Chemical ecology and olfactory behaviour of an aphid parasitoid and a lacewing predator

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Abstract

Female parasitoid (Aphidius colemani) olfactory preference was consistently biased in olfactometer experiments towards the Brassica cultivar on which it was reared when offered other Brassica cultivars as alternatives using either whole plants or detached leaves. Using gas chromatography, differences could be detected in the volatile composition of whole plants and detached leaves of five Brassica cultivars and the volatiles responsible for the fine distinctions the wasps are capable of making between plant cultivars are suggested. By subjecting female wasps to olfactory conditioning protocols, studies were carried out to understand the learning process for individual green leaf volatiles (6-carbon molecules), known to elicit behavioural responses in many insect species. Wasps were exposed to primary alcohols with differing carbon chain lengths in conjunction with aphids in an attempt to condition the wasps to the alcohols. When tested for learning, wasps changed their responses towards alcohols with molecules consisting of between 5 and 6 carbon atoms. The effect of cold temperature on olfactory preference was also investigated. After treating females at 0 °C for 0.5 h or longer, preference for the odour of the Brassica cultivar on which they were reared was lost for one hour after the cold treatment had finished, after which, the preference returned. However, when Brassica or unencountered plant odours were presented with clean air as the alternative choice, females could discern plant odour even immediately after the cold treatment. This suggests that the olfactory and locomotive systems were not altered by cold, whereas responses arising from learning-produced memories appear to have been inhibited temporarily and revealing underlying innate responses. The results indicate that temperature treatments could offer the possibility of dissecting innate and learnt behaviours in these parasitic wasps. The importance of controlling humidity arising from odour choices in olfactometers is also observed. In addition to assessing the parasitoid’s behaviour, various aspects of the role that neomatatabiol, a chemical compound closely related to aphid sex pheromone was investigated in relation to its role in the chemical ecology of the green lacewing Peyerimhoffina gracilis.
Declaration

The work presented in this thesis is entirely my own, it has not been submitted for any other academic qualification and any collaborative work has been specifically acknowledged.

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Chapter 1

General Introduction: Aphids, Parasitoids and Lacewings

1.1 Aphids

1.1.1 Life cycle and general biology

Aphids (Hemiptera: Aphididae) are viviparous phloem-feeding insects that can transmit viruses to the plants they feed upon. There are more than 4700 described aphid species occurring throughout the world mostly living in temperate regions (Dixon et al., 1987; Blackman & Eastop, 2007). Due to a combination of factors that enable them to reach high reproductive rate, aphids can colonize new habitats extremely efficiently. The evolution of parthenogenesis is one of the key factors that lead to aphid success. Most species of aphids alternate between a sexual phase that ensures gene recombination and segregation and a phase of parthenogenetic reproduction that enables biomass growth of the colony where the rates of population increase can be doubled compared to sexual individuals. Being able to reproduce with no need of fertilization shortens generation times since embryos can start developing in embryos of unborn mothers (Blackman & Eastop, 2000; Powell et al., 2006).

Another evolutionary success of aphids is the way in which energy resources are allocated in the colony. Within a same clone, there can be wingless (apterous) or winged (alate) forms. The production of these two forms is triggered by environmental and other factors such as colony density, plant physiology and predator presence. Apterae are normally more fecund and sedentary while alates are less fecund and more mobile. Since production of wings in an individual represents a big energetic cost, aphids allocate resources to reproduction while resources and environmental conditions are favourable and thus increase colony biomass, but when resources start to decline and aphid density increases, energy is allocated differently and winged forms are produced as dispersal forms. Although slow flyers, alate forms take advantage of
wing currents and reach distances of up to hundreds of km (Dixon, 1985; Blackman & Eastop, 2000).

Some aphid species (ca. 10% of all species) alternate between plant hosts during their life cycle (heteroecy) (Williams & Dixon, 2007). This involves regular seasonal migrations between two plant species: the primary host, where sexual reproduction occurs, and the secondary host that is only colonized by parthenogenetic individuals. A generalized host-alternating life cycle can involve an alate parthenogenetic female and male migrate, in the autumn, to the primary host where the former produce sexual oviparous females that lay over-wintering eggs. In spring eggs hatch and give rise to viviparous parthenogenetic females that produce alate forms that migrate to the secondary host which in turn produce alate and apterous forms, and when the days start to shorten in autumn, the cycle commences again. Many variations of the patterns mentioned above are found among the Aphididae. Some species have become entirely parthenogenetic due to genetic or environmental conditions. In other species alternation between two hosts species throughout the year has been lost (Blackman & Eastop, 2000; Powell et al., 2006).

1.1.2 Aphids as pest
Aphids are considered the most important insect pest in agriculture of northern European countries (Birkett & Pickett, 2003) and world-wide there are more than 250 species of the superfamily Aphidoidea that feed on agricultural crops (Blackman & Eastop, 2000). They feed on the phloem sap of the plants they attack, which leads to a loss in plant nutrients and thus productivity. However, the main impact on plant health is caused by them acting as vectors for plant viruses (Dixon, 1973).

1.1.3 The peach-potato aphid Myzus persicae

1.1.3.1 General biology
The peach-potato aphid Myzus persicae Sulzer (Figure 1) is a cosmopolitan species, probably of Asian origin. It is holocyclic, alternating between a primary host, usually Prunus spp., where sexual reproduction occurs and secondary hosts comprising more than 400 plant species where parthenogenetic reproduction occurs (van Emden et al., 1969; Blackman & Eastop, 2000). In the United Kingdom, where the primary host is not widely distributed, M. persicae has become predominantly anholocyclic, overwintering in sheltered sites (i.e. glasshouses) reproducing parthenogenetically throughout the year and only some individuals go through the sexual part of the life cycle (van Emden...
et al., 1969; Blackman, 1976). The development in moderate climates can be fast, with up to 20 generations in one year (Blackman & Eastop, 2000).

![Myzus persicae](image)

Figure 1: Two adult parthenogenetic *Myzus persicae* females and three nymphs in the background.

1.1.3.2 *Myzus persicae* pest status

The peach-potato aphid is a pest on many crops including potato, tomato, lettuce, sugar beet, tobacco, legumes and most brassicas. It causes damage by directly feeding on plants, by honeydew deposits on leaves but the principal damage caused by *M. persicae* is indirect, as this aphid is vector to more than 120 plant virus diseases, including Beet mild yellowing virus, Potato leaf roll virus, Pea enation virus, Beet western yellows virus and Lettuce mosaic virus (Blackman, 1976). Its pest status is enhanced because the diversity of crops they can attack, which is exemplified by the fact that in the United Kingdom *M. persicae* was found in plants from more than 40 families, mainly the Asteraceae and Brassicaceae (Tatchell et al., 1983; Blackman & Eastop, 2007).

1.1.3.3 Control methods of *Myzus persicae*

1.1.3.3.1 Chemical pesticides

Synthetic chemical pesticides have been the solution in the modern world to reduce crop losses. Although 2.5 million tonnes of pesticides are applied worldwide, more than 15% of the potential world food is still being lost exclusively to insect attack.
Nevertheless, it is estimated that without pesticides, crop losses to pests would increase from 15 % to 20 % (Paoletti & Pimentel, 2000) and crop prices would rise as well.

Between 20 and 90% of the main arable crops in Great Britain needed to be treated at least once with insecticides to minimize aphid attack during 2004 (Garthwaite et al., 2004). Control methods for *M. persicae* mainly rely on systemic chemical insecticides which has led to the evolution and selection of various resistance mechanisms in the target species. In the United Kingdom, the efficacy of insecticides used to control *M. persicae* are heavily influenced by the level of resistance of the pest towards these chemicals (Foster & Devonshire, 1999) and the problem has worsened in glasshouse crops since more individuals carry different combinations of the resistance mechanisms (Foster et al., 2003).

### 1.1.3.3.2 Genetically modified crops

A relatively new alternative that began some 20 years ago is the introduction of genetically modified (GM) crops. Plants are engineered to resist diseases and herbicides, taste sweeter, ripen faster, have a higher nutritional value and many other characteristics to produce a better, cheaper product (Paoletti & Pimentel, 2000; Brookes & Barfoot, 2006). Although commercially released in 1996, the use of GM crops has grown in the last 10 years steadily reaching more than 100 million ha of cultivated crops in 22 countries during 2006 (International Service for the Acquisition of Agri-biotech Applications website: http://www.isaaa.org/). Although the advantages of GM crops are still under severe scrutiny and discussion (Poppy, 2000; Clark & Lehman, 2001; van Emden, 2003), they offer a good alternative to chemical controls in many aspects (Poppy & Sutherland, 2004), but they still need to develop further and any decisions taken should be well informed.

### 1.1.3.3.3 Biological control

A technique widely accepted to control insect pests is the use of natural enemies. Populations of all living organisms are subject to some extent, to reduction by natural enemies, process known as natural control, and it has been suggested that around 99% of all potential pests are controlled by their natural enemies. When pests are controlled with human intervention by using their predators, parasites, antagonists and diseases, it is known as biological control (Hajek, 2004). This technique has flourished when chemical pesticides did not work as expected in addition to finding negative environmental and health side-effects.
The strategies that rely on natural enemies can be grouped into four categories: Classical biological control, which is the intentional introduction of an exotic biological control agent for permanent establishment and long-term pest control (Eilenberg et al., 2001). Inundative biological control is a technique directed towards rapid control of pests over a short term without expecting the natural enemy to reproduce. Inoculative biological control on the other hand is focused on the release of natural enemies to control the pest over an extended period, but not permanent. The last technique is the conservation and enhancement naturally-occurring enemies. This technique differs from the previous ones since there is no artificial introduction, the aim here is to conserve the natural enemies of the pest through alternating pesticide use and enhancing the population by providing food, shelter and alternate prey (Hajek, 2004).

Consumer demand for pesticide-free food, costs similar to those of chemical control, ease of application, no safety period between application and harvesting and low resistance in the target species, stimulate the use of biological control methods (van Lenteren, 2003a). This is reflected in number of species commercially available for inundative and inoculative biological control for greenhouse pests, which has increased steadily since the seventies and now there are more than 130 species available (Hajek, 2004). Entomopathogenic fungi, lacewings and parasitic wasps, have been used in biological control of *M. persicae* with varying degrees of success (McEwen et al., 2001; Kift et al., 2005).

### 1.1.3.3.4 Integrated pest management

The reliance on single control methods over the past 50 years has led to difficulties in pest management. Problems raging from target pesticide resistance to environmental degradation led to shifting control techniques to a more conscientious and balanced approach to pest management that reflect cultural, biological and biorational standards (Maredia, 2003; Koul & Cuperus, 2007), as a consequence integrated pest management (IPM) started gaining acceptance. Integrated pest management combines the control strategies mentioned above and prioritizes several factors such as a good understanding of the pest biology and ecology, its interaction with the ecosystem, the importance in society and economics (Norris et al., 2003). To illustrate the importance of IPM, in 2003 5% of the greenhouse area were under IPM at a global scale, with potential to increase to 20% in the next 10 years (van Lenteren, 2003a).
1.2 Parasitoids

1.2.1 General biology

Parasitoids are entomophagous insects widely spread in nature. It is estimated that around 25% of all insects belong to this group. Although there are around 70,000 described species of parasitoids, it is believed that there could be more than 1.5 - 2 million still undescribed. Most of the species belong to the orders Hymenoptera and Diptera but the orders Coleoptera, Lepidoptera, Trichoptera, Neuroptera and Strepsiptera also have representatives (Godfray, 1994; Quicke, 1997). The main characteristic shared by parasitoids is the need for a second insect species to complete their life cycle. While adult parasitoids are free living, larvae use a second insect species as a food source. In most cases it is the adult female that locates a suitable host to lay the eggs in or on, but in some cases the larvae locate the host, a strategy which is more common in dipteran parasitoids and comprises all of neuropteran and lepidopteran parasitoids (Quicke, 1997). Parasitoids can either develop on more than one species, in which case they are known as generalists, or on a single species and they are called specialists. Once the parasitoid larva emerges from the egg, it reaches the host if it is not within it already, and starts feeding. The larvae, in contrast with normal parasites, sooner or later kill the host. There are many variants to the basic model described above, ranging from endoparasitoids or ectoparasitoids, (depending if the larvae is inside the host or only piercing it with its mouthparts) although this condition is not permanent, since there are some examples where an ectoparasitic larvae becomes endoparasitic and vice versa. Some parasitoids can inhibit the hosts’ development, in which case are known as idiobionts, while others known as koinobionts, allow the host to continue developing sometimes until pupation. Also the number of eggs that develops per host varies from one (solitary) to several (gregarious) and if more than one female oviposits on one host, it is called superparasitism (Dorn & Beckage, 2007).

More than two thirds of the hymenopteran species are parasitoids and around 80 % of all parasitoid species belong to the order Hymenoptera. Probably the fact that has enabled ancestral hymenopteran parasitoids to have such an evolutionary success is the highly specialized ovipositor, that enabled them to lay their eggs in hosts which were difficult to reach or pierce with the aid of accessory glands to inject additional substances to paralyze or control the immune response of the host (van Baaren et al., 1995). The origin of parasitism within the Hymenoptera is under discussion, but it has been suggested that a primitive hornet wasp (Siricidae) or similar shifted its feeding habits from mycetophagous to carnivorous. The female wasp would normally lay its
eggs in wood with a specialized ovipositor and its larvae feed on wood decomposed by a symbiotic fungi inoculated into the wood by accessory spore-carrying glands present in the female. The shift to a carnivorous diet would imply probably the females laying their eggs close to a coleopteran larva sharing the same habitat, and the Hymenopteran larvae switching to this much richer food source (Quicke, 1997).

1.2.2 Aphidiid parasitoids

Aphidiid (Braconidae: Aphidiidae) parasitoids are hymenopterous solitary endoparasitoids of aphids. Reproduction in Aphidiidae parasitoids is sexual, but as in all hymenopterans, unfertilized eggs give rise to males and fertilized eggs to females. In addition to this, mated females can either lay unfertilized or fertilized eggs. Females are synovigenic, not all the egg load is present at emergence but some eggs are mature in the oviducts at this time and the rest develop during the females’ adult life. Normally females lay one egg per host, although if another female lays a second egg in the same host, only one larva can complete development. Oviposition is fast, varying from 2 - 9 s depending on the species. The larva undergoes four larval instars in the host and is believed to feed during the first three larval instars on specialized cells called teratocytes, that derive from the embryonic membrane and are released into the hosts’ hemocoel and aid in nutrition and host regulation between other roles (Gopalapillai et al., 2005). Only during the last larval instar does there seem to be an attack on the reproductive organs and other tissues of the host (Hågvar & Hofsvang, 1991).

Just before pupation, the aphid host is killed and only its cuticle is left. The larva then attaches the cuticle to the leaf with a secretion from the silk glands, through an aperture cut on the ventral side, and then a cocoon is spun (in most genera) inside it. The aphid cuticle with the parasitoid pupa inside is commonly known as “mummy” and they can easily be differentiated in an aphid colony because they change colour and shape, characteristics which sometimes can be used to differentiate genus and species (Powell, 1982). After 4 to 9 days, adults emerge through a hole cut in the posterior dorsal side of the mummy. Since adults do not feed on aphids, aphid honeydew is used as a source for nutrients (Hogervorst et al., 2007).

1.2.3 The parasitoid Aphidius colemani

*Aphidius colemani* Viereck (Hymenoptera: Aphidiidae) (= *A. plantensis* Brethes, = *A. transcaspicus* Telenga) (Figure 2) is a small (~ 3 mm) generalist parasitoid exhibiting a wide host range within members of the family Aphididae therefore considered generalist at the species level and specialist at the family level. It probably originates
from Northern India and Pakistan but now its distribution is widespread including North and South America, Australia and various parts of Europe, owing in several cases (British Isles, Norway, Czech Republic, USA) to artificial introductions for biological control programmes (Hofsvang & Hågvar, 1975; Starý, 1975).

Aphidius colemani's host range varies in different parts of the world. While Mediterranean populations are restricted to a couple of hosts, in the tropics populations show a wider host range including aphid species which are found in both areas but are only parasitized in the tropics (Starý, 1975). Tardieux & Rabasse (1986) suggest that this difference in preferences may be due to A. colemani being constituted by a complex of cryptic sibling species.

Aphidius colemani parasitizes larval stages of M. persicae and after stinging and ovipositing; the parasitoid undergoes four larval instars inside the host. During its own last larval stage the parasitoid kills the host, leaving only the cuticle untouched, which is then cemented on to the leaf. It is within this "mummified" enclosure that it spins a cocoon and the pre-pupal and pupal stages occur, after which the adult cuts a circular hole on the dorsal side of the mummy case and then emerges. The time that elapses from oviposition to adult emergence at 20 °C is 14 days (Zamani et al., 2007) and after
they emerge, adults feed on aphid honeydew and are ready to mate a couple of hours after emergence (Hofsvang & Hågvar, 1977).

1.2.3.1 **Aphidius colemani** as biological control agent

*Aphidius colemani* is available commercially since 1992 for biological control of *M. persicae* and the cotton aphid, *Aphis gossypii* Glover, mainly for glasshouse use. It is sold as pupae and recommended rates of release range between 0.15 and 1.5 parasitoids m$^{-2}$ (van Lenteren, 2003b). A recent study (Vasquez et al., 2006) showed that inoculative releases (release rates of 5 adults m$^{-2}$) of *A. colemani* reduced the impact of the cotton aphid on *Chrysanthemum* spp. to the same levels of a chemical pesticide. The authors conclude that although it is still 4.7 times more expensive than a standard pesticide, the use of *A. colemani* is both safer to the environment and to agricultural workers and it is very unlikely that this method will cause resistance in aphids.

1.2.4 Parasitoid host finding behaviour

1.2.4.1 Conceptual models

The interaction between parasitoids and their hosts is under strong selective pressures for the hosts to avoid parasitoids and for the parasitoids to find hosts. Hosts, normally herbivores, have found ways of hiding from parasitoids, and parasitoids use cues related with the host that confer information about its presence, quantity and quality. Traditionally parasitoid’s host-finding behaviour is divided into a series of events categorized according to their spatial scale: A parasitoid seeking for a host firstly has to find a site where it might find a potential host community consisting of probable plants with hosts, after this, at the microhabitat scale, it finds a community of hosts and then the parasitoid goes on to locate an individual host, which it examines and then accepts or not (Vinson, 1976). Vet *et al.* (1990) proposed a more dynamic conceptual model in which a newly-emerged parasitoid’s behaviour depends on a range of innate and learnt responses which can be triggered with different stimuli. The relative importance of each response is dynamic and will be modified through experience to maximize the possibility of host encounters.

Vet & Dicke (1992) proposed the model of “dietary specialization” with regards to infochemical use by natural enemies. This model predicts that the response of parasitoids and carnivores will be affected by the dietary specialization of the predator and that of its host species. Thus predators can be considered as specialists or generalists at the host level and hosts can be considered specialists or generalists at
the plant level. Following this logic, four categories can be distinguished, category A, where predators and hosts have specialist habits, for which only innate responses towards infochemicals are predicted in predator host location. Category B and C consist of predators with generalist or specialist habits respectively, and hosts with specialist or generalist feeding habits, respectively, and the model predicts that predator host-searching behaviour will rely on a combination of innate responses towards common infochemicals at the plant/host level and learnt responses towards specific plant/host infochemicals. The last category, D, comprises generalist predators and generalist hosts, and a random predator host/searching behaviour without use of infochemicals is expected. But recently, a modification to this concept was proposed by Steidle & van Loon (2003) when they reviewed the use of cues (specific, general, innate and learnt) by predators belonging to the categories mentioned above. Their findings only applied to groups A, C and D since B was not included in the review because almost no studies are devoted to this particular group of insects. Contrary to the “dietary specialization” model, the data suggest that all categories use infochemicals regardless of dietary specialization, specialists use specific cues more frequently and generalists use more frequently general cues, the innate use of infochemicals occurs in all predators regardless their specializations and lastly learning occurs frequently in generalist predators and rarely in specialists.

1.2.4.2 Types of host-finding cues

1.2.4.2.1 Sources
Parasitoids have evolved in a multitrophic context and consequently their physiology and behaviour are influenced by elements in all the levels they interact with. Therefore it makes sense to view their host-finding with emphasis on cues that are emitted by the second trophic level (parasitoid’s host/s), the first trophic level (plant/s on which the parasitoids’ host is feeding on) and the signals coming from their conspecifics (Vet & Dicke, 1992).

1.2.4.2.2 Infochemicals
From the whole range of multisensorial cues that a parasitoid can use (i.e. visual, auditory), the most exploited group is in the form of chemical signals, especially at longer distances from the host. Because of the variety of terms used throughout the years to define the chemicals mediating interactions between organisms, they became heterogeneous and confusing. Dicke & Sabelis (1988) proposed an alternative classification based on the costs and benefits of the interaction for both interactants which also included other organisms not involved in the interaction who could be the
producers/emitters of the chemical. They introduced the term “infochemical” to describe
any chemical that could give information in an interaction between two individuals,
evoking in the receiver a behavioural or physiological response that is adaptive to
either one of the interactants or to both. An infochemical can be further divided into a
pheromone, which is an infochemical acting between two individuals of the same
species, or an allelochemical, which is an infochemical that mediates an interaction
between two individuals of different species. There are three types of allelochemicals; a
kairomone, which a reaction adaptively favourable to the receiver but not to the second
organism. An allomone produces an inverse situation where the receiver is not
favoured while the second organism in the interaction is and lastly a synomone
produces an advantageous situation for both organisms. Parasitoids, in their search for
hosts, normally rely on chemical signals which may originate from the plant upon which
the host is feeding, the host itself or chemicals released as a result of interactions
between the host and its food plant or other organisms associated with the host.

1.2.4.2.2.1 Infochemicals from the plant

Plants normally produce a baseline amount of volatiles but when attacked by
herbivores the amount released increases and the composition changes (Paré &
Tumlinson, 1999). Some of these volatiles are used as a source for information by
herbivores looking for plants and are also exploited as kairomones by predators and
parasitoids searching for hosts. The response towards plant volatiles in Aphidiid
parasitoids has been studied in many occasions and *A. colemani*, *A. ervi*, *A. funebris*
and *A. rophalosiphi* have been shown to respond towards the volatiles of undamaged
plants of the same species that their host was reared on but when presented against
the same plant species with aphid attack, parasitoids responded towards this last
alternative and in some cases discrimination is such that they can detect changes in
the volatile blend according to the species of aphids attacking the plant (Powell et al.,
1998b; Storeck et al., 2000; Kalule & Wright, 2004; Lo Pinto et al., 2004). Another
example, the cereal aphid parasitoid *A. rhopalosiphi*, can discriminate between
different cultivated varieties of wheat (Wickremasinghe & van Emden, 1992).

1.2.4.2.2.2 Infochemicals from the host

Kairomones produced by the second trophic level can be as diverse as sex, alarm and
aggregation pheromones, honeydew, faeces and chemicals associated with the host
cuticles. All these infochemicals can be in the form of volatiles when parasitoids are still
away from the source or contact chemicals when they are in the vicinity. In Aphidiid
parasitoids, many studies have shown that chemical cues from the host can influence
their behaviour. In *A. ervi* grown on the aphid *Acyrthosiphon pisum*, host cornicle
secretions and a kairomone on the host cuticle elicit on female parasitoids stinging behaviour (Battaglia et al., 2000) in addition to aphid honeydew (Du et al., 1997) and aphid pheromones (Powell et al., 1998b). Furthermore, in A. rhopalosiphi reared on the aphid Sitobion avenae it is believed that contact kairomones associated with the host cuticle mediate host recognition (Muratori et al., 2006).

1.2.4.2.2.3 Infochemicals from other sources
In addition, microorganisms such as fungi associated with the host and/or the plant may provide chemical information (Martínez et al., 2006; Steiner et al., 2007). Also pheromones from conspecifics may deter parasitoids from superparasitizing hosts, or pheromones from other parasitoid species and predators may affect their host searching behaviour (Godfray, 1994).

1.2.4.2.3 Others factors affecting parasitoid behaviour
Other cues also used by parasitoids, most commonly once they are nearer the host, may include vibrations caused by the host larvae (Fischer et al., 2003) and visual stimuli (Michaud & Mackauer, 1994; Kroder et al., 2007). The visual stimuli influencing behaviour in Aphidiid parasitoids include in A. ervi grown on the aphid Acyrthosiphon pisum yellow paint encapsulated in micro-capillaries elicited oviposition attacks (Battaglia et al., 2000). Also environmental conditions such as atmospheric pressure, wind speed and temperature can also affect the behaviour of parasitoids (Marchand & McNeil, 2000; Kroder et al., 2007).

1.2.4.2.4 The reliability v. detectability problem
As faced with such a variety of stimuli, parasitoids rely on cues that provide information about the host presence, species, density and quality but they also need the cue to be easily detectable. This is often not the case, especially at longer distances since hosts normally produce minute quantities of infochemicals because of their small biomass and also because of selective pressures that suppress production of chemicals that might reveal their location. On the other hand, chemicals associated with the first trophic level, the plant, are released in large amounts but are often not as reliable in conferring information about host quantity/quality as the host-derived ones are but are on the other hand easily detectable because they are produced and released in much greater amounts. This is known as the “reliability-detectability” problem (Vet & Dicke, 1992) and the authors propose three ways in which a parasitoid may overcome this problem. Firstly parasitoids can take a “detour” by detecting more conspicuous signals from host stages different than the one under attack. A second option is to focus their search on chemicals created by the interaction from the host and its food and lastly,
through experience, parasitoids can learn to link stimuli which are easy to detect with reliable but hard to detect stimuli.

1.2.5 The olfaction mechanism

1.2.5.1 Structure

Odour detection in insects occurs in the antennae, where olfactory sensilla are located. Besides olfaction, other sensory modalities such as hygroreception, thermoreception, mechanoreceptors and taste occur in the antennae. The olfactory sensilla are comprised by a variable number of olfactory receptor neurones (ORN), accessory cells and covering cuticular structures, usually with multiple pores that encase the cells. The two main types of olfactory sensilla are “sensilla placodea”, structurally with multiple pores and known to have an olfactory function in various groups of insects (Hansson et al., 1999) including braconid wasps such as the aphid parasitoid *A. rhopalosiphi* (Bourdais et al., 2006). And “sensilla trichodea with wall pores”, that are covered with a smooth cuticle and have several pores are also believed to have an olfactory function (Bleeker et al., 2004; Bourdais et al., 2006).

1.2.5.2 Neuronal pathways

When an airborne odour molecule reaches the cuticle of a sensillium, it reaches the sensory neurone by being adsorbed onto the cuticle, diffusing through the numerous pores and binding to proteins in the lymph. These odour binding proteins (OBPs) selectively (up to some degree) bind to the odour molecules and transport them to receptor proteins in the neurone membrane where odour molecules are released and bound reversibly to one of the transmembrane odour receptor proteins (ORPs) (Mustaparta, 2002). Odour binding proteins, in addition to having a transport function, have a filtering action preventing the ORPs from being in contact with every volatile chemical. Normally one ORN codes for one or sometimes two ORP hence each ORN is highly odour-specific.

The binding of an odour molecule to an ORP triggers the depolarization of the ORN and the nervous impulse is carried through the axon to the brain (Benton, 2006). Olfactory sensory neurones axons merge to form the antennal nerve which terminates in the antennal lobe in the insects deutocerebrum. ORNs that express the same ORP converge into specific structures in the antennal lobe called glomeruli. Glomeruli are constant within species, ranging from around 32 in *Aedes aegypti* to more than 1000 in social wasps and locusts (Hansson & Anton, 2000). The glomeruli are interconnected by a vast array of interneurones and other neurones that take the information to higher
olfactory centres, mainly the mushroom bodies in the protocerebrum, where information is processed, integrated with other sensory inputs, and also where learning takes place (Menzel, 2001; Mustaparta, 2002).

### 1.2.6 Detection of relevant odours

For a herbivorous insect trying to locate the correct plant to feed on, or a parasitoid trying to find a suitable host, the diversity of chemicals encountered creates a true “cocktail” of physiologically active odours among which, many are irrelevant, and only some will indicate the presence or absence of the plant or host. In this task of having to discern the relevant chemical compounds from the unimportant ones, in contrast to responding to the presence or absence of plant-specific compounds, it is now believed that insects respond to specific ratios of ubiquitous compounds (Visser & de Jong, 1988; Bruce et al., 2005). Once the correct ratio of chemicals is detected across several sensilla, insects have to assess if they are being emitted by the same source. It has been found that ORN are frequently organized by function in a same sensillum close together and the same pattern of two specific ORN is repeated many times throughout the antenna (Blight et al., 1995). This would allow insects to detect the correct ratios of chemical compounds across several sensilla and assess the location of the emitting source through a fine spatio-temporal resolution integrated in the CNS (Bruce et al., 2005).

### 1.3 Green lacewings

#### 1.3.1 General biology

Chrysopids (Neuroptera: Chrysopidae), also known as green lacewings belong to the order Neuroptera, which includes lacewings and antlions. With ~ 6000 species in the order, neuropteran insects are holometabolous, oviparous and reproduce sexually. One of their main morphological characteristics is the intricate wing venations pattern and the way in which the wings rest roof-like over the abdomen. Their larvae are normally terrestrial with piercing /sucking mouthparts and have a predatory feeding-habit (Gullan & Cranston, 2005). The family Chrysopidae is one of the most diverse in the order with 20 species recorded in Britain (Donato et al., 2001). Females lay stalked eggs and the larva undergoes three instars before pupation, which takes place within a silken cocoon which can be characteristic of particular taxa. Some species can undergo two or more generations a year. Adults normally feed on nectar and pollen.
and are capable of flight and normally active during dawn/dusk and night (McEwen et al., 2001).

### 1.3.2 Biological control

Green lacewings are considered effective pest management agents since their larvae prey on soft bodied insects, including aphids. Because of their commercial availability and resistance to insecticides, augmentative biological control programmes have been applied in numerous occasions with variable success (Chauhan et al., 2004). The genus *Chrysoperla* is the most important in agricultural biological programmes and many of its 36 recognised species, including *C. rufilabris*, *C. externa* and *C. carnea*, are used in pest control strategies (McEwen et al., 2001).

### 1.3.3 Lacewing chemical ecology

Although a very successful group in terms of biological control results, their behaviour frequently means that they do not settle in the desired location (McEwen et al., 2001) and recent identification of lacewing arrestants and attractants may promote the efficiency of pest control strategies by manipulating natural lacewing populations (Hooper et al., 2002). Still in its early stages, the study of lacewing chemical ecology is moving forward by recent studies. For instance, it has been shown that the lacewings *C. carnea* and *Chrysopa oculata* are attracted in the field towards volatiles emitted by the prey’s host plant. Another study that indicates the potential of manipulating lacewing chemical ecology for enhanced pest control programmes is the recent discovery of a male-produced pheromone in *C. nigricornis* (Zhang et al., 2006).

### 1.3.4 The green lacewing *Peyerimhoffina gracilis*

The green lacewing *Peyerimhoffina gracilis* (*Tjederina gracilis*) Schneider (Figure 3) was first recorded in the UK in the year 1999, when a series of water traps releasing two aphid sex pheromone compounds were set in the field as part of a study to capture male aphids, and surprisingly high numbers of *P. gracilis* males were found in the traps (Donato et al., 2001).
The species originally had a Mediterranean and eastern European distribution although being found in the British Isles may be an indicator of its distribution moving northward, probably because of climatic warming. Adults normally occur between February and December but in this study males have been captured throughout the whole year (section 8.3.1, page 157). Females lay stalked-solitary eggs and there may me up to 2 generations a year (Donato et al., 2001). Morphologically, this lacewing species can be differentiated from other lacewing species in that the tip of the abdomen in females and males is acutely pointed (Aspöck et al., 1980).

1.4 Objectives

On one hand various aspects of the chemical ecology of the parasitoid A. colemani are studied. On the other hand the chemical ecology of the second aphid predator, the green lacewing P. gracilis, are also studied. The general objectives addressed in each experimental chapter are described below:

- The olfactory behaviour of the parasitoid A. colemani is studied towards various closely-related Cruciferae, with the objective to ascertain the parasitoid’s degree of discernment towards five cultivars of the same species of Brassica when reared on one of these cultivars (Chapter 3, page 17).

- The volatile-emission profiles of the five cultivars mentioned above are also studied (Chapter 4, page 70) with the objective to establish qualitative and
quantitative differences which might explain the parasitoid’s behaviour observed in the previous chapter.

- The behaviour of the parasitoid is studied towards a range of molecules with differing carbon numbers with the objective to establish differences in discernment and learning of the parasitoid according to the closenesses of the molecule in question to Green Leaf Volatiles (Chapter 5, page 103).

- The olfactory response of *A. colemani* is also studied after exposure to cold temperatures with the aim to evaluate the affect of cold on the learnt behaviour of the parasitoid in order to obtain a better understanding in differences of learnt and innate behaviours in this generalist parasitoid (Chapter 6, page 103).

- The objective was to obtain a better understanding of using odour sources with different water-vapour release rates and their importance in confounding effects during olfactometer studies (Chapter 7, page 135).

- Lastly, with the objective to gain a better understanding of the a possible link between the behaviour of male green lacewings *P. gracilis* males and a chemical compound closely-related to an aphid sex pheromone is studied through behavioural and analytical-chemistry techniques (Chapter 8, page 148).
Chapter 2
General Materials and Methods

2.1 General procedures
The way in which the parasitoids were cultured, and the different protocols used in the
olfactometer bioassays are generally the same throughout the next chapters and they
are described in this section.

2.1.1 Plants
The plants used for aphid rearing and as stimuli in behavioural experiments were
grown inside a glasshouse at Silwood Park (Ascot, UK) assuring days with at least 16 h
of light provided by two 400 W lamps (Osram Vialox NAV-T Super (Son-T Plus)). The
temperature was maintained between 20 and 35 °C throughout the year. The plants
used were different cultivars of the crucifer Brassica oleracea (Brassicaceae), which
included the cultivar Golden acre of the variety capitata (Linn) Alef (referred to as
cabbage throughout the text), and four Brussels sprout cultivars (variety gemmifera
(Linn) Zenker), which included Bedford-winter harvest (winter harvest), red delicious,
fillbasket and hamlet. For simplicities' sake, regardless of the variety the plants belong
to, they are termed throughout the text as cultivars. The seeds (Suttons, Devon, UK)
were sown in groups of approximately 40 into 9-cm diameter plastic pots with compost
(Levington multipurpose) and after 2 weeks they were transplanted individually into
similar pots. Plants were used when grown to at least 15 cm high either in the
bioassays or to rear aphids and parasitoids.

2.1.2 Aphid culture
Myzus persicae was reared on the Brussels sprout cultivar winter harvest. The culture
was kept inside a controlled temperature room (19 ± 1 °C, 60 ± 5 % relative humidity
and 16:8 L:D photoperiod). Between nine and twelve plants were placed inside
wooden-framed cages (45 x 40 x 45 cm). The sides were covered with a fine mesh and
in one of them the mesh formed a tube through which the cage could be accessed. The
roof of the cage was made of transparent Perspex and the floor was lined with capillary
matting material. The cage was then placed in a plastic tray in which water could be poured in regularly to reach the plants through the matting.

Every three weeks plants infested with aphids were removed from the cages and replaced with clean plants to maintain the culture. To transfer the aphids, some of the infested leaves were detached and placed on the new plants.

2.1.3 Parasitoid culture

Populations of the parasitoid *Aphidius colemani* were maintained on *M. persicae* feeding on winter harvest. The parasitoid culture was held in a controlled temperature room (19 ± 1 °C, 60 ± 5 % relative humidity and 16:8 L:D photoperiod). The wasps were initially obtained as mummies from Syngenta-Bioline (Little Clacton, UK) in 2002 and were kept in the same kind of cages used for the aphid culture. Parasitoids had a constant supply of aphids which ensured enough numbers for the bioassays. Each cage contained between nine and twelve aphid-infested winter harvest plants transferred from the aphid culture and were replaced by new plants with aphids as they became old, under-infested or covered with fungi. Old plants were cut half-way along the main stem and kept inside the cage to allow the emergence of all the parasitoids.

Since October 2005 the culture was maintained in a different way. Instead of replacing the plants by new ones as they became old or under-infested; they were replaced every five weeks. At the end of the five-week period, all the old plants replaced with new plants with fresh aphids and 10 winter harvest leaves with un-emerged mummies were placed over the plants. In this way, the fact that the number of eggs laid by parasitoids peaks in the first two-four days (Hågvar & Hofsvang, 1991), was capitalized upon supplying newly-emerged females. Approximately in the second week the new generation of parasitoids started to emerge and this lasted two more weeks. Throughout the project this method proved to be more reliable in terms of quantity and timing than the previous protocol used.

2.1.4 Olfactometers

2.1.4.1 Detached-leaves olfactometer -version I-

Some of the bioassays were done in four-arm Perspex olfactometers originally designed by Douloumpaka and van Emden (2003) and modified by Vamvatsikos (Vamvatsikos, 2006) (Figure 4 A, B, C and D) to test the response of female aphid parasitoids *A. colemani* towards volatiles from detached leaves. The olfactometer consisted of a circular central arena (i.d. = 5 cm, height = 2.8 cm) with four protruding Perspex tubes (i.d. = 0.7 cm, length = 3.6 cm) on the sides orthogonal to each other.
These tubes protruded into four odour-chambers (arms) which consisted of Perspex tubes (i.d.: 2.4 cm, length = 9.5 cm) where detached-leaves of plants could be placed inside and wasps that moved from the central arena into the arms, were trapped. In the far end of each arm an air filter with activated charcoal (6 g) (Figure 4 C) was attached through which the air entered the system, passed along the arms and continued to flow into the central chamber.

![Diagram](image)

Figure 4: The four chamber olfactometer used to test the olfactory response of *Aphidius colemani* females towards detached leaves of plants. (A) Side view and top view of the olfactometer. (B) Detail of the central chamber, the joints and one of the odour arms. (C) Detail of the Perspex charcoal filter. (D) Detail of the central chamber and one of the odour arms.

A lid covered the central arena and air was exhausted through a Perspex tube (internal diameter: 0.7 cm, length: 4.3 cm) communicated to the central arena through a hole covered with fine mesh on the lid. An air flow meter (GPE Meterate) was attached to the exhaust hole and ensured a constant air flow (1.7 l min$^{-1}$) in the system. The
olfactometer could be disassembled and the parts were washed after every second run with odourless detergent (Lipsol detergent, Bibby Sterilin Ltd. UK) then rinsed with 50% v/v ethanol and lastly rinsed with distilled water. All the components were then dried in an oven at 60 °C. All the parts (air filters, odour arms, central arena and lid) could be detached from each other for easier cleaning.

2.1.4.2 Whole-plant olfactometer

The above olfactometer was modified to use whole plants instead of detached leaves as stimuli. The air flowing into each olfactometer arm originated, instead of detached leaves placed inside the arms, from independent odour sources: each one consisting of a plant enclosed in a multi-purpose PET poly(ethyleneterephthalate) (3.2 l, maximum temperature 200 °C, Sainsbury’s Supermarkets Ltd., London, UK) cooking bag (Figure 5).

Figure 5: Whole-plant olfactometer. (A) A cooking bag was fastened over a plant and a constant flow of humidified clean air was pumped into the bag while air was drawn out of the bag into one of the olfactometers’ arm. (B) The four odour sources provided each of the olfactometers arms a constant air flow, which could be regulated with the flowmeters shown in the background. (C) Olfactometer connected to the flowmeter and the odour sources via Teflon tubing.
A six-week old plant and plastic pot with compost was placed inside a second bigger plastic pot (opening diameter = 13 cm, height = 13 cm) and lifted 8 cm by means of two modified smaller plastic pots (opening diameter = 8 cm, height = 7.4 cm). Aluminium foil was used to cover the compost and the gap left between the bigger and smaller pots. The bigger pot was used as a surface to fasten the plastic bag securely over the plant without damaging it with two rubber bands.

The air pull (id = 1.5 mm, od = 3.2 mm) and air push (id = 4.8 mm, od = 6.3 mm) conducts consisted of Teflon tubing (Adtech Polymer Engineering Ltd, UK) that protruded through the bottom of the bigger pot and extended 33 and 20 cm respectively into the space occupied by the plant when the bag was put in place. Brass fittings (Swagelok, UK) were used to easily detach the pots from the inlet and outlet tubing to allow a better handling while placing the bag over the plant. Each of the four bags with the plants inside were filled with purified (through 55 g of charcoal filter) and re-humidified (through a water bubbler filled with 0.4 l distilled water) air at a rate of 4 l min⁻¹ and carried through Teflon tubing (id = 4.8 mm, od = 6.3 mm) from the pump to the bags so each bag received an "air push" of approximately 1000 ml min⁻¹. A flow meter between the charcoal filter and the humidifier ensured a constant air flow during the experiment. The clean and purified air was drawn out of the bags and into each of the olfactometer arms at a rate of 425 ml min⁻¹ via Teflon tubing (id = 4.8 mm, od = 6.3 mm). Excess air could vent through the non-hermetic seal between the bag and the pot. The Teflon tubing was attached to the olfactometer through modified hermetic plastic lids with brass fittings fitted at the end of each lid that connected with the tubing. A platform was designed to hold the hermetic lids in place with tube clamps since the Teflon tubing had to be bent to be in place which caused the lids not to seal effectively.

The bags that would be used during the day where cleaned from impurities (Stewart-Jones & Poppy, 2006) by baking them during 2 h at 120 °C the previous evening or that same morning and left in the oven until used. The bags where used once and then discarded. Every four runs, all the Teflon tubing was cleaned for at least 2 h with humidified clean air.

2.1.4.3 Detached-leaves olfactometer -version II-

Once the whole-plant olfactometer was built and effective, it was modified further to hold instead of plastic pots with whole plants, Perspex tubes (i.d. = 0.7 cm, length = 3.6 cm) with detached leaves inside. Air was pushed at a rate of 4 l min⁻¹ through an air filter with 55 g of activated charcoal, then through a glass water-bubbler with 0.4 l of distilled water and then pushed into the Perspex tube where the desired sample was
placed. Air was pulled out of this arm at a rate of 425 ml min\(^{-1}\), with the excess air exiting the system through four 1 mm holes drilled into one of the lids covering the openings. The tubing used were Teflon conduct (id = 4.8mm, od = 6.3mm in most of the trajectory and id = 1.5 mm, od = 3.2 mm at some portions) (Adtech Polymer Engineering Ltd, UK). This was an improvement on the first olfactometer (detached-leaf olfactometer -version 1-) since it was possible to administer humidified air in addition to having the olfactory stimuli in independent chambers, which permitted wasps to be counted directly through the Perspex tube, previously wasps had to be found and counted from in between the leaves that served as stimuli.

### 2.1.4.4 Hybrid olfactometer

In some bioassays a combination of the whole plant olfactometer and the detached leaves olfactometer was used to assess the behavioural response of female parasitoids. For this, two of the sources of the whole plant olfactometer were replaced by two sources of the detached leaves olfactometer -version II-. In this “hybrid” setup, whole plants and detached leaves could be presented at the same time to parasitoids.

### 2.1.5 Rotation scheme

To account for irregularities in the olfactometer arms or any visual asymmetry in the wooden box that could bias the wasps, the system was rotated according to a pre-defined rotating scheme. The rotation scheme took into account the position of the odours in the olfactometer-arms and also the position of the olfactometer inside the black-wooden box where the bioassays took place. There were six different placement combinations for the two odours within the four arms. Also, once the odours were placed inside the olfactometer, there were four possible positions in which the olfactometer could be located inside the black box. From these two groups of positions, 24 different combinations could arise. For each of the four positions in the wooden box, two odour placement combinations were randomly selected and this ensured pseudo-randomisation for the rotation scheme. In this way, eight positions were chosen pseudo-randomly to constitute the rotating scheme that was used for all 8 replicates in each of the experimental series. With the second version of the olfactometer, the rotation scheme followed the same logic, connecting the odour sources to the assigned olfactometer arm.

### 2.1.6 Wasps used in the bioassays

Parasitoids used in the bioassays were removed from the cages using a miniature aspirator attached to a modified universal tube (8 x 25 mm). The lid of the container
was modified to screw onto the “air-pull” end of a mini-aspirator (Vamvatsikos, 2006) and had a fine mesh to allow air to flow but prevented the wasps being sucked further in. The bottom of the container had a hole (diameter 9 mm) with a small Perspex tube (90 mm length x 8 mm internal diameter) attached which allowed easier access to the insects in the cage. The far end of the Perspex tube had a small brush (i.e. a few human hairs attached with tape) which helped to sweep the parasitoids that walked on the sides of the cage into the air flow and into the container with no apparent damage.

Once all the adults had been aspirated from the cage into the container in groups of ~70, they were transferred into glass vials (50 x 25 mm) in smaller groups of between 2 and 5 individuals. This was done by allowing males and females to walk along the walls of the container standing upright inside a cardboard cylinder (200 x 40 mm) with only the tip of the small Perspex tube protruding into the light. In this way the parasitoids walked from the darkness towards the illuminated tip of the small Perspex tube and were captured in the glass vials. Once they were in smaller groups, they were transferred individually into new glass vials and this time males and females were identified by the shape of their abdomens (Figure 2). Females were then introduced into the central chamber of the olfactometer in groups of 20 by aspirating them individually, from the glass vials directly into the central chamber. This was done by connecting the aspirator to the end of the central arena exhaust tube and covering all the other holes except one, through which the wasps could be sucked by means of a flexible rubber tube extension. Females were assumed to be mated and with oviposition experience but this was not considered to have a major impact on the behaviour since evidence indicates that virgin females respond equally in olfactometer tests involving plant-odours as mated females (Vamvatsikos, 2006).

2.1.7 Bioassays

All the bioassays were carried out at room temperature (21-27 °C) and at room relative humidity (45 -52 %) without natural lighting. To reduce any external visual stimuli during the bioassay, the olfactometer was placed inside a black wooden box (660 x 560 x 660 mm) illuminated by two high frequency (30 KHz) fluorescent tubes (Philips Tl-D 90 de Lux 18W/965 SLV). A curtain covered the front of the box to prevent visual disruptions during the bioassay. Each replicate lasted for 0.5 h from the moment the olfactometer was placed inside the wooden box and connected to the air pump. Parasitoids were tested in groups of 20. After each run, the numbers of parasitoids in each arm and the numbers that remained in the central arena -the non responders- were counted.


2.1.8 Statistical analyses

2.1.8.1 Bias in choice

The response towards the choices offered in the olfactometer was analyzed using a generalized linear model (GLM) with Poisson errors and a logarithm link function with correction for overdispersed data. The model, in addition to test for effects of the choices offered, also tested for differences between the 8 replicates (heterogeneity between the runs) and for differences between the four arms of the olfactometer. The significance for the first term (i.e. olfactometer stimulus) was assessed by comparing the $\chi^2$ value against tabulated $\chi^2$ values with 1 df and the significance obtained for the second and third terms (run and olfactometer arm) the effect of the replicates against tabulated values with 7 df and the effect for olfactometer arm against tabulated values with 3 df. If more terms were added to the model they are specified in the relevant chapter of the thesis. When terms were found to be non-significant, other than olfactometer stimulus, they were removed from the model and are not mentioned in the results section (JMP programme, version 7.0).

2.1.8.2 Comparison between sets of bioassays

Further analysis on the results of olfactometer tests were carried out by comparing the results across different treatments. The data were analyzed with GLM with a binomial error structure and logit link function was used, with correction for overdispersed data (Crawley, 2002). One comparison took into account the proportion of wasps that did not respond to any of the stimuli and remained in the central chamber of the olfactometer against the combined proportion of the wasps that responded to both stimuli. A second comparison considered only the proportion of wasps that responded to winter harvest against the wasps that responded to the alternative choice. In this way data could be analysed across different treatments taking into account the overall level of response regardless of the choice of the parasitoids or considering the wasps that did show a response. The significance was assessed against tabulated F values and posterior Student t tests were carried out to locate significant differences if any (R programme, version 2.2.1). In this case degrees of freedom are represented as the residual degrees of freedom (residual degrees of freedom = total degrees of freedom – used degrees of freedom).
Chapter 3
Parasitoid Responses to the odours of Crucifer Cultivars

3.1 Introduction

3.1.1 Olfaction in insects
In most insect species detecting airborne chemical compounds plays a major role in survival and reproduction. From locating sexual partners and conspecifics via pheromones to food via kairomones and synomones, all involve the detection of specific chemicals that rule these ecological interactions.

3.1.2 Learning in insects
Learning is the process by which knowledge is acquired about the surrounding world and confers the ability to adapt in a constantly changing environment and increases the fitness of the learner (Giurfa, 2007). Insects, can modify their behaviour based on past experiences and learning is evidenced in many species (Dukas, 2007). For example the honeybee, *Apis mellifera*, has been widely studied in terms of it's cognitive capabilities, and has the ability to learn, among others visual stimuli, odours, textures and spatial locations (Giurfa, 2007). Other insects capable of learning include the fruit fly *Drosophila* spp. (McGuire et al., 2005), the cockroach *Periplaneta americana* (Sakura et al., 2002) and also parasitic wasps, which are known to associate the odour of plants with the presence of hosts (Turlings & Wäckers, 2004).

3.1.3 Associative learning
Associative learning is a mechanism widely found in animals that permits establishing predictive relationships between two or more events. The mechanism permits uncertainty to be minimized resulting in an adaptive behaviour. It can be divided into two major groups, classical conditioning (Pavlov, 1927), on one hand is the process through which animals learn to associate an originally neutral stimulus (conditioned
stimulus, CS) like the sound of a bell, with a biologically relevant stimulus (unconditioned stimulus, US) such as food. The US always results in an unconditioned response (UR), like salivation, which can be elicited with the US if the CS and US are presented together for a number of times. On the other hand, the other way of associative learning is operant conditioning (Skinner, 1938) by which animals learn to associate their own behaviour with a subsequent environmental event.

3.1.4 Learning in parasitoids
Many studies have revealed that parasitoids can learn to link the presence of their host with chemical or physical cues from other sources. As proposed by (Vet & Dicke, 1992), due to the reliability-detectability problem (more in section 1.2.4.2.4, page 28), the ability to associate the host with cues that are easy to detect, in a constantly changing environment, can offer parasitoids a great advantage. Studies on learning received increasing attention over the past two decades with more than 20 parasitoid species shown to use learning as a resource to host and food-finding (Langley et al., 2006). For example, Lewis & Takasu (1990) investigated the host-site and food finding behaviour in the parasitoid *Microplitis croceipes* and concluded that these parasitoids can learn two novel odours associated with separate hosts and food resources and then use this information to make an accurate choice between these odours on the basis of their relative host and food needs.

3.1.4.1 Learning in Aphidiid parasitoids
In Aphidiid parasitoids, Du *et al.* (1997) showed that *A. ervi* learns associatively olfactory cues from plants, host-damaged plants, and the combination of plants and aphids while foraging. Also in *A. colemani*, brief foraging experiences on previously unencountered plant-aphid complexes increased the attraction towards such combinations in wind-tunnel bioassays (Grasswitz, 1998). Storeck, *et al.* (2000) studying *A. colemani*, concluded that cues encountered during foraging and oviposition could modify preferences to plant-aphid combinations in female parasitoids.

In addition to this, the environment in which a parasitoid is reared on can have strong influences in its adult behaviour. Vamvatsikos (2006) showed that *A. colemani* females after emerging from the peach potato aphid reared on the Brussels sprouts cultivars winter harvest or red delicious, based their preference when offered other Brussels sprouts cultivars as alternatives in olfactometer experiments, on the plant cultivar which their aphid hosts were reared on. In a similar way, *A. colemani* showed a positive response towards the cabbage cultivar on which it was reared (Kalule & Wright, 2004) and Storeck (2000) proved that *A. colemani*’s rearing history can have effects on plant
preference, when reared either on rape or Chinese cabbage, the parasitoid preferred the plant species on which it was reared over the second one. Furthermore, Wickremasinghe & van Emden (1992), showed an olfactory preference in *Aphidius rhopalosiphi* for leaves of the wheat cultivar on which it was reared.

This strong associative learning behaviour that Aphidiid parasitoids show towards the volatiles of the plant on which it’s host was reared is striking when considered that according to their experience they are capable of distinguishing between different plant species and different cultivars. The moment in which the parasitoid becomes conditioned, was initially attributed to the emerging adult while cutting the exit hole and becoming in contact with plant derived chemical cues retained on the mummy case. If adults were excised manually from their mummy cases prior to emergence, they did not show the preference for the plant on which they otherwise, when emerged naturally, would show a bias towards (van Emden et al., 1996; Storeck et al., 2000). But recent studies suggest the contrary, *A. colemani* can still detect differences in plant volatiles according to rearing history, even when excised from their mummies (Vamvatsikos, 2006). It has been proposed that the learning mechanism does not occur when the adult cuts its way through the mummy but during immature stages. Gutiérrez-Ibáñez *et al* (2007) suggested that a first stage of conditioning towards plant volatiles occurs during the third larval instar in *A. ervi*. It is believed that the larva comes into contact with the leaf and learns relevant chemicals cues that will later aid in the process of host location. This takes place the moment the larva makes a hole in the ventral side of the mummy to attach it to the leaf. Although novel chemicals encountered during adult foraging can alter these initial preferences (Storeck et al., 2000) the prevailing chemicals that surround the pre-pupal parasitoids, are the main cues that will govern the parasitoid’s behaviour in the initial stages of adult life.

### 3.1.5 Objective and hypothesis

In this chapter the extent to which the learnt cues govern the parasitoid’s behaviour is studied. Taking as a starting point Vamvatsikos’ (2006) experiments through which it was established the fine distinction by the parasitoid *A. colemani* between the plant on which it was reared when presented against other cultivars of the same species, the olfactory behaviour of *A. colemani* females is studied further. The olfactory response of parasitoids with experience on one *B. oleracea* cultivar is tested towards the known cultivar and four other unknown cultivars of the same species. The study focuses on the extent to which experience plays a role when the choices available include, and do not include, the cultivar experienced by the parasitoid. Plants are presented to the parasitoids whole or as detached leaves. It is predicted that because plants may differ
in the ratio of the different compounds emitted, in contrast to an “all or nothing” response, female parasitoids will be able to adapt their response to the cultivar that better matches its previous experience regardless of whether it is presented as whole plant or as detached leaves. The null hypothesis is that parasitoids will not have a differential response towards the five cultivars and will not be able to discern between whole plants vs. detached leaves.
3.2 Materials and methods

3.2.1 Overview
Females were reared on the aphid *M. persicae* feeding on Brussels sprout cultivar winter harvest, removed as adults from the cages and immediately tested in olfactometer bioassays for preference towards five cultivars of *B. oleracea* presented as whole plants and as detached leaves. In all cases eight replicates were carried out for each comparison with 20 female parasitoids in each one.

3.2.2 Olfactometer controls
Since the whole-plant olfactometer (section 2.1.4.2, page 37) was designed for this part of the project, control experiments were carried out to test for biases due to possible defects in the construction of the olfactometer such as air leaks.

3.2.2.1 Empty odour sources
A test with the four empty odour sources in the whole-plant olfactometer was carried out to ensure that the wasps’ response was not biased to any of the sources in particular. Two filter papers (diameter = 70 mm, Whatman, UK) dampened with 0.8 ml of distilled water were placed inside each of the four bags.

3.2.2.2 Whole-plant versus clean air
A second control series contrasting the response towards whole winter harvest plants against humidified clean air (two dampened filter papers, as described above) was also carried out. This control was performed to test if parasitoids would distinguish between these two sources, ensuring the proper functioning of the whole-plant olfactometer.

3.2.3 Response to whole plants
The response of female parasitoids was assessed towards five *B. oleracea* cultivars (winter harvest, hamlet, fillbasket, red delicious and cabbage) in the whole-plant olfactometer. The olfactometer was used in a two-way fashion and every possible comparison involving two different cultivars out of the five cultivars were made. Since the effect of cultivar choice could be biased due to differences in the odour source humidities or in the leaf size of the plant material used (possibly having an impact on volatile concentration), after each run the air humidity coming from each of the odour sources was measured (details in section 7.2.1, page 137) and the leaves were detached, digitally scanned and the resulting image used to calculate the total leaf-surface of the samples (Scion Image for Windows, version Alpha 4.0.3.2).
3.2.4 Response to detached leaves from whole plants

The response of female parasitoids was assessed towards the five *B. oleracea* cultivars mentioned above in the detached-leaves olfactometer -version II-. The olfactometer was used as a two-way and every possible comparison was made involving two cultivars. After each run the air humidity coming from each of the odour sources was measured (details in section 7.2.1, page 137) and the leaves digitally scanned and the resulting image was used to calculate the total leaf-surface of the samples (Scion Image for Windows, version Alpha 4.0.3.2).

3.2.5 Response to whole plants versus detached leaves of the same cultivar

The above bioassays showed that parasitoids were biased towards the winter harvest cultivar, while cabbage was least preferred in the range of responses. It was decided to test the response of the female parasitoid when each of these two cultivars was presented as whole plants and as detached leaves (i.e. the detached leaves of an entire plant) in the same bioassay (section 2.1.4.4, page 39).

3.2.6 Response to whole plants and detached leaves of different cultivars

Following the logic explained above, the response of female parasitoids was assessed towards the cultivars winter harvest and cabbage presented as whole plants or as detached leaves. The contrasts performed were firstly two sources containing the cultivar winter harvest presented as whole plants versus the other two sources containing the cultivar cabbage presented as the detached leaves of an entire plant. Secondly the cultivar winter harvest was presented as the detached leaves of an entire plant in two of the sources versus cabbage presented as whole plants in the other two sources. The “hybrid” olfactometer was set-up as mentioned in section 2.1.4.4 (page 39).

3.2.7 Statistical analysis

3.2.7.1 Bias in choice

Biases in olfactometer responses were analyzed with GLM as explained in section 2.1.8.1 (page 41). The following terms were included in the model: Alternatives offered, olfactometer arm (1 - 4), odour source (1 - 4), position of olfactometer in the in wooden box (1 - 4), leaf surface and air humidity when relevant. Non-significant terms were removed from the model and are not mentioned in the results section.
3.2.7.2 Comparison between sets of bioassays

Comparisons between the overall response and the response towards the plant material were done between sets of bioassays (details in section 2.1.8.2, page 41).

3.2.7.3 Responsiveness

A measure of the response (responsiveness) of parasitoids towards individual cultivars was calculated. Responses towards cultivars presented as whole plants or as detached leaves were calculated as the proportion of responding wasps that were biased towards each of the five cultivars regardless the alternative presented. In this way, the responsiveness could be compared in between cultivars with a GLM with Poisson errors and a logarithmic link function with correction for overdispersed data. The $\chi^2$ value was compared to tabulated $\chi^2$ values with 4 df to detect overall differences and posterior ad-hoc tests between cultivars were done to detect the origin of the differences. Non-significant terms were removed from the model and generally are not mentioned in the results section.
3.3 Results

3.3.1 Control bioassays

3.3.1.1 Empty odour sources

There were no differences ($\chi^2 = 1.27$, $P > 0.05$, df = 3) in the response of the parasitoids towards the arms of the whole-plant olfactometer with empty odour sources (Figure 6).

Figure 6: Response (± 1 S. E.) of female parasitoids in the olfactometer with empty odour sources. No significant differences were found in the levels of response towards the four arms of the olfactometer. Letters above the bars compare the responses across the four arms.
3.3.1.2 Response to whole plant versus clean air

The control bioassay that contrasted a whole winter harvest plant against humidified clean air resulted in the wasps responding towards the arms that contained the plant material ($\chi^2 = 21.06$, $P < 0.001$, df = 1) (Figure 7).

![Bar chart](chart.png)

Figure 7: Response ($\pm$ 1 S. E.) of female parasitoids in the olfactometer towards whole winter harvest plants against humidified clean air. A significant difference was detected towards the arms with the odour sources containing the winter harvest plants. *** ($P < 0.001$) compares the choice of responding females.
3.3.2 Response to whole plants

In the following sections data is presented and analyzed by grouping the bioassays according to one of the cultivars contrasted to the other four. Because of this, bioassays are repeated in the figures and in the statistical analyses.

3.3.2.1 Whole winter harvest plants against the other cultivars

Contrasting whole winter harvest against the other cultivars resulted in wasps showing bias towards winter harvest when offered fillbasket, red delicious and cabbage as alternatives ($\chi^2$ fillbasket = 34.05, $\chi^2$ red delicious = 13.92, $\chi^2$ cabbage = 17.11, $P < 0.001$, df = 1) but no bias was registered when hamlet was offered ($\chi^2 = 0.03$, $P > 0.05$, df = 1) (Figure 8). A lower overall response was found in winter harvest versus red delicious than all the rest ($F = 8.15$, $P < 0.01$, df = 28). There was a significant difference between the response involving hamlet when compared to the other three ($F = 25.29$, $P < 0.001$, df = 28).

![Figure 8: Response (± 1 S. E.) of Aphidius colemani towards whole plants comparing winter harvest against four other whole Brassica cultivars. Only when hamlet was presented as the alternative, there was no distinction between the cultivars. *** (P < 0.001) and ns (not significant; P > 0.05) compare the response of the females towards the two alternatives within each treatment. Uppercase letters compare non-responding females across treatments. Lowercase letters compare responding wasps across the different treatments.](image)
3.3.2.2 Whole hamlet plants against the other cultivars

When whole hamlet plants were presented with the other four cultivars as alternatives, there was a bias towards hamlet when contrasted with fillbasket and red delicious ($\chi^2_{\text{fillbasket}} = 8.57$, $\chi^2_{\text{red delicious}} = 10.34$, $P < 0.01$, df = 1) while the contrast against winter harvest and cabbage were non significant ($\chi^2_{\text{winter harvest}} = 0.03$, $\chi^2_{\text{cabbage}} = 1.47$, $P > 0.05$, df = 1) (Figure 9). The proportion of overall response was not different in between the four different treatments ($F = 2.12$, $P > 0.05$, df = 28). Furthermore, there was a significant difference in the response to plant material between the cultivar winter harvest against the cultivar red delicious ($t = 4.63$, $P < 0.01$, df = 14).

![Figure 9: Response (± 1 S. E.) of *Aphidius colemani* females towards whole plants comparing the cultivar hamlet against the other four whole cultivars in the olfactometer. Female parasitoids were biased towards hamlet only when fillbasket and red delicious were presented as alternatives, when winter harvest and cabbage were the alternative choices, no distinction was evidenced. ** (P < 0.01) and ns (not significant; P > 0.05) compare the response of the females within each treatment. Uppercase letters compare the proportions of non-responding females across treatments. Lowercase letters compare the proportions of responding wasps across the four different treatments.](image-url)
### 3.3.2.3 Whole fillbasket plants against the other cultivars

Wasps presented with whole fillbasket plants were biased towards that cultivar when red delicious and cabbage were presented as choices ($\chi^2$ red delicious = 14.11, $\chi^2$ cabbage = 13.69, $P < 0.001$, df = 1) but were biased towards the alternative cultivars when presented against winter harvest ($\chi^2$ winter harvest = 34.05, $P < 0.001$, df = 1) and hamlet ($\chi^2$ fillbasket versus hamlet = 8.57, $P < 0.01$, df = 1) (Figure 10). When the overall levels of response where compared in between cultivars the treatment fillbasket versus winter harvest was significantly lower than fillbasket versus cabbage ($t = 2.23$, $P < 0.05$, df = 14). When the proportion of wasps that did respond to fillbasket were compared across the different treatments, fillbasket versus winter harvest and fillbasket versus hamlet did not differ ($t = 1.70$, $P > 0.05$, df = 14) whereas fillbasket versus red delicious and fillbasket versus cabbage were similar ($t = 0.28$, $P > 0.05$, df = 14).

![Figure 10: Response (± 1 S. E.) of Aphidius colemani females in the olfactometer towards whole plants comparing fillbasket against the other four cultivars. Female wasps were significantly biased towards winter harvest and hamlet but were biased towards fillbasket when red delicious and cabbage were the alternatives. *** ($P < 0.001$) and ** ($P < 0.01$) compare the response of the females within each treatment. Uppercase letters compare the proportions of non-responding females across treatments. Lowercase letters compare the proportions of responding wasps across the four different treatments.](image-url)
3.3.2.4 Whole red delicious plants against the other cultivars

When presented with whole red delicious plants against the other four cultivars, female wasps were biased to the alternative cultivar in the case of red delicious versus winter harvest, red delicious versus hamlet and red delicious versus fillbasket ($\chi^2$ winter harvest = 13.92, $P < 0.001$; $\chi^2$ hamlet = 10.35, $P < 0.01$; $\chi^2$ fillbasket = 14.11, $P < 0.001$; df = 1) but when presented with cabbage as the alternative, wasps were biased towards red delicious ($\chi^2$ = 6.938, $P < 0.01$, df = 1) (Figure 11). There were no significant difference between the levels of overall response between the four different treatments ($F = 0.38$, $P > 0.05$, df = 28). The proportion of the wasps that responded to red delicious did not differ between the red delicious versus winter harvest, red delicious versus hamlet and red delicious versus fillbasket treatments ($F = 0.03$, $P > 0.05$, df = 21) but red delicious versus cabbage was significantly different than the rest ($F = 9.32$, $P < 0.01$, df = 21).

![Figure 11: Response (± 1 S. E.) of *Aphidius colemani* females in the olfactometer towards whole plants comparing red delicious against the other four cultivars. Parasitoids were biased towards the alternative, except when cabbage was presented, in which case females were biased towards the arms red delicious. *** ($P < 0.001$) and ** ($P < 0.01$) compare the response of the females within each treatment. Uppercase letters compare the proportions of non-responding females across treatments. Lowercase letters compare the proportions of responding wasps across the four different treatments.](image-url)
**3.3.2.5 Whole cabbage plants against the other cultivars**

When the response towards whole cabbage was contrasted against the remaining cultivars, a significant bias was detected towards the alternatives in the cases of winter harvest, fillbasket and red delicious (\(\chi^2\) winter harvest = 17.11, \(P < 0.001\); \(\chi^2\) fillbasket = 13.69, \(P < 0.001\); \(\chi^2\) red delicious = 6.94, \(P < 0.01\), df = 1). When the cultivar hamlet was presented as the alternative, no significant differences were found (\(\chi^2\) hamlet = 0.03, \(P > 0.05\); df = 1) (Figure 12). The overall proportion of response was significantly higher in the response to cabbage versus winter harvest when compared against cabbage versus hamlet and cabbage versus fillbasket (\(F = 8.26, P < 0.01\), df = 28).

When the response towards cabbage was compared between the four categories, the contrast with hamlet received the higher response towards cabbage (\(F = 4.23, P < 0.05\), df = 28).

![Figure 12: Response (± 1 S. E.) of *Aphidius colemani* females in the olfactometer towards whole plants comparing cabbage against the other four cultivars. Parasitoids were biased towards the alternative choice except when hamlet was used, situation that did not elicit a differential response. ***(P < 0.001)**, **(P < 0.01)** and ns (not significant; \(P > 0.05\)) compare the response of the females within each treatment. Uppercase letters compare the proportions of non responding females across treatments. Lowercase letters compare the proportions of responding wasps across the four different treatments.](image-url)
3.3.2.6 Responsiveness to whole plants

The responsiveness towards the odours of each cultivar presented as whole plants was obtained by averaging the response towards one particular cultivar considering the results of the bioassays with the remaining cultivars (Figure 13). Statistical differences were found between the general response to the cultivars ($\chi^2 = 49.90, P < 0.001, df = 4$), the cultivar winter harvest with the greatest response, differed from the response to fillbasket, which had an intermediate response ($\chi^2 = 11.14, P < 0.001, df = 2$). Differences were also found between the responses to hamlet and cabbage ($\chi^2 = 21.51, P < 0.001, df = 2$).

![Figure 13: Average response (± 1 S. E.) of *Aphidius colemani* females towards five cultivars of Brassica presented as whole plants with the other four presented as alternatives. Winter harvest and hamlet received the highest levels of responses followed by fillbasket and red delicious and lastly cabbage. Different letters above bars indicate significant differences between the responses towards the cultivars.](image-url)
3.3.3 Response to detached leaves

3.3.3.1 Detached winter harvest leaves against the other cultivars

When the cultivar winter harvest was contrasted against the other four cultivars and the responses were compared towards the two stimuli, no significant differences were detected when winter harvest was contrasted against hamlet ($\chi^2 = 1.20, \ P > 0.05, \ df = 1$) nor against fillbasket ($\chi^2 = 0.50, \ P > 0.05, \ df = 1$) (Figure 14), but significant differences were found with red delicious ($\chi^2 = 11.57, \ P < 0.001, \ df = 1$) and cabbage ($\chi^2 = 18.63, \ P < 0.001, \ df = 1$). No differences were detected between the four treatments in the overall response towards the cultivars ($F = 0.26, \ P > 0.05, \ df = 28$). The response towards plant material when cabbage was used was significantly different from fillbasket ($t = 3.95, \ P < 0.001, \ df = 14$) and hamlet ($t = 2.71, \ P < 0.05, \ df = 14$).

Figure 14: Response (± 1 S. E.) of *Aphidius colemani* females in the olfactometer towards detached leaves of the cultivar winter harvest against the other four cultivars. Female parasitoids were only biased towards winter harvest when red delicious and cabbage were used as alternatives. *** (P < 0.001) and ns (not significant; P > 0.05) compare the response of the females within each treatment. Uppercase letters compare the proportions of non-responding females across treatments. Lowercase letters compare the proportions responding wasps across the four different treatments.
3.3.3.2 Detached hamlet leaves against the other cultivars

When the responses of female wasps with hamlet as one of the choices and the other four cultivars as alternatives were compared between treatments, no differential response was detected in the contrasts involving winter harvest \((\chi^2 = 1.20, P > 0.05, df = 1)\) red delicious \((\chi^2 = 0.11, P > 0.05, df = 1)\) and cabbage \((\chi^2 = 2.45, P > 0.05, df = 1)\) (Figure 15). Significant differences were detected when fillbasket was used as the alternative \((\chi^2 = 18.57, P < 0.001, df = 1)\). The overall response did not differ significantly between the four different cultivars \((F = 1.48, P > 0.05, df = 28)\). When the response towards the plant material was considered, fillbasket differed from the response involving the cultivars red delicious \((t = 3.02, P < 0.01, df = 15)\) and cabbage \((t = 3.64, P < 0.01, df = 14)\).

![Figure 15: Response (± 1 S. E.) of Aphidius colemani females in the olfactometer towards detached leaves of the cultivar hamlet against the other four cultivars. There was no bias in parasitoid choice for any of the choices except for the bias shown towards fillbasket. *** (P < 0.001) and ns (not significant; P > 0.05) compare the response of the females within each treatment. Uppercase letters compare the proportions of non-responding females across treatments. Lowercase letters compare the proportions of responding wasps across the four different treatments.](image-url)
### 3.3.3.3 Detached fillbasket leaves against the other cultivars

When the response to fillbasket was compared against the other four cultivars, no differences were found in the contrast between fillbasket and winter harvest ($\chi^2 = 0.50$, $P > 0.05$, df = 1) (Figure 16). Significant differences were detected in the contrasts involving hamlet ($\chi^2 = 18.57$, $P < 0.001$, df = 1), red delicious ($\chi^2 = 16.29$, $P < 0.001$, df = 1) and cabbage ($\chi^2 = 7.83$, $P < 0.01$, df = 1). In addition to this, the overall response did not differ between the four cultivars ($F = 0.06$, $P > 0.05$, df = 28) nor the response towards the plant material ($F = 2.13$, $P > 0.05$, df = 28).

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**Figure 16**: Response ($\pm$ 1 S. E.) of *Aphidius colemani* females in the olfactometer towards detached leaves of the cultivar fillbasket against the other four cultivars. Responses were always biased towards the cultivar fillbasket except when winter harvest was presented as the alternative, in which case, there was no bias in the response. *** ($P < 0.001$), ** ($P < 0.01$) and ns (not significant; $P > 0.05$) compare the response of the females within each treatment. Uppercase letters compare the proportions of non-responding females across treatments. Lowercase letters compare the proportions of responding wasps across the four different treatments.
3.3.3.4 Detached red delicious leaves against the other cultivars

No bias in the response was found between red delicious when hamlet was used as the alternative cultivar ($\chi^2 = 0.11, P > 0.05, df = 1$) (Figure 17). But a biases were found when winter harvest ($\chi^2 = 11.57, P < 0.001, df = 1$), fillbasket ($\chi^2 = 16.29, P < 0.001, df = 1$) and cabbage were used as alternatives ($\chi^2 = 9.44, P < 0.01, df = 1$). The overall response did not differ between the four treatments ($F = 0.15, P > 0.05, df = 28$). There were significant differences in the response to plant material between the bioassays involving the cultivar cabbage with the cultivar fillbasket ($t = 4.94, P < 0.001, df = 14$), hamlet ($t = 2.92, P < 0.01, df = 14$) and winter harvest ($t = 4.83, P < 0.001, df = 14$). There were also significant differences in between the cultivar hamlet when compared with the cultivar fillbasket ($t = 2.44, P < 0.05, df = 14$) and winter harvest ($t = 2.30, P < 0.05, df = 14$).

![Figure 17: Response (± 1 S. E.) of Aphidius colemani females in the olfactometer towards detached leaves of the cultivar red delicious against the other four cultivars. The response of wasps was biased towards red delicious only when cabbage was presented as an alternative. *** (P < 0.001) and ns (not significant; P > 0.05) compare the response of the females within each treatment. Uppercase letters compare the proportions of non-responding females across treatments. Lowercase letters compare the proportions of responding wasps across the four different treatments.](image-url)
3.3.3.5 Detached cabbage leaves against the other cultivars

When cabbage was one of the two choices, no bias was detected when hamlet was used as an alternative ($\chi^2 = 2.45$, $P > 0.05$, df = 1) (Figure 18). Significant differences towards the alternative were detected for winter harvest ($\chi^2 = 18.63$, $P < 0.001$, df = 1), fillbasket ($\chi^2 = 7.83$, $P < 0.01$, df = 1) and red delicious ($\chi^2 = 9.44$, $P < 0.01$, df = 1). No significant differences were detected in the level of overall response between the four different treatments ($F = 0.63$, $P > 0.05$, df = 28). When the level of wasps responding towards the plant material was compared between the treatments, a lower response was detected when the bioassay that involved cabbage versus winter was compared to the one that presented cabbage versus hamlet ($t = 2.64$, $P < 0.05$, df = 14).

![Figure 18](image_url)

Figure 18: Response (± 1 S. E.) of *Aphidius colemani* females in the olfactometer towards detached leaves of the cultivar cabbage against the other four cultivars. Responses were biased towards the alternative choice in all comparisons except when hamlet was used as the alternative. *** ($P < 0.001$), ** ($P < 0.01$) and ns (not significant; $P > 0.05$) compare the response of the females within each treatment. Uppercase letters compare the proportions of non-responding females across treatments. Lowercase letters compare the proportions of responding wasps across the four different treatments.
3.3.3.6 Responsiveness to detached leaves

Significant differences were found between the responses towards the five cultivars ($\chi^2 = 41.67, P < 0.01, df = 4$) (Figure 19). Wasps had a higher response towards winter harvest when compared to hamlet ($\chi^2 = 6.28, P < 0.05, df = 2$), red delicious ($\chi^2 = 5.44, P < 0.05, df = 2$) and cabbage ($\chi^2 = 28.33, P < 0.001, df = 2$) but it was no different from the response to fillbasket ($\chi^2 = 0.18, P > 0.05, df = 2$). Wasps responded less to cabbage when compared to hamlet ($\chi^2 = 8.14, P < 0.01, df = 2$) and to red delicious ($\chi^2 = 7.61, P < 0.01, df = 2$). Lower overall results were found in wasps responding to whole plants when compared to detached leaves for the cultivars winter harvest ($\chi^2 = 8.79, P < 0.01, df = 1$) and hamlet ($\chi^2 = 17.31, P < 0.001, df = 1$).

![Figure 19: Average response (± 1 S. E.) of Aphidius colemani females towards five cultivars of Brassica presented as detached plants with the other four presented as alternatives. Different letters above the bars indicate significant differences between the responses towards the cultivars. Winter harvest had the highest response levels together with fillbasket, followed by hamlet and red delicious and lastly cabbage with the lowest level of response.](image-url)
3.3.4 Response to same-cultivar whole plants versus detached leaves

When either winter harvest or cabbage were compared as whole plants and as detached leaves, the response was biased towards the detached leaves ($\chi^2$ winter harvest = 25.69, $P < 0.001$, df = 1; $\chi^2$ cabbage = 27.39, $P < 0.001$, df = 1) (Figure 20). No differences were found when the overall levels of response where compared between the two treatments ($t = 1.73$, $P > 0.05$, df = 1) nor when the response towards the plant material was compared ($t = 0.03$, $P > 0.05$, df = 1).

Figure 20: Average response (± 1 S. E.) of *Aphidius colemani* females towards winter harvest plants or cabbage plants presented as whole plants and as detached leaves in the olfactometer. In both cases a significant response was recorded towards the detached leaves. *** ($P < 0.001$) compares the response of the females within each treatment. Uppercase letters compare the proportions of non-responding females across treatments. Lowercase letters compare the proportions responding wasps across the different treatments.
3.3.5 Response to different-cultivar whole plants versus detached leaves

When different cultivars were presented together, one as whole plants and the other as detached leaves, the response was biased towards the detached leaves of winter harvest when cabbage was presented as whole plants as the alternative ($\chi^2 = 14.46, P < 0.001, df = 1$). But when winter harvest was presented as whole plant and cabbage as detached leaves, no bias in the response was found ($\chi^2$ cabbage = 0.72, $P > 0.05$, $df = 1$) (Figure 21). No difference was found in the overall response between both treatments ($t = 1.12, P > 0.05, df = 14$) but differences were found between the choices of the responders ($t = 3.20 P < 0.01, df = 14$).

![Figure 21](image-url)

**Figure 21:** Average response (± 1 S. E.) of *Aphidius colemani* females towards winter harvest plants and cabbage plants presented either as whole plants or as detached leaves in the olfactometer. Only when whole cabbage plants were presented against the detached leaves of an entire winter harvest plants, there was a significant response towards winter harvest. *** ($P < 0.001$), ns (not significant, $P > 0.05$) compares the response of the females within each treatment. Uppercase letters compare the proportions of non-responding females across treatments. Lowercase letters compare the proportion of responding wasps across the different treatments.
3.4 Discussion

3.4.1 Olfactometer controls

The controls carried out on the whole-plant olfactometer with empty odour sources confirmed that no asymmetries in the construction of the olfactometer were affecting the airflow entering the olfactometer arms. This indicated the new olfactometer design (section 2.1.4.2, page 37) originated from a modification of an existing olfactometer, was adequate to assess *A. colemani* behaviour since the response is even towards the four odour sources when no olfactory stimuli is present. A second control bioassay with whole plants as two of the odour sources and the remaining two containing only a humid air control, confirmed that parasitoids were able to distinguish between two sources and had a clear bias towards plant material.

3.4.2 Response to whole plants and detached leaves of the five different Brassica cultivars

The behavioural response of *A. colemani* females towards the five Brassica cultivars generally resulted in a bias towards the cultivar experienced and a variable selection when presented with a choice between unencountered cultivars. When the response to winter harvest was considered against the other four cultivars as whole plants, a clear distinction occurred in all contrasts except when hamlet was the alternative, in which case no clear distinction was made between the cultivars. When the plants were presented as detached leaves, in addition to not discerning hamlet, parasitoids did not discern fillbasket from winter harvest. This general bias shown in most of the contrasts towards whole plants and detached leaves of winter harvest was not an innate response, since the converse experiments, done on *A. colemani* females reared on *M. persicae* feeding on red delicious, resulted in females responding towards red delicious when offered the choice between red delicious and winter harvest (Vamvatsikos, 2006).

When female parasitoids were presented with a choice between previously unencountered cultivars, their behaviour was less consistent, but some trends became apparent and worthy of consideration. The response towards fillbasket as whole plants was divided, on one hand, when presented with hamlet, parasitoids elicited a clear response towards hamlet, but when red delicious and cabbage were offered, fillbasket was the cultivar of choice. With fillbasket plants presented as detached leaves, the cultivar was preferred over the other three unencountered cultivars. With red delicious offered as whole plant, the choices seemed to be consistent and were evidenced by a clear bias towards the alternative cultivars offered except when cabbage was
presented, choice that elicited in female parasitoids a bias towards red delicious. But when the cultivars were presented as detached leaves, there was a further lack of discrimination towards red delicious and hamlet. A clear trend emerged when parasitoids were tested with contrasts that contained cabbage in one of the choices, resulting in parasitoids making in most bioassays clear choices towards the alternative cultivars offered as whole plants and as detached leaves. The choices invariably were biased towards the alternative cultivar except when hamlet was used, in which case there was no discrimination either for whole plants or for detached leaves. There seems to be clear biases in the behaviour of female parasitoids for the cultivars mentioned above, with winter harvest favoured over most of the cultivars, then followed by fillbasket and red delicious and lastly, the least preferred cultivar was cabbage. But the behaviour of *A. colemani* when hamlet is offered as one of the choices is less predictable. In whole-plant choices, no bias was registered when cabbage was used as alternative but the choices were biased towards hamlet when fillbasket and red delicious were the alternatives. A lower level of discrimination was apparent when the leaves were detached and in addition to not discriminating cabbage, parasitoids did not discriminate red delicious.

Some exceptions emerged in the results for both whole plants and for detached leaves that affect the behaviour of parasitoids illustrating the complicated chemical world that parasitoids interact with. For instance, on one hand, when winter harvest was contrasted against hamlet, there was no bias to any of the choices. On the other hand, when the response towards winter harvest was contrasted against cabbage, there was a clear bias towards winter harvest. But interestingly, when hamlet was contrasted against cabbage, there was no distinction between them. A possible explanation for this behaviour could be, in a simplified way, that hamlet is the cultivar lying in between cabbage and winter harvest in a scale of chemical-emission similarity. Because of this, *A. colemani* is able to distinguish cabbage from winter harvest, both of which are the furthest apart, but there are not enough differences to distinguish hamlet from winter harvest or cabbage. The behavioural results recorded in this study could be due to similarities and differences between the volatile emissions of the unencountered cultivars with the experienced cultivar. Therefore, the relation between winter harvest, hamlet and cabbage, could be that some of the chemicals present in the volatile blend of winter harvest are overlapping with those of hamlet, and the same for cabbage and hamlet, but the overlap ceases to exist once the comparison is made between winter harvest and cabbage (See Chapter 4, page 70).
Female parasitoids experienced during their larval stages, adult foraging and feeding experiences in the rearing cages volatiles of the cultivar winter harvest attacked by *M. persicae* aphids, which most certainly would differ from the emissions of whole plants (Paré & Tumlinson, 1999) and those of detached leaves (Steinberg et al., 1993; Mattiacci et al., 1994). Regardless of this difference, female parasitoids were capable of discerning between most of the cultivars when presented not as plants infested by aphids but as whole plants and detached leaves. It is possible to assume that there is a certain degree of constancy in the volatile emissions of winter harvest when attacked by aphids that is also found in whole plants and in artificially-damaged plants inducing in *A. colemani* females through experience a positive behaviour towards them.

### 3.4.3 Responsiveness

To simplify matters, the “responsiveness”, or an index representing the magnitude of the bias towards the cultivars regardless the alternative used in the bioassays, was calculated. The responsiveness of *A. colemani* elicited generally strongest towards the cultivar experienced during development while the response towards inexperienced cultivars was either similar or lower. This was reflected on one hand when plants were presented whole, with the highest levels of responsiveness recorded for the cultivars winter harvest and hamlet. On the other hand with plant material presented as detached leaves, the highest responsiveness was towards the cultivars winter harvest and fillbasket.

The responsiveness also indicates that there is a rank of preference for previously inexperienced cultivars. Intermediate responses were recorded for the cultivars red delicious and fillbasket (for whole plants) and hamlet (for detached leaves). And the lowest levels of responsiveness were elicited towards the only plant that is not a Brussels sprout but a cabbage (although called “cultivar” instead of variety, for simplicity reasons). When presented as whole plants and as detached leaves, cabbage was invariably the least preferred cultivar. This result is probably evidence of the greater genetic distance with the four Brussels sprouts cultivars and thus a greater difference in terms of volatile chemical composition. The pedigree of the five cultivars used would offer another angle from which the differences in parasitoid behavioural responses could be explained, but unfortunately the seed supplier (Suttons, Devon, UK) was not able to provide any information about this (Ian Prestt, personal communication).

Few studies have dealt with behavioural differences of natural enemies towards cultivars of the same species. Most studies in the subject investigated differences in
the responses of natural enemies after plants had been infested by herbivores. For example, the response of the parasitoid *Anagrus nilaparvatae* was evaluated towards herbivore-induced volatile emissions of different rice varieties and the behaviour correlated with differences in the composition of the cultivars emissions (Yonggen et al., 2006). Also differences were found in the volatile production of four cultivars of the ornamental crop *Gerbera jamesonii* after attack by a spider mite eliciting differential behavioural responses in the predatory mite *Phytoseiulus persimilis* (Krips et al., 2001).

One of the few studies dealing with behavioural differences of parasitoids towards uninfested cultivars of the same species was found in the parasitoid *A. colemani* responding towards different cultivars of cabbage (Kalule & Wright, 2004), and determined that even though there was a preference for the cultivar on which the parasitoids where reared on, there was an order in the preference for other cultivars. Ultimately parasitoids and natural enemies will respond towards those plants emitting herbivore-induced volatiles and probably emissions of whole plants or mechanically-damaged plants will be silenced, from a behavioural point of view, by stronger signals. The study of the behaviour of natural enemies in simplified scenarios which are not necessarily ecologically relevant, offers the possibility to study in simplified systems the basis of behaviour. In this study, plants attacked by aphids were not used because of practical and logistical reasons that made it impossible rearing aphids on five different cultivars. In the future, it would be necessary to assess the behavioural response of the parasitoid towards cultivars with aphid attack to gain a better understanding of how the parasitoids would respond behaviourally.

### 3.4.4 Whole and detached plant material

Studies indicated that parasitoids on one hand are biased towards plants damaged by herbivores when offered an alternative of the same plant but artificially damaged or whole. On the other hand, the response is biased towards plants damaged artificially when presented the alternative of whole plants (Steinberg et al., 1992; Blaakmeer et al., 1994; Geervliet et al., 1996) which is in accordance to the results found in this study. When the response of *A. colemani* was assessed towards whole plants and detached leaves of the same cultivar, invariably female parasitoids were biased towards the choice that contained the detached plant material. The behaviour was probably affected due to the highest concentration of volatiles emitted by the detached leaves: Leaves that are detached from the plant probably emit distress signals resulting in higher levels of emissions as it is known that plants under stress emit higher quantities of volatiles (Paré & Tumlinson, 1999). The possible increase in volatile emissions of detached leaves could be comparable up to some extent to that of plants
attacked by aphids. Studies that entrained volatiles of Brassica plants after at least 24 h of infection indicate that there is a significant increase in volatile emission (Blaakmeer et al., 1994; Mattiacci et al., 1994; Geervliet et al., 1997), in addition to studies showing that the increase of certain volatile compounds starts shortly after damage occurs (Loughrin et al., 1994; Scascighini et al., 2005). Studies with Brussels sprouts have also indicated that there is no qualitative difference in gas chromatography profiles produced in response to herbivory or mechanical damage (Agelopoulos & Keller, 1994; Mattiacci et al., 1994). This could be the case in the present study where a response similar to aphid feeding is elicited by the detached leaves and parasitoids responding accordingly during the bioassay.

In addition to being able to detect what are thought to be quantitative differences in volatile emissions, parasitoids discriminated a whole cabbage plant offered against the leaves of a winter harvest plant, biasing towards this last choice. In this situation parasitoids probably could detect the contrast between choices since winter harvest would mean “quality”, added to the fact that the detached leaves probably emitted a higher amount of volatiles, with the alternative offered having a relatively poor quality and quantity. The parasitoids response seems to be divided when winter harvest whole plants are offered against cabbage detached leaves. In this situation parasitoids probably have the choices of on one hand the “quality” of the known plant but have the “quantity” of the cabbage detached leaves, eliciting no bias towards the choices.

3.4.5 Conclusions

Female parasitoids *A. colemani* can distinguish the cultivar experienced during development from other cultivars. The fact that parasitoids experienced aphid-infested Brussels spouts plants suggests that a parallelism in the volatile-chemical composition between aphid-infested plants, uninfested plants and uninfested damaged plants (i.e. detached leaves). Females also have an order of preference between three unencountered Brussels sprouts cultivars and one cabbage cultivar, with the Brussels sprouts eliciting higher levels of response than the cabbage cultivar. This response could be due to similarities in the quality and quantity of the chemicals emitted by the plants. The data in the current study also suggests that parasitoids are able to discriminate damaged plants from whole plants, probably due to differences in the amounts emissions. Further studies with infested plants would indicate if the range of behavioural responses towards different cultivars of the same species found in this study is retained. In addition, the chemical composition of the volatile emissions of the five Brassica cultivars would offer further understanding of the basis of the parasitoids behaviour (Chapter 4, page 70).
Chapter 4
Volatile Emissions of Brassica Cultivars

4.1 Introduction

4.1.1 Plant chemistry

Most plant taxa produce a fairly similar range of chemical compounds known as “primary plant substances”. This group of chemicals includes compounds such as cellulose, lipids and sugars, which are exclusively involved in plant fundamental physiological processes (Schoonhoven et al., 2005). Additionally, plants produce “secondary plant substances”, which are found in specific taxa and do not have an apparent role in the primary metabolism. This group of chemicals includes compounds such as alkaloids, terpenoids, phenolics and a common group of chemicals known as green leaf volatiles (GLVs) among others. Their role in ecological interactions is fundamental since they can affect health, reproduction rates and behavioural responses of other species. Their role at a multitrophic scale ranges from direct and indirect defences to stimulating activities such as oviposition and herbivory in other species. Secondary plant substances can be induced when insects start feeding on the plant or even contact a leaf surface. Undamaged plants can release secondary metabolites and some of them are small enough to be released as volatile compounds through open stomata in the leaves. In addition many secondary plant substances are released in different ratios when plants are damaged (Schoonhoven et al., 2005).

4.1.1.1 Crucifer chemistry

Crucifers are known for their production of glucosinolates, and their volatile by-products, the isothiocyanates. The glucosinolates are responsible, among other things, for producing the strong flavours that cabbage, broccoli and Brussels sprouts detected by humans as well as in condiments like mustard and wasabi. Glucosinolates are secondary plant metabolites rich in nitrogen and sulphur that through several
biochemical pathways produce a diverse range of compounds such as isothiocyanates, thiocyanates and nitriles which are involved in biological activities ranging from defence against herbivores to promoting the presence of beneficial insects. For example, many herbivore species are intolerant to certain glucosinolates, while other species, such as the aphids *M. persicae* and *Brevicoryne brassicae* have become resistant to them (Collier & Finch, 2007). While some herbivores can be dissuaded from feeding on a plant, some herbivore natural enemies can respond towards them. It has been suggested that the parasitoid *A. colemani* could become positively conditioned to glucosinolates the moment the adult emerges from its pupal case (Douloumpaka & van Emden, 2003). The parasitoid *Diaeretiella rapae* Mackintosh has been found to respond in olfactometer studies towards but-3-enyl isothiocyanate (Blande et al., 2007). The isothiocyanates, a glucosinolate product, comprise volatile compounds produced upon tissue damage in *Brassica* and related species and are thought to be important in plant defence. Another study reported an increased response in field trials of the aphid parasitoid *Diaeretiella rapae* towards genetically engineered *B. oleracea* and *B. napus* with an enhanced production of but-3-enyl isothiocyanate (Bradburne & Mithen, 2000).

### 4.1.2 Objective and hypothesis

In the previous chapter the extent to which the parasitoid *A. colemani* distinguishes five closely-related Brassica cultivars was established. In order to have a better understanding of the basis of this behaviour, the volatile-chemical composition of the five cultivars as whole plants and as detached leaves is assed. It is predicted that differences will be found in the chemical composition of the five cultivars in addition to differences between whole and damaged plants. The null hypothesis is that there will be no differences in volatile chemical composition between the cultivars or between damaged and undamaged plants.
4.2 Materials and Methods

4.2.1 Volatile collection by air entrainment

Headspace volatiles were collected between July 2006 and February 2007 from whole plants and detached leaves of entire six-week-old B. oleracea plants. These included four cultivars of Brussels sprouts: winter harvest, hamlet, fillbasket and red delicious and one cabbage cultivar: golden acre (cabbage) (details on growing conditions in section 2.1.1, page 34).

4.2.1.1 Whole-plant headspace volatile collection

The volatile chemicals were collected from six-week old plants by entrainment onto Tenax TA resin (60/80 mesh, 0.05 g, Supelco) contained in a glass GC inlet liner (8cm long X 0.3 cm ID) between two glass-wool stoppers. These were baked at 180 °C during at least 2 h with a nitrogen flow prior to use. Volatiles were entrained following Stewart-Jones & Poppy (2006). The side and top of the plastic pots containing the plant were covered with aluminium foil leaving a small opening for the plant to protrude. A Multi-purpose PET (poly-ethyleneterephthalate) (3.2 l, maximum temperature 200 °C, Sainsbury’s Supermarkets Ltd., London, UK) cooking bag secured around the pot with two rubber bands was used to enclose the plant and collect the volatile chemicals (Figure 22). The bags were baked for 2 h at 180 °C to remove possible impurities. A small hole was detached in one of the top corners for the glass liner to be fitted and secured with wire. Air filtered through 60 g of activated charcoal was pushed into the bag through Teflon tubing (external diameter: 3.2 mm, internal diameter 1.5 mm) at 1.1 l min⁻¹. This created a positive pressure which inflated the bag and was used to purge the system over 1 h. After the purging, headspace air was pulled through the Tenax at 0.7 ml min⁻¹ during 3 h. The remaining 0.4 ml min⁻¹ that entered the system was vented through the gaps left in between the bag and the pot since the rubber bands did not create a gas-tight seal. Volatile entrainments were carried out in the same glasshouse where the plants were grown (details in section 2.1.1, page 34) between 13:00 and 16:00.

Four replicates where done for winter harvest, cabbage, hamlet and red delicious, five replicates for fillbasket and 18 replicates for controls, which consisted of plastic pot with only compost left for six weeks. When the entrainments had finished, the leaves of each plant where detached and digitally scanned, the resulting image was used to calculate the total leaf-surface of the plant (Scion Image for Windows, version Alpha 4.0.3.2).
Figure 22: Whole-plant volatile entrainer. A six-week old plant was covered with a cooking bag. Filtered air was introduced into the bag and then drawn out of the system under positive pressure through a Tenax filter, where the volatiles were collected during 3 h.

4.2.1.2 Detached-leaves headspace volatile collection
The volatile chemicals produced by detached leaves of the five cultivars of *B. oleracea* were collected by a similar method to the one described above. Leaves were detached immediately before the entrainment took place and were rolled inside a Perspex tube (diameter = 24 mm, length = 95 mm). A lid with a brass fitting (Swagelock, UK) connecting the Teflon tubing with the Perspex tube allowed an air-tight seal for the charcoal-filtered air to enter the system at 1.1 l min⁻¹ and pulled out through the Tenax-filled liner at 0.7 l min⁻¹ (Figure 23).
Chapter 4  Volatile Emissions of Brassica Cultivars

Figure 23: Detached leaves volatile entrainer. Detached leaves were placed in a clean Perspex tube and during 0.5 h charcoal-filtered air was pushed into the system and drawn out through a Tenax-filled liner to collect the chemical volatiles emitted by the leaves.

The Tenax liner was held with two brass fittings: one connecting the lid of the tube to the liner and the other connecting the liner to the Teflon tubing. Excess air could exit the otherwise air-tight system through a hole drilled in the inlet lid (diameter = 1 mm). Parafilm holding the lids to the Perspex tube was used to ensure that the lids did not open because of excess air pressure inside the tube. A purging period of 1 min took place before the volatile collection commenced. Because leaves were detached and it was assumed that volatile emissions of detached leaves would not be constant (Scascighini et al., 2005) throughout the 3 h period the whole-plant volatile collection
lasted, it was decided to use a volatile-collection period 0.5 h. This would represent in the best manner the volatile emissions that parasitoids would encounter during the 0.5 h olfactometer bioassay with detached-leaf material.

Five replicates where done for fillbasket, hamlet, red delicious and winter harvest while four replicates where done for cabbage. Four control replicates were carried out, consisting of an empty Perspex tube. Once the entrainment had finished the leaves where removed from the tubes, scanned and the total leaf-surface calculated as above. Perspex tubes and lids were washed after each entrainment as specified for the detached-leaf olfactometer -version 1- (section 2.1.4.1, page 35).

4.2.2 Chemical analysis of headspace volatiles

4.2.2.1 Gas chromatography analysis

The volatiles collected in the Tenax liners from the headspace of plants and controls were analyzed in Rothamsted Research (Hertfordshire, UK). Samples were placed in a programmed temperature vaporisation unit (PTV) for analysis on a Hewlett-Packard 6890 gas chromatograph (Agilent Technologies, UK) fitted with a non-polar capillary column (HP-1 100% dimethyl polysiloxane, 50 m x 0.32 mm id x 0.82 am film thickness) and a Hewlett-Packard 6890 gas chromatograph (Agilent Technologies, UK) equipped with a polar capillary column (DB-Wax, 30 m x 0.32 mm ID x 0.50 am film thickness). Both gas chromatographs were equipped with a flame ionization detector. Desorption inside the PTV unit was performed using a rapid temperature ramp starting at 30°C (temperature of the PTV injector during introduction of the sample) and then programmed at 16°C s⁻¹ to 220°C. The GC oven temperature was maintained at 30°C for 30 s and then programmed at 5°C min⁻¹ to 120°C, then 10°C min⁻¹ to 240°C. The carrier gas was hydrogen.

4.2.2.2 Identification of chemical compounds

4.2.2.2.1 Retention indexes

A tentative identification was made for the peaks of interest through comparing the retention indexes (RIs) for each relevant peak with a library compiled by Rothamsted Research. The RI for a given compound is a number that indicates its retention relative to the adjacent alkanes used as calibrating standards. RIs and approximate quantities of all chemicals were estimated by the following method: On every gas chromatograph that would be used each week, a 1 µl injection of a solution of n-alkanes (C7-C25) standards diluted in hexane (100 ng µl⁻¹) was analyzed. An average area corresponding to 1 ng was then calculated and the area of peaks and the retention
times of entrained samples were contrasted with the data resulting from the alkanes standards.

4.2.2.2 Confirmation through synthetic standards

To confirm the tentative identification of compounds obtained from the RIs, synthetic compounds (Table 1) were analyzed in conjunction with previously-entrained samples of the cultivar fillbasket (whole plant). For this, solutions of the desired chemical compounds were made (100 ng of each compound at a dilution of 100 ng µL⁻¹ in distilled hexane) and injected into a Tenax liner previously entrained with fillbasket (whole-plant) volatile emissions and analyzed in the gas chromatographs with the polar and non-polar columns mentioned above and with the same temperature regime. The aim was to obtain, at the desired RIs, a clear (not wider) and higher peak indicating a greater amount (in excess of 100 ng when compared with the “normal” fillbasket result).

Table 1: Synthetic chemicals used to confirm the identity of several of the volatile compounds found in the five cultivars entrained as whole plants and as detached leaves.

<table>
<thead>
<tr>
<th>Number</th>
<th>Chemical compound</th>
<th>Laboratory</th>
<th>Purity (%)</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nonane</td>
<td>Aldrich</td>
<td>99</td>
<td>N2,940-6</td>
</tr>
<tr>
<td>2</td>
<td>α-pinene</td>
<td>Aldrich</td>
<td>98</td>
<td>14,752-4</td>
</tr>
<tr>
<td>4</td>
<td>6-methyl-5-hepten-2-one</td>
<td>Botanix</td>
<td>No information</td>
<td>No information</td>
</tr>
<tr>
<td>5</td>
<td>β-pinene</td>
<td>Aldrich</td>
<td>99 +</td>
<td>40,275-3</td>
</tr>
<tr>
<td>6</td>
<td>Myrcene</td>
<td>Fluka</td>
<td>~ 90</td>
<td>No information</td>
</tr>
<tr>
<td>7</td>
<td>Limonene</td>
<td>Aldrich</td>
<td>97</td>
<td>18,316-4</td>
</tr>
<tr>
<td>8</td>
<td>Nonanal</td>
<td>No information</td>
<td>No information</td>
<td>No information</td>
</tr>
<tr>
<td>9</td>
<td>Decanal</td>
<td>Aldrich</td>
<td>95</td>
<td>12,577-6</td>
</tr>
<tr>
<td>10</td>
<td>Undecanal</td>
<td>Aldrich</td>
<td>97</td>
<td>U2202-25G</td>
</tr>
</tbody>
</table>

4.2.2.3 Gas chromatography-mass spectrometry

Additionally, entrained compounds from the cultivars cabbage, fillbasket, red delicious and winter harvest were confirmed by coupled gas chromatography-mass spectrometry (1 sample per cultivar) (carried out by Birkett M. and Mohib A., Rothamsted Research, Harpenden, UK). The samples were analyzed in a Hewlett-Packard gas chromatographer (Agilent Technologies, UK) fitted with a non-polar capillary column (50 m, 0.32 mm id, 0.52 µm film thickness), with a cold on column injector and a
deactivated HP-1 pre-column (50 m x 0.53 mm id). The gas chromatograph oven temperature was programmed from 30 °C (5 min hold) to 250 °C at 5 °C min\(^{-1}\) and the carrier gas was helium. The gas chromatograph was directly coupled to a VG Autospec double-focusing magnetic sector mass spectrometer and integrated data system (Fisons Instruments, UK).

### 4.2.3 Statistical analyses

#### 4.2.3.1 Whole-plant and detached-leaves headspace volatile general analysis

Only those volatiles present in at least three replicates per cultivar and not showing in controls were considered. The total number of chemicals present in each run was averaged for each cultivar entrained as whole plant and as detached leaves separately. In addition, the amount of volatiles produced by each cultivar was considered taking into account the quantities produced in ng h\(^{-1}\) and averages were obtained for each cultivar entrained as whole plant and as detached leaves. Means of the number of compounds and emissions were compared within each treatment (i.e. whole-plant and detached-leaves entrainments) with an ANOVA. A Student \(t\) test was used to compare within cultivars the number of volatile compounds detected and the total emissions between treatments (i.e. whole plants or detached leaves).

#### 4.2.3.2 Leaf area and volatile emissions

The leaf surfaces of whole plants or detached leaves used in the entrainments were compared between cultivars using ANOVA. Since no differences were found, data were pooled and compared between each of the treatments (whole plants versus detached leaves) with a two-tailed Student \(t\) test. In addition, a linear regression was done for each cultivar entrained as whole plant and as detached leaves and compared between treatments with an ANCOVA to assess the effect of leaf size on emissions. Since no differences between cultivars were found, data were grouped together and a two-tailed Student \(t\) test was carried. The \(t\) test compared the slope obtained with a slope equal to zero; a non significant result meant that the factor leaf-size did not have a significant effect on the volatile production (R programme, version 2.4.1).

#### 4.2.3.3 Analysis of individual volatiles

A comparison of the emissions of individual volatiles was carried out for cultivars entrained as whole plants and as detached leaves. A one-way ANOVA was used considering the amounts registered for each chemical compound found in each replicate (represented as the proportion for a particular chemical respective to the total
amount emitted by that replicate). When significant differences were found a Tukey’s multiple comparison test was used to locate the sources of those differences (R programme, Version 2.4.1).

4.2.3.4 Change in emissions of detached leaves compared to whole plants
The change in the emission of a particular chemical after leaves were detached from the plants was compared to the amount found in plants entrained whole. The change was calculated as the average proportion detected for a particular chemical in a particular cultivar entrained as detached leaves, compared with the average proportion detected for the same compound in that cultivar entrained as an whole plant with this last amount standardized to 1.

4.2.3.5 Multivariate analysis
4.2.3.5.1 Canonical variates analysis
Because the data consisted of observations made from five known groups (i.e. the five Brassica cultivars), data could be analyzed with canonical variates analysis (CVA) (GenStat, version 8.0). The analysis creates an axis (canonical variate (CV)) which is a linear combination of the original variates (i.e. the RIs) that maximises between group variability relative to the within group variability. If subsequent orthogonal axes are created, they account for further variation not explained by the initial CV. The loadings of a given CV therefore represent the weights of each original variate in influencing the CV and they are scaled so that the average within-group variability in each CV is 1. The loadings corresponding to roots that are less than one indicate that the original variate has more within group variation than between group variation. The mean scores were represented in a 2-dimensional graph and encircled with a 95% confidence interval assuming multivariate normality of the data. Loadings were divided into three groups. The first two groups represented those loadings with influence on inter-group variability: Group 1 with loadings greater than 10 having the greatest impact on separating groups and group 2 with intermediate loadings between one and 10. Lastly group 3 contained those loadings that influenced intra-group variability. Two analyses were made, one for the five cultivars entrained as whole plants and one for the five cultivars entrained as detached leaves. In both cases all the chemical compounds were considered in the analyses.
4.3 Results

4.3.1 Whole plant and detached leaves volatile emissions

4.3.1.1 Number of chemical compounds

Gas chromatography analysis performed on emissions of whole plants (typical GC output: Figure 24) and detached leaves, revealed the presence of 22 different volatile chemicals in total (found in at least three replicates/cultivar/treatment) and between 5 and 20 volatiles in individual runs with averages per cultivar ranging in between 10 and 17 compounds per cultivar (Figure 25).
Figure 24: Typical gas chromatogram output of the *Brassica oleracea* cultivar fillbasket entrained as a whole plant. Identified chemicals are represented in green, chemicals tentatively identified are represented in blue, unknown compounds are in red and those showing in black are chemicals present in control samples.
No differences were detected in the number of chemical compounds produced within the cultivars entrained as whole plants ($F = 0.297$, $P > 0.05$, df = 16), nor as detached leaves ($F = 1.003$, $P > 0.05$, df = 19). But the number of compounds detected was lower for detached leaves compared to the resulting number for whole plants in the cultivars fillbasket ($t = 4.183$, $P < 0.01$, df = 8), hamlet ($t = 3$, $P < 0.05$, df = 7), red delicious ($t = 3.513$, $P < 0.01$, df = 7), winter harvest ($t = 2.767$, $P < 0.05$, df = 7) while there was no statistical difference in cabbage ($t = 1.334$, $P > 0.05$, df = 6).

**Figure 25:** Average (± 1 S.E) number of compounds present in each Brassica cultivar entrained as whole plant or as detached leaves. Uppercase letters inside the bars compare cultivars entrained as whole plants, lower case letters inside the bars compare cultivars entrained as detached leaves. **(P < 0.1), *(P < 0.05) and ns (P > 0.05) compare between the same cultivar entrained either as whole plant or as detached leaves.
4.3.1.2 Quantities emitted

The average emission produced per cultivar of whole plants and detached leaves ranged between 0.4 and 15 ng h\(^{-1}\) (Figure 26). There were no significant differences in the total amount of volatiles emitted by the five cultivars when entrained as whole plants (F = 1.601, P > 0.05, df = 16) with averages ranging between 0.3 and 0.9 ng h\(^{-1}\). No differences were detected between the cultivars when entrained as detached leaves (F = 2.07, P < 0.05, df = 19) with averages ranged between 3 and 15 ng h\(^{-1}\). A greater amount of volatiles was emitted by the cultivars entrained as detached leaves when compared to the same cultivar when entrained as a whole plant in the case of cabbage, fillbasket, hamlet and winter harvest (\(t\) cabbage = 4.34, P < 0.01, df = 6; \(t\) fillbasket = 10.35, P < 0.001, df = 8; \(t\) hamlet = 3.250, P < 0.05, df = 7; and \(t\) winter harvest = 3.210, P < 0.05, df = 4) but no differences were detected for red delicious (\(t\) = 2.499, P > 0.05, df = 4). On average a 20-fold increase in volatile emissions was detected when plants were entrained as detached leaves.

![Figure 26](image)

Figure 26: Total average amount (± 1 S.E) of volatiles emitted by the five cultivars of Brассicas entrained as whole plants or as detached leaves. Detached leaves emitted a greater amount of volatiles than whole plants. Uppercase letters compare amounts of cultivars entrained as whole plants, lower case letters compare amounts of cultivars entrained as detached leaves and ** (P < 0.1), * (P < 0.05) and ns (P > 0.05) compare the emissions of the same cultivar entrained as a whole plant or as detached leaves.

4.3.1.3 Leaf area and volatile emissions

No significant differences were found in leaf sizes between the different cultivars in the whole plants (F = 3.002, P > 0.05, df = 16) nor in the detached leaves treatments (F =
1.041, P > 0.05, df = 19). With cultivar data grouped together, a higher average leaf surface was detected in the whole plants ($\bar{X} = 237.2$, S.E. = 15.31 N=21) when compared against the detached leaves ($\bar{X} = 185.9$, S.E. = 6.535 N=24) ($t = 3.22$, $P < 0.01$, df = 43). The leaf area did not have an effect on the amount of volatiles produced by each cultivar with whole-plants data pooled together ($t = 1.71$, $P > 0.05$, df = 19) with no differences in between cultivars (ANCOVA, $F = 1.57$, $P > 0.05$, df = 11). Data of plants entrained as detached leaves resulted in no differences in the amount of volatiles produced by each cultivar ($t = 0.67$, $P > 0.05$, df = 22) in addition no differences were detected between the five cultivars (ANCOVA, $F = 0.46$, $P > 0.05$, df = 14) (Figure 27).
Figure 27: Volatile emissions of cultivars entrained as whole plants or as detached leaves according to the total leaves area. Leaf size did not have an effect in the amount of volatiles produced.
4.3.2 Analysis of individual volatiles

A total of 22 different volatile compounds were detected in at least 3 of the entrainments per cultivar/treatment. The average amounts per cultivar ranged from 0 to > 6 ng h\(^{-1}\) (Table 2). Ten compounds could be identified through matching with synthetic standards and through confirmation with gas chromatography coupled with mass spectrometry. Those compounds were: α-pinene, 6-methyl-5-hepten-2-one, nonane, β-pinene, myrcene, limonene, decanal, nonanal, and undecanal. Thirteen were tentatively identified through matching the RI obtained with an RI database compiled in Rothamsted Research (Harpenden, UK) and the possible identities for the unknown compounds are proposed: (E)-2-hexenal, (Z)-3-hexen-1-ol, 5-methyl-3-methylene-5-hexen-2-one, (+)-sabinene, (Z)-3-hexen-1-yl acetate, octan-1-ol, 2-phenylethanol, 2-ethylbenzaldehyde, linalool oxide, dimethyl tetrasulfide and β-selinene. Two compounds could not be identified: unknown1 and unknown2.
Table 2: Average amounts of the relevant chemical compounds of the five cultivars of *Brassica oleracea* entrained as whole plants or as detached leaves. The names of the compounds that could be successfully identified are represented in green font, those that only a tentative identification was made are in blue font, and those that could not be identified are in red- font. Studies in which the same compound was found in volatile entrainments of Brussels sprouts and/or cabbage plants: ∞ = Blaakmeer, et al. (1994), ‡ = Bukovinszky, et al. (2005), * = Geervliet, et al. (1997), @ = Mattiacci et al. (1994), # = Mattiacci et al. (2001b), † = Smid et al. (2002).

<table>
<thead>
<tr>
<th>Retention Index</th>
<th>Chemical compound</th>
<th>Studies in which the compound was also found</th>
<th>Cabbage</th>
<th>Cabbage Fillbasket</th>
<th>Average amount (ng h⁻¹)</th>
<th>Hamlet</th>
<th>Red delicious</th>
<th>Winter harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td>820</td>
<td>(E)-2-hexenal</td>
<td></td>
<td>Whole (n=4) Detached (n=4) 0.059 ± 0.038, 0.146 ± 0.073</td>
<td>0.07 ± 0.034</td>
<td>-</td>
<td>0.01 ± 0.001, 0.064 ± 0.088</td>
<td></td>
<td></td>
</tr>
<tr>
<td>840</td>
<td>(Z)-3-hexen-1-ol</td>
<td>♦ @ * †</td>
<td>0.04 ± 0.4, 1.697 ± 0.322</td>
<td>0.02 ± 0.005, 2.847 ± 0.796</td>
<td>-</td>
<td>1.14 ± 0.62, 3.184 ± 1.319</td>
<td></td>
<td></td>
</tr>
<tr>
<td>870</td>
<td>5-methyl-3-methylene</td>
<td></td>
<td>0.10 ± 0.037, 0.13 ± 0.066</td>
<td>0.11 ± 0.021</td>
<td>-</td>
<td>0.03 ± 0.021, 0.226 ± 0.144</td>
<td></td>
<td></td>
</tr>
<tr>
<td>900</td>
<td>nonane</td>
<td></td>
<td>0.06 ± 0.004, 0.137 ± 0.08</td>
<td>0.07 ± 0.021</td>
<td>0.007 ± 0.001, 0.335 ± 0.183</td>
<td>0.006 ± 0.002, 0.364 ± 0.221</td>
<td>0.005 ± 0.001, 0.346 ± 0.221</td>
<td></td>
</tr>
<tr>
<td>925</td>
<td>(Z)-3-hexen-1-yl acetate</td>
<td></td>
<td>0.04 ± 0.009, 0.223 ± 0.095</td>
<td>0.02 ± 0.007, 0.25 ± 0.129</td>
<td>0.04 ± 0.009, 0.159 ± 0.057</td>
<td>0.03 ± 0.006, 0.354 ± 0.151</td>
<td></td>
<td></td>
</tr>
<tr>
<td>945</td>
<td>α-pinene</td>
<td>♦ @ * #</td>
<td>0.04 ± 0.002, 0.002 ± 0.002</td>
<td>0.004 ± 0.002</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001, 0.002 ± 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>950</td>
<td>6-methyl-5-hepten-2-one</td>
<td></td>
<td>0.05 ± 0.003, 1.363 ± 0.592</td>
<td>0.004 ± 0.002, 0.209 ± 0.583</td>
<td>0.005 ± 0.002, 0.554 ± 0.122</td>
<td>0.002 ± 0.001, 3.091 ± 1.652</td>
<td></td>
<td></td>
</tr>
<tr>
<td>970</td>
<td>(+)-sabinene</td>
<td>♦ @ * †</td>
<td>0.04 ± 0.011, 0.223 ± 0.095</td>
<td>0.02 ± 0.007, 0.25 ± 0.129</td>
<td>0.04 ± 0.009, 0.159 ± 0.057</td>
<td>0.03 ± 0.006, 0.354 ± 0.151</td>
<td></td>
<td></td>
</tr>
<tr>
<td>975</td>
<td>β-pinene</td>
<td>♦ @ *</td>
<td>0.016 ± 0.001</td>
<td>0.002 ± 0.002</td>
<td>0.003 ± 0.000</td>
<td>0.003 ± 0.000, 0.003 ± 0.006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>984</td>
<td>myrcene</td>
<td>♦ @ *</td>
<td>0.051 ± 0.02, 0.17 ± 0.011</td>
<td>0.316 ± 0.041</td>
<td>-</td>
<td>0.051 ± 0.013, 0.031 ± 0.024</td>
<td>0.036 ± 0.006</td>
<td></td>
</tr>
<tr>
<td>987</td>
<td>(Z)-3-hexen-1-yl acetate</td>
<td></td>
<td>0.04 ± 0.031, 0.438 ± 0.15</td>
<td>0.228 ± 0.096, 5.567 ± 1.135</td>
<td>0.007 ± 0.001, 6.689 ± 2.019</td>
<td>0.002 ± 0.001, 4.285 ± 2.186</td>
<td>0.130 ± 0.077, 6.409 ± 2.872</td>
<td></td>
</tr>
<tr>
<td>1025</td>
<td>limonene</td>
<td>♦ @ *</td>
<td>0.02 ± 0.003, 0.212 ± 0.047</td>
<td>0.163 ± 0.05, 0.436 ± 0.095</td>
<td>0.322 ± 0.034, 0.364 ± 0.157</td>
<td>0.131 ± 0.03, 0.789 ± 0.485</td>
<td>0.113 ± 0.021, 0.219 ± 0.111</td>
<td></td>
</tr>
<tr>
<td>1058</td>
<td>octan-1-ol</td>
<td>*</td>
<td>0.006 ± 0.001</td>
<td>0.001 ± 0.001</td>
<td>0.001 ± 0.001, 0.008 ± 0.008</td>
<td>0.001 ± 0.001, 0.023 ± 0.014</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1085</td>
<td>nonanal</td>
<td>@ # †</td>
<td>0.02 ± 0.009, 0.271 ± 0.039</td>
<td>0.053 ± 0.023, 0.244 ± 0.071</td>
<td>0.025 ± 0.002, 0.536 ± 0.252</td>
<td>0.019 ± 0.004, 0.834 ± 0.377</td>
<td>0.011 ± 0.002, 0.663 ± 0.153</td>
<td></td>
</tr>
<tr>
<td>1087</td>
<td>2-phenylethanol</td>
<td>♦ @ #</td>
<td>0.02 ± 0.019</td>
<td>0.014 ± 0.007, 0.015 ± 0.015</td>
<td>0.032 ± 0.005, 0.095 ± 0.035</td>
<td>0.018 ± 0.007, 0.05 ± 0.027</td>
<td>0.018 ± 0.007, 0.186 ± 0.066</td>
<td></td>
</tr>
<tr>
<td>1106</td>
<td>unknown1</td>
<td></td>
<td>0.019 ± 0.009</td>
<td>0.013 ± 0.004</td>
<td>0.03 ± 0.003, 0.059 ± 0.045</td>
<td>0.014 ± 0.003</td>
<td>-</td>
<td>0.011 ± 0.003, 0.015 ± 0.015</td>
</tr>
<tr>
<td>1121</td>
<td>unknown2</td>
<td></td>
<td>0.007 ± 0.009</td>
<td>0.006 ± 0.002</td>
<td>0.006 ± 0.002</td>
<td>0.02 ± 0.002, 0.028 ± 0.028</td>
<td>0.003 ± 0.002</td>
<td>-</td>
</tr>
<tr>
<td>1118</td>
<td>2-ethylbenzaldehyde</td>
<td></td>
<td>0.004 ± 0.001</td>
<td>-</td>
<td>0.002 ± 0.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1152</td>
<td>linalool oxide</td>
<td>♦</td>
<td>0.006 ± 0.003, 0.008 ± 0.003</td>
<td>0.013 ± 0.001</td>
<td>0.004 ± 0.001</td>
<td>0.004 ± 0.001</td>
<td>-</td>
<td>0.002 ± 0.001</td>
</tr>
<tr>
<td>1156</td>
<td>decanal</td>
<td>♦ # †</td>
<td>0.033 ± 0.002</td>
<td>0.076 ± 0.001</td>
<td>0.029 ± 0.003</td>
<td>0.027 ± 0.009</td>
<td>0.015 ± 0.003</td>
<td>-</td>
</tr>
<tr>
<td>1196</td>
<td>dimethyl tetrasulfide</td>
<td></td>
<td>0.009 ± 0.003</td>
<td>0.006 ± 0.002</td>
<td>0.002 ± 0.000</td>
<td>0.003 ± 0.001</td>
<td>0.001 ± 0.001</td>
<td>-</td>
</tr>
<tr>
<td>1289</td>
<td>undecanal</td>
<td></td>
<td>0.006 ± 0.004, 0.04 ± 0.025</td>
<td>0.011 ± 0.005, 0.041 ± 0.018</td>
<td>0.002 ± 0.000</td>
<td>0.005 ± 0.002, 0.089 ± 0.033</td>
<td>0.002 ± 0.001, 0.104 ± 0.028</td>
<td></td>
</tr>
<tr>
<td>1490</td>
<td>β-selinene</td>
<td></td>
<td>0.009 ± 0.003</td>
<td>0.005 ± 0.002</td>
<td>0.005 ± 0.001</td>
<td>0.003 ± 0.001</td>
<td>0.003 ± 0.001</td>
<td>0.004 ± 0.001</td>
</tr>
</tbody>
</table>
4.3.2.1 Emissions of individual volatiles

The five cultivars entrained as whole plants showed significant differences in the relative amounts of (Z)-3-hexen-1-yl acetate emitted ($F = 3.01$, $P < 0.05$, df = 16) (Figure 28) with winter harvest and fillbasket emitting similar amounts ($P > 0.05$) but higher amounts than hamlet and red delicious ($P > 0.05$). No differences were found in the emissions of the other chemical compounds (for simplicity reasons, chemicals are represented with their RIs) ($F_{820} = 1.08$, $F_{840} = 1.85$, $F_{870} = \text{absent}$, $F_{900} = 1.13$, $F_{935} = 0.16$, $F_{965} = 1.31$, $F_{970} = 1.76$, $F_{975} = 1.15$, $F_{984} = 1.65$, $F_{1025} = 0.9$, $F_{1054} = 1.09$, $F_{1058} = 0.91$, $F_{1085} = 1.59$, $F_{1087} = 0.91$, $F_{1096} = 0.53$, $F_{1118} = 1.33$, $F_{1152} = 1.76$, $F_{1186} = 0.67$, $F_{1196} = 1.3$, $F_{1289} = 1.49$, $F_{1490} = 1.44$, $P > 0.05$, df = 16).

Significant differences were found in cultivars entrained as detached leaves in (Z)-3-hexen-1-ol ($F = 4.66$, $P < 0.01$, df = 19) with the cultivar hamlet emitting more than cabbage ($P < 0.05$). In addition, (+)-sabinene had significant differences in the amounts emitted ($F = 4.65$, $P < 0.01$, df = 19) with cabbage emitting a higher amount than hamlet ($P < 0.05$) and (Z)-3-hexen-1-yl acetate ($F = 3.53$, $P < 0.05$, df = 19) with cabbage emitting lower amounts than fillbasket ($P < 0.01$), hamlet ($P < 0.01$) and red delicious ($P < 0.01$) but a similar amount to winter harvest ($P > 0.05$). There were no differences found between the remaining volatile compounds ($F_{820} = 2.189$, $F_{870} = 1$, $F_{900} = 1.39$, $F_{935} = \text{absent}$, $F_{965} = 2.39$, $F_{970} = 2.39$, $F_{975} = \text{absent}$, $F_{984} = 1$, $F_{1025} = 0.84$, $F_{1054} = 1.60$, $F_{1058} = 1.18$, $F_{1085} = 2.45$, $F_{1087} = 2.50$, $F_{1096} = 0.94$, $F_{1118} = \text{absent}$, $F_{1152} = \text{absent}$, $F_{1186} = \text{absent}$, $F_{1196} = \text{absent}$, $F_{1289} = 1.56$, $F_{1490} = \text{absent}$, $P > 0.05$, df = 19).
Figure 28: Average amounts (± 1 S.E.) of individual volatiles represented as the proportion each compound contributed to the total amount obtained for that cultivar entrained as whole plant (whole) or as detached leaves (cut). ‘ns’ (P > 0.05), * (P < 0.05) and ** P < 0.001 denote the comparison between cultivars entrained. Compounds are represented with their retention indexes and with names of those compounds with confirmed identities in green font, tentative identities in blue font) and unknowns compounds in red font. ‘abs’ denotes compounds not found in the analysis.
4.3.2.2 Whole plants and detached leaves volatile production

Cultivars entrained as whole plants were generally dominated by the presence of limonene (~30% of the blend) (Figure 29 and Figure 30), followed by myrcene (~20% of the blend) (+)-sabinene and (Z)-3-hexen-1-yl acetate (~10% of the blend). In detached-leaves entrainments, the main compounds included (Z)-3-hexen-1-yl acetate followed by (Z)-3-hexen-1-ol and 6-methyl-5-hepten-2-one in lower proportions with similar ratios of these compounds in the cultivars fillbasket, hamlet, red delicious and winter harvest.

Figure 29: Relative amounts of the 22 chemical compounds in the blends for the five cultivars entrained as whole plants or as detached leaves. Names of chemicals which were identified are in green font, those that were tentatively identified are in blue font, and unknowns are in red.
Figure 30: Relative amounts (± 1 S.E.) of the main volatile compounds present in quantities greater than 10% in at least one of the five Brassica cultivars entrained as whole plants or as detached leaves. Whole-plants had generally limonene and myrcene as the main components in the volatile blend, while the main components in cultivars entrained as detached leaves were (Z)-3-hexen-1-yl acetate followed by (Z)-3-hexen-1-ol and 6-methyl-5-hepten-one. Chemicals which were identified are in green font, those that were tentatively identified are in blue font.
4.3.2.3 Change in the emissions of detached leaves compared to whole plants

The individual volatiles emitted by plants entrained as detached leaves were compared to amounts emitted by whole plants. The analysis showed a 130-fold increase in the emission of (Z)-3-hexen-1-yl acetate in the cultivar red delicious and a 90-fold increase in the cultivar hamlet (Figure 31). Also a 40-fold increase in 6-methyl-5-hepten-2-one in winter harvest with the remaining cultivars increasing the amount produced as well. Also (Z)-3-hexen-1-ol, nonane and undecanal emissions were higher when the leaves were detached off the plants. Consistent decreases in the emissions of the volatiles limonene and 2-phenylethanol (~ 5 times) were detected across the cultivars.
Figure 31: Change in the amounts of each chemical compound emitted by the five Brassica cultivars when entrained as detached leaves compared to the amount emitted by whole plants. Chemicals which were identified are in green font, those that were tentatively identified are in blue. The increases in emissions in red delicious of 6-methyl-5-hepten-2-one (45-fold), hamlet and red delicious of (Z)-3-hexen-1-yl acetate (hamlet: 97-fold and red delicious (127-fold)) have been overscaled to show minor compounds.
4.3.3 Multivariate analysis

Canonical variates analysis carried out on all 22 chemical compounds represented as proportions revealed significant differences for all the cultivars entrained as whole plants and as detached leaves (Figure 32). In both analyses five distinct groups, each one significantly different from the other, were formed.

![Diagram A: Canonical variates analysis for whole plants](image)

**Figure 32:** Canonical variates analysis stressing the differences between the five cultivars of Brassica (winter = winter harvest, red = red delicious) entrained as whole plants (A) or as detached leaves (B). Circles around the crosses represent 95% confidence intervals. Five different groups comprising the five cultivars were conformed for whole plants and for detached leaves.
The CVA carried out on the volatile compounds emitted by whole plants, indicates according to the first canonical variate, that the chemicals with most weight (group 1) in determining between-group variation where undecanal, dimethyl tetrasulfide, 6-methyl-5-hepten-one, \((E)\)-2-hexenal, unknown1, limonene, unknown2, \(\alpha\)-pinene and \(\beta\)-pinene (Figure 33). Still influencing the inter-group distances but belonging to group 2 with a lesser impact in separating groups was registered for the volatiles \((Z)\)-3-hexen-1-ol, decanal and 2-ethylbenzaldehyde. The remaining chemicals: nonane, \((+)-\)sabinene, octan-1-ol, \(\beta\)-selinene, linalool oxide, nonanal, \((Z)\)-3-hexen-1-yl acetate, 2-phenylethanol and myrcene had loadings lower than one, resulting in their contribution towards intra-group distances. Lastly 5-methyl-3-methylene-5-hexen-2-one was not present in the whole plants entrainments therefore the loadings was equal to 0.

Figure 33: Relative weight of each chemical compound in separating the cultivars. CVA loadings < 1 indicate that the original variates had more between-group variation than within group variation. CVA loadings < 1 indicate greater contribution of the original variate towards within-group variation. Chemical compounds represented in green are those with confirmed identities, compounds in blue represent those whose identity is suggested and red compounds are unknowns.
For cultivars entrained as detached leaves, according to the first canonical variate the most influential volatiles in determining inter-group distances were: octan-1-ol, unknown1, myrcene, undecanal and 5-methyl-3-methylene-5-hexen-2-one. Volatiles that resulted in group 2, with intermediate weightings were nonane, limonene, (+)-sabinene and (Z)-3-hexen-1-yl acetate. Those chemicals belonging to group 3, with the lowest loadings that contributed to intra-group variability were 2-phenylethanol, unknown2, nonanal, (E)-2-hexenal, (Z)-3-hexen-1-ol, 6-methyl-5-hepten-one. Lastly β-selinene, linalool oxide, 2-ethylbenzaldehyde, decanal, β-pinene, α-pinene and dimethyl tetrasulfide had loadings equal to 0 since they were not found in the detached leaves entrainments.
4.4 Discussion

4.4.1 The chemical composition of cultivar emissions

4.4.1.1 Individual compounds

The volatile blends of whole plants were dominated by the presence of limonene, myrcene, (Z)-3-hexen-1-yl acetate, (+)-sabinene and decanal. Plants entrained as detached leaves were dominated by (Z)-3-hexen-1-yl acetate, (Z)-3-hexen-1-ol 6-methyl-5-hepten-2-one, limonene, sabinene and nonanal. All of these compounds have been found in previous studies in which the composition of Brussels sprouts emissions was studied through air entrainments (Blaakmeer et al., 1994; Mattiacci et al., 1994; Geervliet et al., 1997; Mattiacci et al., 2001a; Smid et al., 2002; Bukovinszky et al., 2005) therefore although (Z)-3-hexen-1-yl acetate identity was not confirmed, it is probable that it is the compound in question.

A comparison of the data obtained in the current study with those in the literature, showed that out of the 22 chemicals found here, 10 were found previously while the other 12 were not. This difference could be due to failures in identifying some of the chemicals and also due to the presence of chemicals previously unregistered in the other studies. For example Mattiacci (2001a) found only 11 compounds in whole Brussels sprouts plants (cultivar not specified) while Bukovinszky (2005) found 49 compounds in unharmed plants (cultivar cyrus). Probably the cause of this variation resides, in addition to the differences in the cultivars used, in the methods employed in each study, since the type of polymer used to capture the volatiles, the age of the plants and lighting conditions could have affected differentially the results of each study.

Since no isothiocyanates were found in this study, it is possible to speculate that some of the tentatively-identified compounds or the unknowns could have been them. But it has been found that isothiocyanates in cabbage plants are released only upon damage by the lepidopteran caterpillar *Pieris rapae* (Agelopoulos & Keller, 1994). Therefore it is possible that damaged plants will not emit isothiocyanates unless they are damaged by a herbivore. Elicitors present in herbivores saliva (Kessler & Baldwin, 2002) when in contact with crushed cells in the site of herbivory on the leaves might trigger the production of these glucosinolate derivates.

Emissions of few of the volatile compounds were significantly different between some of the Brassica cultivars studied. When entrained as whole plants, differences were found in the emission of only one chemical, (Z)-3-hexen-1-yl acetate, which was
produced in similar amounts by fillbasket and winter harvest, while emitted in lower amounts by hamlet and red delicious, and the emissions from cabbage resulted in between the two groups. With cultivars entrained as detached leaves, differences were also found in the emissions of the compound \((Z)-3\text{-hexen-1-yl acetate}\), resulting in cabbage emitting lower amounts than fillbasket, hamlet and red delicious but a similar amount to winter harvest. Differences were also found in the production of the compound \((Z)-3\text{-hexen-1-ol}\) with hamlet emitting more than cabbage, and also \((+)-sabinene\) resulted in different amounts emitted, with cabbage producing more than hamlet. There was a considerable variation in the emissions between replicates in most of the chemicals detected, affecting the significance of the tests. This variation is often a normal finding in studies involving analysis of plant-volatile emissions (Paré & Tumlinson, 1999; Smid et al., 2002). Although in this study every possible precaution was taken to prevent variable results, such as keeping constant the time of the day in which the sampling was carried out, the light conditions, plant size and age; there are other factors such as temperature, the position of the plant and the leaves inside the entrainment bag, that could have affected the final results.

In contrast, when all the chemicals where taken into account through multivariate analysis, significant differences were detected within whole and damaged cultivars. The relevant chemicals responsible for between-group variation differed between the two treatments. On one hand, dimethyl tetrasulfide, undecanal, \((E)-2\text{-hexanal}\) and \(6\text{-methyl-5-hepten-2-one}\) win the CVA were important in separating cultivars entrained as whole plants. On the other hand, octan-1-ol, unknown1, myrcene, undecanal and \(5\text{-methyl-3-methylene-5-hexen-2-one}\) were the relevant chemicals in separating damaged plants. Although statistically significant, the differences found, probably do not mean much from a parasitoids’ behaviour point of view, since the relevant chemicals for the parasitoid would probably not exactly match those identified in this study. Multivariate analysis, although a powerful approach in some cases, in the present study it was found that small changes such as the addition or deletion of one variate had an immense impact in the outcome of the analysis. The fact that there were few replicates (between 4 and 5 replicates per treatment) probably had an effect on variability of the outcome. Therefore, CVA is ideally suited to analyze samples with smaller number of variates involved if few replicates are involved. In this study, although it offered a first insight into the differences between cultivars, it would be useful in the future to narrow down the 22 compounds to the behaviourally active ones and perform this analysis again.
4.4.1.2 Emissions of detached leaves

Generally there was a significant increase in the amount of volatiles emitted by the cultivars when the leaves were detached. An exception to this was red delicious, where no significant increase was detected between both treatments because of the great variability recorded in between the replicates. Another trend that emerged was that the number of compounds emitted by each of the cultivars decreased when the leaves were detached.

Increases in the proportions of (Z)-3-hexen-1-ol, 6-methyl-5-hepten-2-one and (Z)-3-hexen-1-yl acetate offer an insight into the fast response of the plant to the damage effected by detaching the leaves, probably released from accumulated storage sites on the leaf, triggered by the damage (Paré & Tumlinson, 1999; Chamberlain et al., 2000). Generally the increase ranged between 5 and 30-fold. In the case of hamlet and red delicious, a ~ 100-fold increase was detected for (Z)-3-hexen-1-yl acetate because of very small proportions detected when entrainments were done on whole plants. Disregarding these two exceptions, the compound which was produced in the biggest increase after (Z)-3-hexen-1-yl acetate, was 6-methyl-5-hepten-2-one with an average 24-fold increase between cultivars, (Z)-3-hexen-1-ol (~ 6-fold increase) with the compound being present in damaged plants but not in whole plants in the cultivar red delicious. A ~3-fold increase between cultivars was detected for nonane and a ~ 2-fold increase for nonanal and undecanal (but not detected in the cultivar hamlet).

The more volatiles emitted by the damaged plants is in accordance, at least in a general way, with some of the results found in other studies. For example, (Z)-3-hexen-1-yl acetate has been found numerous times in volatile entrainments of herbivore infested Brussels sprouts plants in more quantities than uninfested plants (Blaakmeer et al., 1994; Mattiacci et al., 1994; Geervliet et al., 1997; Bukovinszky et al., 2005). Also, excised leaves of maize, Zea mays, resulted in a 2.5 to 8-fold increase in volatile emissions compared to leaves attached to the plant (Schmelz et al., 2001) and an increase in the production of the chemical compounds (Z)-3-hexen-1-yl acetate, linalool and E-β-farnesene was found in damaged maize (Turlings & Tumlinson, 1992). Other studies found increases in the green leaf volatiles (Z)-3-hexen-1-ol, (Z)-3-hexen-1-yl produced by Pieris brassicae-infected cabbage plants (Scascighini et al., 2005), and changes were registered when cotton plants (Gossypium hirsutum) were attacked by beet armyworms (Spodoptera exigua) (Loughrin et al., 1994).
4.4.2 Behavioural data in the light of the GC analysis

Volatile emission analysis of the five Brassica cultivars resulted in significant differences in the range of chemicals present in the five cultivars. Some of the detected differences in emissions could be the basis of parasitoids discernment between the cultivars. It has been suggested that crop-plants, originating from a subset of few individuals and product of years of artificial selection, could have resulted in a loss of variability in the amount and identity of volatiles produced (Pareja et al., 2007).

Although this is probably the case with *B. oleracea*, as suggested by behavioural experiments on the parasitoids *D. semiclausum*, where a wild type *B. oleracea* was preferred over a commercially available conspecific (Bukovinszky et al., 2005), the behavioural data in the present study suggests that in most of the cultivars tested there is enough variability for the parasitoids to distinguish between them, although it is still unknown what similarities these commercial cultivars keep with feral Brussels sprouts.

Parasitoids and other predators such as coccinelids can distinguish between different uninfested cultivars of the same species, although in most cases the preference shifts to plants attacked by herbivores when these are offered as alternatives (Elzen et al., 1986; Dicke et al., 1990; Rapusas et al., 1996; Rietdorf & Steidle, 2002). In the present study, based on the dissimilarities in volatile profiles, parasitoids were able to discern between various cultivars presented as whole plants and/or detached leaves. Furthermore, the least preferred cultivar (i.e. cabbage) when mechanically damaged elicited a similar bias in choice when offered with the most preferred cultivar (i.e. winter harvest) when offered whole. The changes in volatile production observed in detached plants and the behavioural response of the parasitoids suggests that it could be comparable in terms of volatile profile to the volatile profile of a plant attacked by herbivores.

Many of the volatiles found in this study are believed to be involved in parasitoid host-location. It is suggested that (Z)-3-hexen-1-ol could play an important role in the initial steps of host location and then other compounds could be responsible for a more specific response (Wei et al., 2007 ). Also, electrophysiological responses were recorded from the parasitoids *C. glomerata* and *C. rubecula* towards some of the chemicals reported here: 

<table>
<thead>
<tr>
<th><em>E</em>-2-hexenal</th>
<th><em>Z</em>-3-hexen-1-ol</th>
<th>(Z)-3-hexen-1-yl acetate</th>
<th>limonene</th>
<th>nonanal</th>
<th>decanal</th>
</tr>
</thead>
</table>

In a second study showed that the parasitoids *C. marginiventris*, *Microplitis rufiventris*, and *Campoletis sonorensis* elicit electrophysiological responses to more than 20 volatiles emitted by maize (*Zea mays*), cotton (*Gossypium herbaceum*), and cowpea (*Vigna unguiculata*) plants after...
attack by the common caterpillar pest *Spodoptera littoralis*, including linalool, (Z)-3-hexen-1-yl acetate, myrcene, (E)-2-hexenal and (Z)-3-hexen-1-ol (Gouinguené et al., 2005). Behavioural responses have been recorded for parasitoids to single chemical compounds. The volatiles nonanal, decanal, limonene and 6-methyl-5-hepten-2-one elicit a response in the *Pieris* sp parasitoids *C. glomerata* and *C. rubecula* (Smid et al., 2002). Du et al. (1998) found that the parasitoid *A. ervi* responded behaviourally and electrophysiologically to some of the volatiles emitted by the broad bean, *Vicia faba* attacked by the aphid *Acyrthosiphon pisum*, which included 6-methyl-5-hepten-2-one, (Z)-3-hexen-1-ol, (Z)-3-hexen-1-yl acetate and linalool. Also the aphid parasitoid *A. funebris* responded in behavioural experiments to the chemical (Z)-3-hexen-1-yl acetate (Pareja et al., 2007). Although it is difficult to say if all of the above would be involved in the behavioural response of female *A. colemani*, it is possible to speculate that, (Z)-3-hexen-1-yl acetate, (Z)-3-hexen-1-ol and (+)-sabinene, could be involved in the response towards the cultivars since significant differences were detected in these three compounds between some of the cultivars.

Not all the volatiles found in the present study will be involved in driving the responses registered in the previous chapter. Although in the multivariate analysis, behaviourally active and inactive compounds were considered, the results are in accordance with the behavioural results from the previous chapter. The CVA analysis resulted in less distance between the five cultivars when damaged than when whole. The proximity in chemical profiles of the cultivars as demonstrated by the CVA of detached leaves in addition to the similarity in the relative amounts of some of the compounds registered in the five damaged cultivars (section 4.3.2.2, page 89), would have an impact on the discernment of the parasitoids. The same trend is observed in the results registered in *A. colemani* response. The responsiveness towards whole plants (section 3.3.2.6, page 56) shows five overlapping groups, and the responsiveness towards damaged leaves (section 3.3.3.6, page 62) displays only three non-overlapping groups, difference that could impact in the parasitoids’ behaviour.

The fact that very few differences were detected in individual volatile compound analysis and that significant differences between the cultivars were detected in the CVA, would indicate that the parasitoids are probably eliciting responses upon differences in the ratios of the volatile blends (Bruce et al., 2005) between the cultivars in contrast to using individual volatiles. Two of the chemical compounds responsible for determining between-group variation of whole plants were (E)-2-hexanal and 6-methyl-5-hepten-2-one. The fact that these two compounds elicited behavioural responses, in addition to electrophysiological responses in other parasitoid species makes it possible
to speculate that female *A. colemani* are using at least, these two compounds to discriminate between the cultivars.

Unfortunately it the present study it was not possible to perform electrophysiology to narrow down the 22 compounds to those that could have behavioural activity. A technique such as gas chromatography coupled with electroantennography (GC-EAD) would have allowed this. But because the volatiles were entrained onto the porous polymer “Tenax TA”, which requires thermal desorption to analyze the samples, GC-EAD could not be done. The GC setup in Rothamsted Research only allows samples in liquid form to be coupled with the electroantennography. With volatiles trapped onto another porous polymer such as Poropak-Q, which can be eluted with a solvent (solvent desorption) the GC-EAD analysis would have been possible. Initial trials with Poropak resulted in very low amounts of volatiles captured, thus it was decided to use the porous polymer Tenax, which has a better trapping efficiency for the kind of volatiles that would be captured. In future studies it would be fruitful trying out various porous polymers in which solvent desorption can be carried out and determine which captures sufficient quantities of plant emissions.

### 4.4.3 Conclusions

Significant differences were found in emissions of the five cultivars when analyzed as whole plants and as detached leaves. Individual compound analysis resulted in one compound for whole plants ((Z)-3-hexen-1-yl acetate) and three for detached plants ((Z)-3-hexen-1-ol, (+)-sabinene and (Z)-3-hexen-1-yl acetate) differing significantly between some of the cultivars, suggesting that they might be involved in the behavioural response. In contrast to the few differences found in individual compound analysis, multivariate analysis resulted in cultivars significantly differing between each other for both whole-entrained plants and detached-entrained leaves, in accordance with the behavioural results in Chapter 3 (page 42). The detached-leaves entrainments resulted in a closer distribution between the cultivars of the CVA analysis, probably product of the increase in the proportion of emission of some of the compounds such as (Z)-3-hexen-1-yl acetate, (Z)-3-hexen-1-ol and 6-methyl-5-hepten-2-one to similar levels. This could be a possible explanation to the lower resolving power of parasitoids elicited in the behavioural experiments to the five cultivars when presented as detached leaves compared to the discrimination showed towards whole plants. The results of the CVA also suggest, in conjunction with previous studies on parasitoid chemical detection, that *A. colemani* could be detecting ratios of *(E)*-2-hexanal and 6-methyl-5-hepten-2-one in addition to (Z)-3-hexen-1-yl acetate, (Z)-3-hexen-1-ol and (+)-sabinene and other chemicals when discriminating between the cultivars. Further
investigations in the subject such as coupling gas chromatography with recordings of the electrophysiological activity of the antenna would enable to narrow down the volatiles detected in this study to the behaviourally active ones and gain a better understanding of the parasitoid behaviour and chemical detection capabilities.
Chapter 5
Volatile Molecular Structure and Parasitoid Learning

5.1 Introduction

It is generally believed that parasitoids have evolved to use chemical cues which are both reliable (i.e. offer some certainty of the host presence and possibly host-patch quality) and detectable (i.e. may be detected by the sensory capabilities the parasitoid possess). This may be regarded as the reliability-detectability problem (Vet & Dicke, 1992) (more in section 1.2.4.2.4, page 28), as it is known that both characteristics are negatively correlated, precisely because of selection acting on the host species (Powell et al., 1998a).

In this scenario, plants have also evolved mechanisms to defend themselves against herbivory. As confirmed in the previous chapter, damage or stress generally triggers physiological changes on the plant that increases volatile emissions. This result can be achieved either by synthesising chemical defences such as toxic proteins or metabolites that directly affect the herbivore’s development or by emitting an induced volatile blend that on one hand prime neighbouring plants to activate defences before being attacked themselves (Baldwin et al., 2006) and on the other hand, increase the number of natural enemies that respond towards the plants (De Moraes et al., 1998; Dicke, 1999; De Boer et al., 2004). These volatiles may contain information on the identity of the herbivore, making them reliable for a predator and also the larger biomass of the plant, compared to the one of the insect, makes the volatiles detectable to the carnivores (Vet & Dicke, 1992).

5.1.1 Green leaf volatiles

The bouquet of chemicals produced by plants that enables them to indirectly take action against herbivore attack normally includes those known as green-leaf volatiles (GLVs). This group of chemicals mainly consists of simple compounds originating from
the enzymatic degradation of plant lipids which are converted into a range of six-carbon molecules such as alcohols, aldehydes and acetates (Paré & Tumlinson, 1999; Engelberth et al., 2004). These chemicals occur in all plants, but in different proportions according to species (Visser, 1986). As evidenced in this study (Chapter 4, page 70), GLVs are present in the volatile blends in the five Brassicas analyzed since compounds such as \((E)-2\text{-hexenal}, (Z)-3\text{-hexen-1-ol}\) and \((Z)-3\text{-hexen-1-yl acetate}\) were found in most of the plants. Normally undamaged plants emit low quantities of GLVs, but when leaves are damaged either manually or by herbivore activity, the release rates of these volatiles increases (Whitman & Eller, 1990; Dicke, 1999). This change in release rates is also reported within this study where important increases in the amounts emitted in the GLVs \((Z)-3\text{-hexen-1-ol}\) and \((Z)-3\text{-hexen-1-yl acetate}\) were recorded after leaves were detached.

5.1.2 The recognition of plant volatiles by parasitoids

Since plants emit complex mixtures of volatiles, sometimes up to several hundred compounds, insects have evolved specialized olfactory systems that allow them to detect suitable environments for their needs. It is currently believed that insects recognise appropriate plants by detecting specific ratios of some of these compounds in the blend in contrast to species-specific compounds (Bruce et al., 2005). Examples of this are the highly specific GLV-detecting neurones found in the Japanese scarab beetle, *Phyllopertha diversa* (Hansson et al., 1999) and the discrimination shown in the parasitoid *Microplitis croceipes* which can learn and later differentiate between simple volatiles that differ by two carbon atoms and also can make a distinction in the position of functional groups within similar molecules (Meiners et al., 2002).

The effects of GLVs on the third trophic level have been reported in several occasions. One of the first studies that suggested the relationship between plants and parasitoids through GLVs was done by Whitman and Eller (1990) in which female parasitoids of the species *Microplitis croceipes* and *Netelia heroica*, oriented to individual GLVs in flight tunnel bioassays. Later studies showed that when individual GLVs present in cabbage were presented in a Y-tube olfactometer, natural enemies of the diamondback moth *Plutella xylostella*, *Trichogramma chilonis* and *Cotesia plutellae* elicited significant responses towards them (Reddy et al., 2002). Also the behavioural response of the leafminer parasitoid *Opius dissitus* towards plant-produced GLV \((Z)-3\text{-hexen-1-ol}\) suggest that this chemical might be one of the compounds involved in initial host location in this parasitoid species (Wei et al., 2007). In addition, it is believed that female parasitoid *A. colemani* could rely on certain GLVs to find suitable oviposition sites (Chapter 4, page 70).
Even though plants emit GLVs and insects respond towards them, generalist parasitoids have to find hosts that feed on different plant species which in turn can emit different kinds of volatiles. Parasitoids have overcome this by evolving the ability to learn chemical cues that are related with the presence of their host such as honeydew, excreta and mutualistic organisms (Madden, 1968; Poppy et al., 1997; Buitenhuis et al., 2004). Generally the compounds to which female parasitoids are exposed to during feeding or oviposition prime her to later orient to those chemicals (Lewis & Takasu, 1990; Olson et al., 2003). Even though it is known that receptor neurones are specialized in detecting several kind of volatiles such as sex pheromones (Field et al., 2000), it is believed that insects innately detect GLVs mainly because the receptor neurones are tuned to some extent to a wide variety of six-carbon compounds (Hansson et al., 1999). Therefore it is possible to hypothesise that *A. colemani* females will learn more easily the chemical compounds that are within the range of the six-carbon molecules because of the co-evolution of the parasitoid sensory system and the GLVs.

5.1.3 Objective and hypothesis

The aim in this section is to assess the relevance that slight changes in molecular structure can have on the behaviour of the parasitoid *A. colemani*. The behavioural responses towards a range of ten individual alcohols, with differing number of carbon atoms (1-10), are assessed in olfactometer bioassays in female parasitoids with and without experience of the alcohols. Since GLVs are generally molecules with 6 carbons, it is predicted that parasitoids will be able to learn and to discriminate easier those volatiles that are closest to the 6-carbon molecule. The null hypothesis is that there will not be change in the parasitoids behaviour after experiencing the alcohols.
5.2 Materials and Methods

5.2.1 Wasp training procedure

A protocol was designed to establish if female parasitoids were able to learn olfactory cues under laboratory conditions. A reward was administered by allowing female parasitoids to oviposit on 4th instar or adult aphids during 0.5 h with a constant flow of air with the desired olfactory stimuli. Artificial diet grown-aphids were used to provide a means by which parasitoids could have a reward (i.e. oviposition) while experiencing an odour to which they could be conditioned. This was done in the “conditioning chamber” (Figure 34, A, B and C).

Figure 34: Conditioning chamber used to train female parasitoids. (A) Chamber with the odour arm attached. (B) Chamber with parasitoids and aphids inside before attaching the tube with the olfactory stimulus arm. (C) Top and side view of the conditioning chamber.
In some training protocols, the aphids used were winter harvest-reared *M. persicae* (section 2.1.2, page 34), but in most, aphids were reared on an artificial diet (Dadd & Mittler, 1966). The diet consisted of amino acids, sugars and vitamins prepared in the laboratory. These aphids have been reared in this way for over 25 years but are consistently smaller than the aphids grown on plants (van Emden & Kifle, 2002). Even so, populations of the parasitoid *Aphidius colemani* can be successfully reared on diet aphids (Vamvatsikos, 2006). Aphids were treated with the desired odour by applying approximately 10 nl with a 1 µl micro-capillary filled with the odour in liquid form to 40 aphids. Female parasitoids would still make oviposition attempts on these aphids, although it is not known if any eggs were laid (personal observation). The aphids were introduced into the conditioning chamber followed by 20 female parasitoids, which were aspirated one by one into the chamber using the same method in which they were introduced into the olfactometer (details in section 2.1.7, page 40). The chamber with the aphids and the parasitoids was left for 0.5 h in the wooden box. During this time a steady flow of air (425 ml min⁻¹) was pulled into the chamber through 6 g of activated charcoal with an air pump.

In addition to aphids being topically treated with the desired stimuli, the odour was administered by placing a micro-capillary inside the Perspex tube (i.d. = 0.7 cm, length = 3.6 cm) connected to the conditioning chamber (Figure 35). Micro-capillaries were held with hair curl clips (Superdrug Stores, UK) cut at 15 mm from the base, and positioned at a 45° angle with respect to the horizontal without touching the Perspex tube. Once the “training” phase had finished, parasitoids were freed from the conditioning chamber by opening the lid inside a Perspex cage (20 x 20 x 20 cm) where they could fly freely and could be aspirated into the central chamber of the olfactometer as explained above (section 2.1.6, page 39).

![Figure 35: Alcohol dispenser used to train and test the response of female wasps.](image)
5.2.2 Primary alcohols used in conditioning trials

The objective was to test the behavioural response of female parasitoids towards ten primary alcohols with and without experience of them. The alcohols used differed structurally in the amount of carbon atoms in the molecule, one carbon atom being the smallest molecule and ten carbon atoms the largest (Table 3). Of the ten alcohols used, one of them, 1-hexanol, is found among GLVs.

Table 3: Primary alcohols used in the bioassays.

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Number of carbon atoms</th>
<th>Purity</th>
<th>Brand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>1</td>
<td>99.8</td>
<td>Analar</td>
</tr>
<tr>
<td>1-Ethanol</td>
<td>2</td>
<td>99.86</td>
<td>Hayman</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>3</td>
<td>≥99.80</td>
<td>Aldrich</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>4</td>
<td>≥99</td>
<td>Sigma</td>
</tr>
<tr>
<td>1-Pentanol</td>
<td>5</td>
<td>≥99</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>6</td>
<td>99</td>
<td>Aldrich</td>
</tr>
<tr>
<td>1-Heptanol</td>
<td>7</td>
<td>≥99.0</td>
<td>Fluka</td>
</tr>
<tr>
<td>1-Octanol</td>
<td>8</td>
<td>99</td>
<td>Sigma</td>
</tr>
<tr>
<td>1-Nonanol</td>
<td>9</td>
<td>98</td>
<td>Aldrich</td>
</tr>
<tr>
<td>1-Decanol</td>
<td>10</td>
<td>99</td>
<td>Aldrich</td>
</tr>
</tbody>
</table>

5.2.2.1 Issues with alcohol volatilities

One problem that arose while preparing the bioassays was the difference in volatilities of the alcohols. The alcohol with the heaviest molecule, 1-decanol had the lowest volatility within the range of ten alcohols, around 6000 times less than the alcohol with the lightest and smallest molecule, methanol. This constituted a problem because the amount of alcohol “offered” to the parasitoids during the 0.5 h that the training phase lasted would be ideally similar between the alcohols. The problem was partly solved by dispensing the alcohols from micro-capillaries of different volumes (Drummonds Scientific Co, USA) which basically differ in the internal diameter. This offered a range of different sized surfaces from which the alcohols could evaporate. With the micro-capillary half-filled and standing at a 45° angle, the surface contributing to most of the evaporation is the bottom one (the upper one can be neglected, since no liquid is in contact with it and the molecules exiting the upper orifice by evaporation should be minimal if compared with the amount being evaporated in the lower one). Following this logic, the micro-capillaries with the largest surfaces were used to dispense the alcohols of lowest volatilities and vice-versa (Table 4).

It was decided to use the micro-capillary that would closest match the amount of 1-hexanol that evaporated from a 1 µl capillary during 0.5 h since trial trainings indicated
that parasitoids got positively conditioned to this concentration of volatiles. The volatilities were measured for each of the alcohols by placing four micro-capillaries filled with the alcohols in the olfactometer arms with the air flow set at 425 ml min\(^{-1}\). The capillaries were left enough time to get at least 10 mm evaporation from the column. The volatilities for each alcohol were finally calculated by averaging the amount of alcohol evaporated from the four capillaries per unit of time. Unfortunately due to manufacturing restrictions, not all the micro-capillaries could be matched with all the alcohols according to this scheme. This happened when volatilities became too high and it was not possible to obtain capillaries with such a small opening-surface. In this situation, the smallest surface available was used. This occurred for methanol and 1-ethanol where the alcohols evaporated before the end of the bioassay, so a longer 1 µl micro-capillary (long) was used since it had smaller internal diameter. Another exception was in the case of 1-decanol where two 100 µl micro-capillaries were used to compensate for the low volatility. In addition to this, it was not possible to control the speed of evaporation from the topically-applied alcohol on the aphids.

Table 4: Theoretical and experimental relative volatilities of each of the alcohols respective to 1-hexanol. The micro-capillaries used in the bioassays are also represented with the exposure areas of each one and the relative size with respect to a 1 µl micro-capillary.

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Theoretical relative volatility respective to 1-hexanol</th>
<th>Measured relative volatility respective to 1-hexanol</th>
<th>Volume of micro-capillaries used (µl)</th>
<th>Surface of exposure (mm(^2))</th>
<th>Change in surface respective to a 1 µl micro-capillary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>+ 108</td>
<td>+ 36</td>
<td>1 (long)</td>
<td>0.016</td>
<td>-2</td>
</tr>
<tr>
<td>1-Ethanol</td>
<td>+ 50</td>
<td>+ 30</td>
<td>1 (long)</td>
<td>0.016</td>
<td>-2</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>+ 17</td>
<td>+ 25</td>
<td>0.5</td>
<td>0.008</td>
<td>-4</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>+ 5</td>
<td>+ 9</td>
<td>0.5</td>
<td>0.008</td>
<td>-4</td>
</tr>
<tr>
<td>1-Pentanol</td>
<td>+ 2</td>
<td>+ 4</td>
<td>0.5</td>
<td>0.008</td>
<td>-4</td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.032</td>
<td>1</td>
</tr>
<tr>
<td>1-Heptanol</td>
<td>- 2</td>
<td>- 3</td>
<td>2</td>
<td>0.061</td>
<td>+2</td>
</tr>
<tr>
<td>1-Octanol</td>
<td>- 6</td>
<td>- 8</td>
<td>10</td>
<td>0.243</td>
<td>+8</td>
</tr>
<tr>
<td>1-Nonanol</td>
<td>- 37</td>
<td>- 23</td>
<td>100</td>
<td>0.864</td>
<td>+27</td>
</tr>
<tr>
<td>1-Decanol</td>
<td>- 60</td>
<td>- 52</td>
<td>2 x 100</td>
<td>1.728</td>
<td>+55</td>
</tr>
</tbody>
</table>
5.2.3 Bioassays

5.2.3.1 Testing the protocol with strawberry odour

Previous studies have shown that parasitoids learn associatively the odours of various artificial flavours (Iizuka & Takasu, 1998; Olson et al., 2003). To ensure that the set-up was adequate to impart positive experiences to female parasitoids, tests were made with a liquid form of strawberry artificial food flavouring (Create Flavours, North Somerset, UK).

The response of female parasitoids was tested after various protocols with the odour of a strawberry flavouring against clean air. Firstly, the behavioural response of inexperienced female parasitoids was tested towards the strawberry flavour against clean air over 0.5 h in the olfactometer (treatment: inexperienced). Secondly, the response was tested only after females had been in the conditioning chamber over 0.5 h with a constant flow of air mixed with the strawberry odour (treatment: strawberry odour alone). For this, the strawberry food-flavouring was dispensed from a cut (25 mm long) 100 µL micro-capillary half-filled with the liquid and placed in the Perspex tube of the conditioning apparatus (Figure 35, page 107). In a third bioassay, parasitoids were exposed to aphids reared on winter harvest plants together with a flow of the strawberry odour over 0.5 h (treatment: strawberry odour + plant-aphids). Lastly, in a fourth bioassay, aphids reared on the artificial diet were offered to parasitoids at the same time that the odour flowed through the conditioning chamber during 0.5 h (treatment: strawberry odour + diet-aphid). When parasitoids were subjected to experiences with aphids and the strawberry odour, the flavouring was also applied topically to aphids. Approximately 10 nl aphid\(^{-1}\) of flavouring were applied with a 1 µl micro-capillary to 40 aphids). Females would still make oviposition attempts in these aphids, though it was unknown if egg were laid (personal observation). After the training period, parasitoids were tested in the detached-leaf olfactometer used in a two-way fashion with the strawberry flavour placed in two arms of the olfactometer and the other two arms containing two empty micro-capillaries. Bioassays commenced 10 min after the. Eight replicates were carried out with twenty female parasitoids in each.

5.2.3.2 Response of inexperienced females to the primary alcohols

The behavioural responses of inexperienced female *Aphidius colemani* towards the ten primary alcohols were assessed in the olfactometer in ten individual bioassays. Females were left in the conditioning chamber with a clean air flow during 0.5 h. It is important to state that no aphids were placed inside the conditioning chamber during the 0.5 h since it is possible that the presence of aphids in conjunction with a flow of
clean air, could have positively conditioned the parasitoids towards the clean air, “hiding” the response of an inexperienced female towards the alcohol once the behavioural response was tested.

After the period in the conditioning chamber, the behavioural response of the females was tested towards one of the ten alcohols in the olfactometer -version I-. Micro-capillaries were filled with the desired alcohol following the protocols explained above and allowed to evaporate inside the olfactometer arms over 0.5 h. In every bioassay only one alcohol was used against a control of empty micro-capillaries. The micro-capillaries filled with alcohol were introduced in two arms of the olfactometer while the other two arms contained empty micro-capillaries.

5.2.3.3 Response of experienced females to the primary alcohols

The behavioural response of *A. colemani* was tested in the olfactometer with 1 of the ten alcohols. Prior to the bioassay, females were subjected to the conditioning protocol over 0.5 h, with a flow of one of the then alcohols (the same one as tested in the olfactometer) and diet-reared aphids. It was decided to use aphids reared on the artificial diet since the level of significance the responses of parasitoids that were trained to the strawberry flavour, was slightly higher (although not significant) when compared to the result of the training with aphids reared on plant (P < 0.01 vs. P < 0.05) (Figure 36, page 112). Ten individual sets of bioassays were carried out, one with each of the ten primary alcohols.

5.2.4 Statistical analysis

5.2.4.1 Bias in choice and difference between treatments

The bias in the choice of female parasitoids and the difference between different treatments was analyzed as described in section 2.1.8 (page 41).

5.2.4.2 Affect of experience on the behavioural response

In order to evaluate the effect that experience with an individual caused in the behavioural response of female parasitoids, the response of inexperienced females towards one alcohol was compared to the response of experienced females towards that same alcohol. On one hand the effect on the proportion of non-responding wasps assessed and on the other hand the effect was assessed on the proportion of wasps that responded towards the alcohol. In this way it was possible to detect increases or decreases in the response of female wasps towards the alcohols after they had the experience. The statistical method used for this is explained further in section 2.1.8.2 (page 41).
5.3 Results

5.3.1 Testing the protocol with strawberry odour

The response of inexperienced females and those that experienced only the strawberry odour, resulted in wasps eliciting no bias towards the strawberry odour nor towards the clean air ($\chi^2$ inexperienced $= 0.18$, $\chi^2$ strawberry $= 0.46$, $P > 0.05$, df = 1) (Figure 36). In contrast, parasitoids that had access to plant- or diet-reared aphids in concurrence with the odour of strawberry, were biased towards these arms ($\chi^2$ plant-aphids + strawberry $= 5.70$, $P < 0.05$; $\chi^2$ diet-aphids + strawberry $= 8.02$, $P < 0.01$; df = 1). When the proportion of non responders was compared between treatments, there were no significant differences ($F = 1.61$, $P > 0.05$, df = 28). When the proportion responding to the strawberry odour was compared across treatments, significant lower responses towards the strawberry odour were detected for inexperienced, strawberry odour alone treated wasps compared to that of plant-aphid and the diet-aphid experienced females ($t = 3.84$, $P < 0.001$, df = 30).

![Figure 36](attachment:image.png)

Figure 36: Response of A. colemani females with no experience with aphids or strawberry odour, with strawberry odour alone, with strawberry odour together with aphids reared either on plants or artificial diet. When the strawberry odour was presented with aphids, parasitoids became positively conditioned to the odour. ** ($P < 0.01$), * ($P < 0.05$) and ns (not significant; $P > 0.05$) compare the response of the females within each treatment. Uppercase letters compare the proportions of non-responding females across treatments. Lowercase letters compare the differences in proportions across the four different treatments of the females that responded towards the choices.
5.3.2 Response of inexperienced females to the primary alcohols

The responses of inexperienced females towards the ten alcohols varied (Figure 37) and females significantly moved to the arms free of the alcohols in the cases of 1-propanol ($\chi^2 = 4.67$, $P < 0.05$, df = 1), 1-pentanol ($\chi^2 = 20.09$, $P < 0.001$, df = 1), 1-heptanol ($\chi^2 = 14.74$, $P < 0.001$, df = 1), 1-octanol ($\chi^2 = 4.94$, $P < 0.05$, df = 1), 1-nonanol ($\chi^2 = 4.15$, $P < 0.05$, df = 1). Non-significant responses were recorded in the cases of methanol ($\chi^2 = 0.02$, $P > 0.05$, df = 1), 1-ethanol ($\chi^2 = 0.92$, $P > 0.05$, df = 1), 1-butanol ($\chi^2 = 0.01$, $P > 0.05$, df = 1), 1-hexanol ($\chi^2 = 2.25$, $P > 0.05$, df = 1), and 1-decanol ($\chi^2 = 0.35$, $P > 0.05$, df = 1). The proportion of non responders did not differ significantly between the treatments ($F = 1.61$, $P > 0.05$, df = 70). When the proportions of wasps responding to the alcohol was compared between the treatments, the proportion of wasps responding towards 1-pentanol was lower than the rest ($F = 2.41$, $P < 0.05$, df = 70).

![Diagram of responses of inexperienced female Aphidius colemani towards ten primary alcohols against an alternative of clean air. In general females moved towards the arms of the olfactometer that did not contain the alcohol. *** (P < 0.001), ** (P < 0.01), * (P < 0.05) and ns (not significant; P > 0.05) compare the response of the females within each treatment. Uppercase letters compare the proportions of non-responding females across treatments. Lowercase letters compare across the different treatments the differences in proportions of females responding to the alcohol.](image-url)
5.3.3 Response of experienced females to the primary alcohols

The response of female parasitoids that had experienced one of the alcohols in conjunction with diet-reared aphids, was biased towards the arms that contained clean air in methanol ($\chi^2 = 15.28, P < 0.001, \text{df} = 1$), 1-ethanol ($\chi^2 = 10.85, P < 0.001, \text{df} = 1$), 1-propanol ($\chi^2 = 14.36, P < 0.001, \text{df} = 1$), 1-butanol ($\chi^2 = 24.22, P < 0.001, \text{df} = 1$), 1-hexanol ($\chi^2 = 6.04, P < 0.001, \text{df} = 1$), 1-heptanol ($\chi^2 = 13.52, P < 0.001, \text{df} = 1$), 1-octanol ($\chi^2 = 21.04, P < 0.001, \text{df} = 1$) and 1-decanol ($\chi^2 = 24.85, P < 0.001, \text{df} = 1$) (Figure 38). The wasps treated with 1-pentanol ($\chi^2 = 5.57, P < 0.05, \text{df} = 1$) and 1-nonanol ($\chi^2 = 3.25, P > 0.05, \text{df} = 1$) did not elicit differential responses towards any of the two choices offered. The proportion of wasps that did not respond was significantly higher in the 1-octanol and 1-nonanol treatments when compared with methanol, 1-propanol and 1-butanol (variables grouped together, $t = 3.17, P < 0.01, \text{df} = 38$). In addition, there were no differences across the 10 different treatments in the proportion of wasps responding towards the alcohols ($F = 0.72, P > 0.05, \text{df} = 70$).

Figure 38: Response of female *Aphidius colemani* after being exposed to airborne alcohols and diet-reared aphids. Females were generally biased towards the clean-air alternative. *** ($P < 0.001$), * ($P < 0.05$) and ns (not significant; $P > 0.05$) compare the response of the wasps within each treatment. Uppercase letters compare the proportions of non-responding females across treatments. Lowercase letters compare across the four different treatments the differences in proportions of females responding to alcohol.
5.3.4 Affect of experience on the response to the primary alcohols

The response towards the alcohols of inexperienced females was compared to the response of experienced ones. When wasps were exposed to 1-hexanol, the amount of non-responding wasps decreased (Figure 39 and Table 5) while when wasps were exposed to 1-pentanol the proportion of wasps that responded towards that alcohol increased after the experience. In the other treatments the experience did not have an effect on the response of the females.

![Graph](image)

Figure 39: Change in the proportion of non-responders and those that responded to the airborne alcohol after the training period. The proportion of non-responding wasps decreased when 1-hexanol was used and females increased their response towards 1-pentanol after the training. * (P < 0.05) and ns (not significant; P > 0.05) compare the difference in the pre- and post-experience response.

![Table](image)

Table 5: t-Statistic and P values for the change in the proportion of non-responding wasps and wasps that responded towards the alcohol after experiencing them.

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Change in proportion of non responding wasps after experience</th>
<th>t</th>
<th>P</th>
<th>Change in proportion of wasps responding to the alcohol after experience</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol</td>
<td>-</td>
<td>1.65</td>
<td>&gt; 0.05</td>
<td>-</td>
<td>2.13</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>1-ethanol</td>
<td>-</td>
<td>0.51</td>
<td>&gt; 0.05</td>
<td>-</td>
<td>1.47</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>1-propanol</td>
<td>-</td>
<td>1.54</td>
<td>&gt; 0.05</td>
<td>-</td>
<td>0.62</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>1-butanol</td>
<td>-</td>
<td>1.11</td>
<td>&gt; 0.05</td>
<td>-</td>
<td>1.63</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>1-pentanol</td>
<td>-</td>
<td>0.89</td>
<td>&gt; 0.05</td>
<td>increased</td>
<td>2.23</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>1-hexanol</td>
<td>decreased</td>
<td>2.86</td>
<td>&lt; 0.05</td>
<td>-</td>
<td>0.34</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>1-heptanol</td>
<td>-</td>
<td>0.31</td>
<td>&gt; 0.05</td>
<td>-</td>
<td>0.03</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>1-octanol</td>
<td>-</td>
<td>0.91</td>
<td>&gt; 0.05</td>
<td>-</td>
<td>0.28</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>1-nonanol</td>
<td>-</td>
<td>1.86</td>
<td>&gt; 0.05</td>
<td>-</td>
<td>0.61</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>1-decanol</td>
<td>-</td>
<td>1.69</td>
<td>&gt; 0.05</td>
<td>-</td>
<td>1.84</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>
5.4 Discussion

5.4.1 Response towards the strawberry flavour

The response of female parasitoids with no experience in the conditioning chamber and the response of females that had experienced the strawberry odour by itself were not significantly biased towards the odour of the flavour or the clean air the behavioural trials. These results suggest that female *A. colemani* were not conditioned to a similar type of chemical compound present in the flavouring odour that was present in the rearing cages (i.e. as one emitted by winter harvest or *M. persicae*) and that female parasitoids do not have an innate response towards the artificial strawberry odours.

Contrastingly, when the odour of the flavour was presented together with aphids in the same environment, it affected *A. colemani* behavioural response by biasing it towards the odour stimuli. Regardless the origin of the aphids, after encountering and ovipositing on plant-reared or diet-reared aphids during 0.5 h, females responded positively towards the strawberry odour. Many examples in the literature show the learning capabilities of parasitoids (Olson et al., 2003; Rains et al., 2006), but Lewis and Takasu (1990) conducted the first study that established the learning capabilities of parasitoids could reach comparable levels of adaptability shown by honeybees and rats. In this study, the authors reported that female parasitoids *Microplitis croceipes* learned artificial odours (vanilla and chocolate) associated with the host and food resources that later could be used to find the host or the food more efficiently. In a similar way, in the present study the response evidenced after the training with the strawberry odour, shows that *A. colemani* females can modify their behaviour if exposed during 0.5 h to a novel odour. The process taking place is probably associative learning, through which parasitoids can link the strawberry odour with the presence of hosts, showing that the setup used is adequate to train females to an inexperienced odour.

5.4.2 Response of inexperienced females to the primary alcohols

The response of inexperienced female parasitoids towards the ten alcohols tested in the bioassays was variable but with the common trend: Parasitoids did not respond significantly towards the alcohols. In six of the treatments (1-propanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol and 1-nonanol) females made a significant choice towards the tubes that contained the clean air, which is indicative that parasitoids posses receptors that are sensitive to these alcohols. In the other four bioassays females were not biased to any of the choices (methanol, 1-ethanol, 1-butanol, and 1-
decanol). This response could be, most probably, the result of females detecting the odour of the alcohol but not eliciting a differential response to any of the choices, since a second scenario where females lack the appropriate odour receptor neurones to detect the particular odourant, is discarded because of the results obtained in females that underwent the training period, showing that after the training with the alcohols in question, females were biased to clean air when offered the choice of the alcohol, indicating that there are receptors that are sensitive to the particular alcohol molecules. Nevertheless, the response of inexperienced females towards the alcohols showed that they did not have innate (or previously learnt) responses towards the alcohols.

5.4.3 Response after training to the primary alcohols

Several studies have dealt with the way in which parasitoids, once learnt, discriminate between several similar molecular structures. For example, the parasitoid *M. croceipes* needs a difference of at least two C-units for successful discrimination between structurally-similar chemical compounds (Wäckers, 1994) and it appears this (i.e. 1-hexanol v 1-octanol) is done at the level of the central nervous system rather than in the sensilla (Stopfer et al., 1997). In the present investigation the intent was to evaluate differences in the learning performance of female parasitoids towards similar structured molecules to assess any correlation between the way parasitoids learn and the GLVs, which are six-carbon molecules.

When females were exposed to the alcohols after the training period, wasps elicited significant responses towards the arm with the clean air in eight of the ten bioassays. Although the training protocol worked for the volatiles of strawberry, resulting in parasitoids learning by classical conditioning (Pavlov, 1927) (see section 3.1.3, page 42 for more details) to the odour of strawberry by associating an originally neutral stimulus with a biologically relevant stimuli, when alcohols were used, the trend seen in the behaviour of inexperienced females, was accentuated by females responding to the clean-air choice in previously non-significant choices. These were the cases of the treatments involving methanol, 1-ethanol, 1-butanol, and 1-decanol. In addition, as observed in inexperienced females, responses to clean air were recorded for the rest of the alcohols except with 1-pentanol and 1-nonaol where females did not elicit a bias in their choice. The results suggest that rather than offering a positive experience, the training for some reason, imparted a negative experience in most of the situations, eliciting and accentuating the negative response initially seen towards the alcohols.

Parasitoids are known for their learning capabilities but failure in learning to certain volatiles has been recorded in other studies. After a training period which was
otherwise proven to impart positive learning experiences to *M. croceipes*, parasitoids failed to respond towards 1-nonanol and 1-decanol (Meiners et al., 2002), although electrophysiological responses were detected towards these compounds (Ramachandran & Norris, 1991). This could partially explain the results found in this study, but would only address the responses towards the alcohols larger or smaller than 1-hexanol.

When the responses elicited by females after the training period were compared with the responses elicited by inexperienced females, two significant changes were detected. The number of females found in the olfactometer arms increased significantly when treated with 1-hexanol and increased their response towards 1-pentanol. The fact that the only change in behaviour was detected in 1-hexanol and in one of the neighbouring molecules could be an indicator of the higher learning capacity or sensitivity of wasps around the six-carbon molecule. Electrophysiological responses of antennal sensilla of the parasitoid *M. croceipes* were found to be maximum with 1-hexanol when compared with decreases or increases in the carbon chain length of primary alcohols (Li et al., 1992). In addition highly adaptable learning capacity has been shown in behavioural studies towards 1-hexanol and structurally similar molecules such as 1-hexanal, 2-hexanol and 3-hexanol in the parasitoid *M. croceipes* (Meiners et al., 2002) and even in non-parasitoid organisms, such as the cockroach *P. americana*, positive responses towards various six-carbon molecules were shown to be elicited after learning experiences (Sakura et al., 2002). The results obtained in this study, at least towards 1-hexanol and structurally neighbouring molecules, were not as expected and could be due to a malfunctioning of the protocol. Even though the adequate functioning was assessed with strawberry flavourings, and evidence of a change in the behaviour was found, it is possible that parasitoids might need less time exposed to the odour. Lewis and Takasu (1990) found that the number of experiences increased the ability to choose correctly a particular odour in a parasitoid, and it is possible that shorter (or longer) and spaced training events could have rendered different results. Also changing the way in which alcohols are dispensed, such as diluting them, could give fruitful results. For example, the cockroach *P. americana* learned the odour of 1-hexanol at a low and high concentration (a filter paper with 20 µl of a 1 and 100 µg µl⁻¹ respectively of 1-hexanol diluted in liquid paraffin), but failed to do so with an intermediate one (a filter paper with 20 µl of a 5 µg µl⁻¹ solution of hexanol diluted in liquid paraffin) (Sakura et al., 2002).
5.4.4 Conclusions

Inexperienced parasitoids displayed a response towards the clean-air alternative rather than towards the alcohols. The same trend, but more pronounced was observed when females underwent a training experience with the alcohol. When the responses towards the alcohol was compared before and after the training period, a statistically significant increase of responding wasps was detected when 1-hexanol was used, in addition to a statistical significant increase of females that responded towards 1-pentanol. Although it was not possible to find a correlation between the carbon chain length and the behaviour of the parasitoid, it is believed that the results recorded in this study can be improved and a better understanding of the parasitoids learning abilities can be achieved with modifications in training protocol.
Chapter 6
Parasitoid Memory Loss and Innate Olfactory Preferences

6.1 Introduction

6.1.1 Learning

The enormous capacity for associative learning found in many parasitic wasp species can be explained by the need to locate suitable hosts in constantly changing environments (Godfray, 1994). They have evolved to utilise a variety of cues that derive from different trophic levels, including those cues produced by the host itself such as excreta and pheromones, and also infochemicals from plants and mutualistic organisms (Buitenhuis et al., 2004; Martínez et al., 2006) (more in Chapter 1, page 17).

Learning to associate cues with the presence of an appropriate host is one of several ways in which a parasitoids fitness can be increased (Dukas & Duan, 2000). There are some 20 examples of hymenopterous parasitoid species that show learning capabilities (Langley et al., 2006) and aphid parasitoids have been shown to respond robustly towards various plant volatiles according to their previous experience (van Emden et al., 2002; Powell & Pickett, 2003; Lo Pinto et al., 2004).

As shown in this study (Chapter 3, page 42) and in many others (Douloumpaka & van Emden, 2003; Bilu et al., 2006), females of the generalist aphid parasitoid *Aphidius colemani* respond to the odour of the plant cultivar or species on which they were reared over other species/cultivars. Although it has been suggested that when the imago emerges from the mummified aphid, it encounters and becomes conditioned to relevant chemicals cues, a novel hypothesis suggests that the imago may become conditioned even while still inside the mummy (Vamvatsikos, 2006) or even during the larval stages (Gutiérrez-Ibáñez et al., 2007) (in more detail in section 3.1.4.1, page 43).

Despite the fact that this initial conditioning is strong, it can be overridden by oviposition encounters or contact with aphid mummies, when females adult of *A. colemani* and *A. ervi* learn the odour of the plant-host complex on which they encountered hosts (Du et
al., 1997; Storeck et al., 2000). An interesting study which involved learning processes in *A. colemani* females (Storeck, 2002), allowed contact of parasitoid females with mummy cases reared on other plant varieties and then these newly-acquired memories were disrupted with cold temperatures. After these treatments, female parasitoids retrieved the memory that had been stored prior to the new experience, revealing a layering in memory storage and retrieval.

### 6.1.2 Innate responses

Experience seems to be of crucial importance in host-searching behaviour in parasitoids, but innate responses can also play an important role. The behaviour of the parasitoid *A. ervi* responding to aphid sex pheromones in the field and in the laboratory is attributed to genetic factors (Hardie et al., 1994; Poppy et al., 1997). Also Geervliet *et al.* (1996) detected differences in the innate responses of the parasitoids *Cotesia glomerata* and *C. rubecula* towards various plant species. The relative importance of innate and learnt responses seems to depend on the level of specificity of the parasitoids in question. It is argued that generalist parasitoids, having a broad host-range, tend to rely mostly on learnt cues, but there is evidence that they could also rely innately on general cues common to several plant-host complexes during initial host-foraging experiences and later learn specific cues once the plant-host complex has been found (Steidle & Johannes, 2002). To what extent parasitoids use innate or learnt cues to locate appropriate hosts is largely unanswered.

### 6.1.3 Cold and memory

The influence of cold in parasitoids has been recorded on numerous occasions but mainly focusing on longevity, reproductive success and survival of the adults insects after the cold treatments (Hofsvang & Hågvar, 1977; Whitaker-Deerberg et al., 1994; Hallman & Denlinger, 1998; Legrand et al., 2004). For instance, a recent study established that extreme temperatures can have impacts on morphology of antennae sensilla when applied during the development of the parasitoid *A. rhopalosiphi* (Bourdais et al., 2006) while a second study documented how cold affects energetic reserves and survival rates in adult *A. colemani* parasitoids (Colinet et al., 2006).

Cold temperatures have also been used as a tool to study memory dynamics in several insects species such as the honeybee (Hammer & Menzel, 1995; Menzel, 2001) and to some extent in *Drosophila* spp. (Xia et al., 1997; Xia et al., 1998) but until now it has received little attention in parasitoids (Ueno & Tanaka, 1996; Storeck, 2002; Kaiser et al., 2003; McKay & Broce, 2004). When a learning event takes place, a memory that can later be retrieved is formed in the insect brain. When this memory-formation
process takes place, it is consolidated in a two-way process: The first stage is a short- 
term process which does not require protein synthesis and therefore during this period 
the memory is particularly vulnerable to interference, the advantage is that it can be 
accessed immediately after the learning event took place. When a memory is in this 
stage of consolidation, it is generally referred to as short-term memory. The second 
stage, termed long-term memory, is a slow process that involves protein synthesis 
(Alloway, 1972; DeZazzo & Tully, 1995) and is stable.

6.1.4 Objective and hypothesis
The fact that newly-emerged Aphidiid parasitoids seemingly have already learnt 
olfactory stimuli, makes studying innate olfactory preferences a difficult task and the 
idea of obtaining naive females to study innate preferences through disrupting any 
memory that parasitoids could have through cold treatments is studied in this chapter.
The extent of the memory loss is also investigated. It is predicted that cold 
temperatures will affect the olfactory behaviour of parasitoids. The null hypothesis is 
that females will have an identical response to odours of plant material when exposed 
to cold when compared to untreated females.
6.2 Materials and methods

Female parasitoids were subjected to cold treatments of different temperatures and durations after which their behavioural olfactory response was tested towards volatiles emitted by different plant material in the olfactometer.

6.2.1 Bioassays and plant material

The stimuli presented to the female wasps were odours from freshly-detached cultivars of *B. oleracea*. Two Brussels sprouts cultivars, winter harvest and red delicious were used, and also the cabbage cultivar golden acre (cabbage). The detached-leaf olfactometer -version1- (section 2.1.4.1, page 35) was used in all the bioassays in a two-choice fashion by placing inside two of the arms 5 - 7 leaves of one plant cultivar and in the two remaining arms the same amount of leaves of another cultivar. When greenhouse-grown wheat (*Triticum aestivum* var. Ashby) was used, around 30 - 40 (12-cm long) stems were cut from 4-week old plants (30-cm tall), folded in half and placed inside the olfactometer arms. When lime tree (*Tilia x europea*) or lupin (*Lupinus sp.*) leaves were used, seven leaves of lime tree and one leaf of lupin were cut from plants located in Silwood Park gardens (Ascot, UK), 5 min previous to the bioassays. In some bioassays a contrast was made between plant material against clean air, in which case, two filter papers (Whatman, Ø = 42.5mm) dampened with 200 µl of distilled water were placed inside the odour arms. Control trials showed that the levels of water-content in the air inside an olfactometer arm produced by a dampened filter paper compared with the amounts of leaves used for each plant were similar (more in Chapter 7, page 135).

6.2.2 Temperature treatments

Female *A. colemani* were aspirated into modified 200 µl Eppendorf tubes in groups of approximately 20 and exposed to the temperature treatments. Around forty holes (Ø = 0.4 mm) were made in the lower half of the Eppendorf tubes to allow air circulation during the treatment and also allowed the females to be aspirated into the tubes. The inside of the Eppendorf lid housed a small cotton-wool ball dampened in distilled water. Survival ranged between 100 % (n = 176) at 20 °C for 24 h and 90 % (n = 178) at 0 °C for 24 h.

For temperature treatments at 20 °C, Eppendorf tubes containing wasps were placed inside glass vials (5 x 2.5 cm) inside a light-tight box (7 x 5 x 3 cm) and placed in a temperature-controlled cabinet. In treatments where the temperatures were below 20 °C, a thermoelectric module was used. This Peltier device was modified to hold an
aluminium block (0.9 x 3.5 x 10.5 cm) which was attached to the one of the ceramic plates and the heat was removed from the opposite plate by means of a constant flow of cold water (Lees, 1986) (Figure 40 A and B). In this way the temperature of the metal block could be controlled by changing the amount of current circulating through the ceramic plates. Five small holes (Ø = 0.7 cm) were drilled 2.3 cm into the metal block, which fitted the 200 µl Eppendorf tubes with the wasps inside. A sixth hole (Ø = 0.3 cm, 0.7-cm deep) housed a probe (TX-0023, Gemini Data Loggers Ltd, UK) which monitored the temperature during the treatments. The device was inside a Polystyrene box (27 x 27 x 27 cm) with 200 g of Silica gel in a Petri dish to reduce humidity and condensation. The temperature control was ± 0.5 °C (Figure 40 C)

Figure 40: Peltier device used to impart temperature treatments to female parasitoids Aphidius colemani. (A) Polystyrene box with a Petri dish containing Silica gel and the power source. (B) temperature of the metal block that contained the Eppendorf tubes with parasitoids and the thermometer’s probe to monitor the temperature. (C) Temperature readings of the Peltier device over 24 h offering a ± 0.5 °C control.
6.2.3 Temperature protocols

Females used in all the experiments were taken directly from the rearing cages where the association to the odour of the cultivar winter harvest that they experience during rearing, emergence and possibly during oviposition, would be strong (Vamvatsikos, 2006). The association with the plant was tested immediately after the females were removed from the rearing cages as a control (0 h treatment), and 24 h later (20 °C-24 h treatment). To establish the affect of low temperatures on the olfactory behaviour of the parasitoids, females were exposed to 5 °C (5 °C-24 h treatment) and 0 °C for 24 h (0 °C-24 h treatment). Immediately after the treatments (within 15-20 min acclimatization to ambient temperature), wasps were tested for preference between winter harvest and cabbage-leaf odour in the olfactometer.

6.2.4 Affect of 0 °C on the general olfactory behaviour

To establish to what extent females that underwent 24 h at 0 °C (0 °C–24 h treatment) would have their behaviour altered, the preference towards three B. oleracea varieties odours (winter harvest, red delicious and cabbage) and wheat were tested individually against clean air. A control series was also carried out with wasps tested immediately after being removed from the rearing cages.

6.2.5 Affect of duration of exposure to 0 °C

Further studies were conducted by testing the preference of female wasps towards winter harvest against cabbage after being chilled at 0 °C for 12 h, 6 h, 1h, 0.5 h and 0.1 h.

6.2.6 Affect of time elapsed after cold treatment

Instead of testing the behavioural response after an acclimation period of 15 min, females were tested 1 h after the temperature treatment had finished. The response to winter harvest against cabbage was tested after females were chilled at 0 °C for 1 and 24 h (0 °C - 1 h + 1 h and 0 °C - 24 h + 1 h, respectively) only after being left for 1 h at room temperature (20 °C - 25 °C) in the central chamber of the olfactometer to acclimatize prior to the bioassay.

6.2.7 Statistical analysis

6.2.7.1 Bias in choice and comparison between sets of bioassays

Biases in olfactometer responses were analyzed with a GLM as explained in section 2.1.8.1 (page 41). The following terms were included in the model: Alternatives offered, olfactometer arm, and position of olfactometer in the in wooden box. Non-significant
terms were removed from the model and generally are not mentioned in the results section. Comparisons between overall responses and duration of exposure to cold are explained in section 2.1.8.2 (page 41).

6.2.7.2 Affect of duration of exposure

The response of female parasitoids after being exposed to 0 °C for different times was analyzed as for the comparisons between the overall responses and responses to plant material explained in section 2.1.8.2 (page 41) but with one difference: Instead of considering the different treatments as categorical data, the time that wasps spent in the cold (from 0 to 24 h) was considered as a continuous variable. Therefore instead of comparing individual treatments between each other, it was possible to assess an effect of the time exposed to 0 °C on the proportion of overall response towards the plant material and on the proportion of wasps that exclusively responded to winter harvest.
6.3 Results

6.3.1 Temperature protocols

In the 0 h and 20 °C-24 h treatments, the number of wasps that responded to the arms that contained winter harvest leaves was significantly greater than the number of wasps that responded to the volatiles of cabbage leaves ($\chi^2$ 0 h treatment = 27.25, $\chi^2$ 20 °C-24 h treatment = 20.71, $P < 0.001$, df = 1) (Figure 41). After the 5 °C-24 h treatment wasps still responded significantly more to winter harvest ($\chi^2 = 4.43$, $P < 0.05$, df = 1) while in the 0 °C-24 h treatment, females did not show a bias ($\chi^2 = 0.60$, $P > 0.05$, df = 1) and the response was affected in such a way that the heterogeneity between the replicates in this last treatment was significant ($\chi^2 = 22.80$, $P < 0.001$, df = 7). The proportion of non-responding females was significantly lower in the 0 h and the 5 °C-24 h treatment when compared to the other two treatments ($t = 4.213$, $P < 0.001$, df = 28). In addition, females that did respond and were biased to winter harvest when contrasted to cabbage, was significantly higher in the 0 h and 20 °C-24 h treatments than in the other two treatments ($t = 3.707$, $P < 0.001$, df = 31).

![Figure 41: Proportion (mean ± 1 S.E.) of Aphidius colemani females responding to detached winter harvest or cabbage leaves after being removed from the rearing cages (0 h, n = 145), after being held for 24 h at 20 °C (20 °C-24 h, n = 160), 5 °C (5 °C-24 h, n = 157) and 0 °C (0 °C-24 h, n = 138). Only at 0 °C there was no bias in the choice, in the other treatments wasps were biased to winter harvest, *** (P < 0.001), * (P < 0.05) and ns (not significant; P > 0.05) compare the response within each treatment. Uppercase letters compare the proportions of non-responding wasps across treatments. Lowercase letters compare across the four treatments the differences females responding to winter harvest against those responding to cabbage.](attachment:figure41.png)
6.3.2 Affects of 0 °C on the general olfactory behaviour

When females where tested immediately after being removed from the rearing cages, the response was significant towards the arms containing the leaves in all six treatments ($\chi^2_{\text{winter harvest}} = 35.31$, $\chi^2_{\text{red delicious}} = 17.61$, $\chi^2_{\text{cabbage}} = 34$, df = 1, $\chi^2_{\text{wheat}} = 27.3$, $P < 0.001$; $\chi^2_{\text{lupin}} = 9.06$, $P < 0.05$; $\chi^2_{\text{lime tree}} = 3.87$, $P < 0.05$; df = 1) (0 h in Figure 42). There were significant differences between the six plant-treatments in the number of non-responding wasps with a higher proportion of non-responding wasps in lupin compared to wheat, cabbage, winter harvest and red delicious ($t_{\text{wheat}} = 2.42$, $P < 0.05$, df = 14)) and between lime tree compared to cabbage, winter harvest and red delicious ($t = 2.63$, $P < 0.05$, df = 14). No differences were detected within the number of responding wasps that were biased towards the plant ($F = 0.93$, $P > 0.05$, df = 42).

When parasitoids were treated at 0 °C during 24 h, females significantly moved into the arms that contained the plant leaves ($\chi^2_{\text{winter harvest}} = 19.54$, $\chi^2_{\text{red delicious}} = 19.37$, $\chi^2_{\text{cabbage}} = 7.01$, $\chi^2_{\text{lupine}} = 26.42$, $P < 0.001$; $\chi^2_{\text{lime tree}} = 8.18$, $P < 0.01$; $\chi^2_{\text{wheat}} = 4.72$, $P < 0.05$, df = 1) (0 °C-24 h in Figure 42). There were significant differences in the number of non-responding females between the six different treatments. Lime tree was different from wheat, cabbage, winter harvest and red delicious ($t_{\text{wheat}} = 2.97$, $P < 0.01$, df = 14) and lupin differing from winter harvest, cabbage and red delicious ($t_{\text{winter harvest}} = 3.00$, $P < 0.01$, df = 14) lastly wheat had a higher level of non-responders than red ($t = 2.27$, $P < 0.05$, df = 14). No significant differences were found in the females that responded towards the plant material ($F = 1.25$, $P > 0.05$, df = 42).

When the response in each plant-treatment was compared before and after being subjected to cold, the proportion of non-responders did not differ significantly between them ($t_{\text{winter harvest}} = 0.02$, $t_{\text{red delicious}} = 0.59$, $t_{\text{cabbage}} = 0.08$, $t_{\text{wheat}} = 0.36$, $t_{\text{lupin}} = 0.80$, $t_{\text{lime tree}} = 1.77$, $P > 0.05$, df = 14) and the responses to the plant material did not differ in the winter harvest, red delicious wheat lupin and lime tree treatments ($t_{\text{winter harvest}} = 0.442$, $t_{\text{red delicious}} = 0.029$, $t_{\text{wheat}} = 1.97$, $P > 0.05$, $t_{\text{lupin}} = 0.72$, $t_{\text{lupin}} = 0.56$, $P > 0.05$ df = 14) but between the two cabbage treatments, the response towards cabbage leaves was higher in the wasps tested immediately after being removed from the culture cages ($t = 2.37$, $P < 0.05$, df = 14).
Figure 42: Proportion (mean ± 1 S.E.) of *Aphidius colemani* females responding to a choice of plant leaves against clean air. Parasitoids were either tested after being removed from cultures or after being held for 24 h at 0 °C. Choices were winter harvest, red delicious, cabbage, wheat, lime tree and lupin against a choice of humidified clean air (n = 160 in each treatment). In all instances there was a significant preference within each treatment towards the olfactometer arms that contained plant leaves (*** = P < 0.001, * = P < 0.05). Uppercase letters compare the proportions of non-responders (i.e. responders towards plant material combined with responders towards clean air against non-responders) between the wasps tested immediately after removing them from the rearing cages or after treated for 24 h at 0 °C (0 °C-24 h). Lowercase letters compare the proportions of females that responded towards the plant material between the treatments within each of the two temperature regimes.
6.3.3 Affects of duration of exposure to 0 °C

Females did not show a significant bias towards the arms containing cabbage or winter harvest after being exposed to 0 °C for 0.5 h or more ($\chi^2$ 0.5 h = 0.002, $\chi^2$ 1 h = 0.76, $\chi^2$ 6 h = 1.60, $\chi^2$ 12 h = 0.67, P > 0.05, df = 1) (Figure 43). Treatments 0 h (0 h treatment = 27.25, P < 0.001, df = 1) and 24 h ($\chi^2$ = 0.60, P > 0.05, df = 1) are included for the purpose of comparison. The only treatment (in addition to the 0 h treatment) that elicited significant responses between the choices was the 0.1 h treatment where more females responded to winter harvest than the alternative ($\chi^2$ = 8.57, P < 0.001, df = 1). Further analysis showed a significant decrease in the amount of overall number of wasps responding with the time exposed to 0 °C (F = 15.41, P < 0.001, df = 56) and a significant decrease in the response towards winter harvest as time exposed to cold increased (F = 4.57, P < 0.05, df = 56) (Figure 44). The results of the 0 h treatment and the 0 °C+24 h treatment were included for this statistical analysis.

![Figure 43: Response of Aphidius colemani females after different exposure times to 0 °C and tested in the olfactometer offering a choice of winter harvest or cabbage detached plant-material. Females were not biased to either of the choices offered in treatments ≥ 0.5 h (ns (not significant) = P > 0.05; *** = P < 0.001).](image)
Chapter 6 Parasitoid Memory Loss and Innate Olfactory Preferences

Figure 44: Overall response to winter harvest and cabbage (A) \( P < 0.001, df = 56 \) and response towards Brussels sprout leaves only (B) \( P < 0.05, df = 56 \) of female wasps according to time of exposure to 0 °C. Female parasitoids were chilled for different time periods at 0 °C and their response towards winter harvest was assessed against cabbage. The lines represent the fit of the generalized linear model.

6.3.4 Affect of time elapsed after cold treatment

When the females were tested in the olfactometer with a choice of winter harvest or cabbage odours after a 1 h acclimatising period at room temperature following a 1 h (1 h +1 h treatment) or a 24 h (24 h +1 h treatment) exposure at 0 °C, there was a significant preference towards the arms containing the winter harvest plants \( P < 0.001 \) (Figure 45). Further analysis showed no significant difference in the overall proportion of responding wasps \( t = 0.463, P > 0.05, df = 14 \) nor in the females that responded towards winter harvest \( t = 0.93, P > 0.05, df = 14 \) between the two treatments. In addition, the two treatments had higher overall responses when compared with the 0 °C + 24 h treatment at tested immediately \( t = 4.21, P < 0.001, df = 14 \) for the 1 h+1 h treatment and \( t = 4.00, P < 0.001, df = 14 \) for the 24 h+1 h treatment) and also a higher level of response towards winter harvest in the 1 h+1 h treatment \( t = 2.57, P < 0.05, df = 14 \) but no difference in the 1 h+24 h treatment in the response towards winter harvest \( t = 1.87, P > 0.05, df = 14 \). When compared with insects tested directly from culture conditions, the overall response did not differ \( t = 0.49, P > 0.05, df = 14 \) for the 1 h+1 h treatment and \( t = 0.98, P > 0.05, df = 14 \) for the
24 h+1 h treatment), while the responses towards winter harvest were also similar ($t = 0.70$, $P > 0.05$, df = 14 in the 1 h+1 h treatment and $t = 1.69$, $P < 0.01$, df = 14 in the 24 h+1 h treatment).

![Figure 45: Proportion (± 1 S.E.) of Aphidius colemani females that responded to the plant leaves after an acclimatizing period of 1 h at room temperature following an exposure to 0 °C either for 1 h or 24 h. In both treatments, significantly more females were biased winter harvest odour (*** = $P < 0.001$). Uppercase letters compare the proportions of non-responders (i.e. responders towards plant material combined with responders towards clean air against non-responders) between the wasps tested immediately after removing them from the rearing cages or after treated for 24 h at 0 °C (0 °C-24 h). Lowercase letters compare the proportions of females that responded towards the plant material between the treatments within each of the two temperature regimes.](image-url)
6.4 Discussion

Parasitoids can make robust distinctions between odours from closely-related plant species and this allows them to orient towards the volatile chemicals experienced during the early stages of adult life (Storeck et al., 2000; Douloumpaka & van Emden, 2003). This is possibly done through detecting differences in the proportions of certain chemical compounds (Bruce et al., 2005). In the present study *Aphidius colemani* females preferentially oriented towards the leaves of the plants they were reared on even after 24 h without contacting either the host-plant complex or the host (0 h and 20 °C-24 h treatments). This is not surprising since memory retention times in several insects has been found to be of > 24 h (Kester & Barbosa, 1991; Du et al., 1997) and even up to weeks (Gandolfi et al., 2003b). Although after exposure to 5 °C for 24 h (5 °C-24 h treatment) the parasitoids’ response towards winter harvest was significantly lower than in the control, they could still distinguish between the two Brassica odours. But a further drop of 5 °C (0 °C-24 h treatment) did induce a change in behaviour, causing the parasitoids to cease to respond positively to the natal odour. This olfactory behavioural alteration was detected when parasitoids were exposed to 0 °C for periods longer than 0.5 h and the response towards the winter harvest leaves waned with increasing exposure times, and additionally it was only detected within the first hour that the cold treatment had finished.

When the parasitoids response was tested in a broader odour recognition trial, by offering them a choice of plant leaves against clean air, there was a clear and consistent bias towards the arms that contained plant material, even after the most severe chilling treatment. This, first of all suggests that females exposed for 24 h at 0 °C did not have their locomotor system affected, therefore the lack of preference towards winter harvest in the previous experiments cannot be attributed to that. Secondly, the leaves used in these treatments are all from hosts of *M. persicae*, which suggests that the response evident in this stage could be due to innate mechanisms towards common chemicals present in the Brassicas used in the experiments. Brussels sprout plants are known to emit volatile chemical blends behaviourally active to the parasitoids *Cotesia glomerata* and *C. rubecula* (Mattiacci et al., 1994; Geervliet et al., 1997; Mattiacci et al., 2001b; Smid et al., 2002) and to the diamondback moth parasitoid *Diadegma semiclausum* (Bukovinszky et al., 2005). Although some of these studies used plants attacked by the herbivore caterpillar *Pieris brassicae* and the diamondback moth *Plutella xylostella* so the volatile composition differed from that of uninfested plants, some of these behaviourally active compounds could be released by the leaves used in the present study, as suggested in Chapter 4 (page 70). Some of
these volatile compounds could permit a clear (innate) response of the parasitoids when the contrast between the two choices is high (Brassicas versus clean air), but when the clean air control is replaced by another Brassica odour the choices become similar enough that the parasitoids cannot discriminate after exposure to 0 °C.

In the present study the cold treatments probably did not alter sensilla morphology, because the treatment was applied during adulthood and not developmental stages, so the observed change in behaviour is most likely due to temporary changes in the central nervous system. The documented temporary amnesia in recently acquired memories (within 20 min of acquisition) produced through cold anaesthesia in *Drosophila* sp (Xia et al., 1998) could be an indicator of what is occurring in *A. colemani*. The cold treatments could have affected fine recognition responses that where acquired through learning processes. These responses are labile and prone to being disrupted by cold, while innate ones could be less affected by cold temperature.

The present study offers a first insight into what might be the dissecting of learnt and innate behaviours through cold treatments, which in turn may offer a tool to study innate preferences in systems where obtaining naive individuals is experimentally difficult or impossible. Further work must be brought forward to this area and assessing the existence of different affects of cold on newly-acquired memories and on “old” memories would be one step. This should help understand the basis of the behavioural alterations shown in this study.

### 6.4.1 Conclusions

The present study suggests that the response of the parasitoid was modified with an exposure time of more than 24 h at 0 °C, while a more general response to plant volatiles was not affected by the cold temperature. In addition, responses arising from experience appear to have been inhibited temporarily and revealing underlying innate responses. The results indicate that temperature treatments could offer the possibility of dissecting innate and learnt behaviours in parasitic wasps.
Chapter 7
Hygroreception in Olfactometer Studies

7.1 Introduction
To maintain a stable water balance, insects react and move to preferred areas according to the water vapour content in the air. Whenever water vapour regulation is achieved behaviourally, it is attributed to cells dedicated to detecting changes in humidity (Tichy, 2003). Insect hygroreceptors appear to be restricted to a small number in distal portions of the antennae and consist of a poreless sensilla with a thermoreceptive cell along with a pair of hygroreceptive cells that react to the same stimulus but oppositely; a dry cell that increases its response with decreasing humidity and a humid cell that decreases its response with the same change in humidity (Altner & Loftus, 1985; Tichy & Loftus, 1996). Hygro and thermoreceptive sensilla, known as “sensilla coelonica type II”, have been found in male and female braconid wasps such as the aphid parasitoid A. rhopalosiphi (Bourdais et al., 2006) and the Pieris parasitoids C. glomerata and C. rubecula (Bleeker et al., 2004). Generally the way in which humidity affects insect behaviour depends on the individual’s physiological state. Hygropositive and hygronegative responses in the beetle Oryzaephilus surinamensis change if it has been previously starved and dehydrated (Arbogast & Carthon, 1972). Under normal conditions the cockroach, Periplaneta americana, prefers lower humidity, but the preference is reversed if dehydrated (Yokohari & Tateda, 1975).

Because of the diverse results that humidity can have on the behaviour of insects, hygroreception should not be disregarded in olfactometric behavioural studies, especially in situations where the alternatives offered as choices in the olfactometer differ in their water vapour release rates. Normally studies that use these kinds of assays do not quantify the differences in humidity when the sources offered could differ in their water-vapour release rates. A choice of plant material against an alternative choice of air or synthetic chemical compound illustrates this asymmetry. Out of 11
studies found to assess insect behaviour with presupposed asymmetric contrasts, 5 of the studies increased the air humidity that entered the arms of the olfactometer by allowing it to flow through a ‘bubbler’ or dampened cotton-wool but then failed to assure that the plants did not increase the humidity further (Table 6). On the other hand, 6 of the studies did not increase the initial humidity or compensate in the alternative arm of the olfactometer with a control for humidity. Although most of the studies standardize the physiological state of the insects by feeding and providing water before testing them, it does not ensure that their response will not be dictated by the humidity of the sources rather than by the odour.

Table 6: Olfactometer studies where humidity was not appropriately controlled for.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Insect/s tested</th>
<th>Olfactometer type</th>
<th>Humidifier</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant</td>
<td>Vs.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brassicas</td>
<td>Air</td>
<td>Diadegma semiclausum</td>
<td>Y-tube</td>
<td>Humid cotton-wool</td>
</tr>
<tr>
<td>Maize</td>
<td>Air</td>
<td>Cotesia marginiventris, Microplitis rufiventris</td>
<td>Six arm</td>
<td>Humified air</td>
</tr>
<tr>
<td>Maize</td>
<td>Air</td>
<td>Cotesia marginiventris, Microplitis rufiventris</td>
<td>Six-arm</td>
<td>Bubbler</td>
</tr>
<tr>
<td>Brassicas</td>
<td>Air</td>
<td>Aphidius colemani</td>
<td>Petersen</td>
<td>Distilled water</td>
</tr>
<tr>
<td>Maize</td>
<td>Air</td>
<td>Cotesia marginiventris</td>
<td>Six arm</td>
<td>1 L bubbler</td>
</tr>
<tr>
<td>Lima bean</td>
<td>Synthetic compound</td>
<td>Phytoseiulus persimilis</td>
<td>Y-tube</td>
<td>No</td>
</tr>
<tr>
<td>Brussels sprouts</td>
<td>Artificial diet</td>
<td>Aphidius colemani</td>
<td>Four arm</td>
<td>No</td>
</tr>
<tr>
<td>Broad bean</td>
<td>Air</td>
<td>Aphidius ervi</td>
<td>Y-tube</td>
<td>No</td>
</tr>
<tr>
<td>Tea shoots</td>
<td>Air</td>
<td>Aphidius sp, Chrysopa sinica, Coccinella septempunctata</td>
<td>Y-tube</td>
<td>No</td>
</tr>
<tr>
<td>Centaurea nigra</td>
<td>Pot + soil</td>
<td>Aphidius funebris</td>
<td>Y-tube</td>
<td>No</td>
</tr>
<tr>
<td>Broad bean</td>
<td>Air</td>
<td>Aphidius ervi</td>
<td>Y-tube</td>
<td>No</td>
</tr>
</tbody>
</table>

7.1.1 Objective and hypothesis

The objective in this section of the thesis is to quantify the changes in water vapour content of air flowing arising from different substrates, as well as assess the effects of filtering the air and compensating for humidity through different techniques. Also the behaviour of the parasitoid is assessed towards different humidities. The hypothesis is that different substrates placed in the airflow of an olfactometer will affect the humidity of the air and that parasitoids will be biased towards higher humidities.
7.2 Materials and methods

7.2.1 Hygrometry

To quantify the humidity released into the air-stream of the olfactometer by Brussels sprout leaves, a hygro-thermometer (Extech Instruments, US, model 445713, Range 10% to 99%, ± 5%, ±1°C) was used (Figure 46). It measures temperature and relative humidity (RH) in sensors located in the display-case and in a probe. The hygro-thermometer was used in conjunction with a setup that allowed measuring the water-vapour content of a sample of air coming from any of the three olfactometers used in this study.

Figure 46: One of the setups used to measure changes in air water-vapour content due to changes in the substrates used. A hygrometer probe was fitted inside a Perspex tube (arm) where the water-vapour content of the incoming air could be measured. Temperature and humidity could be monitored both inside the arm and in the chamber where the apparatus was placed.

For this, the hygrometer’s probe was placed inside a Perspex tube (i.d. = 0.7 cm, length = 3.6 cm) and the humidity of the air flowing past the probe could be monitored. A circular arena as the one used in the parasitoid conditioning chamber (section 5.2.1, page 106) was attached to one end of the tube and this in turn connected to a vacuum
pump producing an airflow at a rate of 425 ml min⁻¹. The other end of the Perspex tube could be connected to either an air filter containing 6 g of activated charcoal (simulating the detached-leaf olfactometer -version 2-), or to one of the terminals that otherwise would be attached to the arm of the whole-plant olfactometer or the detached-leaves olfactometer -version 2- (details in 2.1.4.2 further details). In this way it was possible to measure the effects on humidity of a sample “treated” with air circulated through 0.4 l of water and/or filtered through 55 g of activated charcoal or air which was only filtered through 6 g of activated charcoal.

The system was placed inside a wooden box with artificial light (details in 2.1.7) and a constant air flow (425 ml min⁻¹) was applied for the duration of the experiment. In different experiments, air could either be filtered through 6 g of activated charcoal, 55 g of activated charcoal or firstly through 55 g of activated charcoal and secondly humidified through 0.4 l of distilled water. In addition, the affects of different sources (i.e. dry filter paper, damp filter paper, detached leaf/leaves and whole plants) on the air water-vapour content in an olfactometer arm could be measured.

In all instances humidity was measured and analyzed as RH. In initial data analysis humidity was converted to “saturation deficit”, which is an indicator of the absolute amount of water in a sample of air (Lees, 1986; Rebora et al., 2007). But because humidity measurements were taken over several months and room temperature varied, water absolute amounts changed considerably due to differences in room temperature (~ 7 °C). Therefore it was decided, in order to minimize the effect of room temperature, to subtract the room RH from the arm RH and thus reduce the variability.

Once the data was acquired, the leaves (either detached leaves or whole plants) were digitally scanned and the resulting image was used to calculate the total leaf-surface of the samples (Scion Image for Windows, version Alpha 4.0.3.2).

7.2.2 Affect of time on humidity of various sources

The humidity and temperature recordings were taken both inside the Perspex tube and the wooden box every 5 minutes over 25 min. The air entering the circuit was previously filtered through 6 g of activated charcoal and the following substrates were placed inside the Perspex tube: (1) a dry filter paper (Whatman, diameter = 42.5 mm) (treatment: dry filter paper), (2) a filter paper dampened with 200 µl of distilled water (treatment: damp filter paper), (3) one leaf (treatment: 1 leaf), (4) three leaves (treatment: 3 leaves) and (5) all the detached-leaves from six-week old Brussels sprout plant (hamlet cultivar) (treatment: detached leaves of entire plant). Samples were
introduced into the Perspex container immediately after dampening or detaching. Four replicates were carried out for each treatment and measurements commenced after samples had been 5 min in the arms with air flowing.

7.2.3 Dry versus humidified air

The change in humidity was measured from samples treated with either air entering the system previously filtered through 55 g of activated charcoal (dry air) or filtered through the same amount of activated charcoal and then bubbled through 0.4 l of distilled water (humid air). The change in air water-vapour content was measured for dry air and humid air for the following samples: (1) a dry filter paper (Whatman, diameter = 42.5 mm) (treatment: dry filter paper), (2) the same kind of filter paper dampened with 200 µl distilled water (treatment: damp filter paper) (3) all the detached leaves of a six-week old hamlet plant (treatment: detached leaves of entire plant) and (4) a whole six-week old hamlet plant (treatment: whole plant). Because the previous set of measurements resulted in no effect of time on air humidity-content, a single measurement of the RH was taken 10 min after the air had started to flow. Five replicates were done for each treatment. The leaf surface of treatments involving plant material was also measured as described above.

7.2.4 Parasitoid response to humid and dry air

The response of parasitoids was studied in the 4-way olfactometer with a choice of dry filter paper (Whatman, diameter = 42.5 mm) in two of the arms and the same kind of filter paper dampened with 200 µl distilled water in the other two arms. Air entering the system was filtered through activated charcoal (6 g). Two treatments were carried out; one with parasitoids removed from the rearing cages and tested immediately in the olfactometer and a second treatment to assess if parasitoids exposed to 0 °C during 24 h (details in 6.2.2) could detect changes in water vapour content.

7.2.5 Statistical analysis

7.2.5.1 Affects of time on humidity of various sources

A linear regression was done for each treatment to assess the effect of time on the humidity content of the air in the different sources. To assess the effect of time on air water-content, the slope of each treatment was compared to a slope equal to zero with a two-tailed Student t test. Differences between treatments were compared between treatments with an ANCOVA and a posteriori ad-hoc two-tailed t-tests (R programme, version 2.2.1).
7.2.5.2 Dry versus humidified air
Data on the effects of dry and humidified air on different sources were analyzed within an ANOVA with posterior Tukey's Multiple Comparison Test to detect differences between the treatments (JMP, version 7.0).

7.2.5.3 Parasitoid response to humid air
The analyses carried out are explained in section 2.1.8 (page 41).
7.3 Results

7.3.1 Affect of time on humidity of various sources

When the effect of time was evaluated over the humidity of various sources there was no change in the amount of water vapour released by the five sources (t dry filter paper = 0.50, P > 0.05; t damp filter paper = 1.73, P > 0.05; t 1 leaf = 1.72; P > 0.05; t 3 leaves = 1.06, P > 0.05, df = 1; t detached leaves of entire plant = 0.76, P > 0.05, df = 1;) (Figure 47).

![Graph showing water-vapour contents of different substrates placed in an olfactometer arm with air entering the system filtered through 6 g of activated charcoal. The substrates used consisted of a dry filter paper, a damp filter paper, one detached leaf, three detached leaves and the detached leaves of an entire Brussels sprouts plant. Within each treatment there were no effects of time on the humidity of the air. Significant differences were found between some of the treatments in their water-vapour release rates.](image_url)

Figure 47: Water-vapour contents of different substrates placed in an olfactometer arm with air entering the system filtered through 6 g of activated charcoal. The substrates used consisted of a dry filter paper, a damp filter paper, one detached leaf, three detached leaves and the detached leaves of a an entire Brussels sprouts plant. Within each treatment there were no effects of time on the humidity of the air. Significant differences were found between some of the treatments in their water-vapour release rates.

Significant differences between the water vapour released by some of the sources were found (ANCOVA, F = 120.57, P < 0.001, df = 10). A posteriori analysis revealed that a damp filter paper and the entire detached-plant did not emit significantly different amounts of water vapour (t = 0.50, P > 0.05, df = 1) whereas these two treatments were significantly different from the treatments with a dry filter paper and one leaf (t = 6.92, P < 0.001, df = 1), which in turn produced a similar change in air water-vapour...
content with no significant differences between them ($t = 0.50$, $P > 0.05$, df = 1). Lastly, three leaves produced an intermediate amount of water vapour, which was different to a damp filter paper and the entire detached-plant ($t = 2.78$, $P < 0.01$, df = 1) and it was higher compared to the water vapour produced by one leaf and a dry filter paper ($t = 3.08$, $P < 0.01$, df = 1).

The mean leaf surfaces for each of the three treatments where as follows: one leaf (16 cm$^2$, S.E. = 1.8), three leaves (88 cm$^2$, S.E. = 4.8), entire detached-plant (205 cm$^2$, S.E. = 14, mean leaves per plant= 8, S.E. = 1.2). A logarithmic behaviour was considered to be the best fit to describe the system since a limit in air water vapour content would be reached once air-water vapour reaches saturation (Figure 48).

![Figure 48: Change in air water-vapour content due to different surfaces of detached Brussels sprouts leaves. A logarithmic behaviour was considered to be the best fit to describe the system.](image-url)

$y = 9.3388 \ln(x) - 19.787$

$R^2 = 0.7018$
7.3.2 Dry versus humidified air

Significant differences were found in between the treatments ($F = 117.31, P < 0.001, df = 4$) (Figure 49). The humidity of the four treatments increased significantly after the air was circulated through 0.4 l of distilled water (Tukey’s Multiple Comparison Test, dry filter paper: $P < 0.01$, damp filter paper: $P < 0.01$, entire detached-plant: $P < 0.01$, whole plant: $P < 0.01$). When the humidity from the dry filter paper with dry air was considered as a baseline, a significant increase in humidity was detected when a damp filter paper, an entire detached-plant or an whole plant were introduced into the airflow ($P < 0.01$). A further increase from the dry filter paper (with dry air) base line was produced by humidifying the filtered air ($P < 0.001$) and there was a further increase by the addition of either a damp filter paper or the plant material ($P < 0.01$). The increase in humidity after introducing a ‘damp’ source (i.e. damp filter paper, entire detached-plant or an whole plant) into the flow was of ~10 % RH, and the increase produced by the different sources once the air was humidified compared to that of the same sources in dry air, in three treatments (dry filter paper, damp filter paper and entire detached-plant) was of ~20% RH, while in the whole plant treatment a smaller increase of ~10% RH was registered.

![Figure 49: Change in air water-vapour content from 4 different substrates with air circulated through 55 g of activated charcoal (dry air) or air circulated through the same amount of activated charcoal and then bubbled through 0.4 l distilled water (humid air). In every case, this change produced a significant increase in humidity content. Different letters above the bars indicate significant differences between the treatments.](image)
7.3.3 Parasitoid response to humid and dry air

The response of female parasitoids when removed from rearing cages and tested immediately after with a choice of dry and humid air, was biased towards the arms of the olfactometer with the humid filter papers ($\chi^2 = 17.86$, $P < 0.001$, df = 1) (Figure 50). Females tested in the olfactometer after 24 h at 0 °C were also biased towards the arms with the damp filter paper ($\chi^2 = 51.68$, $P < 0.001$, df = 1). There was no difference between the number of non-responding wasps of both treatments ($t = 0.33$, $P > 0.05$, df = 14) nor in the proportion of females that responded towards the humid air ($t = 1.86$, $P > 0.05$, df = 14). The increase in RH in the humid arms of the olfactometer was ~ 20% when compared with the dry arms.

Figure 50: Response of female parasitoids towards a choice of humid (filter paper dampened with 200 µl of distilled water) and dry (dry filter paper) air causing an increase of ~ 20 % RH. Wasps were tested immediately after removal from the rearing cages or subjected to a 24 h treatment at 0 °C. In both cases, the response was biased towards the arms with humid air. *** ($P < 0.001$) compares the response of the females towards the two choices within each treatment. Uppercase letters compare the proportions of non-responding females across treatments. Lowercase letters compare across the different treatments the differences in proportions of females responding to the humid air.
7.4 Discussion

The humidity of the air circulating with samples placed in the air stream remained stable throughout the 25 min it was measured. A wet filter paper is a good control for humidity when the leaves of a six-week-old Brassica plant –whole or detached- are presented in the olfactometer since its water-vapour release rates are similar over at least 25 min. When measurements of air water-vapour content were taken with air filtered through 6 g of activated charcoal and allowed to flow into a tube with only a dry filter paper, the measurements recorded indicated a humidity slightly higher, but not significant. The numbers of leaves used did have a significant effect on air humidity, with the humidity from 3 leaves being significantly lower than that from all the leaves of a six-week-old plant but it is probable that further leaf additions would not have increased the humidity much over that level.

Although filtering the air through 6 grams of activated charcoal probably did not affect the humidity of the air that entered the closed olfactometer air circuit, a tenfold increase in the amount of filtering material had significant effects by drying the air below the room’s humidity. This could be explained by the fact that the filtering substrate, in addition to retaining volatile impurities from the air, acts as a water-vapour retention substrate (Wiig, 1949). When humid sources were placed in the dry air stream, significant increases in humidity were detected and humidifying this dry air through a water bubbler increased the water-vapour content significantly. Additions of moist substrates to this humid airflow further increased the water content emphasizing the fact that there should be a careful evaluation of changes in humidity in bioassays where there might be differences in water contents between the substrates used, even in those where the air is treated with humidifiers. Probably the best solution to this problem would be to humidify the air sufficiently as to increase the water vapour pressure to levels close to saturation, in this way no further increments would be possible due to additions of humid substrates. With this in mind, measurements were made with a 1 l bubbler instead of a 0.4 l one, and similar RHs increases were detected in both situations (~ 20 % RH; t = 0.66, P > 0.05, df = 6). Further increases in water volume might have the desired effects and modern air humidifiers such as those used for domestic purposes could offer a solution to humidity control.

Water and temperature are the two main abiotic factors governing the distribution of terrestrial organisms, and insects are prone to water loss due to their small body size (Addo-Bediako et al., 2001) and have evolved behavioural mechanisms to adapt to this. For example, the cockroach, *P. americana*, has been found to discriminate differences as low as 7.5% RH (Doi & Toh, 1992) and electrophysiological recordings
of moist and dry cells in the hygroreceptive sensilla responded to increments and decrements of 1% RH (Tichy, 2003). In the stick insect, Carausius morosus, a similar response was recorded in single humid and dry cells, detecting differences between 6.3% and 3.1% RH, respectively (Tichy, 1987). Sensilla believed to be involved in hygroreception have been found in the distal part of the antenna of male and female A. rhopalosiphi (Bourdais et al., 2006). These type of sensilla, called “sensilla coeloconica type II”, have a distinctive “doughnut shape” with a “peg” protruding from the centre. It is possible that they are also responsible for the behaviour of the parasitoid A. colemani recorded in this study, by which females show a bias towards the arms with the higher humidity discriminating differences of 20% RH.

In cockroaches it has been found that responses due to changes in humidity are explained by the physiological state and deprivation of water correlates with hygropositive responses and water was not correlated with hygronegative responses (Doi & Toh, 1992). In contrast, the haematophagous bugs Triatoma brasiiliensis and T. infestans hygopreference as adults is always inclined towards dry environments (Roca & Lazzari, 1994; Guarneri et al., 2002) and move towards areas with relative humidities close to 0%. This behaviour can be explained by their blood-feeding habit, which requires them after having a blood meal, losing high volumes of water contained in the blood and this is accomplished in part by moving to areas where low RH will promote body water loss. In contrast to this extreme behaviour in the adults, 4th instar nymphs of T. brasiiliensis prefer, after ecdysis, sites with up to nearly 90% RH (Roca & Lazzari, 1994; Guarneri et al., 2002). In the present study the physiological state of females was unknown, but presumably, the females tested immediately after removal from the cages, would not have been under extreme dehydration since there is water available. Although it is less certain the physiological condition of the wasps that underwent the chilling treatment, which probably did not feed although provided with water for 24 h previous to the testing. Although their metabolic rates would have been slow in the cold temperature, there would still be activity and presumable water loss. If it is assumed that females tested immediately after removal of the rearing cages were not under hydric stress, the fact that they did orient towards the more humid choice in the olfactometer, suggests that insects do not have to be water deprived to seek for a relatively more humid environment. In fact, although physiologically, dehydrated insects can detect smaller differences in humidity than non-dehydrated ones, there is evidence that a water-satiated insect will still prefer humid environments over dryer environments (Guarneri et al., 2002).
7.4.1 Conclusions

The present suggests that humidity should be a factor to be controlled for in olfactometric studies. In addition to the hygropositive response elicited by female parasitoids *A. colemani* in the present study, many insect species have been shown to respond positively and negatively to changes in humidity, therefore slight variations in humidity could have confounding effects in behavioural assays. The influence of humidity in insect behaviour should not be overlooked in behavioural olfactory studies in which the choices offered could differ in water-vapour release rates. In this study, a full comprehension of *A. colemani’s* hygroreceptive system was not intended, nor was the interaction between hygro- and chemo-reception systems but the aim was to highlight the consequences that changes in humidity arising from the choices offered in olfactometer studies could have. Chemoreception and hygroreception have always been treated separately and studies must be carried out to investigate the interaction between these two sensory modalities and how they are integrated in the central nervous system in order to further understand the relative contributions of both in insect behaviour.
Chapter 8

Lacewing Chemical Ecology

8.1 Introduction

The role that chemical communication may play in the Chrysopidae is poorly understood. Among the few reports mentioning the occurrence of chemical communication in the chrysopids, there is a defensive secretion (Zhu et al., 2000; Zhang et al., 2004) identified as (Z)-4-tridecene. The only record of a sex pheromone produced by males to attract females was found in *C. perla* lacewings (Wattebled et al., 1978), but unfortunately the compound was not identified. The first pheromone identified in any lacewing species was the male-produced (1R, 2S, 5R, 8R)-iridodial (Zhang et al., 2004), which was extracted from lacewing-cuticular washings and proved to be electrophysiologically active and traps in the field caught males of the golden eyed lacewing *Chrysopa oculata* Say. The authors identified the compound as a male-aggregation pheromone. Recently the same compound was found to have a pheromonal role in *C. nigricornis* and was produced by males (Zhang et al., 2006).

In addition to the active compounds recently discovered with a role in lacewing intra-specific communication, lacewings respond to chemicals produced by different trophic levels. Induced plant volatiles, on one hand, are known to affect behaviour of adult carnivorous lacewings. For example, the herbivore-induced plant volatile methyl salicilate catches the green lacewings *C. nigricornis* and *C. oculata* in field trials (James, 2003; James, 2006). On the other hand, several aphid predators, such as obligate parasitoids of aphids, use their prey pheromones to increase the possibility of encounters (Hardie et al., 1994) and lacewing species are known to respond in the field and in electrophysiological recordings towards (4aS, 7S, 7aR)-nepetalactone and (1R, 4aS, 7S, 7aR)-nepetalactol, two components of aphid sex pheromones (Boo et al., 1998; Zhu et al., 1999; Hooper et al., 2002). Donato *et al.* (2001) reported that neomatatabiol ((1R, 4S, 4aR, 7S, 7aR)-dihydronepetalactol), a closely-related chemical to (1R, 4aS, 7S, 7aR)-nepetalactol, caught *P. gracilis* lacewings in water traps. Most of the individuals trapped were males, raising the question if the compounds tested in the traps could be components of lacewing sex pheromones.
Chrysopid lacewings are not known to lek, but in several species of ant lion (Neuroptera: Myrmeleontidae), a related group to the lacewings, lekking behaviour is common in males, a possibility that cannot be discarded in *P. gracilis*, though females would be expected to be found associated with this behaviour. The fact that pheromone-producing glands have been found in the abdomen of the golden eyed lacewing, *C. oculata*, and the role of the male-aggregation pheromone recently proposed (Zhang et al., 2004) makes it possible to speculate the pheromonal function for neomatatabiol or an analogous compound could have in *P. gracilis*.

### 8.1.1 Objective and hypothesis

The effect of neomatatabiol on *P. gracilis* behaviour and its possible origin is addressed in this section of the thesis. The Silwood Park *P. gracilis* population is monitored with traps releasing neomatatabiol in the field over 36 months. The response of *P. gracilis* males towards neomatatabiol and a second compound, (Z)-4-tridecene, registered in cuticular washings analyses, are assessed in the behavioural assays. Also the effect of neomatatabiol applied topically on the abdomen of lacewings is assessed. Lastly, individuals caught in the field are used to identify possible pheromones through cuticular washings. It is predicted that neomatatabiol will be found on male lacewings cuticular extracts and that a strong bias towards the compound will be found in olfactometer bioassays but not when combined with (Z)-4-tridecene, since this compound is believed to have a defensive role. In addition, it is believed that topical neomatatabiol could affect lacewing behaviour and survival. The null hypothesis is that neomatatabiol will not be found in cuticular washings and that the compounds tested in bioassays will not affect the lacewing behaviour or survival.
8.2 Materials and Methods

8.2.1 Lacewing captures

8.2.1.1 Trapping method

Lacewings were captured in the field with traps made out of two transparent plastic bottles (Figure 51 A and C). The top of a 2 l mineral-water bottle was cut at 11 cm from the neck. The cut-off top was then inverted and introduced into the bottom-half of the bottle. In this way a funnel was created that leads the insects into the trap. Two of these modified bottles were held together with wires, resulting in one of the halves with the funnel pointing upwards and half pointing downwards, a gap of 10 cm separated both halves.

Figure 51: Trap used to capture live lacewings in the field. (A) Shows one of the traps hanging from a pine tree, this also shows a wire mesh cage used in some of the trappings. (B) Shows the cage (13 X 13 X 17 cm) in which the lacewings, once captured, were kept until later use in bioassays. (C) Illustrates a detail of the trap.
Ten mg of neomatatabiol (provided by A. M. Hooper, Rothamsted Research, Harpenden, UK) (details of synthesis procedure (Hooper et al., 2002)) diluted in 50 µl of diethyl ether were placed in a glass dispenser (Chromacol amber glass vial, 08-CPV (A)) and left for the diethyl ether to evaporate. The vial was covered with a lid with a hole (Ø = 1 mm) to allow volatile emission. The vial was suspended with a wire held from the top-half of the trap. In this way, a lacewing reaching to the trap could enter either the top- or bottom-half or of the trap and be captured alive. Traps were hung from branches of a Bhutan Pine (*Pinus wallichiana* Jackson) (Figure 51A) at breast height, at least 2 m apart in Silwood Park gardens (Ascot, Berkshire) and checked every 2-4 days from October 2004. The number and sex of the lacewings trapped was recorded and most of them were transferred into a Perspex cage (13 X 13 X 17 cm) (Figure 51 B) with food (water:yeast:sugar, 10:2:4) and water for later use in laboratory bioassays.

### 8.2.1.2 Annual monitoring

Two traps were kept during the year round on the Bhutan pine. Also, between August - October 2004, June - November 2006 and June - September 2007, 3 (for the first two periods) and 4 (for the last period) additional traps were placed in neighbouring vegetation (within a circle of 5 m of the Bhutan pine) hanging from branches at breast height. These traps were used to collect additional lacewings in order to use in bioassays. Monitoring of lacewing catches was calculated monthly by averaging the number of lacewings caught divided by the number of traps out in the field that month.

### 8.2.1.3 Additional data collected

The number of lacewings captured in the top- and the bottom- halves of the traps were also recorded. Temperature data was collected with on-site data loggers (Tiny talks, Gemini data Loggers, UK) throughout the year since March 2005 to September 2007 with a sampling frequency of 0.5 h and monthly averages with maximum and minimum temperatures were obtained. Monthly rainfall data from March to December 2005, January to December 2006 and January to September 2007 was obtained from Silwood Park’s meteorological station (Jim Culverhouse personal communication).
8.2.2 Behavioural assays

8.2.2.1 Olfactometer

The olfactory response towards various compounds was performed on field-captured male lacewings. A glass “Y” tube olfactometer (130 mm base, 70 mm arms, 13 mm internal diameter) (Figure 52) with frosted joints (50 mm long) (P. A. Brooks, Glass blower, Oxon, UK) was used the bioassays. The frosted joints allowed an easy connection of the olfactometer to the joints which were already attached to the air-carrying hoses. The end of the main arm of the olfactometer was connected through a silicon tube (id = 10 mm, od = 12 mm) to a pump that pulled the air at a rate of 1.7 l min⁻¹.

Figure 52: Y-tube olfactometer used to test the response of the lacewings towards various chemical compounds. (A) Olfactometer and the air filtering set-up. (B) Smoke test to show that the turbulence in the junction was not excessive. (C) Junction with the smoke flowing. (D) Olfactometer.
The air entering the olfactometer through the short arms of the “Y” was previously filtered through 55 g activated charcoal and re-humidified through 40 ml of distilled water (Figure 52). Smoke tests (smoke was created with Smokestream V2.0, Source Manufacturing, UK) were carried out to assess any turbulences in the junction of the two arms (Figure 52, C). A control assay was carried out with the olfactometer with no odour stimuli in either of the arms, and no bias was detected in the choice of lacewings ($\chi^2 = 0.23$, $P > 0.05$, df = 1, n = 25). Odours were presented in glass dispensers (Chromacol amber glass vial, 08-CPV (A)), with a 1 mm hole drilled in the lid. The top of the dispenser was inserted into a hole cut in the silicon tubing at ~ 1 cm from where the glass joint was fitted.

Lacewings collected from field traps were left to feed in a Perspex cage (Figure 51, B) inside a controlled light and temperature cabinet (20 ± 1 °C and 20:4 L:D photoperiod) for at least 24 h and then used in the bioassays. One lacewing was introduced into the base of the Y tube and immediately after, the joint replaced. The bioassay were carried out under the same temperature and lighting conditions described in section 2.1.7 (page 40). Bioassays started immediately after this and the lacewing was left 5 min to respond, a choice was counted as such when the lacewing entered an arm and it’s head reached an imaginary line set 5 cm from the Y junction. The insects were tested once and then discarded (freed in the field, at least 300 m away from the traps). Between 26 and 47 replicates were carried for each contrast on different days with temperatures ranging between 22 and 25 °C. Every second run the olfactometer was washed with Lipsol detergent, (Bibby Sterilin Ltd. UK), rinsed with 50% v/v ethanol and rinsed again with distilled water. All the components were then dried at 60 °C for at least 15 min. The position of the odours alternated every two runs.

### 8.2.2.2 Chemicals used

Six sets of comparisons were carried out (Table 7) involving neomatatabiol (10 mg neomatatabiol diluted in 50 µl of diethyl ether (ether)). Because gas chromatography analysis resulted in cuticular washings having high amounts of (Z)-4-tridecene, a secretion found in other lacewing species as well, it was decided to test the effect of this chemical (10 mg of (Z)-4-tridecene diluted in 50 µl of ether) on the behaviour of male lacewings. In addition, the combination of the two compounds mentioned above (5 mg neomatatabiol diluted in 25 µl of ether and 5 mg of (Z)-4-tridecene diluted in 25 µl of ether) was used to assess the behaviour of male lacewings. Lastly, the response towards a control (50 µl of ether) was also assessed. Although all of the ether was evaporated before closing the lid of the dispenser, in future references, the response of the lacewings towards this control will be referred to as the response towards “ether”.

Dispensers with compounds were prepared every 4 weeks regardless of use. Once the solution was placed in the dispenser, the lid was left open for 10 min at ambient temperature to let the ether evaporate, and then the lid was put in place. Volatiles could exit the dispenser through the 1 mm hole drilled in the lid. In between uses dispensers were covered in Parafilm M (Wisconsin, USA) and stored in the cold (~ -5 °C) until future use.

Table 7: Chemicals used in olfactometer bioassays with male lacewings *Peyerimhoffina gracilis* captured in the field.

<table>
<thead>
<tr>
<th>Chemical compound</th>
<th>(Z)-4-tridecene + Neomatatabiol</th>
<th>(Z)-4-tridecene</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neomatatabiol</td>
<td>Tested</td>
<td>Tested</td>
<td>Tested</td>
</tr>
<tr>
<td>Control</td>
<td>Tested</td>
<td>Tested</td>
<td>-</td>
</tr>
<tr>
<td>(Z)-4-tridecene</td>
<td>Tested</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

8.2.2.3 Statistical analyses

8.2.2.3.1 Bias in choice

The bias in choice was analysed with a $\chi^2$ test on the total count of individuals that responded to each choice. The $\chi^2$ value obtained was compared with tabulated $\chi^2$ values with 1 df.

8.2.2.3.2 Comparison of non-responding wasps

Lacewings that did not respond to any of the choices were compared between the treatments with a generalized linear model with a binomial error structure. Further analysis on the results of olfactometer tests were carried out by comparing the results across different treatments. The data were analyzed with GLM with a binomial error structure and logit link function was used (R programme, version 2.2.1). The comparison took into account the proportion of lacewings that did not respond to any of the choices after 5 min since the assay was started. The significance was assessed against tabulated F values and posterior Student $t$ tests were carried out to locate significant differences if any. In this case degrees of freedom are represented as the residual degrees of freedom (residual degrees of freedom = total degrees of freedom – used degrees of freedom).
8.2.3 Effect of topical neomatatabiol on male lacewings

Observation trials with lacewings in contact with pure neomatatabiol (i.e. a drop on a Petri dish) resulted in males behaving in an unusual manner. Shortly after being introduced in the Petri dish, males started contorting their whole body and shaking in an unusual manner, in addition to contacting neomatatabiol with their mouthparts. Thus, it was decided to explore this unexpected behaviour further. In a preliminary trial it was shown that male lacewings would not eat neomatatabiol. Therefore it was decided to apply neomatatabiol topically on the body of the lacewings. Neomatatabiol diluted in acetone (0.1 mg neomatatabiol/µl of acetone) was applied on the abdomen in two different amounts: 0.05 mg (applied as 0.5 µl of solution: treatment: “neomatatabiol 0.05 mg”) and 0.1 mg (applied as 1 µl of solution, treatment: “neomatatabiol 0.1 mg”). Additionally, two control treatments were carried out: 0.5 and 1 µl of acetone (treatments: “acetone 0.5 µl” and “acetone 1 µl” respectively). Once the treatment was applied with a 1 µl micro-capillary on 15 males per treatment, lacewings were individually placed in transparent glass vials (height: 5 cm, diameter: 2.5 cm) with a gauze secured with an elastic band over the opening. Lacewings were observed once every day over five days.

8.2.4 Cuticular washings

8.2.4.1 Trapping methods

Analyses of cuticular washings of field-trapped individuals was performed to assess the production of neomatatabiol and related chemicals such as nepetalactone, or dihydronepetalactone by *P. gracilis* males. To discard the possibility that the lacewings trapped in the field could have direct contact with the neomatatabiol in the glass vials and confound the results, modified traps were set up with a cylindrical metal wire-mesh cage (3 X 6 cm, mesh size: 0.5 mm) surrounding the dispenser (Figure 51, A). During preliminary field trials it was observed that previous to falling into the trap, lacewings may come into contact with the hole in the lid of the dispenser as if trying to gain access into the dispenser. Thus the protection permitted the volatiles to move freely but prevented contact between the lacewings and the dispenser. In addition, traps with traceable neomatatabiol labelled with deuterium (d-neomatatabiol) (provided by A. M. Hooper, Rothamsted research, Harpenden, UK) were used to rule out contamination on the lacewings due to malfunctioning of the caged dispensers and to rule out endogenous production of the chemical compound.
In total, four different setups (neomatatabiol, protected neomatatabiol, d-neomatatabiol and protected d-neomatatabiol) were used to collect lacewings for cuticular washing analysis. One of the traps used was set up in the field throughout the year (neomatatabiol) and the remaining traps were set out in the field on the same Bhutan pine where the previous traps were located and monitored every 2-4 days from July 10, 2005 to September 19, 2005.

8.2.4.2 Analyses of cuticular washings

Between one and five lacewings captured in each trap were preserved in 5 ml of hexane for chemical analyses. Gas chromatography and gas chromatography-mass spectrometry were done as in sections 4.2.2.1 and 4.2.2.2.3 (page 75 and 76 respectively) (A. M. Hooper, Rothamsted Research, Hertfordshire, UK). The only difference was that the hexane used to obtain the cuticle washings, was filtered through glass-wool (lacewings were kept in the hexane for periods ranging between 3 and 5 weeks) and 1 µl of this filtered hexane was injected directly into the gas chromatograph. Because the aim of the investigation was to prove the presence or absence of neomatatabiol or any analogous compounds, the analysis was only qualitative, and no attempt was made to establish quantitative data of the resulting chemicals.
8.3 Results

8.3.1 Lacewing captures

8.3.1.1 Annual monitoring

A total of 567 male *P. gracilis* were captured throughout the 36 months the traps were set up in the field (Figure 53). *Peyerimhoffina gracilis* was the only species captured through the trial. Since October 2004 to October 2005 there were uninterrupted monthly captures except in December 2004, when no captures were recorded. As of November 2005 lacewings were only recorded since June to September 2006 with a few individuals caught in January. From October 2006 to July 2007 no lacewings were caught. In 2007 lacewings were only recorded during August and September, when lacewings were found regularly in the trap. In the two full years that the traps were set out in the field, 264 lacewings were caught in 2005 and 205 lacewings in 2006. In successive years a considerable drop in captures occurred, with only 71 lacewings caught in 2007.

![Graph showing average monthly captures of male *Peyerimhoffina gracilis* males per trap during part of 2004, 2005, 2006 and part of 2007. Only male lacewings were caught. Numbers in parenthesis along the month indicate the number of traps set out in the field that particular month. In successive years a decrease in lacewing captures was detected with 264, 205 and 71 lacewings caught in 2005, 2006 and part of 2007 respectively.](image)
8.3.1.2 Additional data

8.3.1.2.1 Position in trap
The number of lacewings captured in the top-half of the traps was significantly higher than those captured in the bottom-half (Figure 54). Out of a total of 387 lacewings, 305 were captured in the top-half and 82 in the bottom-half ($\chi^2 = 128.5$, $P < 0.001$, df = 1).

Figure 54: Percentage of male *Peyerimhoffina gracilis* caught in the top-and bottom-halves of the traps. Significantly more lacewings were captured on the top-half. *** ($P < 0.001$). Numbers above bars indicate number of lacewing captured.
8.3.1.2.2 Temperature and rainfall data

Temperatures in the field were recorded from March 2005 to September 2007 (Figure 55). The hottest summer was in 2006 with the highest average temperature (20.8 °C) and highest maximum temperature (36 °C) recorded in July. February 2006 had the lowest average temperature (3.3 °C) recorded in the monitored period. The average monthly temperatures of the summer months (June, July, August and September) of 2007 resulted in the lowest of the three summers recorded (16.2 °C) with 17.0 and 17.6 °C in 2005 and 2006 respectively. Monthly rainfall data (Figure 56) generally varied between 30 and 90 mm per month. It is worth highlighting the very dry April in 2007 (1.8 mm) and the high levels of rainfall recorded in July (158 mm).

![Figure 55: Average, maximum and minimum monthly temperatures in Silwood Park from March 2005 to September 2007.](image1.png)

![Figure 56: Monthly rainfall data from March 2005 to September 2007 in Silwood Park.](image2.png)
8.3.2 Behavioural assays

Presenting male lacewings with neomatatabiol, (Z)-4-tridecene, the mixture of neomatatabiol and (Z)-4-tridecene and the ether control treatment, elicited variable responses (Figure 57). The only choices that elicited a significant response in the lacewings towards either of the alternatives were neomatatabiol vs. ether, where a response was registered towards neomatatabiol ($\chi^2 = 4.23$, $P < 0.01$, df = 1). Also the comparison between neomatatabiol vs. (Z)-4-tridecene + neomatatabiol elicited a bias towards neomatatabiol ($\chi^2 = 4.50$, $P < 0.01$, df = 1). In other assays, lacewings were not biased to either of the choices: neomatatabiol vs. (Z)-4-tridecene ($\chi^2 = 0.76$, $P > 0.05$, df = 1), (Z)-4-tridecene vs. ether ($\chi^2 = 1.58$, $P > 0.05$, df = 1), (Z)-4-tridecene vs. (Z)-4-tridecene + neomatatabiol ($\chi^2 = 0.22$, $P > 0.05$, df = 1) and (Z)-4-tridecene + neomatatabiol vs. ether ($\chi^2 = 0.73$, $P > 0.05$, df = 1). Comparison between non-responding lacewings resulted in differences between the treatment neomatatabiol v ether with less non-responding lacewings than in neomatatabiol v (Z)-4-tridecene ($t = 2.09, P < 0.05$, df = 93) and (Z)-4-tridecene v ether ($t = 2.49, P < 0.05$, df = 90).

![Figure 57: Response of male lacewings in a Y-tube olfactometer towards different combinations of neomatatabiol, (Z)-4-tridecene and a diethyl ether control. Choices with a $\chi^2$ test ($^* = P \leq 0.05$, ns = not significant = $P > 0.05$). Non-responders were not included in the statistical analysis. Neom (neomatatabiol), Tridec ((Z)-4-tridecene). Numbers inside the bars indicate total number of lacewings in each category.](image-url)
8.3.3 Effect of topical neomatatabiol on lacewing survival

Survival was affected by the highest dose of neomatatabiol when applied on the abdomen of male lacewings. Within two days of applying the highest dose, lacewings lay on their sides having sporadic spasms (shaking and turning) and most of the time, their abdomen was bending backwards. More than 60% of the lacewings died between the 2nd and 3rd day of application. After the 5th day most of the lacewings returned to a normal state. The two control treatments and 0.05 mg of neomatatabiol did not affect lacewing survival (Figure 58). Sporadic spasms were seen during the first two days in this last treatment in two of the 15 lacewings treated.

![Figure 58: Effect of topical neomatatabiol on survival of lacewings over a five-day period.](image)

Two known amounts of neomatatabiol diluted in acetone and acetone controls were applied on the abdomen of lacewings. The highest dose, 0.1 mg reduced lacewing survival to less than half, while acetone controls and 0.05 mg of neomatatabiol, did not have any effects.
8.3.4 Cuticular washings

8.3.4.1 Trapping methods

The trap containing neomatatabiol without the gauze enclosure resulted in more captures than all the other trapping methods, with nearly 80 insects (Figure 59), with the remaining traps capturing between 6 and 19. The traps with protected neomatatabiol and unprotected d-neomatatabiol resulted in nineteen lacewing captures each throughout the sampling period. The trap with the protected d-neomatatabiol resulted in only six lacewings captured. No statistics was carried to compare differences in captures between the treatments out since only one trap per treatments was used. All the lacewings captured were males.

![Figure 59: Number of lacewings captured in each of the four kinds of traps in 2005: unprotected odour dispensers containing either neomatatabiol and d-neomatatabiol in addition to protected dispensers containing neomatatabiol and d-neomatatabiol were used. The highest captures were recorded in unprotected neomatatabiol. Between 1 and 5 lacewings captured from each trap were used to obtain cuticular washings and carry out chemical-composition analyses.](image-url)
8.3.4.2 Analysis of cuticular washings

The gas chromatography analyses of cuticular washings of *P. gracilis* males caught in traps with unprotected neomatatabiol and unprotected d-neomatatabiol had traces of neomatatabiol and d-neomatatabiol respectively (Figure 60). The traps with normal neomatatabiol resulted in traces of normal neomatatabiol in addition to a derivative of the compound, dihydronpetalactone, while the trap containing d-neomatatabiol resulted in traces of d-neomatatabiol and d-dihydronpetalactone. Cuticular washings of lacewings obtained through the four trapping methods had traces of tridecadiene, (Z)-4-tridecene, palmitic acid, linoleic acid and oleic acid. Retention times of gas chromatography traces differ in between the four different treatments because they were done at different times, with up to two years difference, nevertheless traces are comparable and differences are evident.

![Gas chromatography analysis](image)

**Figure 60:** Gas chromatography analysis of cuticular washings of *Peyerimhoffina gracilis* males caught in the field with different trapping methods: Traps contained either neomatatabiol or neomatatabiol labelled with deuterium. In addition, dispensers were either protected with a mesh cage or unprotected. Neomatatabiol was only found in lacewings captured in traps with unprotected dispensers. (Z)-4-tridecene was found in lacewings captured in the four types of traps.
8.4 Discussion

8.4.1 Lacewing captures

8.4.1.1 Annual monitoring

The only lacewing species caught throughout the three years was *P. gracilis* and all were male. *Peyerimhoffina gracilis* overwinters as adults (Donato et al., 2001), and due to colder and shorter days low numbers or none were captured during the coldest months (November, December, January and February), although some individuals were captured despite the low temperatures and shortening days in November 2004 and January and February 2006. In March 2005, what could be the end of the overwintering stage was reflected by the increase in catches in this year. It has been documented that individuals of a mountainous strain of *P. gracilis* are induced into diapause with short days (8:16 L:D) and it was interrupted with longer days (12:12 L:D) (Grimal, 1988). In 2006 lacewings captures started in June, with the exception of a low number of lacewings captured in January. The following year, 2007, an even later start in captures was recorded. In this year lacewings were caught during the months of August and September representing a 75 % drop from the two previous years. The low number of lacewings recorded this last year could have been due to the relatively high rain fall and low temperatures recorded during the whole year. It also could be the successive trapping having an effect in the lacewing population, although it is believed otherwise, since most of the lacewings were freed in the field once they were used in behavioural bioassays. Only a few males were killed for the cuticular washing analysis and the topical neomatatabiol assays. A fact that supports that the hypothesis of it being unique year rather than a decreasing trend, is the number of lacewings captured in the Rothamsted Insect Survey suction trap located in Silwood Park (Ascot, UK), which was considerably lower in 2007 (68 captures from April to September) than in 2005 and 2006 (576 and 1138 captures respectively from April to September) with *C. carnea* being the dominant species found (personal observation).

Another important factor worth mentioning is that during the months of highest number of captures, several attempts were made to capture *P. gracilis* individuals from the vegetation in Silwood Park. This is normally done by beating the foliage of trees and bushes and capturing any insects that fly away with the help of an entomologist net. With the hope of finding females, attempts were made and several species of lacewings were caught but unfortunately none were *P. gracilis*. This is intriguing since there are reports of *P. gracilis* being captured by beating branches in France (Grimal, 1988).
In previous studies it has been documented that male lacewings *C. septempunctata* and *C. japonica* are attracted to the plant *Actinidia poligama*, that naturally produces the compound neomatatabiol (Hyeon et al., 1968). Also, *P. gracilis* has been previously captured in field trials with this traps containing this compound (Donato et al., 2001; Hooper et al., 2002). Although there seems to be a trend that the Silwood Park *P. gracilis* population could be declining, prolonging the monitoring would indicate if 2007 consisted of a “bad” year or if there is a negative trend as indicated by the results.

8.4.1.2 Captures in top-half versus bottom-half of the traps

The difference in captures between the two halves of the trap is worth mentioning. A greater number of lacewings were caught in the top-half of the traps when compared with the bottom-half. Possible explanations for this could be that once the lacewings are lured to the trap, they move around the area and possibly due to phototaxis or geotaxis, move upwards. Insects generally use several means of orientation and positive responses to light have been reported in many insect species including ants, aphids and parasitoids (Wehner et al., 1996; Hajong & Varman, 2002; Storeck, 2002) and possibly these factors could be guiding *P. gracilis* to the upper trap. Another explanation could be the way in which the traps were constructed: The wire from which the dispenser hangs is attached to the top-half of the trap, and the lacewings, when they reach the trap possibly first land on the glass vial and then tend to walk rather than fly (personal observation). The wire offers them a bridge, that by following it are led into trap. In the future it would be interesting, in order to prove this, to build a new version of the trap in which two wires hold the dispenser, one leading to the top of the trap and one to the bottom. Also the set up of video cameras recording the captures would help to determine the behaviour of the lacewings once they reach the trap.

8.4.2 Behavioural assays

When the behavioural response of field-caught males was tested in the olfactometer towards different contrasts between neomatatabiol, (Z)-4-tridecene the combination of both and ether, a significant response towards neomatatabiol was observed when the alternative was ether or (Z)-4-tridecene + neomatatabiol indicating that neomatatabiol elicits a bias in the behavioural response of *P. gracilis* males at a close range. In addition to neomatatabiol also eliciting electroantennogram activity in *P. gracilis* (Hooper et al., 2002). This positive response observed in the field towards the neomatatabiol was confirmed in the present laboratory assays.

An interesting behaviour was observed during some of the olfactometer bioassays where neomatatabiol was involved: Once the lacewing was introduced into the
olfactometer and started walking upwind, it released a brown secretion from the tip of
the abdomen leaving a thin trace (1 - 2 cm long) on the glass surface. The possibility of
it being excreta is partly discarded since generally faeces are produced as very small
dark brown pellets (personal observation). (1\text{R}, 2\text{S}, 5\text{R}, 8\text{R})\text{-Iridodial was discovered as}
a male-produced aggregation pheromone in the goldeneyed lacewing, \textit{C. oculata},
believed to be produced in elliptical glands present between the third and eighth
abdominal sternites of the males (Zhang et al., 2004) and the production of a defensive
secretion in the anterior part of the thorax has been documented in \textit{C. carnea}
(Zhu et al., 2000). It remains unknown the purpose -if any- of this particular secretion. It may be
fruitful to perform chemical analyses to establish its composition and also perform
electronic microscopy in case glands are found in this area. It still remains unknown if
neomataatabiol can effectively trigger the behaviours stated above, or if it is merely a
product of the bioassay (i.e. stress produced by the manipulation).

Surprisingly, when other comparisons were carried out (i.e. neomataatabiol vs. (\text{Z})-4-
tridecene, (\text{Z})-4-tridecene vs. ether, (\text{Z})-4-tridecene vs. (\text{Z})-4-tridecene + neomataatabiol
and (\text{Z})-4-tridecene + neomataatabiol vs. ether), no bias was observed to either of the
choices. The prediction in these assays was that \textit{P. gracilis} males would avoid the (\text{Z})-
4-tridecene and be biased towards neomataatabiol. But surprisingly, when (\text{Z})-4-
tridecene was presented as the alternative to neomataatabiol, no bias towards either
choice was recorded. Probably the dose used of (\text{Z})-4-tridecene was behaviourally
irrelevant or with both compounds presented together, lacewings become erratic
without eliciting a behavioural discernment between the two choices. Another study
recorded an avoidance behaviour towards 500 µg of this compound in \textit{C. carnea}
(Zhu et al., 2000). Unfortunately the study does not specify the way in which it was
administered into airflow of the olfactometer. The amount of (\text{Z})-4-tridecene used was
based on the amount of neomataatabiol placed in the dispensers used in the field and in
olfactometer, which resulted in positive responses, therefore it was assumed that it
would be a good amount to use as a starting point. Unfortunately there were not
enough lacewings to do further behavioural assays with different concentrations. If this
line of research is continued, it would be interesting to asses the effect of lower and
higher doses of (\text{Z})-4-tridecene on the behaviour of male lacewings.

\textbf{8.4.3 Effect of topical neomataatabiol on lacewing survival}

Control treatments of acetone and the lowest dose of neomataatabiol (0.05 mg) did not
affect lacewing survival when applied topically. But when this dose was doubled and
applied on the male lacewings, they commenced moving their abdomen immediately
after the application and more than half of the lacewings died between the second and
third day. The neomatatabiol applied topically at this concentration was absorbed through the cuticle and although it is highly improbable that lacewings would become in contact with such high dose of this compound in nature, it is now established that lacewings become firstly intoxicated and later a high percentage of the lacewings cannot tolerate the compound resulting in the death after two days of a continuous uncoordinated movement.

8.4.4 Cuticular washings

8.4.4.1 Different trapping methods

Lower number of insects were recorded the traps with protected neomatatabiol, protected d-neomatatabiol and unprotected d-neomatatabiol when compared to unprotected neomatatabiol. Since only one replicate of each treatment was done, no solid conclusions can be drawn but speculations can be made about the effects of the different trapping methods on the behaviour of male *P. gracilis*. For instance, lower captures might have resulted in protected dispensers because of the caging itself possibly did not permit the lacewings to have a closer access to the compound. This restriction could have affect the overall behaviour of the males, such as not searching as long and as intensively as they would in the normal traps. An observation that favours this hypothesis is that while the unmodified traps were being checked for captured lacewings, several times (~ > 5 times every year) males were found with their heads in the lid of the dispenser remaining in this position for up to several minutes. This close contact with the compound could be crucial for the lacewings to enter the traps. As for the effect that d-neomatatabiol could have in the number of captures, it is unclear. Isotopes have been used in several occasions to study the biosynthetic pathways of pheromones (Choi et al., 2002; Kim et al., 2005), but when it comes to behavioural responses in insects, isotopes have rarely been investigated. There are few examples of increased and decreased responses towards the chemicals once the molecule is modified (Renou & Guerrero, 2000).

8.4.4.2 Analysis of cuticular washings

The chemical analyses of cuticular washings of lacewings captured from the four types of traps, resulted in five compounds being recorded in every sample. Three of the compounds: Palmitic acid, linoleic acid and oleic acid, are believed to be triglycerides from the cuticle of the lacewings. The other two compounds, (Z)-4-tridecene and tridecadiene, could have a different function. (Z)-4-Tridecene has been found in *C. carnea*, for which a defensive role is attributed (Zhu et al., 2000) and also, a similar compound, 1-tridecene was found to be produced by male *C. oculata*, and elicited an
arrestment behaviour in bioassays in addition to reducing captures in field traps (Zhang et al., 2004). This compound has been previously recorded in *P. gracilis* when headspace entrainments of males resulted in the compound in question being found (A. M. Hooper, unpublished data). The fact that (Z)-4-tridecene and similar compounds have been found in two other lacewing species in addition to being found in the present investigation in *P. gracilis*, suggests that it could be widespread defence strategy in chrysopids.

Analysis of washings from males obtained from traps that had unprotected dispensers resulted in the presence of neomatatabiol. Further analysis showed traces of d-neomatatabiol only from lacewings collected from traps with this compound, indicating that volatiles adhere onto the cuticle of the lacewings. Furthermore, lacewings caught in protected traps had no traces of normal neomatatabiol, confirming that the chemical compound is being picked up from the dispensers and that it is not being produced by the lacewings. Dihydronepetalactone was found in lacewings caught in unprotected traps with neomatatabiol and d-neomatatabiol. This compound is an oxidation product of the neomatatabiol picked up from the dispensers that is metabolized and through enzyme activity, oxidized (A. M. Hooper, personal communication). Although the exact metabolic pathway is unknown, is apparent from analyses of lacewings captured in protected traps that lacewings do not appear to produce neomatatabiol naturally.

The results of the present study suggest that males do not produce neomatatabiol or a similar compound. The scenario where female *P. gracilis* produce a pheromonal compound similar to neomatatabiol to promote the presence of males is more plausible. The similarity of neomatatabiol to aphid sex pheromones may be only incidental. The fact that adult lacewings are not predatory and that only males have been found in the traps, supports the above and makes it highly improbable that the compound is being used as a kairomone to locate aphid prey. In addition, when observation trials were performed with two males placed in a glass vial and a drop of neomatatabiol on a filter paper, it was not uncommon to see that the two males would adopt a mating position (curving the abdomens and opposing the tips); leading to believe that neomatatabiol could have some role in sexual behaviour. This behaviour was not seen in males placed in vials without neomatatabiol. Although female-produced pheromones have not been found in lacewings, the increasing number of studies stressing the importance of pheromonal communication in various lacewings species suggests the possibility of a female-produced pheromone. Air-borne vibrations are normally used by lacewings at close-range (McEwen et al., 2001; Henry et al., 2002); therefore it could be that pheromones attract males at larger spatial scales.
Future investigations under different scenarios (e.g. headspace entrainments of individual and grouped male lacewings) and the acquisition of female *P. gracilis* to carry out behavioural assays and chemical analyses would help clarify the role of chemical communication in *P. gracilis* ecology.

### 8.4.5 Conclusions

The high number of *P. gracilis* males captured in the field and some of the results in the olfactometer assays suggested that there could be a male aggregation role for neomatatabiol. But analysis of cuticular washings suggested the possibility that neomatatabiol is not produced by male lacewings. In addition, the compound \((Z)-4\)-tridecene was observed in cuticular washings. Such compound is believed to be a defensive secretion against predators in other lacewing species, although behavioural bioassays did not show conclusive results, possibly because the dose was not behaviourally significant. More research is needed in this area, especially to elucidate the role of neomatatabiol in *P. gracilis* chemical ecology. Obtaining live males and females to establish an ongoing culture should help.
Chapter 9
General Discussion

9.1 Main findings
The main findings of each experimental chapter are enumerated below:

- Objective: The olfactory behaviour of the parasitoid *A. colemani* was studied towards various closely-related Cruciferae, with the aim to ascertain the parasitoid’s degree of discernment towards five cultivars of the same species of Brassica when parasitoids were reared only on one of these cultivars and the offered whole plants and/or detached leaves.

  **Findings:** Female parasitoids ranked their preference towards the five Brassica cultivars according to their previous experience. A significant level of discrimination was found for both whole plants and detached leaves of the five cultivars.

- Objective: The volatile-emissions of the five cultivars mentioned above were also studied to establish qualitative and quantitative differences which might explain the parasitoid’s observed behaviour.

  **Findings:** Compounds such as (E)-2-hexanal, (Z)-3-hexen-1-yl acetate, (Z)-3-hexen-1-ol, 6-methyl-5-hepten-2-one and (+)-sabinene could be used by female *A. colemani* to discriminate. However analyses of volatile profiles of the five cultivars did not result in clear-cut differences between the compounds although differences were found with multivariate statistical analysis.

- Objective: The behaviour of the parasitoid was also studied towards a range of molecules with differing carbon numbers with the aim to establish differences in discernment and learning of the parasitoid according to the similarity of the molecule in question to Green Leaf Volatiles.

  **Findings:** After experiencing alcohols with molecules having between 5 and 6 carbon atoms, females modified their behaviour, suggesting that there could
be a higher sensitivity in learning molecules of this size rather than with 1-4 and < 10 carbon atoms.

- Objective: In order to obtain a better understanding in differences of learnt and innate behaviours in this generalist parasitoid, the olfactory responses of A. colemani females were studied after exposures to cold temperatures.
  
  **Finding:** What is believed to be the learnt component in the olfactory response of parasitoids was temporarily inhibited by cold, while responses to plant volatiles, believed to be innate, were not altered by cold exposure.

- Objective: Obtain a better understanding of using odour sources with different water-vapour release rates and their importance in confounding effects during olfactometer studies.
  
  **Findings:** Differences in humidity that arise by asymmetries in water vapour-release rates of odour sources used in olfactometer studies of phytophagous insects should be properly controlled for in olfactometer studies.

- Objective: To gain a better understanding of the a possible link between the behaviour of male green lacewings P. gracilis males and a chemical compound closely-related to an aphid sex pheromone is studied through behavioural and analytical-chemistry techniques.
  
  **Findings:** Male-lacewing cuticular washings showed that individuals do not produce neomatatabiol or an analogous compound de-novo. The strongly sex-biased response observed in field captures could be the due to the similarity of the compound to a female sex-pheromone. In addition, the production of what could be believed to be an alarm pheromone was detected in the cuticular washings.

### 9.2 Discussion

#### 9.2.1 Behaviour, parasitoids and lacewings

A behavioural repertoire is affected by genes, environment, physiology and experience (Vet et al., 1990), and the different sensory modalities that interplay add another level of complexity making the understanding of how these factors interact in forming the final behaviour, a challenge. The present study was intended to gain a better understanding of how one of the factors (i.e. volatile chemicals) contributes to the behaviour of a parasitoid species and a green lacewing species, giving rise to more questions than it gave to answers.
In the initial chapter of this work five objectives were set up to investigate. Four of them were exclusively related with the behaviour of the parasitoid *A. colemani* and one of them was to determine certain aspects of the chemical ecology of the green lacewing *P. gracilis*. The first objective related to the behaviour of the parasitoid, was aimed at establishing the extent of discernment of the parasitoid caused by previous plan-experience. As for plant-volatile emissions, different plant species normally result qualitatively in the largest contrasts (Dicke, 1999) and emissions within one plant species can vary according to the herbivore species attacking the plant (Turlings et al., 1998) and according to the genetic variability between the cultivars of a same species. In this context, it was established that the parasitoid adjusts its behaviour to what could be considered relatively small variations in volatile emissions of plant cultivars of the same species (Chapter 3 and Chapter 4, pages 42 and 70 respectively), in most cases favouring the odours of the Brassica variety experienced previously. The strength of the initial experiences of the parasitoid is evidenced by the constancy to which it responds to the plant its aphid hosts were reared on. The experiences that parasitoids undergo at emergence and subsequent foraging has a marked effect in their adult behaviour, and interestingly parasitoids can translate what was learnt in the rearing cages, to different scenarios not comparable at a first sight with it, such as healthy plants and/or cut leaves from healthy plants. Interestingly, parasitoids when faced with what are believed to be reliable cues (i.e. volatiles from the experienced cultivar) against easy-to-detect cues but from an unencountered cultivar (i.e. cabbage) the response was not a clear-cut choice, hinting that parasitoids could be facing the reliability-detectability dilemma in which the larger amount of volatiles emitted from the cut leaves of cabbage (quantity) encounter the “quality” of the volatiles emitted by winter harvest.

In this same context, the second objective was to establish qualitative and quantitative differences that could help explain the parasitoid’s behaviour towards the Brassica cultivars. Although it still remains unknown the volatile profiles of these varieties when attacked by aphids, the basis of this behaviour seems to reside in the presence of compounds such as 

\[(E)-2\text{-hexanal}, (Z)-3\text{-hexen-1-yl acetate, (Z)-3-hexen-1-ol, 6-methyl-5-hepten-2-one and (+)-sabinene, and there seems to be a constancy in the chemicals that allows the parasitoid to favour the Brassica variety on which it previously had encountered aphids. Future experiments with Brassicas infected with aphids reflecting the rearing scenario and determining their volatile profiles are needed to comprehend what chemical information imparted as a learning experiences the parasitoid is acting upon. Also gas chromatography combined with electro-
antennograms using *A. colemani* would show which compounds are the bio-active ones.

In the third experimental chapter of this thesis, the conditioning behaviour of the parasitoid was investigated in more detail (Chapter 5, page 103). The objective was to gain a better insight into the discernment behaviour of parasitoid with respect to certain learnt chemical volatiles. While in previous chapters it was observed that parasitoids could maintain a preference for previously encountered plant volatiles in substantially different scenarios, in this chapter the aim was to establish differences in learning and discernment according to the similarity of the experienced compound to Green Leaf Volatiles. Therefore the behaviour towards this widespread group of plant volatiles consisting of 6-carbon molecules, known as GLVs was used as a base to investigate the parasitoid learning behaviour. Results hinted that there might be a higher sensitivity in learning compounds with 5-6 carbon atoms in their molecular configuration, while compounds having more or less carbon atoms in their linear configuration did not elicit significative changes in the behaviour of the parasitoid. This suggests that parasitoids could be more prone to learn certain molecular configurations, like in this case, structures closely-related to GLVs, fact that is supported by the results obtained in Chapter 4, where it was observed that many of the volatiles emitted by the Brassicas consisted of 6-carbon molecules. It is believed that in the future results could be improved with changes in the training protocol. For instance, shorter training sessions and a longer interval between this session and the testing phase might render positive results and reflect the parasitoids learning capabilities in a better way (G. Poppy, personal communication).

In the fourth objective of the thesis, cold was used as a tool to establish if cold may help dissecting innate responses from learnt ones (Chapter 6, page 120). After exposing female parasitoids to 0 °C for 0.5 h or longer, the preference for the odour of the Brassica cultivar on which they were reared was lost for one hour after the cold treatment had finished, after which, the preference returned. But when plant odours were presented with clean air as the alternative choice, females could discern plant odour, suggesting that the olfactory and locomotor system were not altered by the cold exposure, whereas responses arising from learning processes appear to be inhibited temporarily and in turn revealing underlying innate responses. In an ultimate goal, not reached in this thesis, it would be interesting to know in this generalist parasitoid where learning plays a central role, up to what extent innate preferences contribute to the behaviour of the parasitoid. For this, these initial experiments need to be complemented with bioassays in which experiences are imparted in a controlled way.
and later exposures to cold could help taking this subject further, in addition to using completely naïve individuals, which is a concept difficult to reach in individuals where it has been demonstrated that learning occurs even in stages prior to adulthood (Villagra et al., 2007).

The degree to which responses are tied to experience and the variety of responses shown by *A. colemani* in this study are striking. *Aphidius colemani* is a generalist parasitoid and its hosts can be found on many different plant species, which makes the sensory cues that it encounters many and diverse, suggesting a highly plastic behaviour (Olson et al., 2003; Steidle & van Loon, 2003). The results evidenced in this study show that *A. colemani*, being a generalist parasitoid, has a highly adaptable behaviour and can learn and recognize molecules close to the range of the 6-carbon configuration as displayed by GLVs. To reach more robust conclusions in terms of the importance that learning has in the ecology of this parasitoid’s innate responses, and how these could influence the parasitoid’s behaviour, further investigations are need.

Other studies have shown the flexibility in *A. colemani* female’s olfactory behaviour (Storeck, 2002; Bilu et al., 2006; Vamvatsikos, 2006) but other sensory modalities have also been shown to be adaptable in Aphidiid parasitoids. In this context, the above experimental chapters gave rise to noticing the importance that another sensory input, humidity, could have in confounding results of olfactometer studies arose unexpectedly (Chapter 7, page 135). Experiments showed that humidity has to be considered and controlled for carefully in olfactometer experiments in order not to confound responses thought to be triggered by chemical information with what could be the result of variations in humidity release-rates of the sources used. This chapter offers an initial insight into a problem that might occur more frequent than initially considered, but research needs to be taken further in experiments where hygroreception is integrated with chemoreception in order to obtain a holistic view of the parasitoid’s behaviour.

The last objective, was to investigate various aspects of the role that neomatatabiol, a chemical compound closely related to an aphid sex-pheromone, could has in relation to the chemical ecology of the green lacewing *Peyerimhoffina gracilis* (Chapter 8, page 148). Analysis of cuticular washings hinted the possibility that neomatatabiol is not produced by male lacewings, since no signs of this compound was found in gas chromatography trances. In addition, an interesting discovery was made when the compound (Z)-4-tridecene was observed in these cuticular washings: Such compound is believed to be a defensive secretion against predators in other lacewing species. Unfortunately behavioural experiments did not show conclusive results as to whether
this compound could produce an aversive behaviour, possibly because the dose was not behaviourally significant. More research is needed in this area, especially to elucidate the role of neomatatabiol in *P. gracilis* chemical ecology. The role that neomatatabiol could have in lacewing chemical ecology is yet to be fully understood.

### 9.2.2 Summary of ideas

The main ideas of the thesis are summarized below (Figure 61, Figure 62 and Figure 63). Since some of the objectives set out to investigate differed quite substantially from each other, it was decided that the best way to capture the essence of the research carried out would be to represent the ideas in three separate blocks.

**Figure 61:** Diagram summarizing the resulting ideas arising from the studies of the parasitoid *Aphidius colemani*’s chemical ecology.
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Figure 62: Diagram summarizing the ideas resulting from the studies of the relevance of humidity in olfactometric studies.
9.2.3 A practical point of view

A better understanding of the basis of the olfactory and other types of behaviour in biological control agents can lead to better control strategies (Vet et al., 1990). *Aphidius colemani* is believed to be a potentially effective control agent and has been used in many occasions against several economically important pests with varying degrees of success (Messing & Rabasse, 1995) and knowing the extent to which this parasitoid species and other natural enemies modify their responses in unencountered chemical scenarios, in addition to having a clear knowledge of where the borders lie in the parasitoids discernment when variations occur, is crucial to exploit at a maximum the innate and experience-related behaviours of parasitoids in biological control programmes.

One practical aspect of control strategies that may be improved in commercially-available parasitoids and natural enemies is through exploiting their learning capabilities. The insectaries where these are reared consist of environments very
dissimilar to the ones in which the they will be released to perform as control agents, and the lack of contact with the semiochemicals and other types of cues that will be present in the 'real' environment is normally a factor that causes reduced efficiency once the parasitoids are released (Vet et al., 1990; Gandolfi et al., 2003a). It has been suggested that parasitoids could be trained to specific synthetic kairomones prior to their release to overcome this problem (Hare et al., 1997; Grasswitz, 1998; Powell et al., 1998b; Blande et al., 2007). In this study it has been shown that various green leaf volatiles could be responsible for eliciting responses in *A. colemani* (Chapter 4, page 70), and further investigations could lead to elucidating which ones are relevant and how they might be used in beneficial ways in order to manipulate the behaviour of the parasitoids. Further studies in memory retention times, number of training protocols needed to achieve a reliable response and the factors affecting decisions in systems where subtle variations due to intrinsic plant-factors such as age and abiotic factors such as lighting conditions and/or hydric conditions (Takabayashi et al., 1994), will help establishing how they might affect behaviour helping to elucidate the chemical ecology of *A. colemani* and parasitoids in general. Also the importance of learning in other predatory species used in biological control programmes, such as lacewings, is yet to be investigated.

Another aspect that is exploited is the innate responses of natural enemies and pests towards pheromones and allelochemicals. The use of pheromones in biological control programmes has been gaining acceptance and importance since 1991, when the first successful control of the codling moth, *Cydia pomonella*, was achieved by disrupting the mating of the moth by placing traps with the codling moth sex pheromone "codlemone" (Witzgall et al., 2007). The use of pheromones has proved to be successful in moths. But the use of parasitoid-sex pheromones has been relatively poorly studied, although it has been suggested that they could be used to attract males to sites where females were previously attracted by kairomones (McNeil & Brodeur, 1995; McClure et al., 2007). The manipulation of existing parasitoid populations has rendered positive results in many occasions mainly by achieving retention of individuals in target areas and by enhancing oviposition rates (Lewis & Martin, 1990). Also aphid sex-pheromones are used to promote the temporal coexistence of parasitoids with their aphid hosts in crucial moments when pest numbers are still low in the beginning of the season when colonizing crop fields (Powell et al., 1998b). Responses towards aphid-pheromone compounds such as (4aS,7S,7aR)-nepetalactone have been found in parasitoids (Hardie et al., 1994) and could be useful in such manipulation techniques. The study of innate responses towards plant volatiles...
is especially difficult in insects that have a learnt component in their behaviour even before emergence from the pupae, as suggested by Gutiérrez-Ibáñez (2007) for the parasitoid *A. ervi*. This could be the case for other parasitoid species (Gandolfi et al., 2003b; Vamvatsikos, 2006) and obtaining a better insight into the innate bases of behaviour, especially in relation to plant-volatile emissions, by using cold exposures as shown in Chapter 6 (page 120) can have potential to shed light into the study area.

The use of infochemicals to manipulate lacewing populations has been proposed on a number of occasions and methyl salicylate has been found to be a powerful attractant for the goldeneyed lacewing *C. oculata* and *C. nigricornis* and has been suggested as a key compound to monitor populations of beneficial insects or to improve pest-control methods involving lacewings (James, 2006). Combination of methyl salicylate with the male-produced aggregation pheromone (1R, 2S, 5R, 8R)-iridodial could offer improved results since a synergistic effect between both has been found (Zhang et al., 2004).

Although the specific role of neomatatabiol in the chemical ecology of *P. gracilis* remains unknown, it suggests that pheromones in lacewings could be more widespread than previously thought and the prospects of using them to enhance biological control programmes is encouraging.

Another relatively new technique that most surely will benefit from further knowledge in aspects of insect biology, is genetically modified (GM) crops which has been implemented in the past decade offering many advantages over conventional crops (more in section 1.1.3.3.2, page 20). One of the main positive aspects of using GM crops is that it offers the advantage of antagonizing the action of the herbivore through the production of specific insecticidal proteins. In addition, another way in which GM crops can be useful is through the control of certain plant attributes such as physical characteristics, the production of pollen and nectar in addition to volatile emissions. Modifying these can have the potential of enhancing the performance of the third trophic level in biological control programmes (Cortesero et al., 2000). Recent studies with transgenic plants have shown potential through volatile-emission manipulation for keeping herbivores away and attracting natural predators. The production of terpenoids has been modified (Degenhardt et al., 2003; Kappers et al., 2005) in order to attract parasitoids. But due to the fast learning capabilities of insect parasitoids, it has been suggested that these kind of manipulations might offer fruitful results (Turlings & Ton, 2006) provided that these signals are promoted in attacked plants rather than in plants devoid of herbivores. A situation that would only mean natural enemies wasting their energy searching for non-existent hosts (Poppy & Sutherland, 2004). Genetically modified crops can offer new and promising prospects in advancement of biological
control techniques, but manipulating natural enemies with enhanced plant characteristics still has to go a long way.

Obtaining a thorough understanding of the direct and indirect consequences that changing plant attributes can have on the ecology of organisms is essential to avoid unexpected risks (Poppy, 2000; Poppy & Sutherland, 2004). Also having a clear knowledge of how the addition of large amounts of synthetic infochemicals can affect the ecology and organisms is primordial. Situations where different species respond to the same compound can happen more than expected. As suggested by some of the results in this study, a potential situation where pheromonal compound is used to manipulate one species, but a second species becomes affected (i.e. lacewings), is not a scenario difficult to imagine. Thus, a good knowledge on the behaviour of natural enemies in a laboratory scale is basic but studies at a broader spatial scales (i.e. field) are essential.
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