WAGENINGEN UNIVERSITY

LABORATORY OF ENTOMOLOGY

# Host discrimination by *Cotesia glomerata* (L.) (Hymenoptera: Braconidae), a parasitoid of *Pieris brassicae* (L.) (Lepidoptera: Pieridae), as affected by experience

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| No: | 09.04 |
| Name: | Linda Heilmann |
| Period: | January 2004 – July 2004 |
| Thesis: | F050-707 |
| 1e Examinator: | dr. ir. Joop A. van Loon |
| 2e Examinator: | dr. Nina E. Fatouros |

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# 1. Introduction

## 1.1. Host discrimination and superparasitism

The ability of parasitoids to distinguish between healthy and parasitized hosts and/or between hosts with different numbers of parasitoid larvae is called host discrimination (van Lenteren *et al*., 1978; Van Lenteren, 1981). Host discrimination at close range has been found for an estimate of 150-200 species of hymenopteran parasitoids (van Lenteren, 1981). Since host discrimination is so common, it probably is the result of natural selection whereby individuals which are able to discriminate between healthy and parasitized hosts have an advantage over individuals which can not discriminate.

For solitary parasitoids this advantage is more obvious than for gregarious ones. Solitary wasps can only develop successfully as a single parasitoid larva per host because the second larva will almost always die during intraspecific competition (Van Alphen & Visser, 1990). Gregarious parasitoids, like *Cotesia glomerata* (L.) (Hymenoptera: Braconidae), the parasitoid wasp studied, on the other hand can successfully develop in a brood of more than one larva in a single host (Godfray, 1994). Though there is a trade-off between the number of offspring and the size of an individual wasp, total offspring fitness may increase to an optimum brood size (Gu *et al*., 2003; Harvey, 2000). This optimum can be reached by a single clutch by a single female wasp or by two or more clutches by one female or conspecific females. This is true as long as the time interval between ovipositions is not too long, since then the larvae of the second clutch may be outcompeted by the older larvae which may have used up some of the resources (Godfray, 1994). The second female can adjust the sex ratio to a higher proportion males to limit the negative effects of a larger brood on their reproductive success (Gu *et al*., 2003; Tagawa, 2000). Parasitizing already parasitized hosts is called superparasitism, and has been found for many parasitoid wasps including *C. glomerata* (Van Lenteren, 1981; Van Lenteren *et al*., 1978; Van Alphen & Visser, 1990; Le Masurier, 1990). In the field superparasitism rates of 20-30% for *C. glomerata* on *Pieris* hosts were found (Gu *et al*., 2003; Tagawa, 1992), indicating that superparasitism is quite common in this species.

Superparasitism was long thought to be in contrast with host discrimination, but now it is known that superparasitism and host discrimination go together: wasps which discriminate between healthy and parasitized hosts can still superparasitize instead of rejecting a host. This is also the case for *C. glomerata*. The ability of *C. glomerata* to discriminate between parasitized and unparasitized hosts is shown by longer oviposition times in unparasitized hosts (Ikawa & Suzuki, 1982), rejection of parasitized hosts (Le Masurier, 1990), smaller clutches laid in parasitized hosts (Kusano & Kitano, 1974; Tagawa, 1992) and preference behaviour for unparasitized hosts from a distance (Fatouros *et al*., 2005). Despite this ability to discriminate, superparasitism is common for

*C. glomerata*, indicating that in some circumstances this might be adaptive. Naïve wasps may superparasitize because they do not yet have an idea of the proportion parasitized and unparasitized hosts in their environment. Parasitizing the first host they encounter even if already parasitized, restraints wasps to reject hosts in an environment with a low proportion of unparasitized hosts (Van Lenteren, 1981). Or as Van Alphen & Visser (1990) stated, at least some offspring is secured by not discriminating.

Other situations in which it is likely that superparasitism is adaptive is when wasps only encounter parasitized host for a long time or when the overall density of hosts in the environment is low (Van Lenteren, 1981; Van Alphen & Visser, 1990; Ikawa & Okabe, 1985; Vos & Hemerik, 2003; Stephens & Krebs, 1986). The reproductive success of parasitoid wasps is believed to be a function of the number of eggs per host/patch and the time needed to find and parasitize a host (Stephens & Krebs, 1986). If the search time increases, to optimize total reproductive success per time unit, it may be beneficial to oviposit parasitized hosts and lay smaller clutches in them than to lengthen search time more by searching for unparasitized hosts. The encounter of many parasitized hosts indicates a low proportion of unparasitized host which is reflected in longer search times between hosts. Also a low overall density of hosts is characterized by long search times. That search time can have an influence on the behaviour of parasitoid wasps was shown for *C. glomerata*. Wasps were found to lay more eggs when the time interval between oviposition experiences increased (Ikawa & Suzuki, 1985).

To facilitate host discrimination parasitoids need reliable cues which indicate that a host has been parasitized. For long it was thought that wasps can only determine the state of a host on encounter by changes in hemolymph composition after oviposition (Ganesalingam, 1974; Hoffmeister, 2000; Nufio & Papaj, 2001) or by marking pheromones left by other females (Godfray, 1994; Van Lenteren *et al*., 1976). Fatouros *et al*. (2005) showed that *C. glomerata* already in the first phase of host searching can discriminate hosts by using plant volatiles. Also *Apoanagyrus lopezi* (Hymenoptera: Encyrtidae) may use plant volatiles to discriminate between parasitized and unparasitized mealybugs from a distance, though volatiles from the second trophic level alone also trigger host discrimination in *A. lopezi* (Van Baaren and Nénon, 1996; Souissi *et al*., 1998). The study on *C. glomerata* shows that plant volatiles can be herbivore-state specific (=parasitized or unparasitized) and that wasps can respond to these differences (Fatouros *et al*., 2005). The importance of this in the process of host searching is explained in the next chapter.

## 1.2. Host searching by *Cotesia glomerata*

### 1.2.1. Host microhabitat location

In a natural environment the studied tritrophic system, *Brassica oleracea* L. var. *gemmifera* (Brassicaceae) - *Pieris brassicae* L. (Lepidoptera: Pieridae) – *C. glomerata* does not stand on its own, but is part of a much larger ecosystem with numerous interactions between all kinds of species. *Cotesia glomerata* faces the seemingly impossible task to find its small host in this collection of odours, colours and shapes. Several steps in host searching can be identified. First of all, parasitoid wasps have to find the host microhabitat (habitat location phase). Then parasitoids have to find the host within this microhabitat (host location phase) and have to determine whether the host is suitable for parasitization. Finally the host is parasitized or rejected (host acceptance phase) (Vet *et al*., 1995).

Because finding a host is directly linked to the reproductive success, it is expected that natural selection has led to optimal host searching strategies in each step. As said above, one important factor that determines the reproductive success is the time needed to find and parasitize a host. Time can only be spent once and a long search time for a single host or clutch could mean there is no time left to find more hosts or for example to find food. It is therefore likely that strategies have evolved that limit search time in every step (Stephens & Krebs, 1986).

To limit search time wasps need both reliable and detectable cues (Steinberg *et al*., 1993; Vet & Dicke, 1992; Vet *et al*., 1991; Dicke, 1999). Herbivore-derived stimuli are direct indicators of host presence and are therefore the most reliable cues for a parasitoid wasp. However although these cues are reliable, they are hard to detect from a distance. First, herbivores are under constant selection pressure to avoid attracting predators and parasitoids and therefore probably have evolved to emit minimal amounts of species-specific volatiles. In addition, plant-emitted blends are much more abundant due to the larger plant biomass and the amount of herbivore-derived volatiles is probably negligible in the total volatile blend. Plant- or host microhabitat-derived cues on their turn are not very reliable indicators of host presence, because not all food plants have to be infested (Vet & Dicke, 1992).

So parasitoids are faced with a problem: the most reliable cues are not detectable and the most detectable cues are not reliable. To overcome this reliability-detectability problem, parasitoids have evolved several strategies (Vet *et al*., 1991). First they can make use of an infochemical detour, or in other words they use the more distinct information from infochemicals of other herbivore stages. Also they may focus on stimuli associated with the interaction of the herbivore and its food (e.g. herbivore-induced volatiles). And third, they may learn to link easy-to-detect stimuli to reliable but hard-to-detect stimuli.

Female *C. glomerata* wasps use the second and third strategy to optimize host searching (Mattiacci *et al*., 2001; Steinberg *et al*., 1993; Geervliet *et al*., 1996; Blaakmeer *et al*., 1994; Geervliet *et al*., 1998b; Bleeker *et al*., 2006). In this paragraph the second strategy is treated. The third strategy is discussed in §1.3.

All plants emit volatiles, but upon damage by herbivores extra volatiles are emitted, partly due to the release of green-leaf volatiles from the damaged area and partly due to induction of herbivore-induced volatiles (Dicke *et al*., 1990; Turlings & Tumlinson, 1992; Mattiacci *et al*., 2001; Poecke *et al*., 2001). The green-leaf volatiles are emitted locally right upon damage, while the induced volatiles take a while before being emitted (Turlings *et al*., 1998; Scascighini *et al*., 2005), but can be systemically emitted throughout the whole plant. Systemically induced plants were found to produce 2.5 till 30 more volatiles (depending on the compound) than newly-infested plants (Mattiacci *et al*., 2001). The induction of herbivore-induced volatiles is triggered by contact of regurgitant from the host gut with the wounded plant tissue (Turlings *et al*., 1992, 1993a, 1995). An active compound in the regurgitant of *P. brassicae* is the enzyme ß-glucosidase (Mattiacci *et al*., 1995). The composition of the induced volatile blend can differ both quantitatively and qualitatively from intact plants or plants damaged by other means. In the blend of herbivore damaged Lima bean and corn compounds have been identified that are not present in the blend of intact or mechanically damaged plants (Dicke *et al*., 1990; Turlings *et al*., 1990). The differences in cabbage and Brussels sprouts in particular, are probably mainly quantitative (Mattiacci *et al*., 1994; Agelopoulos & Keller, 1994a; Geervliet *et al*., 1997).

Many parasitoids are attracted to the source of herbivore-induced plant volatiles from a distance (e.g. Agelopoulos & Keller, 1994b; Mattiacci *et al*., 2001; Powell *et al*., 1998; van Poecke *et al*., 2001). Naïve *C. glomerata* wasps were ten times more attracted to systemically induced leaves than to newly-infested leaves without herbivore-induced volatiles (Mattiacci *et al*., 2001) and preferred herbivore-infested plants over non-infested plants (Sato, 1979; Steinberg *et al*., 1993; Mattiacci *et al*., 1994; Scascighini *et al*., 2005, Geervliet *et al*., 1996; Blaakmeer *et al*., 1994).

Since induced volatiles are direct indicators of host presence they are more reliable than non-induced volatiles. Of course, the more specific the volatiles, the more reliable they are. Some studies have shown that the differences in herbivore-induced volatiles are not only plant-specific, but can also be herbivore-species (Du *et al*., 1996; Powell *et al*., 1998), herbivore-stage (Takabayashi *et al*., 1995, 1998) and herbivore-state specific (Fatouros *et al.,* 2005). Female *C. glomerata* wasps discriminate between different plant species (Geervliet *et al*., 1996) and even between plants with parasitized and unparasitized hosts (Fatouros *et al.,* 2005), but no discrimination between different herbivore-species (Geervliet *et al*., 1996; Shiojiri *et al*., 2000b) and herbivore-stages was found (Mattiacci & Dicke, 1994). If wasps are capable of perceiving the differences between different herbivore stages, states and species, it gives them the possibility to

already in-flight determine the kind of host present in the host microhabitat and to make a decision whether to land or to continue searching for another microhabitat. Aborting the search in this early stage saves time since in vain searching or ovipositing in step two (host location within the microhabitat) and three (host acceptance) in the host searching behaviour is prevented.

### 1.2.2. Host location and host acceptance

Once landed in a host microhabitat, *C. glomerata* females start walking slowly while touching the leaf with its antennae. When wasps encounter leaf damage caused by host larvae feeding they really get excited which is shown by raising their wings (Sato, 1979). In this phase of host searching host-related cues such as host saliva, faeces and silk stimulate wasps as well as leaf damage (Sato, 1979; Mattiacci & Dicke, 1995a).

Because of the close-range the information about the host is more specific and therefore more reliable than at long-range when only plant volatiles are detectable. In-flight *C. glomerata* does not discriminate between plants infested by different host-species or different hosts-stages, but it does after entering the host microhabitat, still without direct host contact (Mattiacci & Dicke, 1995a; Shiojiri *et al*., 2000a). Based on the information found in the direct environment of the host, the wasp can decide whether to continue searching or to abort the current search. In case it continues searching within the patch and the wasp encounters a host, the host acceptance phase begins.

Upon encounter with the actual potential host caterpillar, *C. glomerata* first contacts it with its antennae for an external examination. When the host is approved, it inserts its ovipositor in the body wall of the caterpillar for an internal examination. After up to two seconds of probing behaviour during which eggs are never laid, the wasp keeps its ovipositor inserted for a further 5-20 seconds to oviposit its eggs or it rapidly withdraws the ovipositor and walks away (Le Masurier, 1990). Withdrawing after the initial probing period is thought as a sign of rejection.

Even in this late stage of host searching, rejecting a host can save time that is otherwise spent on unsuccessful ovipositing. Besides saving time it can also prevent wastage of eggs (Van Lenteren, 1981).

## 1.3. Learning

### 1.3.1. Learning in parasitoid wasps

Before emergence, parasitoids already have contact with the host they developed in and emerge from and may learn certain characteristics about their host during this phase (pre-adult learning).

This learning may influence the wasp's adult behaviour, but it is hard to distinguish learned behaviour in the pre-adult from truly naïve behaviour (Turlings *et al*., 1993b; Vet & Groenewold,

1990; Vinson, 1998). Therefore wasps are called naïve when they have not had an obvious experience yet.

Wasps are supposed to emerge with an innate set of responses to environmental cues. These responses largely determine the wasp's innate behaviour. Vet *et al*. (1990, 1995) developed a response potential model in which all stimuli are ranked according the potential response they evoke. Strong natural selection is mainly expected when stimuli are constant between generations and therefore it is expected that these stimuli evoke strong innate responses with little variation. The opposite is expected for stimuli that change a lot in between generations (Vet *et al*., 1990, 1995).

Besides their innate responses to stimuli, parasitoid wasps, like many other insects, have the ability to change their innate responses to stimuli during their life span by learning. Broadly two forms of learning can be distinguished, priming or sensitization and preference learning (Turlings *et al*., 1993b).

Priming makes wasps more responsive to stimuli in general after exposure to an innately recognized environmental stimulus (Turlings *et al*., 1993b). The effect of priming is immediate and short lived (Turlings *et al*., 1993b), though in *C. glomerata* sensitization can still be effective after one day as was shown by Bleeker *et al*. (2006). In some studies priming or sensitization was found to be solely responsible for the enhanced response of parasitoid wasps (Dicke *et al*., 1990; Kaiser & Cardé, 1992). In the case of preference learning, wasps learn to (better) respond to experienced odours. A form of preference learning is associative learning. This form of learning may take longer to establish, but also is retained longer than sensitization. For *C. glomerata* a long term memory trace consolidated within four hours (Smid *et al*., 2007) and was still present after five days (Bleeker *et al*., 2006). Associative learning as well as sensitization can occur in every stage of host searching behaviour (Vinson, 1998), but here the focus lies on learning in the first phase of host searching, the host habitat location phase.

During associative learning unconditioned stimuli (US) are associated with conditioned stimuli (CS). Most research has been performed on rewarding unconditioned stimuli, but the US can also be unrewarding. The latter can result in a negative association (Vet *et al*., 1995) and may result in a reduced responsiveness (McAuslane *et al*., 1991). The weaker the innate response to the CS and the stronger the innate response to the US, the stronger the effect of learning is expected to be (Vet *et al*., 1995). In host searching, the host or its by-products are associated with the experienced plant odour and this odour can subsequently be used as a cue in host searching. In §1.2.1 this was already mentioned as a strategy to overcome the reliability-detectability problem. The response to detectable and unreliable plant-odours may initially be weak or non-existing, but are learned to be a reliable indicator of host presence. Indeed many parasitoid wasps, including *C.*

*glomerata*, can learn to associate plant odours to an experience (for reviews see Vet *et al*., 1995; Dicke, 1999).

Two kinds of associative learning can be distinguished: α-conditioning which occurs when already existing (weak) innate responses are altered and ß-conditioning which occurs when new stimuli are learned (Vet *et al*., 1990). By α-conditioning preferences within the existing stimulus set can be developed, while with ß-conditioning the stimulus set can be expanded for example with new plant odours (Vet *et al*., 1991). ß-conditioning means that individual wasps are not genetically bound to certain stimuli.

Learning comes with both energetic (formation and maintenance of a memory) and ecological (time needed to optimize the response and vulnerability to mistakes) costs. Because of the costs, learning is only expected when the benefits compensate for the costs (Vet & Dicke, 1992; Stephens, 1993). This is thought to be true for generalists which are born with a broad range of responses to different host species. For generalists the environment can change a lot among generations of wasps while the environment within the life span of a single wasp can be quite constant. It is then when learning is expected the most (Stephens, 1993). Because of the many innate responses wasps may spend a lot of time searching for non-existing hosts. When they find a host, this host may be a good predictor of other hosts of the same species. This may especially be true for *C. glomerata* searching for *P. brassicae*, since *P. brassicae* selects stands of the same plants to lay its eggs on (Le Masurier, 1994). By learning the experienced odour and subsequently searching for this odour (temporary specialization), generalist wasps can substantially decrease their search time compared to naïve wasps. Indeed, better learning capabilities are found for the generalist *C. glomerata* than for the closely related specialist *C. rubecula* (Geervliet *et al*., 1998b; Bleeker *et al*., 2006).

Different learning strategies may be used separately, but also can be used simultaneously. *Cotesia glomerata* for example is capable of learning new plant odours and even shift their preference to formerly unattractive plant odours after experience with the plant-host system (Geervliet *et al*., 1998b). Bleeker *et al*. (2006) found that this preference shift of plant species after an oviposition experience with the newly learned odour partly can be contributed to associative learning and partly to sensitization. Besides ß-conditioning and sensitization also α-conditioning was found for *C. glomerata*. Naïve *C. glomerata* females are equally attracted to plants infested by *P. brassicae* and *P. rapae* feeding on Brussels sprouts. However, after a few experiences female *C. glomerata* wasps learned to prefer *P. brassicae* over *P. rapae* infested plants (Geervliet *et al*., 1998b).

The unconditioned stimulus in the last study consisted of a contact experience with a leaf on which both hosts had been feeding. This experience was sufficient for wasps to change their behaviour, so no oviposition experience or physical contact with the host was needed (Geervliet *et al*., 1998b). Also *C. rubecula* and *C. marginiventris* showed increased responsiveness to infested plants without

contacting the host itself, indicating that host-derived cues can act as an unconditioned stimulus in associative learning or sensitization (Turlings *et al*., 1989; Dmoch *et al*., 1985). The strength of this US may, however, be lower than an oviposition experience (Vet & Groenewold, 1990; Dmoch *et al*., 1985).

### 1.3.2. Completeness of the information

Though learning can improve host finding and discrimination of parasitoid wasps (see Vet *et al*., 1995; Turlings *et al*., 1993b; Dicke, 1999 for reviews), wasps could specialize on a single odour too quickly. A single host encounter may not provide the wasp with adequate information about what is available within its foraging area (Turlings *et al*., 1993b). The wasp may miss potential host microhabitats or hosts. More experiences might be needed to get an accurate view of the environment. Indeed it is observed for several parasitoid species that several experiences are required for wasps to change their behaviour (Geervliet *et al*., 1998b; Eller *et al*., 1992). Waiting too long before specializing can also be costly. Wasp may then spend a lot of time foraging in unprofitable patches which can have a direct impact on the wasp's reproductive success.

A solution for the problem may be to generalize the learned odour. Smith (1993) hypothesized that especially when differences between two conditioned stimuli are minimal, after a rewarding experience with one of them it can be adaptive to respond to both stimuli because the experienced CS also could predict rewards connected to a second closely similar CS. Vet *et al*. (1998) found for *L. heterotoma,* a parasitoid wasp on *Drosophila* species, that wasps after a rewarding oviposition experience on one of two similar substrates showed a preference for both substrates. By generalizing the learned odour, *L. heterotoma* wasps prevent missing hosts on the very similar substrate, but at the same time do not waste any time searching on substrates that differ much more and probably do not contain any hosts. If *L. heterotoma* fails to find a host in the generalized odour (=unrewarding experience) it spends almost no time in the next patch with the same odour (Papaj *et al.*, 1994). So wasps generalize the experienced odour till it is shown that this odour is not profitable. This shows that the completeness of the information can be important in the "decision"of the wasp to show discriminative behaviour.

In the current study the ability of *C. glomerata* to discriminate between plants infested with parasitized and unparasitized hosts was examined. Since the odours of these two plants are likely to be very similar, *C. glomerata* may need a "positive" oviposition experience as well as a "negative", unrewarding one to optimize preference behaviour towards the plant with the unparasitized hosts. The aim of this study was to examine whether complete information indeed makes that the preference of *C. glomerata* for the plant with the unparasitized wasps is increased.

### 1.3.3. Order of the information

Another factor that may influence the effect of learning on the behaviour of wasps is the order in which experiences are received. Different studies have resulted in contrasting conclusions with regard to the order of the experiences. In the study by Geervliet *et al*. (1998b) on the ability of *C. glomerata* to discriminate between different host species, learning was shown to enhance the preference of *C. glomerata* for *P. brassicae* after oviposition experiences on both *P. brassicae* and *P. rapae*. The order in which these experiences were given did not seem to affect this preference. In a study about patch profitability, however, Geervliet *et al*. (1998a) found that the first experienced host density has a larger effect on the preference behaviour of *C. glomerata* than the second experienced host density. In contrast, female *Leptopilina boulardi* wasps prefer the last odour learned (De Jong & Kaiser, 1992; Kaiser & De Jong, 1993). It is not quite understood what cause(s) lie(s) behind these differences. The aim of this study was to gather further insight in the effect of the order in which different experiences are received on the behavioural choices of *C. glomerata*.

## 1.4. Previous research

This study follows on the study of Fatouros *et al*. (2005) who studied the ability of the solitary parasitoid *Cotesia rubecula* (Marshall) (Hymenoptera: Braconidae) and the gregarious parasitoid *Cotesia glomerata* (L.) (Hymenoptera: Braconidae) to discriminate between plants fed upon by either parasitized or unparasitized hosts (*Pieris rapae* and *Pieris brassicae* (Lepidoptera: Pieridae) from a long distance. It was found that *C. rubecula*, after a short experience with a piece of Brussels sprouts leaf infested by healthy hosts which had been removed immediately prior to the experiment, preferred to land on plants with unparasitized caterpillars in a two-choice bioassay in a windtunnel setup. This was the case for both plants with herbivore damage and mechanical damaged plants treated with host regurgitant. Also *C. glomerata* was found to prefer plants treated with the regurgitant of unparasitized caterpillars after the short experience with the damaged leaf.
Fatouros *et al*. (2005) released female wasps of *C. glomerata* from an herbivore damaged leaf in the windtunnel and it is not clear whether the shown in-flight host discrimination was naïve or learned. The innate response of wasps may have been enhanced by sensitization and/or associative learning. In the present study it was examined whether the host discrimination from a distance was also shown by naïve wasps. Since it is thought that superparasitizing the first encountered host may be the best strategy for naïve wasps, no such innate host discrimination was expected for *C. glomerata*.

# 2. Research questions

a) Do female *C. glomerata* wasps with a) no experience (naive); b) a contact experience with the plant-host system (leaf-experienced); c) a single "positive" oviposition experience in a unparasitized *P. brassicae* (incomplete experience); d) a single "positive" oviposition on a unparasitized *P. brassicae* and a "negative" oviposition experience in a parasitized *P. brassicae* (complete experience), all on *B. oleracea*, show a preference for *B. oleracea* plants with healthy hosts over plants with parasitized *P. brassicae* hosts?

b) Do female *C. glomerata* wasps that oviposited in both an already parasitized *P. brassicae* host and an unparasitized host feeding on *B. oleracea* have learned by these experiences to (better) discriminate between *B. oleracea* plants with healthy hosts and parasitized hosts than wasps which received only a "positive" oviposition experience?

c) Do female *C. glomerata* wasps which got a "positive" oviposition experience in their host *P. brassicae* followed by a "negative" oviposition experience differ in their ability to discriminate between *B. oleracea* plants with unparasitized hosts and plants with parasitized hosts from female *C. glomerata* wasps which first received a "negative" oviposition experience followed by a "positive" one?

# 3. Materials and methods

To examine if *C. glomerata* is able to discriminate between parasitized and unparasitized caterpillars feeding on a host plant without making direct contact with the caterpillars or their (by)products, two-choice windtunnel experiments were performed whereby the wasps could choose between a leaf with parasitized caterpillars and a leaf with unparasitized caterpillars.
To test the influence of learning on the behaviour of the wasps, different treatment groups of wasps were established that had had different experiences.
Below the preparations for the bioassays are described for the plants, herbivore and the host caterpillars. At the end of this chapter the bioassay itself is described.

## 3.1. Plant treatment

### 3.1.1. Rearing

Brussels sprouts, *Brassica oleracea* L. var. *gemmifera* (Brassicaceae) were reared in a greenhouse at 20-25 °C, 50-70% relative humidity and a 16L:8D photoperiod.

### 3.1.2. Bioassay preparation

Forty-eight hours prior to the bioassay, 20 parasitized or unparasitized *P. brassicae* larvae (see §3.2.2) were transferred to the second or third leaf (counted from above) of an intact Brussels sprouts plant. Because Mattiacci *et al*. (2001) showed that volatiles emitted were leaf- and plant-age dependent, the plants used for the bioassay were of similar age (40 till 47 days), containing five till seven leaves, and were selected to be as similar as possible in their architecture, especially within one bioassay.
One day before the bioassay it was checked if the length of the circumference of the damaged area(s) on both plants was similar. In case one plant showed substantially more damage than the other, some caterpillars were removed from the plant with the most damage.
Just prior to the bioassay the damaged leaves with the caterpillars of each treatment still on it (leaf with parasitized caterpillars = PAR; leaf with unparasitized caterpillars = UNPAR) were excised and the petioles were placed in glass vials filled with water.

### 3.1.3. Treatment after the bioassay

Directly after the bioassay, the caterpillars were removed and the leaves were taped with transparent tape on a white sheet of paper. Because of the natural curvature of the leaves this often resulted in tearing of the leaves. These places were indicated by an arrow for later recognition. Also the date and the treatment given were written down on the paper. The leaves

were scanned (resolution 75 dpi, size 600x824 pixels) and saved as a .TIF file. Scion Image software (URL 1) was used to determine values (in pixels) for the surface and outline of the leaf and the surface and outline of the damage done by the caterpillars. The exact method in Scion Image has been described in Appendix I.

## 3.2. Caterpillar treatment

### 3.2.1. Rearing

Stock colonies of *P. brassicae* L. (Lepidoptera: Pieridae) were reared on Brussels sprouts in a climate room (23±5° C; 50-70% relative humidity; L16:D8). *Pieris brassicae* eggs were obtained by introducing a *B. oleracea* plant in a cage with adult *P. brassicae* for 24 hours to allow egg deposition. After about five days *P. brassicae* caterpillars hatched from the eggs.

### 3.2.2. Preparation for the windtunnel bioassay

Four days (96 hours) before the bioassay, a small clutch of early 1st instar *P. brassicae* caterpillars on a piece of Brussels sprouts leaf in a Petri-dish was offered to five 5-8 days old *C. glomerata* female. The wasps used for this purpose came directly from the rearing cage or were used in the bioassay earlier on (see discussion of confounding factors, chapter 5.1). It was observed which caterpillars were stung by the wasps and after six ovipositions from each wasp, it was removed from the leaf. The 36 supposedly parasitized caterpillars were then transferred to a clean plant of *B. oleracea* on which they were reared till 48 hours prior to the bioassay (see §3.1.2). On another plant 36 unparasitized caterpillars were reared. The plants were kept in the same climate room (20-25 °C, 50-70% relative humidity, 16L:8D).

### 3.2.3. Treatment and inspection after the bioassay

The parasitized caterpillars used for the PAR-treatment in the bioassay were reared in a rearing cage in a climate room and after approximately a week when the larvae of *C. glomerata* egressed from the caterpillars and made cocoons, it was checked whether the caterpillars had indeed been parasitized. The number of cocoons per caterpillar was counted.

## 3.3. Parasitoid treatment

### 3.3.1. Biology of Cotesia glomerata

*Cotesia glomerata* is a gregarious larval endoparasitoid of non-hidden Lepidoptera (Laing & Levin, 1982). Under laboratory circumstances *C. glomerata* is known to be capable of parasitizing and successfully developing in the Large and Small Cabbage White butterflies, *Pieris brassicae* (L.) and

*Pieris rapae* L. and, to a lesser extent, the green veined white *Pieris napi* (L.), with a preference for *P. brassicae* (Brodeur *et al*., 1996, 1998). In the field, Dutch *C. glomerata* wasps were found to parasitize both *P. brassicae* and *P. rapae* with a larger number of offspring yielded from *P. brassicae* (Geervliet *et al*., 2000).

*Cotesia glomerata* adults can successfully develop in all five larval instars of *P. brassicae* (Laing & Levin, 1982), but prefer to parasitize 1$^{st}$ and 2$^{nd}$ instar larvae (Brodeur & Vet, 1996). Older hosts show a fierce defensive behaviour which may actively prevent oviposition. In addition the rate of encapsulation is higher in older hosts (Brodeur *et al*., 1995). Under laboratory conditions per oviposition on average 20 to 40 eggs are laid (Ikawa & Okabe, 1985; Gu *et al*, 2003). Field observations of clusters in excess of 60 eggs per host most probably originate from superparasitism (see §1.1; Gu *et al*., 2003; Tagawa, 2000). Parasitoid larvae develop in the host and feed on host hemolymph while the host continues its feeding on the host plant (Gauld and Bolton, 1988). After two instars, the parasitoid wasps emerge from the host's fifth instar and spin clustered cocoons which are attached to the leaves. Male wasps emerge from the cluster of cocoons first and pheromones on the cocoons attract males from other clusters even before the females emerge (Tagawa & Kitano, 1981). Female wasps are ready to mate immediately after emerging (Laing & Levin, 1982) and Tagawa & Kitano (1981) found that mating takes place within 5 minutes after emergence. After mating, females fly off and start searching for hosts (Laing & Levin, 1982). Their life span is thought to be three to five days in the field (Geervliet *et al*., 1997). Within this time female wasps have to find one or several hosts to deposit (part of) her 500-2200 eggs (Vos & Vet, 2004).

### 3.3.2. Rearing

*C. glomerata* L. (Hymenoptera: Braconidae) wasps were reared in a greenhouse (20-25°C; 50-70% relative humidity; L16-D8). The wasps were obtained from colonies that originated from individuals collected in Brussels sprouts fields in the vicinity of Wageningen. Female wasps were allowed to parasitize first instar caterpillars of *P. brassicae* reared on *B. oleracea* plants. The cocoons of the emerged *C. glomerata* wasps were collected and reared in a large rearing cage with water and honey as a food source for the wasps after they had emerged (approximately five days after egression from the host). This cage was kept in the same climate room (20-25 °C, 50-70% relative humidity, 16L:8D) as the plants with caterpillars, but were kept as far away from each other as possible.

An extra source of *C. glomerata* cocoons came from the rearing of the parasitized caterpillars used in the bioassay. These caterpillars were reared in plastic boxes having a lid provided with a gauze cover in the climate room mentioned above. Fresh *B. oleracea* leaves were provided every day as a food source. Approximately one week after the bioassay the larvae of *C. glomerata* did egress from

the caterpillars and made cocoons. These cocoons were collected and added to the cocoons collected from the greenhouse rearing.

### 3.3.3. Bioassay preparation

For the bioassay 1-4 day-old female wasps were used, which were sexed 24 hours before the bioassay. Five groups of wasps, divided over two series of experiments (series 1: group a-c; series 2: group c-e), were established that had undergone different treatments 24 hours prior to the bioassay. Treatment group three was used in both series as a control group. The different treatment groups could be distinguished based on the experiences the wasps had had (see below). After the wasps had undergone their treatment, per group they were transferred to a small glass cage (15x15x15 cm) in which they stayed overnight in the climate room. Water and honey was provided. Thirty minutes before the first bioassay of that day the cages were transferred to the bioassay environment (see §3.4 for a description).

### 3.3.4. Treatment groups

**a. Naïve females (Na)**

Naïve females had not had any obvious experience with stimuli from the host or the plant-host complex before the bioassay.

**b. Females with an experience just prior to the bioassay (L)**

Prior to the bioassay, leaf-experienced females got the same treatment as naive females. At the start of the bioassay, however, the leaf-experienced females were released on a glass microscope slide on with a small, freshly excised, piece of a *B. oleracea* leaf (approximately 0.5 x 0.5 cm) from which feeding first instar caterpillars had just been removed instead of being released from a glass vial (see bioassay set-up, §3.4) like the other treatment groups.

**c. Females with an incomplete experience (IC)**

Twenty-four hours before the bioassay the female wasps in this group were allowed an oviposition experience in a *P. brassicae* 1$^{st}$ instar caterpillar that was feeding on a *B. oleracea* leaf. To achieve this, the wasps were transferred from the large rearing cage to a piece of leaf in a Petri-dish containing unparasitized *P. brassicae* larvae. Each wasp was observed to parasitize a single caterpillar and, after it was observed to have finished the oviposition, the wasp was transferred to a small glass cage.

**d/e. Females with a complete experience (CUP and CPU)**

One day prior to the bioassay the female wasps in these groups got two experiences with a time interval of one hour: one "positive" and one "negative" experience. The difference between the two groups, CUP and CPU, was the sequence of the experiences. Wasps in the CUP-group first got a "positive" experience followed by a "negative" one. In the CPU-group the "negative" experience was given first.

The "positive" experience consisted of the same treatment the wasps with an incomplete experience received. The wasps were transferred to a piece of *B. oleracea* leaf in a Petri-dish containing unparasitized 1$^{st}$ instar *P. brassicae* and then they were observed to parasitize a single caterpillar. The "negative" experience consisted of an experience with a late L2 caterpillar which had been parasitized three days earlier when it was still in its early L1 stage. Also for this experience the wasps were transferred to a Petri-dish containing a piece of *B. oleracea* leaf, though this time with (supposedly) parasitized caterpillars only. After ovipositing a wasp was removed and transferred to the small overnight cage. In case a wasp did not oviposit within one minute after being released or in case a wasp was only probing, the wasp was excluded from further use.

## 3.4. Bioassay

Flight experiments were conducted in a wind tunnel set up as described by Geervliet *et al*. (1994) at 24±5°C, 50-70% relative humidity, 0.7 klux and with a laminar wind speed of 0.2 m/s. The two differently treated leaves, PAR versus UNPAR, were placed as odour sources at the upwind end of the wind tunnel. The distance between the two leaves was approximately 14 cm.

*C. glomerata* wasps of the different treatment groups were alternately released in the middle of the release cylinder at the downwind end of the wind tunnel (60 cm from the odour sources) to be tested. Per series all three treatments were tested at the same day to compensate for possible differences between days in e.g. barometric pressure.

A "choice" was recorded when the wasp completed a flight, so landed on one of the two odour sources. After landing on either one of the odour sources, wasps were removed from the leaf before they had contact with the actual hosts. A "no choice" was recorded when the parasitoid landed anywhere else in the tunnel besides the release cylinder or the odour source, or if the wasp remained longer than 10 minutes in or on the release cylinder. with Every wasp was only used once, between 4 and 14 wasps per group were tested per day. In total 94 wasps per treatment group of series 1 were tested and 60 wasps per treatment group of series 2.

The odour sources were exchanged after every 2 wasps of each treatment to compensate for possible asymmetric aspects of the setup.

## 3.5. Statistical analysis

The difference (%) in (damaged) leaf surface and (damaged) leaf outline between the two leaves PAR and UNPAR within a bioassay was calculated by the formula $(L_{most}/L_{fewest})*100$, whereby $L_{most}$ stands for the number of pixels of the leaf with the highest number of pixels and $L_{fewest}$ for the leaf with the fewest pixels. So the first being the leaf with the larger surface-damage or damage-outline and the latter the leaf with the smaller damage.

Choices of the wasps between the UNPAR- and PAR-treatment within a test were analysed by using a one-sided binomial test (Siegel, 1956). It was chosen for a one-sided test since Fatouros *et al*. (2005) demonstrated previously that unparasitized caterpillars were preferred over parasitized ones. The null hypothesis was that there was no difference in the number of wasps landing on both odour sources with the alternative of more wasps landing on the UNPAR-treatment.

Differences in choice distribution among the tests within each series were analysed with 2x3 contingency tables using Chi-square statistics. If the Chi-square showed differences between the tests within a series, the tests within a series were analysed pair wise using Chi-square statistics with a Bonferroni correction (Bonferroni, 1935) to find which tests differed from each other. The null hypothesis was that the choice distribution in each test was similar, while the alternative hypothesis was that learning altered the choice distribution among treatment groups.

Significance levels were P=0.05 for all tests.

# 4. Results

## 4.1. Bioassay set-up

### 4.1.1. Parasitization rate

It turned out in all but one bioassay the majority of the caterpillars on the PAR-leaf had been parasitized, with an average of 87 % (min 71.4%; max 100%) of the caterpillars being parasitized (Appendix II). In more than half (8 out of 15) of the bioassays this was 100%. The bioassay with zero percent parasitized caterpillars on the PAR-leaf was excluded from further analysis.

### 4.1.2. Clutch size

The size of the clutches of the parasitized caterpillars used for the PAR-leaf were found to have an average of 18.3, varying from an average of 14.7 till 22.9 per bioassay (Appendix II). This resembles other findings (Ikawa & Okabe, 1985; Gu *et al*., 2003) and was not considered a reason for discussion.

### 4.1.3. Quantification of feeding damage

Despite the removal of unparasitized caterpillars from the UNPAR-leaf a day prior to the bioassay it was found that in most bioassays (11 out of 14) more leaf surface was eaten of the UNPAR- than of the PAR-leaf. On average 51% more leaf surface was eaten of the UNPAR- than of the PAR-leaves (64% in series 1 and 39% in series 2). The variation between bioassays was high with differences in surface area eaten ranging from 10 till 191% (Appendix II).

In all but one bioassay the outline of the damage of the UNPAR leaf was larger than the outline of the PAR leaf with differences ranging from 4 till 95% and an average of 38% difference (30% in series 1 and 44% in series 2; Appendix II). In the one bioassay where the outline of the eaten area of the PAR-leaf was larger than of the UNPAR-leaf, the difference was 21%. Although it was recognized that the differences were large and maybe of influence on the choice of the wasps, no bioassays were excluded for further analysis, since this would have resulted in a shortage of repeats.

## 4.2. Bioassay results

The results of the choice distributions within and among tests are summarized in figure 1.

### 4.2.1. Choice distributions within a test

It was found that wasps with an incomplete experience in the first series landed more on the leaf with the unparasitized *P. brassicae* (P=0.034; N= 77; binomial test; appendix III) than on the leaf with the parasitized caterpillars. In the second series wasps with the same incomplete experience also showed a preference, although the P-value was just above the 5% level (P=0.056; N=48; binomial test; appendix III) for one of the leaves. When both groups of wasps with an incomplete experience in series 1 and 2 were pooled and analysed together, a preference was found for the UNPAR-leaf (P=0,006; N=125; binomial test; appendix III).
In other treatment groups no significant preferences were found.

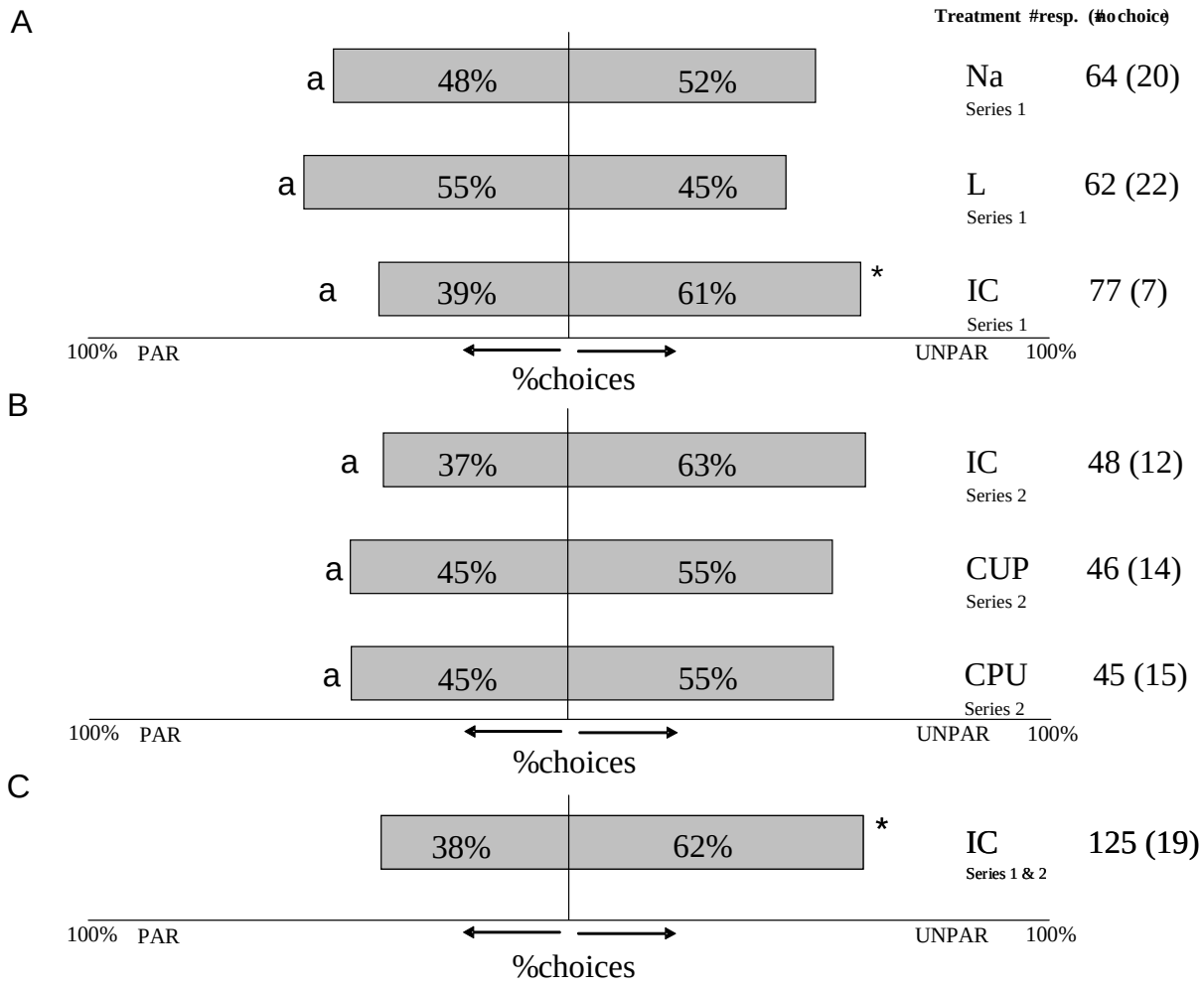


*Figure 1.* Choice distributions of responding *C. glomerata* females between Brussels sprouts leaves infested by unparasitized (UNPAR) and parasitized (PAR) *P. brassicae* with different experiences; (a) series 1; (b) series 2; (c) total of incomplete experiences. Bars with the same character indicate no significant differences in parasitoid choices between treatment groups. Asterisks indicate a significant difference within a choice test: * $P<0.05$

### 4.2.2. Choice distributions among tests

No differences in choice distribution among treatment groups were found either in series 1 or 2.

# 5. Discussion and conclusions

## 5.1. Discussion of confounding errors

During the test period some unforeseen factors may have influenced wasps, caterpillars and/or plants. Firstly, during series two, the stock colony of *C. glomerata* was infested with hyperparasitoids. The effect of parasitism on *C. glomerata* is unknown, but it may have influenced the behaviour of wasps in the bioassay.

As a result of the hyperparasitoids of a new colony of *C. glomerata* was started which resulted in a shortage of wasps in the experimental period. To increase the number of wasps that could be tested, naïve and leaf-experienced wasps used in the bioassay in the current study and naïve wasps used in the study by Bleeker *et al*., (2006)were reused to parasitize hosts which infested the odour sources. Since wasps did not have had an oviposition experience, the experiment is not thought to be influenced by this reuse.

In the first half of the experiments (series 1: day 1-7 and 11) some problems arose with the climate control in the climate room. The temperature rose till 30 degrees and half of the wasps prepared for the second bioassay-day died overnight. The surviving wasps were tested, but their behaviour may have been influenced by the temperature increase. The plants used as odour sources on the third bioassay-day experienced the temperature for about four hours and were not visibly affected at the beginning of the bioassay. However, during the bioassay it seemed that the leaves withered. As soon as this was observed, testing was finished that day, but it can not be excluded that the behaviour of wasps already tested was influenced by potential differences in volatile emission of these leaves compared to leaves at other days. Finally, in the preparation for bioassay-day four, the plants on which caterpillars fed between 96 and 48 hours prior to the bioassay were visibly affected by the higher temperature. The plants, hosts and wasps for bioassay-day five and six were held in another climate room till the temperature problems were solved.

## 5.2. Specificity of the plant volatiles

In-flight, incompletely experienced wasps preferred leaves with unparasitized hosts over ones with parasitized hosts. This confirms that the volatile blend of *B. oleracea* is state-specific and that wasps can use these volatiles to discriminate between host-states from a distance as was found by Fatouros *et al.* (2005). This ability enables wasps to save precious search time and thereby potentially enhance their reproductive success (Stephens & Krebs, 1986).

Plants may also take advantage from emitting state-specific volatiles. Seed production of *Brassica nigra* plants was found to increase when infested by parasitized *P. brassicae* compared to plants infested with unparasitized hosts, but to decrease with increasing brood sizes (Smallegange *et al.,* 2008). Plants therefore may benefit from attracting parasitoid wasps when infested by unparasitized hosts, but in addition also from informing wasps that hosts have already been parasitized and thus reducing superparasitism.

Despite several studies on the headspace of Brussels sprouts (Smid *et al*., 2002; Blaakmeer *et al*., 1994; Mattiacci *et al*., 1994), it is still unclear which compounds or what compound in the volatile blend are/is used by *C. glomerata* to discriminate between herbivore-damaged, intact or mechanically damaged plants. The latest understanding is that *C. glomerata* may use changes in the ratio between herbivore-induced and non-induced compounds (Smid *et al*., 2002). Fatouros *et al*. (2005) compared the headspace of Brussels sprouts treated with the regurgitant of parasitized ($PAR_{reg}$) and unparasitized *P. brassicae* ($UNPAR_{reg}$) for those compounds found to evoke a reaction in the antennae of *C. glomerata* (Smid *et al*., 2002). Overall lower amounts of emitted volatiles were found for the $PAR_{reg}$-treatment and like in the study of Smid *et al*. (2002) differences in the ratios of compounds were found. Reduced amounts of ß-glucosidase in the regurgitant of parasitized *P. brassicae* might be responsible for the differences found in headspace composition between plants infested by parasitized and unparasitized caterpillars (Mattiacci *et al*., 1995).

If qualitative differences exist in the headspace of the $PAR_{reg}$ and $UNPAR_{reg}$, the reaction of the antennae to the responsible compound would not have been examined by Smid *et al*. (2002) and such a qualitative difference may have been overlooked by Fatouros *et al*. (2005). However, since there are costs involved in the production of plant volatiles, it is not likely that plants have evolved a strategy of producing new volatiles while a reduction in induced volatiles might be sufficient.

## 5.3. Differences between the this study and the study by Fatouros *et al.* (2005)

Wasps with a short leaf-experience just prior to the bioassay did not show discriminative behaviour between the PAR- and UNPAR-treatment. Also the responsiveness of wasps was not enhanced compared to naïve wasps (74% and 76% respectively) even though this was found by Geervliet *et al*. (1998b) and was the main reason for Fatouros *et al*. (2005) to provide wasps with a leaf-experience.

The non-discriminative behaviour of *C. glomerata* after the leaf-experience is in contrast of the finding of Fatouros *et al*. (2005) that leaf-experienced wasps prefer the $UNPAR_{reg}$ treatment. Though it was tried to copy the bioassay set-up there were some differences between the current study and the study of Fatouros *et al*. (2005).

Probably the most striking difference is that Fatouros *et al*. (2005) used mechanically damaged plants treated with gut regurgitant whereas in the current study plants with actual feeding damage

were used. Fatouros *et al*. (2005) used plants treated with regurgitant to control the damage between plants with parasitized and unparasitized caterpillars since healthy caterpillars were found to eat about 20% more than parasitized ones. Here, despite the removal of some caterpillars one day before the bioassay on average even a larger difference in feeding was found with 51% more leaf eaten by healthy caterpillars (64% in series 1). Though these visual cues could have influenced the wasps (Wäckers & Lewis, 1994; Sheehan *et al*., 1993; Geervliet *et al*., 1994), volatiles emitted by the plant through the edges of the damage were thought to be of more importance. Also the difference in damage-outline was substantial with an average difference of 38% (30% in series 1). *Cotesia glomerata* prefers plants with higher host density, probably due the larger damage and subsequent increase in released plant volatiles (Le Masurier, 1994; Geervliet *et al*, 1998a). So in theory quantitative differences between the plants in the bioassay could well have influenced the behaviour of the wasps. However, in practice the amount of damage of plants with healthy hosts was higher, so instead of preventing wasps of discriminating, it would be more likely that this difference enhanced host discriminating behaviour. It therefore does not explain the observed discrepancy between this study and the study by Fatouros *et al*. (2005).

Another difference was the age of caterpillars used in the bioassay. Fatouros *et al*. (2005) used the regurgitant of fifth instar larvae while in this study at the time of the bioassay the larvae feeding on the leaves were late second instar larvae. Maize plants infested by early instar *Pseudaletia separata* emit large amounts of specific herbivore-induced volatiles, while plants infested by late instars do not (Takabayashi *et al*., 1995). However, Mattiacci & Dicke (1995b) did not find a preference difference of *C. glomerata* to second or fifth instar larvae on Brussels sprouts from a long distance, not even after experience with either one of them. It may be that the plant volatiles emitted by Brussels sprouts are not stage-specific; that *C. glomerata* is not capable of perceiving stage-specific differences in the volatile blend; or that wasps choose not to discriminate. The end result is that *C. glomerata* females do not discriminate between plants with different caterpillar instars and therefore it can be concluded that this difference most probably do not account for the different behaviour of *C. glomerata* females in the two studies.

The plants used in the present study were younger than the ones used by Fatouros *et al*. (2005) with about half the number of leaves. Mattiacci *et al*. (2001) showed that plant age could affect the behaviour of *C. glomerata*. In young plants systemic induction was most detectable in older leaves, while in older plants systemic induction occurred in leaves of all ages. It was argued that because young leaves of fast growing plants like *B. oleracea* have a lower risk of being eaten by herbivores, it may not be adaptive for plants to invest in the production of such volatiles. This theory was supported by the finding that *P. brassicae* prefers feeding on systemically induced leaves even though these leaves might be of lower nutritional value (Mattiacci *et al*., 2001). Since in the present study relatively young leaves on young plants were used compared to the ones used by

Fatouros *et al*. (2005), the absolute amount of systemically induced volatiles may have been lower in the present study. Mattiacci & Dicke (1995b) found a threshold for plants treated with different quantities of regurgitant. The quantity of at least 20 L2 was needed for *C. glomerata* to prefer leaves treated with regurgitant over mechanically damaged leaves. Even though *C. glomerata* may use the ratio of induced volatiles in the total volatile blend and not the absolute quantitative amount, it still has to detect the small amounts of induced-volatiles present in the blend. Innate or leaf-experienced wasps may not be sensitive to amounts beneath a certain threshold, while this sensitivity may be enhanced in oviposition-experienced wasps.

Finally the timing and duration of the applied damage differed. In the study by Fatouros *et al*. (2005) the plants were once-off mechanically damaged and treated with regurgitant 18-24 hours prior to the bioassay. In the present study the damage was more gradual, starting one day prior to the bioassay and still continuing during the bioassay. Both methods are thought to be sufficient to induce systemic herbivore-induced volatiles, but in the present study larger amounts of green leaf volatiles released right upon damage were supposedly present during the bioassay, while in the study by Fatouros *et al*. (2005) these volatiles may not have been present anymore or were present in lower amounts due to the long interval between the induction of the damage and the bioassay. The ratio of induced volatiles relative to the total blend may have been lower in the present study while absolute quantities probably are not affected by this difference in methodology. Naïve *C. glomerata* are expected to be more sensitive to higher ratio differences since these represent potentially higher reproductive offspring. The percentage of responsiveness found in both studies, however, shows the opposite. Responsiveness in the study of Fatouros *et al*. (2005) was 55% while in the present study 74% of the wasps responded.

## 5.4. Host discrimination in naïve wasps

The difference in findings between the study of Fatouros *et al*. (2005) and the present study complicate drawing conclusions about the innate ability of *C. glomerata* to discriminate between plants with unparasitized and parasitized host larvae from a distance. The naïve wasps in this study do not seem to differ in their behaviour from leaf-experienced wasps for which Fatouros *et al*. (2005) found a preference. It may be that any methodological issues influencing the leaf-experienced wasps also influenced the naïve wasps and that wasps do innately discriminate between PAR- and UNPAR-plant odours. On the other hand it could be that the results for naïve wasps are trustworthy and that innate *C. glomerata* do not discriminate, either because they still have to learn the difference in odour or because they "choose" not to discriminate because superparasitism can be a good strategy. For now, it can only be hypothesized what would be most adaptive for naïve *C. glomerata*.

As a starting point, the high responsiveness of naïve wasps shows that wasps are highly attracted by herbivore damaged *B. oleracea* plants, as was already shown in several studies (Mattiacci *et al*., 1994, 2001; Geervliet *et al*., 1998b; Blaakmeer *et al*., 1994). Geervliet *et al*. (1996) found that this attraction was plant-family specific rather than plant-species specific. The circumstances between generations of wasps can change dramatically with regard to host species availability as well as to the plants the hosts feed on. In addition, plant volatiles vary greatly among plant species, plant parts, different growth stages and different genotypes (for review see Turlings & Wäckers, 2004). C. *glomerata*, although mostly parasitizing *P. brassicae,* is a generalist with their host species feeding on different crucifer species which makes a broad innate specialization on the plant-family, but not on the plant-species level useful.

The volatiles used by *C. glomerata* to determine whether a host is parasitized are thought to be more specific than the ones used to determine if a plant is herbivore-damaged. For innate wasps to discriminate between different host states on different plant species, the differences in PAR- and UNPAR-plant odour should be constant among crucifer species, which seems unlikely seen the high variability in plant volatiles. Rather wasps learn the probably subtle differences after they have found a potential host and learn the odours associated with it. This way, they can focus on the differences associated with a single host species and a single plant species. So although biologically *C. glomerata* females are capable of perceiving quantitative differences in the volatile blend, it is thought that innately wasps do not use this ability but rather respond to all herbivore-induced volatiles emitted by crucifer species. This will lead them to a host-infested plant where they encounter hosts if still present on the plant. In a close range, naïve *C. glomerata* females, though they accept both parasitized and unparasitized hosts (Ikawa & Suzuki, 1982; pers. observation), do discriminate between hosts upon contact (Ikawa & Suzuki, 1982), which makes it possible to learn to associate the experienced host-state to the experienced odour.

## 5.5. Host discrimination in incompletely and leaf-experienced wasps

Overall incompletely experienced wasps in the present study were found to prefer the UNPAR-treatment, though it must be noted that in the second series the preference for the UNPAR-treatment of wasps was not significant, although nearly. The difference in results in the first and second series might be due to a large behavioural variability of parasitoid wasps. In the second series the percentage of wasps preferring the UNPAR-plant-odour was similar to the first series, but the lower number of wasps tested in the second series in combination with the lower responsiveness of wasps in the second series made that the preference was non-significant. The lower responsiveness might be contributed to a day-to-day variation as was also found by Steinberg *et al*. (1993) and might be due to barometric differences (Appendix II) or might be contributed to variability in plant odour.

The single "positive" experience probably was sufficient to induce preference behaviour towards the experienced odour. On the other hand no differences could be found among the different treatment groups in series 1 (N, L and IC1), from which one may conclude that the enhancement of the response for UNPAR-plant odours only is small or that the enhancement is large, but similar for both odour sources. That is if associative learning is the mechanism behind the change.

If the enhancement is only small, one may retrospectively conclude that the innate response to the UNPAR-plant odour was already considerable. The oviposition experience represents a strong innate response and according to the potential response model (Vet *et al*., 1990, 1995) the CS then should have been strong too to evoke only a small change in response. Such an innately strong response makes sense if it is assumed that wasps use the same cue to find hosts in general on crucifers and to discriminate between different host-states. The innate response to odours associated with parasitized host infested plants is probably considerable in this case. One could say that the innate responses to herbivore-damaged plants have to be fine-tuned by experience to the found plant-host system.

If the increase in response to the experienced odour after the oviposition experience was large, generalisation may have resulted in preference behaviour without the existence of differences among the treatment groups. Other plants, even if they belong to the same species and are infested by the same host species and state as the one experienced, may vary in their volatile blend from the experienced plant. Too prevent narrowing their search too much and thereby missing suitable hosts, wasps may generalize the experienced odour to a certain degree till it is clear that no such hosts are available in the generalized odours, as was found for *L. heterotoma* (Vet *et al*., 1998). *Leptopilina heterotoma* did not show a preference for either one of the odours in contrast to *C. glomerata* which preferred the experienced odour. However, such a preference does not exclude generalization, since it could be that the effect of the experience was stronger for the experienced odour than for the generalized odours. Such a mechanism was found for *Cotesia marginiventris*, which innately showed little responsiveness to and no preference for either cabbage loopers feeding on cotton or army worm feeding on maize, but after experience with either one of the plant-host systems, preferred the experienced odour and were also more attracted to the other non-experienced odour. No actual oviposition experience was needed for this learning to take place, contact with frass and damaged leaves was found sufficient (Turlings *et al.,* 1989). That an oviposition experience is not always required was also found by Geervliet et al. (1998b) for *C. glomerata*. If host by-products are state-specific it is not unlikely that they can also act as the US in host discrimination learning and this may explain the preference found by Fatouros et al. (2005) for the $UNPAR_{reg}$-treatment. The effect of this learning, however, is expected not to endure as long as the effect of associative learning with an oviposition experience as the US (Vet *et al*., 1995),

which seems functional since an oviposition is proof of the presence of a host, while host by-products are very likely to indicate host presence, but do not offer a hundred percent certainty.
Either with a weak or strong innate response to the experienced odour, the shown preference behaviour probably is weak and may easily be changed by another experience (see §5.6.1), which is thought to be adaptive since a single experience with a host does not "tell" the wasp what the proportion and density of unparasitized hosts is in the environment. Specializing on the experienced odour therefore may be risky, because it could result in long search times in an environment with low total numbers of hosts or a high proportion of parasitized hosts. However, if the preference is weak it is also likely to wane quickly if no other unparasitized hosts are found (Vet *et al*., 1990).
It must be noted that it is unlikely that *C. glomerata* encounters just a single unparasitized host since *P. brassicae* lives in clusters and an encountered host is likely to predict more similar hosts in the vicinity. It is remarkable that wasps already after a single oviposition experience do discriminate between PAR- and UNPAR-plant odours since this raises the expectation that after ovipositing in a full cluster of unparasitized *P. brassicae* the preference for the experienced odour is not easily overruled anymore. This seems not adaptive at first sight since the state of caterpillars in a single cluster also does not predict the proportion of unparasitized hosts in other clusters. On the other hand, after finding a cluster of *P. brassicae*, the need for *C. glomerata* to find more hosts may have decreased because many eggs are deposited. *Cotesia glomerata* can presumably take the risk of not finding more hosts after their initial success. But why should they invest in learning the experienced odour in the first place? It might be that the other host of *C. glomerata*, *P. rapae* does not lay it eggs in clusters and that for this host learning might be highly adaptive. *Cotesia glomerata* may have evolved to learn the experienced odours and may not be able to "switch off" this ability when it encounters *P. brassicae*.
Besides associative learning as described above, sensitization can also have influenced the wasp. Bleeker *et al*. (2006) found that after an oviposition experience in unparasitized hosts, the general responsiveness to plant odours was increased compared to naïve wasps. Due to the enhanced responsiveness, behavioural variability is expected to be decreased which is for example shown in more direct flights to plant-host complexes (McAuslane *et al*., 1991; Eller *et al*., 1992). If naïve wasps differ in their responses towards plants infested by healthy and parasitized wasps but do not discriminate because of their variability in behaviour, sensitization may just be the push they need to show discriminative behaviour. Sensitization alone, however, does not explain the results found for wasps with a complete experience (see §5.6.2). It is thought that sensitization may increase host finding success in general, but not necessarily in-flight host discrimination as well.

## 5.6. Host discrimination in wasps with a complete experience

### 5.6.1. Effect of a second experience

Wasps with a complete experience did not prefer either one of the odour sources and there is no indication that they would if the low number of wasps and the day-to-day variation were taken into account. So it seems that a "negative" experience erases the effect of the "positive" experience.

If wasps would perceive the parasitized host as unrewarding it is expected that such an experience would enhance the response to rewarding odours and/or induce avoidance behaviour of the experienced odour. The opposite seems to happen: instead of strengthened preference behaviour for the UNPAR-treatment, no preference is found anymore after a complete experience (CUP). From this it is concluded that wasps do not perceive the "negative" experience as negative, like *L. heterotoma* did for unoccupied patches (Papaj *et al*., 1994), but at the most as less rewarding than an oviposition experience with a healthy host. An additional observation that supports this is that wasps got visibly excited by the encounter with a parasitized host (pers. observation)

The perception of parasitized hosts as "less positive" is thought to benefit *C. glomerata* since superparasitism in certain situations can result in a higher total offspring than specializing on healthy hosts alone. One such a situation is when the proportion unparasitized hosts in the environment is low. In this study wasps, after two experiences, have experienced one parasitized and one healthy host. The chance that in such a situation many hosts have already been parasitized in the foraged area is still large. Only after more encounters with unparasitized hosts, wasps can be confident that there are many healthy hosts available and may it be save to reject parasitized hosts completely. Till then responding to UNPAR-plant odours as well may result in a higher total offspring fitness. This is supported by the finding of Ikawa & Suzuki (1982) that when in a close-range experiment wasps were offered a certain number of unparasitized hosts in a row followed by a single parasitized host, the more unparasitized hosts had been experienced the higher the percentage of rejection and the lower the mean oviposition time was.

Though superparasitism can be advantageous, the reward level of the oviposition experience (US) is supposed to be lower than when parasitizing a healthy host. This may result in a weaker increase in response, though the results of this study suggest similar response enhancements for odours associated with "positive" and "negative" experiences, since the behavioural shift towards preferring the UNPAR-treatment after a "positive" experience seems to be completely erased by the "negative" experience. However, it could well be that there were differences which only did not show in the behaviour of wasps as quantified in this study, which might therefore not be suitable to show such differences.

### 5.6.2. Order of the experiences

No support for either a stronger effect of the first or second experience was found in this study. Wasps from the CUP-treatment group responded in a way very similar to wasps from the CPU-treatment group both in the percentage of wasps responding and in the percentage of responding wasps flying to either one of the odour sources. In fact, out of the 60 wasps tested in each group only one wasp less responded in the CUP-treatment group and the percentages of wasps flying to PAR-and UNPAR-plant odour were the same with 45% and 55% respectively.

These results show that the preference behaviour of incompletely experienced wasps could not be solely contributed to sensitization. If it could, the effect would still be expected after the second experience, though not to change the behaviour towards not preferring either one of the odour sources.

From these results it may be concluded that sensitization did not have a strong effect on the memory strength of the first experienced odour (Geervliet *et al*., 1998a), at least not strong enough to evoke a behavioural change. If the first experience would have a larger effect than the second one, this is expected to result in a higher proportion of wasps from the CUP-treatment group landing on the UNPAR-treatment than wasps from the CPU-treatment group, which is not the case. The difference between the present study and the study by Geervliet *et al*. (1998a) in which it was found that the first experience was most important for the subsequent behaviour of *C. glomerata*, may have been that naïve wasps in the present study already showed a high innate responsiveness to both odour sources, while the innate response in the study of Geervliet *et al*. (1998a) was low. Since responsiveness can never exceed a hundred percent, the initial high response may have limited the response increase in the current study.

Also no stronger effect of the second experience as found by De Jong & Kaiser (1992) for *Leptopilina boulardi* was found. *Leptopilina boulardi* was conditioned by placing females in a certain odour for 20 minutes in the presence of 300-500 first instar host larvae. Many oviposition experiences in a certain odour follow on many experiences in another odour. The repeat of experiences with short time intervals in the presence of the second odour might inform *L. boulardi* that many hosts can be found in the experienced odour, or in other words that this odour is a good predictor of hosts. At the same time the memory of the first learned odour is not reinforced and is likely to wane (Vet *et al*., 1995). As a result the memory for the second odour may become stronger than the memory of the first odour (Vet *et al*., 1995; Menzel, 1999).

A stronger response to the secondly learned odour may be adaptive for *L. boulardi*, but it is thought that this would not be the case for *C. glomerata*. *Cotesia glomerata* wasps only experienced each odour once within an hour. This might inform the wasp that hosts may not be very abundant and that parasitized hosts are present, though not in what proportion. This would be a good reason to search for both odours. Additionally, in a natural environment, the order in which

they receive the experiences is likely to be mere coincidence with only two experiences, so it does not seem adaptive to prefer the second odour irrespective of the nature of this odour. If the last odour is experienced repeatedly and the first odour is not reinforced, it may well be that *C. glomerata* like *L. boulardi* also prefers the second learned odour probably due to the waning of the first odour.

## 5.7. Conclusions

The finding of Fatouros *et al.* (2005) that *C. glomerata* is able to discriminate between healthy and parasitized host larvae by using volatile cues was confirmed. Whether this is an innate ability or has to be learned remains to be further investigated. Either way, learning was found to affect the preference of *C. glomerata*. After ovipositing in a healthy host, the preference shifted towards the experienced odour, but the effect of this learning was easily cancelled by a second "negative" oviposition experience. The same was the case if the "negative" experience was given first. This shows that the "negative" experience is at the most perceived as less rewarding instead of negative, though more research is needed to examine how the response changes after a "positive" or "negative" experience are proportioned to each other.

The seemingly wipe out of the first experience by the second one regardless of the order in which the different experiences were received, shows that the responsiveness was not only increased by a general increase in response to plant odours, but that responses to certain odours were altered more than others, most probably by associative learning. The different learning mechanisms, however, could not be unravelled by this study.

It is likely that a single experience only induces a small shift in preference which may be just enough to induce a behavioural shift in the windtunnel. Under natural circumstances there will be a much greater variability and it is likely that (many) more experiences are needed for *C. glomerata* to show a steady preference with a low variability to the most profitable odour. The gregarious way of living of *P. brassicae* may contribute to this.

## 5.8. Recommendations

The recommendations are restricted to behavioural studies without exact knowledge of the volatiles used by *C. glomerata* in host discrimination from a distance. The identification of these volatiles would open an array of new possibilities for research on host discrimination. This, however, would be too much to discuss here.

The effect of an oviposition experience on in-flight host discrimination depends on the innate behaviour of *C. glomerata*. A two-choice bioassay as in the study of Fatouros *et al*. (2005) with regurgitant, but without the leaf-experience may answer the question if wasps can perform host

discrimination in-flight innately, but only if wasps are able to discriminate. If wasps do not show preference behaviour in a two-choice bioassay, this namely does not mean that wasps can not discriminate. The differences between responses may not be high enough to show a preference or wasps may choose not to discriminate. To avoid these problems, it is recommended to carry out no-choice windtunnel experiments with either PAR- or UNPAR-treatments as odour sources, though there is a risk that responses to both odour sources are too high to determine a difference between them (responsiveness can never exceed 100%). A no-choice bioassay may also provide further insight in the effect of the leaf-experience just before the bioassay.

No-choice bioassays may also show the differences in effect of rewarding and less rewarding ovipositions experiences. More insight may also be gained by varying the number of "positive" and "negative" experiences in two-choice bioassays. How many "positive" experiences are needed to overrule the effect of a single "negative" experience and the other way around? This could shed light on the relative strength of both experiences. Also it may do more justice to the natural environment of the wasps to present the experiences with *P. brassicae* in groups since *C. glomerata* will almost always encounter clusters instead of single caterpillars.

Experienced wasps in the present study received their experiences one day prior to the bioassay. Therefore the time between the experiences and the bioassay was quite long, while the single experiences with each odour may not have induced a very strong and stable memory. In a natural environment after not encountering a host for twenty-four hours it might be adaptive for wasps to superparasitize and quick waning may have been favoured by natural selection. It could be that in the present study no preference behaviour was found simply due to the long time-interval between the experiences and the bioassay. It is recommended to test this by varying the time-interval.

Last some recommendations on the methodology are given. In the present study a large variability in the behaviour of wasps was found which may be due to day-to-day variations, differences in strength among odour sources in general and on different days, and a natural variability in wasps. To minimize variability and to get trustworthy results it is recommended that in future studies wasps are tested on many different days; odour sources are only used for a limited number of test wasps; plants treated with regurgitant are used; and that a large number of wasps per treatment is tested (at least more than the 84 used in the first series in the present study).

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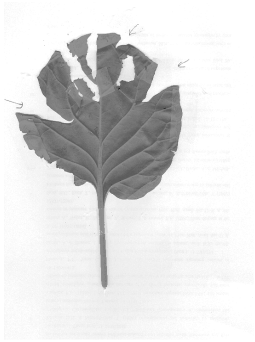
URL 1. Scion Image <http://www.scioncorp.com/> (08-03-2007)

# Appendix I. Determining leaf surface, outline and damage in Scion Image

1. File → Open file

Opening the scanned leaf (fig 1).

2. Process → Convert to greyscale



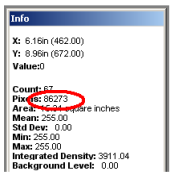
3. Options → Threshold

4. The outline of the leave where the damaged reached the edge of the leave was restored.

**Determining the leaf surface**

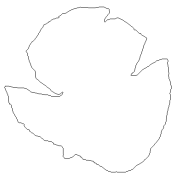
5. The damaged areas were filled up with black restoring the original leaf surface. The petiole was removed. At places where the damage reached the edge of the leaf the original edge was estimated.

6. The leaf area was selected
7. Analyse → Measure
8. The number of pixels was read in the info window

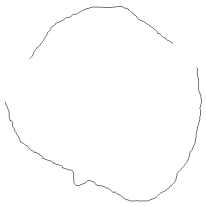


**Determining the Leaf outline**

5. Process → Binary → Make Binary
6. Process → Binary → Outline



7. Removing the lines were the leave was torn.



8. Options → Threshold
9. Selecting the area
10. Analyse → Measure
11. The number of pixels was read in the info window

**Determining the damage outline**

5. Process → Binary → Make Binary
6. Process → Binary → Outline

7. The outline of the leave not directly attached to damage was removed.

8. Options → Threshold
9. The leaf area was selected
10. Analyse → Measure
11. The number of pixels was read in the info window

**Determining the Damage surface**

Step 5 till step 7 are the same as for determining the damage outline.

8. The damaged areas were filled up with black.

9. Options → Threshold
10. The leaf area was selected
11. Analyse → Measure
12. The number of pixels was read in the info window

## Appendix II. Bioassay set-up data

| Bioassay day | N | % parasitized *Pieris brassicae* on PAR-leaf | Average clutch size | Difference in surface-damage (%) | Difference in damage-outline (%) | Air pressure (hPa) |
|---|---|---|---|---|---|---|
| 1 | 12 | 100 | 14.8 | 111* | 121 | 1021 |
| 2 | 8 | 71.4 | 16.7 | 110* | 150 | 1032 |
| 3 | 14 | 100 | 17.5 | 269 | 145 | 1022 |
| 4 | 12 | 100 | 18.1 | 151 | 115 | 1030 |
| ~~5~~ | ~~10~~ | ~~0~~ | | | | |
| 6 | 12 | 100 | 18.1 | 188 | 113 | 1001 |
| 7 | 14 | 90 | 16.8 | 201 | 162 | 1006 |
| 8 | 8 | 100 | 20.3 | 292 | 196 | 1013 |
| 9 | 4 | 93.3 | 15.6 | 120 | 119 | 1007 |
| 10 | 10 | 83.3 | 18.3 | 122 | 157 | 1025 |
| 11 | 12 | 83.3 | 20.6 | 158 | 104 | 1023 |
| 12 | 9 | 90.9 | 21.4 | 127 | 172 | 1013 |
| 13 | 10 | 100 | 16.1 | 144* | 147 | 1027 |
| 14 | 8 | 100 | 22.9 | 116 | 104 | 1007 |
| 15 | 11 | 100 | 21.0 | 140 | 111 | 1002 |

*The PAR-leaf had more damage than the UNPAR-leaf

## Appendix III. Binominal test, differences within treatment groups

| | | Category | N | Observed Prop. | Test Prop. | Asymp. Sig. (1-tailed) |
|---|---|---|---|---|---|---|
| Na | PAR | <= .5 | 31 | .48 | ,50 | .451(a) |
| | UNPAR | > .5 | 33 | .52 | | |
| | Total | | 64 | 1.00 | | |
| L | PAR | <= .5 | 34 | .55 | ,50 | .263(a) |
| | UNPAR | > .5 | 28 | .45 | | |
| | Total | | 62 | 1.00 | | |
| CUP | PAR | <= .5 | 21 | .46 | ,50 | .330(a) |
| | UNPAR | > .5 | 25 | .54 | | |
| | Total | | 46 | 1.00 | | |
| CPU | PAR | <= .5 | 20 | .44 | ,50 | .276(a) |
| | UNPAR | > .5 | 25 | .56 | | |
| | Total | | 45 | 1.00 | | |
| IC1 | PAR | <= .5 | 30 | .39 | ,50 | .034(a) |
| | UNPAR | > .5 | 47 | .61 | | |
| | Total | | 77 | 1.00 | | |
| IC2 | PAR | <= .5 | 18 | .38 | ,50 | .056(a) |
| | UNPAR | > .5 | 30 | .63 | | |
| | Total | | 48 | 1.00 | | |
| ICtot | PAR | <= .5 | 48 | .38 | ,50 | .006(a) |
| | UNPAR | > .5 | 77 | .62 | | |
| | Total | | 125 | 1.00 | | |

a Based on Z Approximation.

# Appendix IV. Chi square test, differences among treatment groups

**Series 1**

treatment * choice Crosstabulation

| | | | choice | | Total |
|---|---|---|---|---|---|
| | | | PAR | UNPAR | |
| treatment | Na | Count | 31 | 33 | 64 |
| | | Expected Count | 30.0 | 34.0 | 64.0 |
| | L | Count | 34 | 28 | 62 |
| | | Expected Count | 29.0 | 33.0 | 62.0 |
| | IC1 | Count | 30 | 47 | 77 |
| | | Expected Count | 36.0 | 41.0 | 77.0 |
| Total | | Count | 95 | 108 | 203 |
| | | Expected Count | 95.0 | 108.0 | 203.0 |

Chi-Square Tests

| | Value | df | Asymp. Sig. (2-sided) |
|---|---|---|---|
| Pearson Chi-Square | 3,579(a) | 2 | .167 |
| Likelihood Ratio | 3,595 | 2 | .166 |
| N of Valid Cases | 203 | | |

a 0 cells (.0%) have expected count less than 5. The minimum expected count is 29.01.

**Series 2**

treatment * choice Crosstabulation

| | | | choice | | Total |
|---|---|---|---|---|---|
| | | | PAR | UNPAR | |
| treatment | IC2 | Count | 18 | 30 | 48 |
| | | Expected Count | 20.4 | 27.6 | 48.0 |
| | CUP | Count | 21 | 25 | 46 |
| | | Expected Count | 19.5 | 26.5 | 46.0 |
| | CPU | Count | 20 | 25 | 45 |
| | | Expected Count | 19.1 | 25.9 | 45.0 |
| Total | | Count | 59 | 80 | 139 |
| | | Expected Count | 59.0 | 80.0 | 139.0 |

Chi-Square Tests

| | Value | df | Asymp. Sig. (2-sided) |
|---|---|---|---|
| Pearson Chi-Square | ,748(a) | 2 | .688 |
| Likelihood Ratio | ,752 | 2 | .687 |
| N of Valid Cases | 139 | | |

a 0 cells (.0%) have expected count less than 5. The minimum expected count is 19.10.