrouillieren, Trophallaxis, Pheromone, die Rate eingehender Ressourcen und Tänze sind Mechanismen, die vermutlich individuelle Tätigkeitsausübungen regulieren. Derzeitige Methoden für Verhaltensstudien in Honigbienen sind unzureichend, um die notwendigen Daten zur Aufdeckung der Mechanismen zu erlangen, welche der individuellen Ausübung von Tätigkeiten insbesondere innerhalb des Bienenstocks unterliegen. In dieser Arbeit wurde das computerbasierte und automatisierte Bee Behavioral Annotation System (BBAS) etabliert. BBAS kann kontinuierliche Verhaltensdaten von bis zu 1000 Arbeitsbienen gleichzeitig erzeugen. Es wurde gezeigt, dass BBAS basierend auf diesen Verhaltensdaten automatisch und zuverlässig Begegnungsverhalten von Arbeitsbienen in kleinen Gruppen erkennen kann. Begegnungsverhalten umfassen Antennieren, Betteln, Anbieten und Trophallaxis. Diese Verhalten sind vermutlich bedeutend für den Informationsaustausch zwischen Arbeitsbienen und ihre individuelle Ausübung von Tätigkeiten. BBAS kann Trophallaxis basierend auf der charakteristischen Dauer dieses Begegnungsverhaltens zuverlässig von Antennieren, Betteln und Anbieten differenzieren. Des Weiteren wurde in dieser Arbeit ein möglicher Mechanismus aufgedeckt, der dem individuellen Informationszugriff auf die Aufgabenanforderungen kleiner Gruppen von Arbeitsbienen unterliegt. Unabhängig von der Gruppengröße besuchten alle Arbeitsbienen wiederholt die verschiedenen Aufgabenbereiche, den Brut-, Pollen- und Honigbereich einer Bienenwabe. Das individuelle Besuchsverhalten verringerte sich durch Einschränkungen der Mobilität auf der Wabe, während dieses nur geringfügig an die Aufgabenanforderung angepasst wurde. Das individuelle Besuchsverhalten der Aufgabenbereiche erzeugt ein stabiles Muster von Besuchen der Aufgabenbereiche auf Guppenebene. Diese Ergebnisse deuteten an, dass jede Arbeitsbiene individuell und kontinuierlich durch Besuche der Arbeitsbereiche auf Informationen der Aufgabenanforderung zugreifen kann. Des Weiteren deuten sie an, dass Honigbienen kollektiv Aufgabeninformationen durch wiederholte, individuelle Besuche verschiedener Aufgabenbereiche erhalten.

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