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# OLFACTORY BEHAVIOURS ASSOCIATED WITH HOST-APHID LOCATION IN A GENERALIST PARASITOID WASP

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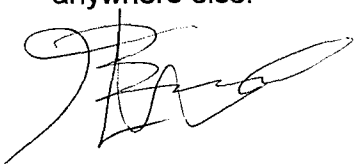
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# Abstract

Olfactory responses of the generalist parasitoid wasp, *Aphidius colemani,* were studied to provide insight into the origin of behavioural preferences. Bioassays were conducted in an olfactometer and insects were offered choices between leaf odours from different plant species and different varieties of the same species (i.e. Brussels sprout). After the parasitoids emerged from peach potato aphids (*Myzus persicae)* that had been reared on Brussels sprout (*Brassica oleracea* L. var. *gemmifera*) variety Bedford-Winter harvest or Red delicious, they preferred the leaf odour of that sprout variety over the odour of others. When the parasitoids emerged following excision of pupae from aphid mummies, they retained their preference for the odour of the plant on which the mummies were formed when they were reared on Winter harvest but not on Red delicious. Parasitoids that emerged from hosts reared on a chemically-defined artificial diet, preferred several plant-leaf odours to clean air but did not distinguish between different sprout varieties regardless of the plant of origin of their mothers. The validity of these results was confirmed parasitisation success in a host-selection study. When diet-reared parasitoids were allowed to contact sprout-reared aphid mummies for 24 h, they developed a preference for the sprout variety on which those mummies had been reared. There also appears to be a maternal effect on preference in that, if the mother of diet-reared parasitoids was raised on Winter harvest, a single mummy from that same variety provided a large enough stimulus to elicit preference for Winter harvest odour over that of a second sprout variety, Red delicious. However, in order to switch preference to the other sprout variety, Red delicious, experience of a single mummy was not enough and five were needed. Thus, the parasitoids are influenced by post-emergence experience of the chemical cues contacted on the cuticle of the mummies. The results are discussed in relation to what seems to be a hierarchy of preference determination in the adult parasitoid from innate (i.e. plant odour preference over purified air), through maternal influence in the ease at which preference can be learned, as well as the overriding post-emergence learning.

# Declaration

The work presented in this thesis is entirely my own and have not been submitted anywhere else.



Panagiotis Georgios Vamvatsikos



Professor Jim Hardie

(PhD supervisor)

# Dedication



To my grandmother Angeliki Tsakmakli who has passed away on the day I was taking written examinations held by The State Scholarship Foundation (I.K.Y), Athens, Greece (Saturday, 19th May 2001). I will always remember her words implanted in my mind since I was a pupil at school:

"When you grow up, you should make efforts to become a pillar of the society in order to contribute positively to its progress".

The present study is a small sample of my efforts and I will continue trying… "Farewell dear grandma. I will always remember you".

Greek translation/Ελληνική μετάφραση:

Στην γιαγιά μου Αγγελική Τσακμακλή η οποία έφυγε από κοντά μας την ημέρα που έδινα γραπτές εξετάσεις που διοργανώθηκαν από το Ίδυμα Κρατικών Υποτροφιών (I.K.Y.), στην Αθήνα (19 Μαϊου 2001). Θα θυμάμαι πάντοτε τα λόγια της που μου έχουν εμφυτευθεί στο μυαλό από τότε που ήμουνα μαθητής στο σχολείο:

"Όταν ενηλικιωθείς, θα πρέπει να κάνεις προσπάθειες να γίνεις ένα αξιοσέβαστο μέλος της κοινωνίας έτσι ώστε να συμβάλλεις θετικά στην πρόοδο αυτής". Η παρούσα μελέτη αποτελεί ένα μικρό δείγμα των προσπαθειών μου και θα συνεχίσω να προσπαθώ... " Ώρα σου καλή αγαπημένη μου γιαγιά. Θα σε θυμάμαι πάντα".

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# CHAPTER 1: Introduction and literature review

## 1.1 Introduction

During the last decades of the 20th century, following large outbreaks of pests due to the development of resistance to pesticides, growers and researchers have come to realise that future success in pest management can best be achieved through an ecological approach to control, within the framework of Integrated Pest Management (IPM). The latter focuses on the integration of relevant ecologically-sound tactics of pest management, including biological control, environmental management, genetic manipulation and behavioural manipulation methods while the pesticides are employed sparsely and synergistically with these methods. Biological control through the action of beneficial natural enemies is perhaps the most ancient of all forms of insect pest control. Two major features of natural enemies are essential to their effectiveness: retention in target areas and efficient location and attack of target hosts or prey (Prokopy & Lewis, 1993).

The present study deals with two insect groups of great economic importance in terms of agricultural production. Aphids are a worldwide pest group and parasitic hymenoptera form a very diverse group (a million species or more) within the insect class (Quicke, 1997) including promising candidates for aphid biological control, with some of the species already commercialised.

Research on parasitoids has focused on behavioural and ecological aspects to shed some light on the mechanisms by which the parasitoids respond towards external stimuli. A better understanding of the different factors that influence the behaviour of natural enemies is essential for developing manipulation procedures for their effective performance as biocontrol agents (Salt, 1958). For example, behavioural methods of managing pests using parasitoids may involve the use of chemical stimuli, physical stimuli, or both (Vinson, 1985).

Such mechanisms can often provide the 'tricks' which we can introduce to the pest-parasitoid interaction to improve the efficiency of parasitoids for biological control (van Emden, 1996). The potential of discovering parasitoid-mechanisms for successful pest manipulation is obvious in ecological and economic terms as development of resistance mechanisms comparable to pesticides was less likely to occur. However, selection pressure on defence mechanisms in host-parasitoid interactions can also influence resistance/immunity through an evolutionary process (Kraaijeveld *et al.*,

2002) and the probable cost of resistance may arise, for example, from genetic variation of the natural enemies against the defence mechanisms of their hosts (Ferrari *et al.*, 2001).

The present study is relevant to the control of aphid pests with indigenous parasitoids. The aim is to investigate the host-selection behaviour of aphid parasitoids, (in particular the commercially available *Aphidius colemani*, Hymenoptera, Aphidiinae) and obtain some knowledge about possible mechanisms that could lead to the manipulation of parasitoids for biological control. The specific objectives of this study to date are:

1. To investigate host location and preference of aphid parasitoids over four offered odour choices (different plants) after they have been reared on one of them and
2. To compare the aphid-parasitoids responses but towards offered odours of four different varieties of the same plant species (i.e. Brussels sprouts, Brassicaceae).
3. To investigate how any preference is learned.

## 1.2 Biology of aphids

In the Insecta, aphids (Aphididae) are considered as a successful group of pests as they can infest and cause severe damages to many crops. Although predominant in the temperate regions aphids have a worldwide distribution. Many species are pests of agricultural and horticultural crops (Blackman & Eastop, 2000). All species feed on plant sap and most of them are small and inconspicuous insects. Parthenogenetic reproduction (they do not require fertilization for the development of their embryos) and polyphenism (the development within a species of several different adult morphs) are main characteristics of aphids (Dixon, 1973).

Aphids transmit more plant viruses than any other group of animals, and it is likely that aphids do more damage by transmitting viruses than by direct feeding on the plants (Swenson, 1968; Ng & Perry, 2004; Ng *et al.*, 2005). The growth of an infested plant is usually abnormal and stunted. The aphids remove nutrients from the plant and their toxic saliva is translocated in the phloem. The production of honeydew can favour development of sooty moulds on leaves (Summers *et al.*, 2004). Aphid feeding can also induce galling, tissue senescence and leaf curling (Miles, 1989).

### 1.2.1 *Myzus persicae* (Sulzer)

The green peach-potato aphid, *Myzus persicae* (Sulzer), is found throughout the world. It is probably of Asian origin like its principal primary hosts *Prunus* spp. (Blackman & Eastop, 2000). The earliest reference to this species dates back to early 19$^{th}$ century from Australia (Wilson *et al.*, 2002). It is highly polyphagous and one of the most important insect pests of the world (van Emden *et al.*, 1969). Both cyclical parthenogen and obligate parthenogen genotypes of *M. persicae* have been identified in the field (Vorburger, 2006). Besides directly damaging valuable agricultural and horticultural crops in the field as well as in the glasshouse, this species is a very important virus vector of more than 100 plant viruses to various families including Brassicaceae (Kennedy *et al.*, 1962; Nault, 1997).

This aphid can be a pest at low densities and is often too scarce to be a reliable food source to sustain a rapid increase of a natural-enemy population. The life cycle of *M. persicae* has been extensively reviewed (van Emden *et al.*, 1969; Blackman & Eastop, 2000; Vorburger, 2006). , *Myzus persicae* can be abundant at the transplant stage of brassica plants but otherwise they seem to prefer old and senescing leaves (van Emden & Wearing, 1965; van Emden *et al.*, 1969).

## 1.3 Biology of aphid parasitoids

The biology of parasitoids has been well documented (Clausen, 1940; Askew, 1971; Hågvar & Hofsvang, 1991; Godfray, 1994; Quicke, 1997; Hochberg & Ives, 2000). The term parasitoid was first used by (Reuter, 1913) to distinguish organisms that are parasitic only during their immature stage and which generally cause the death of their host, from more typical parasites. There are two main groups of hymenopterous parasitoids of aphids, the Aphelinidae (Chalcidoidea) and the Aphidiinae (Ichneumonoidea: Braconidae). The latter, include the most common and economically important genera such as *Adialytus*, *Aphidius*, *Diaeretiella*, *Ephedrus*, *Lipolexis*, *Lysiphlebus*, *Monoctonus*, *Pauesia*, *Praon* and *Trioxus*. More than 400 species are known and most of them occur in the temperate and subtropical regions of the world. The Aphidiinae are small insects and the adult colouration varies considerably from black or dark brown with yellowish, orange or yellow-brownish patterns on the abdomen (Starý, 1988).

### 1.3.1 Life cycle of Aphidiinae

The Aphidiinae are strictly endophagous parasitoids of aphids. They have generally four larval instars but only the final stage larva kills the aphid host (Cloutier &

Mackauer, 1979; Polaszek, 1986). Before completing its development the larva spins a cocoon inside, or in the case of *Praon* spp. under, the empty aphid cuticle. Gradually, the aphid skin becomes indurated and the typical "mummy" develops. Prepupal, pupal and adult stages develop within the mummy. The adults cut an emergence hole in the aphid cuticle.

Oviposition usually occurs soon after the emergence of the female, irrespective of mating and feeding. The parasitoids seem to feed on aphid honeydew (Starý, 1988) and the production of honeydew from parasitised aphids increases (Cloutier & Mackauer, 1979). Studies have shown that feeding on aphid honeydew increases the longevity and/or fecundity of the parasitoids (England & Evans, 1997; Wäckers, 2001; Lee *et al.*, 2004). Oviposition procedure is typical. After contact has been made with the host aphid by antennal tapping, the female stands on erect legs and bends her abdomen forwards under the thorax to drill the aphid with her ovipositor. One egg is usually deposited in each individual host. Reproduction is mainly biparental and unfertilised (haploid) eggs give rise to males (arrhenotoky) while fertilised (diploid) eggs produce females (thelytoky) (Starý, 1970).

The developmental rate of a parasitoid species is influenced by temperature and may take from two to several weeks (Champbell *et al.*, 1974; Rohne, 2002). It also depends on the aphid instar at parasitisation. In the earlier instars, development of parasitoid larva is delayed while in the later instars it is accelerated (Cloutier & Mackauer, 1979; Rohne, 2002) .

### 1.3.2 *Aphidius colemani* Viereck (Hymenoptera: Aphidiidae)

*Aphidius colemani* Viereck is a cosmopolitan species and occurs in most parts of the world. It originates from the transition area between the Mediterranean and Central Asia (Starý, 1975). The optimum temperature for the development of *A. colemani* ranges between 20-25°C (Gueanoui, 1991).

It is already commercialised for aphid biological control in greenhouses because of its high reproductive potential and short development time. it has the ability to parasitise successfully cotton aphid, *Aphis gossypii* (Glover) and green peach-potato aphid, *M. persicae.* It has been used successfully for the biological control of these insects in glasshouse vegetable crops (e.g. cucumber) in Mediterranean regions, the Netherlands and the United Kingdom by maintaining pest populations below economic thresholds (van Schelt *et al.*, 1990; Gueanoui, 1991; Bennison & Corless, 1993).

As *A. colemani* is polyphagous (generalist parasitoid), it can also parasitise various other aphid species including *Aphis fabae* (Scopoli) (Volke & Stadler, 1991),

*Rhopalosiphum padi* (L.), *R. maidis* (Fitch) and *Toxoptera aurantii* (Fonscolombe) (Messing & Rabasse, 1995).

## 1.4 The searching behaviour of parasitoids

Parasitoids are of immense importance in natural and agricultural ecosystems where they influence or regulate the population density of many of their hosts. Much research on parasitoids has been stimulated by the frequent success in biological control programmes (Godfray, 1994). Many species have been released to combat agricultural pests and while effective control is not guaranteed, huge savings, both in financial and human terms, have resulted from successful programmes.

### 1.4.1 Parasitoid-plant relationship

A study on plant-parasitoid interactions between host and non-host plants using Y-tube olfactometer bioassays showed that not only was the host plant preferred over the non-host but also infested host plants elicited the stronger parasitoid response compared with the response to the uninfested ones (Gohole *et al.*, 2003). Furthermore, their study showed that the pupal parasitoid *Dentichasmias busseolae* (Heinrich) could not discriminate between volatiles emitted from infested *Zea maize* (L.) plants (the host plant) with either of the two herbivore species in question, *Chilo partellus* (Swinhoe) and *Busseola fusca* (Fuller), and overall the volatiles emitted from the plant-herbivore complex elicited the stronger parasitoid response.

However, evidence has been presented that when a parasitoid offered a plant odour against clean air significant responses to the plant odour can be recorded. In particular female parasitoids *Apanteles kariyai* (Watanabe) responded positively not only to uninfested leaves of the lepidopteran larvae *Pseudaletia separate* (Walker) host plant species such as corn (*Zea mays* L.) or rice (*Oryza sativa* L.) in a Y-tube olfactometer but also to the odours from uninfested leaves from non-host plants such as mulberry (*Morus bombycis* KOIDZ.), dandelion (*Taraxacum japonicum* KOIDZ.), Welsh onion (*Allium fistulosum* L.) and (Kidney bean (*Phaseulous vulgaris* L.) when each one of them tested against clean air. This response to the non-host plant odours is a special case of plant-parasitoids interactions which is known as "misunderstanding" (Takabayashi *et al.*, 1991).

### 1.4.2 Parasitoid-host relationship

(Godfray, 1994) reviewed recent information of successful parasitism, and divided the process into host-habitat location, host location, host acceptance and host suitability. Earlier, (Vinson, 1985) suggested a larger number of divisions, for example, he divided host acceptance into examination, probing, drilling and oviposition. This model emphasises a static hierarchical view of parasitoid behaviour.

A more dynamic model was proposed (Lewis *et al.*, 1990; Vet *et al.*, 1990) according to which the stimuli will vary in their information content and the parasitoid should respond to the stimulus most closely associated with the host. Thus, the stimuli may have the information content of a volatile chemical produced by the host, a chemical associated with the faeces, and so on down to the lowest ranking stimuli, which might be associated simply with the host habitat. In particular, after a parasitoid finds a host on a food plant, it might use volatile chemicals associated with that food plant in future host location and a latent response to the food plant is promoted through experience (Geervliet *et al.*, 1994).

### 1.4.3 Cues from the host plant

An extensive review on parasitoids' behaviour suggested that they are attracted to their potential hosts by both physical and chemical cues, which induce the parasitoids congregate in the area where the hosts are located. The chemical cues can be derived generally into those from a) the host, b) the host's products (e.g. honeydew, faeces), c) the host-plant complex and d) the host's plant food source (Vinson, 1985).

Moreover, a recent review has showed the important role of the quality of the host's food plant on parasitoids and preys illustrating examples of how a good or bad quality diet may affect such tri-trophic interactions and in particular the increase of population, the growth and survival ratio, the sex ratio, the oviposition preference or the formation of a defence mechanism against natural enemies (Awmack & Leather, 2002).

The importance of chemical cues (semiochemicals) in parasitoid searching behaviour is well known (Vinson, 1976; Noldus, 1989; Du *et al.*, 1996; Vet, 1999) and they can affect parasitoid behaviour in a number of ways. It was observed that the gravid ichneumonids *Coccophagus turionellae* responded positively to the odour of pine trees, which is the food-plant source of its host the pine shoot moth *Rhyacionia buoliana* (Denis & Schiffermuller) (Thorpe & Caudle, 1938). It has also been found that the ichneumonid *Itoplectis conquisitor* (Say) which also attacks *Rhyacionia buoliana* preferred the odour of Scots pine (*Pinus silvestris*) to a far greater degree than to the

odour of Red pine (*Pinus resinosa*), and this is also reflected by the degree of parasitism found in the field (Arthur, 1962).

The host location by the parasitoid *Diaeretiella rapae* (M'Intosh), which attacks a number of aphid species, including *M. persicae*, especially those on crucifers, has been studied and it has been demonstrated that the parasitoid responded to volatile mustard oils released by the host plant. They also found that the presence of cabbage plants growing nearby increased the parasitism of *M. persicae* on sugar beet plants because parasitoids were attracted to the area by the crucifer volatiles (Read *et al.*, 1970). Herbivore-induced responses of plants serve also as a reliable estimate of the host density in a patch (Turlings & Wäckers, 2004). A study suggested that levels of volatiles emmited by a plant infested with aphids is related to the duration of infestation (Du *et al.*, 1998). Extensive use of aphid induced cues by parasitoids to adapt patch exploitation behaviour was recently demonstrated (Tentelier *et al.*, 2005).

However, odours emanating from plants may not always induce parasitoid responses. It has been showed that low rates of parasitism of the larch sawfly (*Pristiphora erichsonii* Hartig) by the tachinids *Bessa harveyi* (Townsend) and *Drino bohemica* (Mesn.) found in mixed forests as compared with pure stands of larch occurred because the odour of the host plant was masked by the volatiles of many different plant species (Monteith, 1960).

Much more evidence for the importance of host-plant odours has been obtained from behavioural studies with Y-tube olfactometers to show positive responses to wheat in both sexes of two parasitoids of cereal aphids *Aphidius uzbekistanicus* (Luzhetski) and *Aphidius ervi* (Haliday), though only females responded positively to the odours of aphids (Powell & Zhi-Li, 1983).

Using also a Y-tube olfactometer, it has been demonstrated that *A. colemani* females prefer the host plant on which they have been reared (*M. persicae* on Chinese cabbage or oil seed rape) and plant chemical cues are important through a process of learning by subsequent foraging experiences (Storeck *et al.*, 2000). In another example, chemical cues emitting from plants (semiochemicals) are also important in the leafminer parasitoid *Pholetesor bicolor* (Nees) as they evoke a positive response in its probing behaviour on damaged sites by its host *Phyllonorycter pomonella* without the presence of the host itself or host's by-products (contact kairomones) be necessary (Dutton *et al.*, 2000). However, a recent review has argued that there may be a difference in responses elicited by semiochemicals (infochemicals and kairomones) between generalist and specialist parasitoids and their dependence on a specific diet in order to demonstrate these responses cannot be accepted in general as all arthropods are expected to use infochemicals to a certain degree (Steidle & van Loon, 2003).

### 1.4.4 Reliability – detectability theory

During the different phases of host location, parasitoids can use infochemicals derived directly from their host or the substrate on which the host lives (Vinson, 1985; Steidle & van Loon, 2003) and such cues can differ in their reliability and detectability (Vet & Dicke, 1992; Wäckers & Lewis, 1994). Thus herbivore-induced plant volatiles can be used as host-location cues (for review see Dicke, 1999). The important roles of long-range and short-range chemical cues in host location by insect parasitoids is well known (Vinson, 1985). Short-range cues emitted from the host and its by-products (e.g. frass) are reliable indicators of host presence (Rojas *et al.*, 2006) but these cues are difficult to detect (Vet *et al.*, 1991; Turlings *et al.*, 1993; Wang & Messing, 2003) since herbivores have evolved to emit low levels of such cues in order to be inconspicuous to their natural enemies.Some hosts, however, may become conspicuous through their pheromone communication which makes them detectable not only by others of their own species, but also by their parasitoids (Read *et al.* 1970; Hardie *et al.* 1991). On the other hand, herbivore-induced stimuli as plant volatiles (long-range cues) can be easier to detect although they cannot be so reliable and precise about where hosts are located. Nevertheless, parasitoids can detect quantitative differences in volatiles emitted from herbivore-infested plants initially utilising this information to assess patch profitabilities in their habitat and subsequently to discriminate between different host densities (Geervliet *et al.*, 1998). Following herbivore attack, not all plants respond in the same manner and the induced release of volatiles can differ between plants of the same species under attack of different herbivore species (Powell *et al.*, 1998).

Thus, the reliability and detectability of cues present in the environment appears to be a complex problem that natural enemies have to solve for successful host location (Vet *et al.*, 1991). In addition, Vet *et al.* (1992) suggested three possible ways that adult wasps may employ to overcome the complexity: a) Parasitoids use specific, detectable chemical signals (infochemical detour) from host offspring that will not be parasitised (Wiskerk *et al.*, 1993), b) Feeding herbivores induce plant volatiles often specific to each herbivore species, which are detectable by the parasitoids (Steidle & van Loon, 2003) and c) Using the ability to learn (associative learning) parasitoids can improve their searching efficiency when they relate available cues (Bleeker *et al.*, 2004). However, Vinson (1998a) reviewed these ideas and while he agreed that cues may indeed deviate in their detectability and reliability properties, he also argued that this matter may not be the only, or most important critical issue that has induced alternative host-location strategies in insect parasitoids.

Since the foundation of the reliability – detactebility theory several studies investigated the idea. For example, in a study of two different tritrophic systems the parasitoids *Cotesia kariyai* and *Cotesia glomerata* attacking *Pseudaletia separta* and

*Pieris rapae* lepidoptean hosts on corn plants solved the host-location problem in different ways: a) *C. Kariyai* responded to specific plant volatiles emmited after damage by its host and b) since *C. glomerata* responded to both naturally and artificially infested plants it seems to use specific volatiles from uninfested plants in combination with general plant volatiles as well as those from infested plants to locate its hosts (Takabayashi *et al.*, 1998).

Using the *Drosophila-Leptopilina* insect-parasitoid system in dose-response experiments with aggregation pheromones, the parasitoid's chemotactic response was in accordance with the reliability-detectability criteria and was influenced by substrate quality (Wetheim *et al.*, 2003). In another study, Wiskerke *et al.* (1993), showed that *Leptopilina heterotoma* (Thomson), a larval parasitoid of *Drosophila*, overcomes the reliability-detectability problem by identifying a volatile aggregation pheromone of adult flies as the detectable source of information to locate and attack *Drosophila* larvae. Another solution to the problem has been shown with the larval parasitoid *Cotesia glomerata* which uses infochemicals from herbivore-damaged plants as host location cues while infochemicals from the hosts or its by-products seemed not to be utilised as much by this parasitoid species (Steinberg *et al.*, 1993).

Moreover, it has been suggested that there are evolutionary pressures for pupal parasitoids, facing the reliability-detactability problem, to find alternative host-location strategies (Vet *et al.*, 1995). Many insect species use some sort of vibrational communication (Cocroft & Rodriguez, 2005) and parasitoids can use vibtration to locate hosts. Indeed, larger adult females have an improved searching efficiency as they can produce higher intensity vibrational signals (Otten *et al.*, 2001). In addition, it has been demonstrated that vibrational sounding by the pupal parasitoid *Pimpla (Coccygomimus) turionellae* (L.) uses its ability to produce vibrations to locate well-hidden simulated hosts (cigarette filters) in the absence of both chemical and visual cues (Wäckers *et al.*, 1998). In such cases the parasitoid may locate its host via echolocation (Vinson, 1998b) but such location mechanisms may be difficult to utilise by insect parasitoids in the field due to the complexity of the surrounding environment (background noise) and presumably can mask vibratory signals triggered by the host (Casas *et al.*, 1998).

Thus, any signals that can improve parasitoid searching efficiency appear to be of major importance, particularly given the short adult lifespan (Vet & Dicke, 1992; Conti *et al.*, 2004). Specific signals like kairomones (chemical cues that benefit only one of two interacting organisms i.e the receiver - parasitoid) and synomones (chemical cues that benefit both the interacting organisms i.e the emmiter - plant and the receiver - parasitoid) (Vet & Dicke, 1992). The advantage of kairomones is that they appear to be reliable and specific signals but with limited detectability by insect

parasitoids from a long distance (Fujiwara *et al.*, 2000; Gouinguenè *et al.*, 2003). In contrast, synomones are plant volatiles emmited in much greater amounts than kairomones depending on the scale of herbivore infestation. As a consequence, synomones are more detectable but less reliable than kairomones (Colazza *et al.*, 2004a,b). The interaction between kairomones and synomones (hexane or ether rinses of *Toxoptera aurantii* aphid cuticle and volatiles from infested tea plants, respectively) was shown to improve parasitoid responses to its host-plant complex (Han & Chen, 2002). Furthermore, synomones are specific, depending on the invading herbivore species and they can spread systemically to insect-free parts of a host plant (Guerrieri et al., 1999) and plant-to-plant aerial and/or root communication can trigger analogous responses to uninfested plants in close proximity to thos infested (Chamberlain *et al.*, 2001; Pickett & Poppy, 2001; Guerrieri *et al.*, 2002).

### 1.4.5 Learning

#### *1.4.5.1 Parasitoid learning of chemical cues*

Parasitoid learning behaviour can be affected by experience at various stages of their life cycle. Sometimes cues appear to be learned by the immature insect, which affect their responses as adults, such as during emergence from the cocoon, a parasitoid may initially focus on cues that guided their mothers to the host or on the plant odours associated with the host (Bogahawatte & van Emden, 1996; Douloumpaka & van Emden, 2003). However, experiences during the adult stage appear to have a greater effect on the insects' responses. In addition, it seems that generalists parasitoids have a greater ability to learn and discriminate volatiles from host-plant complex using Brussels sprout and cabbage plants in particular (Geervliet *et al.*, 1998), in comparison with specialists parasitoids, and it has been suggested that they have set responses to certain plant odours (Vet & Dicke, 1992).

#### *1.4.5.2 Learning by immature parasitoids*

The idea of larval learning in insects is relatively old. At the beginning of the last century it was suggested that herbivorous beetles selected oviposition sites on plant species similar to the ones on which they had been reared (Hopkins, 1917). The idea that the experience of the larvae might effect a neural change in the subsequent adult is known as Hopkins' host selection principle (HHPS). Some years later it was demonstrated that the ichneumonid parasitoid *Venturia canescens* (Gravenhorst) showed a strong positive response towards the odour of its natural host but there could

also be an induced response towards the odour of an unusual host (Thorpe & Jones, 1937). This influence of larval experience on adult behaviour was termed "preimaginal conditioning".

Convincing demonstrations of preimaginal conditioning in various insect groups are few. Early demonstrations of preimaginal conditioning typically involved inducing food or host preferences in adult insects by exposing them as larvae to a novel food source or host (Thorpe, 1939; Hershberger & Smith, 1967). These studies have been criticised for failing ensure sufficient isolation of the emerged adults from the preimaginal environment (Jaenike, 1988).

(Corbet, 1985) gave a possible explanation for the above phenomena. Adults were responding to a "chemical legacy" of the larval environment, and the induced behavioural change was acquired in the early imaginal rather than the preimaginal stage. The "chemical legacy hypothesis" suggested that, "since traces of the larval diet can be found within the body of the imago and within the pupal cuticle as well as on the surface of the pupa, one cannot be sure that no contaminants from the larval environment are present in the environment of the emerging adult". For example, the braconid *Microplitis demolitor* (Wilkinson) will respond to cowpea odour if reared on hosts *Helicoverpa zea* (Boddie) feeding on real cowpeas, but not if reared on artificial diet containing no cowpea material. The preference to odour of food plants of the host disappears if the parasitoid pupa is removed from its cocoon, which is constructed by the parasitoid itself (Hérald *et al.*, 1988).

However, it has been suggested that in the case of the chalcidoid parasitoid *Dahlbominus fuscipennis* (Zetterstedt) and its host (the pupae of the European spruce sawfly *Gilpinia hercyniae* (Hartig) prior to late stage of the host's pupal development the cocoon can carry infochemicals through a diffusion process from the inside to the silk surrounding the cocoon (contact kairomone) which leads to hosts recognition by the parasitoid (Rostas *et al.*, 1998).

Furthermore, the larval stage of the host as well as its by-products (frass and silk) can also influence parasitoid searching behaviour. The parasitoid *Cotesia glomerata* (L.) responded less to silk and frass extracts of fifth stage larvae of its host *Pieris brassicae* (L.) on cabbage plants while showed increased olfactory responses to the host's by-products extracts from the first larvae instars (Mattiacci & Dicke, 1995). In addition, it has been also showed that extraction from silk gland secretion from late stage larvae failed to elicit positive responses in *D. fuscipennis* (Rostas *et al.*, 1998).

(Wickremasinghe & van Emden, 1992), using Y-tube olfactometer tests, found that adult parasitoids of the family Aphidiidae (Hymenoptera) responded positively to the odour of the plant on which aphid mummies containing them had been collected. In particular, they showed that upon emergence from the aphid mummy, adult female

*Aphidius rhopalosiphi* (DeStefani Perez) when offered volatiles from two different wheat cultivars in the olfactometer, showed a clear preference for the odour of the cultivar on which they had developed. This study could be evidence either for "chemical legacy hypothesis" or Hopkins' "host selection principle" (HHPS), because a change of adult behaviour had been induced by chemical cues experienced by the larvae.

*A. rhopalosiphi* females reared on the aphid *Metopolophium dirhodum* (Walk.) on two wheat cultivars ("Maris Huntsman" and "Rapier") responded positively to the odour of the cultivar on which their host had initially been reared when the parasitised aphids were moved to the alternative cultivar at least 6-8 days after parasitisation (van Emden *et al.*, 1996). That this was not a case of HHPS was shown by the fact that positive responses in favour of either cultivar disappeared when the parasitoids emerged from pupae excised from the aphid mummies. Thus, any positive responses recorded in natural emergence must have been due to chemicals being contacted by the parasitoid on the skin of the mummy or while biting its way out of the mummy case.

*Aphidius colemani* females appear to have an innate oviposition preference for *Aphis gossypii* over *Myzus persicae* as hosts but they also use, to some extent, chemical information aquired from the hosts on which they are reared. In this instance, parasitoid rearing on *Myzus persicae* hosts was not sufficient to induce a preference for *M. persicae* but the preference was reduced (Messing & Rabasse, 1995). Similarly, *Diaeretiella rapae* wasps exhibited a higher attack rate on the specialist crucifer aphid host *Lipaphis erysimi* (Kaltenbach) rather on the generalist *M. persicae* and the rearing experience, on either aphid species, did not have a major influence on the observed responses of this parasitoid (Blande *et al.*, 2004). Furthermore, it was suggested that *A. colemani* from artificial diet-reared *M. persicae* responded positively to odours of the plant on which their parents hosts had been reared but only when their aphid hosts had experienced the same plant (Brussels sprout or Cabbage) for 48 h at an early stage of the parasitoid's development (Douloumpaka & van Emden, 2003). That study showed again that the adult parasitoid encountered the "early" chemical cue while cutting its way out of the mummy. Parasitoids from pupae excised from the mummy case, showed no such preferences. However, when such adults that had emerged excision of pupa from the mummy cuticle were offered whole mummies to handle for 24 h gained a preference for the plant odour on which the aphid mummy had developed (Vamvatsikos & van Emden, 2002). Thus, it appears that the mummy must contain the chemical information that determines the plant-odour preference of the emerged parasitoids.

That work confirmed earlier evidence that even the seemingly innate responses may derive from chemical information which may be available for parasitoids "emergence conditioning", but subsequent foraging experiences can override the initial

information and may change the parasitoid's preferences completely (Storeck *et al.*, 2000). These examples demonstrate that there is no clear evidence for HHPS but the principle cannot totally be discounted.

### *1.4.5.3 Learning by mature parasitoids*

It appears that pre-adult experience generally has only a minor effect on adult host-searching behaviour, compared to adult experience (Vet, 1983; Drost *et al.*, 1988; Sheeham & Shelton, 1989). For example the eucoilid parasitoid of *Drosophila, Leptopilina clavipes* (Hartig) is normally attracted to fungi. However, if reared on hosts in a fruit medium containing yeast, the adult wasp will also orientate toward decaying fruit (Vet, 1983). The braconid parasitoid *Diaeretiella rapae* normally attacks aphids on crucifers but is more responsive to potato plants if it is reared on hosts feeding on potato (Sheeham & Shelton, 1989).

Experience can influence responses in two ways. It can cause a general increase in the numbers of females responding, when brought about by exposure to the host or a host product (sensitisation) but it can also alter a female's preference for specific cues (Turlings *et al.*, 1993).

The adult gregarious parasitoid *Cotesia congregata* (Say) is only sensitive to learning plant odours for a few hours after emergence (Kester & Barbosa, 1991). Also, a general increase in responsiveness to odours of host faeces was observed for *Microplitis croceipes* (Cresson) after these parasitoids were allowed to oviposit in *Heliothis zea* (Boddie) larvae on different plants (Eller *et al.*, 1992). Even contact with hosts on plant-based artificial diet increased responsiveness to faeces from plant-fed hosts.

The second way experience can influence responses is by altering a female's preference for specific cues (Turlings *et al.*, 1993). In olfactometer choice tests, that *Cotesia marginiventris* (Cresson) preferences altered for specific cues, exhibiting minor shifts in preference in favour of the odour of the plant-host combination it had experienced (Turlings *et al.*, 1990). In addition, a single experience increased the preference when females *M. croceipes* offered choices between the odours of faeces of hosts that were fed on different diets (Eller *et al.*, 1992). When the experience was repeated two or four times, the parasitoids exhibited a significant preference for the odour of the faeces they had experienced, even if they strongly preferred the alternative odour before experience or after only one experience. Experience after emergence with the host or host by-products can increase insect numbers responding to an odour (priming) (Turlings *et al.*, 1993; Vet *et al.*, 1995) and the insects

subsequently learn to respond to the specific odours that they encountered during that experience (preference).

#### *1.4.5.4 Associative learning*

The establishment, through experience, of an association between two stimuli, or between a stimulus and a response, is often suggested as a learning mechanism known as "associative learning". For example, the parasitoid *Bracon mellitor* (Say) exhibited ovipositor probing in response to a novel compound (methyl parahydroxy-benzoate) added to the artificial diet used to rear their host the boll weevil *Anthonomus grandis* (Boheman) and it was suggested that this was a "classical" form of associative learning (Vinson, 1977). (Wardle & Borden, 1989) found that the polyphagous ectoparasitoid *Exeristes roborator* (Fabricius) learned apple odours after the parasitoids had experienced the odour during encounters with hosts.

*Leptopilina heterotoma* (Thomson) females, parasitoids of *Drosophila* species, could learn to respond to a novel odour (Vet & Groenewold, 1990). They concluded that this novel odour can be used by the parasitoid in microhabitat selection through a process of association under the presence of the host itself (a contact with the host products or oviposition experience). As a result of the association, the parasitoids will respond to the novel odour as well and use it as cue in subsequent host-searching efforts (Takasu & Lewis, 2003).

Cues associated with bean plants (*Vicia faba* L.), host-damaged plants (pea-aphid *Acyrthosiphon pisum*, Harris) and plant-host complexes affected the foraging behaviour of the aphid-parasitoid *A. ervi* demonstrating another case of associative learning (Du *et al.*, 1997). Moreover, the researchers did not observe any changes in parasitoid responses when oviposition experience occurred on aphid hosts isolated from the plants, strongly indicating that the learning process took place at the plant level. A number of studies on different tritrophic systems have shown that one day's oviposition experience on a plant is adequate for parasitoid learning to occur (Du *et al.*, 1996; Guerrieri *et al.*, 1997; Poppy *et al.*, 1997; Powell *et al.*, 1998; Girling *et al.*, 2006). Yet, the information obtained by the learning process through oviposition experience may be lost after a short period of time (Fujiwara *et al.*, 2000). This finding is in agreement with (Keasar *et al.*, 2001) who suggested that the learning stimulus following oviposition will probably gradually decrease with time and in order to maintain the enhanced parasitoids' foraging momentum, further experience is required.

In some cases, associative learning may not only be the result of an odour associated with an oviposition experience (reward stimulus) but could also be resulting after a contact with the host's by-products when the latter is likewise offered as a reward (Perez-Maluf & Kaiser, 1998). These researchers have also investigated the

effect of mating experience on searching behaviour of *Leptopilina boulardi*, a *Drosophila* parasitoid which is not influencing the odour learning neither does an oviposition experience alone before the odour is introduced.

However, the mating status can positively affect subsequent parasitoid foraging behaviour of some species but not in the case of *Lysiphlebus testaceipes* (Cresson) which as virgin females spent less time on an aphid infested host-plant and the rate of attack is reduced compared with the mated females. In addition, mated females could superparasitise more aphids than virgins (Michaud, 1994). Furthermore, the barometric flux may affect the male aphid-parasitoid *Aphidius nigripes* (Ashmead) responsiveness to females and reduce their mating behaviour in variable environmental conditions (Marchand & McNeil, 2000).The mating status can also influence adult longevity as well as does the source of the food uptake. For example, virgin females of *Bathyplectes curculionis* (Thomson) have extended longevity than mated females when they offered floral food or water. This difference was not evident when a honey solution was the food source as both sexes showed similar and increased longevity (Jacob & Evans, 2000).

#### *1.4.5.5 Conditioned stimuli (CS) and unconditioned stimuli (US)*

Since associative learning constitutes one of the strategies parasitoids employ to overcome the reliability – detactability problem (Vet *et al.*, 1991) it is expected that they can learn to link stimuli originated from their hosts (unconditioned stimuli) to stimuli from the parasitoids' habitat (conditioned stimuli). Except for associative learning, the learning process of a novel stimulus with a reward that induces insect responses to the novel odour is often called as operant conditioning (Vet & Croenewold, 1990; Vet *et al.*, 1995). However, associative learning is the major mechanism that parasitic wasps utilise to link highly detectable conditioned stimuli to highly reliable unconditioned stimuli (for reviews see: Papaj & Lewis, 1993; Turlings *et al.*, 1993; Godfray, 1994; Vet *et al.*, 1995; Hilker & Meiners, 2006). Thus, when a neutral conditioned stimuli (CS) that is ignored or effects a weak response is paired with non-neutral unconditioned stimuli (US) known to elicit a recognizable behavioural response, then such a response is induced by the originally neutral conditioned stimuli (CS) as well, this learning mechanism is a classical form of "Pavlovian" conditioning (Pavlov, 1927; Papaj & Prokopy, 1989; Guerrieri *et al.*, 1997).

The important role of conditioning, defined as pre-adult experience in parasitoids' host-location mechanism, has been demonstrated in several studies (Wickremasinghe & van Emden, 1992; Poppy *et al.*, 1997; Storeck *et al.*, 2000; Rodriguez *et al.*, 2002; Douloumpaka & van Emden, 2003; Blande *et al.*, 2004). These

studies also confirmed previous related evidence of the major influence of genes (i.e. innate responses) on parasitoids' behaviour (Messing & Rabasse, 1995). McGuire *et al.* (2005) reviewed studies on *Drosophila melanogaster* genetics and olfactory learning and conditioning (short-term memory) while Skoulakis & Grammenoudi (2006) in their review made a clear distinction between such genes according to their functionality in two categories: firstly genes which are necessary for the development of neurones controlling the conditioned responses in the behaviour of *Drosophlila* and secondly those involved in the biochemistry of these neurones. Furthermore, Schwaerzel *et al.* (2004) identified specific genes in adult *D. melanogaster* that link a conditioned odour stimulus (dopamine and octopamine) to an unconditioned stimulus (shock – i.e. aversive conditioning and/or sucrose – i.e. appetitive conditioning) and how this information is transferred at the level of the neurone. Aversive conditioning has been shown in individual *D. melanogaster* flies when cold temperature (US) was associated with odours of synthetic chemicals (CS) (Phelan *et al.*, 2001).

A response evoked after contact with food resourses (i.e. sucrose) is frequently refered to as reward (Papaj & Prokopy, 1989). Parasitoids demonstrate complex learning mechanisms and they can also associate sugar sources with host foraging. Indeed, Wäckers *et al.* (2002), using the larval parasitoid *Microplitis croceipes,* presented evidence of appetitive conditioning utilising sucrose solution (US) and the novel odour of 1-octanol (CS) and suggested that the work is another classical example of "Pavlovian" conditioning. Olson *et al.* (2003) developed a specific experimental protocol separating 'seeking' (i.e area searching) and 'coiling' behaviour (i.e. behaviour observed prior of host attack) using food (sucrose solution) and host frass respectively as unconditioned stimulus. These authors demonstrated that the appearance of stereotyped parasitoid behaviours such as seeking and coiling (one or the other) depended on the unconditioned stimulus i.e. food or host linked to the odour used as the conditioned stimulus.

Furthermore, the pupal parasitoid *Pimpla luctuosa* (Smith) can learn to associate novel odours such as vanilla and strawberry (CS) not only during oviposition experience (US) but also while feeding on the host's haemolymph (US) (Iizuka & Takasu, 1998). Also, parasitic wasps learn to respond to vanilla odour when this odour served as a conditioned stimulus and was associated with oviposition experience or antennating host frass (unconditioned stimulus) not only at 30 min after the learning experience but also 24 h later (Takasu & Lewis, 2003). Meiners *et al.* (2003) demonstrated that the recognition and olfactory learning of individual compounds within odour blends is possible after experience with an odour mixture during oviposition but it can be affected by the blend's background concentration.

Associative learning and conditioning can also occur in honey bees when the conditioned stimuli (CS) are followed by unconditioned stimuli (US) (reviewed by Menzel, 1999). In addition, recognition of single or complex odours, order of stimuli (CS and US) experienced and time appears critical (Menzel, 1999; Phelan *et al.*, 2001; Menzel *et al.*, 2001). In the context of the studies from Takasu & Lewis (2003) antennation of sugar food source (US) or intake by honey bees proboscis (US) can be linked to an odour (CS) (Bitterman *et al.*, 1983). Also, similar to Meiners *et al.* (2003), there is evidence that it is the presence or absence, rather than a change in concentration (CS) that plays an important role in *Apis mellifera* honey bees (Laloi *et al.*, 1999) and that there is a hierarchy in odour recognition by honey bees (Laloi *et al.*, 1999; Laloi *et al.*, 2000). In addition, the recognition hierarchy in individuals depends on the their learning ability (Laloi *et al.*, 1999) and they can also learn complex odour mixtures (Laloi *et al.*, 2000).

Moreover, honey bees can learn to distinguish between similar odours by generalisation of experienced components since it is difficult to encounter exactly the same odours while foraging for food sources (Smith, 1993; Peltz *et al.*, 1997). During early adult life (4-6 days) the young bees exhibit an enhanced responsiveness to olfactory learning (Morgan *et al.*, 1998). To study olfactory learning in honey bees the technique of the classical conditioning of the proboscis extension response (PER) can be employed, a method relatively old but efficient (Takeda, 1961) by which honeybees can link an odour stimulus (CS) with a sucrose source (US) and an easily observable behavioural response.

For example, using PER method it was shown that honey bees can generalise between two pollen odours, oilseed rape and field bean, distinguish one from the other and learn to link each type of pollen with sucrose reinforcement (Cook *et al.*, 2005). Conditioning with the PER method has shown that honey bees can discriminate between two positively rewarded odours after they had initially generalised between similar odours within a mixture (Paldi *et al.*, 2003) and even sublethal pesticide toxicity can be assessed using olfactory learning in *A. mellifera* (Decourtye *et al.*, 2005). In another study using olfactory responses of conditioned (PER) and naïve honey bees, there was no evidence suggesting preimaginal conditioning and only learning by young individuals was observed (Sandoz *et al.*, 2000).

# CHAPTER 2: General material and methods

## 2.1 Laboratory conditions

All the experiments were performed in a laboratory with no natural light between October 2002 and August 2003. The relative humidity fluctuated between 65 and 75% and the temperature ranged between 18 and 27 °C.

## 2.2 Glasshouse conditions

All plants used for experiments and maintaining the insect cultures were grown in a glasshouse maintained at 20-30 °C on all but some of the hottest summer days when temperatures could rise above 30 °C but rarely above 35 °C. Additional light was supplied from Brinkman Pootlamps containing an Osram Vialox NAV-T Super (Son-T Plus) 400 W bulbs placed approximately 3 m from plants to ensure at least 16 h light/day. For plant protection from the summer sun greenhouse shading (Coolglass, Enfield, UK) was mixed with cold water and applied to the glass panels with a manual sprayer.

## 2.3 Plant material

The plants used were from the family Brassicaceae. Brussels sprouts (Suttons seeds, Torbay, UK), cultivar 'Bedford-Winter harvest', cultivar 'Bedford-Fillbasket', cultivar 'Hamlet', cultivar 'Red delicious' (all in the group of *Brassica oleracea* L. var. *gemmifera* Zenker) and Cabbage cultivar 'Golden acre' (*Brassica oleracea* L. var. *capitata* (L.) Alef) (Kings seeds, Kelvedon, UK). Dwarf broad beans - *The Sutton*, *Vicia faba* (*L.*), (Johnsons seeds, Boston, UK) were also grown from seed in the glasshouse. All the above mentioned seeds were untreated.

Seeds were sown into 9-cm-diameter plastic pots containing Levington Multipurpose Potting Compost (Godalming, UK). Two weeks after sowing the seedlings (except broad beans which were planted individually) were transplanted to individual 9-cm-diameter pots. Seeds were sown every week to ensure that a continuous supply of plant material was available at a suitable growth stage (40-45 days after sowing).

The plants were examined carefully at 2-3 day intervals and any infested plants, mainly aphids and thrips during the summer, were discarded. Thus, a pest-free

environment or low infestation level was maintained in the glasshouse throughout the year.

## 2.4 Cages

Both aphid and parasitoid cultures were kept in wooden-frame cages – based on Type I (Adams & van Emden, 1972, Fig. 2.1). Each cage was 45 x 40 x 45 cm with nylon mesh vertical sides, which on the front side formed a sleeve to allow access inside the cage. Transparent 5-mm-thick Perspex glass (Aquarius Plastics Ltd., Guilford, UK) was used for both roof and base; an additional thin wooden frame on the front formed a door. All wooden parts were varnished for durability.

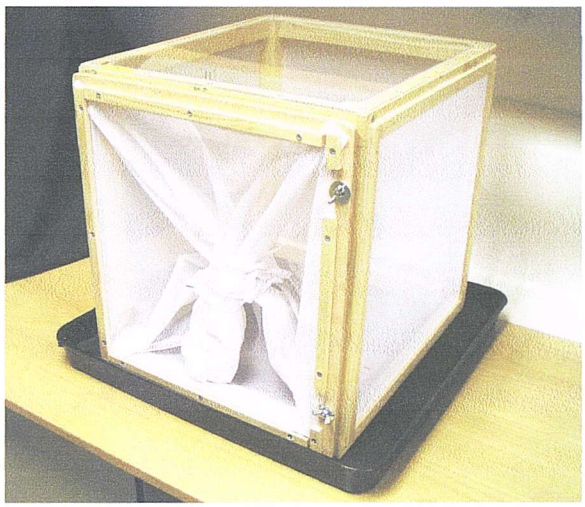


**Fig. 2.1** A wooden framed cage

In addition, capillary matting material (Geerings of Ashford, Ashford, UK) was placed between the wooden-frame and Perspex base protruding 2-5 cm around the cage's base. Finally the cages were placed in square (55 × 55 × 5 cm height) black plastic gravel trays (Steward Plastics Ltd, Croydon, UK). This allowed the user to water from outside the cage with the water passing through the capillary matting into the cage (Fig. 2.1). Watering was required two or three times a week and was achieved with minimal disturbance to the insect cultures.

Old, fallen leaves were removed weekly from the capillary matting surface to prevent fungal development. The cages were washed thoroughly every three/four months and the wooden body of the cage was also cleaned with 50% ethanol.

## 2.5 Insect cultures

The aphid and parasitoid cultures were kept in separate controlled temperature and humidity (CT) rooms.

### 2.5.1 Aphid culture

The green peach-potato aphid, *Myzus persicae*, was maintained on Brussels-sprout plants, cultivars 'Bedford-Winter harvest' and 'Red delicious' and kept at 18±1 °C, 60±5% relative humidity and LD 16:8 h photoperiod under artificial cool white light provided from four units with (4 × 2 = 8) fluorescent lamps (Philips, product code: 871150063219740, Master TL-D Super 80 58W/840 SLV). The aphid cultures were maintained in cages with 9 plants (40-45 days old) in each.

### 2.5.2 Parasitoid culture

The parasitoid, *Aphidius colemani*, was cultured on aphid hosts reared as above, except where stated in the experimental procedure. The parasitoids were kept in a CT room at 19±1 °C, 75±5% relative humidity, LD 16:8 h photoperiod under artificial cool white light (section 2.5.1). The initial parasitoid culture was established by using a commercially available product named 'Aphiline C' (Syngenta-Bioline, Little Clacton, UK, product code: 0102G). The original host for *A. colemani* was the melon or cotton aphid *Aphis gossypii* (Glover) with its host plant being various species within the family Curcubitaceae (Dr. R. GreatRex, Syngenta-Bioline, personal communication). At least two generations of parasitoids were allowed to establish on *M. persicae* on Brussels-sprouts prior to using them for experiments. Five cages (as in section 2.5.1) of *A. colemani* were maintained to ensure a continuous supply of parasitoids and mummies. Ten mated female parasitoids were introduced to parasitise the aphid-infested plants at the start of a new culture. A battery-operated aspirator (Maplin Electronics Ltd, Barnsley-Wombwell) was used to handle adult parasitoids (Fig. 2.2).

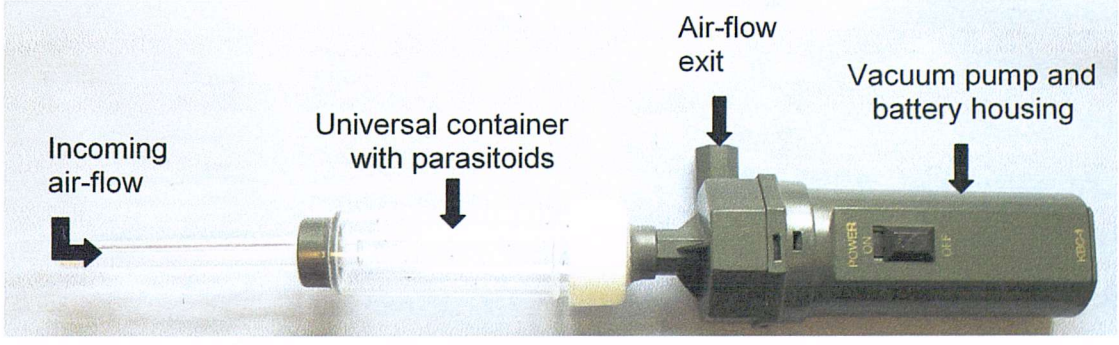



**Fig. 2.2** The battery-operated aspirator and the custom-built collection chamber

Parasitoid density was maintained at low levels in order to prevent a male-biased sex ratio developing and decline of the culture (Douloumpaka, 2000). For most of the time, this was achieved as parasitoids were removed for bioassays and then discarded. A female-biased sex ratio tended to occur at the start of a culture.

## 2.6 Parasitoid selection for bioassays

### 2.6.1 Parasitoids collected directly from the culture

The parasitoids which were used for the initial bioassays emerged from aphids reared entirely on Brussels sprouts, cultivar 'Bedford-Winter harvest' and collected from the culture cage with the aspirator. In this case the age of the parasitoids was not known precisely.

### 2.6.2 Parasitoids emerging from mummies isolated from plant material

Mummies which had developed a smooth, shiny integument and light brown to dark silvery-gold colour (late-stage mummies: one or two days before emergence) were removed from plant leaves using a soft paintbrush and placed on damp filter paper in a 5.5-cm-diameter Petri dish. Petri dishes were placed in a small separate Fisons' environmental cabinet (51 × 60 × 48 cm) away from any plant material or any other cultures with mean daily temperature at 20±1 °C under artificial light (LD 16:8 h) provided from four white coated fluorescent lamps (General Electric, F15W/35, T8 ø 26 Standard Halophosphate, product code: 29531) and observed daily. The newly-emerged adults were collected, paired (unless otherwise stated) and allowed access to honey solution (20% in distilled water) for 24 h. They were tested the next day at 24-48 h old.

### 2.6.3 Parasitoids emerging after dissection from mummies

Douloumpaka (2000) described a method for extraction of parasitoid pupae from the ventral side of the mummy. In this study a similar technique was developed to minimise damage to the excised pupa as well as to be time efficient. The mummies were dissected from the dorsal side, using two pairs of fine forceps (Innox no.5), which were gently squeezed, on the back of the mummy body thus creating a fold with the mummy's cuticle. With both pairs of forceps the middle section of the fold was then grasped and the soft mummy skin was pulled gently apart to avoid injuring the pupa inside.

The pupae were kept until emergence on filter paper above damp cotton wool in 5.5-cm-diameter Petri dishes. Keeping the cotton wool damp at all times was of critical importance as otherwise the excised pupae became desiccated and died. The Petri dishes were placed in the environmental cabinet (section 2.6.2) and covered with tissue paper to diffuse the direct light reaching the pupa and simulate the skin of the mummy (Douloumpaka, 2000). The mummies were collected according to their colour (dark colour) before natural emergence occurred. If the late stage collected mummies for excision were identified correctly, the success of the method was up to 75% (see Table 4.1) matching Storeck *et al.* (2000). The newly-emerged adults were collected, paired, allowed access to honey solution for 24 h, and tested the next day at 24-48 h old.

### 2.6.4 Sexing and mating of parasitoids

Following collection with the aspirator the parasitoids were anaesthetised with $CO_2$ (van Emden *et al.*, 2002) and then placed, in batches, under Petri-dish lids on a white-paper background to ensure good contrast with the parasitoids' dark colour. Once recovered from anaesthesia, females (Fig. 2.3) could be identified with a naked eye by their long pointed ovipositors and parasitoids were separated into male/female pairs in cylindrical glass vials (2.5 cm diameter × 5 cm height). The vials were stoppered with cotton wool soaked in 20% honey-water solution, as a food source, and left for 24 h to ensure mating. However, anaesthesia with $CO_2$ was used only for some earlier experiments (chapter 3, section 3.3.1) and not for the later ones where, another technique had been developed (section 2.7.2) which made anaesthesia unnecessary. Virgin adults used in subsequent experiments emerged from mummies isolated in separate vials.




**Fig. 2.3** Ventral view of a male (left) and a female (right) parasitoid (microscope: WILD Heerbrugg, Switzerland, M5-71576 - digital camera: Olympus C730UZ)..

## 2.2 Olfactometer techniques

### 2.2.1 Olfactometer equipment

Two 4-way olfactometers were constructed to test the response of *A. colemani* to volatile plant compounds (Fig. 2.4). Both were made from 4 cylindrical Perspex tubes (9.5 × 2.5 cm internal diameter) forming the test-odour chambers (Douloumpaka & van Emden, 2003).

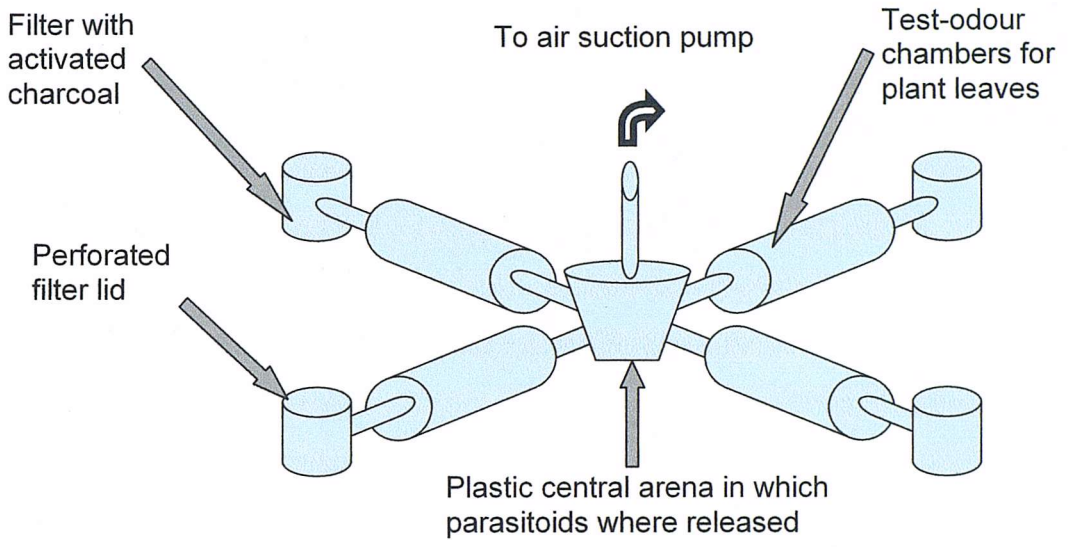



**Fig. 2.4** Construction of the olfactometer 1 (after Douloumpaka and van Emden 2003) (not to scale)

Each of these chambers was connected to the central arena by four smaller pieces of Perspex tubing (4 × 0.5 cm internal diameter). These small tubes protruded 2 cm into the odour chambers but were flush with the internal surface of the central arena.

The central arena consisted of a 30-ml conical-shaped translucent plastic pot (4.5 cm top, 3.7 cm bottom diameter × 3 cm height) and this construction is described as **olfactometer 1**. A plastic lid covered the pot firmly forming an air-tight central arena. The lid was fitted with a small tube (0.5-cm-internal diameter) connected to an air-flow meter (Jencons-PLS, Forest Row, UK) and an electric suction pump (DYMAX 30, Charles Austen Pumps Ltd., Byfleet, UK). The pump provided an adjustable, air flow with almost no fluctuation (± 1 ml/min).

The open end of each odour chamber was connected to an activated charcoal filter (BDH-Merck Ltd., Poole, UK) to purify incoming air and all parts of the apparatus were sealed using hot melt glue (Screwfix Direct, Yeovil, UK). Each filter contained 6 grams of activated charcoal and it was covered with a perforated lid (2.5-cm diameter). Seventeen 1-mm-diameter holes were drilled through each lid 3 mm apart and on 4

centred axes, 45° one from the other (1 hole at the central point and the others around it) forming symmetrical radial shape. The apparatus is shown in (Fig. 2.5).

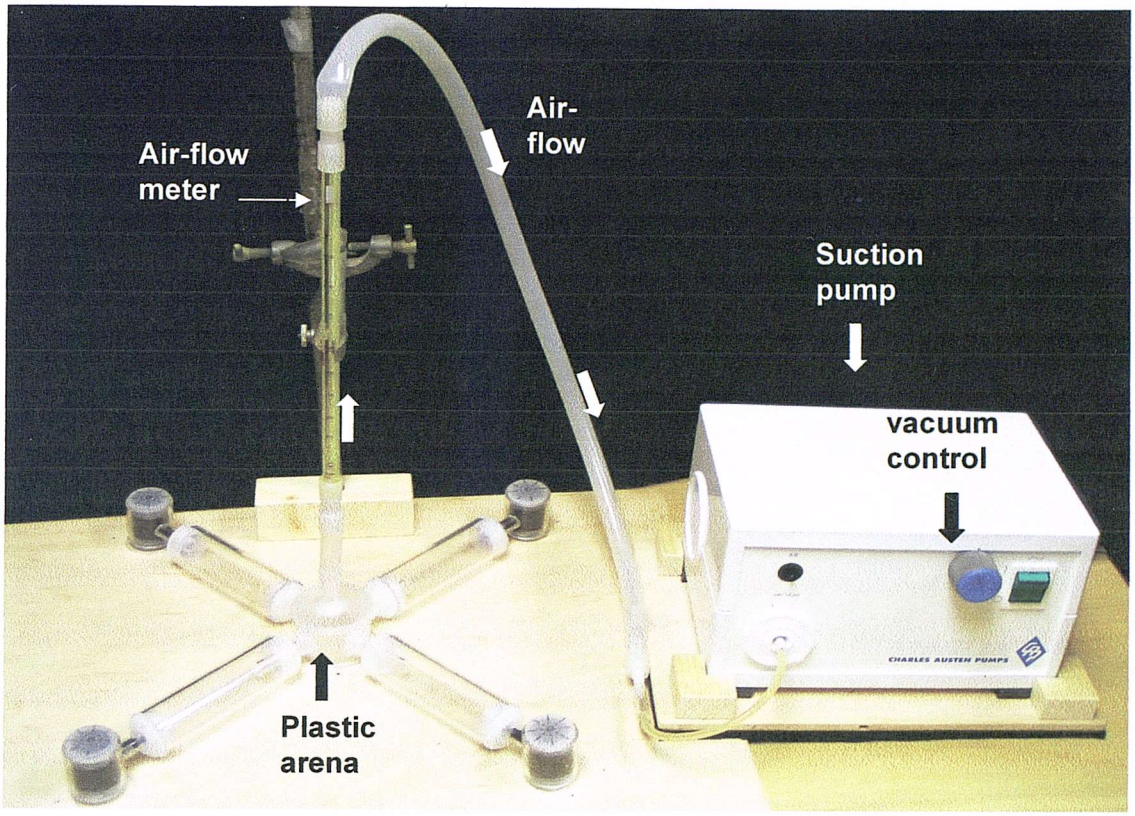



**Fig. 2.5** A 4-way olfactometer 1 system with the central plastic arena connected to the air-flow meter and the vacuum pump.

The olfactometer was placed inside a black wooden box (66 × 56 × 66 cm) where the bioassays took place, but the pump was placed outside the black box to isolate any vibration. Inside the box, the relative humidity fluctuated between 65% and 75%, the temperature ranged between 18 °C and 27 °C and all the experiments were carried out under artificial cool daylight provided from two high frequency >30 KHz lamps (Philips, product code: 871150061079940, TL-D 90 de Lux 18W/965 SLV). A black curtain was firmly attached to the front of the box to ensure constant light conditions and preventing any disturbance from movement in the laboratory during experiments.

Air was drawn into the olfactometer's central arena through the charcoal filters and the odour chambers. Smoke tests showed smooth (almost no turbulence) airflow into the arena at a flow rate of 1500 ml/min. However, subsequent tests indicated that more insects responded in a flow rate of 1700 ml/min where some turbulence was observed.

A modified construction constituted **olfactometer 2** (Fig. 2.6) and two of these were made. A cylindrical Perspex tube was used to construct the central arena (4.5-cm

internal diameter × 2.5-cm height). The top and base were also made from Perspex material. The lid was air-tight and protruded 3 mm into the arena.

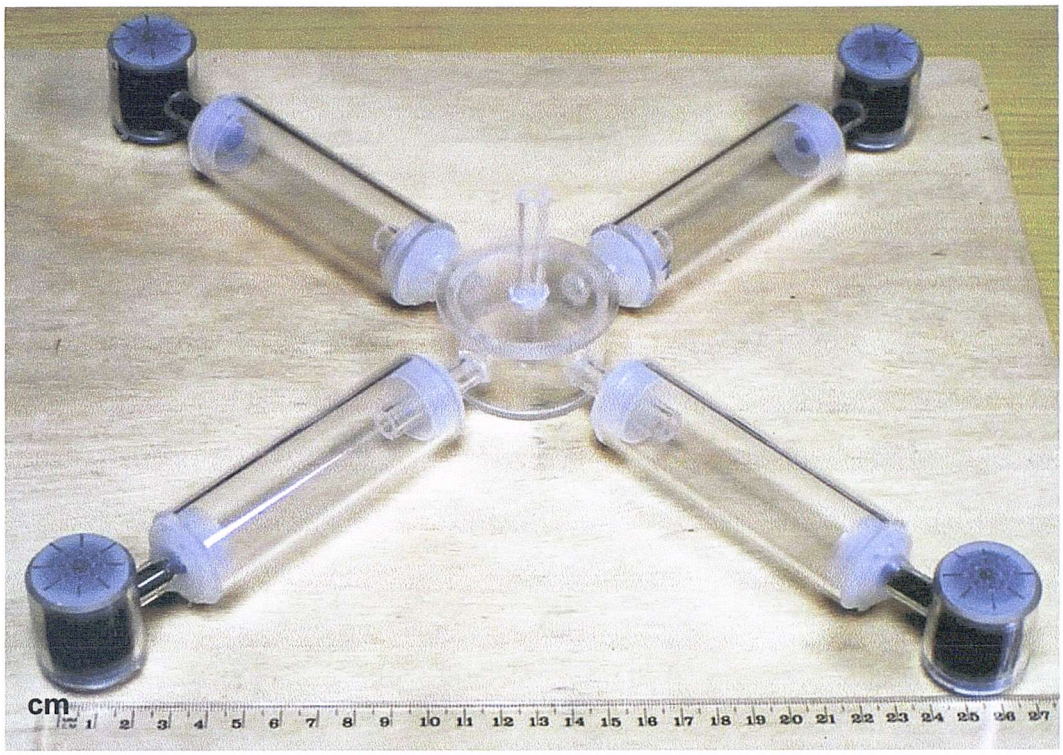



**Fig. 2.6** Olfactometer 2, a modification of the 4-way olfactometer 1 with the parts of the central arena constructed from solid Perspex glass.

### 2.2.2 Olfactometer bioassays

Initially, the parasitoids were anaesthetised with $CO_2$ (section 2.6.4) and tapped into the central arena forming single-sex batches of 14-25 (as available) with each individual parasitoid used only once. However, this procedure was difficult and time consuming.

In subsequent experiments the number of parasitoids involved in each bioassay was fixed at 20. In order to standard the procedure of inserting the parasitoids in the arena a novel technique was developed. After mating took place (section 2.6.3), single sex parasitoid groups were drawn into the central arena of the olfactometer by using the olfactometer as an extension of the vacuum pump by connecting the aspirator to the arena's lid using a 5 mm diameter plastic pipe (Fig. 2.7). Three of the connecting tubes were stoppered with cotton wool while a piece of the same plastic pipe was attached to the 4$^{th}$ open tube and used to draw the insects in the central arena. Thus, anaesthesia with $CO_2$ at this point, was avoided.

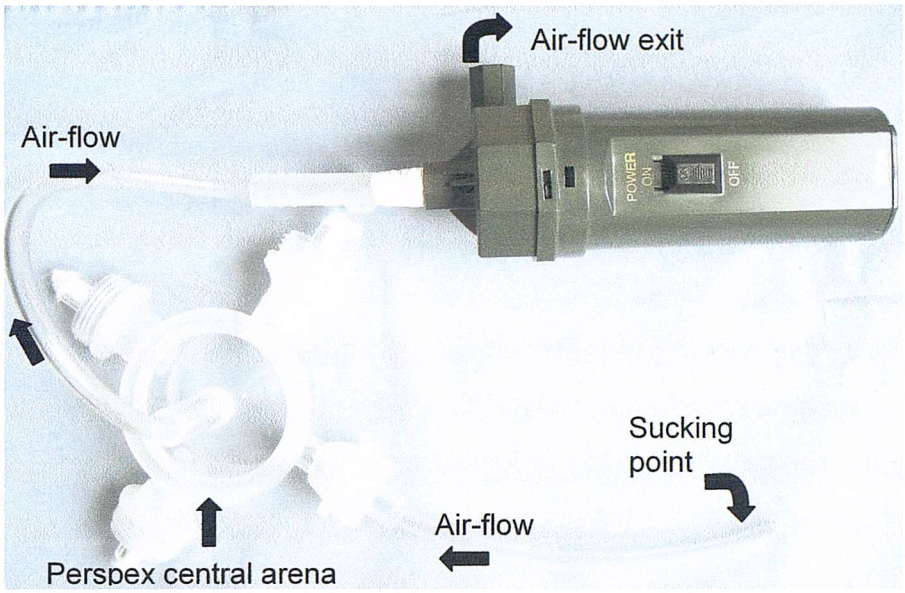



**Fig. 2.7** The parasitoid-collection system

The test-odour chambers were attached, the pump (positioned outside of the black box) turned on and the box made light tight. After 30 min, parasitoids which had entered the odour chambers were counted including those that had walked half way or more into the side connection tubes. For all the experiments of this study, 30 min bioassays were carried out for the following reasons: firstly, to reduce the high number of non-responders seen in preliminary observations, secondly, it proved a convenient time interval between replicates as 30 min was just enough to prepare the next replicate for testing (using a 2$^{nd}$ olfactometer) and finally, as the side-connection tubes protruding 2 cm into each of the odour chambers the chances of insects returning to the central arena, after entering these chambers, were low (Douloumpaka & van Emden, 2003).

The size of the leaves used in bioassays varied in each replicate but each leaf was maximum 8-cm long by 10-cm wide. Only leaves from non-infested plants used for all the experiments. Every leaf was rolled and placed within the odour chamber (one inside the other) using a pair of forceps (stainless steel) with smooth-flat tips taking extra care not to damage them during this procedure. Damaged leaves were discarded. Initially, each odour-chamber contained 2-3 leaves and the usual practice was to use medium size leaves (approximately 4-6 cm long by 5-7 cm wide). Later on, more plant material was used (3-7 leaves) for experiments. The curly small leaves (from the top of the plant) were also used. As smoke tests indicated that the most of the air stream was blowing through the middle of each odour- chamber the small leaves were placed so as to occupy the central space and so increase the turbulence in the airflow.

The whole system (central arena and odour chambers) was rotated 90° clockwise after each replicate to cancel out any directional bias in the apparatus (Fig. 2.8). The four odour chambers were numbered (1-4) in order to facilitate the rotating process.

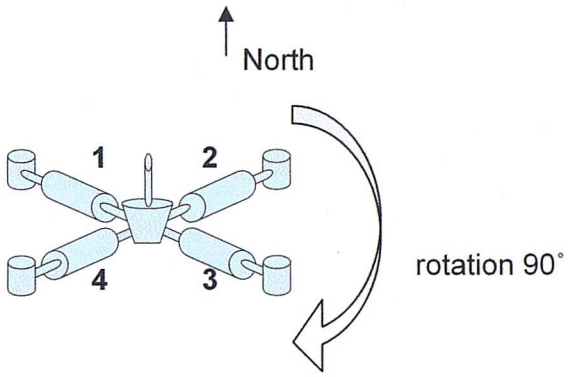



**Fig. 2.8** Rotation of the whole system (central arena and numbered test chambers) after each replicate.

After two replicates, all parts of the olfactometer (except the charcoal filters) were rinsed with 50% ethanol and soaked in 10% lipsol liquid (scentless detergent) overnight. They were then rinsed thoroughly with both warm tap water, distilled water and dried in a glass oven. The used charcoal was replaced with activated one after the completion of each experiment. The used charcoal was replaced with fresh activated charcoal after the completion of each experiment.

## 2.3 Diet-reared aphids

### 2.3.1 Synthesis and making procedure of the artificial diet

A culture of *Myzus persicae* (Sulzer) had been maintained at the University of Reading on a chemically-defined artificial diet (Mittler and Dadd, 1966) since 1976. In our laboratory we used double-processed tissue culture water for the diet preparation (Sigma-Aldrich Company Ltd, Poole, UK, product code: W3500). This was the only change we made to the diet (compared to what was used by Douloumpaka, 2000), when we started making it in the laboratory (Table 2.1).

**Table 2.1** Composition of artificial diet (after Mittler and Dadd, 1966) and the adding sequence of the chemicals followed after Douloumpaka (2000).

| Chemical Compound | Required Quantity | Ordering Quantity | Catalogue Reference | Company- Page Number |
|---|---|---|---|---|
| Double processed tissue culture water | 500 ml | 500ml | W-3500 | Sigma 2115 |
| Sucrose crystalline Analar (15g/100ml) | 75g | 1000g | 10274 5C | BDH 2-672 |
| Potassium hydrogen orthophosphate | 3.750g | 500g | 29608 4J | BDH 2-491 |
| Magnesium sulphate | 0.615g | 5Kg | 25162.361 | BDH 2-343 |
| L-Tyrosine | 0.200g | 100g | 371562R | BDH 2-714 |
| L-Asparagine monohydrate | 2.750g | 100g | A 8381 | Sigma 221 |
| L-Aspartic Acid | 0.700g | 100g | A 9256 | Sigma 222 |
| L-Tryptophan | 0.400g | 5g | T 0254 | Sigma 2051 |
| L-Alanine dextro-rotary | 0.500g | 100g | 37006 5B | BDH 2-27 |
| L-Arginine monohydrochloride | 1.350g | 500g | 37016 6F | BDH 2-58 |
| L-Cysteine hydrochloride,hydrate | 0.200g | 100g | 37055 3M | BDH 2-184 |
| L-Glutamic acid | 0.700g | 500g | 37102 4T | BDH 2-267 |
| L-Glutamine | 0.750g | 500g | 37107 7J | BDH 2-267 |
| Glycine | 0.400g | 100g | 44449 2A | BDH 2-269 |
| L-Histidine | 0.400g | 25g | H 6034 | Sigma 1022 |
| L-Isoleucine | 0.400g | 5g | I 2752 | Sigma 1149 |
| L-Leucine | 0.400g | 25g | L 8000 | Sigma 1204 |
| L-Lysine-monohydrochloride | 0.600g | 100g | 37129 3P | BDH 2-338 |
| L-Methionine | 0.200g | 5g | M 9625 | Sigma 1309 |
| L-Phenylalanine | 0.200g | 100g | P 2126 | Sigma 1608 |
| L-Proline | 0.400g | 25g | P 5607 | Sigma 1713 |
| L-Serine | 0.400g | 25g | S 4311 | Sigma 1846 |
| L-Threonine | 0.700g | 5g | T 8625 | Sigma 1980 |
| L-Valine | 0.400g | 25g | V 0500 | Sigma 2090 |
| L-Ascorbic acid (Vit. C) | 0.500g | 100g | 44006 3N | BDH 2-59 |
| Aneurine Hydrochloride (Vit. B) | 0.0125g | 100g | 44005 5N | BDH 2-692 |
| Riboflavin | 0.0025g | 25g | 44088 4T | BDH 2-565 |
| Nicotinic Acid | 0.050g | 100g | 44069 2N | BDH 2-431 |
| Folic acid | 0.0025g | 100g | 44043 6W | BDH 2-256 |
| (+)-Pantothenic acid (calcium salt) | 0.025g | 100g | 44074 4Y | BDH 2-449 |
| Inositol (meso) inactive | 0.250g | 100g | 38044 3M | BDH 2-308 |
| Choline choride | 0.250g | 100g | 42014 2B | BDH 2-165 |
| EDTA Fe-Na3 chelate pure | 0.0075g | 100g | EDFS | Sigma 787 |
| EDTA Zn-Na2 chelate pure | 0.004g | 100g | E 4886 | Sigma 786 |
| EDTA Mn-Na2 chelate pure | 0.004g | University of Reading* | | |
| EDTA Cu-Na2 chelate pure | 0.002g | 100g | EDCU2S | Sigma 786 |
| Pyridoxine hydrochloride (Vit B6) | 0.0125g | 25g | 44086 5Q | BDH 2-546 |
| D-Biotin-crystalline | 0.0005g | 1g | 44011 4H | BDH 2-71 |

***Note:** Due to the small quantities of EDTA Mn-Na2 required in this study, this chemical was granted from the University of Reading, but it can be ordered:

| Chemical Compound | Ordering Quantity | Catalogue Reference | Company |
|---|---|---|---|
| EDTA Mn-Na2 chelate pure | 100g | JTL704-5 | www.vwrsp.com |

### 2.3.2 Storage conditions and diet viability

After preparation of 500 ml of diet, 25-ml aliquots were kept frozen at -40°C in polyethylene sample vials (Fisher Scientific, product number: 03-338-1F) in until required (Fig. 2.9). The diet could be kept under these conditions and used for a period up to 6-8 months after which it deteriorates to the degree that inhibits aphid reproduction and development.

The first symptom that the frozen diet had deteriorated was the formation of a thin brown layer on the surface (Fig. 2.9). This indicated that the diet had to be replaced soon. However, it was still usable (at least once) after this layer was removed, while still frozen.

The second sign is that the culture starts to decline and the birth rate gradually declines until they stop reproducing. Late stage symptoms consist of aphid body colour change from light green to pale yellow and brown and eventually the culture dies out. However, the loss of the culture can be prevented with freshly prepared diet. This is the only way that the culture can be recovered (Elizabeth A. Wild, University of Reading, personal communication, 2002).

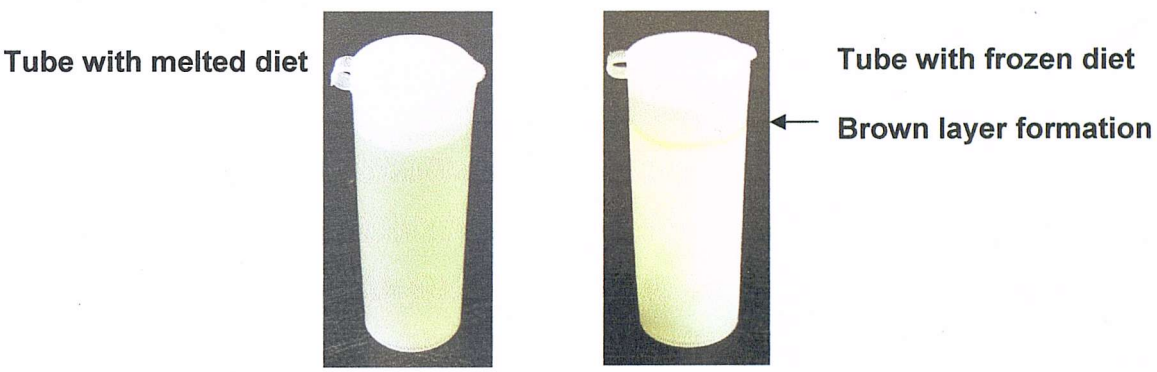



**Fig. 2.9** Vials where the frozen diet was stored.

### 2.3.3 Diet sterilization and sachet preparation

Prior of the construction of diet sachets, the diet was removed from the freezer and allowed to melt. Both the liquid diet and the Parafilm M® membrane used to make the sachets were sterilized. The first step was to assemble Swinney® filter holder system (product code: 406038223, 239000806 and 239207190 respectively, distributor in UK: VWR International, Poole. Manufacturer: Pall Corporation, Michigan 48103-9019; Fig. 2.10) The system was sterilized by autoclaving at 121 - 123 °C for 30 min prior to each use.

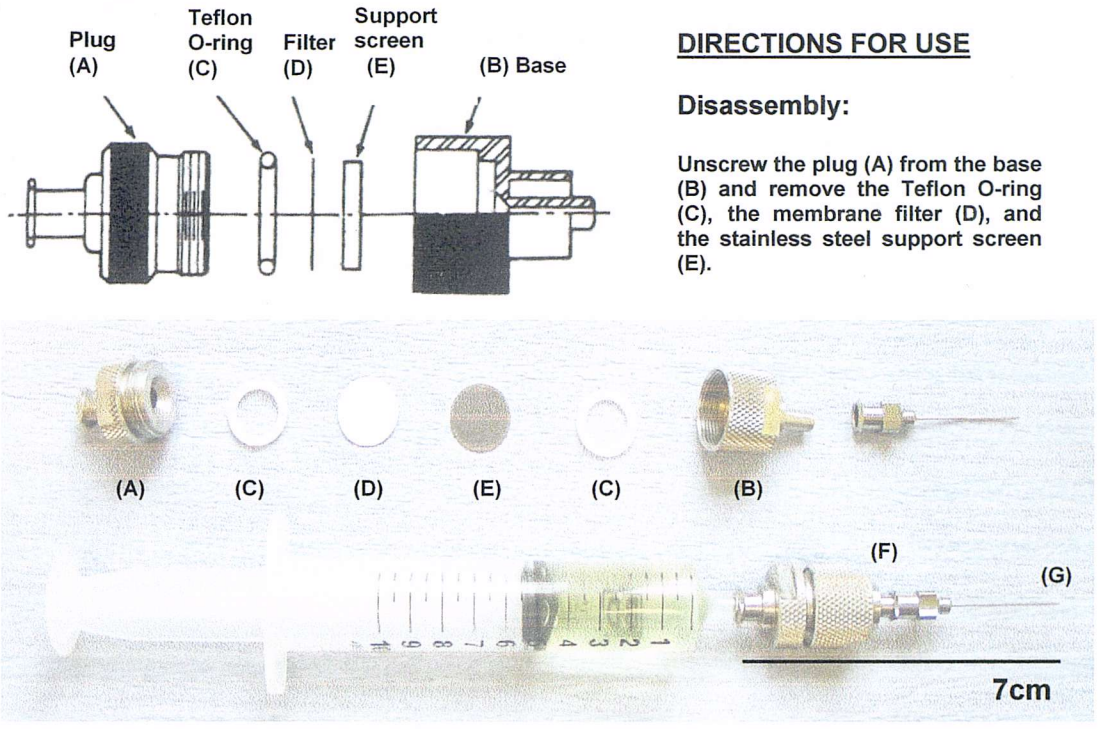



**Fig. 2.10** Swinney® filter holder system (source: Instruction manual, Gelman Instrument Company which merged with Pall corporation in 1997) connected to a 10 ml plastic syringe containing artificial diet. (D) Supor® Membrane Disc Filters, (Pore size 0.45 µm, diameter 13 mm), (F) Filter Holder Swinney® (Stainless steel, diameter 13 mm), (G) needles, (Stainless steel, size No 15, 23G, 25 mm long).

Secondly, diet sachets were prepared on cylindrical Perspex tubes (2.5 cm internal diameter × 2.5 cm high) covered with a stretched membrane (Parafilm M® all-purpose laboratory film – Pechiney Plastic Packaging, Menasha, WI 54952, USA). Using a laminar flow cabinet, sterilization of the top sachet's surface took place after exposure under UV light for 45 min. After that, approximately 20 drops of diet were squeezed through the filter holder (previously sterilized) and sealed with a 2$^{nd}$, sterilized stretched membrane. Thus, the compressed diet created a thin layer between the two pieces of Parafilm M® and covered most of the sachet's surface. Then, the sachets were stored in a freezer at -40 °C. Each sachet attached to the Perspex tube formed a rearing cylinder (Fig. 2.11) which will be referred from now on as sachet.

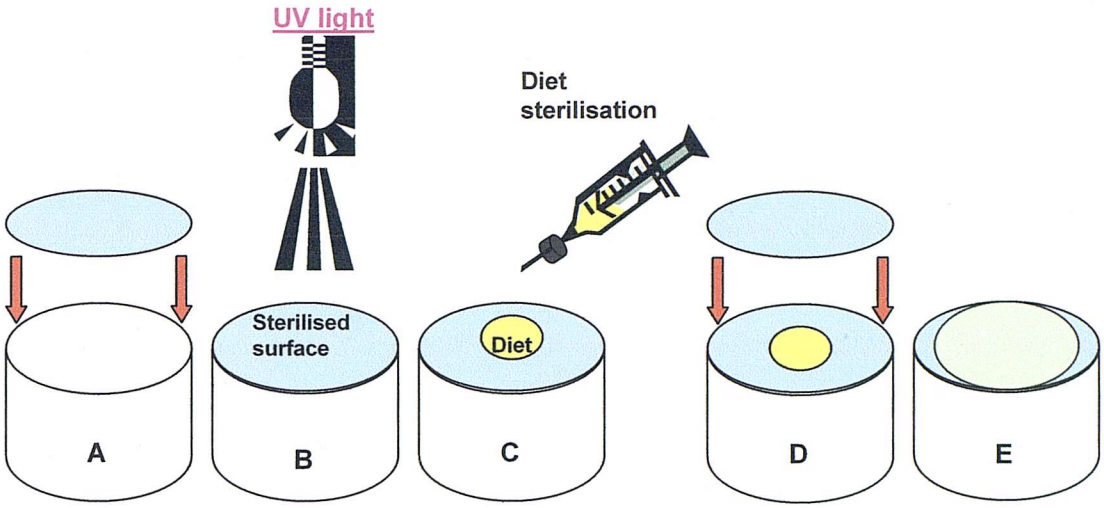



**Fig. 2.11** Schematic diagram (A → B → C → D) showing the preparation of sachets/rearing cylinders with diet (not to scale).

### 2.3.4 Culturing aphids on artificial diet sachets

The aphid culture was started when sachets were allowed to unfreeze just prior to their use. A small number of aphids were removed initially from a culture of aphids feeding on artificial diet (Mittler and Dadd 1966) and then transferred onto fresh artificial diet sachets constructed on Perspex rearing cylinders (Fig. 2.12). This culture was the original aphid clone that had been maintained on the artificial diet at the University of Reading since 1976 (Professor Helmut F. van Emden, University of Reading, personal communication) and has been studied previously (van Emden, 1988; van Emden & Andrews, 1997; van Emden & Kifle, 2002). After adult aphids were transferred into the sachets they were returned back the Fison's environmental cabinet (Section 2.6.2) where, then, left to feed (upside down mimicking the way they usually feed on leaves), grow and reproduce for 2 days under constant conditions (Fig. 2.12).

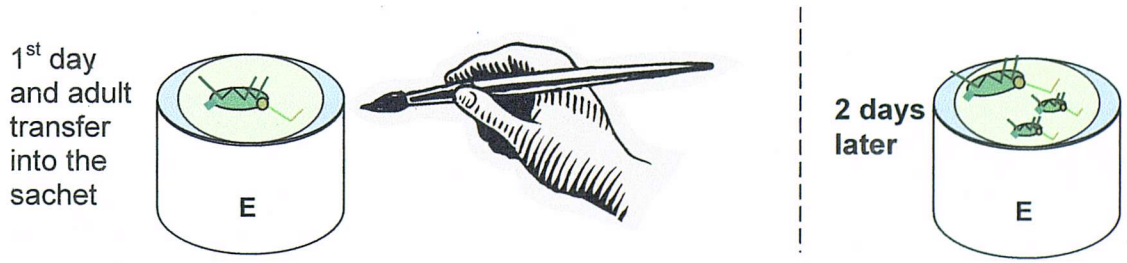



**Fig. 2.12** Schematic transfer of adult on artificial diet sachet to start the culture (not to scale)

At that time, the upper membrane of the 2-day-old sachet was removed by piercing and pulling it aside with a needle (Fig. 2.13). The old diet was allowed to drip and the remaining droplets were wiped away by gently pressing the sachet on tissue paper. A fresh unfrozen sachet was placed on the top of the old one and the newly formed double rearing cylinder was returned to the Fisons' cabinet for another 2 days. The aphids now at the bottom, diet-less section of the rearing cylinder rapidly climb to the top and commence feeding (Fig. 2.13).

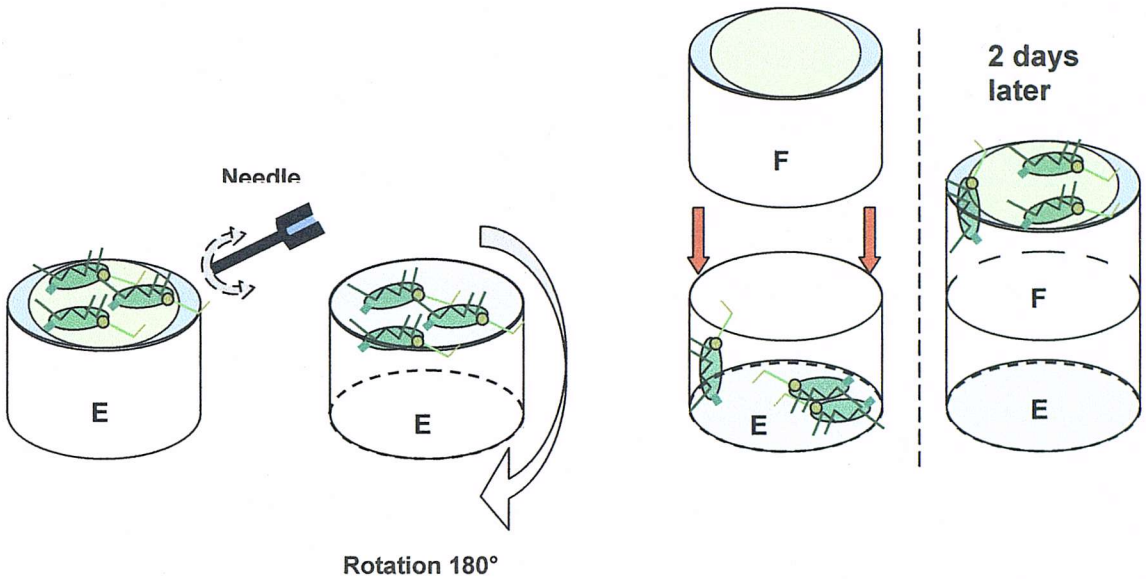



**Fig. 2.13** Schematic step by step (E → F) replacement of sachets with diet (not to scale).

This diet-replacement procedure was repeated every 2 days with the preparation of frozen sachets procedure taking place approximately every two weeks. Overall, the culturing procedure was successful (Fig. 2.14). In fact, this was the first time that the culture has survived for more than six months in a laboratory other than the one at the University of Reading (Professor Helmut F. van Emden, University of Reading, personal communication).

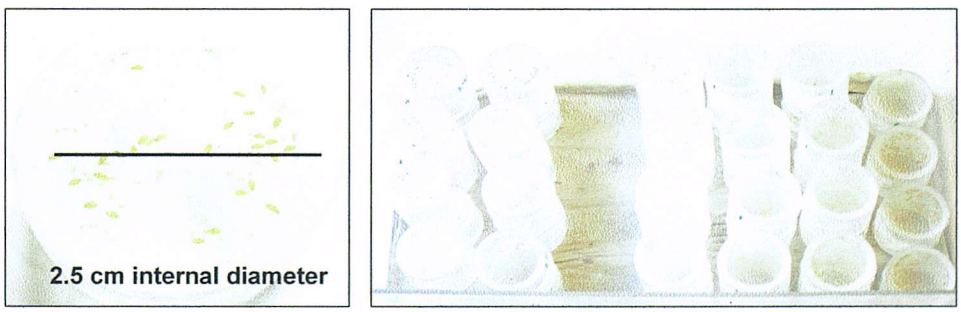



**Fig. 2.14** An individual sachet and multiple sachets in the culture.

# CHAPTER 3: Olfactory responses and olfactometer comparisons

## 3.1 Introduction

Several studies have demonstrated that learning clearly influences searching behaviour as emerging parasitoids showed a preference towards the plant on which their development occurred (Wickremasinghe & van Emden, 1992; van Emden *et al.*, 1996; van Emden *et al.*, 2002; Douloumpaka & van Emden, 2003; Kalule & Wright, 2004). Thus, in order to confirm this behaviour for the present system, the parasitoids' responses were tested to three different plant odours and clean air. In addition, olfactometers 1 and 2 (section 2.7.1) were tested for efficiency and directional bias. It must be noted that when the words distinguish, discriminate or differentiate are used they indicate that the parasitoids show a significant response to an odour offered with the olfactometer system. Considering the design of the olfactometers it is unlikely that the wasps can travel in and then out of an odour chamber during the bioassays. Thus, the first odour chamber that they enter is deemed to be the choice made.

## 3.2 Materials and Methods

### 3.2.1 Parasitoid responses to different odour choices in a 4-way protocol Preliminary experiment.

Adult female and male parasitoids collected from cultures reared on *Myzus persicae*-Winter harvest host-plant complex (HPC) were sexed, paired for 24 h and tested, after they were anaesthetised with $CO_2$, in single-sex groups in olfactometer 1 using a four-choice protocol (sections 2.6.1, 2.6.2 and 2.7.2). The volatile choices offered were emitted from leaves of a) Brussels-sprouts, cultivar 'Bedford-Winter harvest', b) Cabbage, cultivar 'Golden acre', c) Dwarf broad beans, cultivar 'The Sutton' (see section 2.3) (two from the family Brassicaceae and a third non Brassica) and d) Clean air (control). Thus, the parasitoids could choose between the odour emanating from the host's food plant and three alternative chambers.

The plants used for this experiment were approximately the same age (40-50 days after sowing) when the leaf size was suitable to fit in the olfactometer's odour chambers (i.e. the leaf's blade was not longer that 8.5 cm excluding the length of

petiole). Uninfested plants used for the bioassays (section 2.3) and the leaf blades were cut at the joining point with the petiole just prior to the tests. Two-three leaves were placed in the odour chambers so as to cover their internal surface. Care was taken to minimize mechanical damage to the leaves during the procedure as it is known that damaging leaves can change the volatile profile (Geervliet *et al.*, 1994; Agelopoulos *et al.*, 2000).

The whole system (central arena and odour chambers) was rotated 90° clockwise after each replicate to cancel out any asymmetry in the apparatus (section 2.7.2). Any directional bias and the efficiency of the cleaning procedure were also tested (10 replicates for both females and males) with all of the four chambers containing cotton wool. The age of the parasitoids was not known (section 2.6.2) and it was most likely that the parasitoids had experienced the host-plant microhabitat and also oviposition as they were collected directly from the culture cage. Only olfactometer 1 was used for the preliminary experiment.

#### *3.2.1.1 Statistical Analysis*

The data for the preliminary experiment (sections 3.3.1.1, 3.3.1.2) were analysed with the statistical package of "S-Plus" using Analysis of Variance (Anova) for a comparison between more than three means (four different categorical variables/odour choices) with different number of insects used within replicates. The data collected in this experiment were in the form of counts in which the response variable refers to the parasitoids, found in each category/odour chamber. Count data could be best analysed using GLMs (generalised linear models) in "S-Plus" (Crawley, 2002) allowing the specification of a variety of different error distribution to deal effectively with common problems arising from count data like zeros which are difficult to transform and non-normally distributed errors (Poisson errors in this case). Significant differences were revealed by contrasts (Crawley, 2002). The same method was applied to analyse the responses for both the males and females tested for this preliminary experiment. The parasitoids counted in the central arena were not included in the statistical analysis and regarded as showing "no response" (non-responders). However, the data for these are presented.

### 3.2.2 Parasitoid responses to two odour choices

The procedure that was followed for the handling of the parasitoids was similar to that described above (section 3.2.1) but anaesthesia with $CO_2$ was not used from this point onwards in the present study (section 2.6.4). However, in this case, three to

seven leaves (instead of two to three in the preliminary experiment) were placed in such a way in each test chamber so as to occupy its central internal space in an attempt to increase the number of the parasitoids responding to plant odours in the olfactometer system. Twenty individuals formed each replicate for all of the subsequent bioassays. In addition, the four-way olfactometer 1 was used as a two-way device with two out of the four test chambers containing leaf material from the same Brussels-sprout variety. Thus, apart from the rotation of the whole system 90° clockwise between replicates, the chambers containing the same odour were also interchanged every four replicates (Figs 3.1, 3.2). The responses of the female *A. colemani* to four Brussels-sprout varieties were tested in all the possible two by two combinations. For example, if the chambers next to each other contained the same odour they were arranged as in Fig. 3.1and then rearranged as in Fig. 3.2.

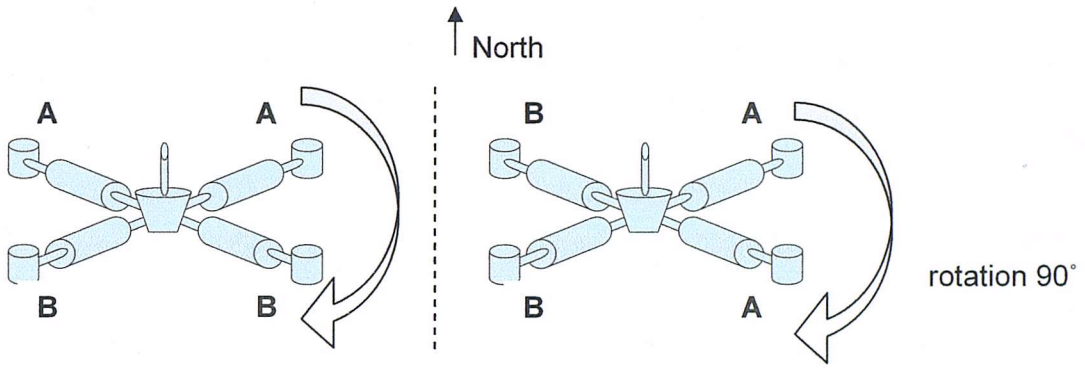



**Fig. 3.1** Rotation of the whole system central arena and test chambers after each replicate (for the first 4 replicates). A, B different odours/choices.

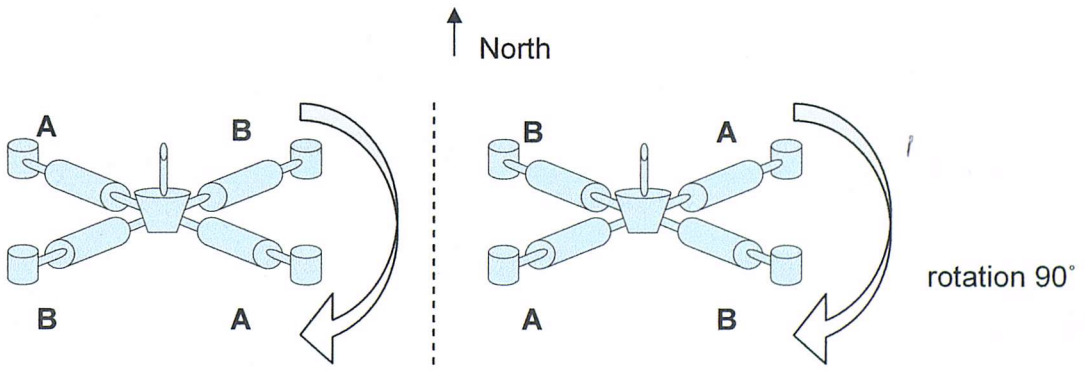



**Fig. 3.2** Rotation of the whole system central arena and test chambers after each replicate (for the next four replicates). A, B different odours/choices.

#### *3.2.2.1 Statistical Analysis*

The data obtained separately for each comparison were subject to $\chi^2$ tests. The overall choice ($\chi^2$ for d.f.=1) on the total number of parasitoids that responded was only accepted if accompanied by a non-significant heterogeneity ($\chi^2$ for d.f.=n-1) between replicates (n) (Wickremasinghe & van Emden, 1992). The heterogeneity was used to check for any inconsistencies between the replicates performed. It is analogous to residual sum of squares in an analysis of variance in that a significant value would indicate a treatment-replicate interaction, i.e. that the effect of treatment varied significantly between replicates. The number of parasitoids which showed no response was not included in the statistical analysis but the data are presented.

### 3.2.3 Female responses to the odour of Winter harvest and comparison between the olfactometer 1 and 2.

The method used for the bioassays was similar to the previous experiment (section 3.2.2). One difference was that the female parasitoids were tested against the odour emanating only from three to seven leaves (consistent within the odour chambers for each replicate) of Winter harvest which were placed in each numbered chamber (1-4). Also, the total number of replicates performed was 16 for each olfactometer. Each replicate involved 20 individuals and thus, the total number of parasitoids tested was 320 for each olfactometer. The data obtained for the parasitoids responded to Winter harvest odour were analysed with the statistical package of "S-Plus". An additional $\chi^2$ test was carried out to compare the total number of non-responders i.e. number of parasitoids remaining in the central arena of each olfactometer.

### 3.2.4 Control – Testing olfactometer 2 for directional bias and cleaning procedure efficiency. Female responses to clean air

Olfactometer 2 was also tested for directional bias and mated female parasitoids were offered clean air (control) in all four chambers (section 3.2.1). The data obtained were analysed with the statistical package of "S-Plus" (section 3.2.1.1).

## 3.3 Results

### 3.3.1 Parasitoid responses to different odour choices in a 4-way protocol - Preliminary experiment

#### *3.3.1.1 Control - Testing olfactometer 1 for directional bias and cleaning procedure efficiency. Female and male responses to clean air*

The majority of female and male parasitoids, 84.3% and 87.7%, respectively, showed no response and remained in the central arena. Those that entered the numbered chambers (cotton wool-clean air) showed no significant differences (Fig. 3.3) either for females (chambers:1-4, 4.2%, 4.9%, 3.6% and 3%) or males (chambers:1-4, 4.2%, 1.7%, 4% and 2.4% respectively). Each replicate involved 17-23 insects.

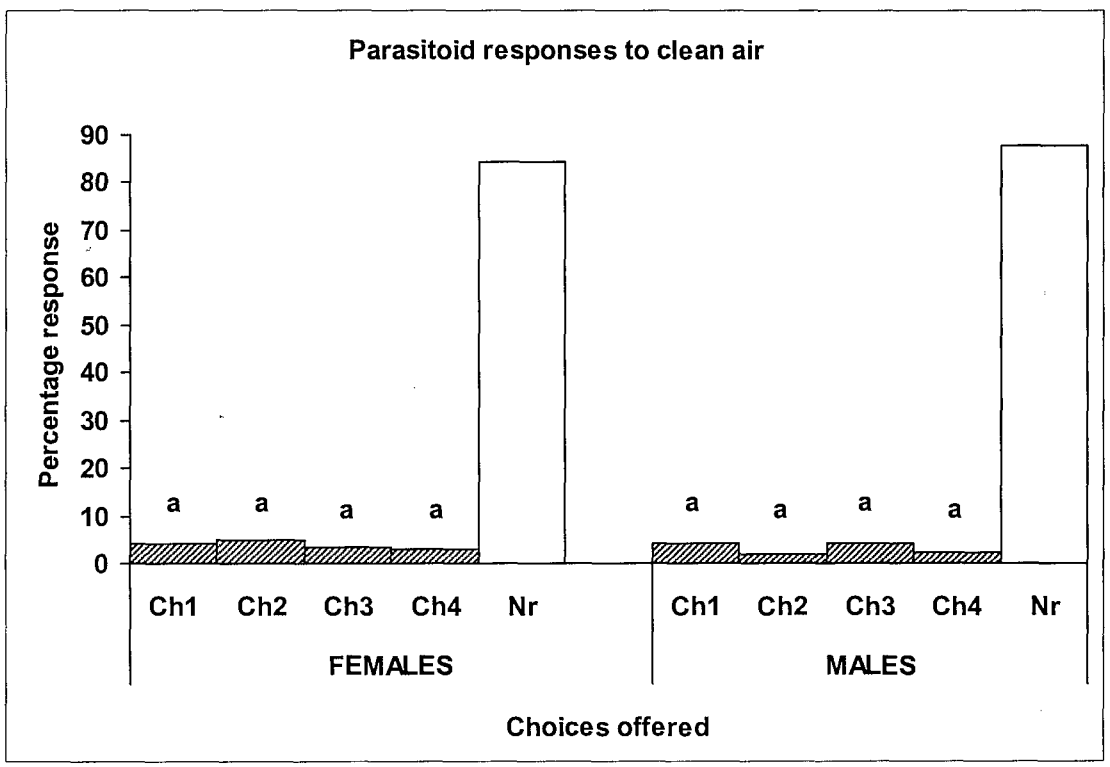



**Fig. 3.3** Percentage responses of *Aphidius colemani* emerging from *Myzus persicae* reared entirely on Brussels-sprout cv Winter harvest. Ch1,2,3,4: chambers 1,2,3,4 respectively, Nr: No response. Means with the same letter above the column are not significantly different (P>0.05) both for females and males. n=10 replicates and 211 insects tested for each sex.

#### *3.3.1.2 Female and male responses to plant odours*

A significant response to Brussels-sprout odour (Wh: Winter harvest) was recorded in females. Although as many as 56.5% of the parasitoids remained in the central arena (no response), 20.9% positively responded to Brussels-sprout odour (Fig. 3.4), the highest response when compared with responses to the odours of Cabbage,

Clean air and Broad bean (4.6%, 6.9% and 11.1%, respectively) and a significant preference was recorded for females. In this experiment, fewer males than females were found in the odour chambers after 30 min with 79.2% of the tested males remain in the central arena (no response) (Fig. 3.4). Within the offered choices a statistically significant response, 8.1%, to Brussels sprout odour was recorded when compared with the responses to Cabbage and Broad bean (2.7% and 3.5%, respectively). However, the response of males to Brussels sprout was not significantly different from the 6.5% recorded to Clean air. Each replicate involved 14-25 both for male and female parasitoids.

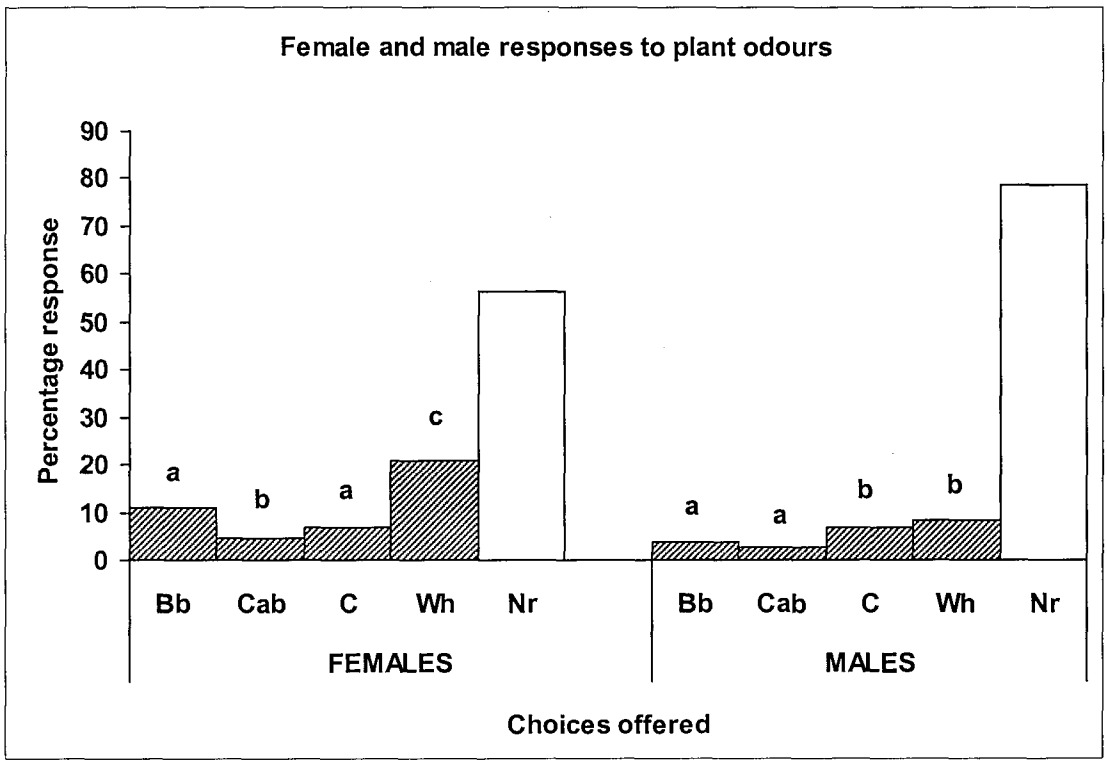



**Fig. 3.4** Percentage responses of *Aphidius colemani* emerging from *Myzus persicae* reared entirely on Brussels-sprout cv Winter harvest. Odours (from left to right): Bb: Broad bean, Cab: Cabbage, C: Clean air, Wh: Winter harvest and Nr: No response. Means with the same letter above the column are not significantly different ($P>0.05$). Level of significance between the different letters (females: a:b $P<0.01$, a:c $P<0.001$ and b:c $P<0.001$) and (males: a:b $P<0.05$). 561 females, n=29 replicates and 370 males, 20 replicates.

In view of the preference shown by female parasitoids in the previous experiment, the sensitivity and specificity of their responses was examined by comparing the Winter harvest odour to clean air and three Brussels-sprouts varieties/odours (Fillbasket, Red delicious and Hamlet) in a two-odour choice protocol.

### 3.3.2 Parasitoid responses to two odour choices

Brussels-sprout odour, of any variety tested, was preferred to clean air (Fig. 3.5, Table 3.1) and the odour of the variety on which the parasitoids had been reared (Winter harvest) was preferred over the alternative odour of Brussels-sprout varieties (Fig. 3.6, Table 3.2).

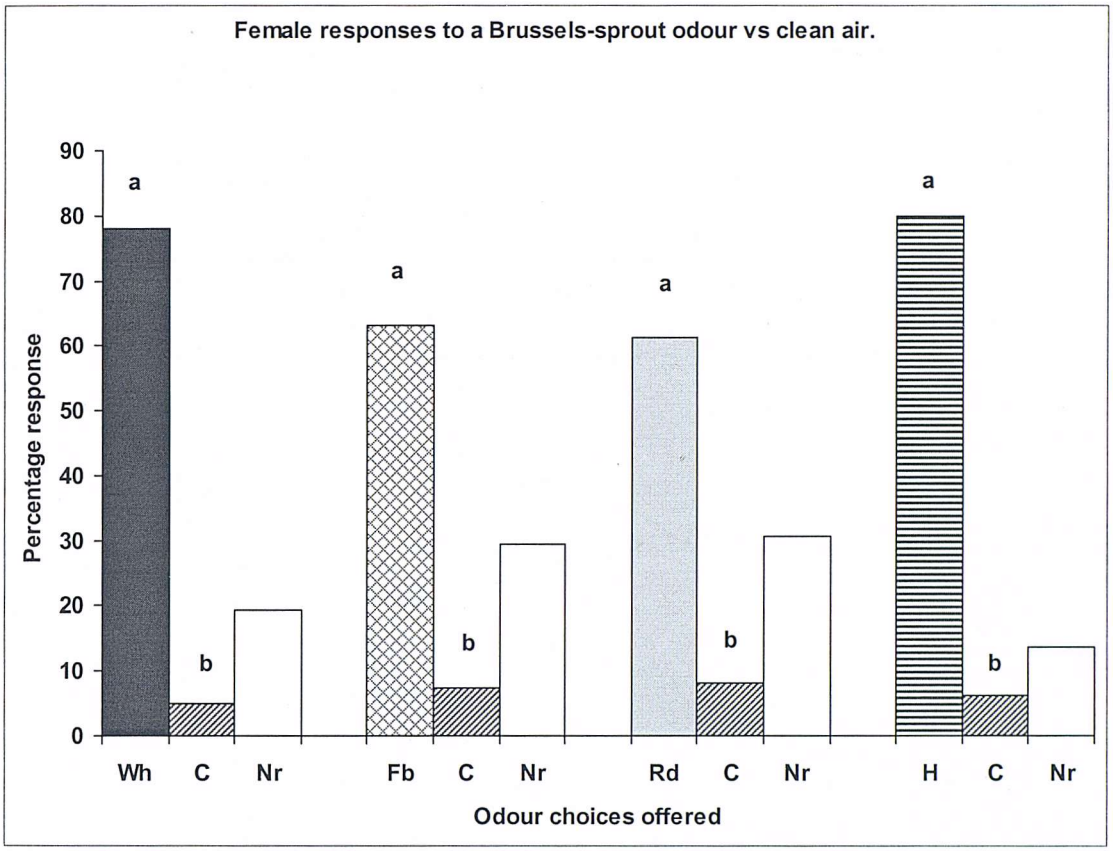



**Fig. 3.5** Percentage responses of *Aphidius colemani* emerging from *Myzus persicae* reared entirely on Brussels sprout cv Winter harvest and collected from the culture. Odours (from left to right): Wh: Winter harvest, C: Clean air, Nr: No response, Fb: Fillbasket, Rd: Red delicious and H: Hamlet. Means with different letter above the column are significantly different (comparison only within each odour test, i.e. variety versus control, Table 3.1). n=8 replicates, 20 insects per replicate.

**Table 3.1** Comparisons only within each sprout variety versus clean air. HPC= the host-plant complex (*Myzus persicae*-Winter harvest complex).

| Parasitoid collection from | Odour leaf material | No. of (*n*) replicates | Totals being compared for 1df | Choice $\chi^2$ (1df) | Heterogeneity $\chi^2$ (n-1df) |
|---|---|---|---|---|---|
| HPC | Wh vs C | 8 | 125 vs 8 | 102.92 *** | 2.45 ns |
| HPC | Fb vs C | 8 | 101 vs 12 | 70.10 *** | 4.63 ns |
| HPC | Rd vs C | 8 | 98 vs 13 | 65.09 *** | 2.65 ns |
| HPC | H vs C | 8 | 128 vs 10 | 100.90 *** | 1.01 ns |

***P<0.001, ns:P>0.05

However, while a significant preference was recorded for Winter harvest when compared with Hamlet the significance level (P<0.05) could not be accepted as the heterogeneity was significant (Table 3.2) indicating that the response obtained between the replicates was inconsistent. Thus, this result was considered not valid.

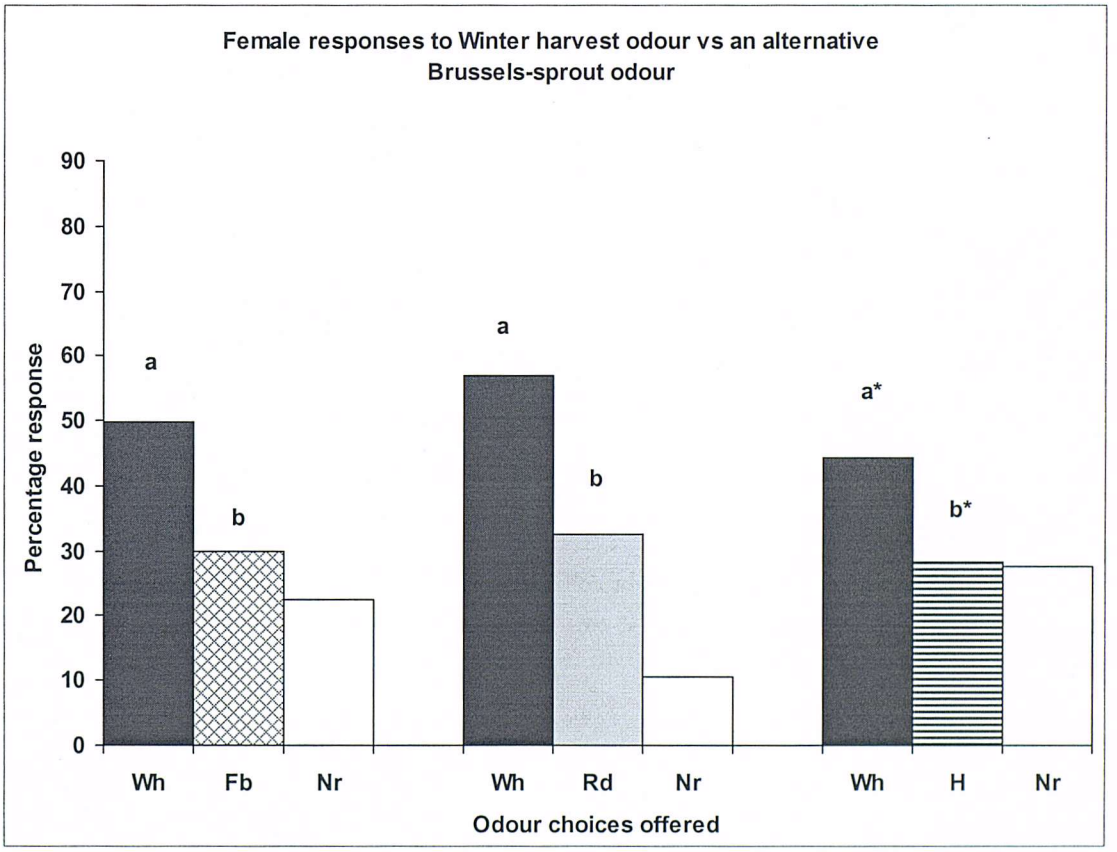



**Fig. 3.6** Percentage responses of *Aphidius colemani* emerging from *Myzus persicae* reared entirely on Brussels sprout cv Winter harvest. Odours (from left to right): Wh: Winter harvest, Fb: Fillbasket, Nr: No response, Rd: Red delicious and H: Hamlet. Means with different letter above the column are significantly different (comparison only within each the odour of Winter harvest and the alternative one, respectively). a*:b*

refer to significantly different means but with the heterogeneity also significant (Table 3.2). n=8 replicates, 20 insects per replicate.

**Table 3.2** Comparisons between Winter harvest against each of the other three sprout varieties. HPC= the host-plant complex (*Myzus persicae*-Winter harvest).

| Parasitoid collection from | Odour leaf material | No. of (*n*) replicates | Totals being compared for 1df | Choice $\chi^2$ (1df) | Heterogeneity $\chi^2$ (n-1df) |
|---|---|---|---|---|---|
| HPC | Wh vs Fb | 8 | 79 vs 48 | 7.57 ** | 7.02 ns |
| HPC | Wh vs Rd | 8 | 91 vs 52 | 10.64 ** | 11.98 ns |
| HPC | Wh vs H | 8 | 71 vs 45 | 5.83 * | 28.39 *** |
| | | | | *P<0.05, **P<0.01,***P<0.001, ns:P>0.05 | |

### 3.3.3 Female responses to the odour of Winter harvest and comparison between the olfactometer 1 and 2.

The significant heterogeneity observed in a comparison in the previous experiment (section 3.3.2) and construction inconsistencies led to the redesign of the olfactometer central arena in an attempt to overcome these concerns (e.g. plastic lid not quite air tight, difficulties in making connecting Perspex tubes flush with the inside arena wall as well as problems with gluing them together in a stable manner – the connecting tubes occasionally came unglued during bioassays). A single odour test (Winter harvest) was conducted and olfactometer 1 (plastic arena) and 2 (Perspex arena) were compared (section 2.7.1).

The majority of the parasitoids entered the odour chambers containing Winter harvest leaves and there were no significant differences between chambers (Fig. 3.7) olfactometer 1, (Wh:1-4, 19%, 23.4%, 20.7% and 16.6%) and olfactometer 2, (Wh:1-4, 25.7%, 23.5%, 20.4% and 22.8%). Of the individuals tested using the olfactometer 1, 20.3% remained in the central arena while this percentage was reduced significantly to 7.6% in olfactometer 2 (Table 3.3).

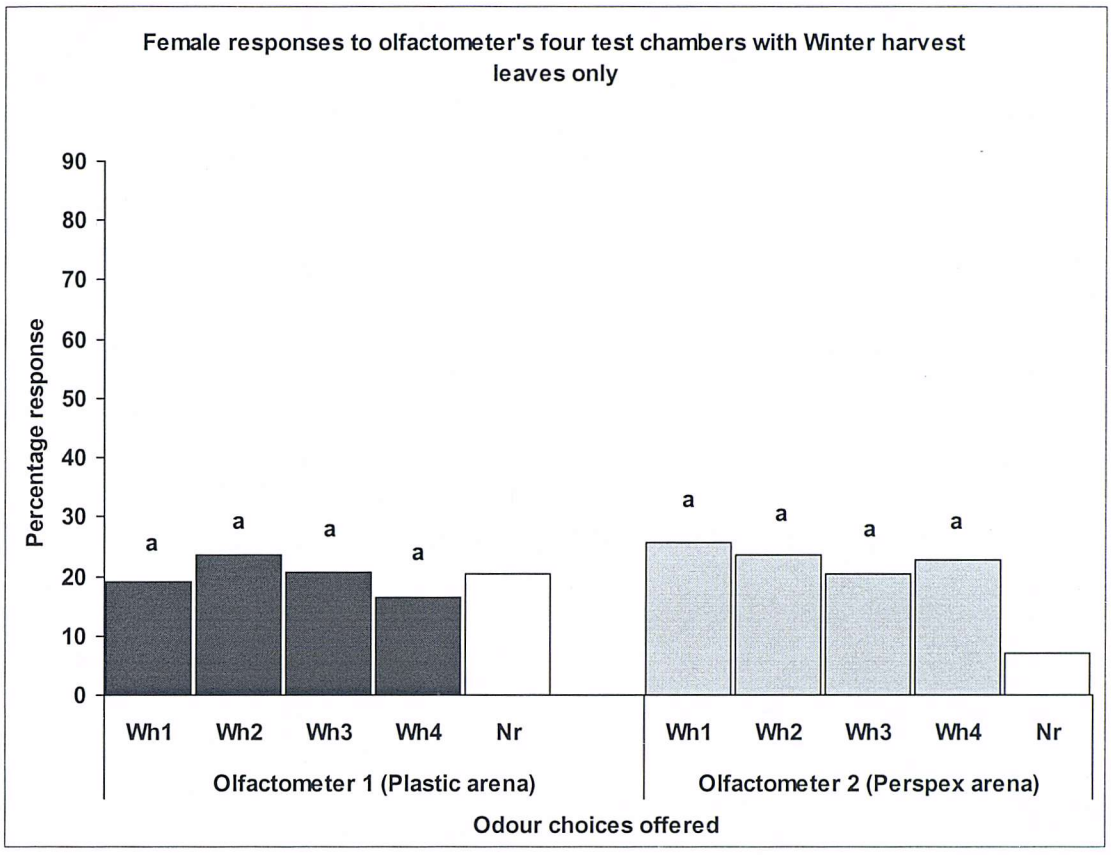



**Fig. 3.7** Percentage responses of female *Aphidius colemani* emerging from *Myzus persicae* reared entirely on Brussels sprout cv Winter harvest. Odours (from left to right): Wh: Winter harvest, Wh1,2,3,4 odour emanating from chamber1,2,3,4 in each olfactometer design, respectively. Nr: No response. Means with the letter a above the column are not significantly different (comparison only within each olfactometer). n=16 replicates for each olfactometer, 20 insects per replicate.

**Table 3.3** Comparison between the number of insects responding to plant odours and the non-responders in the olfactometer 1 and 2. HPC= the host –plant complex (*Myzus persicae*-Winter harvest complex). n=16 replicates for each olfactometer.

| Parasitoid collection from | Odour leaf material: Winter harvest in all odour chambers | Category | Totals being compared | Total chi square $\chi^2$ (1df) |
|---|---|---|---|---|
| HPC | Olfactom.1 vs Olfactom.2 | responders | 255 vs 295 | 20.69*** |
| HPC | Olfactom.1 vs Olfactom.2 | non-responders | 65 vs 25 | |
| | Olfactom.= Olfactometer | ***P<0.001 | | |

### 3.3.4 Control – Testing olfactometer 2 for directional bias and cleaning procedure efficiency. Female responses to clean air

The data analysis did not reveal any significant responses of female parasitoids (Fig. 3.8) towards any of the chambers (chambers: 1-4, 5%, 5%, 8.1%, 5.6%, respectively) while the non-responders (76.3%) represented the majority of insects after 30 min in the olfactometer.

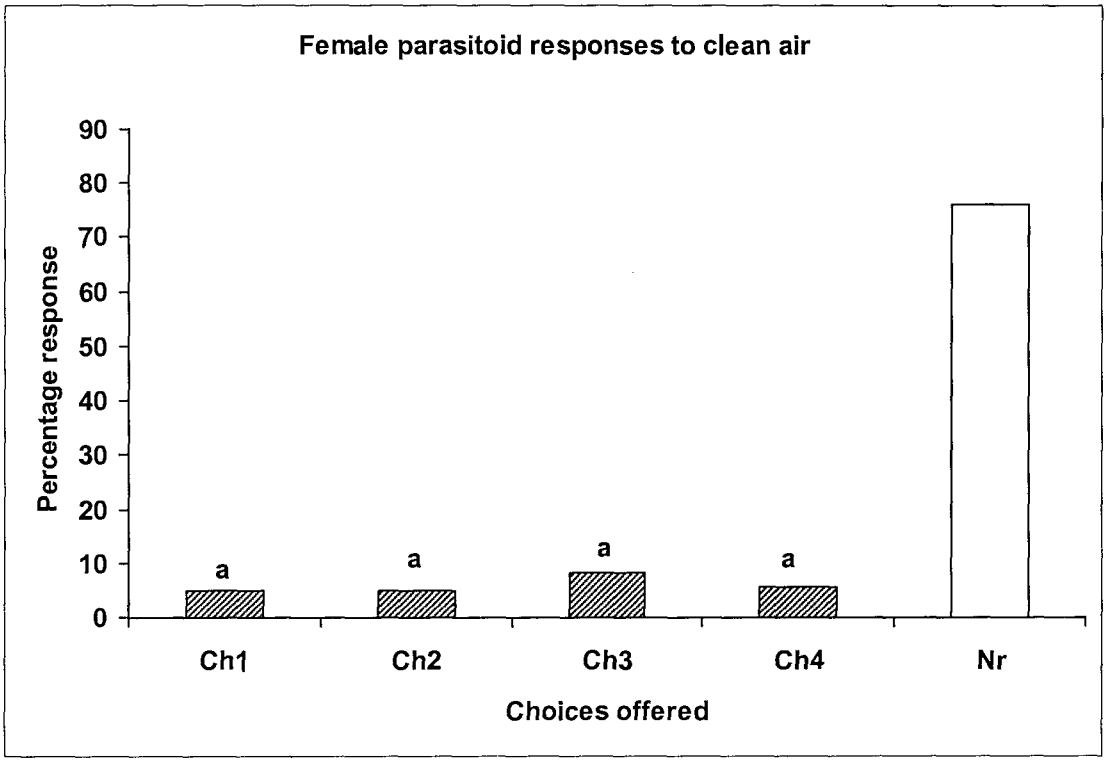



**Fig. 3.8** Percentage responses of mated female *Aphidius colemani* emerging from *Myzus persicae* reared entirely on Brussels-sprout cv Winter harvest. Ch1,2,3,4: chambers 1,2,3,4 respectively, Nr: No response. Means with the same letter above the column are not significantly different (P>0.05). n=8 replicates, 20 insects per replicate.

## 3.4 Discussion

No bias was detected after cleaning olfactometer 1 (Plastic arena) as there were no significant differences between the numbers of insects in the four clean-air chambers offered to either sex, illustrating no directional bias and a suitable cleaning procedure. In addition, considering the male responses in the preliminary experiment to plant odours it was decided to conduct further experiments only with females despite the fact that some studies have shown that males also distinguish between plant odours (Storeck *et al.*, 2000; Douloumpaka & van Emden, 2003). For example, in the current study it was intriguing that significantly more males responded to clean air than cabbage or broad bean odour and the fact that the non-responders for both males and

females were more than 50% of the parasitoids tested. The difference in the number of replicates (29 and 20) performed for females and males, respectively, came from the fact that males were more often found dead in the glass vials (section 2.6.4) where they were placed to mate with females over 24 h before the bioassays in the olfactometer. However, it was assumed that all females tested were mated as it was observed that mating usually occurs a few minutes after the parasitoids were placed in the glass vials.

The current data showed that significant responses could be obtained when the insects were offered the choice between any of the four varieties of Brussels-sprout against clean air, irrespective of which variety their host was feeding. In real agroecosystems the parasitoids suffering a lack of hosts and plants on which they developed may respond to volatiles from other plants. *Aphidius colemani* is a generalist parasitoid wasp and has the capacity to learn which enables it to exploit a variety of resources or habitats while still maintaining a high searching ability and achieving optimal rates of oviposition. It has previously been shown that if odour of four different plant species (that were not the lepidopteran host's food plant), tested with *Apanteles kariya* individually in an olfactometer against clean air, the parasitoids show a significant response to the plant odour. This is known as the theory of "misunderstanding" which refers to these non-specific types of plant-parasitoid interactions (Takabayashi *et al.*, 1991). The results presented may provide further evidence of this theory. Although the four Brussels-sprout varieties belong to the same plant species the data indicate that the positive responses to a variety/plant odour over clean air may be innate as similar significant responses recorded in all comparisons.

A preference was evident for the Brussels-sprout variety Winter harvest on which the parasitoid developed over other varieties, except in the case of Hamlet where the result was confounded by heterogeneity between runs indicating inconsistency of parasitoid responses between replicates and that these two varieties might had similar volatile profiles. It was anticipated that experiments using the Hamlet variety in subsequent olfactometer bioassays possibly will reveal whether this hypothesis should be investigated more thoroughly. Furthermore, volatiles shared between Winter harvest and Hamlet varieties might be worth identifying since it seems difficult for *Aphidius colemani* to differentiate between these two Brussels sprout varieties, but this idea was not developed further. Unfortunately, Hamlet is currently no longer commercially available but Hamlet seeds could be acquired for research purposes (Suttons seeds, Torbay, UK, personal communication).

When the two olfactometers were studied in terms of parasitoid responses, it was noted (anecdotally) that increasing the amount of leaves presented in the odour chambers as well as the redesign of the central arena increased the bioassay's

efficiency. The percentage of non-responders dropped significantly with the use of olfactometer 2 (Perspex arena) demonstrating an improved construction and the numbers of non-responders compare favourably with those (20-45%) of previous studies (Storeck *et al.*, 2000; Douloumpaka & van Emden, 2003). Olfactometer 2 was therefore selected for use in further experiments.

The experiments in this chapter revealed the sensitivity of parasitoid preferences and assisted efforts to improve the bioassay protocol.

# CHAPTER 4: Effect of pre- and post- emergence manipulation of *Aphidius colemani*

## 4.1 Introduction

Adult female parasitoids have the ability to learn and respond to plant cues in their environment. For example after experiencing the odour of a plant alone or the plant-host combination, experience with the host products and mummified hosts or oviposition experience (Kester & Barbosa, 1991; Turlings *et al.*, 1993; Du *et al.*, 1997; Fujiwara *et al.*, 2000) and in particular, in this learning process, contact with the host or a mummified host can often elicit preference for the odour of any plant (Vet & Groenewold, 1990; van Emden *et al.*, 1996; Storeck *et al.*, 2000). Such chemical cues could positively influence parasitoid responses towards the odour of a plant, even distinguishing between wheat cultivars (Wickremasinghe & van Emden, 1992). Furthermore, all the relevant information about parasitoid learning has been reviewed providing evidence that there are chemical cues on or in the cuticle of the host "mummy", in the case of aphids that induce parasitoid preference to specific plant odours (Godfray, 1994). Such a mechanism could increase the probability of parasitoids locating plants on which hosts may be found.

In this chapter, host-plant preference was initially examined for females collected straight after natural emergence in the host-plant environment (with Winter harvest or Red delicious as the plant of origin). However, the main aim was to investigate the olfactory response of *A. colemani* females having no experience of the odours emanating from the plant-host environment at emergence. Thus, mummies were taken from the plant of origin and isolated in Petri dishes prior of the emergence of the adult parasitoid. Whether the parasitoids retain their ability to respond to the plant on which mummy formation took place could then be examined. The tritrophic system in question was A. *colemani* adult parasitoids emerging from *M. persicae* hosts reared on Brussels sprouts in the following treatments:

A) Natural emergence:

a) Mummies reared on plant culture throughout parasitoid development - Mated females (culture-collected adults),

b) Mummies isolated away from the plant of origin:

i) Mated females (emerging in Petri dishes),

ii) Virgin males and females (emerging individually in glass vials),

B) Excision of pupae prior emergence:

Mummies isolated away from the plant of origin but pupae surgically removed from the mummified host - Mated females (emerging in Petri dishes).

The above treatments were designed to study the effect of reducing/excluding, one by one, the available experiences in terms of pre- and post-natal learning but also to examine whether virgin parasitoids demonstrate different response patterns to those allowed to mate. Furthermore, to investigate whether a learning hierarchy exists and to what extent this might influence the behaviour of developing/emerging parasitoids from the aphid hosts/mummies.

## 4.2 Materials and Methods

The method used for the subsequent bioassays was detailed in the previous chapter and olfactometer 2 was used (section 3.3.2).

### 4.2.1 Natural emergence – mated females (culture-collected adults)

Initially, responses of parasitoids emerging naturally from *Myzus persicae*-Winter harvest host-plant complex in culture cages were examined by comparing the Winter harvest odour and three other plant odours (Red delicious, Cabbage and Broad bean) to clean air. In addition, parasitoid responses to Winter harvest versus Red delicious odour were investigated when the parasitoids developed on aphid-hosts feeding on either Winter harvest or Red delicious Brussels sprout varieties. Parasitoids collected from culture on Red delicious that had been reared on this variety for more than 15 generations were tested for olfactory preference. An overall $\chi^2$ test as well as a $\chi^2$ test for heterogeneity (section 3.2.2.1) was carried out to compare the number of females responding in a two-way protocol (section 3.3.2).

### 4.2.2 Natural emergence – mated females (emerging in Petri dishes)

Late-stage mummies (dark coloured mummies formed on Winter harvest – brown and black) were collected from the Winter harvest variety and placed on damp filter paper inside Petri dishes isolated from the host-plant complex. The Petri dishes were checked daily and the emerged parasitoids were mated and tested after the handling procedure was completed (sections 2.6.2, 2.6.3, 2.6.4).

### 4.2.3 Natural emergence – virgin males and females (emerging separately in glass vials)

To obtain virgin adults, late-stage mummies were place individually into small glass vials (10-mm diameter × 50-mm high) with plastic lids. The vials were checked daily and after emergence, single-sex groups of 20-25 parasitoids were transferred into larger vials (2.5-cm diameter × 7.5-cm high) and allowed access to honey solution for 24 h (section 2.6.2).

### 4.2.4 Emergence after excision of pupae – mated females (emerging in Petri dishes)

Parasitoid emergence after excision was possible (section 2.6.3) and parasitoid pupae were removed from the cuticle of late-stage mummies one to three days (i.e. approximately pupal stages d, e, f, g, Fig. 4.1) before natural adult emergence occurred. Excised pupae surviving excision at stages d-g were used in this study and the emerged adults in Petri dishes were mated for 24 h prior to any tests.

The bioassays conducted included comparisons of Winter harvest odour against clean air, the three sprout varieties (Fillbasket, Red delicious and Hamlet) and also against two other plant odours, Cabbage and Broad bean. In addition, after excision and emergence, the adult female parasitoids were given access to whole mummies (light coloured early stage mummies – yellow and brown) formed on a sprout variety other than that on which the test parasitoid was reared (Winter harvest or Red delicious) during the 24 h mating procedure. A number of studies have dealt with the determination of larval stages in aphid parasitoids and it is now generally accepted that three larval instars occur (see review by O'Donnell, 1987). It must be noted that the larval and pupal stages presented above (Fig. 4.1) are an empirical classification which serves solely the purpose of the current study and all insects excised at stages a, b, c died after some development towards late-stage pupae, but before adult emergence. The survival of 356 parasitoids excised at stages d, e, f, g was recorded and the data are presented (Table 4.1).

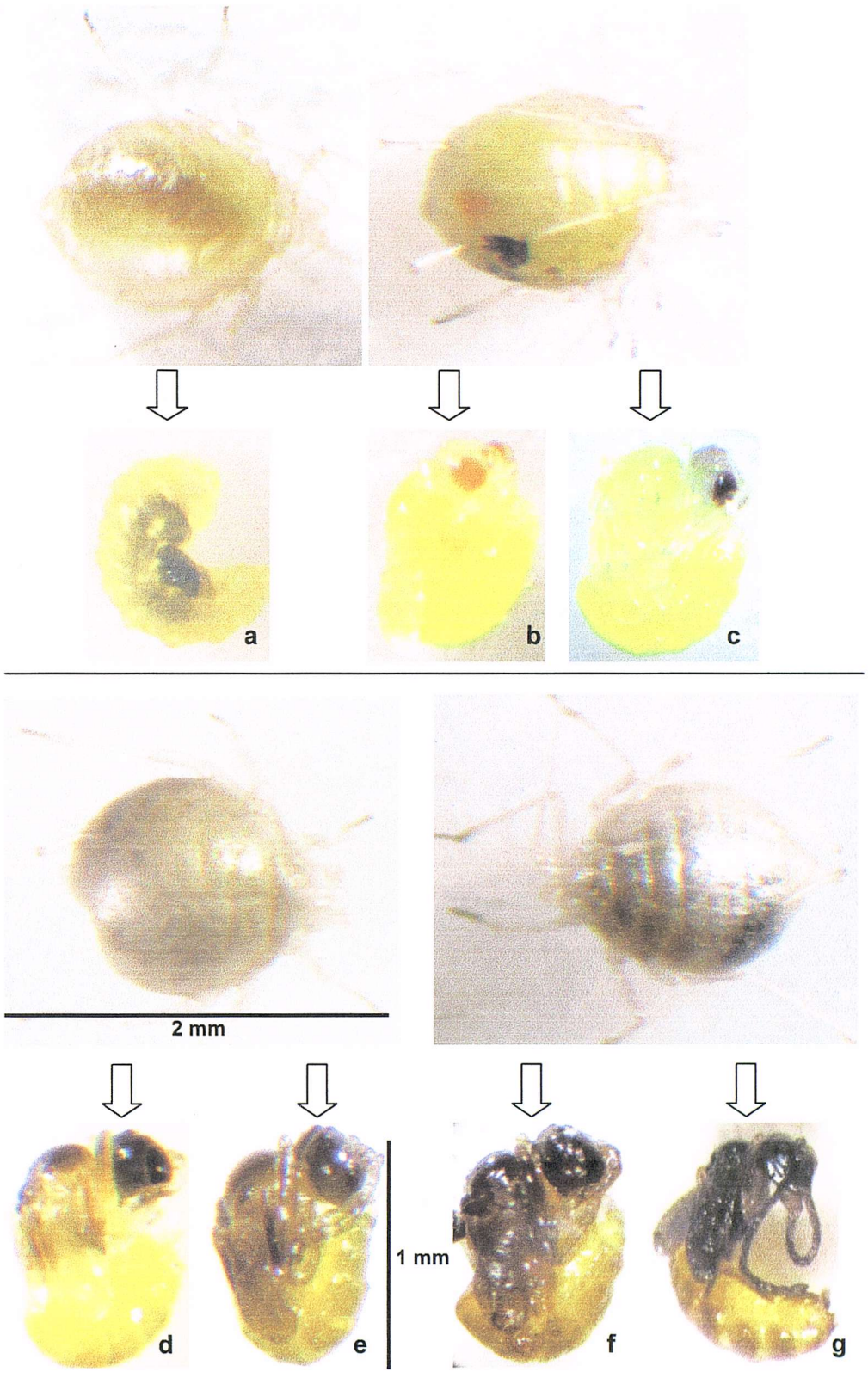
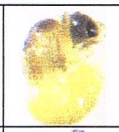
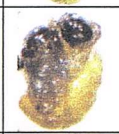



**Fig. 4.1** The photographs corresponding to the letters a, b, c, d, e, f, g represent consecutive stages of larval (a) and pupal (b - g) development as revealed after excision from the mummies shown above them (microscope: WILD Heerbrugg, Switzerland, M5-71576 - digital camera: Olympus C730UZ).

**Table 4.1** Emergence success after excision of different stages of the developing pupa from mummified aphid hosts.

| Stage of pupal development | | excised pupae - (uninjured pupae) | emerged imago | Emerged Overall % | Sex | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | ♂ | % | ♀ | % |
| d. white antennae brown thorax brown head |  | 37 - (34) | 15 | 40.5 | 7 | 46.6 | 8 | 53.3 |
| e. white antennae brown thorax black head |  | 76 - (70) | 49 | 64.5 | 24 | 48.9 | 25 | 51.1 |
| f. white antennae black thorax black head |  | 94 - (87) | 81 | 86.2 | 44 | 54.3 | 37 | 45.7 |
| g. black antennae black thorax black head |  | 149 - (131) | 125 | 83.9 | 67 | 53.6 | 58 | 46.4 |
| **Totals:** | | **356 (322)** | **270** | **75.8** | **142** | **52.6** | **128** | **47.4** |

The third column refers to the total number of mummies excised and the numbers inside the parenthesis represent the number of pupae removed from mummies and were not visibly injured during the procedure of excision (section 2.6.3). Injured pupae were those which had been stabbed with the forceps during excision and those with legs, wings or antennae missing or damaged. All the stages (d, e, f, g) were used in this study but (g) and (f) were mainly targeted throughout as excision was most successful.

## 4.3 Results

### 4.3.1 Responses of mated female parasitoids after natural emergence (culture-collected adults)

All plant odours tested were preferred to clean air (Fig. 4.2, Table 4.2) and followed similar results to previous experiments (section 3.2.3). Furthermore, when parasitoids were allowed to choose between odours of Winter harvest and Red delicious, a significant preference to the odour of the variety on which the parasitoids had been reared was evident over the alternative sprout odour (Fig. 4.3, Table 4.3).

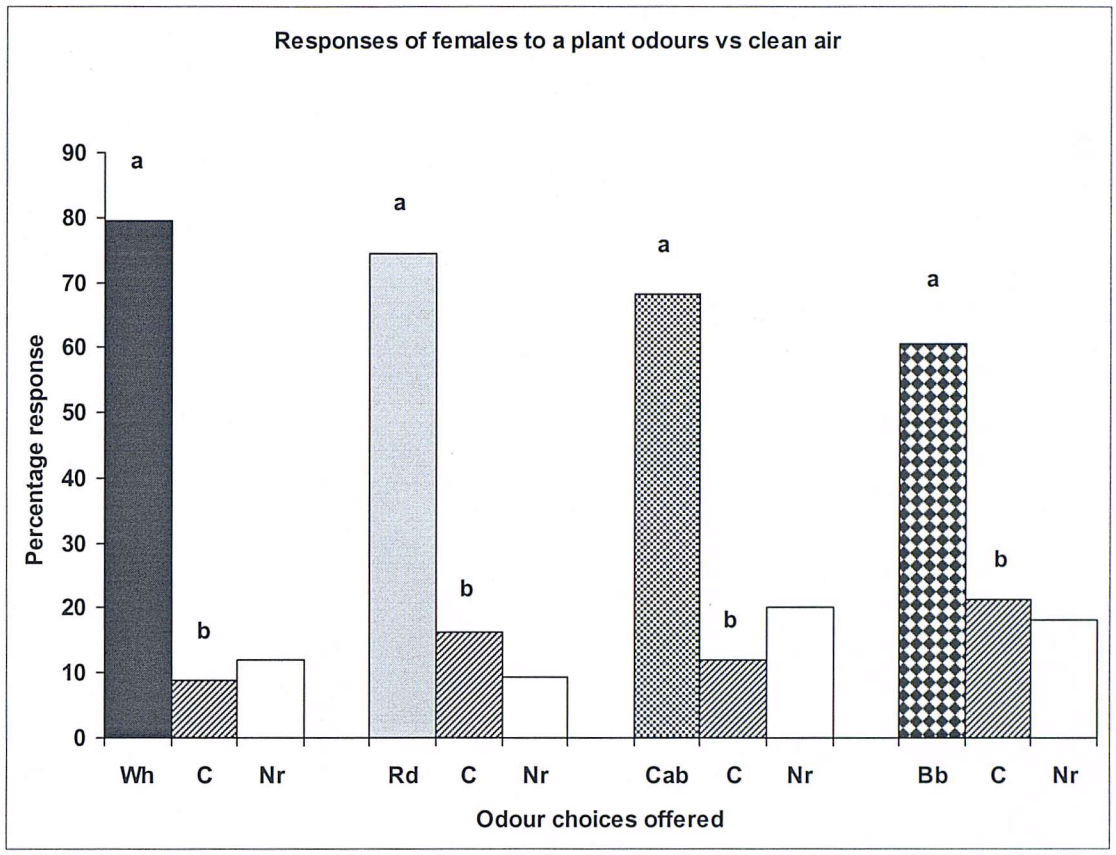



**Fig. 4.2** Percentage responses of mated female *Aphidius colemani* emerging from *Myzus persicae* reared entirely on Brussels sprout cv Winter harvest. Odours (from left to right): Wh: Winter harvest, C: Clean air, Nr: No response, Rd: Red delicious, Cab: Cabbage and Bb: Broad bean. Means with different letter above the column are significantly different (comparison only within each odour test, i.e. plant versus Clean air, Table 4.2). n=8 replicates, 20 insects per replicate.

**Table 4.2** Comparison of mated females *A. colemani* between plant/sprout variety odours and clean air. HPC= the host-plant (*Myzus persicae*-Winter harvest) complex.

| Parasitoid collection from | Odour leaf material | No. of (*n*) replicates | Totals being compared for 1df | Choice $\chi^2$ (1df) | Heterogeneity $\chi^2$ (n-1df) |
|---|---|---|---|---|---|
| HPC | Wh vs C | 8 | 127 vs 14 | 90.56 *** | 2.15 ns |
| HPC | Rd vs C | 8 | 119 vs 26 | 59.65 *** | 2.59 ns |
| HPC | Cab vs C | 8 | 109 vs 19 | 63.28 *** | 3.98 ns |
| HPC | Bb vs C | 8 | 97 vs 34 | 30.30 *** | 2.52 ns |

***P<0.001, ns:P>0.05

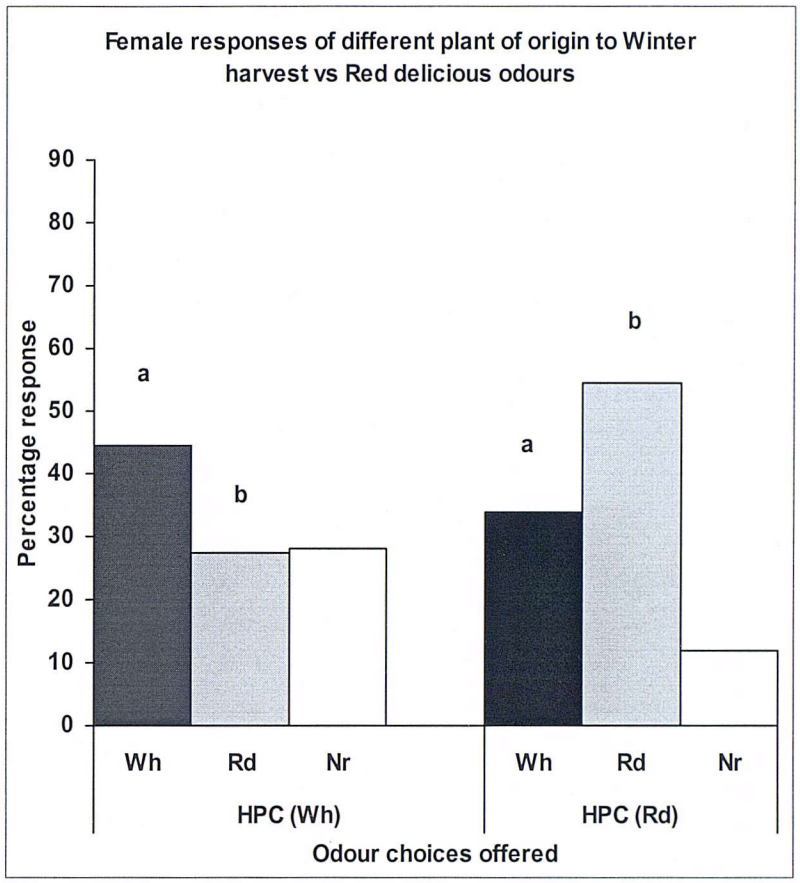



**Fig. 4.3** Percentage responses of mated female *Aphidius colemani* emerging from *Myzus persicae* mummies formed on Brussels sprout cv Winter harvest [HPC(Wh)] or Red delicious [HPC(Rd)]. Odours (from left to right): Wh: Winter harvest, Rd: Red delicious; Nr: No response. Means with different letter above the column are significantly different (comparison only within each odour test, i.e. Winter harvest versus the alternative choice, Table 4.3). n=8 replicates, 20 insects per replicate.

**Table 4.3** Comparison between sprout varieties using mated parasitoids of different origin. HPC (Wh)= host-plant (*Myzus persicae*-Winter harvest) complex. HPC (Rd) = host-plant (*Myzus persicae*-Red delicious) complex.

| Parasitoid collection from | Odour leaf material | No. of (*n*) replicates | Totals being compared for 1df | Choice $\chi^2$ (1df) | Heterogeneity $\chi^2$ (n-1df) |
|---|---|---|---|---|---|
| HPC (Wh) | Wh vs Rd | 8 | 71 vs 44 | 6.34 * | 8.06 ns |
| HPC (Rd) | Wh vs Rd | 8 | 54 vs 87 | 7.72 ** | 11.32 ns |

*P<0.05, **P<0.01, ns:P>0.05

### 4.3.2 Responses of mated female parasitoids after natural emergence from mummies isolated from the plant (emerging in Petri dishes)

In the bioassays where a sprout variety odour was run against clean air the parasitoids showed significant responses ($P<0.001$) to the plant (Fig. 4.4).

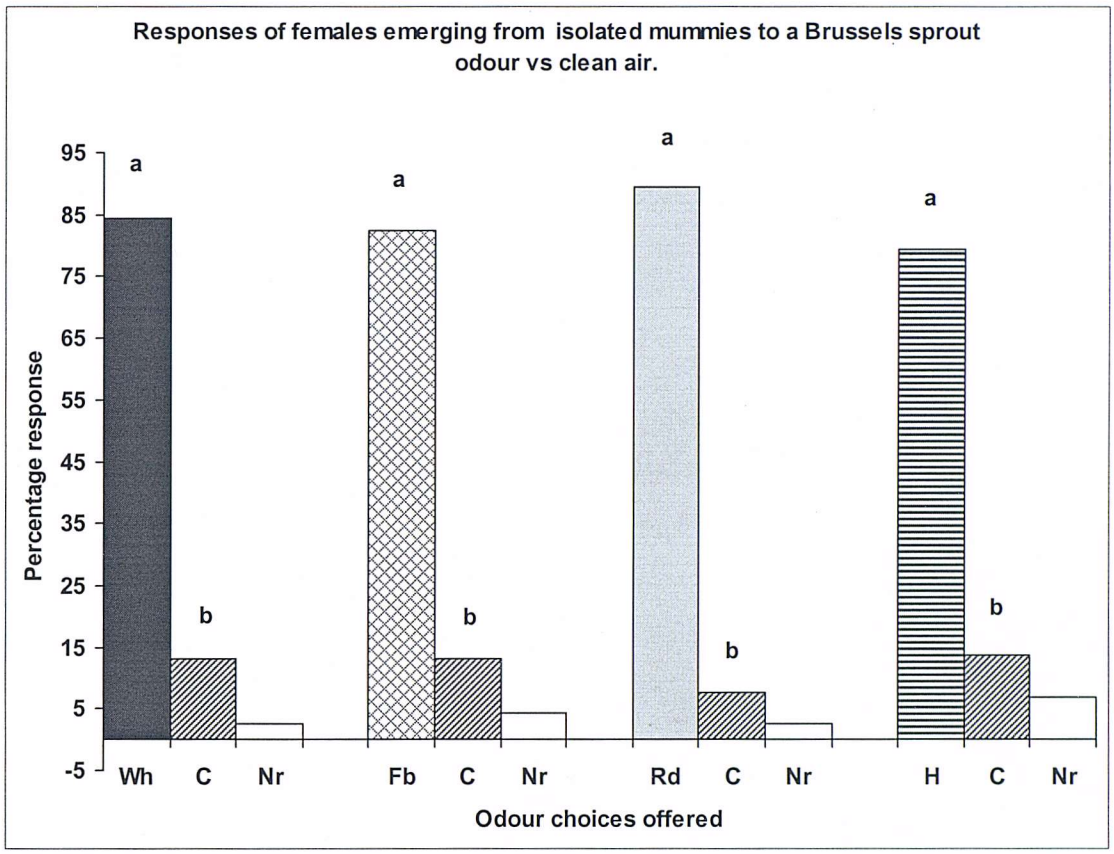



**Fig. 4.4** Percentage responses of mated females *Aphidius colemani* emerging from *Myzus persicae* mummies formed on Winter harvest and isolated in Petri dishes. Odours (from left to right): Wh: Winter harvest, C: Clean air, Nr: No response, Fb: Fillbasket, Rd: Red delicious and H: Hamlet. Means with different letter above the column are significantly different (comparison only within each odour test, i.e. variety versus Clean air, Table 4.4). n=8 replicates, 20 insects per replicate.

**Table 4.4** Responses of mated females *A. colemani* emerged in Petri dishes to odours from four Brussels sprout varieties. I= isolated mummies, emergence away from Winter harvest on which they were reared, 24-48 h old

| Parasitoid collection from | Odour leaf material | No. of (*n*) replicates | Totals being compared for 1df | Choice $\chi^2$ (1df) | Heterogeneity $\chi^2$ (n-1df) |
|---|---|---|---|---|---|
| I | Wh vs C | 8 | 135 vs 21 | 83.31 *** | 4.92 ns |
| I | Fb vs C | 8 | 132 vs 21 | 80.53 *** | 3.73 ns |
| I | Rd vs C | 8 | 143 vs 12 | 110.72 *** | 0.88 ns |
| I | H vs C | 8 | 127 vs 22 | 73.99 *** | 2.48 ns |

***P<0.001, ns:P>0.05

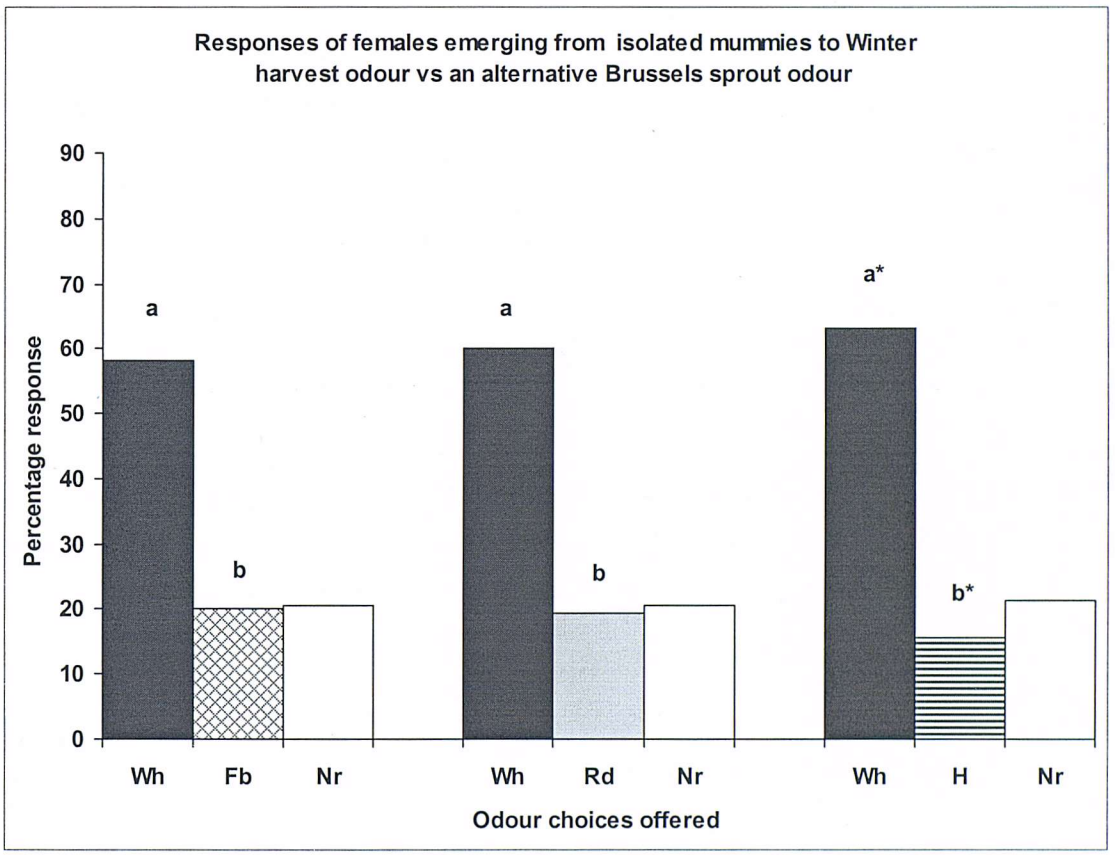



**Fig. 4.5** Percentage responses of mated females *Aphidius colemani* emerging from *Myzus persicae* mummies formed on Winter harvest and isolated in Petri dishes. Odours (from left to right): Wh: Winter harvest, Fb: Fillbasket, Nr: No response, Rd: Red delicious and H: Hamlet. Means with different letter above the column are significantly different (comparison only within each odour test, i.e. Winter harvest versus the alternative choice). a*:b* refer to significantly different means but with significant heterogeneity (Table 4.5). n=8 replicates, 20 insects per replicate.

**Table 4.5** Responses of mated females *A. colemani* emerged in Petri dishes to Winter harvest versus an alternative Brussels sprout variety. I= isolated mummies, emergence away from Winter harvest on which they were reared, 24-48 h old

| Parasitoid collection from | Odour leaf material | No. of (*n*) replicates | Totals being compared for 1df | Choice $\chi^2$ (1df) | Heterogeneity $\chi^2$ (n-1df) |
|---|---|---|---|---|---|
| I | Wh vs Fb | 8 | 93 vs 32 | 29.77 *** | 10.93 ns |
| I | Wh vs Rd | 8 | 96 vs 31 | 33.27 *** | 7.02 ns |
| I | Wh vs H | 8 | 101 vs 25 | 45.84 *** | 18.76 ** |
| I | Wh vs H[1] | 6 | 88 vs 12 | 57.76 *** | 6.84 ns |

**P<0.01, ***P<0.001, ns:P>0.05

[1]Alternative calculation, omitting 2 replicates where responses to the two odours (Winter harvest vs Hamlet) were equal (8 vs 8 and 5 vs 5, respectively).

The positive response to Winter harvest ranged between 58-63% but the mean percentage of non-responding individuals was coincidentally low and consistent (21% in all cases). When Winter harvest odour was tested against the other three Brussels-sprout varieties the parasitoids showed clear significant responses to Winter harvest except when tested against Hamlet (Fig. 4.5, Table 4.5). The response to the odour of Winter Harvest was higher than to that of Hamlet, but there was heterogeneity between runs. Inspection of the data showed that none of the replicates had a larger number of parasitoids responding to the odour of Hamlet. The cause of the heterogeneity was that, in two replicates, an equal number of parasitoids responded to both odours. The heterogeneity disappeared if these two replicates were omitted (Table 4.5, alternative calculation). In view of this, and the very large difference in the overall response to the two odours, the overall preference for the odour of Winter Harvest is considered valid.

#### *4.3.2.1 Further observations on naturally-emerging parasitoids isolated from plants*

Isolating aphid mummies from the plants should prevent the emerging adult females from exposure to plant volatiles and the possibility of learning cues through contact with the aphid hosts, their by-products and oviposition experience. However, it is interesting that oviposition attack responses were observed in the Petri dishes with the emerging females. No data were recorded but the females were showing a "stinging" behaviour towards whole mummies and possibly gaining experience by contacting the surface of the mummies. The female wasps were attacking the whole mummies in a similar way to attempts to insert eggs into the aphid-host (i.e. firstly

tapping the mummies with the antennae and then seemingly inserting or attempting to insert the ovipositor).

### 4.3.3 Responses of virgin male and female parasitoids after natural emergence from isolated mummies (emerging individually in glass vials)

When offered the odour of Winter harvest leaves versus clean air, both virgin male and female parasitoids demonstrate a significant response to the sprout plant odour (Figs 4.6, 4.7 and Tables 4.6, 4.7). Furthermore, when Winter harvest odour was tested against the other three Brussels-sprout odours/varieties significant parasitoid responses were recorded towards the sprout plant of origin (Winter harvest) for both males and females in all cases.

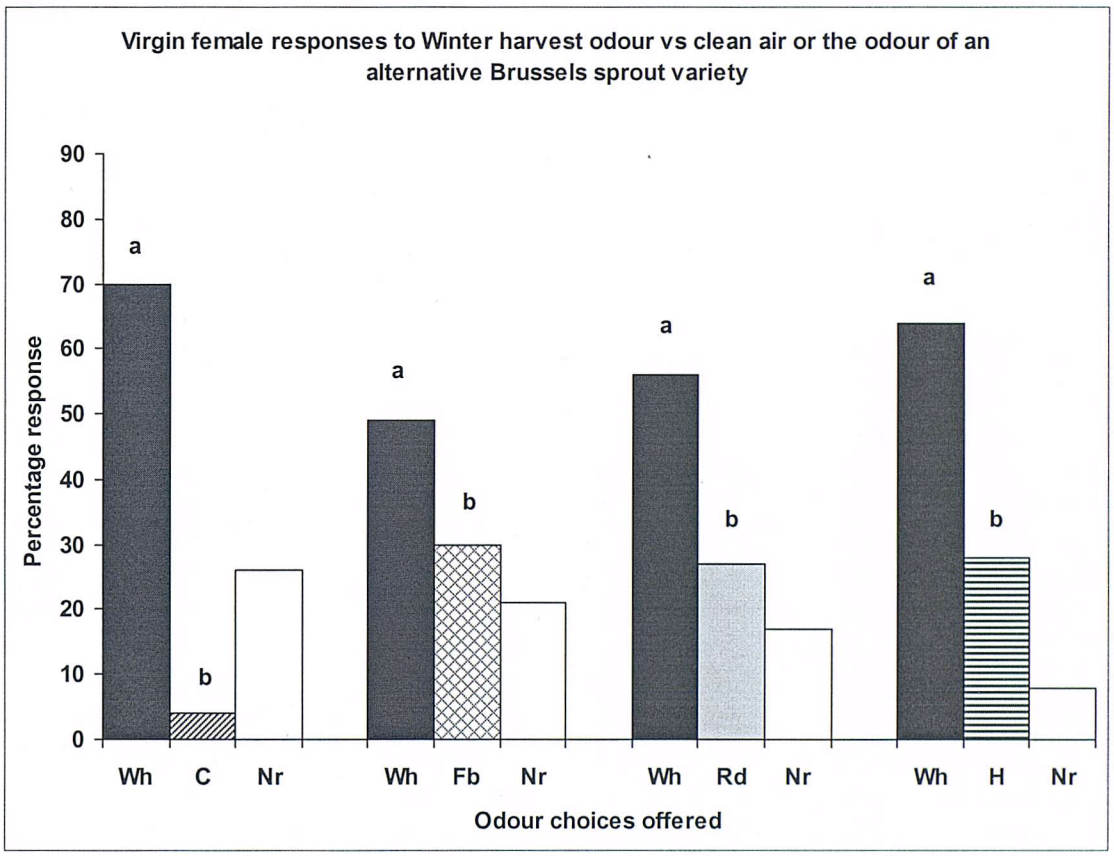



**Fig. 4.6** Percentage responses of virgin female *Aphidius colemani* emerging from *Myzus persicae* mummies formed on Winter harvest and isolated in stoppered glass tubes. Odours (from left to right): Wh: Winter harvest, C: Clean air, Fb: Fillbasket, Nr: No response, Rd: Red delicious and H: Hamlet. Means with different letter above the column are significantly different (comparison only within each odour test, i.e. Winter harvest versus the alternative choice) (Table 4.6). n=8 replicates, 20 insects per replicate.

**Table 4.6** Responses of *A. colemani* virgin females to different odours, i.e. mummies formed on Winter harvest, isolated in glass tubes, female parasitoids emerging individually and tested as virgins, 24-48 h old.

| Parasitoid condition | Odour leaf material | No. of (*n*) replicates | Totals being compared for 1df | Choice $\chi^2$ (1df) | Heterogeneity $\chi^2$ (n-1df) |
|---|---|---|---|---|---|
| virgin | Wh vs C | 8 | 114 vs 6 | 97.20 *** | 1.34 ns |
| virgin | Wh vs Fb | 8 | 80 vs 48 | 8.00 ** | 11.26 ns |
| virgin | Wh vs Rd | 8 | 90 vs 40 | 19.23 *** | 9.51 ns |
| virgin | Wh vs H | 8 | 106 vs 35 | 35.75 *** | 9.74 ns |

**P<0.01,***P<0.001, ns:P>0.05

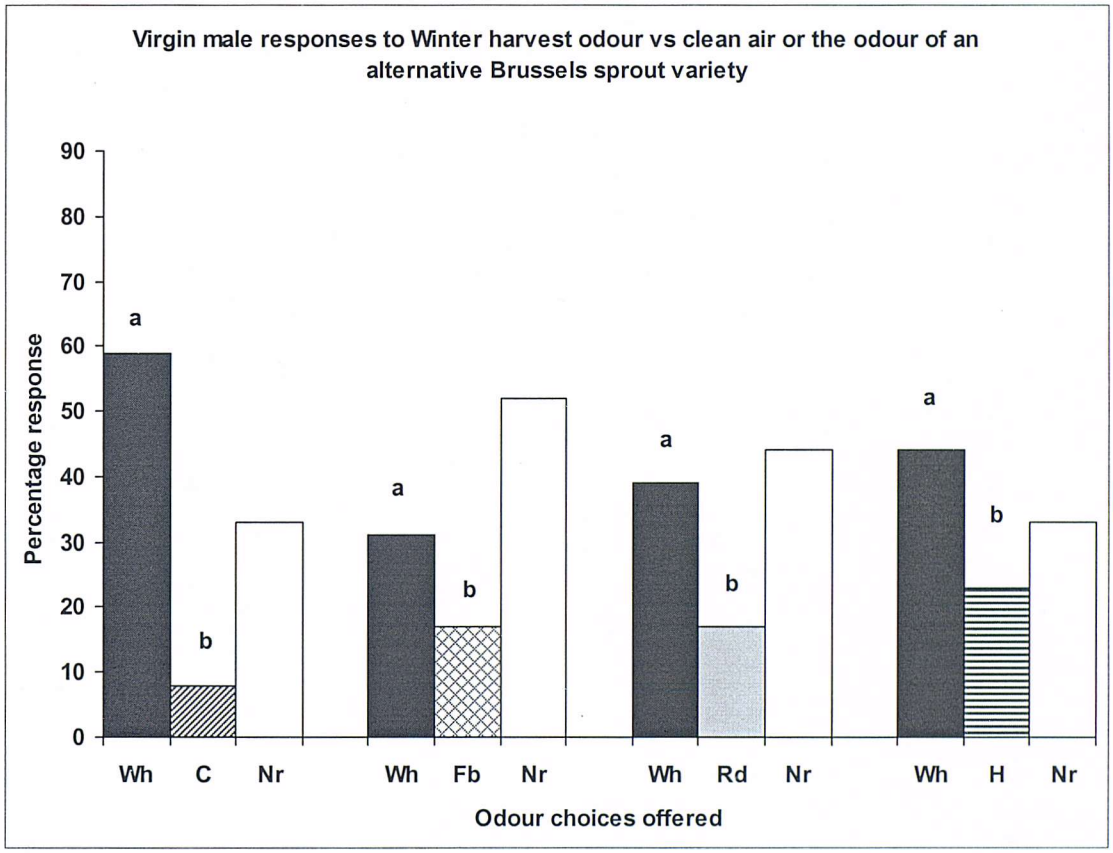



**Fig. 4.7** Percentage responses of virgin male *Aphidius colemani* emerging from *Myzus persicae* mummies formed on Winter harvest and isolated in stoppered glass tubes. Odours (from left to right): Wh: Winter harvest, C: Clean air, Fb: Fillbasket, Nr: No response, Rd: Red delicious and H: Hamlet. Means with different letter above the column are significantly different (comparison only within each odour test, i.e. Winter harvest versus the alternative choice) (Table 4.7). n=8 replicates, 20 insects per replicate.

**Table 4.7** Responses of *A. colemani* virgin males to different odours, i.e. mummies formed on Winter harvest, isolated in glass tubes, male parasitoids emerging individually and tested as virgins, 24-48 h old.

| Parasitoid condition | Odour leaf material | No. of (*n*) replicates | Totals being compared for 1df | Choice $\chi^2$ (1df) | Heterogeneity $\chi^2$ (n-1df) |
|---|---|---|---|---|---|
| virgin | Wh vs C | 8 | 94 vs 16 | 55.31 *** | 6.84 ns |
| virgin | Wh vs Fb | 8 | 53 vs 20 | 14.92 *** | 8.69 ns |
| virgin | Wh vs Rd | 8 | 59 vs 21 | 18.05 *** | 11.84 ns |
| virgin | Wh vs H | 8 | 71 vs 41 | 8.04 ** | 9.40 ns |

**P<0.01,***P<0.001, ns:P>0.05

On the whole, a greater percentage of virgin females responded (i.e. were found in the odour chambers; 74-92%) than virgin males (48-67%) although in all cases the wasps showed significant preference for the odour of Winter harvest (plant of origin) over clean air (P<0.001) or over the other sprout varieties (P<0.01; Figs 4.6, 4.7). Also, the total number of virgin females (n=160 – Table 4.6) and virgin males (n=160 - Table 4.7) responders versus non-responders significantly differed (P<0.001; Table 4.8) in all comparisons but one, i.e. when Winter harvest odour was tested against clean air.

**Table 4.8** Responses of *A. colemani* virgin males and females to different odours, i.e. mummies formed on Winter harvest, isolated in glass tubes, parasitoids emerging individually and tested as virgins, 24-48 h old.

| Parasitoid condition | No. of (*n*) replicates | Bioassays performed | Numbers of responders / non-responders | | | Total $\chi^2$ (1df) |
|---|---|---|---|---|---|---|
| | | | Females (♀) | vs | Males (♂) | |
| virgin | 8 | Wh vs C<br>Nr | 120<br>40 | vs | 110<br>50 | 1.55 ns |
| virgin | 8 | Wh vs Fb<br>Nr | 128<br>32 | vs | 73<br>87 | 40.47 *** |
| virgin | 8 | Wh vs Rd<br>Nr | 130<br>30 | vs | 80<br>80 | 34.63 *** |
| virgin | 8 | Wh vs H<br>Nr | 141<br>19 | vs | 112<br>48 | 15.88 *** |

***P<0.001, ns:P>0.05

Wh: Winter harvest, Fb: Fillbasket, Rd: Red delicious, H: Hamlet, Clean air.

### 4.3.4 Responses of mated female parasitoids emerging after excision of pupae from aphid mummies (emerging in Petri dishes)

Female parasitoids showed a significant preference for the plant odour of origin, Winter harvest, (Table 4.9) over all the other alternative odours except when Winter harvest was tested against Hamlet. In that case, not only were the choices not significant but the heterogeneity was also significant. Furthermore, in all of the comparisons in Fig. 4.8 the percentage of female non-responders ranged between 21 and 45%. The percentage of non-responders was similar to the percentage responding to the plant of origin (i.e. Winter harvest) when the latter was tested against Cabbage or Broad bean odour.

When adult females were obtained after excision of Winter harvest mummies and were given access to whole mummies formed on an alternative sprout variety (Red delicious) during the 24-h mating procedure, it was found that five mummies, but not one mummy, were enough to elicit significant change in parasitoid olfactory responses towards the sprout odour on which the mummies were formed (Fig. 4.9, Table 4.11). This was also true when mummies reared and collected from Red delicious plants and the excised parasitoids tested after given access to one and five whole mummies from Winter harvest plants (Fig. 4.10, Table 4.12). Although, the response to the odour of Winter harvest, on which the accessed mummies were formed, was significant in both bioassays performed in the case of adults that allowed access only to one mummy the result was associated with a significant heterogeneity and thus, it was not considered valid. In addition, a significant preference to the Red delicious odour was obtained from adult emerging naturally from isolated mummies formed on Red delicious and a preference for this sprout variety was also evident when adults emerging in isolation were tested against clean air. However, no preference was evident when the parasitoids were allowed to choose between two sprout odours (Red delicious and Winter harvest) after excision but had no contact with Winter harvest mummies.

#### *4.3.4.1 Further observations on female parasitoids emerging after pupal excision*

It was planned to test excised males for olfactory preference but before, during and after the bioassays it was observed that they were forming clusters in the central arena. It seemed that the males were trying to copulate with each other and as soon as two of them were engaged in this kind of interaction others joined. This was a major problem as few males entered the odour chambers while the majority of them remained in the central arena (in groups of 2-15 insects out of the total 20 wasps placed in the arena for a single test) showing little response or in most cases no response at all. Thus, no data are presented in the current study with excised males.

Newly-emerged female parasitoids from excised pupae exhibited oviposition attack responses (as previously noted towards isolated mummies) towards the excised but non-emerged pupae this time, which were present in the same Petri dish.

The total number of naturally-emerged females (n=160 - Tables 4.4, 4.5) and females emerged after excision of pupae (n=160 - Table 4.9) responders versus non-responders significantly differed ($P<0.05$; Table 4.10) in all comparisons despite the fact that when Winter harvest odour tested against Hamlet odour in both cases (Tables 4.5, 4.8) the data showed that the heterogeneity $\chi^2$ was significant (i.e. inconsistent responses between replicates). Also, naturally-emerged female wasps showed a greater percentage response (i.e. 79-97% were found in the odour chambers) than females emerged after excision of pupae (68-79%) although in all cases the wasps showed significant preference for the odour of Winter harvest (plant of origin) over clean air ($P<0.001$) or over the other three sprout varieties ($P<0.05$; Figs 4.4, 4.5 and 4.8).

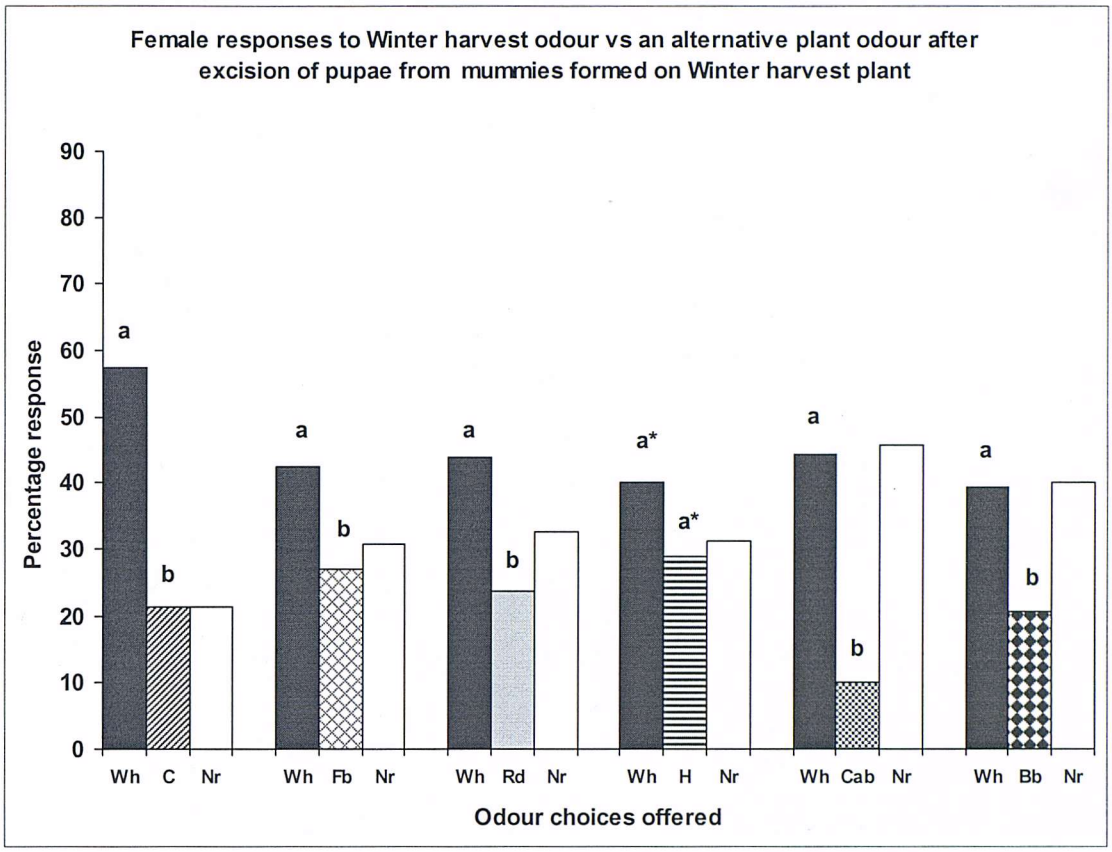



**Fig. 4.8** Percentage response of excised, mated females *Aphidius colemani* emerging after removal of pupae from *Myzus persicae* mummies formed on Winter harvest plants and isolated. Odours (from left to right): Wh: Winter harvest, C: Clean air, Nr: No response, Rd: Red delicious, H: Hamlet, Cab: Cabbage, Bb: Broad bean. Means with different letter above the column are significantly different (comparison only within each odour test, i.e. Winter harvest versus the alternative choice). a*:b* refer to significantly different means but with significant heterogeneity (Table 4.9). n=8 replicates in each case, 20 insects per replicate.

**Table 4.9** Responses of excised, mated females *A. colemani* to plant odours. E= excised parasitoids (pupa removed from mummy case) emerging in Petri dishes away from Winter harvest on which they were reared, 24-48 h old

| Parasitoid collection from | Odour leaf material | No. of (*n*) replicates | Totals being compared for 1df | Choice $\chi^2$ (1df) | | Heterogeneity $\chi^2$ (n-1df) | |
|---|---|---|---|---|---|---|---|
| E | Wh vs C | 8 | 92 vs 34 | 26.70 | *** | 10.71 | ns |
| E | Wh vs Fb | 8 | 68 vs 43 | 5.63 | * | 14.00 | ns |
| E | Wh vs Rd | 8 | 70 vs 38 | 9.48 | ** | 3.92 | ns |
| E | Wh vs H | 8 | 64 vs 46 | 2.95 | ns | 31.33 | *** |
| E | Wh vs Cab | 8 | 71 vs 16 | 34.77 | *** | 3.96 | ns |
| E | Wh vs Bb | 8 | 63 vs 33 | 9.38 | ** | 4.58 | ns |

*P<0.05, **P<0.01, ***P<0.001, ns:P>0.05

**Table 4.10** Comparison of total mated *A. colemani* females responding in the olfactometer versus the non-responders (Nr). I = isolated mummies, emergence in Petri dishes away from Winter harvest on which they were reared, 24-48 h old. E= excised parasitoids (pupa removed from mummy case) emerging in Petri dishes away from Winter harvest on which they were reared, 24-48 h old.

| Parasitoid collection from | No. of (*n*) replicates | Bioassays performed | Numbers of responders / non-responders | | | Total $\chi^2$ (1df) | |
|---|---|---|---|---|---|---|---|
| | | | Isolated | vs | Excised | | |
| I or E | 8 | Wh vs C | 156 | vs | 126 | 26.88 | *** |
| | | Nr | 4 | | 34 | | |
| I or E | 8 | Wh vs Fb | 125 | vs | 111 | 8.68 | ** |
| | | Nr | 35 | | 59 | | |
| I or E | 8 | Wh vs Rd | 127 | vs | 108 | 5.79 | * |
| | | Nr | 33 | | 52 | | |
| I or E | 8 | Wh vs H | 126 | vs | 110 | 4.14 | * |
| | | Nr | 34 | | 50 | | |

*P<0.05, **P<0.01,***P<0.001, ns:P>0.05

Wh: Winter harvest, C: Clean air, Fb: Fillbasket, Rd: Red delicious, H: Hamlet.

Furthermore, the mating condition (mated or virgin) made no difference to the numbers of females responding in the bioassays. This became apparent when a comparison on the overall numbers of responders and non-responders (i.e. adding the numbers in the four dual-choice bioassays, Wh vs C, Wh vs Fb, Wh vs Rd and Wh vs

H) of mated female parasitoids (534 and 106, Table 4.10) with the total number of virgin females (519 and 121, Table ), revealed that the distribution was not significantly different ($\chi^2$=0.18, for 1 d.f., P>0.05).

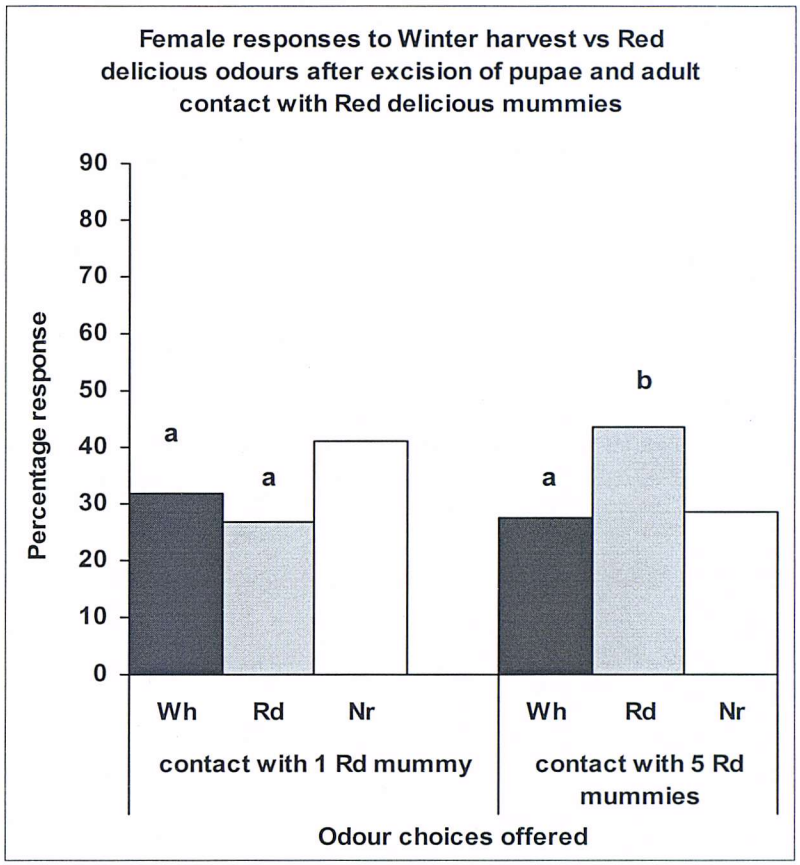



**Fig. 4.9** Percentage response of excised females *Aphidius colemani* emerging from *Myzus persicae* mummies formed on Winter harvest plants and isolated in Petri dishes. Odours (from left to right): Wh: Winter harvest, Rd: Red delicious, Nr: No response. Means with different letter above the column are significantly different (comparison only within each odour test, i.e. Winter harvest versus the alternative choice) (Table 4.11). n=8 replicates in each case, 20 insects per replicate.

**Table 4.11** Responses of excised, mated females *A. colemani* to Brussels sprout varieties. EC= parasitoids emerging away from Winter harvest plants on which they were reared, 24-48 h old, but allowed contact for 24 h with mummies formed on Red delicious, (1) and (5) mummies respectively.

| Parasitoid collection from | Odour leaf material | No. of (*n*) replicates | Totals being compared for 1df | Choice $\chi^2$ (1df) | Heterogeneity $\chi^2$ (n-1df) |
|---|---|---|---|---|---|
| EC | Wh vs Rd(1) | 8 | 51 vs 43 | 0.68 ns | 12.33 ns |
| EC | Wh vs Rd(5) | 8 | 44 vs 70 | 5.93 * | 1.79 ns |

*P<0.05, ns:P>0.05

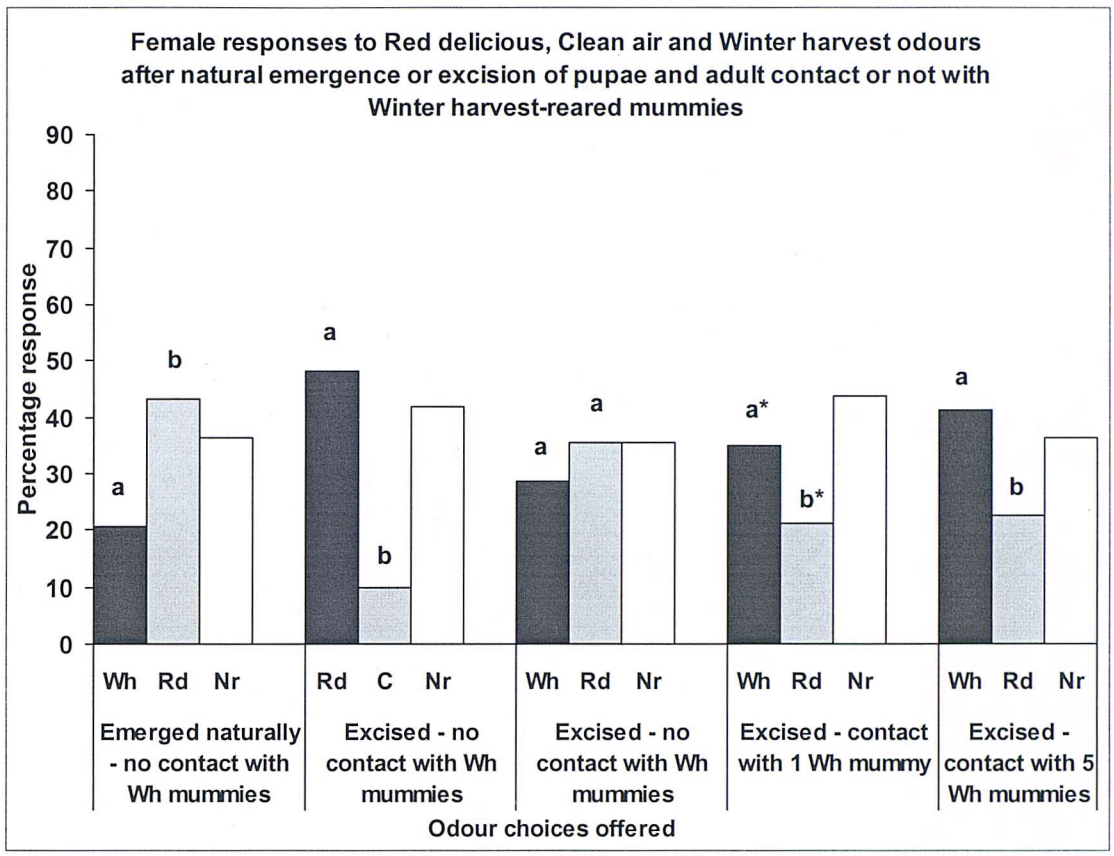



**Fig. 4.10** Percentage response of naturally emerged or excised females *Aphidius colemani* emerging from *Myzus persicae* mummies formed on Red delicious plants and isolated in Petri dishes. Odours (from left to right): Wh: Winter harvest, Rd: Red delicious, Nr: No response, C: Clean air. Means with different letter above the column are significantly different (comparison only within each odour test, i.e. Winter harvest versus the alternative choice). a*:b* refer to significantly different means but with the heterogeneity also significant (Table 4.12). n=8 replicates in each case, 20 insects per replicate.

**Table 4.12** Responses of excised, mated females *A. colemani* to Brussels sprout varieties. I-HPC:Rd = isolated mummies, emergence away from *Myzus persicae*-Red delicious complex on which they were reared, 24-48 h old, EC-HPC:Rd= parasitoids, 24-48 h old, emerging after excision of pupae from Red delicious-reared mummies and contact or not for 24 h with mummies formed on Winter harvest, (1) and (5) mummies respectively.

| Parasitoid collection from | Odour leaf material | No. of (*n*) replicates | Totals being compared for 1df | Choice $\chi^2$ (1df) | Heterogeneity $\chi^2$ (n-1df) |
|---|---|---|---|---|---|
| I-HPC:Rd | Wh vs Rd | 8 | 33 vs 69 | 12.71 *** | 7.41 ns |
| I-HPC:Rd | Rd vs C | 8 | 77 vs 16 | 40.01 *** | 3.40 ns |
| EC-HPC:Rd | Wh vs Rd | 8 | 46 vs 57 | 1.17 ns | 4.33 ns |
| EC-HPC:Rd | Wh(1) vs Rd | 8 | 56 vs 38 | 3.45 ns | 18.14 * |
| EC-HPC:Rd | Wh(5) vs Rd | 8 | 66 vs 36 | 8.82 ** | 7.07 ns |

*P<0.05, **P<0.01,***P<0.001, ns:P>0.05

## 4.4 Discussion

### 4.4.1 Responses of mated female parasitoids after natural emergence (culture-collected adults)

Parasitoids collected directly from the culture showed a clear preference to any sprout/plant odour tested against clean air (Winter harvest, Red delicious, Cabbage and Broad bean respectively), simply confirming the response patterns observed in chapter 3 using the earlier olfactometer design (Plastic arena) and similar methodology. Also, *Aphidius colemani* parasitoids showed a preference to the sprout odour (Winter harvest or Red delicious) on which their aphid-hosts and mummies developed and thus, the influence of the sprout variety of origin on their olfactory responses became apparent. Olfactometer tests revealed that behavioural responses of another aphid parasitoid, *Aphidius rhopalosiphi*, can be influenced by the particular cultivar of wheat on which its aphid host, *Rhopalosiphum padi*, was feeding (Wickremasinghe & van Emden, 1992). In another study with the same system it appeared that there was a critical point in time (after parasitisation and prior mummification) depending on the day of development on a wheat variety where the larvae developing in parasitised living aphid hosts can learn (or not) the chemistry of the alternative cultivar on which they have been transferred. This critical point was 6-8 days under glasshouse conditions where pupation of the female parasitoid took place

between 7 and 8 days. Observations on adult parasitoids, after natural emergence, demonstrated the fine mechanisms involved in switches of parasitoid behaviour (van Emden *et al.*, 1996). In addition, from mummy formation to natural emergence, odours from the surrounding environment can be absorbed on the cuticle of the mummy and play a major role in influencing the adult parasitoid olfactory responses since the wasps can experience chemical cues present when biting their way out of the mummy cuticle (van Emden *et al.*, 2002). Furthermore, in a comparison between two different tritrophic systems (*Aphidius ervi – Acyrthosiphon pisum* on alfalfa or *Sitobion avenae* on wheat) it was shown that an initial preference for the host-plant complex on which they were reared was not apparent after an oviposition experience was offered on a different host-plant complex. The latter oviposition experience had overridden the rearing experience (positive responses shown in a wind tunnel as oriented flights) (Daza-Bustamante *et al.*, 2002).

### 4.4.2 Natural emergence – mated females (emerging in Petri dishes)

Adults emerging from mummies removed from the plant also showed a clear preference to any of the Brussels-sprout variety/odour offered versus clean air. It must be noted, that the percentage of non responders was below 7% in all comparisons against clean air showing consistency and demonstrating the efficiency of the methodology used. Moreover, the adult females emerging from mummies in Petri dishes showed similar response patterns to those in the previous chapter (section 3.2.3) obtained after collection as adults from the culture cages using olfactometer 1 (Plastic arena). The data collected (section 4.2.3) revealed that parasitoid preferences appear once more dependent on the rearing history of their aphid hosts. In all the comparisons between the four Brussels-sprout odours/varieties the female parasitoid preferred the odour of host's food plant (Winter harvest) in agreement with previous findings on the olfactory responses of *A. colemani* mated females to other Brassica plants (Kalule & Wright, 2004) following similar methodology of mummy isolation in glass vials.

The oviposition attacks by adult females on whole mummies observed in Petri dishes were unexpected but were observed towards yellow and dark coloured mummies. As far the author is aware of, there are no previous reports of this in the literature. However, it is known that visual cues can influence the oviposition behaviour of aphid parasitoids even prior to physical contact (Michaud & Mackauer, 1994; Battaglia *et al.*, 1995). Considering that yellow pigment can evoke oviposition attack responses by *Aphidius ervi* parasitoids (Powell *et al.*, 1998; Battaglia *et al.*, 2000) seems to be not surprising that similar oviposition attacks observed in the present

study as the parasitoid may respond to the yellow and dark coloured (brown or black) whole mummies (Fig. 4.1, section 4.2.4). For example, the specialist parasitoid *Aphidius rhopalosiphi* demonstrated an ovipositional attacking preference to yellow over brown *Sitobion avenae* aphid hosts (Ankersmit *et al.*, 1986) and *Aphidius ervi* females showed an ovipositional attacking preference to green over brown *Acyrthosiphon pisum* aphid hosts (Powell *et al.*, 1998).

Another possible explanation might be that experience of the surface of the mummy alone could elicit parasitoid responses since it could contain chemical cues (secondary plant chemicals for example) derived by the feeding of the aphid-host on the plant of origin or the volatiles present during mummification and thus facilitating the learning process by the emerging adult wasps. Indeed, there is evidence of learning at the time of emergence with cues encountered on the mummy's cuticle which could induce host-plant preference. Experience of the mummy case during/after emergence is sufficient to evoke significant responses of the female parasitoids to the odour of Winter harvest (plant of origin). Experience could be gained not only while cutting their way out during emergence but also after contact with the mummy's cuticle after emergence (van Emden *et al.*, 1996; Storeck *et al.*, 2000). It was suggested that tomato volatiles, from neighbouring plants, had been absorbed on the surface of the mummy and encountered by the emerging adults influencing subsequent parasitoid preferences (van Emden *et al.*, 2002). It has also been shown that moulted skins of the host *Acyrthosiphon pisum* elicited positive oviposition attack responses by *Aphidius ervi* parasitoid wasps for up to ten days providing evidence of contact ovipositional kairomone(s) on the aphid skin (Battaglia *et al.*, 2000) and it has been demonstrated that this parasitoid showed similar responses to contact kairomones in the host cuticle (Powell *et al.*, 1998). The observed antennal tapping on mummies in the current study may facilitate the detection of contact kairomones from mummy cuticle involved in parasitoid olfactory responses the same way these cues are detected from the cuticle of moulted aphid (Battaglia *et al.*, 2000). In another study involving *Aphidius colemani* wasps, it was suggested that cues derived from the aphid cuticle were not volatile and the wasp needed to be close for the recognition, probably via antennal contact chemoreceptors (Le Ralec *et al.*, 2005).

### 4.4.3 Responses of virgin male and female parasitoids after natural emergence from isolated mummies (emerging individually in glass vials)

The majority of experiments in the present thesis involved mated parasitoids. However, both virgin male and female parasitoid preferences were also studied in this chapter despite the intriguing result for males obtained previously in chapter 3 in a 4-

way protocol (section 3.3.1.2). In the present study, the results obtained in a 2-way protocol showed that both virgin male and female wasps exhibited a significant preference towards Winter harvest odour (the plant of origin) than to an odour from another sprout variety or clean air in all dual-choice bioassays performed in the olfactometer indicating the influence of the plant of origin on their olfactory preferences overall but females seemed to exhibit greater percentage responses than males. However, virgin parasitoids of either sex could respond similarly to plant odours against clean air. Though significantly more females than males responded to sprout odours, indicating a greater searching efficiency in females than males, as found in another study in which mated females and males were used (van Emden *et al.*, 2002).

Virgin and mated aphid parasitoids such as *Aphidius smithi* Sharma and Subba Rao, *Ephedrus californicus* Baker, *Monoctonus paulensis* (Ashmead), *Praon pequodorum* Viereck, and *Lysiphlebus testaceipes* Cresson may have differences in their foraging behaviour and it is known that the mating status appears to have an influence on their attacking and parasitisation rate (Michaud, 1994). Inexperienced virgin *Habrobracon hebetor* (Say) female wasps which parasitise lepidopteran *Ephestia kuehniella* Zeller larvae showed no responses but mated females demonstrated oriented flights in wind tunnel bioassays towards host frass while an oviposition or a contact reward increased subsequent responses of both virgin and mated wasps (Parra *et al.*, 1996). Host-searching behaviour is intensive in female hymenopteran parasitoids that carry a high egg load (Minkenberg *et al.*, 1992) and could be mediated through mating which can serve as a prophylactic mechanism from unfertilised egg depletion in virgin females *Leptopilina boulardi* (Barbotin, Carton & Kelner-Pillaut) and it has shown that mating itself or combined with an oviposition reward could not affect the percentage of females learning of a specific odour (Perez-Maluf & Kaiser, 1998). However, in the present study while a reward (oviposition or contact) was not utilized, the statistical analysis on the overall combined data between isolated but mated female and virgin female parasitoids revealed no significant difference in their responses indicating that mating status did not play a major role in the olfactory behaviour of this parasitoid species.

### 4.4.4 Responses of mated female parasitoids emerging after excision of pupae from aphid mummies (emerging in Petri dishes).

Female parasitoids were allowed to emerge from excised pupae (i.e. after removal of the late-stage pupa from the mummy cuticle) aiming to exclude any chemical information that could be learnt during the latter stages of pupal development and by the adult cutting its way out from the mummy cuticle and influence adults'

behavioural responses (van Emden *et al.*, 1996; Storeck *et al.*, 2000; van Emden *et al.*, 2002; Douloumpaka & van Emden, 2003). The goal was to investigate to what extent chemical information present in the aphid mummy could be obtained during larval/pupal development within the mummy and subsequently affect adult behaviour eliciting positive responses to odours of Brussels sprout on which the herbivore host was feeding.

In the present study, for mummies formed on the Winter harvest variety the data provided evidence of conditioning of parasitoid behaviour prior to adult eclosion as females emerging from excised pupae showed similar response patterns to those obtained after natural emergence. It is possible that chemical information was retained somehow in the parasitoid pupa and this is enough to elicit a positive response to the odour of the plant of origin. Thus it might be that the parasitoid's behaviour could be conditioned during its development, i.e. during the larval growth in the aphid host up to late-stage pupa, without contact with host/plant cues as an adult (Poppy *et al.*, 1997). However, excision seemed to have an effect on behaviour of Red delicious-reared parasitoids and changed a preference for the plant of origin in naturally-emerged females to a random choice in those emerging from excised pupae (Fig. 4.10, first and third histograms from the left).

None of the previous studies using excision of parasitoid pupae from aphid mummies (van Emden *et al.*, 1996; Storeck *et al.*, 2000; van Emden *et al.*, 2002; van Emden & Kifle, 2002; Douloumpaka & van Emden, 2003) investigated the behaviour of adult parasitoids in olfactory tests comparing plant odours and clean (purified) air. Nevertheless, studies using other olfactometer systems but naturally-emerging wasps have shown that parasitoids respond significantly to odours emanating from non-host plants compared to clean air (Takabayashi *et al.*, 1991). The fact that adult parasitoids, obtained after excision of aphid mummies, continue to show positive, and perhaps innate, responses to various plant odours over clean air confirmed the persistence of this behavioural pattern observed also in the previous chapter for naturally-emerged parasitoids.

The responses recorded after excised, inexperienced parasitoids were allowed access to whole mummies of different sprout of origin, in the current study, simply demonstrate the extent of learning ability of *A. colemani* female parasitoids. The adult parasitoids, which were obtained from Winter harvest or Red delicious-formed mummies after excision, failed to demonstrate positive olfactory responses to Winter harvest odour over Red delicious odour and vice versa when the wasps were given experience of one whole mummy of the alternative origin (Figs 4.9, 4.10 and Tables 4.11, 4.12). Thus, a single mummy seemed to be a weak stimulus i.e. not enough to elicit a significant preference. Although, adult female responses were not significant

there was a change of the behavioural response (Fig. 4.9, Table 4.11) in that a previously recorded significant response to Winter harvest odour (Fig. 4.8, Table 4.9) became non-significant after contact with one Red delicious-formed mummy (when pupae were excised from Winter harvest-formed mummies). However, when a stronger stimulus was provided (i.e. five mummies) it was enough to evoke significant responses to the odour matching the latest contact experience prior the bioassays conducted (Figs 4.9, 4.10 and Tables 4.11, 4.12). The remarkable capacity of learning in parasitic wasps has been demonstrated in several studies (Papaj & Prokopy, 1989; Vet & Groenewold, 1990; Poppy *et al.*, 1997; Battaglia *et al.*, 2000; van Emden *et al.*, 2002).

# CHAPTER 5: Parasitoid responses after emergence from artificial diet-reared hosts

## 5.1 Introduction

A chemically-defined synthetic diet was developed by (Dadd & Mittler, 1966) aiming for optimal development and maintenance of *Myzus persicae* although it did not fully replicate the balance of compounds in the phloem sap of host plants (Mittler, 1972). In the present study, this same diet was used to continue an original *M. persicae* culture started from a single aphid 9th February 1976 and run continuously on artificial diet from that date (van Emden, personal communication, 2003). van Emden *et al.* (1996) had suggested that this model system could be used in behavioural studies with natural enemies since aphids devoid of specific secondary plant chemistry (compared with aphids feeding on plant leaves) could be obtained.

Although larger aphids might represent better quality hosts, they are better able to defend themselves and Aphidiidae parasitoids tend to favour 2nd and 3rd aphid instars for oviposition. Thus, fewer mummies are formed when older, larger aphids are exposed to parasitoids (Starý, 1988). Female *Aphidius colemani* prefer to oviposit in 1st-instar *M. persicae* reared on eggplants (*Solanum melongena* (L.) cv. Bonica) and this results in mummification during the 4th larval instar or adult stage (Perdikis *et al.*, 2004) but they can show a preference for large *Aphis glycines* (Matsumura) on soybean plants (*Glysine max* (L.) Merrill) (Lin & Ives, 2003).

Diet-reared aphids have a slow growth rate and are smaller when compared, instar to instar, with insects feeding on plants in the same environmental conditions (van Emden, 1988; van Emden & Andrews, 1997), and parasitoid size is directly related to host aphid size (van Emden, 1995; Chau & Mackauer, 2001). It has been demonstrated that oviposition by *A. ervi* in intermediate nymphal stages (2nd, 3rd and 4th instars) of *M. persicae* provide the best percentage parasitisation when the aphid hosts were feeding on artificial diet (Colinet *et al.*, 2005). Diet-reared *M. persicae* reach 3rd and 4th instar between the 6th and 8th day of development (van Emden & Kifle, 2002) and these stages were mostly used for parasitisation on the artificial diet in the present study. They were regarded as approximately equivalent in size to 2nd and 3rd instars developing on plants. In addition, parasitoids ovipositing in small aphids feeding on plants tend to produce a male-biased sex ratio (Godfray, 1994; Quicke, 1997) and this is also true for small diet-reared aphid instars (van Emden & Kifle, 2002). However, it

was shown that the parasitoids emerging from diet-reared aphids (2nd, 3rd and 4th instars) results in c. 1:1 ratio of males and females (Douloumpaka, 2000).

In this chapter the olfactory response of female wasps was investigated after emergence from aphid hosts reared on chemically-defined artificial diet. The hypothesis was that the wasps should not differentiate between plant odours offered in the olfactometer as the diet was considered to have no associated plant odour and consisted of mainly sucrose, amino acids and vitamins without any specific secondary plant chemistry (e.g. glucosinolates). It should be noted that in order to get parasitoid offspring from diet-reared aphids, their mothers (or grandmothers) were collected from the culture of *M. persicae* on Brussels sprout, variety Winter harvest.

## 5.2 Materials and Methods

Prior to conducting bioassays using wasps emerging from artificial diet-reared aphids, the duration of the host life cycle (i.e. birth to reproducing adults) was determined in Fisons' environmental cabinets set at 20±1°C and LD 16:8 (sections, 2.6.2, 2.8). For this purpose, approximately seventy adult diet-reared aphids were placed on fresh diet sachets and left for 24 hours to obtain 1st instar offspring. The 1st instar larvae were removed and placed in groups of 10 on five diet sachets (50 1st instars in total) and that day was considered as the 1st day of development. These aphids were reared (section 2.8) and observed daily, while the diet was changed every two days, up to the point they became adult and started to reproduce. The data are shown in Fig. .

The 1st offspring were observed on the 9th day of development while the majority (66%) of the insects tested completed their life cycle during the 10th and 11th day inclusive (Fig. 5.1). Smaller percentages (12%, 14%, 6%) were recorded on 9th, 12th and 13th day respectively, though all of them had reproduced by day 14 (2%). In view of these results, and also the method of culturing aphids on diet (i.e. changing of sachets every two days, section 2.8), the protocol for rearing parasitoids from diet-reared hosts began with oviposition in aphids aged between days 6 and 8, a period which corresponded to 3rd and 4th instar larvae (van Emden & Kifle, 2002).

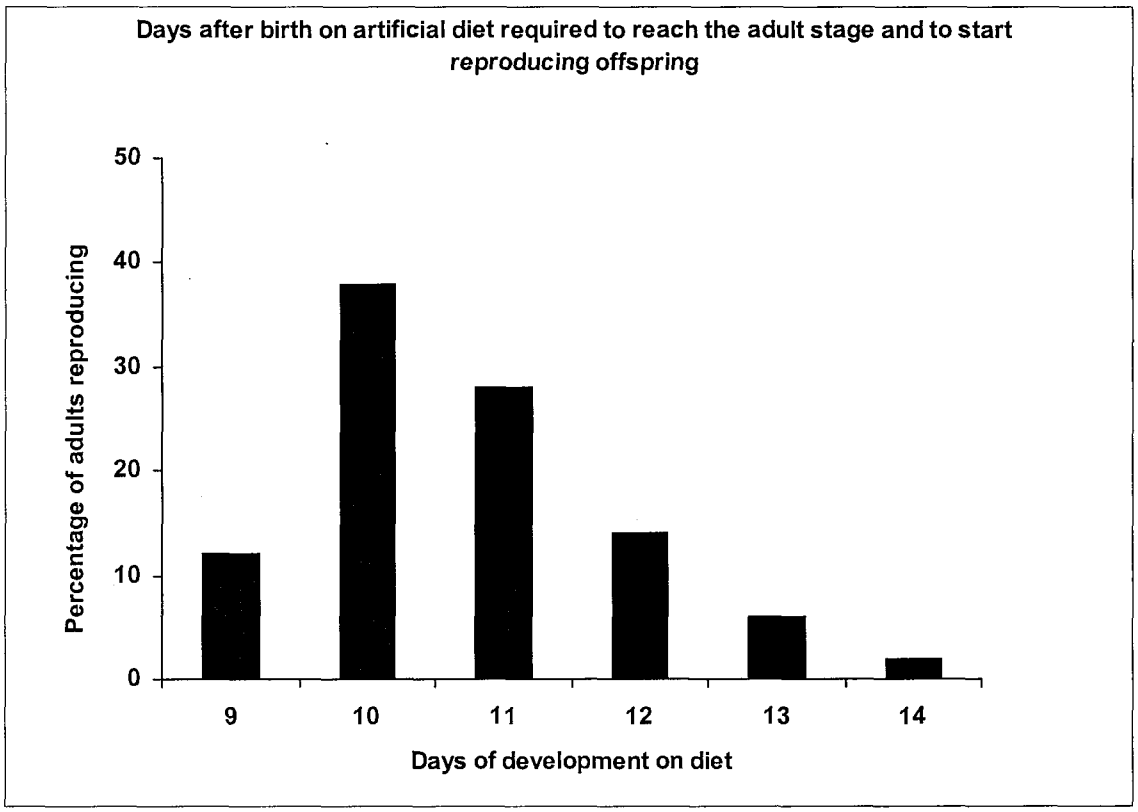



**Fig. 5.1** Percentage of aphids commencing reproduction versus time after birth (n=50).

The procedure developed to obtain 3rd and 4th instars involved several steps. Initially adult diet-reared aphids were transferred from the stock culture to four fresh diet sachets (70-90 in each one) and left to produce offspring for 2 days. The adults were then removed and transferred to 4 fresh diet sachets to continue reproduction. . The old diet sachet with the newly-produced larvae (0-2 days old) was then drained and replaced with a fresh diet sachet, as usual, and the aphid larvae allowed to feed over the next two-day period until the next diet-sachet replacement (section 2.8). Four fresh sachets were used every two days to start a new culture of instars until parasitisation was initiated (Fig. 5.2).

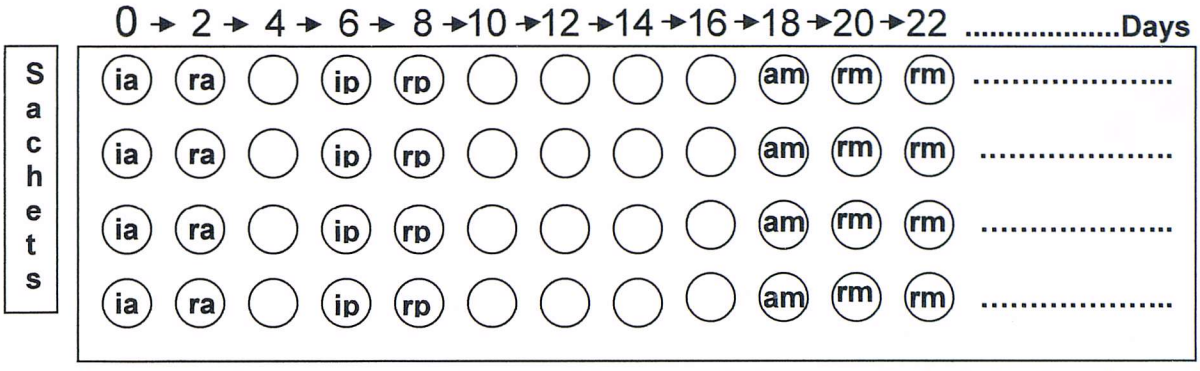



**Fig. 5.2** Schematic representation of 4 diet sachets manipulation/culturing along with parasitisation as well as mummies formation/collection with respect to time (days). ia: introduction of adult aphids, ra: removal of adult aphids, ip: introduction of adult wasps, rp: removal of adult wasp, am: mummies starting to appear, rm: removal of mummies.

After the third change of sachet (i.e. 6-8 days) the insects had reached the appropriate size/stage for parasitisation. One mated (section 2.6.4) female wasp (mother), collected from the culture, was introduced and the old sachet was immediately covered and sealed with a fresh one entrapping the parasitoid with the aphids inside. The old sachet was then drained and the wasps were left to continue oviposition until the next diet-sachet change (48 h later) when they were discarded. The culturing of the parasitised aphids was continued and the first appearance of mummies occurred 10-12 days later (Figs 5.2, 5.3).

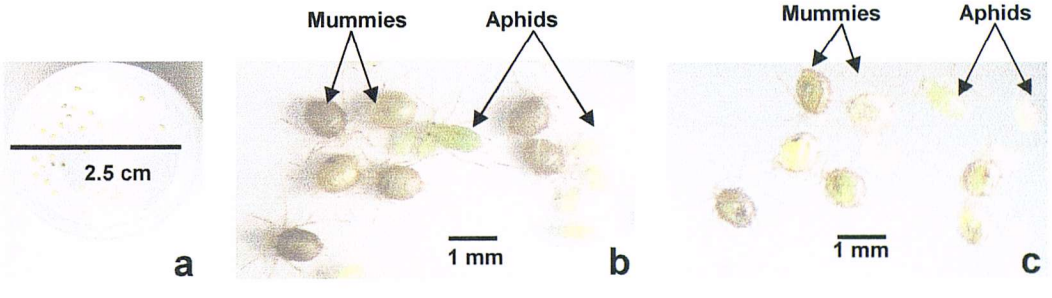



**Fig. 5.3** Development of diet-reared aphids into mummies attached on Parafilm M® – a,b,c photographs taken after draining of diet, a) diet sachet with mummies formed on Parafilm M® (ventral view), b) dorsal and c) ventral view of aphid mummies and live aphids developing on diet (microscope: WILD Heerbrugg, Switzerland, M5-71576 - digital camera: Olympus C730UZ).

The mummies formed on the Parafilm M® of the diet sachet were removed using a soft paintbrush, dipped in distilled water, to detach them. They were placed on damp filter paper in 5.5-cm-diameter Petri dishes pending emergence (section 2.6.2). It should be noted that the mummies were not collected upon initial appearance but 2-4

days later when the mummified cuticle had hardened and the parasitoid had pupated. It was convenient to collect the mummies when changing the diet sachets as they remained attached to the old diet sachet while the other host aphids move upwards to feed on the fresh diet sachet.

Mummy formation continued for up to sixteen days after they were first seen in the Perspex rearing cylinder. The harvesting was continuous until no further mummies were observed over an additional 6-day period (i.e. three consecutive changes of the diet). Any aphids that survived on the sachets, after the collection of the last mummy, were used again with the same culturing and parasitisation method. Approximately 60-80 diet sachets (i.e. 15-20 days) were required to complete a parasitisation and mummy-harvesting cycle on the diet for the four sachets with which the procedure started initially. Furthermore, at the same time that the procedure of obtaining parasitoids was initiated, the numbers of a) aphids offered for parasitisation, b) mummies harvested, c) emerged parasitoids and d) the sex of the emerging adults were recorded (Fig. 5.4) to determine the resources required.

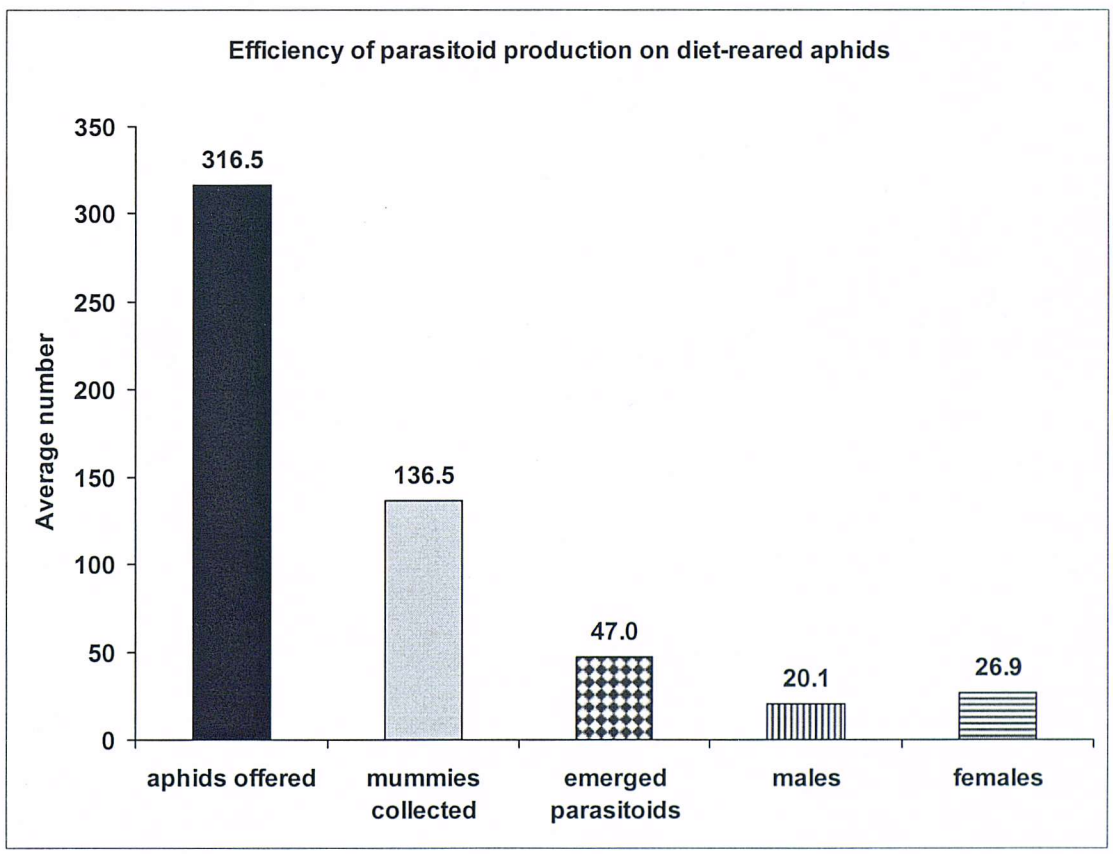



**Fig. 5.4** Average number of aphids needed to obtain enough female wasps for 2 olfactometer bioassays (n=20 rearings).

Data accumulated from 20 rearings, each comprising four sachets with 70-90 aphids suitable for parasitisation aphid instars resulted in 6330 aphids being offered for

parasitisation with almost half (2730) of them becoming mummified. Only 939 parasitoids emerged (from 34.4% of the collected mummies) with 402 males and 537 females.

Overall, the culturing method for parasitoids on artificial-diet aphids was successful as long as the diet aphid culture appeared healthy. Changing to fresh diet every 2 days provided optimal conditions as assessed by the following protocol. Two hundred aphids (4$^{th}$ instar) were allowed to feed on old diet (i.e. replaced every 2 days but left for 2 days after removal from the freezer at 20±1 °C and LD 16:8) and all died within twenty days with few progeny born (Fig 5.5). In a separate culture with ten 4$^{th}$ instars simultaneously initiated but on fresh diet (i.e. replaced every 2 days but immediately after thawing from the freezer) resulted in the production of more than one hundred aphids in twenty days. The results presented illustrate the importance of changing and replacing the diet with fresh sachets consistently at short time intervals to maintain the diet culture in good condition and a two-day period was adopted for the purpose of current study.

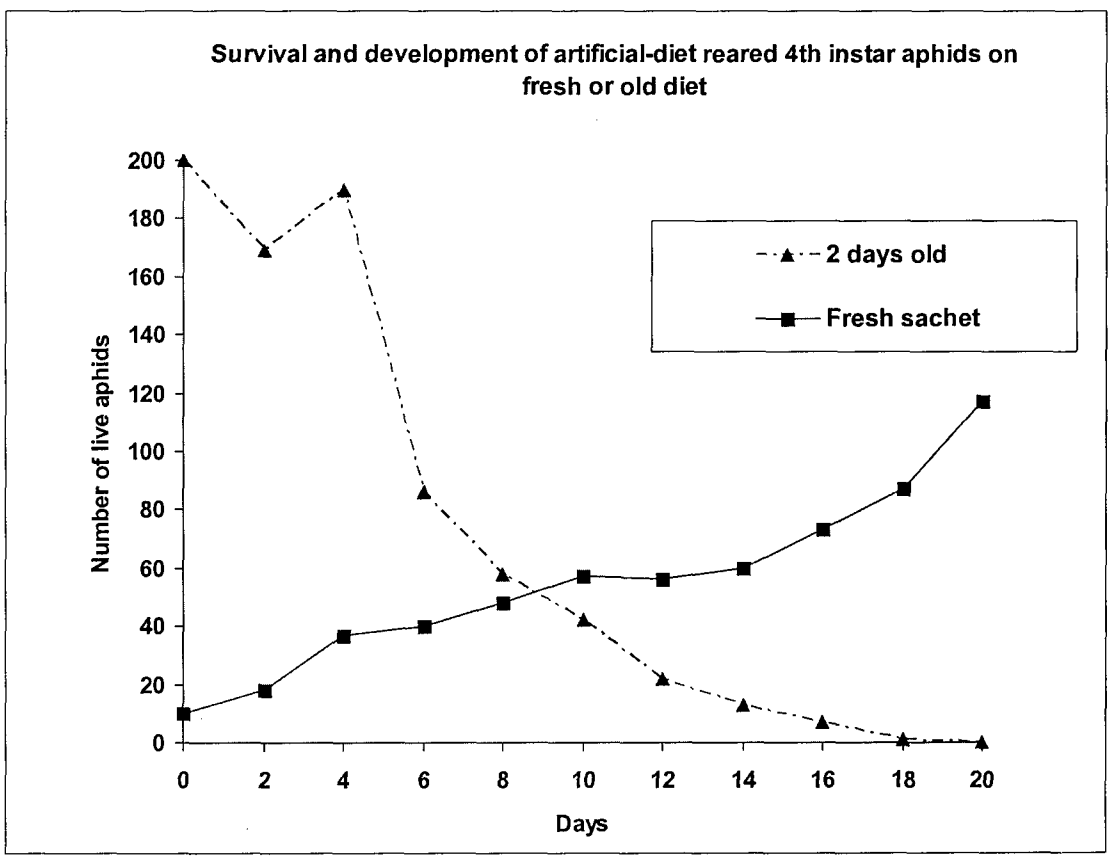



**Fig. 5.5** Comparison of aphid cultures reared on fresh or old diet sachets.

Olfactometer bioassays were run as in the previous chapters (sections 3.3.2, 4.1.2) but only 10 mated females (not 20 as in previous chapters) formed each replicate (due to

the low numbers collected from the diet, Fig. 5.4). Also, a single male was used to mate with two females for 24 h when occasional female-biased ratios occurred. An overall $\chi^2$ test, as well as a $\chi^2$ test for heterogeneity (section 3.2.2.1), was carried out to compare the number of females responding in a two-way protocol after natural emergence or emergence following pupal excision. Second generation diet-reared parasitoids were also used.

## 5.3 Results

### 5.3.1 Responses of naturally-emerged parasitoids

Leaf odours of the plant species and varieties tested, were preferred to clean air (Fig. 5.6, Table 5.1) and followed similar response patterns to those in the two previous chapters (sections 3.2.3, 4.3.3). However, when parasitoids were allowed to choose between the two sprout odours of Winter harvest and Red delicious no significant preference to the odour of the Winter harvest (on which the parasitoid mothers had been reared) was evident (Fig. 5.7, Table 5.3).

Experience (section 4.4.2) in the form of one mummy from a Winter harvest plant was sufficient to bias the olfactory responses of diet-reared parasitoids to the Winter harvest odour (Fig. 5.7, Table 5.3). However, when mummies reared on the non-maternal Red delicious sprout variety were experienced, not one but five mummies were required for a significant response to be evoked towards Red delicious odour.

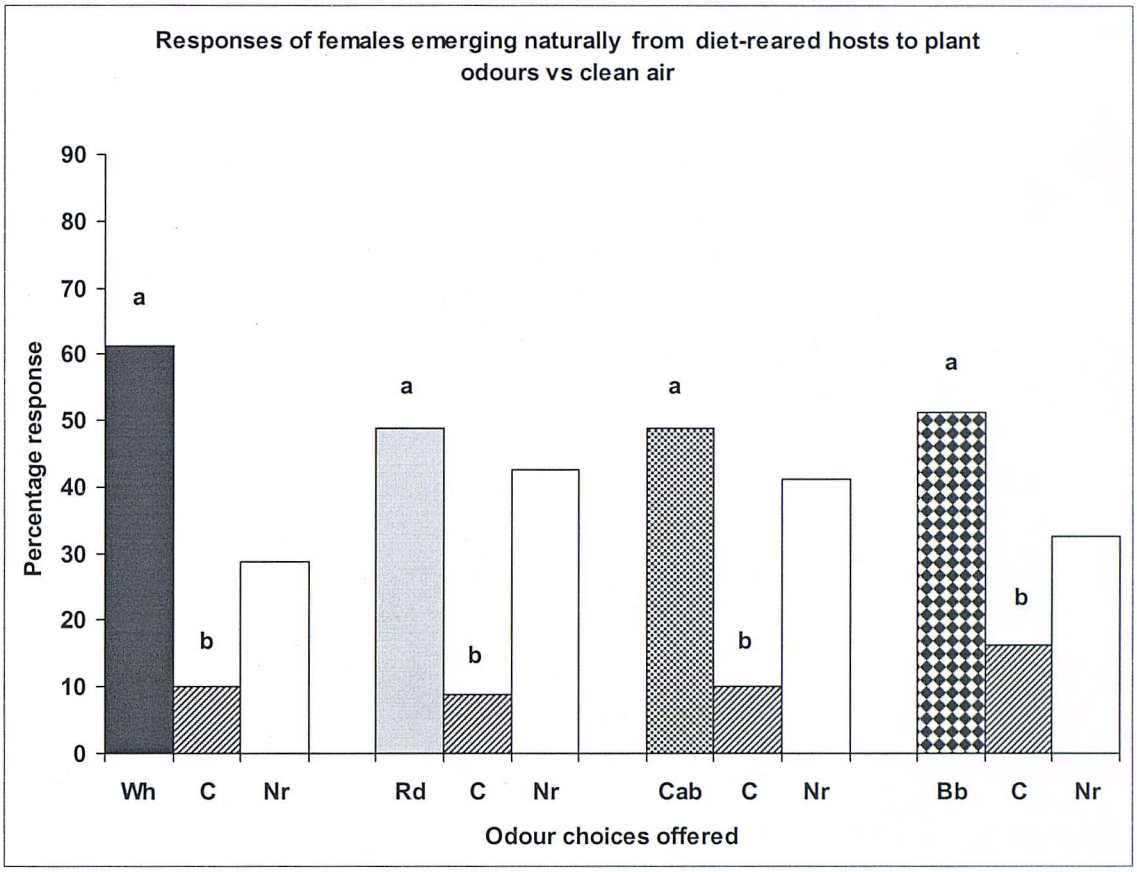



**Fig. 5.6** Percentage responses of female *Aphidius colemani* emerging naturally in Petri dishes from mummies formed on artificial diet. The mother parasitoid used for parasitisation of diet-reared aphids was collected from HPC: Winter harvest-*Myzus persicae* host-plant complex. Odours (from left to right): Wh: Winter harvest, C: Clean air, Rd: Red delicious, Nr: No response, Cab: Cabbage, Bb: Broad bean. Means with different letters above the column are significantly different (P<0.05) (comparison only within each odour test, i.e. plant versus Clean air, Table 5.1). n=8 replicates, 10 insects per replicate.

**Table 5.1** Responses of female *A. colemani* emerging naturally from diet-reared aphid hosts between plant odours and clean air. D= diet reared parasitoids.

| Parasitoid collection from | Odour leaf material | No. of (*n*) replicates | Totals being compared for 1df | Choice $\chi^2$ (1df) | Heterogeneity $\chi^2$ (n-1df) |
|---|---|---|---|---|---|
| D | Wh vs C | 8 | 49 vs 8 | 29.49 *** | 3.43 ns |
| D | Rd vs C | 8 | 39 vs 7 | 22.26 *** | 3.27 ns |
| D | Cab vs C | 8 | 39 vs 8 | 20.45 *** | 11.28 ns |
| D | Bb vs C | 8 | 41 vs 13 | 14.52 *** | 8.40 ns |

***P<0.001, ns:P>0.05

Overall, a greater percentage of plant-reared females responded (i.e. were found in the odour chambers; 82-91%) than diet-reared females (52-72%) (Table 5.2) although in all cases the wasps showed significant preference for the plant odour over clean air ($P<0.001$; Figs 5.4, 4.2). Also, the total number of plant-reared (n=160 – Table 4.2, section 4.3.1) and diet-reared (n=80 – Table 5.1) female responders versus non-responders (Table 5.2) significantly differed in all comparisons but one, i.e. when Winter harvest odour was tested against clean air. Female parasitoids emerging from diet-reared hosts were mated and allowed to parasitise diet-reared aphids. Similar to the 1st-generation insects above, their offspring (2nd generation reared on diet hosts) responded significantly to a sprout odour against clean air but could not differentiate between the odour of Winter harvest and Red delicious varieties (Fig. 5.8, Table 5.4).

**Table 5.2** Comparison of *A. colemani* females (reared on aphid hosts feeding on plant or diet) responding in the olfactometer versus the non-responders(Nr). HPC= wasps collected from plant culture, (*Myzus persicae*-Winter harvest host-plant complex), D= wasps collected from diet culture (mothers from *Myzus persicae*-Winter harvest complex).

| Parasitoid collection from | No. of (*n*) replicates | Bioassays performed | Numbers of responders / non-responders | | Total $\chi^2$ (1df) |
|---|---|---|---|---|---|
| | | | Plant reared | Diet reared | |
| HPC or D | 8 | Wh vs C | 141 | 57 | 1.54 ns |
| | | Nr | 39 | 23 | |
| HPC or D | 8 | Rd vs C | 145 | 46 | 15.10 *** |
| | | Nr | 35 | 34 | |
| HPC or D | 8 | Cab vs C | 128 | 47 | 3.845 * |
| | | Nr | 52 | 33 | |
| HPC or D | 8 | Bb vs C | 131 | 44 | 7.95 ** |
| | | Nr | 49 | 13 | |

*P<0.05, **P<0.01,***P<0.001, ns:P>0.05

Wh: Winter harvest, Rd=: Red delicious, Cab: Cabbage, Bb: Broad bean, C: Clean air.

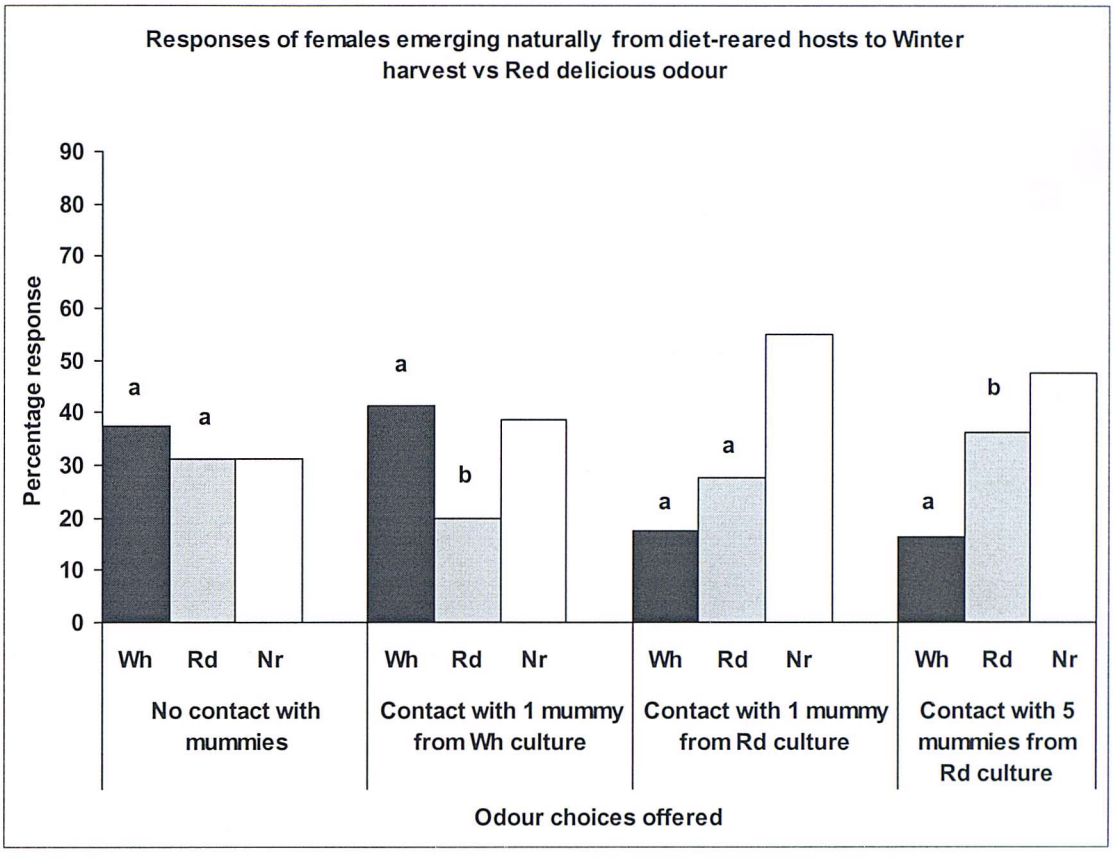



**Fig. 5.7** Percentage responses of diet-reared female *Aphidius colemani* which upon natural emergence were given access or not to mummies of different origin (i.e. formed on plants). The mother parasitoid used for parasitisation of diet-reared aphids was collected from HPC: Winter harvest-*Myzus persicae* host-plant complex. Odours (from left to right): Wh: Winter harvest, C: Clean air, Rd: Red delicious, Nr: No response. Means with different letters above the column are significantly different ($P<0.05$) (Table 5.3). n=8 replicates, 10 insects per replicate.

**Table 5.3** Responses of female *A. colemani* emerging naturally from diet-reared aphid hosts to Winter harvest vs Red delicious odour. D= diet reared parasitoids. 1WH=contact with 1 mummy formed on Winter harvest. 1RD or 5 RD= contact with 1 or 5 mummies formed on Red delicious, respectively.

| Parasitoid collection from | Odour leaf material and origin of mummies contacted | No. of (*n*) replicates | Totals being compared for 1df | Choice $\chi^2$ (1df) | | Heterogeneity $\chi^2$ (n-1df) | |
|---|---|---|---|---|---|---|---|
| D | Wh vs Rd | 8 | 30 vs 25 | 0.45 | ns | 13.67 | ns |
| D | Wh vs Rd (1WH) | 8 | 33 vs 16 | 5.90 | * | 9.70 | ns |
| D | Wh vs Rd (1RD) | 8 | 14 vs 22 | 1.78 | ns | 7.96 | ns |
| D | Wh vs Rd (5RD) | 8 | 13 vs 29 | 6.10 | * | 10.69 | ns |

*P<0.05, ns:P>0.05

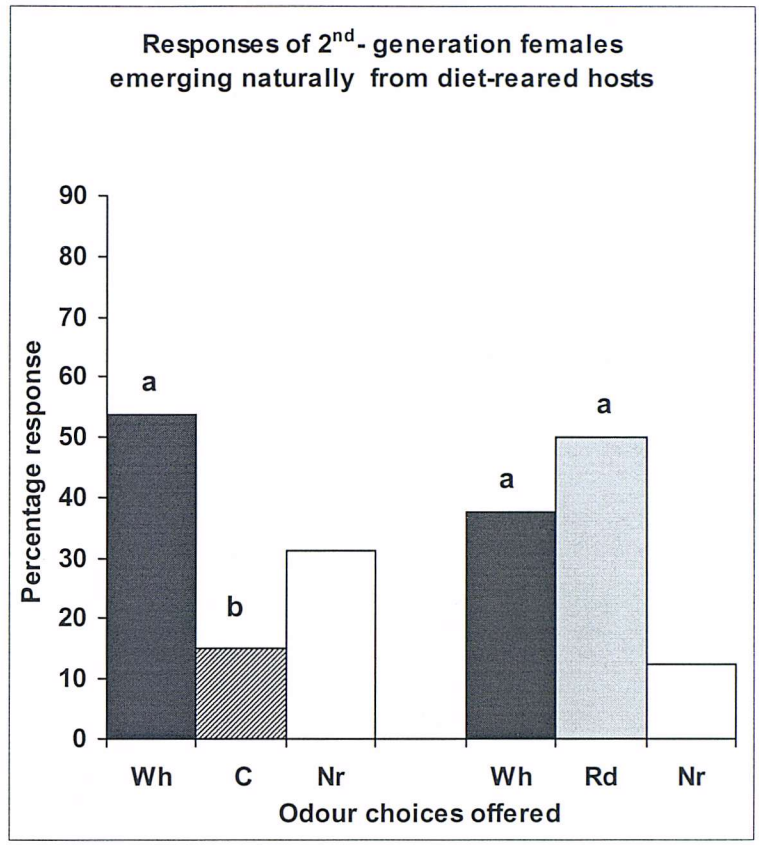



**Fig. 5.8** Percentage responses 2nd-generation female *Aphidius colemani* emerging from mummies formed on artificial diet. The grandmother parasitoid used for parasitisation of diet-reared aphids was collected from HPC: Winter harvest-*Myzus persicae* host-plant complex. Odours (from left to right): Wh: Winter harvest, C: Clean air, Rd: Red delicious, Nr: No response. Means with different letters above the column are significantly different (P<0.05) (comparison only within each odour test, i.e. Winter harvest versus the alternative choice, Table 5.4). n=8 replicates, 10 insects per replicate.

**Table 5.4** Responses of 2nd-generation female *A. colemani* emerging from diet-reared aphid hosts to Winter harvest vs Red delicious odour. D= diet reared parasitoids.

| Parasitoid collection from | Odour leaf material | No. of (*n*) replicates | Totals being compared for 1df | Choice $\chi^2$ (1df) | Heterogeneity $\chi^2$ (n-1df) |
|---|---|---|---|---|---|
| D | Wh vs C | 8 | 43 vs 12 | 17.47 *** | 11.04 ns |
| D | Wh vs Rd | 8 | 30 vs 40 | 1.43 ns | 7.47 ns |

***P<0.001, ns:P>0.05

### 5.3.2 Responses of parasitoids emerging following pupal excision

Female parasitoids, excised from diet-reared mummies, did not show an olfactory preference to the odours of either Winter harvest or Red delicious varieties when they had no contact with mummies or contact with five mummies formed on artificial diet (Fig. 5.9, Table 5.5). However, significant preferences were observed when adults were given access to five mummies formed on Winter harvest or Red delicious varieties. Access to a single mummy, from either sprout variety, was not enough to elicit preference but in these assays the heterogeneity was significant, indicating inconsistency of responses between replicates.

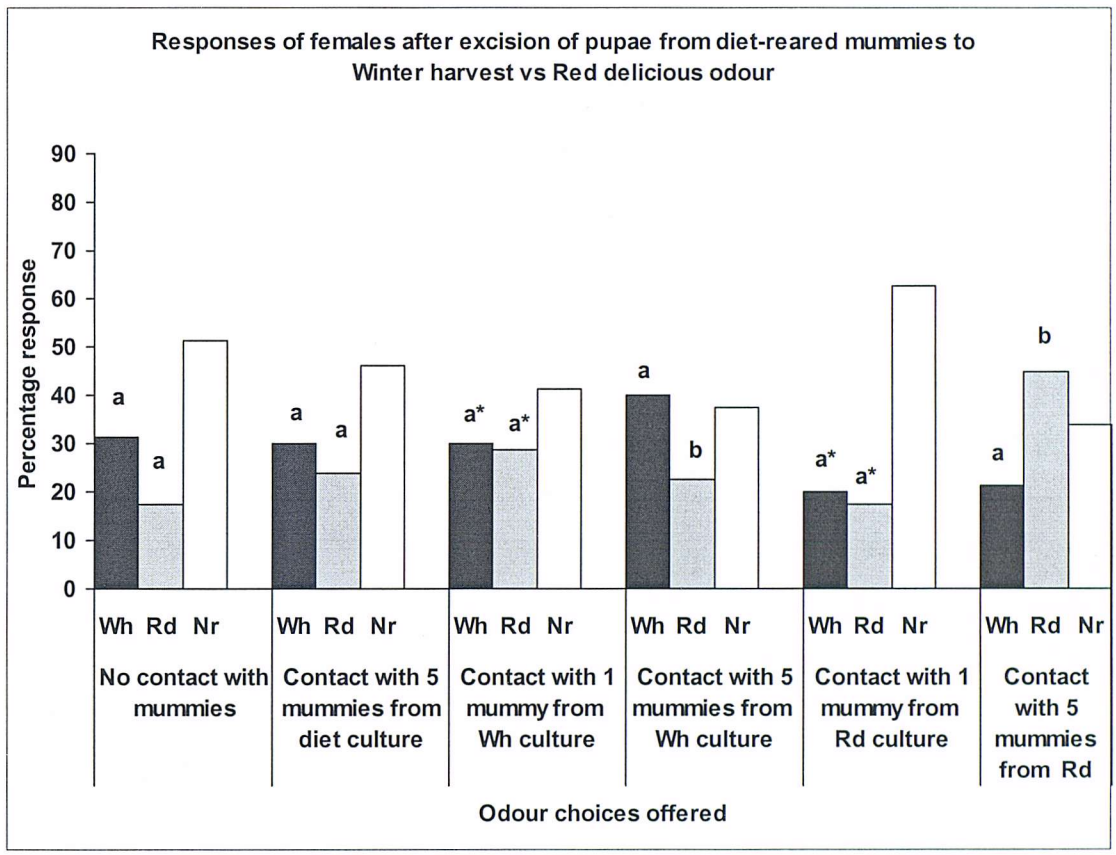



**Fig. 5.9** Percentage responses of female *Aphidius colemani* after excision from mummies formed on artificial diet. The mother parasitoid used for parasitisation of diet-reared aphids was collected from HPC: Winter harvest-*Myzus persicae* host-plant complex. Odours (from left to right): Wh: Winter harvest, Rd: Red delicious, Nr: No response. Means with different letters above the column are significantly different ($P<0.05$) and * indicates significant heterogeneity between replicates (comparison only within each odour test, i.e. Winter harvest versus Red delicious, Table 5.5). n=8 replicates, 10 insects per replicate.

**Table 5.5** Responses of female *A. colemani* emerging from diet-reared hosts after pupal excision to Winter harvest vs Red delicious odour. D= diet reared parasitoids. 5D=contact with 5 mummies formed on artificial diet. 1WH or 5WH= contact with 1 or 5 mummies formed on Winter harvest respectively. 1RD or 5 RD= contact with 1 or 5 mummies formed on Red delicious, respectively.

| Parasitoid collection from | Bioassays performed and origin of mummies contacted | No. of (*n*) replicates | Totals being compared for 1df | Choice $\chi^2$ (1df) | Heterogeneity $\chi^2$ (n-1df) |
|---|---|---|---|---|---|
| D | Wh vs Rd | 8 | 25 vs 14 | 3.10 ns | 2.73 ns |
| D | Wh vs Rd (5D) | 8 | 24 vs 19 | 0.58 ns | 8.16 ns |
| D | Wh vs Rd (1WH) | 8 | 24 vs 23 | 0.02 ns | 14.96 * |
| D | Wh vs Rd (5WH) | 8 | 32 vs 18 | 3.92 * | 12.76 ns |
| D | Wh vs Rd (1RD) | 8 | 16 vs 14 | 0.13 ns | 15.10 * |
| D | Wh vs Rd (5RD) | 8 | 17 vs 36 | 6.81 ** | 6.10 ns |

*P<0.05, **P<0.01, ns:P>0.05

## 5.4 Discussion

Rearing parasitoids on diet-feeding aphid hosts was difficult as the culture crashed c. 2 times a year (i.e. the diet-reared aphids stopped reproducing and their colour changed from shiny yellow-green to brownish with a large number eventually dying). The first, and usually the only thing, one can do to resolve the problem is to prepare a new batch of artificial diet. If successful, the timescale usually required for the fully recovery of the culture, in the conditions used in this study, can cause a couple of months delay to built up the aphid numbers before starting using the culture again for experiments. Some of the difficulties in the rearing procedure are also listed in chapter 5 (Section, 5.2) and care was taken to maintain constant rearing procedures and storage conditions. However, the precise causes of culture crashes still remain a mystery (Elizabeth A. Wild, University of Reading, personal communication).

Despite the difficulties of rearing *A. colemani* wasps on artificial-diet feeding aphid hosts and the low emergence rate obtained, the results indicated that there were clearly innate responses in these parasitoids to general plant odours. Naïve generalist parasitoids (with no adult foraging experience) responded to general cues (e.g. green leaf volatiles) (Hoballah & Turlings, 2005) or Brassica volatiles after mechanical leaf damage (Storeck *et al.*, 2000). Naïve female generalists, can also respond innately during host-finding process to specific volatile cues (i.e. aggregation pheromones from

male hosts *Rhyzopertha dominica* (Fabricius)), not previously experienced by these parasitoids which were reared on an alternative host species *Sitophilus granarious* (L.) (Steidle *et al.*, 2003). In addition, an innate behavioural response to infochemical cues (i.e. cues deriving from host-plant/prey complex) was found in both generalist and specialist parasitoids (Steidle & van Loon, 2003).

In chapter 4, parasitoid behavioural responses appeared to be influenced when encountering plant chemicals during their development or upon adult emergence. In this chapter it was assumed that such an influence was not present and the parasitoids would be naïve as their development occurred entirely in an artificial system without any plant odours present. The only plant association for insects reared on hosts on artificial diet was the plant on which the mothers or grandmothers developed. The data showed that, wasps with different rearing histories (host's feeding medium) can respond similarly to plant odours against clean air always preferring the plant odour. When Winter harvest was the sprout variety of origin (HPC) the analysis revealed that diet-reared parasitoids responded as strongly as those reared on HPC while in all the other comparisons HPC-reared wasps showed significantly stronger responses than diet-reared ones. This result may indicates when the mothers of HPC/diet-reared parasitoids have the same plant of origin they are similarly conditioned to its odour (if they were tested over clean air).

When *A. colemani* females were given the choice between two sprout odours (Winter harvest and Red delicious), following natural emergence from diet-reared hosts, they did not differentiate between them even though the mother had been reared on Winter harvest. Similar response patterns were observed with 2$^{nd}$ generation females (mother reared on diet). First and 2$^{nd}$ generation parasitoids do, however, show preference to plant odours over clean air. This observation may indicate an innate response to plant odours.

Contact with a single mummy formed on Winter harvest, which may carry volatiles on its cuticle matching the mother's plant of origin, by naturally-emerging diet-reared parasitoids born from Winter harvest-reared mothers elicited significant responses to the odour of this sprout cultivar. Experience of five but *not* one Red delicious mummy resulted in a significant olfactory preference towards the latter sprout odour and indicates the presence of a maternal effect. That is, the insects appear to be "primed" and more readily associate with the mother's plant of origin than a different plant variety. These responses were similar to those of plant-reared parasitoids in that experience of five, but not one mummy was needed to learn another sprout variety odour in the previous chapter. In a future study, the determination of olfactory preferences of female parasitoids obtained from diet-reared mummies could also include adult parasitoids that upon emergence are offered non-parasitised aphid

moulted cuticles reared on Winter harvest or a different brassica variety (Fillbasket, Red delicious, and Hamlet) to handle for 24 h. This would help to identify whether the parasitoids are imprinting to the aphid or its own tissue. However, care must be taken to collect honeydew-free exuviae from moulting aphids.

Parasitoids obtained after excision of pupae from diet-reared mummies showed responses similar to those obtained from naturally-emerged parasitoids from diet-reared hosts. There was no preference for sprout variety when tested straight after excision or after contact of diet-reared mummies. This, again, indicated that plant-related chemical cues, possibly transferred from the mother parasitoid via the egg or more generally introduced into the host were absent or at a level not sufficient to affect subsequent olfactory responses. The response to experience for insects obtained after excision differed from those allowed to eclose naturally in that a single mummy from Winter harvest did not elicit preference. Experience of 5 mummies was needed to effect preference for the maternal Winter harvest as well as for Red delicious. It should be noted that there appears to be higher numbers of non-responders from diet-reared hosts than plant-reared hosts and there were high levels of heterogeneity when the parasitoids experienced single mummies which may be attributed to the rearing procedure on an artificial medium and its effects on the responses of the emerging insects.

For example, *Exeristes roborator* (F.), a generalist ectoparasitoid of larval lepidopterans, that had experienced an artificial habitat demonstrated reduced subsequent attacking and host-searching response activity in the natural habitat, but there was no significant change of preference towards either of these habitats (Wardle & Borden, 1991). Parasitic wasps emerged from diet-reared larval hosts not only exhibited a significant reduction in successful emergence from these hosts (Reis *et al.*, 2003) but also reduced responses to odours from a host-plant complex (Drost *et al.*, 1988; Hérald *et al.*, 1988; Gandolfi *et al.*, 2003). In addition, the poor host-seeking behaviour of parasitoids was evident (a reduced percentage parasitisation) when they emerged from hosts fed on artificial diet and were then released into a natural environment (Godfray, 1994).

The data in the current study show that female *A. colemani*, reared in aphid hosts fed on artificial diet for many generations, do not show the odour preference of their mothers, or grandmothers, reared on hosts fed on plants. They do, however, retain a preference for plant odours over clean air choice which may be an innate response. There is also an indication that a maternal effect is present, at least in naturally-emerging females. That is, they more readily learn the odour of the plant variety on which the mother was reared than that of a different variety. When adults were excised from the diet-reared mummies, this 'priming' of a learned response

appeared to be lost but the high numbers of non-responders and heterogeneity between replicates is a confounding factor. Nevertheless, the mechanisms inducing odour preference are complex.

# CHAPTER 6: Parasitisation under laboratory conditions

## 6.1 Introduction

Carnivorous arthropods are known to use volatiles emitted from plants, which are often induced by herbivore damage, when foraging for hosts (Steidle & van Loon, 2003). Herbivory can cause changes in a plant's volatile profile (Dicke & van Loon, 2000) which can be specific for herbivore species, different larval instars and the parasitoids associated with such hosts (Guerrieri *et al.*, 2002; Gouinguenè *et al.*, 2003). Such specific volatile blends may also emanate from different varieties of a plant species after feeding by the same herbivore species (Gouinguenè *et al.*, 2001).

Many studies have demonstrated that parasitoids in multitrophic interactions can differentiate among herbivore-induced cues while searching for insect hosts (Hoballah *et al.*, 2002) and this ability may be enhanced by learning of volatile cues from the surrounding rearing environment (Papaj & Lewis, 1993). Plant volatile emission after herbivore attack can elicit significantly different olfactory responses in the aphid parasitoid *Aphidius ervi* when comparing odours from uninfested *Acyrthosiphum pisum*-infested broad bean leaves. In addition, continuous aphid feeding can increase the quantity of volatiles produced by the plant (Du *et al.*, 1998). In another tritrophic system, using odours emitted by mechanically-damaged or caterpillar (*Pieris brassicae*)-infested Brussels sprout leaves, it was shown that the latter were preferred by the parasitoid *Cotesia glomerata* (L.) in a glasshouse flight-chamber bioassay and chemical analysis confirmed different headspace cabbage leaf volatile concentrations (Mattiacci *et al.*, 1994).

In the three previous chapters (3, 4 and 5) the olfactory responses to plant odours of *A. colemani* parasitoids have been studied using aphid-free plant leaves which where placed in an olfactometer apparatus. The present study, investigated the extent that previously revealed olfactory movement preferences could be associated with oviposition preference for a host-plant/prey complex when parasitoids were released in a cage after manipulating their emergence/rearing condition.

## 6.2 Materials and methods

Wooden-frame cages (section 2.4) have been used and all of them were kept in a CT room at 19±1 °C, 75±5% relative humidity, LD 16:8 h photoperiod under artificial

cool white light (sections, 2.5.1 and 2.5.2). Two undamaged Brussels sprout plants (40-45 days after sowing) were placed inside each cage (one of each sprout variety, Winter harvest and Red delicious). Each plant was then infested with 30 intermediate but mixed size ($2^{nd}$- to $4^{th}$-instar larvae) *Myzus persicae* taken from two aphid cultures reared on these varieties. Thus, Winter harvest-reared aphids were placed on Winter harvest plants and Red delicious-reared aphids were placed on Red delicious plants. Initially, the two plants were placed at opposite corners within the same cage. All the 30 aphids were placed on the top surface of a single leaf of each variety and care was taken to infest leaves approximately of the same size, orientation and plant height (mid canopy). The aphids were placed initially on a single leaf and a systemic release of volatiles due to herbivore feeding could be induced, which can trigger positive responses from natural enemies experienced with host or host-related cues (Powell *et al.*, 1998). In the present study, the aphids were left to feed on each sprout variety for 24 h which has been shown to be enough time for the plants to emit parasitoids detectable herbivore induce plant volatiles (Du *et al.*, 1996). Five mated female (section 2.6.4) *Aphidius colemani* parasitoids were then released in the centre of each cage, allowed to oviposit for 24 h and then discarded. Thus, the parasitoid aphid ratio tested was 1:6. After each replicate, fresh plants were added and the distribution of treatments was rotated 90° clockwise. The parasitoid manipulation/treatments prior to release were:

1. HPC, parasitoids collected directly from the host-plant complex after naturally emerging from aphids feeding on the plants (i.e. mummies formed on either Winter harvest or Red delicious sprout varieties and remained on the plant until natural emergence occurred),
2. E, excised parasitoids (pupa removed from mummy case – mummies initially formed on plants and then transferred into Petri dishes) emerged in isolation away from the vicinity of the host-plant complex,
3. D, naturally-emerged parasitoids from diet-reared aphids (mummies initially formed on diet and then transferred into Petri dishes) isolated away from the vicinity of any host-plant complex.

The parasitisation of *A. colemani* was measured as a percentage of the number of aphids mummified (out of 150 aphids, i.e. 30 per replicate x 5 replicates) on each variety no later than 21 days following the introduction of parasitoids (as this was the period after which no further formation of mummies observed, personal observation). Thus, the percent parasitism of the aphids that were transferred at the beginning of the experiment was measured.

The data obtained separately for each comparison were subject to $\chi^2$ tests. An overall $\chi^2$ test as well as a $\chi^2$ test for heterogeneity (section 3.2.2.1) was carried out to compare the number of mummies formed on each sprout variety.

## 6.3 Results

When the origin of the parasitoids was the Winter harvest-*Myzus persicae* complex, naturally-emerging adult females and females emerging after removal of pupae parasitoids showed a significant oviposition preference for hosts on Winter harvest plants over those on Red delicious but the latter it was associated with a significant heterogeneity between runs and the result was not considered valid (Fig. 6.1, Table 6.1).

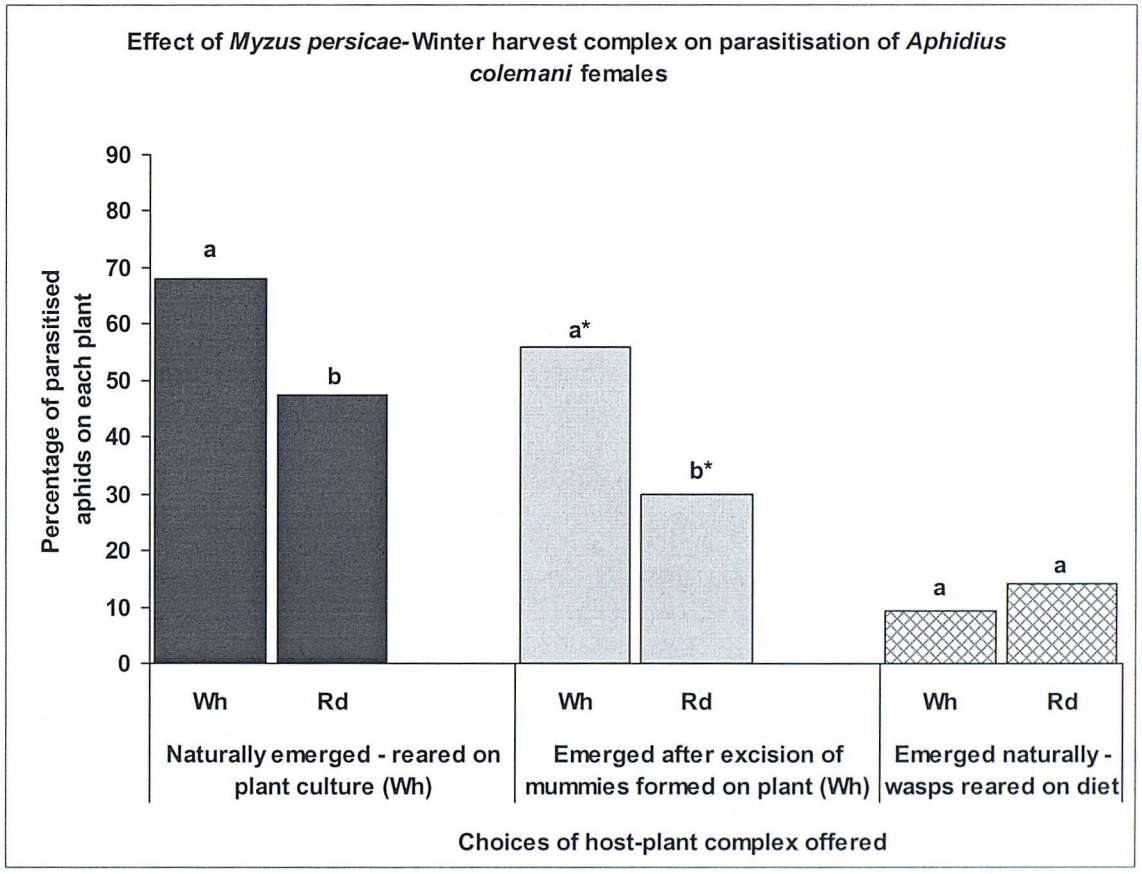



**Fig. 6.1** Parasitisation by female *Aphidius colemani*. Origin of test aphids: hosts were feeding on Winter harvest (Wh) and Red delicious (Rd), prior relocation and parasitisation on these sprout varieties, respectively. Parasitoids that emerged naturally and after pupal excision from Winter harvest-reared hosts/mummies or emerged from diet-reared hosts were given access to the host-plant complexes: *Myzus persicae* - Winter harvest and Red delicious. The mother parasitoid used for parasitisation of diet-reared aphids was collected from *Myzus persicae* - Winter harvest host-plant complex. Means with different letter above the column are significantly different ($P<0.05$)

(comparison only within each treatment, Table 6.1). a*:b* refer to significantly different means but with significant heterogeneity (Table 6.1). n=5 replicates, 5 female wasps and 30 aphids on each sprout variety were used per replicate.

**Table 6.1** Parasitisation (number of formed mummies) by *Aphidius colemani* females on plants. HPC(Wh)= naturally-emerged parasitoids collected directly from the *Myzus persicae* - Winter harvest host-plant complex cultures, E(Wh)= excised parasitoids (pupa removed from mummy case) emerging in Petri dishes. D(Wh)= naturally emerged parasitoids from diet-reared aphids (parents reared on *Myzus persicae*-Winter harvest host-plant complex).

| Parasitoid source | No. of replicates | Choice of host-plant complex, number of mummies formed Wh vs Rd | Choice $\chi^2$ (1df) | Heterogeneity $\chi^2$ (n-1df) |
|---|---|---|---|---|
| HPC(Wh) | 5 | 102 vs 71 | 5.55 * | 2.34 ns |
| E(Wh) | 5 | 84 vs 45 | 11.79 *** | 18.85 *** |
| D(Wh) | 5 | 14 vs 21 | 1.40 ns | 3.85 ns |

*P<0.05, ***P<0.001, ns:P>0.05

However, when diet-reared parasitoids were allowed to choose between the two oviposition sites no significant parasitisation preference for a host-plant complex was apparent even though the previous generation had been reared on Winter harvest. In addition, when the total number of mummies formed between different treatments was compared, the diet-reared parasitoids exhibited significantly the lowest parasitisation (less than 14% on both sprout varieties) over the other two treatments (HPC and E) which differed significantly from each other (first three rows of Table 6.3).

On the other hand, when the origin of the parasitoids was the Red delicious-*Myzus persicae* complex a significant preference was seen only from naturally-emerged parasitoids and this was towards the hosts on Red delicious (Fig. 6.2, Table 6.2). Parasitoids emerging after pupal excision or naturally-emerging from aphids feeding on diet did not show any significant oviposition preferences. Once more, when the total number of mummies formed between different treatments was compared, the diet-reared wasps showed a significantly lower parasitisation than those from plant-reared hosts (HPC and E) which did not differ as in the previous experiment (last three rows of Table 6.3).

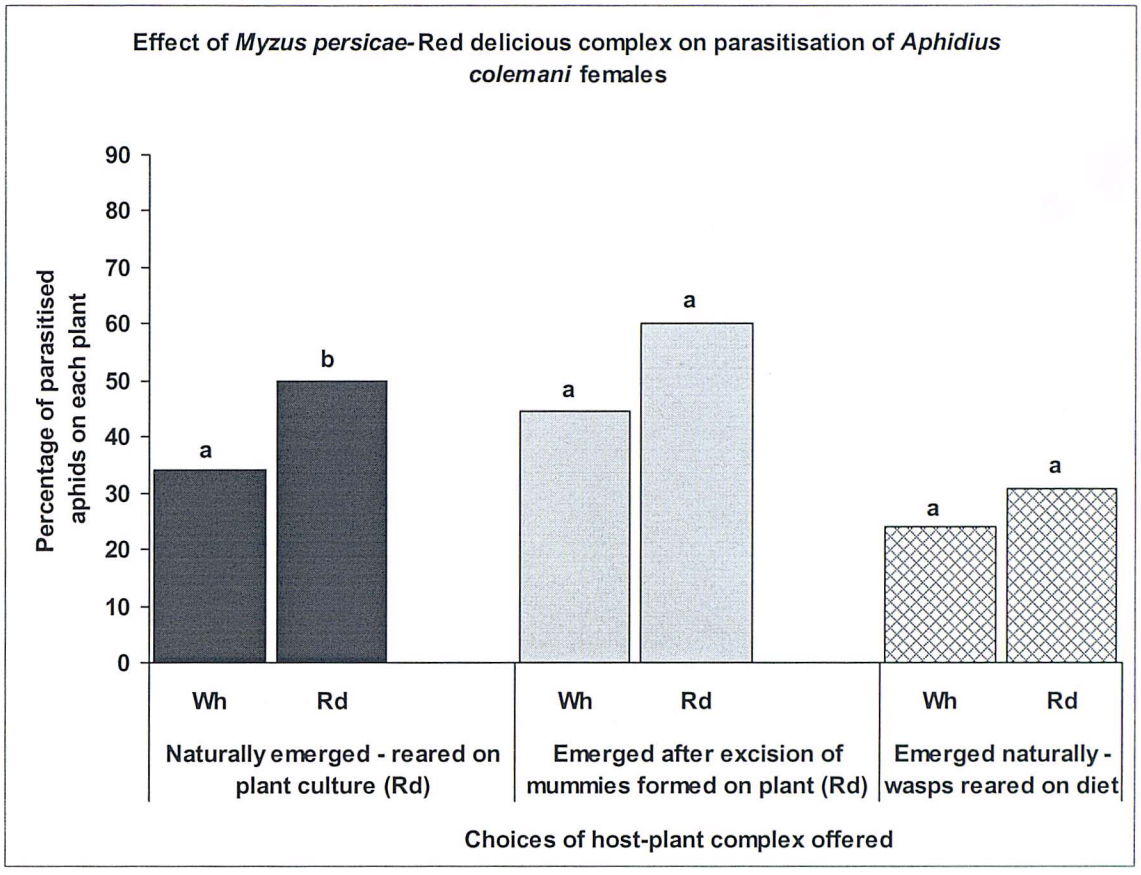



**Fig. 6.2** Parasitisation by female *Aphidius colemani*. Origin of test aphids on plants: hosts were feeding on Winter harvest (Wh) and Red delicious (Rd), prior relocation and parasitisation on these sprout varieties, respectively. Parasitoids that emerged naturally and after pupal excision from Red delicious-reared hosts/mummies or emerged from diet-reared hosts were given access to the host-plant complexes: *Myzus persicae* - Winter harvest and Red delicious. The mother parasitoid used for parasitisation of diet-reared aphids was collected from *Myzus persicae* - Red delicious host-plant complex. Means with different letter above the column are significantly different ($P<0.05$), (comparison only within each treatment, Table 6.2). n=5 replicates, 5 female wasps and 30 aphids on each sprout variety were used per replicate.

**Table 6.2** Parasitisation (number of formed mummies) by *Aphidius colemani* females on plants. HPC(Rd)= parasitoids collected directly from the *Myzus persicae*-Red delicious host-plant complex cultures. E(Rd)= excised parasitoids (pupa removed from mummy case) emerging in Petri dishes. D(Rd)= naturally-emerged parasitoids from diet-reared aphids(parents reared on *Myzus persicae*-Red delicious host-plant complex).

| Parasitoid source - origin | No. of replicates | Choice of host-plant complex, number of mummies formed: Wh vs Rd | Choice $\chi^2$ (1df) | Heterogeneity $\chi^2$ (n-1df) |
|---|---|---|---|---|
| HPC(Rd) | 5 | 51 vs 75 | 4.58 * | 3.12 ns |
| E(Rd) | 5 | 67 vs 90 | 3.37 ns | 0.16 ns |
| D(Rd) | 5 | 36 vs 46 | 1.22 ns | 5.96 ns |

*P<0.05, ns:P>0.05

**Table 6.3** Comparison between treatments of total parasitisation (number of formed mummies) by *Aphidius colemani* females of the same origin on both varieties (Winter harvest and Red delicious). HPC=parasitoids emerging from the host-plant complex. E=excised parasitoids(pupa removed from mummy case) emerging in Petri dishes. D=emerging parasitoids from diet-reared aphids.

| Parasitoid origin | Comparison of different treatments - offsprings emerging from: | Total number of mummies formed on both sprout varieties (Wh+Rd) per treatment respectively: | Total $\chi^2$ (1df) |
|---|---|---|---|
| mother reared on Winter harvest (Wh) culture | HPC vs E | 173 vs 129 | 6.41 * |
| | HPC vs D | 173 vs 35 | 91.55 *** |
| | E vs D | 129 vs 35 | 53.87 *** |
| mother reared on Red delicious (Rd) culture | HPC vs E | 126 vs 157 | 3.39 ns |
| | HPC vs D | 126 vs 82 | 9.31 ** |
| | E vs D | 157 vs 82 | 23.53 *** |

*P<0.05, **P<0.01,***P<0.001, ns:P>0.05

Further analysis of the results presented in Figs 6.1, 6.2 involved total parasitisation by parasitoids reared on different varieties but of the same treatment (HPC, E and D). Thus, Winter harvest-*Myzus persicae*-reared parasitoids emerging directly from culture perform significantly better than the parasitoids reared on Red delicious-*Myzus persicae* complex (i.e. resulting in formation of significantly more

mummies on the host-plant complexes) (Table 6.4). However, this significant tendency disappeared after adult emergence following pupal excision. For parasitoids emerging from diet-reared aphid hosts, the maternal host-plant complex of the parasitoid appeared to influence successful mummification. In the current study, parasitoids from the different treatments (including the diet-reared parasitoids) successfully parasitised and completed development in *Myzus persicae* (personal observation).

**Table 6.4** Comparison between treatments of total parasitisation (number of formed mummies) by *Aphidius colemani* females from different origins on both varieties (Winter harvest and Red delicious). HPC= parasitoids emerging from the host-plant complex. E= excised parasitoids(pupa removed from mummy case) emerging in Petri dishes. D= emerging parasitoids from diet-reared aphids.

| Parasitoid origin | Comparison of different treatments - offsprings emerging from: | Total number of mummies formed on both sprout varieties (Wh+Rd) per treatment: | | | Total $\chi^2$ (1df) | |
|---|---|---|---|---|---|---|
| mother reared on Winter harvest (Wh) or Red delicious (Rd) culture | HPC(Wh) vs HPC(Rd) | 173 | vs | 126 | 7.38 | ** |
| | E(Wh) vs E(Rd) | 129 | vs | 157 | 2.74 | ns |
| | D(Wh) vs D(Rd) | 35 | vs | 82 | 18.88 | *** |

**P<0.01,***P<0.001, ns:P>0.05

## 6.4 Discussion

Observations on herbivore-induced volatile emission and interactions with natural enemies revealed using Y-tube olfactometers under controlled environments may not reflect accurately the situation present in the field (Takabayashi *et al.*, 1994). Such studies have attracted some criticism about their simplicity and value in a ecological and economic context (Hunter, 2002). However, many studies have investigated the performance of natural enemies under controlled conditions while field studies in this area are fewer. Research under laboratory conditions has been often used to provide quantitative estimates of parasitism (as measured by aphid mummies) and more easily reveal the potential of natural enemies as biological control agents. Thus, the percentage of aphid parasitisation in cage experiments under laboratory conditions and/or in glasshouses can be used as an indicator of biological control efficiency these results should be confirmed by experiments in the field and glasshouses in order to be meaningful in economic terms and on a larger scale. Field experiments using wheat plants artificially infested with *Diuraphis noxia* (Mordvilko ex

Kurdjumov) aphids demonstrated the effectiveness of parasitoids (*Aphelinus asychis* Walker, *Aphelinus varipes* (Foerster), *Diaeretiella rapae* (McIntosh) and *Aphelinus matricariae* (Haliday) and predators species (the coccinellid *Coccinella septempunctata* L. and the syrphids *Episyrphus balteatus* De Geer, *Metasyrphus corollae* Fabricius and *Sphaerophoria scripta* Linnaeus) assessed, in reducing the aphid populations in closed-caged and open-caged or uncaged plants (Hopper *et al.*, 1995). Another study in which caged and uncaged barley plants were used, showed that aphid densities of *Diuraphis noxia* and *Rhopalosiphum padi* were greater in caged than uncaged plants and while parasitism was low in both cases, the observed reduction in the number of aphids on barley plants was due to the increase of the aphid fungal pathogen, *Pandora neoaphidis* (Basky & Hopper, 2000).

A number of predator and parasitoid species have been used synchronously in cages and it has been demonstrated that *Diuraphis noxia* aphid populations were reduced in comparison with cases that wheat aphid-host plants were free of any natural enemies (Mohamed *et al.*, 2000). An additive effect by using both parasitoids and predators on the reduction of aphid population may not always be evident since cages containing parasitoids only, showed greater percent parasitism when compared with cages containing both these natural enemies and this could be also attributed to predation (Ferguson & Stiling, 1996).

Parasitism can be affected by the number of mummies in relation to an aphid population and the number of hosts available in relation to a parasitoid population. A correlation between the number of *Schizaphis graminum* Rondani mummies observed on grain sorghum plants and the parasitoid *Lysiphlebus testaceipes* (Cresson) showed that the aphid numbers started declining when the mummies accounted for 20-30% of the aphid population (Fernandes *et al.*, 1998). A linear correlation has also been demonstrated in low aphid densities (i.e. < 50 aphids/cage) between the number of *Rhopalosiphum maidis* (Fitch) aphid and its parasitoid *Lysiphlebus delhiensis* (Subba Rao and Sarma) since parasitisation of hosts increased over time and this correlation became better with increasing host density (Mishra & Singh, 1991).

Such relationships may occur in other tritrophic systems as well. The percentage parasitism of *Bemisia tabaci* (Gennadius) whitefly from its parasitoids *Eretmocerus* spp. and *Encarsia* spp. was positive correlated with pest density in cages with cotton plants when 4-8 parasitoids/plant were released (Otoidobiga *et al.*, 2004). The parasitoid:aphid ratio seems to be an important factor affecting the parasitisation levels of the host. When the parasitoid *Diaeretiella rapae* was used with the cabbage aphid, *Brevicoryne brassicae,* in cage experiments (parasitoid:aphid ratio 1:5), the resulting parasitisation was not enough to control the aphid population and only an increased ratio (1.2:1), combined with successive parasitoid releases, could

successfully control the aphid population. Similar results were obtained when a parasitoid:aphid ratio of 1:4 was used in a greenhouse. Although, placing 'banker' cabbage plants with mummies and aphids (mummy:aphid ratio 1:1) in a broccoli field resulted in the dispersal of the parasitoid but not to the successful control of the pest. Thus, it was speculated that more releases were needed but relevant data were not provided in that study and the possibility of banker aphids on cabbage migrating to broccoli plants was not studied either (Zhang & Hassan, 2003).

In addition to the parasitoid:aphid ratio, host suitability plays an important role for insect parasitoids (Vinson & Iwantsch, 1980) and their effectiveness may rely on being at the right place at the right time (Powell *et al.*, 1998). Oviposition behaviour is dependent on genetic and also epigenetic factors such as experience and conditioning and the latter may override any parasitoid innate preferences. For example, *A. colemani* females reared on either *A. gossypii* or *M. persicae* have showed an innate oviposition preference for the *A. gossypii* aphid host in the laboratory (Messing & Rabasse, 1995) while *Aphidius ervi* parasitoids with high genetic homogeneity were shown to adjust their host-searching behaviour according to previous oviposition experiences associated with the rearing history of aphid hosts (*Acyrthosiphon pisum* on alfalfa and *Sitobion avenae* on wheat) (Daza-Bustamante *et al.*, 2002). On the other hand, adult female aphid parasitoids, *Ephedrus cerasicola* Starý, were allowed to select between two plants (cucumber and swede) infested with two different species (*Aphis gossypii* and *Myzus persicae*) a higher production of mummies on the cucumber plants was observed whichever host was present. This occurred even though the parasitoids were reared on *Myzus persicae* feeding on swede plants, and cucumber plants were preferred, in terms of oriented flights after parasitoid release in cages, even when both plant-choices available were uninfested (Hågvar & Hofsvang, 1994).

In the case of *A. colemani*, populations from different continents or regions can vary in their host range and may not complete development in common aphid species (Messing & Rabasse, 1995). A successful switch from one host species to several others (development to adult in 9 out of 14 insect hosts offered) has been also demonstrated for *A. colemani* reared initially on *Schizaphis graminum* Rondani on wheat plants under laboratory conditions (Elliott *et al.*, 1994).

Furthermore, parasitoid olfactory responses could be influenced by experience acquired at or just after emergence of *Aphidius rhopalosiphi*, reared on aphid hosts, from the environment around the host-plant complex. When these wasps were reared in cages on *Metopolophium dirhodum* (Walker) aphids feeding on wheat plants in a glasshouse, the presence or absence of tomato or marigold plants affected positively subsequent olfactory parasitoids responses under laboratory conditions (van Emden *et al.*, 2002). Experience of host and host-derived cues can further enhance the

responsiveness of *A. ervi* (Powell *et al.*, 1998), *Cotesia plutellae* (Potting *et al.*, 1999) but also *A. colemani* (Grasswitz, 1998) compared to naïve parasitoids. However, it has been demonstrated *A. colemani* females with oviposition experience could not differentiate between aphid-infested and uninfested plants in their responses (Lo Pinto *et al.*, 2004).

In the present study, olfactory responses of *A. colemani* revealed in the three previous chapters (3, 4 and 5) will have an influence on females searching for suitable oviposition sites inducing preferences relevant to their rearing history. This was evident when the host-plant complex was the *Myzus persicae*-Winter harvest since naturally-emerging parasitoids or those emerging after pupal excision showed a preference for the host-plant complex on which they were reared. That is, in the present study there was a greater degree of parasitism found on the *Myzus persicae*-Winter harvest host-plant complex than on the *Myzus persicae*-Red delicious host-plant complex. However, there are physical differences in leaf colour between the plants and the behavioural mechanisms that differentiate between the host-palnt complexes may not be purely olfactory. Although, the conditioning of the parasitoids seems that did not disappear after excision this significant result was associated with significant heterogeneity between replicates and thus it was not accepted as significant.

In the case of *Myzus persicae*-Red delicious complex the fact that only naturally-emerging parasitoids showed a clear preference to the variety they were reared on seemed to indicate that the plant variety on which the parasitoid host is feeding may play initially a vital role by inducing oviposition preferences. In this case, however the excision process had a clear effect on adult oviposition preference and parasitoids appeared no longer able to differentiate between the two host-plant complexes. It is interesting that the trend of oviposition preference obtained in the experiments of this study was similar to the olfactory responses obtained using uninfested leaves in an olfactometer (chapter 4).

Despite the observation that parasitisation from females reared on diet (mothers were reared on *Myzus persicae*-Red delicious complex) was significantly greater than in the previous experiment (when the mothers were reared on the *Myzus persicae*-Winter harvest complex), it was again lower than the other two treatments indicating that diet-reared hosts can adversely affect successful mummification of the emerging parasitoids. Diet-reared parasitoids failed to demonstrate any oviposition preference for a host-plant complex in a similar way they have failed to demonstrate olfactory preferences for a host-plant odour in other studies (Storeck *et al.*, 2000; Douloumpaka & van Emden, 2003) and the results obtained in chapter 5.

The data presented in this chapter showed that the sprout variety itself may significantly affect the performance of parasitoids as *Myzus persicae*-Winter harvest

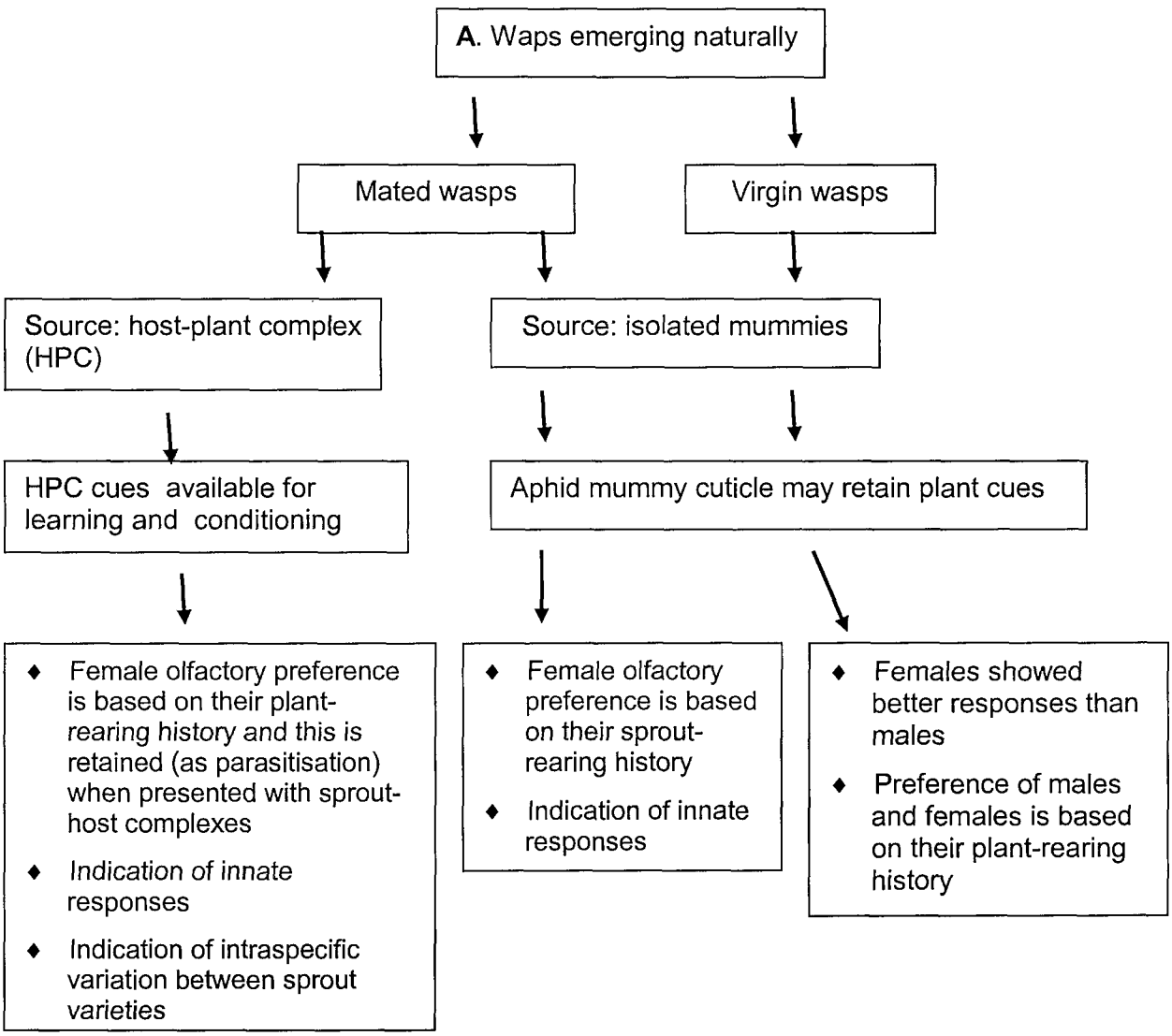
reared parasitoids performed better than those reared on *Myzus persicae*-Red delicious, in terms of the total number of mummies observed on these plants. It was intriguing that the inverse seems to be true for maternal influence on diet-reared parasitoids while no effect for the parasitoids emerging after pupal excision was uncovered.

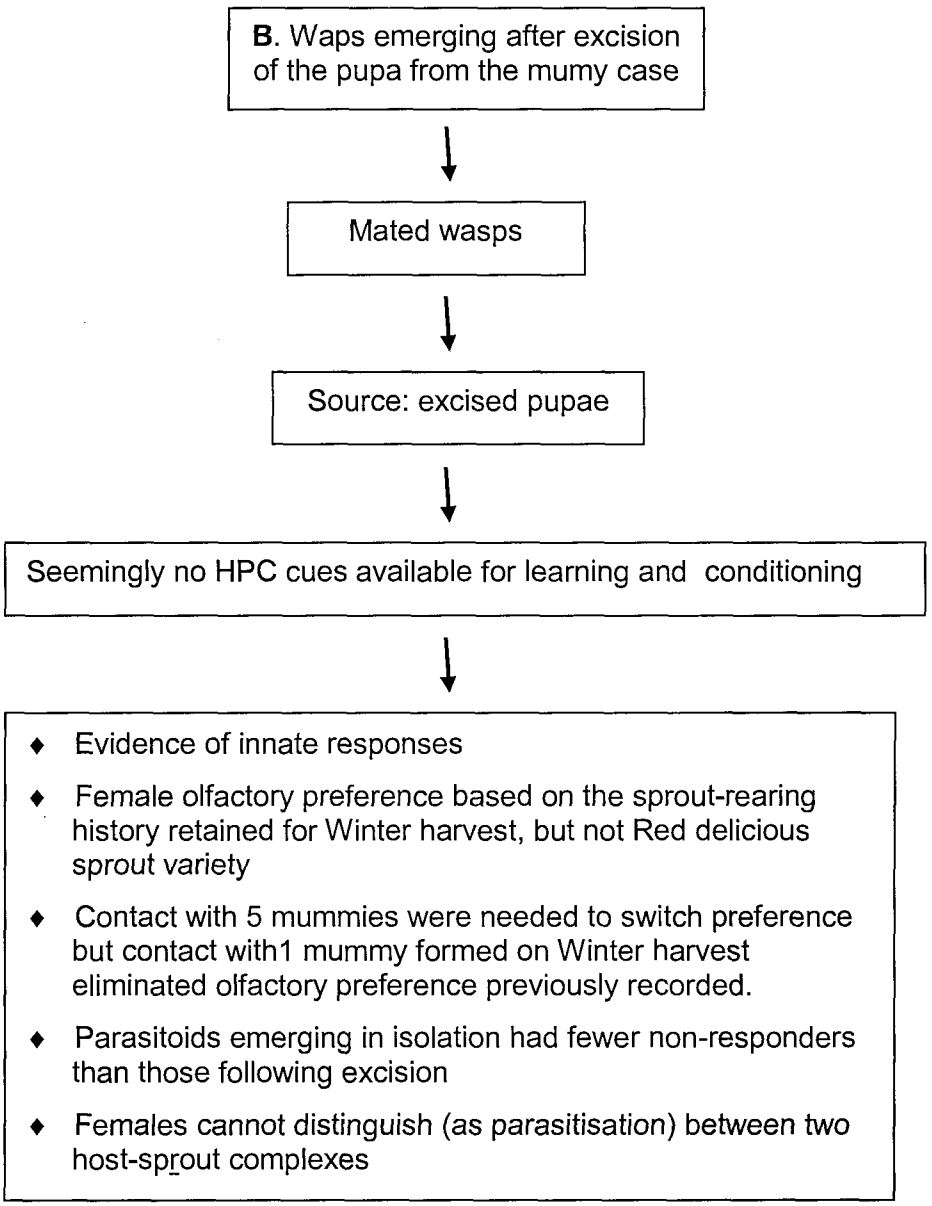
Overall, it is likely the observed interactions under controlled laboratory conditions may be of little importance in field conditions when the variation cannot be controlled (Takabayashi *et al.*, 1994) but it could important when cultivating crops in protected environments such as glasshouses. In addition, the reduction in parasitisation by diet-reared adult parasitoids clearly indicates that this rearing method would add nothing to effective biocontrol in the field or glasshouse. It is, however, unlikely that commercial production of aphid parasitoids will ever develop using diet-reared hosts (Fig 5.4; Chapter 5), although, it is a useful experimental tool.

# CHAPTER 7: General discussion

## 7.1 Summary

The work in the present sudy demonstrates the enormous infuence of rearing history in the olfactory responses of the parasitoid *Aphidius colemani* and the following diagrams (**A**, **B** and **C**) illustrate the key findings:




B. Waps emerging after excision of the pupa from the mumy case
Mated wasps
Source: excised pupae
Seemingly no HPC cues available for learning and conditioning
♦ Evidence of innate responses
♦ Female olfactory preference based on the sprout-rearing history retained for Winter harvest, but not Red delicious sprout variety
♦ Contact with 5 mummies were needed to switch preference but contact with1 mummy formed on Winter harvest eliminated olfactory preference previously recorded.
♦ Parasitoids emerging in isolation had fewer non-responders than those following excision
♦ Females cannot distinguish (as parasitisation) between two host-sprout complexes

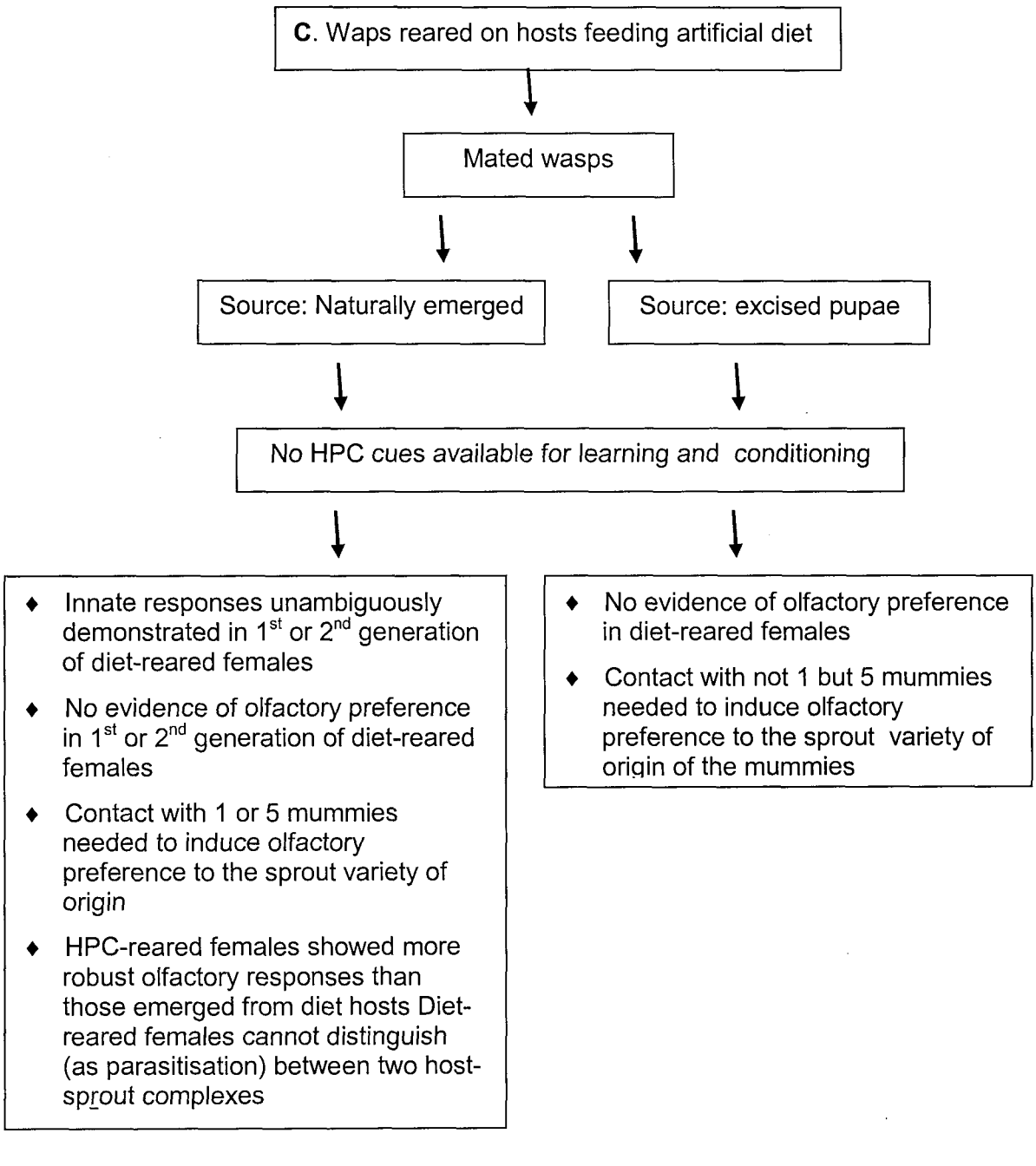



**Fig. 7.1** Schematic representations (**A**, **B** and **C**) of olfactory responses of *Aphidius colemani* parasitoids taking into account their physiological condition, handling methods, mating status, and rearing medium.

## 7.2 Assessment of techniques

Previous studies on olfactory preferences of aphid parasitoids have involved anaesthesia at various stages during the handling procedure as it is difficult to manipulate adult parasitoids but the experience acquired in parasitoid handling can reduce the need for anaesthesia. Brief exposure to $CO_2$ may not be lethal, whereas brief chilling can be (van Emden *et al.*, 2002) and in that study anaesthesia was

considered inevitable, even just before the bioassays, to facilitate and reduce the handling time. However, as adverse effects on insect behaviour and learning may occur after anaesthesia with $CO_2$ (Nicolas & Sillans, 1989), it was only used in the beginning of protocols in the present study (i.e. for sexing adult wasps 24 h prior to bioassays) (section 3.3.1). A technique of drawing the parasitoids into the central arena was developed to eliminate anaesthesia at that stage (section, 2.7.2), which was efficient in terms of time and adult parasitoid losses at this final stage of the experimental procedure.

In the present study, detached leaves from plants of the same age were used with the leaves excised just before the beginning of each test but the volatile emissions may not be constant during a single bioassay. Leaves excised just prior to the bioassays should have the same quality as the leaves of the whole plant for a limited period of time (Steinberg *et al.*, 1992).

All the plants used for maintaining the insect cultures and for the olfactometer bioassays were grown together in a glasshouse, without any partition but on separate benches for different plants and/or sprout varieties. Insect (aphid and parasitoid) cultures on different sprout varieties (Winter harvest or Red delicious) were kept in cages placed in the same controlled temperature and humidity room. It is known that neighbouring plants can affect the olfactory responses of *A. colemani* (van Emden *et al.*, 2002) but it was not possible to grow each plant species/variety in isolation and the robustness of the olfactory responses indicates that it was not a problem in the current study.

## *7.3* General conclusions and parasitoid behavioural mechanisms

*Aphidius colemani* is commercially available as biological control agent. A better understanding of the role played by inherited traits and the influence of adult learning experiences is vital for developing behavioural manipulation strategies. The current study investigates the olfactory responses of this aphid parasitoid to plant odours attempting to give insights to the parasitoid's searching behaviour. Initially parasitoid responses were investigated after natural emergence within the parasitoid culture and further adult preferences were identified after mummy isolation from the plant, pupal removal from mummy cuticle and artificial-diet-reared aphid hosts. Thus, in this study the parasitoid behaviour was investigated using a different approach since the available experience was gradually excluded from the tritrophic system used, the aphid-host *M. persicae* and Brussels sprout cultivars from the family Brassicaceae.

Parasitoids that emerge naturally can employ many strategies to locate their hosts (Powell, *et al.*, 1998). The parasitoids more readily search for suitable hosts after

they have satisfied nutritional and mating requirements (Vinson, 1998). As soon as they emerge naturally they can experience in the surrounding habitat the presence of general plant odours and herbivore-induced synomones using them as conditioned stimuli (CS) which they can associate with contact of host and its by-products (i.e. unconditioned stimuli US) (Vet *et al.*, 1995; Hilker & Meiners, 2006). Parasitoid responses after direct collection from plant culture demonstrate a clear preference for the odour of the Winter harvest variety, the plant of origin. The age of the parasitoids collected directly from the culture cages was not known and after emergence they could have possibly contacted the aphid host on the plant, its products and they may have had oviposition experience, factors that can offer learning experience that influence parasitoid responses (Steinberg *et al.*, 1993; Du *et al.*, 1997; Storeck *et al.*, 2000). In this context, oviposition experience offers the opportunity for further reinforcement of conditioning and the learned stimuli used for host habitat location (Poppy *et al.*, 1997; Girling *et al.*, 2006). Kalule & Wright (2004) reared *A. colemani* on three cabbage cultivars and after natural emergence from mummies isolated off the plant (i.e. placed into small vials) the parasitoids showed a preference for the cabbage variety on which they were reared. In that case, aphid-infested whole plants were used as the odour source.Storeck *et al.*, (2000) showed that whilst naturally-emerging *A. colemani* had an olfactory preference for the natal plaqnt, either Chinese cabbage and oilseed rape, no preference was detected in adults emerging after pupal excision. However, the researchers used only aphid-infested leaves whereas in the present study only uninfested detached leaves were used in the bioassays to test the limits of parasitoid sensitivity to olfactory cues since infested plants can induce greater parasitoid responses than uninfested ones (Bukovinszky *et al.*, 2005). No preference was shown for adult wasps emerging after pupal excision also in three other studies (van Emden *et al.*, 1996; van Emden *et al.*, 2002; Douloumpaka & van Emden, 2003). Those studies used similar experimental systems to those used in the present study but there were certain differences in protocols. Pupal excision was performed from the ventral, rather than the dorsal side of the mummy used in the present study which made the process faster (i.e. removal of 1 pupa per minute). Other differences involved the olfactory stimuli used and certain aspects in methodology, for example using anaesthesia ($CO_2$), different types of olfactometers, whole plants or detached leaves as well as the time allowed for the parasitoid to respond to given odours (10 *vs* 30 min).

The data from naturally-emerged and excised parasitoids demonstrated that, naturally-emerged females respond more readily than females emerging after excision of pupae, in all comparisons conducted for olfactory preference and parasitisation, indicating that the excision process might have affected subsequent female responses to the odour choices offered in the olfactometer or perhaps providing evidence of a

learning hierarchy of the developing parasitoid pupa in the aphid host. The existence of a learning hierarchy and innateness in behavioural responses could stem from the concept of dietary specialization and infochemical use in natural enemies (Vet & Dicke, 1992). According to this concept the degree of innate or learned responses of carnivores (in both specialists and generalists parasitoids and predators), is based on the extent of the dietary specialization on their host/prey species which can be also specialists or generalists on host plants or feeding substrates. However, this theory was modified in that learning is used mostly by generalists and all carnivores (both specialists and generalists) are expected to use chemical cues for foraging by learning or innate mechanisms (Steidle & van Loon, 2003).

When wasps were liberated inside cages to locate, parasitise available hosts and make a choice between two host-sprout complexes they were facing the reliability-detectability problem (Vet *et al.*, 1991). Several steps have been previously identified for successful parasitism (Vinson, 1985; Godfray, 1994). Since experience can improve the abilities of female parasitoids in host selection (Drost, et al., 1988; Vinson, 1998) the more experienced adults (i.e. naturally emerged in the host's habitat) not only made a choice towards the plant on which they were reared (Vet & Dicke, 1992; Steidle & van Loon, 2003) but also displayed the highest number of mummies formed on the plants compared to those resulting from parasitoids emerged after excision and from hosts reared on artificial diet.

When parasitoids emerged from diet-reared hosts it can be assumed that the major mechanism female wasps can use for host-habitat location is their genetic predisposition (innate responses) (Messing & Rabasse, 1995) which can be modified by experience and conditioning to host-related stimuli (Poppy *et al.*, 1997). Inexperienced parasitoids may exhibit innate responses but it is assumed that the range as well as the intensity of such responses are limited (Vinson, 1998). In the present study the overall responses to odour seem to be weaker than those from naturally-emerged wasps since the percentage responding is smaller. The data collected also show that induced alterations in the olfactory behaviour of parasitoids is evident when contact with mummies is allowed.

A plant odour was always preferred over clean air even when parasitoids developed in diet-reared hosts for a second generation. This result was obtained in a consistent manner and clearly demonstrates that such responses to general plant odour may be innate. Innate preferences of *A. colemani* revealed (chapters 3, 4, and 5) could be also found in other tritrophic systems. For example, white mustard, Brussels sprout, a feral crucifer species and malting barley odour were all preferred in Y-tube bioassays over clean air by *Diadegma semiclausum* (Hellén) a parasitoid of *Plutella xylostella* L. moths (Bukovinszky *et al.*, 2005) which were similar responses to those

previously described in the literature as theory of "misunderstanding" (Takabayashi *et al.*, 1991).

Adult responses may be influenced by the larval rearing environment (Hopkins, 1917) in that a memory of the larval food can be retained by adult insects (Thorpe & Jones, 1937; Thorpe, 1939). However, it also seems that the adult parasitoid can learn the host's plant odour upon eclosion of the imago from the pupa (Corbet, 1985; Hérald *et al.*, 1988; Vet & Groenewold, 1990; Turlings *et al.*, 1993) and the distinction between pre- and post-imaginal environments is difficult (Jaenike, 1982; Jaenike, 1988).

According to Hopkins' host selection principle (HHSP) (Hopkins, 1917) insect species exhibit a persistent preference for the host species they are reared on for many generations and perhaps chemical cues present in the host species can be transferred from the larva to the adult stage and influence the adult behaviour after emergence from the host on or in which the adult developed. Despite the evidence provided to support this theory in its early days (Thorpe & Jones, 1937; Thorpe, 1939; Hershberger & Smith, 1967) the implications of this theory on the pre-imaginal conditioning of insects have been studied further and provide no evidence for the theory (Courtney & Kibota, 1990; van Emden *et al.*, 1996).

Alternative theories have arisen and the "neo-Hopkins host selection principle", suggests that exposure of imagos to a specific host can affect adult subsequent responses (Jaenike, 1983). It should also be taken into consideration that responses may originate from influences during the larval stage of the parasitoid or contamination of the larva/pupa with traces of chemical cues present in the host insect and can influence adult responses which is known as the "chemical legacy hypothesis" (Corbet, 1985). The latter theory may be applicable in the lepidopteran situation with *Trichoplusia ni* and *Plutella xyllostella* larval feeding experiences on artificial diet and cabbage leaves while HHSP could also be considered (Akhtar & Isman, 2003).

Barron (2001), suggested that it may be difficult to distinguish between responses originating from the larval or the early adult experiences during the insect's development (Corbet, 1985). The latter, could be only assessed in the present study if it was feasible to obtain adult wasps after excision of the larva prior pupation. To exclude, however, the possibility of chemical legacy the larva could be rinsed with water or other solvents (Akhtar & Isman, 2003) but the removal of chemical traces that can be found within the pupal cuticle, the body of the imago and the surface of the pupa (Corbet, 1985) cannot be a certainty. Adult, rather than larval conditioning can offer a greater influence on behaviour of insects (Barron & Corbet, 2000). This was shown in longhorned beetles, *Dectes texanus,* on sunflower and soybean plants and no evidence of HHSP was found since the larval host plant did not influence adult oviposition behaviour (Michaud & Grant, 2005). In addition, no evidence supporting the

HHSP principle was found in the aquatic weevil *Euhrychiopsis lecontei* Dietz when feeding larvae on northern or Eurasian watermilfoil *Myriophyllum spicatum* L. (Solarz & Newman, 2001).

Douloumpaka and van Emden (2003) suggested that the parasitoid-produced silk may have chemical cues that affect the imago on eclosion. The silk surrounds the pupa inside the aphid cuticle and is likely to remain there after pupal excision. Another possible source of chemical information is the pupal cuticle. The results of current study after excision, may indicate cues on the silk or the pupal cuticle could have elicited the significant response in Winter harvest-reared parasitoids but not in Red delicious-reared insects. In some parasitoid wasps the host's larval instars may be important affecting adult parasitoid responses and silk and frass cues elicit searching behaviour of *Cotesia glomerata* (Mattiacci & Dicke, 1995).

Chemical cues associated with the prepupal integument may diffuse from inside the cocoon to the outside where they could be recognised as contact kairomones by the parasitoid *Dahlbominus fuscipennis*. In this case silk gland secretions of the parasitoid larvae failed to evoke positive adult responses of this parasitoid, indicating cues were not present in the silk glands, but the host's silk affected behaviour (Rostas *et al.*, 1998). In other parasitoid species it is possible that their own cocoon silk may act as a reservoir of host cues. A simple experiment to test this hypothesis could involve the use of another parasitoid species *Praon spp.*, which spins the silky cocoon outside and separate from the aphid body. This could provide further insights for the comparison of the conditioning potential towards certain plant odours as in previous experiments of the present study. Bioassays with excised female parasitoids could examine whether contact provided with the empty mummy case, the silk or moulted aphid cuticle could be among possible sources of chemical information utilised by aphid parasitoids as olfactory stimuli. However, a similar series of experiments to those listed in this chapter would be needed to form the basis of *Praon spp.* olfactory responses first.

Parasitism can alter the chemical composition of the parasitised host in favour of the developing parasitoid and silk comprises a possible source of kairomones mostly for pupal parasitoids since little work has been done on the silk production by parasitoid larvae (Quicke, 1997). Conditioning, according to chemical legacy Corbet (1985), may also apply in this context since contamination with cues present in the silk is possible as the developing larva and early stage pupa may contact chemical stimuli on the internal surface of the mummy's cuticle which can be absorbed by the developing parasitoid via the larval/pupal epidermis in minute quantities before excision. However, the preliminary experiment on the excision of the initial larval/pupal stages of developing parasitoids inside the mummy showed zero survival and thus no adults

were obtained. Nevertheless, a suitable method could be developed in the future and extend these studies.

Another study, (van Emden *et al.*, 1996), using wheat cultivars, suggested that there was no evidence for aphid parasitoid olfactory responses that support Hopkins' host selection principle (HHSP) but the current study, using Brussels sprout varieties, provided supporting data (section 4.3.4). Thus, female parasitoids reared on Winter harvest but excised as pupae retain a preference for plant of origin, however, similar studies on those reared on Red delicious, do not. Why the result differs between the sprout varieties remains unknown. This finding might also further corroborate the "chemical legacy hypothesis" based on "neo-Hopkins host selection principle" but the Hopkins's principle itself cannot be totally discounted. For example, the induction of host-plant olfactory preferences have recently been demonstrated in granary weevils with the evidence presented favouring more the Hopkins host selection principle rather than the chemical legacy or the neo-Hopkins principle (Rietdorf & Steidle, 2002). In addition, following a previous study which provided evidence that pre-exposure to a narrow range of menthol concentrations can influence subsequent responses of fruit flies (Barron & Corbet, 1999), true pre-imaginal learning has been shown recently in a ecto-parasitoid wasp where an alien cue (menthol) appears to be recognised by the adults when their larval stages where exposed to this volatile compound (Gandolfi *et al.*, 2003). Although, the latter seems to be the best demonstration of HHSP in recent years it fails to state whether the pupae were removed from the host on pupation. If not, contact with menthol absorbed on host cuticle is possible. Overall, the Winter harvest results are in agreement with some recent studies (Barron & Corbet, 1999; Barron, 2001; Rietdorf & Steidle, 2002; Akhtar & Isman, 2003; Gandolfi *et al.*, 2003) which support HHSP while the Red delicious results agree with others in not favouring HHSP (van Emden *et al.*, 1996; Solarz & Newman, 2001; Michaud & Grant, 2005). Thus, almost a century after HHPS was suggested it is still debated.

The Winter harvest variety was initially chosen for the present study in order the results would be comparable to the work Douloumpaka and van Emden (2003) whereas the choice of Red delicious and the other two sprout varieties was done by chance. The variation in the responses recorded between different sprout varieties under similar adult manipulating conditions and within each experiment may indicate some intraspecific variation in the crucifer odours (Bukovinszky *et al.*, 2005) used in the current study. That is, Winter harvest may possess different 'strength' than Red delicious in terms of parasitoid olfactory responses. The current study emphasises the conclusive remark by Barron (2001) that a clear definition of what can, or cannot be, explained by the original Hopkins principle is needed.

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