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UNIVERSITY OF CALIFORNIA SAN DIEGO

The Role of Visual Learning in Honey Bee Foraging and Communication

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biology

by

Bahram Kheradmand

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2019

The Dissertation of Bahram Kheradmand is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California San Diego

2019

DEDICATION

This work is dedicated to two people. One I once knew, and one I someday will.

EPIGRAPH

Into the darkest night I fly, and I can hear your dance for me.
The bodies' scents, as they go by, swim with me, in this brimming, simmering sea.

Are you my child? Are you my mother? Come here and sit with me a while!
To see how we are one another. Oh come here mother, come here child!

Crumpled into an abstract trope, Into the strangest eyes I peer.
I am a part of you, I hope. You are a part of me, I fear.

To keep you whole, myself I'll sever; into the scorching sun I fly.
So you can get to live forever, but someday soon I will die.

Death is no hindrance for the soul, if soma sing each other's song.
Steal my idea as yours I stole; our lives together will be long.

Now let me be you, and you be me; love at its deepest darkest form.
Dark, as the night that sets us free. Deep, as the wisdom of the swarm.

Into the darkest night I fly, and in my chest I house a gleam.
The brightest day is in my eye, and in my wake I muse a dream.

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PREFACE

Life is about finding ways. All of life exists as a result of finding ways to sustain, prosper and reproduce. Throughout the billions of years of life on earth, the way that organisms have behaved is the result of, and a major contributor to how successfully they live. As a social species, we humans need to communicate in order to coordinate our behavior. This forms an integral part of our lives. We also need to learn many things so that we can survive better in an ever-changing environment. But we are not alone. Many species share those constraints with us humans, and as a result, they have evolved similar behaviors.

The work presented here was done in hopes of achieving a task that is too big for a few researchers to accomplish. A task that is the core endeavor of many scientists in many different fields. The task of understanding how behavior emerges in the interplay of the many parts within and outside an organism. Understanding how a brain the size of a grain of rice can set about complex behaviors that resemble that of our own: navigation, selecting food sources, language, exploration and learning rules. We hope that by understanding that small brain, we can learn something about a much larger brain, our own. To understand how we understand.

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Chapter 1 is a reformatted reprint of the material as it appears in Kheradmand, B., & Nieh, J. C. (2019). The Role of Landscapes and Landmarks in Bee Navigation: A Review. *Insects*, 10(10), 342. The dissertation author was the primary investigator and author of this paper.

Chapter 2, has been submitted for publication of the material as it may appear in Kheradmand, B., Chan, S., Richardson-Ramos, I., Nelson, C., & Nieh, J. C. “Statistical learning in honey bees.” The dissertation author was the primary investigator and author of this paper.

Chapter 3, in full, is a reformatted reprint of the material as it appears in Kheradmand, B., Cassano, J., Gray, S., & Nieh, J. C. (2018). Influence of visual targets and landmarks on honey bee foraging and waggle dancing. *Insect science*. The dissertation author was the primary investigator and author of this paper.

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FIELDS OF STUDY

Animal behavior

Communication in social insects

Learning and memory

ABSTRACT OF THE DISSERTATION

The Role of Visual Learning in Honey Bee Foraging and Communication

By

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Doctor of Philosophy in Biology

University of California San Diego, 2019

Professor James C. Nieh, Chair

Learning visual information is crucial for many animal species. Honey bees are a social species that forage daily and rely on visual information to navigate from nest to food sources and back. The ever-changing environment demands foragers to learn and adapt to new conditions in order to efficiently exploit available resources. By training honey bees to artificial flowers, we investigated how changing the visual characteristics of the food source (shape and color, surrounding landmarks, distance from the hive, and timing of rewards at visually distinct sources) leads to behavioral changes in honey bees. We show that bees rely heavily on shape

and color of food sources and surrounding landmarks to decide where to land. They reduce their efforts in recruiting other bees to the same food source when the landmarks around the food source change. We also demonstrate that bees can learn temporal regularities in the profitability of different visual patterns. Finally, we propose a new method to consistently measure and annotate the waggle dance of honey bees to better understand how the measurement of distance by flying foragers shapes this remarkable example of referential communication.

General Introduction

This dissertation investigates how the visual input of animals affects their behavior. Our model organism of choice is the honey bee, a flying insect known for its vision, foraging behavior, social lifestyle, and learning abilities. The four chapters in this dissertation follow the course of a forager honey bee's flight to its feeding location, how it behaves at the feeders when encountering familiar or unexpected visual stimuli, and how it communicates to other bees about the food source when it returns to the hive. Each chapter is being published as a separate paper, and thus we provide an introduction for each chapter. However, this section serves as a summary of the rationale for each of the chapters and how they connect together to form this dissertation.

Chapter 1 is a review of the visual stimuli that bees use to navigate. It discusses the different types of information extracted from visual input (such as colors, shapes, directions and distances) and how they are used in bees' navigation. Different strategies for navigation, and hypotheses about how bees mentally represent locations are discussed, and evidence for different hypotheses is presented. We propose a model for how different types of visual information are combined to create a unique search image to pinpoint a specific location: a hierarchy of stimuli codes different aspects of the visual field and their respective saliences and allows matching the view from the current position with memories of familiar locations.

Chapter 2 explores the ability of bees to associate different food sources with rewards at different times. In particular, we investigate how feeders of different appearances can be linked to the order in which they provide a sugar reward. To test the ability of bees to learn the temporal pattern of changing profitability, we trained them to two feeders with different visual

appearances (A and B) situated next to each other. We tested an alternating pattern of rewards. On the first visit, pattern A was rewarding while pattern B was not. On the second visit, pattern B was rewarding while pattern A was not. On the third visit, pattern A was rewarding while pattern B was not, etc. This alternation continued for 32 visits, and landing choices of bees upon arrival to the location of the feeders was recorded. We found that bees landed on the feeder with the reward significantly more often than expected by chance. A negative control experiment showed that this learning effect is not due to any olfactory cues by the sucrose reward or scent marking by bees. A positive control experiment showed that bees can learn when the two feeders have an unchanging order of holding rewards (A is always rewarding while B is always not rewarding). Thus, we demonstrate the ability of bees to find order in a sequence of stimuli that occurs on a temporal scale of a few minutes.

Chapter 3 deals with the relative importance of different types of visual information used by navigating bees. Different cues are used on different parts of the path. Once the bee is close to the target, landmark information becomes particularly influential. Because bees can use distance memories to assess how close they are to the target, we wished to study the relative importance of memory and visual cues. We first trained them to a feeder of a particular shape and color at a particular distance. We then tested them in a setup that gave them conflicting distances or shapes and colors. This allowed us to determine the preferences. We found that landmark memories have a much stronger influence on landing behavior of foragers than memories.

We then tested the effect of different landmarks near the food source upon landing behavior. We trained bees to a feeder on a stand under a large blue umbrella. After a few hours, we separated the umbrella and the feeder stand by a few meters and observed how the returning foragers reacted to this unexpected change in the relationship between the feeder and the

conspicuous landmark nearby. Bees originally struggled to find the feeder, even though it was only a few meters away from the umbrella. After learning the new location in relation to the umbrella, they still preferred to use the umbrella to get from their hive to the general proximity of the feeder, and then to steer towards the feeder. The initial confusion in finding the feeder not only manifested itself in longer trip durations, but also diminished their motivation to advertise this food source upon returning to the hive by communicating its location to their nestmates. This communicatory behavior is further studied in chapter 4, and the importance of the distance measurement of bees on their flight is examined in detail.

Chapter 4 investigates the waggle dance: a series of circular movements that returning foragers perform at the hive to advertise the location of food sources to other foragers. This behavior is a key contributor to the success of honey bees in exploiting newly emerged food sources. Through the waggle dance, a forager returning from a flower can indicate the location of the flower by moving in a particular orientation while shaking her abdomen (wagging) for a specific duration of time, thereby indicating the direction of the resource (coded in the direction of the waggle phase) and the distance to it (coded in the duration of the waggles). After the waggle phase, the dancer turns and roughly returns (usually in a semi-circular arc) to where she started wagging and then repeats the wagging motion while pointing in the same direction. Longer food source distances result in dances with more waggles, but as the distance decreases, fewer and fewer waggles are required to indicate the distance. As the distance becomes extremely short (less than 50 meters), the forager stops wagging but continues to move in semi-circles to indicate that a profitable food source exists nearby. We studied the differences in the dances produced for different distances and provided the first clear definitions for the different movements that dancers perform and how to measure them.

Taken together, these four chapters dissect some of the key behaviors that forager honey bees demonstrate. These behaviors all involve processing the visual information gathered on the foraging trips (landmarks and distance). We examine how changes in these stimuli and their relationship to rewards affects the behavior of the foragers on their trips, and their communicatory signals back at the hive. We find that bees are rapidly able to adapt their behavior to changes in the environment to maximize exploiting available floral resources.

Chapter 1: The role of landscapes and landmarks in bee navigation: a review

Abstract

The ability of animals to explore landmarks in their environment is essential to their fitness. Landmarks are widely recognized to play a key role in navigation by providing information in multiple sensory modalities. But what is a landmark? We propose that animals use a hierarchy of information based upon its utility and salience when an animal is in a given motivational state. Focusing on honeybees, we suggest that foragers choose landmarks based upon their relative uniqueness, conspicuousness, stability, and context. We also propose that it is useful to distinguish between landmarks that provide sensory input that changes (“near”) or does not change (“far”) as the receiver uses these landmarks to navigate. However, we recognize that this distinction occurs on a continuum and is not a clear-cut dichotomy. We review the rich literature on landmarks, focusing on recent studies that have illuminated our understanding of the kinds of information that bees use, how they use it, potential mechanisms, and future research directions.

Introduction

What are landmarks?

A major and fascinating question in the study of animal cognition is how animals identify, remember, and use landmarks to navigate. Reliable navigation is crucial for animals that migrate or have nests; hence, and multiple species have evolved different strategies to find their way to food sources and back to their home. Sophisticated strategies and neural

mechanisms have therefore evolved to facilitate navigation. For example, depending on their natural history and ecology, many species need spatial memories to guide them on their paths. These navigational strategies use multiple sensory modalities, including vision, olfaction, proprioception, electroreception, and magnetoreception. A recurring theme is that animals usually orient themselves towards their goals with multiple, redundant forms of information and prioritize this information according to its reliability and salience (relative conspicuousness to the observer in a given motivational state) (Bradbury & Vehrencamp, 1998).

For example, each environment offers multiple pieces of visual information, ranging from the position of celestial objects above the skyline, to the colorful petals of a flower. Animals can use these features to orient themselves in the correct directions at different points along their routes. As an animal explores its surroundings, it comes across entities that are salient in some manner. The presence of these entities is used to help identify and define the location at which they are found (Horridge, 2009, pp. 200; O'Keefe & Nadel, 1978, pp 7-8). It is not known how animals perceive their goals relative to or nested within an overall landscape, but animals functionally use these entities at different stages along a path to reach a goal. These entities are classically referred to as landmarks (Collett, 1992). In contrast, we reserve the term "landscape" to refer to more global cues that are the combination of multiple landmarks. The dichotomy between landmarks and landscapes is not absolute and depending on the exact sensory stimuli, animals may perceive them as a continuum.

In many cases, it is unclear if a single stimulus aspect is enough to describe an entity or if entities consist of multiple stimuli that animals can recognize and use. The latter is quite likely since entities are real world objects with multiple characteristics. In the study of animal navigation and, more recently, machine vision, the term landmark has been used in multiple contexts with different meanings for different organisms (Gillner, Weiß, & Mallot, 2008). Here,

our goal is to review the different senses in which the term landmark has been used and how landmarks and landscapes are used by honey bees. However, we find it useful to include some studies of other insects that navigate using landmarks in order to compare and contrast with honey bees (Freas, Fleischmann, & Cheng, 2019; Knaden & Graham, 2016). We focus primarily on examples drawn from vision but have tried to define our overall concepts broadly to encompass multiple sensory modalities.

How have landmarks been described?

One of the earliest questions posed by researchers was if a location is recalled in terms of the overall visual input at that location, or if a small number of distinct visual stimuli are enough to identify a location (O'Keefe & Nadel, 1978). In other words, animals may recall specific places based upon a visual gestalt (an organized whole that is greater than the simple sum of its parts: the gestalt hypothesis), or can they use a smaller subset of the visual information available (key feature hypothesis). Early experiments by Tinbergen showed that digger wasps use local landmarks close to the nest entrance while disregarding more distant landmarks (Tinbergen, 1932). In this case, and in multiple others, the entire visual gestalt is not necessary. Similarly, the classical way in which honey bee researchers (von Frisch, 1967) train foragers to a food source by moving a feeder over multiple forager visits to a desired location exploits the preference of foragers for familiar local landmarks (the visual appearance and odor of a feeder and its surrounds) over more distant landmarks.

Relevantly, Anderson (Anderson, 1977) showed that a rather simple model consisting of only a few landmarks in different parts of a bee's field of view can explain the searching behavior of the bee in an experimental arena, reinforcing the claim that simplified features, not an entire complex landscape, are sufficient for successful navigation. Bees can therefore simplify a complex visual scene by extracting key features such as color, size, orientation, and

distance. However, multiple elements, including the landscape, can also play a role. Cartwright and Collett (Cartwright & Collett, 1983) showed that bees returning to a food source tend to search for it in areas where the overall image projected on their eyes most closely matches their memories, a process referred to as “picture matching”. They increased the size of the landmarks and showed that bees would search at a farther distance where the apparent size of the landmarks was the same as they experienced during their training.

As noted above, landmarks comprise a very diverse category of objects. Some goals such as the hive and food sources may be conspicuous enough to be regarded as landmarks themselves. However, some goals may be relatively inconspicuous until the animal approaches them and thereby require guiding landmarks along the way. A potentially useful classification for landmarks is therefore to distinguish them by how their size changes as the observer moves. Some landmarks will significantly change in apparent size (as defined by the visual acuity and discrimination ability of a given animal) as the animal approaches (closer landmarks) and others such as mountains or certain features at the horizon will not appreciably change in apparent size over the route (distant landmarks). These very distant landmarks are part of the overall landscape (Towne, Ritrovato, Esposito, & Brown, 2017; Degen et al., 2016) and have also (under a slightly different context) been called “global cues” (Avarguès-Weber, Dyer, Ferrah, & Giurfa, 2015).

In our definition, we therefore classify celestial objects as part of the landscape, not as a landmark per se. We recognize that there is disagreement about how to classify celestial objects that guide navigation. Dyer and Gould (Dyer & Gould, 1983) call the sun a celestial landmark. Because the sun moves, this definition is at odds with Horridge (Horridge, 2009) who argues that the landmark gives identity to a specific location. Rather than create new terms, we instead define “near” versus “far” landmarks using the distinction of perceived size changes.

We note that this concept is not limited to vision, but could apply to other senses that experience substantial change (“near”) or little change (“far”) over the scale of an animal’s route, namely magnetoreception (Gagliardo, Ioalè, Savini, & Wild, 2006) and chemosensation (Dittman & Quinn, 1996).

This idea of classifying landmarks by using changing stimuli relative to the receiver is not new. Collett, Harland, and Collett (Collett, Harland, & Collett, 2002) defined two types of landmarks, both of which provide stimuli that change as the receiver approaches: “isolated” landmarks are singular entities that can be reached and rapidly passed. In contrast, “boundary” landmarks are continuous and the animal can move alongside or through them (Menzel et al., 2019). This approach suggests that panoramic landscape information helps with guiding an animal’s overall orientation, whereas reachable landmarks define points in space. Bees distinguish between very similar views of a complex natural landscape (Dyer, Rosa, & Reser, 2008) or similar artificial stimuli (Avarguès-Weber, Portelli, Benard, Dyer, & Giurfa, 2010). It is not clear if bees generalize different views of the same landmark based on a cue that is recognizable from any direction. The alternative is that a landmark could only be used in navigation when perceived from a specific angle.

The relationship of a landmark to the goal is another important aspect of defining landmarks. This relationship could be in terms of distance, a specific orientation, or a specific order of appearance between the goal and one or more landmarks (Chittka, Geiger, & Kunze, 1995; Huber, Couvillon, & Bitterman, 1994). If the goal is much less conspicuous than its surrounding objects, the relationship between the landmark and the goal is a key factor in defining the location, whereas if the goal is comparably conspicuous, it may be the only visual cue needed to find it within a short range.

Why is it so difficult to define landmarks?

It is difficult to pinpoint what animals learn in a visual learning assay when multiple aspects of a stimulus may be salient. Bees can use a single object (or a specific arrangement of a few objects) as a landmark, but objects have multiple features, and the feature that a bee uses can depend on other objects in the environment. This occurs because a cue is useful when it helps to distinguish the correct behavioral choice from an incorrect one, and this depends on the informational content of the scene (Caduff & Timpf, 2008). Hertz (Hertz, 1931) showed that bees trained to a feeder with a specific pattern would still land on and feed at a feeder with a different pattern if it shared some common features with the original pattern. Dyer et al. (Dyer et al., 2008) demonstrated that such acceptance happens with complex images, but if two images were offered during training, one always paired with reward and the other with lack of reward, bees learned to distinguish highly similar images.

A proposed definition for landmarks

We define a landmark as an object or set of objects with useful and salient features that animals in a given motivational state can use to navigate towards a goal. What features are useful depends on the landmark and its immediate surroundings, since the utility of these features (individually or combined) is a function of their uniqueness, conspicuousness, stability (Zeil, 2012), and context. Context includes both the relationship of objects to each other within a landscape (inter-landmark context) and the relationship of features' information, potentially covering multiple sensory modalities, for a given object (intra-landmark context). For example, solar information is generally useful and reliable for navigating bees, and honey bees prioritize using the position of the sun, deduced from direct sighting or via sky polarization patterns (henceforth referred to as e-vector) to orient. However, landmarks also help bees orient, particularly when other sources of information are unavailable (Dyer & Gould, 1981; Towne et al., 2017).

Uniqueness and conspicuousness of features allow easier recognition of the landmark. Unstable, moving objects do not typically serve as landmarks unless they are the goal (such as a moving feeder during training, an admittedly non-natural context). General landmarks that are present throughout a landscape (a river or a hedge) may be used differently from local (point) landmarks at the goal (Collett, 1992). Menzel et al. (Menzel et al., 2019) show multiple examples of guidance by continuous landmarks, in line with older experiments (von Frisch & Lindauer, 1954; Dyer & Gould, 1981) in which bees prioritize using landmark information over celestial cues (sun and e-vector) when the hive is located near such landmarks. A diverse set of experiments show that the effect of an elongated landmark such as a forest edge is strongest when compass direction and the visual features of the landmark are congruent. This effect supports Collett, Harland and Collett's (Collett, Harland, & Collett, 2002) suggestion that landmarks can be classified as isolated/local vs. boundary/context. It is unclear whether landmark identity is defined solely based on visual (and other sensory) features or, in part, by the relation to specific locations in space.

Representations of space in honeybees

A location in space can be defined in multiple ways. The most abstract way, used by humans and perhaps some animals who perform long-range migrations, is to define absolute space. This requires using reference points that are large-scale stimuli such as the earth's magnetic field or its rotational axis and thus requires very accurate sensory input to measure the positions of the global reference points. At a smaller scale, space can be represented with reference to smaller stimuli, such as hills or trees. When an animal needs to learn more than one location, it can either learn visual properties of each location separately and, potentially, learn a direct route between them. This mechanism is referred to as specialized route memory or egocentric navigation, and the animal knows where it is based solely upon its current view.

Another possibility is that the animal also forms a connection between the two locations, forming a vector with a specific direction (compared to global or local reference points) and a specific distance, so that it can navigate between those two points without having to learn the details of the route that connects them. This mechanism is known as generalized landscape memory, (Menzel, Brandt, Gumbert, Komischke, & Kunze, 2000) or allocentric navigation. Why might generalized landscape memory be beneficial? As more locations are learned, if they are learned separately, the animal can only travel between them using known paths. But if they are learned in connection to each other, it is possible that knowing the spatial relationship between multiple locations allows the animal to make shortcuts and travel novel routes between known locations (interpolation) or to find the way to a known location from an unknown location (extrapolation).

Tolman (Tolman, 1948) proposed the concept of a cognitive map, a system of coding space which uses relational information to build a representation. This representation could “link together conceptually parts of an environment which have never been experienced at the same time” (O’Keefe & Nadel, 1978) and several species, including bees, are claimed to possess cognitive maps (Menzel et al., 2011; Menzel & Greggers, 2015, but see Bennett, 1996). Some experiments provide evidence that bees take such novel shortcuts (Cheeseman et al., 2014b; Gould, 1986). However, in some cases, animals may be using simpler mechanisms (Bennett, 1996) (see below) to perform the same tasks, and it could be difficult to reject alternative hypotheses. When other strategies could account for novel shortcuts (Hoinville & Wehner, 2018), cognitive maps are difficult to demonstrate given that multiple experiments are required to exclude all other possibilities (Cheung et al., 2014, but also consider Cheeseman et al., 2014a), and this hypothesis remains actively investigated and debated. Research on humans suggests that Euclidean space is not naturally derived from visual experiences and that a

cognitive graph made from paths between locations explains human navigation better than a cognitive map (Warren, 2019).

Honeybees are a unique model organism for studying spatial representations since their waggle dance depends on the visual memories that they form on their trips. Follower bees also develop a spatial representation based on the tactile input from the dancers (Rohrseitz & Tautz, 1999). Whether all three codings of space (guiding the forager's navigation, the dancer's movements, and the follower's exploration) are rooted in the same spatial representation is yet to be determined.

Different types of navigation

Honeybees navigate using disparate visual cues at different parts of their route. Upon exiting the hive, the bee has access to limited stimuli: landmarks around the hive and direction information from the e-vector. She can use these cues to travel in the correct direction. On her route, she can compare the real-time visual input of near and far-range landmarks to memories of points on the route on her previous trips and maintain her heading while keeping track of how far she has travelled. Once near her floral destination, she can use short-range landmarks to pinpoint her target (Collett, Chittka, & Collett, 2013). These navigational strategies broadly fall into three categories: alignment image matching, positional image matching, and path integration (Collett et al., 2013). At each part of the path, if the necessary visual information is available, bees can use it to navigate.

Alignment image matching provides a simple way of comparing current views or cues extracted from a view with memories from previous trips. The animal then tries to maximize the match through corrective movements. If the bee is not too far from known areas where familiar features are abundant, this strategy allows her to stay on course. Positional image

matching works by extrapolating the current location from differences between relationships among parts of the current view image and the same relationships in memories of views. The only requirement for positional image matching is that the panoramas (landscapes) of the novel area and the known areas share sufficient items in common. Alignment and positional image matching are not necessarily achieved in different ways, rather, they are different scalings of using memorized visual information to orient towards a goal. Path integration does not require landmarks, but only a source of directional reference point (offered by the sun and its e-vector) and a source of odometry (optic flow or stride counting). By keeping track of the path segments taken, the animal can ideally integrate its current location at any moment and find a homing vector (Collett et al., 2013).

The relative accuracy of these three strategies depends on the situation. Path integration has relatively low error at shorter distances and in novel areas but can result in larger errors when orienting near the goal because the remaining vector is much smaller than the accumulated error. Alignment image matching requires familiarity with the route but can guide the animal with minimal error, and can be learned in a single trip. Landmarks clearly play key roles in alignment and positional image matching strategies, but can also be combined with path integration to create short vectors for segments of the entire route (von Frisch, 1967). We will discuss the contributions of landmarks to each of these strategies below.

Snapshots

A snapshot is a set of extracted features of part of or the entire visual field (and perhaps other sensory inputs) at a location. Although it is possible that an image is memorized in whole, it is more likely that only its prominent features are extracted and memorized. Bees learn sets of visual snapshots (Zhang, Lehrer, & Srinivasan, 1999) and use them in different ways to guide their behavior at different points on their trips (Collett, 1996), like ants (Judd & Collett, 1998).

When using snapshots, a landmark can help a bee to recall a vector or to maintain a steady heading by holding the landmark at a position in its field of view (preferably frontal) by flying closely to the left or right of it.

Collett, Harland, and Collett (Collett, Harland, & Collett, 2002) argue that a landmark does not necessarily lead to the recall of a certain location, and that panoramic contexts are more reliable than single landmarks that are single entities because using panoramic contexts could reduce the risk of misidentifying or missing a landmark. What a context consists of is not explicitly defined, but could be quite broad, including time of day and motivational state (Pahl, Zhu, Pix, Tautz, & Zhang, 2007). However, their experimental design suggests that the general patterning of the environment (stripes versus dots) can create a context. Perhaps large and distant objects could also serve as contexts.

Vectors

A vector is a linear path starting from a specific point and ending at a destination. It has a given distance and direction relative to the reference points that the animal uses, such as landmarks or the e-vector. For example, ants recall different vectors depending upon the visual landmarks that they encounter (Harris, De Ibarra, Graham, & Collett, 2005).

Vectors can be used in multiple strategies. A vector could connect the nest and different food sources or lead from each of them to mid-path points from which other vectors could provide guidance. An appropriate panoramic context could activate a local vector (M. Collett et al., 2002), and a sequence of these vectors would then allow the animal to travel a non-linear path by segmenting this path into shorter linear sub-paths. Alternatively, bees can learn complex flight maneuvers and perform them in a fixed sequence (Collett, Fry, & Wehner, 1993).

Using vectors does not exclude using snapshots because the two concepts are complementary. Moving in the correct direction will inevitably lead the animal to the next snapshot location where the next vector can be activated without a need for the last vector to be completely executed. Experiments with bees flying in a tunnel show that foragers looking for a missing feeder search in the correct distance after passing the last landmark on their route (Srinivasan, Zhang, & Bidwell, 1997) and that the total image motion (optic flow) determines the distance communicated in the waggle dance (Dacke & Srinivasan, 2007; von Frisch, 1967). A similar behavior is observed in desert ants when multiple path segments of different lengths and directions are used to reach a food source (Collett & Collett, 2015; Bolek, Wittlinger, & Wolf, 2012), although it is difficult to exclude the role of landmarks at the turning points.

Features and the sensory world of the animal

Although we have defined landmarks (see above), a strict definition is elusive because there is no consensus on what the brains of different animals consider to be the signature or signatures of a location or object. Bees have a very capable visual system, one that has evolved to solve multiple problems. However, it is difficult to provide bees with stimuli to dissect perfectly their navigational strategies. The gestalt and snapshot hypotheses suggest that multiple parts of a scene can help code space. Specific cues within an image, which we refer to as features, could be extracted separately through multiple feature detectors, and the coincidence of these features may encode the memory of a location. Examples of features include total area, center point of an area, total edge length, average local orientation, radial or tangential edges, colors, etc. However, anatomical and physiological studies of the eyes and optic lobes of insects have elucidated some of the feature extraction mechanisms, and behavioral tests have dissected the relative importance of different features and how animals use them in hierarchies.

Two hypotheses about what visual information bees remember are parameter-based memory and pictorial memory (Gould, 1987). In parameter-based memory, a series of measurements of different features are summed together to create a final score for a scene. Later, if a scene has a similar score, it indicates a match. Pictorial memory, however, must encode the relative position of the elements of the scene. Gould (Gould, 1984) argued that since relative position plays a role in pattern recognition, pictorial memory, and not parameter-based memory, is how bees memorize scenes. As an intermediate hypothesis, it is possible that different areas of the eye sense the presence of one or a few specific cues in a parametric manner and that matching occurs when several areas simultaneously receive input sufficiently similar to the memorized information (Lehrer, 1998).

Feature hierarchies

Many experiments have tried to tease out the relative importance of different visual features. Gould and Towne (Gould & Towne, 1988) reviewed the relevant literature during the 1960-1980's and provide a list of features that bees prefer to use in order of presumed importance: 1) relative color inputs of areas, 2) the distribution of line angles, and 3) and the distribution of borders.

Horridge (Horridge, 2009, chapter 9) offers a different prioritized list based on several experiments with primarily black and white patterns: 1) total area, 2) position of the center of area, 3) total modulation, 4) radial edges, 5) average local orientation, 6) positions of hubs, and 7) tangential edges. Large black circles were preferred over radial spokes or parallel edges, and symmetry in a pattern of bars was preferred over the edge orientations of symmetrical patterns. Recent studies demonstrate the ability of honey bees to learn complex visual tasks using different categories of visual stimuli (reviewed in Borst & Helmstaedter, 2019).

When landmark information and vector based navigation provide conflicting information, landmarks seem to be more important (Srinivasan et al., 1997; Vladusich, Hemmi, Srinivasan, & Zeil, 2005; Kheradmand, Cassano, Gray, & Nieh, 2018, but see Menzel et al., 2010). Prominent landmarks can override e-vector information (von Frisch & Lindauer, 1954, but see Menzel et al., 1990), and it is possible that landmark-based directional information (computed on the return flight) affects the navigation and the waggle dance of foragers (De Marco & Menzel, 2005). Motivation could play a role in how foraging bees infer and use their spatial information. When a bee is released after three hours of waiting at a feeding spot, it takes off in the opposite direction from the nest, as if trying to get from the nest to the food source (Dyer, Gill, & Sharbowski, 2002) but bees trained to two feeders at different times of day can still find their way back from a feeder even at times not associated with feeding (Menzel, Geiger, Joerges, Müller, & Chittka, 1998). This demonstrates that time-specific route memories related to time-specific rewards were still available and could be used at other times.

At a higher scale, one could ask about the relative importance of different strategies of foraging in dissimilar environments (Figure 1.1). In novel environments, path integration seems to overcome landmark information (Chittka, Kunze, Shipman, & Buchmann, 1995). This suggests that landmark information may take longer to process or form in a salient manner, but path integration is a robust way of navigating over a short range. It has been suggested that all different strategies and features can be used simultaneously in a heterarchy, weighted by their salience and information content (Wehner, Hoinville, Cruse, & Cheng, 2016).

Orientation flights and returning to known locations

Bees perform a stereotypical flight associated with visual learning of a location. During nest orientation flights (Becker, 1958), and when learning the location of a food source upon departure (Lehrer, 1991; Gould, 1988), they face their goal and fly side-to-side and back-and-

forth in a series of expanding arcs (Osborne et al., 2013). During these learning flights, they are thought to extract and memorize useful visual information about important cues. Subsequently, bees likely refer to these memories and compare the memorized scene with real-time visual input: to reach the goal, they can minimize the difference between the memory and the visual input (Zeil, Hofmann, & Chahl, 2003). The bee remembers not only the visual cues associated with the goal itself, but the general composition of the immediate surrounding (learned during smaller and lower flight arcs) and even distant panoramic information (in the larger and higher flight arcs). The bee potentially associates a direction (compared to a reference point, such as e-vector) with the memories of that viewpoint. Orientation flights are important for bees that are new to an area (Capaldi & Dyer, 1999). Bees can find their way back to the hive from much farther than their expected area by using only a short learning flight near the hive (Degen et al., 2016). When bumblebees leave one source and try to find another food source, they start by performing a learning flight at the first source and then an optimized expansion of the search area to find new flowers. This flight has a sequence of arcs with increasing diameters and these back and forth motions form loops (Lihoreau, Ings, Chittka, & Reynolds, 2016; Zeil, 1993). The same behavior is observed during the first learning flights around the hive by new foragers and by misplaced bees trying to figure out where they are. When approaching a food source, bees learn the apparent size of nearby landmarks. However, it is only after the learning flight, during departure, that they also learn the distance to these landmarks (Lehrer & Collett, 1994). Bees acquire the overall three-dimensional structure of the goal area during turn-back-and-look behavior (Lehrer & Bianco, 2000) but they learn the color, shape, and size of landmarks during arrival at the food source.

When bees arrive at a target, they first aim at a landmark and associate it with their stored view. Their trajectory then brings them from the landmark to the goal so that the goal can

be reached by image matching. They then move in a way such that the landmark matches the position and size on the retina that it had during the learning flights. (Collett & Rees, 1997). Bees also use self-induced image motion to pinpoint the goal by matching the depth of field information computed from parallax of different areas of their panorama (Lehrer, 1996). Upon return, they approach the goal by matching the parallax from self-motion to the parallax during learning flights (Collett et al., 2013) in the absence of textural and contrast cues (Dacke & Srinivasan, 2008). By controlling the image motion rate within a small range, bees ensure smooth flights with slower flight speed as they approach and land on their goal (Srinivasan, Zhang, Lehrer, & Collett, 1996).

Different sensory modalities

Olfaction

Odors can inform honey bee searching for and returning to food sources (von Frisch, 1967). For example, odors trigger recall of specific food sites by triggering odor-associated landmark memories (Reinhard, Srinivasan, Guez, & Zhang, 2004). Honeybees mark their nest entrance, as well as food sources, with Nasonov pheromone and other volatiles to attract other bees to these goals (Fernandez & Farina, 2001; Free & Williams, 1983). Similarly, stingless bees create and follow odor trails that consist of odors deposited every few meters (Jarau, Hrnčir, Zucchi, & Barth, 2004). In some cases, these odor trails may extend from the nest to the rewarding food source, but it seems that such trails provide an outbound route to food, not information directing bees back to their nest, though this remains to be tested (Lindauer & Kerr, 1960). Wasps migrating to a new nest site orient to odors deposited on the edges of leaves along the path to the new nest site (Jeanne, 1981). Odor trails also play a major role in the orientation of multiple ant species and act as olfactory landmarks guiding workers to resources and back to the nest (Hölldobler & Wilson, 1990; Steck, Hansson, & Knaden, 2009).

Magnetoreception and mechanosensation of electrical charges

In the realm of landmarks that are located very close to or at the goal, there is evidence that bumble bees (Clarke, Whitney, Sutton, & Robert, 2013) and honey bees (Greggers et al., 2013) orient via mechanosensory hairs to the electrostatic fields generated by floral resources and learn these fields, opening up the possibility that they could also use these senses to recognize their nests (Lihoreau & Raine, 2013). There is data suggesting that honey bees may sense magnetic fields (Liang, Chuang, Jiang, & Yang, 2016; Lambinet, Hayden, Reid, & Gries, 2017; Baddeley, Graham, Philippides, & Husbands, 2011; Wystrach, Mangan, Philippides, & Graham, 2013) and that disrupting their ability to sense the earth's magnetic field reduces their homing abilities (Ferrari, 2014). Ants have been shown to actively use the magnetic field to orient towards their goals (Fleischmann, Grob, Müller, Wehner, & Rössler, 2018). Additional studies would be revealing.

Multimodality

Landmarks and landscapes typically provide multiple types of information. Ants use both visual and chemical cues in their orientation towards food (Aron, Beckers, Deneubourg, & Pasteels, 1993). Cataglyphis ants can be trained to use visual, olfactory, magnetic, tactile, and vibrational landmarks to find their nest entrance (Buehlmann, Hansson, & Knaden, 2012; Freas et al., 2019). Visual and olfactory cues play an important role for allowing carpenter bees to recognize their nest aggregations (Ostwald, Shaffer, Pratt, & Fewell, 2019). Focusing on landmarks, stingless bees use both visual and chemical cues to orient towards rewarding food sources (Sánchez, Nieh, & Vandame, 2011). In bumble bees, the presence of a specific landmark odor can enhance discrimination of color cues and thereby the use of color as a local landmark

(Kunze & Gumbert, 2000). Bumblebees learn the differences between floral scent patterns, and subsequently transfer this learning to differences in visual patterns (Lawson, Chittka, Whitney, & Rands, 2018). In these examples, the modalities likely have different saliences depending upon the species and context. For example, honey bees may pay more attention to the odors and colors of flowers than to other landmarks, although this varies with bee race (Lauer & Lindauer, 1971). Similarly, Gould and Towne (Gould & Towne, 1988) conclude that odors are more important than colors, and both are more important than landmarks and patterns, but others have suggested equal or independent effects of each type of stimulus (Couvillon & Bitterman, 1988).

Landmarks in other insects

The solitary bee (*Epicharis metatarsalis*) uses visual cues to find its nest entrance (Inouye, 2000). In stingless bees (*Tetragonisca angustula*), homing bees orient towards the visual appearance of the nest from about 1 m away, likely corresponding to their ability to visually distinguish this landmark (Zeil & Wittman, 1993). This example also illustrates the role of salience and reliability in information use.

Wasps perform orientation behaviors that are like the turn-back-and-look behavior of bees learning visual landmarks. Wasps also face the food item or nest site and fly back and forth in arcs that increase in radius over time. They will likewise circle high above the site to be learned while flying away, presumably to gain a better view of landmarks to use as snapshots (Richter, 2000, Zeil, 1993).

Both wasps (Polistinae) and species of stingless bees (Meliponini) that deposit odor marks, tend to deposit these odor marks on conspicuous objects along the marked path (Richter, 2000; Nieh, 2004; Nieh, Contrera, Yoon, Barreto, & Imperatriz-Fonseca, 2004). These are examples of visually assisted chemotaxis in which insects modify a landmark with a signal to

make it more conspicuous, just as multiple vertebrates species deposit scent marks on visually conspicuous landmarks (Bradbury & Vehrencamp, 1998). Bees can also deposit cues, not just signals, on landmarks. Bumble bees and stingless bees leave footprint odor cues (Ayasse & Jarau, 2014, Jarau et al., 2004) on food sources, though, unlike signals, such marks are a byproduct of visitation.

Ants that follow odor trails shape their trails along edges and other features (Hölldobler & Wilson, 1990). The way in which these physical edges shape the path is similar to how landscape edges can influence the flights of bees (von Frisch, 1967). *Melophorus bagoti* ants can learn two different routes, one for the outbound and one for the inbound journey, and these routes are direction-specific (Wehner, Boyer, Loertscher, Sommer, & Menzi, 2006). Similarly, *Formica japonica* ants use landmarks differently if they are displaced at different points along their round trip (Fukushi & Wehner, 2004). These results suggest that the same landmarks or views trigger different responses depending on the context or motivational state.

Neural bases of landmark memory

How are features detected and memorized and used to guide future behavior? The concept of feature detectors as local neural circuits that process simple sensory inputs was suggested in the 1950's (Martin, 1994). Relatively recently, the detailed anatomical and functional studies of the optic lobes of diverse insects (bees, ants, flies, etc.) have begun to find these feature detectors (Borst, Haag, & Reiff, 2010; Zeil, Ribi, & Narendra, 2014; Hempel de Ibarra, Vorobyev, & Menzel, 2014; Avarguès-Weber, Mota, & Giurfa, 2012). The circuitry of simple feature detectors in the eyes (Paulk, Phillips-Portillo, Dacks, Fellous, & Gronenberg, 2008; Mota, Gronenberg, Giurfa, & Sandoz, 2013; Mertes, Dittmar, Egelhaaf, & Boeddeker, 2014), memories of visual features in the mushroom bodies (Plath et al., 2017; Van Nest, Wagner, Marrs, & Fahrback, 2017), and movement planning and representing spatial orientation

in the central complex (Honkanen, Adden, Da Silva Freitas, & Heinze, 2019; Seelig & Jayaraman, 2015) provide promising research directions.

In fruit flies, landmark orientation and path integration vector information are processed in the ellipsoid body, a part of the central complex (Seelig & Jayaraman, 2015). In bumblebees, different populations of neurons in the lateral protocerebrum show preferential color and motion sensitivity (Paulk, Dacks, Phillips-Portillo, Fellous, & Gronenberg, 2009), and some wide-field cells of the lobula can recognize the particular motion signatures of landmarks (Mertes et al., 2014). However, the mechanisms by which different headings are merged with different stimuli, including landmarks, when the animal is moving towards a goal, are not well understood in insects. Advances in neural recordings in virtual reality environments have opened up new possibilities for delineating the intricate interactions between different cues in simple and complex environments (Zwaka et al., 2019) and using such virtual reality can facilitate our study of learning in action in an extremely controlled manner (Buatois, Flumian, Schultheiss, Avarguès-Weber, & Giurfa, 2018; Olsson et al., 2011; Rusch et al., 2017) and allow us to test alternative hypotheses about representations of space, such as the cognitive graph (Warren, 2019), in bees.

Recent findings and future directions

As new neuroanatomical and behavioral data are discovered, new models have been built that closely mimic and account for the choices of navigating animals' using realistic stimuli (Cope, Sabo, Vasilaki, Barron, & Marshall, 2017; Müller, Nawrot, Menzel, & Landgraf, 2018; Dyer et al., 2008; Ardin, Peng, Mangan, Lagogiannis, & Webb, 2016). These models provide insight into the computations that navigating animals perform and allow us to test different hypotheses on the importance of visual features. In developing these models it is crucial to consider and balance the types of input bees see in the real world and how they actively respond

to abstract and simplistic stimuli (Zeil & Boeddeker, 2007; Boeddeker, Mertes, Dittmar, & Egelhaaf, 2015) that provide greater control but perhaps lesser relevance.

An important question is whether landmarks are remembered as part of a panorama, as separate entities, or as features. Bees learn to generalize features and categorize stimuli (Benard, Stach, & Giurfa, 2006; Stach, Benard, & Giurfa, 2004) and they may generalize the salient features of a landmark from different orientations and treat it as a unique entity.

How many different sets of snapshots and vectors can a bee learn? In an interesting example, receiving ambiguous polarization patterns drives bees to dance for multiple vectors even within the same dance (Evangelista, Kraft, Dacke, Labhart, & Srinivasan, 2014) although perhaps navigational vectors are coded in a different way than waggle dance vectors (Dacke & Srinivasan, 2008, but see Menzel et al., 2011 arguing for a unified vector representation). Research on ants navigation should help elucidate this topic (Baddeley, Graham, Philippides, & Husbands, 2011, Wystrach, Mangan, Philippides, & Graham, 2013).

If we next consider the path that a bee takes, do they learn landmarks en route to a goal in the same way that they learn landmarks at or very close to the goal? Are floral memory and landmark memory separate processes? Do bees expect to see the next landmark in the sequence when they get close to where the vector from the previous landmark led them? These questions have been the topic of many studies, but definitive proof for some of the competing hypotheses is still lacking. Methodological advances in cognitive and physiological aspects of vision are paving the way for answering them.

Finally, one might ask about the broader salience of understanding bee navigation. This information is useful, not only for understanding how bees navigate, but, more generally, for understanding navigation in other animals, since mammals and insects share multiple

mechanisms of feature detection (Borst & Helmstaedter, 2015). Studying how bees navigate could help us understand the general mechanisms that have evolved to solve the problem of animal navigation and could bioinspire the development of better machine vision strategies for multiple applications, such as robots that navigate using view-based information and emulate the active vision strategies of animals (Lehrer & Bianco, 2000). Characterizing the continuum of landmarks and landscapes and the features used to identify locations will be a central part of this effort.

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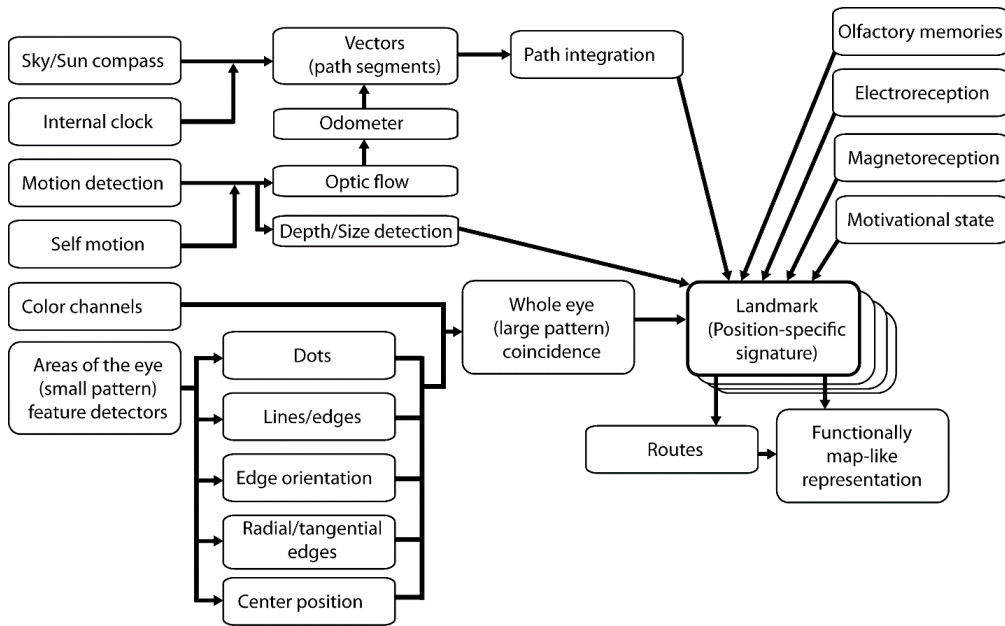


Figure 1.1. Different sensory stimuli and processing pathways for landmark detection and spatial navigation. At each location, the coincidence of multiple sources of sensory input is merged and combined into a signature. Each signature may be functionally attached to a navigational motor output such that a particular path is chosen by an animal in a specific motivational state. Multiple landmarks and routes may be combined to form a functional allocentric representation of the environment. Other cues and processing pathways play roles in guiding the navigation of bees, but we have limited our focus to the ones most studied.

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Chapter 2: Statistical learning in honey bees

Abstract

Anticipating upcoming events can increase fitness by allowing animals to decide the best course of action. Humans and many other animals with large, complex brains can learn sequences of events and thereby anticipate rewards through statistical learning. However, the time scale at which many sequences are learned is short. Events separated by a few seconds are easily learned, and events separated by longer delays are typically more difficult to learn. Here, we show that honey bees (*Apis mellifera*) can learn a sequence of two food sources alternating in profitability every few minutes. Bees can predict and land on the feeder that will be rewarding on their next approach statistically more frequently than chance, and their performance improves with experience. These results challenge the common time intervals in which statistical learning and reinforcement are thought to be effective and show that a relatively small brain can learn sequences of visual patterns.

Introduction

Survival and fitness depend on learning how to behave optimally in changing environments (Johnston, 1982; Mery & Kawecki, 2002). Many environmental changes follow regular patterns, and the order in which events occur is predictable. Sequences of external stimuli represent specific circumstances that require specific behaviors in response. For example, a series of vocal signals in a particular order has a distinct meaning (Janik, 2013), and navigation often involves knowing one's locations based on receiving a series of stimuli (Foster & Knierim, 2012). Similarly, sequences of successive actions result in specific outcomes and

the success of these behaviors is a direct result of the order in which the subunits are performed. For example, a series of complex manipulations of a food source makes food available to foragers (Morgan, Whitehorn, Lye, & Vallejo-Marín, 2016), building a nest involves collecting materials and putting them together in a particular manner (Hinde & Matthews, 1958; Lewarch & Hoekstra, 2018), and grooming behavior is performed by cleaning different body parts in a particular order (Seeds et al., 2014). Making a time-dependent connection between the subunits of stimulus or response sequences in order to represent or perform them allows animals to reduce uncertainty (Dall, Giraldeau, Olsson, McNamara, & Stephens, 2005; Inglis, 2000).

Statistical learning is the ability to detect regularities (co-occurrence patterns, frequent transitions, etc.) in sensory input (Armstrong, Frost, & Christiansen, 2017). *Sequence learning* is a form of statistical learning in which regularities in the order of events, stimuli, or actions are learned. Humans rely on sequence learning for many cognitive abilities that are hallmarks of intelligence: language, pattern recognition, predicting outcomes, and preparing for complex motor patterns (Dehaene, Meyniel, Wacongne, Wang, & Pallier, 2015). Human infants are capable of learning specific statistical correlations within auditory and visual input (Bulf, Johnson, & Valenza, 2011; Saffran, Johnson, Aslin, & Newport, 1999). Some types of sequence learning even occur without conscious awareness of the sequence learned (Brady & Oliva, 2008; Fiser & Aslin, 2002). Muscle contraction patterns such as those used for swinging a golf club for putting the ball and playing a song on the piano are examples of sequences that humans can learn to excel at (Abrahamse, Jiménez, Verwey, & Clegg, 2010; Maxwell, Masters, & Eves, 2000; Verwey & Wright, 2014).

Many animals show signs of sequence learning, and some species learn relationships between many events and solve problems requiring multiple steps: primates (Biro & Matsuzawa, 1999; Conway & Christiansen, 2001; Martin, Biro, & Matsuzawa, 2017), crows

(Gruber et al., 2019; Taylor, Elliffe, Hunt, & Gray, 2010), and parrots (Pepperberg, 2007), are a few examples. Landmark-guided navigation requires orienting towards specific landmarks at specific points *en route* to goals in different time scales: for seasonal migrations (Chapman, Reynolds, & Wilson, 2015; Hedenström et al., 2007), daily foraging trips (Casaux & Bertolin, 2018; Chameron, Schatz, Pastergue-Ruiz, Beugnon, & Collett, 1998; Macquart, Latil, & Beugnon, 2008), and maze learning (Lucon-Xiccato & Bisazza, 2017; Sharp, Miller-Cahill, Riccio, & Fountain, 2018; S. Zhang, Mizutani, & Srinivasan, 2000). Classical conditioning is perhaps the simplest case of sequence learning, in which one stimulus predicts next stimulus. Many animals with relatively simple brains, such as *Caenorhabditis elegans* and *Aplysia californica*, can form such simple associations between stimuli and rewards (Carew, Walters, & Kandel, 1981; Nishijima & Maruyama, 2017) and other animals, such as dogs and honey bees demonstrate even higher-order classical conditioning, in which the first stimulus in a chain of interlinked stimuli can trigger a learned response (Bitterman, Menzel, Fietz, & Schäfer, 1983; Brogden, 1939).

Not all sequences are composed of subunits that immediately follow each other. The ecology of an animal determines the time scales and the type of sequences that it needs to learn (Dukas, 1998). In most laboratory experiments, the conditioned and unconditioned stimuli may overlap, immediately follow each other, or follow after a very brief delay of a few seconds (Bitterman et al., 1983; Stahlman & Blaisdell, 2011; Zentall, Wasserman, & Urcuioli, 2014). In many human experiments on visual sequence learning, the interval between the images shown ranges between a tenth of a second to a few seconds (Rosenthal, Andrews, Antoniadis, Kennard, & Soto, 2016; Shafto, Conway, Field, & Houston, 2012). If stimuli are presented with long time intervals in between, short term memories may not be able to sustain stimulus-evoked activity

before the next stimulus is presented, and thus the link between the two stimuli may be more difficult to form (Santolin & Saffran, 2018).

Little is known about the presence of such learning capabilities in animals (Garcia, Ervin, & Koelling, 1966). Latent learning could be conceptualized as one such ability, and many animals seem to be able to form memories with delayed rewards or punishment, particularly when navigating (Menzel, 1993). On the other hand, rapid sequences may lack the temporal separation to lead to increased learning. It has been shown that extremely short (a few seconds) intertrial intervals could result in lower learning levels than intermediate (a few minutes) intertrial intervals, and massed learning protocols may result in lower learning levels than spaced learning protocols (Menzel, Manz, Menzel, & Greggers, 2001).

Honey bees, especially foragers, need to learn many sequences about timing and spatial distribution of floral food sources. Flower nectaries replenish at different speeds and replenishment rates can depend on time of day (Castellanos, Wilson, & Thomson, 2002; Corbet, Kerslake, Brown, & Morland, 1984). Therefore, learning where and when to visit next is beneficial for foragers, and bumblebees have been shown to be able to measure and time reward production by flowers (Boisvert, Veal, & Sherry, 2007). Bees may thus have evolved the ability to recognize and remember sequences in which the time interval between stimuli is on the order of minutes (Cnaani, Thomson, & Papaj, 2006). At shorter time scales, bees can retain a visual stimulus and compare it to an upcoming stimulus in order to perform a delayed match to sample task, where the time interval between the two stimuli is on the order of less than 8 seconds (Shaowu Zhang, Bock, Si, Tautz, & Srinivasan, 2005). Furthermore, bees can learn rules based on multiple visual stimuli occurring in a rapid sequence (S. W. Zhang, Lehrer, & Srinivasan, 1999). They can also learn long-term patterns on the order of hours, such as one food source being rewarding during morning and another (or the same) food source being rewarding in the

afternoon (Pahl, Zhu, Pix, Tautz, & Zhang, 2007). However, this ability could perhaps be explained better by circadian rhythms (or a combination of the Sun's position and landmarks acting as long-term contextual cues), and by attenuation of the drive to visit the food source once it becomes unrewarding. Honey bees learn complex sequences of movements (Collett, Fry, & Wehner, 1993) and bumblebees can form specific routes in which they visit several flowers in a particular order, traplining behavior (Saleh & Chittka, 2007). Bees may therefore have the ability to recognize and memorize sequences that are slower than those tested in conditioning or maze learning experiments, yet faster than circadian rhythms.

We hypothesized that bees are capable of learning relatively simple, minute-long sequences. To test this hypothesis, we had individual bees (without access to social cues) feed at a pair of feeders with different visual patterns of which only one was rewarding on any given trip. The key difficulty of this task was that no single pattern was consistently rewarding. The bee needs to learn that *the pattern that was not rewarding in the previous visit will become rewarding at the next visit*. If bees can learn this sequence competently, we expect them to land on the feeder that is rewarding on each visit and to make the correct decision more promptly as they gain experience.

Materials and methods

During Spring and Summer 2018 and Summer 2019, we trained 43 European honey bees (*Apis mellifera ligustica*) from ten different colonies located in an apiary at the UCSD Biological Field Station in La Jolla, California. We conducted one trial a day, usually from 9:00 AM to 3:00 PM. Each trial had two phases, a training phase and a testing phase. In the training phase, one feeder (details below) was placed in front of a hive and, once several bees were

feeding on it, was transferred to a white circular platform (diameter 25 cm) on a tripod of the same height as the hive entrance. The tripod was slowly moved away from a hive entrance to ensure that a few bees would visit it continuously (methods of von Frisch (von Frisch, 1967)). Once the tripod was about 25 m away from the hive, we stopped moving it, set its height to 1 m, marked all arriving bees, and recorded how often each returned to the feeder within the next 30 min to choose a reliable forager, defined as the bee that returned most frequently. Because bee feeder choice can be influenced by the presence of other bees (Horna Lowell, Morris, Vidal, Durso, & Murphy, 2019), after selecting and marking the focal bee, we used a manual aspirator to capture all other bees, including subsequent recruits. Once the selected focal bee returned to the feeder at least three times without other bees present, we began the testing phase by removing the training feeder, cleaning the feeder platform (details below), and putting out two clean testing feeders (with different visual patterns, detailed below) on the platform, only one of which was rewarding at any given moment. The two feeders were separated by 10 cm. Each feeder was composed of a feeder plate and an overlay with a pattern drawn on it.

Feeder plates

The feeder plate was a transparent square acrylic block (3.9 cm × 3.9 cm × 1.8 cm) with a cylindrical well in the center (1 cm wide, 0.8 cm deep) containing either the rewarding (2.5 M analytical-grade sucrose prepared in deionized water, about 1 ml and refilled frequently) or the non-rewarding solution. We tested two different non-rewarding solutions: neutral (distilled water) or aversive (3 M NaCl prepared in distilled water, following Aguiar et al. (Aguiar, Roselino, Sazima, & Giurfa, 2018)). Each bee only experienced one of these non-rewarding solutions. We merged the data for the bees exposed to neutral (N = 14) and aversive (N = 6) solution treatments since their results were very similar and the bees did not behave differently upon landing on the feeder plates. Other studies show that aversive stimuli may not increase

learning (compared to water) when the reward is already strong (Aguiar et al., 2018; Muth, Cooper, Bonilla, & Leonard, 2018).

Overlays with visual patterns

We used different patterns for the training and testing of the bees. The patterns were drawn with a black marker (Sharpie Magnum, Model #: H-384BL) on square plastic overlays consisting of a transparent square polyester sheet (Staples transparency film, Model #: 21828) with the same size as the feeder plate (3.9 cm × 3.9 cm) and with a small hole at the center (diameter = 4 mm) that bees could not crawl through but could extend their proboscis through to drink. For the training phase, we used a pattern with a ring and a cross (Fig. 2.1 top panel, left). For the testing phase of each bee, we used two patterns: two concentric rings and an 8-sided asterisk (Fig. 2.1 top panel, right). These patterns have been widely used in similar experiments with honey bees (Frisch, 1967; Shaowu Zhang et al., 2005) because bees can easily distinguishable between them.

The sequence experiment

In the testing phase, bees (N = 20) would experience the sequence of alternating rewards between the two patterns (Fig. 2.1 bottom panel). Once the satiated bee left for the hive, we would remove the overlays and clean them, then place them on the other feeder plate and wait for the bee to return. Bees would consistently visit the feeder from 10 AM to 2 PM and this corresponded to about 32 visits during that period (see below).

Honey bees are extremely sensitive to scents, and they frequently scent mark profitable food sources. To avoid biases due to scent marking, the entire platform was covered with a clean white paper towel that we replaced after every eight visits to avoid any potential scent mark deposition on the platform. We cleaned the overlays on which the bee landed after every visit

and replaced the feeder plates after every eight visits, since bees rarely touched the feeder plates or the platform. To clean the overlays, we dipped them in clean water and wiped them twice with a clean paper napkin. This removed most of the scent marks and uniformly distributed any remaining scents between the two overlays because bees were equally rewarded on both feeders in an alternating sequence (see negative control experiment below).

To remove other potentially distinguishing visual cues in the feeder plates, both feeders always contained identical volumes of liquid. Since bees can use landmark information to identify food sources, we rotated the location of the feeders on the platform in a pseudorandom manner to prevent bees from using landmark information to choose a feeder. Thus, the only information bees could reliably use to determine the rewarding feeder was the patterns on the overlays and its sequence. If the bees can use the visual patterns and learn the alternating sequence of reward, we predicted that they would perform better than chance in the sequence experiment. To rule out alternative hypotheses, we ran two control experiments.

Negative control experiment for scents

To test if bees were orienting to odor cues potentially provided by the sugar solution or their own scent marking of the feeders, we ran a series of negative control trials (N = 11). We used two feeders as before, but the rewarding and non-rewarding feeder plates had identical visual patterns (the ring-cross). If bees could smell the sucrose or their own scent marks on the feeder plate, they should significantly prefer the rewarding feeder. We followed the same procedures as in the sequence experiment to remove olfactory, visual, and social cues.

Positive control experiment for floral constancy

Honey bees are floral constant: they tend to visit the same flower species over an extended period of time, as long as it stays profitable (Grüter, Moore, Firmin, Helanterä, &

Ratnieks, 2011). However, some bees, known as scouts (Biesmeijer & De Vries, 2001), may seek novel food sources when familiar food sources are not predictably profitable. If our sequence experiment selected for scouts, they could explain alternating choice as a form of novelty seeking. To test for floral constancy, we ran a series of positive control trials (N = 12). In each trial, we offered bees two feeders with different visual patterns, but one of them was always filled with sucrose solution and the other one was always filled with distilled water. Thus, bees should easily have been able to learn the always rewarded pattern. We used the same procedures as in the sequence experiment to remove olfactory, visual, and social cues. However, instead of recording 32 visits per bee, we recorded 20 visits. We expected to observe the effect of constancy within fewer visits, as multiple other studies have shown that bees can easily and rapidly learn to distinguish between different visual patterns when only one pattern is rewarding (von Frisch, 1967).

If bees prefer the feeder that resembles their last rewarding food source, they would perform worse than chance in the sequence experiment and better than chance in the positive control experiment. Whereas if they prefer the novel choice, they would perform better than chance in the sequence experiment and worse than chance in the positive control experiment.

Measures of sequence learning other than the first landing choice

When the focal bee visited the feeders, it would hover above the platform and visually inspect the two feeders before landing on one. A well-trained bee would land then walk towards the hole at the center of the overlay and taste the solution by extending its proboscis into the hole. If it landed on the incorrect feeder, upon tasting the non-rewarding solution, it would hop off and hover above the platform again. Sometimes, even after landing on the correct feeder, the bee would hop off and inspect the feeders again without tasting the contents. We measured the number of hops and what proportion of hops resulted in correct landings before feeding. For

each bee, we also measured the duration of each trip. For our sequence learning analysis, we only used the first landing choice on each visit since only this first choice should be entirely dependent on memories acquired on the previous trips.

Analysis

Depending on weather and the focal bee's motivation, we collected data from a given forager for 125 to 210 minutes. To allow maximal use of our data from different bees, we limited all our statistical analyses to the first 32 visits of each bee. This way, all bees have equal effects on the tests, and each bee can offer enough visits to realistically show its ability to learn. We used the R software, version 3.4.3 to run all statistical tests (R Core Team, 2017). Package "lme4" was used for the mixed effects logistic regressions.

To assess whether the bees in the three experiments performed better than random choice, we ran one-sample t tests on our entire dataset, comparing the proportion of first landings on the correct feeder to 0.5 (null expectation probability). Furthermore, we ran a Fisher's exact test to see if the bees in the sequence experiment performed better than the bees in the negative control experiment in the second half of the visitations (visits 17-32) when we expect differences in performances to be larger because of learning. We used paired t tests to assess any improvements in performance between the first half and the second half of the sequence and negative control experiments for (paired for each bee).

We used a mixed effects logistic regression to test if correct choices are a function of time. Each bee's data consist of 32 correct/incorrect landing choices, thus the visits of each bee were treated as repeated measures for the binomial output. A power analysis showed that a sample of 20 bees learning to choose the correct pattern at an asymptotic odds ratio of 2 to 1

(0.667 correct choice probability) within 22 visits would have a high probability ($\beta=0.86$) of showing significant results ($\alpha=0.05$) in a similar logistic regression.

We used linear regression to assess the effect of experience on hopping behavior and on how many of landings prior to feeding (on a single trip) were on the correct feeder for each bee. These two regressions used average hopping behavior for all bees for each visitation. We also used linear regression to test the effect of overall correct first landing rate on hopping and the effect of visitation frequency on average first landing success for each bee. In these two regressions, each bee was treated as an independent unit, but all visits of each bee were pooled together. For more details on our data analysis, please refer to the supplementary material. To assess whether the performance of individual bees was better than random, we performed binomial tests on successes of each bee, compared to an expected probability of 0.5.

Results

Bees were able to learn the alternating rewards sequence. They visited the feeder frequently (range: once every 3.9-6.6 min). In the sequence experiment, bees trained to alternating rewards ($N = 20$) had an overall success rate of 0.584, significantly higher than chance (one-sample t test, $t_{19} = 5.54$, $P < 0.0001$). The learning effect in the sequence experiment was stronger during the latter half of visitations (visits 17-32, when learning is expected to be greater) with an overall success rate of 0.634 (one-sample t test, $t_{19} = 5.67$, $P < 0.0001$). In these later visits, bees performed better in the sequence learning experiment than in the negative control experiment (Fisher's exact test, success rates of 0.634 versus 0.539 respectively, $P = 0.044$, Fig. 2.2). Bees in the sequence experiment improved at landing on the correct feeder with

successive visits (Figs. 2.3 and S2.2, logistic regression, success as a function of visit numbers (as repeated measures for each bee), $z = 2.17$, $P = 0.030$).

Our bee-odor removal procedures were effective. In the negative control experiment, bees trained to two identical patterns ($N = 11$) had an overall success rate of 0.534, not significantly different from chance (one-sample t test, $t_{10} = 1.75$, $P = 0.11$). As expected, bees also exhibited floral constancy. In the positive control experiment, bees trained to constant rewards ($N = 12$) had an overall success rate of 0.704, significantly higher than chance (one-sample t test, $t_{11} = 6.01$, $P < 0.0001$) and much higher than the negative control in their second half of visits (Fisher's exact test, $P < 0.001$).

Most bees were very active and after their first landing on either feeder, would often fly off and land again on the same feeder or the other feeder before drinking from the rewarding feeder. Per bee, the average number of landings before drinking ranged from 1.65 to 3.40 for the bees in the sequence experiment. There was no correlation between visit number and how many times a bee flew off and landed again before drinking (linear regression, $F_{1,30} = 2.28$, $P = 0.141$). However, there was a nearly significant improvement on the ratio of landings on the correct feeder as a function of visit number (linear regression, $F_{1,30} = 3.74$, $P = 0.063$). The average first-landing success rate of each bee did not correlate with how many times (on average) it switched between feeders before being rewarded (linear regression, $F_{1,18} = 2.07$, $P = 0.168$). There was no correlation between average trip duration and average correctness of individuals (linear regression, $F_{1,18} = 0.144$, $P = 0.709$). Figure 2.4 summarizes these results.

As expected, individuals had different learning curves (Figs. S2.1 and S2.3) and some were much better learners (range 23-15 correct choices in 32 visits, corresponding to binomial test p values ranging 0.02-1).

Discussion

Bees were able to learn our alternating sequence task, although the effect size was not very large (odds ratio of correct to incorrect first landings for visits 25-32 was 1.58). Tests with similar binomial choices have shown comparable responses from individual honey bees, where success rates are increasing steadily at the population level but definitive learning effects are not readily observable at an individual level within 30 trials (Giurfa, Zhang, Jenett, Menzel, & Srinivasan, 2001; Howard, Avarguès-Weber, Garcia, Greentree, & Dyer, 2019).

It is unlikely that bees performed better than chance in the sequence experiment by learning cues such as sucrose odor, visual difference between sucrose solution and water feeders, or scent marks on the feeder. Our negative control experiment shows that bees do not perform significantly better than chance in the absence of the pattern sequence even if potential bee scent marks survived our cleaning process. Furthermore, if bees had had access to a reliable odor cue, we expect that they would have used that cue to find the sucrose feeder (instead of the visual pattern sequence), performing much better than what we observed in the sequence experiment results. Odors are important cues for foragers (Gould & Towne, 1988) and bees demonstrate very high success rates even when trained with odors at low concentrations (Bitterman et al., 1983; Wright & Smith, 2004).

In our sequence learning experiment, we randomized feeder location on the platform to reduce the effect of visual landmarks on landing behavior (Kheradmand, Cassano, Gray, & Nieh, 2018), but could have confused the bees and diminished their ability to focus on the correct visual cue. Increased uncertainty lowers learning in many animals (Anselme, 2018). Bees in the positive control experiment had a much higher percentage of landing correctly (70%) than in the sequence experiment (58%). However, this percentage of correct choices is much lower than the 85-90% typically reported in studies where one choice is always rewarding and

the other always unrewarding (Gould & Towne, 1988; van Hateren, Srinivasan, & Wait, 1990). Testing the effect of randomizing the location on choice accuracy would be interesting for future studies to examine given that a standard safeguard against site bias and side preference involves swapping feeder locations or visual stimuli after each bee visit (Scheiner et al., 2013).

There is substantial variation in animal behavior and learning (Gomez-Marin & Ghazanfar, 2019; Raine & Chittka, 2012). Not all bees learn the sequence successfully. As the costs of making an incorrect decision increase, one expects to find stronger selection for accurate choices. The artificial flowers in our experiment were only separated by 10 cm, a negligible distance for bees to cover, either in time or in energetic costs. Thus, it may not be surprising that bees performed only somewhat better than chance at a fairly difficult sequence task (58%) and much better (70%) in the positive control experiment. There are several possible explanations for the relatively poor performance of our bees in the sequence experiment. Bees may learn the correct rule (“the pattern that rewarded me last time will not reward me this time”) but suffer from a poor ability to remember which pattern they last visited because they have been repeatedly and equally rewarded at both patterns. They may make mistakes by failing to distinguish between the patterns on some visits. Individual behavioral preferences may play a role such that bees with a strong tendency to explore novel food sources (scouts) could score well without learning the rule, especially in high-uncertainty conditions. However, foragers that have a strong tendency that stick to recently rewarding food sources (as shown in our positive control experiment) should find the alternating sequence more difficult. Fruit flies have a tendency to turn in an alternating manner in a maze with two sequential turns (May & Wellman, 1968), but the effect has a different time scale than the alternating sequence in this study, and may be a side effect of the fly trying to reorient to its original heading.

Even humans can fail to learn the abstract rules governing certain stimulus transitions, but still develop correct statistical biases towards certain transitions (Spiegel & McLaren, 2006). Statistical learning occurs in many different contexts and forms, and at present, no single mechanism explains how particular stimuli or action sequences are learned (Thiessen, 2017). Although the architecture of the insect brain suggests the plausibility of statistical learning in insects (Huerta, 2013), our study is the first to demonstrate the ability of an insect to learn visual sequences at this temporal scale.

We conclude that bees can learn a simple alternating sequence of rewarding visual patterns, an ecologically feasible scenario that honey bees may face in their daily foraging, since floral nectar rewards decrease as a result of consumption and can gradually replenish. Thus, statistical learning is not limited to animals with large brains such as mammals, suggesting that perhaps the ability to learn sequences is a basic function of all nervous systems (Arena, Calí, Patané, Portera, & Strauss, 2015). Our results show that honey bees can learn a sequence with intervals lasting much longer than a few seconds, an ability that is relatively poorly understood (Garcia et al., 1966). The honey bee is a fascinating model for animal cognition (Avargues-Weber, Mota, & Giurfa, 2012; Menzel, 2012; Srinivasan, 2010) and the relative simplicity of its nervous system may help us discover the mechanisms by which complex tasks can be solved with simple solutions.

Acknowledgement

Chapter 2, has been submitted for publication of the material as it may appear in Kheradmand, B., Chan, S., Richardson-Ramos, I., Nelson, C., & Nieh, J. C. “Statistical learning in honey bees.” The dissertation author was the primary investigator and author of this paper.

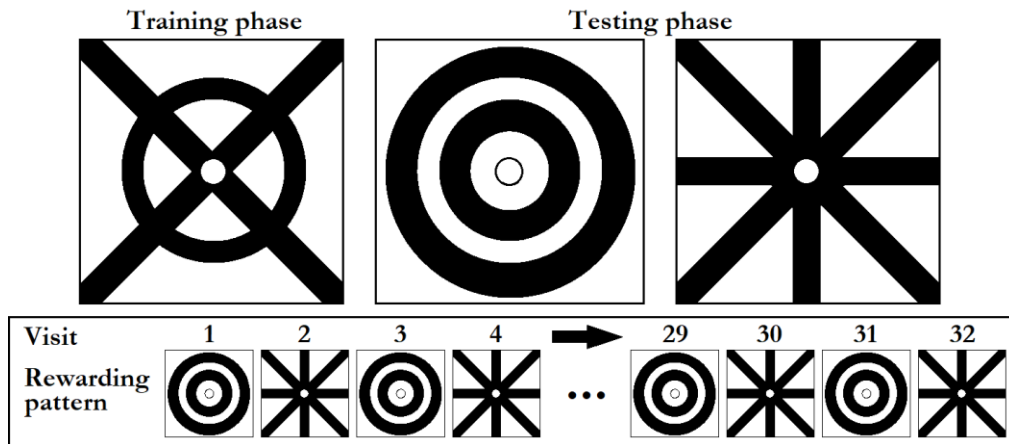


Figure 2.1. Feeders and patterns used in training and testing phases. The visual patterns (top) were drawn on plastic overlays and set over the feeder plates. The pattern on the left was used in the training phase and the patterns on the right were used in the testing phase. The bottom panel shows the sequence (the rewarding pattern on each visit).

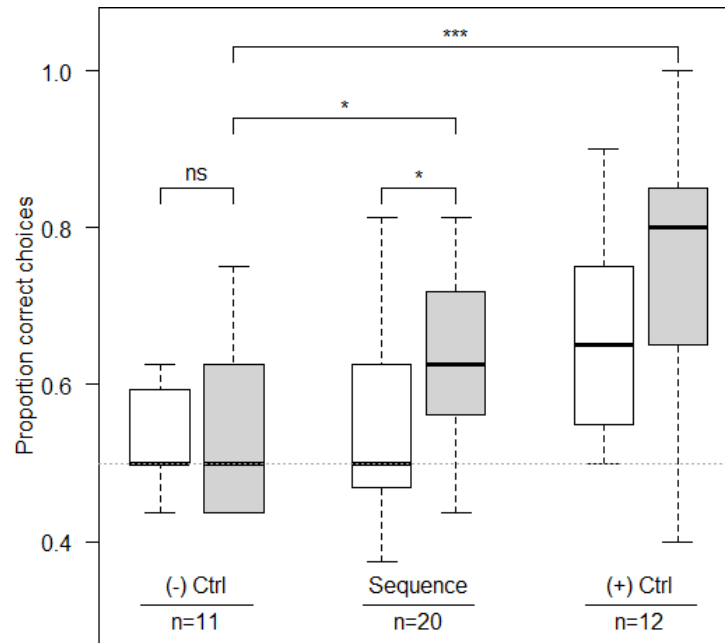


Figure 2.2. Bees learning the sequence with two different patterns are correct significantly more often than bees trained to two identical patterns. Twenty bees were tested for 32 visits with two different patterns being rewarding in an alternating sequence (center two groups). Eleven bees were tested with two identical patterns being rewarding in an alternating sequence for 32 visits, serving as a negative control for any odor cues (leftmost two groups). Twelve bees were tested with two different patterns, one constantly rewarding and one constantly unrewarding for 20 visits (rightmost two groups), demonstrating the ability of bees to distinguish the patterns. White bars represent the first half of visits and the grey bars represent the second half. Bees performed better in the second half of the visits than the first half in the sequence experiment (paired t test, $t_{19} = -2.79$, $P = 0.012$), but not in the negative control experiment (paired t test, $t_{10} = -0.3$, $df = 10$, $P = 0.76$). In the second half of their visits, the bees in the sequence (Fisher's exact test, $P = 0.044$) and the positive control experiments (Fisher's exact test, $P < 0.001$) had significantly more correct landing choices than the bees in the negative control experiment. The three bars on the right are all significantly different from 0.5 (all three one-sample t tests, $P < 0.005$).

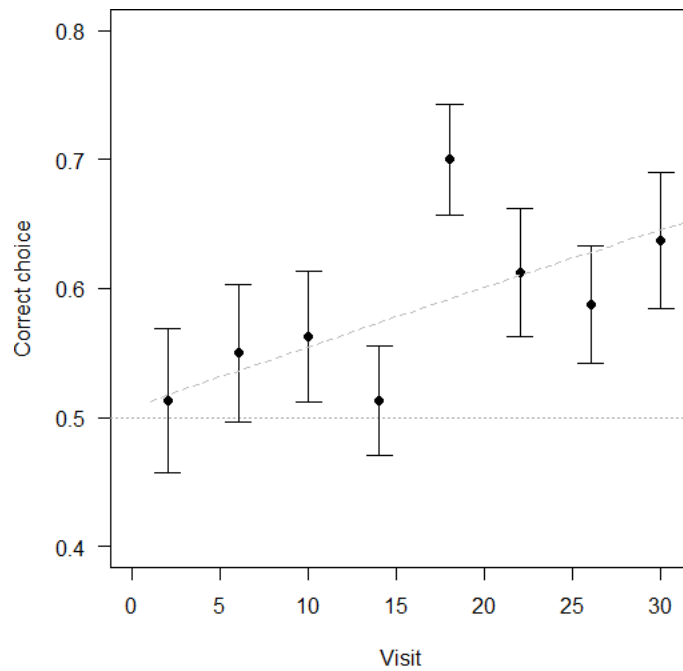


Figure 2.3. Correct landings increase as bees gain more experience. Each point represents the average of 4-visit binning of each bee's correct choices. The solid grey line shows average chance expectation of 50% success and the dashed grey trendline shows the logistic regression success as a function of visit numbers (repeated measures for each bee, $z = 2.17$, $P = 0.030$). Error bars represent standard error for 4-visit bins for bees tested on the visual pattern sequence ($N = 20$), although this is used only for visualization purposes and the data are not analyzed in bins.

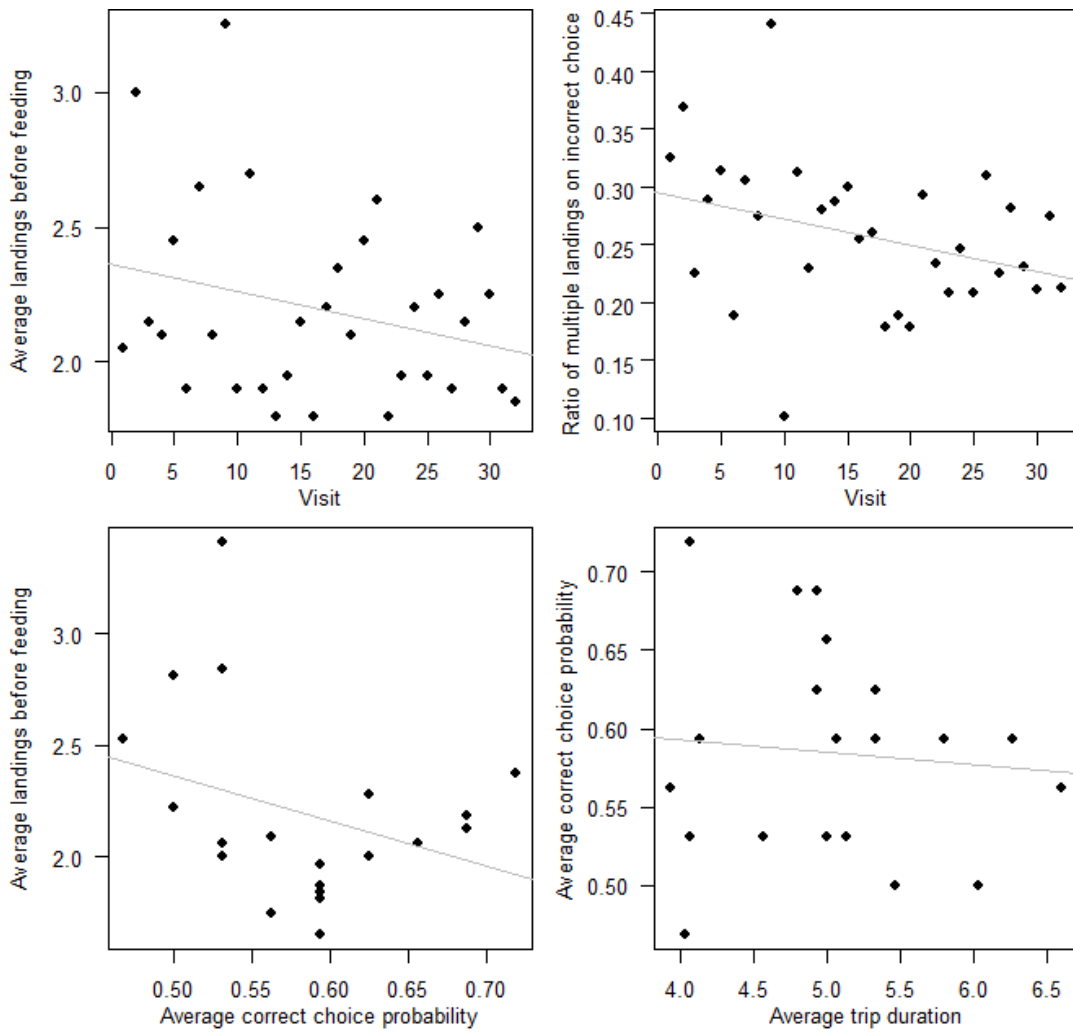


Figure 2.4. Bee landing behavior does not change significantly through experience. 4a) No correlation found between experience and number of landings before feeding (averaged for all bees on each visit, $P = 0.141$). 4b) Weak correlation found between experience and proportion of landings on the incorrect feeder (averaged for all bees on each visit, $P = 0.063$). 4c) No correlation found between correctness of first landing choice and number of landings before feeding for each bee. Each bee is represented as one point on this graph ($P = 0.168$). 4d) No correlation found between average trip duration and correctness of first landing choice. Each bee is represented as one point on this graph ($P = 0.709$).

Supplementary figures

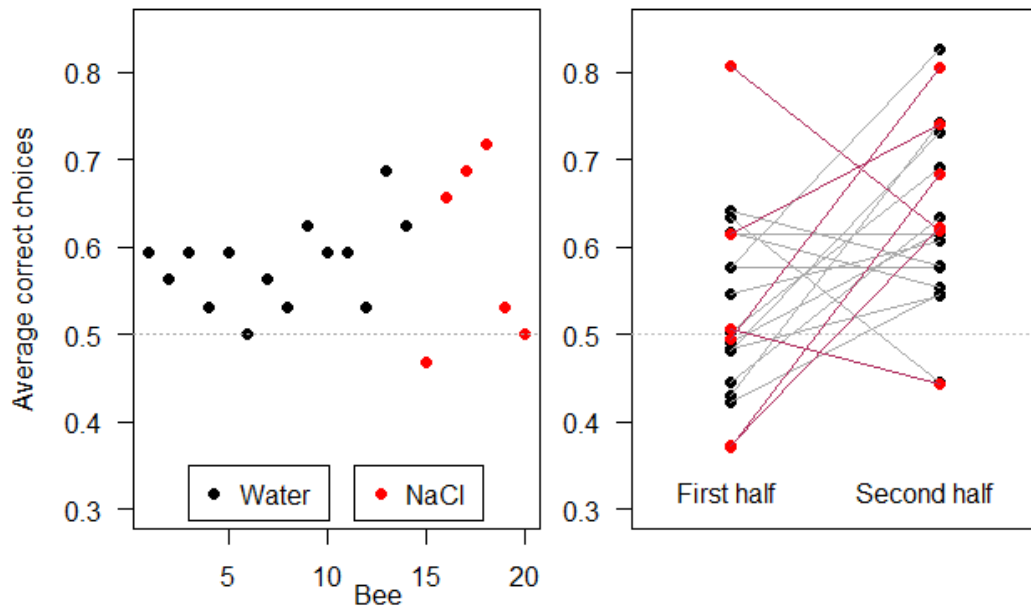


Figure S2.1. Success rates for each bee for the sequence experiment. Each dot represents the proportion of correct first landings for each bee on its 32 visits. The dotted grey line shows the average chance expectation of 50% success. The black dots represent bees that received distilled water as their non-rewarding solution, whereas the red dots represent bees that received 3M NaCl as their non-rewarding solution. The panel on the left shows average success of each bee averaged over 32 visits. The panel on the right shows each bee's performance during its first half and second half of the visits. Bees performed better in the second half of the visits than the first half (paired t test, $t_{19} = -2.79$, $P = 0.012$)

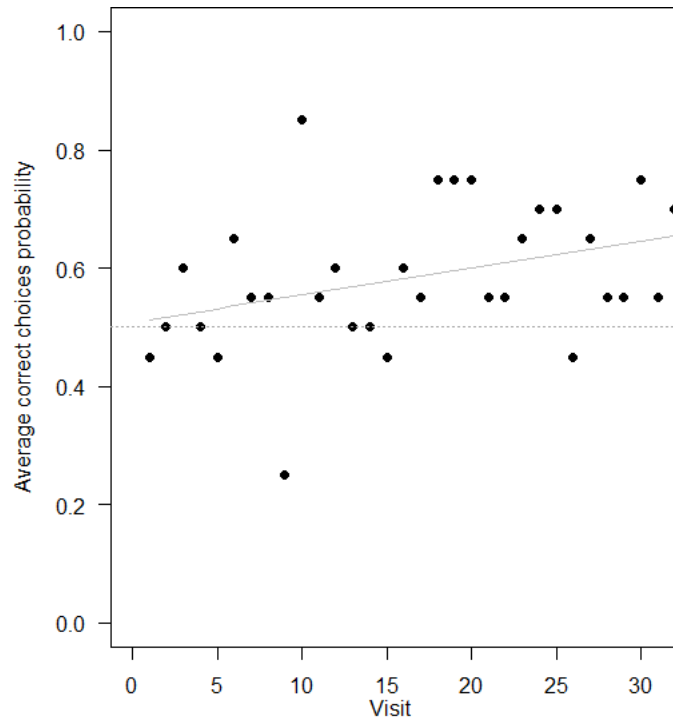


Figure S2.2. Overall learning in the population. Correct choices increase significantly as the bees continue visiting the feeders (grey solid trendline based on logistic regression, success as a function of visit numbers as repeated measures for each bee, $z = 2.17$, $P = 0.030$). The dashed greyline shows average chance expectation of 50% success.

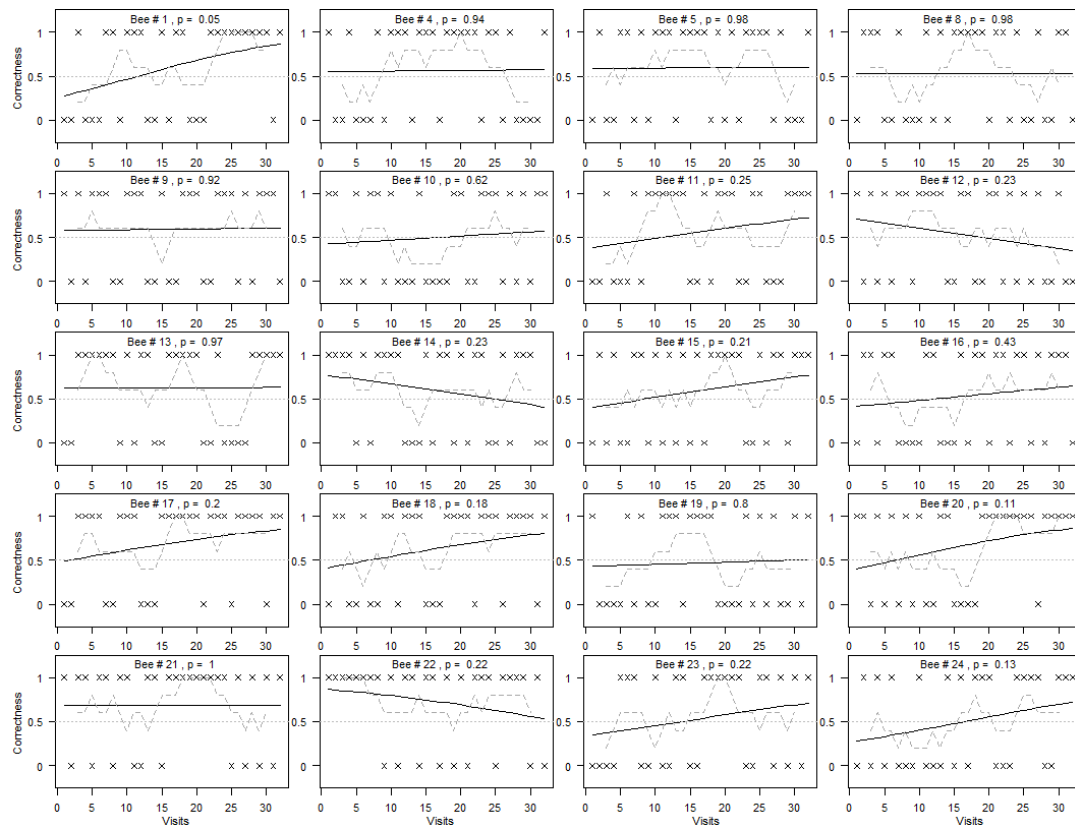


Figure S2.3. Performance of individual bees. Each panel depicts the choices of each bee in the sequence experiment. Bee identity and the p value for the logistic regression on a bee's learning during the visits is shown above each panel. The black solid trendline shows the logistic regression, the grey dashed trendline shows the 5-visit moving average of correct visits for each bee, and the flat grey dotted line shows 50% correctness expected by chance. Bees 2, 3, 6, and 7 did not return more than 30 visits and were not included in any of our analyses.

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Chapter 3: Influence of visual targets and landmarks on honey bee foraging and waggle dancing

Abstract

Animals use diverse sensory stimuli to navigate their environment and to recognize rewarding food sources. Honey bees use visual attributes of the targeted food source, such as its color, shape, size, direction and distance from the hive, and the landmarks around it to navigate during foraging. They transmit the location information of the food source to other bees if the food source is highly rewarding. To investigate the relative importance of these attributes, we trained bees to feeders in two different experiments. In the first experiment, we asked whether bees prefer to land on (a) a similar feeder at a different distance on the same heading or on (b) a visually distinct feeder located at the exact same location. We found that, within a short foraging range, bees relied heavily on the color and the shape of the food source and to a lesser extent on its distance from the hive. In the second experiment, we asked if moving the main landmark or the feeder (visual target) influenced recruitment dancing for the feeder. We found that foragers took longer to land and danced fewer circuits when the location of the food source or a major landmark associated with it changed. These results demonstrate that prominent visual attributes of food sources and landmarks are evidently more reliable than distance information and foraging bees heavily utilize these visual cues at the later stages of their journey.

Introduction

Animals depend upon learning environmental cues to exploit resources. They use multiple modalities and learn multiple stimuli, including visual, olfactory, tactile, and acoustic

attributes of the resource or its immediate surrounding (Bingman & Cheng 2005, Gould & Gould 2012). This learning is particularly important for central place foragers, animals that navigate from their nest to target resources and back (Dyer & Gould 1983, Wehner et al 1996, Able 2001). To achieve this complex task, animals use multiple navigation strategies. Birds, mammals, and insects such as honey bees can path integrate by calculating the overall direction and distance between a starting point and a destination by memorizing the directions and distances of path segments (Mittelstaedt & Mittelstaedt 1982, Muller and Wehner 1988, Chittka et al 1995, Etienne et al 1996, Stone et al 2017). Such navigation can rely on visual cues, such as the position of moving celestial objects, and visual attributes of the resource and fixed, conspicuous objects nearby (Greene & Cook 1997, Collett & Graham 2004).

Honey bees can measure direction from solar information (Rossel & Wehner 1984) and distance, mainly from integrating the amount of visual motion, optic flow, passing by their eyes (Esch & Burns 1995, Srinivasan et al 1997, Dacke and Srinivasan 2008). A few animals, such as humans and perhaps even honey bees (Menzel & Greggers 2015, but see Cruse & Wehner 2011), form a cognitive map of the environment and assess their location on this map-like representation. A very common strategy is to also use landmarks, objects that are easily recognizable, conspicuous, spatially persistent, and in the proximity of target. The size, color, shape, and patterns of these landmarks, of objects en route, and the resource itself can all be used (Cartwright & Collett 1982, Dyer 1996).

Foraging bees are excellent models of animal navigation because they make multiple return trips per day to good resources (von Frisch 1967). Bees develop diverse memories from multiple modalities, and it is known that some memories are preferred over others due to a hierarchy of relevance and reliability that allows bees to use available information to forage

efficiently (Gould & Towne 1988). We investigated the relative importance of two of these memories: distance information and visual attributes of the food source.

We hypothesize that bees rely on the memories of features that are associated with a food source, such as its exact shape and color, rather than memories of features that are different for different individual resources of the same type, such as their distance or direction. We only tested the effect of distance compared to visual features of the target in our experiments. Researchers train bees to a specific location by gradually moving the feeder away from hive entrance. This demonstrates that once bees learn the visual features of a feeder, they can follow it around as it moves as long as the moving speed is slow and the food source is conspicuous (von Frisch 1967). Many studies have shown the dominance of landmarks in finding food sources, and the target itself is one of the most important visual cues used in honey bee navigation (von Frisch 1967, Cartwright & Collett 1982, Chittka & Geiger 1995).

Honey bees are also a useful model for studying navigation because of their ability to communicate the location and the quality of a food source to nestmates via the waggle dance (von Frisch 1967). Bees forage optimally and therefore minimize foraging trip duration and flower manipulation time while advertising the resources that have the highest returns to other bees (Waddington & Holden 1979, Wells & Wells 1983, Pyke 1984). For highly profitable resources, bees increase the number of dance circuits that they perform and visit the food source more frequently (von Frisch 1967). This perceived profitability depends on several factors, such as the hunger state of the hive (which is contingent upon past and present resource availability), current resource quality, colony need for the resource (Seeley 1992), overall hive activity levels, and even the weather and plant species being visited (von Frisch 1967). Thus, the repetition of waggle dance circuits by an individual dancer integrates multiple sources of information and is

a readout of individual forager judgments while also reflecting colony need and food availability.

The ephemeral nature of floral resources requires individual foragers and the colony to constantly seek out new food sources. Bees are known to dance for newly-discovered food sources that maintain profitability over the course of a few visits (von Frisch 1967), but we asked if a new food source might be considered more valuable by a forager that has repeatedly visited the same food source for an extended time. In other words, will novel food increase dance effort? Because bees consider multiple properties of a nectar resource such as its flow rate, sugar concentration, and distance from the hive in determining food quality (Dyer 2002), we wished to test a manipulation that would not alter the net energy that bees could obtain from the resource significantly. We therefore changed the landmarks surrounding the food to test if this would alter dancing. Specifically, we tested if associating a relatively inconspicuous food source with a nearby conspicuous landmark would result in bees relying on the landmark and if separating the food source from the landmark by a very short distance would change forager visits and dance effort.

We therefore conducted two experiments. The first tested the relative importance of visual properties of the target and its perceived distance from the hive on forager bees' landing choice. Given the importance of visual attributes of the target established by the first experiment, in our second experiment we explored whether altering a major landmark associated with a food source would alter foragers landing behavior and their perceptions of food value, as measured by the number of dance circuits performed by each forager upon returning to the nest and the number of visits per unit time to the food source.

Materials and Methods

Experiment 1: Visual properties of a target versus its distance

We performed these experiments in June-October 2016. We used 10 colonies of *Apis mellifera ligustica* located in an apiary at UCSD Biological Field Station in La Jolla, California. We trained bees to a feeder in a tunnel that amplified the optic flow they perceived. On each day, we began training by putting the feeder close a focal hive. We used a new focal hive each day, but it was possible that bees from other hives could visit the feeder and become trained to the tunnel. However, this should not have influenced our results since our goal was to ensure that we used bees from multiple colonies to ensure adequate colony-level replication.

Tunnel

Our tunnel was 9.5 cm wide, 18 cm tall, and 8 m deep. At its 8 m end, the tunnel was blocked with white cardboard. The tunnel rested on 80 cm tall sawhorses and was positioned in an open field such that no overhead landmarks were visible to bees inside the tunnel. Tunnel walls were lined with alternating 2.5 cm black and white vertical stripes on the sides. The tunnel floor also had the similar stripes perpendicular to its long axis (Fig. 3.1A). The tunnels were covered by a thin black mesh on the top that allowed bees to see the sky, UV light, and polarized light patterns, but forced bees to fly through the tunnel to access the feeder (Fig. 3.1). Before each test, tunnel walls and the mesh were cleaned with water to remove odor marks. Although this cleaning method may not have removed all odors marks, the tunnels were built in 1.2 m long segments that were frequently and randomly shuffled during the training phase to avoid odor mark accumulation that could guide bees to the feeders. Most importantly, prior to testing of each bee, the wall segments were cleaned and shuffled again.

Artificial flowers (feeders)

We used two types of feeders distinguished by their distinct colors and shapes (Fig. 3.1 C & D). The first type was a bright yellow cuboid (3.9 cm * 3.9 cm * 1.8 cm) with a cylindrical hole in the center (1 cm wide, 0.8 cm deep) holding the sucrose (hereafter referred to as YS, short for Yellow Square). The second type was bright blue circular Petri dish (diameter 5.2 cm, height 1.4 cm) painted blue on the outer side and bottom and was covered with an aluminum mesh (hereafter referred to as BR, short for Blue Round). These feeders were filled with 2.0 M unscented sucrose during the training phase. During the testing phase, we used empty unscented feeders that were placed at one or more of three possible distances in the tunnel: 2.4 m, 4.8 m, and 7.2 m. Based upon the ease with which bees oriented to and landed on these feeders, they were evidently easily noticed by bees inside the tunnels, and served as targets to which bees would navigate.

Training

Following standard methods (von Frisch 1967), bees were trained to a feeder placed on top of a flat white disc (25 cm radius) on a tripod (80 cm high). The tripod was initially set up at the entrance of a focal hive and gradually moved to the tunnel entrance. We then placed the feeder inside the tunnel, resting on a transparent plastic stand (4 cm high, as shown in Fig. 3.1C & D) and slowly trained the bees to fly 4.8 m inside the tunnel. Bees then visited the feeder for at least 2 h until they were proficient in flying through the tunnel to reach the feeder. At the beginning of this 2 h period, bees were individually marked on their thoraces with different colors of acrylic paint. On average, each bee visited the feeders every 7-10 min, which resulted in 12-15 total visits at the feeder's final location per bee before testing. In all conditions except one, the training feeder was YS, and in one condition (designed to test for innate color preference), the training feeder was BR (Table 3.1).

Testing conditions

In each testing condition, artificial flowers of certain types were put at different distances in the tunnel (Table 3.1). Based on the options, bees landed at the feeder at the distance to which they had been trained (4.8 m), the closer feeder (2.4 m), the more distant feeder (7.2 m) or did not land at all during 2 min (see below). To begin the testing phase, we allowed one bee to enter the tunnel and then blocked the tunnel with a piece of white cardboard, thereby limiting our assay to one bee at a time and preventing the choices of other bees from potentially influencing the behavior of the focal bee. A choice was defined as a bee landing on a specific feeder or not landing on any feeder during the time it spent inside the tunnel. The landing choice, flight time until landing, and behavior prior to landing was recorded by two observers. Observers moved frequently along the length of the tunnel to avoid becoming landmarks. Each condition was tested on 20 different bees, except for condition 0, which was tested on 29 bees. In total, we tested 189 bees (approximately 17 bees per colony). Based on previous observations, we knew that bees had a wide range of visit rates. During 2 hours, a few bees visited the feeder inside the tunnel more than 20 times, while some had fewer than 10 visits, and most bees returned around 15-18 times. By randomizing the testing condition for each bee, and testing a large number of bees in each condition over multiple days, we ensured that differences between bees in different conditions were not due to variation in visit rates.

Most bees landed on feeders, but a few bees landed on the tunnel floor or on the mesh covering the tunnel. If a bee continued to fly toward a feeder and landed on it, we included it in our data. We gave each bee 2 min to land on a feeder. We chose this time limit based on average flight time of around 30 s that our bees took to reach the feeder when it was placed at the 4.8 m tunnel position during training. Any bee that landed elsewhere, or that failed to land within 2 min, was scored as not landing. We used a 2 min limit to test as many bees as possible before they became unmotivated due to waiting at the tunnel entrance. We refer to the setup of the

feeders with a three-letter code that specifies color and position. For example, condition 7, in which there was a BR feeder at 4.8 m and a YS feeder at 7.2 m is abbreviated as “_BY” (the underscore refers to no feeder at the first position of 2.4 m).

Experiment 2: Novelty and motivation

From June to August of 2017, foraging honey bees from two different two-frame observation hives each containing approximately 2000 bees (von Frisch 1967) were trained to a small, clear 2.0 M unscented sucrose feeder in the early morning (around 8:00 to 9:00 am), and individually painted on the feeder. The target feeder was a transparent Petri dish (diameter 4 cm, height 1 cm) covered with aluminum mesh and filled with sucrose solution placed on top of a 1 m high tripod. We did not use odors during training to emphasize the visual features of the large local landmark: a bright blue umbrella (Wondershade™, Portable Umbrella, Royal Blue, Model number: MEWS21A-WS2RY) 1.5 m radius, height set to approximately 1.8 m, Fig. 3.3A) that accompanied the feeder throughout the training phase, and considerably changed the panorama of the foraging grounds due to its conspicuous height and color. In preliminary trials, we found that bees could easily learn the appearance of this blue umbrella and use it as a landmark in an otherwise relatively visually monotonous environment, a dirt road bordered by a chain link fence on one side and by bushes of approximately the same height and foliar density on the other side. We individually marked and trained bees to a site 80 m from the focal colony and recorded the number of times each bee visited the feeder within each 15 min period. We also noted if bees immediately landed or hesitated when they came within 1 m of the feeder. At the observation hive, a second observer counted the number of dance circuits each bee performed for the food source.

The experiment consisted of a before-move phase (2 h) and an after-move phase (1.5 h). At the beginning of the after-move phase, we separated the large landmark from the feeder

by 6 m, a distance far enough to confuse the bees but not discourage them from searching for the feeder around the umbrella. This is a very short distance relative to the normal flight distances (>6 km) of a foraging honey bees, (Beekman and Ratnieks, 2000), and therefore likely incurred only a slight increase in energy expenditure. We either moved the umbrella (large local landmark) to the side and retained the feeder and tripod (target) at the training position or kept the umbrella at the training position and moved the feeder and tripod to the side (Fig. 3.3B). Of the twenty-one bees that were successfully trained and continued to visit and dance, ten were from colony 1 and eleven from colony 2.

Statistical analyses

Experiment 1: Visual properties of a target versus its distance

To calculate the probabilities of landing if bees matched target image (shape and color) or distance, we used a multinomial model (Riefer & Batchelder 1988) with the following assumptions. First, on each feeder bees can decide to land or not land. Second, to simplify the model, bees were assumed to land (or not land) only at three locations: 2.4 m, 4.8 m, and 7.2 m. At each location, the bee checks if the distance or the target match its prior memory and then lands with a certain probability. We calculated a third possibility, which is a baseline tendency of bees to land at any given spot, because bees may tend to land on any conspicuous object. An example decision tree is shown in Fig. 3.2.

We built a decision tree for each test condition (Fig. S3.1) and ran simulations with different parameter level combinations to optimize our estimates of landing probabilities based upon whether bees used target image or distance memory. Each of our three parameters were tested in the range [0-1] with intervals of 0.025, resulting in $41 \times 41 \times 41 = 68921$ parameter combinations. For each combination, we simulated 20 model bees that behaved with the given

probabilities for each of our conditions (except condition 0, the color control). We then calculated the difference between our model and actual results for each condition and used a cost function (sum of squares of distances over all conditions) to assess the accuracy of the estimated behavior for each parameter combination. We then chose the combination (out of 68921 combinations) that minimized the cost function. We repeated this process 1000 times and obtained the distribution of each of our three parameters. Our experimental conditions were designed to enhance the estimation power of our model by creating highly different decision trees (Fig. S3.1) for the bees. For bees that landed within our 2-min interval, we compared the duration of flights between different conditions with Student's t test. We applied the Dunn-Sidak correction ($k = 2$) for multiple tests with the same data, denoting results that pass this test as "DS". We used the R software, version 3.4.3 (R Development Core Team) to build this model and to run all statistical tests. Graph 6b was drawn using package ggplot2, and all other graphs and statistical tests were performed using the core package.

Experiment 2: Novelty and motivation

To focus on the effect of the separation, we looked at the eight visits before and eight visits after moving the landmark. We chose a limited set of visits to equitably compare the before and after behavior of each bee, given that all bees did not perform the same number of visits before and after separating the landmark. We selected eight visits because it corresponds to 1 h, on average, provides a sufficient number of data points per bee for analyses. Other studies (De Marco & Farina 2001, Gil et al 2007) show that bees can change their dance effort rapidly and they can learn reward amounts and rapidly change their foraging behavior accordingly. We expect flexibility in dance behavior to be rapid, otherwise it will may be of limited use to the dance followers.

We compared the number dance circuits per visit performed by bees (averaged per bee) before and after the separation of the large landmark and the target with a Repeated Measures Analysis of Variance (ANOVA) in R. In this model, the number of each bee's dance circuits for each visit was the repeated measure (each bee is a subject with multiple visits), and type of separation (feeder moved away or landmark moved away) and experimental phase (before or after separation) were fixed effects. Visit rates in different 15 min periods were compared with a Repeated Measures ANOVA, with the 15-min period being the repeated measure for each bee, and time as a fixed effect.

Results

Experiment 1: Visual properties of a target versus its distance

As expected, bees preferred the correct target at the distance that they were trained to (4.8 m). In condition 3 (BY_), bees seemed to fly over the blue feeder without even pausing to explore this newly-added, conspicuous object. However, bees relied upon visual memories of the target much more strongly than on distance memories when the visual attributes and the distance of the target were experimentally separated. Bees would land on a feeder with 0.88 probability if the shape and color matched their training period, independent of its distance from tunnel entrance. Conversely, their landing probability at the 4.8 m feeder was 0.25, regardless of the shape and color of the feeder. The baseline landing probability was low (0.10). Model parameters are shown in Table 3.3 and Fig. 3.4.

Bee flight time in the tunnel was influenced by distance and the location and appearance of food sources along the flight path (Fig. 3.5). Bees spent less time flying when the feeder they landed on was closer to the entrance: comparing conditions 3 (BY_) and 5 (YB_) ($t = -3.93$, df

= 24.2, $p = 0.0006$ DS) or comparing conditions 3 (BY_) and 7 (_BY) ($t = 1.56$, $df = 29.8$, $p = 0.13$). They also spent more time when they could not find a feeder associated with the correct feeder type: comparing conditions 6 (_YY) and 8 (_BB) ($t = 8.33$, $df = 8.6$, $p < 0.0001$ DS) or comparing conditions 2 (YY_) and 4 (BB_) ($t = -3.16$, $df = 10.1$, $p = 0.010$ DS).

The landing choice of the bees did not depend on the color and the shape of the targets they were trained to. Bees in condition 0 (trained to BR feeder and tested at “YB_”) performed with similar probabilities as other bees (Pearson’s Chi-squared test, $\chi^2 = 6$, $p = 0.1991$). Flight time also did not differ between feeder types (YS or BR) because bees were equally able to find their training feeder, regardless of type, when the opposite type appeared along the way (conditions 0 (YB_) and 3 (BY_), $t = 0.10$, $df = 36.4$, $p = 0.92$).

Experiment 2: Novelty and motivation

The large local landmark (the umbrella) drew more attention from bees than the target (feeder and tripod). Even when the feeder stayed at the same location and the umbrella was moved away, bees always checked under the umbrella first, and, upon finding no feeder there, began to search at increasingly greater distances from the umbrella until they found the feeder. In the 15-min period immediately after landmark separation, bees had fewer visits to the feeder due to visiting the umbrella (effect of 15-min observation period on visit rate, $F_{2,36} = 3.74$, $p = 0.033$, $n = 19$). However, bees rapidly learned the new spatial relationship between the large landmark and the target and their visit rates returned to comparable levels ($t = -0.686$, $df = 109.7$, $p = 0.494$) after about 15-30 min (Fig. S3.3). Bees spent an average of 7.7 min searching after landmark separation, and this search time did not depend upon the type of separation ($t = -0.776$, $df = 13.8$, $p = 0.451$, $n = 18$).

Overall, bees changed their dancing effort when the target and the umbrella were separated (Fig. 3.6) but this was due to the temporary decrease in dancing immediately after the separation. Bees danced fewer circuits per trip in the period after separation, as shown in Fig. 3.6A and 3.6C (effect of phase, $F_{1,19} = 5.03$, $p = 0.037$). It did not make a difference if the small inconspicuous target (feeder and tripod) was moved away from the large local landmark (umbrella) or vice versa, as shown in Fig. 3.6C (effect of interaction of Phase*Item Moved, $F_{1,19} = 0.86$, $p = 0.36$). However, this change was small (14% when feeder was moved away, and 12% when the umbrella was moved away).

There was variation in dancing motivation, but individuals were consistent. Bees that danced more before the move, also danced more after the move ($r = 0.832$, Fig. 3.6D). As expected, bees differed in terms of the number of dance circuits they produced (effect of bee identity, $F_{19,288} = 9.674$, $p < 0.0001$).

Discussion

Our results demonstrate that the reliance of bees on visual memories of the target is very strong, much stronger than their distance memories. We also show that changing a major landmark near a food source decreased their waggle dancing, likely due to decreased visitation because they consistently flew directly towards the major landmark. Foragers took longer to land when a food source's appearance, landmarks associated with it, or its location changed.

In experiment 1, bees showed a strong preference for the visual attributes of the food source, rather than its memorized spatial location as determined by its distance. We did not test for the effect of direction, which might also be an important stimulus and a focus for future studies. Considering the small amount of other visual features available to bees in the tunnels,

due to absence of other landmarks, feeder shape and color were likely the only prominent characteristics that bees could rely on, making the feeder a prominent visual target. Tunnels provide amplified optic flow and their minimal design might push bees to use distance information to compensate for route images, but we did not see a strong effect of landing on the food source at the correct distance if it had the incorrect shape and color. Despite the intense optic flow, bees chose to ignore it when they had access to reliable visual cues from the target. Chittka & Geiger (1995) showed that bees not only measured distances but counted landmarks to learn where a feeder is located. Menzel et al (2010) radar-tracked bees to test if bees use distance memories in conjunction with landmarks. They concluded that bees predominantly rely on the distance memory, although the number of landmarks on the path to the foods source is also used by some bees. Srinivasan et al (1997) showed that bees can use landmarks to make their navigational distance memory more accurate, but this does not necessarily translate into a more accurate waggle dance signal duration. Our experiments demonstrate that, when presented with highly conspicuous visual cues, bees primarily rely upon the shape and color if they must choose between the distance memory and image memory. It is important to note that in our experiment, as well as most related studies, researchers train bees from a hive to a given feeder by moving the feeder gradually away from the hive. This may motivate bees to rely on target shape and color instead of its location since having a moving feeder means that the location of the target is not associated with reward whereas its visual attributes are associated with reward at all times.

Bees rely on different cues at different parts of their route. Early stages depend mainly on the direction of flight, obtained from polarization patterns of the sky, whereas the later stages are guided by approaching specific landmarks and by the distance memory (Collett 1996). Further investigation of spatial information of the target and its visual attributes can elucidate

how and when each of these factors becomes the most reliable navigational cue, and the role of directional information. Open-field experiments would allow us to test these questions with a variety of distances and directions. However, the tunnel setup has the advantage of providing researchers with the opportunity to carefully observe each bee's exploration and landing choice(s) and to exclude the potential influencing presence of other foragers.

In experiment 2, our goal was to determine if local landmarks play a role in orientation (as in Tinbergen's 1935 classic landmark learning experiment with beewolf wasps) and also in determining the perceived profitability of a food source. A potential natural scenario would be the shifting or displacement of a major piece of foliage around a rewarding food source or emergence of a new flower. Trained foragers took only a few minutes of exploration near the major landmark to find the feeder. This behavior suggests that they form a visual memory from the feeder, but this information is not prioritized if the visual memory of a more conspicuous landmark (the large blue umbrella) can guide them to the food source. This result does not mean that bees ignore direction and distance to return to a food patch, but that within a patch, landmarks are highly reliable as landing cues. Our results therefore reinforce prior studies demonstrating that bees rely on several types of landmarks for their navigation to rewarding food sources (Cartwright & Collett 1982, Gould 1996). They use large and conspicuous landmarks en route to or adjacent to the food source, and they also use the visual attributes of the resource itself.

Food quality and quantity (von Frisch 1967, De Marco & Farina 2001), food competition (Nieh, 2010), overcrowding (Lau and Nieh 2010, Nieh 1993, Thom et al 2003), and predator presence (Jack-McCollough & Nieh 2015, Tan et al 2016) can all influence the number of waggle dance repetitions per forager. However, less is understood about whether waggle dancing by individuals will fluctuate when food sources are constantly profitable over long

periods of time, since most studies have involved manipulating food quality or quantity (Raveret Richter & Waddington 1993, De Marco et al 2005) rather than monitoring the long-term variations of individual bees' dance effort for an unchanging feeder. Our results show that dancers are mostly consistent over the duration of the day, but on average, changing the landmarks associated with a resource led to a short-term decrease in their dancing effort. This is likely due to the increased searching effort of foragers that did not find the food source next to the conspicuous landmark. This should decrease the profitability of the food source for that longer trip. Profitability depends primarily on the net energetic gain from the feeder, so even if the distance to the feeder is estimated to be higher due to high optic flow, shorter flight times elicit higher dance rates (Shafir & Barron 2009). Alternatively, but not exclusively, the absence of a conspicuous landmark could lower a forager's motivation since receiving the reward at the food source is associated with approaching the landmark. There is considerable variation between different bees' number of waggle circuits per foraging trip, and the decline in dancing effort may not lead to a substantial change in recruitment.

Our experiment required us to use bees that were highly motivated to return to and dance for our food source during training and test phases. The dancing motivation of foragers can be quite variable (von Frisch 1967), but foragers that are consistently motivated to dance are likely important colony sources of foraging signaling. The number of returns to the feeder did decline right after the separation, but this could be a result of bees taking longer to find the food source, not from a reduction in their willingness to search for the food.

These results suggest that familiar food sources are preferred to novel types of resources at a familiar location. Foragers must select between forming new spatial memories and forming new food-type preferences through new search images (Goulson 2000), and the salience of each of these cues may play a role in the ontogeny of a forager's path throughout its lifetime. An

interesting and open question is therefore how bees choose the landmarks they rely on, what exactly constitutes landmark conspicuousness, and how this influences navigation routes (Lindauer & Warwick 1958, von Frisch 1967).

Acknowledgement

Chapter 3, in full, is a reprint of the material as it appears in Kheradmand, B., Cassano, J., Gray, S., & Nieh, J. C. (2018). Influence of visual targets and landmarks on honey bee foraging and waggle dancing. *Insect science*. The dissertation author was the primary investigator and author of this paper.

Tables

Table 3.1. The locations and types of feeders used in testing the role of distance and visual attributes (shape and color) of the target feeder (experiment 1). We used either Yellow Square (YS) or Blue Round (BR) feeders. In each condition (except condition 1), two feeders are located at two out of three possible distances in the tunnel. The three-letter abbreviations are provided in the first column with the underscore indicating the position without a feeder. For example, YB_ references a condition in which the yellow and blue feeders are respectively at the beginning and middle positions with no feeder at the tunnel end.

Condition	Feeder at 2.4 m	Feeder at 4.8 m	Feeder at 7.2 m
0 (YB_)	YS	BR	No feeder
1 (_Y_)	No feeder	YS	No feeder
2 (YY_)	YS	YS	No feeder
3 (BY_)	BR	YS	No feeder
4 (BB_)	BR	BR	No feeder
5 (YB_)	YS	BR	No feeder
6 (_YY)	No feeder	YS	YS
7 (_BY)	No feeder	BR	YS
8 (_BB)	No feeder	BR	BR

Table 3.2. Where bees landed in tests of distance vs. target features (experiment 1). Each landing corresponds to a single bee trained to a yellow square (YS) feeder at 4.8 m except condition 0, in which bees were trained to a blue Round (BR) dish at 4.8 m) and tested in the presence of feeders indicated by the three-letter abbreviations. The three-letter abbreviations indicate feeder colors and positions. For example, “YB_” refers to a condition in which the yellow and blue feeders are respectively at the beginning (2.4 m) and middle (4.8 m) positions and no feeder is present at the end of tunnel (7.2 m). In condition 0, we tested 29 bees. In all other conditions, we tested 20 bees per condition.

Condition	Landing at the correct distance	Landing at the incorrect distance	No landing
0 (YB_)	16	8	5
1 (_Y_)	20	0	0
2 (YY_)	6	14	0
3 (BY_)	18	2	0
4 (BB_)	6	3	11
5 (YB_)	0	20	0
6 (_YY)	20	0	0
7 (_BY)	4	8	8
8 (_BB)	3	2	15

Table 3.3. The parameters of the distributions from our multinomial model for experiment 1. Each distribution consists of 1000 combinations of three parameters that resulted in the smallest cost function out of the 1000 iterations of all combinations.

Parameter	95% lower bound	Median	95% upper bound
p(Landing Baseline)	0.000	0.100	0.200
p(Landing Correct Target)	0.725	0.875	0.950
p(Landing Correct Distance)	0.050	0.250	0.425

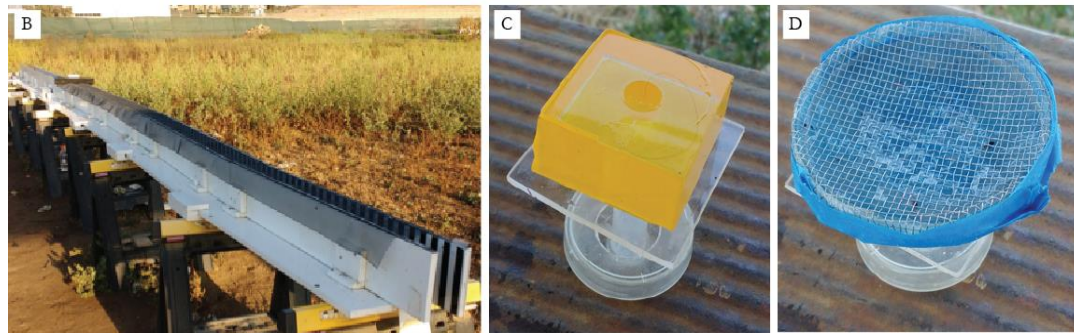
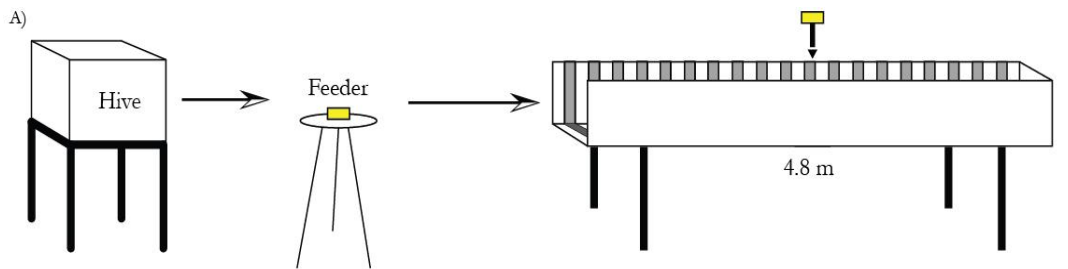


Figure 3.1. The setup for testing the roles of distance vs. target image (experiment 1). A) Bees were trained from each hive to the tunnel entrance and then into the tunnel. B) View of the tunnel set-up with the mesh on top. C) Yellow Square (YS) feeder. D) Blue Round (BR) feeder. The transparent stands are set under the feeders when the feeders are in the tunnels and are identical.

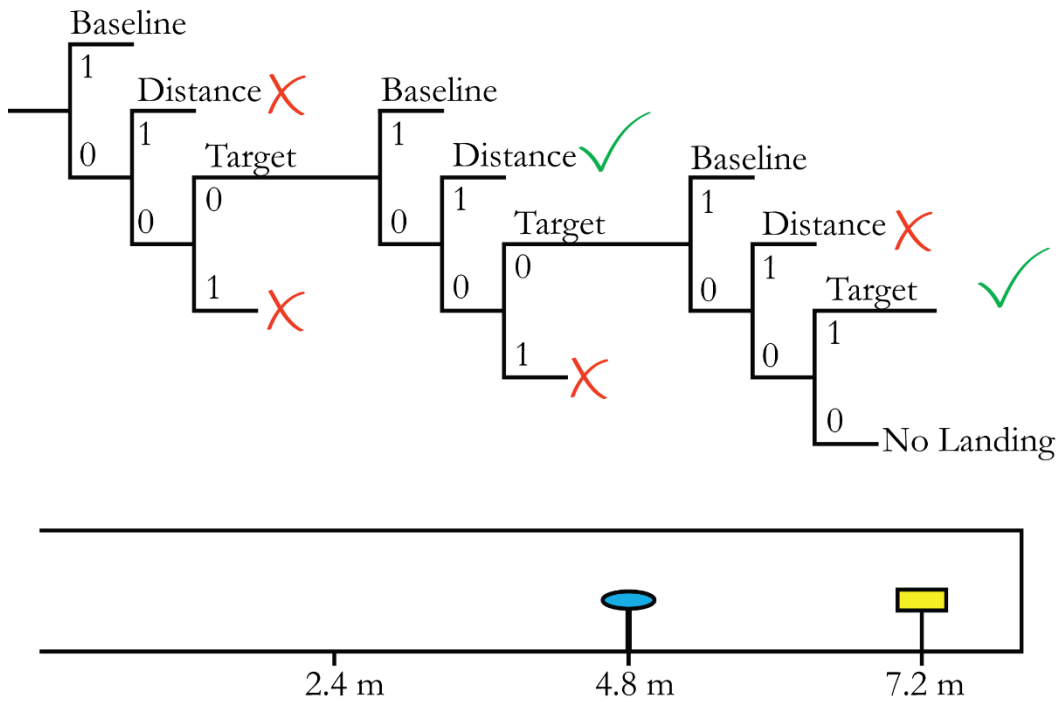


Figure 3.2. Example decision tree for condition 7 (No feeder at 2.4 m, Blue Round feeder at 4.8 m and Yellow Square feeder at 7.2 m, abbreviated as “_BY”). As the bee flies through the tunnel, it passes over three distinct segments. In the model, the bee checks at each segment if it wants to land due to a baseline landing tendency (in all segments), if it wants to land due to a match between its distance memory and current distance (only at 4.8 m) or due to matching the target it has been trained to and the current feeder (any feeder that looks yellow and is cuboidal). Its landing because of a match is determined by the respective probability: $p(\text{Landing}|\text{Baseline})$, $p(\text{Landing}|\text{Correct Distance})$ and $p(\text{Landing}|\text{Correct Target})$. In this example, the bee ignores the first segment, ignores the BR feeder, and lands on the YS feeder.

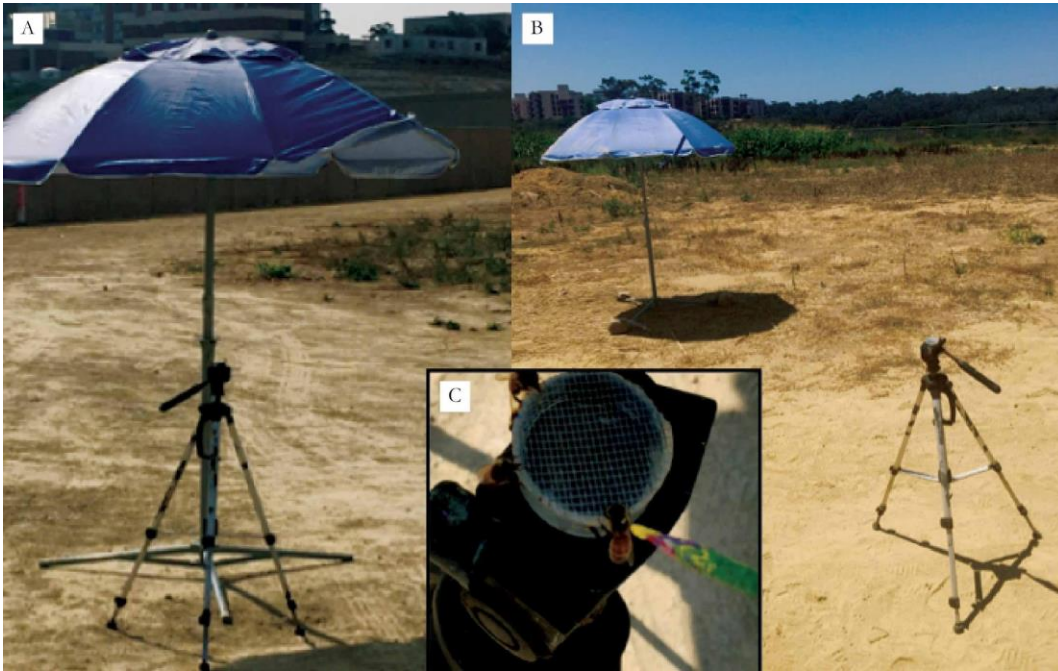


Figure 3.3. The setup of the novelty and motivation experiment (experiment 2). A) Before the separation and throughout the training phase, the major landmark (umbrella) is directly above the visual target (feeder on top of a tripod). B) After the separation, the tripod and the feeder are at the same location, but the umbrella has been moved about 6 m away to the south of the feeder while bees fly east to get from their hive to the feeder. C) Close-up showing bees on the unscented feeder. One bee is being marked with a yellow dot.

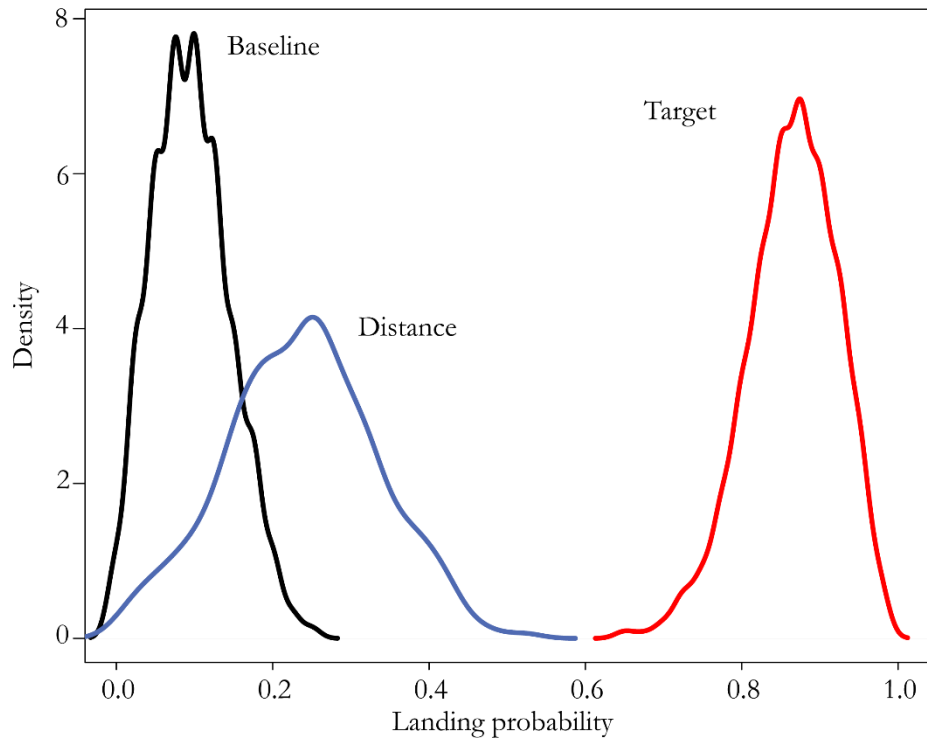


Figure 3.4. Parameter distribution densities for each matching parameter's landing probability. The black line represents the distribution of the baseline landing probability anywhere in the tunnel. The green line shows the distribution of landing probability on the feeder at the correct distance. The red line represents the distribution of landing probability on the target feeder with the correct shape and color. The distributions are not completely smooth since our parameter ranges were quantized at intervals of 0.02.

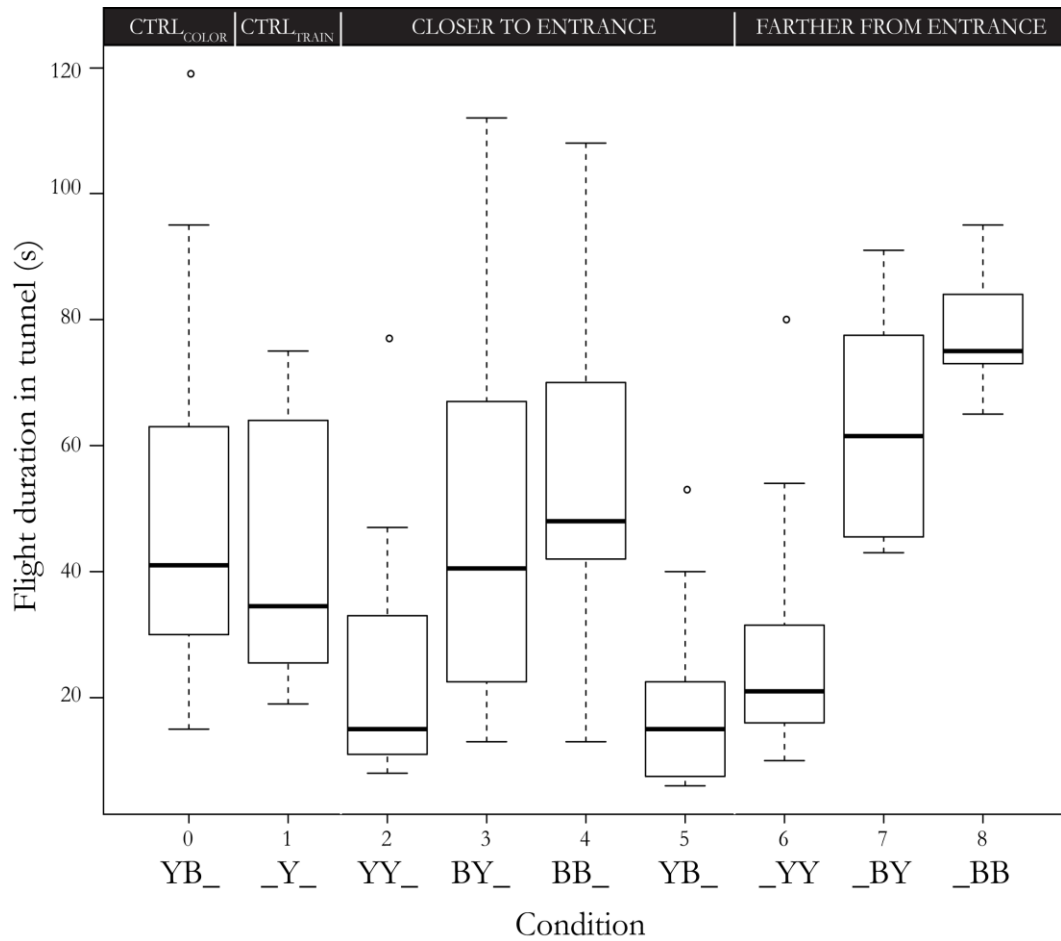


Figure 3.5. Results of experiment 1: latency to land for each test condition. Bees took less time to travel down the tunnel and land on the feeders if the feeders were closer to the entrance and were of the same type as the training feeder. The three-letter abbreviations indicate feeder colors and positions. For example, “YB_” refers to a condition in which the yellow and blue feeders are respectively at the beginning (2.4 m) and middle (4.8 m) positions and no feeder is present at the end of tunnel (7.2 m). Condition 0 serves as a control for color bias of bees. Condition 1 serves as a control for landing affinity of bees. In conditions 2 through 5, an extra feeder is added at 2.4 m. In conditions 6 through 8, an extra feeder is added at 7.2 m. Table 3.2 lists the details of feeders, training methods, and landing choices.

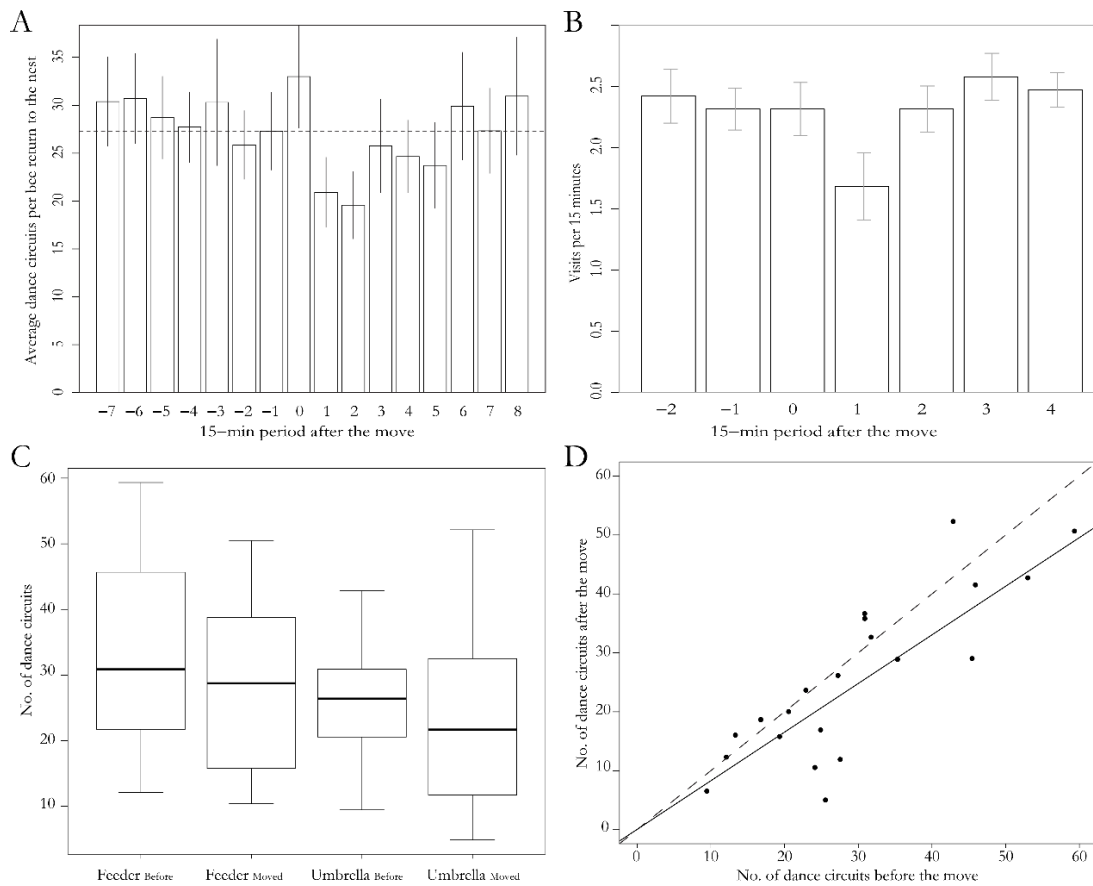


Figure 3.6. Effects of landmark displacement on bee waggle dancing (experiment 2). A) Dancing effort per trip declines as a result of separating the food source from the prominent landmark, but recovers after around 30 minutes. Separation occurs between periods 0 and 1. B) The number of visits per 15-minute period declines right after the separation of the feeder and the umbrella. However, after 15 minutes, bees have learned the new location of the feeder and visits return to their previous levels. C) The type of move (feeder or umbrella moved away) did not change alter the number of dance circuits ($n = 11$ bees for feeder moved and $n = 10$ bees for umbrella moved). D) Bees that danced more circuits before the feeder was separated from the landmark also continued to dance more after the separation. However, 62% of bees danced fewer circuits after the move. Each dot represents one bee's average number of circuits before (on x axis) and after (on y axis) the separation. Solid line: regression line $y = 0.85x + 0.313$, Dashed line: identity line $y = x$.

Supplementary figures

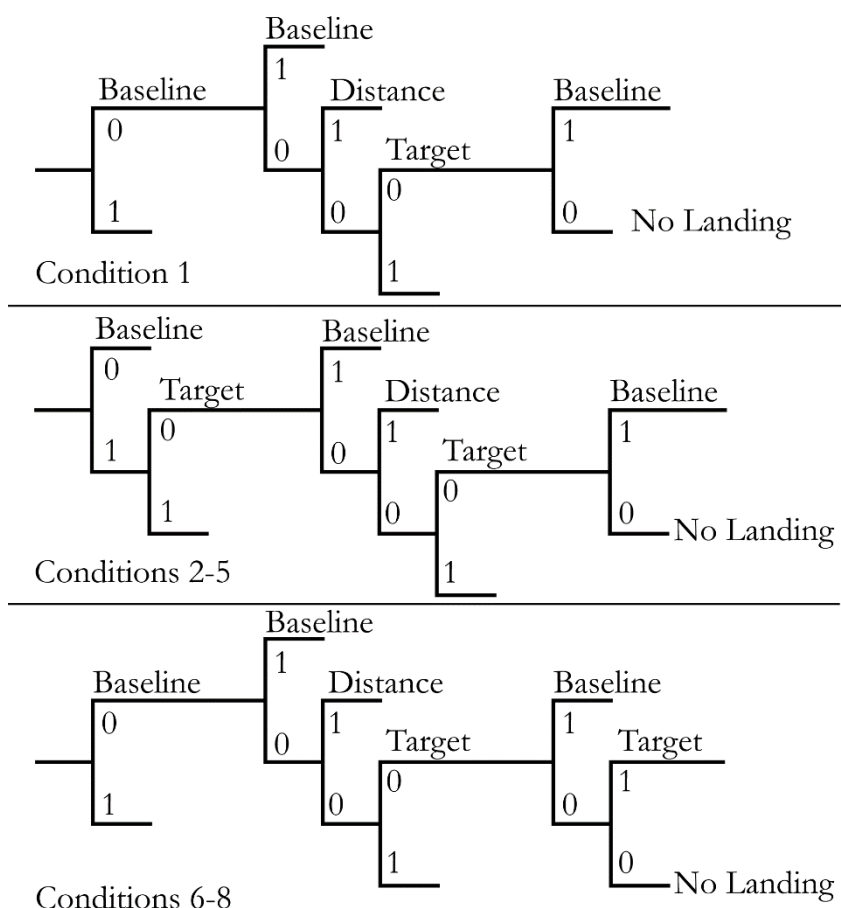


Figure S3.1. Decision trees for each test condition in experiment 1. Table 3.2 lists the details of feeders, training methods, and landing choices. Condition 1 contains only one feeder similar to the one the bees have been trained to, at the location that bees have been trained to. In conditions 2 through 5, an extra feeder is added at 2.4 m and the feeder at the correct distance (4.8 m) is either kept the same or changed to a Blue Round feeder. In conditions 6 through 8, an extra feeder is added at 7.2 m and the feeder at the correct distance (4.8 m) is either kept the same or changed to a Blue Round feeder. All three potential feeder positions offer the Baseline landing, but only the middle feeder location (4.8 m) has the distance memory matching option. Depending on the condition, each feeder can offer a matching image (when it offers a Yellow Square feeder).

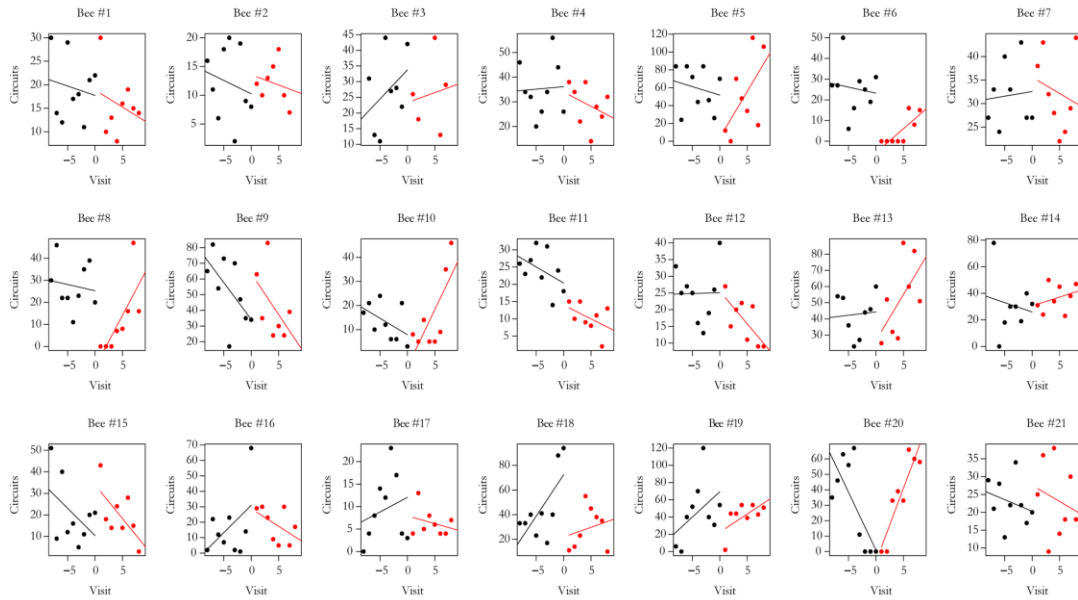


Figure S3.2. The number of dance circuits by individual bees during each visit back to the nest before and after the major landmark was moved in experiment 2. The move occurred between visits 0 and 1 and thus negative and positive visits respectively show visits before and after the landmark was moved. The circuits danced for each visit is the sum of all dance circuits that a returning forager performed during her time in the hive before flying again to the feeder. A figure-8 movement thus would count as two circuits. Black dots refer to the circuits danced after each visit prior to the separation of the major landmark from the feeder, and the black line is the linear regression for those dots. Similarly, red dots refer to the circuits danced after each visit following the separation of the major landmark from the feeder, and the red line is the linear regression for those dots.

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Chapter 4: Kinematics of the waggle-round dance continuum of honey bees

Abstract

Foraging honey bees recruit their nest mates to profitable food sources with a complex referential signal, the waggle dance, which communicates information about the direction, distance, and quality of the food source. Distance and direction are encoded in a wagging phase that is marked by lateral abdominal oscillations, wing vibrations, and often sound, which occurs between circling movements. Dances for food sources at very short distances (<50m) from the nest typically lack this wagging, and thus dances for nearby resources have been called the round dance. The transition between round and waggle dances occurs in a continuum but how this transition precisely occurs is poorly understood. Although the dances are recognized and essentially defined by their shapes, there has been no quantitative analysis of how these shapes change with increasing distance to the resource. We show that the transition between round and waggle dances is gradual and observable in many aspects of the dance. We present a single algorithm that applies to both round and waggle forms and allows for easy annotation of circuits, wagging phases, and other features of the dance based on tracked position information of dancing bees in the hive. Our algorithm clarifies the terms used to describe the dance and should facilitate the automatic detection and decoding of dances, a technique that has wide utility in understanding honey bee ecology, pollination services and potential pesticide exposure.

Introduction

The dances of honey bees have inspired generations of ethologists. Upon returning from rewarding locations such as pollen- or nectar-rich flowers, potential nest sites, and sources of

water, a honey bee communicates these locations to other bees by walking in circles (also referred to as circuits) and shaking its abdomen and wings (von Frisch, 1967). This behavior, called the waggle dance, is a multimodal signal. The dancer gives out nectar samples, the smell of the flower she has visited (Farina, Grüter, & Arenas, 2012; von Frisch, 1967) and cuticular hydrocarbon odors that excite other bees and stimulate them to exit the hive (Thom, Gilley, Hooper, & Esch, 2007). Tactile cues in the form of antennal contacts (Ai, Okada, Sakura, Wachtler, & Ikeno, 2019; Cholé et al., 2019; Rohrseitz & Tautz, 1999), vibrations in the substrate (J. Tautz & Rohrseitz, 1998; Juergen Tautz, 1996) and an air jet produced by beating the wings when wagging (Michelsen, Kirchner, & Lindauer, 1986; Michelsen, Towne, Kirchner, & Kryger, 1987; Wenner, 1962) are other ways in which the dancer gathers an audience for herself in the dark nest.

The waggle dance was originally defined by the presence of lateral abdominal waggles that the dancer performs. The dancer walks in a rather straight line during these segments, referred to as “waggle runs.” The other segments, during which the dancer is walking in semicircular arcs without wagging, are called “return runs” (Fig. 4.1 top panel, reproduced from Esch, 1961 shows the waggle dance. See Figs. 4.3 and S4.1 for other representations of example waggle dances).

A location of interest is coded in a polar coordinate system, with a direction and a distance. The direction element can point directly to the resource when the dance is performed on a horizontal surface (in *Apis florea*, Dyer, 1985) or can be transposed relative to gravity on a vertical comb, with a movement directly upwards indicating that the food source is in the direction of the sun and other angles communicating location relative to the sun’s azimuth (*A. mellifera*, *A. cerana*, and *A. dorsata*, Fig. 4.1, Dyer, 2002; Edrich, 1977).

The distance element is encoded in waggle run duration, sound duration, or physical distance covered during the wagging phase. Since these three measures are intertwined, we will focus on waggle run duration as a reliable and often-used proxy for the distance signal (Dyer, 2002). As the distance to the location of interest is increased, the duration of the waggle run, and parameters that are correlated with it (the air jet behind the dancer, number of wags, the distance walked during the waggle run, etc.) also increase (Kirchner, Lindauer, & Michelsen, 1988; von Frisch, 1967). The change is rather linear in the low to medium range distance signals, but as the distance to the location of interest increases (upwards of 4.5 km) the waggle duration reaches an asymptote (~5 s, von Frisch, 1967). On the lower end of the distance range, however, there is a limit on wagging precision. Therefore, distances shorter than 25-40 m are coded in a waggle-less type of dance, called the round dance. Since there is no waggle run in round dances, it was assumed that they lack not only distance information, but also direction information. Although with very high variability, both distance and direction information (Gardner, Seeley, & Calderone, 2008; Kirchner et al., 1988) are coded in the round dance, demonstrating that there is a single continuum of waggle to round dances. By training bees to intermediate distances (5-200 m), this continuum is easily observable, and the transition (Fig. 4.1 bottom panel, reproduced from von Frisch, 1967) was coarsely described by Karl von Frisch (von Frisch, 1967). Thus, it has been proposed to not use the discrete terms round vs. waggle dance, and see this behavior as occupying a continuum called the “waggle dance” (Gardener et al. 2008). However, the ends of this continuum are fairly well captured by the terms “round” and “waggle” dance, and we will use these terms to facilitate our discussion and analysis of this striking behavior. As von Frisch reported, different subspecies of bees vary in terms of their transitional dances, and *Apis mellifera carnica* transition in the figure-8 manner (Fig. 4.1 bottom panel, top

row) whereas *A. m. mellifera* and *A. m. ligustica* transition in the sickle manner (Fig. 4.1 bottom panel, bottom row).

Bees dance in frequently fluctuating conditions, for example due to crowdedness of the dance floor or imperfections in the flatness of the wax floor (Juergen Tautz, 1996). Therefore, the dance behavior, although easily distinguishable by its circular and repetitive nature, is highly variable. This variation is complemented by slower but also frequent changes in the environment, like temperature fluctuations, clouds blocking the sun hence changing the visual input to the bees, the sun moving, and flowers running out of nectar or pollen. Different bees vary in how they dance because of differences in motivation, which can be due to different genetic backgrounds, prior experiences of food quality, interactions with dance followers and nectar-receiving bees (unloaders). The information in the dance is more accurate as the advertised distance increases (Schürch & Ratnieks, 2015; von Frisch, 1967), and the rate of the error is explained by many factors (Beekman, Doyen, & Oldroyd, 2005; Couvillon, Phillipps, Schürch, & Ratnieks, 2012; Weidenmüller & Seeley, 1999).

The waggle dance also contains information about food source (or nest site) quality, coded in two different ways. Firstly, the number of repetitions (circuits) that a motivated dancer performs is correlated to how profitable it has assessed the food source to be, and this effort in turn leads to sending more recruits to the target (von Frisch, 1967). Secondly, the return runs are shorter as a result of faster walking speed when food quality is high (T. D. Seeley, Mikheyev, & Pagano, 2000), demonstrating that not all information in the dance is coded in the waggle run portion.

All of these sources of variation render the task of defining the waggle dance continuum more difficult, and the vast majority of descriptions of the waggle dance either focus on the waggle run or only describe the dance in a qualitative manner. Traditionally, determining if a

dance is waggle or round is done subjectively by researchers. Fully formed waggle dances, for high-quality food sources over 200 m away from the hive, have remarkable periodicity both in wagging frequency and the spacing of sequential waggle runs. But this clear trend starts to fade in shorter distances, and sometimes, even experienced observers cannot easily determine when a circuit starts or ends. Likewise, the process of measuring the parameters of the waggle run, which heavily depends on selecting a few waggle runs out of many (Couvillon, Pearce, et al., 2012), is a process that could suffer from experimenter bias, even by experienced researchers.

Furthermore, annotating the dance behavior is a time-consuming and laborious task. As a result, several recent studies have automatized waggle run detection and parametrization (Kimura, Ohashi, Okada, & Ikeno, 2011; Landgraf, Rojas, Nguyen, Kriegel, & Stettin, 2011; Wario, Wild, Rojas, & Landgraf, 2017). These studies have focused on detecting dances with clear waggle runs, which produce visual signals easily detectable by algorithms and humans alike. However, the round dance, short waggle runs, and transitory dances have been largely ignored in these pioneering efforts. This is perhaps because even after determining a dance as a round dance or a waggle dance based on presence of any wagging behavior, defining the different phases of the circuits (waggle runs, return runs, or when a circuit starts and ends) is not a trivial task, especially for the intermediate dance types. Fig. S4.7 (reproduced from a tracked dance in Jensen & Michelsen, 1997) depicts wagging interspersed with round dancing, and is a suitable example of why defining circuits may be difficult.

In this study, we aim to define the waggle-round dance continuum in clear terms, while preserving the characteristics by which human observers detect waggle dances. Human observers, even experienced bee researchers, often do not know exactly what parameters they use to separate wagging motion from other types of jitter in bee movies. This lack of clear

definitions manifests strongly when playback of movies is sped up or slowed down and the familiar 11-17 Hz waggle motion is distorted.

Previous descriptions of the dance relied heavily on the ability of observers to detect circling motions and lateral oscillations without having a clear definition. Although the waggle phase has been studied extensively, its primary definition in the literature is simply when the side to side movement of the abdomen at around 11-17 Hz is performed. The most detailed definitions come from studies aimed at creating a robot bee that can reproduce the dance (Landgraf et al., 2011), but even those studies focus only on the waggle phase. The waggle run is indeed the portion that contains the most information (Michelsen, Andersen, Storm, Kirchner, & Lindauer, 1992), however, other segments of the dance are also informative and we aimed to take a more detailed look at them.

Our goal was to develop an algorithm for detecting and parsing the different phases of the round-waggle dance continuum and thereby come up with rather strict functional definitions that include the original meanings of these behaviors (and their subunits) as defined by pioneering researchers. This algorithm would assist researchers share a common platform for counting and determining the characteristic movements in the dance, as well as helping us understand what movements are done and how the dance behavior is performed (Barron & Plath, 2017).

Methods

Bees and flight paths

During the summers of 2016 and 2018, we trained over 25 honey bees (*Apis mellifera ligustica*) from two different colonies located in an apiary at the UCSD Biological Field Station

in La Jolla, California. About 3-5 dancing bees were trained from each colony on each day to avoid overcrowding the dance floor. Our dance data were collected between 9:00 AM to 5:00 PM in observation hives containing around 3000 bees. Bees were trained to 2.5 M sucrose feeders at different distances (10 m, 25 m, 50 m, 100 m, 200 m, 300 m) in the field following the methods described in von Frisch (1967).

Video capture

Our videos were recorded on a GoPro Hero 4, at 60 frames per second with 1920*1080 pixels. However, the characteristic fisheye effect of GoPro movies needed to be removed in GoPro studio software, forcing us to automatically down sample our videos to 30 frames per second. This down sampling may have resulted in some loss of movement detection in the 10-30 Hz range if such movements coincided with the inter-frame interval. The camera was held by hand, and the cameraperson tried to keep the bee at the center of the image without changing the distance between the camera and the bee in order to avoid changes in magnification. We chose videos each containing between 3 and 12 circuits of dancing, including over 40 samples (each) of well-formed waggle runs, round circuits, and circuits of transitory dances.

Tracking

We aimed to focus on parameters easily obtainable from position information of the dancer over the course of dance, namely, the x and y coordinates for one body part (or more) for each frame. We used the Tracker software (Brown, 2017) to manually track dancing bees. For each frame of the video, we tracked the x and y coordinates for multiple points, as shown in Fig. 4.2: the tip of the head (H), middle of thorax (T) and the tip of the abdomen (A). To compensate for camera motion, we used a comb vertex reference point (R) visible throughout the entire video and a size control spot (S) on the same hexagon as (R) but two vertices away

(the width of a cell). We tracked all bees manually to ensure precision, as currently no software exists that can reliably track the tip of the abdomen of bees.

Manual annotation

Prior to developing an algorithm to characterize the dances, we categorized our videos based on the distances at which the bees foraged. We expected to detect round dances in distances under 60 m, and waggle dances in distances upwards of 90 m. Observers (experienced in watching and identifying waggle dancing based upon hundreds of hours of direct observation) watched the circuits in each video and gave each video a subjective score for wagging strength (also referred to as dancing vigor in the literature) between 0 and 1, where 0 denoted no waggles and 1 denoted strong waggles, in 0.25 increments. To develop our algorithm, we selected approximately a quarter of data in a representative manner. The rest of the data were later used as a test group to avoid over-fitting. For the training data, the frames in which bees waggled was annotated. Table S4.1 lists our sample sizes for the different experimental conditions.

Defining the dance

Many parameters can be used to describe the round-waggle dance continuum and we explored two main categories: time-independent geometric parameters (arcs, radii, body axis angle, body-axis angle variance (proxy for wagging amplitude), etc.) and time-dependent geometric parameters (walking speed, wagging duration, return phase duration, body-axis angular change, etc.). We used these parameters to independently define circuits and waggle phases. We then analyzed the temporal relationship between waggles and circuits by observing at what point during the circuit the waggle run (or in some cases two runs within a circuit) occur. We developed an algorithm in R and executed on RStudio (R core team, 2017, RStudio team,

2015) to detect circuits, waggle runs, and return runs. We used the following R packages: *plot3D*, *polyclip*, *geometry*, *pracma*, *circular*, and *knitr*.

Isolating circuits

A circuit can be defined in several ways for round or waggle dances, each with its own advantages and disadvantages. Circuits can be defined by measuring the change in the body-axis angle in consecutive frames, but the variation in body-axis angles is high and even more so during the waggle phase, rendering this measure ineffective (Fig. S4.2). If circuits are defined by sharp turns in the dancer's trajectory, then circuits in unidirectional round dances lacking sharp turns will be difficult to define. If a circuit is defined by approximately 360° circles starting at a specific absolute body-axis (head to tail) angle relative to the horizontal axis of the frame, leftward turns and rightward turns of waggle dances (which happen at different angles) would induce high error in circuit detection due to the variability of total turning angles in a circuit. Similarly, defining the circuits based on the relative angle of the line connecting the bee to the average position on the comb (absolute centroid) introduces errors when the bee is meandering between circuits and when the circuits are not centered on the same point, as in the case of alternating waggle circuits. Defining the circuit using the waggle movement at the beginning or the end would not be feasible for round dances. Since at the end of a waggle run, the direction of rotation for the next semicircular return run is determined, we expect to see the waggle run in a circuit at its final frames (Fig. 4.2, bottom left panel).

Since all these circuit definitions have shortcomings, we combined multiple methods and assigned them a hierarchy for optimal circuit detection. First, to determine the angle of rotation for a given frame, we defined a center of rotation by averaging the x and y coordinates for a large number of frames (corresponding to up to 2 seconds) before and after the given frame (Fig. 4.2, bottom right panel). We then calculated the angle of rotation using the slope of the

line connecting the abdomen to the center of rotation. We used the abdomen tip to maximize the changes observed in our parameter because the abdomen typically moves in a greater arc than the head or thorax (as shown in Fig. 4.3 middle and bottom panels). The incremental increase and decrease in the angle of the line between the abdomen tip and the centroid offered us a reliable curve with peaks and valleys on frames where one circuit ended and a new one started (Fig. S4.3). We removed short-term fluctuations in this curve by smoothing it using a locally estimated scatterplot smoothing (LOESS) function, and then removed any small peaks or valleys that occurred too close to other prominent peaks and valleys. To avoid having small peaks due to waggle oscillations and any noise from recording or tracking of the data, we applied a low-pass filter to the position data (keeping only the movements below 10 Hz), obtaining a smoother but high-fidelity version of the trajectory. The parameters used for finding the peaks and valleys were optimized on our training data set. Next, if the dancer kept rotating beyond 360° in one direction, we assigned a circuit to each 360° rotation. This ensured that both waggle and round dance circuits can be detected with high accuracy. Table 4.1 summarizes some of the features of a circuit that can be used to define and detect it.

Automatic waggle detection

Wagging motion can be defined in several ways. The most common element of the waggle, observable in our movies (30 FPS), is the sideways abdominal oscillations. Previous studies have shown the range of frequencies for the wagging movements to fall in the range of 11-17 Hz (Landgraf et al., 2011; von Frisch, 1967). During the waggle, we expect a slower walking speed since bees walk at a slow pace when abdominal wagging occurs (Jürgen Tautz, Rohrseitz, & Sandeman, 1996).

We applied Fast Fourier Transform (FFT) to parse out wagging motions of the abdomen tip near 13-15 Hz (algorithm A: wagging around 13-15 Hz). We also compared this

algorithm to a simpler algorithm that only looked at precisely 15 Hz periodicities of the abdomen tip (algorithm B: wagging at exactly 15 Hz). Given that our movies are filmed at 30 Hz, exact 15 Hz motion is easily calculated by subtracting displacements between every other (alternating) frame within a short window of frames (Fig. S4.4). We found that for the FFT and the 15 Hz algorithms, windows of 16 and 7 frames, respectively, optimized detecting short waggles without detecting false waggles (based upon observations of the waggle dance and the subjective scoring of waggle amplitude, see above). The output of each algorithm is a waggle score for every frame, and this score approximates waggle amplitude in the given window of frames. This score is objective (in contrast to the subjective scores given by observers) but does not separate waggle oscillations from non-waggle oscillations in the same frequency range, such as tremble movements resulting from low nectar concentration (T. Seeley, 1992). It is possible to control for tremble-like noise in the data by monitoring the jitter of the thorax or the head as a baseline for sideways motion. Dances with high lateral thorax or head oscillations are not suitable for waggle detection.

These algorithms have different benefits and shortcomings. Including a wide range of frequencies in waggle movement calculation (with FFT) allows detecting waggles that are not exactly at 15 Hz, but also would introduce error from oscillations that may arise from other behaviors such as tremble dances or shaking signals with similar frequencies. Both algorithms produce a waggle score that increases as a function of the amplitude of abdominal oscillations within the given frequency range. Thus, a weak waggle at the central frequency of the range can have a score similar to a strong waggle at peripheral frequencies of the range. Recording at extremely high frame rates (upwards of 120 frames per second) would allow filtering waggle scores in a narrower frequency range, but even then, other behaviors that cause shaking at the same frequency will be picked up by the algorithm. To increase the accuracy of the FFT

algorithm, we applied a high-pass filter (keeping only the 10-15 Hz movements) to the position data of each bee, then looked at the magnitude of the 13-15 Hz bin in each 16-frame window.

Since the score obtained from the FFT is not a linear function of waggle amplitude (due to including a range of frequencies other than wagging frequency, and the different weighting for frequencies farther from the mid-bin frequencies), we use the exact 15 Hz score, a linear function of the abdomen displacement, to control for the amplitude of each waggle detected using the FFT score. These two measures are congruent in strong wagging motions but only the FFT can find weak waggle motions such as those occurring in dances for shorter distances (Fig. S4.5). Defining waggle runs based on waggle scores requires putting constraints on the scores to increase the sensitivity and specificity of the scoring system. Table 4.2 summarizes some of the important constraints for defining and detecting waggles.

Although we tracked three points on the body of each bee, only the position of the abdomen tip was used to gauge the timing of circuits, waggle activity, dance radius and the area covered by the dance. The movement of the abdomen tip is biologically more relevant, so all figures show abdomen positions, unless noted otherwise. We used the head and abdomen positions in each frame to measure the orientation of the dancer's body. Head and thorax movements can help control for tremble-like movements, but we did not study it in this project. Note that the linear regressions in the results are a simple way of looking at the variation in the dance, and our data do not satisfy all assumptions of linear regressions. However, we have used linear regression as the simplest benchmark tool to coarsely assess the performance of our algorithm. Further analyses on the data will consider the identity of the dancers, the unique conditions of dancing for each return to the nest, and other potential factors.

Results

Visualizing the dance continuum

Bees perform the waggle dance in many different forms. Although the waggle phase is mostly linear, the return phase can take many different shapes. Round dances also take many forms although they follow a rather circular shape. Fig. 4.3 showcases a few of these circuit shapes. Our bees (*A. m. ligustica*) mainly transition in the sickle manner (Fig. 4.3 middle panel, similar to the transition in Fig. 4.1 bottom panel, bottom row).

Circuits

Due to the high variation in the shape of return runs, finding a perfect definition for circuits proved challenging. However, our algorithm detects almost all circuits without a major issue, even when the dancer takes detours from her expected circular path or suddenly turns in an acute angle (Fig. S4.3). The low-pass filter removes traces of waggles, making the circuit-definition an independent process from waggle detection. However, strong waggle dances with strictly alternating directionality are easier to split into circuits due to the clean change in the direction of turning. A summary of key circuit measures is given in table 4.3.

Dance radius increased as a function of flight distance (Linear regression, $F_{1,120} = 9.92$, $P < 0.01$) but area covered by circuits of the dance was the same across flight distances (Linear regression, $F_{1,120} = 1.00$, $P > 0.1$), as shown in Fig. S4.6. Most waggles occur during the early stages of defined circuits (Fig. 4.6 bottom panel). Walking speed is lower during return runs compared to waggle runs (paired t-test, $t_{29} = 2.27$, $P = 0.03$). A summary of circuit characteristic is shown in Figs. 4.5 and 4.6. Our data set and R code are available online, and all dances can easily be plotted in RStudio. Some examples of individual circuits, and a few examples of traces of a few consecutive circuits are depicted in Fig. S4.8.

Waggles

The transition between the waggle dance and the round dance is gradual. As expected, the duration of the waggle runs detected by our algorithm increases as the distance flown to the feeder increases (Fig. 4.6 top panel, bottom 2 graphs, Linear regression, $F_{1,120} = 6.317$, $P = 0.013$), as does their amplitude (Linear regression, $F_{1,120} = 73$, $P < 0.001$). The waggle scores correlate strongly to the subjective scores by experimenters (Linear regression, $F_{1,120} = 45.36$, $P < 0.001$) and waggles detected by the FFT algorithm closely resemble waggle detection by human observers (Figs. 4.6 and 4.7). However, the variability of the dances for any given distance is staggering.

Discussion

Our study quantifies and shows the gradual change from the round dance to the waggle dance, not only in terms of distance signal (waggle phase), but many other aspects of the dance, such as the area covered by the dancer, the amplitude of wagging, and walking speed. Our results complement and expand on previous efforts that described the waggle dance in more detail but did not focus on the transition between round and waggle dancing (Landgraf et al., 2011; Michelsen et al., 1992; Jürgen Tautz et al., 1996).

We introduce a new approach to quantifying waggle dances. Our algorithm is able to find circuits in the round-waggle dance continuum with minimal error. It also detects and scores waggle motions and can estimate the duration and direction of each waggle run. Our results corroborate previous findings by other researchers showing that even round dances demonstrate signatures of oscillations (Gardner et al., 2008; Kirchner et al., 1988). Our algorithm can easily be modified to automatically annotate higher frequency videos, and we predict that its accuracy

will improve with higher quality videos. However, even for low-quality recordings with a moving background, by giving a simple tracking of a bee's abdomen to our algorithm, one can objectively detect the basic elements of the waggle dance and quantify them without bias. Furthermore, our code facilitates measuring other properties of the dance not discussed here (e.g. variability in the direction signal, duration of the dance, changes in the circuits of a dance as a function of dancing time and experience, etc.).

Combining our methods with efforts by other labs to track all individuals in a colony simultaneously, and with development of better image recognition algorithms that allow for tracking not only a few points on the main body segments, but also tracking limbs and antennae, it would soon be feasible to understand how the waggle dance behavior is executed in everchanging conditions of the crowded hive. The high variability observed between and within the circuits of dance performed by different individuals demands further studies into the biomechanics of the round-waggle dance.

Our algorithm should allow us to explore the evolution of bee dance signals, which likely share a common origin and evolved via ritualization and signal diversification. Honey bees perform multiple dance-like behaviors, such as the tremble dance, the buzz run, and the shaking signal (von Frisch, 1967). These signals, interestingly, also contain waggle-like movements, but it is difficult to objectively quantify how waggle-like they are. Our algorithm should enable us to measure objectively how waggle-like these dances are and begin to test the hypothesis that honey bees use waggle-like behaviors as a form of sensory exploitation. In multiple signals, these wagging motions may draw the attention of receivers and point to a common evolutionary origin of such signals. More broadly, we could apply this algorithm to compare the food recruitment dances of different honey bee species who likewise share the

waggle dance and similar dance like behaviors, but in which the transitions between round and waggle dancing and the links with multiple other signals remain essentially unknown.

Acknowledgements

Chapter 4, in part, is currently being prepared for submission for publication of the material. Kheradmand, B., Lam C., & Nieh, J. C., “Kinematics of the waggle-round dance continuum of honey bees.” The dissertation author was the primary investigator and author of this material.

Tables

Table 4.1. Defining characteristics of a dance circuit.

Parameter	Rationale	Range (unit)
Angular change during circuit	A circuit is defined by walking in a circle.	180-450 (°)
Minimum walking speed	If too slow, not dancing.	3 mm/s
Duration	A circuit cannot be too short or too long (depending on walking speed).	Minimum 0.67 s Maximum 4.0 s
Rotation direction	Left and right, alternating or constant.	Clockwise/Counterclockwise
Radius	Average radius may depend on waggle duration.	Minimum 5 mm
Area covered during circuit	Large enough to attract followers, but not so large to lose follower attention	Minimum 50 mm ²

Table 4.2. Defining characteristics of a waggle run.

Parameter	Rationale	Range (unit)
Waggle frequency	In lower frame rates, similar frequencies are difficult to distinguish	13-15 Hz
Minimum time between waggles	Two waggles cannot occur immediately one after another	0.5 s
Amplitude	All waggle motions are on the continuum, but a minimum wagging score can be defined to distinguish between strong and weak waggles	Based on our current data set, the difference between weak and strong waggles is close to a score of 140-150 (from the FFT function).
Maximum walking speed	If the waggle run is too fast, oscillations may be due to walking on uneven combs	60 mm/s

Table 4.3. Average measures of circuits. The data are shown in Figs. 4.4-4.6.

<u>Distance</u>	<u>10 m</u>	<u>27 m</u>	<u>52 m</u>	<u>105 m</u>	<u>200 m</u>	<u>300 m</u>
Duration						
<i>Circuit</i>	1.39 s	1.42 s	1.16 s	1.85 s	1.58 s	1.86 s
<i>Waggle run</i>	0.30 s	0.31 s	0.35 s	0.40 s	0.45 s	0.48 s
<i>Return run</i>	1.09 s	1.11 s	0.81 s	1.45 s	1.13 s	1.38 s
Radius	15.3 mm	13.1 mm	14.1 mm	18.6 mm	17.3 mm	19.0 mm
Angular Change	312.5°	268.3°	234.2°	290.4°	277.0°	260.9°
Walking speed	mm/s	mm/s	mm/s	mm/s	mm/s	mm/s
<i>Waggle run</i>	28.92	15.3	16.4	26.5	11.4	12.35
<i>Return run</i>	26.26	17.6	14.3	23.2	13.8	11.13
Area covered	205.1 mm ²	186.3 mm ²	130.3 mm ²	163.5 mm ²	171.3 mm ²	166.3 mm ²

Table 4.4. Measures of wagging. The data are shown in Figs. 4.4 & 4.7.

<u>Distance</u>	<u>10 m</u>	<u>27 m</u>	<u>52 m</u>	<u>105 m</u>	<u>200 m</u>	<u>300 m</u>
Waggle duration	0.30 s	0.31 s	0.35 s	0.40 s	0.45 s	0.48 s
Waggle score (FFT)						
<i>Minimum</i>	35	52	58	114	77	67
<i>Average</i>	67	110	75	205	160	196
<i>Maximum</i>	99	144	86	365	361	335

Figures

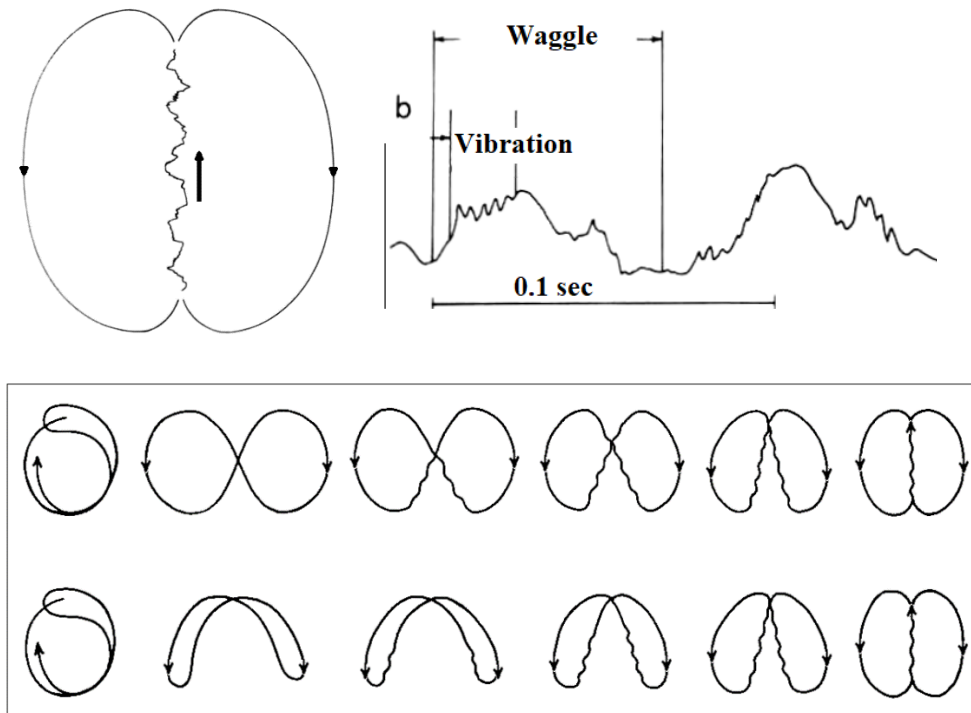


Figure 4.1. The waggles dance is defined by lateral abdominal waggling in the 11-17 Hz range (top left panel) and thoracic vibrations in the 200-300 Hz range (top right panel, Reproduced from Esch, 1961). Transitions between the round dance and the waggles dance as flight distance increases (bottom panel, from left to right) are gradual. The dances in the top row transition via the CO-form, performed by Carniolan honey bees. The dances in the bottom row transition via the sickle form, performed by European dark honey bee and Italian honey bees. Redrawn from von Frisch, 1967.

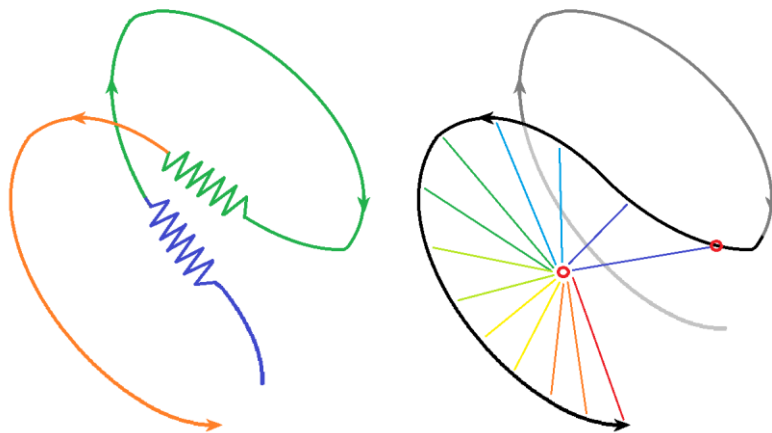
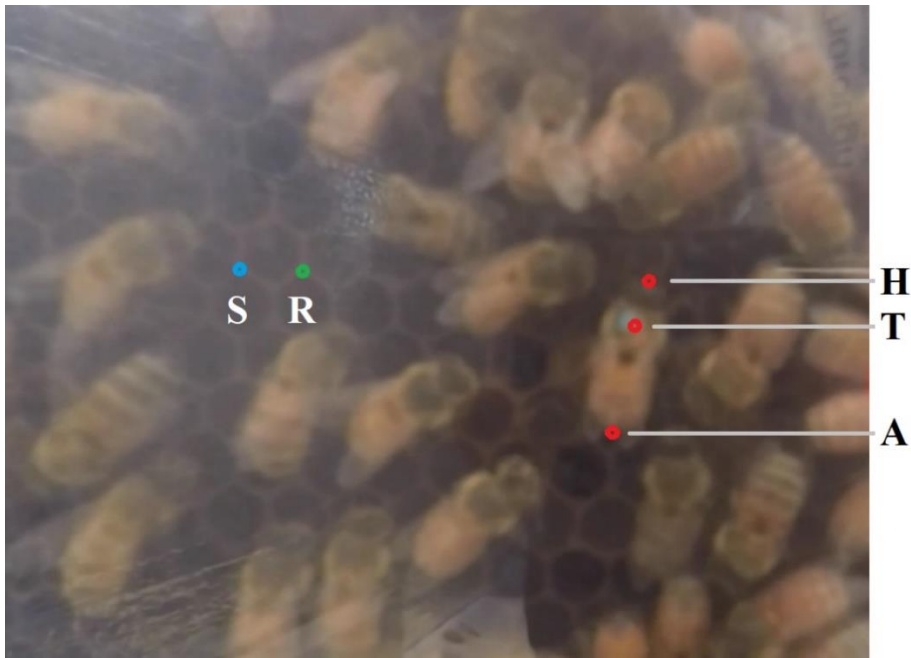


Figure 4.2. Tracking of the bee and defining circuits. The top panel shows the points tracked on each frame. Based on the x and y coordinates of tracked points, circuits are defined as the segments of movement in which the bee is turning in the same direction (clockwise or counterclockwise), as shown in the bottom left panel. This allows us to define a centroid for the turning movement, and use it to measure the circuit's angular change, its radius and the area covered by it, as shown in the bottom right panel.

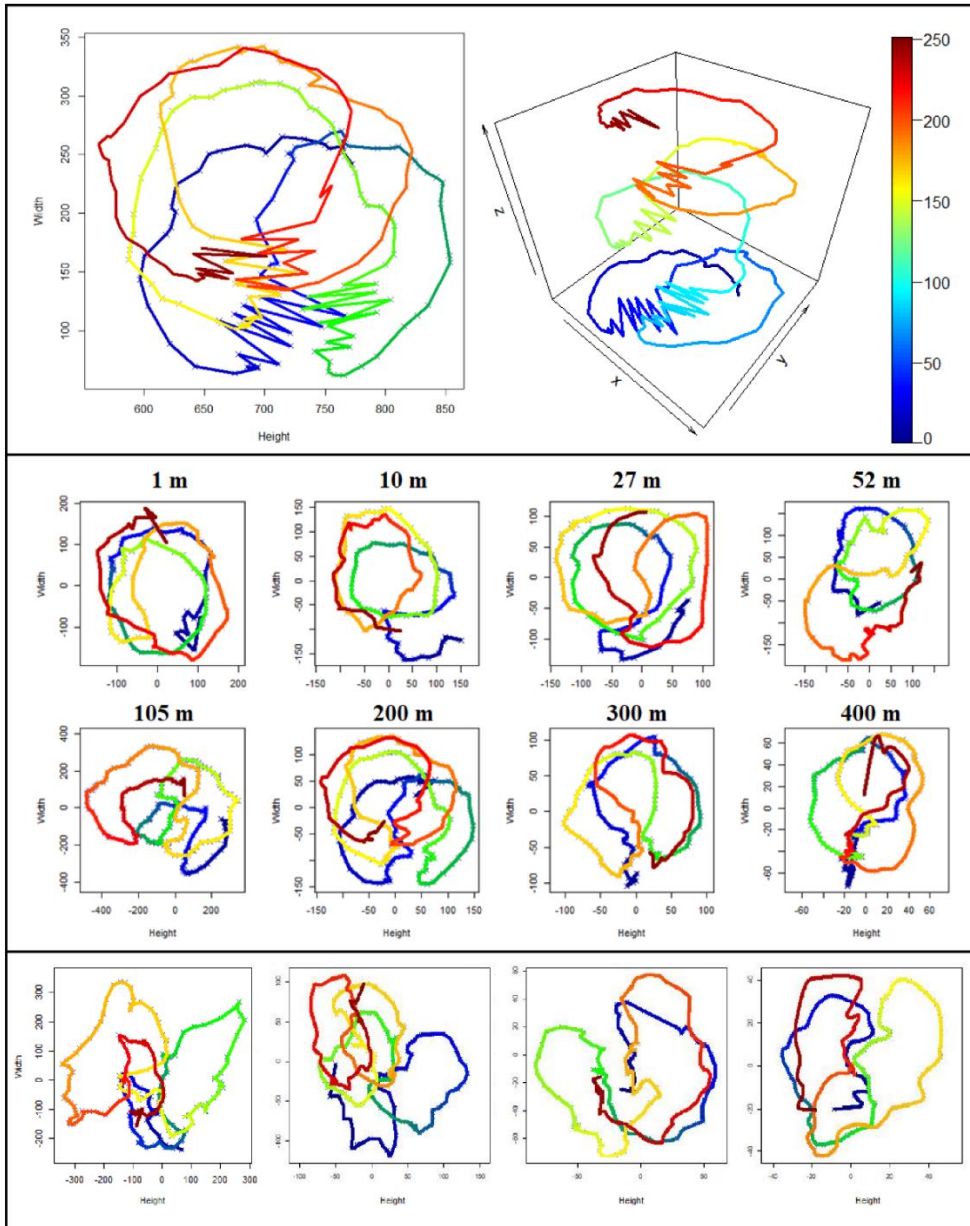


Figure 4.3. The top panel visualizes the dance in 2D and 3D with colors indicating time (frame number). The same track is shown, tracing in 2D and 3D the movements of the abdomen tip of a forager returning from a feeder located 200 m south of the hive around noon time. Time is represented in the z-axis and also in color.

The middle panel shows example tracks for abdomens of dancing bees foraging at different distances. The color of the line corresponds to time (going from dark blue to green to yellow to orange to dark red).

The bottom panel shows the tracks for the heads of the last four bees in the plot above. Note that dance shape is strongly controlled by which body part we track.

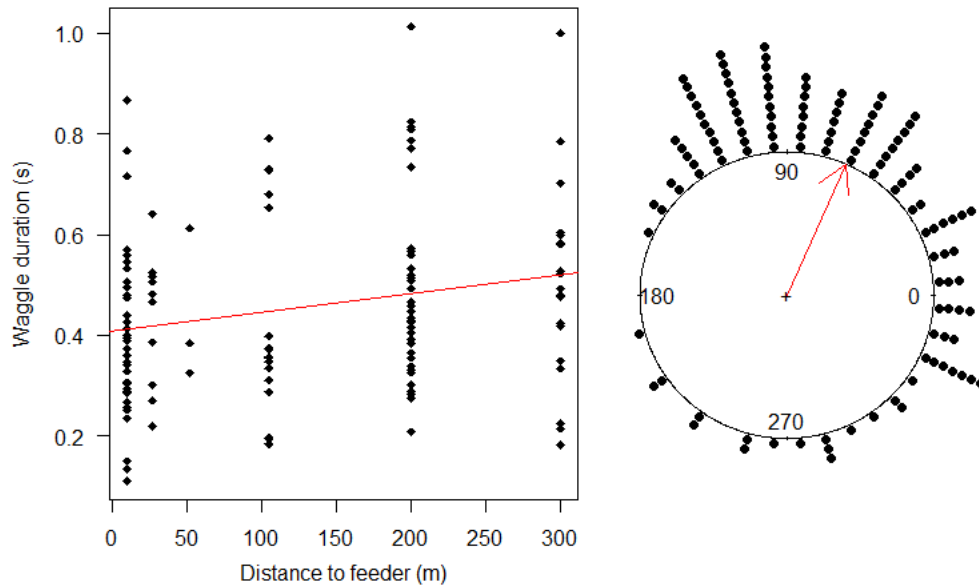


Figure 4.4. Distribution of waggle durations (left panel) and directions (right panel). Waggle duration is significantly higher at higher flight distances. The high variation in dance angles is due to the different times of day for the same feeder and having feeders at different directions relative to the nest.

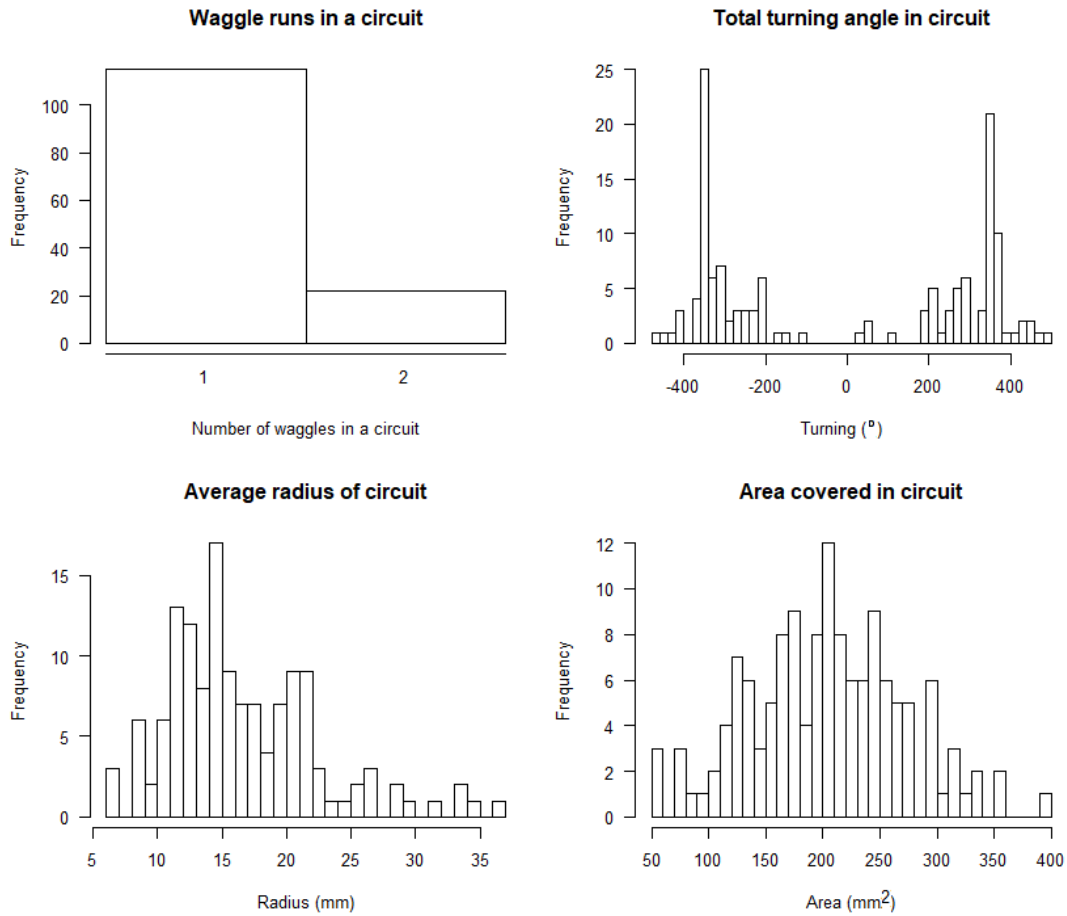


Figure 4.5. Distribution of circuit measures for all distances combined. Most circuits only have one waggle (irrespective of waggle amplitude), but a minority have 2 waggle motions (top left). In most circuits, the dancer completes at least three quarters of a full turn before starting the next circuit (top right). Most dances have a radius approximately one to two bee lengths (bottom left). Average dance area is approximately a circle with one bee length radius. Note that these are the radii and areas covered by the tip of the abdomen, and the same measures for the front of the head or the middle of the thorax may have different sizes.

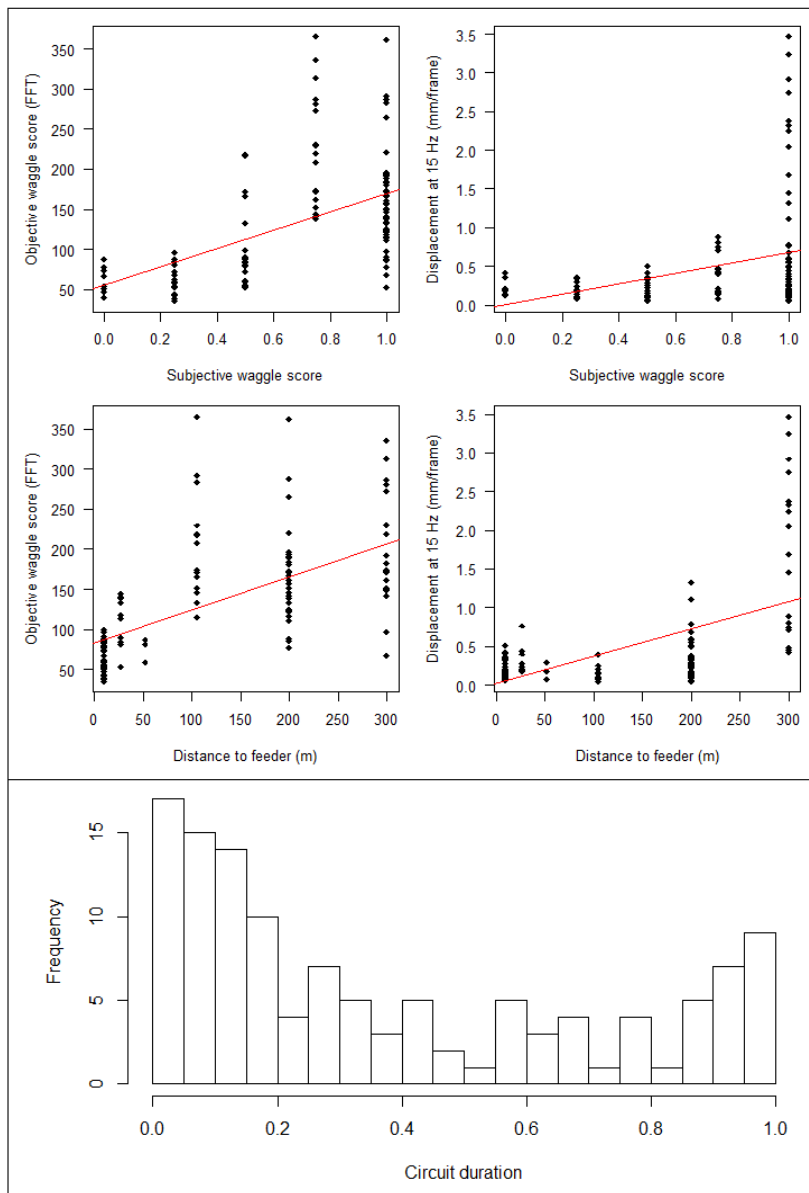


Figure 4.6. Properties of waggles. Top panel: There is a strong correlation between observers' subjective waggles score and both the objective waggles score by the algorithm using FFT (top left) and the algorithm looking at 15 Hz displacement of the abdomen tip (top right). Both scores strongly correlate to the flight distance of the bee, although their correlations are not distributed in the same manner (bottom left and right). Bottom panel: Waggles occur mostly at the beginning or the end of circuits. Our circuit definition depends on the angles of rotating around the centroid, and since the waggles run is rather straight overall, it has a small effect on turning direction. But the waggles are often preceded or followed by a sharp turn and because of that, most waggles are found either at the beginning or the end of the defined circuits.

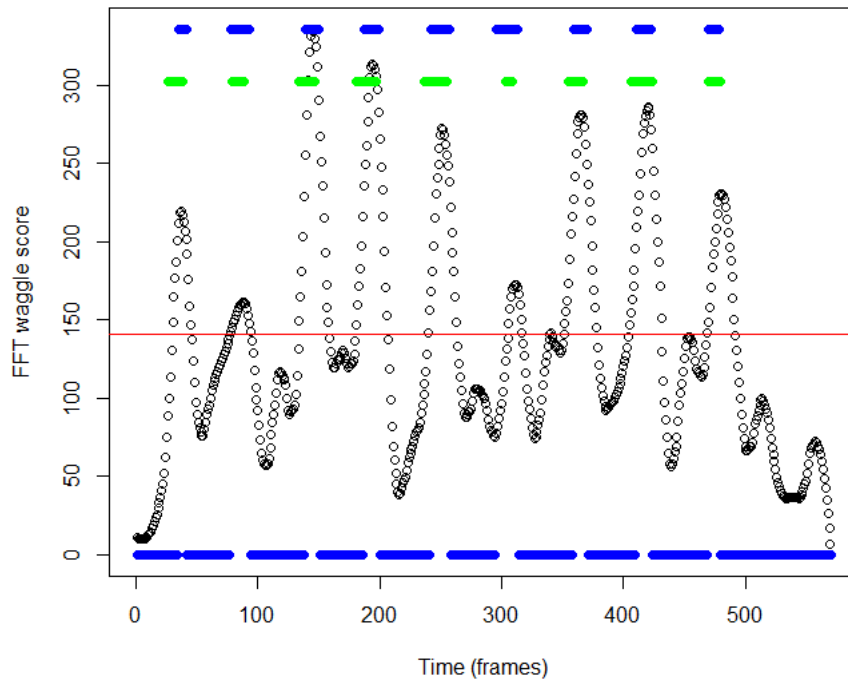


Figure 4.7. An example of the waggle detection results. The blue dots represent frames in which a human observer detected a waggle. The green dots represent the frames in which the algorithm detects a waggle. The red horizontal line is the minimum acceptable waggle score to identify waggle peaks from other noise, based on the distribution of the waggle scores throughout the video. The scores given by the FFT function are smoothed prior to finding peaks.

Supplementary tables

Table S4.1. Sample sizes for different dances included in our analysis. One bee was tracked per video. In total, 31 videos, averaging 8.45 s (7589 frames total) were analyzed.

Distance	Videos
10 m	12
27 m	3
52 m	1
105 m	3
200 m	8
300 m	3
400 m (60 Hz)	1 (steady camera)

Supplementary figures

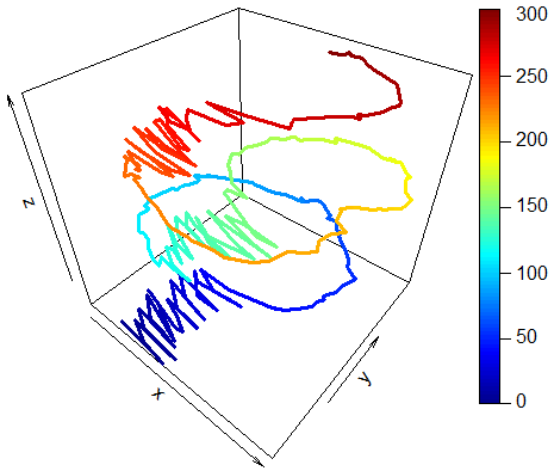


Figure S4.1. A waggle dance for 400 meters, recorded at 60 FPS.

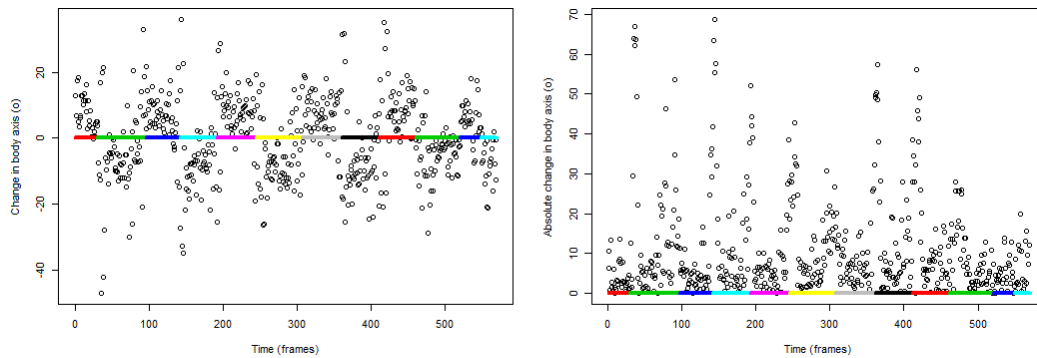


Figure S4.2. Determining circuits using body-axis change between frames is error-prone, especially during the waggle phase (left panel). In fact, the increase of the variation of this body-axis change could be used as a signature for waggles, although it is not as robust as FFT in determining waggles (right panel).

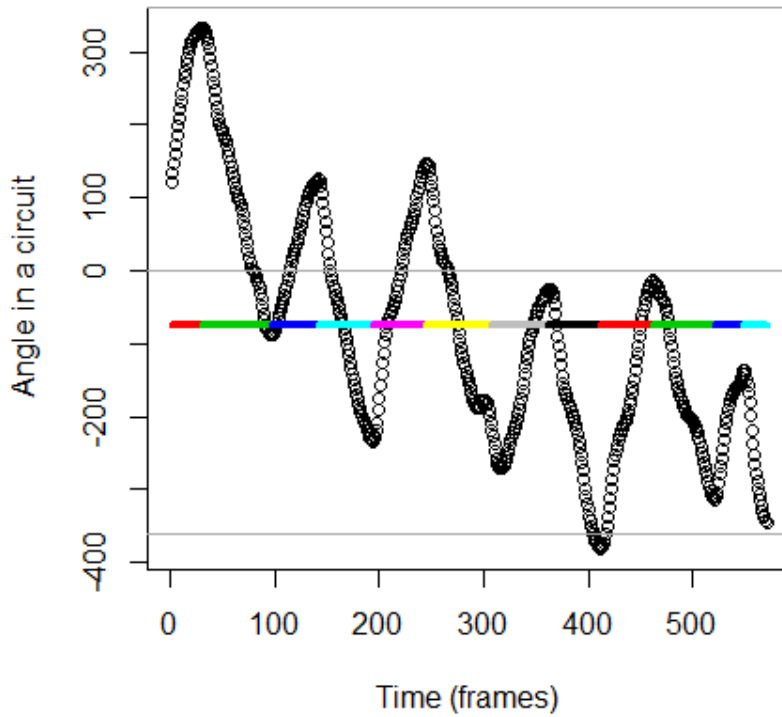


Figure S4.3. Change in angle of rotation around a centroid allows accurate detection of circuits. The angles of rotation are shown in black circles and circuits detected by the algorithm are shown in the horizontal colored bar, where each color represents a circuit. Local maxima and minima can sometimes lead to small errors, as in the border between the yellow and the grey circuits.

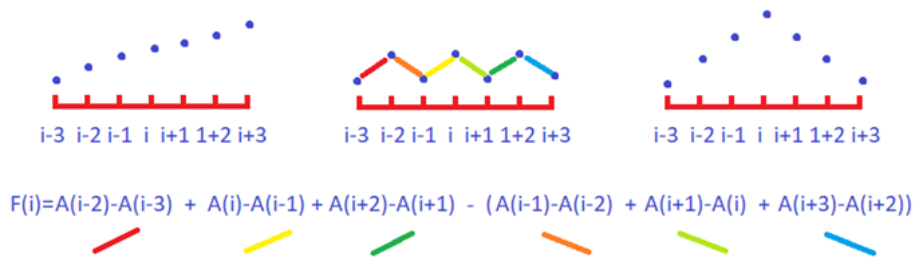


Figure S4.4. Waggle scoring function for 15 Hz (exact). Over the 7-frame window, linear motion and slow turns will cancel each other out, but displacements at 15 Hz will add up and create a detectable waggle signature.

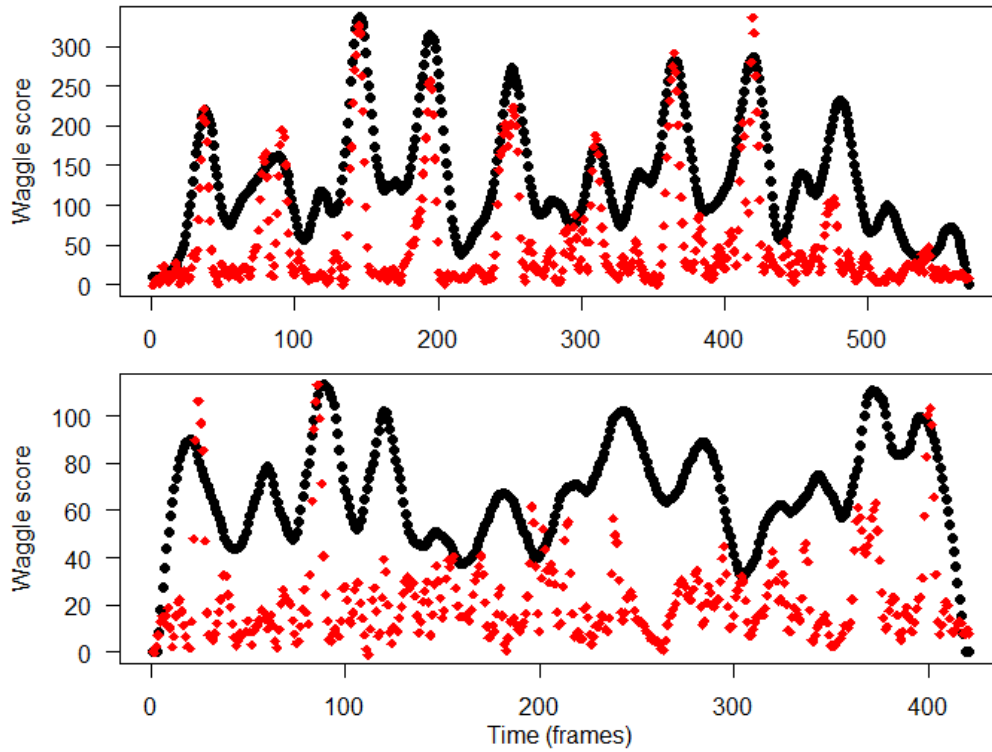


Figure S4.5. Comparing the waggle scores obtained from the FFT (13-15 Hz) and the exact 15 Hz functions. The black circles are the smoothed FFT waggle score. The red diamonds are unsmoothed 15 Hz waggle score (abdomen tip displacement), scaled to the FFT score. Although the scores are comparable in strong wagging videos (top), weaker waggles are best detected by the FFT function (bottom).

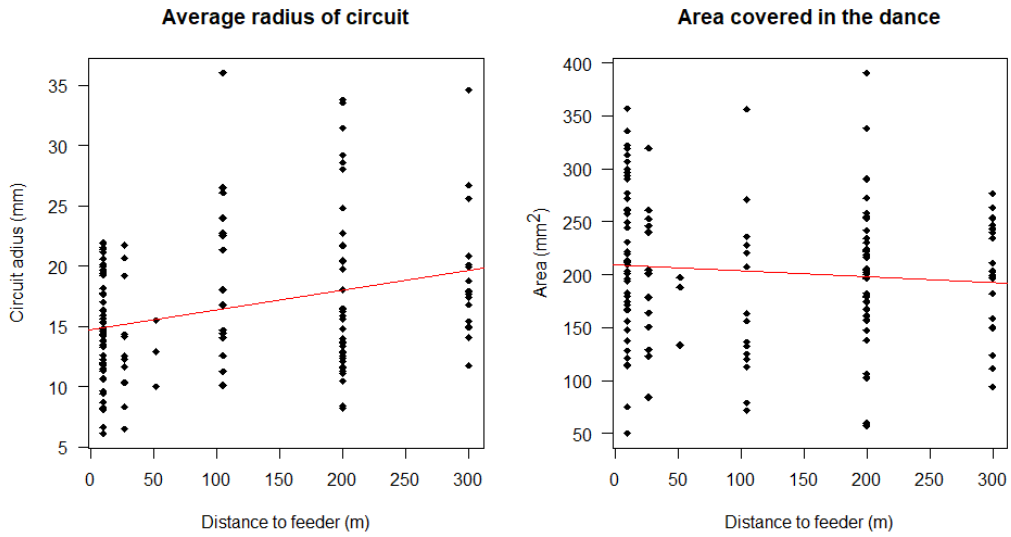


Figure S4.6. Dance radius increases for significantly longer distances but the area covered by the dance does not significantly increase.

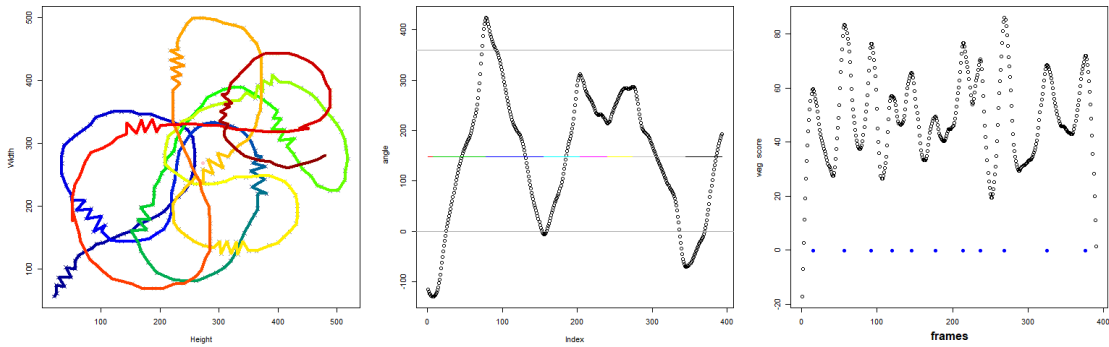


Figure S4.7. Example of circuit detection and waggles motion extraction from a round dance with waggles (data reproduced from Fig. 3 in Jensen & Michelsen, 1997).

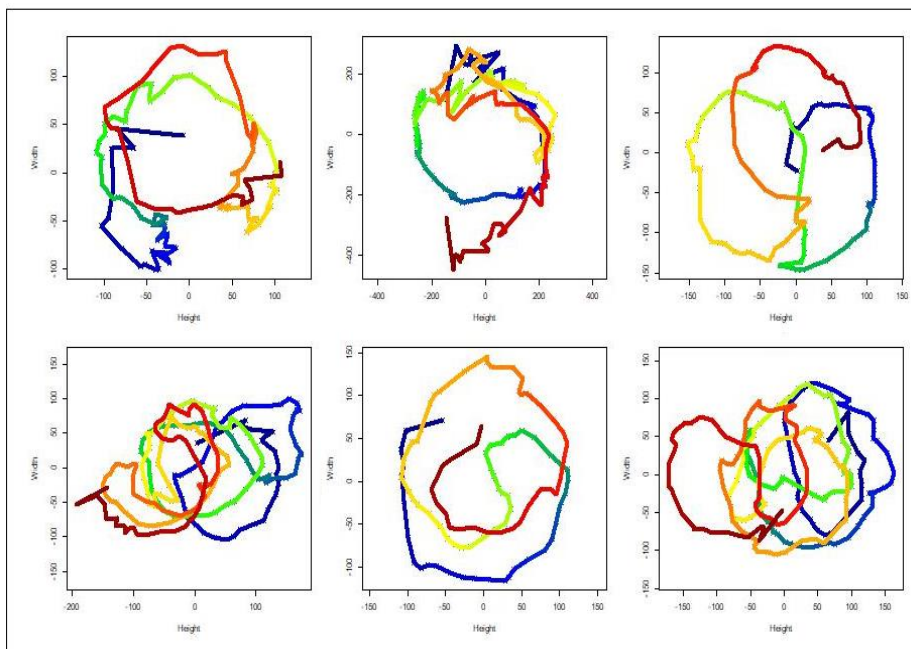


Figure S4.8. More examples of circuits and waggle runs. All dances can be visualized and tested using the R code and the data set provided by the authors.

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