OLFACTORY LEARNING IN A PARASITIC WASP

MPhil Dissertation

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"IT IS ONLY IN THE LAST 10 YEARS THAT THE PARASITOIDS ABILITY TO LEARN HAS BEEN TRULY APPRECIATED"
(GODFRAY, 1994).

ABSTRACT

In numerous past studies, parasitic hymenoptera species have demonstrated sophisticated learning abilities particularly when associating chemical odours with a reward (e.g. Lewis and Takasu, 1990; Turlings et al., 1993a). This type of learning is termed associative learning and can be defined as "The process by which animals learn about casual relationships between events and behave appropriately as a result" (Dickinson, 1980).

The parasitic wasp *Cotesia plutellae* is part of a tritrophic system in which it utilises chemical cues from the food plant of its host, *Plutella xylostella*, in order to locate this host more effectively. This study investigates how behaviour changes as a response to the learning of these volatile chemicals. Initial experiments determine whether learning of chemical odours from the Chinese cabbage occurs and how learning modifies preference for a particular odour. The study then focuses on which particular chemicals in the profile are important for learning and whether chemicals outside the insect's natural foraging range can be learnt.

The results suggest that learning does occur in this species and that actively released plant volatiles play a particularly important in role. The sesquiterpene (E)-ß-caryophyllene may be a key component in learning, although it is likely that a combination of chemicals are more effective. *Cotesia plutellae* appears to learn a novel chemical vanillin, which does not normally occur in its foraging range. Bioassays showed that as well as spending more time in the appropriate odour field, experienced parasitoids may decrease their velocity and increase meander. Applications of this research are discussed.

ACKNOWLEDGEMENTS

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CHAPTER 1: INTRODUCTION AND OVERVIEW

1.1 Aims and objectives

This project investigates the learning of volatile chemicals in a species of parasitic wasp. The main aims are as follows:

- Firstly, to confirm that the learning of volatile plant chemicals occurs in the laboratory population of parasitic wasp, *Cotesia plutellae* used in this study

- Then to Investigate, how learning modifies innate preferences for different types of plant odours

- Which chemicals are important for learning in the odour profile of a plant under host attack

- Whether *Cotesia plutellae* is able to learn a novel chemical, which is not typical of its natural foraging range.

Experiments involve direct observations or computer software analysis of experienced individuals to see how attracted they are to plant odours and this will be compared with naïve controls and wasps which have had only a sensitisation experience. Particular chemicals important in learning and the ability to learn novel chemicals will be explored in detail by testing the response of *Cotesia plutellae* to three selected chemicals from the profile of infested Chinese Cabbage before and after experience. An extra side preliminary study discussed in the APPENDIX, works in conjunction with computer sciences to create a

programme, which can analyse physiological changes occurring in the insect brain due to learning. This uses data from another species of hymenoptera – the honeybee (*Apis mellifera*).

1.2 Introduction

1.2.1 An Introduction to Parasitoids

Parasitoids can be described as 'insects whose larvae develop by feeding on the bodies of other Arthropods, usually insects, resulting in the death of the parasitoid's host' (Godfray, 1994). They are sometimes viewed as an intermediate between predators and parasites because, like predators, they always kill the host that they attack and like parasites they require just a single host to mature. Parasitoids are found within several of the insect orders including the diptera and coleoptera and a few even exist in the neuroptera, but most are found throughout the hymenoptera in the parasitica division (Askew, 1971). These insects are known as the parasitic wasps.

The lifecycle of a parasitic wasp can be divided into four stages: egg, larva, pupa and adult and they are holometabolous (show complete metamorphosis). The adult female lays her eggs either directly onto a host or in its immediate vicinity using her ovipositor. Hosts are commonly other insect species and the juvenile stages are more often attacked, although, some wasps also lay eggs inside the egg or pupal stage of their host. The life histories of parasitic wasps can be very different to one another and it is important to define these characteristics. For example, endoparasitoids develop on the inside of their host's body whereas ectoparasitoids live externally with their mouthparts buried in the host's body and parasitoids can share a host (gregarious) or feed alone (solitary).

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1.2.2 Cotesia plutellae and its Host

This study uses parasitic wasp species *Cotesia plutellae* which is a solitary, endoparasitoid of moth species *Plutella xylostella,* Linnaeus (Lepidoptera; plutellidae). It is generally reported to be host specific to *P.xylostella* larvae although it may occasionally attack other lepidopterous hosts (Verkerk & Wright, 1996). *C.plutellae* can develop on all four larval instars of *P.xylostella*, although 2nd instar is the most suitable for development (Talekar & Yang, 1991).

The parasitoid has a life cycle of around 13 days. It spends 4 days in the pupal stage and will then emerge in its adult form and mate (adults can survive for 28-32 days). Once mated, the adult female will search for a *P.xylostellla* host in which she will oviposit her eggs. The eggs hatch into larvae, which feed off the inside of the host for 8 days until they emerge and pupate (Figure1).

Figure 1:The lifecycle of Cotesia plutellae (right) and its host Plutella xylostella (left). The

adult parsitoid lays its eggs inside the larval host (Photographs from www.new.agri.co.uk).
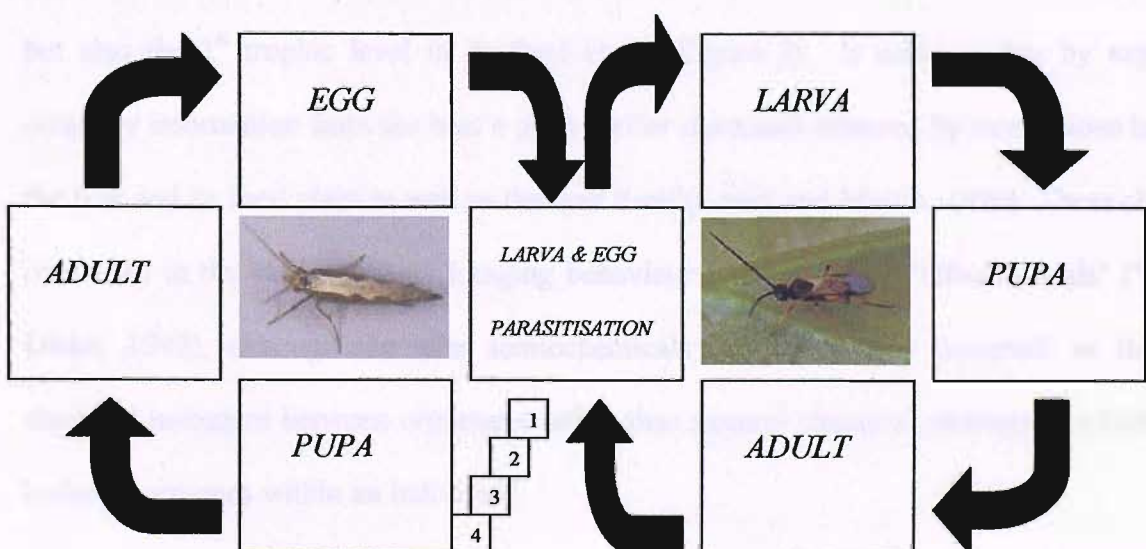
P.xylostella is a major pest of cruciferous vegetables and is widely distributed on a global scale feeding on a large range of host plants (Sarfraz et al., 2005). *P.xylostella* goes through 4 larval instars, which can be determined by measurement of their head capsule under a microscope (see methods section). Each larval instar will live for a variable amount of time ranging from 1.83 days to 4.18 days.

The larva will pupate for around five days emerging as an adult moth with a life span of 16 days. The adults will then mate and the female will lay many yellow eggs on its plant host (Figure 1). The young larvae feed from the inside of the plant causing a characteristic net-like damage.

C. plutellae is a useful model species as it is a large parasitoid and is simple to rear in captivity. It also shows sophisticated learning capabilities and is important in the control of a major pest species (Potting et al., 1999).

1.2.3 The Tritrophic System

C. plutellae is part of a tritrophic system in that it interacts not only with the 2nd trophic level but also the 1st trophic level in its food chain (Figure 2). It achieves this by exploiting olfactory information from the host's plant and/or chemicals released by interactions between the host and its food plant as well as the host itself (Lewis and Martin, 1990). These chemical cues used in the stages of host foraging behaviour can be termed 'infochemicals' (Vet and Dicke, 1992), although the term semiochemicals is more widely accepted, as these are chemical messages between organisms rather than general chemical messages - which could include hormones within an individual.

Figure 2: The Tritrophic System. C. plutellae detects chemicals from the 1st trophic level (plant) as they are 'DETECTABLE'. By LEARNING to ASSOCIATE these chemicals with the 2nd trophic level (host) they become more 'RELIABLE'.

B. rapa   1st   **P. xylostella**   2nd   **C.plutellae**   3rd
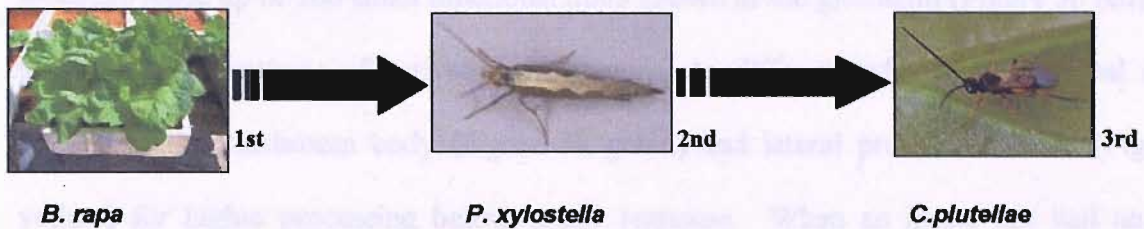
Natural selection can greatly influence the way in which parasitoids utilise these semiochemicals and 'indirect' search strategies may be adopted. Hosts produce chemicals which provide reliable cues to their whereabouts, but as they have evolved to remain inconspicuous, they are not always 'detectable' apart from during times when they need to be conspicuous to one another (e.g. when communicating intraspecifically). Information from the host's plant can predict the host's presence (Vet and Dicke, 1992) - this may not be as 'reliable' as chemicals from the host itself, but as it is in the interest of the plant to be detected, it may be more 'detectable'. This concept is known as the reliability-detectability hypothesis, the dilemma that the parasitoid faces as to which volatiles to utilise, can be overcome if it learns to associate a particular plant volatile profile with a host by associative learning, making that cue more 'reliable.'

1.2.4 Odour Detection and Processing

When an insect detects an odour it is received by chemosensory olfactory receptors on the antennae. Each receptor is housed within a hair and surrounded by sensillum lymph. Odour

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molecules enter the hairs through tiny pores in the cuticle and a signal is passed down odorant relay neurones (ORNs) through the antennal nerve and then the olfactory nerve to the brain. In the brain, the signal is first processed by the antennal lobes (Figure 3a). The Antennal lobes are made up of 160 small functional units known as the glomeruli (Figure 3b red) which show different patterns of activation in response to different odours. The signal is then relayed to the mushroom body (Figure 3b green) and lateral proto-cerebellum (Figure 3b yellow) for higher processing before motor response. When an insect has had an odour learning experience, physiological changes with regards to the odour processing in the insect's brain and possibly also its antennal sensitivity occur (Vet et al., 1990).

Figure 3:The Insect Brain. *a) Cross section of a honey bees brain and b) flow diagram of higher brain processing in a hymenopteran insect (Galizia et al.2003)*
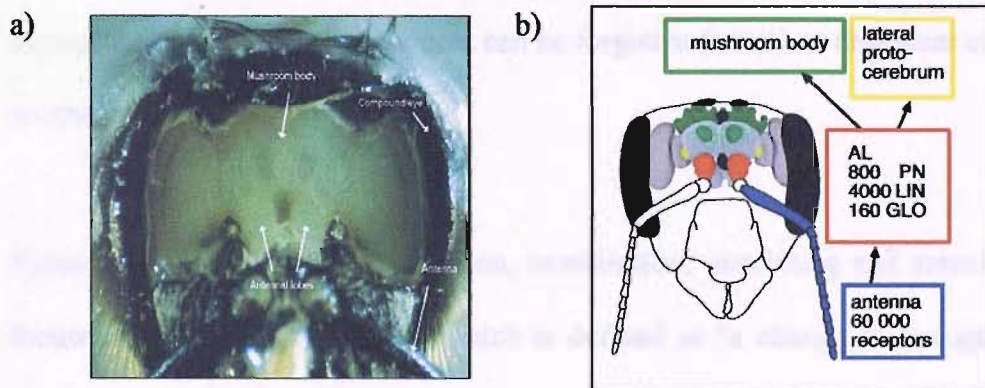
In the brain, glomeruli activation patterns and intensity change in response to the learned odour and studies have attempted to image and map these changes. From this they have produced functional models describing the learning process at a physiological level (Galizia & Menzel, 2001; Sasche & Galizia, 2001; Smid et al., 2003; Sandoz et al., 2003).

1.2.5 An Introduction to Associative Learning

It is difficult to give a satisfying definition of learning as scientists from the fields of physiology, neurobiology, psychology, ethology and behavioural ecology all study learning with slightly different perspectives. A broad definition describes learning as "a change in state resulting from experience" (Shettleworth, 1998). This differs from instinct, which can be defined as "behaviour, which is expressed in complete form the very first time performed and is relatively insensitive to experience of any kind." (Shettleworth, 1998).

Papaj and Prokopy (1989) and Jaenike and Papaj (1992) applied the following criteria to learning, thereby avoiding the broad definitions: 1. Behaviour can change in a repeatable way through experience. 2. Behavioural change is gradual with continued experience up to an asymptote and 3. Learned responses can be forgotten (wane) or disappear as a consequence of another experience.

Types of learning include habituation, sensitisation, imprinting and associative. This study focuses on associative learning, which is defined as "a change in strength of a connection between a stimulus processing centre and a response generating centre" or "the process by which animals learn about casual relationships between events and behave appropriately as a result" (Dickinson, 1980). Behaviour tends to be adjusted for events that are more likely to happen due to their common occurrence in the past. It involves the making of a connection between a conditioned stimulus or CS (which initially evokes no response in the organism) to an unconditioned stimulus or US (with a measurable response).

Associative learning is sometimes termed Pavlov's conditioning as it was first demonstrated experimentally by Pavlov in dogs, using a bell as the conditioned stimulus and food as the US, which could be measured by a salivating response. Once a connection between the bell and the food had been made by presenting them simultaneously, the dog would respond to the sound of the bell alone. This project will work on the same principles using the US in the form of plant or synthetic volatiles and the CS as a host reward.

Sensitisation is also a form of learning but is not the same as associative learning and has been defined as 'non-associative learning in which the progressive amplification of a response follows repeated administrations of a stimulus' (Bernays & Chapman, 1994) It is the opposite of habituation. In this case it may be the increased response by an insect to an odour prior to exposure to that odour but does not involve any association of the odour with a reward.

1.2.6 Learning in Parasitic Wasps

Past studies have strongly suggested that insects have a remarkable ability to learn, showing skills of odour learning as sophisticated as those in the rat in some studies. The first demonstration of such associative learning of odours in insects was using honeybees (Menzel and Bitterman, 1983). Much of the early research on associative learning was in fact carried out on bees for example, using antennal stimulation with an odour (CS) paired with a sucrose reward (US), bees eventually responded with proboscis extension to the odour alone.

More recently, research in learning has been carried out on other members of the hymenoptera such as the parasitic wasps, which show particularly advanced capabilities. Arthur (1966, 1967) was the first to demonstrate associative learning in parasitic wasps, using *Itoplectis*

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conquisitor (Say; Ichneumonidae). He created artificial shelters of various shapes and colours containing hosts and found that once a parasitoid had located a shelter in a particular shape or colour, it preferentially examined similar shelters.

The full extent of the effects of parasitoid learning on foraging was not recognised until the early 80's, although a few anecdotal examples of learning in parasitoids were known (Thorpe and Jones, 1937; Arthur 1971; Vinson, 1977). It is now generally accepted that patterns of parasitoid foraging are determined by the interactions of genetic, physiological, environmental and experiential factors (Lewis and Takasu, K. 1990; Vet and Groenewold, 1990; Poppy et al., 1997; Kester and Barbosa, 1991; Drost and Carde, 1993; Vet and Dicke 1992; Turlings et al., 1993a).

1.2.6.i Factors affecting learning

Learning may be advantageous in a changing environment as it allows the insect to adapt more rapidly to changes in food source by modifying its search strategy. Therefore the severity of the environment can determine whether insects are able to learn quickly (Thiel et al, 2003; deJong and Kaiser, 1991).

If the environment is controlled then any individual variation in learning must be a result of genetic variation (Gu and Dorn , 2000) or phenotypic plasticity – a change in physiology due to associative learning. Physiological changes can effect foraging decisions, for example the nutritional state of the insect effects its sensitivity to particular odours. Lewis and Takasu (1990) trained *M.croceipes* to associate one chemical with food and another with hosts. When hungry the insect orientated towards the chemical associated with food whereas when it was

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well fed it preferred the chemical associated with hosts. This phenomenon could be related to changes in receptor sensitivity to chemicals (Davis and Takahashi, 1986). In addition egg load can also affect the wasp's foraging decisions. For example a female parasitoid that still has all of her eggs near the end of her life has a very high motivation to oviposit and will subsequently accept any host she comes across (Rosenheim & Rosen, 1991).

1.2.6.ii Pre adult Learning

Several behavioural studies have suggested that learning can occur when the parasitoid is still in the pupa (Cortesero et al., 1994) and this learning is called 'pre-emergence learning' or 'preimaginal conditioning.' (Thorpe and Jones, 1937; Vinson, 1977; Smith and Cornell, 1979; Vet, 1983; Luck and Uygun, 1986; Sheenan and Shelton, 1989). This type of learning was first demonstrated by Thorpe and Jones (1937) using parasitoid species *Venturia canescens* (Gravenhorst; Ichneumonidae) following the Hopkins Host Selection principle – 'the observation that many adult insects demonstrate a preference for the host species on which they themselves developed as larvae (Hopkins, 1916). Pre-emergence learning could be explained by the chemical legacy hypothesis (Corbett, 1985), which states that traces of chemicals from hosts are carried over from larvae to adults or cause neural changes during development that persist in the adult and directly effect the sensitivity of the insect to these chemicals. Lewis and Tumlinson (1988) found that a water soluble non-volatile contact kariomone in the frass of host larvae served as a key stimulus in learning by *Microplitis croceipes* (Cresson; Brachonidae) even if the odour were novel.

1.2.6.iii Adult Learning

Evidence suggests that adult learning generally contributes more to foraging success than pre-adult learning (Vinson, 1977; Sandlan, 1980; Vet, 1983; Drost et al, 1988; Sheehan and Shelton, 1989; Turlings et al., 1993a).

After a host and host plant experience *Leptopilina heterotoma* (Thompson; Eucoilidae) walked faster and straighter and spent more time in upwind movements in response to their host plant odour than inexperienced individuals and were generally more consistent and less variable in their behaviour (Papaj and Vet; 1990). Du et al., 1997 reported that experience increased the orientation and landing responses to host induced volatiles and undamaged plant volatiles in an aphid parasitoid species.

Turlings 1989, discovered that host contact was not required and contact with host frass and damaged leaves was sufficient for learning to occur and responses to host-related odours to improve, actual encounters with hosts were not necessary. Subsequent experiments showed that an oviposition reward gave a stronger learning response than just mere exposure to host frass and that decay in the level of response was much more rapid when oviposition was absent (Vet and Groenewold, 1990). The optimum number of oviposition experiences varies between species and usually the experiment must be performed fairly soon after the experience as in the absence of continued experience parasitoids tend to 'forget' what they have learned (Papaj and Vet, 1990). The condition of the plant target involved is also an important factor as in *M. croceipes* there appears to be an innate preference for old rather than freshly damaged plants. This may be an adaptive feature as the chemicals produced later on are a direct response to the caterpillar's saliva (McCall et al., 1993).

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There is also evidence that some adult parasitoids learn host plant chemical cues during emergence from the cuticle of the dead host, known as the 'mummy' due to these chemicals being detectable on the cocoon material or host cuticle - this is known as emergence conditioning. In Y-tube experiments a generalist parasitoid *Aphidius colemani* showed a preference for the host-plant complex, which it had been reared on. This preference was not seen when pupa was dissected from its suggesting that the mummy was providing chemical cues utilised in adult learning (Storeck et al., 2000).

1.2.6.iv Learning in Cotesia plutellae

Cotesia plutellae has been the subject of several behavioural studies and shows odour learning abilities.

Potting et al. (1999) demonstrated that mechanically damaged plants were attractive to *C. plutellae*, as were host-damaged plants (with the hosts removed). Females which had experienced an oviposition were offered a choice between mechanically damaged and host damaged plants in wind tunnel tests and spent the most time on plant- host complex or host-damaged leaves suggesting that they had a greater affinity for learning host-induced volatiles.

Liu and Jiang (2003) used a Y-tube method to show that plant preferences by *C. plutellae* could be altered by experience. Parasitoids had an innate preference for Chinese cabbage over Common cabbage but with a foraging experience on Common cabbage preferences were altered in favour of this host plant.

CHAPTER 2: DO INSECTS LEARN AND HOW DOES LEARNING MODIFY PREFERENCE?

2.1 Introduction

Several studies using parasitic wasps in 4 –arm olfactometer experiments have shown that an oviposition experience can alter a parasitoid's natural preference for an odour due to associative learning (Micha et al. 2000; Turlings et al., 1989). Lepidopteran parasitoid *C. marginiventris* has an innate preference for host plant corn (*Zea mays* L.) over cotton (*Gossypium hirsutum* L.) but this was modified by the experience of ovipositing in a host on a cotton plant. A general increase in response to plant volatiles after the experience was seen but this was due to sensitisation and not learning (Turlings et al., 1989). Micha et al. (2000) report similar findings in which preference for an oat plant complex by aphid parasitoid *Aphidius rhopalosiphi* (DeStefani-Perez; braconidae) was altered by a wheat experience, so that there was no longer a preference between the two. Research using *Cotesia plutellae* showed that an innate preference for Chinese cabbage over Common cabbage could be reversed by a foraging experience on common cabbage (Jiang, 2001).

This chapter involves two main experiments both of which use 4 –arm olfactometer bioassays. The first will investigate whether *Cotesia plutellae* can learn plant volatiles from host plant Chinese Cabbage. If this was occurring, wasps are likely to spend more time in the odour field of the host when they are experienced than in the control odour fields. It will also be determined which types of plant chemicals may be important in learning by providing plant targets with different damage types in order to create the odour fields. The second

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experiment will investigate whether learning experiences can modify an innate preference for an odour. Wasps will be given a choice between two plant odour fields to see whether experience on one type of target increases time spent in this odour field relative to the other odour field. Any changes in walking behaviour with experience will be observed by monitoring velocity, meander and visits to the odour field before and after the experience.

2.2 Materials and Methods

2.2.1 General Methodology

2.2.1.i Insect Culture and Selection

Thirty *Brassica rapa* seeds were sown once a week in 9cm diameter circular pots containing sterile compost and were watered daily until they reached approximately 15cm in height. They were used for *P. xylostella* food material or in bioassay experiments as targets. All Chinese cabbage plants were grown in a glasshouse maintained at a temperature of around 20°C. Lighting was both natural and artificial.

A new rearing system was set up in which a continuous supply of parasitoids and host larvae were produced. *C. plutellae* and *P. xylostella* larvae were kept in cages made of clear Perspex especially designed for the culture. Each cage measured 30cm³ and had nylon mesh on two sides for ventilation (Figure 4a). The adult *P.xylostella* were kept in a larger cage measuring 60cm³ in a separate room to avoid unwanted parasitisation of larvae. The culture room was kept at 25°C (+/-5) with 50-60% humidity and a 16L: 8D photoperiod.

Figure 4a (right): Photograph of one of the rearing chambers.
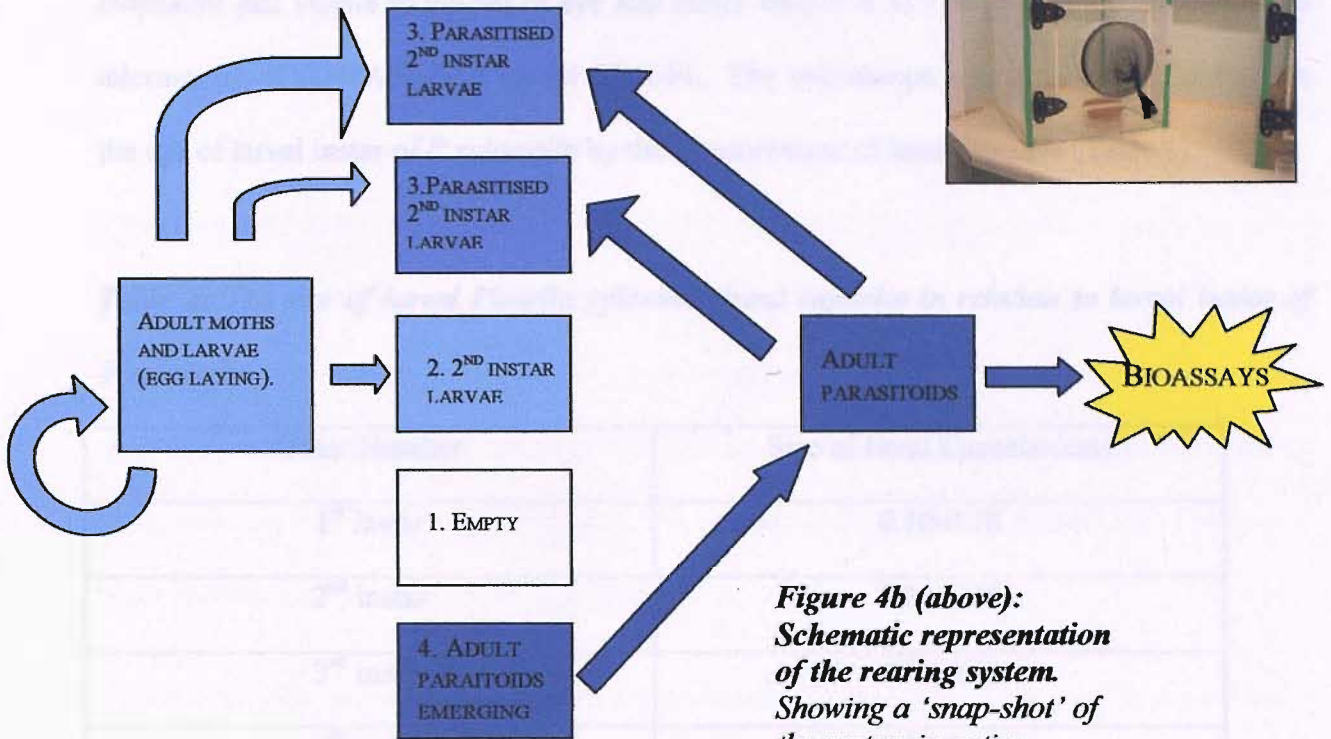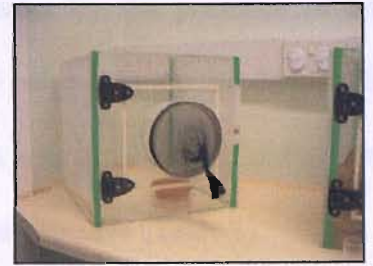
Figure 4b (above): Schematic representation of the rearing system. Showing a 'snap-shot' of the system in action.

The rearing system consisted of 6 smaller chambers and 1 larger chamber. The adult moths in the larger chamber were fed on 70% honey solution every second day and given plants to lay their eggs on and for larva to feed on. A parasitisation was carried out once per week in which two Chinese cabbage plants covered in 2nd instar larvae were placed in one of the smaller chambers to acclimatise for 24 hours. 3 day old mated female parasitoids were then introduced using a battery powered pooter and left to parasitise the larvae for 24 hours. Parasitized larvae increased their food consumption and therefore were provided with new food plants daily. Any larvae, which did not develop into parasitoid pupae and emerged into moth adults, were transferred into the larger adult moth chamber. Emerging adult parasitoids were transferred into an empty small cage and fed 70% honey solution every second day. They were either used in bioassays and then destroyed or used for parasitisations (Figure 4b).

Female parasitoids were distinguished and separated from males by the presence of an ovipositor just visible to the naked eye and easily visible at x15 using a binocular dissection microscope, KYOWA optical model SDX-PL. The microscope was also used to determine the age of larval instar of *P.xylostella* by the measurement of head capsules (Table 1).

Table 1: The size of larval Plutella xylostella head capsules in relation to larval instar of P.xylostella

Instar Number	Size of Head Capsule (cm)
1st instar	0.10-0.18
2nd instar	0.20-0.30
3rd instar	0.33-0.48
4th instar	0.50-0.65

2.2.1.ii Bioassays Using Behavioural Software

All bioassays were performed between 23-26°C under artificial lighting conditions.

Several methods for behavioural bioassays were available. So preliminary trials were made to see which method was the most appropriate for use with this particular parasitoid species. Both wind tunnels and glass house flight chambers have been reported as reliable methods in past behavioural experiments and more realistic of natural conditions (Steinberg et al., 1992). However, a day-to day variation in response is found in the glass house and wind tunnel due to decreasing barometric flux (Steinberg et al., 1992). Preliminary trials produced variable results when using this method and therefore alternative options were considered. As this

experiment does not focus on natural selection, repeatable data is more important than mimicking natural conditions.

Olfactometers are a useful way of obtaining close range, reliable data. A Y-tube set-up was not appropriate in this case as it did not function well with the behavioural software available and does not create contiguous odour fields (Vet, 1983). They also do not provide statistically reliable data (there is a high chance of error as only two choices). In a 4 –arm olfactometer only 25% of non-responding animals will end up in the odour field by chance, thus either fewer animals need to be used to obtain the same statistical power or a higher statistical power is achievable for a given number of animals.

Figure 5: A: The four arm olfactometer used in this study, with 4 distinct odour fields and central area. B: The Psion Workabout
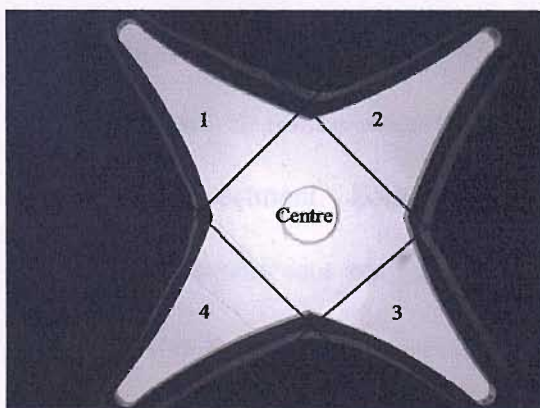
A)

B)
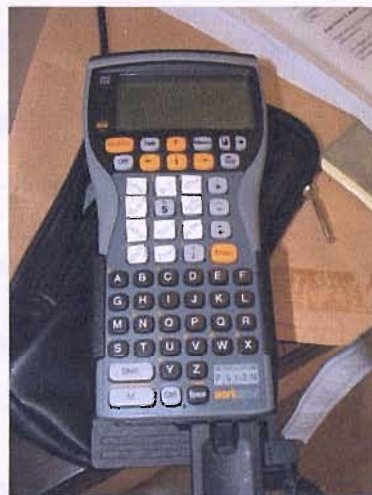
All bioassays used 4 –arm olfactometers, similar to the set-up described in Vet et al. (1983) which was developed from the original design for the study of sex pheromones (Pettersson, 1970). The arena was divided into five areas – four distinct odour fields and a central area where all of the odour fields merged (Figure 5a). Parasitoids were introduced into the centre and allowed to walk or fly freely within the olfactometer, they were monitored for 5 minutes before being removed using a pooter. For the first 2 experiments, the arms had air flowing into them through rubber piping at 300ml/min. Odour fields were created by pumping air through large cylindrical jars (400x200x5mm) containing the target or through empty jars for the controls. The system was illuminated from above with a artificial light source.

Four different Chinese cabbage target types were selected as targets, mechanically damaged (MD), host damaged (HD), host plant complex (HPC) and undamaged (UD). MD plants were cut 10 times using scissors immediately before experiments. HD plants had 10 larvae of various instar applied an hour before the experiment, which were then removed just previous to the beginning of the experiment. HPC had 10 larvae applied an hour before the start of the experiment which remained on the plant throughout the experiment and UD did not have any sort of prior treatment. Some studies suggest that applying larvae only an hour before the start of the experiment may not be long enough to ensure release of herbivore –induced volatiles, this is discussed in more detailed on page 38.

Some of the parasitoids were termed 'experienced' and these individuals were given a 5-minute experience on either a host-plant complex (HPC experienced) or a mechanically damaged leaf presented in conjunction with a host (MDP Experienced), just prior to the experiment inside a plastic cylindrical chamber (50x100x2mm). All parasitoids were given 1

hour to acclimatise to the conditions of the experimentation room and were supplied with honey solution whilst acclimatising and stored in glass tubes.

Data was analysed using various statistical tests, all of these were carried out using SPSS for windows 2.0.1 and with the aid of 'Choosing and Using Statistics – a Biologists Guide (Second Edition)' by Calvin Dytham, 2003.

2.2.2 Measuring Time Spent in Single Choice Plant Odour Fields

A configuration was designed using the Noldus Observer version 3 software which programmed certain keys on the Psion Workabout (Figure 5b) to represent particular areas in the arena. It was transferred to the psion workabout via a serial cable. Data recording was then carried out manually using the Psion Workabout.

The plant targets (UDP, MDP, HPC, HDP) were placed inside a bell jar with air circulating through into one of the four arms of the olfactometer, the other three olfactometer arms had clean air blowing through. Wasps were either experienced (2.3.2) or naïve and they were monitored using 5-minute bioassays. This was repeated 20 times for each target-treatment combination, so that 160 individuals were tested in total (table 2).

Table 2: Experimental design for Experiment1. Showing treatments (vertical) targets (horizontal) and number of wasp replicates for each combination.

	HPC	HDP	MDP	UDP
Experienced	20	20	20	20
Naïve	20	20	20	20

To insure randomisation of results, a block design of testing was used in which only 5 of each target-treatment combination was used in a consecutive sequence. The choice of olfactometer odour arm was also randomised to avoid sampling bias using an online randomiser (http://www.randomizer.org). The observer software elementary statistics were then used to calculate time spent in each area as a percentage and in seconds.

T-tests were used to statistically analyse differences between time spent in odour fields when naïve and experienced and after the data had been Arcsine Transformed, separate one-way ANOVAs were used to compare naïve and experienced treatments for each separate target type.

2.2.3 Measuring Behaviour and Time Spent in Dual Choice Plant Odour Fields

Data was recorded automatically using Ethovision which can be defined 'an integrated system for automatic recording of activity, movement, and interactions of animals' (Noldus et al., 2001). It used a video camera (pointing directly onto the arena from below) to take 2.5 frames per second and analysed the movement of the animal by calculating the changes in pixels in each frame. The object was set at a minimum detection of 10 pixels so as to eliminate any background interference.

In this experiment, odour fields were created using the following three combinations:

1. Undamaged plant vs. Mechanically damaged plant.

2. Mechanically damaged plant vs. Host plant complex

3. Water control with larvae vs. Host plant complex.

Odour passed into two of the four arms which were changed every 5 replicates and selected randomly using the online randomiser, odours were presented from adjacent arms and opposite arms depending on which arms were randomly selected each time.

Water control with larvae consisted of a small petri dish (7cm diameter) filled with distilled water and 10 2nd instar larvae were contained in another petri dish covered with nylon mesh. Wasps were either naïve, experienced as described in section *2.2.3* or exposed. 'Exposed' wasps had been in the odour field for 5 minutes but without the presence of a host reward. If wasps increase their response to an odour after only exposure it is assumed that the insect is not learning the odour but has become sensitised to it (see section *1.2.5*).

20 individuals were tested for each treatment and target combination (Table 3) and time in the odour field as well as velocity, meander and number of visits, were calculated by the Ethovision software. 2-way ANOVAs with multiple treatment levels were used to test for differences in time spent in the odour field by wasps with three treatments and a post-hoc Sidak test used to determine where significant differences occur. Independent t-tests were then used to compare the velocity, visits to the odour field and meander when wasps were naïve and experienced.

Table 3: Experimental design for Experiment 2. Showing target combinations (horizontal),

treatments (vertical) and number of wasp replicates for each combination.

Treatment	MD vs. UD	MD vs. HPC	Larval water vs. HPC
Naïve (baseline)	20	20	20
Mechanical damage exposed	20	20	0
Various experience treatment	MD experienced 20	HPC experienced 20	HPC experienced 20

2.3 Results

2.3.1 Results of Single Choice Experiments

2.3.1.i Wasps Spent More Time in the Odour Field after an Experience

Figure 6: A comparison of mean percentage time spent in the HPC odour field, centre and each of the control zones of the olfactometer by naïve and experienced parasitoids regardless of target type.
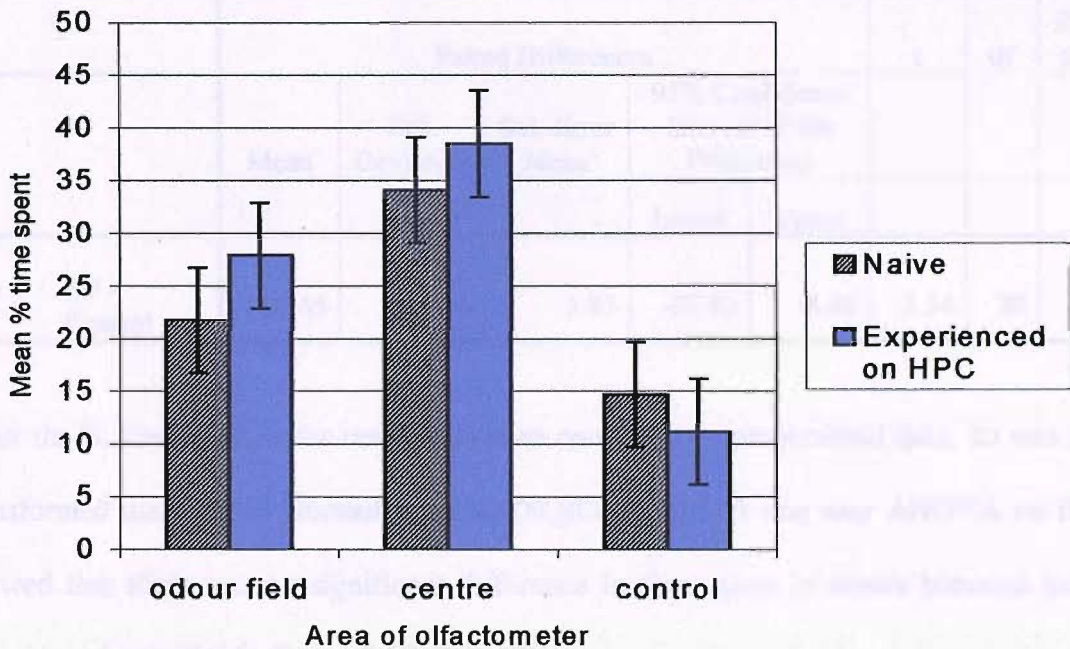
Wasps tend to spend more time in the odour field and less time in the control zones when experienced than when naïve. Although time in centre remains relatively constant there is a small increase in time spent there when wasps were experienced (Figure 6). A paired samples t-test was carried out to test for differences between time spent in the odour field and the control field when wasps were firstly naïve and then experienced.

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There is no significant difference in time spent in the odour field and control field when wasps are naïve (t_{38}=1.65, P=0.107) P>0.05. There is a significant difference in time spent in the odour field and control field when wasps are experienced (t_{38}=3.54, P=0.001) P=0.001(Table 4). Therefore naïve wasps do not show any significant response to the odour, whereas experienced wasps do.

Table 4: Results table for a paired samples t-test comparing time spent in the odour field and control by experienced wasps

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	OF - Control	-20.65	36.86	5.83	-32.43	-8.86	-3.54	39	.001

After the % time spent in the centre had been converted to proportional data, its was arc-sine transformed using SPSS (formula: ARSIN(SQRT(prop))). A one way ANOVA on this data showed that there was no significant difference in time spent in centre between naive and experienced parasitoids ($F_{78,79}$=0.57, P=0.571).

2.3.1.ii Wasps Spent More Time in the HPC Odour Field after a HPC Experience

Figure 7: Percentage time spent in odour field by naïve and experienced parasitic wasps in response to the four different targets.
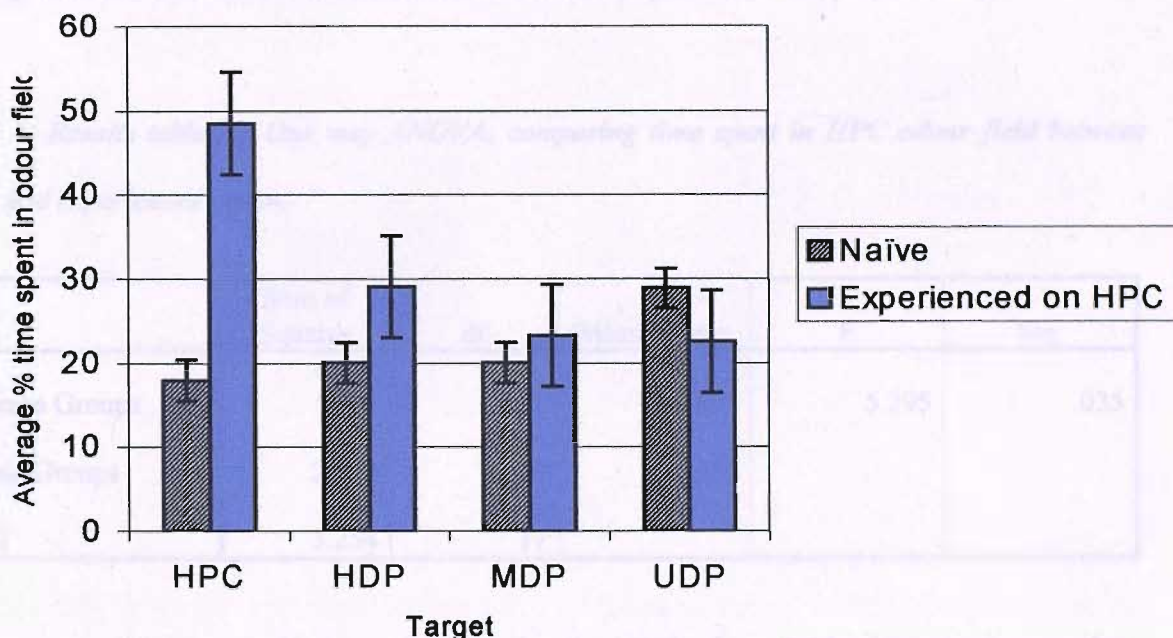
Time spent in odour field is generally higher in experienced individuals compared to naive, especially when responding to a host plant complex. Response from naïve parasitoids stays relatively consistent across target groups spending around 20-25% of their time in the odour field, whereas response from experienced parasitoids is more variable. The least difference is seen in response to mechanical damage.

After the % time spent in the odour field had been converted to proportional data, its was arc-sine transformed using SPSS (formula: ARSIN(SQRT(prop))). A One-way ANOVA was conducted on each target type testing for a significant difference in the proportion of time spent in the odour field between naive and experienced wasps. There is no significant difference in time spent in the HDP odour field between naïve and experienced wasps

($F_{16,17}$=0.059, P=0.811). There is no significant difference in time spent in the MDP odour field between naïve and experienced wasps ($F_{16,17}$=0.036, P=0.852). There is no significant difference in time spent in the UDP odour field between naïve and experienced wasps ($F_{16,17}$=0.33, P=0.574). There is a significant difference ($F_{16,17}$=5.295, P=0.035) P<0.05, in time spent in the HPC odour field between naïve and experienced wasps (Table 6).

Table 6: Results table for One way ANOVA, comparing time spent in HPC odour field between naïve and experienced wasps.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.809	1	.809	5.295	.035
Within Groups	2.445	16	.153		
Total	3.254	17			

Wasps appear to increase their preferences for the HPC odour field with experience of the HPC but not the HDP, MDP or UDP odour fields.

2.3.1.iii Experience had no significant effect on velocity, meander and visits to the odour field

Figure 8: Comparison of velocity in the odour field and control by naïve and experienced parasitoids.
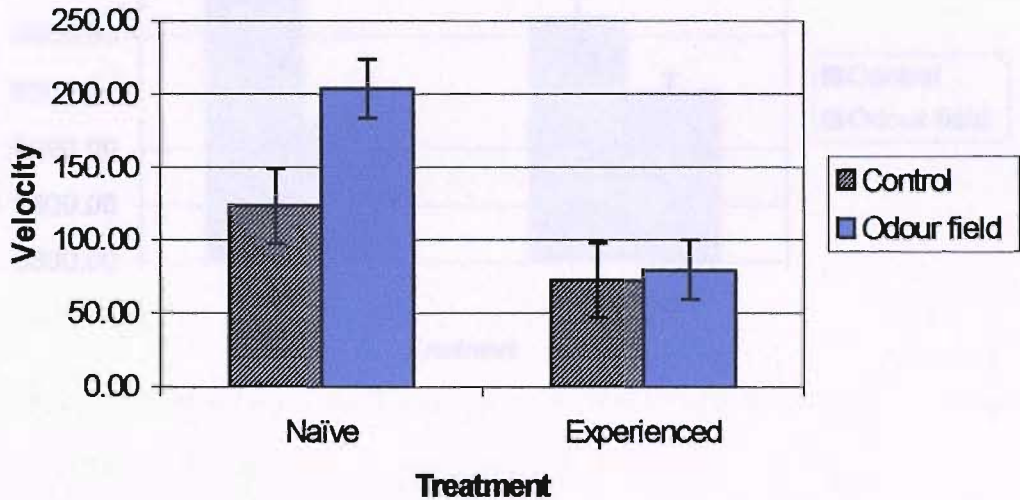
Velocity data from the first experiments was combined in figure 8. Data from experienced wasps in the odour field was totalled, as was the data from naïve wasps. Data in the control zones was also totalled for both naïve and experienced separately and then divided by three to calculate and average velocity in one control zone. There appears to be a general decrease in velocity when the parasitoid is experienced in both the odour field and control but particularly in the odour field. However, this difference is not significant using an independent t-test to compare velocity in the odour field when wasps are naïve and experienced ($t_{114}=0.638$, $P=0.525$).

Figure 9: Comparison of meander in the odour field by naïve and experienced parasitoids.
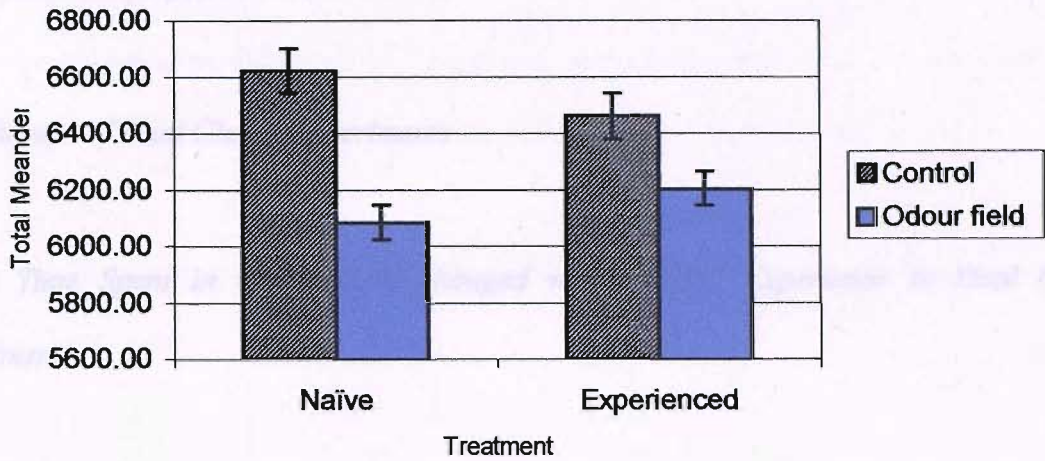
Meander appears to be higher in the control zone than the odour field and this is not significantly altered by experience (Figure 9). Statistical analysis shows there is no significant difference in meander between naïve and experienced wasps (t_{114}=0.920, P=0.360) when using an independent t-test.

Figure 10: Number of times parasitoids walked in and out of the odour field.
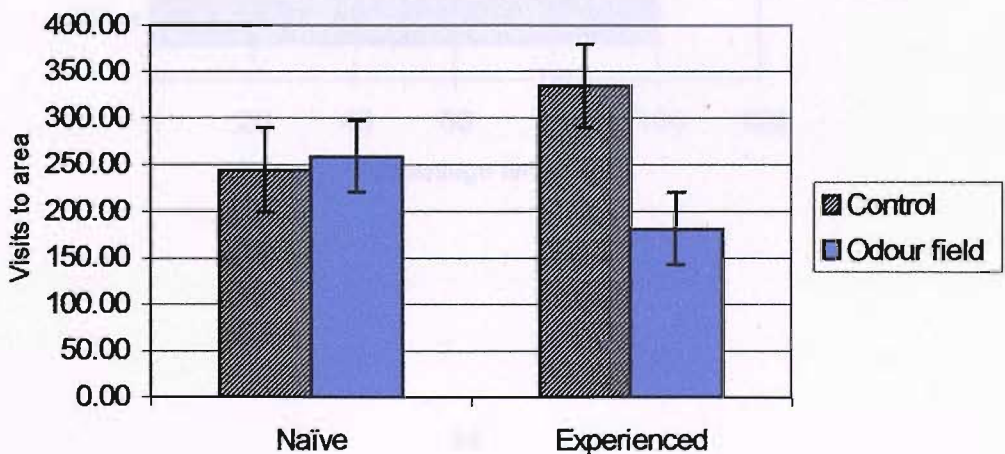
There appears to be a decrease in number of times walking in and out of odour fields for experienced wasps compared to naïve (Figure 10), however an independent t-test showed no significant difference between the number if visits to the odour field made by naïve and experienced wasps (t_{114}=1.310, P=0.193).

2.3.2 Results of Dual Choice Experiments

2.3.2.i Time Spent in Odour field changed with a HPC Experience in Dual Choice Experiments

Figure 11: Percentage time spent in the two different odour fields after treatments

Figure 11a. Percentage time spent in MDP and UDP odour fields after naïve, HPC exposed and MDP experienced treatments.
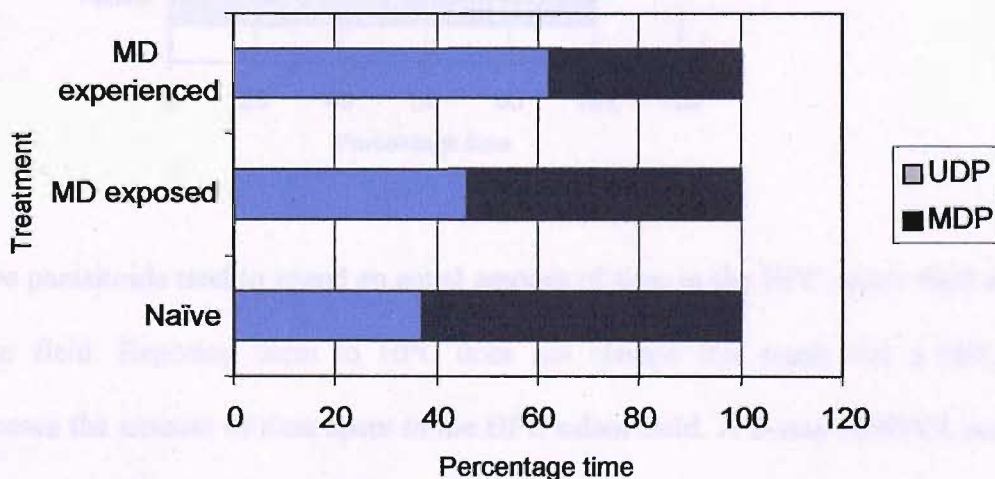
Naive appear to spend more time in the MD odour field but an experience on MD plant does not appear to increase time spent in this area and actually increases time spent in the UDP odour field. After arcsine transformation of proportion data, a two way ANOVA was used to test for differences in time spent in the two odour fields after the three different treatments. There is no significant difference in time spent in the MD odour field and the UDP odour field for naïve, exposed and experienced wasps ($F_{107,106}$ =1.446, P=0.240).

Figure 11b: Percentage time spent in MDP and HPC odour fields after naïve, HPC exposed and HPC experienced treatments.
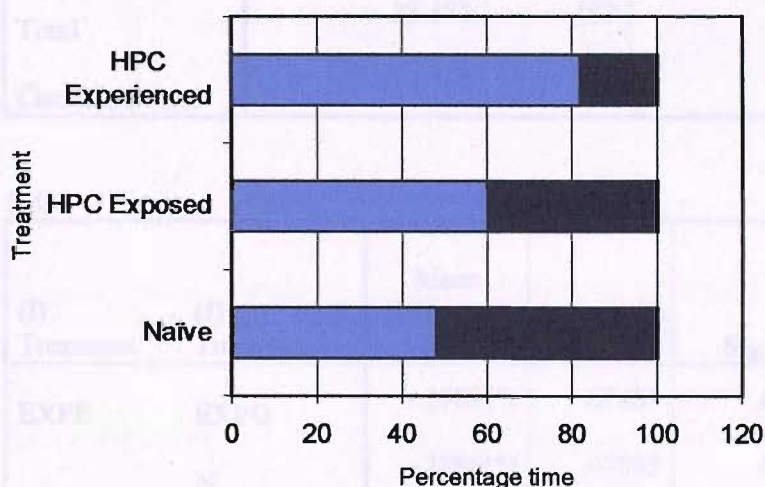
Naive parasitoids tend to spend an equal amount of time in the HPC odour field and the MDP odour field. Exposing them to HPC does not change this result but a HPC experience increases the amount of time spent in the HPC odour field. A 2-way ANOVA was conducted on the data as previously and a Sidak Post-hoc test revealed that there is a significant difference between naïve and experienced wasps ($F_{107,106}$=0.229, P=0.01) P<0.05, but no significant different between naïve and exposed wasps ($F_{107,106}$=0.070, P=0.711).

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Table 7: Results table for Two way ANOVA, comparing time spent in HPC and MDP odour fields with naïve, HPC exposed and HPC experienced treatments. Followed by Sidak Post-hoc test.

Tests of Between-Subjects Effects

Dependent Variable: Proportion

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4.158(a)	5	.832	8.396	.000
Intercept	19.150	1	19.150	193.324	.000
Area	1.862	1	1.862	18.799	.000
Treatment	1.496	2	.748	7.549	.001
Area * Treatment	.510	2	.255	2.574	.081
Error	10.005	101	.099		
Total	33.172	107			
Corrected Total	14.163	106			

Sidak

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
EXPE	EXPO	-.2989(*)	.07489	.000	-.4808	-.1171
	N	-.2286(*)	.07585	.010	-.4128	-.0444
EXPO	EXPE	.2989(*)	.07489	.000	.1171	.4808
	N	.0703	.07320	.711	-.1074	.2481
N	EXPE	.2286(*)	.07585	.010	.0444	.4128
	EXPO	-.0703	.07320	.711	-.2481	.1074

Based on observed means.
* The mean difference is significant at the .05 level.

Experience appears to alter preferences in favour of a HPC over a MDP but doesn't change those for an MDP over an UDP.

2.3.2.ii A HPC Experience Increased Response to a HPC Target but not a LWC in Dual Choice Experiments

Figure 12: Larval water control experiment showing changes in time spent in odour field with a HPC experience.
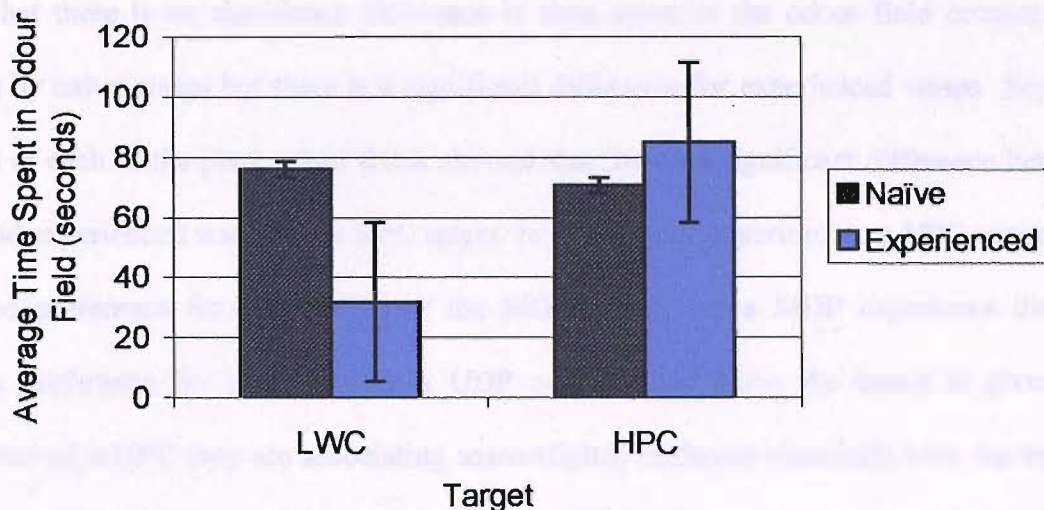
There is innate preference for both odour fields compared to the control, but when given a HPC experience time spent increases in the HPC odour field and decreases in the larval water odour field (Figure 12). This suggests that wasps are innately attracted to the larva and water odour but when given a HPC experience, they are more attracted to the whole host-plant complex and tend to avoid the larva and water odour field.

A set of independent t-tests were carried out on Naïve and Experienced wasps to see if there is a difference between the proportion of time spent in each of the two odour fields. There is no significant difference between the two odour choices for naïve individuals (t_{37}=1.908, P=0.067), but there is a significant difference between odour field choice for experienced wasps (t_{37}=2.072, P=0.045), P<0.05.

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2.4 Discussion

These results suggest that associative learning is taking place during the experience. Naïve parasitoids spent roughly 20% of their time in the odour field whereas experienced individuals spent 30-45% of their time in the HPC and HDP odour fields but only 20% of their time in the MDP and UDP odour fields in single choice experiments. Statistical analysis shows that there is no significant difference in time spent in the odour field compared to controls by naïve wasps but there is a significant difference for experienced wasps. Separate analysis of each of the plant odour fields showed that the only significant difference between naïve and experienced was using a HPC target. In dual choice experiments, a HPC experience increased preference for the HPC over the MDP odour, but a MDP experience did not increase preference for a MDP over a UDP odour field. When the insect is given the experience of a HPC they are associating some slightly different chemicals with the reward than when using a MDP experience and perhaps they are better at learning the odours specific to a HPC. Mechanical damaged plants and undamaged plants will release the same chemicals as the HPC but only the ones caused by physical damage and general green leaf volatiles, not active chemicals responding to host attack. Studies showed that Mechanical damage does not release the same specific chemicals as host damage but if caterpillar regurgitate was applied to these sites then a similar chemical profile is released (Turlings et al., 1995).

One would expect host-damaged plants to be emitting similar volatiles to a plant-host complex and therefore produce similar learning responses, however, experienced wasps did not spend significantly more time in the HDP odour field than naïve wasps. This may be because the wasps are learning the volatiles produced immediately by the feeding of the hosts or alternatively that the host was not left on the plant for a long enough period of time prior to

testing. Turlings et al., 1995 showed that recent herbivore attack on corn seedlings produced a very different chemical profile to plants which had hosts on them for several hours.

Past studies report similar findings of increase in response with experience to a host-plant. For example, after a host and host plant experience *Leptopilina heterotoma* Thomson (Hymenoptera; Eucoilidae) spent more time in upwind movements in response to their host plant odour than inexperienced individuals and were generally more consistent and less variable in their behaviour (Papaj and Vet; 1990).

The second experiments support the theory that HPC released odours are more important in learning than mechanical damage or undamaged volatiles and supports past studies using the same species (Potting et al., 1999). When naïve wasps were given the choice between UDP or MDP targets they innately preferred the mechanical damage and there was no increase in preference for a MDP when the wasps had been conditioned to this plant using a reward. However, when the choice was between a HPC and a MDP and the wasp had been conditioned to the HPC, there was a significant change in preference towards the HPC. MDP volatiles may be too much of a general cue to invest time in learning as mechanical damage would not only be produced by hosts but also by other herbivores. Potting et al. (1999), reported similar findings. In their studies, mechanically damaged plants were attractive to *C. plutellae*, as were host-damaged plants (with the hosts removed). Females which had experienced an oviposition were offered a choice between mechanically damaged and host damaged plants in wind tunnel tests spent the most time on plant- host complex or host-damaged leaves suggesting that they had a greater affinity for learning host-induced volatile profiles.

A control experiment was carried out to ensure that wasps were not learning non-plant-associated cues i.e. the water from the plant or the larvae on the plant. Wasps were given a choice between a Larva and water control and a HPC and experience increased the preference to a HPC and decreased the preference to the LWC. This suggests that wasps are learning plant chemicals or the combination of chemicals from the HPC rather than the chemicals from the larvae and water alone which they will avoid when presented with a HPC alternative. There were no changes in preference when wasps were only exposed to the odour and not given a reward suggesting that sensitisation is not the cause of these changes in preferences. Larvae emit very small amounts of chemical most of the time, the exception being when they are communicating to other larvae using pheromones, in order to remain inconspicuous. Therefore plant chemical cues which are produced in much higher quantities are utilised for larvae detection.

Lewis and Tumlinson (1988) reported that a reward given in the absence of the conditional stimulus (host plant odour) was also ineffective. Vet and Groenewold (1990) also reported that mere exposure to odour did not have an important impact on subsequent behaviour in *L.heterotoma*, showing that sensitization and habituation did not fully account for the observed behavioural changes. They did control experiments by exposing females to the CS Z3-6:OH in the presence of yeast substrate but no US (host reward). These females did not distinguish between a yeast substrate and a yeast+Z3-6:OH substrate in a four-arm olfactometer test. The reward is therefore an essential part of the learning process.

C.plutellae showed an increase in meander and decrease in velocity in the odour field with learning, however these results were not significant with statistical analysis, perhaps due to a small sample size. These results do not support past studies, for example, *Leptopilina*

heterotoma Thomson (Hymenoptera; Eucoilidae) walked faster and straighter in the odour field during a study by Papaj and Vet; 1990.

2.4.1 Discussion Summary

- After a host-plant complex experience, wasps spent more time in the host-plant complex odour field than the controls and modified their preference towards a host-plant complex over a mechanically damaged plant. This suggests that they are learning plant volatiles.

- Wasps appear to learn the volatiles produced in a host-plant complex rather than mechanical damage or undamaged plant volatiles as this may be a more reliable cue for a host's presence. They did not learn the volatiles from a host-damaged plant with the hosts removed, possibly because the hosts did not spend long enough damaging the plant.

- There tends to be a general decrease in velocity with learning and increase in meander in the odour field although this was no statistically significant.

CHAPTER 3 – WHAT IS LEARNT AND CAN ANYTHING BE LEARNT?

3.1. Introduction

Plants give off a complex chemical profile, which changes when they are under attack from a herbivore. Many predators and parasitoids are attracted to these chemicals and exploit them in order to find a host/prey. Studies have shown that uninfested plants treated with herbivore regurgitate release attractive volatiles, suggesting that the elicitor of attractive volatile release can originate from herbivores (Turlings et al., 1993b; Alborn et al., 1997). Several studies have analysed activated host plant volatiles in order to determine which are important in detection and learning using a combination of electrophysiology studies and behavioural studies (Du et al., 1998; Ngi-Song et al., 2000). These Volatiles can be divided into 3 main groups, green leaf volatiles, terpenoids and oximes.

D'Alessandro and Turlings (2005), analysed volatiles from maize under attack by *Spodoptera littoralis* Boisduval, (Lepidoptera; Noctuidae) and the response of parasitoid *Cotesia marginiventris*. They found that terpenes were the dominant volatiles including the sesquiterpenes E-bergamotene and (E)-ß farnesene. Also several green leaf volatiles were detected. Removing these sequiterpenes had no effect on the attraction of naïve females but experienced females preferred the full blend. These appeared important but not vital for attraction. Wei and Kang (2006) analysed the volatiles from bean plants under attack by leaf minor species, coupled with GC-EAD to see which elicited responses in parasitic wasps *Opius dissitus*. (3Z)-hexanyl acetate, (3Z)-hexen-1-ol and (E)-ß-caryophyllene all elicited EAG responses. *Cotesia plutellae* responded to larval frass volatiles dipropyl disulphide,

dimethyl disulfide, allyl isothiocyanate and dimethyl trisulfide, an artificial plant leaf blend and green leaf volatiles of cabbage Z3-6: Ac, E2-6: Ald and Z3-6:OH (Reddy et al. 2002).

Not only can wasps learn to associate these plant chemicals found in their natural environment with a reward, but also novel chemicals, which are not naturally occurring. In 1971, Arthur conditioned parasitoid species *V.canescens* to search for hosts in the presence of chemical geraniol. Vinson (1977) then showed that *B. mellitor* could be conditioned to exhibit ovipositor probing in response to novel chemical methyl parahydroxy-benzoate and some parasitoid females can even be trained to the odour of commercial perfume (deJong and Kaiser, 1991). Olson et al. (2003) discovered that parasitoid *Microplitis croceipes* can associate a wide range of different chemicals outside their natural foraging range with rewards. Wasps were conditioned to associate several structurally diverse chemicals; cyclohexanone, 3-octanone, octanol, diisopropyl aminoethanol, and 2,4 and 3, 4-dinitrotoluene (DNT) as the unconditioned stimulus in a conditioned response. They concluded that the plasticity insects demonstrate in learning, extends beyond the fine-tuning of existing connections but may even involve the creation of new neural connections.

This chapter consists of two experiments. The first explores which chemicals are produced by the host plant, Chinese cabbage and how this chemical profile changes when actively induced by attack from *P.xylostella*. This will help to identify which chemicals are more likely to be important in learning and detection. Behavioural trials will then be conducted using three different volatiles, (E)-ß-caryophyllene, (Z3)-hexenyl acetate (both produced in significant quantities by the plant) and vanillin (a novel chemical not usually found in the foraging range of the parasitoid) to determine whether wasps are able to learn these individual chemicals.

3.2. Materials and Methods

3.2.1 Capturing and Analysing Plant Volatiles from Infested and Uninfested Plants

3.2.1.i Preparation

Five pairs of same age Chinese cabbage plants were analysed to see which volatile chemicals were released in an attack response. One of each pair of same day old plants was infested for 3 days with 4 2nd instar larvae and the other was left uninfested. 2 control bags were also analysed which contained no plants to ensure that they were not producing odours.

Polyester bags were baked in the oven at 110°C for 8 hours and placed carefully over the top of the plants using cloth gloves. The bags were secured round the top rim of the plant pot using lengths of wire but a small gap was left to insert the inlet airflow tube inside the bag. Previous to this, tin foil was used to cover the soil in the plant pots and a small hole was made in one corner of the bag so that the air could pass out. Air was pumped through the bag at 900cm/min for 24 hours so that the plant could acclimatise before starting the entrainment (Stewart-Jones & Poppy, 2006).

3.2.1.ii Entrainment

Headspace samples were trapped on Tenax TA (50 mg, mesh 60-80, Supelco, Bellefonte, USA) held in injector liners (l. 81mm, o.d. 5mm, i.d. 3.2mm) by plugs of silanised glass wool. Before use, liners were washed with redistilled diethyl ether (5 ml) and baked under a slow flow of purified nitrogen (225°C, 2 hr). Liners were then fixed to the corner of bags using a

twist of gardening wire and sampling was started at 900 cm^3/min. Headspace was drawn through the adsorbent at 600 cm/min whilst inlet flow was maintained at 900 ccm^3/min. Entrainments ran for 7 h after which liners were sealed in glass tubes and stored in the freezer (-22°C) until analysis.

3.2.1.iii Analysis by Coupled Gas Chromatography-Mass Spectrometry (GC-MS)

Entrainment samples were thermally desorbed using an Optic 2 programmable injector (Anatune, Cambridge, UK) fitted to an Agilent 6890 GC coupled to a Hewlett-Packard 5972 Quadrupole Mass Selective Detector that ionised by electron impact (70eV). Injector conditions were equilibrated (30 s) then ramped from 30°C to 220°C at 16°C/s, carrier gas was helium (8psi constant) and the injector was operated in split mode (1:2). The capillary column was a BPX5MS (SGE, Australia; 30 m x 0.25 mm i.d., 0.5 µm film) and oven temperature was held at 30°C for 2 min then programmed at 4 °C/min to 200°C then 10°C/min to 250°C and held for 10 min. The MS was 172°C and scanned from mass 33 to 330 2.5 times/sec. Data were captured and analysed by Enhanced ChemStation software (Vers B.01.00).

Volatiles were initially tentatively identified by comparing spectra to those in internal, Wiley275 and Nist 98 databases. Confirmation of identity was achieved for most compounds by comparison of spectra and retention times to those of authenticated standards. For the six compounds that standards were unavailable, probable identifications were determined from Kovats indices and database matches. To avoid problems of background/baseline interference, total ion current was not measured. A target ion was quantified for each compound and validated using a qualifier ion for which a relative response was determined.

Photographs were taken of all of the leaves on each plant so that total leaf area could be calibrated with chemical volume. Leaves were laid on a piece of white paper with a 30 cm ruler and a photograph was taken from above. Photos were then downloaded into ImageJ software and transferred into binary images of the leaf outline, pixels were then calibrated against the ruler in cm and total leaf area calculated using the software (Figure 13).

Chemical production from the unifested and infested plants was then compared using a T-test.

Figure 13: Photograph of the leaves from an entire infested plant and the binary image interpretation from image J used for calculating leaf area.
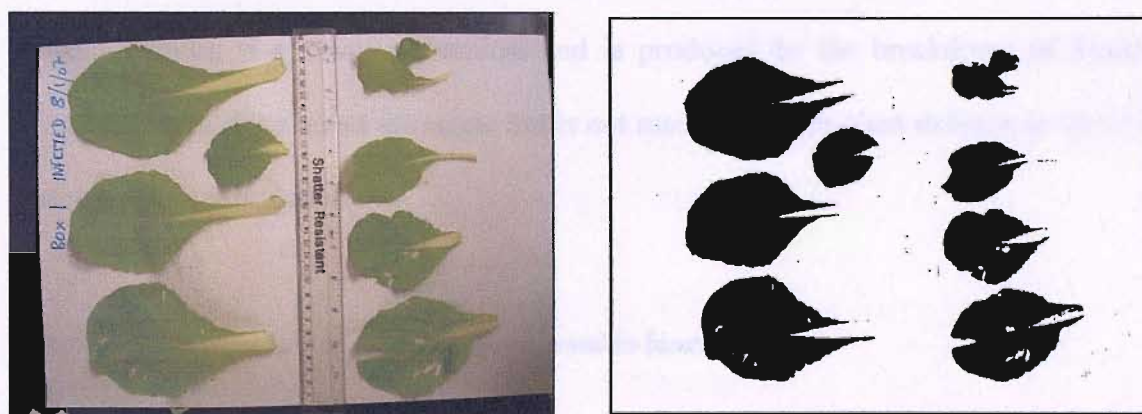
3.2.2 Measuring Parasitoid Response to Three Specific Plant Chemicals Before and After Experience

Chemicals (E)-ß-caryophyllene, (Z3)-hexenyl acetate and vanillin were chosen for treatments and targets for this experiment.

(E)-ß-caryophyllene (Figure 14A) is a natural bicyclic sesquiterpene and is produced in large quantities by plants under attack from insect herbivores. This strong correlation with insect

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feeding has suggested that it is stimulated in response to insect oral secretions which act as elicitors via the modification of signalling hormone jasmonic acid.

(Z3)-hexenyl acetate (Figure 14B) is a long-chain, six-carbon ester and belongs to the family of green leaf volatiles. It is produced much more generally and by plants when they are undamaged, mechanically damaged or infested. It is formed via a different pathway to (E)-ß-cayophyllene – and is produced by autolytic oxidative breakdown of membrane lipids when leaves are mechanically damaged.

Vanillin (Figure 14C) is also a plant chemical but is an aromatic compound found in vanilla beans. Vanillin is a volatile chemical and is produced by the breakdown of lignin. It is sometimes used as an insect attractant but is not associated with plant defence in the tritrophic system used in this study.

Figure 14: The structure of three chemicals used in bioassays
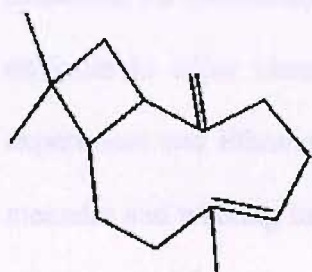
A) (E)-ß-Caryophyllene   B) (Z3)-Hexenyl acetate   C) Vanillin
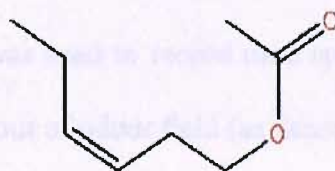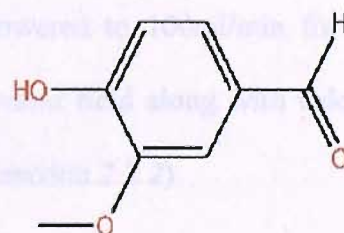
(E)-ß-caryophyllene and (Z3)-hexenyl acetate were featured as they were produced in vast amounts in the entrainment and vanillin was chosen as it is a novel chemical not found in the

insects natural foraging range and has been used before in past studies (Taskasu & Lewis, 1993). The three chemicals were brought from Sigma Aldridge, UK and kept at 0°C when stored.

Female wasps were reared and selected for bioassays as described in section *2.2.1*. Forty-five wasps were tested for each chemical and three different treatments were used (Table 9). Fifteen wasps were naïve and were kept in test tube isolation for 24 hours before the bioassay. They were fed 70% honey solution. Fifteen were placed inside a 3cm x 10cm cylindrical chamber and exposed to the chemical alone without a reward by placing a $3cm^2$ piece of filter paper containing 1μ of the chemical (applied using a Gilson Pipette-man) in the chamber with them for 10 minutes. The remaining fifteen were given an oviposition experience inside the same sized cylindrical chamber for 10 minutes, in the presence of a $3cm^2$ piece of filter paper containing $1\mu l$ of the chemical (applied using a Gilson Pipette-man).

Parasitoids were then observed for 5 minutes in a 4-arm olfactometer with one odour field and 3 controls each time. The odour field had air blowing into it which had passed through a chamber containing a piece of $3cm^2$ filter paper with $1\mu l$ (as Park et al., 2001 state this is sufficient for detection) of the relevant chemical and 10 minutes was left in between each replicate to allow clean air to blow through. Airflow was lowered to 100ml/min for this experiment and Ethovision was used to record time spent in odour field along with velocity meander and walking in and out of odour field (as described in section *2.2.2*).

The odour arm was randomised using online randomizer (www.randomizer.org) and bioassays were performed in a randomised block method so that only 5 of each odour-treatment combination could take place in one session and therefore time of day and time of

week was varied. This block was then repeated 3 times so that there were 15 reps for each treatment-odour combination (Table 8). Independent t-tests were used to test for interactions between treatments and targets.

Table 8: Experimental design for experiment 4. Showing chemical target, treatment and number of replicates.

	(Z3)-Hexenyl acetate	(E)-ß Caryophyllene	Vanillin
Naïve	15	15	15
Exposed	15	15	15
Experienced	15	15	15

The block method used to ensure randomisation of bioassay by alternating treatment, odour and arm every 5 replicates (Table 9).

Table 9: A 'snap-shot' of three days out of the nine days of bioassays demonstrating the method of randomisation.

Day	Number of reps	Treatment	odour	Arm
1	5	Naïve	Hex	2
1	5	Experienced	Car	4
1	5	Exposed	Van	1
2	5	Experienced	Van	3
2	5	Naïve	Car	1
2	5	Exposed	Hex	2
3	5	Experienced	Hex	4
3	5	Exposed	Car	3
3	5	Naïve	Van	3

3.3. Results

3.3.1 Plant Chemical Release Changed with Infestation

***Table 10: Average volume of chemical released by infested and uninfested replicates** (G=green leaf volatile. T=terpenes A=aromatic N=nitrile M=methyl ester)*

Av. Amount of chemical	Type of compound	Uninfested (+/-SE)	Infested (+/-SE)
(Z)-3-hexenol	G	1222.7(+/-623.2)	8241.33(+/-5874.0)
(Z)-2-pentenyl acetate	G	215.33(+/-215.3)	2140.33(+/-1425.7)
1-octen-3-ol	G	1451.33(+/-922.5)	416.83(+/-161,5)
Beta-myrcene	G	25822.67(+/-10587.2)	43078.17(+/-14169.2)
(Z)-3-hexenyl acetate	G	41130.167(+/-26896.09)	268701.17(+/-168326.4)
Hexyl acetate	G	11441.167(+/-10990.9)	5872.83(+/-3163.9)
Limonene	T	320.83(+/-156.1)	279.50(+/-138.6)
(E)-ocimene	T	1228.67(+/-543.1)	1109.83(+/-704.6)
Benzacetonitrile	N	734.67(+/-532.8)	6295.17(+/-2352.5)
Methyl salycilate	M	955.67(+/-643.4)	13520.50(+/-4499.3)
Indole	A	0.00(+/-0)	2353.33(+/-1208.2)
Alpha-copaene	T	771.50(+/-338.0)	5415.83(+/-1961.04)
Beta-elemene	T	669.67(+/-263.0)	3548.00(+/-1239.4)
(E)-ß-caryophyllene	T	24597.00(+/-10677.0)	166068.16(+/-54775.1)

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Alpha-humulene	T	1222.33(+/-484.3)	9249.83(+/-3218.7)
(Z,E)-alpha farnescene	T	8293.33(+/-5650.3)	4433.50(+/-1117.9)
(E,E)-alpha farnescene	T	13472.50(+/-3142.7)	32028.33(+/-14526.5)
Unknown sesquiterpine	T	205.17(+/-131.9)	1591.50(+/-632.2)
TMTT	T	1012.17(453.1)	6643.33(+/-2829.7)

Table 10 lists the mean peak areas of the main chemicals, which were produced by infested and uninfested Chinese Cabbage plants. The standard errors are often large suggesting that the peak areas vary between replicates. Most of the chemicals produced are terpenes and green leaf volatiles and two chemicals (E)-ß-caryophyllene and (Z3)-hexenyl acetate appear to be produced in large quantities.

Figure 15: a) Average peak area for each of the 19 volatiles released from Chinese Cabbage, comparing infested and Uninfested.
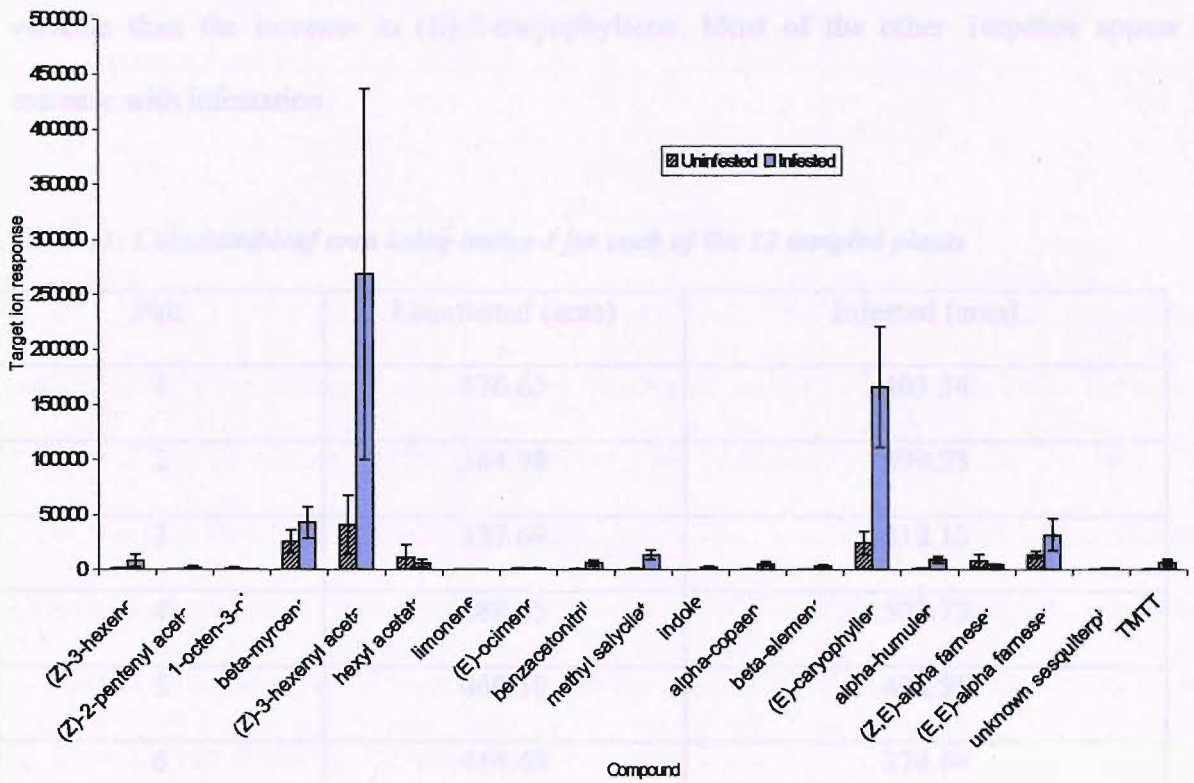
b) A modified version of graph 15a using a smaller scale so that the chemicals produced in smaller quantities can be seen more clearly.
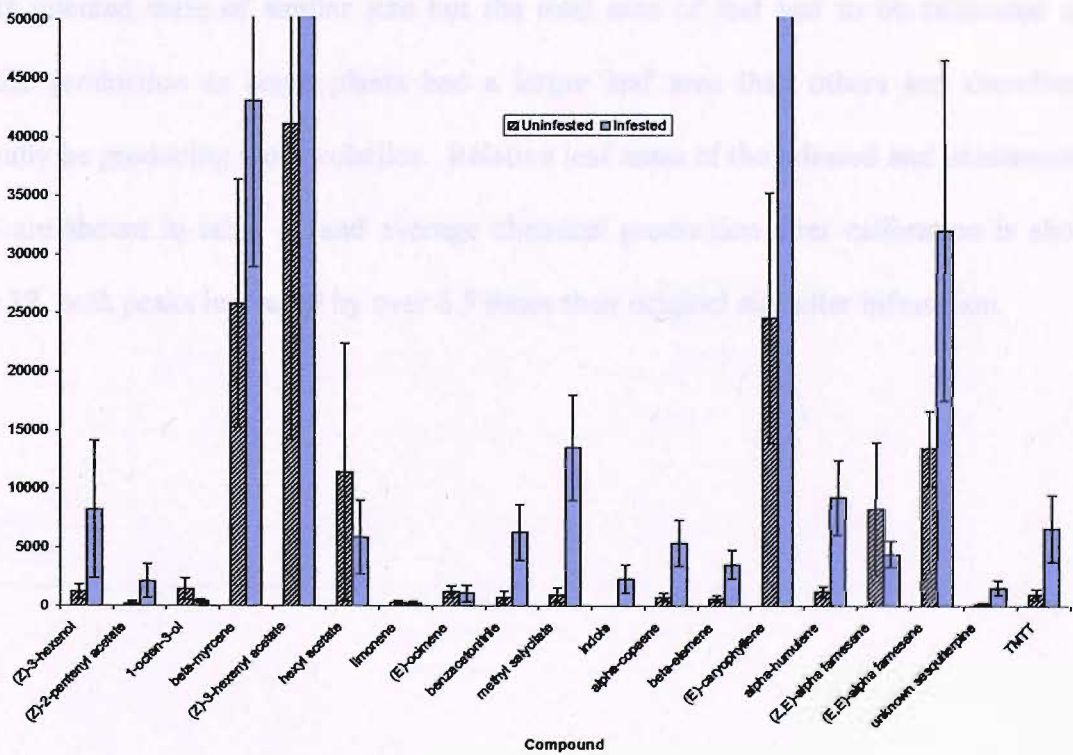
In figure 15a, the largest two peaks are (E)-ß-caryophyllene and (Z3)-hexenyl acetate. The increase in production of (Z3)-hexenyl acetate with infestation appears to be much more variable than the increase in (E)-ß-caryophyllene. Most of the other Terpenes appear to increase with infestation.

Table 11: Calculated leaf area using image J for each of the 12 sampled plants

Pair	Uninfested (area)	Infested (area)
1	376.65	403.34
2	344.98	399.25
3	327.69	418.13
4	388.85	503.72
5	460.10	434.55
6	414.49	374.64

Plants selected were of similar size but the total area of leaf had to be calibrated against volatile production as some plants had a larger leaf area than others and therefore may naturally be producing more volatiles. Relative leaf areas of the infested and uninfested plant pairs are shown in table 11 and average chemical production after calibration is shown in table 12, both peaks increased by over 6.5 times their original size after infestation.

Table 12: Average quantities of (E)-ß-caryophyllene and (Z3)-hexanyl acetate after calibration against total leaf area.

	Uninfested	Infested
Caryophyllene	20187.71	144237.09
Hexanyl acetate	33586.41	221101.78

When analysing these results statistically with a paired samples t-test, there is a significant difference between infested and uninfested for (E)-ß-caryophylene (t_5=2.7, P=0.043) P<0.05 (Table 12). There is no significant difference for (Z3)-hexanyl acetate (t_5=1.4, P=0.229), perhaps due to the variable results for infested plants (See error bar on Figure 15b). Although (Z3)-hexenyl acetate is produced by Chinese cabbage in large quantities it is not consistently elevated by infesting the plant and the elevation observed could have been due to plant stress or damage when setting up the entrainment.

*Table 13: **Statistical analysis using a paired samples t-test comparing the (E)-ß-caryophylene produced by infested and uninfested pairs of plants.***

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Uninfested - Infested	-124049.2	112540.6	45944.5	-242153.4	-5945.2	-2.70	5	0.043

3.3.2 Time Spent in (E)-ß-caryophyllene Odour Field Increased with Experience

Figure 16: Time spent in (E)-ß-caryophyllene odour field and control zones after exposure and experience treatments.
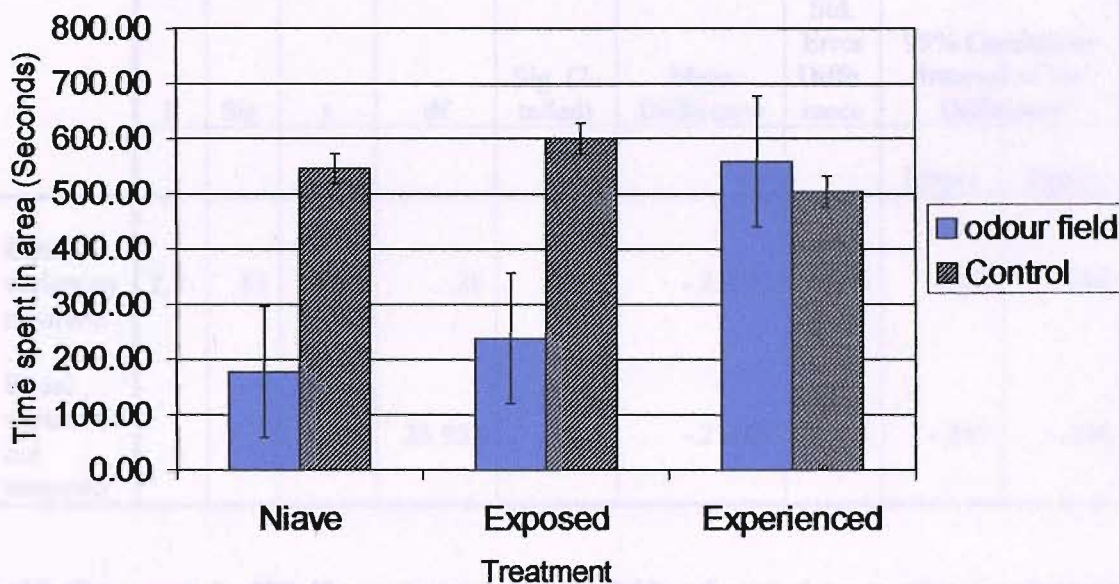
Wasps appear to avoid the odour field when naïve or exposed, however time in the odour field increases in relation to the control once it has had a learning experience with this chemical (Figure 16). An independent samples t-test was performed comparing proportion of time spent in the (E)-ß-caryophyllene odour field by naïve and experienced wasps. There is a significant difference (t_{26}=4.11, P=0.00) P<0.001 between naive and experienced wasps (Table 14).

Table 14: Results of the independent samples t-test, comparing proportion of time spent in (E)- β-caryophyllene odour field by naïve and experienced wasps.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Prop	Equal variances assumed	2.4	.13	-4.11	28	.000	-.21105	.0513	-.316	-.106
	Equal variances not assumed			-4.11	25.953	.000	-.21105	.0513	-.317	-.106

Figure 17: Time spent in (Z3)-Hexenyl acetate odour field and control zones after exposure and experience treatments.
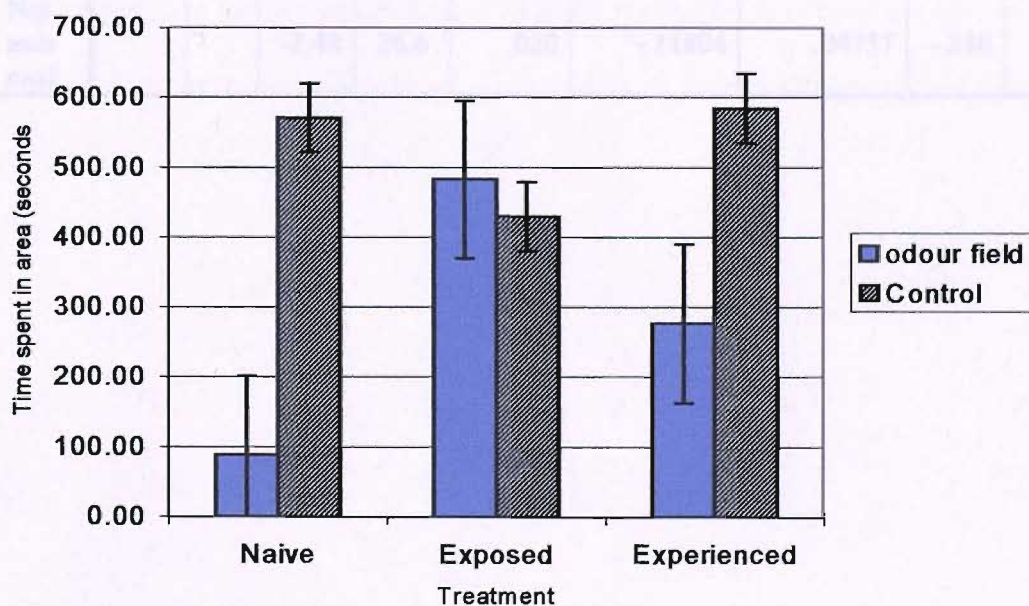
Wasps appear to avoid the (Z3)-hexenyl acetate odour field when naïve and experienced, although when they are exposed there is an increase in time spent in the odour field (Figure 17). When tested using an independent samples t-test, there is a significant difference in proportion of time spent in the odour field by naïve and experienced wasps (t_{26}=2.481, P=0.020) P<0.05 (Table 15).

Table 15: Results for independent samples t-test, testing for a significant difference in the proportion of time spent in the (Z3)- hexanyl acetate odour field by naïve and experienced

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lowe	Upper
Prop	Equal variances	3.55	.07	-2.48	28	.019	-.11804	.04757	-.216	-.020
	Not assumed			-2.48	26.6	.020	-.11804	.04757	-.216	-.020

Figure 18: Time spent in Vanillin odour field and control zones after exposure and experience treatments.
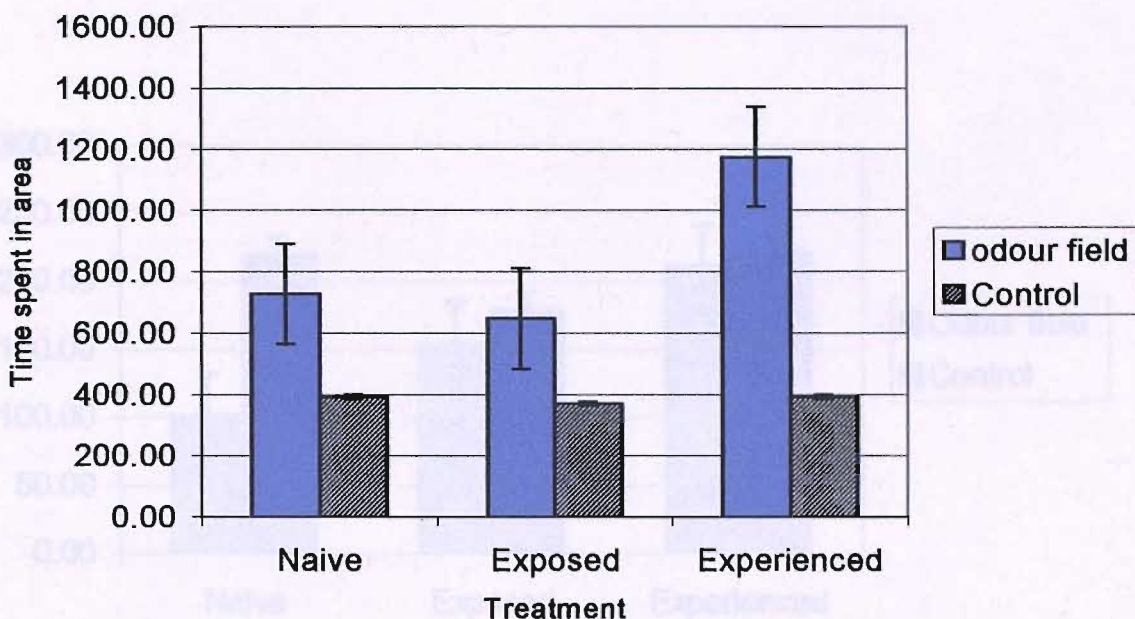
Wasps prefer the vanillin odour field slightly when naïve or exposed but time spent in the odour field increases more dramatically in relation to the control once the wasp has had a learning experience of this chemical (Figure 18). When comparing proportion of time spent in the odour field by naïve and experienced wasps using an independent t-test the results were not significant ($t_{26}=1.132$, $P=0.267$).

3.3.3. Visits to Odour Field Changed with Experience

Figure 19: Total visits to (E)-β-caryophyllene, (Z3)-hexenyl aceteate and Vanillin odour field and control zones with three different treatments.
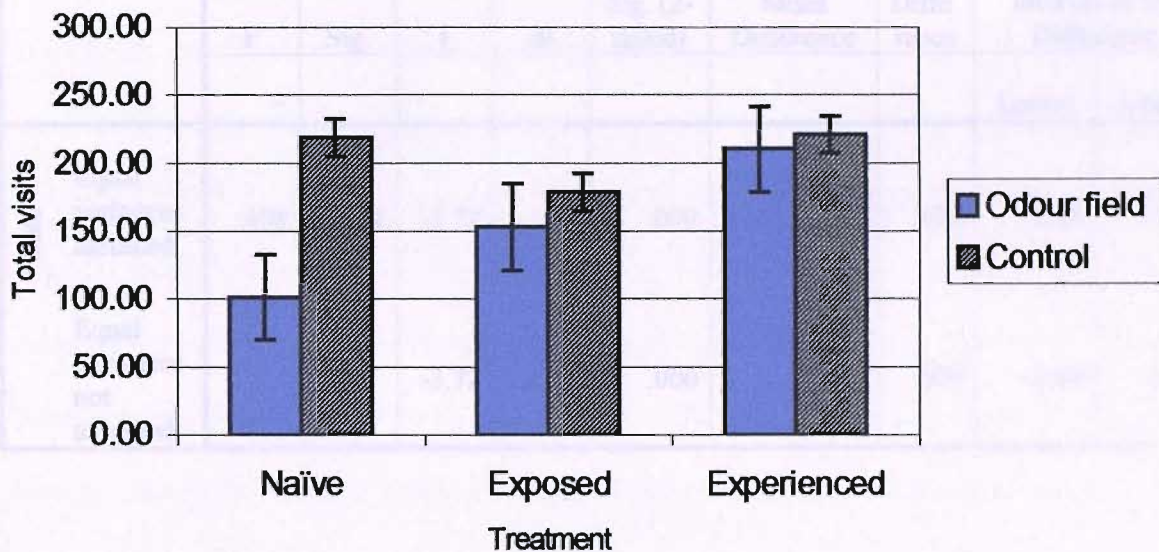
The number of visits to the odour field and control zones were combined for all three experiments in 3.3.2. The number of visits to the odour field appears to increase with experience and there is an overall increase in walking in and out of all odour fields with experience. There is a significant (t_{88}=3.72, P=0.00), P<0.001 difference in visits to the odour field by naïve and experienced wasps (Table 16).

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Table 16: t-test comparing number of visits to the odour field when wasps are naïve and experienced.

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Data	Equal variances assumed	.408	.524	-3.72	88	.000	-2.53	.679	-3.88	-1.18
	Equal variances not assumed			-3.72	87.8	.000	-2.53	.679	-3.88	-1.18

3.4 Discussion

The results suggest that wasps are able to learn these single volatile chemicals from the odour profile and appear to learn (E)-ß-caryophyllene 'better' than (Z3)-hexenyl acetate. In past studies, a blend of chemicals tends to elicit more of a response in parasitic wasps (Wei and Jang , 2006) and therefore blends of our 19 identified chemicals may elicit more response in *Cotesia plutellae*. As plants can only produce a limited number of chemicals, using blends of different quantities allows them to produce a more specific chemical cue. In a previous study, using a blend of nine chemicals from the host, the plant and host-plant interactions meant that wasps *(C. kariyai)* could learn the blend at lower concentrations than they did when using a smaller range of just host-specific plant chemicals. Hence, both the host-induced and

nonspecific volatile compounds appear to be important for females to learn the chemical cues

in host location (Fukushima et al. 2004).

Further experiments could be conducted to examine which particular odour combinations are

important in learning rather than just testing single chemicals. Also, different concentrations

need to be examined as the three chemicals have different volatilities (Table 17). As they

were used as targets in the same concentration, this difference in volatility was not taken into

account, Vanillin is a larger molecule and has the highest boiling point and is therefore less

volatile than (E)-ß-caryophyllene. It may have lasted longer on the filter paper before

evaporating whereas the other chemicals may have evaporated off more quickly. On the other

hand, we would expect there to be less vanillin in the air than (E)-ß-caryophyllene and (Z3)-

hexenyl acetate as it is less volatile so perhaps the concentration of the (Z3)-hexenyl acetate

was too high in comparison.

Table 17: The boiling point and atomic mass of 3 plant volatiles.

Chemical	Size of molecule (rmm)	Boiling Point (°C)
(E)-ß-Caryopyllene	204.35	129-130
(Z3)-Hexenyl acetate	142.20	75-76
Vanillin	152.15	170

The terpene, (E)-ß-caryophyllene may be important in learning as this volatile elicited the

most significant learning response of the 3 tested. This volatile is associated with active

production in plants and increases dramatically when Chinese Cabbage is attacked by the

host, making it a more reliable source of information for indicating host presence. The active

chemical profile may be specific to the particular host inducing it and parasitoids may even be able to discriminate between the profile induced by parasitised larvae and unparasitised larvae due to differences in the amount of volatile release (Fatourous et al., 2005).

Turlings and Tumlinson (1990) found that Fresh feeding damage results in a significant release of (Z)-3-hexenal, (E)-2- hexenal, (Z)-2-hexenol, (E)-2-hexenol and (Z)-3-hexenyl acetate. These volatiles, also known as "green leaf volatiles" are the only compounds detected at this stage. After several hours, however, emission of large amounts of terpenoids were observed. Terpenoids may therefore be a more reliable signal of the presence of a host as the host is required to feed for several hours before they are produced in significant amounts. The terpenoids could only be induced by caterpillar saliva and not by mechanical damage alone (Turlings et al., 1995).

(Z3)-hexenyl acetate is a green leaf volatile, which is much less specific and more widely encountered. It is produced in relatively high quantities even when the host is not present and is also released during mechanical damage. The increase in (Z3)-hexenyl acetate with infestation appears to be very variable between entrainments making its increase statistically insignificant for the 5 replicates analysed, perhaps this is variability is due to plants being knocked and mechanically disturbed when setting the experiment up. As both undamaged plants and mechanically damaged plants do not reliably indicate the presence of a host, it makes sense for the wasps to invest more time in learning reliable cues. Exposing the wasps to the chemicals did not increase their time spent in the odour field apart from when using (Z3)-hexenyl acetate. This may therefore be an important chemical in sensitisation of the insect.

The wasps appeared to learn the novel chemical vanillin which is not typical of their foraging range but is a plant chemical and is structurally related to chemicals that they will regularly encounter. This demonstrates that more host-specific wasps can in fact modify their foraging strategies to adapt to a changing environment by associating a novel plant odour or conditioned stimulus with a host reward.

Further experimental development is required for this chapter, as time was limited and many questions still remain unanswered. Experiments involving learning responses to chemical blends and a wider range of volatiles would be useful as well as investigation of different chemical concentrations and quantities.

3.4.1. Discussion Summary

- Chinese cabbage releases 19 main detectable volatiles. Two of which appear to be produced in significant quantities, (E)-ß-caryophyllene and (Z3)-hexenyl acetate increasing with infestation.

- *Cotesia plutellae* appears to learn (E)-ß-caryophyllene in bioassays but avoids the (Z3)-hexenyl acetate odour field possibly because ß-caryophyllene is a more reliable cue. It also appears to learn novel chemical vanillin.

CHAPTER 4: GENERAL DISCUSSION

It is thought that generalist wasps learn so that they can focus on the particular resources available in the surrounding habitat at any particular time. *Cotesia plutellae* appears to be able to not only focus in on odours from its specific host plant which are likely to be the most reliable and change its innate preference accordingly, but also increase its response to new chemicals in a changing environment.

Wasps tended to learn the active chemicals from herbivore damaged plants, (E)-ß-caryophyllene in this case, as these are conspicuous and can be reliable. In fact, signals from herbivore damaged plants are further enhanced by the fact that the chemical emissions are not limited to the damaged sites. For corn seedlings, the induced compounds are released throughout injured plants and unharmed leaves of damaged plants show an increase in the release of terpenoids (Rose and Tumlinson, 2004). Plant signals are more detectable than host volatiles as the amount of volatiles produced per hour by the plant is much greater than the amounts normally seen in insect pheromone communication. Only a few nanograms of sex pheromone per hour can be detected, compared to several micrograms of a particular substance emitted by one corn seedling (Turlings et al., 1990).

Research has recently focused on the concept of reliability of host plant cues, whether or not the volatiles emitted are specific to the host and whether the parastioid is able to distinguish

between hosts in this way. It is likely instead that host searching behaviour is determined by a combination of species-specific profiles and learning experience, as in this study and others there is an increase in response to host-plant volatiles after experience and this effect was shown to last at least for several hours in *C. marginiventris* (Turlings et al., 1990).

If learning of plant chemicals is important in the field, it is likely that signals would be emitted during the time of day when natural enemies are most likely to forage. Work has been done to confirm whether or not the timing of volatile release is in tune with the parasitoids needs. The plants give off the strongest signals during the photoperiod when natural enemies tend to forage and the parasitoids often use other signals such as close range chemical contact, visual and vibrational cues in conjunction with herbivore induced volatiles from the host plant (Turlings et al., 1995).

4.1 Method Critique and Future Directions

Figure 20: Ethovision images from part 3.2.1.ii of this study showing a wasp's walking path when it is HPC experienced (red) and naïve (black) in response to a HPC odour coming from the bottom left hand arm of the olfactometer
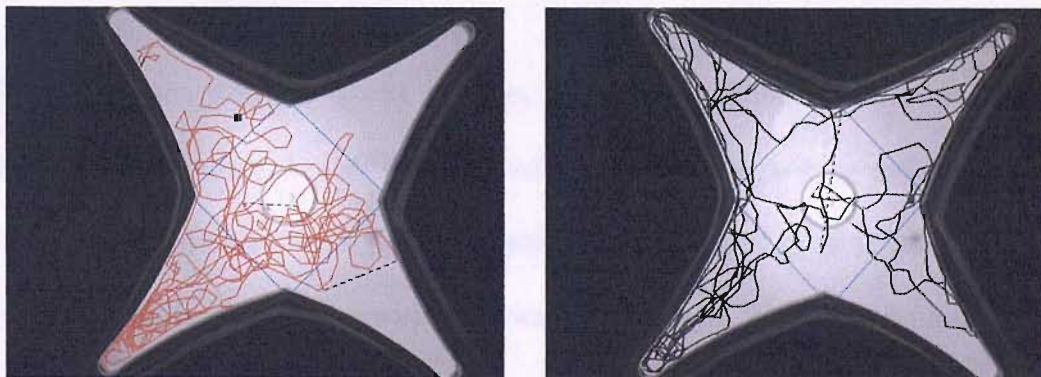
The results satisfy the first of the three learning criteria (Papaj and Prokopy, 1989) mentioned in *1.2.5* which is '1. Behaviour can change in a repeatable way through experience'. Wasps tend to walk for more time in the odour field when experienced and replication suggests that this change in behaviour is significant. Figure 20 is an image of the path walked by a nave parasitoid in comparison to an experienced one recorded by ethovision – the difference in behaviour is very apparent as the experienced wasp is walking in a much more specific pattern. It would be interesting to continue experiments on *Cotesia plutellae* to satisfy the remaining two criteria.

To explore whether 'behavioural change is gradual with continued experience up to an asymptote' experiments could be designed to increase the length of the learning experience or number of experiences, to see whether there is a relationship between this and the amount of time spent in the odour field. The final criterion, 'learned responses can be forgotten (wane) or disappear as a consequence of another experience,' could be investigated by increasing the time between experience and testing to see whether the wasps 'forget' what they have learnt. 'Distractor' experiences could also be given in between using different odours to see whether they interfere with learning.

The assumption was made that volatiles produced in large amounts by host damage are important in learning. A more reliable method would have been to use electroantennography to see which volatiles were eliciting a positive electrophysiological response in wasps and this could be used in conjunction with behavioural bioassays. Wei & Jang (2006) used GC-EAD to analyse volatiles from bean plant damage and found that chemicals (Z3)-hexenyl acetate and (E)-ß-caryophyllene elicited responses in parasitic wasp *Opius dissitus*.

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In most of the behavioural bioassays in the project, behaviour was variable between individuals due to background noise during the experimental technique, so replicate number needed to be high in order to achieve significant results. This variability could be due to a number of factors such as atmospheric pressure changes and genetic variability. Behaviour is often unreliable because it is a physical output of a complicated neurological process, which has to travel from the insects sensory system, to the brain and then induce a motor relay response. Detecting associative learning at the sensory level may be a more reliable way of interpreting it.

Firstly, electroanntenography (EAG) would determine for sure whether or not the insect is detecting the chemical and changes in antennal depolarisation responses to naïve and learnt volatiles could determine whether antennal sensitivity is effected by learning. Secondly, images could be taken of the insect brain to see whether learning alters neuronal processing. This could then be mapped and modelled for interpretation (APPENDIX A).

4.2 Applications

4.2.1 Ecological

Parasitoids are of immense importance to natural ecosystems as they can regulate the population density of many of their hosts and therefore have the potential to be important in successful biological control programmes. Vet and Groenewold (1990) also discuss implications of parasitoid learning in pest management and suggest that:

1. 'Aberrant learning during culturing should be avoided to prevent interference with learning of target stimuli.

2. The potential to respond to target stimuli should be improved through learning prior to release.

3. Maximal responses to target stimuli should be maintained by providing the right reinforcements during the foraging process.'

Poppy et al., (1997) also discuss the implications in biological control. 'The influence of experience during development (conditioning) or after adult emergence on behavioural responses to semiochemical cues could provide opportunities for "priming" parasitoids to preferentially forage for specific hosts by means of learning and/or conditioning.' Papaj and vet (1990) showed that learning increases foraging efficiency using *L. heterotoma* and systemically arranged apple-yeast and mushroom baits in a forest. Experienced wasps found traps faster and were more likely to find the habitat they had experienced. Encouraging results for the planned application of releasing pre conditioned insects.

Mitchell, 2002 describes how the diamond back moth has become such a pest of cruciferous crops that many growers have abandoned cabbage production in favour of other less profitable crops and they are investigating ways in which to manage this without relying on conventional pesticides. Field trails involving the release of large numbers of *C. plutellae* in cabbage fields were carried out and parasitism was found to be related to numbers released although other species were more effective in destroying the larvae.

4.2.2 Detection

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Knowledge gained from research into the processing of olfactory information in insects could be used in the development of more sophisticated electronic chemical detectors. A recent invention - the E-nose (Cyranose) has 32 sensors that analyze smells and create a smell print and match it to a database. It works in much the same way as the animal olfactory system, a small number of receptors can analyse many more odours. A dog's nose is however in general much more sensitive than any electronic nose that we can put together, but like all sensing systems there are things which a dog cannot smell because its sensors are just not sensitive to it (www.iit.html). Insects have a more sensitive olfactory system than any mammal so could be important in providing information for an effective electronic detector.

Several studies have demonstrated that E-nose may be used to diagnose pneumonia, sinusitis and lung cancer. Its ability to take in and analyse patient smells may also enable it to detect cerebral spinal fluid, liver disease, diabetes and other conditions (John Crawford, University of Aberdeen). It could be a medical tool to enhance human smelling ability just as a stethoscope enhances hearing. NASA is using E-nose to recognise compounds like ammonia onboard space stations, which can become dangerous at only a few PPM if a leak occurs. E-nose uses 16 different polymer films specially designed to conduct electricity when stray molecules are absorbed onto these films, the films expand slightly and that changes how much electricity they conduct. Each one reacts to each substance or analyte in a slightly different way and the varied changes in 16 films produce a distinctive and identifiable pattern (http://science.nasa.gov).

The insects themselves could also be used in the detection of minute traces of chemicals. For example, in the moth *Spodoptera littoralis*, a change in heartbeat frequency can be triggered when fewer than six molecules of a key pheromone component hit the antennae of the insect.

Thus, the insects themselves might be most capable of informing us on which are the key substances that they use in their behaviour (Angioy et al., 2003). An interesting article in the New York Times (14.05.02) described how the US government is hoping to use bees to sniff out explosives near security check points by conditioning them with sugar-water rewards. Pentagon research scientists claim that 99 % of the time, bees swarm around explosives and are better than dogs at detecting particular odours and can be trained in much the same way. More recently (2005) University of Montana researchers (DAPRA funded) have been using these ideas to train bees to detect land mines. W.J.Lewis firstly devised a handheld container with a hole through which outside odours are wafted. Inside, bees previously conditioned to the odour of an explosive are monitored with a tiny camera linked to a computer. The system registers when the insects cluster near the hole, showing that they are detected the odour of the explosive. They then release the bees and use a radar-like system that bounces laser light off them to show where they tend to cluster. The team was able to locate several defused land mines at a U.S. Army test site. However, there are some limitations such as bees are easily distracted and will go for their nearest preferred target (http://www.mindfully.org). The success of this effort will depend on the ability to understand and utilize the sensory signals and sensorimotor behaviour displayed and to exploit the neural biomechanical control circuitry used for foraging, mate identification, and predator avoidance.

The short lifecycle and genetic diversity of these organisms, along with the apparent speed with which they can be trained, offers potential benefits of flexibility and convenience to the science of olfactory learning, and the science of biological detectors (Olson et al., 2003).

4.2.3. Pure Science and Other Organisms

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Insects have been used as model organism in the study of many homologous human systems as it provides a simpler and more available research tool. Research into the processing of olfactory information along with learning and memory particularly at the cellular level, could be usefully applied in the investigation of similar processes in vertebrates. The olfactory system has become an important system for neurobiological function and sensory processing and research into the structure and function of the insect olfactory system has often preceded similar investigations in vertebrates and has been important in the formulation of general principles (Hansson; 2002).

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APPENDIX – Side Project with Computer Sciences Students

Work was done in conjunction with computer science students in order to investigate how the neurological changes during learning could be modelled. Odours can be represented by specific spatio-temporal combinatorial patterns of activated glomeruli (Hildebrand & Shepherd, 1997). For example, Sachse and Galizia (2001) measured spatio-temporal odour patterns in the glomeruli of the antennal lobe of the honey bee (*Apis mellifera*, Luc Viator *(Hymenoptera; Apidae)*) and found that each odour evoked a spatio – temporal odour pattern of excited and inhibited glomeruli. This pattern changed with learning (Figure 21).
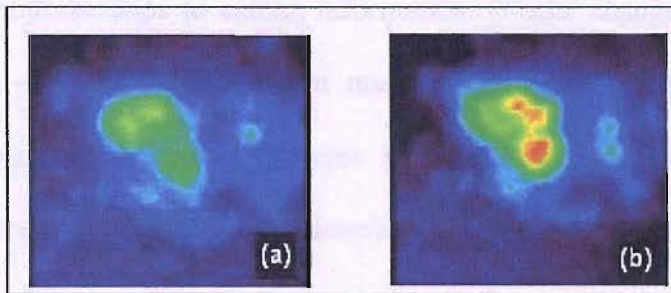

Figure 21: Calcium images of a honeybee's glomeruli a) without a learning experience b) with a learning experience (from Sachse&Galizia; 2001).

Smid et al. (2003), produced a 3 dimensional model of the glomeruli of two closely related parasitic wasp species by staining the glomeruli and using confocal microscopy and then processing stacks of optical sections which were processed by computer software. In the same year, Sandoz et al., (2003) used calcium imaging to show that each different odour evoked a different pattern of glomerular activity which was symmetrical between sides and highly conserved in naïve animals. Galizia and Menzel (2001) devised a functional atlas of the antennal lobe using the odour evoked activity patterns of the glomeruli. They show how this alters with response to an odour which has been paired with a reward using a surface plot of activity.

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During the course of this project, work was carried out with three Masters engineering students from computer sciences department and biological advice given for their project. Their task was to identify areas of activation using images of a bee's brain (provided by Dr. J. Hildebrandt) and model the changes occurring mathematically. This can then be applied to *Cotesia plutellae* once brain-imaging data becomes available and any changes occurring as a result of learning can be identified.

Computer models of the glommeruli were created using Feature Extraction and only the important areas where activity was present were focused in on. This was achieved using image processing which is an area of computing in which images are modified by algorithms to perform steps to extract information. Colour changes were interpreted by numerical data and computer models were made (Figure 22). These models could be very useful in comparing activation changes between naïve and experienced wasps and looking for mathematical relationships describing a learning activity.

Figure 22: An image produced by the Masters students showing the mean colour change produced by calcium imaging, throughout a video clip of a bees antennal lobe whilst responding to a conditioned odour (Abuelgasim et al., unpublished).