OLFACTORY LEARNING, ITS DEVELOPMENT AND CHANGING ROLE IN HONEYBEE (*APIS MELLIFERA*) BEHAVIOUR

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Abstract

The honeybee (*Apis mellifera*) is capable of showing a wide variety of cognitive tasks, and can be readily conditioned in the laboratory to specific odours, paired with a sucrose reward, using the proboscis extension reflex (PER) learning paradigm. This thesis aims to establish any differences in the behavioural parameters of this olfactory learning.

A strong, repeatable methodology is developed, and this specificity of the learning, tested by training bees to different odours, provides a useful model of other phenomenon important in learning theory, such as overshadowing, blocking, massed and spaced training effects, and habituation. The research also indicates a circadian rhythm in the olfactory learning, which is linked to the field, where food sources are only available during certain periods of the day.

A new technique was developed to investigate long term captivity and the effects this has on olfactory learning and homing abilities. In both these different, but crucial, learning criteria's, captivity played no significant effect, suggesting that the long term memory of the honeybee is a stable, and not easily disrupted entity.

The behavioural and developmental stages of the dynamic honeybee colony were examined, to identify any differences in learning in bees aged 1-24 days old. Bees younger than 15 days of age did not show comparable learning to adult foragers, despite having a fully mature olfactory neural pathway.

Similarly, PER learning of different castes was researched, with nurse, guard, forager, and precocial forager bees being studied. The results showed that there exists a heirachy in olfactory learning with nurse and guard bees exhibiting learning lower than foragers and precocious foragers. This suggests the social role of the bee, and the interaction between behavioural maturation within its complex society, is a major determinant of olfactory learning ability.

The effects of the season are also examined to see if the levels of learning are constant over the year. Learning was reduced in the summer months, with an increased learning in the winter, which is related to the available forage and the hive demography.

The experiments reported show that by using just one example of bee learning, insights into the mechanisms of learning and memory can be sought. The olfactory system of the honey bee is particularly well researched, and thus, bees can be easily used as a tool at all levels of enquiry from molecular and cellular studies to behavioural genetics, anatomy and physiology.

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CHAPTER 1:

1.0 General introduction

1.1 The honeybee

The honeybee (*Apis mellifera*) is perhaps the most successful pollinator and exploiter of flowers, with these Hymenoptera species inhabiting every niche available over Europe, Africa, the Americas and spreading into Asia (Gould & Gould, 1995). The most common bee species is *Apis mellifera*, which is a temperate region species, followed by *Apis cerana*, the Indian honeybee, *Apis florea*, the dwarf honeybee, and finally *Apis dorsata*, the giant honeybee. All these species have particular niches to which they are most suited and they coexist with other insect species, and even with each other.

The honeybee has a rich behavioural repertoire, which includes individual as well as group traits, and this is unequalled in the animal kingdom. This has made the honeybee an excellent study animal, being examined from a host of different angles, from the behavioural ecologist to the doctor studying allergic reactions. All this has provided an in-depth and detailed account of the honeybees life and characteristics. This fascination with the honeybee dates back to prehistory, as rock paintings of man harvesting honey from colonies in trees have been found dating from 8,000 - 15,000 years ago, with this perhaps being the earliest known example of man exploiting the honeybee.

The honeybee colony consists of three different honeybee types, the queen, the drone and the workers. The workers number about 10,000 in the winter and this rises to 50,000 or more in the summer. This society also includes 200 to 1,000 male drones in the summer, which are killed off or evicted from the hive at the end of the summer. Each of the honeybee individuals has a specific job to undertake, with the queen being

the only sexual female in the colony, mating only once in her lifetime with the only other sexual members of the colony, the male drones. These drones are much larger than the female workers, having much larger eyes, a larger body and shortened antennae. Finally, the workers, who can be subdivided into the different task which they perform. These may follow a sequential progression, or the worker may remain with the same task for their entire lifetime. See table 1.1 for a breakdown of the behaviours and the ages that these may appear (taken from Winston, 1994, p91).

From the queen laying the egg, to the honeybee emerging from the comb, the female worker bee takes 21 days, compared to 37 days for the male drones. The average honeybee lives for only 36 days in the summer, but may live for over 6 months during the winter (overwintering bees) (Hooper, 1991). In a study by Seeley (1978), the queen lived for 1 year in 79 % of unmanaged hives, 26 % for 2 years and virtually no queens survived for 3 years. However, Bozina (1961) reported that 35 % of queens in normal colonies lived 4 - 6 years, and he also noted that he had had three queens survive for 8 years or more, and Hooper (1991) reports that the average lifespan of the queen in his colonies was 3 - 4 years.

The demands on the bee to show specific behaviours varies with developmental stage, as newly emerged bees' duties include cleaning the hive, and feeding larvae. As the individual ages, it takes on more complex behaviours, such as guarding and foraging. It is to this extent that within a honeybee colony, there exists a definite division of labour, with distinct collections or castes of honeybee who perform different functions (Page & Robinson, 1991). Young bees tend the brood and guard the hive entrance, whilst the older bees forage (Free & Brand, 1977). This division of labour is very plastic, with the jobs designated to each caste being sustained for a period of time, depending on the hives' demographics and needs.

Task	Age range (days)	Mean age (days)	Reference
Cell cleaning (early)	0-52	9.0	Winston & Punnett, 1982
	1-25	6.2	Seeley, 1982
	1-25	---	Sakagami, 1953
	1-26	---	Lindauer, 1952
	1-5	---	Rosch, 1925, 1927
	1-21	5.3	Perepelova, 1928a
	1-30	9.7	Smith, 1974
Capping brood	3-7	4.8	Seeley, 1982
	2-26	---	Sakagami, 1953
	1-26	6.3	Smith, 1974
	1-19	---	Kolmes, 1985a, b, c
Tending brood	1-52	12.6	Winston & Punnett, 1982
	2-26	12.8	Lindauer, 1952
	6-13	8.6	Rosch, 1925, 1927
	6-16	9.2	Perepelova, 1928b
	1-26	9.3	Smith, 1974
	1-13	6.5	Seeley, 1982
	1-26	---	Kolmes, 1985a, b, c
	2-31	---	Sakagami, 1953
Queen tending	1-49	17.1	Winston & Punnett, 1982
	1-10	5.5	Seeley, 1982
	1-52	10.7	Allen, 1960
Receiving nectar	8-14	11.2	Rosch, 1925, 1927
	10-22	14.9	Seeley, 1982
	5-28	---	Sakagami, 1953
	1-17	---	Kolmes, 1985a, b, c
Handling pollen	12-25	16.2	Seeley, 1982
	1-33	---	Sakagami, 1953
Comb building	1-52	15.2	Winston & Punnett, 1982
	2-52	15.8	Rosch, 1925, 1927
	1-17	---	Kolmes, 1985a, b, c
	0-34	---	Sakagami, 1953
Cleaning debris from hive	2-20	13.9	Perepelova, 1928a
	10-23	14.7	Rosch, 1925, 1927
	9-16	11.3	Seeley, 1982

Task	Age range (days)	Mean age (days)	Reference
Cell cleaning (late)			
Cell walls	1-21	13.3	Perepelova, 1928a
Smoothing edges	1-21	11.0	Perepelova, 1928a
Capping removal	4-21	18.2	Perepelova, 1928a
Ventilating	1-25	14.7	Seeley, 1982
	1-61	19.0	Winston & Punnett, 1982
	1-19	---	Kolmes, 1985a, b, c
Patrolling	0-60	15.5	Winston & Punnett, 1982
	1-27	10.3	Seeley, 1982
Resting	0-69	19.2	Winston & Punnett, 1982
	1-27	9.1	Seeley, 1982
Guard duty	4-60	22.1	Winston & Punnett, 1982
	10-46	---	Sekiguchi & Sakagami, 1966
	11-25	---	Butler & Free, 1952
	7-23	14.9	Moore, Breed & Moor, 1986
First orientation flight	5-15	7.9	Rosch, 1925, 1927
	4-65	25.7	Winston & Punnett, 1982
	7-12	8.9	Seeley, 1982
First foraging trip	3-65	25.6	Winston & Punnett, 1982
	10-34	19.5	Ribbands, 1952
	10-32	20.1	Ribbands, 1952
	9-35	19.2	Ribbands, 1952
	20-41	30.2	Lindauer, 1953
	5-39	18.3	Sakagami, 1953
	10-27	20.6	Seeley, 1982
	10-59	37.9	Winston & Ferguson, 1985
	10-34	19.5	Rosch, 1925, 1927
	7-43	---	Sekiguchi & Sakagami, 1966

Table 1.1 Ages at which tasks are performed by workers.

This behavioural caste development has been found to be regulated by juvenile hormone

(JH) (Rutz, Gerig, Willie & Luscher, 1976; reviewed by Robinson, 1987a, b), which is

produced in the bee brain by the corpora allata (Rachinsky & Hartfelder, 1990), with the rate of JH synthesised increasing during the lifespan of the honeybee. JH was first discovered by Wigglesworth (1934, 1936) and has been known to be critical for the regulation of both metamorphosis, reproductive maturation and behaviour in insects (Koeppe, Fuchs, Chen, Hunt, Kovalick & Briers, 1985; Bownes, 1986; Wyatt, 1991). While the numbers of bees in each of the castes are variable, the behaviours of each can be readily identified. There also exists differences in the rate at which bees progress through the age castes, some showing a precocious behavioural development, while others mature at a slower rate (Sekiguchi & Sakagami, 1966; Nowogradski, 1983). This development induced by JH can also be artificially manipulated, whereby foragers can be made to revert to nursing behaviours, and vice-versa. This is performed by removing one caste from the hive, creating a vacuum for that particular behaviour in the colony, which is then filled by some of the remaining honeybees who either revert to that role, or precociously develop the vacant caste behaviours. The manipulation can also be done by application of JH analogues either orally or topically (Robinson & Ratnieks, 1987; Robinson, 1987).

The age demographics of the bees vary throughout the year, placing a different emphasis on the requirements of the hive. In winter, as the queen stops laying eggs, the hive population is all of a similar age, these are long-lived over wintering bees, whereas in spring, as the queen begins to lay her eggs in earnest, and these bees start to emerge, the demographics are shifted markedly towards young bees. The summer populations display a Normal distribution, with fewer younger and older bees, and a large population of middle-aged bees, and in autumn it returns to a greater proportion of older bees.

1.2 Why study invertebrate behaviour ?

Honeybees present a unique opportunity to study invertebrate behaviours, as they are readily accessible, easy to maintain, simple to train, and possess a rich behavioural repertoire (Ferneyhough & Ray, 1999). Invertebrates in general offer several important advantages for understanding the basic principles of learning and memory, as they are useful to test the generality of both behavioural theories of learning and the underlying physiology and biochemical mechanisms. The invertebrate nervous system is more amenable to physiological and biochemical manipulations than the vertebrate nervous system, and this therefore allows easier access to experimentation. The behaviour of invertebrates is also generally less complex and more reflexive than vertebrate behaviour, and the genetic analysis of behaviour is easier to influence, with great advances being made with studies on the fruitfly (*Drosophila melanogaster*) (Bolwig, Del Vecchio, Hannon & Tully, 1995; Yin, Del Vecchio, Zhou & Tully, 1995; Rohrbrough, Pinto, Mihalek, Tully & Broadie, 1999) and the nematode (*Caenorhabditis elegans*) (Wen, Kumar, Morrison, Rambaldini, Runciman, Rousseau & Van Der Kooy, 1997; Krieger & Breer, 1999; Morrison, Wen, Runciman & Van Der Kooy, 1999). This combination of the accessible nervous system, genetic manipulation and relative ease and speed of training makes invertebrates an attractive group in which to investigate learning and memory. Perhaps the pinnacle of invertebrate evolution are the Hymenoptera, which includes both the social insect species, the ants and honeybees. The majority of what honeybees are capable of behaviourally, is due in the most part to their excellent sensory systems, and how each of these evolutionary aspects contributes to the extensive behavioural capacity of the honeybee. One such element of this is the visual system which the bee possesses.

1.3 Visual system of the honeybee

Much of the behavioural repertoire of the honeybee, outside of the hive, is visually guided, and like nearly all invertebrates, the eyes of the honeybee are compound, with the workers having about 4500 facets, the queen about 3500, and drones, who must be able to identify the queen on mating flights, having 7500. Each facet makes up the vision of the honeybee, and is pieced together to make a whole image. Snodgrass (1956) estimated that the honeybee brain receives about 1 % as many connections as a humans eye provides, and like humans, the visual system in bees is in colour, and is trichomatic, but the three primary colours have shifted towards shorter light wavelengths (Daumer, 1958; Von Frisch, 1967; Silberglied, 1979; Kevan, 1983). This discovery of colour vision in honeybees was made by Karl von Frisch (1914), also the bees eyes are especially sensitive to ultraviolet light, as well as to green and blue light. The honeybee is able to learn all colours, but learns violet the fastest (400 - 420 nm wavelength), and blueish green the slowest (about 490 nm wavelength), with blue, yellow, bee purple, and ultraviolet in between (Menzel, 1967a). This ultra-violet range eyesight allows bees to see patterns on flowers invisible to the human eye. Bee pollinated flowers have a dark spot in the centre, and frequently have lines leading from the petal edge to the centre. These lines are further enhanced as the green foliage surrounding the flowers is detected as grey by the bees vision. As well as this form of light being 'seen', the honeybee can also detect polarised light, via the ocelli on the top of the bees head, which enables the honeybee to navigate. Menzel (1990) reports that the honeybee uses this polarised light as a guide for long-distance orientation in flight to guide the bee on flights between the hive and a patch of flowers. However, Menzel (1990) further states that this form of light is not learned as a substrate for food. Research by Erber (1982), Fischer (1973) and Vogt (1969) has shown that slowly rotating vertical sectors, moving grids or flashing

light stimuli presented to the bee as it approaches a feeding place are also not learned. This is in accord with research carried out during this thesis research (not reported) whereby a slowly rotating black and white drum, and also a strobe light were attempted to appetitively condition honeybees, both unsuccessfully. A possible reason for this inability to learn these parameters to a food reward is that in nature the hovering bee is subject to a great number of different visual stimuli, such as vertical and horizontal moving patterns, and varying intensities of light. The honeybee perceives this bombardment of stimuli at the feeding place, and so does not learn them, as there are more salient parameters present, such as odour, colour, distance and orientation from the hive, of which to learn. Bees can be trained to learn these factors in non appetitive paradigms such as phototaxis, scanning behaviour, depth perception and landmark orientation, but they do not learn them as food cues (Menzel, 1990).

1.4 Colour learning

The concept of colour vision in the honeybee was first discovered by von Frisch in the early 1900s as he trained bees to a dish containing sucrose, which was placed on red coloured card. He then set up a series of dishes each on different coloured card, and found that the bees always went to the trained colour, red. However, when he then tested them with different shades of grey along with the red card, the bees randomly landed on any dish, it was from this that he reasoned that bees could not distinguish red, as it is outside of their spectral vision. Von Frisch then carried out a range of experiments and established the range of the honeybees' visual spectrum. Opfinger (1931) later showed that colour was learned only on the approaching flight, and not the leaving, as the reward stimulus of the nectar has not yet been experienced, so the colour may not be worth remembering. Erber (1972, 1975a, b, 1976), Menzel (1983), and Menzel and

Erber (1972, 1978) have since narrowed this time window down to show that this learning is actually the colour seen in the last two or three seconds before landing, and not the colour seen whilst feeding or departing the flower. The rate at which the colour is learned and consolidated has also been investigated (Gould, 1984), with between three and five trials required for the colour to give a 90 % accuracy, with this figure rarely achieving levels above 95 %. These results suggest that the colour learning first enters a short term memory before being consolidated into the long term memory. In further experiments examining colour learning, Gould (1984) reported that naive recruit bees preferentially landed on violet coloured flowers, however this is not statistically absolute, as even in the presence of a violet flower, a naive bee may sometimes choose a yellow, blue or white flower, which has implications in an evolutionary sense, as not all flowers are violet.

In a report by Srinivasan, Zhang and Zhu (1998), bees were trained to an odour, and a colour, and then during the test trials the bees had to navigate a maze which had the trained odour at the entrance. The bee then had to recall the colour, which was an indicator of the exit. The results proved significant, showing that honeybees are capable of linking sights to smells, and most probably do this in the field, where a qualitative choice is required.

1.5 Olfaction

The location of the sense of smell in the honeybee is on the protruding antennae (Gascuel, Devaud, Quenet & masson, 1995; Gascuel, Kopysova & Masson, 1998), where they serve orientation in the most efficient way, as the honeybee is able to move and adjust the direction in which the antennae point, to locate the bearing of a particular odour. The antennae can be divided into three main parts, the scape (or basal stalk),

which is the portion that is attached directly to the headcase, the pedicle, and the flagellum, the latter of which is the site of olfaction. This discovery of the olfactory sense on the antennae was carried out by amputation of the antennae in combination with training to odours, by von Frisch (1919) who trained free flying bees to a particular odour, and then amputated the whole or part of their antenna and observed their responses. He noted that trained bees with whole antennae hovered by the trained odour before landing, however amputee bees hovered by all the odours and landed by chance on any dish. These initial experiments have since been repeatedly demonstrated by Marshall (1935), Frings (1944), Fischer (1957), and Dostal (1958).

The flagellum itself consists of ten segments containing sensilla, which sense the odour. There are seven types of sensilla, which are situated in pores within the antenna, and these pores being covered with hairs or cones which protrude from the antennae. These sensilla are named thus (von Frisch, 1993);

1) Sensilla trichodeum, is a small thick walled hair, evenly distributed in great numbers over the eight distal flagella segments.

2) Sensillum trichodeum, a thick walled peg, not very numerous, and found on segments three to ten predominantly.

3) Sensillum trichodeum olfactorium, a slender thin walled peg, has pores present which allow penetration of stimulants, and these sensilla are innervated by 5 - 10 nerve cells. They are numerous, and distributed evenly over the eight flagella segments, having about 8 - 9,000 on each antenna.

4) Sensillum basiconicum, are olfactory cones, with large thin walls, innervated by 16 - 20 sensory cells, and there are 100 -150 on each antenna.

5) Sensillum placodeum, a pore plate organ, innervated by 16 - 20 receptor cells, there are about 3,000 on each antenna.

6) <u>Sensillum coeloconium</u>, a pit cone, and is innervated by a single neuron, and is situated on the upper and lower antennal segments.

7) <u>Sensillum ampullaceum</u>, a pit cone, innervated by a single neuron, more numerous than sensillum coeloconium, and congregates around them, the numbers on each antenna are 236.

The pore plates are probably the location of the olfactory sense, as they are more predominant on the male drones, whose sense of smell is of fundamental importance when seeking a queen in the nuptial flight. On one antenna, the worker has 3,000 pore plates, on the drone there are 15,000. Lacher and Schnieder (1963) recorded the excitatory potentials from individual receptor cells, and found that the pore plates were the only sensilla to respond to odourants. They also found that individual sensory cells on a pore plate respond to various odours, but not to them all, and no two sensory cells respond precisely to the same selection of odourants, each one having a different 'reaction spectrum'. This apparently infinite combination of reaction spectra is no doubt the physiological basis for the discrimination by the honeybee of so many different odours. Once the honeybee has detected a floral odour, it then can begin to feed, by extending its proboscis.

1.6 The proboscis

As the name suggests, the proboscis extension reflex is the reflex of proboscis extension in response to contact or anticipation of a food reward. The proboscis is a portion of the mouthparts of the honeybee which are required to manipulate many different substrates, and have many different functions, such as grooming, removing debris, and cutting and shaping propolis. The actual mouthparts are made up of paired mandibles which are attached to either side of the head, and the proboscis, consisting of the maxillae and

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labium. The proboscis, like the mouthparts also has many different functions, with its major capacity being to ingest liquids, principally nectar, honey and water. The proboscis is made up of eight structures which are connected to the base of the mouth, with the lateral maxillae and central labium jointed to enable the whole proboscis to be folded into the mouth cavity in a Z - shape when not extended (see figure 1.6). When the proboscis is fully extended, the galeae of the maxillae and the labium form a tube around the glossa (the tongue) (Winston, 1979). It is the size of this structure that determines the species of flowers that the bee can attend, as the proboscis length differs in different races of bee (Winston, 1994). The glossa is densely haired, and there is a flagellum through which liquids are absorbed and transported into the mouth (Michener & Brooks, 1984). These liquids are also pumped by muscles at the base of the glossa, and the movement of the liquids is aided by forwards and backwards movements of the glossa, as well as capillary action.

1.7 The olfactory pathway

Each antennae possesses about 230,000 chemoreceptors, which project to 156 glomeruli in the antennal lobe (Flanagan & Mercer, 1989a, b). The antennal lobe in the insect is the functional analogue to the olfactory bulb in mammals (Boeckh, Distler, Ernst, Hosl & Malun, 1990). These then join to about 4,700 local interneurons, and about 1,000 projection neurons, which exit the antennal lobes in one of three ways, eventually innervating the mushroom bodies. The olfactory pathway (see figure 1.7) ultimately ends here, and there is evidence that this may be the site of long term memory storage (Menzel, Erber & Masuhr, 1974), as the volume of these structures increases with the complexity of the behaviour. These observations were made during early research into this area by Alten (1910), Holmgren (1916), Peitschker (1911), Hanstrom (1928)

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Figure 1.6: The proboscis mouthparts, and the Z-shaped configuration they fold into under the mandible
Taken from Winston (1995), p 20 and Roubik (1992), p66

copyrighted images removed

Figure 1.7: The olfactory pathway in the honeybee brain
Taken from Gould and Gould (1995), p163

Pandazis (1930) and Gossen (1949). The number of cells in the mushroom bodies is also much greater in the female worker than the male drone (40 % to 24 % respectively of all brain cells) (Witthoft, 1967), as the female worker exhibits more intricate behavioural repertoires, for example, flower handling, foraging, feeding larvae, than the male drones, whose life is spent mainly in the hive, except for one brief flight to inseminate the queen bee on the nuptial flight. The density of the synapses in the mushroom body is also high, which is indicative of species exhibiting complex behaviours (Vowles, 1955; Goll, 1967; Schurmann, 1970, 1972).

The mushroom body is formed by 170,000 local neurons, called the Kenyon cells (Mobbs, 1982; Witthoft, 1967), and it is these cells that receive inputs from the projection neuron tracts in the upper part of the calyx. Other parts of the calyx receive inputs from other sources, such as the visual neurons in the collar of the calyx, and visual and olfactory neurons on the basal ring. The two lobes of the mushroom bodies and the alpha and beta lobes connect the output neurons with their own input regions, the lateral protocerebellum, the contralateral mushroom body and many other brain regions (Menzel, Durst, Erber, Erchmuller & Hammer, 1994; Rybak & Menzel, 1993). The anatomy of this olfactory system has been well established (Galizia, Joerges, Kuttner, Faber & Menzel, 1997; Joerges, Kuttner & Menzel, 1995) showing a surprisingly early nervous system maturation by the second day post emergence (Gascuel & Masson, 1991), compared with the maturation of behaviour, which is relatively slow, taking up to twenty-one days for the bee to become a mature forager. Menzel (1990) states that the olfactory conditioning is retarded within the first day of emergence, and only reaches final levels during the second and third day. This is probably due to developmental processing within the antennal lobe, as Masson and Arnold (1987) have shown that electrical activity in the chemosensory pathway and the maturation of the antennal glomeruli structure does not reach the adult stage before the

second to fourth day post emergence. Research by Allan, Slessor, Winston and King (1987) also indicates that the olfactory system of the honeybee does not mature within the first three days post emergence.

Specific behaviour responses are in a large part based on the already established responses, but the specificity of response is also achieved through learning (Heinrich, 1984). The bee seems to have an innate search image (Kevan & Baker, 1983) which develops into a learned search image through experience. The term 'search image', should be used with care however, as it does not relate to a specific neural pathway, similarly, the prefix 'innate' should also be used with caution, as it has never been shown that a bee already has a built in comprehension of flower shape or colour.

The honeybees' whole ecology is reliant on flowers and their temporal cycle of opening and offering nectar which changes constantly, through minutes, hours or days, and thus, the honeybee has to relate these changes to their own behaviour and time.

1.8 Time linked learning

As well as honeybees having colour vision over a specific spectrum, they have also been demonstrated to possess a keen time sense. This was noticed by Forel in 1910, when he noted that bees possessed a sense of time, as they visited a feedstation he had unknowingly set up (jam on his toast, at the same time every morning), and the bees continued to visit, even when he did not have morning breakfast. This sense is usually governed by celestial cues, such as the sun (Beier 1968; Beier & Lindauer, 1970; Beling, 1929; Frisch, 1987; Frisch & Aschoff, 1987; Kleber, 1935; Moore & Rankin, 1983; Wahl, 1932). However, in the honeybee, an animal intrinsically linked to flowers, a rhythm of food supply can also act as a time dependent variable, as flowers produce nectar at set time intervals during the day (Gould & Gould, 1995). Bees can be easily

trained to visit a food site for instance in the south at one time and another food station in the west at a different time (Menzel, 1990). They have also been trained, over a long time period, to land on one particular artificial flower petal at one time of day and another at a separate time (Gould, 1988).

Von Frisch (1967) reports that cues and landmarks are linked to time, as bees learn to fly toward a set feeding place at a certain time of day and to expect a reward at a set array of cues (Bogdany, 1978; Gould, 1987a; Koltermann, 1971). Menzel (1990) argues that these experiments do not imply that this conditioning is a time linked effect, as this behaviour only occurs after differential conditioning over an extended period. In a study by Bogdany (1978) bees were trained to a compound of colour, shape and odour at time linked intervals. However, when the bees were then tested on a changed compound, they showed a preference for the odour, overshadowing the two other factors. Indeed, Muhlen (1987) demonstrated in a series of experiments that there is a heirachy of salient cues, with odour being highest, followed by colours and patterns, and finally by surface structures.

1.9 Homing

In addition to learning specific time periods, the most important cue to learn in a honeybees lifetime is the hive location, and honeybees are classed as central place foragers (Menzel & Muller, 1996), in that they continually return to one place, the hive. They navigate this complex learning task via a reference system based on celestial compass information, such as the sun and polarised light, as well as route specific landmarks (Collett, 1993; Wehner, 1992). Wehner (1984) whilst studying the desert ant *Cataglyphis fortis*, suggested that this social insect modifies its innate template of solar movements by continuous monitoring of the suns position whilst out of the nest. This is

the same method employed by honeybees, as they too need to adjust their innate template of solar movements, as the sun constantly moves throughout the days and months of the year. They do this by modifications during learning processes, whilst out of the hive (Dyer & Dickinson, 1994). The flight path back to the hive can be made by taking a compass point from the suns' position, and along with the flight distance, estimate the direction and range back to the hive (Wehner & Wehner, 1990), added to this are landmark memories (Von Frisch, 1967; Dyer & Gould, 1981; Von Frisch & Lindauer, 1954). These hypotheses have since been supported by Cartwright and Collett (1983, 1987) using flight path observations and model calculations.

There exists contentious literature as to whether the honeybee utilises and possesses a cognitive map of its locale, with Gallistel (1990) and Gould (1984, 1986b, 1995) suggesting that honeybees do, and Menzel and Muller (1996), Wehner (1992) and Wehner and Menzel (1990) maintaining they do not. The central issue appears that honeybees, when trained to two separate points, one in the morning and one in the afternoon, when then tested midway between the two times, the two paths are integrated as suggested by Menzel et al (1995a, b), and not as a map-like memory (Gallistel, 1990; Gould, 1986b). Menzel and Muller (1996) suggest that bees when presented with this problem refer to different memories in a hierarchical order. Gould (1984) carried out another experiment, whereby bees were trained to a feedstation set up on a rowing boat in a lake. He also set up a similar station on the shoreline, and found that although bees were seen to dance the lake feedstation location, no bees actually attended that station, whereas, the shoreline station was frequently visited. Gould hypothesised that the bees are referring to their internal map of the foraging area, and so when they do this, the map say 'water - danger', and they therefore do not visit. Another reason could be that the honeybee is transferring the odour of the lake back to the hive, and this is what is being ignored by the recruited bees. What remains in all this contentious literature is

that there still remains a great deal to understand and interpret as to how bees 'map' and travel through their environment.

1.10 Honeybee navigation

As the sun is the method by which honeybees primarily navigate, a series of experiments has been reported to show this. In the first, (Von Frisch, 1950) foragers were trained to a feedstation, with few landmarks, and then the hive was moved overnight to a new location. The next morning, the hive entrance was opened, and the foragers were noted as to where they visited, as there were feedstations set out on the four main compass points. Von Frisch showed that trained honeybees foraged in the trained direction, as they had in the old site. A further method involved actually moving foragers whilst they were visiting a feedstation, with the direction of the departing forager then noted, and in these experiments, Brines (1978) observed that the departing bee flew in the direction the individual came from, rather than the direction of the hive. A further technique also involves moving the hive, but in a more ambitious way. The foragers are trained as before to a feedstation, and the hive is moved overnight, but over greater distances, such as across an ocean, or a continent, or in an eloquent study, across the equator, after having been trained in Sri Lanka, and then moved to India (Lindauer, 1957). As before, when the hive was reopened the next morning, feedstations are set out in the compass directions, but the sun was in a very different part of the sky. Lindauer noted that the foragers when released flew in the direction they had originally been trained to, even though the sun was in a different place in the sky.

When the sun is covered, and the sky overcast and therefore, polarised light is too diffuse, landmarks themselves come more into play. In a series of experiments reported by von Frisch and Lindauer (1954), foragers were trained to visit a feedstation placed

along a shoreline of a lake (west to east). Then, the hive was relocated to a different shoreline (north to south), and the vast majority of bees visited the feedstation positioned along the shoreline, rather than the correct compass direction, showing that when one method of navigation has been removed, the honeybee possesses a backup system.

1.11 Associative and non-associative learning.

Psychologists have divided types of learning into two categories, nonassociative and associative learning. Nonassociative learning is where a repeated presentation of a stimulus leads to an alteration of the intensity of response. This form of learning is considered to be one of the simplest forms of learning as the subject does not learn anything new, but simply the innate response to a situation is modified. The two most widely researched areas of nonassociative learning are habituation and sensitisation, both of which are examined in the next chapter. This thesis however, is more focused on the second form of learning, associative, and classical conditioning in particular. Associative learning consists of a behaviour modification, with the association of two or more events. In this form of learning the animal learns to do something more advantageous or different. Nonassociative learning may also be the building blocks for the types of more complex behaviours shown in associative learning (Groves & Thompson, 1970; Hawkins & Kandel, 1984; Razran, 1971). This learning also covers different forms of learning, such as classical, instrumental, and operant procedures, where responses are associated with stimuli, consequences and other responses.

1.12 Why use the classical conditioning proboscis extension reflex ?

As previously stated, this thesis is primarily concerned with classical conditioning, whereby an originally neutral stimulus, the conditioned stimulus (CS), is paired with a second stimulus that is known to produce a response, the unconditioned stimulus (US). This US elicits an unconditioned response (UR). For example in the honeybee, the US is usually a sucrose solution, and the UR is proboscis extension, for the bee to feed. There are two forms of classical conditioning, appetitive, and aversive. As the name suggests, the appetitive classical conditioning is where a food reward is the US, whereas aversive classical conditioning is where a brief event such as electric shock follows the CS. As reported earlier, this thesis only uses appetitive classical conditioning.

The main reason classical conditioning is used as a comparative tool is due to the ease of methodology with which to study either freely moving, restrained, semi-intact invertebrates, or even isolated portions of the nervous system. The majority of the research into classical conditioning concentrates on its effects on the nervous system and the changes that occur within it.

How this classical conditioning relates to the actual daily routines of an invertebrate's life is perhaps best illustrated by the honeybee, as they must learn landmarks, orientations and food sources, reviewed in the previous sections. To operate in a constantly changing environment an animal must learn new behaviours, as well as recalling reflexive responses in the new context. Many insects extend their proboscis reflexively when their receptors (antennae, mouthparts or tarsae) come into contact with a sucrose solution, and it is the same for honeybees. Minnich (1932) and von Frisch (1919) first found that honeybees can be conditioned to visual and olfactory cues with paired sucrose presentations, this was later developed by Frings (1944) and also Kuwabra (1957) and Takeda (1961).

Another reason why this classical conditioning is important is that it may unlock the answer to the cellular mechanisms of learning, this linked with the easy access to the invertebrate nervous system has a potent attractiveness to those researching neuroscience. This technique was first developed by Frings (1944) to determine thresholds in blow flies and now this proboscis extension technique has become the most popular method for classical conditioning in the honeybee (Menzel & Muller, 1996; Couvillon & Bitterman, 1980; Erber, 1981; Gerber & Smith, 1998; Pelz, Gerber & Menzel, 1997; Smith, 1991; Bhagavan, Benatar, Cobey & Smith, 1994; Bitterman, Menzel, Fietz and Schafer, 1983; Menzel, 1989, 1990; Menzel & Bitterman, 1983; Gerber, Gerberjahn, Hellstern, Klein, Kowalsky, Wustenberg & Menzel, 1996; Gerber, Wustenberg, Schutz & Menzel, 1998). The procedure allows excellent control of many parameters such as inter-trial interval, CS - US intervals, stimulus intensity and stimulus duration. It is also possible to compare the performance of laboratory based bees with free flying subjects. As well as the behavioural aspects of this learning, the physiological and biochemical aspect of this learning can also be analysed. The main limitation of this technique is that of the physical condition of the subject as bees restrained for greater than 24 hours are prone to mortality. This is not surprising as the honeybee is very active and confinement to a static position must be very stressful. Linked to this is the problem of satiation to the animal with repeated sucrose feedings, thereby negating it's effect as an US. A new method for maintaining bees in the laboratory for greater periods of long term captivity and PER experiments are reported later in this thesis.

1.13 Experiments using free flying bees in the field

Prior to the laboratory based classical conditioning PER learning paradigm, the initial technique used to train bees was developed by Karl von Frisch (1914) whereby bees are trained to visit an experimental station from the hive, where they are trained to distinguish targets by colour, odour or position. The main advantages of this technique over laboratory bound experiments are versatility and convenience, as well as basic apparatus, natural situations, and because the experiments can continue for several hours or days. This is perhaps unique in the invertebrates as the honeybee does not satiate in the field, as they can return to the hive to unload before returning to the test area. However, this technique also has its limitations, as the honeybee is in control of the inter-trial interval and also stimulus control. The prevailing weather conditions may also interrupt the experiments, as bees are unable to fly in cold weather, and it is impossible to undertake physiological or biochemical investigations. This method is also operant in nature, as the honeybee must first do something of its own accord in order to gain the reward, this is in contrast to the classical, or Pavlovian, conditioning of the PER olfactory learning in the laboratory. This form of PER learning therefore enables the experimenter to gain more control over the various parameters with olfactory conditioning. The free flying bees and the restrained laboratory subjects are exposed to the same stimuli however, and the honeybee must perform the same operations in both, it is with this in mind that results gained in the laboratory can be transposed to those expected in the field.

1.14 Olfactory behaviour in the honeybee

The olfactory capabilities of the honeybee have been well documented, and are important in the bees ability to inform its nest mates about potential food stocks (Greggers & Menzel, 1993). The odour of the flower it has just visited has been shown to adhere to the waxy hairs of the bees' body (von Frisch, 1967). This is communicated, along with the direction and distance, via the dance (von Frisch, 1923). The transmitting of the odour as a marker for a particular flower has added significance, as bees cannot communicate colour, but can carry home the scent. The sensitivity of the olfactory perception is also important in the dark hive, where qualitative choices need to be made about the food stores coming in. These olfactory stimuli are closely related to proboscis extension both at the flower and within the hive (Menzel, 1985).

Historically, von Frisch (1919) showed that bees possess a sense of smell located on the antennae, this was discovered by amputation of the antennae, in combination with training to odours. The sense of smell was determined in the field by setting out a row of 24 boxes, each containing a different odour, with one containing the trained odour. The bees only flew to the box containing the training odour, even when the box was moved, and when the box was removed from the rest the bees did not enter any of the other boxes. The same author also found that not all odours were learned at the same rate. He found that patchouli, with it's camphor-like smell, and other fetid odours were difficult to condition, as well as man-made odours. Aufsess (1960) also found that bees learned insect-pollinated flower odours better than bird-pollinated flowers. This implies that the bees are more readily compliant to learning biologically significant odours than to artificial ones. These studies involved the use of free flying subjects, but it is easier to use restrained bees for reflexive conditioning, as it allows a greater control of parameters, and shows no adverse effects on the learning (Menzel & Bitterman, 1983).

1.15 Proboscis extension reflex learning

The characteristics of the proboscis extension reflex (PER) in restrained honeybees remains one of the most prolific learning paradigms in the bee literature (Bitterman et al, 1983; Menzel et al, 1974; Menzel, 1985, 1989, 1990). It is also useful for studies of the physiology of memory, as after only three rewards, associated with a particular odour, the association is retained for the lifetime of the bee. The individual characteristics of olfactory learning have also been established for PER (Menzel, 1990), for example, long term retention, generalisation of odours and learned responses, and forward and backward conditioning. These examples of invertebrate learning compare favourably with the more well established vertebrate learning (Kesner & Olton, 1990). The phenomenon of PER has long been known as a learning paradigm, Ribbands (1955) conditioned bees to water, and this was repeated by Kuwabra and Takeda (1956). Frings (1944) conditioned to cumarin, and Kuwabra (1957) conditioned to light. These experiments, along with Takeda (1961) involved restraining the bee, by either, glueing to a substrate, clipping the wings, or securing in small tubes. The effects this has on such a social insect have also been investigated (Menzel & Bitterman, 1983) and this research showed no adverse effects on the levels of learning, compared to free flying subjects. The restraining technique has an added advantage in that it allows presentations of stimuli to be more exactly controlled. Also, precise physiological manipulations and measurements during the learning are possible (Menzel et al, 1974; Erber, 1980). Given the increased attention on PER learning and the importance of olfactory learning per se, current research has focused on PER at a molecular and cellular level. This is however to the detriment of investigating how the bee learns and behaves, i.e. at the behavioural and ecological level, and it is to this end that this thesis is addressed.

Worker forager bees learn the association between pollen and nectar with visual (Menzel, 1967a, b; Gould, 1986a), spatial relations (Dyer, 1991), olfactory (von Frisch, 1967), and tactile (Kevan, 1987) stimuli offered by the flower. These factors are constantly changing, as a forager visits different flower species. However, the correlation is rapidly learnt, and thereafter, the forager can efficiently locate and harvest from that particular flower species for life. A two phase memory has been proposed for this learning (Menzel, Greggers & Hammer, 1993), whereby the choice behaviour is stored initially in a short term (working) memory, which is then replaced by a specific long term (reference) memory. This is backed by Erber (1975a, b) who showed that short term memory is quickly lost if it was not reinforced within minutes. He proposed that the loss while in the short term memory prevents the bee from entering information about an uneconomical flower into its long term memory. Erber (1976) further split the memory into four distinct phases. The first (sensory storage) lasts only a few seconds. The second is sensitive to the disruption of neural activity and lasts approximately seven minutes. The third is sensitive only to CO_2 narcosis, and lasts between three and fifteen minutes. The fourth phase is the long term memory, it begins at fifteen minutes and is resistant to any narcosis. Menzel (1979) also concluded that the short term memory might be a kind of information storage area, allowing further refinement of incoming information, to be stored in the long term memory. Earlier, Menzel and Erber (1978) and later repeated by Erber, Masuhr and Menzel (1980) and Erber (1981), they removed an area of the headcase, to expose the brain, and by cooling areas after olfactory conditioning, located the pathway of short term to long term memory, identified the neurones that were altered during learning, showing the susceptibility to impairment of learning after conditioning. He cooled areas of the brain using fine metal probes. The learning lasted approximately three minutes in the antennal lobes, seven minutes in the alpha lobes, and after ten minutes in the calyx. After this period it was impossible to

disrupt the learning, as it had entered a long term memory store. They showed by this process that consolidation was a moveable entity as the time after conditioning increased. Initially only the olfactory lobe, attached to the antenna utilised during the training was affected, then this spread to the alpha lobe of the mushroom body, and finally into the calyces of the mushroom body. It is here that the long term memory is formed and cannot be disrupted (see figure 1.7 for a diagram of this process).

CHAPTER 2:

2.0 Methods and materials

One of the central tenets to this thesis is the method by which the bees will be conditioned and tested. As olfaction is the most important sense to the honeybee, being the method by which it negotiates the learning and communication of potential food sources, this was the sense we exploited, utilising the proboscis extension reflex (PER) learning paradigm. In this thesis we also attempted to examine other learning paradigms, such as conditioning to a flashing light or a rotating pattern, both these proved unsuccessful, mainly due to the number of trials that were required for satisfactory levels of response. Menzel (1985) reported a 90 trial experiment to train to a flashing light, only obtaining responses of 40 %. Kuwabra (1957) and Masuhr and Menzel (1972) also reported that PER conditioning to visual stimuli is only successful in about 30 % of bees and is very slow, so we considered this to be too extreme, and so decided to remain with olfactory conditioning. This conditioning tool is widely used throughout honeybee olfactory learning research, with its relative ease of use, replication, and speed of completion, yet the actual components of this procedure are not universally standard, with inter and intra laboratory variation (Gerber & Smith, 1998; Pelz et al, 1997; Smith, 1991; Bhagavan et al, 1994; Bitterman et al, 1983; Menzel, 1989, 1990; Menzel & Bitterman, 1983; Gerber et al, 1996, 1998), which may cause confusion, and even promote misleading results. It was consequently decided that each constituent of the method should be broken down to assess the efficacy of each part. The initial methods of capturing, storing and training of the honeybees were adapted from those developed by Bitterman et al (1983), and Menzel (1990). Both these

well used methods acted as a starting point, as they were heavily referenced, and appeared to give reliable and reproducible results.

The several populations of honeybees used throughout this thesis were taken from various hives situated around Oxford Brookes University, Wolverhampton University, Henley-on-Thames, Worcester and Chipping Norton. All these colonies were fully stocked, having between 40,000 to 80,000 bees housed in Modified National hives (Hooper, 1991) and were of good breeding, being the more placid tempered Italian strain, *Apis mellifera ligustica*. However, in some of the chapters, honeybees were taken from mini nucleus hives, where populations of 5000 or less were maintained, and wherever these are used, it is stated within the text.

The individual components of the procedure are detailed in the following sections;

2.1 Methods of capturing honeybees

The majority of reported research fails to fully specify the actual method used to capture the honeybees, with basic sentences such as 'the subjects were caught from the hive entrance' (Erber et al, 1980; Menzel & Bitterman, 1983; Bitterman et al, 1983; Buckbee & Abramson, 1997; Gerber et al, 1998) being stated, which gives no actual indication of the capture method. At the outset of these experiments several methods of collecting were compared to obtain the best method.

2.1.1 Queen catcher method

It was this vague background that generated our initial method of capture, which was to observe the hive entrance, allowing the bees to exhibit their natural behaviour, entering and leaving the hive. After a certain time, when the bees had become accustomed to our

presence, and were no longer inquisitive about us, a spring levered queen catcher was swept from left to right across the entrance of the hive, grasping as many subjects as possible, which was usually about twenty. The major drawback to this method was that the bees caught could not be individually identified to be of any one specific caste, and would more often than not contain a mixture of guards and foragers. Another negative aspect of this method was that the sweeping action across the hive entrance caused an alarm response, with guard bees sensing this as an attack (Hooper, 1991). This disruption of the hive caused a mass exodus of the bees from the hive to defend and attack the intruder. It was decided quite quickly to cease this method !! and to find a less disturbing and more accurate capture technique.

2.1.2 Forceps method

This next method of sampling was that individual bees were tracked and captured using a pair of forceps. Once a returning forager was identified, by having a pollen load on its corbiculae, the forceps are opened and the bee was grasped by the hind legs. This method was by far the most accurate, as it allowed the sampler to individually select which bees were chosen for the experiment. For instance, only guards bees could be caught, by observing their behaviour with incoming bees, and just sampling these bees from the hive entrance.

2.1.3 Vial method.

Another less disruptive method used (Abramson, 1990) was to place a 25 cm^3 vial onto the flight board, to allow the bees to enter the tube of their own will (see photographs 1, 2 and 3). The main drawback to this method was that it was very time consuming, and

Method of capturing honeybees using the vial method

Photo 1: Vial placed on the flightboard of the hive
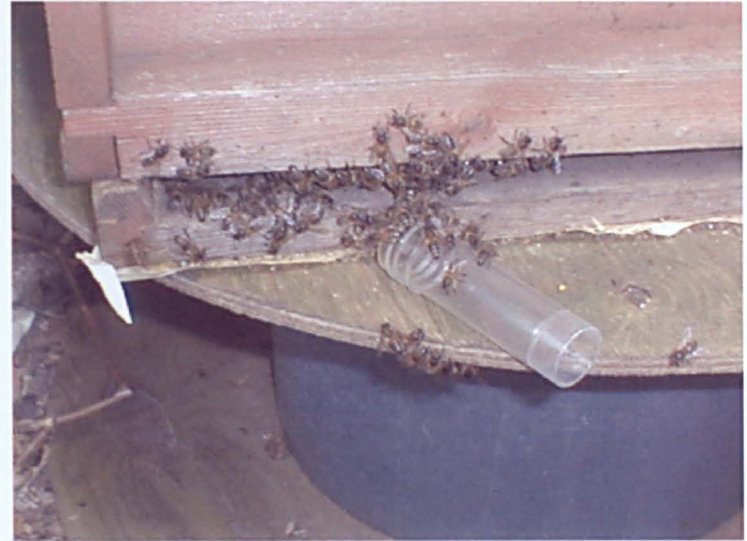
Photo 2: Honeybees entering the open vial

Photo 3: Captured honeybees in the vials

although the vials could be manually placed over foragers, guard bees were more often caught, as they are naturally inquisitive to any foreign body at the hive entrance. It was therefore common practice, in my experiments, unless otherwise stated in each chapter, to use the forceps method, which allowed the most accuracy in identifying the bees tested.

2.2 Methods of storing and holding caught honeybees

2.2.1 Glass jar

After sampling the bees with the queen catcher method (see section 2.1.1), the caught bees were placed in a 500 cm³ glass jar, allowing the bees free movement. There was a major drawback to this method however, as a humid atmosphere was soon generated due to the bees respiring. This condensation caused some bees to become wet, these remaining at the bottom of the jar. It was also observed that these bees also failed to recover from the anaesthetic when returned to the laboratory, perhaps due to the spiracles of the bees becoming blocked. The number of bees caught at any one time in this glass jar method ranged from forty to one hundred.

2.2.2 Vials

In an effort to reduce this build up of condensation, and taking into account the protocols of Bhagavan et al (1994) who stored their captured bees in 15 cm³ vials, and Buckbee and Abramson (1997) who stored their bees in 'loosely sealed glass vials', we decided to develop a method using freely available 25 cm³ plastic vials. The amount of bees stored at any one time was greatly reduced using these vials, with a maximum of

eight bees in each vial (see photograph 3). This eliminated the condensation forming, whilst also allowing the bees room to manoeuvre. Both these methods also had important implications for the following component of the capture and conditioning methods.

2.3 Methods of anaesthesia

The majority of papers using the proboscis extension reflex in the laboratory anaesthetise the subjects using various methods of hypothermia, such as placing the bees in vials, then storing them in an ice-water bath until the subjects became inactive (Bhagavan et al, 1994; Buckbee & Abramson, 1997), placing in a refrigerator for 6 - 8 minutes (Erber, Gray & Lorenzen, 1980), or simply cooling until the bees became inactive (Menzel & Bitterman, 1983; Bitterman et al, 1983; Gerber et al, 1998). Other techniques such as CO_2 narcosis and other anaesthetics have been implicated in affecting subsequent learning experiments (Ebadi et al, 1980; Erber, 1976). So therefore, in our research, the subjects were placed in a 4 °C fridge as this seemed the easiest and least expensive method (see photograph 4).

2.3.1 The glass jar method of anaesthesia

Whilst using the 500 cm^3 glass jar, the large volume of air that needed to be chilled was too great a volume for the 4 °C refrigerator, where it required a total of one hour, followed by thirty minutes in the freezer compartment. Even this quite extensive method did not totally disable the bees, as they only became sluggish, so the jar was returned to the 4 °C compartment for a further ten minutes. This method had a seriously adverse effect on the conditioning of the bees when they recovered from the anaesthetic. The

Methods used to anaesthetise and restrain the honeybees

Photo 4: Vials containing the honeybees placed in a 4 °C fridge

Photo 5: Tubes, masking tape and forceps used to restrain the bees

Photo 6: Anaesthetised honeybees, about to be restrained

Photo 7: High sided holding tray used to store the honeybees

freezing process used with the glass jar caused some bees to die, presumably due to their haemolymph freezing, as these subjects may have been directly on the wall of the glass jar, and so again we quickly ceased to use this first method.

2.3.2 The vial method of anaesthesia

The next protocol was to place the 25 cm^3 vials containing the captured subjects in the 4 °C refrigerator for ten minutes. This length of time was the optimum time for all the bees in each vial to become totally immobile, and did not appear to affect the bees ability to recover, and subsequent conditioning trials. Whilst under the anaesthetic in this second method, a very small percentage of the bees did not recover (less than 1 %), and it could be suggested that these bees had died due to natural senescence, this could especially be true if those honeybees tested were mature foragers.

2.3.3 Chilling

The method of chilling to 4 °C was similar to the methods used by Erber et al (1980), Menzel and Bitterman (1983) and Bitterman et al (1983), but quite contrary to the method of Abramson (1990) where the bees were placed in an ice bucket for a few minutes, until slightly anaesthetised, and then manipulated. To examine if these methods altered the learning characteristics, an experiment was undertaken to compare the levels of learning after differing chilling techniques. The results showed that cooling to 4 °C for ten minutes showed no significant difference from the levels shown when the bees are chilled on ice for a short period. It was therefore decided to keep this method as the standard in my experiments, and so bees were anaesthetised at 4 °C for ten minutes in every experiment.

2.4 Methods of restraining honeybees

To enable olfactory conditioning in the laboratory, the subjects have to be immobilised in some way to allow the odour delivery to be presented accurately and consistently. It is therefore necessary to restrain the honeybees, and we decided to use a restraining method adapted from experiments carried out by Bitterman et al (1983). This restraining technique used small brass tubes into which the bees are secured with tape, but due to the high expense in the brass tubes, our research customised Eppendorf tubes, where the bottom of the tubes were cut off with scissors. These tubes had a height of 2 cm, and a diameter of 1 cm (see photographs 5 and 6).

The anaesthetised bees were held with a pair of forceps and passed head first through the tube until their head and thorax emerged from the top of the tube. A thin piece of masking tape was then placed across the top of the tube, securing the bee lightly between the thorax and abdomen. This method prevented the subject from escaping, but also allowed it free movement of its head, antennae and thorax. This was in contrast to previous reported research (Bhagavan et al, 1994; Menzel & Bitterman, 1983; Bitterman et al, 1983; Gerber et al, 1998), who only allowed the head and mouthparts to protrude from the top of the tubes, however Erber et al (1980) also allowed the thorax to protrude. Our research allowed the front pairs of legs to extend so the bee can perform antennal cleaning, as Buckbee and Abramson (1997) have suggested that a build up of sucrose on the antennae affects the subsequent conditioning, and allowing the bees this extra freedom appears to have no other adverse effects on the learning.

At the start of our experiments when the bees had been removed from the large jar, and were dying (due to the condensation), we were concerned that the air was not circulating the abdomen of the bee, so the back of the Eppendorf tubes were cut away to expose the

dorsal side of the bee. However, this method was not continued as it posed too great a risk to the experimenter of being stung from the exposed abdomen. Reverting back to closed tubes did not however increase the mortality of the bees and once the method using the large storage jar had stopped, then the rate of mortality was vastly reduced to negligible levels. Once each subject had been restrained, they were placed into a high sided holding tray, ready for conditioning (see photograph 7). This tray had Blu-tac on the floor, and each space was serially numbered, to facilitate monitoring and following individual bees responses and performances.

2.5 Recovery interval prior to training

Many researchers allow some time for the bees to recover from the anaesthetic, so, in this research, an arbitrary time of two hours was decided upon. Menzel (1990), Bhagavan et al (1994), Menzel and Bitterman (1983), Buckbee and Abramson (1997), Gerber et al (1998) and Bitterman et al (1983) all fed their experimental bees to satiation, prior to storing them overnight in a cool dark place before testing, to allow habituation to the test apparatus. However, in our experiments, it was found that increasing the time between restraining and testing was detrimental to the health and condition of the bees, causing greater mortality.

In other experiments reported later in this thesis, where bees were removed from their tubes after training, it was not possible to follow individual bees, so each bee was serially numbered with a small plastic disc, glued onto the thorax.

2.6 The concentration of the sucrose reward

The concentration of the food reward which is the unconditioned stimulus is an important factor in associative conditioning, as too low a concentration leads to a failure in the association as it will not be reinforced. This is due to the energy needed to provoke a response being less than that obtained by the reward. However, too high a concentration of sucrose also has a negative effect on the conditioning, as the viscosity of the liquid is too great for the bees to feed. In chapter 5.0 the effects of motivation and sucrose concentration are examined, which also reflects the experiments examining the effects of sucrose concentration on the levels of learning reported by Couvillon and Bitterman (1980) and Loo and Bitterman (1992). The majority of previous research into olfactory conditioning using sucrose solutions as the appetitive reinforcer use either 1.25 M (Gerber et al, 1998), 1.5 M (Bhagavan et al, 1994), 2.0 M (Gerber et al, 1996), 50 % (Couvillon & Bitterman, 1984; Abramson & Bitterman, 1986; Buckbee & Abramson, 1997), 40 % (Menzel & Bitterman, 1983; Bitterman et al, 1983) or a 30 % (Couvillon & Bitterman, 1984) solution. It was therefore decided in this thesis to employ a 50 % sucrose solution which is also equivalent to 1.46 M solution, and this concentration is the level that correlated the closest with the majority of other research, thereby enabling us to positively compare our results.

2.7 The concentration of the odour

Although many researchers fail to indicate the odour concentrations in their methods, it was decided to have a concentration just perceptible by the experimenters, as this would be sensed greater to the more highly sensitive olfactory system of the honeybee. In a study by Pelz, Gerber and Menzel (1997), the effects of training to two different

concentrations of one odourant found that the honeybees were unable to discriminate between them. However, they did find that stronger concentrations did form stronger associations than low concentrations, but bees cannot treat two different concentrations of one odourant as qualitatively different stimuli. This may be due to the honeybees not needing to discriminate between the same odour in nature, as the odour of the flower they are foraging is the odour of the flower, and this will naturally fluctuate depending on the bees' vicinity to the site of pollen production, and hence odour. In another study by Bhagavan and Smith (1997), they reported that honeybees are able to discriminate between the same odour concentrations, but only from a high concentration to a low one, but not from a low to a high concentration.

The scents used in olfactory learning experiments differ wildly, with odours as diverse as there are researchers, with odours such as hexanol and 1-hexanol (Bhagavan et al, 1994), proprionic acid (Gerber et al, 1998), jasmine (Couvillon & Bitterman, 1984), rosemary (Erber et al, 1980), cumarin (Frings, 1944), cinnamon (Buckbee & Abramson, 1997), citral (Takeda, 1961) and carnation (Gerber et al, 1996; Menzel & Bitterman, 1983; Bitterman et al, 1983). In this research, we chose the odour geraniol, as this has been used by other researchers (Menzel & Bitterman, 1983; Bitterman et al, 1983), and was an odour readily accessible. This odour has also been characterised as a component of the honeybee Nasonov pheromone (Pickett, Williams, Martin & Smith, 1980), which is a recruitment pheromone.

The method of odour delivery has also generated differing approaches, Abramson (1990) and Gerber et al (1996) in their research, used actual flower petals in a syringe to act as the scent that was delivered to the bee, but the usual method of odour delivery is to place a few drops of the odour liquid onto a strip of filter paper within a syringe, which is then depressed a stated volume, directed at the honeybee. However, in our research, we developed a different method of odour delivery, whereby the odour

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concentration used was 6 drops of the geraniol oil into 100 cm³ distilled water. The odoured water was contained within a 150 cm³ conical flask, stoppered with a rubber bung, containing two holes. Through one of the holes ran a glass tube into the solution, the other hole also had a glass tube running through it, but this ended above the surface of the solution (see photograph 13). The method by which this apparatus was used was to exhale steadily, and blow into the tube that went into the solution, which forced the displaced, geraniol scented air, out of the other tube and in the direction of the bee. The concentration of geraniol (6 drops in 100 cm³ distilled water), was kept constant throughout all experiments and the conical flask was prepared with a fresh supply every two weeks. In some of the later experiments a different odour was required, so we chose a peppermint scent, which was also prepared to the same concentration of six drops in 100 cm³ distilled water.

2.8 Inter-trial interval between trials

The inter-trial interval (ITI) is the time that elapses between one trial and the following trial. This can take the form of a continuous sequence (massed training) or through defined time sequences, such as repeating every five minutes (spaced training). In chapter 3.0, the comparison between massed and spaced training is investigated, but for the majority of the experiments in this thesis, spaced trials, with an ITI of at least 10 minutes was the norm. Researchers have varied in these inter trial intervals, using times such as 5 minutes (Menzel & Bitterman, 1983), 6 minutes (Bhagavan et al, 1994), 8 minutes (Gerber et al, 1996), 10 minutes (Hammer & Menzel, 1995; Bitterman et al, 1983; Buckbee & Abramson, 1997), 17 minutes (Gerber et al, 1996) and 20 minutes (Gerber et al, 1998). The ITI in the experiments reported remained at a minimum of 10 minutes, as Erber et al (1980), Menzel (1979), and Smith (1991) have observed that

short term memory (STM) is not consolidated into long term memory (LTM) before seven minutes. With this memory consolidation in olfactory learning it is known that response levels show a two phase time course, with a reduction in learning at 2 - 3 minutes after training (Menzel, 1990). Proboscis extension in the time preceding this 2 - 3 minutes seems due largely to sensitisation, whereas in the longer term, associative memories are formed (Hammer & Menzel, 1995; Menzel, 1990). So we decided to err on the cautious side, and chose 10 minutes to be the minimum ITI, and although this was the most frequent ITI, many of the experiments had differing ITI's, and hence, in each experiment, it is indicated to allow for any future comparison.

2.9 Bee boxes used to hold captive honeybees

The restraining technique (section 2.4) was only suitable for experiments which are for a maximum of 24 hours, as the immobile bees cannot manoeuvre themselves, and this slightly artificial restraining, affects the survivorship of the subjects. So, a further method needed to be developed, whereby subjects could be conditioned, and then kept captive for longer periods, for testing in long term retention studies. So, a 'bee box' was designed that allowed the bees to be stored, and then once again be restrained for later retention tests. These boxes were made of perspex, dimensions 7.5 cm by 7.5 cm by 9.5 cm (see photographs 8, 9, 10 and 11), having removable sides and top. In each of the sides were 45 air holes, with the boxes having two sliding trays, which made it possible to feed the bees, with a previously prepared sugar candy.

To ascertain if these boxes affected the honeybees in any negative way, we performed a series of experiments to discover the best technique for storing these boxes, and also if the length of time the bees were kept captive affected the honeybees ability to return back to their hive upon release.

Bee boxes used to hold honeybees for long term captivity experiments
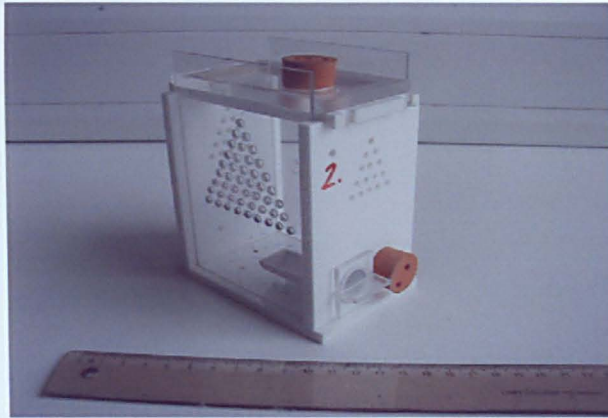
Photo 8: The dimensions of the bee box
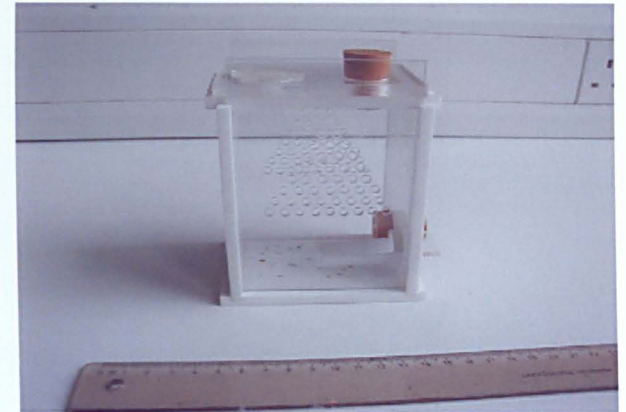
Photo 9: The dimensions of the bee box
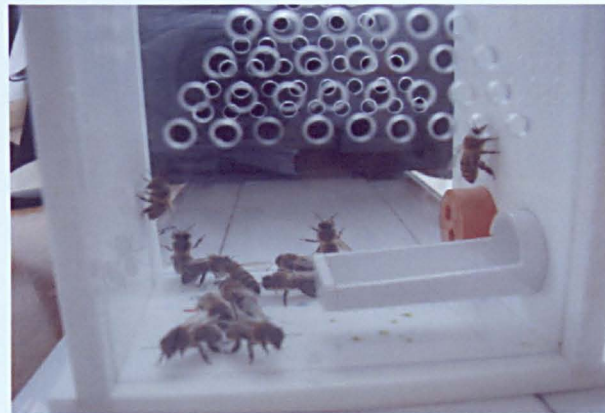
Photo 10: Honeybees in the bee box
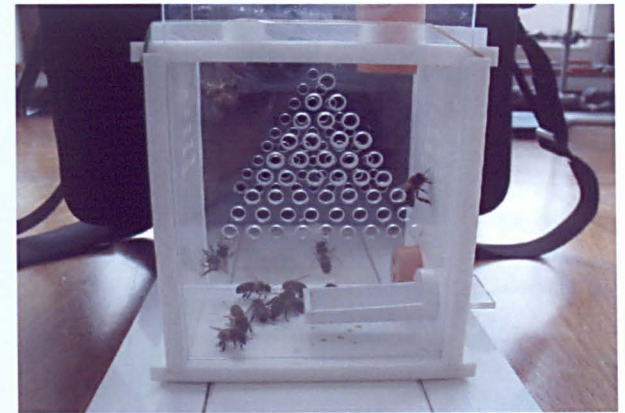
Photo 11: Honeybees in the bee box

2.9.1 Testing the bee boxes for survival of honeybees in the laboratory

In a preliminary experiment the effects of cooling, restraining, testing, then cooling once again and captivity affected the survivorship of the subjects.

2.9.2 Method

The honeybees were caught and restrained as described in section 2.11, with the initial experiment consisting of three paired learning trials, with a constant inter trial interval, after which the bees are given a 2 hour consolidation period to allow the learning to be consolidated into the long term memory (Ebadi et al, 1980; Erber et al, 1980). These bees were then re-chilled to 4 °C for 10 minutes, unstrapped, and placed in groups of 20 to 30 in the bee boxes. In each of these boxes was a tray of bee candy, which was changed daily, these boxes were then placed in a dark ventilated area. The bees were kept captive in these boxes for either 2, 4, 7, or 9 days. After this captivity period the bees were removed from the boxes into 25 cm^3 vials, and anaesthetised at 4 °C as before. The bees were again restrained in the tubes as previously described, ready for testing.

2.9.3 Results

We found that bees given a second procedure of anaesthetic, restraining, and testing, showed no difference in the retest than bees just given one procedure.

2.9.4 Determining the best method to store the bee boxes

In a second experiment we captured 37 bees, anaesthetised them, and placed them in bee boxes, storing 20 bees in the light, and 17 bees in the dark. The results showed that 47 % of the light kept bees died, compared to no mortalities for the bees stored in the dark. This was most probably due to stress on the bee, in that they are not used to remaining in the light all the time, as the dark hive environment is a more natural atmosphere for the bees, so it was therefore decided to always keep the bees stored in the dark for any great period of time. This remained standard in all future experiments using bee boxes that are described in this thesis.

2.9.5 Determining captivity effects on homing abilities

In another experiment the effects of captivity on homing behaviour was studied, this later went on to form a chapter in itself (chapter 6.0), however, a preliminary experiment is described below.

2.9.6 Methods

In all experiments, bees were caught from the entrance of the hive and placed in groups of either 5 or 6 in glass vials, which were then transported to the laboratory which took a maximum of 15 minutes. The vials were placed in a refrigerator at 4 °C for 10 minutes, which anaesthetised the bees. After the 10 minutes the bees were transferred to clear perspex boxes (7.5 cm by 7.5 cm by 9.5 cm) in groups of 25 or less. Upon release, one experimenter stayed with the box to ensure the bees all flew and also to note the general direction in which each of the bees departed. The second experimenter waited at the

hive entrance to count the returning bees, which were identified by having a coloured non-toxic paint spot on their thorax. This second experimenter stayed in position for a maximum of 60 minutes, or until 100 % of the bees had returned to the hive. The boxes were kept in the dark, and only brought out into the light when the supplies of bee candy were changed (daily), or the bees were to be released.

2.9.7 Results

On all these preliminary experiments with the homing behaviour, and the effects the bee boxes and captivity played on this, 90 - 100 % of the bees returned to the hive.

2.9.8 Conclusions

There appeared to be no adverse affects of storing bees in these boxes, both from a natural behaviour, or from a survivorship perspective. These boxes also did not seem to affect the learning processes of the bees, with long term retention of the olfactory learning paradigm.

2.10 Controls to the experiments and comparison of the hives used throughout this thesis

It was important in these studies to be able to generate controls to the experiments, to indicate that the effects shown by the bees were due to learning, or natural behaviours, rather than just being a coincidence. It was also important to show that the levels of learning from the different hives we used were of an equal standard, as Brandes et al (1988) reported that honeybees could be selected for 'good and poor learners'.

2.10.1 Method

At any one time, up to seven hives were used for the experiments, so it was therefore decided to look at the levels of learning abilities to see if there was any difference between the colonies. A study of the most used hives, based at Oxford Brookes University was undertaken.

2.10.2 Results

There were four hives in total, and there was a difference, which tallies with Brandes et al (1988) who found that you could select for genetic variability in learning in honeybees. We consequently only used the hive which showed the highest levels of learning (hive 2).

2.10.3 Homing abilities of honeybees released outside or within their foraging range

In a further experiment whilst using the bee boxes (section 2.9), we needed to know if the honeybees just returned to the nearest hive, or they showed a specificity to one particular hive, their own.

2.10.4 Method

Two groups were used throughout the experiments as controls, the first were bees captured from the hives at Oxford Brookes University, anaesthetised, then released 10 metres from the hive, the same day. These were compared to honeybees captured and

treated identically to the Oxford Brookes University bees, but these bees were from hives situated 35 kilometres from the Oxford hives, in hives from Chipping Norton, Oxfordshire.

2.10.5 Results

All of the bees released from the Oxford Brookes hives reached and entered the hives they had been taken from. This was in stark contrast to the bees captured from the Chipping Norton hives, where not one bee entered the available hives. This result thereby eliminated any possible cues that may be invisible to human perception of the location of the hives, either visual or olfactory.

2.11 The finalised method

On each test day, a random sample of bees were taken from the entrance of hives, the bees were collected individually with a pair of forceps, which ensured only specific, identifiable subjects were sampled. The captured bees were placed in glass vials of volume 25 cm^3, with between five and eight bees per tube. The bees were taken back to the laboratory where they were anaesthetised by chilling the tubes to 4 °C in a refrigerator for ten minutes (Ribbands, 1950; Erber, 1976; Robinson, 1984). Bees were removed from the tubes whilst still anaesthetised, and secured into plastic tubes 2 cm high, with 1 cm diameter, by pinching the bee between its thorax and abdomen with masking tape, and then adhering the ends of the tape to the top of the tube. This allowed the bee free head movement, with proboscis, antennae, and front pair of forelegs outside of the tube. Such restraint of the subjects has the advantage of allowing better control of training variables, than is possible with free flying bees (Menzel, 1989).

The overall sample of bees varied, but was always between twenty-five to seventy bees, and were given 60 - 120 minutes to recover from the anaesthetic, in a well ventilated laboratory (Takeda, 1961), at room temperature (20 - 25 °C), although Batson, Hoban and Bitterman (1992) gave the bees only fifteen minutes to recover, and found no ill effects or loss in learning.

The criteria for a bee to be included in the experiment was;

i) It survived the anaesthetic and recovery time.

ii) It did not give a proboscis extension reflex to the conditioned stimulus alone.

iii) It took the sucrose solution readily.

Each bee was given three learning trials, with at least a ten minute interval between each trial to allow for consolidation of the information (Bitterman et al, 1983). This method of training has been shown to have no adverse effects on the learning performance, as the results gained are closely matched by results gained from free flying subjects (Menzel & Bitterman, 1983).

At the trial, each bee was moved from its troop to a different area of the laboratory to prevent odour detection by the other subjects (see photograph 12 and 13). The bee was then given an odour or conditioned stimulus for six seconds, the last second of which was accompanied by a 50 % sucrose solution reward, given by a syringe (5 cm^3 barrel, 19 gauge needle) to the antennae to elicit a proboscis extension (following Bitterman et al, 1983) then to the proboscis for feeding. This was continued for a further six seconds after the odour had ceased.

The odour was applied by blowing air into a 100 cm^3 conical flask containing 60 cm^3 distilled water with six drops of geranium oil (an aromatherapy product). The increased gas volume was then expelled via another tube at the top of the flask. The tubing delivering the odour was positioned 2 cm from the bee, and aimed at the base of the tube holding the bee, so as not to blow directly onto the bee and therefore act as a mechanical

Photo 12: Restrained honeybee about to be conditioned
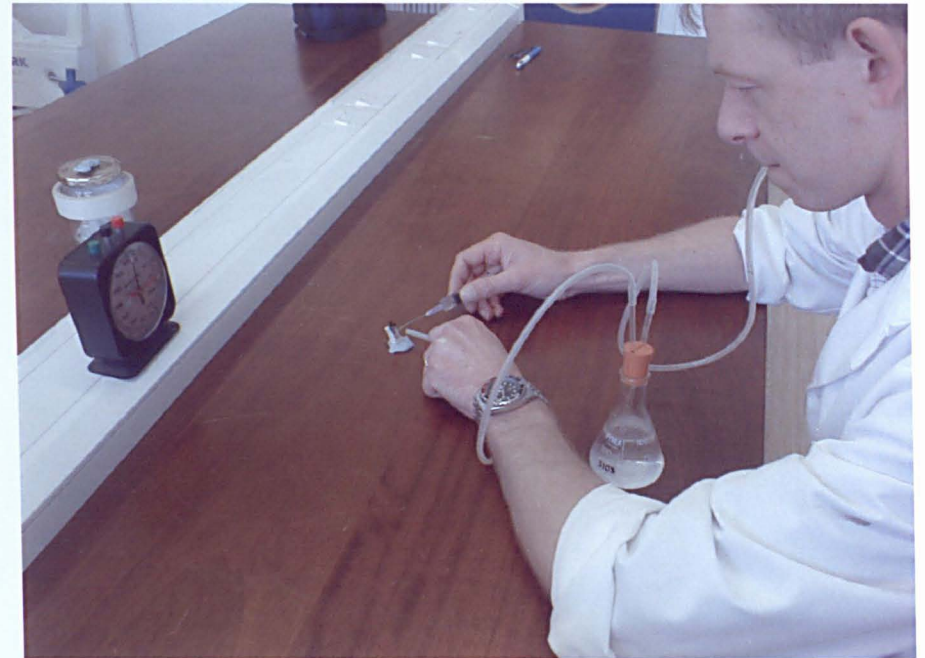
Photo 13: Olfactory conditioning apparatus

as opposed to a chemical stimulus. Each bee was given its stimulus in turn and the time was noted as the inter trial interval, which was kept constant throughout the three trials. The probability of PER, was calculated using the following equation (Menzel & Bitterman, 1983):

Probability = $\underline{\text{Number of bees in trial showing response}}$

Number of bees in the trial

Figure 2.11: Flow diagram of the standardised PER training protocol for our research group (Ray & Ferneyhough, 1997a, b, 1999; Ferneyhough & Ray, 1999).

Capture

↓ minimum of time

Storing

↓ minimum of time

Anaesthesia

↓ 10 minutes at 4 °C

Restraining

↓ 2 hours

Scent given

↓4 seconds

Sucrose given

↓ 10 minutes

Scent given

↓ 4 seconds

Sucrose given

repeat until experiment has been completed
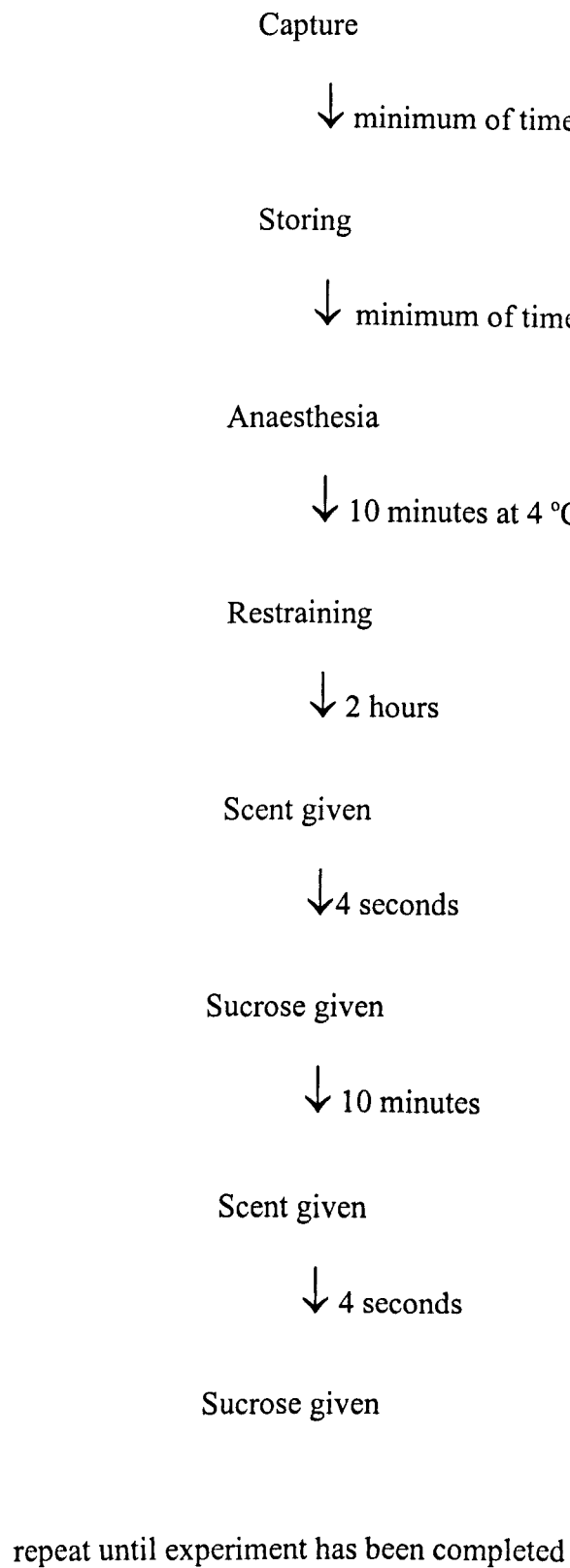
Table 2.12 Olfactory learning methods used by various laboratories investigating PER

Authors	Capture	Storing	Anaesthesia	Restraining	Time before testing	Sucrose conc.	Odour	I.T.I (mins)
Abramson & Bitterman, 1986	NR	NR	NR	NR	NR	50%	NR	NR
Bhagavan et al, 1994	NR	placed singly in 15 ml vials	ice-water bath until inactive	antennae and mouthparts free	fed, then left overnight in cool, dark place	1.5 M	Hexanol 1-Hexanol	6
Bitterman et al, 1983	from hive entrance	NR	cooled for a few minutes until inactive	head protruded	fed, then left either in morning-afternoon or afternoon-morning	20-40%	Geraniol Carnation	10
Buckbee & Abramson, 1997	from hive entrance	loosely sealed glass tubes	place on ice to reduce movement	metal tubes with head protruding	one day before testing	50%	Cinnamon	10
Couvillon & Bitterman, 1984	NR	NR	NR	NR	NR	30% & 50%	Jasmine	NR
Erber, Masuhr & Menzel, 1980	from hive entrance	NR	6-8 minutes in fridge	glass tubes, head, legs and abdomen not restrained	NR	NR	Rosemary	NR
Frings, 1944	NR	NR	NR	NR	NR	NR	Cumarin	NR
Gerber et al 1996	NR	NR	NR	NR	NR	2 M	Basswood Carnation	8 17
Gerber et al, 1998	from hive entrance	NR	cooled for a few minutes until inactive	head protruded	fed, then left in morning-afternoon or afternoon-morning	1.25 M	Propionic acid	20
Hammer & Menzel, 1995	NR	NR	NR	NR	NR	NR	NR	10

Authors	Capture	Storing	Anaesthesia	Restraining	Time before testing	Sucrose conc.	Odour	I.T.I (mins)
Menzel, 1989	NR	NR	NR	NR	NR	NR	Geraniol Carnation Orange	NR
Menzel, 1990	NR	NR	NR	NR	NR	NR	Cumarin	NR
Menzel & Bitterman, 1983	from hive entrance	NR	cooled briefly	head protruded	fed, then left overnight in cool, dark place	40%	Geraniol Carnation	5
Ray & Ferneyhough, 1997a, b & 1999	from hive entrance	placed in groups of 6 in 25 ml vials	cooled to 4C in fridge 10 mins	head and thorax protruding	left for 2 hours	50%	Geraniol	10
Takeda, 1961	NR	NR	NR	NR	NR	NR	Citral Hydroxy-citronellal	NR

NR = not reported.

CHAPTER 3:

3.0 Investigation of parametric characteristics of olfactory learning

This chapter aims to show that the results gained in our laboratory from the methods detailed in the previous chapter and adapted from Bitterman et al (1983) and Menzel (1990) give equivalent results to those of other laboratories investigating PER olfactory learning. Thus, the following characteristics of PER learning were investigated; overshadowing, blocking, pre-US exposure, random presentation of the CS and US, spaced and massed trial effects, habituation, extinction, and generalisation. Also explored was the 'one trial learning' aspects of the conditioning suggested by some authors (Gerber et al, 1996; Menzel, 1990; Erber 1975a, b, 1981; Menzel & Muller, 1996; Erber et al, 1980).

All these parametric characteristics have parallels in the vertebrate literature (Groves & Thompson, 1970; Pearce, 1997) with reviews by Bitterman (1988), Menzel (1990), Menzel, Hammer, Braun, Mauelshagen and Sugawa (1991), Menzel et al (1993), and Hammer and Menzel (1995) linking these to honeybee behaviour, and as such give an interesting and easily accessible insight into this form of learning in the honeybee, and can act as a model for the vertebrate literature (Robinson, Fahrbach & Winston, 1997). Olfactory learning in the honeybee has been demonstrated in many laboratories, using a variety of techniques (Bitterman et al, 1983; Menzel, 1990; Erber et al, 1983; Bhagavan et al, 1994; Erber et al, 1980; Buckbee & Abramson, 1997; Gerber et al, 1998; Ray & Ferneyhough, 1997a, b, 1999); different odours (Bitterman et al, 1983; Menzel, 1989, 1990; Menzel & Bitterman, 1983; Couvillon & Bitterman, 1984; Bhagavan et al, 1994; Erber et al, 1980; Buckbee & Abramson, 1997; Gerber et al, 1996; Ray & Ferneyhough, 1997a, b, 1999), differing sucrose reward concentrations (Bitterman et al, 1983;

Abramson & Bitterman, 1983, 1986; Menzel & Bitterman, 1983; Couvillon & Bitterman, 1984; Bhagavan et al, 1994; Buckbee & Abramson, 1997; Gerber et al, 1998; Ray & Ferneyhough, 1997a, b, 1999), inter trial intervals (Bitterman et al, 1983; Menzel & Bitterman, 1983; Bhagavan et al, 1994; Buckbee & Abramson, 1997; Gerber et al, 1996; Gerber et al, 1998; Hammer & Menzel, 1995; Ray & Ferneyhough, 1997a, b, 1999), and also the time the bees were stored before the experiments commenced (Bhagavan et al, 1994; Menzel & Bitterman, 1983; Bitterman et al, 1983; Buckbee & Abramson, 1997; Gerber et al, 1998; Ray & Ferneyhough, 1997a, b, 1999). Thus, it is important to compare the methods used in this thesis with those employed in other laboratories to demonstrate clearly the parameters of the learning in comparison with those reported by other researchers.

3.1 The effects of overshadowing in olfactory learning

Pavlov (1927) was the first scientist to describe overshadowing, when he suggested whilst experimenting with his dogs, that the presence of one stimulus may affect the conditioning of another. It has since been shown in other vertebrates (Pearce, 1997) and in honeybees (Couvillon & Bitterman, 1980, 1982; Couvillon, Klosterhalfen & Bitterman, 1983). Rescorla and Wagner (1972) proposed that the stimuli compete with each other for a pre-determined amount of associative strength, which is linked to the nature of the reinforcement. The stimulus that is the more salient then gets the greater share of association, overshadowing the other stimulus. However, Hull (1943) suggested one stimulus such as an orange colour alone, may be functionally very different to an orange colour and jasmine odour compound, indicating that overshadowing may be nothing more than a generalisation decrease. Couvillon and Bitterman (1982) found some evidence in honeybees in support of Hulls work. In their research, bees responded

after training to an orange/jasmine compound, very little to the colour alone or the odour alone, but responded strongly to the compound, this finding is comparable to findings with vertebrate species (Farthing & Hearst, 1979; Miles & Jenkins, 1973). In all the experiments described above, the compound stimulus is composed of two dissimilar stimuli, for example a colour linked with an odour or an odour linked with a mechanical stimulus. Smith and Cobey (1994) tested binary mixtures of odourants and found that bees trained to certain odour compounds, then responded equally to them both individually. However, when trained with an aldehyde/alcohol compound and then tested to each individual odour, they gave greater responses to the aldehyde. This may be due to the occurrence of aldehydes being more prevalent in natural forage than the presence of alcohols.

With overshadowing using two different forms of stimuli, one may be more salient to the subject than the other. For instance, in honeybees, odour is a more salient cue of food than colour, as odour can be passed on as information to the hive, whereas colour cannot. The odour of a flower to a honeybee is an ecologically relevant stimulus due to the learned association between it and the nectar reward. These differing odours have been shown to stimulate different regions of the honeybee brain (Laurent, 1996; Joerges, Kuttner, Galizia & Menzel, 1997) with the brain images showing different regions responding to different odour mixtures.

3.1.1 An investigation into overshadowing using two odours

In the following experiment we chose two distinct odours (geraniol and peppermint) as the stimuli, rather than an odour and a mechanical stimulus, such as stroking the antenna, to keep the conditioned stimuli in the same sensory modality, and also so that

the compound could be delivered simultaneously, without a delay between the two presentations.

3.1.2 Method

Honeybees were caught from the hive entrance and placed into vials, and then returned to the laboratory where they were chilled to 4 °C for 10 minutes, and then restrained in small plastic tubes, as previously described in the methods chapter (2.11). The bees were then allowed a 2 hour recovery period from the anaesthetic, and then the training commenced. In a series of spaced trials, separated by a 10 minute inter trial interval, each bee was given an odour delivery accompanied by a sucrose reward, first touched to the antennae to elicit a proboscis extension, then to the proboscis. Each honeybee was allowed to drink from this solution for a further 4 seconds (10 seconds in total for each association). After the first trial, an extension of the proboscis to the odour alone was classified as a positive response. The first three trials were reinforced to the compound of peppermint and geraniol, and were delivered using the equipment as detailed in chapter two. In the following three trials, the bees were divided into two groups, one of which was tested with geraniol odour alone, the other with peppermint odour alone (see figure 3.1.2 for a breakdown of the training procedure).

Both these groups were tested unreinforced, with the same 10 minute inter trial interval. The final sequence in this experiment consisted of a seventh trial whereby the original compound of odours was presented to the bees.

Figure 3.1.2 The overshadowing training procedure

Trial 1: Peppermint/Geraniol mix

↓

Trial 2: Peppermint/Geraniol mix

↓

Trial 3: Peppermint/Geraniol mix

↙   ↘

Group A   **Group B**

↓   ↓

Trial 4: Peppermint   Geraniol

↓   ↓

Trial 5: Peppermint   Geraniol

↓   ↓

Trial 6: Peppermint   Geraniol

↘   ↙

Trial 7: Peppermint/Geraniol mix
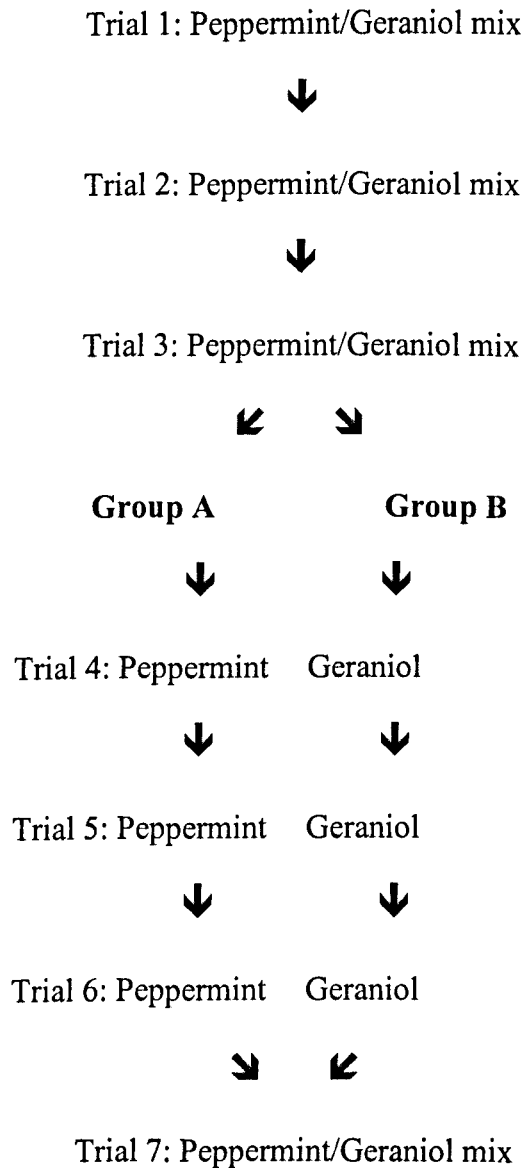
3.1.3 Results

The results (see figure 3.1) showed that over half of the bees gave a PER response to the

training odours after 3 trials, with 58 % responding on the third trial. However, on the

fourth trial, the bees tested, unreinforced to geraniol alone reduced their response to

only 20 %, which fell to 0 % on the third test trial (trial six overall). This could be due

to an extinction in the learning (see chapter 3.8), however, this is in stark contrast to the

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Figure 3.1. Overshadowing effect in PER conditioning
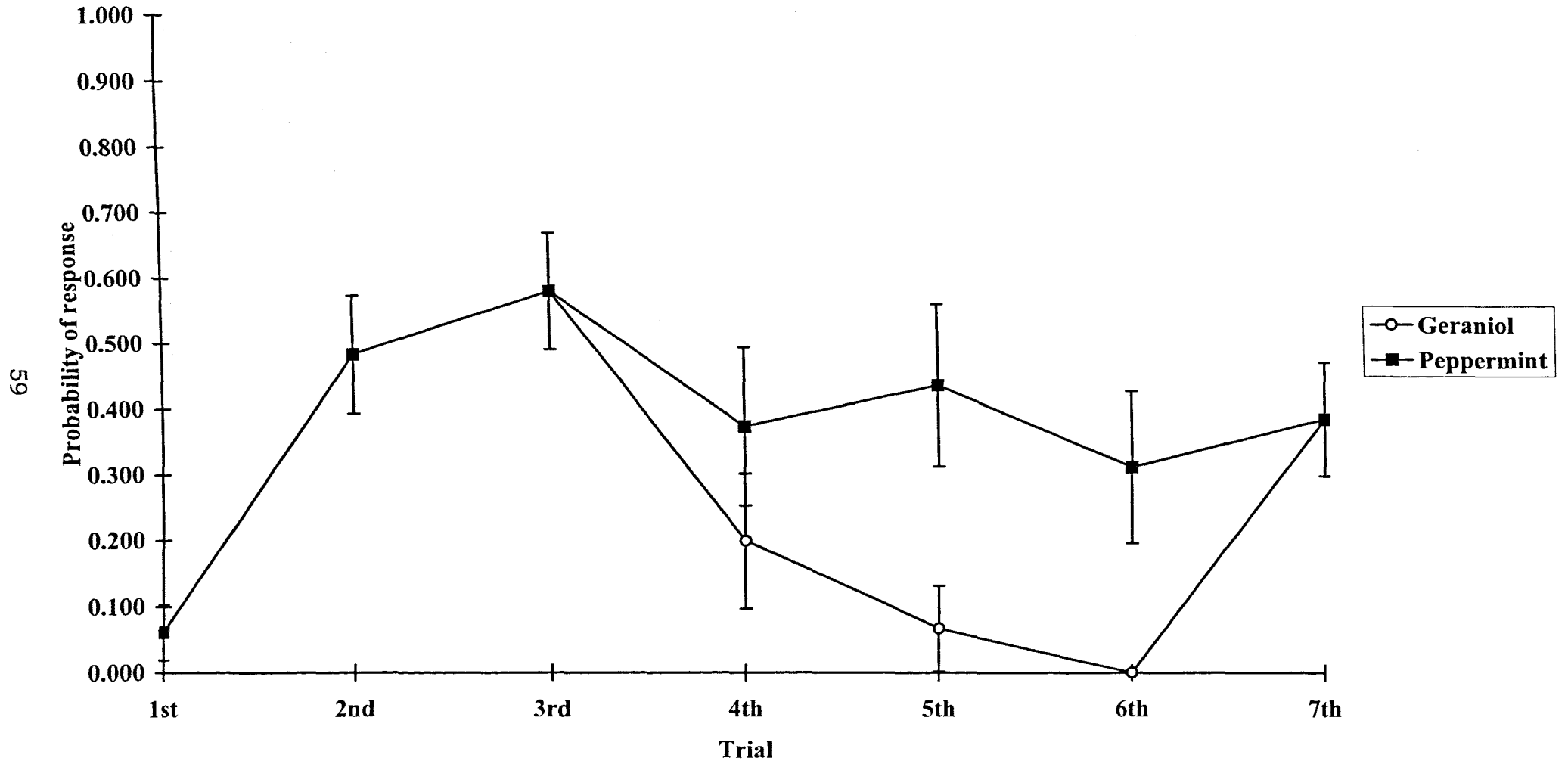
bees tested with peppermint, where 37 % responded on the first test trial (trial 4 overall), with 31 % still responding on the third test trial (trial 6 overall). These results presented suggest that peppermint odour overshadows the learning of geraniol odour. As an interesting addition to this experiment, when the bees were once again tested with the original training compound odour, the level of response increased in both groups to 39 %, this was less than for the initial paired learning trials, but was still an increase on the previous unpaired trials with the single odours.

The peppermint odour may overshadow the geraniol odour due to it being a more salient and pungent odour. As to the experimenter, it was noted that peppermint was the stronger of the two odours, even though equal quantities of the two were added to the delivery flask. Another explanation for the results could be that the peppermint odour was a novel stimulus which the honeybee had not previously experienced, and was therefore of greater importance to learn, as the bees may have come across the geraniol odour while foraging (Gerber et al, 1996).

3.1.4 Conclusion

In conclusion, this overshadowing experiment has shown that the method of my training agrees with the literature, with overshadowing taking place in a compound stimulus with one odour becoming more salient than another.

3.2 Blocking in olfactory learning

In associative learning, the term blocking demonstrates that animals do not necessarily associate a conditioned stimulus with a reinforcement if the stimulus is presented with a second conditioned stimulus (Thorn & Smith, 1997). So, blocking allows the animal to

select the learning to the most salient cue at the expense of novel ones. The blocking phenomenon was first described by Kamin (1968, 1969) in rat associative conditioning, and has also been found in other vertebrates and invertebrates (Sahley, Rudy & Gelperin, 1981; Smith & Cobey, 1994; Smith, 1996, 1997). Both blocking and overshadowing experiments are used to identify the content of conditioned stimuli (Kamin, 1968; Rescorla & Wagner, 1972; Rescorla, 1988). It has also been shown that blocking is independent of the type of US, with both appetitive (Kamin, 1968, 1969) and aversive (Ross, 1985) conditioning. Within the honeybee olfactory learning literature, blocking has been investigated with Menzel (1990) stating that blocking has little or no effects in PER conditioning, and that the results he obtained were in accord with other researchers (Couvillon et al, 1983). As in overshadowing, blocking consists of training to compounds of stimuli, however with blocking, the subjects are trained to two individual stimuli, such as an odourant, and a mechanical stimulus. These are initially presented separately, then they are presented as a compound (all reinforced), on the following test trials, the stimuli are once again presented separately but unreinforced. In olfactory learning in the honeybee, the blocking phenomenon is well known at the neuroanatomical level (Hammer & Menzel, 1995; Menzel & Muller, 1996) and Thorn and Smith (1997) have also shown that honeybees require stimulation to both antennae to enable blocking of odours, with the dynamics of the interactions being complex within the honeybee brain.

3.2.1 An investigation into the effects of blocking in olfactory learning

In our experiments honeybees were trained to either geraniol or peppermint odour, then trained to a geraniol / peppermint compound and finally tested with either peppermint or geraniol alone. Although Menzel (1990) gave 8 learning trials to each stimuli and

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compound, in this report, only three spaced trials were given, as three spaced trials to an odour has been shown to maintain an amnesia resistant long term memory (Erber, 1976).

The blocking experiments undertaken by Couvillon et al (1983) were with freely flying honeybees, the bees in our experiments however, were restrained in the laboratory.

3.2.2 Methods

As in the previous experiment, honeybees were caught from the hive entrance and transported back to the laboratory in vials, and treated as previously described (see chapter 2.11). Prior to the experiment commencing, the bees were allowed a two hour recovery period to increase their hunger and therefore willingness to learn, and also to enable the effects of the anaesthesia to subside. The conditioning procedure for the blocking experiment was that prior to the first three trials, the bees were divided randomly into two groups. The first group were given three reinforced trials to peppermint, with the US being a 50 % sucrose solution, the second group were trained in an identical way but to a geraniol odour, with each trial having an inter trial interval of 10 minutes. After each group had been trained to their respective odours, they were given a one hour consolidation period, to allow the association to assimilate into the long term memory (Menzel, 1990). The next three trials (trials 4 - 6 overall) were then with a compound mixture of equal measures of geraniol and peppermint, again these trials were reinforced with the 50 % sucrose solution, having the same inter trial interval, to ensure all the parameters were standard. After these last three trials, the bees were given a further one hour consolidation period. To test for a blocking effect in the learning, the next trial (trial 7) given was an unreinforced test to the opposite odour to which they were originally trained (see figure 3.2). Therefore, the bees initially trained

to peppermint in the first three trials were tested with geraniol, and conversely, the geraniol trained bees were tested with peppermint. Finally, on the eighth trial, each of the groups was tested unreinforced to their original training odour, i.e. the peppermint group 1 to peppermint odour, and the geraniol group 2 to geraniol odour.

3.2.3 Results

The results obtained (see figure 3.2) agree with those presented by Menzel (1990), who stated that no significant blocking effect of the learning was apparent. However, this may be due to the method by which the compound was delivered, as in the experiment described above, a mixture of the two odours was presented as the CS, rather than a combination of different stimuli, such as an odour and a mechanical stimulus (Pelz et al, 1997). In both our groups, the levels of learning in the first three trials showed the expected learning curve. However, it should be noted that the peppermint trained group did not show the levels of response seen with the geraniol trained group. Both groups, when trained to the compound mixture, gradually decreased their responses, even though the trials were reinforced. On the test trial (trial 7) with the untrained odour, the levels of response were much reduced when compared to the following eighth trial, which was to the original trained odour. These results therefore indicate that no substantial blocking of the original olfactory learning has taken place, hence agreeing with the findings of Menzel (1990) and Couvillon et al (1983).

Figure 3.2. The effects of blocking in olfactory learning
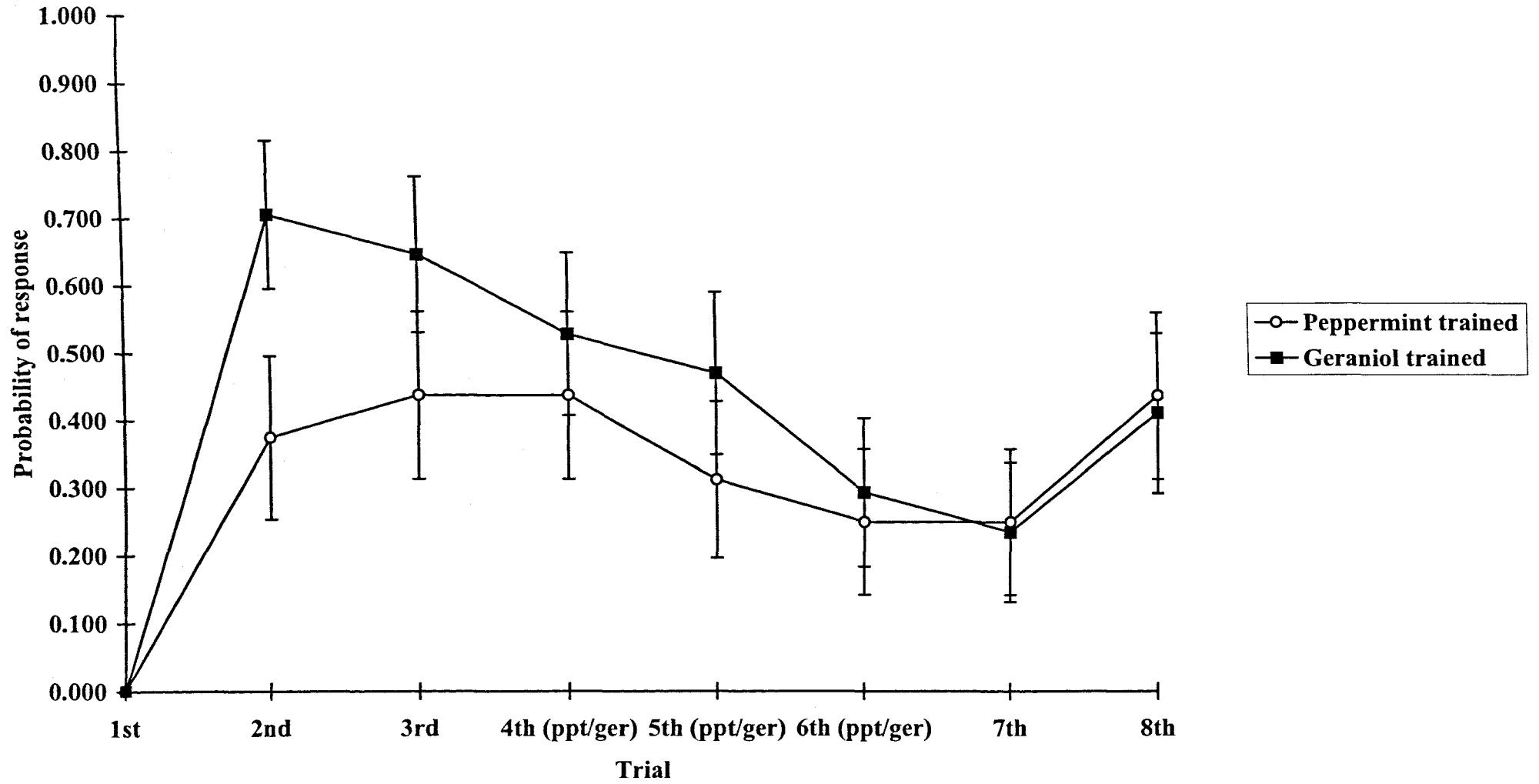
3.3 Pre-US exposure effects on olfactory learning

In these experiments, the experiments of Abramson and Bitterman (1986) were repeated, although in their experiments the bees were free flying subjects, compared to our laboratory, where we once again used restrained subjects in the laboratory. Another variation from the study by Abramson and Bitterman (1986) was that they used an aversive shock avoidance learning paradigm, whereby the bees visited a sucrose rewarded dish for 10 trials prior to pairing this with an electric shock. Bitterman et al (1983), in another study, used restrained honeybees, and found that pre-exposure of four trials to the US alone slightly retarded the subsequent learning, as there was 'somewhat more resistance to acquisition'. In the vertebrate literature, pre-exposure to a lone stimulus is termed latent inhibition (Lubrow, 1973), whereby prolonged exposure to the CS reduces the ability to then condition using that stimulus. However, exposure to the US immediately prior to conditioning is called the pre-US exposure effect.

3.3.1 Methods

In the experiments reported, bees were captured and restrained as described previously (chapter 2.11) and were given three preliminary trials, whereby the bees were just presented with the US (a 50 % sucrose reward), in isolation from the training odour. This pre-US exposure should interfere with the olfactory conditioning, as previous contact with the US alone affects and impairs the saliency and incentive to learn. The way in which the training was undertaken was that an initial 3 trials of the US alone were presented, each spaced by a 10 minute inter trial interval, which remained constant throughout the experiment. These first three trials with the US were such that the antennae were touched with the sucrose solution, and the honeybee was permitted to

feed for a maximum of 10 second period. The following three trials were paired associations with a CS (geraniol odour).

3.3.2 Results

The results (see figure 3.3) showed that pre-exposing the honeybees to a US alone reduced the response to the odour on the paired CS-US trials (trial 4 to trial 6), with only 20 % of the honeybees providing a positive response of a proboscis extension, compared to over 70 % for the controls, who had six CS - US paired trials. These results suggest that a pre-exposure to the unconditioned stimulus does have a negative effect on the response in subsequent paired learning trials. These results agree with the literature (Bitterman et al, 1983; Abramson & Bitterman, 1986; Lubrow, 1973) whereby a pre-exposure to a US or CS does affect subsequent learning.

3.4 The effects of random presentation of CS and US

As an important control factor to demonstrate the associative component in this learning, it must be clearly demonstrated that a single presentation of either a conditioned or an unconditioned stimulus alone does not elicit a proboscis extension, and in this chapter the random presentation of the conditioned stimulus (CS) or the unconditioned stimulus (US) alone is presented. This demonstrates that no learning occurs, as no associations are formed between the two stimuli with the subject. The experiments reported here (see figure 3.4) agree with those of Bitterman et al (1983), that repeated presentations of a CS or US reduces acquisition of subsequent conditioning.

Figure 3.3. Pre-US exposure effects on olfactory conditioning
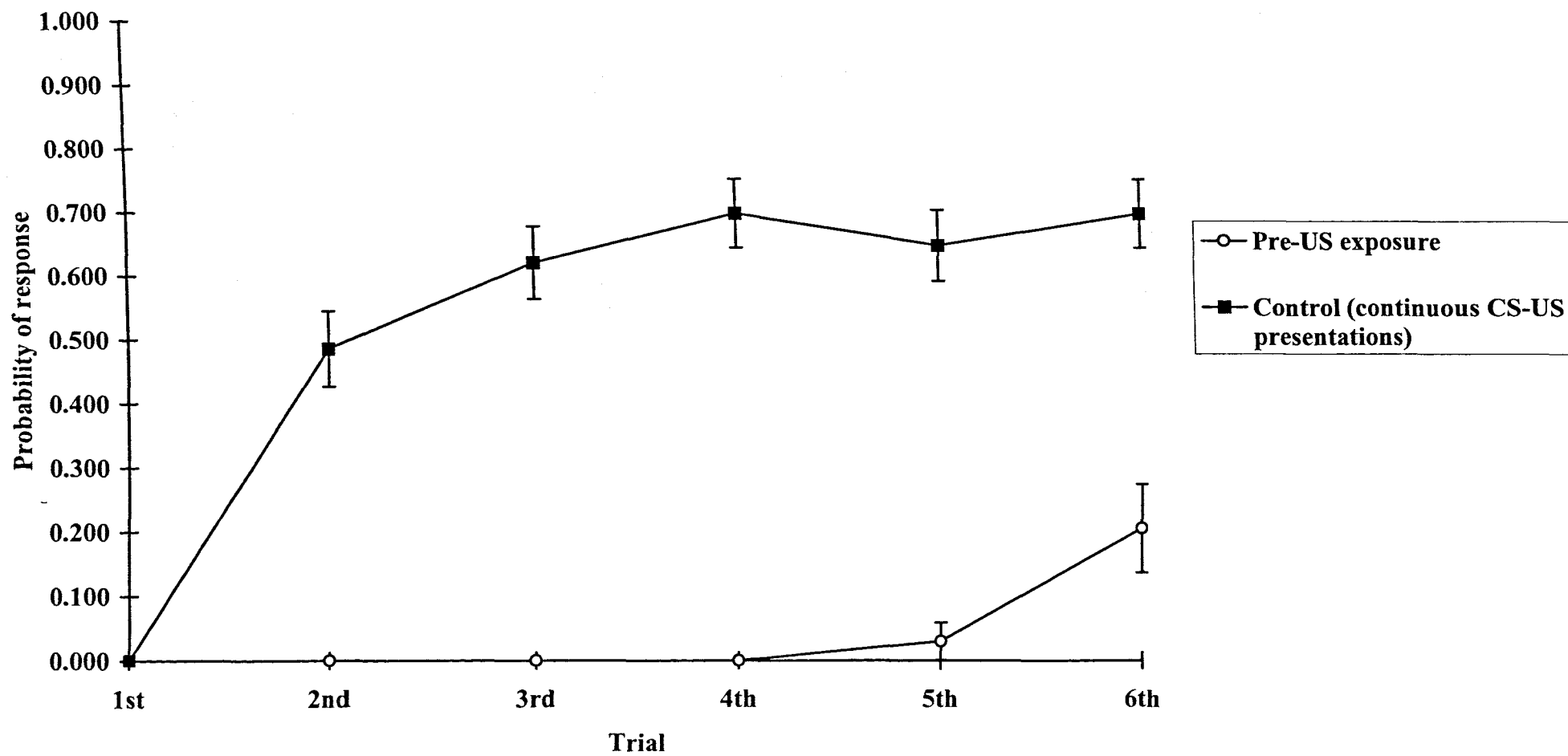
3.4.1 Method

Honeybees were captured and restrained as previously outlined (see chapter 2.11), and were then given 6 random presentations of the CS or US. The actual order that the subjects were given was CS, US, US, CS, CS, US, this sequence was decided upon using an experimenter who had no previous knowledge of the experiment. Following these unpaired presentations of the CS and US, two paired trials of CS-US were presented, to ascertain if the learning had been impaired.

3.4.2 Results

The results (see figure 3.4) showed that the six random presentations of each of the stimuli only yielded a maximum response of 13 %, with a more common response of 0 %, and further, on the following two paired training trials, no response to the odour was evidenced. These results suggest that the previous random presentations of the CS or the US have disrupted the learning, as the honeybee has experienced a pre-stimulus exposure prior to associative conditioning, as described in chapter 3.3.

3.5 Habituation in the honeybee

As PER is an appetitive component of the olfactory learning pradigm, using a repeated trials experimental procedure, similar to habituation, there is a need to establish that the honeybees are not becoming habituated to the presentation of the odour, and this section addresses this phenomenon. Habituation has been called the simplest form of learning, with a repeated stimulus causing a decrement in response (Groves & Thompson, 1970). It is a different form of learning to the PER paradigm, but it does share a similarity in

Figure 3.4. The effects of randomly presenting the CS or US alone
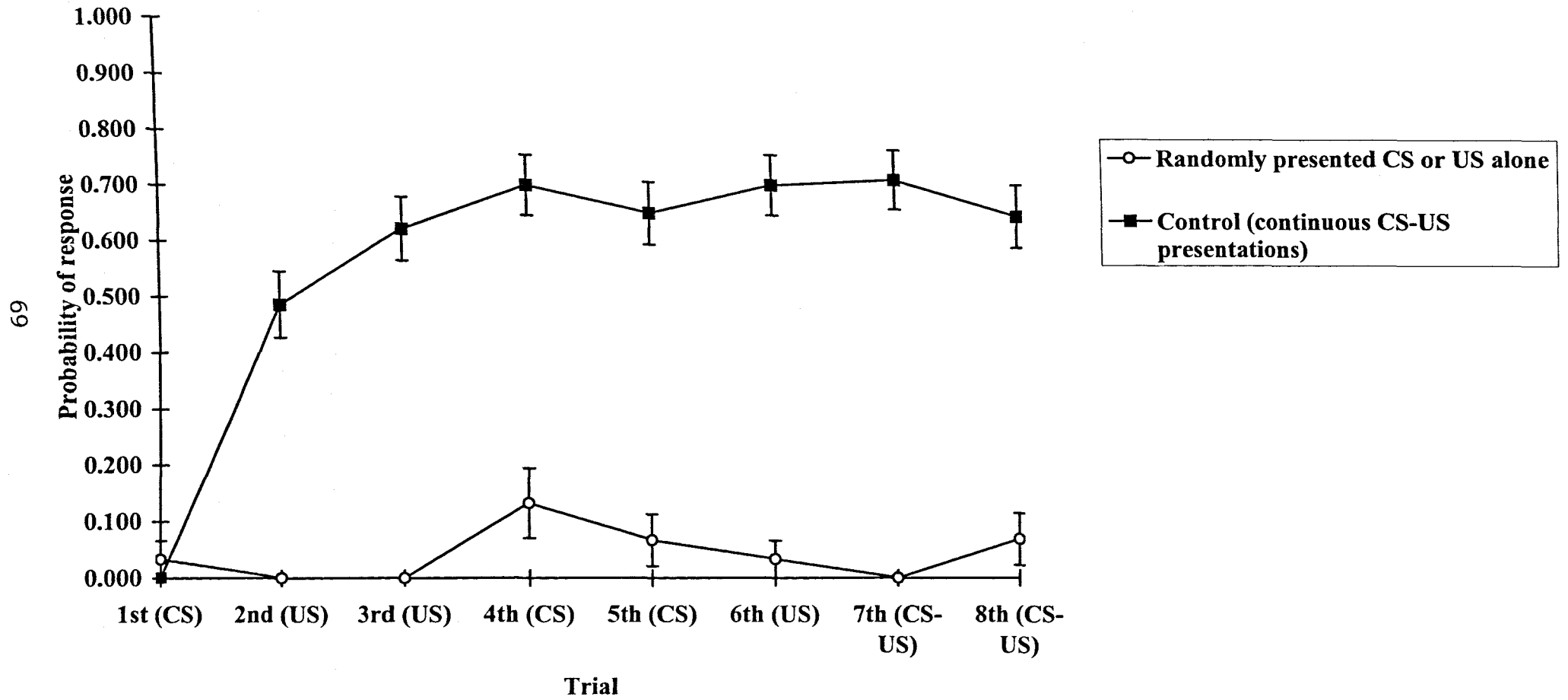
that there are repeated presentations of the stimulus. The difference between these two forms of learning, is that in olfactory learning, there is a conditioned association being formed, compared to habituation, where there is a single repeated stimulus presentation. Habituation is a widespread phenomenon, being demonstrated in many animals from primitive Paramecia (Jennings, 1906), Pacific sea anemones (Logan, 1975), stickleback fish (Peeke & Veno, 1973), land snails (Ray, 1998), and rabbits (Whitlow, 1975) as well as in honeybees (Menzel & Muller, 1996), with Braun and Bicker (1992) stating that habituation to repeated sucrose stimulations of the antennae develops quickly for low sucrose concentrations, compared to slowly for higher concentrations. Bicker and Hahnlein (1994) also established that a single habituation session led to a short lived effect (less than 10 minutes), with multiple sessions leading to longer-lasting effects (about 25 hours). True habituation has also been further defined when a novel dis-habituation trial is presented, which then elicits an increased response to the new stimulus. In this experiment, the appetitive response of the proboscis extension to a sucrose reward was the stimulus to one antenna, and the dis-habituation stimulus was to the other antenna.

3.5.1 Method

Honeybees were caught and restrained as previously described (see chapter 2.11), then fed a 50 % sucrose solution to satiation 2 hours prior to the experiments start. A droplet of 50 % sucrose solution was given to the left antenna, with an inter trial interval of 3 seconds, until the proboscis extension failed to occur for 5 consecutive trials, this was the habituation protocol. This was defined as the pre-test. A dis-habituation (change of stimulus) trial was presented which was a 50 % sucrose solution to the opposite (right) antenna. The test was then to see how many trials were needed to rehabituate to the 50

% sucrose to the left antenna, this was undertaken 10 minutes after the pre-test. Any animals showing no habituation were discarded from the experiment (protocol from Bicker & Hanhlein, 1994).

3.5.2 Results

The results (see figure 3.5) showed that it took an average of 9 trials to habituate, compared to 8 to re-habituate after the dis-habituation trial. This intimates that the honeybees do habituate to the presentation of the US to the antenna, by showing a decrement in the response, but only slightly. However, this mild form of habituation can then be dis-habituated by a presentation to the opposite antennae, giving a true habituation. There is a danger that the honeybee when being trained using the PER paradigm, may become habituated to the stimulus, however, this would not be the case, as an association component of this learning, the conditioned and unconditioned stimuli, has been formed.

3.6 Spaced and massed trial effects in PER conditioning

The majority of research into massed and spaced trials has been involved with the manipulation of the inter trial interval (ITI). Ray (1998) trained the land snail, *Helix aspersa*, to an antennal withdrawal reflex to habituation, under a massed and spaced training schedule. In the massed training schedule, each snail was given a tactile stimulus (CS) to the antennae at a constant ITI of 30 seconds until habituation been reached. In the spaced trials, each snail was given two presentations separated by 30 seconds, then a 4 minute interval, the two presentations 30 seconds apart, this proceeded until habituation to the stimulus had occurred. Upon retest, either 12 or 24 hours post

Figure 3.5. Habituation of an antennal response to a sucrose presentation
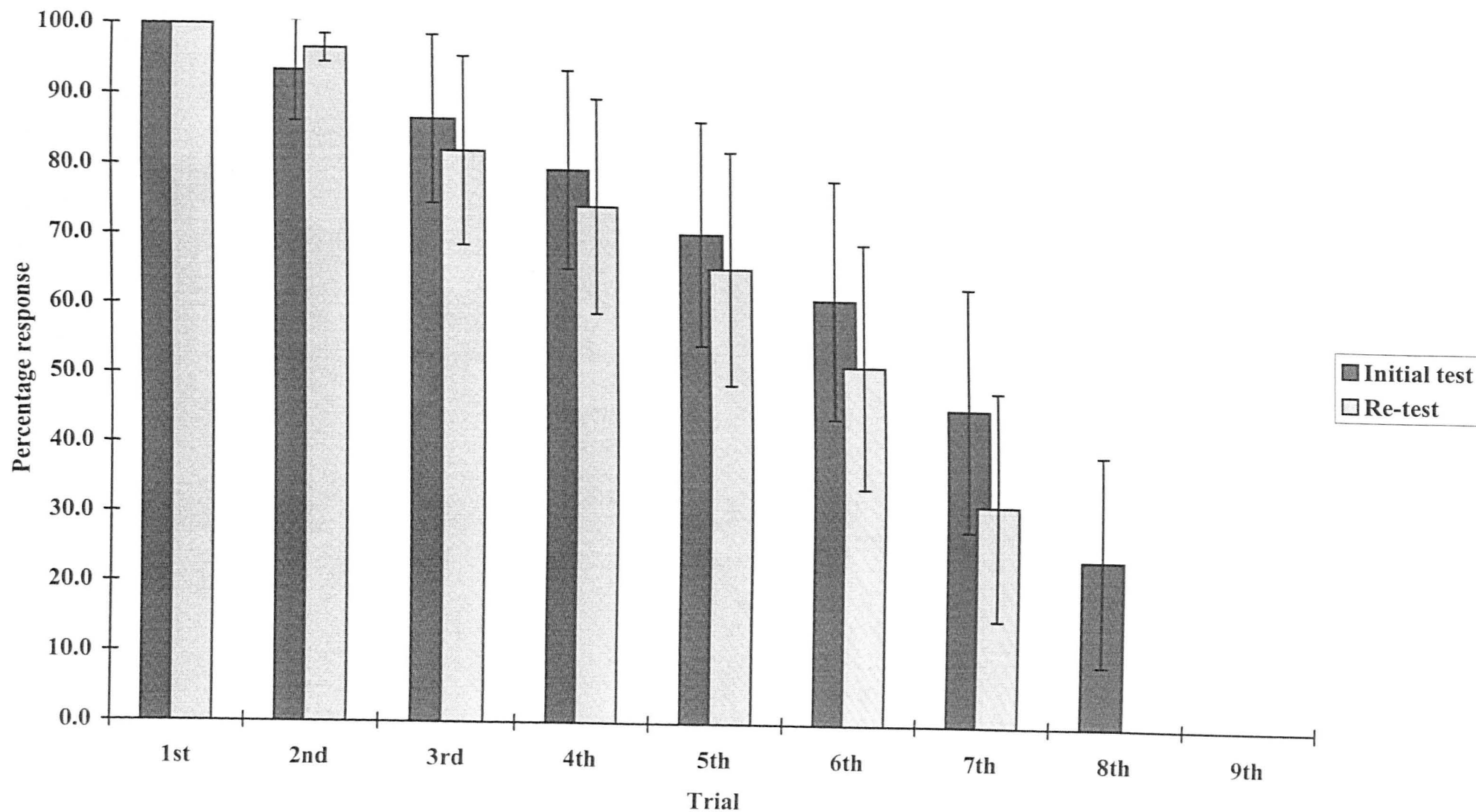
training, the stimulus was delivered at 30 second intervals irrespective of the initial training. After 12 hours there was a significant difference between the two groups, and also with the 24 hour retest groups. Thus, when the number of trials to habituation showed no difference between groups, indicating no short term habituation, but there is a long term habituation response between massed and spaced training.

Spaced trials are when CS - US presentations are given in a regular order, such as every 10 minutes, as opposed to massed training where each trial is presented after the previous one. In the literature (Carew, 1996; Gerber et al, 1998; Smith, 1991) spaced trials lead to more rigid long term retention than massed trials. With the spaced trials, it has been indicated that the learning is more long lasting and is consolidated to the long term memory, compared to massed training, where this forms only a short term memory. In honeybees, the spaced trials have at least 8 minutes between each individual trial (Erber, 1976), who showed by cooling different parts of the bee brain after conditioning, the pathway of memory formation, and he also found that after a certain time that the memory could not be disrupted by anaesthesia, and had been consolidated into the long term memory of the bee.

3.6.1 Olfactory learning using spaced trials

3.6.2 Method

The bees in this particular experiment were caught from the hive entrance, and were brought back to the laboratory and anaesthetised and restrained as previously described (see chapter 2.11). The bees were trained to a geraniol odour, and were initially given three training trials, with an inter trial interval of 14 minutes. After the three trials, a one

hour consolidation period was allowed, and then three further unreinforced trials were given to test for long term retention.

3.6.3 Results

The results of this experiment (see figure 3.6.3) showed that on the third trial there was a high level of response rate, achieving a probability of response above 0.6 on this trial. Again, after the consolidation period, the next three trials each gave a similar response rate, thus indicating that the learning had been consolidated and was stored into the long term memory of the honeybee, as previous research has shown that the memory time course is such that amnesia resistant long term memory is formed after 8 minutes (Brandes et al, 1988; Erber, 1975a, b, 1976).

3.6.4 Olfactory learning using massed trials

Massed trials are where the training is almost the opposite to spaced training, in that the trials are continuous, one after the other, with no long inter-trial interval.

3.6.5 Method

In the experiments undertaken, the inter-trial interval was set at one minute. The bees were given an initial five reinforced trials, followed by a 30 minute consolidation period, then tested further with five unreinforced trials. The experimental design was as previously reported (see chapter 2.11).

Figure 3.6.3. The effects of massed and spaced training on PER learning
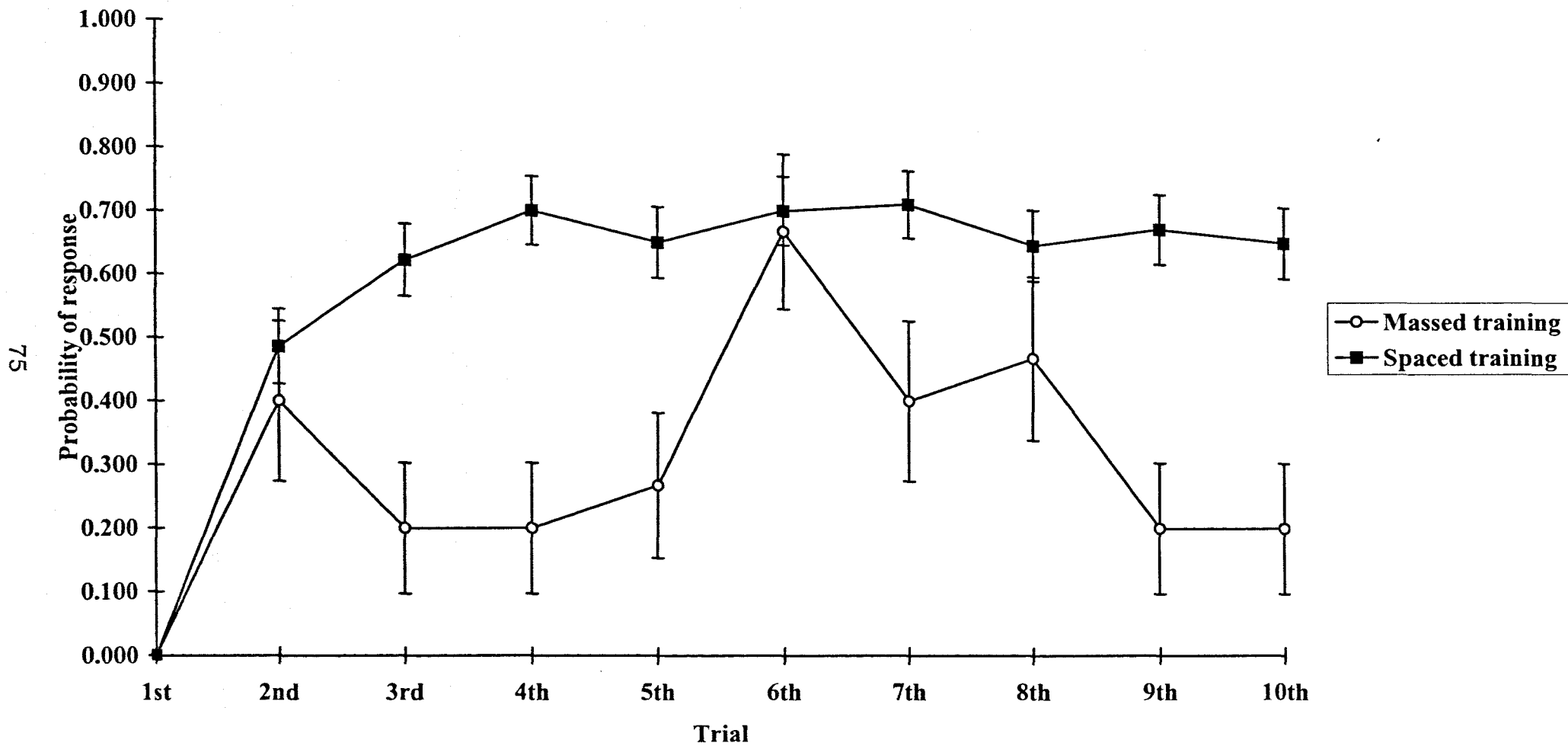
3.6.6 Results

The levels of learning (see figure 3.6.3) were low after the fifth trial, however the response rate increased markedly on the first test trials (trial 6), suggesting that the learning had been consolidated. However, the following trials showed the learning just to a transient effect, with the response rates decreasing to only a 20 % of the subjects. These results are analogous to those reported by Smith (1991), in which bees were trained to an olfactory cue and then tested over a varying time period for retention, and to ascertain the time course for the memory. They found, along with others (Erber et al, 1980; Menzel, 1979, 1983) that there was a learning up to 3 minutes, then an unstable memory up to 7 minutes. Olfactory learning after this period was unaffected by anaesthetic or cooling of the brain. In our experiments, we are obviously dealing with this first form of the memory, and it is interesting to note that following the training, the retention tests are initially high, then fall quite steadily, suggesting an extinction of the learning (see chapter 3.7).

3.7 Extinction of olfactory learning

Extinction plays on important role in any animals learning patterns, with extinction relying on the fact that an unreinforced paradigm is no longer necessary to be consolidated into the long term memory (Couvillon & Bitterman, 1980, 1984). It would be inefficient for a set response to occur if the information was no longer relevant, such as no more food being available from a flower source, or a threat no longer being viable. Therefore, the learning needs to be extinguished in order for more salient and necessary cues to be learned. In the honeybee literature, Takeda (1961) stated that extinction would occur with presentations of a series (about 10) of non-reinforced stimuli, with a

spontaneous recovery observable on the following day. However, Wenner and Johnson (1966) state that after a second day of unrewarded trials, no recovery will occur. This extinction of the leraning is also evident in the rodent literature (Ison, 1962; North & Stimmel, 1960; Senkowski, 1978) and an explanation for this is that the rats performance is dirputed by frustration stemming from unrealised anticipaion of a reinforcement (Amsel, 1958, 1962).

3.7.1 Two experiments to determine extinction of olfactory learning

In this chapter, two experimental designs were developed to test for an extinction in the acquisition and retention of the olfactory learning. In the first experiment, the subjects were given one CS-US presentation followed by unpaired CS presentaions alone, to generate an extinction effect, and in the second, a series of protocols was designed, to establish when extinction would occur after a set number of initial training trials.

3.7.2 Method

In these investigations, the bees were captured and restrained as described in chapter 2.11, and were then given a sinlge CS-US presentation, followed by CS deliveries until the levels of response were reduced, the bees being given a total of 10 trials with a nine minute ITI.

3.7.3 Results

Figure 3.7.1 shows the response rates decreasing from a high of 64 % on the second and third trials to only 12 % on the 10th trial, indicating that an extinction type curve (Pearce, 1997) of the response rate did occur in this experiment. To further examine this phenomenon, a series of experiments was deisgned where bees were initially trained to either one, three or six paired conditioning trials, and were then presented with just the odour, without reinforcement until the response rate reduced to nil.

The results (see figure 3.7.2) showed that the bees given 1 reinforced training trial took a further 6 trials to exhibit an extinction of the learning. The group given 3 trials required a further 8 trials to cease responding, and finally, the groups given 6 initial training trials extinguished after only 3 unreinforced trials.

3.8 Is the proboscis extension reflex in the honeybee one trial learning ?

3.8.1 Introduction

Learning in free flying honeybees both at the flower and at artificial feed stations is subject to many different learning parameters, such as distance and orientation from the hive, flower colour and odour. The ecological success of the honeybee has enabled the bee to associate a high nectar source with a flowers specific odour, the location of which can then be communicated to the hive. However, does the bee just learn this association after one visit, or does it take more to specify which odour is associated with the food reward ? Honeybees need to learn and remember which floral odour gave the highest food reward to be able to communicate this to her sisters. Although a bee visits many

Figure 3.7.1. Extincton of PER with repeated exposure to CS without reward following a single conditioning trial
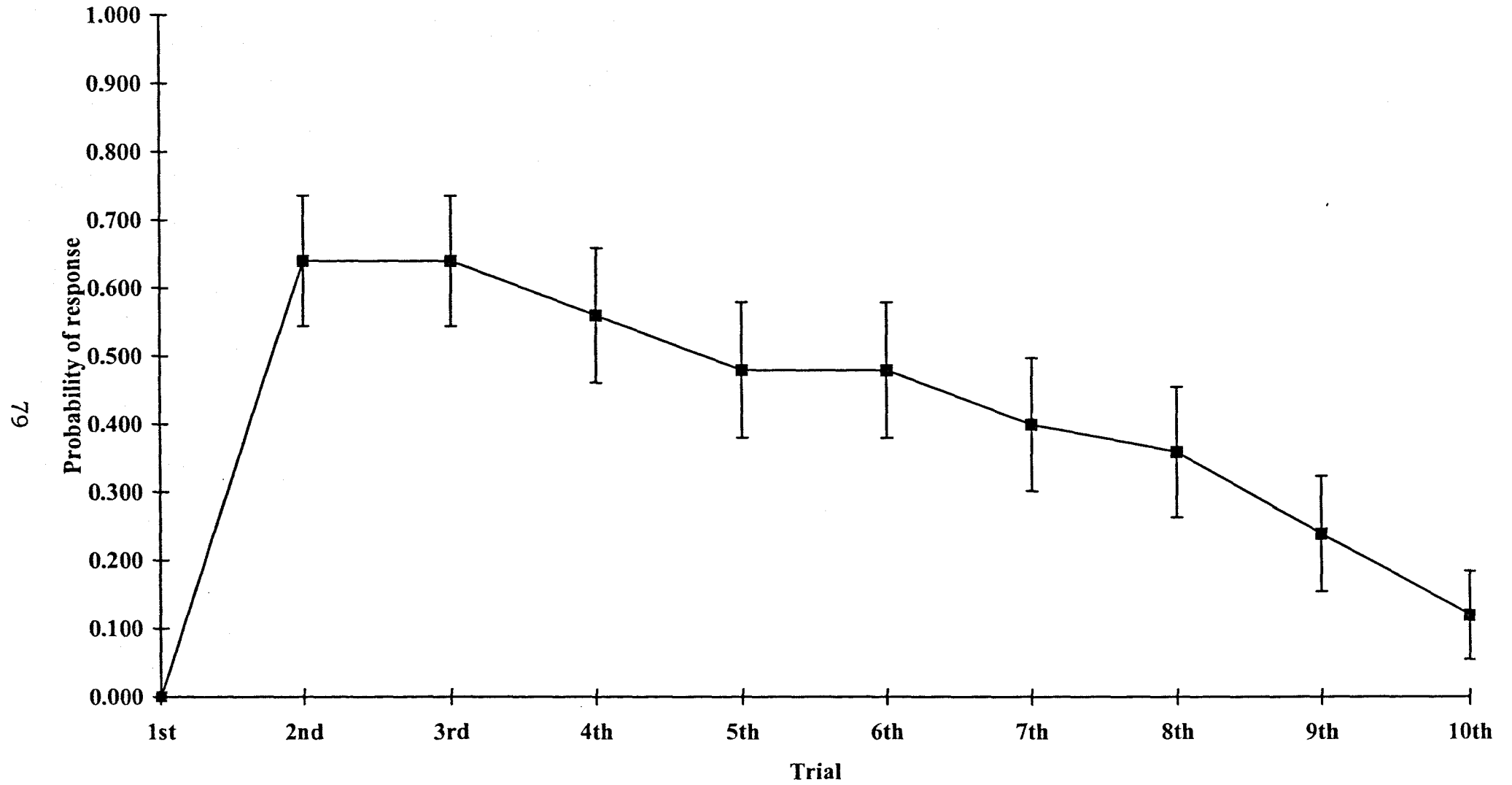
Figure 3.7.2. Extinction of PER learning following 1, 3 or 6 conditioning trials

80
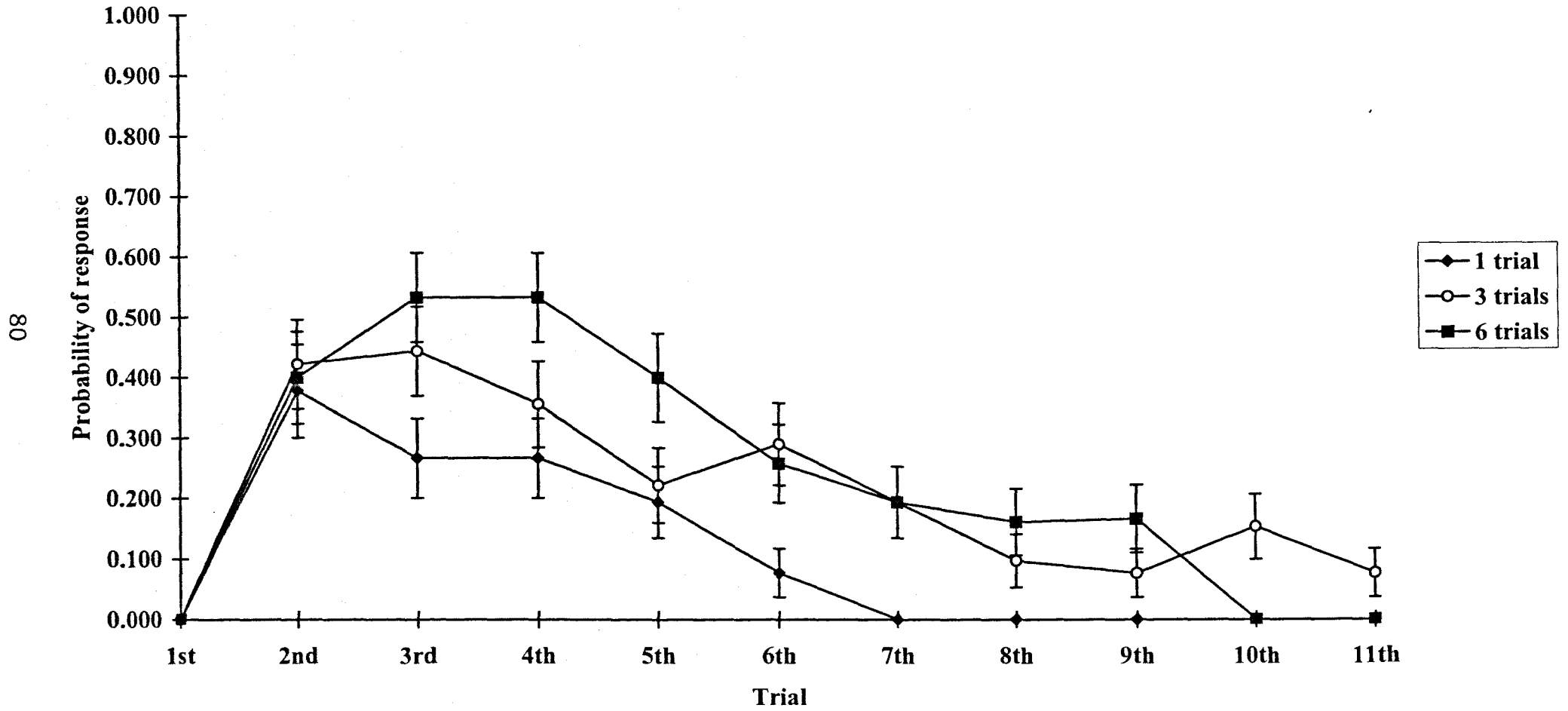
flowers on a foraging trip, it only remembers and communicates the most profitable source (Gould & Gould, 1995).

The proboscis extension reflex (PER) has generated a copious amount of literature in bee learning, being used in genetic, pharmacological and anatomical investigations of behaviour (Hammer & Menzel, 1995; Menzel & Muller, 1996). The majority of the studies into proboscis extension reflex (PER) learning assumes it to be one trial learning. Erber (1981) reported that "Depending on the season up to 90 % of a population of bees learns the conditioned response after one learning trial", Gerber et al (1996) says "CS - US....association after one learning trial". However, Menzel (1990) is more cautious stating that the learning is one trial, subject to a list of conditions, with a single learning trial significantly altering the behaviour, to give a 50 % - 80 % response level. However, he qualifies this by stating that early memory, after a single learning trial, is particularly sensitive to extinction and reversal learning, whereas consolidated memory is more resistant (Menzel, 1979). It has also been shown that a memory trace is not susceptible to narcosis, electroshock or cooling after seven minutes, following a single learning trial (Erber, 1975a, b; Erber et al, 1980), although they only tested with the trained odour, and not a novel one. In the field, Menzel and Bitterman (1983), report that free flying bees pretrained to visit an odourless feeding place, need only one exposure to a flower-like odour to select it with accuracy, when given a choice between two others. Menzel et al (1974) stated that after a single presentation of a CS - US, 30 % - 50 % of honeybees will extend their proboscis upon further presentations of odour alone. Menzel and Muller (1996) also state that a "single pairing of odour as CS and sucrose as US changes the PER probability from < 10 % to levels > 60 %", and then go on to say that "because bees generalise between CSs, the response to CS- is initially high, and reduces as learning progresses". This is probably due to olfactory learning not being a one trial learning paradigm, hence the bees initially generalise to odours with

this waning, as more associated trials are presented. Hammer and Menzel (1995) tested the time course of memory decay after 1 and 3 learning trials, and found that the one learning trial decayed by more than 50 % after 72 hours. Smith (1991) reports that although the association may be formed after one trial, two to three are required to attain asymptotic response levels. Menzel and Muller (1996) and Hammer and Menzel (1995) also both stress that experience over trials influences learning.

As some laboratories agree that this olfactory learning is one trial, others either qualify the statement, or say the learning is more than one trial learning, it therefore generates the question "is olfactory proboscis extension reflex learning one trial ?'.

As olfactory PER in the honeybee has long been assumed to be one trial learning, it has been related to other one trial learning paradigms studied in vertebrates. Rose, Gibbs and Hambley (1979) and Cherkin (1972) used chicks, Clayton and Krebs (1998) used marsh tits and jackdaws, and Garcia and Koeling (1966) used rats. These vertebrate studies usually involved food aversion, whereas the PER learning is an odour-reward learning. Food aversion in the honeybee in the sense it is used in the vertebrate literature has not been shown, with Menzel (personal observation) reporting that lithium chloride (LiCl), often used in vertebrate aversive conditioning, has no effect on the bee. Aversive conditioning does exist in the wild, where honeybees do not harvest the alfalfa plant as it has a trip mechanism which knocks the bee off the plant, but if bees are placed in fields containing only this crop, they have no alternative, and they learn to manipulate the petals, and to gain nectar from the side. Electric shock has also been used for aversion studies in honeybees (Balderama et al, 1987), but this was not one trial learning, as the number of trials required for the association to be consolidated, was greater than one. Abramson and Bitterman (1986) used a signalled avoidance and electric shock, and again found, this was not one trial learning.

The odours used also differ, with carnation and geraniol (Menzel & Bitterman, 1983; Bitterman et al, 1983) being used the most, but also citral and hydroxycitronellal (Takeda, 1961) have been used. These scents may differ with respect to the bees' 'acceptance' of it, with the 'artificial' odours being foreign to the bee, which may more readily learn the more 'ecologically' significant odours, which relate to flower cues. The methods in which the bees are kept prior to the experiment may also affect the performance of the bee. Takeda (1961) kept subjects 1 - 2 days before feeding, others kept them overnight (Bitterman et al, 1983) before testing. This may increase the motivation of the bee to learn, but it may also indirectly affect the result (Ferneyhough & Ray, in prep.) with the condition of the honeybee suffering, as well as the motivation of the honeybee being affected.

This chapter tests the hypothesis that PER conditioning was not one trial learning, by training the bees to one scent, then testing them with a novel scent after 1, 2, or 3 learning trials. There seems to be confusion within the literature as to the nature of the learning in PER olfactory in terms of when it is learnt, presented is a qualification of this phenomenon.

3.8.2 Materials and methods

Two honeybee colonies housed at Oxford Brookes University were where the sample subjects originated, the experiments being carried out in the winter months due to the more reliable and elevated PER levels (Ray & Ferneyhough, 1997a).

The PER techniques were adapted from Menzel (1990), and the methods are reported as in chapter 2.11. This procedure was the standard method, however, the experiments differed in the following ways.

3.8.3 Testing for one, two or three trial learning

3.8.4 Method

All the trials were spaced with a constant ITI, the number of subjects being 110.

Group X was given a reinforced geraniol trial, then tested with the novel odour, peppermint, which was unreinforced. Following this, a further 3 reinforced geraniol trials.

Group Y were given 2 reinforced geraniol trials, then an unreinforced peppermint, followed by two reinforced geraniol.

Finally group Z were given 3 learning trials with geraniol odour, reinforced, and then on the fourth trial an unreinforced peppermint odour was presented, followed by a reinforced trial on the fifth, to geraniol.

3.8.5 Results

In the first experiment the bees were tested either on their second, third, or fourth trials with an unreinforced, novel odour, following at least one reinforced conditioning trial. These results suggest that the learning was not a one trial learning paradigm, as the levels of response shown by group X (see figure 3.8.5) are equivalent to levels shown by both groups Y and Z. These two groups were given two reinforced trials with geraniol, suggesting that bees in group X are generalising to the odour stimulus, as the association between the conditioning stimulus (CS+) and the unconditioned stimulus (US) had not been consolidated into the long term memory (LTM). The levels of learning after this trial with unreinforced stimulus (CS-) does however show comparable learning to the other groups, showing that these bees are not a rogue result. This

Figure 3.8.5. Presenting a novel odour (peppermint) following 1, 2 or 3 conditioning trials to geraniol odour
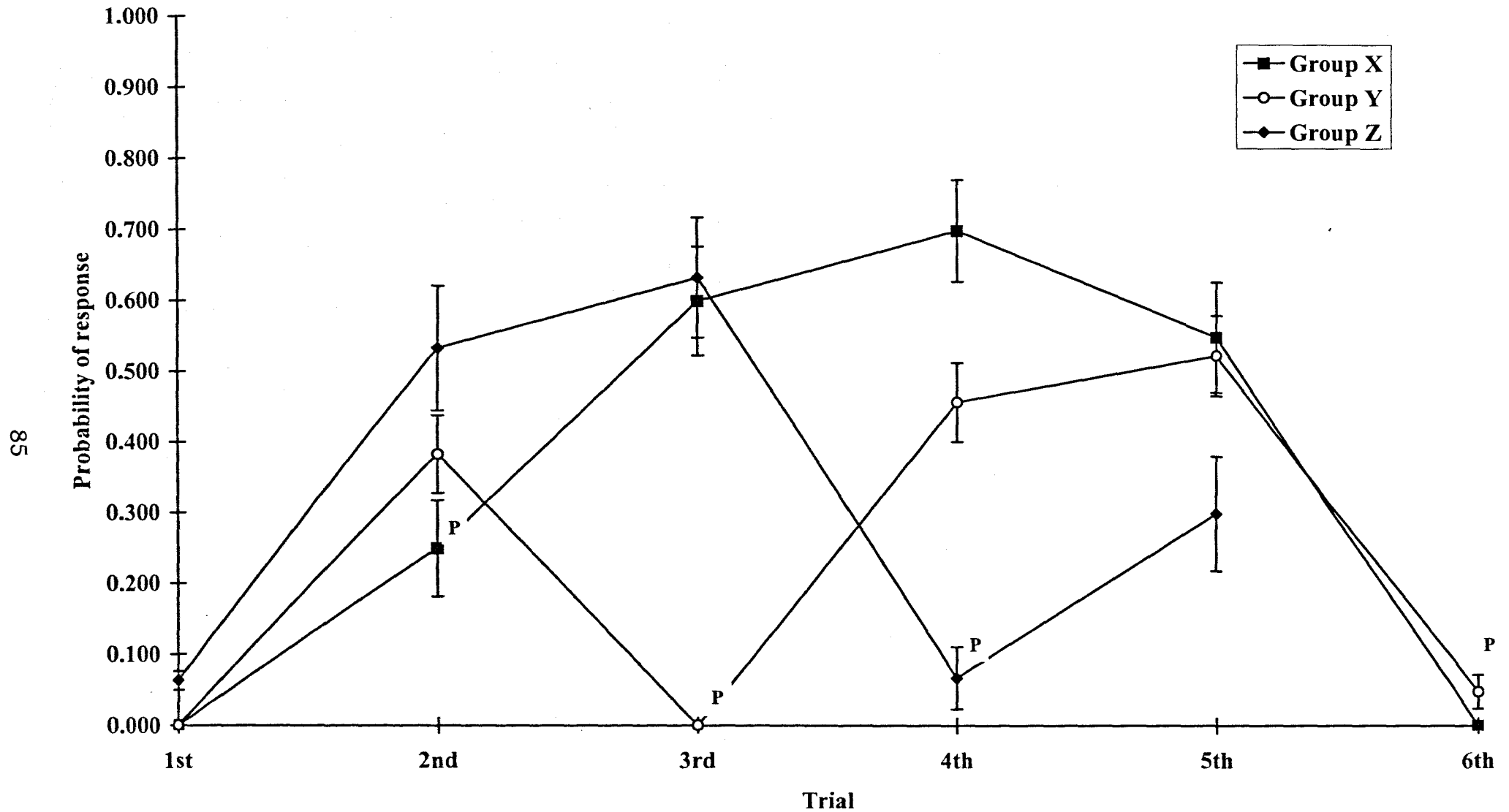
P = Peppermint presentation.
G = Geraniol presentation.

Group X = G, P, G, G, G.
Group Y = G, G, P, G, G.
Group Z = G, G, G, P, G.

experiment was further supported by groups Y and Z, who were given a CS- on the third and fourth trials, and who did not respond, suggesting that the CS+, US association has been formed and again showed that the PER olfactory learning paradigm was not a one trial learning paradigm, but requires at least two trials for the learning to become consolidated into the LTM. Not presented is a further trial with CS- (peppermint) after the five trials reported, which showed that the learning returned to a probability of 0, indicating that learning had become consolidated, retention was evident due to the LTM, and was a specific piece of learning of the CS+ odour (geraniol). The 'transient effect', reported by Bitterman et al (1983) in the learning may actually be due to the subjects having not yet completely learned the association, in that they require more than one trial. So this transient effect may just be a short term generalisation to any odour. This is seen in figure 3.8.5, where after one trial, followed by a novel odour, a response was gained this did not occur after two trials. These results are also in accord with Smith (1991) where he reports that honeybees need to generalise in order to minimise mistakes, by passing over similar floral odours that could contain a nectar reward. He goes on to suggest that a generalisation gradient may be apparent, so that bees do not just learn one odour, as they could visit a flower just depleted of its nectar reward. This ties in with the olfactory PER paradigm being more than one trial learning, as initially it is generalisation, but after further trials, or visits, it becomes a specific piece of learning.

3.8.6 Presentation of a novel odour, after three conditioning trials

3.8.7 Method

After the three initial learning trials a one hour consolidation period was given, after

this, the bees were divided into two groups (A and B).

Group A were once again tested unreinforced with geraniol, followed by a presentation

of peppermint, and finally another presentation of geraniol.

Group B had an unreinforced presentation of peppermint, followed by two further

presentations of geraniol, unreinforced, after the one hour consolidation period.

This experiment was carried out with 70 honeybees.

3.8.8 Results

The aim of the second experiment was to show that this learning does not lead to

generalisation between odours. In this second experiment, a one hour consolidation

period after the third trial was given (see figure 3.8.8). The subsequent testing of a novel

odour on the first or second trial after consolidation was carried out, with the data

suggesting that after three trials, the association of the PER paradigm has been stored in

the LTM. This was shown by the testing of the novel odour, in this case peppermint

(after geraniol as the CS+). In both the fourth and fifth trials, the subjects showed a large

decrease in response, probably not due to fatigue or the association failing to

consolidate, as further testing with the CS+ restored the levels of learning to the

previous probability before the one hour consolidation period.

Figure 3.8.8. The effects of presenting a novel odour (peppermint) after a one hour consolidation period (between the 3rd and 4th trial)
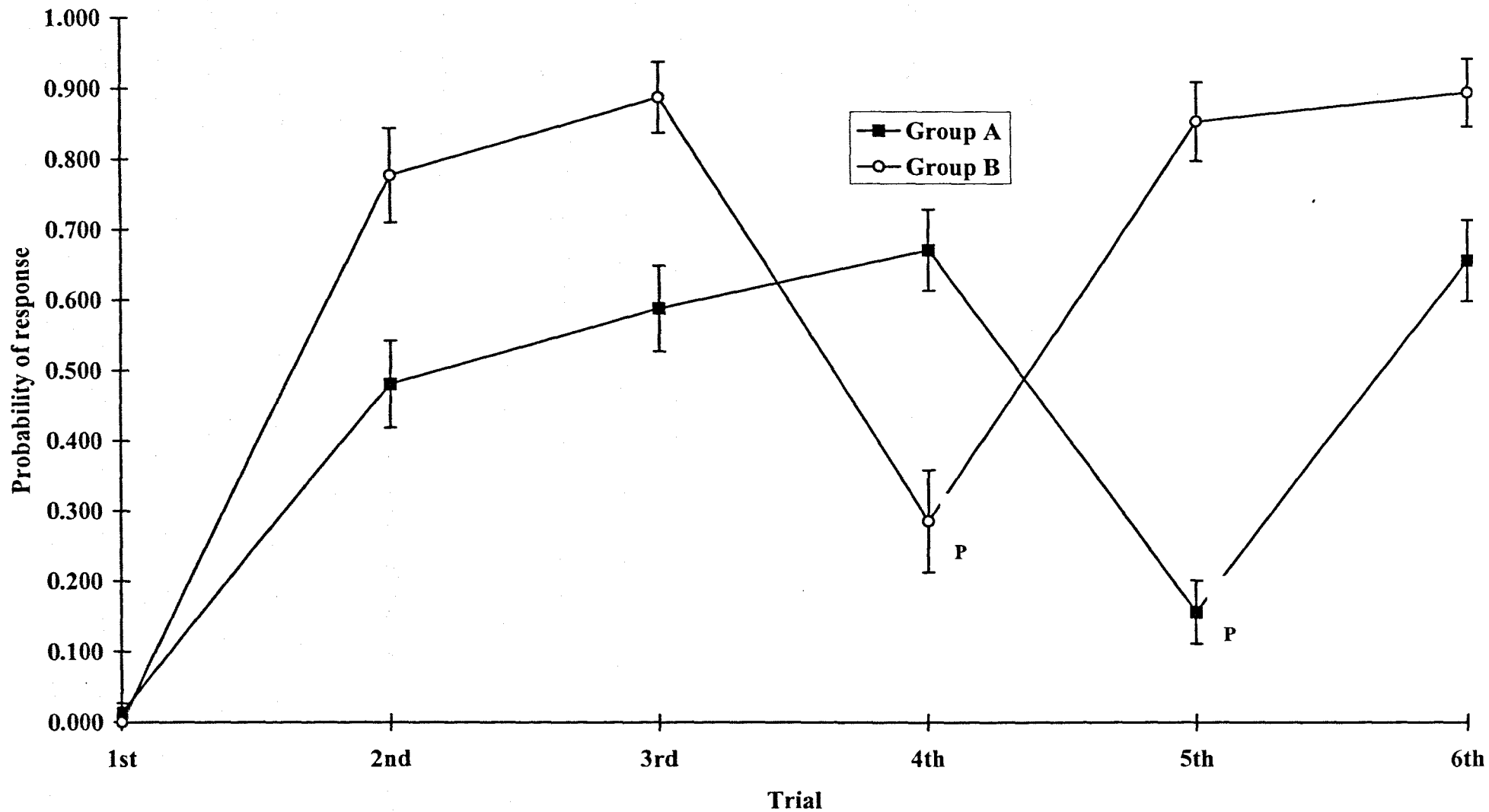
P = Peppermint presentation.

G = Geraniol presentation.

Group A = G, G, G, G, P, G.

Group B = G, G, G, P, G, G.

3.8.9 Discussion

The original question to whether this olfactory learning is a one trial learning paradigm can now be addressed following the experiments reported. If PER olfactory learning is one trial, then this suggests that generalisation could not occur, as the honeybee will have made the specific association between the odour and the reward, on a single trial, and hence, subsequent trials with a novel odour would not elicit a response as a firm association would have been made. However, if this form of learning is not one trial, then the bee could theoretically indicate any flower it had visited whilst foraging, thus negating any potential exploitation of the food source. Also, if generalisation was occurring in the bees' world, it would make it almost impossible for the bee to carry out its normal behaviour, as every odour would be met with an extension of the proboscis, whether this be a flower or a nestmate. So, there must exist a generalisation gradient (Smith, 1991) whereby the honeybee must initially generalise to ensure obtaining a nectar reward, but this generalisation gradient must reduce, enabling the bee to learn an odour that associates with a food reward, and it is this information that is taken to the hive. These comparisons between the laboratory established results and what occurs in field based experiments cannot therefore be compared in this context, as a bee does not use one trial learning in the field, as it visits the same flower patch many times before returning to the hive, i.e. more than one trial, and many experiments have shown the accuracy of the dance to flowers or artificial feed stations that the bee has just visited (von Frisch & Lindauer, 1961). If the bee just visited a flower once, then on its return to the hive, it may not communicate the source, as it had not learned the association, it would not therefore be practical for the bee to just visit one flower on a foraging trip, before returning to the hive. Menzel and Bitterman (1983) pretrained free flying honeybees to visit an odourless feeding place, and then found that they required only

one exposure to a flower-like odour to select it with accuracy, when given a choice between two others. This is an inconclusive piece of evidence, as the bees had previous experience of the feeding place, rather than just one trial only with the odour, as recent evidence has reported that honeybees link sights with smells (Srinivasan et al, 1998). From the results reported, the initially high response to CS- shows that it was not one trial learning, as the bees showed a response, i.e. they generalised to the novel, untrained odour. This decreases with further presentations of the CS- as it was not reinforced, making it 'uneconomical' for the bee to respond, and therefore there is an extinction of the response. These results are backed by those found in Menzel and Muller (1996) and Hammer and Menzel (1995), who stated that the initially high response to CS- reduces as learning progresses. Menzel (1990) reinforced one group with four trials and another group with eight trials, then tested with a novel odour for a further four or eight trials, and found the bees did initially generalise, but this decreased as the amount of presentations of CS- increased, with response rates returning to 0 after six trials. This showed that the PER olfactory learning paradigm cannot be one trial learning, as generalisation between stimuli is occurring. However, the levels of response to CS- after four or eight reinforced trials to CS+ would have been expected to be lower than those recorded, as our results indicated (figure 3.8.5).

The protocols in which the honeybees are handled and prepared could also affect the learning, and the interpretation of the results. Takeda (1961) kept his bees for 1-2 days before feeding them, presumably to increase motivation to learn. However, this would no doubt have a negative effect on their health, and make them more likely to give erroneous results due to them responding to any stimulus for a food reward. Other researchers have used short inter trial intervals of five minutes (Menzel & Bitterman, 1985; Hammer & Menzel, 1995), which have been shown to be in the period of transition from short term memory to long term memory (Erber et al 1980), and so a

further test of a CS- would not be a true test, as it is only testing short term memory, and not consolidated learning.

In conclusion, from our results, there are two overriding findings, the first is that the learning is not one trial, as generalisation cannot occur as well, you can't have your one trial cake and eat the generalisation slice too !! Secondly, the bees do learn an association between a CS+ and a US on the first trial, but this becomes specific to that training odour only with subsequent trials. Therefore, PER olfactory learning is not one trial learning, but is at least a two trial learning paradigm.

3.9 Overall conclusions to the replications of parametric characteristics

The purpose of this chapter to was to ensure that the methods by which the capture and training of the honeybees from the colonies used in this thesis provide results which are directly comparable with the established literature and research into the proboscis extension reflex olfactory learning paradigm.

3.9.1 Overshadowing

With the overshadowing, the results obtained indicated that this particular phenomenon did take place, with one odour having a greater salience than another.

3.9.2 Blocking

The results supported the findings of both Menzel (1990) and Couvillon et al (1983), that no significant blocking effects were observed.

3.9.3 Pre-US exposure

Pre-exposure to the unconditioned stimulus prior to training did show a negative effect on the subsequent training, again, this agreed with the research by Bitterman et al (1983) and Lubrow (1973).

3.9.4 Random presentations

A protocol of randomly presenting the conditioned and the unconditioned stimuli, independently, to the honeybee, does disrupt the learning processes. Not pairing the CS with the US, means that an association is not formed, with little or no response being evidenced.

3.9.5 Habituation

There were signs of an habituation to the learning, but not as clearly defined as found in other research (Bicker & Hahnlein, 1994; Braun & Bicker, 1992; Menzel & Muller, 1996). There did appear to be an habituation response, but this was not found to be statistically significant.

3.9.6 Massed and spaced trials

The training utilising a spaced trials technique, with an inter trial interval above 7 minutes, gave the strongest acquisition and retention curves, as this has been reported to be the time required for the learning to be consolidated into the amnesia resistant long term memory (Erber, 1975a, b, 1976; Erber et al, 1980; Gerber et al, 1998)

Massed training led to lower less reliable response rates, due to the learning only being a transient effect, which showed no long term retention.

3.9.7 Extinction

This section indicated that extinction of the learning was possible when the subjects were each given a set number of unreinforced trials, following reinforced training trials. Again, this was in accord with the literature.

3.9.8 One trial learning ?

In this final part of this chapter, the data presented suggest that the olfactory learning is not a one trial learning paradigm, but requires at least two trials for it to be consolidated into the long term memory. Generalisation was also not a clear cut phenomenon, and as suggested by Smith (1991) may indeed have a gradient, which reduces with further experience to the reinforced stimulus.

3.9.9 Conclusions

In conclusion, the methods presented and used in this thesis give equivalent results to the literature, which can be replicated, and therefore utilised to form the backbone to this thesis without providing erroneous or unqualified data.

CHAPTER 4:

4.0 The effects of time linked learning in the laboratory

4.1 Introduction

The behaviour and activity of many vertebrates and invertebrates is regulated by

circadian rhythms, controlled by an internal clock (Aschoff, 1955). Such control

manifests itself behaviourally in many ways, such as mating (Immelmann, 1980),

feeding (Sanchez-Vazquez & Tabata, 1998) and general activity (Roberts, 1965). For

the honeybee, an animal governed by a circadian rhythm (Koltermann, 1974), much of

its behaviour is regulated by such temporal organisation (Renner, 1960), with a sense of

time being crucial for its ability to exploit a specific food source at a specific time

(Moore, Siegfried, Wilson & Ranke, 1989). This time sense is also advantageous for the

bee, to facilitate more efficient foraging strategies, with many flowers yielding their

greatest pollen or nectar at a specific time of day. The ability of bees to remember a

particular time is consequently linked to their food source, nectar from flowers, which

have a defined time when their nectar flow and concentration is at its greatest

(Michener, 1974), this obviously differs from species to species, but the bee must

remember the time of day it visited a particular flower when it elicited the highest nectar

concentration (Menzel & Erber, 1972; Lee & Bitterman, 1990; Loo & Bitterman, 1992).

Hence, this sense of time is also interlinked with the bees' motivation to learn and

memorise a particular set of instructions as to where the flower is located from the hive,

and the concentration of nectar yields at particular times (Gould & Gould, 1995). Many

researchers have shown honeybees able to link a particular behaviour with a specific

time of day, with Gould (1987a) training bees to land on a particular petal of an artificial

flower at a particular time of day, showing the bee stored actual flower landing behaviours with specific times of day. In 1910, Auguste Forel recorded that bees visited his table at midmorning and afternoon tea, returning at these specific times on successive days, and only ceasing to appear after no food was left out for a few days. Koltermann (1971), in a more controlled study, fed honeybees at a field feedstation where food was initially available constantly. After bees became familiar with the feedstation, it was placed on a scented card, at two specific time intervals. 24 hours later, the bees had a choice between the trained odour, or a novel scent at the feedstation, both of which were unreinforced. The bees consistently preferred the trained scent with increased activity around the two trained times. These data suggest an internal mechanism for this time linked learning as the experiment was conducted indoors, under constant illumination, thereby eliminating any external stimulus, such as sun position. Additional support for such an internally controlled 24 hour circadian rhythm was provided by Renner (1960) who trained bees in France to a feedstation at a particular time, and then transported the hive to New York. Upon release the following day, they appeared at the feedstation 24 hours after their last feed, instead of 29 hours, which would have been the correct time in New York, if they were using the sun's position. Bees have also been trained to five different temporal relationships with different food rewards (Beling, 1929; Wahl, 1932). Martin, Lindauer and Martin (1983) found that free flying bees once trained at a particular time will then, the next day, respond better to testing at that time rather than an earlier or later time. Beling (1929) tried to train bees to feedstations unsuccessfully to a 19 hour or a 48 hour cycle, he could only entrain a 24 hour rhythm.

The training time to test interval reported in many papers (reviewed in chapter 3) is for the honeybees to be captured from the hive, fed, and then kept overnight, for the conditioning to begin the following day (Menzel & Bitterman, 1983; Gerber et al, 1996;

Gerber et al, 1998). Other researchers have caught the subjects either in the morning to be trained in the afternoon, a delay of about six hours, or have been caught in the afternoon, and trained the following morning, a delay of about twelve hours (Bitterman et al, 1983). Other researchers have used considerably longer delays, with Gerber et al (1998), restraining the bees for up to 4 days after conditioning, feeding once a day with sucrose solution. These bees showed long term retention when trained with inter trial intervals of 20 minutes and 1 minute in the initial learning trials. With these long capture to test intervals, a motivation factor may become apparent, with the subjects who are left for long periods without food, perhaps becoming more likely to respond due to an increased motivation, or an alternative hypothesis to this could be that a circadian rhythm is becoming apparent.

There is some contention between laboratories, as to whether there is a circadian time linking in memories, with Menzel (1990) suggesting that there is no evidence of any further function of time, such as an automatic circadian rhythm of memory formation or memory retrieval, and that the time of day does not rank very high in the hierarchy of learned cues. However, Koltermann (1974) had previously shown that the long term memory of the honeybee is strictly time linked for a number of different kinds of food signals. It must be stated that all these reported experiments were carried out using freely flying honeybees.

This chapter addresses the effects of time linked learning on laboratory based olfactory conditioning, with bees conditioned to specific odours at specific times of the day. The subjects were subsequently tested for retention of the olfactory learning at either the same time of day, i.e. 24 hours later, or at 21 or 27 hours post training. Previous studies into time of day learning have used free flying honeybees, who are also allowed to forage naturally outside of the training regime. The study reported has taken this learning from the field into the laboratory, which offers a more controlled environment

in which to investigate the individual aspects of the learning and the time linked learning that may underpin this learning. The laboratory setting also allows us a dissection of the interaction between the conditioning stimulus and time. No systematic study of this learning has previously been carried out in the laboratory, and it is to this end that this chapter is addressed.

In the experiments reported in this chapter, we aim to ascertain if there is a circadian link with the time the honeybees are trained, with the subsequent re-tests at various time intervals. This attempts to show that honeybees, when conditioned in a laboratory environment, are more sensitive to the time of day this association is formed, and will respond greater at these training times, when tested the following day.

4.2 General methods

Collection and restraining techniques were as reported in chapter 2.11, with a total of 251 bees being collected and divided into three separate training schedules. All bees were initially given three reinforced training trials, with geraniol as the CS.

The inter-trial interval was also kept at a constant 20 minutes, with the training trials conducted between 1200-1300 GMT.

The bees were kept together in the same high sided plastic tray, but were split into three groups, one third were tested between 0900-1000, 21 hours after the initial training, another third at 1200-1300, 24 hours after the initial training, and the final group at 1500-1600, 27 hours later.

4.3 Training and testing honeybees at specific times

An initial experiment was conducted to ascertain if there was a difference in retention of learning between groups of bees trained at a specific time of day in the laboratory, then tested at the same time the following day, 24 hours later, or after 21 hours or 27 hours.

4.3.1 Method

The bees were each given 3 initial learning trials, all reinforced with 1.5 molar sucrose solution, as detailed above. The following day, on the 4th to 6th trials, the subjects were presented with the odour (CS) alone, without the sucrose reinforcement. This was to test the long term retention of the olfactory learning and to assess if the time taken to retest affected the retention levels. This experiment mimics those carried out with free flying bees (Koltermann, 1971, 1974; Moore et al, 1989; Menzel & Erber, 1972; Lee & Bitterman, 1990; Loo & Bitterman, 1992; Martin et al, 1983), in that a specific time window has been trained, and the following 'test' day the previously rewarded feedstation is empty, or in this experiment, there is no sucrose reinforcement, only the trained odour. As a control, a group of bees followed the protocol as above, but the 4th to 6th test trials followed on without a days break, with the same inter-trial interval.

4.3.2 Results

Bees in all groups readily learned the association, however, it is interesting to note that the levels of learning were low, with only a probability of 0.4 after the third trial. This is probably due to the time of year these experiments were undertaken (Ray & Ferneyhough, 1997a).

At retest, it is evident that there is an initial increase in the response on the fourth trial, followed by a decrease or plateau in the subsequent fifth and sixth test trials (see figure 4.3). However, the bees tested after 21 hours showed a decrease in response on their fourth trial ($\chi^2 = 3.93$, p = 0.047, d. f. = 1, between bees tested after 21 and 24 hours). This is also exhibited by the control bees, where extinction of the response occurs after trial 4. The group tested at the same time of day as the original training elicited the greatest PER response at retest on the fourth trial, but was only slightly higher than the group tested after 27 hours. This unexpected high response by the bees tested after 27 hours could be due to motivational factors, due to not feeding for 27 hours. These bees also retained their high PER levels on the subsequent unreinforced fifth and sixth trials, showing no extinction, which is in contrast to the group who were trained and tested 21 and 24 hours after their initial training trials ($\chi^2 = 2.11$, p = 0.146, d. f. = 1, between the groups tested after 24 and 27 hours, on the sixth trial).

The bees tested at the same time of day as the original three training trials initially gave a high response to the fourth trial, then extinction began to occur. On the sixth trial, the response levels were equivalent to the levels of learning observed after the three initial training trials. The downward direction of the learning curve suggests the beginning of the extinction of the association, as the CS is no longer accompanied by a sucrose reward. With the group tested 21 hours after they were trained, a slight decrease in the response was evidenced on the fourth trial, and by the fifth and sixth trials, the learning had decreased to almost 0. The response of this group could be due to the bees being tested 21 hours after training and so this does not readily associate itself to a 24 hour time linked learning.

Figure 4.3. Probability of responding to an olfactory stimuli at specific times the next day, following initial training at 1200-1300
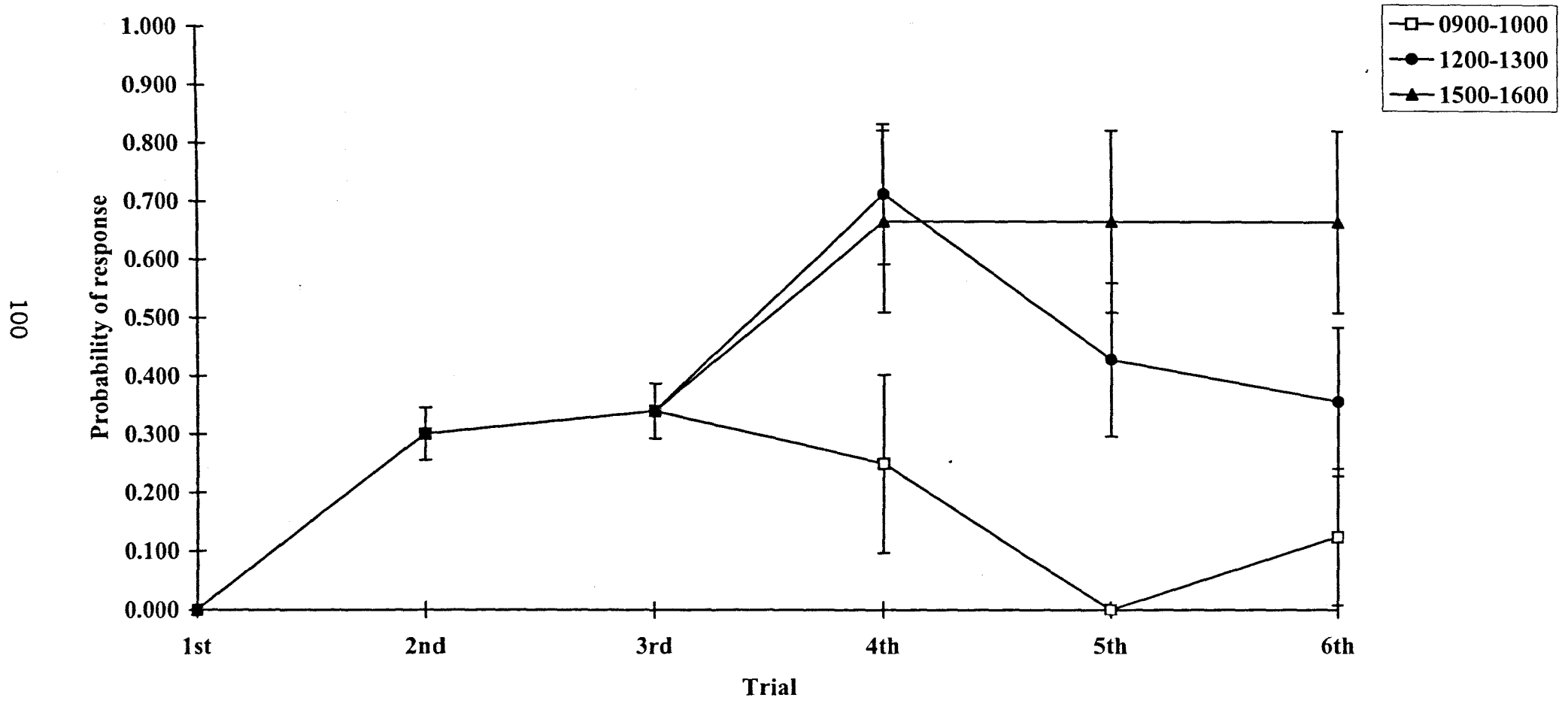
4.3.3 Summary

In summary, bees tested 24 or 27 hours after they were initially trained, show an increased response on the first testing trial (the fourth trial), followed by a decrease or stabilisation of the response. This could be due to the 24 hour tested bees responding, as they were tested at the same time of day as they were trained and were therefore showing a 24 hour time linked effect. With the 27 hour retest group a motivational factor, due to the length of time between feeding could be responsible for the increased response, even though the fourth to sixth trials were unreinforced. The bees tested after 21 hours showed signs of extinction of the learning on the subsequent test trials, as they were being tested earlier than the time they were trained. This response is also that exhibited in the control group.

4.4 The potential effects of hunger on PER testing

The aim of this section was to address any interaction of motivation, with retention of learning, and so the following study was conducted.

4.4.1 Method

The design involved three initial learning trials, between 1200-1300, as detailed in the method above with trial 4 the following day being reinforced, but trials 5 and 6 were unreinforced, these tests were all to the training odour geraniol. Again, the bees were split into three groups for the re-test, at either 21, 24 or 27 hours after the initial three olfactory learning trials. All the trials were spaced trials to the same constant inter-trial interval of 20 minutes.

The fourth trial would initially test for a retention of the learning, and as it was reinforced it will eliminate any increased motivation due to hunger. The reinforcement associated with the fourth trial at the varying times may also form a new time link with the odour, with the bees associating the new time after 21, 24 or 27 hours with the sucrose reward.

4.4.2 Results

After the initial three trials, around 50% of the bees gave a PER response (see figure 4.4). On the fourth trial, the groups tested 24 and 27 hours after the initial three training trials again increased their learning. This is in contrast to the honeybees tested after 21 hours who gave response rates equal to their initial 2nd and 3rd training trials ($\chi^2 = 7.66$, $p = 0.0057$, d. f. $= 1$, between groups tested after 21 and 24 hours on the fourth trial), and their response rates remained at these levels (of about 50%), with no apparent extinction of the learning. On the subsequent unreinforced fifth and sixth trials, the learning responses of each of the 21, 24 or 27 hour tested groups remained at the levels of learning equivalent to those elicited on each of their fourth trials. This suggests that the bees had made a new time linked association on the fourth trial. The results in figure 4.4, when compared with those in figure 4.3, imply that there is a motivational factor playing a role, with the results from figure 4.3 showing an extinction effect after the unreinforced fourth trial, compared to experiment 4.4, where there is a stabilisation in the response rates after the fourth reinforced trial. Although the motivation factor appears to have been countered, a new time linked association may have been made, with this new association being more salient to the honeybee than the original association learned the previous day.

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Figure 4.4. Probability of responding to an olfactory stimuli after training at a specific time (1200-1300)
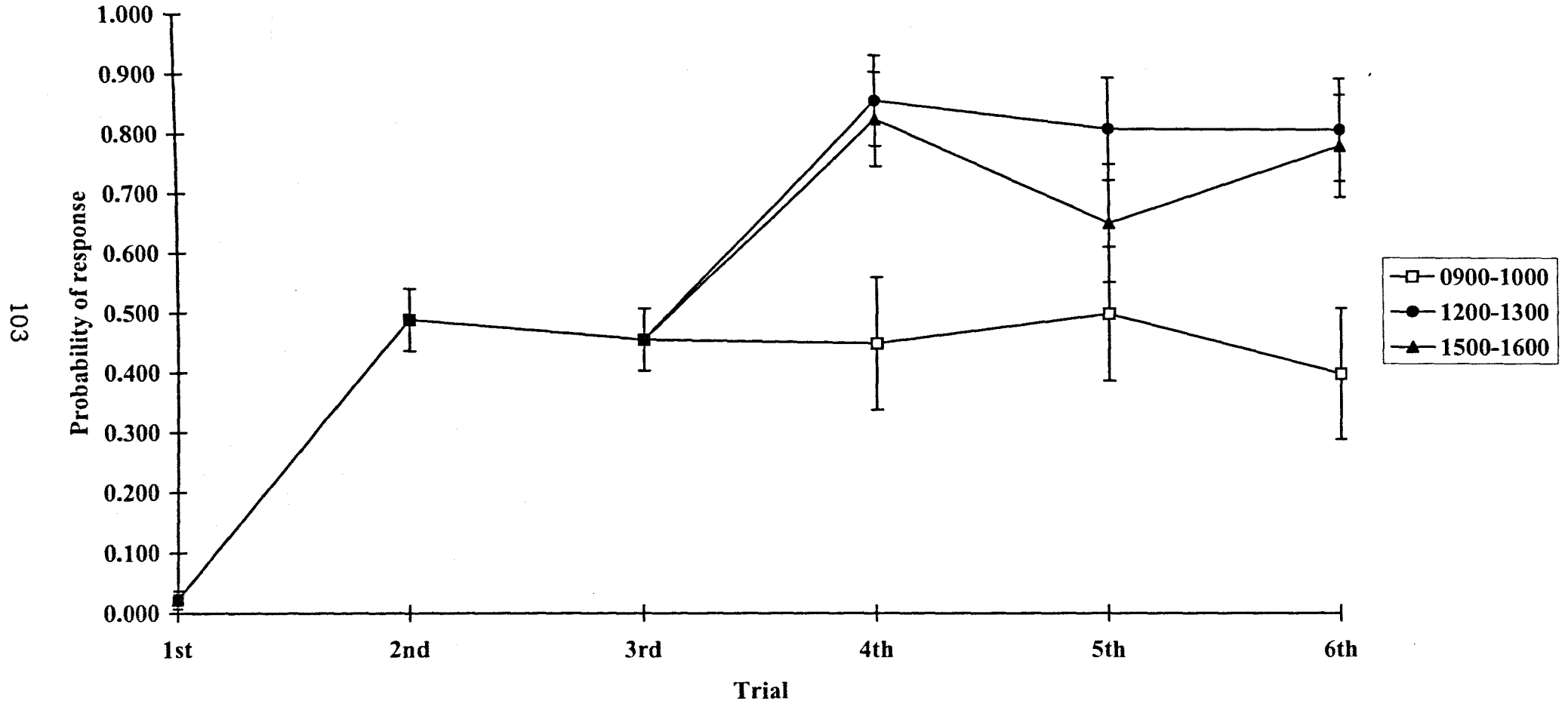
4.5 Conditioning over a specific period, then testing the following day with a novel odour

Now that there appeared to be a time linking of the learning, we carried out a third experiment, which examined if the bees were responding specifically to the trained odour, or if they would respond equally to an untrained, novel odour. This experiment was designed to illustrate if the honeybees were simply generalising their responses to any odour, when tested the following day.

4.5.1 Method

The protocol of the experiment was as before, in that a group of bees were trained, with three reinforced geraniol odour trials, and tested the following day after either 21, 24 or 27 hours. This experiment differed in that the fourth trial was reinforced, to reduce any increased motivation to exhibit the learned behaviour, but a novel unreinforced odour, peppermint, was presented on the fifth and sixth trials.

4.5.2 Results

After the three initial training trials the levels of learning were very low, with a probability of 0.3 after the second trial, which decreased slightly to 0.2 following the third training trial (see figure 4.5). On the fourth trial, the following day, bees tested after 21 hours exhibiting low levels of retention, equivalent to those seen on the third trial. The bees tested at the same time of day as the original training elicited a marked increase in the learning ($\chi^2 = 8.22$, p = 0.0041, d. f. = 1, between the 21 and 24 hour groups on the fourth trial), up to a probability of response of 0.8, with the group tested

Figure 4.5. Probability of responding to a novel olfactory stimuli the following day after PER conditioning
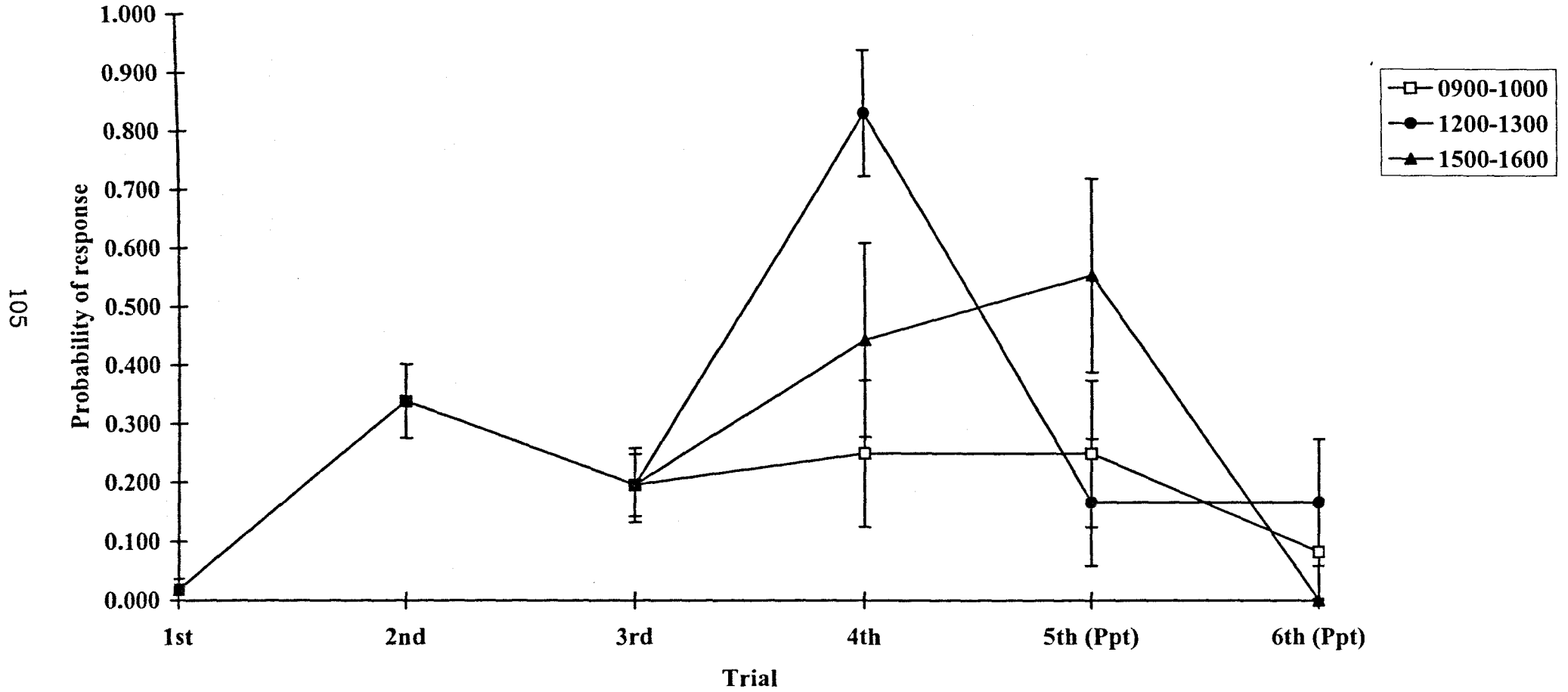
after 27 hours showing a slight increase in the response, as was evidenced in the previous two experiments. This fourth retention trial indicates a time linked association between the odour and reward. The next two trials tested for a generalisation of response to a novel, unreinforced odour to further test this specific response to the trained odour. The bees retested after 21 hours responded at an equal rate to their previous fourth trial, even though it was to a novel odour, suggesting a generalisation response. The bees tested after 27 hours, however, showed an increase in the response to the novel odour ($\chi^2 = 3.5$, p = 0.061, d. f. = 1, between the groups tested after 24 and 27 hours, on the fifth trial; $\chi^2 = 2.11$, p = 0.146, d. f. = 1, between the bees tested after 21 and 27 hours on the fifth trial), suggesting that this group were generalising their response to the novel odour, and may be increase their response due to an increased motivation. Bees tested at the same time of day as the original training showed a large decrease in their response ($\chi^2 = 2.7$, p = 0.097, d. f. = 1, between the fourth and fifth trials) showing no generalisation to the novel peppermint odour.

The learning abilities of the bees tested after 21 or 27 hours should not vary from the bees tested after 24 hours, as they were genetically matched bees from the same hive.

4.5.3 Conclusions

In conclusion therefore, time linked learning has been shown in the laboratory, but a motivational factor may be affecting the results, whereby the honeybees are artificially increasing the response due to hunger.

Figure 4.5.1 shows the combined data of all three experiments, up to the fourth retention trial, to show any time linked learning. The fourth trial was to test at either 21, 24 or 27 hours after the three initial learning trials. What is evidenced is that the group tested after 21 hours showed no increase in response from the third trial. Bees tested 24 hours

Figure 4.5.1. The effects of training honeybees at 1200-1300, then testing them at varying times the following day, using the data from the previous three experiments
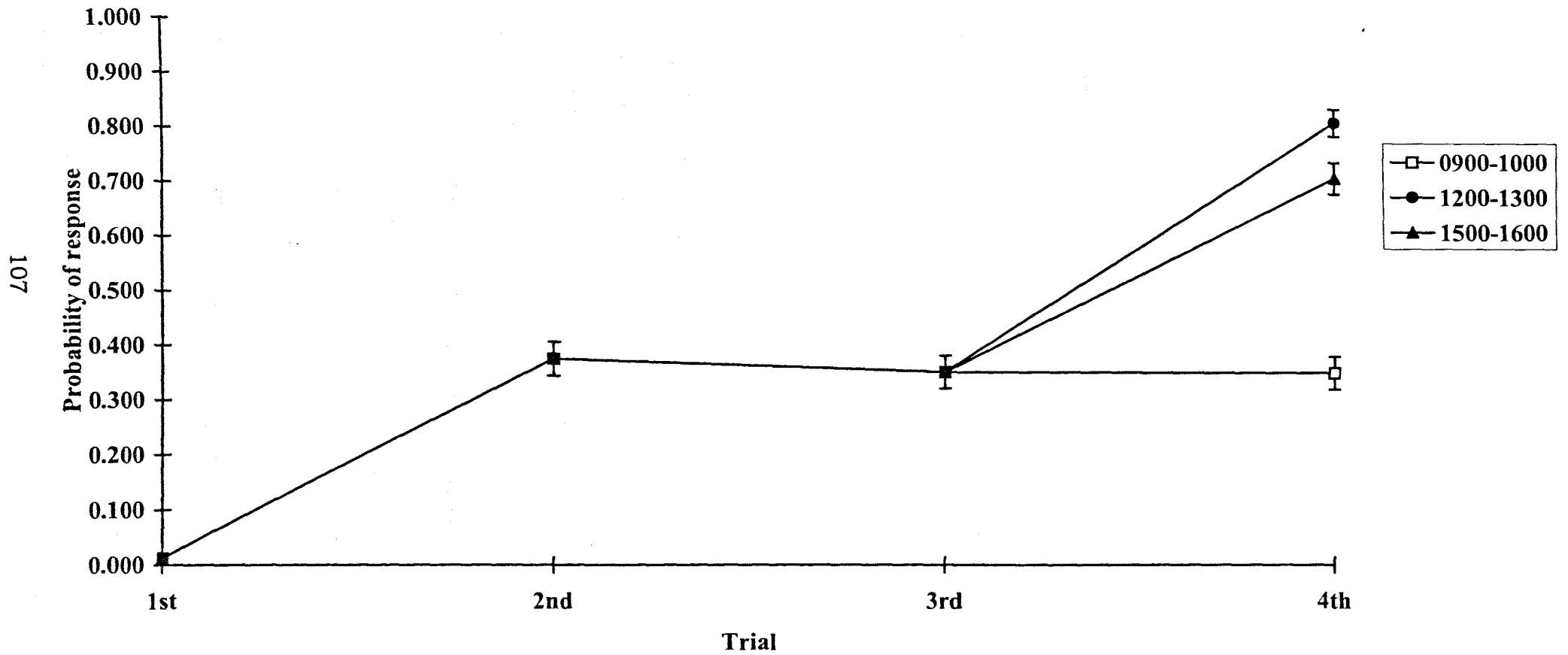
after they were initially trained, showed a significantly different increase in their response ($\chi^2 = 20.63$, p < 0.0001, d. f. = 1, between groups tested after 21 and 24 hours, on the fourth trial). Bees tested 27 hours after training also show a significantly increased response on the fourth trial, but not to the same levels as the bees who were tested after 24 hours ($\chi^2 = 3.32$, p = 0.068, d. f. = 1). Finally, there is also a difference between the two groups that were tested either 21 hours or 27 hours after they were trained ($\chi^2 = 7.85$, p = 0.0051, d. f. = 1).

The results indicate that bees tested either 21 or 27 hours post training show a reduced response level compared to bees tested on a 24 hour interval. This points to the inference that bees tested at the same time of day as the original olfactory training show greater responses due to the bees having a 24 hour time linked circadian rhythm, and not a 21 or 27 hour rhythm.

4.6 Discussion

Earlier field based research using free flying honeybees highlighted the importance of time linked learning in honeybee behaviour (Koltermann, 1971, 1974; Moore et al, 1989; Menzel & Erber, 1972; Lee & Bitterman, 1990; Loo & Bitterman, 1992; Martin et al, 1983). These effects are confirmed here in these laboratory studies with confined animals. The mechanisms of this laboratory time linked learning is analogous to that shown in free flying bees as the bees exploit food sources with the maximum efficiency and minimum effort, flying to flowers only when food is being produced (Koltermann, 1974).

Two main factors emerge from our results, the first of these is that all the bees showed a positive conditioned response effect, exhibiting levels of olfactory learning over the three initial learning trials. The second factor is that of motivation, as the bees were not

fed for anything from 21 to 27 hours after the three training trials, and before the test trials. This is not unusual in the literature where bees are routinely starved overnight prior to the experiment beginning (Bitterman et al ,1983; Menzel & Bitterman, 1983; Gerber et al, 1996; Gerber et al, 1998), to ensure equal levels of hunger in each of the subjects. Our results however, suggest that the larger time differences between feeding has a negative effect on the results, increasing generalisation and being deleterious to the experiment. Increased motivation has become a major factor in these data, as the experiments where bees are trained to field feedstations, at specific time windows are allowed to undertake natural foraging behaviour. These free flying bees are satiated for the remainder of the day, having learned the time linked association for food availability. The bees tested in the laboratory however, were given no other opportunity to feed.

The bees in these experiments did show parametric characteristics seen in other experiments, such as acquisition of the association, consolidation and long term retention.

In the first experiment (4.3) a sense of time was intimated in the results, however, due to between 21-27 hours since feeding the motivation has increased the response rate of the subjects. Levels of the response in bees tested 21 and 24 hours after the initial training are reduced, showing an extinction of the learning as these trials are unreinforced. The bees tested after 27 hours responded at a constant rate as the motivation has enhanced the learned behaviour. The next experiment (4.4) addressed this motivation factor by feeding the bees on the fourth trial. The response levels stabilised on the subsequent unreinforced 5th and 6th trials, and proboscis extension did appear to be due to the time linking of training to testing interval. The bees tested at the same time of day as the original olfactory training responded greatest, followed by the 27 hour test group, the finally the bees tested after 21 hours. The fifth and sixth trials were unreinforced, and

response levels remained constant, perhaps due to the motivation factor being countered, with the CS-US association being reinforced. In the final experiment (4.5), the bees tested after 21 and 24 hours showed no generalisation of their response to the novel peppermint odour, whereas the group tested after 27 hours did generalise their response on the fifth trial. This could be due to this group not learning the association, and the motivation factor causing these bees to generalise their response.

Further experiments to advance this research could be to train the bees in the laboratory for two cycles of 24 hours i.e. 6 trials, and then test after 21, 24 or 27 hours. Would this reinforce the time linking to a greater extent than the data reported here. Also, as the activity of the honeybee is under the control of the prevailing weather conditions, with bad weather or low temperatures confining the bees to the hive, when the weather next becomes favourable, the bees need to recall the flowers locations. Therefore, do they visit the same flowers last visited, and at a time linked to when they previously visited the location.

Motivation does affect the honeybee's time sense, when conditioned and tested at specific controlled times. The association has been demonstrated to be in the long term memory after three learning trials (Hammer & Menzel, 1995; Menzel & Muller, 1996), so the association between the odour, time and reward is a specific learning task. Honeybees do exhibit a time linked learning effect in the laboratory, which seems to be on a 24 hour circadian rhythm. However, care should be taken so as not to allow a motivation effect to mask the results. These results appear to back up the literature of training bees to a specific time at a feedstation in the field and this further supports the research by Menzel and Bitterman (1983), who found no difference in the learning between laboratory based and free flying honeybees.

4.7 The time course of memory formation

This research can also be viewed in conjunction with the time course of memory formation, whereby the actual time taken for the learning to progress from a working memory to an amnesia resistant long term memory is important. The honeybee has been shown to possess a short term memory (STM), intermediate term and a long term memory (LTM), and the time course between them has been measured (Erber, 1975a, b, 1976), whereby the STM was erased using experimental procedures such as narcosis, cooling or weak electroconvulsive shocks. The STM is coded in an ordered neural activity, whereas the LTM has more stable structural and biochemical substrates (Menzel et al, 1974).

In a further experiment, following Smith (1991), we decided to examine the actual time course of the memory formation, from short term to intermediate term, to log term memory, as proposed and demonstrated by Menzel (1979, 1983), Erber et al (1980) and Brandes et al (1988). They suggest that the memory takes the following time course, up to three minutes, the honeybees have a short term working memory, which changes from three to five minutes into a labile intermediate memory which is sensitive to cooling and is easily disrupted, and finally, after five to seven minutes, a more robust long term memory has been formed which cannot be disrupted by cooling.

4.7.1 Method

The experimental design was adapted from those by Brandes et al (1988) and Smith (1991), whereby the bees are given a single training trial, followed by a retention test a set inter-trial interval later.

The honeybees were caught and restrained as detailed in chapter 2.11, and then were allowed a three hour recovery period, which also served to increase the motivation to learn, due to an increased hunger. The bees were tested on three consecutive days, and were randomly divided into eleven groups. The inter-trial intervals used were 30 seconds, 1, 2, 3, 4, 5, 6, 7, 8, 10 and 14 minutes, these groups covered the short, intermediate and long term memory areas, and the subjects were each given a single training trial to a geraniol odour, paired with a 50 % sucrose solution.

4.7.2 Results

As can be seen in figure 4.7, the levels of learning exhibited by the subjects after 30 seconds was the same as the responses after 7 minutes and up to 14 minutes. This suggests that this is the asymptote of the learning for these particular bees, with these response rates of almost 70 % being in stark contrast to those exhibited between 1 minute up to 5 minutes. There is a reduction in the response rate to just under 6 % after 2 minutes, which proved to be significantly reduced from all the other groups ($\chi^2 = 3.2$, $p = 0.07$, d. f. = 1, comparing the 2 minute group against the 3 minute group, who were the group exhibiting the next lowest response rates), and the responses were much reduced when compared against the groups after 7 minutes, $\chi^2 = 14.568$ ($p > 0.0001$, d. f. = 1).

4.7.3 Conclusions

These results show that there does appear to be a short term memory effect, followed by an intermediate labile memory, and finally a long term memory. This bi-phasic effect is in agreement with the results gained by other researchers (Erber et al, 1980; Menzel,

Figure 4.7. Time course of memory formation
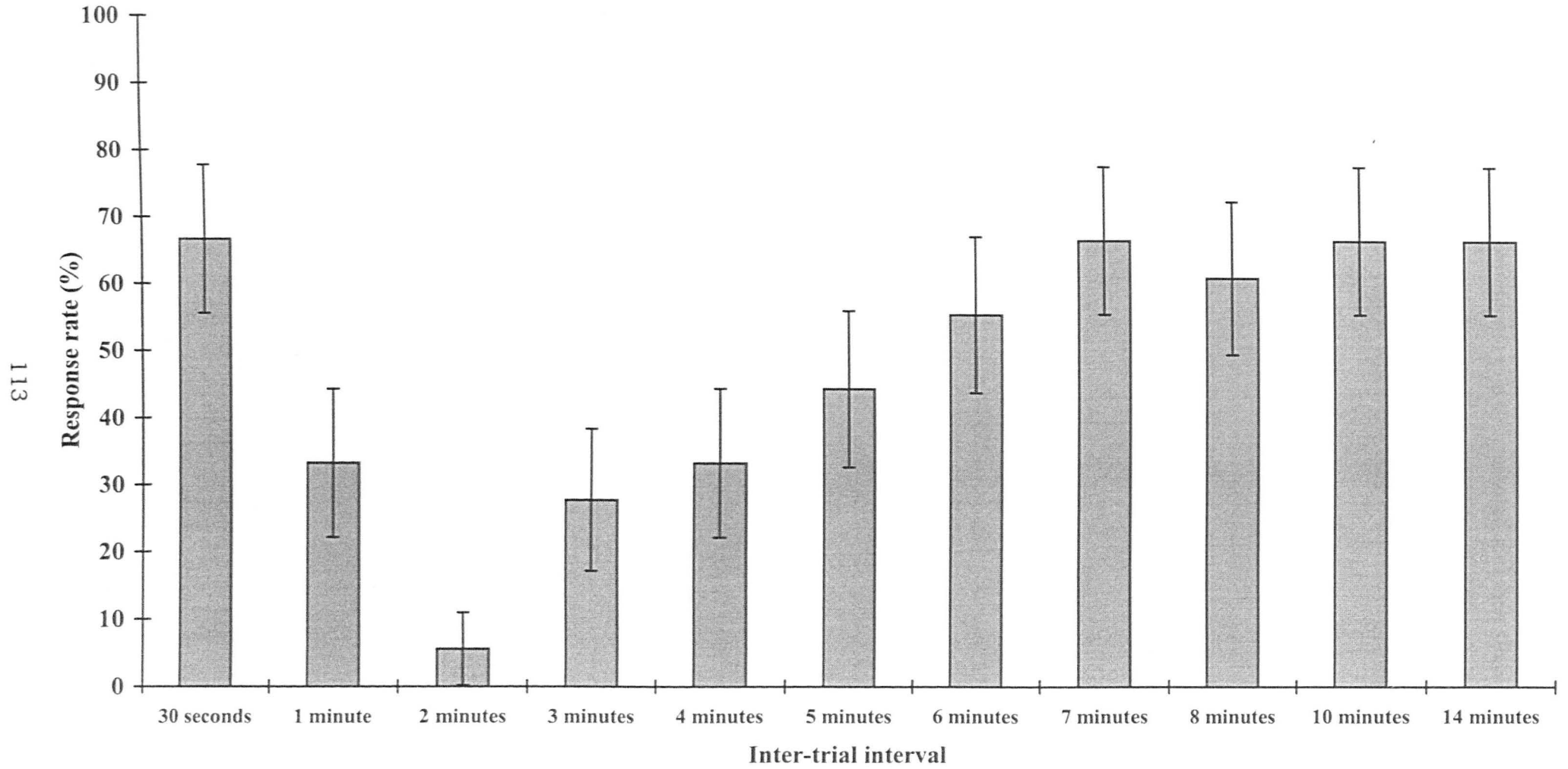
1979, 1983; Brandes et al, 1988; Smith, 1991), however, the results presented in this chapter show an initial short term memory up to 2 minutes, and then a labile memory up to 6 minutes, which is a slightly longer intermediate term than the literature suggests, which may be due to the time of year in which the bees were tested (see chapter 9). The biological interpretation of these findings are that a STM is erased if a different experience is made within about 30 seconds and Menzel (1968) found that honeybees had an average visit time per flower of 3 seconds, and an average landing time of 10 seconds. In sparser growing flower patches, the STM needs to be able to be corrected at longer intervals, and if these are unrewarded visits, then the memory will be erased, and a new memory will be formed (Menzel, 1982, 1983).

CHAPTER 5:

5.0 Motivation

5.1 Introduction

In this chapter, the effects of different concentrations of sucrose solution on olfactory learning are examined. Further, experiments investigating if there was an optimal concentration for PER training are studied.

PER conditioning remains a widely used learning paradigm in bee research. However, considerable variation exists between laboratories and training protocols (as reported in chapter 2.5), not least of which is the case of the sucrose concentration used as the reward. Historically, the concentration of sucrose used in laboratory, and field studies of PER conditioning is 1.5 molar (Menzel & Erber, 1972; Abramson & Bitterman, 1986; Brandes et al, 1988; Lee & Bitterman, 1990; Smith & Cobey, 1994; Bhagavan et al, 1994; Abramson, Aquino, Silva & Price, 1997). Other concentrations have also been used. Concentrations as low as 0.88 M (30% sucrose) (Dukas & Real, 1993; Sandoz, Roger, Pham-Delegue, 1995; Sigg, Thompson & Mercer, 1997), and 1.17 M (40% sucrose) (Couvillon & Bitterman, 1982; Menzel & Bitterman, 1983) have successfully been used, as well as more concentrated solutions such as 2.0 M (Koltermann, 1974; Menzel et al, 1974).

Free flying honeybees have a wide threshold range for sucrose solutions, which is equivalent to the concentration of nectar that they forage in the field, this ranges from 0.063-0.125 M to 1-1.5 M (von Frisch 1993). These observations were based on the occurrence of the dance in the hive to alert sister bees of a food source. Von Frisch later increased the sucrose concentrations gradually at a feedstation from 0.19 M to 2 M, in 6

steps, over a 3.5 hour period. The percentage of bees dancing increased from zero for 0.19 M, 53 % for 0.375 M, 73 % for 0.5 M, and 100 % for 2 M sucrose. Although, as von Frisch states, these figures have no general validity, as they were carried out in the field and were just general observations, but they do show the effect of increasing the sucrose concentration and its effect on the occurrence of dancing, and recruitment to the food source. This chapter addresses;

a) If bees have an optimal sucrose concentration for PER conditioning and

b) Do higher value rewards i.e. greater molarities, produce better learning.

The studies were conducted using a standard training paradigm to facilitate comparison between reward salience within the laboratory.

5.2 General methods

Capture methods and restraining were kept as standard, as reported in chapter 2.11, the bees remained in their holding tray until the experiment commenced, three hours later. In the previous chapter, the effects of learning at particular times of the day was investigated, and so to expand on from those results, the next area to examine was the motivation of the subject to learn, and so a series of experiments was undertaken to examine the effects of differing sucrose concentrations.

5.3 Varying sucrose concentrations and olfactory learning

5.3.1 Method

A total of 266 bees were caught and restrained as in the general method (chapter 2.11), and these were then split into eight groups, testing, over three trials, with the following

sucrose molarities; 0.5 M, 1.0 M, 1.5 M, 2.0 M, 2.5 M, and 3.0 M. In each group, the control was distilled water, i.e. 0 M sucrose.

In an initial experiment subjects were starved for either two or six hours before commencing the experiment. The results of this showed no difference in the initial levels of conditioning and retention, or in a response to a novel, untrained odour. So, the following experiments were briefly that the bees were captured in the morning of the experiment and brought into the laboratory where they were anaesthetised and restrained in plastic tubes. Three hours later, the bees received three training trials of odour and reward and the probability of PER was calculated.

5.3.2 Results

The results suggest there are no differences between the molarities of sucrose and the responses of the honeybees (see figure 5.3). The molarity that produced the greatest PER response of 0.7, after 3 trials, was 1.0 M and the lowest response was given for the 2.5 M concentration, with a probability level of 0.55. In the experiment reported, each bee was only exposed to one sucrose concentration as is usual in PER conditioning. It would be interesting to research the bees responses if they were trained and tested with a variety of differing sucrose concentrations, for example, the 0.5 M bees were only offered that concentration, but may have responded to a greater extent if they were then trained and tested with a higher concentration to see if their response increased. This would be a very complex experiment to undertake in the laboratory as each group of bees has to be trained to each different concentration, so that they each receive 21 trials with seven different sucrose concentrations. This would undoubtedly cause fatigue, and certainly satiate them ! Perhaps this is an example of a laboratory based

Figure 5.3. The effects of different sucrose concentrations on olfactory conditioning
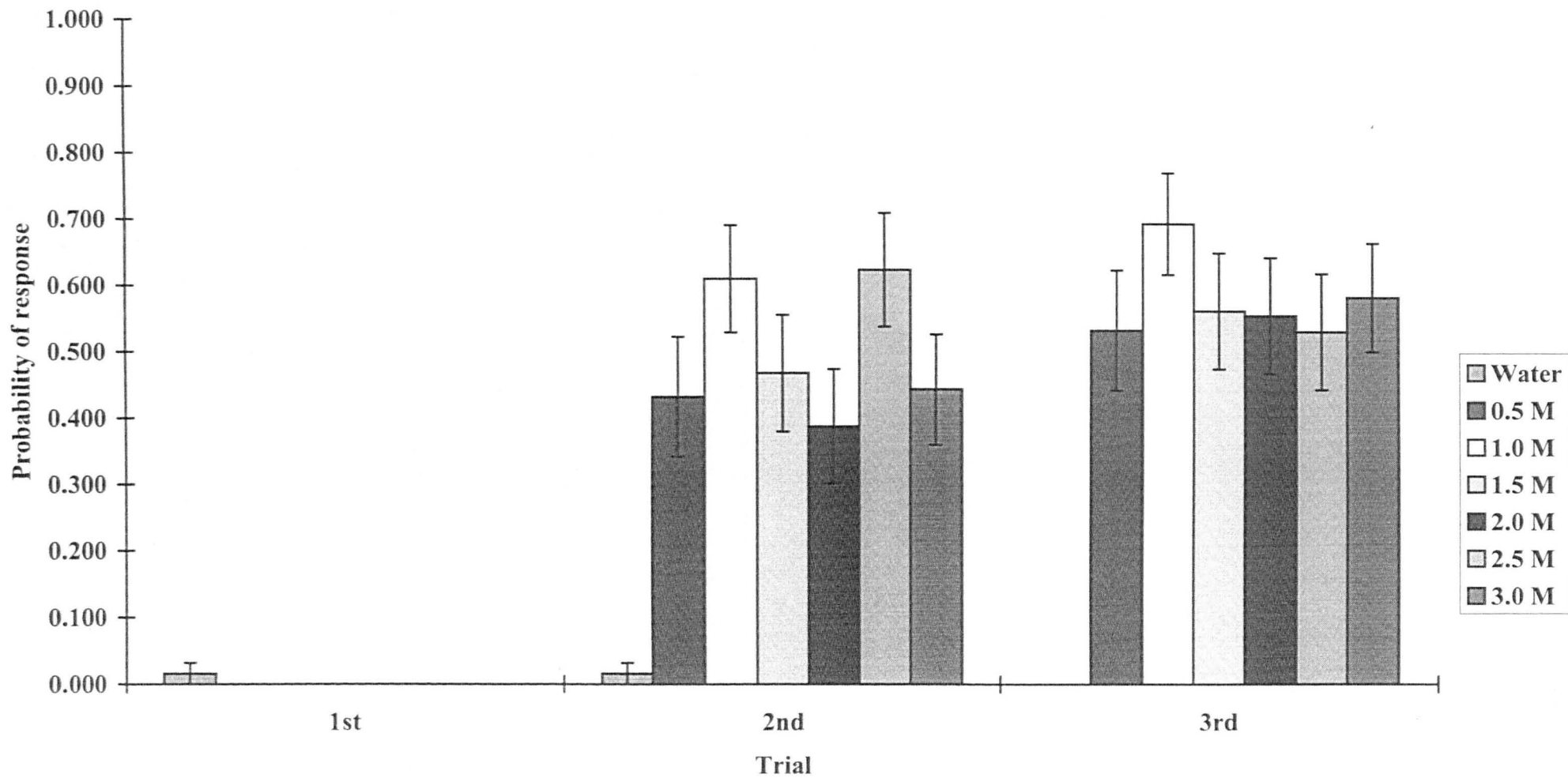
experiment that is better carried out with free flying bees in the field, as it is easier to see if the concentrations are sufficient, as the bees will return to the food source.

5.4 General discussion

Greggers, Kuttner, Mauelshagen and Menzel (1993) also state that the most important measure of profitability of a food source is the time spent licking per visit or trial, with the sucrose solution concentration being of secondary importance.

In the experiments using different sucrose concentrations, no firm conclusions can be brought, due to the imperfect experimental procedure. If anything, the honeybee subjects appear to show no variation with the differing sucrose molarities, and indicate no response to pure water. This is in contrast to Loo and Bitterman (1992) who did find that the bees visited more concentrated sucrose feed stations. Again though, these experiments were with free flying bees, who had previously experienced a lower molarity of sucrose. In our experiments, they were laboratory bound, and only encountered one concentration.

The results show that using a standard laboratory protocol for PER conditioning, the quality of reward i.e. sucrose concentration is without effect on olfactory learning in the laboratory. Bees appear to learn just as well with low concentrations as with high. This finding will facilitate comparative assessment of learning reported across laboratories using different reward strengths and further shows the remarkable strength of this learning paradigm. The results also suggest that further research is needed into the motivational aspects of bee learning in both field and laboratory preparations.

In conclusion, these results add to research by Menzel and Bitterman (1983), who compared the learning between laboratory based and free flying honeybees, and found no difference. This, however, cannot be said for our experiments into the bees'

motivation to learn using differing sucrose molarities, as free flying subjects produce

different results to our laboratory based studies.

CHAPTER 6:

6.0 Long term captivity effects on olfactory learning

6.1 Introduction

The honeybee has a prodigious memory, capable of memorising a visit to a flower for its lifetime (Menzel, 1990; Dukas & Visscher, 1994; Gould & Gould, 1995). However, little research has focused on the effects of captivity in a laboratory environment on this remarkable long term memory of the honeybee, specifically the olfactory memory. All this, despite the fact that laboratory based studies of such memory, with PER remaining the most prolific bee learning paradigm. If the amount of time the honeybee is captive is detrimental to the subsequent performance of the bee on learning experiments, then this will lead to inaccurate assumptions about bee olfactory learning and its manipulations, and could lead to inter and intra laboratory variability in the literature.

One of the major features of bee learning is the production of a stable and enduring memory with relatively few trials. From field studies, olfactory learning has been suggested to last a lifetime after 3 trials (Menzel, 1990; Menzel & Muller, 1996; Hammer & Menzel, 1995). A review of the PER literature reveals the captive time of the bees prior to memory testing (training time to test) varies in many reported papers. Similarly, studies of long term retention requires bees to remain restrained, which may influence both learning acquisition and retention. A frequently cited method is for the honeybees to be captured from the hive, fed, and then kept overnight, for the conditioning to commence the following day (Menzel & Bitterman, 1983; Gerber et al, 1996; Gerber et al, 1998). In similar research, bees have been caught, either in the morning to be trained in the afternoon, or in the afternoon, and trained the following

morning (Bitterman et al, 1983). The longest captive period was carried out in the experiments by Gerber et al (1998), where the bees were kept restrained for up to 4 days following conditioning, being fed once a day with a sucrose solution. These bees showed long term retention after 4 days, when trained with inter trial intervals of 20 minutes and 1 minute in the initial learning trials.

When honeybees are held captive in the laboratory for a period prior to training, this may affect the motivation to learn, as the subjects hunger increases and this may lead to a generalising of response to any presented stimulus. The experiments reported in this chapter develop a technique for holding bees in the laboratory in plastic bee boxes, with food available ad lib, thus providing a method to assess long term retention of PER without retaining the bees in a restraint. These methods are quite artificial when compared to the majority of experiments carried out in the field, as the bees are allowed to remain in their hives, whereas in the experiments reported here, the bees are removed from their hive and placed in plastic 'bee boxes', which were devoid of the social interactions with a full hive community. This storage method prevented the honeybees from communicating any learned associations, with this social isolation (although the bees were kept in boxes with on average 25 other bees) perhaps causing a precocious development of the bees (Ray & Ferneyhough, 1997b, 1999), a phenomenon further explored in the following chapters.

6.1.1 Method

Bees were collected from the hive entrance and restrained as previously described. The bees were then given a 2 hour recovery period, after which they were presented with conditioning trials. During this period, each bee was numbered with a small plastic disc, glued onto the thorax, this enabled each bees learning performance to be followed

throughout the experiment, and also to monitor any mortalities that may occur. The experiment consisted of 3 learning trials, with a constant inter-trial interval, after which the bees are given a 2 hour consolidation period to allow the learning to be consolidated into hypothermia resistant long term memory (Ebadi et al, 1980; Erber et al, 1980). These bees were then re-chilled to 4 °C for 10 minutes, unstrapped, and placed in groups of 20 to 30 in clear perspex boxes (dimensions 7.5 cm by 7.5 cm by 9.5 cm), as previously described in chapter 2 (see photos 8 - 11). In each of these boxes was a small tray of bee candy, which the bees had ad lib access to, it was changed daily, and the boxes were placed in a dark, well ventilated area. The bees were kept captive in these boxes for either 2, 4, 7, or 9 days. After this captivity period, they were removed from the boxes, into 25 cm³ vials, and anaesthetised at 4 °C as before, and then restrained in the tubes, ready for testing.

We have found that bees given a second procedure of anaesthetic, restraining, and testing, showed no difference in the retest, than bees given a single cycle of anaesthesia and restraining. The bees were given a 2 hour recovery period, and then a fourth unreinforced test trial was presented, which tested the long term retention of the initial association. The fourth test trial was carried out at the same time of day as the original trials, to compensate for any time of day effects (Ferneyhough & Ray, in prep), with the same inter-trial interval. The next trial was reinforced to reaffirm the association, the sixth trial was unreinforced to test for learning. A seventh trial was unreinforced to a novel odour, peppermint, this was presented with no reinforcement to assess any generalisation to the stimuli. Finally, an eighth trial to the original conditioned stimulus was presented to retest the established learned association. For a breakdown of the testing, see figure 6.1.

1st trial, geraniol reinforced

↓

2nd trial, geraniol reinforced

↓

3rd trial, geraniol reinforced

---------------------------------- captivity of 2, 4, 7 or 9 days

4th trial, geraniol unreinforced

↓

5th trial, geraniol reinforced

↓

6th trial, geraniol unreinforced

↓

7th trial, peppermint unreinforced

↓

8th trial, geraniol unreinforced
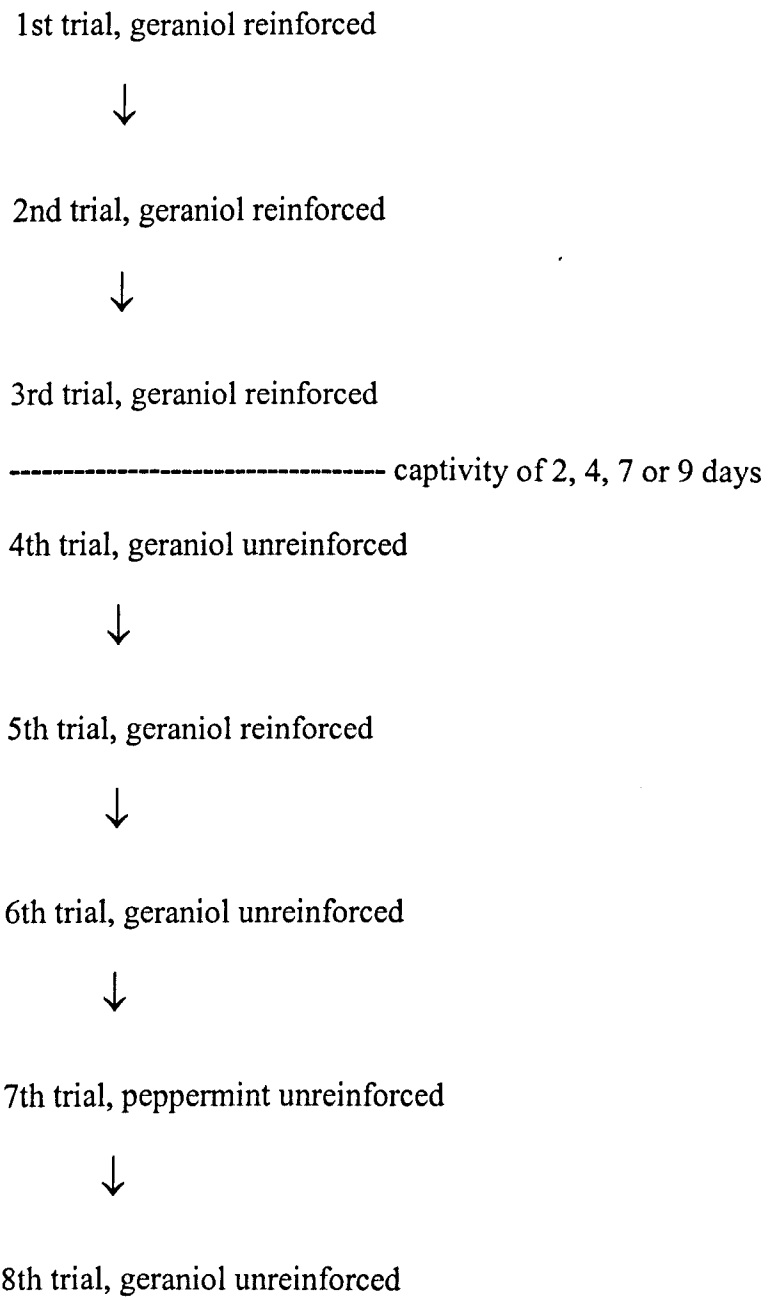
Figure 6.1. Breakdown of training and testing given to bees, before and after training.

6.1.2 Results

In the initial three learning trials, levels of learning were equal to those reported in both this and other laboratories (Bitterman et al, 1983; Gerber et al, 1998; Menzel, 1985, 1990; Takeda, 1961), and there was no significant difference between any of the groups. This trend continued to the first unreinforced retention test, with the levels of response equivalent to those presented after the third trial. This result indicates that the learning had been consolidated into the long term memory, and as such suggests that captivity does not affect the long term retrieval of the learned association. There were exceptions to this however, as bees captive for 9 days showed a reduced response on their retention trial, but this did not prove to be statistically significant.

Following the fifth (reinforced) trial, the responses on the (unreinforced) sixth trial increased suggesting learning. This was apparent for each of the four groups (see figure 6.1.2), but only the 9 day captive bees gave a significant result ($\chi^2 = 5.8$, p = 0.016, d. f. = 1).

On the following generalisation trial, when each group was presented with the novel peppermint odour, a significant reduction in the learning was apparent, when compared to their previous trial (trial 6), with 2 day ($\chi^2 = 56.5$, p < 0.0001, d. f. = 1), 4 day ($\chi^2 = 14.6$, p < 0.001, d. f. = 1), 7 day ($\chi^2 = 24.6$, p < 0.00016, d. f. = 1), and 9 day ($\chi^2 = 36.1$, p < 0.0001, d. f. = 1).

With further analysis, examining the differences between each group on the generalisation trial, only one of the results proved to be significant, and this was when they were compared to the group captive for 7 days. This result appears odd, as if the 7 day group showed a greater increase, the 9 day group would also be expected to show an increase due to the greater captivity period. The most plausible explanation for these data could be that the 7 day captive group consistently gave the highest PER responses

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Figure 6.1.2. The effects of long term captivity on olfactory memory
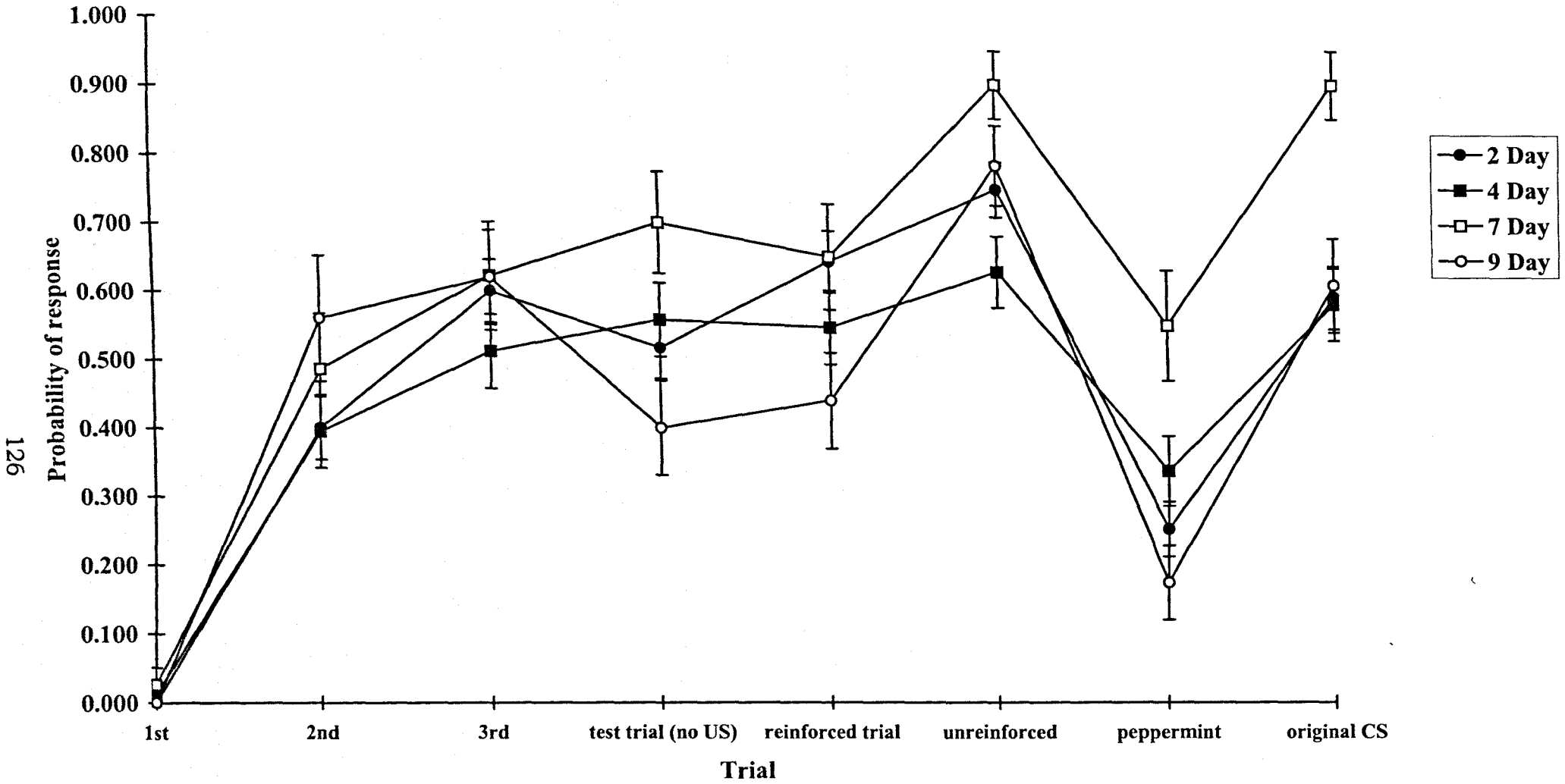
after the initial three training trials, and so these high PER responses may just be continuation of that, compared to the slightly lower response rates of the other groups. Finally, on trial 8, which was to the original conditioned odour (geraniol), the levels once again return to levels presented on trial 6 and before, as these trials were also using geraniol as the CS. Each group once again significantly increased their response levels, from the previous 7th trial with 2 day ($\chi^2 = 26.0$, p < 0.0001, d. f. = 1), 4 day ($\chi^2 = 7.7$, p < 0.001, d. f. = 1), 7 day ($\chi^2 = 6.1$, p = 0.014, d. f. = 1) and 9 day ($\chi^2 = 9.1$, p < 0.003, d. f. = 1).

6.1.3 Conclusions

From the results, there appears to be no negative effects on olfactory learning and memory after long term captivity, thus supporting similar conclusions with free flying honeybees, where over-wintering bees remain in the hive for 2 - 6 months (depending upon the severity of the winter), to re-emerge and forage on flowers previously visited for up to 6 months earlier (Lindauer, 1960). The long term captivity also does not appear to increase generalisation effects to a novel odour, implying that the original piece of learning was specific. The captivity for long periods, such as 9 days, which can amount to 25 % of the bees life (assuming an average forager bee lives for 36 days (Hooper, 1991)) does not affect the subsequent retention of consolidated learning, and such long periods may be encountered in the wild, due to heavy persistent rain fall, or an unseasonable cold period. These results therefore offer a powerful substantiation of PER learning as a valuable technique in honeybee learning and memory research. Further, these findings offer a novel way to maintain bees in the laboratory for other studies of long term retention.

Storing honeybees in an artificial environment such as the bee boxes, where they are deprived of the complex social organisation of the hive, does not appear to affect olfactory learning and memory. This method of captivity may therefore be a useful tool for housing bees away from the hive, in experiments where manipulation of intact bees is necessary. So, although their individual behaviour may have been affected at a chemical level, there was no effect on the PER olfactory learning capabilities. With this in mind, it may be of interest to monitor the juvenile hormone (JH) titres of each bee during these captivity periods, to see if the JH decreases or increases, and thereby affect the behaviours of the bees, as JH levels have been shown to be the chemicals responsible for behaviour changes in honeybees (Robinson, Page, Strambi & Strambi, 1989; Robinson & Ratnieks, 1987; Robinson, 1987a, 1987b; Robinson & Huang, 1998). As this chapter so far has demonstrated that an acquired, plastic piece of learning, such as olfactory conditioning, was unaffected by long term captivity in an unnatural environment, we then decided to investigate a more 'hard-wired' and essential piece of learning, which was the location of the hive. The olfactory PER learning was also a laboratory based paradigm, so would a more natural piece of learning be equally unaffected ?

For the honeybee, the exact position of the hive is crucial information that is reinforced daily, with failure to re-locate proving fatal. We also wanted to investigate if capturing and retaining bees in these unnatural bee boxes would also affect or disrupt the memory processes, as bees have no previous experience of being unable to fly back to the hive and surviving.

6.2 Long term captivity effects on homing behaviour

6.2.1 Introduction

The concept of a cognitive map in the honeybee has generated an extensive literature since being proposed by Gould (1986b), however, conflicting ideas between laboratories have developed with some claiming bees do possess a cognitive map (Gould, 1986b), while others refute such a concept (Wehner & Menzel, 1990; Dyer, 1991; Menzel, Geiger, Joerges, Muller & Chittka, 1998). The stability of any map must be relatively adaptable and plastic, as it is possible to move a hive and the bees will learn the new location. Visscher and Dukas (1997) and Hooper (1991) report that up to 20 % of bees on their first foraging flight fail to return to the hive, this may be due to weather conditions, fatigue, or simply failing to recall the location of the hive. It is therefore apparent that the location of the hive is not innately hardwired, but has to be learned (Gould & Gould, 1995). Indeed, young bees perform play flights around the hive area to become familiar with local landmarks and the position of the hive relative to the sun (Anderson, 1977; Hooper, 1991; Capaldi & Dyer, 1999; Capaldi et al, 2000). Young bees have also been shown to take a number of these orientaion flights prior to foraging, ranging from 1 - 18, with a mean of 5.6 +/- 0.29 (Capaldi et al, 2000). In the majority of the research reporting on homing behaviour, the bees are trained to a feeder, then displaced from either the feeder or from the entrance to the hive, and their ability to fly back to the hive is assessed. In the majority of experiments, the bees are caught and released the same day (Chittka, Geiger & Kunze, 1995; Dyer, 1991; Dyer, Berry & Richard, 1993; Gould, 1986), with the most recent of these studies (Menzel et al, 1998) the bees were kept captive for a maximum of 20 minutes. Von Frisch (1993) captured bees for a few minutes while displacing them from feeders at 10 metres to 600 metres

from the hive, and assessed their ability to return to the hive. In another experiment, Gould (1995) held the bees captive in their hive for 2 hours by closing the hive at 11 a.m. and reopening it at 1 p.m., to observe if the change in sun position altered the bees perception of the location of a trained feeder, the bees still found the feeder.

Menzel (1968) prevented bees from flying out of the hive for up to 14 days and found that they still visited a previously trained feedstation. Similarly, Lindauer (1960a, b) observed that bees confined to their hive, due to cold weather, for 6 weeks after the last field feeding, were seen to dance in the hive, signalling the direction of their last feeding place. He also noted in 1963, that bees trained to a feedstation, came back to the same feeding place 173 days after a winter period of non-flying. This is a prodigious learning retention when considering that during swarming, the bees leave the old hive, and learn a new position, even though this may be only 1 metre from the old hive, illustrating that the memory of the hive location is a very plastic, adaptable system.

Of fundamental importance to a honeybee's behaviour is the precise location of its hive, yet the characteristics of this learning and its stability in the memory remains anecdotal. In beekeeping practice, there is a golden rule of moving hives which states that "hives may be moved under 3 feet or over 3 miles" (Menzel, 1990), this rule is important so that bees will not come into contact with their old foraging sites, recognise them, and attempt to return to the position of their old hive. A moving distance of over 3 miles is necessary in the active foraging season, but in the winter months, or over a single week of frost, where no flying occurs, the hive may be moved within the same apiary (Hooper, 1991). This suggests that being held captive over 7 days decays the memory of the hive location to such an extent that hive position has to be re-learned or at least re-established, prior to foraging. Also, if common landmarks are removed, such as high grass being cut down, this causes some of the bees to drift to other hives, and some loss of older foragers, unable to adjust to the altered landscape (Dyer, 1996). Despite this

fascinating form of learning and memory, rivalling that of pigeons, research has been limited to restraining bees in their own hives, with all it's attendant cues. This chapter further explores long term memory of hive location by removing and maintaining bees away from the hive, into the laboratory.

The experiments reported take a different approach to previous experiments, where bees are trained to a feeder, then caught and displaced. In this report, the bees are caught from the hive entrance, displaced, and then released after a captivity period at varying distances and directions from the hive. Would these long periods decay the memory ?

6.2.2 Methods

Reported are a series of experiments whereby the time kept in captivity was either 2, 3, 6, or 8 days, these bees were then released from different distances from the hive, or from different compass positions.

In all experiments, the bees were caught and restrained as described previously in chapter 2.11. The vials that contained the captured bees were placed in a refrigerator at 4 °C for 10 minutes, which anaesthetised the bees. After the 10 minutes the bees were transferred to clear perspex boxes (7.5 cm by 7.5 cm by 9.5 cm) in groups of 25 or less. The boxes had good ventilation, with 45 air holes drilled in each side. These boxes were kept in the dark and only brought out into the light when the supplies of bee candy were changed (daily), or the bees were to be released. Upon release, one experimenter stayed with the box to ensure the bees all flew and also to note the direction in which each of the bees departed. The second experimenter waited at the hive entrance to count the returning bees, which were identified by having a coloured non-toxic paint spot on their thorax. This second experimenter stayed in position for a maximum of 60 minutes, or until 100 % of the bees had returned to the hive.

Two groups were used throughout the experiments as controls, the first were bees captured from the hives at Oxford Brookes University, anaesthetised, then released the same day. There was a 100 % return to the hive, with a further control being bees caught from hives situated 35 kilometres from the Oxford hives, and here, all these bees failed to find and enter the Brookes hives, thereby eliminating any possible cues that may be invisible to human perception of the location of the hives, either visual or olfactory.

6.2.3 The effects on the homing ability of honeybees after storing the bee boxes in the light or dark

As the hive is inherently dark, the first experiment investigated the effects daylight had on the bees kept captive in the bee boxes, to ascertain the least stressful conditions in which to store them. As reported earlier in section 2.9.4, we had already ascertained that the best method for storing the bee boxes was in the dark, however, we also wanted to examine if these storage criteria affected the homing abilities.

6.2.4 Method

Bees were captured as described above, and placed in the well ventilated perspex boxes. The bees were then divided into two groups and stored either in the dark or on the laboratory bench, with clear sight of the natural day and night rhythm, for a total of three days. On subsequent release, 10 metres from the hive, but out of visual sight of the hive, the bees made circular orientation flights, then proceeded in a definite direction, as described in the literature (Gould, 1986; Menzel et al, 1990; Wehner et al, 1990; Dyer, 1991).

6.2.5 Results

Only 30 % of the bees stored in the light returned, compared to 77 % of the dark stored bees ($\chi^2 = 5.53$, p < 0.02, d. f. = 1) (see figure 6.2.5), this suggests that being kept in an unnaturally light environment adversely affects the homing behaviour of the honeybee. Bees spend the majority of their time in the dark hive, and so spending more time in the light of day may become too stressful to the bees and they cannot navigate their way back to the hive. It was therefore decided to store the bee boxes in a dark environment during the following experiments.

This result has important implications for other researchers who require honeybees for long term retention experiments or for when honeybees need to be kept for periods longer than a day. It also suggests that there is a need to imitate as close as possible the hive environment in the laboratory, which would also help to relieve any stress to the subjects.

6.3 Releasing captured bees from different directions

A second experiment was conducted to explore how a short term period of captivity (2 days) would affect the homing ability when released from different compass positions, and different surroundings, such as a built up area, grassland, or close to a wood, as the bee may not have previously visited these locations.

6.3.1 Method

Four groups of honeybees (25 in each group) were captured from the hive entrance and remained captive in the dark for 2 days, prior to release from four different compass

Figure 6.2.5. Bees released from 10m after being kept in captivity for 3 days
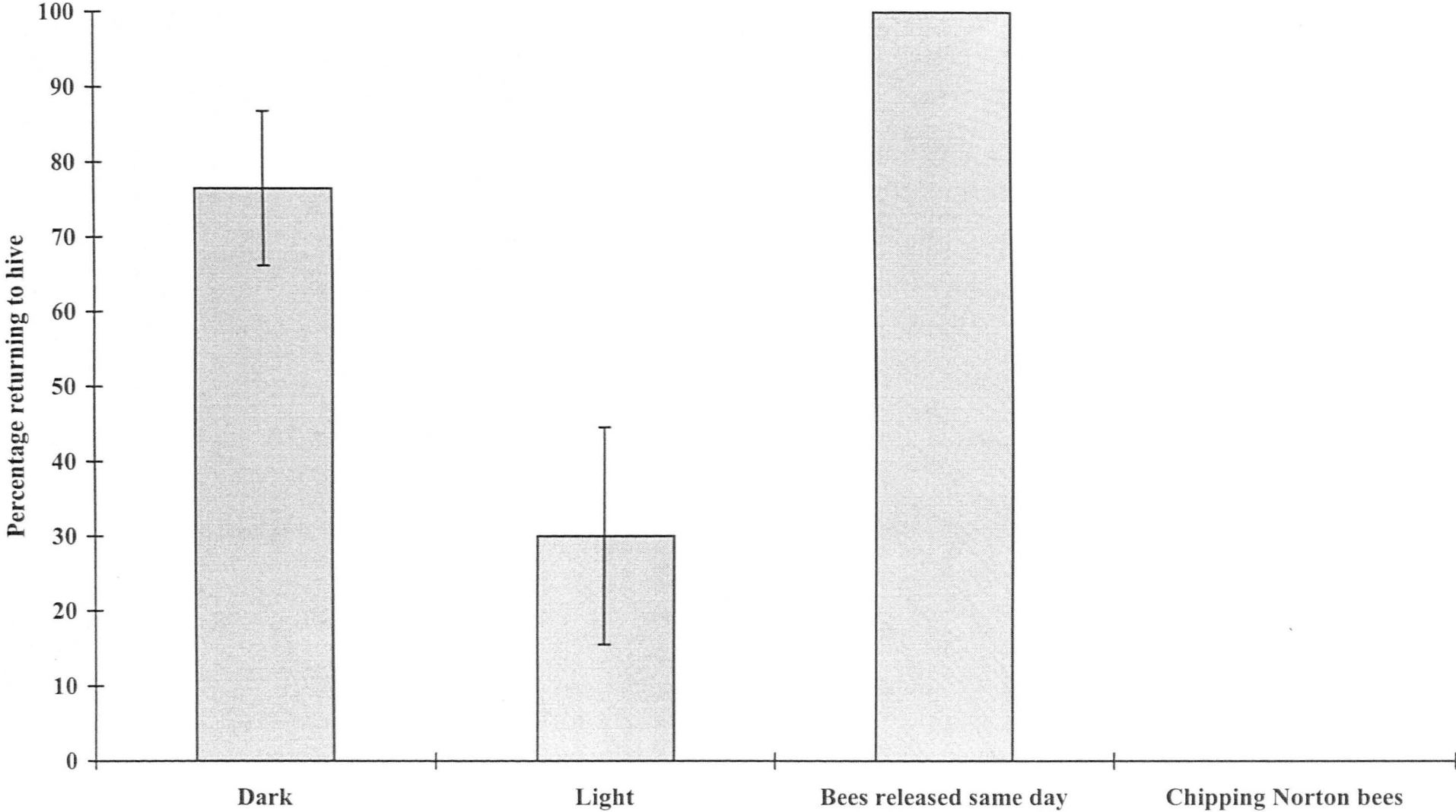
positions. Each position was also situated at different distances from the hive, ranging from 10 metres to 700 metres. The bees that were caught from the hive were all foragers, having returned to the hive from successful foraging trips, identified by having visible pollen loads on their corbiculae.

6.3.2 Results

Upon release, there was no difference in the homing abilities of the bees with 100 % returning to the hive from each direction and distance. This suggests that a 2 day captivity period from the hive does not decay the memory of the hive location and bees are sufficiently familiar with the release sites to return to the hive.

6.4 Homing after longer term captivity periods

In the previous experiment, bees were capable of returning to the hive after a period of 2 days in captivity. A further experiment was carried out, where bees were held captive for 2, 3, 6 or 8 days, to ascertain if longer captivity periods affected the homing behaviour.

6.4.1 Method

Bees were caught as before and divided into four different captivity period groups and stored in the dark. The bees were released 10 metres from, but out of sight of the hive, in an area with large landmarks, such as a large tree stump, glasshouses and a Tarmac car park.

6.4.2 Results

There was no significant difference between each of the different captivity times, with an average returning rate of 79 % after all captive periods (see figure 6.4.2). These data suggest that the bees were all familiar with the immediate surrounding hive environment and had no real difficulty in re-establishing their homing behaviour.

6.5 Releasing honeybees from varying distances after various captivity periods

In light of the results from the two previous investigations, a final experiment was designed to examine if there was any interaction between the length of captivity and difficulty of the homing task (i.e. the distance from the hive).

6.5.1 Method

Bees were captured from the hive entrance and held captive as described previously, having been split into one of six groups. These six groups were as follows:

Captivity time	Release distance (metres)		
2 days	55	110	500
3 days	55	110	500
8 days	55	110	500

They were all released in a westerly direction, and the release times were the same time of day as they were caught. These release sites were within the experienced foraging area (approximately 2 kilometres) normally associated with foragers (von Frisch, 1993).

Figure 6.4.2. Proportion of bees returning to the hive after being kept captive for varying periods
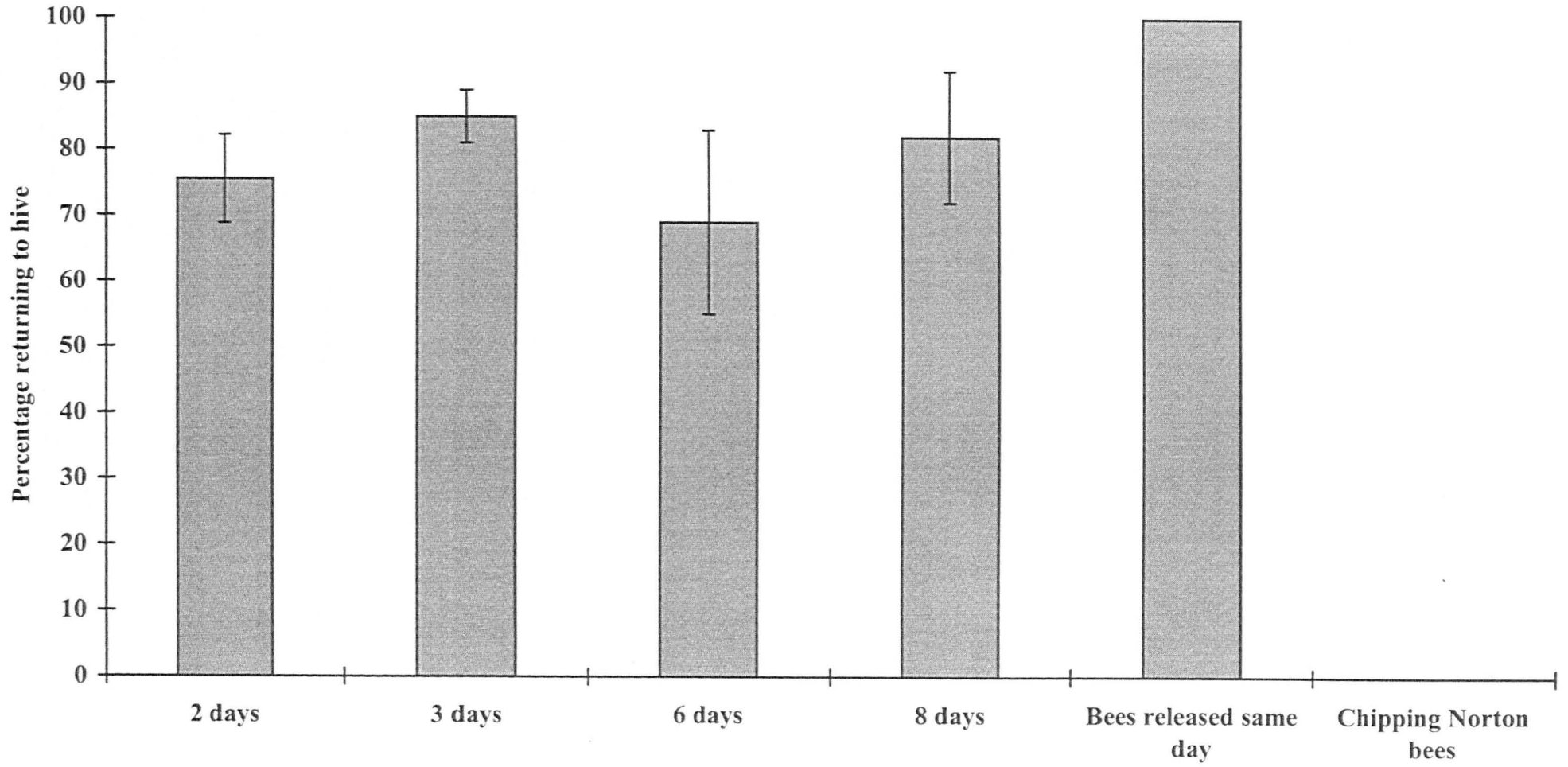
Each of these distance locations was out of vision of the hive, but within familiar landmarks.

6.5.2 Results

As in the previous experiments, the shorter captivity periods of 2 days and 3 days were not significantly different from each other at either the 55 metre or 110 metre release sites (see figure 6.5.2), but they were significantly better when released from the 500 metre site ($\chi^2 = 4.63$, $p < 0.04$, d. f. = 1) where all the bees returned to the hive. An interesting finding was that although the 3 day captive bees returned to the hive at a steady rate irrespective of the distance of the release site, the 2 day captive bees were significantly better when released at the 500 metre site than both the 55 metre ($\chi^2 = 2.82$, $p < 0.1$, d. f. = 1) and the 110 metre site ($\chi^2 = 3.77$, $p < 0.06$, d. f. = 1). This may be due to the bees tested from this hive having a greater foraging area than 55 or 110 metres from the hive. In contrast to these groups, bees kept captive for 8 days, showed a significantly reduced return across all three distances, when compared to the 2 and 3 day captive bees. These are significantly worse than the 2 day captive bees at either 55 metres ($\chi^2 = 5.38$, $p < 0.02$, d. f. = 1), 110 metres ($\chi^2 = 2.43$, $p < 0.12$, d. f. = 1), and 500 metres ($\chi^2 = 6.35$, $p < 0.012$, d. f. = 1). However, it is interesting to note that some bees (32 %) did return from distant sites after such long periods.

There appears to be a dramatic reduction in the memory recall of hive location between 3 and 8 days, and when the bees are released from a distance greater than 10 metres from the hive (see figure 6.5.2). Whether this is due to memory loss, inability to fly, (although all bees departed from the bee boxes with apparent ease) or isolation from the social organisation of the hive is unclear at present, and requires further examination, and is the subject of ongoing research in our laboratory.

Figure 6.5.2. Proportion of honeybees returning to the hive following varying captivity periods and release distances
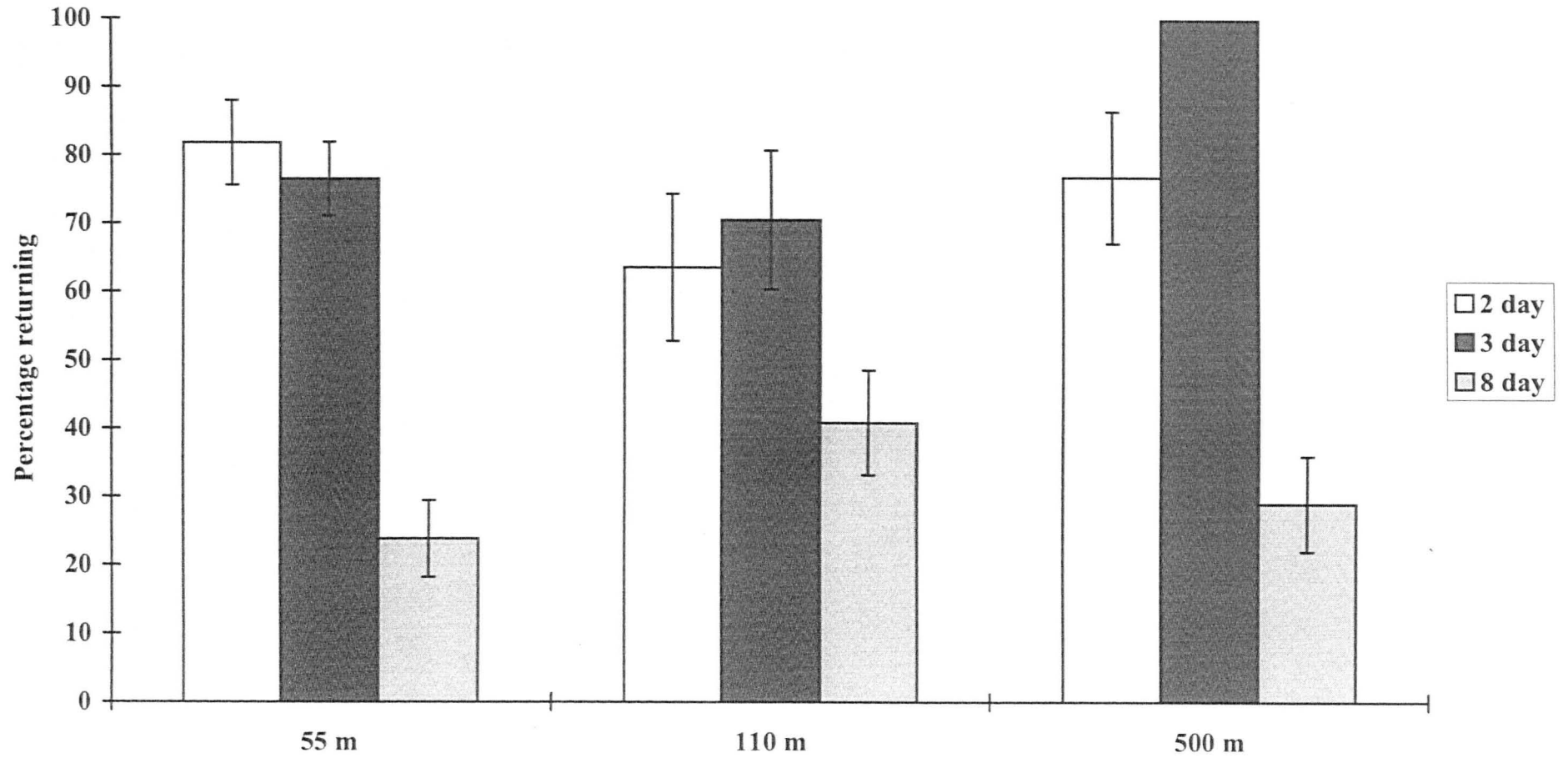
6.6 Discussion

In these experiments, although the bees were socially isolated from the hive, and were unable to interact with the queen or with brood, the memory of hive location, and hence the homing behaviour, was not seriously reduced up until a captivity period of 8 days, and only when these were released from distances greater than 55 metres from the hive. We have shown that keeping bees in daylight seriously affects the homing ability of the honeybee, whereas, keeping them in constant dark, mimicking confinement to the hive due to adverse weather, does not affect this behaviour, as in seen in field experiments and observations (Lindauer, 1963).

The majority of honeybees retained for 2, 3, 6 or 8 days, and then released close to the hive showed a high returning rate. In these experiments, the release distances were all relatively close to the hive (10 metres), perhaps intimating a keen knowledge of the immediate surrounding area, possibly consolidated during playflights or during defecation. However, when the release distances are increased from 10 metres to 55 metres and up to 500 metres away, combined with a captivity period of 8 days, the homing ability begins to decrease. Almost 70 % of these 8 day captive bees failed to return to the hive, over the three distances. This longer period may force the honeybees to alter their behaviour, as they have had no social interaction with either other castes of bee or the queen, which may influence the caste/developmental behaviour they exhibit (Robinson & Huang, 1998; Ray & Ferneyhough, 1997b, 1999), with these adult foragers reverting their behaviours to that of nurse bees, caring and feeding the other bees present.

In conclusion, captivity does not affect a plastic memory, such as olfactory learning, or a more fundamental memory such as hive location. Socially isolating honeybees, and retaining them in captivity for up to one third of their lifetime, appears to have little

negative effect on their homing behaviour, when the release sites are relatively close to the hive. However, bees kept for 8 days and released from distances greater than 50 metres seriously reduce the homing ability of the honeybee. These results suggest some functions of olfactory memory and hive location are difficult to disrupt by displacement and long term captivity, but the more hard wired memory of hive position can be disrupted when captivity periods are long, together with distant release points.

A further experiment to discover the age at which this behaviour first becomes apparent would form an interesting addition to this research and is currently being studied in this laboratory.

CHAPTER 7:

7.0 The effects of age on olfactory learning and memory

7.1 Introduction

The olfactory system of the honeybee matures very quickly, 2 - 3 days after emergence from the comb (Michener, 1974), and it plays a key role throughout adult life in terms of food location and association and inter-individual communication (Bhagavan et al, 1994). The plasticity of the olfactory system is a principal element in the success of honeybees as a species and the role each individual plays in its social group. Despite the prolific literature on PER conditioning in forager bees, little work has concentrated on such learning at other stages of the adult bees' life cycle.

From emergence, adult bees progress through a defined series of duties both inside and outside the hive (see table 1.1). Their initial task is as nurse bees involved in cleaning comb, feeding larvae, manipulating wax and processing honey. From this stage the maturing adult progresses to guard duties involved in protecting the colony from infiltration by other bees and finally, the bee will commence its life as a forager bee provisioning the colony with food and water. The role olfactory learning plays in each of these stages will vary immensely and, consequently, motivational variables for learning involving the conditioning of the PER. Recent research suggests no difference in PER learning with age or caste differentiation after 10 days of age (Bhagavan et al, 1994). This chapter investigates PER conditioning from day 1 post emergence and relates age variability in PER to caste development by manipulating hive environmental variables for development.

7.2 Methods

Frames containing sealed brood at various stages of development were removed from four well established colonies housed in the Oxford area. Each frame was placed individually in a humidified air incubator maintained at 29 °C located in the laboratory and investigated at twelve hour intervals. All bees emerging from the comb within the twelve hours were colour marked on their thorax to denote their age. Bees at 1, 2, 3, 5, 6 and 10 days were collected from the incubator for subsequent PER training. Each age group of bees was compared to a group of experienced forager bees collected from the hives supplying the frame.

In all, fifty bees were collected within each age criteria and placed in glass vials each containing 3 - 5 bees of the same age. The vials were then placed in a 4 °C refrigerator for 10 minutes to anaesthetise the bees, and the protocol for immobilisation and restraining was as in chapter 2.11. Each bee was being given a series of four paired odour/sucrose presentations with an intertrial interval of 20 minutes. A fifth trial served as a retention test conducted unreinforced 24 hours after the initial four training trials. The adult forager control groups were all collected returning to the hive from successful foraging excursions and were collected and trained in an identical way to the age group bees.

7.3 The effects of age on olfactory conditioning with honeybees aged up to 10 days

An initial experiment investigated the effects of age of bees post emergence on PER conditioning up to 10 days of age. Olfactory conditioning was investigated in terms of acquisition of learning and retention and compared to adult forager bees. As there was

no difference in results between donor hive colonies within any age group all data was grouped across hives.

7.3.1 Results

As can be seen from figure 7.3 a profound difference in PER conditioning and retention can be seen with age. Adult forager levels of olfactory conditioning were not evidenced until the bees were 10 days of age. Honeybees at 5 and 6 days of age did acquire the conditioning but fewer bees exhibited the response in the first four training trials. Long term retention was evidenced in these groups but not in the younger bees.

An age effect on acquisition and retention of PER learning was evidenced in all four test populations, this is of interest as bees maintained up to 10 days of age in an enclosed incubator with other younger bees to attend, should all be within the same behavioural caste, yet age does vary both acquisition and retention of the conditioning. All emerged bees up to and including six days old in the test population were observed actively involved in cleaning the comb and in feeding larvae in uncapped cells on the frame, yet 10 day old bees showed much less interest in the comb and spent proportionately more time flying off the comb. In small colonies of bees maintained without active foragers, often, nurse bees will commence foraging precociously (Robinson, 1987a). However in the present experiment there was no opportunity for the test bees to forage as the incubator remained closed to the outside environment throughout their captivity time. It could be suggested that the increased exploratory behaviour of the 10 day old bees may reflect precocious foraging or guard activity.

Figure 7.3. Olfactory conditioning in honeybees of varying ages, compared to adult foragers
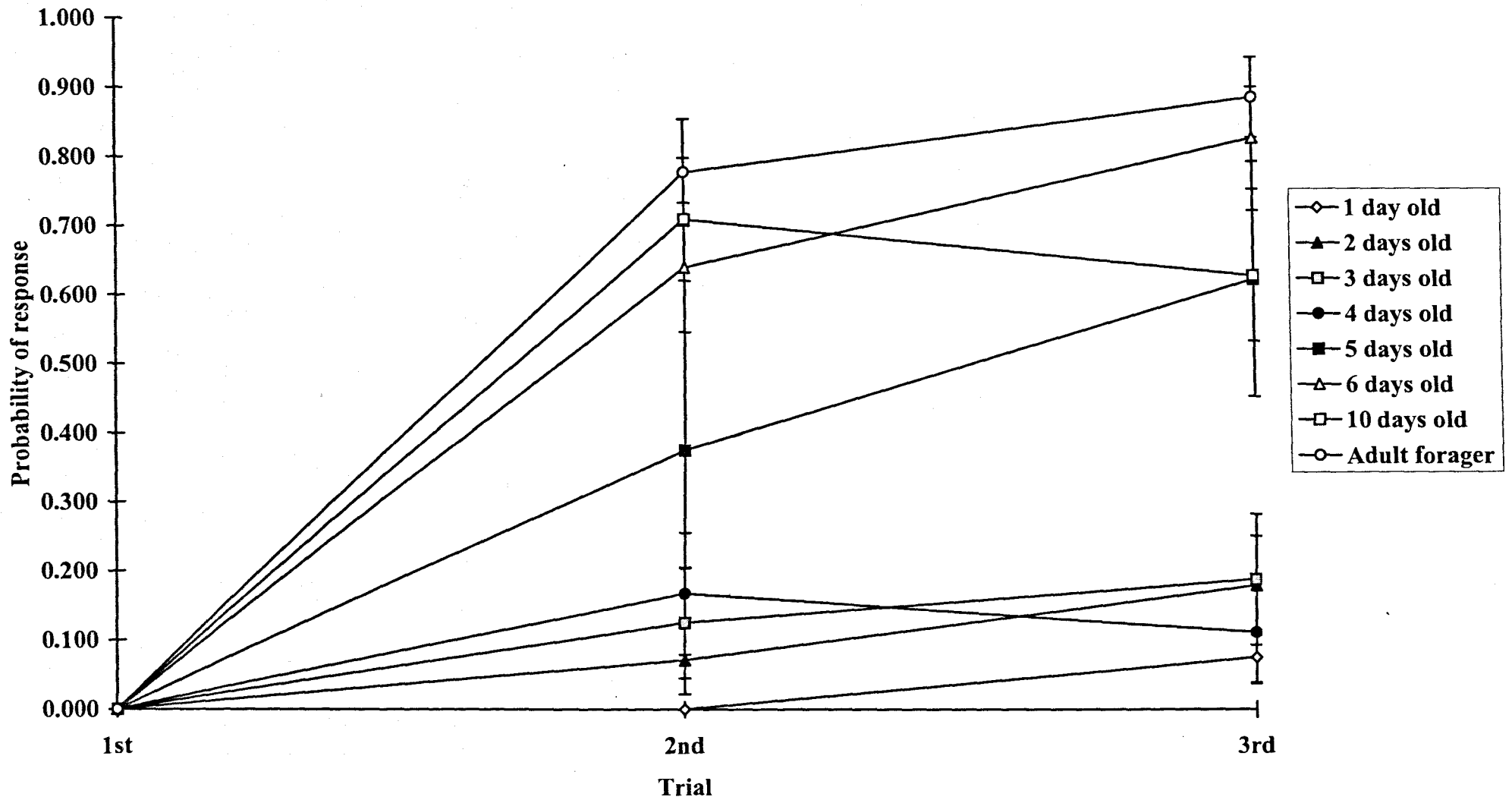
7.4 Olfactory conditioning of honeybees, aged from a full hive colony

To further investigate the findings from the previous experiment (7.3), a second experiment was undertaken using the same hive colonies but marking bees emerging from a comb incubated in the laboratory to identify age, then replacing these bees in the home colony.

7.4.1 Method

Four hundred and eighty bees were marked upon emergence and immediately re-introduced to the full hive colony. On days 1, 3, 5, 10, 16 and 24 the hive was opened and 30 of the age marked bees removed for testing. The method for capture and restraining was as reported in chapter 2.11.

7.4.2 Results

PER conditioning showed a very different age related development in this situation (see figure 7.4), with bees up to 10 days of age showing very poor or no PER learning, at 16 days when most of the tested bees were observed involved in guard duties some conditioning was evidenced but this was inferior to 24 day olds, 90 % of which were observed actively foraging for food or returning to the hive with pollen loads. Thus the development of PER conditioning was slower than in the artificial environment of the laboratory incubator. Further, both this and the previous experiment intimate the importance of individual bees progression through the caste system and its relationship with PER conditioning rather than maturation of the nervous system per se.

Figure 7.4. Olfactory conditioning in honeybees of known age, housed in a full hive colony
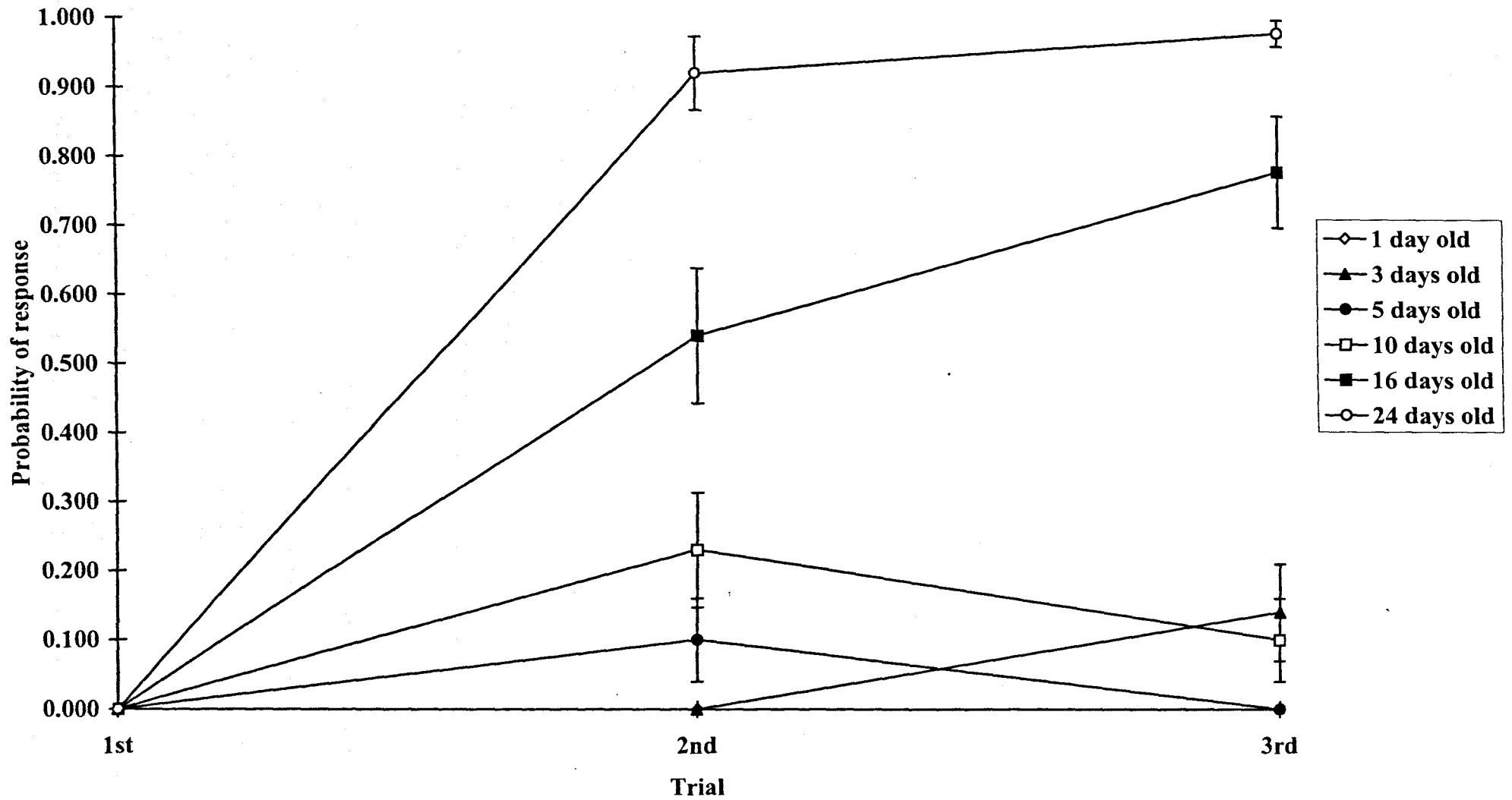
7.5 The effects of specific behaviours on olfactory conditioning

To explore the hypothesis raised by the previous studies, a final experiment investigated if the olfactory learning evidenced by bees of known age varied in terms of caste specific behaviour at the age of testing.

7.5.1 Method

Two brood frames were removed from the same hive and placed in individual incubators. One group remained on the brood comb until 2 to 3 days of age, the second group was removed from the comb within 12 hours of emergence and placed in an incubator under identical conditions but containing an empty comb thus minimising the opportunity for this cohort to show nursing behaviour. Marking, training and testing of bees was as reported in the general method.

7.5.2 Results

Figure 7.5 shows a greater PER conditioning in the group who could not fulfil their nurse bee activities. This result suggests that PER conditioning is not solely a function of maturation of nervous system, but rather an interaction of such maturational variables and the specific role the bee performs within the colony. Thus, colonial variables such as artificial rearing environments, so often used in anatomical studies of bee brain development, may influence PER conditioning by affecting caste specific behavioural development.

The above results are of particular interest in light of recent findings that bees housed in a small colony (2000 bees) show no age effects on PER conditioning after 10 days of

Figure 7.5. Comparison of PER learning in adult foragers, 4/5 day old precocious foragers, and 4/5 day old nurse bees
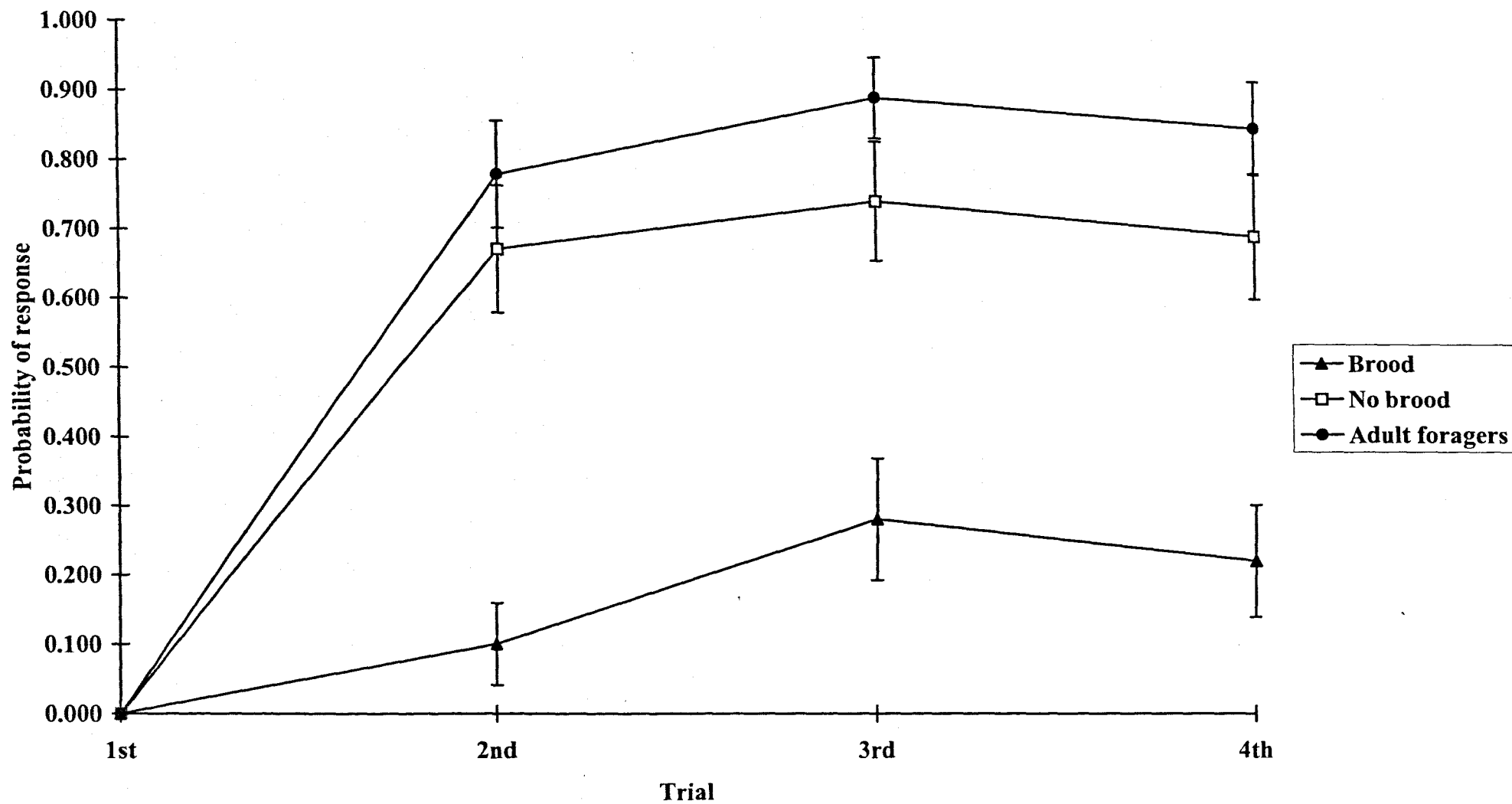
age, and caste has no effect in the same test population (Bhagavan et al, 1994). Bees less than 10 days of age are shown to be inferior on PER conditioning tests when raised within the home hive estimated to contain some 60,000 bees, also showing a slow development of PER conditioning when raised in the laboratory in the absence of mature castes. Interestingly in this situation the bee behaviour in terms of caste development is changed, comparing the results gained in section 7.3 and 7.4. This suggests the behavioural role within the colony is a more powerful influence on olfactory learning than maturation of the nervous system. The experiment reported in 7.4 further explored this finding in that prevention of normal caste behaviour in young (nurse) bees can produce PER conditioning equivalent to later stages of development. Studies of precocious behavioural development of honey bees are well advanced in terms of endocrinological control (Robinson, 1987b) with juvenile hormone titre being implicated in such development, unfortunately, comparable investigations of learning in such precocious bees is in its infancy.

As bees progress through the caste system of the colony there will necessarily be great changes in the role of olfactory learning. Behaviour and presumably motivational factors appear to be of critical importance in determining olfactory conditioning of PER. How this is reflected in terms of the neurological correlates of such learning awaits further research.

The above results provide an interesting heuristic to further exploration of bee olfactory learning and memory and their behavioural and neuroendocrine correlates.

7.6 Conclusions

Age affects PER conditioning in honeybees with their PER conditioning abilities having a relatively slow onset compared to the rapid maturation of the bee nervous system. The

development of this learning seems related to the maturational role the bee is performing within its colony rather than age per se, thus manipulations of hive behavioural dynamics affect learning abilities of individual bees. The results suggests the changing behavioural demands placed on bees as they progress through the defined behavioural castes influence the saliency of olfactory signals toward subsequent behavioural rewards.

CHAPTER 8:

8.0 Behavioural development and olfactory learning

8.1 Introduction

The honeybee is renowned for its ability to learn and remember a variety of task in all sensory modalities when tested both free flying in the field (Gould, 1986) and restrained in the laboratory (Menzel & Bitterman, 1983), with most adult bees requiring only one trial to acquire this learning and show excellent long term retention. However, some variability in PER conditioning is intimated in the literature (Menzel et al, 1974; von Frisch, 1967), unfortunately this is substantiated by little systematic research. Several laboratories report seasonal variation in PER conditioning (Menzel et al, 1974, p178; Menzel, 1990; von Frisch, 1967, p244) despite the fact the bees are trained and tested in the laboratory with little variation in conditions. One variable which may account for some seasonal variability and, of general interest to all bee learning research, is the well defined and variable roles each animal performs within the colony at various stages in its development (Page & Robinson, 1991). Similarly, the number of bees performing specific colony related tasks (castes) in any one month of the year varies according to colonial requirements (Hooper, 1991).

Newly emerged adult bees commence a period of hive duties including feeding immature stages, comb maintenance and construction and only emerge from the hive to defecate. From approximately 15 days of age, bees will commence play flights to orientate themselves with the location and characteristics of the hive and begin guard duties, with their foraging role commencing approximately at 21 days of age (see table 1.1). Thus, it could be hypothesised that motivational factors within olfactory learning

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with a sucrose reward will vary dependent upon the specific role of the bee within the colony.

In the majority of the PER literature experimental subjects are collected from the hive entrance, thus, the population of bees tested should include bees at a variety of developmental stages including forager bees returning and departing the hive, young bees on play flights, and guard bees protecting the hive entrance. Bhagavan et al (1994) reported no effect of behavioural caste on olfactory learning investigated in a population collected from a small constructed nucleus colony of bees containing approximately 2000 animals.

Development of bee behaviour through the different castes does show variability (Page & Robinson, 1991) and does not appear to be rigidly defined temporally (Robinson, 1987a, b). It is also possible to manipulate the number of bees actually performing a specific caste role, which in turn affects the role other bees perform within the hive (Robinson, 1987a, b) for example, if there are few active adult forager bees, younger bees will commence foraging activity earlier in adult life. Similarly, over-wintering bees revert to some hive duties after an active foraging season because of the absence of younger colony mates to perform these roles. Such caste reversal and precocious development appear to be under the physiological control of juvenile hormone (Robinson & Ratnieks, 1987) and reflect the dynamics of the colonial needs. Thus, a nucleus colony may not completely reflect behavioural variances in olfactory conditioning found in an intact colony of bees who's numbers may vary between 50,000 and 80,000 individuals in the height of the European and north American season.

In an effort to further extend the studies of Bhagavan et al (1994), a series of experiments were conducted in our laboratory to investigate the effects of specific behavioural caste on PER conditioning from several well established complete hive colonies. Further, we also manipulated behavioural caste by altering population

numbers and noted the effects of such manipulations on the behavioural caste on their resultant PER learning abilities.

8.2 Methods

8.2.1 Bee caste determination and collection

A total of one hundred bees from each behavioural caste were collected from four separate hive colonies. All bees were collected on the day of testing, caste was determined by the following behavioural and physical appearances of the bees at the time of collection:

a) Nurse Bees - bees inserting their heads into one or more larval cells for more than 20 seconds (collection of nurse bees was conducted with hive briefly open with brood comb partially removed to facilitate accurate identification of this caste).

b) Guard Bees - bees observed at the hive entrance lifting the anterior part of their body when directly approaching other bees entering the hive colony.

c) Forager Bees - bees returning to the hive entrance with pollen loads on their corbiculae.

Guards and foragers were collected from the flight board at the hive entrance. The collection and subsequent testing of all castes was conducted on the same days with the same collection to test interval.

Following identification of behavioural caste, bees were placed in groups of three to five in a glass vial and removed to the laboratory, where they were processed as described in chapter 2.11. Each bee was given a series of three paired odour / sucrose presentations with an inter-trial interval of 20 minutes. A fourth trial served as a retention test which was conducted, unreinforced, 24 hours later.

8.3 Comparison of learning between three different honeybee behavioural castes

Learning curves for each caste of bees were compared to adult forager bee learning curves. The training of all castes was conducted simultaneously in groups of 5 - 10 bees from each caste at each training sessions. As there was no difference between hive colonies on the learning curves for each caste or over different days, data is presented as grouped data for each behavioural caste.

8.3.1 Results

In figure 8.3.1, the nurse bees gave a much reduced response rate when compared to the adult forager bees, which lasted up to the retention test the following day. These bees failed to achieve levels of 25 % over the learning trials, compared to the adult foragers who gained levels of over 80 % after a single trial.

In figure 8.3.2, guard bees also showed a reduced response rate in comparison to the adult foragers, and their retention test responses were also slightly reduced.

8.3.2 Discussion

A definite effect of behavioural caste on olfactory PER conditioning was found in all the hives tested and on all training days. Nurse bees, whose principal duties at time of capture were care of brood, feeding larvae and young bees, showed little olfactory conditioning and no long term retention over a 24 hour interval. Whereas, over 70 % of adult forager bees tested at the same time required only one pairing of geraniol with the sucrose reward to show learning, and similarly showed excellent retention of this learning over 24 hours.

Figure 8.3.1. Comparison of olfactory learning in nurse bees and adult forager bees
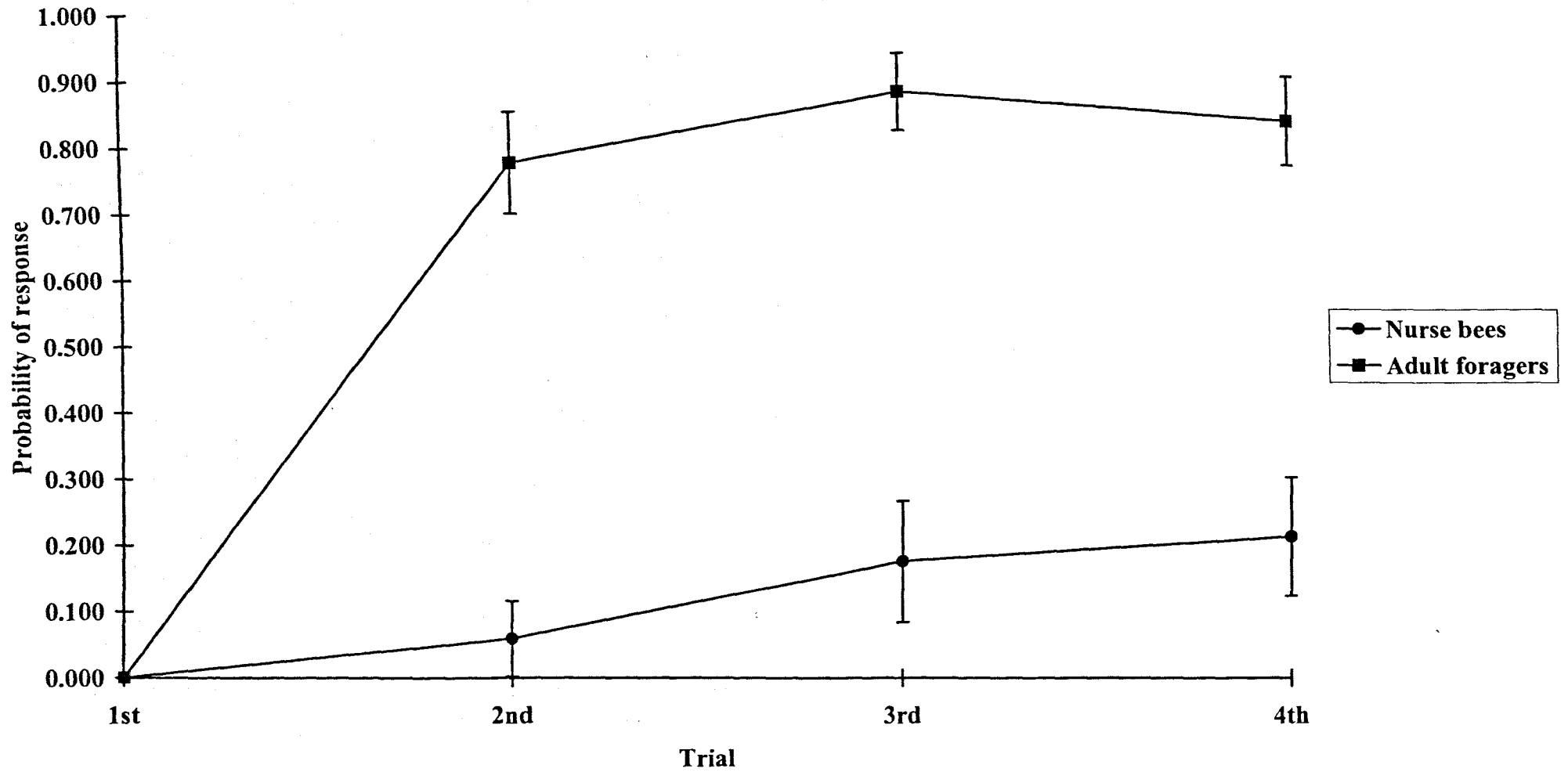
Figure 8.3.2. Comparison of olfactory learning in guard bees and adult forager bees
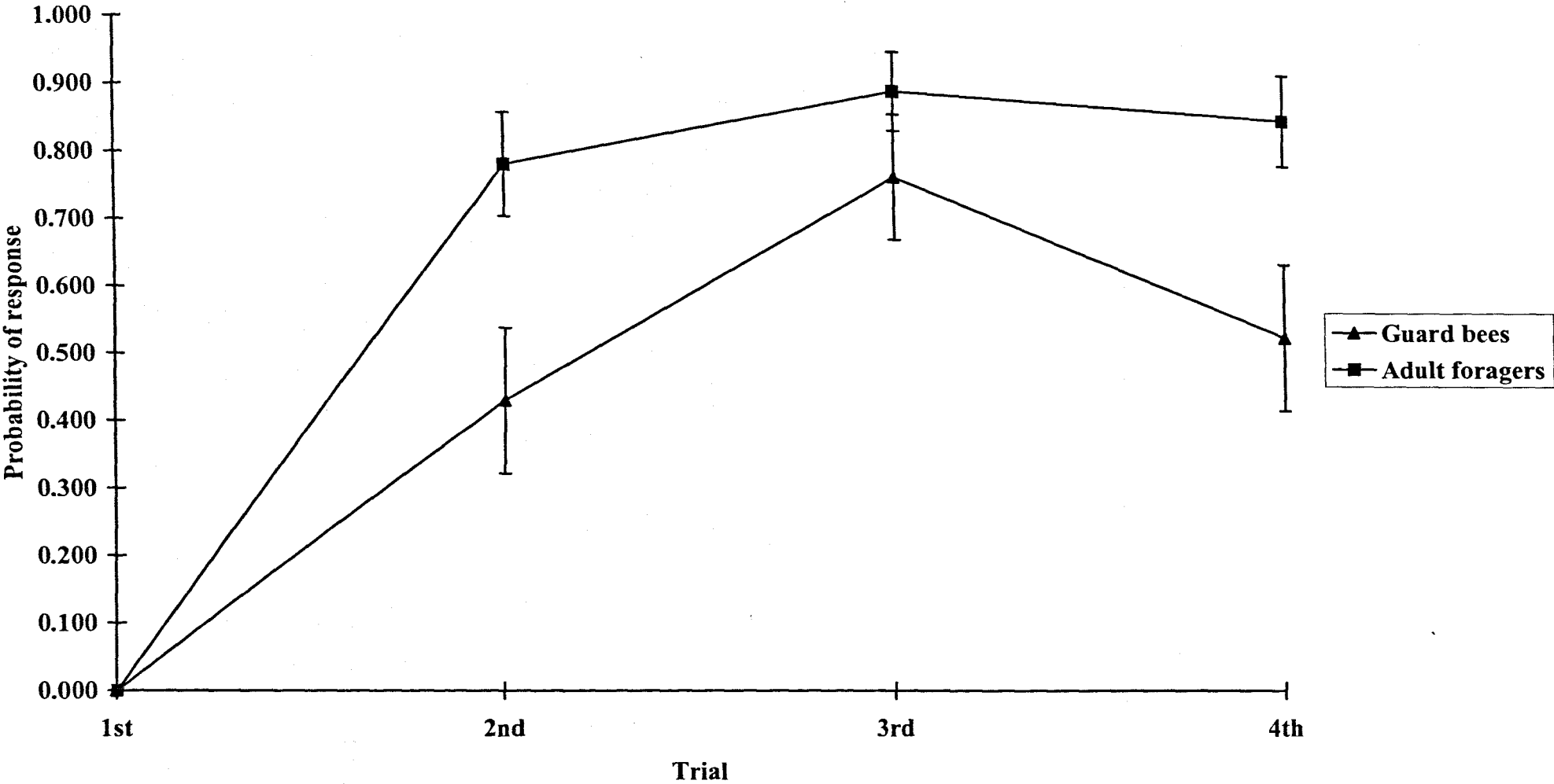
Guard bees showed an interesting learning curve compared to their forager control group, with the guard bees showing some learning, but were less efficient in this learning. Fewer guard bees showed one trial learning, requiring three trials to reach the same response probability as forager bees. Further, less long term retention was evidenced from guard bees. Given the role of odour in kin recognition for guard duties these results are surprising, however, odour recognition for this behavioural caste is not normally associated with food. Despite their well developed and employed sense of olfaction, olfactory learning rewarded by sucrose does occur in guard bees, but requires more trials to reach asymptote, and shows greater variability and less long term retention.

8.4 Precocious development and olfactory learning

In an effort to expand these findings a second experiment was conducted to investigate whether such caste variation in PER conditioning is the product of

a) maturation of the insect nervous system, or

b) a consequence of the specific behavioural role the bee is currently undertaking and thus the product of the different behavioural demands placed on the bees olfactory system.

As a consequence of swarming, seasonal variations (see chapter 9) or bee keeping practice, very young bees are often forced to forage in the absence of more mature adult bees. This drastically reduces the amount of time such bees spend on hive related tasks in their development. Bees as young as four days of development may be found foraging from nucleus hives containing a queen but no adult forager bees (Michener, 1974; unpublished observations).

Taking these findings into account, a second experiment was undertaken, comparing PER conditioning between an adult forager population with a group of four to six day old bees made to forage very precociously due to the absence of other forager bees.

8.4.1 Methods

One hundred adult forager bees were collected and restrained as previously described (chapter 2.11) from two well established hives in the Oxford area. The criteria for selection in this caste remained as bees returning to the hive with pollen loads evident on their hind legs.

Precocious foragers were produced from bees emerged from sealed brood comb in a humidified air incubator, maintained at 29 °C. Once a bee emerged from the comb it was colour coded as to the exact emergence time and placed in one of two small mini-nucleus colonies;

a) Mini-nucleus one contained bees of all behavioural castes including mature forager bees.

b) Mini-nucleus two containing only young (day 0 - day 6) bees.

After 4 - 5 days in such conditions, bees in colony two with no adult forager bees commenced foraging duties and could be observed returning to the mini-nucleus hive with evident pollen loads. No such activity was observed from the marked bees in colony one. A total of one hundred identified precocious foragers (4 - 5 days old) were collected from mini-nucleus two on the criteria of returning to the hive with full pollen loads. A further 100 bees between the same ages 4 - 5 days were collected from mini-nucleus one. Restraint and training procedures were as reported in the first experiment.

8.4.2 Results

Figure 8.4.1 illustrates the comparison between adult forager, 4´- 5 day old nurse bees from colony 1, and 4 - 5 day old precocious foragers from colony 2. The 4 - 5 day old nurse bees show typical low response rates as seen in the previous experiment, and also for the same ages tested in chapter 7. However, bees of equal age, but made to precocially forage exhibit PER responses equivalent to adult foragers, and even show good long term retention after 24 hours (trial 4).

8.4.3 Discussion

The adult forager bees showed a typical learning curve for PER conditioning found in this and other laboratories, with 80 % of bees requiring only one trial to show the response in subsequent testing, with excellent long term retention in trial 4 after 24 hours.

Interestingly, 4 - 5 day old bees made to forage precociously by manipulating the hive environment also showed excellent acquisition of the learning, 50 % of these bees requiring only one trial to show learning in subsequent testing and with comparable retention at 24 hours to adult mature forager bees (see figure 8.4.1). Genetically matched 4 - 5 day old bees (coming from the same brood as the precocious forager bees) showed little learning across trials with no substantive retention, which are in accord with the findings of the previous experiment.

These data further suggest a difference in caste on PER olfactory conditioning and that this variation in learning ability is not dependent upon temporal maturation of the nervous system through the natural behavioural hierarchy. Olfactory learning ability on this task appears dependent upon the specific caste system the bee is currently employed

Figure 8.4.1. Comparison of PER conditioning in adult foragers, nurse bees and nurse bees made to forage precocially
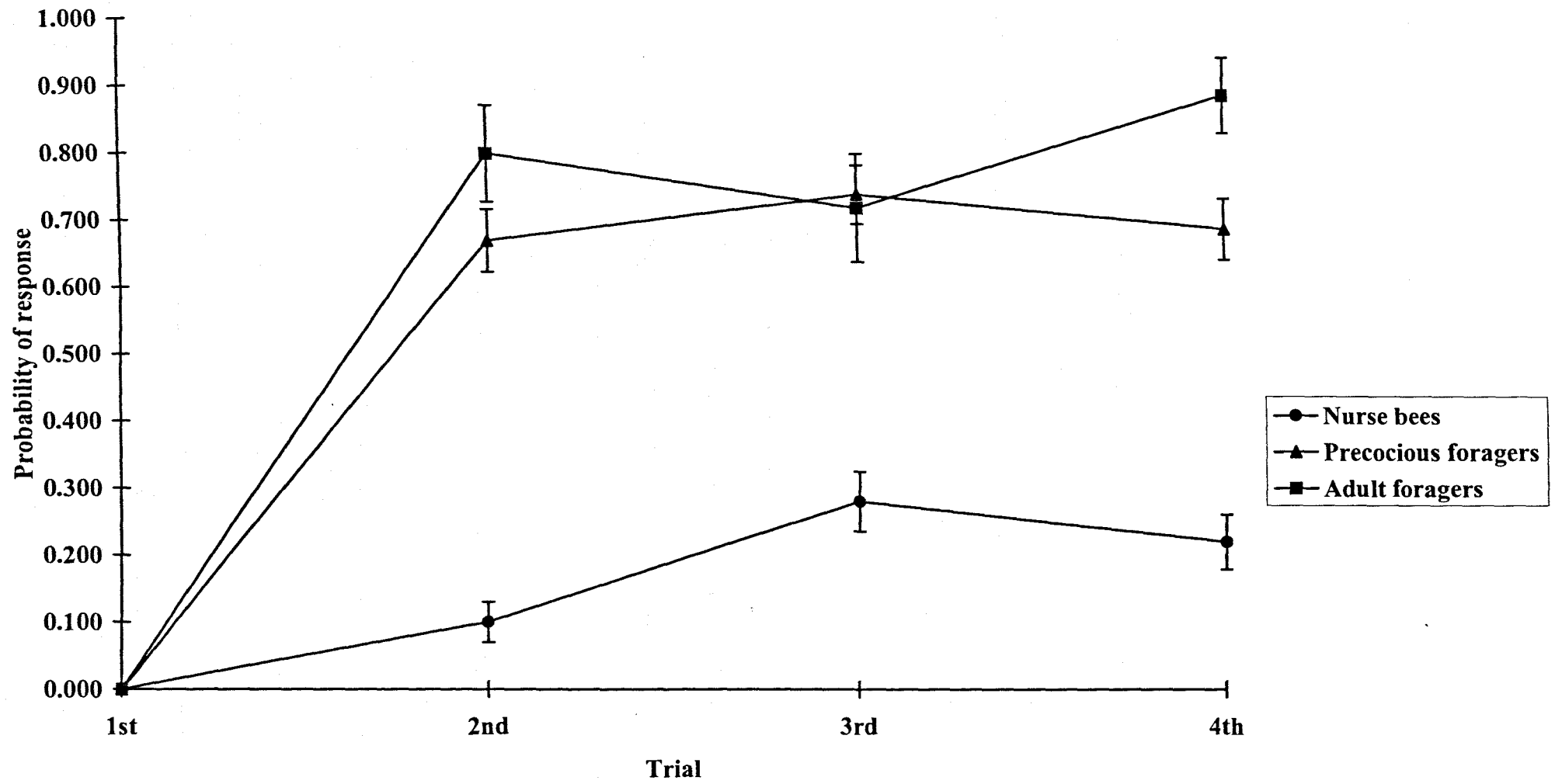
in within its hive society, rather than the temporal maturation of the nervous system. These results confirm the importance of foraging behaviour on olfactory conditioning of PER, and suggests that honeybee behaviour may be a powerful determinant of bee brain maturation in general and the olfactory systems maturation in particular.

8.5 General discussion

The results show a clear variability in PER conditioning between bees engaged in different behavioural castes, when castes are collected from intact mature hives with nurse bees showing little or no PER learning or memory. The ability to show PER conditioning appears to emerge across all our experiments as the bees progress on to guard duties. The guard bees show more variable and generally slower acquisition of the conditioning and inferior long term retention tested at 24 hours, compared to adult forager bees who show excellent acquisition and retention of this task as has been reported in other laboratories (Gould & Towne, 1988; Menzel, 1985), and these results as a whole are not in accord with those reported by Bhagavan et al (1994). Bhagavan et al (1994) reported no caste specific variability in PER conditioning assessed from an artificially manipulated colony of some 2,000 bees, although their data does suggest in one cohort guard bees showed better one trial learning than either foragers or nurse bees, yet in a second cohort, guard bees showed inferior learning to either foragers or nurse bees. Unfortunately, their data does not extend beyond the first learning trial or investigate long term retention of the learning, both of which show a caste variation in our studies.

The hives investigated in the present study were investigated over the summer months where the resident population of bees may be in excess of 60,000 bees. In colonies of

hive bees with insufficient numbers of any specific caste the behavioural roles of the castes may become blurred by the need of the colony as a whole (Robinson, 1987a, b). Indeed, the experiment which employed a small mini-nucleus colony (8.4), devoid of mature foragers, shows an extreme case of such caste specific behavioural change. This may explain the disparity in our results with those of Bhagavan et al (1994) on the initial learning trial, and illustrates the need for further investigation into this interesting effect.

The variability between castes on PER conditioning when collected from intact hives may explain some of the variations reported by laboratories employing this learning paradigm (Menzel et al, 1974, p178; Menzel, 1990; von Frisch, 1967, p244). Similarly, care should be taken on collecting and testing of bees on PER learning to avoid contamination of data from caste differences particularly in repeated trial learning experiments.

Artificial manipulation of bee behavioural development as illustrated in the experiment reported in section 8.4 provides an interesting insight into the relevance of olfactory plasticity with the highly specific behavioural roles bees undertake at different stages of their life cycle. Very young bees normally engaged in nursing behaviour are incapable of PER conditioning, yet if they are required to commence activities outside the hive such as foraging they then show excellent PER conditioning, comparable to adult forager bees. These results suggest that olfactory conditioning in honey bees is more dependent on the relevance of olfactory plasticity to the specific role the animal is undertaking in the colony rather than a function of pre-specified brain maturation.

The role olfaction plays within each highly specified behavioural caste of a bee community is highly variable. Newly emerged adult bees commence their first duties within the hive immediately upon emergence. Their first role normally involves cleaning of the frames and progresses through feeding larvae, manipulating wax and

processing honey. While some of these later duties undertaken by nurse bees undoubtedly involve olfactory components, little plasticity within this role would be required to successfully complete these tasks.

As bees progress to duties outside the hive as guard bees, the demands placed upon the olfactory system begin to change. Guard bees must remember the colony odour to differentiate between members of its own colony and intruder or robber bees. That some drifting of individuals between hive colonies sited close together does occur (von Frisch, 1967; Hooper, 1991) suggests that guarding does involve some errors of detection. Similarly, bee recognition does not solely rely on olfactory recognition, bees attempting to gain access to the hive are differentiated by guard bees on other behavioural criteria e.g. movement and pollen loading (Gould & Gould, 1995). Thus, the role of guard bees may involve some olfactory learning but the pairing of food with changes in olfactory signal as yet has no relevance to the bee, whereas to a forager, this is very important. That guard bees can show some olfactory conditioning with a food reward may well be explained as some guard bees will undoubtedly have commenced some feeding outside the hive as a result of dance following. It is of interest to note the rather poor long term retention of olfactory conditioning in this caste. If guard bees are relying on other foragers to locate food sources they, at their stage of behavioural development, have little need to remember associations of olfactory signals with a potential food source. The major saliency of olfactory stimuli to a guard bee is therefore kin recognition.

The results of artificial manipulations of caste specific behaviour (as seen in section 8.4.2) suggests that the potential to show PER conditioning is already present in bees as young as 4 days old and potentially earlier, yet the relevance of the olfactory stimuli to a food source has little salience to the bees behaviour until it is fulfilling the forager role for the colony.

Experience of the behavioural roles specific to guard and nurse castes does not appear to be a pre-requisite of PER conditioning abilities. Rather, learning and memory on this paradigm is determined by the motivation to associate a specific odour with a food reward. This appears unique to the behavioural roles of bees flying outside the hive to a food source. The role experience of foraging and scouting for food has on PER conditioning is currently under investigation in this laboratory (Ferneyhough & Ray, in prep.).

Honeybees made to forage precocially by the absence of forager bees, do show excellent PER conditioning with only limited foraging experience. This suggests further the powerful influence on this learned behaviour of behavioural demands placed upon bees within the colony rather than developmental age alone. There was no difference between such bees and experienced foragers captured from a mature intact hive (see figure 8.4.1). The maturation of the olfactory system has been extensively researched (Masson, 1977), and by two days of age the antennal lobes and mushroom bodies are fully enervated, and maturation is not dependent on olfactory experience of other bees (Gascuel, 1989). These results show that there is an early maturation of the nervous system and explains the potential for precocial foraging behaviour experienced in our and other laboratories (Robinson, 1987a, b). Anatomical correlates of both precocial foraging and olfactory conditioning may together yield great dividends in furthering our understanding of behaviour development of the insect nervous system.

Further, just as bees can be induced to forage precociously by the behavioural requirements of the colony, similar precocious behaviour can be induced by injections of juvenile hormone analogue (Robinson, 1987a, b). The effects of such manipulations on PER learning and memory are currently being investigated in our laboratory.

The results presented for caste difference in PER conditioning and retention suggest that the appearance of caste specific foraging behaviours, and thus, variation in the

motivation to associate olfactory stimuli with a reward, may explain the reported differences between castes from unmanipulated colonies. These results provide a useful addition to further experimentation with both behaviourally and chemically manipulated bee colonies to further our understanding of the interactions between brain development, experience and behavioural plasticity in the invertebrate nervous system.

CHAPTER 9:

9.0 Seasonal variation of proboscis extension reflex conditioning

9.1 Introduction

Although, the classical conditioning of the proboscis extension reflex remains one of the most prolific learning paradigms in the bee literature, being employed in pharmacological, anatomical and behavioural investigations of bee learning (Hammer & Menzel, 1995), little research has been conducted on seasonal variations in such learning. Indeed, there is some confusion both within and between laboratories, with Menzel (1974) reporting that learning curves are unaffected by season (page 178), however Menzel (1990) later suggests there is a seasonal variation. Other researchers have alluded to seasonal variations in olfactory learning e.g. von Frisch (1993), but did not expand on the matter. Similarly, Gould (1995) found difficulty in training bees on PER conditioning in early July, suggesting the abundance of natural flowers as a possible explanation.

Given the disparate demands placed on bee colonies and their behaviour with the changing season, also the prolific employment of PER conditioning in the literature, we now report a systematic investigation of the effects of season on olfactory learning in England and compare such variations with a similar colony of bees housed in a flight room throughout the year.

9.2 Method

Olfactory learning abilities were sampled from eight colonies of bees housed throughout the English Midlands 1991 - 1993, and four colonies housed at Oxford Brookes University 1993 - 1997. One further colony of bees were sampled for seasonal variation in learning abilities, these were housed indoors in a flight room with no access to the outdoors. This indoor colony was of similar strength and vigour as the outdoor test colonies and were artificially provisioned. Seasonal variation in PER conditioning in this colony is reported.

PER conditioning techniques were adapted from Menzel (1990). On test days a sample of forager bees were collected from the hive entrance and placed in groups of between five and eight bees in glass vials (25 cm^3). Bees were then quickly anaesthetised in their holding vials by chilling at 4 °C in a refrigerator for ten minutes, as previously described (see chapter 2.11). All bees received three training trials with an inter-trial interval of twenty minutes, with the responses to the odour prior to delivery of sucrose reward in trials two and three being counted as a conditioned response. Bees responding spontaneously to odour delivery alone on the first trial were not included in the study. The PER results from each day were then plotted against the corresponding calendar day. Probability of response was employed as data presented were collated from a variety of PER experiments conducted in our laboratory, consequently, the number of bees trained on any one day varied from 50 to 100 bees.

Data for each month consisted of between ten and twenty five experiments conducted on separate days. The indoor colony was sampled for learning ability on between seven and eighteen days per month.

9.3 Results and discussion

The data collected from all outdoor colonies showed no significant difference between colonies on learning abilities, so the data from each colony was collapsed. The levels of PER conditioning obtained in our laboratory appear to be at consistent levels with those of other laboratories (Bitterman et al, 1983; Menzel, 1990; Gerber et al, 1996). Similarly, seasonal variation in learning ability remained consistent over all years investigated, thus a season curve is presented from five years of data, and compared to variation in PER conditioning in an indoor colony sampled over one year (see figure 9.3.1). Also presented are data from the colonies housed outside, showing the daily variation throughout the year, and as can be seen in figure 9.3.2, there is a very variable response, however, when this is collapsed into months (see figure 9.3.3) over the years, there are distinct seasonal patterns each month, and when each month is compared (figure 9.3.4), they show equivalent learning levels. Finally, four typical weeks in each season are presented in figure 9.3.5 (summer - June to September; autumn - September to November; winter - November to February; spring - February to June), it is evident that learning is greater during winter and autumn.

As can be seen in all these graphs, PER conditioning in our laboratory showed a marked seasonal variation in all outdoor colonies. Optimum olfactory learning was achieved in winter months, with poor conditioning levels in early spring and a steady increase in PER conditioning throughout the summer. These results are of particular interest as many researchers may work with bees during the summer months and may find relatively poor levels of olfactory learning. Similarly, these results should emphasise the need for caution when comparing colonies for learning abilities where data is collected at different times of year.

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Figure 9.3.1. Seasonal variation in olfactory learning for colonies housed indoor and outdoor
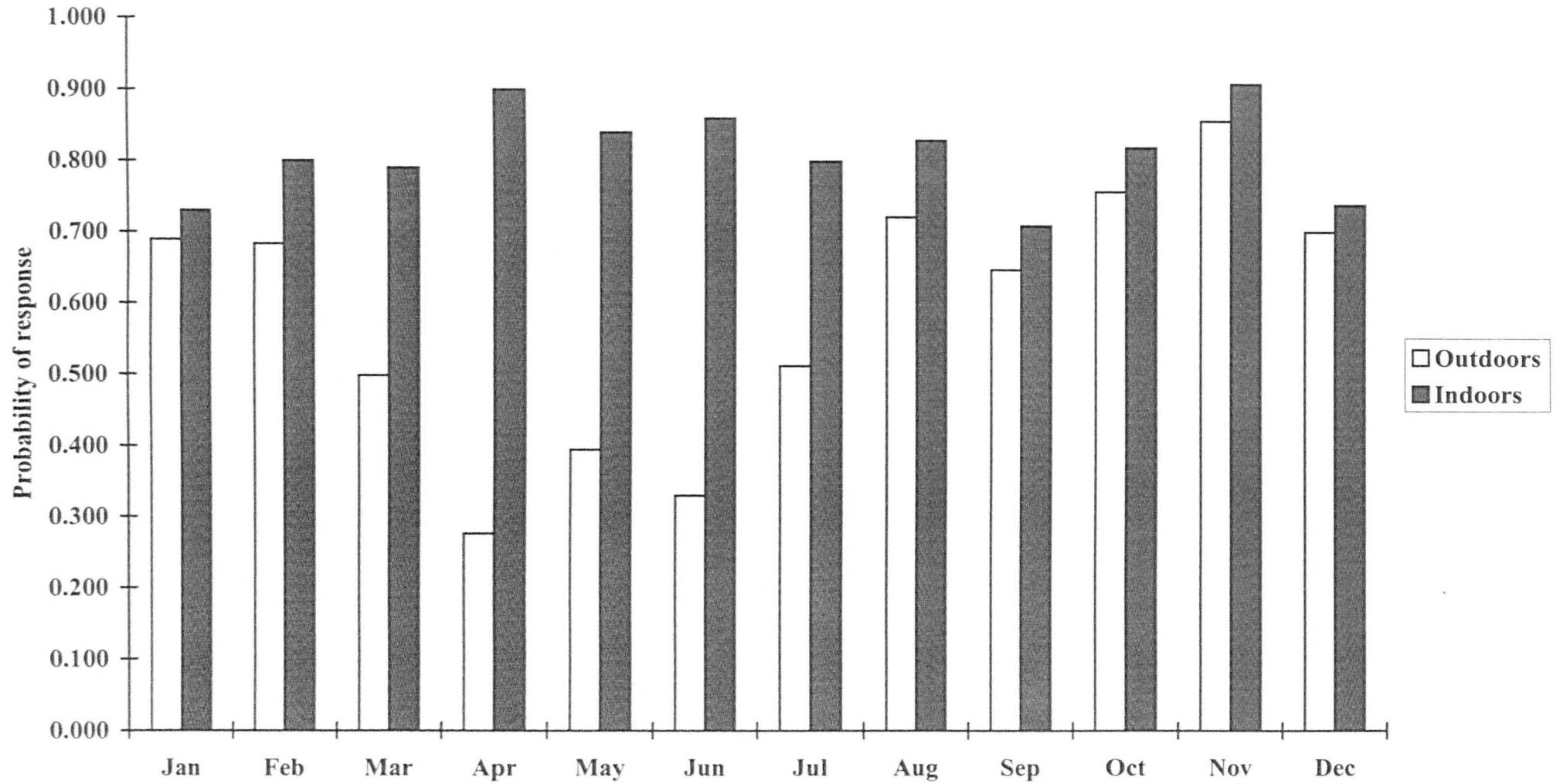
Figure 9.3.2. Olfactory conditioning throughout the year, showing individual days
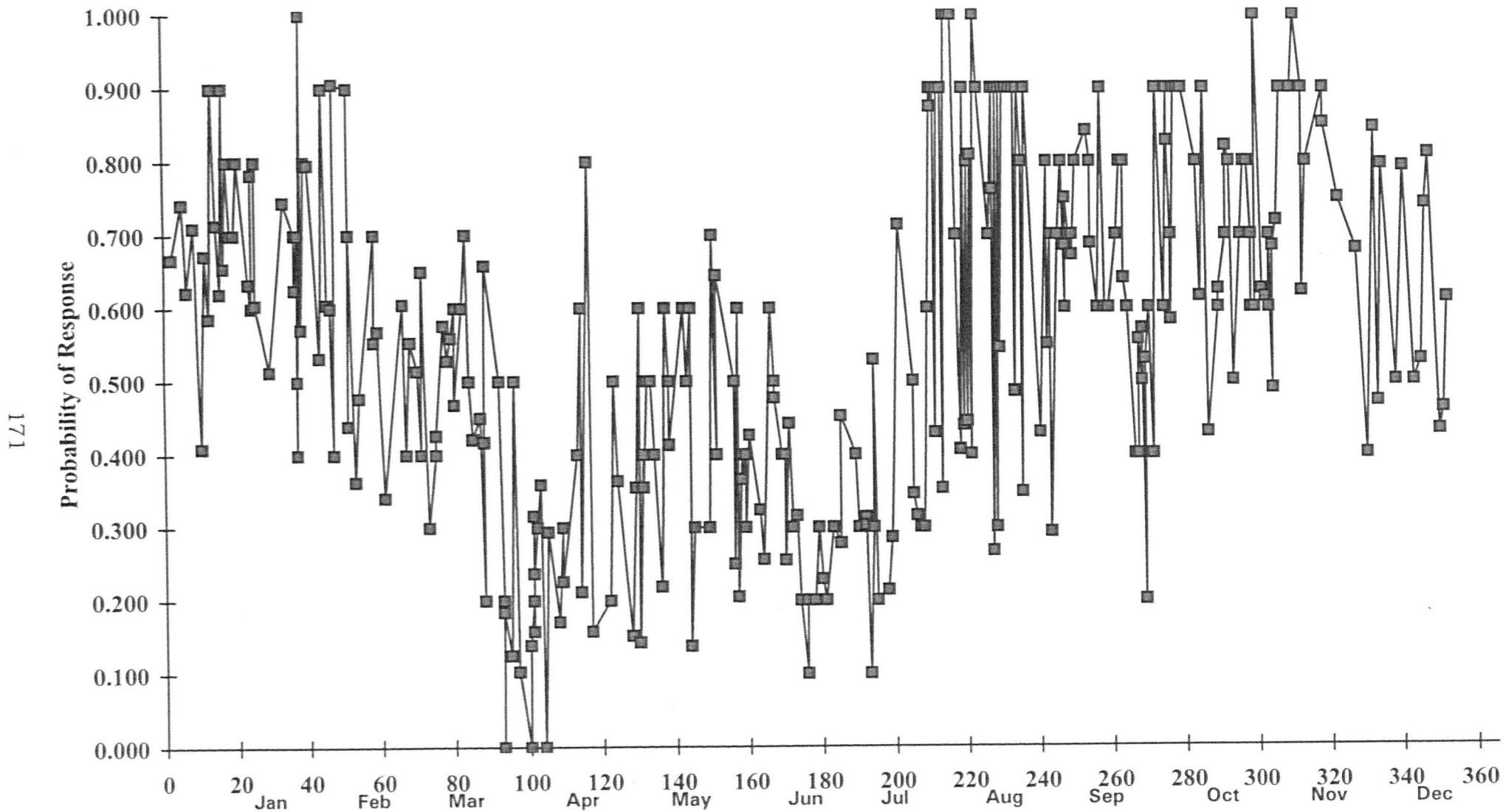
Figure 9.3.3. Seasonal monthly variation over five years
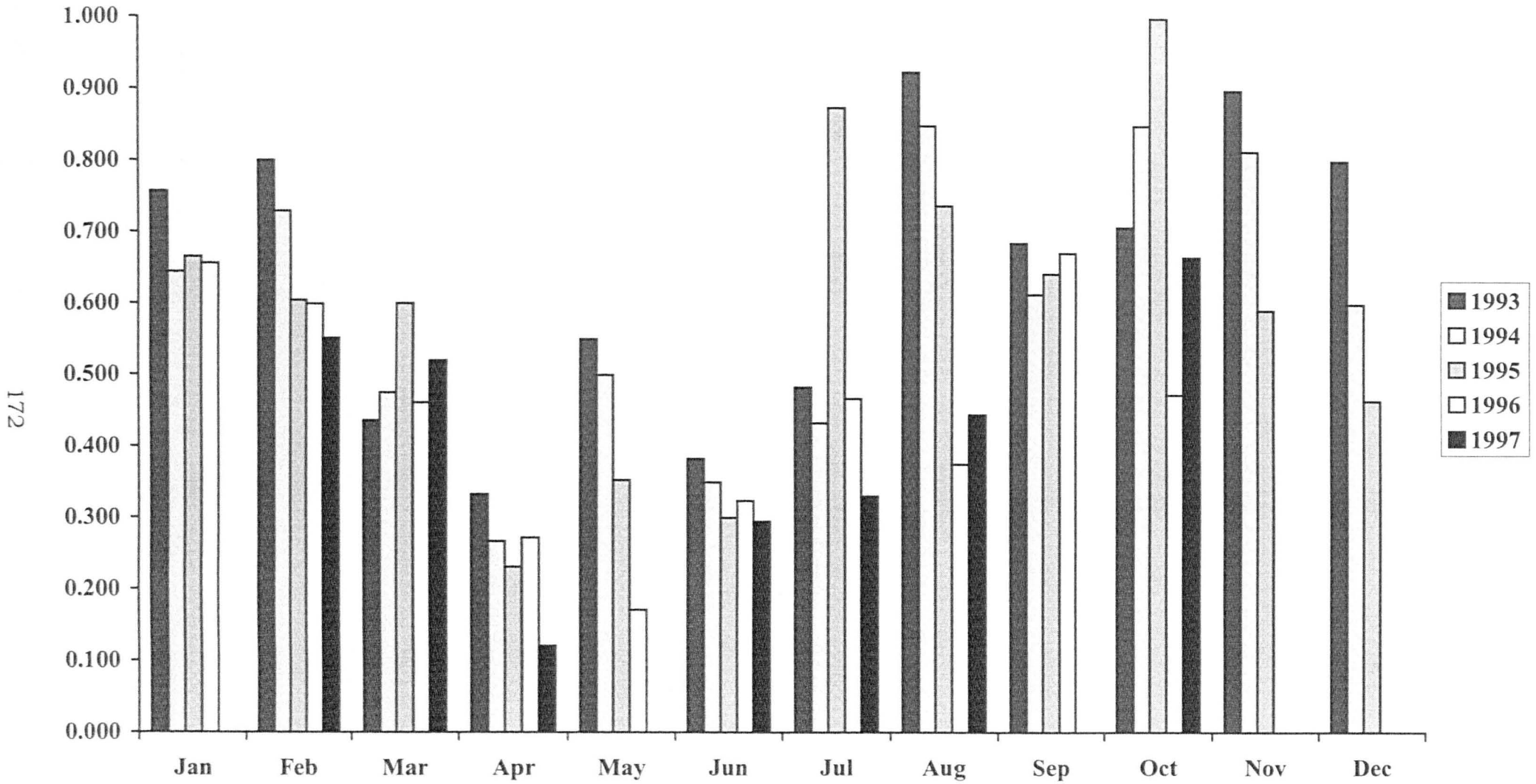
Figure 9.3.4. Monthly variations in olfactory conditioning over all the sampled years
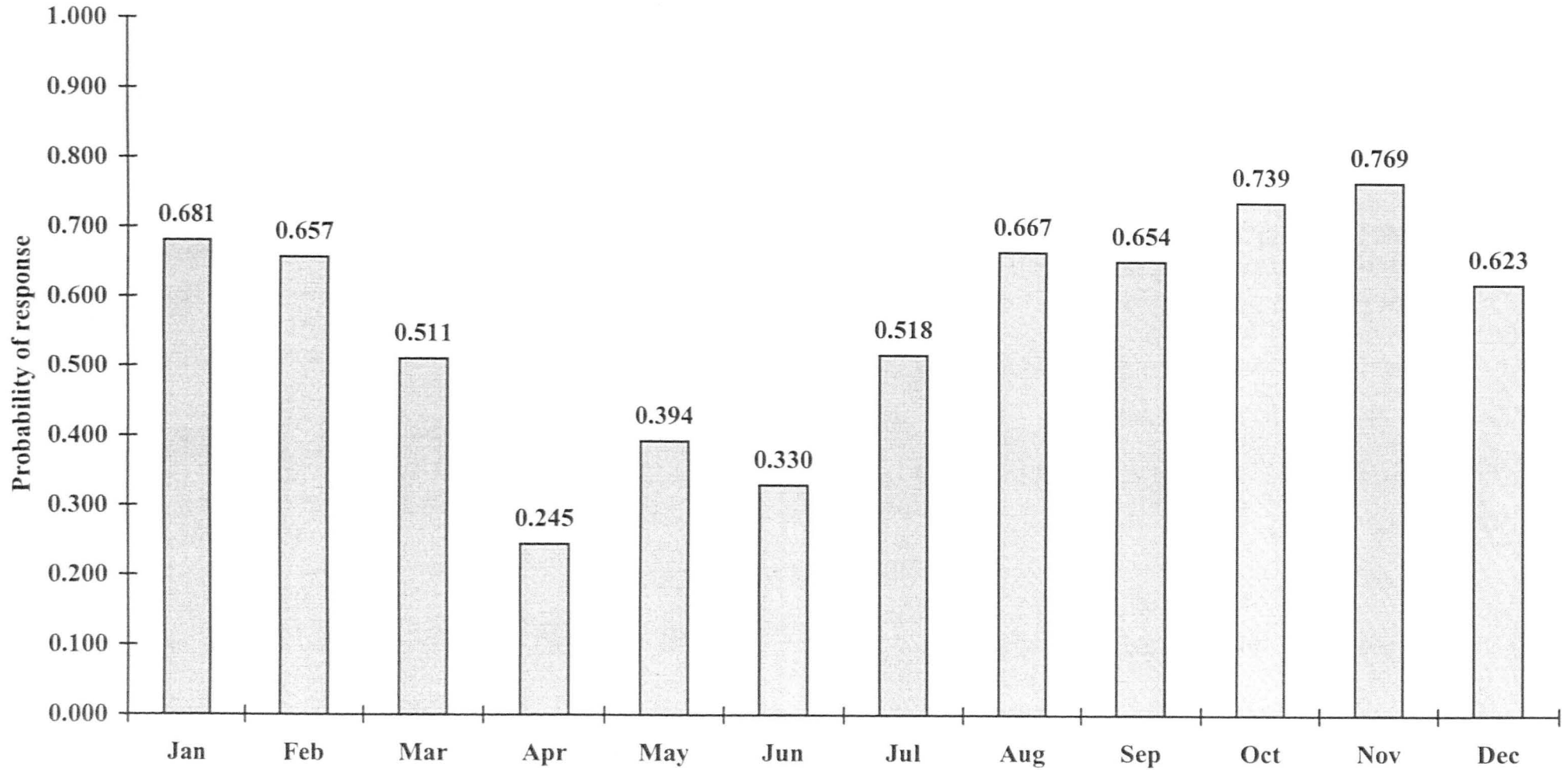
Figure 9.3.5. Typical weekly variation in olfactory conditioning across the four seasons
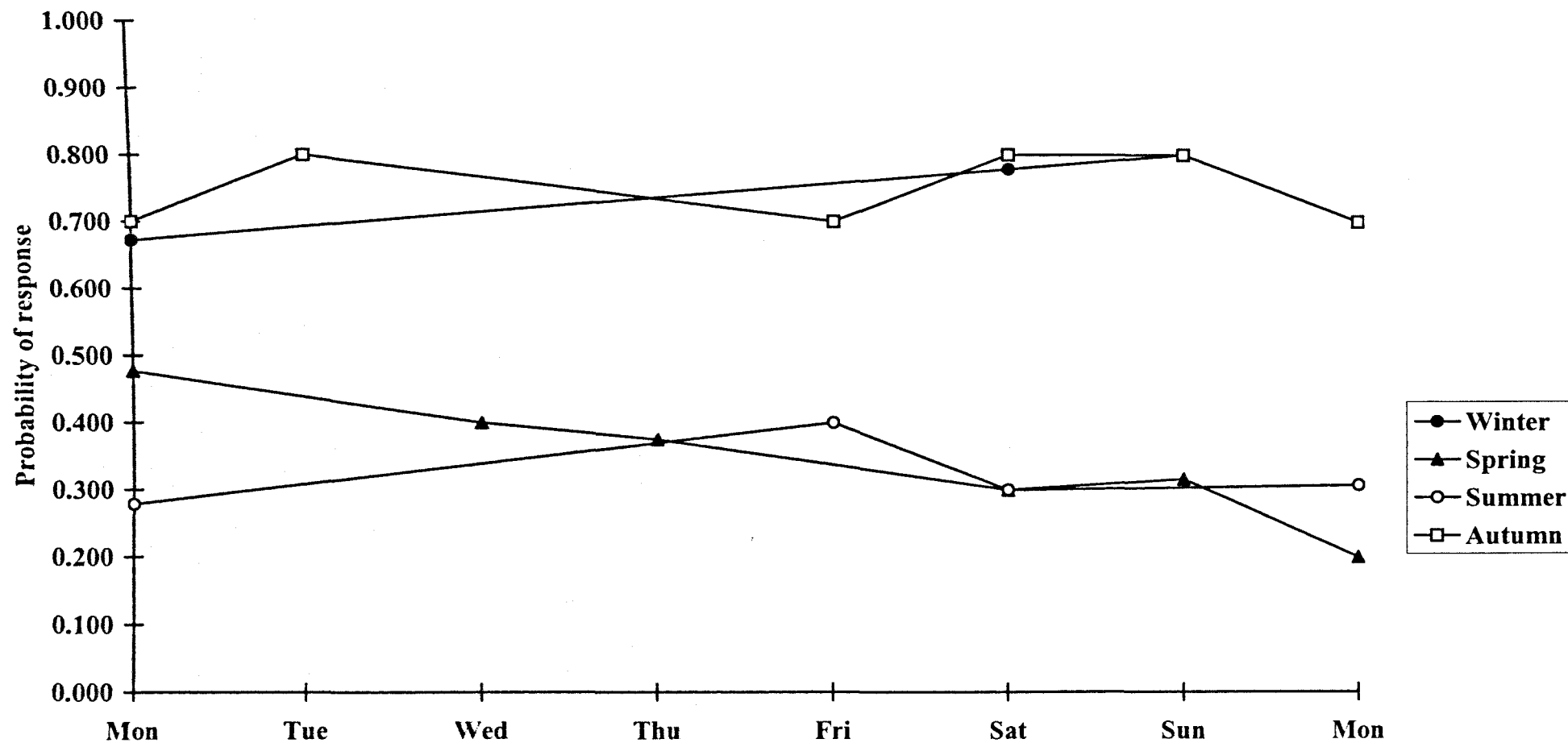
The indoor colony showed no such seasonal variation in PER conditioning. These bees performed a consistently high level of olfactory conditioning throughout the year. Why such seasonal effects occur in outdoor colonies remains the subjects of further research. The availability of natural forage as suggested by Gould and Gould (1995) remains a plausible explanation looking at the distribution of learning over season in our location. This hypothesis would receive further support when we compare the lack of variation in indoor housed colonies which perform at a consistently higher level. These animals experienced minimal variation in the availability of food, and were maintained on a light dark schedule consistent with the season and thus would have similar chronological opportunities for foraging flights as their outdoor equivalents.

In reviewing these data we must remain cognisant of the dynamic nature of bee colonies with season in temperate climates, and the changing demands placed on olfactory plasticity of individuals within the colony. Olfaction plays key roles in bee foraging, communication and kin recognition; with season, the distribution of caste specific behaviour is known to change, thus levels of forage experience, food location or processing experience may similarly vary dependent upon seasonal changes in colony strength and the number of juvenile adult bees in the colony. It is interesting to note that in the indoor colony in the present study the queen remained in lay year round. With no eggs located in many of the outdoor colonies from October to February. Further research is now required on the effects of previous foraging experience on PER conditioning, similarly the effects of caste specific olfactory demands on PER conditioning. Such experiments are currently underway in our laboratory.

Studies of time of year effects on PER conditioning from laboratories with less or more marked seasonal changes would provide a useful heuristic to this interesting seasonal effect.

CHAPTER 10:

10.0 General discussion

Honeybees are complex organisms, highly sensitive to external influences, such as temperature (Hooper, 1991), sun orientation (Von Frisch, 1967), and chemical signals (pheromones and odours) (Agosta, 1992). This complexity is even more remarkable when considering that the honeybee has a brain smaller than 1 mm^3 and has less than 10^6 neurones, which is 1/1000 of the number of neurones in a human retina (Menzel, 1990). Yet, this outstanding insect can perform an impressive array of behaviours, from flower handling (Daumer, 1958; Gould & Marler, 1984; Gould, 1986c; Menzel, 1990) and homing abilities (Gould, 1986; Menzel et al, 1990, Wehner et al, 1990; Dyer, 1991), to caring for its nestmates and communicating the direction and distance of a food source (Von Frisch, 1967; Gould & Gould, 1995; Menzel, 1990).

Honeybees in the field adapt their learning in a way that can be explained in terms of the ecological conditions they face, as they must change their behaviour to the constantly evolving demands of the available food (Heinrich, 1983; Kevan & Baker, 1983; Seeley, 1985). In the laboratory, this behaviour can be 'dissected' into its individual components enabling them to be assessed and examined (Menzel & Muller, 1996; Hammer & Menzel, 1995). One part of the intricate behaviour of the honeybee is olfactory learning, and the harnessed, restrained bee can be used to highlight essential elements of the associative conditioning between food and odour (see table 2.12), and with this information, we are able to form a link between field studies (Von Frisch, 1967; Menzel & Bitterman, 1983; Smith, 1991), laboratory studies (Bitterman et al, 1983; Ray & Ferneyhough, 1997a, b, 1999; Ferneyhough & Ray, 1999), and the cellular

analysis of this behaviour (Menzel & Muller, 1996; Hammer & Menzel, 1995; Fahrbach & Robinson, 1996).

The PER olfactory learning paradigm, utilised throughout this thesis, shows just one example how studies of honeybee learning can give insights into the mechanisms of learning and memory (Menzel & Muller, 1996; Hammer & Menzel, 1995). This form of olfactory learning is rapidly acquired, showing excellent long term retention (Menzel, 1990; Ferneyhough & Ray, 1999), as well as a specificity of the learning which is easily analysed by testing trained bees to alternative odours (see table 2.12). This also provides a useful model for other phenomenon important in learning theory, such as stimulus generalisation (Smith, 1991; Braun & Bicker, 1992; Bicker & Hahnlein, 1994; Menzel & Muller, 1996), massed and spaced training effects (Bitterman et al, 1983), even habituation and sensitisation (Braun & Bicker, 1992; Bicker & Hahnlein, 1994; Menzel & Muller, 1996), as reported earlier in the third chapter.

The olfactory system of the honey bee is particularly well researched (Gascuel & Masson, 1991; Gascuel et al, 1995, 1998) , and thus using this tool, bees can be readily used in neuroscience at all levels of enquiry, from molecular and cellular studies (Hammer & Menzel, 1995; Menzel & Muller, 1996) to behavioural genetics (Page & Robinson, 1988, 1989; Raff, 1994; Krebs & Davies, 1997), and anatomy and physiology (Mobbs, 1985; Menzel & Muller, 1996; Meller & Davis, 1996). The honeybee is not only restricted to olfactory learning, this social insect is capable of numerous other cognitive tasks, comparable to the rodent (Angermeier, 1966; Menzel, 1990; Fahrbach & Robinson, 1996), for example, reported in this thesis (chapter 6) the honeybee is able to re-locate the home hive following displacement in a sealed chamber, and can be used in route and detour learning, shape and colour discrimination (von Frisch, 1967), and with such behavioural plasticity being mediated by a relatively

simple and surgically accessible nervous system, offers an invaluable tool for neuroscience (Ferneyhough & Ray, 1999).

One of the major advantages of honeybees in learning studies, compared to rodents and birds, is the speed of acquisition and retention of the learning (Bitterman et al, 1983). Often tasks are learned in only one trial (Menzel, 1989, 1990), or a few massed training trials, which is of great advantage for a variety of research purposes such as studying the biochemical processes of learning and memory (Withers, Fahrbach & Robinson, 1993, 1995), relative to the temporal characteristics of the training. This proves very difficult in other research animals, such as the rodent who typically require multiple trials over many days to acquire a consolidated memory of the task (Bagnall & Ray, 1999).

Prior to any experiments commencing, a concrete method needed to be established, which could be called upon repeatedly to provide a stable base on which to build, and in the second chapter, this was demonstrated, taking the methods from numerous sources (see table 2.12). Preceding this, no paper had stated exactly how they had performed each component of their olfactory learning experiments, so a detailed methodology had to be developed. This was accomplished, and the reported method in the section 2.11 gave repeatable results, that compared successfully with those gained from other laboratories (see table 2.12).

To validate the methodology used throughout this thesis, these data needed to be more fully analysed to ensure that they were robust enough to be used throughout this thesis, so the final method reported (see section 2.11) was tested against a battery of exhibited components of learning. The learning behaviour in rodents has been thoroughly researched, yet this cannot be fully said about most of the invertebrate learning field, even though there exists a literature of vertebrate learning phenomenon exhibited by honeybees (Bitterman et al, 1983; Couvillon et al, 1983; Couvillon & Bitterman 1980, 1982, 1984; Sigurdson, 1981a, b), a good invertebrate model is required, and the chapter

addressing the parametric characteristics of learning in the laboratory attempted to do this, highlighting the different components of the behaviour.

The first example of a learning parameter was overshadowing, as this has been repeatedly shown in honeybees (Couvillon & Bitterman, 1980, 1982; Couvillon et al, 1983; Rescorla & Wagner, 1972; Smith & Cobey, 1994) as well as in vertebrates (Farthing & Hearst, 1979; Miles & Jenkins, 1973; Rescorla & Wagner, 1972). The results reported indicate that there was an overshadowing, of one odour over another, and this was in accord with the research detailed above.

The next parameter examined was blocking, which allows the subject to learn the most salient cue at the expense of a novel stimulus (Pearce, 1997). Blocking occurs when one stimulus is trained, then a compound of stimuli are presented, and then the original stimulus is presented once again. In the results reported, our data agreed with both the bee and general invertebrate literature (Thorn & Smith, 1997; Menzel, 1990; Couvillon et al, 1983; Hammer & Menzel, 1995; Menzel & Muller, 1996; Sahley et al, 1981; Smith & Cobey, 1994; Smith 1996, 1997) as no significant blocking occurred. However, a quite different result is experienced in the vertebrate literature, where blocking does occur (Kamin, 1968, 1969; Rescorla & Wagner, 1972; Rescorla, 1988). An explanation for this could be provided by Pelz et al (1997) who studied blocking in honeybees and did find an effect, as a compound of odour and mechanical stimuli were delivered. This method is also employed in the vertebrate literature, where one stimulus is presented with a different one, for example a sound and shock. This is in contrast to our research where a compound of odours (i.e. the same family of stimuli) were delivered, and this is perhaps why no blocking was found, as the intermediate odour is perhaps learned as a novel stimulus, and not as a compound of the two.

Pre-exposing the honeybees to the US (sucrose reward), prior to training has the effect of suppressing the subsequent learning (Abramson & Bitterman, 1986; Bitterman et al,

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1983), and has been shown in the vertebrate literature (Lubrow, 1973) as the subjects have had a previous experience of an unpaired reward. The results presented in chapter three agree and corroborate these findings, with levels of learning failing to attain levels above 20 %, compared to above 70 % for the controls.

The effect of randomly presenting the CS and US independently (Bitterman et al, 1983), forms no association between the two stimuli. Again, the results reported are in agreement with the literature, and are further supported by random presentation experiments reported in the vertebrate literature (Pearce, 1997).

The proboscis extension reflex is a repeated trials paradigm, much the same as habituation, but whereas the PER paradigm associates a CS with a US, habituation is a single repeated stimulus. It is an extensive and simple form of learning, where a repeated stimulus leads to a decrement in response, and has been observed throughout the animal kingdom (Jennings, 1906; Logan, 1975; Peeke & Veno, 1975; Ray, 1998; Whitlow, 1975) and has been successfully demonstrated in honeybees (Menzel & Muller, 1996; Braun & Bicker, 1992; Bicker & Hahnlein, 1994). The results reported in chapter 3.5 however were not as conclusive as those previously described in this literature however. There was a definite trend of a decrement in response after the US was presented to the antenna in the initial training, and the number of trials at retest were fewer. These data suggest that habituation was occurring, but were not as definitive as those reported elsewhere.

The inter-trial interval is an important component in olfactory and indeed any form of learning, as the time taken between presenting one stimulus and the next can profoundly affect the outcome of the trial. If the ITI is too short, the association cannot be consolidated into the long term memory, and similarly, if the ITI is too long, then the association is not formed, as the animal's attention has waned (Church & Gibbon, 1982; Gibbon, Church & Meck, 1984; Gibbon & Church, 1984; Gallistel, 1990). With spaced

trials, there is a definite set interval between each presentation, and this form of associative learning leads to long term consolidation of the learning (Carew, 1996; Gerber et al, 1998; Smith, 1991; Ray, 1998). In the honeybee, the time course of memory formation is well known (Erber, 1975a, b, 1976; Brandes et al, 1988; Menzel, 1990; Hammer & Menzel, 1995; Menzel & Muller, 1996), with amnesia resistant long term memory only being formed after 8 minutes (see section 1.7 for the time course and pathway of memory formation in the honeybee). The vast majority of the research reported in this thesis used this spaced training technique, and these gave results directly comparable with those reported elsewhere (see table 2.12). In chapter 3.6, the effects of a massed training schedule were also examined to compare and illustrate that a continuous presentation of a CS - US disrupts the learning processes, and indeed, the result obtained (see figure 3.6.3) showed a much reduced level of learning (Smith, 1991; Erber et a, 1980; Menzel, 1979, 1983) over the trials.

Honeybees need to be able to discriminate in the field, between a stimulus that signifies a rich food source, and one that no longer provides a reward. The method the honeybee employs is an extinction of the learning if a more profitable resource is consequently attended (Takeda, 1961; Wenner & Johnson, 1966; Bitterman et al, 1983; Couvillon & Bitterman, 1980, 1982, 1984). In the field, the honeybee would move off this forage patch in search of a better source, but in the laboratory, where the honeybee is restrained, the subject is unable to do this, so a reduction in the response to the CS is observed. Our results report that in an initial experiment, honeybees were given just one training trial, then the CS alone. This was delivered for up to 10 trials, and the learning curve showed a marked reduction in response. A second experiment gave the subjects either 1, 3 or 6 training trials, then the CS alone. Each of the groups eventually extinguished the learning, by no longer responding, with an average of 6 further trials needed until the response rate reached a level of zero. These results confirm those

gained in other laboratories (Couvillon & Bitterman, 1980, 1982, 1984; Bitterman et al, 1983; Takeda, 1961; Wenner & Johnson, 1966) and also the vertebrate literature into extinction of learning (Amsel, 1958, 1962; North & Stimmel, 1960; Ison, 1962; Senkowski, 1978; Gibbs, Latham & Gormezano, 1978; Hall & Pearce, 1979; Rescorla & Wagner, 1972). These results could be extrapolated to the field, and a honeybee may visit 6 further flowers in a foraging excursions without reward before abandoning that particular area. Indeed, Couvillon and Bitterman (1984) suggest that the extinction of the learning is a product of an unrealised anticipation of the reinforcement. This would form an interesting addition to this research, and could be used to relate laboratory based findings into the field.

In the final section of chapter three, the question of whether olfactory learning was a one trial learning paradigm was investigated. There exists a honeybee literature where the PER paradigm is described as being one trial learning (Erber, 1981; Bitterman et al, 1983; Gerber et al, 1996; Erber et al, 1980; Menzel et al, 1974; Menzel & Muller, 1996) however, Menzel (1979, 1990) and Smith (1991) state that the association, although initially being learned on the first trial is not consolidated into the LTM until the second or third presented trial. In the vertebrate literature, one trial learning does occur (Rose et al, 1979; Cherkin, 1972; Clayton & Krebs, 1998; Garcia & Koeling, 1966) but these are in food aversion studies, and aversive studies in honeybees have shown that it is not one trial learning (Balderama et al, 1987; Abramson & Bitterman, 1986). The research reported here tries to qualify if the PER is one trial learning, with an initial experiment, where honeybees were given either 1, 2 or 3 training trials, then were presented with a novel, unreinforced odour. All but the group given one trial did not respond to the novel odour, suggesting that the one trial group were generalising to the odour, as the association had not been consolidated, intimating that this form of learning was not one trial, but requires at least two trials. To test if the honeybees generalise to odours after

three trials, a second experiment was designed, incorporating a one hour consolidation period. The results demonstrated that after three training trials, the learning had been consolidated into the long term memory, as both groups tested showed a marked reduction in the response to the novel odour. These results hence show that PER olfactory learning is not one trial learning, even though the bees do form an association between the CS and US on the first trial. This is not consolidated into a specific memory for the trained odour until the subsequent trails are presented, i.e. at least two trials.

In conclusion, therefore, it was found that the honeybees obeyed all the expected results when compared with the other laboratories using the PER olfactory learning paradigm (see table 2.12), indicating that our methodology was strong and dependable. Also, that the appetitive learning in honeybees has many characteristics of associative learning shown in mammalian learning studies (Menzel 1985, 1990; Bitterman, 1988).

In the fourth chapter, honeybees were tested in the laboratory as to whether they possessed a time linking of their learning, and from the results there did appear to be an effect. Honeybees were trained over a particular time period, and then tested the following day, either at that same time or three hours later or earlier. The results indicated a pattern, but were not entirely conclusive which could be because the restrained subjects were not allowed to feed during the experiment. This is in direct contrast to experiments examining this phenomenon in the field (Koltermann, 1971; Gould, 1987a; Moore et al, 1989; Loo & Bitterman, 1992; Gerber et al, 1996, 1998) , where the honeybees visit feedstations at set times when food is available, but they are then free to feed ad lib outside of these set times on their normal forage. These free flying bees also set their own CS - US inter trial interval, and US exposure time, whereas with the laboratory based bees, these parameters are all rigidly controlled (see table 2.12). The restrained bees did indicate a time linked effect, responding greater after 24 hours, than the other times tested, and this circadian rhythm mirrors those

experiments demonstrated in free flying subjects, and this is exhibited in nature where the honeybee experiences particular times when flowers elicit a greater nectar flow (Gould & Gould, 1995).

Chapter five examined the effects of increasing the hunger and motivation of the bees to see if this affected the olfactory learning. It was postulated that increasing the concentration of the sucrose reward would increase the ability of the subjects to learn, and perhaps an optimum concentration of sucrose would be established. The results gleaned were not those anticipated though, as there was an experimental error in the design, as each bee was only presented with one choice of sucrose concentration, and so were unaware of any alternative. In the field, there exists a very different observation, as honeybees visit many flowers on their foraging trips, and so sample a large array of different nectar (and therefore sucrose) concentrations (Menzel, 1985), and on their return to the hive, will only communicate the most profitable food source (von Frisch, 1967). In order for this to be tested and exploited further in the laboratory, a more complex and detailed experimental design is needed, and we are currently examining this in our laboratory.

The sixth chapter investigated if socially isolating honeybees had any effect on two quite different memories. These were a newly acquired, novel, olfactory cue and a more essential memory which was the hive location. Both these memories need to be consolidated into the long term memory but for quite different reasons. The odour, as this is the cue used by the honeybee to associate a reward (nectar or sucrose), hence signifying food, and the hive position, as this memory offers the more fundamental benefits of shelter, food, warmth and social contact (Hooper, 1991).

The first section of this chapter specifically tested captured bees for learning of a previously trained olfactory cue, the odour geraniol. After training, the subjects were held in specially designed bee boxes for anything up to 9 days, prior to testing with the

trained odour, as well as further tests with an untrained novel odour, peppermint, which was to examine if the bees generalised to any stimulus. The results showed no adverse negative effects on the learning, memory and retention of the odour after long term captivity. These findings should not be too surprising, as in the field under natural conditions, honeybees have been shown to recall feedstation positions or flower locations six months after confinement in the hive (Lindauer, 1960a, b; Menzel, 1990; Gould & Gould, 1995). Therefore, these laboratory based findings echo those found in the natural situation.

In the second section of this chapter, honeybees were once again subjected to long term captivity, and were then released from various distances and directions to test their homing capabilities. The findings suggested that there were no adverse effects on their ability to re-find their hive, irrespective of captive time, distance or position. The one exception to this were the honeybees that were held captive for the longest period in this experiment, and these 8 day captive groups were found to return in lower numbers than the 2 day, and 3 day captive bees. Although, this was only when the 8 day released bees were from distances greater than 55 metres, there being a high returning rate for distances below this, but 70 % failed to return to the hive when the 8 day captive bees were released from the greatest distance used in these experiments, which was 500 metres.

The explanation for this could be that the released subjects became disorientated, unable to regain their bearings after having been confined away from the hive, in the dark for a considerable amount of time (compared to the lifetime of an adult forager). The bees that were also kept for 8 days but were released closer to the hive may have found their way back, by circling the release area, and becoming aware of the location of the hive. These bee boxes have an unlimited variety of uses, as they do not appear to affect the honeybee behaviour long term, and they can be used in other applications such as

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recovery from surgery (Erber et al, 1980), effects of drugs (Mercer & Menzel, 1982; Bicker & Menzel, 1989; Menzel, Hammer & Sugawa, 1989; Menzel, Gaio, Gerberding, Newrava & Wittstock, 1993), whereby the operated animal could be stored post-operatively in more natural conditions, than being restrained, and colony recognition (Breed, 1987; Page Robinson & Fondrk, 1989; Downs & Ratnieks, 1999), where honeybees could be socially isolated from the hive, yet still allows movement.

In the final three chapters (7, 8 and 9), the effects of age, caste and season were investigated, to examine if they had any effect on the olfactory learning. As the demands on the honeybee change as it ages, so the demands on its' olfactory acuity change (Dukas & Visscher, 1994; Fahrbach & Robinson, 1995; Robinson, Fahrbach & Winston, 1997; Ray & Ferneyhough, 1997a, b, 1999). The development of a complex olfactory memory begins to play an increasingly important role in the life of a honeybee, and her colony (Fahrbach & Robinson, 1995; Robinson et al, 1997). From an initially low level of olfactory learning, when the bee is performing nurse duties (Winston, 1987), such as cell cleaning and feeding larvae, to the period where the bee is carrying out guard duties (Robinson, 1992), vetting the incoming foragers, and other bees undertaking orientation flights, this is when the olfactory sense does not need to be so acute, as the honeybee just has to recognise its kin (Breed, 1987; Page et al, 1989; Breed et al, 1992; Downs & Ratnieks, 1999). However, when the bee begins to forage it needs to commit intricate olfactory and spatial details into its memory, both in the short term and long term (Menzel et al, 1974; Erber, 1975a, b, 1976), in order to alert its nest mates to potentially rich forage sources.

This increasing 'awareness' of the importance of olfactory cues as the bees progresses through the caste system, is not really surprising, because, as it progresses through, it learns more of the importance of the behavioural rewards i.e. pollen and nectar, that are associated with the olfactory stimuli of a particular flower species. This information is

of no use to a very young bee, as it cannot fly, and the paramount needs of the hive, necessitate it to care for the maturing brood (Winston, 1987). It is this altruistic behaviour, that perhaps keeps all the bees from becoming precocious foragers, whereby they mature at an accelerated rate, bypassing the nurse and guard duties. This delayed onset of foraging, where the bee cares, cleans, and guards the colony, may further allow the maturation of the brain (Huang & Robinson, 1992; Fahrbach & Robinson, 1996), and the steady increase in the role of juvenile hormone, which regulates the caste behaviour (Robinson, 1987a, b).

The guard bees long term retention of olfactory stimuli in the laboratory, did not have as high a probability, as the mature forgers (Ray & Ferneyhough 1997a, b, 1999; Ferneyhough & Ray, 1999). This was not surprising due to the role of olfaction in the recognition of kin (Breed, 1983; Breed, Smith & Torres, 1992; Downs & Ratnieks, 1999). However, as the training odour was the floral scent geraniol, it could be argued that as the guards do not have an innate sense of floral smells, they could have an equally high long term retention and learning for the odour of her kin. It should also be noted that the guards do not gain an immediate reward for allowing a bee into the hive, whereas in the PER experiments, they were given a sucrose reward. The reward the guards are given is in the long term, in that their colony is not taken over by another.

In summary, the findings of these studies were that bees only begin to learn to the levels obtained by mature foragers, when they are five days of age and older, however, this was in an artificial hive containing far fewer bees than in a full hive, so a precocious development of behaviours was occurring. In a further investigation, bees from a full hive colony were analysed for when olfactory learning began to appear, and here, it was not until 16 days of age that honeybees started to show levels of response equivalent to adult mature foragers.

Bees of certain castes may vary in their ability to learn in the field, dependant on different behavioural demands at different times of their life cycle within the colony organisation. The caste / age relationship can also be manipulated, producing a precocious development of the bee, which accelerates the learning capabilities, this work needs to be examined in the field to see if the results correspond with the laboratory findings. This plastic behaviour will also be manipulated to force forager bees into nurse duties, and nurse bees into forager duties, with a corresponding study of learning ability.

Nurse bees are not able to learn olfactory conditioning, or store the information in their long term memory. Guard bees do learn, but not to the extent of the mature foragers. This caste system can be manipulated, whereby precocious foragers are produced by reducing the numbers of mature foragers. The levels of learning are not equivalent to the levels seen in their corresponding ages of unmanipulated bees, but are comparable to mature foragers. Again, it is the maturation of the brain together with the behavioural caste that dictates the levels of olfactory learning, although it does appear that it is the caste that has the greater importance (Ray & Ferneyhough, 1999).

Two to three day old nurse bees, when reared on their natural comb, with and without brood present, which allows them to either show their natural nursing behaviour if brood are present, or bees without brood present and were therefore unable to exhibit nursing duties, showed higher learning levels than the identically aged bees allowed to show nurse behaviour. This result shows that it is the act of nursing behaviour that suppresses the olfactory learning. These data show that it is the behavioural role of the honeybee within the colony that is the more powerful influence on olfactory learning, than the maturation of the nervous system (Gascuel et al, 1995, 1998). Behaviour can be altered by manipulating the hive demography, removing a whole caste from a colony,

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such as the nurse bees, causes some foragers to revert back to hive duties, even though they have foraging experience.

The major point to take from both these chapters investigating behavioural development of the honeybee is that it is imperative that all bee researchers need to report the actual caste they are testing in the laboratory, as this will influence the results gained. The actual hive demography also plays an important role, as mini-nucleus colonies may be devoid or severeyl lacking a particular caste, and this could cause a precocious development or regression, affecting the levels of olfactory conditioning (Bhagavan et al, 1994; Ray & Ferneyhough, 1997b, 1999). These differences in the levels of learning between each age and caste suggests that the behavioural demands placed upon the honeybee determines the brain maturation, rather than the actual age of the bee. Although a 25 day old bee may be foraging, another of the same age may be a nurse bee, and although both their nervous systems are the same age, the behaviours they exhibit are very different, which in turn affects the olfactory acuity of the individual, and this behavioural maturation is under direct control of JH. There are differences in the rate at which workers progress through this caste system, with some showing a precocious behavioural development, while others mature slowly (Sekiguchi & Sakagami, 1966; Nowogrodski, 1983). There is also an inter-individual variation in the degree of task specialisation with an age caste, e.g. only a few percent of the workers ever guard the hive (Lindauer, 1952; Moore, Breed & Moor, 1987), also, some bees guard the hive entrance continually for several consecutive days (Moore et al, 1987), while others perform these duties only infrequently. This division of labour within the hive is extremely flexible (Oster & Wilson, 1978; Holldobler & Wilson, 1990), being able to adjust to constant variation in age demography (Fukuda, 1983) and forage availability (Visscher & Seeley, 1982) by continuous adjustments to the numbers of workers in each group (Page & Robinson, 1991). To cope with such adjustments, the

colony must have a very plastic behaviour mechanism, such as atypical age dependent behaviour (Winston, 1987), and increase in the numbers of the workers performing a task (Kolmes, 1985a; Kolmes & Winston, 1988) and a change in the total activity levels (Sekiguchi & Sakagami, 1966; Kolmes, 1985a; Winston & Ferguson, 1985). These changes can lead to this accelerated behavioural development, and precocious foragers begin to appear (Nelson, 1927; Rosch, 1927; Himmer, 1930; Haydak, 1932; Sakagami, 1953; Robinson et al, 1989; Fahrbach & Robinson, 1995), or behaviours may be reversed, so foragers become over-aged nurse bees (Milojevic, 1940; Rosch, 1930; Fahrbach & Robinson, 1995; Ray & Ferneyhough, 1999).

The hormonal control of behavioural development has been researched (Robinson et al, 1989; Huang, Robinson, Tobe, Yagi, Strambi, Strambi & Stay, 1991; Huang, Robinson & Borst, 1994; Robinson, 1992), and there are structural changes in the mushroom bodies of the brain with this behavioural development, with the volume of these structures being significantly larger in foragers than 1 day old bees or nurse bees (Withers et al, 1993, 1995; Durst, Eichmuller & Menzel, 1994). These behaviours can also be monitored with JH titres in the haemolymph, with precocious foragers having a precocially high JH titre, and overaged nurses having a lower titre that their same aged forager sisters (Robinson et al, 1989; Robinson, Strambi, Strambi & Feldlaufer, 1992; Huang & Robinson, 1992). This ability of the honeybee to artificially reverse or progress to different behaviours opens many different possibilities to examine brain maturation and development in the honeybee.

Investigating changes in PER over season (chapter 9), the levels of learning vary throughout the year, with higher probabilities of PER in autumn and winter, and low levels in spring and summer (Ray & Ferneyhough 1997a). This is in stark contrast to the levels of learning seen in a colony kept indoors throughout the year, and fed the same food. Here, the levels of learning do not vary during the year, and remain constant. With

this seasonal variation in olfactory learning, care needs to be taken when reporting results, as this could lead to inter and intra laboratory variations.

The honeybee populations and hive dynamics behave in a predictable seasonal and yearly cycle, this begins with the hive coming out of the dormant winter, the queen begins to lay eggs in early February, and 21 days later these emerge. The queen increases the egg laying rate into March/April, causing a steep rise in the brood population (Hooper, 1991). The brood to adult ratio rises above 1, so even in good weather, the amount of forage brought in will be used up in the maintenance of the colony. The forage brought in is used up so quickly because the number of available adult foragers who aren't nursing the emerging brood is low, and also, the available forage in the early part of the season is of poor quality, with a low sugar content (Hooper, 1991). It is these factors which lead to an increased population of young inexperienced foraging bees, who are not capable of learning to the extent of their older forager sisters (Ferneyhough, Ray & Wilson, 1995; Ferneyhough, Ray & Scutt, 1995). Bees usually begin to forage when they are between 19 - 22 days old, however, newly emerged bees at the start of the season can begin as young as 7 days of age, 2 weeks before the usual onset of this behaviour (Page & Robinson, 1991).

Around May, the queen has completed her main increase in egg laying, and the laying curve begins to plateau. It is at this time of the year, that the hive population is at its youngest, having up to 30,000 developing brood. In spring, the first of the years flowers begin to emerge, and the greatest nectar flow volumes are recorded (Lindauer, 1948). This carries on until the end of June, when the nectar volumes begin to reduce. By the end of June, the potential brood has reduced in size, coinciding with the better quality flora at this time of year (summer), which has a higher nectar sugar concentration than the spring flora (Lindauer, 1948). As a result, the increasing forager population bring back pollen to be stored as honey, as the brood is not present in the same numbers as

earlier in the year. By the end of July, the brood population has been radically reduced, causing a knock on effect of a rapid decrease in the adult population by mid August. After this busy period, coming into late September / October, the hive begins to switch its priority into storing as much pollen and nectar as it can, for the coming winter. This smaller, decreasing population, live on these stores of honey which were built up during late summer and early autumn, throughout the winter. The cycle of the honeybee population begins again when the queen begins to lay new eggs in late winter, and so the honeybee population gradually diminishes until the following spring, when populations increase once more, as the brood begins to emerge (Hooper, 1991).

The year curve can be divided into two distinct periods. August to February, which covers autumn and winter, when the bees are preparing for, and experiencing winter. These bees are all experienced foragers, and hence, 'good' learners (defined by Brandes et al, 1988), with the PER probability consistently above 0.64. This is due to the bees having no natural available forage, and a low ambient temperature reducing their activity. They must feed opportunistically or rely on food stores within the colony. Consequently, in a PER conditioning paradigm, such bees may be more highly motivated to associate an odour with a food reward. The bees which are taken in the winter may give increased PER results, as they have been taken from a relatively cold environment, where their metabolism is much reduced (Crailsheim, 1986), and placed in a warm laboratory, given 50 % sucrose solution, and therefore are more compliant, and willing to learn than May-June subjects where there is also a greater available forage, choice and a more concentrated nectar. The second area covers March to July, where the PER probability fails to rise above 0.5, with the maximum average in July (0.48), coming to the end of the high nectar concentration. The bees at this time of year range from young nurse bees, to older experienced foragers. With perhaps a greater proportion of younger bees after the winter when they begin to emerge. There are distinct peaks in

the year curve which may indicate the natural shift in the hive dynamics. The first peak is in May, when the brood are emerging. The second is in August, where there are an abundance of mature foragers, and the third in November, where there are a majority of over wintering bees. These peaks may also coincide with changes in flora. May is in spring, so a high nectar concentration is available, August is when the late summer plants flower, and November would be when the hive is using its internal food stores. Lindauer (1948, in Gould & Gould, 1995) produced a curve which shows the concentration of sucrose required to switch bees attention away from the natural forage, when this is superimposed onto the year curve (see figure 10.0), the data is brought out of the laboratory, and into the field perspective. Before the two curves cross, in early July, the nectar the bees are obtaining in the field is being preferred to that offered in the laboratory (50 % sucrose solution). However, as the nectar concentration available in the field falls, the laboratory sucrose is of a far higher concentration than that found in nature at this time of the year this coincides with the marked increase in learning, from July into August, and over into autumn and winter. Dukas and Real (1993) state that the variance of nectar affects the learning in bumble bees, whereby the flower species may have differing nectar volumes. This causes the bee to learn at a slower rate than if the flower had an even standing crop of nectar. This would be more prevalent at the start of spring, when flowers bloom at different times. The different nectar volumes are also at the times when nectar is at its highest molarity.

The research reported in this thesis illustrates the rapid and complex behavioural development of the honey bee, and in light of our findings, that the levels of learning is dependent upon the age and caste of the honeybee, and also the season in which the bee is tested, it cannot now be assumed that the learning of experimental subjects is uniform. This leads on that some of the data presented by other laboratories may need to be re-examined. The days of blindly taking bees from a hive for appetitive associative

Figure 10.0. Seasonal variation in olfactory conditioning against the sucrose concentration required to divert honeybees attention away from natural forage
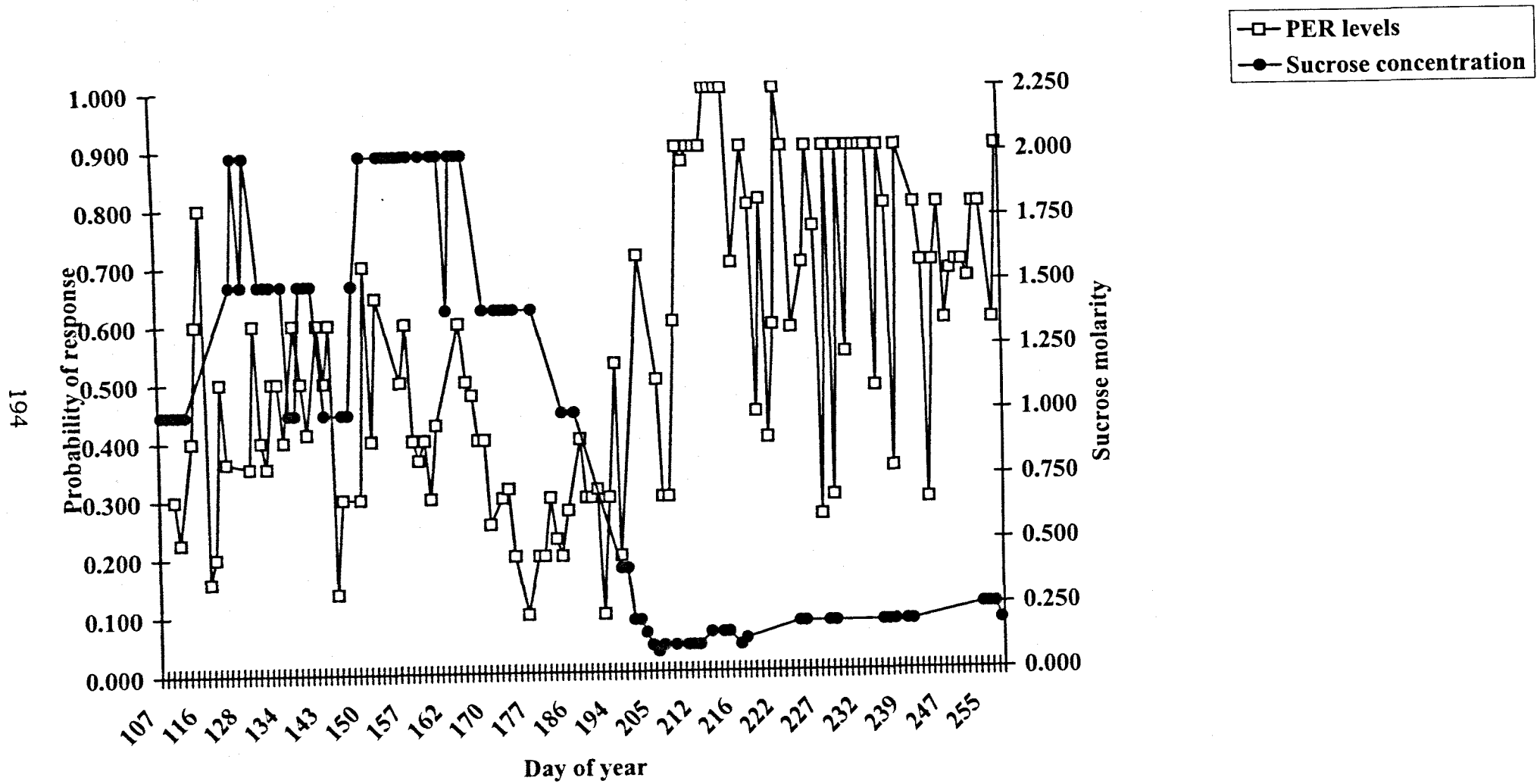
learning experiments, assuming that all the bees were equal needs to be looked at more closely. Also, as the colonies kept indoors throughout the year showed no seasonal variation in learning, further investigations in to this area of keeping bees used for learning experiments should be looked at.

This work will provide useful data on the ecological validity of PER conditioning as a model of olfactory learning, and its development. Further supporting field work will provide an interesting insight into the changing demands placed on the olfactory system of the honeybee and its role in their social organisation and communication. In conclusion, invertebrate learning using honeybees should not be confined to a rushed period over the summer months, but should be expanded throughout the year, specifically over winter, when few laboratories in temperate climates use the available increased learning.

In summary, the research presented in this thesis shows that the honeybee is capable of;

• Exhibiting associative conditioning phenomenons comparable to the vertebrate learning literature, and hence the honeybee can be used as a tool for learning paradigms where a flexible, easily manipulated and accessible model is required.

• The honeybee does possess a time sense, which is based on a 24 hour circadian rhythm.

• Long term captivity of honeybees, where they are allowed free access to food, and are unrestrained, neither seriously affects their olfactory learning or homing abilities.

• Behavioural demands placed upon the honeybee within the hive society due to hive demography, food availability or the time of the year, determines the level of olfactory learning.

• The time of year that the honeybee is trained and tested in the laboratory seriously affects the result obtained, with an increased level of response in the winter months, and a suppressed level of learning in the summer months.

Finally, even though honeybee research has been progressing for nearly 100 years, with new techniques and findings constantly being made, we are only just beginning to understand the potential for this fascinating and complex animal, which has much to offer the fields of neuroscience, ethology, ecology, biochemistry and genetics to name but a few.

The futures bright, the futures yellow and black !!

CHAPTER 11:

11.0 References

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Appendix 1. Publications.