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Navigation and Communication in Social Bees

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

Doctor of Philosophy

in

Biology

by

Megan Alma Eckles

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2012
The Dissertation of Megan Alma Eckles is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2012
DEDICATION

To the amazing group in Panama: Oris and Belkys, for making BCI feel like home; Diomedes, for always checking on me; Hilda, for making every stay easy. To Anna, Christie, Nina, and Ryan, for keeping it fun, even when it wasn’t.

To Bert, for advice, and support, and concern (but mostly the scotch).

To Rafa, for dancing, and talking, and everything else (but also mostly the scotch).

To Meg, for all those late nights, and coffee breaks, and platanos con limón.

To Dina, for that first night spent sitting on your kitchen floor, talking.

To Kristen, for the unending whirlwind.

To Kate and Teague, for bee-sitting, and dog-sitting, and reality checks, and dim sum.

To Femke, for knowing me too well, and Marco, for all the laughter.

To Heather, for her inexplicable dedication.

To Chuck, for paying off those jeans.

To Jane, for making me one of her five. I wish you could have seen this.

To JT, for getting it, and giving me clarity. You’re the best.

To my ‘siblings,’ who always reminded me to laugh it off.

To Tom and Jackie, for making me one of their own. To Barbie and Russ, for the same.

To my Dad, who understood.

To my Mom. For everything. Absolutely everything.
Our treasure lies in the beehive of our knowledge.
We are perpetually on the way thither,
Being by nature winged insects and honey gatherers of the mind.

_Friedrich Nietzsche_

The human mind is a wonderful thing.
It starts working before you’re born, and doesn’t stop
Until you sit down to write something.

_Roger Miller_
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Chapter 3 is currently being prepared for submission for publication of the material. Eckles, Megan A; Nieh, James C. The dissertation author was the primary investigator and author of this material.
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ABSTRACT OF THE DISSERTATION

Navigation and Communication in Social Bees

by

Megan Alma Eckles

Doctor of Philosophy in Biology

University of California, San Diego, 2012

Professor James Nieh, Chair

Bees abound in tropical environments, and the communication of valuable resource locations among nestmates allows them to exploit the diversity of food sources found in this complex environment. Rainforest canopies are a sensory melee of light patterns, odors, colors, and obstacles. How bees have evolved to not only navigate but also to communicate specific pathways through this storm of sensory
information is still under investigation. The true marvel of this system is not just the ability of foragers to successfully pilot through the environment, but their apparent ability to extract and then encode salient navigational information and transmit it to nestmates. Honeybees are known to use a functionally referential communication system whereby environmental information is encoded and then transmitted to a conspecific receiver for decoding. The transformation of sensory information into a communicable signal is most likely a complex cognitive task, yet the evolution of this behavior in bees remains relatively unexplored. Stingless bees (*Melipona* spp.) are an excellent group for studying both navigation and communication: the group consists of numerous and diverse species, foraging strategies, and communication techniques (Roubik, 2006). In comparison, bumblebees (Bombini) have a simpler communication system but also share the same navigational needs during foraging as stingless bees (Meliponini) and honey bees (Apini; Michener, 2000). We examined the visual navigation abilities of one species of bumblebee (*Bombus impatiens*), and one species of stingless bee (*Melipona panamica*). We found that *B. impatiens* is able to information from the spatial density of the visual environment to gauge distance traveled, an ability that honeybees to not appear to possess (Si et al., 2003). We then found that the stingless bee *M. panamica* can use optic flow (the movement of images as they pass across the retina) to gauge not only distance traveled, but also height above the ground. We proceeded to manipulate the visual environment experienced by foraging *M. panamica* such that their vision-based odometers registered a much larger distance than that which the bees actually flew. We found that this information
was then communicated to naïve bees inside the nest, which then flew greatly exaggerated distances in search of food. This result supports the hypothesis that *M. panamica* is referentially communicating inside the nest.

**References.**


RESEARCH ARTICLE

A stingless bee can use visual odometry to estimate both height and distance

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SUMMARY

Bees move and forage within three dimensions and rely heavily on vision for navigation. The use of vision-based odometry has been studied extensively in horizontal distance measurement, but not vertical distance measurement. The honey bee Apis mellifera and the stingless bee Melipona seminuda measure distance visually using optic flow movements of images as they close across the retina. The honey bees gauge height using image motion in the ventral visual field. The stingless bees forage at different tropical forest canopy levels, ranging up to 40 m at our site. Thus, estimating height would be advantageous. We provide the first evidence that the stingless bee Melipona panamica utilizes optic flow information to gauge not only distance traveled but also height above ground, by processing information primarily from the lateral visual field. After training bees to forage at a set height in a vertical tunnel lined with black and white stripes, we observed foragers that explored a new tunnel with no feeder. In a new tunnel, bees searched at the same height they were trained to. In a narrower tunnel, bees experienced less image motion and significantly lowered their search height. In a wider tunnel, bees experienced more image motion and searched at significantly greater heights. In a tunnel without optic cues, bees were disoriented and searched at random heights. A horizontal tunnel testing these variables similarly affected foraging, but bees exhibited less precision (greater variance in search positions). Accurately gauging flight height above ground may be crucial for this species and others that compete for resources located at heights ranging from ground level to the high tropical forest canopies.

Key words: distance, height, navigation, optic flow, stingless bees, visual odometry.

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INTRODUCTION

Insects use the perceived movement across visual fields, or ‘visual flow’, for a number of tasks, ranging from maintaining a straight walking path to regulating flight speed (for reviews, see Srivivasan, 2001; Egelhaaf and Kern, 2002; Srivivasan and Zhang, 2004). Certain ants (Cataglyphis fortis) and wasps (Polistes gallicus) also exploit optic flow for distance measurement, although the manner in which optic flow information is processed varies among taxa (ants (Ronacher and Wehner, 1995; Ronacher et al., 2000), wasps (Ugelini, 1987)). For insects such as social bees that live in complex, three-dimensional environments (Cartwright and Collett, 1982; Lehrer et al., 1988; Srivivasan, 2001; Egelhaaf and Kern, 2002), optical cues appear crucial for successful navigation. Such visual information allows foragers to successfully find, exploit and retrieve resources (Cartwright and Collett, 1982; Lehrer et al., 1988; Srivivasan, 2001; Egelhaaf and Kern, 2002).

Honey bee foragers rely heavily on information from their visual odometer, in some cases more than on learned landmarks (Manzel et al., 2010), which are known to be crucial for navigation and orientation (Cartwright and Collett, 1983; Cartwright and Collett, 1987; Collett, 1992). To dissect out which components of the visual field are most important for navigation, an experimental simplification of the optical environment is helpful (Egelhaaf and Kern, 2002). For example, Srivivasan and colleagues (Srivivasan et al., 1996; Srivivasan et al., 1997) devised an efficient method in which bees forage in a tunnel lined with a black and white pattern to examine visual distance estimation in honey bees (Apis mellifera). By altering the optical environment that bees experienced, they predictively changed the distance that foragers would search inside the tunnel and the distance they communicated through the waggle dance (Esch et al., 2001). These experiments reveal that the honey bee odometer relies on optic flow, the perceived movement of images across the retina (for reviews, see Srivivasan and Zhang, 2004; Esch et al., 2001). More precisely, the speed of angular image motion is integrated over time to gauge distance traveled (Si et al., 2003). Images move faster and farther across the retina when objects are closer to the eye. Thus, when tunnel width decreases, image speed increases, causing foragers to search at shorter distances (Srivivasan and Zhang, 2004). The opposite occurs when tunnel width increases.

Honey bee also appear to use image motion for other flight tasks such as speed control, centering and landing (Baird et al., 2006; Srivivasan et al., 1996; Srivivasan et al., 2000; Srivivasan and Gregory, 1992). Using vertically oriented tunnels, Dacke and Srivivasan determined that honey bees can use optic flow to measure the height of a food reward, but are less precise while searching vertically (Dacke and Srivivasan, 2007). Baird and colleagues found that flying honey bees also modulate their height above ground in response to changes in optic flow (Baird et al., 2006). They did so within their ventral visual field, regardless of the optic flow levels in lateral fields of view. This processing rule makes sense when a bee is flying horizontally for long
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distances, because the vertical field of view provides the only meaningful information for height measurement. However, foragers moving up to the flowering tree canopies of tropical forests can, in some cases, fly nearly vertically to approach those resources. For example, some meliponine bee species lay vertical odor trails to recruit nestmates, and recruited nestmates fly nearly vertically while following those odor marks [Scaptotrigona postica (Lindauer and Kerr, 1960) and Trigona sanguinea (Nieh et al., 2004)]. Meliponines or stingless bees (Hymenoptera, Apidae, Meliponini) are not closely related to honey bees, are twice their age evolutionarily and are a much larger group (Roubik, 2006; Michener, 2007; Roubik and Camargo, 2007; Ramirez, 2010). However, the stingless bees and honey bees are the only highly social bees, and both are found in tropical forests where food is available at different heights, so they likely have similar navigational needs.

Relatively little is known about how meliponines use visual information for navigational tasks. At the nest entrance, hovering Terragona angustula guard bees use variations in optic flow to detect and correct for unintended changes in position due to drifting, displacement by wind or other factors (Zell and Wittmann, 1989; Kelber and Zell, 1990; Kelber and Zell, 1997). Hniric and colleagues used the flight tunnel model to demonstrate that Melipona seminigra can use optic flow cues for distance measurement, and exhibit the same level of search accuracy as honey bees (Hniric et al., 2003). However, it remains unclear whether stingless bees can use optic flow cues for height (vertical) orientation, or whether visual odometry is a general component of behavior in the genus Melipona.

There are reasons to suspect that they can. Stingless bees live in a complex, competitive foraging environment, where resources patches occur throughout the upper canopy and understory (Frankie and Haber, 1983; Roubik, 1993; Roubik et al., 1995). In the tropical forests of central Panama, this canopy can reach over 40 m in height (Croat, 1978). The ability to accurately return to a specific height should therefore be advantageous. Moreover, even in tropical forests, floral resources are seasonally scarce (Roubik, 1989; Croat, 1978) and competition for resources can be intense (Johnson, 1974; Johnson and Hubbell, 1974; Johnson and Hubbell, 1987). Thus, stingless bees have experienced strong selection not only to discover resources and recruit to them but also to do so rapidly and efficiently over a shifting and complex range of heights and micro-environments. We know that Melipona panamica can rapidly recruit nestmates to specific distances and heights in the Panamanian forest (Nieh and Roubik, 1995). Thus, we used this species to examine the previously unexplored question of whether a stingless bee employs optic flow to gauge height and distance during foraging.

MATERIALS AND METHODS

Location and species

We conducted all experiments on Barro Colorado Island in the Republic of Panama, at the Smithsonian Tropical Research Institute’s field station (latitude 9.165575, longitude –79.536718). We collected data from two colonies of M. panamica Cockerell 1912, a cavity-nesting stingless bee that lives in colonies of up to 500–800 individuals (Roubik, 1992) and has a maximum flight range of over 2 km (Roubik and Aluja, 1983). Melipona panamica can accurately recruit nestmates to a specific distance, direction and height without the use of a scent trail (Nieh and Roubik, 1995). Foragers should therefore be able to accurately measure these dimensions. Data collection occurred from 08:30 to 13:00h under cloudy conditions, from August to October in 2006 and 2007, and in August in 2008 and 2009.

Tunnels and training

We mounted a wood training tunnel on wheels (to facilitate its removal during tests) and placed the entrance 5 m from each colony at a 90° angle to the nest entrance. The training tunnel was 20 cm tall by 19 cm wide (interior dimensions) and was 2 m long. The inner walls of the tunnel were painted with a striped, alternating black and white pattern of 8 cm width, perpendicular to the long axis of the tunnel (Fig. 1). We covered the top of the tunnel with black mesh to provide ventilation. To deprive bees of visual landmarks, a uniform white cover was placed 7 cm above the tunnel and extended 5 cm beyond its edges. During data collection, observers remained at least 0.5 m from the tunnel and were in motion in order to avoid acting as landmarks themselves.

There are limits to how precisely an observer can quantify the position of a bee in flight. To reduce possible inacuracies, we recorded which tunnel section each bee was in when it changed flight direction while searching. Marks were made on the outer wall of the tunnel every 10 cm, and the resulting sections (“boxes”) were numbered 1–20 to facilitate data collection (see Srinivasan et al., 1996; Srinivasan et al., 1997). We marked 17–25 bees from each experimental colony with different color latex paint on the thorax and trained them to visit a feeder with unscented 1.5 mol L−1 sucrose solution placed 135 cm inside the 200 cm long tunnel. We offset the feeder from the center of the tunnel so that we could statistically differentiate between directed searching (the mean search position corresponds to the feeder location) and random searching (disoriented bees fly from one end of the tunnel to the other, such that their mean search position corresponds to the tunnel midpoint).

The feeder consisted of an inverted glass jar placed on a grooved dish (see von Frisch, 1967). We recorded each time a bee visited the tunnel feeder and returned to its nest. A bee was considered trained when it had completed 30 trips. After training was complete, bees were allowed to forage freely at the tunnel feeder for at least 45 min before the start of each experiment. At the end of this reinforcement period, the training tunnel was closed, moved 4 m away, and covered with a plastic tarpaulin. Afterwards, the experimental period began with an identical, clean tunnel without any potential odor marks to provide positional information.

Experimental tunnels and data collection

In the experimental period, bees were tested with one of five different tunnel configurations: (1) a tunnel 19 cm in width, identical to the training tunnel; (2) a narrower tunnel, 10 cm in width; (3) a wider tunnel, 40 cm in width; (4) a tunnel with the same dimensions as the training tunnel but lined with 4 cm wide bars half the width of the training pattern bars to test whether bees would count the number
of bees); and (5) a tunnel with the same dimensions as the training tunnel but lined with parallel longitudinal black and white stripes (a control in which usable optic flow is all but eliminated). All tunnels were cleaned thoroughly with 70% ethanol solution between trials to completely eliminate any odor marks. Each tunnel was equipped with a door that could be slid into place to prevent bees from entering. During each experiment, the experimental tunnel was placed in the previous location of the training tunnel, but without a feeder. We allowed only one bee at a time to access the tunnel, so that they would not influence each other’s foraging decisions. Each bee was used only once to avoid pseudoreplication.

Bees exhibited a very clear search behavior in the tunnels. Like honey bees trained to a similar tunnel (Srinivasan et al., 1996; Srinivasan et al., 1997), foragers flew down the center of the tunnel for some distance and then made a series of 180°-deg searching turns (Fig. 1). We recorded where the first, second and third such turns occurred, because honey bees exhibit the most precise turning behavior during these first three turns (Srinivasan et al., 1996; Srinivasan et al., 1997). After her fourth turn, each forager was captured, identified and placed in a holding container until the end of the experiment. Each bee was only tested once in each tunnel configuration. When the queue of bees outside the experimental tunnel dwindled to zero, we re-stimulated foraging by reintroducing a feeder into the training tunnel for 20 min. Experimental periods were punctuated by re-stimulation breaks, as needed, over the course of the study day.

To test for optic flow use during ascending and descending flight, we oriented the tunnel vertically and created a new entrance in the mesh at the bottom of the upright tunnel. The open side of the tunnel faced a white sheet to prevent any use of landmarks. We suspended the feeder from the top of the tunnel with fishing line such that it hung in the center of box 12 (125 cm from the top of the tunnel). Training and data collection followed the same methods used in the horizontal tunnel experiment. In the vertical tunnel, the feeder was gradually raised over the course of multiple bee visits until foragers reached the training position. Bees both entered and exited the tunnel through the entrance at its base. After training, foragers used a hovering flight to ascend to the feeder. Our experiments were considered complete when we had collected data from at least 25 bees per colony (50 bees per experiment).

Data analysis and statistics

For each bee, we calculated the mean search position (MSP), the average of the locations at which bees performed their searching turns. As foragers maintained a fairly consistent flight path through the center of the tunnel (in both horizontal and vertical tunnels), we used the same method as in other studies (Srinivasan, 2001; Herron et al., 2003) to calculate the approximate amount of angular image motion each bee experienced. In this calculation, for every 1 cm of forward motion, the tunnel wall image moves backwards across the retina by an angle of tan(α/2); half tunnel width. We multiply this angle by the total distance flown to estimate total angular image motion. Although this is only an approximation of what the forager actually perceives, it is a useful calculation to compare the levels of apparent image motion in each tunnel configuration and to provide comparisons with the results of other studies.

All data were analyzed using the statistical software JMP 9.0.2. All levels of significance were set as α=0.05. We used a on-sample t-test to compare MSPs with the original training distances. Student’s t-tests were used to compare MSPs, as well as differences in search ranges between axially striped and cross-striped tunnels. We report MSPs as means±s.d. We show MSP data as relative frequency plots to simplify comparison between different tunnel widths. Relative frequencies were calculated as the number of times all bees completed turns in a given tunnel section, divided by the highest number of turns made in any section. Thus, the tunnel section with the most turns was given the relative frequency of one, and each subsequent section received a relative frequency less than one. We performed this calculation to facilitate a visual comparison of where bees concentrated their search in each tunnel.

RESULTS

Optical distance estimation

In a horizontal experimental tunnel with the same width as the training tunnel (19 cm), foragers searched in the area where the feeder was previously located (feeder training location was box 13, MSP 12.97±2.09 boxes, N=53, t102=1.2, P=0.91; Fig. 2A). When bees foraged in a tunnel approximately half the width of the training tunnel (10 cm), they searched significantly closer to the tunnel entrance (MSP 8.87±1.90 boxes, N=110, t109=4.11, P=0.0001; Fig. 2A). Likewise, bees foraging in a tunnel approximately twice the width of the training tunnel (40 cm) searched closer to the end of the tunnel (MSP 17.50±1.44 boxes, N=110, t109=7.25, P<0.0001; Fig. 2A).

We conducted three horizontal control experiments (Fig. 3A). (1) We tested to see whether bees were counting stripes by doubling the tunnel pattern frequency (line width reduced to 4 cm). However, this did not affect forager MSP. Foragers did not search at a significantly different distance compared with the original pattern frequency (MSP 11.33±2.62 boxes, N=108, t107=1.01, P=0.318; versus original feeder location t107=1.76, P=0.08). (2) To test the effect of optic flow, we compared search behavior in axially striped and cross-striped tunnels. Bees foraging in a tunnel lined with axially oriented stripes (which does not provide the visual cues necessary for optic flow) searched significantly closer to the tunnel midpoint than those foraging in a tunnel with cross-stripes (MSP 10.37±2.72 boxes, N=83, t82=23.76, P<0.0001; versus original feeder location t82=5.31, P<0.0001). Thus, bees required optic flow cues to correctly orient. (3) This was also reflected in their search range. When foraging in an axially striped tunnel providing minimal usable optic flow cues, bees exhibited a larger search range (we measured the difference between the maximum and minimum search distance within the first four turns) than bees in a cross-striped tunnel, which provides a high level of optic flow (mean of search range on axially striped tunnel 13.7±7.20 boxes, N=30, mean of search range on cross-striped tunnel 9.4±4.21 boxes, N=53, t49=11.10, P<0.0001).

Optical height estimation

After the tunnel was oriented vertically, forager search behavior followed the same patterns exhibited in the horizontal experiments. Bees foraging in an experimental tunnel with the same width as the training tunnel searched in the area where the feeder was originally located (feeder training box 12; MSP 11.70±1.77 boxes, N=53, t52=0.99, P=0.33; Fig. 2B). When the width of the tunnel was halved, bees searched lower down, closer to the tunnel entrance (MSP 7.86±2.80 boxes, N=103, t102=7.30, P<0.0001; Fig. 2B). When the tunnel width was doubled, bees foraged higher up in the tunnel (MSP 16.29±0.74 boxes, N=109, t108=31.79, P<0.0001; Fig. 2B).

The three vertical control experiments (Fig. 3B) demonstrated that bees were not counting stripes and required optic flow cues. (1) When the tunnel pattern frequency was doubled to test for counting, bees did not search in a significantly different area from that while foraging on the original pattern (MSP 12.01±1.11 boxes, N=107,
Fig. 2. Forager search behavior in the tunnel was similar in the (A) horizontal and (B) vertical experiments. In an empty tunnel (10 cm width) devoid of scent cues but identical to the training tunnel, foragers searched in the area where the feeder was previously located, indicating that they can navigate in the tunnels. In a narrower (20 cm width) tunnel (where image motion is increased), bees searched significantly closer to the entrance, while in a wider (40 cm width) tunnel (where image motion is decreased) bees searched significantly farther down the tunnel.

$t_{1,55}=1.19$, $P=0.28$; versus original feeder location $t_{55}=0.92$, $P=0.36$. (2) When the pattern was changed to axially oriented stripes (providing no visual cues for optic flow), bees began searching closer to the tunnel midpoint (MSP 10.43±1.56 boxes, $N=83$, $t_{55}=10.98$, $P=0.002$); versus original feeder location $t_{55}=5.55$, $P=0.0001$). (3) Lastly, foragers in the axially striped tunnel also covered a larger area range than those in the cross-striped tunnel (mean of search range on axially stripes 17.03±1.96 boxes, $N=30$; mean of search range on cross-stripes 5.64±3.01 boxes, $N=53$, $t_{55}=29.86$, $P=0.0001$).

DISCUSSION
During both horizontal and vertical flight, M. pupalica foraged by using optic flow cues to gauge the distance traveled. This is the first study to demonstrate that a bee moving in a vertical plane can use optic flow cues present primarily in the lateral visual field. When searching in a tunnel without a reward but identical to one in which they were previously trained, bees consistently searched where the reward was located, indicating that they can correctly identify the distance to the food source. Bees that flew in a tunnel half the width...
Height estimation using optic flow

of the training tunnel searched at half the original distance to the reward. Thus, the increased amount of angular image motion perceived per unit distance traveled increased the foragers’ perceived travel distance. Bees that flew in a tunnel twice the width of the training tunnel likewise experienced a decrease in angular image motion and therefore searched at a significantly greater distance (Srinivasan et al., 1996; Srinivasan et al., 1997) and *M. seminigrum* (Hrncir et al., 2003). *M. panormica* foragers do not count stripes. It is unlikely that other cues were responsible for the changes in search behavior observed in the range of tunnel dimensions. The tunnel cover effectively eliminated the use of landmarks. Axial stripes provide no usable optic flow cues (Srinivasan et al., 1996; Srinivasan et al., 1997), and bees were disoriented and traveled over a significantly greater area in axially striped tunnels than in tunnels with optical cues displayed in a perpendicular orientation to the flight axis.

The MSP of foragers in the axially striped tunnel was much closer to the tunnel midpoint (MSP 104 cm) than the training feeder location (135 cm), or to where bees searched in the experimental tunnel that was identical to the training tunnel (MSP 129 cm). This shift in MSP is what one would expect if the bees are disoriented and flying from the tunnel entrance to the terminus and back again, instead of performing a directed search. In this case, their search is bounded by the ends of the tunnel and thus the MSP should be the tunnel’s center. This is why we chose to offset the training feeder location from the center of the tunnel. It is possible that subtle visual irregularities in the axial pattern may have provided a limited amount of optic flow; however, it was insufficient for the foragers to use successfully.

During training in the horizontal tunnel of 19 cm width, bees experienced approximately 6.01 degree of angular image motion for every 1 cm of forward flight. This means they experienced an approximately total of 811.22 degree of image motion during their flight to the feeder in the center of box 13 (135 cm). When tested in the narrower horizontal experimental tunnel, bees experienced 11.3 degree of image motion per cm of forward flight, and would only need to fly 71.79 cm to achieve the same amount of image motion during their training. In the wider horizontal tunnel, foragers experienced only 2.86 degree of image motion per cm of flight, and this would need to fly 283.6 cm to match the amount of image motion they originally learned. Our tunnel length of 2 m was too short to allow for a comparison between this estimate and the actual distances flown by bees during the 40 cm tunnel, although we did find a significant difference between where bees searched in the horizontal 10 cm tunnel and the estimate given by our image motion calculation (MSP_{vertical}=887.30 cm, r=3.36, p=0.0014). As stated previously, these image motion calculations are only estimates, and the difference in search location is one of magnitude only. The image motion calculation also predicts a shorter search distance, as seen in our data (Fig. 2A).

This is the first study to directly address how a stingless bee measures purely vertical displacement. Stingless bees live in an environment where food sources are scattered throughout the forest canopy, so a finely tuned vertical odrometer may be important for navigation to resources. Bees lack the optical structures necessary for depth perception (Esch and Burns, 1995), but both honey bees (Esch and Burns, 1995) and *M. panormica* (Nish and Roubik, 1995) are capable of accurately gauging the heights of food resources. We suggest that foragers are able to exploit the rich optic flow cues from the surrounding forest to simultaneously track their total displacement from the nest and their degree of ascent into the canopy. Stingless bees can fly largely in a vertical plane when laying or following vertical odor trails up into the canopy (Lindauer and Kerr, 1960; Nish et al., 2004). Honey bees are also capable of vertical flight while foraging (Roubik et al., 1999). We have also observed untrained *M. panormica* foragers following a vertical flight path to reach mass flowering trees located between 30 and 40 cm up in the canopy (M.A.L., personal observation).

Although vastly simplified, the tunnel environment does simulate the basic visual environment a bee would experience when flying up into the canopy: the ground and other images below the bee recede in the vertical view, while images in the lateral views remain constant or increase in size as the bee leaves the understory and enters the canopy. The optically dense forest where stingless bees forage provides the perfect environment for foragers to use optic flow from the lateral field of view for height measurement. Hrncir and colleagues found that *M. seminigrum* uses visual information from both the ventral and lateral fields of view (Hrncir et al., 2003), possibly because of the importance of flight height in an area where resources may be vertically stratified (Lehner et al., 1988; Roubik, 1989; Esch and Burns, 1996). We did not directly examine the relative importance of the lateral versus ventral fields of view. Likewise, we did not eliminate visual cues from the dorsal and ventral fields, and thus cannot say that these bees use information solely from the lateral field for height measurement. It is possible that *M. panormica* may exploit all angles of the visual environment in a similar way. However, during vertical flight in a tunnel, the vast majority of image motion occurs in the lateral field of view, suggesting that *M. panormica* is able to exploit visual information when it is concentrated in this visual region. Interestingly, *M. panormica* foragers also demonstrated more precision when searching in a vertical tunnel than in a horizontal tunnel. This may be due to the increased energetics of vertical flight compared with horizontal flight (Dudley, 2000), in which case a more precise vertical search area would limit unnecessary energy loss.

In both honey bees and desert ants (C. fortis), it appears that odometric information is not accumulated when there is no concurrent view of the sky to provide celestial cues (Dacke and Srinivasan, 2008; Sommer and Wehner, 2005; Ronacher et al., 2006). Unlike more open environments such as deserts or temperate forests, the dense canopy of the tropical forest obscures most views of the sky, and what sunlight does penetrate is filtered through dense foliage (Croft, 1978; Leight, 1999). Under these impoverished visual conditions, it would be necessary for any understory-dwelling bee such as *M. panormica* to develop compensatory skills to account for the lack of celestial information. Our results indicate that despite the lack of celestial cues and a relatively low light environment in the dense forest understory, *M. panormica* is able to reliably use optic flow to measure distance traveled. This contrasts with results from honey bees, which suggest that in the absence of sky cues foragers cease to collect information on distance traveled (Dacke and Srinivasan, 2008).
traveled while foraging. In a tropical forest where competition is intense and resources are both spatially and temporally scattered, a refined system for gauging both distance and height may be crucial for stingless bees to rapidly and efficiently exploit assets in their environment.

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Chapter 2

The first critical test of referential communication in a stingless bee

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Summary

A longstanding mystery surrounds the question of whether honey bees are the only animals, aside from humans, capable of encoding and communicating the location of resources, a form of referential communication. Honey bees use the famous waggle dance,¹ but some species in the closely related stingless bees share the ability to recruit nestmates to a precise spatial location.² How they do so remains relatively poorly understood. Referential communication by stingless bees has been hotly debated, with most studies focusing on identifying the signals foragers may be using to encode resource distance, height, and quality. The results of these studies have varied widely and are sometimes contradictory.³ Here, we provide the first direct evidence of referential communication in stingless bees. We manipulated optic flow levels to induce foragers to communicate an exaggerated fictive distance and height to nestmates inside the nest, which were then predictably misdirected to search at greater distances and heights than those visited by the experienced foragers outside the nest. Thus, referential information transfer is not limited to honey bees, but also exists in at least one species in the stingless bees, opening the possibility of studying the evolution of such complex, encoded communication and the mechanisms used.

INTRODUCTION

Honey bees possess the remarkable ability to encode the distance and direction of resources, such as good food patches, in their waggle dances.¹ Following this discovery, Von Frisch’s students, notably Martin Lindauer,⁴ began to search for other
bee species that possess similar abilities and that could therefore shed light on how the
dance language evolved. The current debate surrounds the ability of certain stingless bees to communicate by using encoded signals inside the nest like honeybees do, a system called referential communication. Many stingless bees have an excellent ability to recruit nestmates to a resource location by using odor trials or other guides to direct nestmates. Others, however, leave no clues to a resource’s location outside the nest, and experienced foragers do not seem to ‘lead’ nestmates to new resources. These species may be referentially communicating, although the search for the signals which foragers may be using to encode information has yielded results that are highly variable and occasionally contradict one other. Instead of attempting to identify a referential signaling behavior which may not exist, it would be helpful to first examine whether any information transfer is occurring which cannot be explained by other, less complex communication strategies.

We worked with the stingless bee species Melipona panamica, which is known to precisely communicate specific food location in three dimensions without odor trails, piloting, or any other obvious extranidal behaviors. Upon returning to the nest M. panamica foragers also produce vibrational pulses whose durations correlate with height when bees unload their food to nestmates and with distance when bees make excited movements around the nest after food unloading (Nieh and Roubik 1998). Similar correlations between the distance of food sources and the duration of sound pulses has been reported in M. merrillae, M. quadridiada, M. bicolor, and M. mandacaia. Although a potential encoding mechanism, it remains unclear if this
information is sufficiently reliable due to high variability in the duration of these sound pulses for any given distance. In any case, even showing a strong correlation with limited variability is not sufficient to demonstrate that these sound pulses convey information to recruits. Alternative hypotheses need to be excluded: recruits could also orient to environmental information such as food scent, local environmental doors around the food patch, or simply follow recruiters to the patch.

One successful approach has been used with honey bees. It relies upon creating a predictable mismatch between the location that foragers recruit for (based upon their perception of distance) and the actual location they visit. We recently showed that *M. panamica* is also able to use the amount of image motion which passes over the eye (termed optic flow) to measure distance traveled. Using flight tunnels, we can manipulate the amount of optic flow a forager experiences en route to a feeder, causing it to experience far greater optic flow than it would when flying the same distance in the open environment. This causes the foragers to overestimate the total distance they have flown. If tunnel bees are referentially communicating information to recruits inside the nest, then these novice recruits will act on this exaggerated distance information and will fly much farther than their tunnel counterparts. Any changes in the spatial environment of the tunnel will alter the perceived distance travelled by tunnel bees and thus the distance information being transmitted, which will also manifest itself predictably in the behavior of the recruits leaving the nest.

When marked tunnel bees foraged in a 19cm wide tunnel lined with a stripe period of 8cm, unmarked bees preferentially visited the feeder located 60m away from
the nest, even though other conspicuous feeders closer to the nest were directly in their flight path ($\chi^2 = 251.92$, $p<<0.0001$; Fig 1a). However, when the tunnel width increased to 40cm, most recruits arrived at the feeder 30m from the nest ($\chi^2 = 325.59$, $p<<0.0001$; Fig 1a). Likewise, when the tunnel width dropped to 10cm, recruits began to arrive at the feeder 87m away from the nest ($\chi^2 = 296$, $p<<0.0001$; Fig 1a). This strongly suggests a direct relationship between the optical environment the tunnel bees are experiencing and the information that recruits are using to direct their own search. The behavior of these recruits is what would be expected when tunnel bees are communicating distance information collected by using optic flow: foragers in a wider tunnel experience decreased angular image motion, and would thus communicate a shorter distance than foragers in a narrower tunnel of the same length.

Control experiments where useable optical information in the tunnels was eliminated reinforce these conclusions. We reoriented tunnel stripes axially (running parallel to the long axis of the tunnel), which minimized the amount of optic flow experienced by tunnel bees. Under these new conditions, recruits began to search almost at random, and did not favor any individual feeder ($\chi^2 = 5.13$, $p = 0.23$; Fig 1a). This is consistent with tunnel bees being unable to gather any meaningful optical information from the tunnel, which functionally prevents them from communicating a distance to their nestmates. While it’s unclear exactly how tunnel bees attempt to compensate for this lack of information, it is apparent that recruits are no longer following directed distance information when they leave the nest to search.
An interesting aspect of *M. panamica* behavior and ecology is their precision when foraging at and recruiting nestmates to resources located high above the forest floor. To determine if *M. panamica* may also be referentially communicating information about height, we replicated parts of our previous experiment using a vertically oriented tunnel with single entrance opening at its base. *M. panamica* can use optic flow to measure height as well, and therefore also experience an exaggerated sense of height flown while in these tunnels. Although tunnel bees only flew a total of three meters upward to reach the feeder, recruits preferentially visited a feeder 24m above the ground, passing close by other feeders on their way up ($\chi^2 = 106.10, p<<0.000$; Fig 1b). When the tunnel stripes were reoriented axially, recruits were once again disoriented in their search, visiting all control feeders almost equally ($\chi^2 = 5.01, p = 0.63$; Fig 1b). While still an exaggerated distance, recruits in the height experiments did not search nearly as far away from the nest as did recruits in the distance experiments. This may be due to the fact that bees flew very close to the side of the canopy tower as they ascended, and may have been using visual cues from the structure for optic flow. This would have increased the amount of image motion they experienced when compared to the horizontally-flying bees in the first experiment, and decreased the total distance they would need to ascend before reaching their goal. Due to time and weather constraints, we were unable to replicate the experiments using wider or narrower tunnels. However, these results still strongly suggest that referential communication of height may be occurring in *M. panamica* as well.
When examining whether a species can use referential communication, context is critical. Referential communication in bees appears to increase fitness under conditions where only certain periods of the year provide useable resources, or those resources are distributed in patches throughout the environment such that individuals randomly searching are relatively inefficient. \textit{M. panamica} evolved in a tropical environment where resources are both spatially and temporally patchy,\textsuperscript{12,13} conditions which would make the development of a referential communication system advantageous. This context, when combined with the apparent lack of any signaling between individuals outside the nest, suggests that the simplest explanation for our results may be that these bees are encoding and transmitting information to one another inside the nest.

It took decades of discussion and experimentation before the waggle dance was finally accepted as a referential communication system, despite the fact that the signaling behaviors being used by honeybees to transmit information had already been identified. Researchers who have looked for similar behaviors in stingless bees have yet to find anything as clear and conspicuous as the waggle dance, compounding the difficulty of making a case for symbolic communication in these species. Experiments such as these which look first for evidence that referential communication is actually occurring are an important first step in unraveling which species can use this complex means of communication.
METHODS SUMMARY

A full description of materials and methods, including tunnel and feeder designs, is provided in the Methods and the Supplemental Information.

All experiments began by training fifty marked bees to forage at the far end of a two meter long wooden tunnel lined with black and white stripes to provide image motion. The feeder consisted of a glass jar inverted over a grooved dish (von Frisch ref) and placed on a bright orange disk of paper. During training, the tunnel feeder was filled with 0.3M unscented sucrose solution, which was sufficient to maintain forager interest but not concentrated enough to trigger recruitment behavior (average rate of forager exits over the twenty minutes before feeder placement: 8 bees/minute ± 4 bees, N=603; Average rate of exits for 20 minutes after feeder placement: 9 bees/minute ± 3 bees, N=645). We set up control feeders which were identical to the tunnel feeder at varying distances away from the nest (5m, 30m, 60m, and 87m), but along the same directional axis as the tunnel. Tunnel bees were allowed to forage freely for 30 minutes prior to the start of each experiment, during which time no foragers visited any of the control feeders. This was followed by a 30 minute latency period, where no bees were allowed in the tunnel and no foragers arrived at any of the feeders. During this time, foragers hovered around the entrance to the tunnel, apparently waiting for the entrance to reopen. At the start of each trial, the concentration of sucrose in the tunnel feeder was raised to 2.5M and the tunnel was opened to foragers, which triggered an apparent jump in recruitment (rate of nest-exiting bees after concentration increase: 36 bees/minute ± 11 bees, N=1,303).
Observers monitored which feeders were visited by both marked and unmarked bees. Any bee which landed on a control feeder was aspirated and held in a separate enclosure until the end of the experiment to prevent information being communicated about any feeder other than the tunnel feeder. The tunnel feeder was also monitored, and any unmarked bees who visited the tunnel were also collected in an effort to keep the number of tunnel bees relatively constant. We then varied the width of the tunnel to see if there was a corresponding change in the search behavior of recruits leaving the nest.
SUPPLEMENT: MATERIALS AND METHODS

We conducted all experiments on Barro Colorado Island in the Republic of Panama, in conjunction with the Smithsonian Tropical Research Institute.

**Horizontal Misdirection Experiment**

We collected data from two different colonies of *Melipona panamica* from September to December, 2007-2010. All experiments were conducted under cloudy conditions from 0830hrs to 1400hrs. We trained and marked 25 bees from each colony of *M. panamica* to forage at a feeder consisting of a glass jar inverted over a grooved dish (Design of von Frisch 1967) placed on a circle of laminated orange paper. Each feeder was located at the far end of a wooden tunnel placed at a 90° angle to the nest entrance. Marked bees were trained to the feeder over the course of two hours. During the training period, the tunnel feeder contained 0.3M unscented sucrose solution, which was concentrated enough to maintain the interest of the bees but not so rich that it triggered recruitment to the feeder. The tunnel itself measured 19cm wide by 20cm tall, with a length of two meters. This placed the tunnel feeder an absolute distance of five meters away from the nest. The open top of the tunnel was covered with black insect mesh. The tunnel was always lined with a black and white stripe pattern with a period of 8cm; these stripes were oriented perpendicular to the long axis of the tunnel during experimental trials or parallel to it during control trials. We marked all bees that visited the feeder with nontoxic paint.
We established experimental feeders that were identical to the tunnel feeder at distances of 5m, 30m, 60m, and 87m away from the nest along the same horizontal axis as the tunnel. These feeders were kept covered until the start of each trial. Each feeder was monitored continuously, and all bees that arrived were aspirated and kept in a separate enclosure for the remainder of the experiment. A running census of marked bees that visited the tunnel feeder was also maintained, and any unmarked bees that entered the tunnel were also aspirated in an effort to keep the number of tunnel bees relatively constant.

At the start of each trial, the concentration of sucrose in the tunnel feeder was raised to 2.5M to stimulate recruitment. Trials lasted for one hour, and every 15 minutes the observers monitoring each feeder switched places in order to minimize the influence of scent or visual cues the bees might have learned to associate with each person. Each experiment ended when at least 115 unmarked bees had been aspirated. If this number was reached mid-way through a one hour trial, we continued to collect data until the trial time ended.

Four different experiments were conducted using this protocol: (1) The tunnel matched the dimensions described above; (2) the tunnel was lined with axial stripes; (3) the tunnel was expanded to a width of 40cm; and (4) the tunnel was reduced to a width of 10cm.

**Vertical Misdirection Experiment:**

All experiments were performed on the Lutz Tower, a 48m tall structure located in the Lutz Catchment on the Northeast corner of Barro Colorado Island.
Colonies of *Melipona panamica* were secured to the first platform on the tower, 185cm above the ground. The colony was not placed at ground level in an attempt to minimize the risk of damage from predators or mudslides. The same two colonies were used, and experiments took place from July to September 2008-2009. Trials took place from 0800hrs to 1600hrs and under cloudy conditions.

We placed a wooden tunnel identical to those used in the horizontal experiments on the roof of a storage shed adjacent to the north-east side of the tower. The tunnel was oriented vertically, running parallel to the long axis of the tower. In this configuration, the tunnel entrance at its base was located two meters northeast of the nest entrance and one meter above it. The tunnel consisted of three wooden walls and one mesh wall oriented toward the forest and away from the tower. The top of the tunnel was also mesh to allow for greater penetration of light. We lined the tunnel with a black and white stripe pattern with a period of 8cm, oriented perpendicular to the tunnel’s long axis during experimental trials and parallel to the long axis during control trials. A feeder (consisting of a glass jar inverted over a grooved dish and placed on a colored circle of paper) was suspended 13cm from the top of the tunnel.

We trained a group of 25 *M. panamica* foragers from each colony to the tunnel feeder filled with 0.3M sucrose over the course of three hours. Each tunnel-trained bee was marked with nontoxic paint. We placed test feeders at heights of 5m, 24m, and 47m on the northeast face of the tower, directly over the center of the training tunnel. Each test feeder rested on a 12cm x 23cm piece of plexiglass attached to a horizontal tower support bar such that 20cm of the plexiglass was suspended over the
edge of the tower and visible from the ground. A feeder identical to the one located in the training tunnel was placed on each of these platforms and monitored constantly.

At the start of each trial, the feeder in the tunnel was filled with 2.5M sucrose solution to trigger recruitment behavior. Once again, trials lasted one hour and the observers switched places every 15 minutes. The tunnel feeder and all test feeders were monitored continuously during the trials, and unmarked bees that visited any feeder were aspirated. Marked bees (and other hymenopterans) that visited the test feeders were also aspirated. Experiments ended when at least 115 unmarked bees had visited the test feeders and been aspirated.

Due to inclement weather and time constraints, we only conducted two experiments using this protocol: (1) the tunnel matched the dimensions of the training tunnel, and (2) the tunnel was lined with axial stripes.

Chapter 2 is currently being prepared for submission for publication of the material. Eckles, Megan A; Roubik, David W; Nieh, James C. The dissertation author was the primary investigator and author of this material.
References


2.1a and b

2.1a: 

Figure 2.1a and b. In both the horizontal (a) and vertical (b) experiments, unmarked bees consistently flew to feeders located much farther away than the absolute distance flown by tunnel bees. The distance of the feeder chosen by unmarked bees varied consistently with the size of the tunnel in which marked bees were foraging, indicating that information from tunnel bees was affecting the choices being made by unmarked bees. When the tunnel was lined with axial stripes which provide virtually no optic flow information, unmarked bees were undirected, and no longer focused on any single feeder.
Evidence for a novel visual odometry mechanism in a walking bumble bee (*Bombus impatiens*)

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Abstract

Visual information from the environment is used extensively by bees for navigational tasks, especially distance measurement. While most studies have focused on honey bees, vision-based odometry has also been found in ants, stingless bees, and bumble bees. While the primary visual mechanism for measuring distance is optic flow (the angular speed of images as they pass across the retina), recent studies have found that other aspects of the visual environment may also play an important role in distance estimation. In this study, we examine the effect of the spatial density of the visual environment on the ability of a bumble bee (*Bombus impatiens*) to estimate distance while walking. Foragers were trained to a feeder midway down a tunnel lined with a random black and white square grid pattern. When presented with a clean, empty tunnel, trained foragers consistently searched in the area where the feeder was previously located. However, when the spatial density of the pattern was increased, trained foragers searched significantly farther than the distance they were trained to. Since the levels of optic flow in the tunnel remained constant, foragers were not using optic flow to guide the walking search for food. Foragers are evidently collecting and acting on information about other aspects of the visual environment, such as the spatial frequency of the environment or the number of visual edges passed en route to the goal. These findings suggest that the visual odometer in bees may exploit more information about the environment than previously thought.
Introduction

Bees rely heavily on visual cues for navigation, including distance measurement. Multiple studies have demonstrated that honey bees can use a visual odometer that measures optic flow (the speed at which an image passes over the eye) and integrates it over time to estimate distance traveled (Dacke and Srinivasan, 2008a). The visual odometer was not the first hypothesized means of distance measurement in bees, however. Early work suggested that honey bees used the total energy expended during a flight to a resource as a gauge of distance traveled (von Frisch, 1967; Heran and Wanke, 1952, Heran, 1956). However, recent studies strongly suggest that image motion, specifically the amount of optic flow a honey bee experiences en route to the goal, plays a more critical role in distance measurement, (Esch and Burns, 1995 and 1996; Schöne, 1996; Srinivasan et al. 1997).

One study which provides compelling evidence for the use of visual odometry in honey bees involved training a group of bees to forage in a short, narrow tunnel lined with a random pattern of black and white squares (called a Julesz pattern, Srinivasan et al., 2000). Bees foraging inside the tunnel would perform waggle dances indicating distances as large as 200m, although the absolute distance flown by each bee was only 6m. While flying inside the narrow tunnel, the bees experienced image motion that was much more rapid than what they would experience while flying the same route in the open air, causing the odometer to run more quickly and thus giving the bees an exaggerated sense of distance traveled. To study the properties of this visual odometer, researchers have also measured the search patterns of tunnel-trained
bees in clean, empty tunnels of different widths or lined with different patterns. The search areas of bees foraging in a patterned tunnel form a curve where the peak corresponds to the previous location of the reward. When bees search in a tunnel lined with stripes that are parallel to the long axis, however, they are unable to gauge distance traveled, instead searching uniformly over the entire length of the tunnel. The distribution of areas searched by these bees is almost flat, which is dramatically different from the bell-shaped distribution of search areas for bees foraging in a patterned or non-axially striped tunnel (Srinivasan et al. 1997). This indicates that bees cannot measure translational angular image speed for optic flow and cannot gauge how far they have traveled in the absence of visual cues that run perpendicular to the direction of flight.

Angular image speed seems to be the dominant driver for the visual odometer in bees. However, the visual odometer could be sensitive to other variables in the visual environment, such as contrast levels or the spatial distribution of objects. Si et al. (2003) found that the honey bee visual odometer is insensitive to a broad range of variation in pattern contrast. However, they also found that the odometer will respond to differences in contrast when mean contrast levels drop below 20%. Honey bees trained to fly equal distances over land and over water performed waggle dances indicating that their flight over water was shorter. Researchers hypothesized that this was likely due to the difference in mean contrast levels between the two substrates. As seen from the bee’s perspective, the mean contrast of land was 20% while the mean contrast of water was 9% (Tautz et al. 2004). This sensitivity to differences in the
visual environment means that the odometer is scene dependent, and will change rate as the landscape changes (Barron et al., 2005; Esch et al., 2001). Thus, for recruits to make accurate use of the distance information they receive from experienced foragers, recruits must follow a very similar route as the experienced foragers. By using harmonic radar, Riley et al. (2005) demonstrated that recruits do follow the direction communicated by the experienced bee in her dance. This confirms that recruits and scouts fly over the same terrain and thus experience the same amount of optic flow.

Previous research also indicates that the honey bee odometer does not appear to respond to changes in the spatial frequency of patterns. Si et al. (2003) found that honey bees trained to forage inside a tunnel lined with a pattern of black and white stripes did not signal different distances when the frequency of the stripes was doubled. Similarly, Srinivasan et al. (1991) found that bees flying in a tunnel in which the sides are lined with patterns of different spatial frequencies (changing density of stripes) generally continue to fly equidistant from the sides and along the central axis of the tunnel, although they did find that at certain spatial frequencies there appeared to be a weak correlation between the optic flow estimate and spatial frequency. However, a recent study conducted by Dyhr and Higgins (2010) found that spatial frequency most likely is important in distance measurement in bumble bees, and that previous work focusing on honey bees may not have detected this relationship if it exists solely in the Bombini. In their study, bumble bee (Bombus impatiens), foragers were trained to fly down a short tunnel with a white floor and sides lined with mismatched patterns of different spatial frequencies. They then examined the
centering response of the tunnel bees, a behavior where bees center themselves between objects while in flight by balancing the apparent image speed in each eye. They determined that bees were adjusting their flight paths predictably with changes in spatial frequency. In tunnels where one wall was lined with solid gray and the other was lined with patterns of gradually decreasing spatial frequency, bees adjusted their flight paths such that they flew closer to the gray wall as the pattern on the opposite wall decreased in spatial frequency. Similarly, in tunnels where one side was lined with a high spatial frequency pattern and the other with a low spatial frequency pattern, bees exhibited flight paths that were strongly biased toward the higher spatial frequency wall. In both these cases, bees appeared to perceive the wall with the higher spatial frequency as moving more slowly, thus inducing them to fly closer to increase the image speed and balance image motion in each eye. These results indicate that the visual odometer in *B. impatiens* foragers is responsive to the spatial frequency of the visual environment.

Visual complexity characteristics such as spatial frequency may become more important when images are closer to the eye, where visual resolution is highest. In bees, this would occur when foragers are flying low to the ground or walking, a behavior which is not unheard of in bees. The Amazonian bumble bee, *Bombus transversalis*, uses well-maintained terrestrial trails that are open (roofless) and extend 2-3 m from the nest to gather materials for nest construction (Cameron et al., 1999). Other experiments have also examined the abilities of walking bumble bees. Chittka et al. (1999) demonstrated that *B. impatiens* are able to measure distance using solely
internal means (like *Cataglyphis* ants, Sommer and Wehner, 2004) while walking in complete darkness in an environment without odor cues.

We propose that spatial frequency and other measures of the visual complexity of the environment may play a larger role in visual navigation than previously thought in bumble bees, but under a prescribed set of circumstances. We followed up on the finding that flying *B. impatiens* are sensitive to spatial frequency (Dyhr and Higgins 2010), as shown through their flight centering response. In order to further examine which aspects of the visual environment might also influence the optic flow-based odometer, we trained bees to walk in a small tunnel lined with a complex visual pattern, where their exposure to visual information was extremely high. We chose to focus on spatial density, which is related to spatial frequency but can serve as a separate measure of the visual complexity of an environment. Spatial density may contribute important information to the visual odometer which is not normally observable in experiments where the visual environment is not saturated. We designed an experiment to test if walking *B. impatiens* will alter their search distances when trained to search for food in tunnels of different spatial densities but equal width (where optic flow levels are held constant).

**Materials and Methods**

**Colonies and study sites**

We repeated the experiments under conditions of constant artificial illumination (indoor experiments) and under conditions in which bees could view the
sky from inside the tunnels (outdoor experiments) to determine if access to celestial information affected forager search performance. All indoor experiments were conducted in a windowless lab room, with controlled light (timer controlled 10 hour daylight cycle, 50 W halogen lamps with color temperature=3200 K), temperature (25°C), and humidity (7%). Outdoor experiments were conducted at the UCSD Biology Field Station, located in La Jolla, California with all apparatus (nest box, foraging arena and tunnels) placed outside. We sequentially used two colonies of *Bombus impatiens* from Biobest Biological Systems (Ontario, Canada) transferred into a wood nest box (30 cm x 25 cm x 15 cm) with a clear plastic top covered with an opaque lid to exclude light. Each colony was tested under indoor and outdoor conditions and contained between 250 and 300 bees, which were individually labeled with queen bee tags (Bee Works, Orillia, Ontario, Canada) attached with cyanoacrylate adhesive. The nest box connected to a transparent plastic foraging arena (measuring 280 cm x 50 cm x 25 cm) via a 15 cm clear vinyl tube. Bees generally walked within this foraging arena but also occasionally flew. The colony had unrestricted access to the foraging arena which was supplied daily with 25 ml of unscented 1.0 M sucrose solution and *ad libitum* pollen (freshly ground, collected from honey bees) in small plastic dishes.

We placed tubular tunnels within the foraging arena on 2 cm tall supports, with a ramp leading from the arena floor to the tunnel entrance. Each tunnel measured 5 cm in diameter and 240 cm long. We removed the top of each tunnel to provide a viewing gap 2.8 cm wide. This opening was covered with a UV-transparent clear plastic sheet.
(10 cm x 245 cm). We marked the tunnel edge each cm, out of the bee’s field of view, but close enough to the gap edge to allow accurate distance recording. We lined the tunnels with a randomly generated Julesz pattern of 5mm black and white squares with a spatial density of four squares/cm². The lack of repeating or periodic patterns ensured that bees could not count features or use landmarks to determine their location in the tunnel (Srinivasan et al., 1997). Bees were trained to walk to a small plastic feeder dish (1.5cm diameter, 1cm tall) placed 110 cm down the tunnel that contained a food reward of 1ml unscented sucrose solution (2.5 M), replenished as needed for the duration of each training period. In the lab, a white screen prevented bees in the tunnels from seeing landmarks overhead. In the outdoor experiment, tunnels were placed in an open area such that bees had a clear view of the sky but no visible landmarks.

Training

The first training period for each colony lasted four hours per day, for two or three days prior to the first experimental trial. We recorded the times at which an individual bee entered the tunnel, located the feeder, and exited the tunnel, as well as the number of training trips made per day as a measure of experience. We defined a complete training trip as a forager locating the feeder in the tunnel and then returning directly to the nest. When 25 bees of any experience level were consistently visiting the feeder, the initial training period ended and a trial began. Each trial started with a 40 min retraining period to stimulate foraging in the trained bees, followed by a 40 min test period. After the first 20 min of the retraining period, the first tunnel was
removed and replaced with a clean tunnel (also containing a feeder) to reduce the accumulation of scent marks which bees may have learned to rely on instead of forming their own, independent estimation of the feeder location.

The test period began after we replaced this second tunnel with a third clean tunnel that contained no food reward and no potential bee scent marks. The pattern of the third tunnel either consisted of the same 5mm pattern as the training pattern, a larger 10mm pattern (spatial density of one square/cm²), or blank, white paper which had no printed pattern. Blank paper was included as a treatment to determine if foragers were relying solely on information from the patterned wall of the tunnel for orientation. Patterns were printed on standard stock white paper using Hewlett-Packard black toner on an HP printer. The use of the blank, 5mm, or 10mm test pattern was randomly determined on a given day to discourage bees from “anticipating” the patterns being used. We collected data solely from bees that were alone in the tunnel to eliminate the possibility of social facilitation or other group effects. Trained bees searching for food in the tunnel walked in a straight line and made very distinct 180 degree turns while searching. We recorded the distance (to the nearest cm) of the first four turns made by each bee, following methods used for honey bees (Srinivasan et al., 1997) and stingless bees (Hrncir et al. 2003; Eckles et al., 2012). Bees were individually labeled and each tested only once. Each test period was followed by a 15 minute break before the start of the next trial (beginning with retraining). To determine the effect of training experience, we collected data on all bees that entered the tunnel during the test phase, regardless of their prior experience
in the training tunnel. After each experiment, all tunnels were cleaned with laboratory
detergent and ethanol to remove potential odor marks deposited by bees.

Statistical Analysis

We used JMP v9.0 statistical software to analyze our results. All data met
assumptions of normality as determined through residual analyses after log
transformation. We therefore used parametric tests: Students t-test and analysis of
variance (ANOVA). In our ANOVA, we treated colony as a random effect (Standard
Least Squares Analysis of Variance with EMS algorithm) and test location (indoor or
outdoor), pattern size (5 or 10 mm squares), and experience level (number of training
trips) as fixed effects. We first analyzed full ANOVA models with all interactions,
using reduced models without interactions if the interactions were not significant
(P<0.05). If an interaction was significant, we separately examined the effect of all
factors at both levels of the significant interaction. Because we thereby performed
multiple tests on the same data, we applied the Sequential Bonferroni correction using
the Dunn-Sidak method (Sokal and Rohlf 1995), as appropriate, and report results as
significant (*SB) or non-significant (NSSB). We report average±1 standard deviation
(SD).
Results

To determine if experienced foragers correctly measured distance within the tunnel, we analyzed the MSP data for all bees that completed at least five training trips (Tautz et al. 2003). When presented with a clean, empty tunnel identical to the one in which they were trained, these bees searched in the same area where the feeder was previously located ($T_{34}=-0.66$, $P=0.51$, bees from both colonies pooled). Thus, trained foragers were able to learn the distance to the food source. Experienced foragers searching in a tunnel devoid of patterned walls searched much closer to the midpoint of the tunnel, and not near the location to which they were trained ($T_{34}=1.2$, $P=0.012$), indicating that foragers were no longer able to orient themselves in the tunnel in the absence of visual cues from the patterned walls.

Mean search position (MSP)

We first examined mean search position (MSP). In the full model testing the effect of colony, experience, pattern, and location with all interactions, only one interaction (pattern*location) was initially significant ($F_{1,143}=5.3$, $P=0.03$). In this case, the relatively low $P$-value was not significant after Sequential Bonferroni correction, but further analyses (and thus the correction) were required. No other interactions were significant ($F_{1,140}\leq 1.90$, $P\geq 0.17$). In the reduced model containing the main factors and the pattern*location interaction, colony ($F_{1,142}=1.40$, $P=0.26$) and location ($F_{1,140}=2.02$, $P=0.16$) were not significant.
In this reduced model, there was a significant effect of pattern \((F_{1,143}=75.85, P<0.0001^{*SB})\) such that bees searched on average 15% further down the tunnel for 10 mm as compared to the 5 mm pattern (Fig 1). There was also a significant effect of experience \((F_{1,140}=67.92, P<<0.0001^{*SB})\) such that more experienced bees searched further down the tunnel for both pattern sizes than less experienced bees (Fig 1).

Because of the significant interaction, we also conducted separate analyses to determine the effect of location at the two different pattern sizes (Sequential Bonferroni \(k=3\)). In these separate analyses, there was no effect of location*experience \((F_{1,72}\leq 1.33, P\geq 0.25)\) and no effect of location at the 10 mm \((F_{1,66}=1.39, P=0.24)\) or 5 mm pattern \((F_{1,72}=0.14, P=0.71)\). Thus, there was no significant effect of location in any model, even when analyzed separately for each pattern size. However, there is a strong and significant effect of pattern such that bees searched farther down the tunnel at the larger pattern size (Fig 1).

**Variance in Mean Search Position**

In the full model testing the effect of colony, experience, pattern, and location with all interactions on the variance in mean search position, the only significant interaction was experience*pattern \((F_{1,143}=6.26, P=0.012)\). No other interactions were significant \((F_{1,140}\leq 3.31, P=0.19)\). However, in the reduced model containing the main factors and the experience* pattern interaction, colony, pattern, location, and the interaction were not significant. Experience level was highly significant \((F_{1,143}=34.51, P<<0.0001)\), such that variance was higher for less experienced bees.
**Discussion.**

In a tunnel lined with a lower spatial density pattern, *B. impatiens* foragers searched at significantly greater distances than individuals foraging in a tunnel with a higher spatial density, indicating that the changes in pattern affected their estimation of distance traveled. Instead of measuring the speed of image motion to gauge distance traveled, foragers may be counting visual edges, places where contrast levels change abruptly (such as from black to white). If bees are counting edges between the tunnel entrance and the feeder in a high spatial density environment, they will have to travel much farther to achieve the same edge count when induced to search in an environment with lower spatial density. Evidence for counting in honey bees has been documented previously, but has yet to be examined in any other bee genera (Chittka and Geiger 1995; Dacke and Srinivasan 2008b). However, further work is necessary to identify what exact change in the visual environment foragers are responding to.

The distance between the tunnel walls and the bees’ eyes remained constant across all treatments, so there were no changes in the speed of angular image motion (optic flow) between treatments which could account for the difference in search distances that we observed. However, The optical environment experienced by foragers in the tunnel was much more intense than what they would experience in the open environment, or even in more convention tunnel experiments when there is enough space for the bees to fly. The high levels of optic flow experienced by tunnel
foragers may have allowed them to exploit changes in other aspects of the visual environment (such as spatial frequency or density) which may have otherwise been lost. Spatial frequency is likely also affected when spatial density is increased, since an increase in the density of visual information will most likely also result in an increase in the frequency of visual information passing across the retina. Further studies will be necessary to determine to what extent these two visual features are used independently or concurrently by bumble bee foragers. This reaction to an increase in spatial density may simply be another manifestation of the forager’s ability to use spatial frequency information for navigation. In either case, the results of our study reinforce the findings of Dyhr and Higgins (2010), and indicate that a wide range of visual information contributes to the visual navigation system employed by bumble bee foragers.

*Bombus impatiens* foragers also appear to develop a more precise sense of distance traveled as they gain more experience with the feeder. Most naïve bees avoided the tunnel entrance entirely, and inexperienced foragers that did enter the tunnel tended walk a circuitous path as they focused on inspecting the floor, walls and ceiling of the first third of the tunnel length. Many foragers did not explore far enough to even encounter the feeder. After one or two rewarding encounters with the feeder, however, foragers consistently traveled further down the tunnel, and eventually focused their search in the area where the feeder was previously located. This result is in agreement with other work on social insects which suggests that foragers exhibit
improved performance on certain tasks as their experience and familiarity with the task increases (Chittka and Muller 2009).

Previous work on honey bees and desert ants has suggested that a forager’s ability to learn the distance of a resource using optic flow is severely hindered when they have limited or no access to celestial cues (Dacke and Srinivasan 2008; Sommer and Wehner 2005; Ronacher et al. 2006). In contrast to these studies, the walking foragers in our experiment performed equally well regardless of the presence of absence of a sky view through the top of the tunnel. It is possible that *B. impatiens* foragers are utilizing a different form of visual odometry which is insensitive to the presence or absence of celestial information. This would account both for their ability to accurately locate the previous site of the feeder under both sky conditions, as well as the changes in search behavior exhibited when the spatial density of the optical environment in the tunnel changed. For *Bombus transversalis*, which uses roofless terrestrial trails in the Amazonian forest (Cameron et al., 1999), such an ability would be useful given a relative lack of celestial cues in the forest understory. Possibly, the visual odometer in bumble bees is able to utilize a wide range of visual inputs in order to compensate for the optical vagaries a forager is likely to encounter in the open environment.

While *B. impatiens* individuals do perform short walking trips while maneuvering inside the nest, it is not a behavior commonly seen when foragers are outdoors. During flight, spatial density may not be as useful a metric for measuring distance as the more studied optic flow. However, understanding the full range of
optical information that foraging bees can exploit for navigation will further our understanding of the origins of visual orientation and navigation in social insects.

Chapter 3 is currently being prepared for submission for publication of the material. Eckles, Megan A; Nieh, James C. The dissertation author was the primary investigator and author of this material.
References


Figure 3.1: Foragers that performed more training trips consistently searched at greater distances in the tunnel regardless of pattern size. More experienced bees performed searches on the 5mm pattern that converged on the original feeder location, but searched significantly farther on the 10mm pattern. When searching in a tunnel with no pattern on the walls, foragers were completely undirected in their search.