

SPATIAL COGNITION IN THREE DIMENSIONS



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Thesis submitted to the University of St Andrews for
the degree of Doctor of Philosophy

December 2012

ABSTRACT

To date, most studies of spatial learning have been conducted in the horizontal plane, with few addressing the vertical dimension. I aimed to investigate learning of 3-D locations by wild, free-living hummingbirds and compare them with rats. In my first experiment, I found that hummingbirds can encode a 3-D rewarded location after a single visit. Using a one-dimensional array, I then found that the birds more readily learned a location in a horizontal than in a vertical linear array. However, the ease of learning was a product not only of the orientation of the array but also of its spacing scale. By the end of training, hummingbirds visited the central rewarded flower and the two adjacent flowers more than they visited the distal flowers for all arrays. However, when the array was horizontal and the flowers spaced 30 cm apart, they learned the absolute location of the rewarded flower. In a diagonal array birds learned the 2-D reward location but they chose at random when tested on a vertically or horizontally oriented array. However, when birds trained in the diagonal array were tested on a 180° rotated diagonal array they chose the flower with the same horizontal component as the rewarded flower rather than with the flower with the same vertical component. Finally in order to compare the spatial learning of animals that move in volumes with those who move in two dimensions I trained hummingbirds and rats to a rewarded location in a cubic maze. Although both hummingbirds and rats learned a 3-D location within a cubic maze, hummingbirds appeared to learn the rewarded location as a 3-D coordinate while rats seemed to learn the vertical and horizontal component of the 3-D location independently. In addition, hummingbirds were more accurate in the vertical and rats in the horizontal, which is consistent with their type of locomotion. More experiments in volumetric,

terrestrial and climbing animals are needed in order to determine whether the contrasting search strategies and learning accuracies constitute adaptations to particular spatial niches.

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ACKNOWLEDGEMENTS

I would like to start by thanking Sue Healy for all her support during my entire PhD, especially for reading endless drafts and for all our motivating meetings. I also want to thank Andy Hurly for helping me to plan all my experiments together with Sue in the wonderful atmosphere of the Canadian Rockies, for all the great discussions we had together, for taking us to Table Mountain and of course for all the intensive and enjoyable email exchanges we had towards the end of my thesis. I am also grateful to Jamie Angie for making my rat experiment possible. Thanks to Andy Burnley and Pete Wilcox for building the rats' cubic maze and to Jerico Guzmán for his excellent training on how to handle the rats. I also want to thank Mike Oram for his statistical advice in the few but very inspiring meetings we had and for being a great stats teacher.

Regarding field work I would like to thank Cam Finaly and Ida Bacon for their training on banding hummingbirds and for sharing their experience. I would also like to thank all the people who shared the cabin with me during my three field seasons and for the unforgettable moments we spent together. A very special thank to David Pritchard who was my field assistant during my last field season and who shared all the practical and intellectual excitement of my 3-D experiment.

I would also like to thank CONACYT for funding my PhD, Sue Healy for funding my field expenses, the University of Lethbridge for allowing me to live in their mountain cabin during all my field work, The School of Psychology of The University of St Andrews for its logistical support and funding for conferences, and ASAB for funding me every time I attended to one of their great conferences.

Thanks to Velia and Nacho my Mexican friends living in London for sharing the non-touristic side of that great city which I now feel closer to me because of them, for their hospitality each time I was in London and for visiting me in Scotland. Thanks to all my friends in Edinburgh and St Andrews (in particular, Christelle, David, Lluna, Vilma, Paris, Juergen, Carmen and Melina) because they made my life in the UK such an exciting and

international experience. I would also like to thank to all my friends in biology and psychology with a special mention to Guill, Rachael, Kate, Emily, María and David. To Sally, for our piano and Spanish evenings and to Felicity, for taking me climbing, swimming in and running by the North Sea, and for all our conversations that made anything we did much more special. Thanks to both for all the music nights (together with Jorge). Overall, thanks to all of you for becoming my friends.

Thanks to Jorge, who became my friend and changed my life in St Andrews to a point that we decided to continue our lives together. Thanks for all your help throughout my PhD. Thanks for being the way you are, for all of our adventures and for making me so happy.

I would like to make a very special acknowledgement to all those who were always present, although not physically: To Ileana Abreu, my mum, for teaching me to enjoy life and to be happy. Thank you for teaching me that it is worth it to be free in every sense. In that way, life might be harder but it will also be more interesting and enjoyable. Thanks for visiting me, which I was looking for since I got accepted for my PhD in UK. I would also like to thank my Grandparents and rest of my family: Claudia, Paty, Norma, Rodrigo, Macarena, Ale and Rodri for being part of my life in your very own way and for encouraging me in everything I've done until now. In summary for being the best family anyone could imagine. To Yuriria, who has been always present regardless of where we are. Thanks for keeping our friendship alive. I would also like to thank the rest of my friends back in Mexico who form part of what I am, people with which I have a very special connection. Thanks to Sol, Hugo, Martha, Violeta, Agustina, Germán, René and to all my other friends that make me want to stay in Mexico to spend time together.

I would like to thank the Fuentes Fernandez family for offering me their home with a friendly and relaxing atmosphere ideal for working on my final thesis drafts, for our wonderful trip to the Pyrenees and for being my family in Spain.

“Somos nuestra memoria, somos ese quimérico museo de formas inconstantes, ese montón de espejos rotos.”

Jorge Luis Borges

“It’s a poor sort of memory that only works backwards”

Lewis Carroll

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CHAPTER 1. GENERAL INTRODUCTION

Cognition can be defined as the processes concerned with acquisition, retention and use of information and it influences behavioural traits that themselves affect animal ecology and evolution (Dukas 2004). Spatial learning and memory has played a central role in the development of a general theory of animal cognition not only because of its ecological relevance but also because of the discovery and understanding of its neural correlate, the hippocampus. The neurobiological approach seeks to identify brain areas involved in spatial tasks and to understand how those brain regions encode and retrieve information through the use of animal models (mainly rats) in the laboratory (Kamil 1994; Shettleworth 1998). Cognitive ecologists, on the other hand, try to understand how animals learn about the spatial properties of their natural environment in an evolutionary context. Within this perspective, unrelated taxa that share similar selective pressures are expected to have similar cognitive abilities (convergence) while closely related species faced with different ecological pressures are expected to possess different cognitive abilities (Healy & Hurly 2004).

Navigation, which here I consider to be self-controlled movement in space toward an unseen goal while keeping track of where you are, where you have been and where you are going, is crucial for the survival (foraging, finding their nest, escaping from predators) and reproduction (finding mates) of all non-sessile animals (Sherry 1998). All animals must learn and remember relevant locations to take journeys that minimize risk and energetic cost. These may differ depending on their mode of locomotion. Broadly speaking, animals can represent space in two ways:

egocentrically, whereby locations in the environment are represented relative to the organism (e.g. to my right and below me), and allocentrically, whereby relationships among landmarks in the environment are represented outwith the perspective of the organisms. This latter kind of representation can be used to estimate trajectories regardless of the position or orientation of the animal (Fortin 2008).

Which navigational strategy is used depends not only on the type of representation of the environment but also on the scale of navigation. For example, for large-scale navigation, such as long-distance migrations, animals may use the position of the sun and stars at specific times of the day to orient themselves, or use gradients in the earth magnetic field, perhaps integrated with landmark information as the scale of navigation decreases. At least for birds it is now accepted that different components of avian migration (distance, direction and timing) have a genetic basis (Berthold et al. 2003). Birds also use their sun compass for determining direction in small-scale orientation but the directional information needs to be combined with information from familiar landmarks as shown by data from black-capped chickadees *Parus atricapillus*, which had been thought to use only landmarks to relocate their caches (Sherry & Duff 1996). However, a shift in the locations in which they search after a clock shift suggests that they combine both sources of information. The combination is most clearly seen in that the chickadees' search patterns do not fit the 90° rotation expected from the clock shift or the exact location where the cache would be according to the landmarks. Similarly, in homing pigeons, clock-shift effects are also often smaller than those predicted by the use of sun-compass information alone (Wiltschko et al. 1994). In addition, chickadees need the familiar landmarks to orient themselves using the sun compass because in novel

landmark arrays birds search randomly (Sherry & Duff 1996). An apparent lack of the sun-compass use in novel surroundings has been also observed in scrub jays and pigeons (Wiltschko & Balda 1989; Chappell & Guilford 1995).

In short-range navigation, landmarks, which are the fixed features of the landscape that form their habitat and which can be perceived via any of multiple sensory systems (i.e. visual, odour, auditory and tactile), appear essential to animals (Gagliardo et al. 2001; Meade et al. 2005; Waller & Lippa 2007). Animals use these landmarks for bearings and for gauging distances among the landmarks so as to navigate through their habitat. When landmarks are not available or reliable, animals rely on idiothetic information, which are cues generated by the animal's own movements (e.g. sensory flow, vestibular information), using a navigational strategy known as path integration (Gallistel 1990; Whishaw & Wallace 2003).

Most animals live in a 3-D world regardless of their type of locomotion, which, in turn, determines the extent to which animals can move (i.e. their degrees of freedom of movement) through three-dimensional space. While the horizontal dimension is constituted by two axes parallel to the ground, the vertical dimension involves a single axis perpendicular to the ground and is, therefore, uniquely affected by the force of gravity. Terrestrial animals' vertical movements are constrained by the surface they move on, while arboreal animals (e.g. squirrels, monkeys), despite also moving along surfaces, are far less limited in their vertical moves because they climb. In contrast, flying and swimming animals move with three degrees of freedom because they do not require a surface on which to move i.e. they move in volumetric space (Holbrook & Burt de Perera 2009). However, it is not known whether the

animal's type of locomotion plays a key role in the three-dimensional spatial cognition of different animals.

Three dimensional (3-D) spatial cognition, that is, how animals acquire and remember locations defined by both horizontal and vertical information, is poorly understood. Most of what we know about spatial learning and memory comes from studies in the horizontal plane using a variety of mazes, which have excluded a vertical dimension (Morris 1984; Poucet et al. 1986; Poucet et al. 1988; Eilam & Golani 1989; Packard et al. 1989; Rifa et al. 1992; Healy & Hurly 1995; Tchernichovski & Golani 1995; D'Hooge & De Deyn 2001; Wohlgemuth et al. 2001; Reisel et al. 2002; Sovrano et al. 2002; Royer et al. 2010). Furthermore, for the majority of the spatial experiments rodents have been used as the subjects. Pigeons have been used to some extent, although despite being able to fly, they have typically not been required to during most of the laboratory-based spatial tasks on which they have been tested.

Various spatial studies using insects, however, suggest that the relevance given to the vertical information might be related to the animals' type of locomotion. Desert ants (*Cataglyphis fortis*), which are known for their relatively long-distance path integration, do not use a three-dimensional odometer to do this: ants trained to a food source over undulating terrain and tested in a flat terrain, or vice versa, travelled homing distances that reflected the horizontal distance and not the actual one (Wohlgemuth et al. 2001; Wohlgemuth et al. 2002; Grah et al. 2005). In addition, ants do not remember the order or the distance at which they encounter vertical changes, such that, if they are trained to walk over a flat surface to a food source but forced to descend during a test, ants do not compensate for the change in height by

climbing a slope provisioned during the test (Grah et al. 2007; Grah & Ronacher 2008). From these data, then, it would appear that ants do not use a vertical vector. In contrast, bumble bees (*Bombus impatiens*) learn relative and absolute heights of food sources depending on the context in which flowers are sampled. If they are trained to a single flower they will learn its absolute height. However, if they are trained to a medium-height flower filled with sucrose that is paired with a tall, empty flower and then presented with a choice between a new pair of flowers in which one is at medium-height but the other is now shorter, bees will choose the shorter flower (Wiegmann et al. 2000). Honey bees (*Apis mellifera*) trained to fly to a feeder along 3-D tunnels communicate the total (not just the horizontal) distance travelled to their nest mates, through their waggle dance, suggesting that, in contrast to ants, the odometric signal of bees includes the horizontal and the vertical segments making no distinction between them. However, it is not clear how the recipients of the danced message relocate the 3-D goal without being informed which part of the total distance was horizontal and which vertical i.e. information about the goal's height, or the angle at which to head to the goal (Dacke & Srinivasan 2007). Honey bee scouts might communicate the vertical component in a non-visual modality as has been suggested for other bees: some stingless bees communicate, although it is not known how, to their nest-mates the distance and direction of a food source to which they had been trained (Nieh & Roubik 1998). In some species it appears that information about the height is also communicated (also via an unknown mechanism): for example *Melipona bicolor*, a species that lives in rainforest with variable canopy heights, communicate the height of the feeder at which they were trained to feed (at a canopy level of 12 m) as evidenced from newcomers arriving at the training feeder

and none to a control feeder that was placed at the same distance and direction but a different height. In contrast, *M. mandacaia* newcomers arrived equally to the training and to the control feeders, suggesting that the feeder's height location was not communicated to them. This lack of communicating the height of a resource might be correlated with this species' ecology: it lives in a semi-arid ecosystem with food resources at a relatively constant height (Nieh et al. 2003).

Whether the ability of animals to learn the vertical component of a 3-D location results from the extent at which they move within the three dimensions (i.e. idiothetic cues generated while exploring each dimension), or from the intrinsic properties of their spatial neural correlates is not yet known. The study of the neural basis of navigation has been motivated by the possibility of better understanding the neural basis of human spatial cognition and, like the behavioural experiments of spatial learning and memory, has been based almost entirely on rats performing tests in the horizontal plane. Therefore, little is known about the neural basis of 3-D navigation with a few, fairly recent, exceptions in which the electrophysiological properties of spatial regions of the rat and bat brains have been examined. The rat experiments involved recording rat's place and grid cells while they explored a tilted (30°) box, climbed a vertical wall or climbed a helix (Hayman et al. 2011). In the other hand, bat's place and grid cells were recorded while foraging in a large arena using novel wired and wireless promising techniques for the study of 3-D navigation (Yartsev and Ulanovsky 2011).

In contrast, those studies in which researchers have sought to examine the relationship between variation and ecological demand have involved a diversity of species. The work on food-hoarding birds provides probably the best-documented

example of variation in spatial cognitive abilities associated with a relevant neural substrate, the hippocampus. The size of the hippocampus is positively correlated with a demand for an increased spatial memory in a variety of ecological situations: food hoarding, brood parasitism, migration, homing and home range size (Healy & Hurly 2004). The hippocampus is responsible for memory formation and spatial mapping not only in mammals but also in reptiles, birds and fish (Suzuki & Clayton 2000; Rodriguez 2002; Ulanovsky 2011). Caching birds can remember for hours, days or months the locations of many food items that they have hidden and also have a larger hippocampus than do non-caching birds (Sherry 1985). Caching birds are able not only to remember where they have hidden their food but also when and what they stored, constituting the first demonstration of episodic-like memory in a non-primate species (Sherry 1984; Clayton & Dickinson 1998). The hippocampus is not only the neural correlate for spatial cognition but also a system for the automatic recording of attended experience that enables the encoding, storage and private recollection of experience in a form that would be advantageous to an animal (Morris 2001).

More than three decades of research have demonstrated a role for hippocampal place cells in representing the spatial environment in the brain (O'Keefe & Dostrovsky 1971). When rats move around a horizontal plane, populations of place cells fire at high frequency whenever the animal is at a particular location with respect to the landmarks (i.e. the place field rotates with the cues) in the environment. Neighboring place cells fire at different locations such that, throughout the hippocampus, the entire environment is represented in the activity of the local cell population (O'Keefe 1976; Wilson & McNaughton 1993).

Place cells have been found in a number of mammal species (Robinson 1980; Ono et al. 1993; Ludvig et al. 2004; Ulanovsky & Moss 2007; Pastalkova et al. 2008) and in birds (Siegel et al. 2000). However, more recent studies have shown that place cells are part of a broader circuit for dynamic representation of self-location in which the entorhinal grid-cells are key components of this network providing the elements of a path integration-based neural map (Moser et al. 2008). Grid cells constitute an intrinsic (independent of any input from the environment) metric system that emerges from the periodic firing of entorhinal cortex neurons (Fyhn et al. 2004). If the firing pattern of these neurons (grid cells) were to be plotted it would form a grid that covered the whole environment (Hafting et al. 2005). However, grid cells exhibit different properties when the animal is moving vertically: rats appear to estimate vertical distance at a lower resolution than in the horizontal as the firing fields of grid cells were vertically elongated when rats climbed a vertical wall or a helix (Hayman et al. 2011). This would suggest the vertical component of space is encoded anisotropically, at least in rats.

If rats do indeed encode 3-D space anisotropically we might expect that they might also learn about the vertical and horizontal components of rewarded locations differently. However, this does not appear to be the case. Rats (*Rattus norvegicus*) can learn a rewarding location in 3-D as readily as in the horizontal plane as shown by possibly the first experiment on three-dimensional spatial cognition (Grobéty & Schenk 1992). In that experiment, rats learned a rewarded location in a 3-D maze. When tested after 15 trials of training, in the absence of reward, the rats spent more time searching for the reward in the horizontally adjacent units than they did in the vertically adjacent units. However, in a second test (after 15 extra training trials)

they did the opposite: they spent more time searching for the reward in the vertically adjacent units rather than in the horizontally adjacent units. It is not clear why rats appeared to have changed their search strategy. Perhaps, after 15 training trials the rats were more certain about the vertical component of the rewarded location or they had learned the location as a 3-D coordinate and then spent more time moving horizontally in their first test simply because they prefer moving in the horizontal plane. Rats moved less in the vertical than in the horizontal dimension during the whole training even when one considers that the horizontal is composed of two axes and the vertical of just one (Grobéty & Schenk 1992). It is not clear if the rats' preference for moving horizontally results from their type of locomotion or because they encode vertical information at a lower resolution than that with which they encode horizontal information.

If the horizontal preference of the rats was a result of their constrained vertical movement, we would expect animals that move freely through 3-D space not to exhibit any preference. Fish (*Astyanax fasciatus*) trained to a reward on a rotating Y-maze learned the location in the same number of trials regardless of the orientation of the maze, showing no difference in the rate of learning the vertical and the horizontal components (Holbrook & Burt de Perera 2009). However, when fish were trained to a rewarding location in a Y-maze with both a vertical and a horizontal component (e.g. they had to move up and right) and when tested with the maze rotated, which placed the vertical and horizontal components in conflict, the fish chose the vertical component over the horizontal one (e.g. swam upwards rather than to the right: (Holbrook & Burt de Perera 2009). This would suggest that these fish may not view or treat the vertical and horizontal components of space in a similar

way. Data from field experiments in which hummingbirds have been required to remember spatial locations in 3-D space also seem to suggest that vertical information may be treated differently from horizontal information. For example, rufous hummingbirds (*Selasphorus rufus*), trained to a 3-D location within their territory, relocated more accurately the vertical than the horizontal component of the location when the goal was either moved or removed (Hurly et al. 2010). One can see this relocation in the absence of the reward because hummingbirds will hover in midair at previously rewarded locations, even in the absence of those rewards. Although in that experiment, when the hummingbirds experienced a single rewarding location, they appeared to learn the absolute height, when they were trained to visit two artificial flowers at different heights on a stake, the birds learned the relative height of the rewarded flower and not its absolute location (Henderson et al. 2006). It could be that the birds did not learn the absolute height in the Henderson's et al. (2006) experiment because the two flowers were only 20cm apart. In previous experiments in which the flowers were all at the same height but less than 40cm apart in horizontal space, rufous hummingbirds learned which was the rewarded flower(s) relative to the other flowers (Healy & Hurly 1998). When flowers were further apart than 40cm, birds learned the absolute location of the flowers. In yet another experiment, rufous hummingbirds that were presented with flowers that varied in their height as well as in their horizontal location (i.e. an array of flowers that varied in height) learned the location of the reward in fewer trials than when the flowers were all at the same height (Henderson et al. 2001). Thus, the 3-D component appears to have facilitated learning a location. It does appear that 3-D location learning may be different for the birds in comparison to the way in which they learn

locations in the horizontal plane. However, there has been no explicit testing of this possibility.

In this thesis, therefore, I investigated learning and memory in 3-D in wild free-living rufous hummingbirds to characterize the role that the vertical plane plays in the learning of spatial locations by these birds. By starting from evaluating learning in one dimension I sought to evaluate whether learning in the vertical and the horizontal dimensions were equivalent, and whether learning in 3-D was a product of the birds integrating the vertical and horizontal dimensions by subsequently adding dimensions. In addition I compared the performance by the hummingbirds in a 3-D spatial cognition task with that of a terrestrial mammal, the rat, in an analogous 3-D task.

STUDY SUBJECT

Rufous hummingbirds *Selasphorus rufus* are members of the order Apodiformes all grouped in the polyphyletic family Trochilinae. Rufous hummingbirds are pollinators, with high metabolic requirements although they also carry out the longest bird migration as measured in body lengths. They migrate from southern Alaska to central Mexico after, and prior to, their breeding season (Healy & Calder 2006). During their migration, rufous hummingbirds feed on various flowering plants in a nectar corridor along the Pacific Coast, a corridor that not only supports the rufous hummingbird migration but also the migrations of lesser long-nosed bats (*Leptonycteris curasoae yerbabuena*), white-winged doves (*Zenaida asiatica mearnsii*) and monarch butterflies (*Danaus plexippus*). During their migration, rufous hummingbirds experience a wide variety of habitats from temperate forests,

the Sonora desert, prairies, the Rocky Mountains and the tundra. In these various ecosystems these birds are generalists that feed from flowers that vary greatly in nectar volume and concentration, colour, shape and spatial distribution. Some of these flowers, for example, the flowers in columnar cacti, agave plants and ocotillo (*Fouquieria splendens*), which are among the most abundant in the Sonora desert, also vary in their heights. In contrast, in the prairies most flowers share an approximate height but vary widely in their horizontal coordinates.

When rufous hummingbirds arrive at their breeding ground, male rufous hummingbirds establish feeding territories, which they defend vigorously mainly against male conspecifics but also against any conspecific females and a range of heterospecifics (Hurly et al. 2001). In contrast, females are not territorial during the breeding season. Females build the nest and raise their two chicks, without any help from the males. For the duration of the season, the males are visible and feeding at the feeders very regularly while the females are seen only sporadically. For this reason, all of my experiments were carried out using males only.

In the Westcastle River Valley, Alberta, Canada, which is part of the breeding grounds of the rufous hummingbird, these birds also feed from a variety of flowers that vary in the height at which the flowers can be found (e.g. *Ribes oxycanthoides*, *Lithospermum incisum*, *Castilleja* sp., *Geranium viscosissimum*, *Lonicera involucrate*, *Lonicera utahensis* and *Salix* sp.; Bacon 2010).

Rufous hummingbirds constitute a useful model to study 3-D spatial cognition, both logistically and biologically. The logistic advantages are multiple: (1) the birds readily habituate to humans within very close proximity (1m). This means that experimentation and observation can occur at close quarters and without

the need of a hide; (2) the birds are readily caught for marking, which means they can be individually identified; (3) due to the energy demands for hovering flight and for regulating the temperature of such small bodies, rufous hummingbirds feed frequently (every 10-15 min); (4) males defend their feeding territory very vigorously and exclude female and other male hummingbirds (and frequently heterospecifics).

Rufous hummingbirds, like food-storing birds, prefer to use the spatial arrangement of visual cues around the rewarded location rather than visual features of the goal itself to relocate the reward. When visual and spatial cues are experimentally put into conflict, the hummingbirds seem to prefer to rely on spatial versus visual cues to revisit rewarded locations: hummingbirds appeared to ignore the switching of colour patterns of flowers in an array when returning to a previously rewarded location. Rather, the birds returned to the flower in the previously rewarded location, even though it had the colour pattern of a previously unrewarded flower (Hurly & Healy 1996; Hurly & Healy 2002). However, it is not always clear which of the cues around the rewarded location are used to return to a rewarded location. For example, black-capped chickadees typically use distal landmarks to relocate their caches; however, if they do not find the cache at that site they then use proximal cues (i.e. those cues nearest to the goal) and they can also remember the visual features of the goal. Likewise, rufous hummingbirds will pay attention to the colour of the flowers they visit, although demonstrating that they remember this information can be problematic: if there is any relevant spatial information available, they will use it. The role of the visual information combined with spatial cues is complex; in one experiment when a hummingbird was trained to feed from a

particular flower and a second flower was simultaneously presented, the bird's response appeared to depend on the visual features of this second flower: the birds visited the new flower only when it was the same colour as the flower on which he was trained (Hurly & Healy 1996).

THESIS AIMS:

In the research conducted for my thesis I aimed to investigate learning and memory in three-dimensions using free-living, wild hummingbirds in their breeding range. I wanted to determine whether these birds learn about and use the vertical and the horizontal dimensions of 3-D space in an equivalent way and whether the degree to which an animal uses each of these dimensions influences its learning and accuracy of 3-D goals. My experiments were designed to address the following questions:

1. Can wild, free-living rufous hummingbirds learn a 3-D location after a single experience?

Rufous hummingbirds can learn 3-D locations after multiple visits to that location and they can learn the location of one of several flowers in an array in which all the flowers are at the same height after a single visit. However, it is not clear whether they can learn the height of a reward after a single visit and, therefore, if they will search for the 3-D location in the absence of the goal itself. Although this was the primary reason for asking this question in this first experiment, single visits to rewarding locations share features with the kinds of memories for one-off events that are being examined in an episodic-like memory context. The spatial aspect of episodic-like memory together with the content of the memory of what happened (in

the case of the foraging hummingbird, which flower contained a food reward) and the temporal element (i.e. when in the past the event happened), has been studied in detail in scrub jays (*Aphelocoma coerulescens*) in the laboratory. However, episodic-like memory has rarely been addressed in the wild. In addition, for most spatial tasks performed in the laboratory, including those with scrub jays, the animal can use proximal cues close to find the rewarded location. In contrast, I was interested to see if hummingbirds were able to relocate an absent (un-cued recall) flower in their own territory, testing only the spatial aspect of episodic like memory without.

To answer this question I allowed a hummingbird to feed from an artificial flower once before I removed the flower and placed another flower at some distance from the first flower. The new flower had the same visual features as the first one except that it differed in its height. The location where the bird hovered when it returned to feed on the first flower was recorded. Each bird experienced four different locations in total. If the birds hovered at the location where the flower used to be (now empty) it would show that they can relocate a goal without using the visual features of the goal and it would suggest that the birds can learn and remember a 3-D location after a single experience.

2. Are the vertical and horizontal dimensions of a 3-D location learned equally readily by rufous hummingbirds?

To determine whether the vertical and the horizontal dimensions of a 3-D location might be equivalent from a flying bird perspective, I designed a task to look at spatial learning in a single dimension. I trained hummingbirds to find a central rewarding

location within an array of five artificial flowers mounted on a wooden stake that was oriented either vertically or horizontally. I set the learning criterion to five consecutive first choice visits to the rewarded flower, testing only the birds once they had reached the learning criterion. To determine which cues the birds used to learn the location of the reward, I tested them in the same array on which they had been trained but having displaced the flowers one unit, either up or down, if the array was oriented vertically, or right or left, if it was oriented horizontally. I also trained the birds on each of the array types with two spacing scales between the flowers, small (5cm between the flowers) and large (30cm between the flowers), to determine whether the ease with which the location of the reward was learned and the cues used was a result of the array orientation or whether the spatial scale between the flowers was also relevant.

3. Do rufous hummingbirds learn a 3-D location as an integrated coordinate or do they learn the vertical and horizontal components separately?

To determine whether, when birds learn a rewarding location, they encode, and can retrieve, the vertical and horizontal components separately, I trained hummingbirds to feed from a diagonal flower array which consisted of four artificial flowers mounted on a wooden stake with a 30cm distance between them. The rewarded flower was always located in positions two or three of the four flowers, never at the ends of the array. After a bird had made 40 visits to the array, I tested them with the array presented in three different orientations, keeping the spacing between the flowers constant but displacing all of them either up or down, or right or left. If the birds chose the flowers at the same height and at the same x-y location as that

occupied by the rewarded flower during training, it would suggest that they can retrieve the vertical and horizontal component of a location separately.

Finally, to compare the vertical and horizontal use of space in learning the location of a 3-D reward and the accuracy in relocation of that reward, by a terrestrial and a flying animal, I performed two very similar experiments, in which I tested rats in the laboratory in one and rufous hummingbirds in the field in the other. The question for that experiment was:

4a. Do rats use the horizontal and vertical dimensions to the same extent while learning a rewarding 3-D location? Do they exhibit the same accuracy in both dimensions?

In this experiment, I trained rats to visit a 3-D rewarded location within a cubic maze. Half of the rats were trained using a reference memory task and the other half were trained on a working memory version of the task. In the reference memory task, the reward remained at the same location throughout the experiment, while in the working memory task, the rats had several trials a day at one rewarding location but they experienced a different rewarding location each day. At the end of the training period, rats were tested on the same maze but without a reward. From the tests I determined whether the rats first moved to a horizontally or vertically adjacent location after reaching the previously rewarded location. If the rats first crossed to a vertically adjacent unit this would suggest that they were more certain about the horizontal location of the reward and vice versa. Using the training data, I compared the number of crossings from one unit of the maze to another and the time spent

moving in the three axes (x, y and z). This provided a measure of the way in which the rats used space while learning the location of the reward.

4b. Do hummingbirds use the horizontal and vertical dimensions to the same extent while learning a rewarding 3-D location? Do they exhibit the same accuracy in both dimensions?

I trained hummingbirds to feed from a rewarding location within a cubic maze, which contained an artificial flower on each intersection of the subunits that made up the maze. Once the birds had learned the location of the reward, they were tested with the reward removed. As with the rats, to determine whether birds were more certain of the vertical or horizontal component of the learned 3-D location, in the test I compared the number of birds that crossed to the unit vertically or horizontally adjacent to the previously rewarded location. Also as with the rats I used the training data of the number of crossings of the maze subunits and the time spent moving through each axis (x, y and z) to determine the birds' use of space while learning the location of the reward. Together with the data from Experiment 4a, I could determine whether the rats and hummingbirds used space in a similar way while learning the 3-D location of a reward and whether, in the absence of that reward, their certainty of the location was similar with regard to its horizontal or vertical location.

CHAPTER 2. ONE-TRIAL SPATIAL LEARNING

This chapter has been published in Flores-Abreu, I., T. Hurly and S. Healy (2012) One-trial spatial learning: wild hummingbirds relocate a reward after a single visit. *Animal Cognition* **15**: 631-637. This version differs from the published version in that Figure 3 is now presented separately as Figure 3, 4 and 5, whereas they were published as part of the same figure.

ABSTRACT

Beaconing to rewarded locations is typically achieved by visual recognition of the actual goal. Spatial recognition, on the other hand, can occur in the absence of the goal itself, relying instead on the landmarks surrounding the goal location. Although the duration or frequency of experiences that an animal needs to learn the landmarks surrounding a goal have been extensively studied with a variety of laboratory tasks, little is known about the way in which wild vertebrates use them in their natural environment. Here we allowed hummingbirds to feed once only from a rewarding flower (goal) before it was removed. When we presented a similar flower at a different height in another location birds frequently returned to the location the flower had previously occupied (spatial recognition) before flying to the flower itself (beaconing). After experiencing three rewarded flowers, each in a different location, they were more likely to beacon to the current visible flower than they were to return to previously rewarded locations (without a visible flower). These data show that hummingbirds can encode a rewarded location on the basis of the surrounding landmarks after a single visit. After multiple goal location manipulations, however, the birds changed their strategy to beaconing presumably because they had learned that the flower itself reliably signalled reward.

Keywords: *Selasphorus rufus*, single-trial, spatial recognition, beacon, landmarks

INTRODUCTION

Successful small-scale navigation relies on animals being able to remember a goal's features plus proximal and distal landmarks and to use them either as a single beacon or as associative cues in order to relocate that goal (Gould et al. 2010). In order to relocate a goal, animals may form a memory either of the visual characteristics of the goal itself (goal recognition, beaconing) or of the landmarks surrounding that goal (spatial recognition) i.e. the goal need not be visible. In instances where the goal is very conspicuous, the animal does not need to learn the surrounding landmarks to reach the goal but when it is less conspicuous it may be more efficient for the animal to encode routes to the goal by learning those landmarks. In some cases beacons are used in establishing the spatial recognition system. For example, wood ants *Formica rufa* use beacons as a "scaffold" when learning a route such that when it is learned, removing the beacons has no impact and the ants follow exactly the same route as they had when the beacons were present (Graham et al. 2003). The ants' use of landmarks is context specific and depends on their foraging round-trip stage: they use panoramic landmark cues when experimentally displaced while departing from the nest or from the artificial feeder, whereas they rely on local landmark cues for arrival at either location (Fukushi & Wehner 2004). Rats, too, may use landmarks in a kind of scaffolding: when trained to find an escape platform signalled by a landmark at a constant orientation and distance from the platform they can relocate it in the absence of that landmark (Pearce et al. 1998). Beacons appear to facilitate learning about spatial information in pigeons (Kelly & Spetch 2004) and in humans (Kelly & Spetch 2004) as they do in ants and rats (Pearce et al. 2001; Pearce et al. 2006). Pigeons trained to a single rewarded corner in a rectangular arena with a distinct feature at each corner learn not only the feature

associated with reward but also the geometrical properties, even though these are unnecessary for successful completion of the task (Kelly & Spetch 2004). Finally, Clark's nutcrackers *Nucifraga columbiana*, trained to a single rewarded location in a similar rectangular arena, required that location to be identifiable with a visually-unique landmark: when all of the objects were identical, the birds did not learn the rewarded location. Furthermore, when the landmark information was put in conflict with the geometric information, the birds used the landmark (Kelly 2010).

This facilitation of location learning by the features of the goal itself (used as a beacon) is also seen in wild, free-living hummingbirds *Selasphorus rufus*, which learn the location of a reward faster when it is marked by a location-specific colour than when it is not (Hurly & Healy 2002; Hurly et al. 2010). However, since birds return to the location of the reward regardless of a change in the colour of the flower, it appears that birds encode a goal's location using landmarks in addition to the beacon (Hurly & Healy 2002). This is seen most strikingly when the rewarded flower at which a bird has fed on multiple occasions need not be present for the bird to fly to its location (Hurly et al. 2010). Examination of the birds' flight paths shows that even when the flower is displaced only 2m from the original rewarded location the birds flew first to the original location of the now absent flower before flying to the flower that was present. Furthermore, rufous hummingbirds' accuracy in relocating a rewarded location without being guided by a beacon is related to the size of the flower used for training: accuracy of relocation in three dimensions increases as the flower gets smaller. From now on we will call Spatial Recognition the strategy by which the birds relocate a previously rewarded location in the absence of a beacon, due to the need of identifying the surrounding landmark as in the example mentioned above.

We do not know whether the hummingbirds in the field require multiple experiences to learn a rewarded location, as do the ants and species tested in laboratory experiments. The aim of this study, then, was to determine whether rufous hummingbirds could relocate a location after one rewarded visit. Birds were allowed to feed from an artificial flower only once before we moved that flower to a novel nearby location. If the birds returned to the previously rewarded location, from which the flower was now absent, it would appear that the birds had learned the goal's location (spatial recognition) without needing to use the goal's visual features (beaconing) after a single experience.

METHODS

Study species and site

The subjects used in this experiment were 14 free-living male rufous hummingbirds, *Selasphorus rufus*. The experiment was run from 08:00-19:00 hr Mountain Standard Time in May and June in 2009 in a valley in the eastern Rocky Mountains, 20 km southwest of Beaver Mines, Alberta, Canada, (49° 20' 56.61'' N 114° 24' 38.49'' W). On return from overwintering in Mexico, males set up territories along this valley centred on artificial feeders at an approximate height of 2-3m from the ground containing 14% sucrose. Territorial males were caught, banded and colour marked (with nontoxic waterproof ink on the breast) for individual identification.

Description of experimental arena

The experiments were conducted within the birds' territories, which were about 1 ha in

size (Hurly et al. 2001) and largely forested with multiple open, grassy spaces (Figure 1).

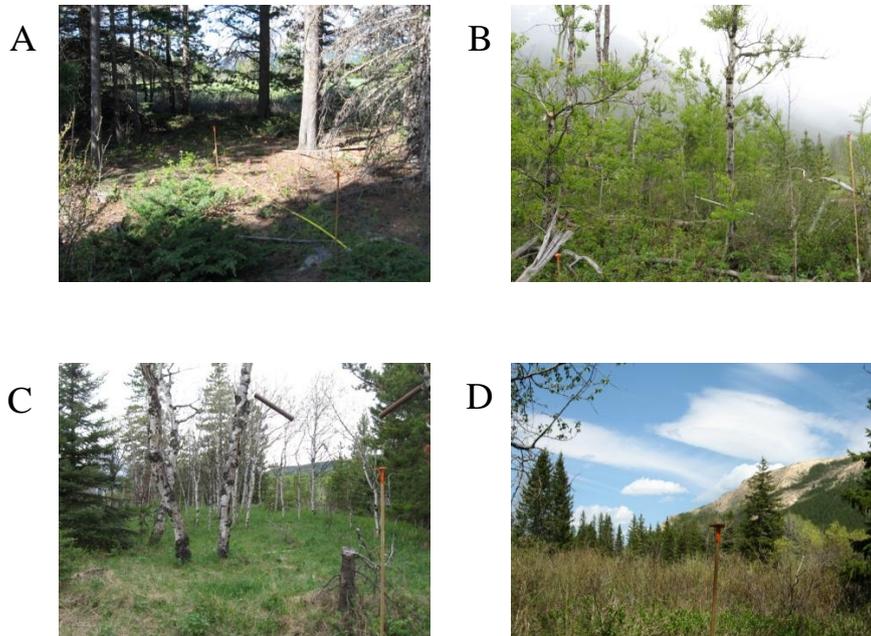


Figure 1 Examples of an experimental arena within a male hummingbird territory

Initial training

To train a bird to feed from artificial flowers containing sucrose, his feeder was lowered in three steps of approximately 1m, until the feeder reached the height of the training flower 60cm (2ft) which was always placed immediately below (at the exact ground position of) the feeder. The feeder was removed either once the bird fed from the training flower or when the bird had fed from the feeder beside the training flower. The experimental flower was made of a 6cm (2.4in) diameter disc of orange cardboard mounted vertically on a wooden stake. This stake was one of three different heights (short: 60cm (2ft) = S; medium: 120cm (4ft) = M; tall: 180cm (6ft) = T). In the centre of the flower was a syringe tip filled with 200 μ l of 25 % sucrose solution. As

hummingbirds fly through three dimensions, we used flowers of different heights so as to change the flower's location within three-dimensional space and not only in the horizontal plane. In this way we could determine whether the birds revisited locations at the correct height as well as the correct x-y location. The bird was allowed to feed twice from the training flower in the same location before the experiment began.

Therefore, whenever a bird hovered at the training flower location this was considered a visit based on two trials. Training took no longer than two hours.

Experimental Procedure

Once the bird had fed twice from the training flower (TF) it experienced three successive one-trial learning presentations in different 3-D locations: three horizontal displacements at a different height each. First, the training flower was removed and the first experimental flower (F1) was placed 2.74m (9ft) from the location of the training flower (Stage 1). After the subject had fed once from F1, that flower was removed and the second experimental flower (F2) was placed 2.74m (9ft) away from the location F1 had occupied (Stage 2). The bird was allowed to feed once from F2, that flower was removed and the third (F3) and last experimental flower was placed 2.74m (9ft) from the location F2 had occupied (Stage 3). All birds experienced the three different flower heights in a pseudo-randomized order. The placement of flowers was either linear, in the shape of an "L" or a diamond (Figure 2) so that successive flowers were not simply further from the location that had been occupied by the training flower.

When a bird flew in to feed from a nearby perch, he could fly directly to the visible flower or to any of the locations previously occupied by flowers. The approximate location of the visits to absent flowers could be recorded when a bird

hovered in the air in the vicinity of a flower's previous location. All visits as well as their order were recorded. All of male's visits to his meadow ended by his feeding at the visible flower. After feeding from a flower, the male perched high up in a tree in his territory, chased other males or displayed to females. Once the bird had fed from F3 the feeder was returned to its original location and the bird's behaviour was recorded for a further foraging bout.

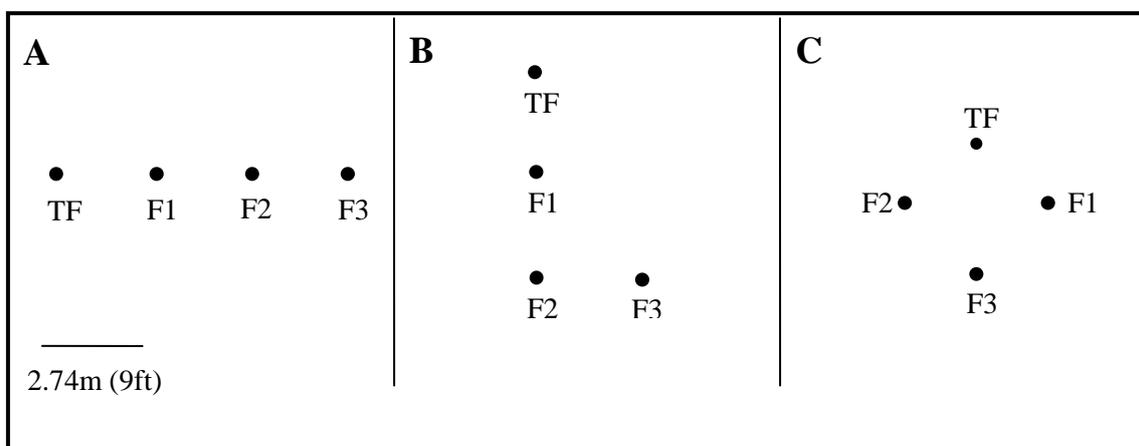


Figure 2 A schematic of the spatial patterns in which flowers were presented: **A)** linear, $n = 3$ **B)** L-shape, $n = 3$ and **C)** diamond shape, $n = 8$. *Solid circles* represent the location of artificial flowers. The bird was only ever presented with a single flower at any one time

RESULTS

All the birds returned to previously rewarded locations, hovering for 1-3sec, in one or more experimental stages. None of the birds hovered at locations other than those at which they had fed at some point in this experiment (the feeder, TF, F1 and F2). Most of them (10/14) required a single visit to relocate a relevant location, returning to it after a single rewarded visit in at least one experimental stage, i.e. F1 and F2, having

previously visited each of these just once to feed. The interval between foraging bouts was 15.0 ± 0.5 (SE) min.

Spatial recognition

A bird could make two kinds of visits when he returned to forage in the experimental area: either to the present flower or to an empty space where there used to be a rewarded flower. We designated all those occasions on which the birds hovered very near (approximately 20cm of the flower's previous location) to the location of an absent flower, spatial recognition, while flying directly to a flower we designated as beaconing. The flower could have been the training flower (TF) or any of the subsequent three experimental flowers (F1, F2 or F3). Hovering at the locations of absent flowers always ended (in less than 10sec) with the bird feeding from the present flower.

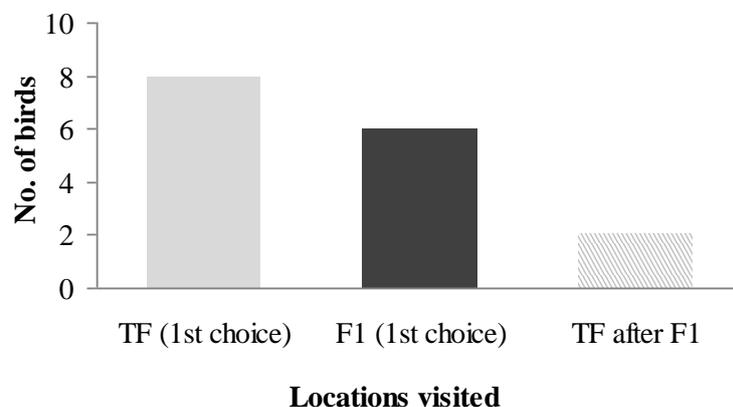


Figure 3 Locations visited during the first experimental stage (when F1 is the visible flower). The *light grey bar* represents the number of birds that used spatial recognition on their first choice (after two visits to TF), The *dark grey bar* represents the number of birds that beaconed to their first choice and the *striped bar* represents spatial recognition used after visiting F1. TF = Training flower, F1 = Flower 1, $n = 14$

When F1 was present 10/14 birds also visited the training flower's location (Figure 3). When F2 was present 12/14 birds visited the locations of either the training flower only (two-trial learning: 3/12 birds) or F1 (with or without visiting TF, single trial learning: 9/12 birds; Figure 4). When F3 was present 8/13 birds visited the locations of either only TF (1/8) or F1 and/or F2 (with or without visiting TF, single trial learning: 7/8; Figure 5). The proportion of birds that performed spatial recognition (regardless of being its 1st choice or not) at any time during a trial was similar throughout the experiment (Figure 6).

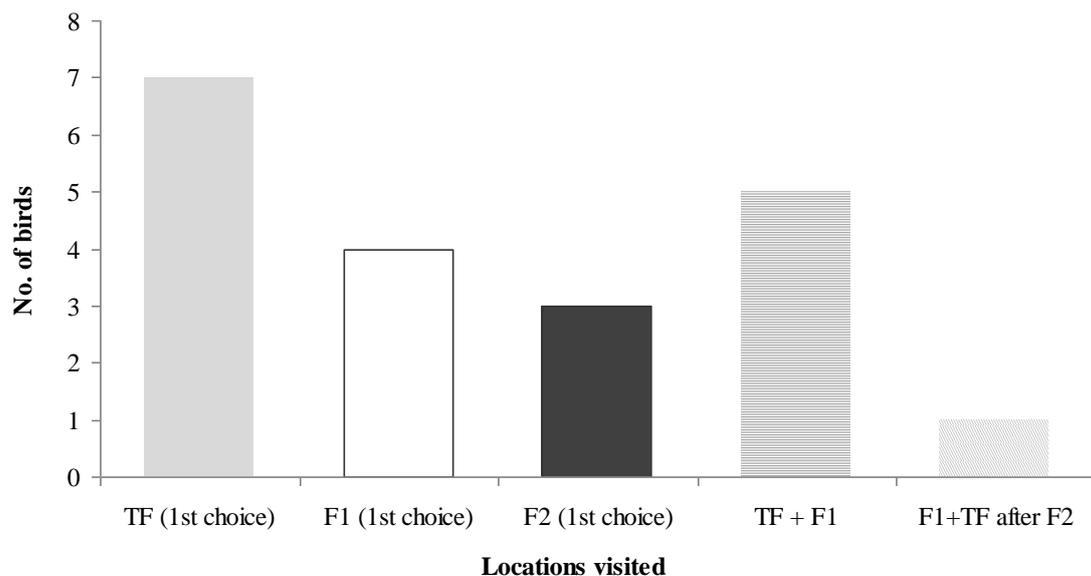


Figure 4 Locations visited during the second experimental stage (when F2 was the visible flower). The *Light gray bar* represents the number of birds that used spatial recognition as their first strategy after two visits to TF, while the *white bars* also represent the number of birds that used spatial recognition but after a single experience. The *dark grey bar* represents the number of birds that beacons for their first choice. The *horizontal striped bar* represent visits to both TF and F1 before beacons was used to visit F2 while the *grey diagonal striped bar* represent visits to F1 and/or TF after visiting F2. TF = Training flower, F1 = Flower 1, F2 = Flower 2, $n = 14$

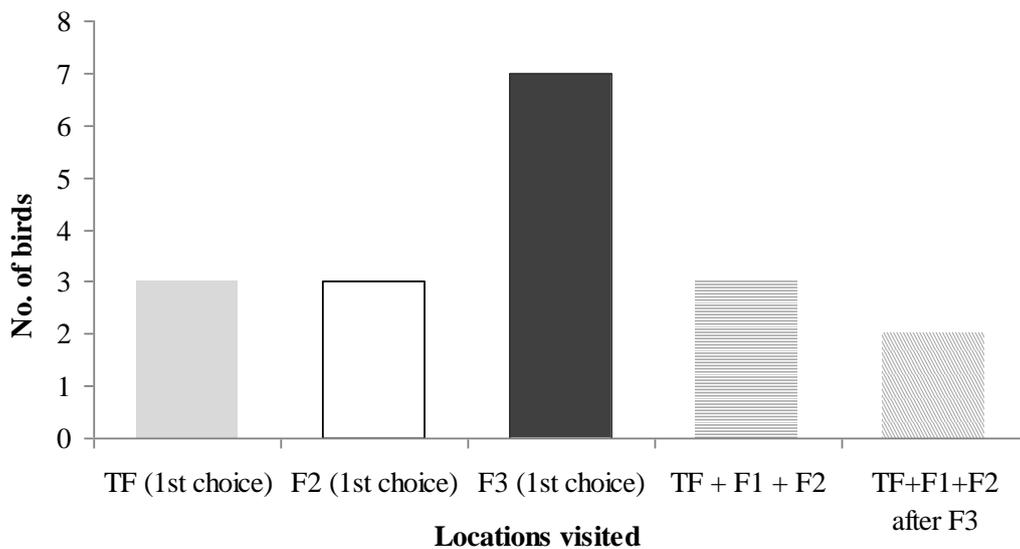


Figure 5 Locations visited during the third experimental stage (when F3 was the visible flower). The light grey bar represents the number of birds that used spatial recognition as their first strategy after two visits to TF, while the white bars also represent the number of birds that used spatial recognition but after a single experience. The dark grey bar represents the number of birds that beacons for their first choice. The horizontal striped bar represents visits to F2, F1 and/or TF after visiting F3. TF = Training flower, F1 = Flower 1, F2 = Flower 2, F3 = Flower 3, $n = 13$

To evaluate the birds' strategy for relocating flowers, we compared the proportion of birds that hovered at one of the flower's previous locations (spatial recognition) as their 1st choice before going on to the actual flower with a chance expectation of the same probability of a visit to each flower location. We did this for each of the three stages. The probability of spatial recognition increases throughout the experiment from 0.5 in Stage 1 to 0.67 and 0.75 in Stages 2 and 3 respectively due to the increase in flower locations (i.e. during Stage 3, chance would be 25% as there were then four possible locations: TF, F1, F2, F3). The number of beaconing visits (as a 1st

choice) was significantly higher than chance only at Stage 3 (Stage 1: $X^2 = 0.286$, $df = 1$, $p = 0.593$; Stage 2: $X^2 = 0.986$, $df = 1$, $p = 0.321$; Stage 3: $X^2 = 5.770$, $df = 1$, $p = 0.016$;

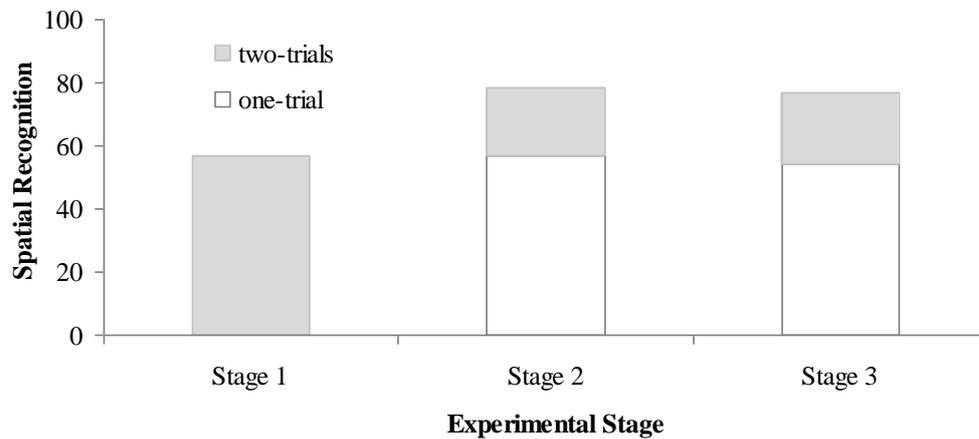


Figure 6 The proportion of birds that used spatial recognition in each experimental stage. One-trial learning refers to visiting the previous location of F1 at Stage 2 or to that one of F1 or F2 in Stage 3. Two-trials refers to visiting the previous location of the training flower at any stage

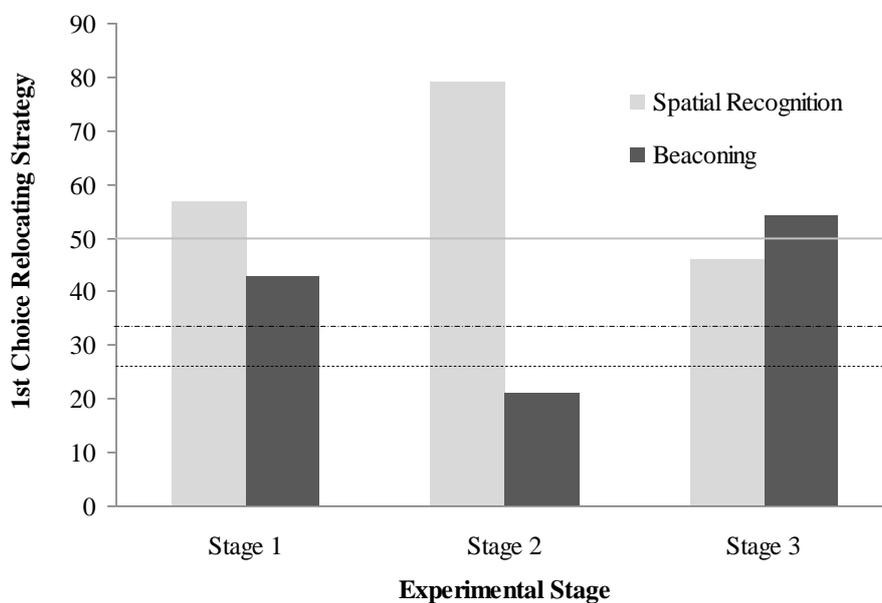


Figure 7 The strategy birds used to visit flowers. The light grey bars represent the proportion of the birds that used spatial recognition as their first choice in all experimental stages. The dark grey bars represent the proportion of birds that used beaconing as their first choice. The grey

line represents the chance level for stage 1 (line A), the dot-dash line the chance level for stage 2 (line B) and the dotted line the chance level for stage 3 (line C), respectively

see Figure 7). In addition, a significant proportion of birds (7/11) switched from spatial recognition during Stage 2 to beaconing in Stage 3 ($X^2 = 8.757$, $df = 1$, $p = 0.004$).

DISCUSSION

Wild, free-living rufous hummingbirds can relocate a goal in the absence of the goal's visual features after a single feeding event. As far as we know this is the first time that spatial recognition, in the absence of any marker at the previously rewarded locations, after a single trial has been demonstrated in the field. In all previous experiments memory has been tested after considerable training or when the beacon remains in place during the one-trial spatial experiments (Brodbeck et al. 1992; Healy & Krebs 1992; Clayton & Krebs 1994; Hurly & Healy 1996; Henderson et al. 2001; Hurly & Healy 2002; Hurly et al. 2010). The hummingbirds' ability to relocate a reward in the absence of a beacon is consistent with the evidence that they attend to other environmental spatial cues in addition to just the features of the goal (Hurly & Healy 1996; Healy & Hurly 1998; Hurly & Healy 2002; Healy & Hurly 2003). We assume that the birds used a combination of landmarks surrounding the goal to do so (e.g. the meadow's geometry, trees, bushes, trunks, rocks, flowers etc.), perhaps as a cognitive map, which is an allocentric representation in terms of distances and directions among items in the environment (O'Keefe & Nadel 1978; Collett & Graham 2004). However, as the experiment progressed the birds appeared to switch from using spatial recognition to using the cues supplied by the visible flower. This finding is consistent with rats

eventually learning to use the beacon signalling a goal's location when the beacon is a more reliable indicator of reward than are the surrounding landmarks (Pearce et al. 1998).

Different brain regions appear to underpin the use of beacons and the encoding of locations with regard to the surrounding landmarks: rats with hippocampal damage appear to rely on beaconing regardless of cue reliability, as do rats with medial lesions to the dorsal striatum (Devan & White 1999). fMRI data recorded during the performance of virtual reality task support a similar spatial recognition/beaconing dichotomy in humans too, in the right posterior hippocampus and dorsal striatum, respectively (Doeller et al. 2008).

The behaviour of the birds during experimental Stage 2 shows that hummingbirds can encode and retrieve the spatial position within their environment after a single experience. In addition, the birds' first choice at Stage 3 suggests both primacy and recency effects: more visits to both TF (primacy) and F2 (recency) locations (6/6 birds) than to F1 (0 birds) appears somewhat similar to the performance of humans on word-list learning and with the performance on spatial and non-spatial tasks by other animals (Bjork & Whitten 1974; Castro & Larsen 1992; Crystal & Shettleworth 1994).

That a rufous hummingbird, while visiting a rewarded flower for the first time, encodes the spatial location and then remembers this information to revisit the flower is a remarkable phenomenon when viewed in an ecological context. Hummingbirds visit hundreds of flowers each day, taking a tiny amount of nectar from each. Given the birds' behaviour in our experiment, it seems possible that the spatial location of each flower is encoded for each visit, even when the flower is empty: rufous hummingbirds

that empty some flowers in an array can avoid these flowers on their next visit and direct their foraging to previously unvisited flowers (Healy & Hurly 1995). If locations of numerous flowers are encoded each day, one must question how much spatial information can be retained and how out-of-date information is eliminated or discounted.

Our experimental design is comparable to those used to test spatial memory in the absence of the beacon e.g. delayed-matching-to-place water-maze tasks (Steele & Morris 1999) and one-trial place memory (Bast et al. 2005) as well as cued recall in rats e.g. place-odour paired-associate task (Day et al. 2003), all of which involve the animal retrieving a location after a single experience. Retrieving a single experience is one of several characteristics of episodic-like memory. Since the ground-breaking experiments with scrub jays retrieving different kinds of stored food, the evidence that birds and rodents have episodic-like memory has mounted (Clayton & Dickinson 1998; Clayton & Dickinson 1999; Clayton et al. 2003; Day et al. 2003; Eacott et al. 2005; Babb & Crystal 2006; Dere et al. 2006; Naqshbandi & Roberts 2006; Eacott & Easton 2007; Roberts et al. 2008; Zinkivskay et al. 2009; Crystal 2010). However, all of these experiments have been carried out in the laboratory. It is not yet clear whether hummingbirds are capable of episodic-like memory but as they can learn the refill rate of flowers (Gill 1988; Henderson et al. 2006), as well as what flowers look like and their locations (Hurly 1996; Hurly & Healy 2002), it may be that they will prove to be a useful system in which to explore episodic-like memory. The fact that they show learning in a trial-unique way is a key aspect for the study of episodic memory. It is possible that if hummingbirds can encode the location of a reward from a single experience they could also distinguish between different experiences (for example: if

the flower they sample was empty or full and if it contained nectar with a high or a low sugar concentration), which would imply that wild birds encoded the “what and where” aspects of episodic-like memory. Such episodic-like memory is yet to be tested in wild hummingbirds.

Acknowledgements

This research was supported by CONACYT (Consejo Nacional de Ciencia y Tecnología), the University of Lethbridge and the Natural Sciences and Engineering Council of Canada. We thank Cam Finlay and Ida Bacon for support in the field and Rosamund Langston and three anonymous reviewers for comments on an earlier version of the manuscript.

CHAPTER 3. ROLE OF AXIS ORIENTATION IN SPATIAL LEARNING

ABSTRACT

Rufous hummingbirds learn about the flowers they forage on, which vary widely in their spatial distribution. These flowers may share a horizontal location but vary in the vertical plane. To date, most studies of spatial learning have been conducted in the horizontal plane, with few addressing the vertical dimension. The aim of this study was to compare the hummingbirds' learning a rewarded location that differed from other similar but unrewarded locations either in only the vertical or in the horizontal axis. We also wanted to determine whether birds could dissociate the vertical and horizontal components of a learned 2-D location. We found that the hummingbirds' learning performance on a linear flower array depended on the orientation of the array and on the spacing distance between the flowers in the array: birds chose the flower in the rewarded location more than any of the other four flowers when the array was oriented horizontally but only when flowers were spaced 30 cm (rather than 5cm) apart. In contrast, when the array was oriented vertically or when the flowers were 5cm apart in the horizontal array, birds visited the rewarded flower and its two adjacent flowers more than they visited the two distal flowers. In addition, which cues are used to learn the rewarded location depended on the spacing scale in the horizontal dimension: at 30cm flower spacing, birds learned the absolute location whereas at 5cm spacing they chose the relative and absolute locations equally. Birds did not reach the learning criterion in the vertical arrays. In the diagonal linear array, birds chose the rewarded flower more than any other. When the array was displaced to either the vertical or the horizontal for the test, the birds chose the flower in the same horizontal location as for the rewarded

training location. Our findings are consistent with hummingbirds encoding the vertical dimension less precisely than they encode the horizontal dimension.

Keywords: *Selasphorus*, hummingbirds, spatial learning and memory, orientation, vertical, horizontal

INTRODUCTION

Much of animal behaviour has a spatial component. For example, territoriality, predator avoidance, provisioning young, and foraging all require animals being able to move through space and to keep track of where they have been, where they are and where they are going (Sherry 1998). Although space itself is three-dimensional, spatial cognition has traditionally been studied in the horizontal plane, using an array of bi-dimensional mazes or arrays in which the animal has either to escape or to relocate a reward in a rather small-scale spatial tasks in an experimental room (i.e. open arena, T-maze, E-maze, radial maze, Morris water maze). Most of these tasks require the animal to navigate across a horizontal surface without moving through a vertical component (Cheng & Gallistel 2005). In a few experiments, the surface itself has been vertical but the task either involved animals learning locations in only two dimensions (Hurly & Healy 1996; Thiele & Winter 2005) or animals being presented with alternative locations that varied only in the horizontal, even though the animals had to move vertically between them (Jacobs & Shiflett 1999). Path integration, which is the capacity to use egocentric cues (generated by the animal's movements) to calculate the updated position of the animal by monitoring its direction and velocity in relation to a start location, and allowing the animal to return to its starting point using the most direct

route in the absence of landmarks (Whishaw & Wallace 2003), has also been traditionally studied only in the horizontal plane in laboratory vertebrates as well as in invertebrates and humans in tests outside the laboratory (Seyfarth et al. 1982; Etienne et al. 1986; Séguinot et al. 1993; Müller & Wehner 1994; Etienne et al. 1996; Drai et al. 2000; Whishaw et al. 2001; Wallace et al. 2002).

The role of the vertical component in estimating the distance travelled was investigated for the first time in desert ants (*Cataglyphis fortis*). Ants did not appear to use a three-dimensional odometer: ants trained to a food source over hilly terrain and tested in flat terrains or vice versa, travelled homing distances that reflected the horizontal distance, and not the actual distance, suggesting they either do not encode, or that they discount their vertical movements (Wohlgemuth et al. 2001; Grah et al. 2005). Ants also do not compensate for vertical changes while relocating their goal: they did not climb up a slope provided so that they might compensate for the distance they were forced to walk down a slope at the beginning of the test (Grah et al. 2007).

To our knowledge, the first explicit attempt to examine the learning and memory of both vertical and horizontal components of a location required rats to relocate a rewarded location within a cubic maze (Grobéty & Schenk 1992). Rats took fewer visits to learn locations on a maze that included a vertical dimension (either a vertical wall or a cubic maze) than they did on a purely horizontal maze (Grobéty & Schenk 1992). In contrast, fish took as many trials to learn a location in the horizontal, as they did a location in the vertical and one in a two-dimensional Y-maze (Holbrook & Burt de Perera 2009). However, rats and fish both may favour vertical over horizontal information: rats trained to a 3-D location on a cubic maze spent more time at the height where the reward used to be than at its horizontal location and when fish were forced to

choose between the vertical and the horizontal components of a location, they chose the vertical (Holbrook & Burt de Perera 2009; Holbrook & Burt de Perera 2011).

Hummingbirds, too, which can relocate the three-dimensional location of a reward after a single experience (Flores-Abreu et al. 2012), after several experiences of a rewarded location were more accurate in returning to the vertical dimension than they were in the horizontal when the reward was removed (Hurly et al. 2010).

These animals could have learned to relocate a three-dimensional location by integrating the horizontal and vertical information throughout training or by learning individually the vertical and the horizontal components. To evaluate whether hummingbirds, as a representative flying animal, learn or treat vertical and horizontal information differently we investigated the role of the array orientation on the learning of a relevant location on a wild hummingbird, *Selasphorus rufus*. To do this, we presented five artificial flowers to the birds in one of two array types: all flowers in a vertical column, in which the horizontal location was the same for all the flowers (vertical array) or in a row, in which all the flowers were at the same height (horizontal array). If the birds find it as easy to learn a flower's location in the vertical as in the horizontal, birds should take a similar number of trials to learn a location on both array types.

In the second part of our study we investigated if the hummingbirds learned the vertical and horizontal components of a two-dimensional location separately and if they prefer to use one over the other if they were forced to choose between them. To do this we trained the birds to locate a rewarded flower in the middle (either position two or position three) of four flowers along a diagonal array oriented at 45°, which meant that each flower was defined by unique x, z coordinates. The birds were tested in three

different four-flower arrays: in the vertical, in the horizontal and in the inverted diagonal. If the birds learn both the vertical and the horizontal components of the location we expect them to choose the corresponding component in the vertical and horizontal tests. If the birds prefer either the vertical or the horizontal over the other, then we expect them to demonstrate that preference in the forced choice in the inverted diagonal test.

METHODS

Subjects

The subjects used in this experiment were 27 wild, free-living male rufous hummingbirds individually identified by a line drawn with a waterproof marker on the white part of their chest. The experiment was conducted in a valley in the eastern Rocky Mountains in Alberta, Canada (40° 20' 56.61'' N 114° 24' 38.49'' W). All birds used in this experiment had taken part previously in other, unrelated behavioural experiments and were, therefore, familiar with feeding from a variety of feeding devices within their territory. The birds used in Experiment 1 did not participate in Experiment 2.

Experiment 1 was run from 08:00-19:00 hr Mountain Standard Time in June and July of 2009 and of 2010 while Experiment 2 was run in June and July of 2010 and of 2011.

This study was carried out with ethical permissions from the University of St. Andrews Ethical Committee, the University of Lethbridge Animal Welfare Committee, Alberta Sustainable Resource Development and Environment Canada.

EXPERIMENT 1

Training

Seven birds were presented with a linear array of five artificial flowers, each of which consisted of a circular piece of cardboard (3cm diameter) pierced by a 0.5 ml microcentrifuge vial in its centre (insert in Figure 1A). The five flowers were attached to a 2.6 m long wooden stake 30 or 5cm from each other, centre to centre. The central flower of the array was filled with 500 μ l of 25% sucrose solution while the other four flowers were left empty.

The wooden stake with the flower array was presented either vertically or horizontally 4m from the location of the feeder. When presented vertically, one end of the stake was pushed into the ground and when presented horizontally the stake was supported by two thin metal legs at a height of 30 cm from the ground (Figure 1).

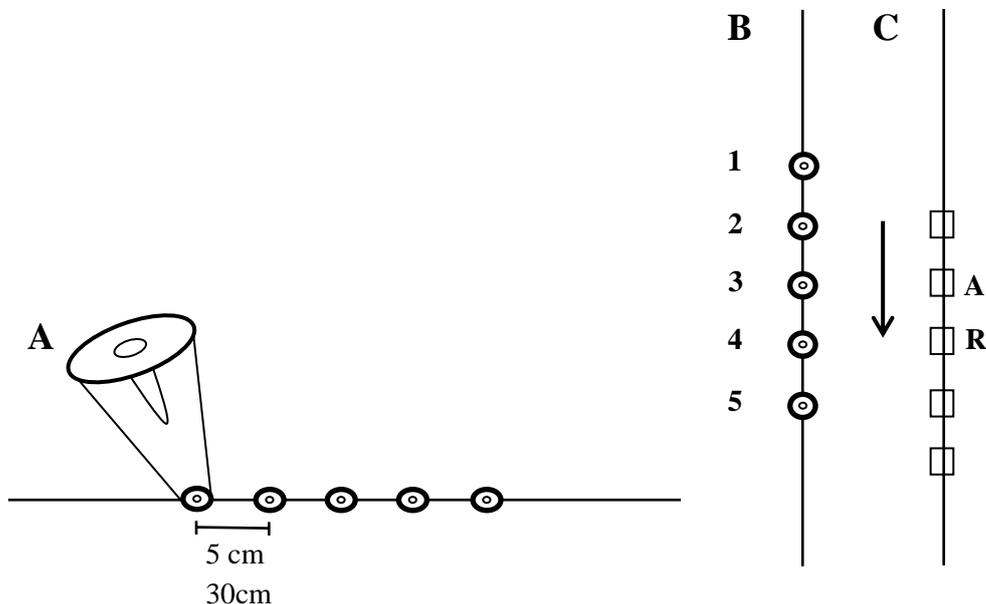


Figure 1 A schematic of the flower array, showing: A) the horizontal array with an insert showing an example of a flower; B) the vertical array, C) the displacement of the flower in the test trial: in this example, the flowers were moved downwards one unit. The squares represent the new flower locations

All seven birds were trained with the array in both dimensions (except for one bird, which experienced only the horizontal array) but the sequence in which they experienced them differed. All of the possible combinations of the array orientation and spacing between the flowers were pseudorandomized and then assigned randomly to the birds. Once a bird had fed from the central flower, the other flowers were filled with water, which the birds dislike.

Subsequently, we allowed birds to visit the array for 70 visits or until they had chosen the rewarded flower as their first choice in five consecutive visits, whichever happened first. Only the birds that chose the rewarded flower five consecutive times as their first choice were presented with a test trial. We recorded the order in which the flowers were visited on each bout during training and tests.

Test Procedure

Once the criterion level (five consecutive first choice visits to the rewarded flower) was reached, all of the flowers were emptied and displaced one unit either to the left or to the right for horizontal arrays or up or down for the vertical arrays (Figure 1C). In the example shown in Figure 1C, following displacement Flower 2 occupied the location previously occupied by the rewarded flower, while Flower 3 occupied the location previously occupied by Flower 4. If the birds chose to visit Flower 2 during the test, it would appear that they had learned the absolute location of the reward, while by visiting Flower 3 it would show that they had learned the relative position of the reward (the central position in array). We categorized the test visits into three types: to the central flower (in the same relative position), to the flower in the absolute location of the

original flower or to any one of the flowers in positions or locations that had not been rewarded.

EXPERIMENT 2

Training

20 birds were trained to feed from a linear flower array using a wooden stake as described in Experiment 1. The difference between the experiments was that in Experiment 2 the array consisted of four artificial flowers 42.4cm apart from each other, to keep the vertical and horizontal distance at 30 cm (Pythagoras Theorem). The rewarded flower contained 500 μ l of 25% sucrose solution and was either Flower 2 or Flower 3 while Flowers 1 and 4 were always empty. The wooden stake with the flower array was presented diagonally (45° with respect to the ground) 4m from the location of the feeder. As in Experiment 1, the unrewarded flowers were not filled with water until the bird had fed once from the rewarded flower. Subsequently, we allowed birds to visit the array for 40 visits or until they had chosen the rewarded flower as their first choice in five consecutive visits, whichever happened first. All birds were presented with three test probes, which only differed in the orientation (vertical, horizontal or diagonal rotated 180°, this latter constituted the conflict test), in pseudorandomized order with 10 extra trials in between each test probe. We recorded the order in which the flowers were visited on each bout during training and tests.

Test Procedure

The test probe arrays consisted of an identical wooden stake with four flowers aligned along the stake as in training but with the location of the stake altered in the following

way (Figure 2): in the horizontal test the stake was presented so that the flowers were in the same horizontal locations but both ends of the stake were now at the same height off the ground while in the vertical test one end of the stake was placed in the same location as occupied by one end of the stake during training so that the flowers were in the same vertical locations as in training but not in the same horizontal locations. In the conflict test the stake was placed as if to form a cross, relative to the stake's location during training. The training array was immediately removed after setting up any of the tests (Figure 2). In the vertical and conflict tests the flowers in the arrays were displaced one unit either up or down to provide the birds with a flower at the same height as that of the rewarded flower during training. In the horizontal and conflict tests the flowers in the array were displaced one unit to one side or the other to provide one flower at the same horizontal location as the reward during training. This displacement allowed us to test whether the birds learnt the absolute height/horizontal location or whether they chose the flower in the correct position relative to the other flowers. The direction in which the flowers were displaced was pseudo-randomized. The distance between the flowers was 42.4cm for the conflict test because it was also presented diagonally, however it was 30 cm for the horizontal and vertical test to keep the vertical or horizontal distance constant. If the bird chose to visit Flower "a" during the vertical test and Flower "b" in the horizontal test, it would show that he had learned the height and the horizontal location of the reward (see Figure 2 C and D); if most of the birds chose "a or b" in the conflict test it would reflect a preference to use the vertical or the horizontal respectively while if half of the birds chose "a" and the other half "b" it would suggest that they do not have a preference.

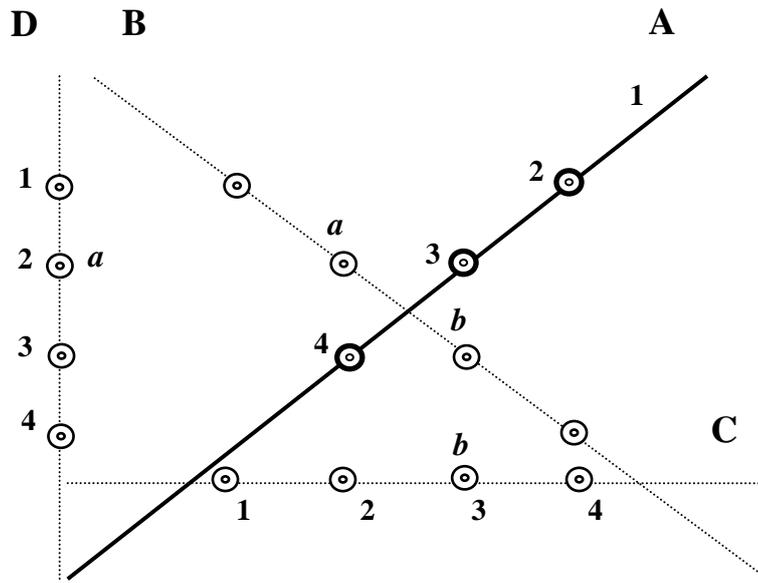


Figure 2 A schematic of the diagonal flower array where the line denotes the wooden stake and the large circles surrounding a small circle represent flowers. A) Training array, B) Conflict test probe, C) Horizontal test probe, and D) Vertical test probe. In this example flower 3 in array A was the rewarded flower. For all of the test probes, flowers *a* and *b* represent respectively the vertical and horizontal component of the previously rewarded location

RESULTS

EXPERIMENT 1

All seven hummingbirds reached the learning criterion (five consecutive first choices to the rewarded flower) on the horizontal array at both spacing scales (5cm and 30cm).

Birds took fewer trials to reach criterion on the 30cm horizontal array than they took on the 5cm horizontal array (a mean and SE of 20 ± 6.9 and 63.6 ± 6.6 trials respectively;

Mann-Whitney; $Z = 2.637$, $p = 0.008$). However, no birds reached criterion on the

30cm spacing scale when the array was vertical while on the 5cm spacing scale 2/6

birds did so after a mean and SE of 44.5 ± 16.5 bouts.

During the horizontal test, all of the birds chose the absolute location in the 30cm spacing scale (Binomial, 30cm scale: (7/7), $Z = 2.268$, $p = 0.016$; Figure 3).

However, in the 5cm test, two birds went to the flower in the absolute location, three to the flower in the relative position and two to neither of these flowers.

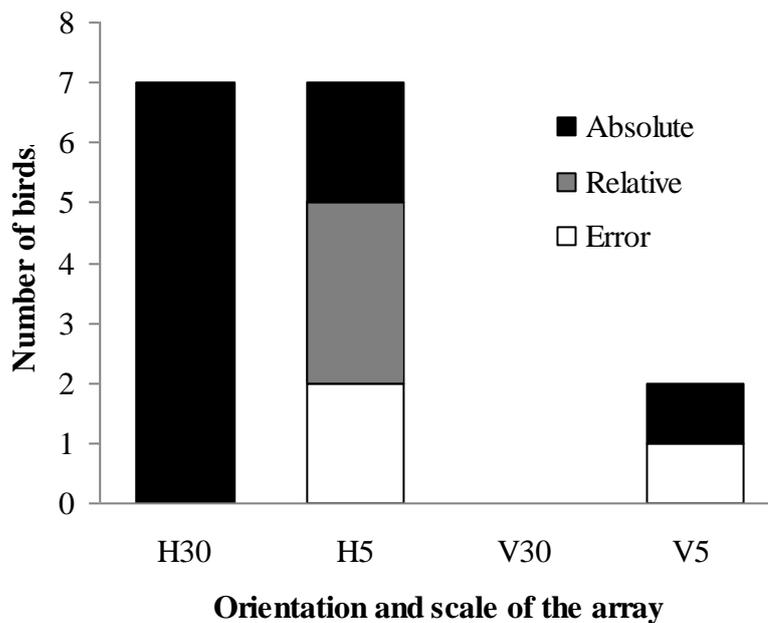
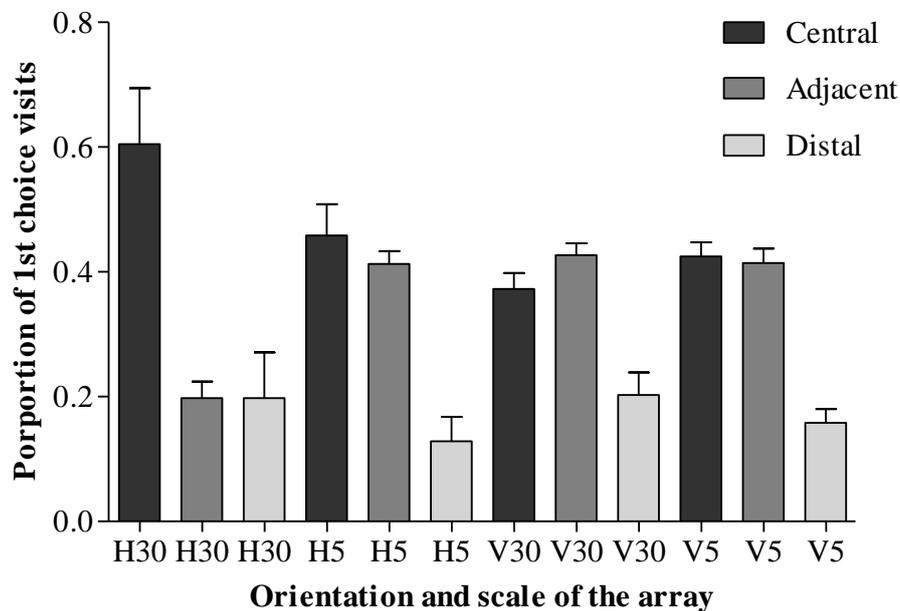


Figure 3 The birds' first choice in the test trial. The data are the number of birds that chose the flower in the absolute location, the flower in the relative position or chose one of the other three flowers (an 'error'). These data are the first choice only made by each bird on the test for each treatment. There are no data for the V30 treatment as none of the birds reached criterion and so none were tested

Because in the vertical training almost no birds reached criterion, we examined the proportion of the total number of 1st choice visits made to each flower type throughout training for both orientations and scales. We combined flowers by their position into three categories (central: Flower 3; adjacent: Flowers 2 and 4; distal: Flowers 1 and 5). Across all four conditions, birds visited the central flower most

frequently followed by visits to the adjacent flower and they visited the distal flowers the least (Figure 4). The magnitude of this effect was dependent on the orientation and scale of the array as reflected by the 2 and 3-way interactions (Repeated-measures ANOVA on first choices; orientation-scale interaction: $F_{1,5} = 11.69$, $p = 0.019$; orientation-scale-flower position interaction: $F_{1,9,9,5} = 6.58$, $p = 0.017$; see Table 1). If we look only at the proportion of 1st choices, it would appear that the birds differentiated the rewarded location from the other two categories of flowers when the array was horizontal and the spacing between the flowers was 30cm. However, it also appears that in rest of the treatments the birds learned to avoid the distal flowers.

A

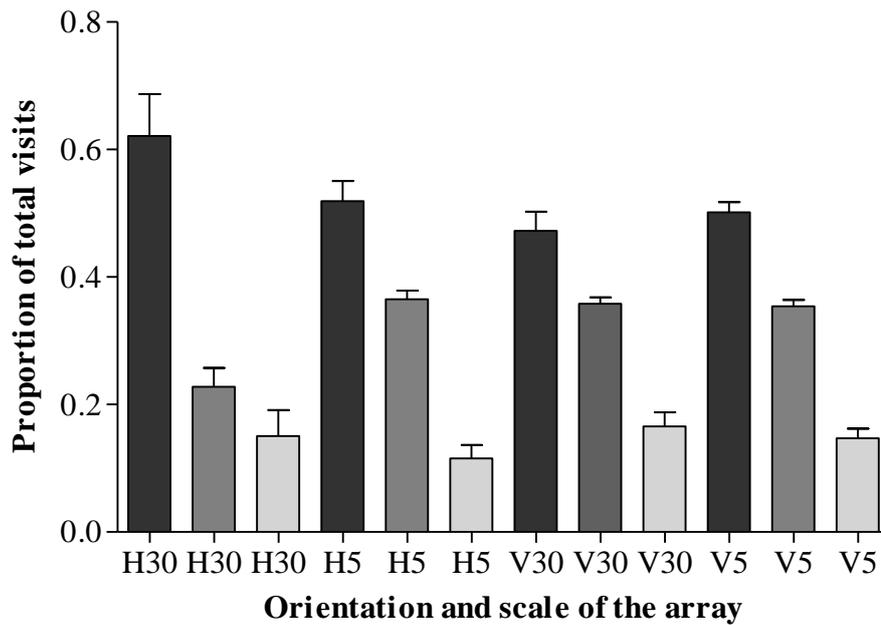
B

Figure 4 The proportion of visits to the central (rewarded), adjacent and distal flowers. The data are the mean (\pm SE) proportion of A) 1st choices and B) total visits to the three types of flowers from seven birds trained at each orientation scale. H30 = Horizontal 30cm, H5 = Horizontal 5cm, V30 = Vertical 30cm, V5 = Vertical 5cm

Table 1. The output from a Repeated Measures 3-factor ANOVA to examine the relative roles of orientation and scale on choice of flower position.

Experiment 1	Factor	<i>F</i>	<i>df</i>	<i>p</i>
First choices	orientation	14.17	1,5	0.013
	scale	1.18	1,5	0.328
	position	23.51	1.2,6.2	0.002
	orientation * scale	11.69	1,5	0.019
	orientation * position	3.32	1.1,5.5	0.122
	scale * position	4.81	1.6,8	0.048
	orientation * scale * position	6.58	1.9,9.5	0.017
	Total choices	orientation	3.34	1,5
scale		1.12	1,5	0.339
position		82.19	2,10	< 0.001
orientation * scale		6.07	1,5	0.057
orientation * position		3.59	2,5.9	0.11
scale * position		2.02	2,6.18	0.207
orientation * scale * position		9.69	2, 5.6	0.021

Huynh-Feldt corrections in *F*-*df* and *p*-value for Sphericity violation

If we consider the mean proportion of total visits, instead of only their 1st choice, birds in the 30cm horizontal treatment clearly differentiated the rewarded flower from both of the other kinds of flowers, and visited those other flowers to the same degree. However, for the other three treatments (H5, V30 and V5) it would appear that the birds visited each of the three categories of flowers differently, depending on their proximity to the rewarded flower: they visited the central flower most, went less often to the adjacent flowers and least of all to the distal flowers (Figure 4B). The magnitude of the difference in visiting each of the three positions was dependant on the orientation and scale (RM ANOVA on total choices; orientation-scale-flower position interaction: $F_{2, 5.6} = 9.69$, $p = 0.021$; see Table 1). If we evaluate the role of orientation in each scale there is an interaction on the number of total visits to each flower position only when the spacing between the flowers in the array is 30cm (2-factors RM ANOVA: scale 30cm orientation, $p = 0.089$; position, $p = 0.002$; orientation * position interaction, $p = 0.046$; scale 5cm orientation, $p = 0.65$; position, $p < 0.000$; orientation * position interaction $p = 0.566$). If we considered both orientations and scales for the total number of visits, the proportion of visits to the central flower is given by an interaction between the orientation and scale of the array (RM ANOVA: orientation, $p = 0.113$; scale, $p = 0.336$; orientation * scale interaction $p = 0.039$). In addition, the proportion of total visits to the adjacent and distal flowers is given by the orientation and scale for the former and by none of them in the latter (RM ANOVA: total visits to Adjacent flowers, orientation, $p = 0.031$; scale, $p = 0.014$; orientation * scale interaction, $p = 0.001$ and total visits to distal flowers: orientation, $p = 0.465$; scale, $p = 0.427$; orientation * scale interaction, $p = 0.621$). Of the distal flowers on the vertical arrays, birds visited them both equally often (5 and 30cm): they did not prefer to visit the higher flowers

(Wilcoxon: number of visits to Flower 1 and 5 in V30 cm: $Z = 1.15$, $p = 0.249$; V5 cm: $Z = 0.73$, $p = 0.465$).

EXPERIMENT 2

Only 13/20 birds attained the criterion and chose the rewarded flower five consecutive times during their training, while the seven remaining birds did not reach criterion within 40 trials. However, all birds chose the rewarded flower more than any other within the array showing that they learned its location (RM 1 factor ANOVA: $F_{3,57} = 23.49$, $p < 0.001$; Figure 5). All of the birds were tested on one of the three probe tests after 40 visits or after reaching criterion. We decided to present the birds with the probe trials after 40 visits because the cumulative visits to the rewarded flower in Experiment 1 reached a plateau at 40 visits. However, we decided to have a learning criterion that allowed us to detect when birds had learned the rewarded location in less than 40 visits because our experiments were limited by time. In addition, there was no difference between the birds that reached the criterion and the ones that did not reach it in their performance during the probe trials. Then they were given ten extra training trials before being tested on each of the two remaining test probes.

In the horizontal and vertical tests birds appeared to choose flowers at random among the four flowers (Binomial Test with an expected proportion of 0.25 and 0.75; Horizontal test: (5/20), $Z = 0$, $p = 0.585$; Vertical test: (7/20), $Z = 0.775$, $p = 0.214$). However, in the conflict test the birds chose the flower with the same horizontal location as that of the flower rewarded during training (Binomial Test with an expected proportion of 0.25 and 0.75; Horizontal location (8/16): $Z = 2.021$, $p = 0.027$, vertical location (1/16): $Z = 1.443$, $p = 0.063$; Table 1; Figure 6). An alternative approach to

analyze the choices to the flower with either the same horizontal or vertical location as the rewarded flower is to consider that the probability to visit each of the four locations is the same (null hypothesis) therefore, the expected proportions are 0.25, 0.25 for the vertical and horizontal locations respectively and 0.50 for both of the extreme locations, 0.25 each. Using this analysis, the birds also appear to have learned the horizontal location of the reward and searched at that site when presented with the inverted diagonal array during the conflict test ($X^2 = 6.375$, $df = 2$, $p = 0.0413$).

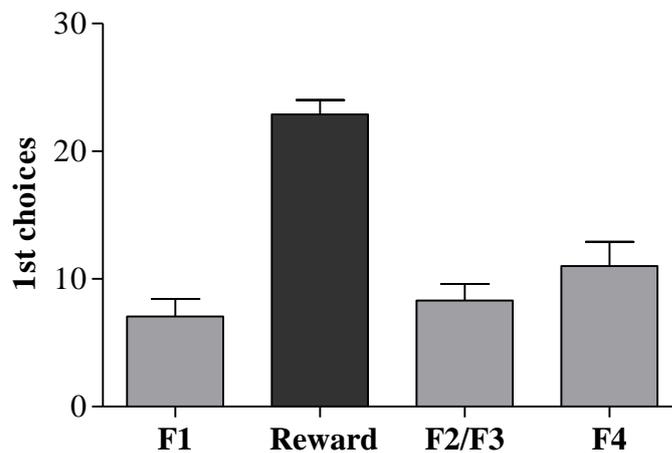


Figure 5 Total visits to each flower in the diagonal (2-D) array. The data are the mean (\pm SE) of the birds' 1st choices to the rewarded flower, the adjacent flower (either F2/F3) and the two distal flowers throughout their whole training. F1 = Flower 1, which was the highest and F4 = Flower 4, which was always the lowest flower. For 10 birds the rewarded flower was F2 and for 10 birds the rewarded flower was F3

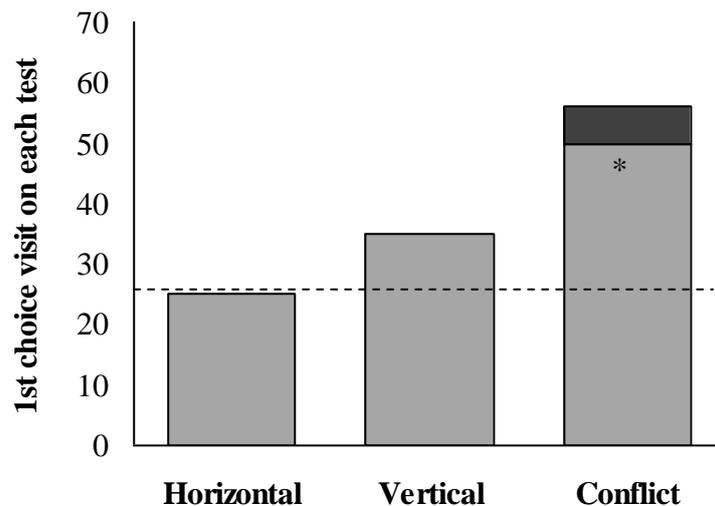


Figure 6 The birds' 1st choice in the three test probes. The data are the proportion of 1st choices to the flower with either the same x-y, or z location in the horizontal and vertical test probes respectively as in training (N = 20). The data for the conflict test come from the 1st choices of 16 birds to the flower with either the same x-y (in grey), or z (in black) location in the inverted diagonal. The dashed line shows chance level. The asterisk * represent a significance level of $p = 0.027$

DISCUSSION

In Experiment 1, hummingbirds learned a flower's location (to criterion) when the linear flower array was oriented horizontally but not when it was oriented vertically. When the spacing scale between the flowers in the array was small (i.e. 5cm) it took the birds more trials to learn which was the rewarded flower than when the spacing was greater (30cm). Furthermore, the number of 1st choices to the reward and adjacent flowers during training on the 5cm spacing arrays did not differ. During the horizontal test at 30cm the birds chose the absolute location of the rewarded flower but when the flowers were only 5cm apart they did not. In the vertical training trials, most birds did not reach the learning criterion and so were not tested. Birds did not reach the criterion because they visited the central and adjacent flowers equally. Although the birds visited

the rewarded flower significantly more often than they visited the other flowers only in the horizontal 30cm array, the fact that birds visited the distal flowers least of all in both orientations and spacing scales would suggest that they had learned the reward was located somewhere within the centre of the array.

In Experiment 2 with the diagonal training array, birds visited the rewarded flower more than they visited any of the other flowers. Although birds visited flowers apparently at random in the horizontal and vertical tests, in the conflict test they chose the flower that matched the horizontal position of the training rewarded flower more than would be expected by chance. This result contrasts with the data from fish trained on a Y-maze. They chose the vertical component when forced to choose between the vertical and the horizontal dimensions (Holbrook & Burt de Perera 2009). An alternative explanation could be that birds chose the horizontal component not because they prefer it, but because they did not learn the vertical component of the rewarded flower. However, this requires that they learned the horizontal component of the reward in the diagonal array. The failure to choose the flower in the appropriate horizontal position in the horizontal test array may have been because the birds did not associate the horizontal test array with the diagonal training array. The test diagonal array, however, appears to have been sufficiently similar to the training diagonal array to have elicited appropriate choices from the birds.

It seems a little surprising that the hummingbirds were more likely to learn the rewarded flower's location in the horizontal axis than to learn its vertical location given that they move freely through three dimensions and that they feed from flowers that vary in height. Rufous hummingbirds feed on flowers in meadows in which rewarding flowers share an average height but not horizontal location, from trees with flowers that

vary in their three-dimensional coordinates to plants with vertical inflorescences (i.e. agaves or columnar cacti flowers). Furthermore, in a previous experiment hummingbirds learned a location in fewer trials if it was defined by a horizontal and vertical component (Henderson et al. 2001) and in another they were more accurate in the vertical than in the horizontal dimension (Hurly et al. 2010). At first sight, our results contrast with the previous finding that birds are more accurate in the vertical than in the horizontal. However, accuracy and ease of learning are not the same. Once birds learn a 3-D location they are more accurate in relocating the vertical than the horizontal component of it. Nevertheless, they seem to learn in less number of trials a location defined only in the horizontal than only in the vertical and even than a 2-D with a vertical and horizontal component. In addition, birds were more likely to learn the absolute height of a rewarded flower in our experiment only when it was coupled to a unique horizontal location (diagonal array) suggesting that the horizontal component facilitated learning the vertical component. When there is not a unique horizontal component to the location, the hummingbirds can learn something of a rewarded location specified only by its vertical location as they learned to avoid the distal flowers in the vertical arrays. As in the Henderson et al. (2006) study, the birds seemed to be able to learn relative positions (to some degree) of the rewarded flowers but not their absolute location.

Finally, there were two ways in which the birds' performance in this experiment was not consistent with the literature. Firstly, although hummingbirds are thought to prefer higher flowers to lower flowers (Blem et al. 1997; Henderson et al. 2001; Henderson et al. 2006), they can also readily learn to avoid higher flowers if they are unrewarded. It may be that height preferences are more clearly seen when sampling

unknown flowers rather than when visiting flowers on multiple occasions, as in our experiments. Secondly, in the horizontal test in Experiment 1 the birds chose the absolute location in the 30cm horizontal test (as did two birds in the 5cm horizontal test), a distance over which in a previous experiment birds chose flowers in the relative position (Healy & Hurly 1998). This is surprising as we would expect that the type of array (linear versus 2-D array) should not affect the ease of learning an absolute location. In contrast all birds learning the absolute location in our study could be a result of the type of array, the intra-array cues being more salient in a 2-D horizontal array as used by Healy and Hurly (1998) than in a linear array as used in this study. However, although the magnitude of the spacing distance at which the birds learned the absolute location differed to previous experiments on a horizontal plane, birds' tendency to visit the central flower still varied according to the spacing scale in the same direction. Our findings are consistent with the earlier data in that the preference for the central flower decreased as the spacing in the array increased.

CHAPTER 4. THREE-DIMENSIONAL LEARNING IN RATS

ABSTRACT

While most animals live in a three-dimensional world, they move through it in different ways. It may be that an animal's locomotion (i.e. terrestrial versus swimming and flying animals) might influence their encoding of their environment. In this study we evaluated the use of three-dimensional (3-D) space by rats by training rats to a rewarded location in a cubic maze using Working and Reference Memory tasks. To compare whether the rats were more accurate in the vertical or in the horizontal dimension, we analysed the rats' movements during training as well as in the orientation of their first movement after reaching the rewarded location on test trials. The rats moved up and down the maze as often as they moved in either of the two horizontal axes (although they moved more in the horizontal when both horizontal axes were combined). However, the rats were more accurate in the horizontal dimension than they were in the vertical and relocated the rewarded location by first finding its horizontal location and then climbing up to its vertical location. Our findings are consistent with the suggestion that rats encode 3-D space anisotropically.

Keywords: spatial cognition, 3-D navigation, rats, spatial learning and memory

INTRODUCTION

Although all animals inhabit a three-dimensional world, not all of them move with the same freedom through that world. Terrestrial animals tend to be restricted in their vertical movements to the surface on which they move in contrast to arboreal, swimming and flying animals, which are able to move relatively freely both horizontally and vertically. Regardless of how animals move in space, they all need to orient themselves and navigate to relevant 3-D locations (i.e. to find food, mates and nests) within their environment. It is then, perhaps, surprising that spatial cognition has been almost entirely studied in a two-dimensional context, whereby animals are typically tested in open-field arenas or a maze of some kind (Morris, 1984; Poucet et al., 1986; Poucet et al., 1988; Packard et al., 1989; Eilam and Golani, 1989; Rifa et al., 1992; Tchernichovski and Golani, 1995; Healy and Hurly, 1995; D’Hooge and De Deyn, 2001; Wohlgenuth et al., 2001; Reisel et al., 2002; Sovrano et al., 2002; Royer et al., 2010). As a result, we still have little idea as to what role the vertical dimension plays in spatial cognition and no idea what impact moving regularly through a 3-D world has on spatial abilities (Holbrook and Burt de Perera, 2011).

The little we do know comes from a handful of studies, all of which show that the vertical component is both attended to and can facilitate the learning of, or memory for, the horizontal component. For example, rats trained to return to a rewarded location with a vertical component (a vertical wall or a surface tilted at an angle of 45°) required fewer trials to learn that location than when they were trained on a floor (Grobéty and Schenk, 1992). Similarly, humans ‘travelling’ in virtual environments and pigeons trained to a rewarded location in an isosceles trapezium-shaped arena required fewer trials to learn the location when the terrain they moved on had a vertical

slope than when it was flat (Restat et al., 2004; Nardi and Bingman, 2009). Finally, in a direct comparison of performance in 2-D and 3-D, rufous hummingbirds *Selasphorus rufus* made fewer errors returning to remembered 3-D locations than they did when returning to remembered 2-D locations (Henderson et al., 2006).

If using a 3-D map increases cognitive load (Jovalekic et al., 2011), animals may learn horizontal and vertical information independently. Species that move through the world differently might also give a different relative weight to each dimension. For example, hummingbirds relocate a single rewarded location more accurately in the vertical dimension than in the horizontal one (Hurly et al., 2010) and the sighted morph of the fish *Astyanax fasciatus*, trained on a Y-maze, when forced to choose one or the other dimension, consistently chose the vertical over the horizontal (Holbrook and Burt de Perera, 2009), even in the presence of landmarks (Holbrook and Burt de Perera, 2011).

Both hummingbirds and fish move freely through a 3-D world. It is less clear whether terrestrial animals, such as rats, would also prefer the vertical to the horizontal. Here, we used a 3-D maze to determine whether rats preferred to move horizontally rather than moving vertically during training and if they relocated a 3-D rewarded location more accurately in the horizontal than in the vertical. In order to determine whether this preference depended on task demands, we trained rats in either a working memory or a reference memory version of the task, whereby the former had four training trials per day with a new location each day and the latter group of rats had four training trials per day with the rewarded location in the same place each day. If rats learn the vertical component before they learn the horizontal component of a 3-D location, as would be expected from the Grob ty and Schenk data (1992) then the rats in

the Working Memory group should be more accurate on the vertical component than they are on the horizontal component. This would not be expected for the Reference Memory rats.

METHODS

Subjects

Experimentally naïve male Lister Hooded rats (N = 12, with a mean \pm SE weight of 276.25 g \pm 2.49) were used in this study. They were housed in groups of four (37 x 57cm cages) on a 12:12 h light: dark cycle and provided with three clear plastic objects on which to climb and a cardboard one in which to hide. Behavioural testing was performed during the light phase. During testing, rats were kept at 90% of their free-feeding body weight. Testing was carried out seven days a week between 9:00 and 18:00 h.

Apparatus

Training and testing was conducted in a cubic maze consisting of 480 white acetal sanded rods (10 cm long, 1 cm diameter) assembled together with grey high-density polyethylene connectors to form 10 cm³ hollow cubic units. These units were attached to each other to form a 5 X 5 X 5 cubic maze. This maze was then placed in a rectangular arena which consisted of a black wooden platform 120 cm X 90 cm, which was itself sitting on a wooden frame 40 cm from the ground (Figure 1A).

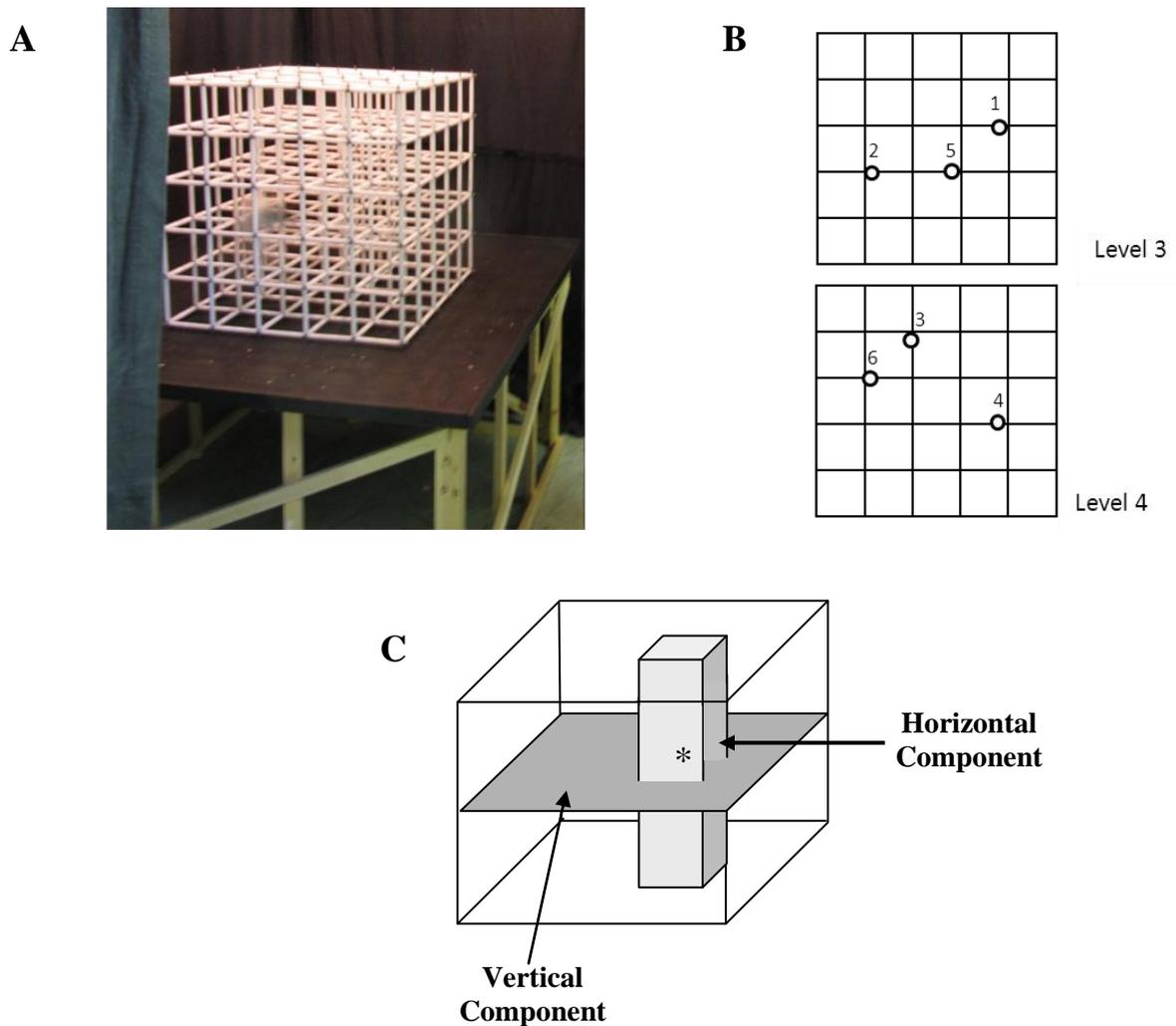


Figure 1 Representations of the cubic Maze: A) a photograph of the 50cm³ cubic maze on the 120 cm X 90 cm platform, B) a diagram showing the rewarded locations, C) a schematic diagram of the cubic maze showing the location of the reward, marked by the asterisk. The dark grey plane represents the level at which the reward was located (vertical component) and the light grey column represents the horizontal component

Habituation

Each rat received three 10-minute sessions for one session per day for three days to allow it to habituate to the open-field arena without the maze. These habituation sessions were followed by two days in which rats were individually placed in the arena for 5min before they were placed inside a white opaque plastic box (25 x 45 x 30cm) for a further 5min where they waited between trials (inter-trial interval). Although only the

rats in the Working Memory group (see below) had to wait in the box between trials, rats in both groups were habituated to the box. While in the box, the rat was given a small food reward. Rats were habituated to the maze over the next three days, during which they could freely forage for food rewards throughout the maze. Food rewards consisted of 0.5cm diameter pieces of sugary paste, which were placed in the junctions of the rods making up the maze. Food was, however, never placed on the bottom level. The total number of food rewards was reduced across the habituation days (from 16 to 4) by decreasing the number of rewards on each of the four maze levels on each subsequent day: four rewards on each of the maze levels on the first day, two rewards on the second day and one reward on the third day. Rats were individually placed in the arena either for 15, 8 or 5min (first, second and third day respectively) or until they found all food rewards. To reduce the possibility that rats would learn a route to the reward, they were placed in the arena at a different side of the maze on each trial at a pseudo-randomized order. Rats got a small food reward (half a Choco hoop) after each trial to motivate them to exit the maze, where they were picked up.

Experimental training and testing

All rats received six days of training with four trials per day followed by one or two test days. Rats were divided into two groups: six rats were assigned to a Reference Memory group and six to a Working Memory group. For the Reference Memory group, each of the four daily training trials was separated by an hour. For the Working Memory group, the interval between trials was two minutes. For both groups a reward (a 0.7cm diameter round piece of the same food reward used during maze habituation) was placed at one location avoiding the edge on either the third or fourth level of the maze.

For rats in the Reference Memory group the reward was always located in the same place for the duration of the experiment, while for the Working Memory group the rewarded location was the same across trials within a day but changed each day (Figure 1B). In the Working Memory group each rat experienced all six rewarded locations across the experiment (one per day) while each rat in the Reference Memory group experienced only one of these six locations. Training was considered complete by the end of Day 6 during which all rats reached the rewarded location in less time than at Day 1.

A test consisted of placing the rat in the arena with the maze un-baited for 10 minutes. Rats in the Reference Memory group were tested twice: on the 7th and on the 10th days. Rats in the Working Memory group were only tested on the 7th day after a training trial to a different location to that of the previous day because the aim of the test was only to make sure the rats were not beaconing to the reward. In contrast the test for the Reference Memory group also evaluated for how long did the spatial memory lasted and if it improved or decreased with time.

The maze was cleaned and rotated between trials and tests to prevent rats from using odour and visual cues to relocate the reward. All trials and tests were video recorded and all data for analyses were scored from the videos.

Task Acquisition

In order to evaluate the rats' ability to learn a three-dimensional location we counted the number of cell crossings and the time taken to reach the rewarded location. We then compared these two measures in both rat groups across days and trials. In addition to building the rats' learning curves, we compared both the difference in performance

between the groups in the change in the number of crossings (Δ_c) from Trial 1 to Trial 2 for Day 1 as well as in the time to reach reward (Δ_t) so as to evaluate whether the duration of the interval between trials made any difference on the rats' performance. The only difference between the two groups in Day 1 was the length of the interval between Trial 1 and Trial 2, one hour for the Reference Memory group and 2 min for the Working Memory group. Therefore, a greater reduction in the time taken to find the reward between Trials 1 and 2 in the Reference Memory task than in the Working Memory task might suggest that the length of the interval is critical for memory consolidation.

Use of three dimensions of space

To evaluate the use of 3-D space, we counted their unit crossings and time spent solving the maze for the three axes "x, y and z" independently across all days where "x" corresponded to right/left, "y" to forward/backward with respect to the video camera and "z" corresponded to up/down. To estimate the number of crossings we only considered the head of the rat because its body occupied two or three (if stretched and standing in two feet) cubic units. Each time the head of the rat traversed a bar separating two units it was considered a "crossing". Then we compared the horizontal "x + y" with the vertical "z" components. To determine whether the rats' use of space changed with experience (as they learned the rewarded location) we estimated a parameter so that we could compare their behaviour across days and between groups. We named this parameter *use of space* and it was calculated by dividing the number of vertical unit crossings by the total number (vertical and horizontal) of crossings, expressing the relative proportion of vertical movements with respect to all movements.

We also calculated the use of space in time, by dividing the time spent on vertical crossings by the total time spent inside the maze. For example, if the rat made 4 vertical and 8 horizontal crossings its *use of space* would be 0.25. Thus, if a rat's *use of space* was > 0.5 , the rat spent more time moving vertically than it did horizontally, whereas if the *use of space* was < 0.5 the rat spent more time moving horizontally than vertically. This is because as the experiment starts after the rat entered the first level. From any location in the first level the rat could potentially make a crossing in "x", "y" or "z". If the rat was placed at a corner within the first level, there were 4 available on each axis. Therefore, if the rat *use of space* is guided only by chance we would expect the same number of crossings in the three axes. However, if the rat's use of space is influenced by its way of locomotion we would expect more "x" or "y" crossings because rats are terrestrial animals.

Search Strategy

To evaluate whether the rats preferred to relocate the rewarded location using one of the dimensions more than the other we compared the number of crossings and time spent in the reward vertical complement (reward "z") versus the reward horizontal complement (reward "x, y") and compared them within groups. However, we transformed these data into proportions because there were 25 units in the horizontal component and only 20 units in the vertical component (Figure 5C) that were considered as the rewarded location. The 20 units from the vertical component were the four units surrounding the reward on each of the five levels because the reward was located on an intersection therefore, it could be equally reached from the four surrounding units. In the other hand the 25 units from the horizontal component constitute the whole horizontal plane in

which the reward was located. We estimated the *strategy* the rats used by dividing the proportion of crossings in the reward's vertical component by the proportion of crossings in the reward's horizontal and vertical component (Figure 1C). If the *strategy* value was > 0.5 it suggests that rats searched more at the horizontal component than at its vertical component. In contrast, if the *strategy* value was < 0.5 it suggests that the rats searched at the reward's vertical component more than at its horizontal component. If the *strategy* value = 0.5 the rats did not relocate the reward by following a consistent strategy. In addition, to explore if the rats reached the vertical or the horizontal component first we determined what each rat did in each training trial during the six days (excluding the first trial of Day 1 in the Reference Memory group and the first trial in all Days in the Working Memory group) and test trials.

Accuracy

To determine the accuracy with which the rats reached the rewarded location on the test day and to assess whether rats were more accurate in the vertical than in the horizontal plane, we looked at the rats' first cell crossing after they had reached the rewarded location and found it to be empty. We also compared the mean number of units crossed at the reward's horizontal plane versus at the reward's vertical plane. If rats searched on the horizontal plane of the reward we could assume that they learned more about the height of the reward than about its horizontal complement, whereas if they searched in the vertical plane of the reward, this would show that they had learned the horizontal better than its vertical complement. The bottom level of the maze was not considered for the analyses. Previous studies have excluded the top level while including the ground level because the mazes in those studies were raised from the ground. In

contrast, our cubic maze was placed on the floor so the ground floor was not equivalent to the rest of the levels. Rats were always placed on the ground and the experiment started when they first climbed within the maze as in previous studies using a cubic maze.

Analyses

The training and test data were extracted from the videos using a program written specifically for our experiment in IDL (Interactive data language), a data visualization software that allowed the user to code every rat movement and to transform it into a 3-D coordinate associated with a time component. The output of that program was used as the input for another IDL program that labelled every coordinate with respect to the relevant horizontal and vertical proximity map for the reward. The total number of crossings and total time spent in the maze were calculated as well as for each of the axes (“x, y, z”) separately. In addition, it estimated the use of space, the search strategy and the accuracy measured in time and number of crossings.

The mean number of crossings and the mean time to reach the rewarded location was compared across training days and trials in both groups using linear mixed models (LMM) for the three parameters, performance, use of space and search strategy as the independent variable with rat as a random factor. We tested the residuals of these three parameters for normality after running the model and all of them were normal except the performance data, which became normal after being log-transformed. For the paired and un-paired comparisons we used non-parametric tests because the data were not normally distributed.

RESULTS

Task Acquisition

The performance of the rats in the two groups improved with experience. In the Reference Memory group the mean number of crossings and mean time to reach the reward decreased across days but not across trials within days (LMM across trials; Crossings: $F_{3,113} = 2.163, p = 0.096$; Time: $F_{3,113} = 1.704, p = 0.170$; across days; Crossings: $F_{5,113} = 10.353, p < 0.001$; Time: $F_{5,113} = 12.842, p < 0.001$). The number of crossings to reach the target decreased from the Day 1 to Day 3 (Figure 2).

In the Working Memory group the mean number of crossings to reach the reward differed in trials and days (LMM across trials; Crossings: $F_{3,91} = 3.462, p = 0.020$; Log (time): $F_{3,91} = 5.789, p \leq 0.001$; across days; Crossings: $F_{5,91} = 3.794, p = 0.003$; Log (time): $F_{5,91} = 5.732, p \leq 0.001$). The time and number of crossings to reach the reward decreased from the second to the third trial (Figure 3).

If we compare the time and number of crossings to reach the reward in Trial 1 and 2 of Day 1 both decreased more in the Reference Memory group rats than in the rats in the Working Memory group. The difference in time and number of crossings to reach the reward between Trials 1 and 2 was greater in the Reference Memory group than they were in the Working Memory rats (Mann-Whitney; time: $Z = 2.562, p = 0.010$; crossings: $Z = 1.845, p = 0.065$; Figure 4 shows difference in time).

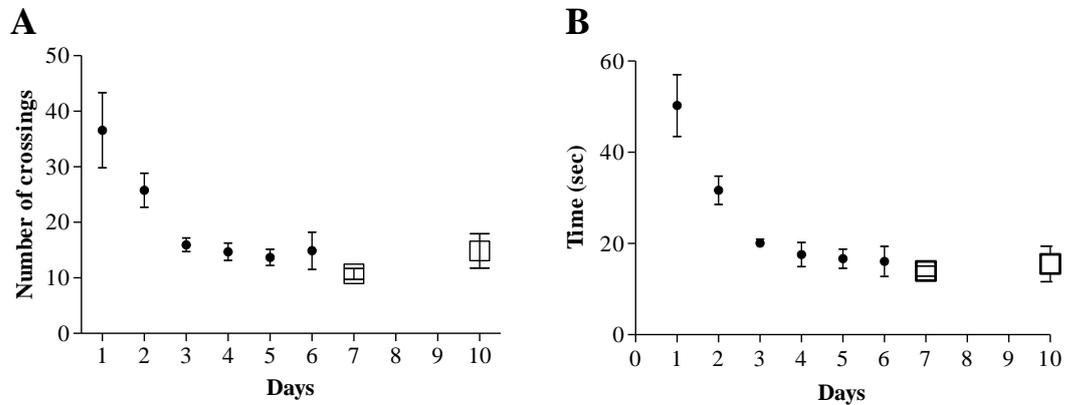


Figure 2 Performance of the Reference Memory group across days as measured by A) the number of unit crossings and B) the time to reach the reward. The data are means (\pm SE) of six rats, with each animal contributing data from four trials except for the tests, which was a single trial for each. The two open squares represent Test 1 = Day 7 and Test 2 = Day 10

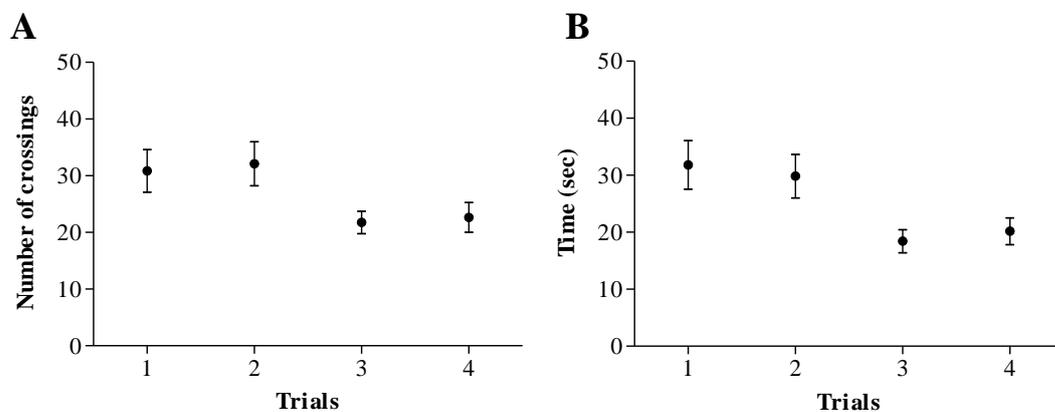


Figure 3 Performance of the Working Memory group across trials as measured in A) the number of unit crossings and B) time to reach the reward. The data are means (\pm SE) of six rats, each contributing data from six days. The test data are not included

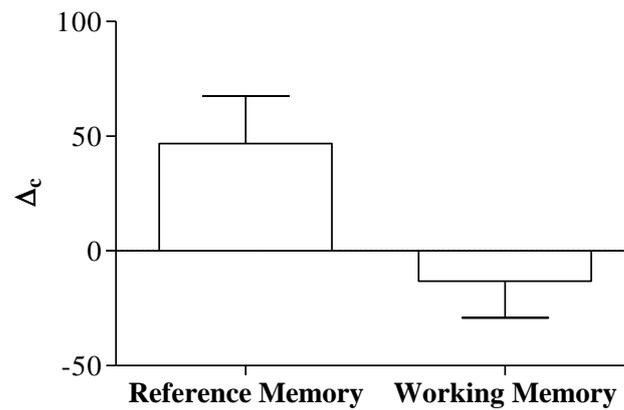


Figure 4 The difference in performance between Trials 1 and Trial 2 of Day 1 for the Reference versus Working Memory groups, measured in the number of crossings to reach the reward after a single trial. Δ_c = Number of crossings in Trial 1 - Number of crossings in Trial 2, $n = 6$

Use of three-dimensional space

Throughout the experiment, both groups of rats moved more in the horizontal plane than they did in the vertical. Although the rats in the Reference Memory group crossed more units and spent more time moving in the “z” axis than either in “x” or “y” (Friedman; crossings: $X^2(2) = 51.599, p < 0.001$; time; $X^2(2) = 17.376, p < 0.001$). However, they made more crossings and spent more time in the horizontal “x + y” than in the vertical “z” dimension (Wilcoxon; crossings: $Z = 7.159, p < 0.001$; time: $Z = 5.558, p < 0.001$; Figure 5A and B). Rats in the Working Memory group crossed slightly fewer units in the “x” axis than in either the “y” or “z” axes but spent an equal amount of time in all three axes (Friedman; crossings: $X^2(2) = 6.986, p = 0.030$; time: $X^2(2) = 0.717, p = 0.699$). Therefore, they also crossed more units and spent more time moving in the horizontal plane than in the vertical axis (Wilcoxon; crossings: $Z = 10.084, p < 0.001$; time: $Z = 7.942, p < 0.001$; Figure 6A and B).

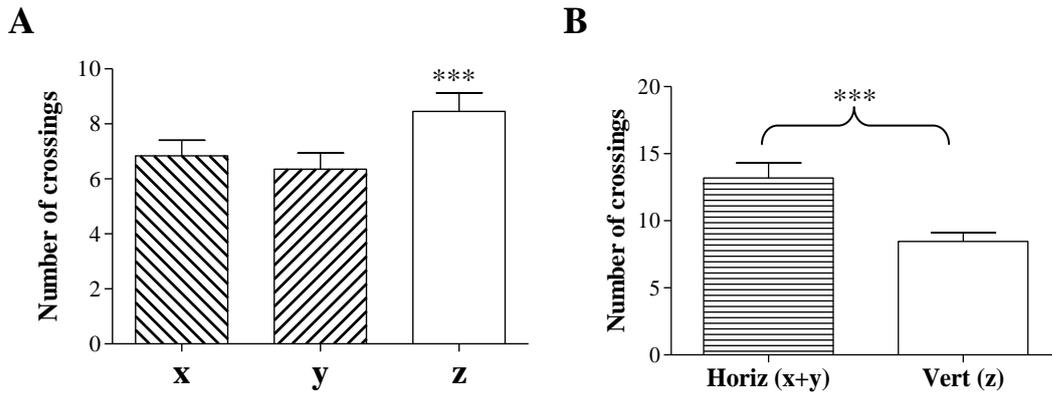


Figure 5 The use of space by rats in the Reference Memory group. The data are the mean number of crossings A) on each axis (“x”, “y” and “z”), and B) in the horizontal and vertical dimension. For both figures the data come from the mean measures (\pm SE) of six rats, each contributing data from six days with four trials per day. The shading in the bars represents horizontal crossings. The asterisks *** represent a significance level of $p < 0.001$

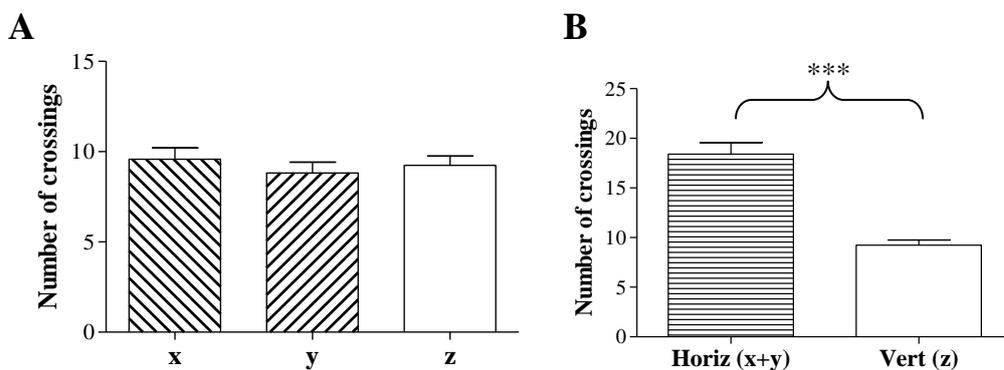


Figure 6 The use of space by the rats in the Working Memory group. The data are the mean number of crossings A) on each axis (“x”, “y” and “z”), and B) in the horizontal and vertical dimension. For both figures the data come from the mean measures (\pm SE) of six rats, each contributing data from six days with four trials per day. The shading in the bars represents horizontal crossings. The asterisks *** represent a significance level of $p < 0.001$

To determine whether experience influenced the rats’ *use of space* we evaluated it across days and trials in both groups. The *use of space* by the Reference Memory

group remained constant across trials and days (the rats moved more in the horizontal than they did in the vertical; LMM; time, trials: $F_{5,113} = 1.202, p = 0.313$; days: $F_{5,113} = 0.553, p = 0.736$; number of crossings, trials: $F_{5,113} = 0.222, p = 0.881$; days: $F_{5,113} = 1.612, p = 0.166$). In contrast, although the *use of space* by the rats in the Working Memory group did not change across trials (number of crossings: $F_{3,91} = 0.458, p = 0.713$; time: $F_{3,91} = 1.125, p = 0.343$), both measures decrease their horizontal movements across days (number of crossings: $F_{5,91} = 2.549, p = 0.033$; time: $F_{5,91} = 2.279, p = 0.053$).

Search Strategy

Both groups of rats followed a similar search strategy: rats searched more in the horizontal complement of the reward than in its vertical complement. Rats in the Reference Memory group crossed more units and spent more time in the reward's horizontal component than in its vertical one (Wilcoxon; proportion number of crossings: $Z = 7.314, p < 0.001$; proportion of time: $Z = 6.416, p < 0.001$; Figure 7A). The rats in the Working Memory group also crossed more number of units as well as spent more time within the reward's horizontal component (Wilcoxon; proportion number of crossings: $Z = 4.504, p < 0.001$; proportion of time: $Z = 3.111, p = 0.002$; Figure 7B).

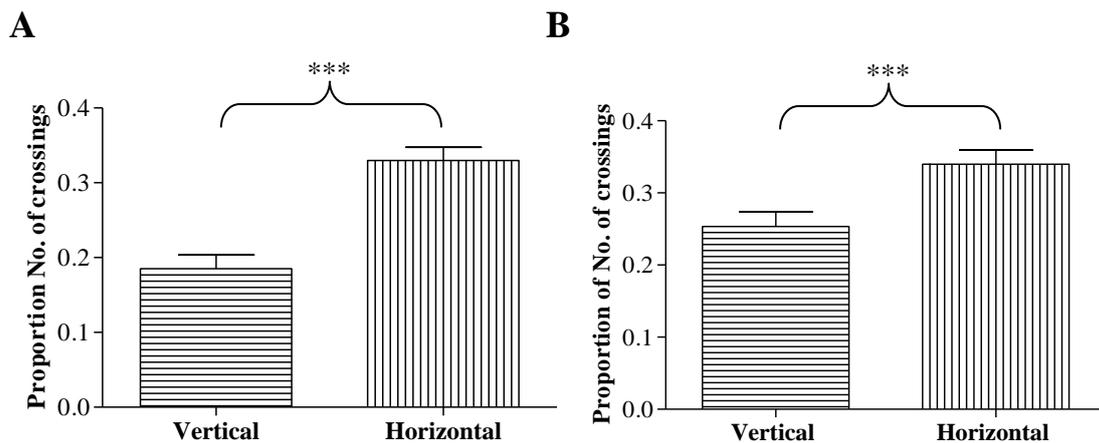


Figure 7 Search Strategy across the training sessions by A) Reference Memory group rats and B) Working memory group rats. The data for each figure are means (\pm SE) of six rats, each contributing data from six days with four trials per day. The bars on the left hand side of each figure represent horizontal crossings at the reward's level and the vertical lines in the right hand side bars of each figure represent vertical crossings at the reward's column. The asterisks *** represent a significance level of $p < 0.001$

The magnitude of the *search strategy* was > 0.5 for both groups of rats across days and trials respectively i.e. rats made more crossings at the reward's horizontal complement than at its vertical complement (Figure 7 and 8). The *search strategy* in the Reference Memory group remained constant across trials and days (LMM; trials, proportion of time: $F_{5,113} = 1.435$, $p = 0.236$; proportion of crossings: $F_{5,113} = 0.663$, $p = 0.179$; days, proportion of time: $F_{5,113} = 1.565$, $p = 0.176$; proportion of crossings: $F_{5,113} = 1.255$, $p = 0.288$; Figure 8A). The *search strategy* in the Working Memory group remained constant across days and trials (LMM; trials, proportion of time: $F_{3,107} = 0.308$, $p = 0.820$; proportion of crossings: $F_{3,107} = 0.473$, $p = 0.702$; days, proportion of time: $F_{3,107} = 0.853$, $p = 0.516$; proportion of crossings: $F_{3,107} = 0.887$, $p = 0.493$; Figure 8B). 4/6 rats in the Reference Memory group reached the vertical and the horizontal component first an equal number of times (Binomial Test with an expected proportion

of 0.5, 0.5). All rats in the Working Memory group reached the vertical and the horizontal component first an equal number of times (Binomial test with an expected proportion of 0.5, 0.5; Figure 9; see Table1).

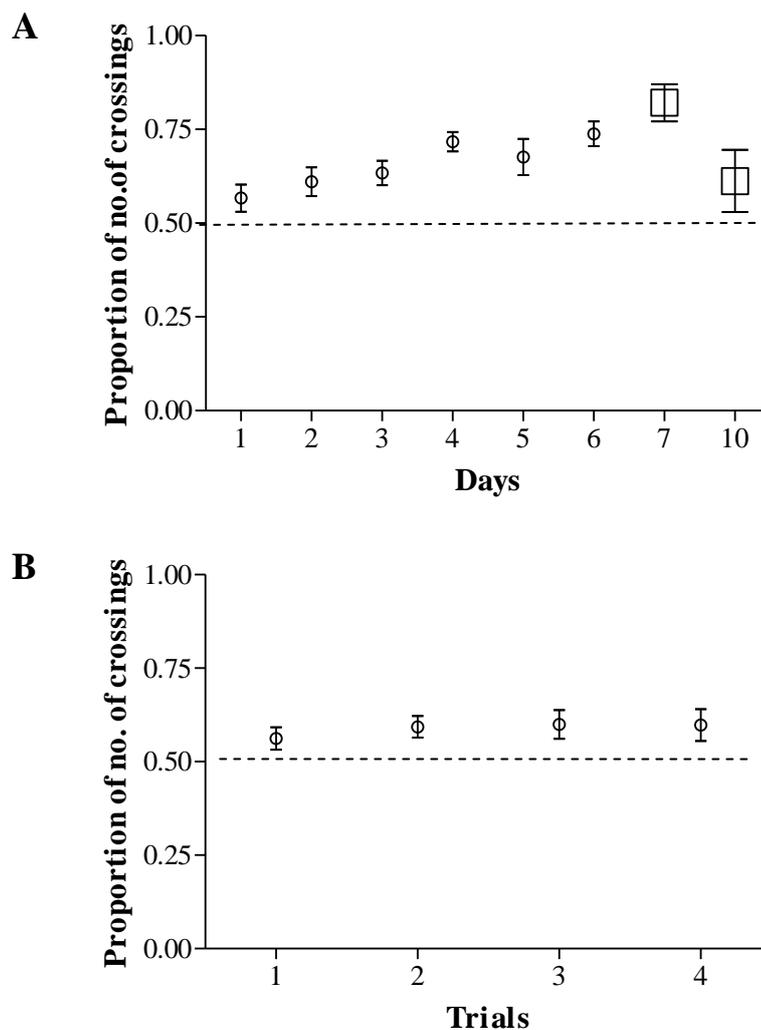


Figure 8 Search Strategy of the rats in the A) Reference Memory group, and B) Working Memory group. The search strategy was estimated by dividing the number of crossings in the reward's vertical component by the crossings in the reward's vertical and horizontal components. The data for each figure are means (\pm SE) of six rats, each contributing data from four trials per day for the Reference Memory group, and six days per trial for the Working Memory group. The dotted line indicates an equal number of movements in the vertical and horizontal reward's components

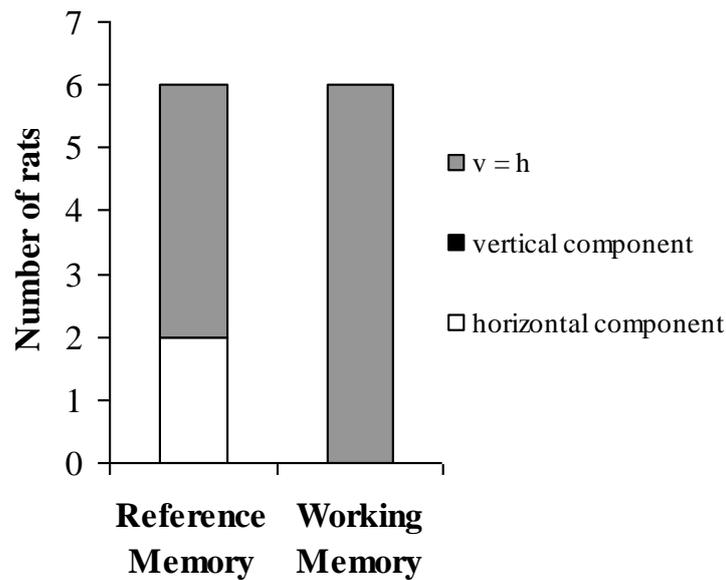


Figure 9 Preference for reaching the vertical or the horizontal component of the rewarded location first during the whole training in both groups of rats. Data are the number of rats that found first either the vertical or the horizontal component consistently throughout training as well as the number of rats that reached the vertical and horizontal component first an equal number of times ($v = h$)

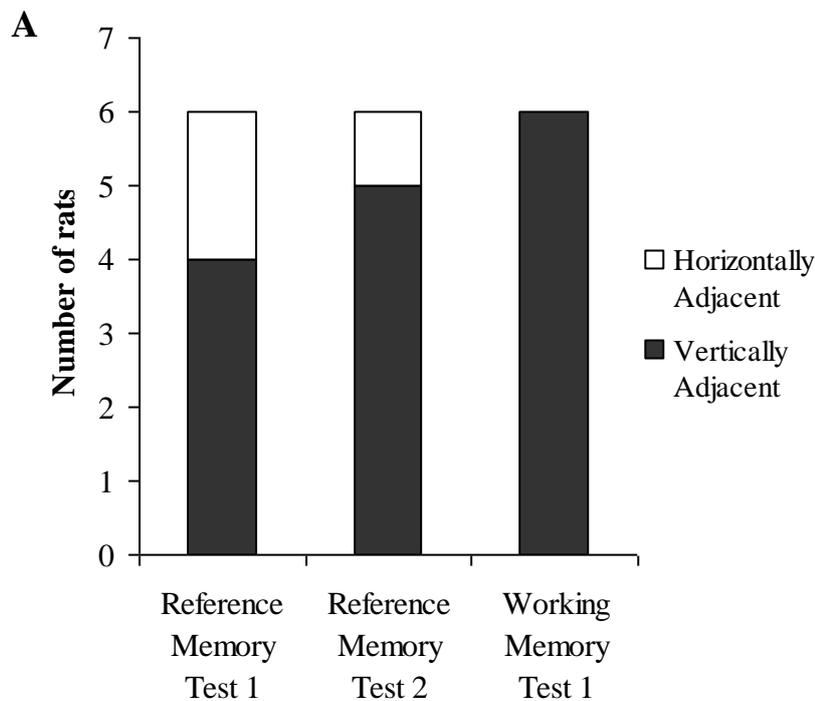
Table 1. Comparison of vertical and horizontal components search order in both group of rats. A non-significant comparison indicates that the rats were as likely to reach the vertical component of the rewarded location first as they were to reach the horizontal component of the rewarded location first.

	Z	p
Working Memory	1.84	0.064
	0.71	0.48
	1.46	0.143
	0.24	0.82
	< 0.001	1
	0.92	0.36
Reference Memory	2.4	0.015
	2.4	0.015
	0.8	0.43
	1.6	0.12
	1.6	0.12
	< 0.001	1

Test: Binomial with 0.5, 0.5 expected proportion

Accuracy

In the test both groups of rats moved vertically once they reached the empty rewarded location, although it was only significant for the second test of the Reference memory group (Reference Memory group, Day 7 Test: Binomial with an expected proportion of 0.33, 0.67 (4/6), $Z = 1.32$, $p = 0.097$; Day 10 Test: Binomial (5/6), $Z = 2.188$, $p = 0.014$; Working Memory: Binomial (6/6), $Z = 3.056$, $p = 0.001$; Figure 10A). The rats in the Reference Memory group reached the rewarded location in a mean (\pm SE) of 11.3 ± 0.92 crossings in their first test and in a mean (\pm SE) of 15.7 ± 3.5 while the rats in the Working Memory group reached the rewarded location in a mean (\pm SE) of 19.8 ± 6.7 (Figure 10B). However, the difference in the number of crossings to reach significance between both group of rats in their first test is not significant (Wilcoxon: $Z = 12$, $p = 0.26$) nor the difference between the first and second test in the Reference Memory group (Wilcoxon: $Z = 12$, $p = 0.22$).



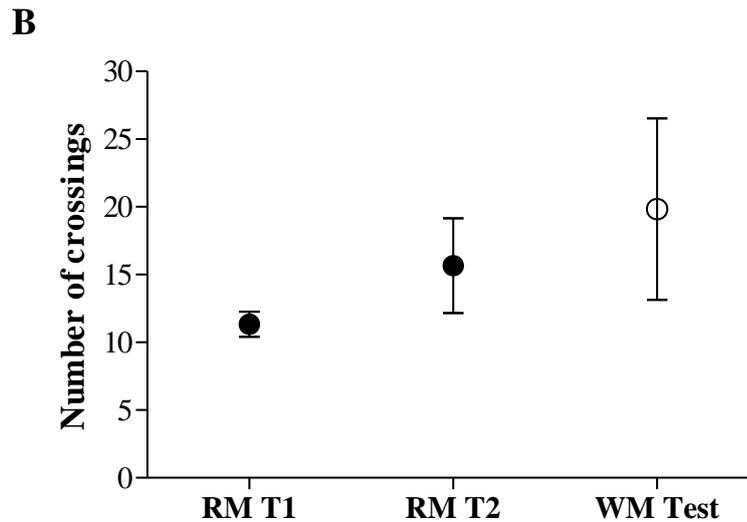


Figure 10 Test results for both the Reference Memory and Working Memory groups of rats. A) The rats' 1st choice as measured by the number of rats that moved either to a vertical adjacent location or to a horizontally adjacent location. B) Rats' performance measured in the mean number of crossings (\pm SE) to reach the reward's location during the test. The filled circles represent the data for Tests 1 and 2 for the Reference Memory (RM T1) group, $n = 6$ and the clear circle represents the data for the Working Memory (WM T1) group $n = 6$

DISCUSSION

Rats learned a three-dimensional location after only a few rewarded experiences. However, they were more accurate in their memory for the horizontal component of that location than they were for its vertical component: once they had reached the empty rewarded location in the test, they searched up or down rather than to either side of that location. When the "x" and "y" data were combined and compared with the "z" data, rats moved more horizontally than vertically while exploring the cubic maze. However, when we compared the rats' movement on the three axes independently, rats moved equally (Reference Memory group) in all dimensions or slightly more in the vertical (Working Memory group). So, although rats normally do not move through lattice-like

structures, when they experience a situation in which they can move freely in 3D, they are as likely to move up or down as they are to go forward or backward or to turn to the left or to the right. When returning to the rewarded location, rats in both groups searched at the horizontal component of the reward more than at its vertical one. It would appear then, then, that rats are more certain about the location of the reward in the horizontal plane than in the vertical.

The greater accuracy of our rats in the horizontal plane is consistent with a previous study in which rats trained to a single location in a similar 3-D task spent more time in the units vertically adjacent to the reward's location than they did in the horizontally adjacent units during the test at the end of the experiment (Grobéty and Schenk, 1992). The rat's horizontal greater accuracy is also consistent with the few 3-D electrophysiological data: the hippocampal place cells and entorhinal grid cells firing fields of rats climbing up either a pegboard or a helix were vertically elongated relative to the firing fields of animals moving around in a horizontal plane which is considered a sign of lower accuracy (Hayman et al., 2011). This vertical elongation of the firing fields may mean that rats encode the vertical dimension with a lower resolution than they do the horizontal dimension and thus are representing 3-D information anisotropically (Hayman et al., 2011).

The greater accuracy of encoding the horizontal information of a 3-D location, rather than the vertical, may be due to the rats moving more in the horizontal than in the vertical dimension. In our experiment, rats moved twice as much horizontally as they did vertically during training, which might be expected given that there are two dimensions (x and y: backwards and forwards, from left to right) to the horizontal plane and only one (z: upwards and downwards) to the vertical. However, when we look at

their movements in each of the three dimensions, we found that the rats moved equally in all three. It is not straightforward to compare our data with those from other studies in which there was either no explicit division of the horizontal axes (Grobéty and Schenk, 1992) or where the rats were free foraging rather than learning a 3-D spatial location (Jovalekic et al., 2011). The magnitude of the difference that Grobéty and Schenk (1992) found in movement between the horizontal and vertical axes suggests that rats in their experiment might have moved less in the z-axis than in either the x or y-axes (Grobéty and Schenk, 1992) as did the rats in a free-foraging study (Jovalekic et al., 2011). It is also not clear whether the requirements of learning a location differs from those imposed by free foraging, where no specific location is learned (Jovalekic et al., 2011). In the free-foraging task, the animal was required to collect six rewards from each level of the maze, which may mean that it was more efficient for the rats to follow a layer-by-layer search strategy. This was not a strategy that would have been useful for our rats.

Although it is not clear whether rats prefer to move horizontally or vertically, it does seem that they are more certain of the horizontal complement of the reward than they were of its vertical complement, which differs from preferences for, and accuracy in, the two dimensions in swimming and flying animals. Despite fish (*Astyanax fasciatus*) moving freely in 3-D, when forced to choose between the vertical and the horizontal, they preferred the vertical (Holbrook and Burt de Perera, 2009; Holbrook and Burt de Perera, 2011). It is possible that these fish can measure depth very accurately due to them having a swim bladder, which is sensitive to pressure and this may also be why they prefer the vertical component of a task (Taylor et al., 2010). Hummingbirds, which cannot so readily measure absolute height, can, in the

appropriate training and testing conditions, be more accurate in relocating the vertical dimension of a reward than its horizontal one (Hurly et al., 2010).

Learning the height of the reward first, and subsequently its horizontal complement, by rats, has been interpreted as an energy-saving strategy because it minimizes the vertical movements the animal has to make (Grobéty and Schenk, 1992). However, although rats trained to a single 3-D location spent more time at the reward's horizontally adjacent units than at its vertically adjacent ones when tested after three days of training, after three further days, they did just the opposite (see Figure 4 in Grobéty and Schenk, 1992) suggesting that their certainty of the reward's vertical complement had declined relative to their certainty of its horizontal complement. If rats do learn a 3-D location in two steps, first the vertical and then the horizontal, we expected the rats in the Working Memory group to have been more accurate in the vertical than in the horizontal during their test. However, all of the rats relocated the horizontal component first on the test trial.

Although it is not clear whether rats learned one of the dimensions of 3-D space first, we can be sure that rats did not prioritize the vertical complement over the horizontal as fish appear to (Holbrook and Burt de Perera, 2009; Holbrook and Burt de Perera, 2011). Like rats trained on a detour task on a cubic maze (Jovalekic et al., 2011), our rats appeared to prioritize the horizontal information. This order of dimensional preference has been interpreted as an example of temporal discounting, where animals execute the low-cost, easier section of a journey first in case it is interrupted before completion (Jovalekic et al., 2011). The search strategy used by rats from both groups in our experiment is consistent with temporal discounting and could also be interpreted as an energy-saving strategy because our rats always began a trial at

the bottom of the maze. Therefore, by reaching the horizontal complement of the reward first they lowered their vertical movement. The possibility that terrestrial animals attempt to lower costs by the way they move through 3-D space to rewarded locations is supported by evidence that humans too, head first to the horizontal complement and then climb (Büchner et al., 2007).

Although both the Working and Reference Memory groups of rats showed a similar use of 3-D space and search strategy, they do not seem to learn at the same speed. The Reference Memory rats trained with intervals of an hour between daily trials appeared to have learned the rewarded location in a single trial, whereas the Working Memory rats, which had a two-minute interval between their training trials, required two trials to learn the location of a reward. It seems likely that the better performance of the rats in the Reference Memory group is due to these animals having a longer time to consolidate their 3-D spatial learning (McGaugh, 2000; Dudai and Eisenberg, 2004).

Having a 3-D mental map together with the capacity to update one's position has been thought to be cognitively much more costly not least because rotations in 3-D interact and are order dependant. As a result, Jovalekic et al. (2011) have suggested that rats (and probably all animals) encode space in the form of stack 2-D maps rather than as 3-D volumetric maps. They also suggested that a layered 2-D map would reduce an animal's cognitive load keeping track of its position and its heading direction should be less complex in 2-D (Jovalekic et al., 2011). Testing cognitive load is far from straightforward but it may be that comparing the use of horizontal and vertical space and accuracy for finding a 3-D location in the same task between animals that usually

move in 2-D (such as rats) with those that usually move through 3-D will be a useful approach.

CHAPTER 5. THREE-DIMENSIONAL LEARNING IN FREE-LIVING HUMMINGBIRDS

ABSTRACT

Animals move within their environment to a very different extent depending on their mode of locomotion. While terrestrial animals perform relatively limited vertical displacements, swimming and flying animals move far more freely in three-dimensions (3-D). As a result, animals may differ in how they learn and remember spatial locations. However, most of what we know comes from 2-D experiments in the horizontal plane. In this study, therefore, we evaluated the use of three-dimensional (3-D) space by wild hummingbirds (*Selasphorus rufus*) when returning to a rewarded location within a cubic maze. To compare whether the birds were more accurate in the vertical or in the horizontal dimension, we analysed the birds' movements during training as well as the orientation of their first location sampled after reaching the empty rewarded location on test trials. The birds moved up and down the maze as often as they moved in either of the two horizontal axes (although they moved more in the horizontal when both horizontal axes were combined). However, the birds sampled fewer locations in the vertical axis than in either of the two horizontal axes. Once they learned the location of the reward, birds returned to it directly without reaching either its vertical or its horizontal component first, and they were more accurate in the vertical dimension than they were in the horizontal. Our findings suggest that birds might not only use 3-D space differently from the way it is used by terrestrial animals but they may also encode 3-D space differently.

Keywords: spatial cognition, 3-D navigation, hummingbirds, flying, swimming

INTRODUCTION

Although most animals live in a three-dimensional (3D) world, they move through it to different extents. For example, terrestrial animals tend to move more horizontally than they do vertically, while arboreal, swimming and flying animals may move relatively equally in the horizontal and vertical planes. To date, the vast majority of research on navigation and spatial cognition has addressed the question of how animals return to rewarded locations in the horizontal plane, largely ignoring the use of a vertical component (Morris, 1984; Poucet et al., 1986; Poucet et al., 1988; Packard et al., 1989; Eilam and Golani, 1989; Rifa et al., 1992; Tchernichovski and Golani, 1995; D'Hooge and De Deyn, 2001; Reisel et al., 2002; Sovrano et al., 2002; Royer et al., 2010).

Therefore, little is known of how animals use and represent the 3-D space in which they forage. The few studies that have incorporated the vertical dimension to a horizontal coordinate suggest that it can play a significant role in spatial learning (Grobéty and Schenk, 1992; Jacobs and Shiflett, 1999; Henderson et al., 2001; Thiele and Winter, 2005; Henderson et al., 2006; Holbrook and Burt de Perera, 2009; Hurly et al., 2010; Holbrook and Burt de Perera, 2011).

The first study to include the vertical component explicitly in spatial experiments showed that rats learned a rewarded location in fewer trials if the location had a vertical component (on a climbing wall or a surface tilted at an angle of 45°) than if the reward was located on the floor of a maze (Grobéty and Schenk, 1992). This suggests that adding vertically relevant information to a horizontal location can facilitate spatial learning. Similar effects have been seen in birds and humans: hummingbirds were better at avoiding emptied flowers when flower locations included relevant vertical information; humans pedalling on a bicycle simulator coupled to a

tilted virtual environment made fewer navigation errors than when the virtual landscape was placed on a flat surface; and pigeons learning a location on a tilted arena made fewer errors than when the arena was flat (Henderson et al., 2001; Restat et al., 2004; Nardi and Bingman, 2009). As with the relative paucity of behavioural data addressing 3-D use, there are, almost no relevant neural data, although firing fields of hippocampal place cells of rats exploring a tilted platform are oriented by the slope of the platform (Jeffery et al., 2006). In addition, rat's and bat's place and grid cells have been recorded while climbing a vertical wall as well as a helix and flying during foraging in a large arena respectively (Hayman et al., 2011; Yartsev and Ulanovsky 2011).

Despite knowing that animals attend to vertical information and that it can facilitate learning a location, it is not clear that animals that move through 3-D space differ in their use of vertical information when compared with terrestrial animals. The few data we have might suggest that these different groups of animals may, indeed, differ in their use of vertical information. For example, rats made more horizontal than vertical moves when trained to a rewarded location as well as on a free foraging task in a cubic maze (Grobéty and Schenk, 1992; Jovalekic et al., 2011) while fish (*Astyanax fasciatus*) trained to a rewarded location in a 3-D Y-maze (in which one of the arms was pointing down and the other up) learned to swim either down/right or up/left according to where the reward was located. However, when the vertical and horizontal components were put in conflict in a test, the fish preferred to swim to the previously correct vertical component rather than to the previously correct horizontal component i.e. if the fish had been trained to find the reward up and left in the maze, following maze rotation, they chose to swim up and right rather than down and left (Holbrook and Burt de Perera, 2009; Holbrook and Burt de Perera, 2011).

The aim of this study, then, was to compare hummingbirds' use of 3-D space and their accuracy in the vertical and the horizontal dimensions of a 3-D maze with that of rats trained in a very similar 3-D maze (see Chapter 5). To do this we trained free-living hummingbirds to feed from a 3-D array of artificial flowers to determine whether they would move preferentially in any of the three axes of space when relocating that reward. As hummingbirds can learn a single 3-D location in one trial (Flores-Abreu et al., 2012) and they remember better the vertical dimension of the location than its horizontal dimension (Hurly et al., 2010), we expected the birds to move equally in both dimensions but to learn the vertical component sooner than the horizontal component and to be more accurate in the vertical than in the horizontal.

METHODS

Study species and site

The subjects used in this experiment were eight free-living male rufous hummingbirds *Selasphorus rufus*. The experiment was run from 07:00-19:00 hr Mountain Standard Time in June in 2011 in a valley in the eastern Rocky Mountains, 20 km southwest of Beaver Mines, Alberta, Canada, (49°20'56.61'' N 114°24'38.49'' W). On return from overwintering in Mexico, males set up territories along this valley centred on artificial feeders containing 14% sucrose. Territorial males were caught and colour marked with nontoxic waterproof ink on the chest for individual identification.

Apparatus

Training and testing were conducted using a cubic maze made of wooden dowel (5.0 mm diameter). Each side was 75cm in length with 4 perpendicular dowels such that the

entire cube contained 27 smaller cubic units each measuring 25cm per side. At each junction we placed a 200 μ l microcentrifuge vial. All 64 locations had a coloured paper ring around the opening of the vial. The colour of the ring (orange, red, blue or green) was the same across all of the locations for each bird but varied among birds. The maze itself was raised off the ground by approximately 30cm (Figure 1). As the rewarded locations were never those situated at the edges of the maze there were eight possible reward locations and each bird was presented with a different rewarded location. The 3-D location of the reward can be thought of as being specified by a component that is correct along the vertical axis (height or z-coordinate) as well as a component that is correct in the horizontal plane (x,y-coordinates). Hereafter, I will refer to these with the terms “vertical component” and “horizontal component”.

Initial Training

All birds were first trained to feed from a pink artificial flower, which consisted of a 3cm diameter pink cardboard disc and a vial in the centre. The vial contained 25% sucrose solution and the whole flower was fixed to the top of a wooden stake (height from the ground: 60cm). The maze contained a 3cm pink disc at the rewarded location and was placed within a bird’s territory approximately 5m away from the feeder. The flower on the stake was moved towards the cubic maze in 1m steps each time the bird fed from it until either the bird fed from the flower within the maze or from the flower immediately beside the maze (no more than 5cm). At this point the flower was removed. Once the bird fed from the rewarded location within the maze three times, the pink disc was replaced by a ring of the experimental colour (i.e. orange, blue, red or green) corresponding to the rest of the flowers in the maze which were then filled with

water, which the birds find distasteful. Once all 63 non-reward flowers were filled with water and the pink disc removed from the target the experiment proper began.

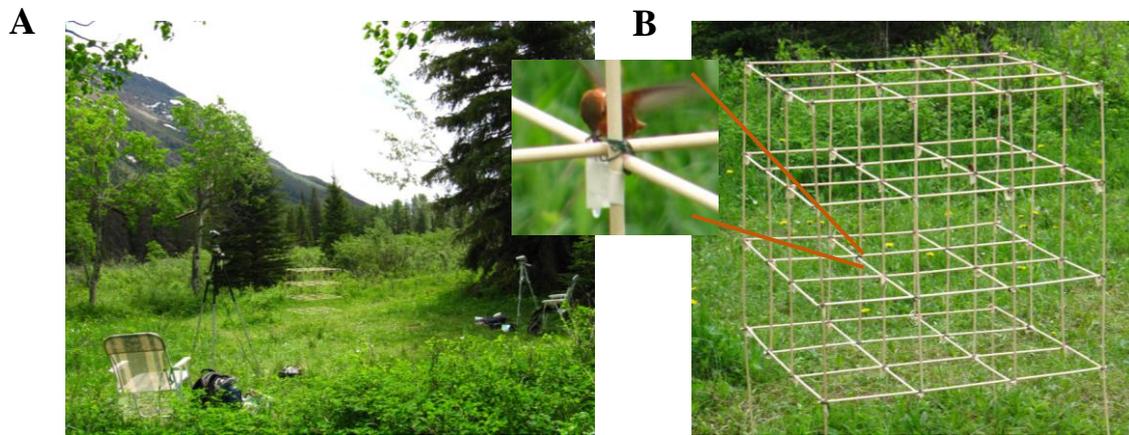


Figure 1 Pictures of the experimental arena and of the apparatus. A) A territory of one experimental bird with the maze secured to the ground and two video cameras to film the experiment. B) The maze (3 x 3 x 3 lattice 75 cm³ cube) with an eppendorf attached to every junction (64 in total). The insert shows a male rufous feeding from the rewarded vial

Experimental training and testing

A bird was allowed to visit the array and to visit any of the 64 flowers in the array.

Typically, he would leave the maze once he had found and drunk from the rewarded flower. The rewarded flower was then refilled. Each bird experienced a single rewarded location from the eight used (Figure 2A). The experiment continued until the bird reached our learning criterion, which consisted of three consecutive visits to the array during which he first visited the rewarded flower first. Once the bird reached the learning criterion, he received a single test trial. On this trial, the reward flower was removed from the maze while all of the other flowers remained in place. All learning bouts and test trials were video recorded and all of the data for analysis were taken from the videos rather than being recorded live. Due to incomplete video, one bird was not

included in the analyses of the latency to reach reward, number of crossings and sampling. However, the data for that bird was included for the analyses of the number of trials to reach criterion as well as in the 1st choice test trial, both of which were recorded live. We video-recorded the duration and number of crossings (i.e. flying horizontally or vertically to an adjacent unit of the maze), as well as flower sampling (i.e. flying horizontally or vertically from one flower to the next one), and the number of flowers sampled.

Task Acquisition

We first evaluated the birds' ability to learn a rewarded 3-D location by determining whether their performance improved across their learning bouts. To do this, we quantified the number of crossings, the time taken and the number of flowers sampled until the birds reached the rewarded flower and compared the average of the first three trials (after the pink disc was removed) with the average of the last three trials. The number of trials to reach criterion excluded the three first visits when the pink disc signalled the rewarded flower but included the three direct visits to the reward.

Use of the three dimensions of space

To evaluate the birds' use of 3-D space we counted and compared their unit crossings, their latency between entering the maze and probing the rewarded flower and their flower sampling for the three axes "x, y and z" independently across all trials where "x" corresponded to right/left and "y" to forward/backward, both with respect to the video camera while "z" corresponded to up/down crossings. Then we compared the horizontal "x + y" with the vertical "z" components. In addition, to be able to compare the birds

use of the vertical axis and of the horizontal plane using the same units (cm) we estimated the total 3-D distance to the flowers sampled from the rewarded one during the training trials using the rewarded location as a reference point. In this way we estimated the vertical and horizontal components of the total 3-D distance from the rewarded flower to the next flower sampled in cm using the Pythagoras Theorem.

Search Strategy

To determine the path that the birds followed to find the reward during training we evaluated whether the birds searched preferentially at the vertical (“z”) or horizontal (“x, y”) component of the reward. To do this we looked at the number of crossings and time spent in the vertical and horizontal components of the maze occupied by the reward (Figure 2C and D). The reward’s vertical component (i.e. all with the same “z”) consisted of the nine units, all on the same horizontal plane. For determining the horizontal component, the bird could probe the reward from any of the four units around it. Therefore, we considered that the reward’s horizontal component consisted of 12 units (four per level; Figures 2B and D). We transformed these data into proportions for analysis.

Accuracy

To determine the accuracy with which the birds reached the location of the reward on the test trial and to assess whether birds were more accurate in the vertical or the horizontal plane, we looked at the first location the birds visited after they had found the rewarded location to be empty. If birds consistently visited a reward’s horizontally adjacent flower (i.e. the locations immediately to the right/left or forward/backward

from the rewarded location = 4 locations) it would suggest that they had learned the height (z) of the reward better than they had learned its location in the horizontal (x - y) plane. On the other hand, if a bird's first choice was consistently to a vertically adjacent location (i.e. the locations immediately above and below the rewarded location = 2 locations) it would then suggest that he had learned the horizontal components of the reward's location better than he had learned the vertical component. We also looked at the total number of flowers visited during test trials and the distance from each of these flowers to the reward to determine if the birds' sampling patterns. In this way we could compare the vertical and horizontal components of the distance to the reward as we did for the first choices of the training trials.

Analyses

The training and test data were extracted from the videos using a program designed specifically for our experiment in IDL (Interactive data language), data visualization software that allowed us to code each of the bird's movements and to transform them into 3-D coordinates associated with a time component. The output of that program was used as the input for another IDL program that labelled every coordinate with respect to the relevant horizontal and vertical proximity map for the reward so that we could identify which movements were within the reward's vertical (same z) or horizontal (same x , y) component. The total number of crossings and total time spent in the maze were calculated as well as for each of the axes (" x , y , z ") separately. In addition the program calculated the total number of flowers sampled and the order and location of each of them. All data were analysed with non-parametric statistics as the data were not normally distributed.

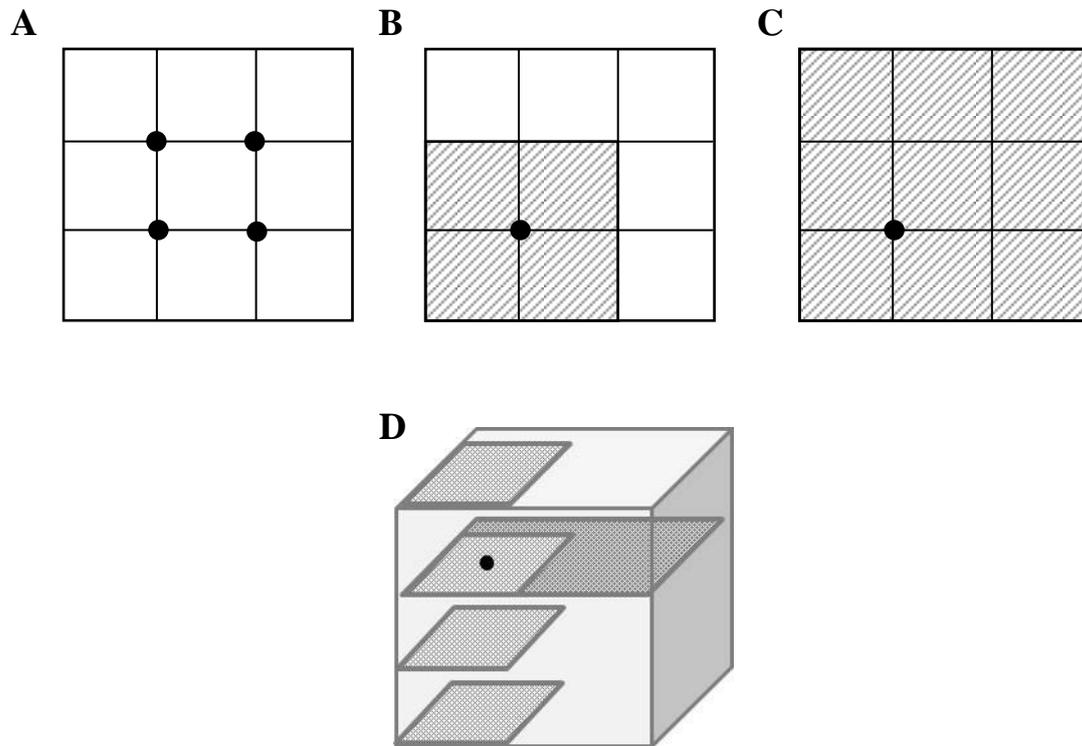


Figure 2 Diagram of rewarded locations within the cube A) the black circle shows the rewarded locations: the four internal intersections in the second or third level of the cube, but only one was used per bird. B) An example of which units were considered to be the x-y reward's location. A bird could feed from the flower from any of the four shaded units. The reward's horizontal location included the same four units in every level of the maze therefore, 16 units in total. C) The reward's vertical location included all nine units at the level where the reward was located. D) Full maze showing z and x-y reward's location

RESULTS

Task Acquisition

All birds reached criterion in a mean and SE of 12.4 ± 1.8 trials. At the beginning of the experiment the birds crossed more units, spent more time and sampled more flowers before reaching the reward than they did at the end of the experiment (Wilcoxon;

Crossings: $Z = 2.95$, $p = 0.003$; Time: $Z = 3.38$, $p = 0.001$; Flowers: $Z = 3.63$, $p = 0.001$;
Figure 3).

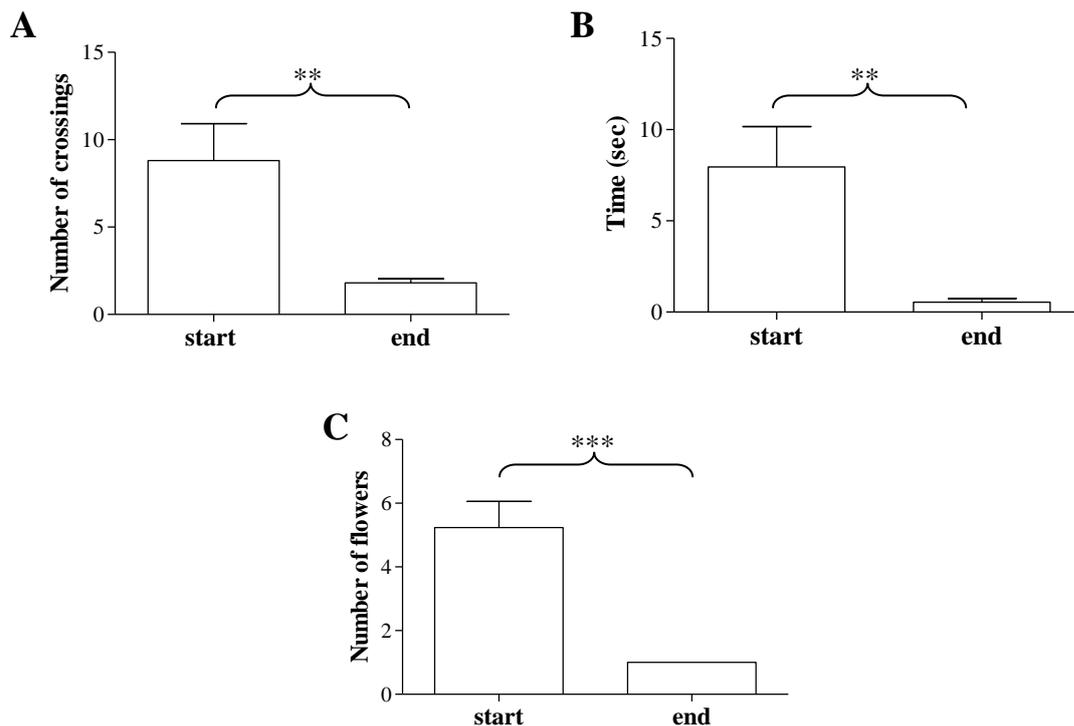


Figure 3 Learning a rewarded 3-D location. All figures represent a comparison of the birds' performance at the start and end of the experiment. The data are the mean of the first three trials versus the mean of the last three trials measured in number of A) crossings, B) seconds and C) flowers sampled until reaching the reward. For all figures the data come from the mean measures (\pm SE) of seven birds. The number of asterisks represents a significance level of ** $p < 0.01$ and, *** $p < 0.001$ respectively

Use of the three dimensions of space

The birds made an equal number of crossings and spent the same amount of time flying in each of the three axes (x, y, z; Friedman; crossings: $X^2(2) = 0.309$, $p = 0.857$; time:

$X^2(2) = 2.59, p = 0.274$; Figure 4 A and C). However, when the movements through both the horizontal axes are summed and then compared ($x + y$) to the movements in the vertical (z) axis the birds crossed more units and spent more time flying horizontally than they spent flying vertically (Wilcoxon; Crossings: $Z = 4.209, p < 0.001$; Time: $Z = 4.957, p < 0.001$; Figure 4 B and D). In contrast, birds sampled fewer flowers by flying vertically between them than horizontally. This was true both when we compared the three axes independently as well as when we added the two horizontal axes ($x + y$) and compared them with the vertical (z) axis (Friedman: $X^2(2) = 7.769, p = 0.021$; Wilcoxon: $Z = 5.04, p < 0.001$; Figure 4 E and F). In addition, the 3-D distance from the reward to the first flower location is mainly given by the horizontal component of the distance. For the last four training trials, the horizontal components of the distance from the rewarded location to the first flower location sampled was greater than the vertical distance (Wilcoxon: $Z = 2.86, p < 0.004$; Figure 5).

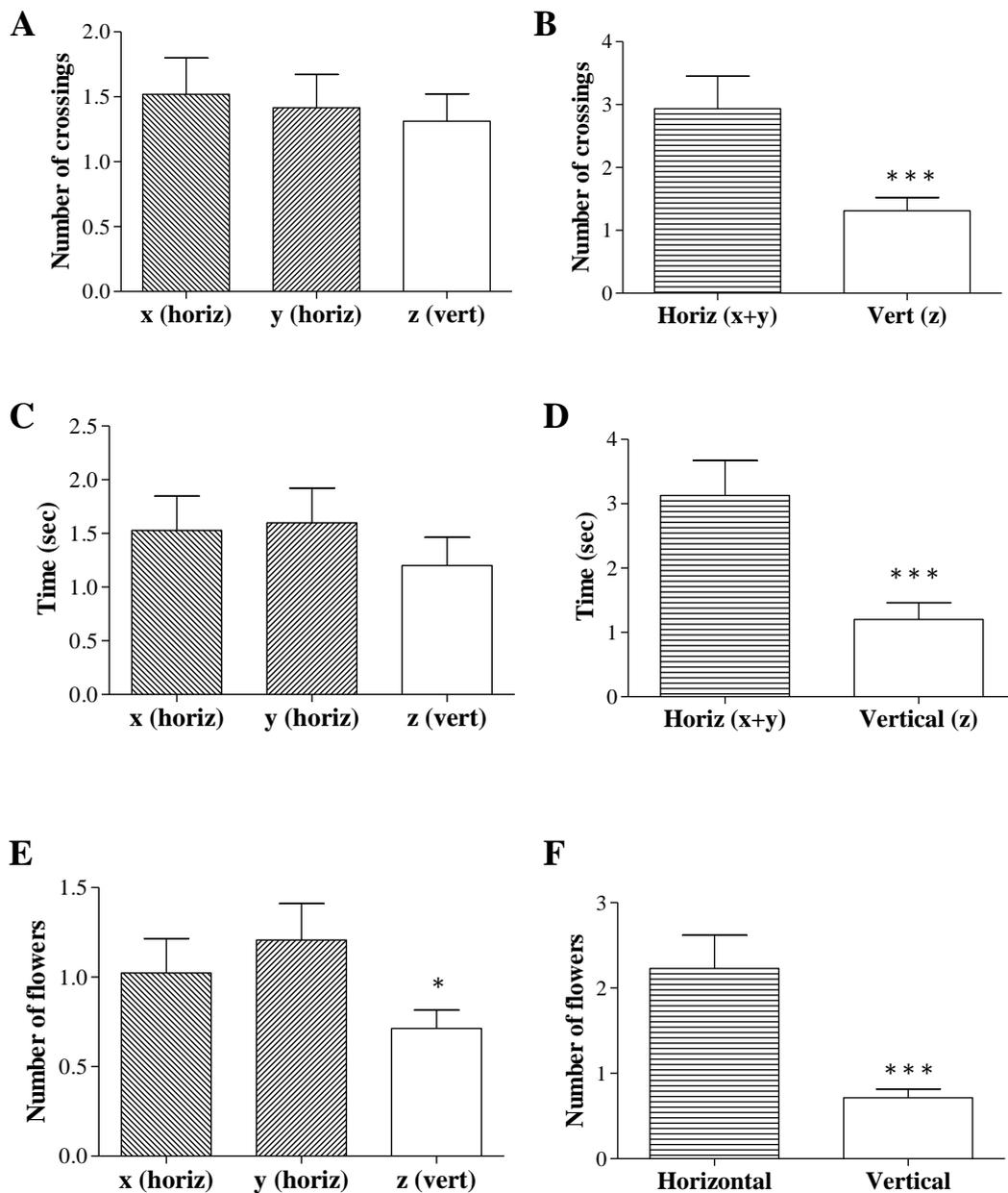


Figure 4 The use of space. The data are the mean number of crossings: A) in each axis, and B) in the horizontal and vertical dimension, the mean time spent C) in each axis, and D) in the horizontal and vertical dimension. The number of flowers sampled in E) each axis, and F) in the horizontal and vertical dimension. For all figures the data come from the mean measures (\pm SE) of seven birds, each contributing data from 7-18 trials. The shading represents horizontal moves. The asterisks represent significance levels of $* = p < 0.05$, and $*** = p < 0.001$

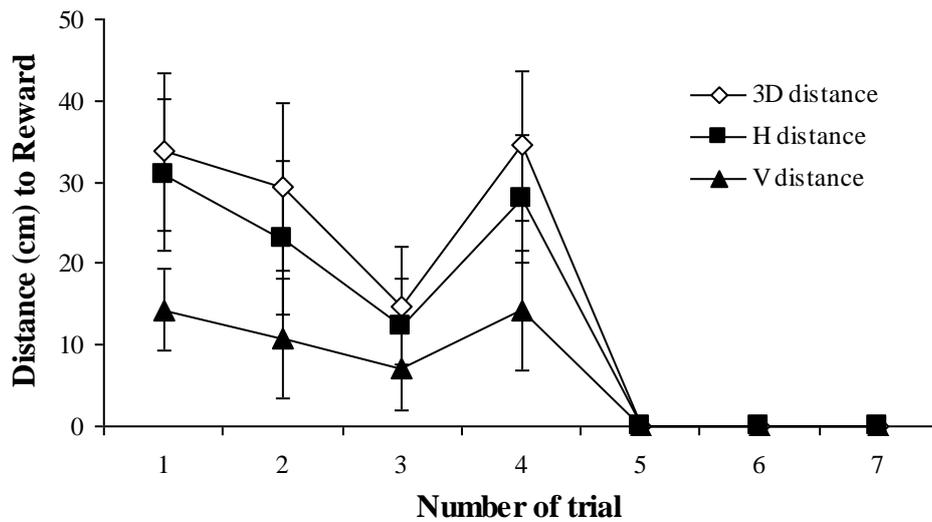


Figure 5 The distance in cm from the rewarded location to the first flower sampled in the last seven training trials. The data come from the mean measures (\pm SE) of seven birds, each contributing with one data point per trial

Search strategy

Birds could have reached the vertical component of the reward first and once there, fly to the rewarded location itself or they could have done the opposite. Each strategy would result in a different number of crossings for each component. However, birds crossed the same number of units within the reward's vertical component as they did within its horizontal component (Wilcoxon; proportion of crossings: $Z = 1.062$, $p = 0.288$). However, birds sampled a greater number of flowers in the horizontal component than they did in the vertical component even though there were more flowers in the vertical than in the horizontal component (Wilcoxon: $Z = 6.177$, $p < 0.0001$; Figure 6).

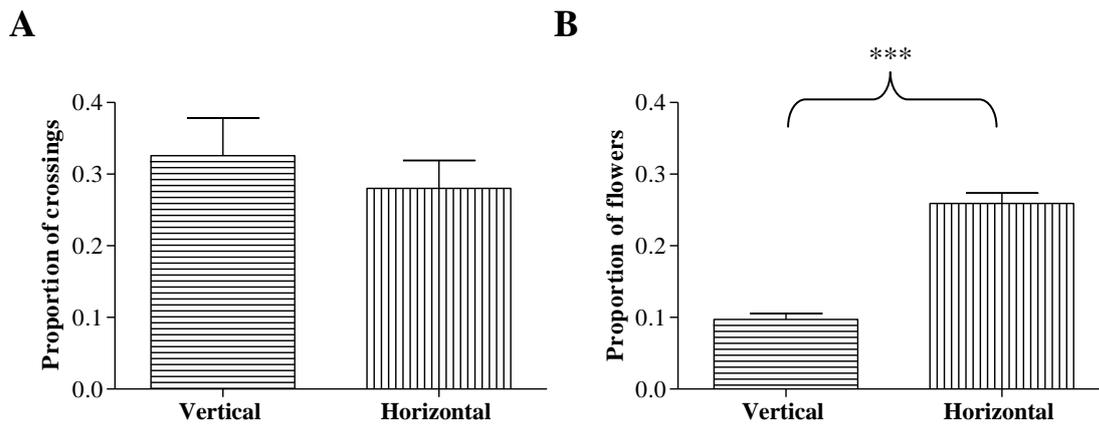


Figure 6 The Search Strategy used. The data are the mean (\pm SE) proportion of A) the number of crossing in the reward's vertical and horizontal components, and B) of the flowers sampled in the reward's vertical and horizontal components. For both figures, the data come from the mean measures (\pm SE) of seven birds, each contributing data from 7-18 trials. The direction of the lines within the bars represents the direction of crossings. The asterisks *** represent a significance level of $p < 0.001$

Accuracy

In the test trials 7/8 birds followed the shortest path (crossing 1 to 3 units) to the rewarded location. Most birds (6/8) did not stop to sample any flower before reaching the rewarded location. When the birds did not find a reward at the rewarded location, they moved horizontally and sampled an adjacent flower (Figure 7).

If we consider that birds could visit a horizontally and a vertically adjacent flower with the same likelihood, they chose a horizontally adjacent flower suggesting they were more certain about the vertical (Binomial with an expectation of 0.5, 0.5 (7/7), 2.268, $p = 0.016$). However, if we consider that there are three dimensions to flower choice (x, y and z) and thus use an expectation of 0.333 and 0.667, this results in a non-significant tendency for birds to choose a location that is horizontally adjacent to

the rewarded location, when they find the rewarded location empty (Binomial (7/7), $Z = 1.455$, $p = 0.061$; Figure 7A). We also calculated the metric distance from the rewarded location to the first flower the bird sampled after finding the reward location empty. The birds first sampled a flower that was closer to the rewarded flower's location in the vertical component (a mean of 25 cm from the reward) than it was in the horizontal component (a mean of 56 cm; Wilcoxon: $Z = 2.83$, $p = 0.005$). After finding no flower at the rewarded location, the birds visited a mean (\pm SE) of 4.75 ± 0.73 flowers. Across those visits, the birds did not preferentially visit flowers in the reward's vertical or horizontal component (Wilcoxon; proportion of flowers: $Z = 0.848$, $p = 0.396$; Figure 7B), however, not only did birds visit flowers on their first choice that were closer in the vertical than in the horizontal to the empty rewarded location but they continued to visit flowers in this plane for their second choice. By the time they made a third choice, the flower they visited was at a similar vertical and horizontal distance from the rewarded location (Wilcoxon; second choice: $Z = 2.25$, $p = 0.024$; third choice: $Z = 1.09$, $p = 0.276$; Figure 8).

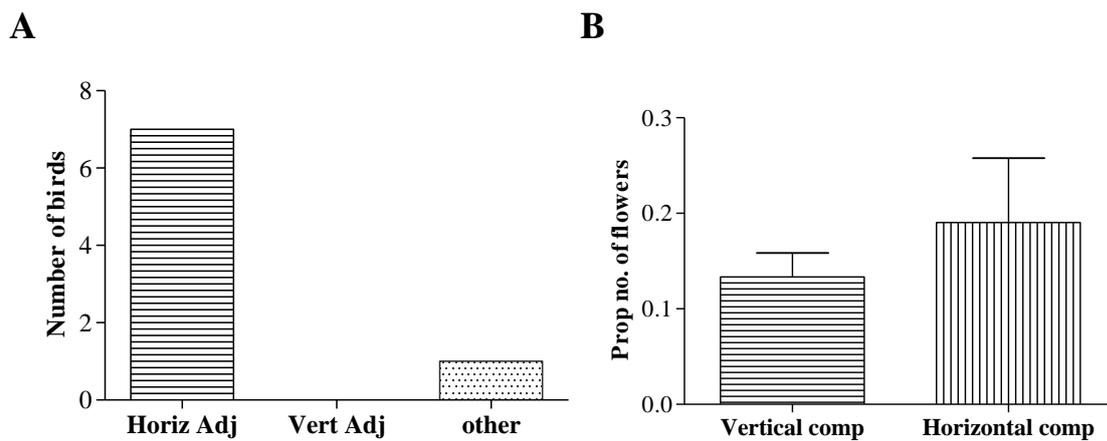


Figure 7 The location of flowers sampled after getting no reward by birds during the test. A) Birds 1st choice during the test and B) Mean (\pm SE) proportion of the number of flowers sampled during the test in the reward's vertical and horizontal components. The data come from the mean measures (\pm SE) of seven birds, each contributing with a single trial. The direction of the lines within the bars represent the direction of the birds' movements. Horiz Adj = Horizontally adjacent to the reward, and Vert Adj = Vertically adjacent to the reward

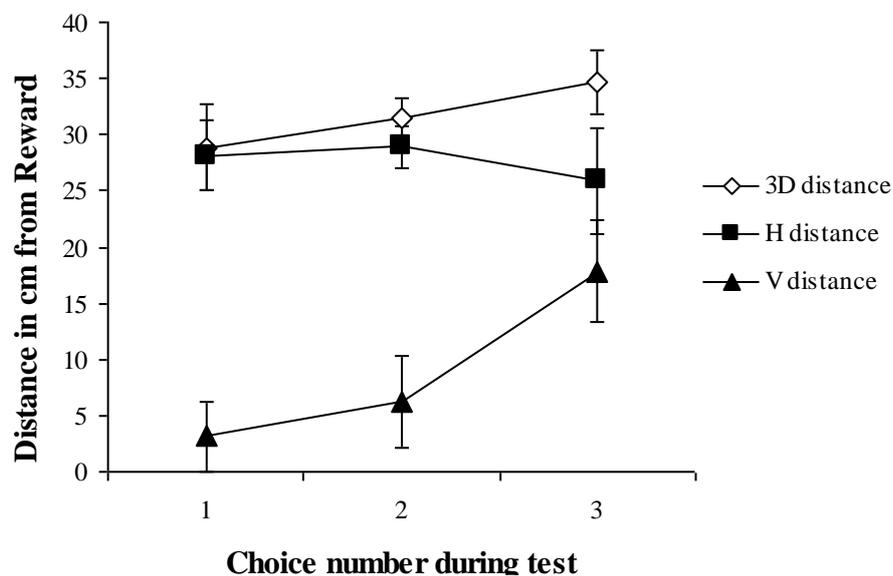


Figure 8 Distance in cm from the rewarded location to all flowers sampled during trials. The data come from the mean measures (\pm SE) of eight birds, for the first and second choices and seven in the third choice

DISCUSSION

The hummingbirds in this experiment learned a 3-D location after 12 rewarded experiences. When the reward was subsequently removed all but one of the birds flew directly to that, now unrewarded location. After hovering at the empty location, all but one of the birds flew to sample a horizontally adjacent flower. While learning the rewarded location, the birds sampled fewer flowers by flying vertically between the flowers than by flying horizontally. For an animal both horizontal axes are equivalent while the vertical axis is unique because it is parallel to the force of gravity. However, as axes are conceptual constructs that we can use to quantify the animal movements in 3-D space therefore, we used alternative methods to evaluate animal's use of 3-D space, we also compared the bird's movements through the three axes independently. When examined in this way, birds moved equally in each axis but still sampled fewer flowers in the vertical axis than in either of the horizontal axes. We also estimated the total 3-D distance for each of their first three choices in the test trial: birds chose flowers closer to the reward's height than to its x,y coordinate.

The location of the flowers that the birds choose to visit first during the test, when the rewarded flower had been removed, shows what they learned about the spatial location of the reward. First, they flew to the 3-D location without stopping at any alternative flowers. Then, by consistently choosing a horizontally adjacent flower (they could go to any of the 63 remaining flowers) they appear to be much more certain about the reward's height than about its x,y location.

The greater vertical accuracy of these hummingbirds is consistent with what we know about the accuracy and preference of animals that move freely in 3-D.

Hummingbirds trained to feed from an artificial flower got closer to the flower's

original height than to its original horizontal location when the flower was removed (Hurly et al., 2010). Similarly, cave fish prefer to use the vertical information of a learned location when forced to choose between the vertical and the horizontal components of a learned location (Holbrook and Burt de Perera, 2009; Holbrook and Burt de Perera, 2011). The hummingbirds may remember the vertical component of a location more accurately because this component is easier to learn than is the horizontal component because only distance from the ground is required to relocate a location in the vertical which consists of a single axis while learning a location in the horizontal requires both distance and direction to be encoded (Hurly et al., 2010). For fish it may be especially easy to learn the vertical component of a location due to them being sensitive to pressure changes via their swim bladder (Taylor et al., 2010). If learning a location in the vertical was easier than learning a coordinate (x-y) in the horizontal plane we would expect birds and fish to learn the vertical component of a 3-D location before they learn its horizontal location as well as being more accurate in the vertical component in tests. However, ease of learning does not appear to explain why fish, at least, prefer, or are more accurate, in the vertical: fish learned a location in the vertical and horizontal axes equally quickly when trained and tested in a Y-maze in the same orientation (Holbrook and Burt de Perera, 2009). The data from the hummingbirds (described in Chapter 3) would also not support an ease of learning explanation for the vertical preference and accuracy in that species.

The greater accuracy of recall in one dimension than in another raises the question of whether the animals learn each component independently. Hummingbirds can learn a 3-D location in the wild, in a single trial (Flores-Abreu et al., 2012). In the current experiment, during training and during the test the birds found the rewarded

location by crossing the same proportion of units in the reward's level (z) as for its horizontal location (x - y), which suggests these birds might have learned the location of the reward as an integrated 3-D coordinate rather than having learned each component independently. In contrast, rats exhibited a horizontal-vertical relocation strategy by reaching the x - y coordinate of the reward before they started climbing up followed by reaching the reward's location (Büchner et al., 2007; see Chapter 4).

The hummingbirds did not move more in the vertical, they sampled fewer flowers in the vertical and yet, in the test, when the rewarded flower was removed, they were more certain about its vertical component than of its horizontal component. This contrasts with all of the rat data from tests of memory for 3-D locations, including from a test in which the rat had to learn the location of a reward in a maze that was very similar to the one we used to test the hummingbirds (Chapter 4). It seems plausible, then, that flying animals either pay more attention to, or encode more effectively, vertical information than they do horizontal information. Although it would not be easy to test, we might expect that the firing fields of their grid cells not to be vertically elongated but horizontally elongated. While significant technological development would be required to test this possibility on hummingbirds this question might be more readily addressed with bats for which the required technology has been already developed (Ulanovsky & Moss 2007; Yartsev et al. 2011).

CHAPTER 6. GENERAL DISCUSSION

For much of the research described in this thesis, I investigated spatial cognition in three-dimensions using free-living, wild male rufous hummingbirds in their breeding range. I compared the birds' learning performance in the horizontal and the vertical dimensions and evaluated the potential facilitation between the two sources of information by transforming a one-dimensional array to a two-dimensional array. I also tested whether hummingbirds would remember a 3-D location after a single trial. Finally, I evaluated whether the animal's type of locomotion affected the extent to which they moved and the ease with which they learned a location in 3-D by comparing the performance of wild hummingbirds with laboratory rats in a very similar experiment. I also compared the use of the horizontal and vertical dimension of 3-D space as well as the accuracy to which these two components are learnt in two species with contrasting natural use of space: hummingbirds, which move in volumes, and rats, which are primarily terrestrial.

MAIN EMPIRICAL FINDINGS

My results show that although rufous hummingbirds can remember a 3-D location after a single trial they may not learn about the vertical and horizontal dimensions of 3-D space in an equivalent way. To learn a location that is specified only by its vertical dimension seemed to be harder for the birds than it was for them to learn a location in the horizontal dimension, as measured by finding that birds did not reach the criterion level that I set for them when they were trained using a purely vertical array (i.e. they did not flew directly and probed the rewarded flower for five consecutive times). In addition, the birds visited the rewarded and its adjacent flowers equally, suggesting,

they did not learn that only the central flower was rewarded (or could not learn that with sufficient accuracy). However, when the training array was placed diagonally birds chose to visit the rewarded flower first more than any other, which suggests that adding horizontal information to a purely vertical array can facilitate the learning of locations within it.

Perhaps counter intuitively, although they found learning a solely vertical location more difficult than learning a solely horizontal location, when learning a 3-D location, birds seemed to learn that location as an integrated 3-D coordinate rather than by learning independently the vertical and horizontal components and retrieving them together. In other words, they learn the absolute 3-D location. Therefore I would not expect birds to generalize their rewarded experience, for example, to a location with the same height but different horizontal coordinate. In addition, they learn the vertical component of a 3-D location more accurately than its horizontal component. If the birds had been able to retrieve the horizontal and vertical components separately, I would have expected them to choose the flowers in the appropriate vertical or horizontal positions in the respective test trials. However, birds chose flowers at random in the vertical and horizontal tests even though they had learned the bi-dimensional rewarded location within the diagonal array. The suggestion that hummingbirds learn 3-D locations in an integrated fashion is further supported by the experiment in which I showed that birds hovered in mid air at the location where they had experienced a reward once, instead of flying directly, a couple of meters away, to the new location of the flower. Similarly, when hummingbirds are trained to a single rewarded location and eventually the goal is removed or moved a couple of meters away from it, they will fly to the location previously occupied by the flower (Hurly et al. 2010).

The cubic maze experiments allowed me to compare whether the degree to which an animal uses each of the three dimensions influences their learning of a 3-D location. The degree to which an animal uses each of the dimensions during the experiment could result from the animal's natural use of space according to its type of locomotion and the ecological niche it occupies or from the particular spatial cognitive demands of the task. If the use of space in both cubic experiments reflected the natural use of space of the animal we would expect birds to move equally in the three axes and rats to move less in the vertical axis than in any of the two horizontal ones. However, rats and birds moved equally in the three axes suggesting that their use of space in a particular task depends more on the task than on the animal's locomotion. In addition, if the animal had learned the rewarded location as a 3-D coordinate in contrast to learning its vertical and horizontal components independently, we would expect it to move towards its location without consistently reaching its vertical or horizontal first. However, if the animal learned the 3-D location by learning its vertical horizontal component independently they might reach one component first and once there move towards the reward. Birds moved towards the rewarded location without first reaching its vertical or horizontal location (they reach both at the same time) while rats reached either the vertical or the horizontal component first. This suggests that birds might have learned the rewarded location as an integrated 3-D coordinate and that rats might have learned the vertical and horizontal components independently.

I compared a terrestrial and a flying animal, which each make very different use of 3-D space in the wild. We might, therefore, expect their spatial cognitive abilities to have been shaped very differently by natural selection. I attempted to design the experiment so as to allow both of them to use 3-D space to a similar extent during the

task and my results suggest that if terrestrial animals are put in a situation in which they can move equally through the three dimensions as volumetric animals do, they will as well. However, the way in which each type of animal learned a 3-D location seems to reflect their natural type of locomotion. The strategy used by the hummingbirds to relocate the rewarded location in the cubic maze would suggest that they had learned the 3-D location in an integrated way because they crossed a similar number of horizontal and vertical units to reach the reward's location. The rats, however, crossed more units in the horizontal than in the vertical location of the reward (i.e. searched more among possible locations in the rewarded column than at the rewarded level, see Chapter 4, Figure 1C). The rat's searching strategy could be explained in two possible ways: 1) rats learnt a 3-D location by remembering its vertical and horizontal components to different extents, or, 2) rats prefer to solve the less costly part of the journey first. This behaviour is considered to be typical of temporal discounting, which rats exhibit during free-foraging and detour tasks (Jovalekic et al. 2011). These two options are also not mutually exclusive. In the absence of a reward, rats go to the rewarded location and then search vertically as it would be expected if they had acquired both the vertical and horizontal information about the rewarded location but at a different resolution. In contrast, if the rat's strategy is mainly a product of temporal discounting we would expect rats not only to reach the horizontal component of the reward first but also to minimize their vertical movements during their whole training because those movements are supposed to be more costly. Rats reached the vertical and horizontal component first an equal number of times and when we compared the number of moves per axis, rats did not move less in the vertical than in any of the horizontal axes suggesting that rats learned the 3-D rewarded location by independently learning its

vertical and horizontal components. Otherwise, it would have not been possible for them to reach them independently. The fact that rats did not move less in the vertical than in the horizontal axes provides evidence against temporal discounting explaining my results.

Taken together, my results suggest that the use and acquisition of the vertical and the horizontal dimensions of space by birds and rats is different. The difference in 3-D spatial cognition between rats and birds might be a product of their evolution. The contrasting cognitive selective pressures that terrestrial and flying animals experience might lead to differential cognitive abilities that would represent adaptations to moving over a surface versus freely in 3-D.

Has natural selection influenced spatial cognitive abilities?

From all cognitive traits studied in an evolutionary framework, the best example is still probably that of the extensive work on spatial memory and its neural correlate in caching birds (Sherry 1985; Healy & Hurly 2004). Comparative studies using sibling species that vary in their spatial demands show that: 1) food storing birds show a better spatial memory than their closely related non-caching species (Balda & Kamil 1989; Pravosudov & Clayton 2002; Shettleworth 2003) and 2) they also exhibit a larger relative hippocampus volume, which is the brain structure involved in spatial cognition between other functions (Krebs et al. 1989; Healy & Krebs 1993; Healy et al. 1994; Sherry 1998). Although these birds move and store food in 3-D, that component of their spatial ability has never been addressed. Indeed, 3-D spatial cognition has been little investigated either in captivity or in the wild. It is not known if closely related species living in environments with contrasting spatial requirements (e.g. tree versus

ground squirrels) have different 3-D cognitive abilities. My study is one of the first attempts to compare the ability to learn about 3-D spatial locations in contrasting animals: a volumetric and a terrestrial species. Therefore, the differences found could also be due to their phylogenetic history (one being a mammal and the other a bird). However, as it appears that fish learn and remember 3-D locations in a way that resembles the hummingbird more than it does the rat suggests that this variation in this aspect of spatial cognition could constitute an adaptation to a volumetric niche.

Although my experiments were not designed to explicitly test the evolution of spatial cognition, my findings could help to understand what forces may influence the evolution of potential spatial cognition adaptations. In particular, I was interested in whether the animal's type of locomotion influenced its learning and memory in 3-D space. Potentially, evolving to live in volumetric space has not only enabled fish, birds and other animals that move in this way to move freely within 3-D space but also to use 3-D spatial information differently from the way in which terrestrial animals use 3-D information. In my cubic maze experiment I allowed both birds and rats to move within 3-D space at the same extent to evaluate if the proportion of movements on each of the three axes would reflect their natural use of space: whether birds would move equally in the three axis, as they do in the wild, and rats move less in the vertical axis than in any of the horizontal ones as shown in a previous learning experiment (Grobéty & Schenk 1992). If animals are cognitively as well as morphologically adapted to their way of locomotion we might expect that volumetric animals would learn 3-D locations in an integrated way while terrestrial ones would acquire the vertical and the horizontal components of a location independently.

Indeed, my data show that rufous hummingbirds remembered a 3-D location after a single rewarded experience and as predicted for being volumetric animals, they appear to have learned 3-D locations in an integrated way. In contrast, rats learn the vertical and horizontal components of the 3-D location independently. However, both rats and birds make about the same number of movements in each of the three axes. It seems plausible that whether an animal has evolved to move in volumes or over a surface might have influenced their 3-D learning more than the animal's use of space (i.e. rats and birds moved equally in the three axes). However, it is also possible that within-life experience of moving through volumes in the way that birds and fish do leads those animals to pay more attention to the vertical component than do animals that experience moving through volumes to a much lesser degree.

One way to test whether 3-D spatial cognition of volumetric and terrestrial animals is a result of convergent evolution would be to determine whether other terrestrial, flying and swimming animals follow the same pattern as rats, hummingbirds and fish. If that were the case we would expect different lineages such as bird and fish to share 3-D cognitive mechanisms. Cave fish, *Astyanax fasciatus*, have proved to be a good animal model to study spatial cognition in volumetric animals therefore it would be worth determining their use of 3-D space while being allowed to move freely within it as rats and hummingbirds were. Whether cave fish would use the three axes of space to a similar extent, and/or also exhibit greater accuracy in the vertical component of a learned rewarded location than in the horizontal component. This kind of experiment could also be adapted to other flying, climbing and terrestrial animals. Fox squirrels for example, being arboreal and highly visual rodents that can be individually identified and trained for behavioural experiments in its natural environment (Jacobs & Shiflett 1999),

would constitute good candidates to investigate 3-D spatial learning and memory in a similar scenario. Studying 3-D spatial cognition in mammalian lineages with different use of space (e.g. bats, squirrels, purely terrestrial species) would be useful to determine whether the difference we observe in 3-D spatial cognition constitutes an adaptation to being volumetric or whether all mammals, regardless of their habitat and locomotion, share behavioural and neural learning mechanisms for dealing with 3-D space.

However, it would also be appropriate to contrast these comparisons with experimental manipulations of the experience of animals moving through 3-D space. One might, for example, restrict fish or birds to a horizontal plane during development or provide a greatly enhanced 3-D environment for a terrestrial animal like the rat before testing their preference for, and accuracy in, the horizontal over the vertical.

Is 3-D space a sum of its parts?

Most of what we know about spatial cognition comes from experiments on the horizontal plane, which is made up of two components (namely, x and y). In contrast, the vertical dimension (z) has been considered unique because it is parallel to the force of gravity and, in consequence, moving up and down imposes a higher energetic cost than does moving in the horizontal (Grobéty & Schenk 1992). If 3-D space is the sum of its constituent parts we would expect that, 1) learning a location in a single dimension would require fewer trials than learning a rewarded location in a 2-D or 3-D array and, 2) it should be possible for the animal to retrieve the horizontal and vertical components independently.

Perhaps surprisingly, the data from the experiments described in Chapter 3 showed that, for the hummingbirds, learning a location within a linear flower array

appeared to be constrained by the array orientation. Adding a horizontal dimension to the locations in the vertical array (i.e. placing the array diagonally instead of vertically) appeared to enable learning both components of the rewarded location. However, the test data (where they chose the horizontally appropriate location) would suggest that the birds had not actually learned the vertical component. One explanation is that they did not transpose that information to the test, perhaps because the test array looked too different from the training array. Further work needs to be done to discriminate between these possibilities. The apparent failure to choose the vertical location in the test is consistent with fish using vertical information when forced to choose between the vertical and the horizontal components in a conflict test but failing to choose the vertical component in a vertical test after being trained to a location on a diagonally oriented Y-maze. Furthermore, fish required the same number of trials to learn a rewarded location in 2-D (by rotating the Y-maze) as they did in the 3-D version (Holbrook & Burt de Perera 2009). How the animals determine the vertical location is also not yet understood. While it is possible that at least some fish might use their swim bladder to detect depth, the most obvious possibility for the birds is that they use visual cues. This is yet another question that requires more work.

While my results suggest that volumetric animals (hummingbirds, at least) learn 3-D locations as integrated coordinate, the birds seem to be more certain about the vertical component of the rewarded location than of its horizontal component. This contrasts with hummingbirds not being able to learn a location in a vertical axis and the fact that, in the 2-D array, they prefer the horizontal location. The birds' apparent impairment could be the result of an anisotropic encoding of the vertical and horizontal dimensions as has been proposed for rats (Hayman et al. 2011). However, when the

relevant location involves the three spatial dimensions, their vertical impairment disappears because birds learn 3-D locations in an integrated way. Another possible explanation that is unrelated to 3-D is that their apparent impairment to learn accurately within the vertical might be related to the scale at which the cues/rewards were spaced in my experiments. If this were the case, increasing the spacing of the elements should result in a better performance.

On the other hand, rats learn 3-D locations by encoding the vertical and the horizontal components independently, not in an integrated way as hummingbirds do. Although rats are supposed to encode a vertical distance with a lower resolution, my results suggest that they might be able to learn a location within a purely vertical array. However, we would expect them to learn in a vertical array less accurately than in a purely horizontal array, or to require a wider spacing between the elements.

Do animals that move in volumes encode 3-D space anisotropically?

It has been suggested the acquisition of information in the vertical and in the horizontal dimensions is anisotropic (Grobéty & Schenk 1992; Hayman et al. 2011; Jovalekic et al. 2011). The neural correlate in rats, used to estimate distances in the vertical seems to have a much lower resolution than in the horizontal and therefore learning a location in the vertical should be considerably less accurate than in the horizontal (Hayman et al. 2011). The conclusion that 3-D space is encoded anisotropically, at least in rats, is based on the finding that rat's grid cells exhibit an elongated firing pattern when the rat explores a vertical plane (as well as a helix structure), and therefore, rats would be expected to encode poorly (inaccurately) distances in the vertical dimension (Hayman et al. 2011). Although this prediction has not been explicitly tested, rats do appear to learn

more accurately the horizontal component of a 3-D location than they do the vertical component (Chapter 4; Grob ty and Schenk, 1992). At odds with anisotropic encoding are the data from Grob ty and Schenk's (1992) experiment showing that rats learned the vertical component of the 3-D location before learning its horizontal location. It seems that it would be useful to train and test rats in a variety of 3-D apparatus before reaching any strong conclusion with regard to accuracy of behavioural or neurophysiological encoding of 3-D space.

My findings suggest that while rats may encode 3-D space anisotropically, animals that move in volumes may not. If we consider that the avian brain and the mammal brain are homologous (Jarvis et al. 2005), then the hummingbirds' impairment to learn on vertical linear arrays could result from their grid cells having elongated firing fields as do those of rats. It has been suggested that the anisotropic encoding of 3-D space might be a constituent characteristic of all vertebrate brains (Jovalekic et al., 2011). One way of addressing whether all animals exhibit an anisotropic encoding of 3-D space regardless of their locomotion type would be to record from grid cells of flying animals. If the grid cells' firing fields constitute the neural correlate for the observed difference in the vertical versus horizontal accuracy of a 3-D location in birds and rats, we would expect, for example, bats to have a grid cell firing pattern more similar to birds than to rats regardless of them being more closely related to rats.

In addition to the electrophysiological studies, behavioural studies are also necessary to test whether the anisotropic encoding of 3-D space is a specifically mammalian feature found in all terrestrial animals or if it is really how all vertebrates acquire information about space. From my results one possibility is that volumetric animals encode 3-D space as an "x, y, z" coordinate and not as a vertical plus a

horizontal component. This is consistent with birds exhibiting such a poor learning of the height of a rewarded location within a vertical axis but being more accurate at finding the vertical than the horizontal component of a 3-D location. They could be using their grid firing fields to estimate vertical distance in a purely vertical array while they could use a different strategy when learning a 3-D location.

CONCLUSIONS

Hummingbirds appear to learn 3D locations as an integrated “x, y, z” coordinate in a single trial and not as an independent horizontal and vertical component. Whether the vertical and horizontal dimensions are equivalent for terrestrial animals it remains to be tested although neurobiological findings would suggest that they should be better at learning in the horizontal than in the vertical due to a higher resolution of their distance metric neural correlate. This is supported by rats learning more accurately the horizontal component of a 3-D location than the vertical one.

Animal navigation in 3-D seems to be different to navigating horizontally. Learning the vertical and the horizontal components of a location do not seem to be equivalent neither for terrestrial nor for flying animals. The possibility that the anisotropic encoding of 3-D space is related to the animal’s mode of locomotion needs further electrophysiological and behavioural investigation. For example, is the elongation of firing fields of grid cells correlated with a poor accuracy in remembering the vertical component of a 3-D location. The prediction that learning in rats is impaired in the vertical also remains to be tested. More generally, further testing of the use of 3-D in a range of species would seem feasible and timely.

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