# STUDY OF TWO CONDITIONING METHODS OF PARASITOIDS USED IN BIOLOGICAL CONTROL PRIOR TO INUNDATIVE RELEASES IN APPLE ORCHARDS

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## ABSTRACT

The rosy apple aphid, Dysaphis plantaginea Pass., is a major pest in apple orchards in Belgium. Two micro-hymenopteran parasitoids Aphidius matricariae Haliday and Ephedrus cerasicola Stary are potentially capable of controlling this pest. However, when released in orchards the adult parasitoids tend to disperse. Based on the results of previous work it is proposed that the reason is that the artificial diet in which they are raised does not contain the odors that enable the parasitoids to identify the habitat where there are suitable aphid hosts. To optimize the control and make it economically effective it is important to reduce dispersal. This might be done by conditioning parasitoids during rearing by exposing them to the odors associated with orchards. The odor used in this study was a hydrodistillate of apple leaves, the main host-plant of the targeted aphid. Two methods of conditioning were used: 1) soaking mummies of the parasitoid in p ure hydrodistillate, 2) including a defined concentration of hydrodistillate in the artificial diet used for rearing the parasitoid. They were exposed to either a dilution of 100×, 200× and 1000× in two consecutive generations. The response of adult parasitoids to odors was determined using a dual choice olfactometer. Of the E. cerasicola conditioned by soaking the mummies 70–75% of individuals were attracted to the odor of plants infested with D. plantaginea compared to the 55–60% in the controls. The A.matricariae that were conditioned by feeding them artificial diets containing different concentrations of apple odor for one or two generations, showed no preference for the odor of apple leaves. Second generation adults of E. cerasicola exposed to a 200 times dilution of pure hydrodistillate showed a more marked response to apple odor.

**Keywords:** *Dysaphis plantaginea*, *Aphidius matricariae*, *Ephedrus cerasicola*, hydrodistillate, olfactometer, artificial diet

## Introduction

The rosy apple aphid, *Dysaphis plantaginea* (Pass.) (Homoptera: Aphididae) is one of the most serious pest of apple in Europe (Hill 1987). This aphid severely damages apple trees. It causes the leaves to curl and drop prematurely, twigs to distort and impedes natural "fruit drop" as consequence the trees produce many misshapen apples. Vast quantities of honeydew are also produced on which sooty moulds develop.

Even at low population densities, the economic damage can be considerable and treatments are recommended when only 1% of the flower buds are infested in early spring (Graf et al. 1999). *D. plantaginea* is most commonly controlled by spraying aphicides before the trees start to bloom and when fundatrices are hatching (Wyss 1999). The low tolerance for this aphis has resulted in intense spraying campaigns across Europe and resulted in the aphid becoming resistant to pesticides (Delorme 1998).

Mass releases of micro-hymenopteran parasitoids in orchards could be an interesting alternative method for controlling aphids. We have carried out field trials to test the effectiveness of this method of control using a mixture of two parasitoid species: *Aphidius matricariae* Haliday and *Ephedrus cerasicola* Starý. The result was that there were fewer colonies of aphids in the plots treated where parasitoids were released than in the control plots. It was noticed, however, that adult parasitoids tend to disperse out of the field, possibly because they do not recognize either the habitat or the host.

Therefore it was decided determine if the prior experience of a parasitoid could be used to condition it to search for a specific host (Poppy 1997; Wardle 1986; Hare 1997; Vet 1990; Powell 1998). Vet (1990) suggests that the failure of some augmentative releases of natural enemies is due to incorrect learning during rearing. Parasitoids are usually reared on artificial diet devoid of plant odors. Such odors allow parasitoids to identify the habitat of their aphid hosts. It is known that parasitoids reared on an artificial diet are less responsive to the odors of the host-plants of their aphid host (Douloumpaka and van Emden 2003; Bourdais, pers.com.).

Adult parasitoids can be conditioned by exposing them to a particular aphid host or particular plant (Douloumpaka et al. 2003; Turlings et al. 1993; Hérard et al. 1988; Drost et al. 1988; Hare 1996; Lewis et al. 1990; Storeck et al. 2000; Vet et al. 1998). Although learning in the adult stage is important, several studies have also revealed conditioning can occur before or at adult emergence (Takemoto et al. 2009; van Emden 2008). Some researchers speculate that the preference for an odor is established in the larval stage through the opening attachment of the mummy (Gutierrez-Ibanez 2007; Hagvar and Hofsvang 1991a) or at the adult stage during the emergence from the mummy, when the newly emerged adult parasitoid is exposed to chemical stimuli emanating from the aphid cuticle (van Emden et al. 2002; Storeck et al. 2000). Plant chemical cues experienced by the mother are transmitted to offspring by a process of marking at oviposition. However, this effect is weak and needs be reinforced by exposure to the odor of the aphid's host plant. Also as the host plant the aphid feeds on has an effect on parasitoid behavior it is likely that odors present in aphid food pass through the cuticle (Douloumpaka and van Emden 2003). Finally, conditioning before or during the emergence enables parasitoids to select environments similar to those in which they develop (Douloumpaka and van Emden 2003; van Emden et al. 1996). Therefore the ability to learn by experience has enabled generalist parasitoids to employ a flexible host searching strategy (Grasswitz and Paine 1993).

Thus it is hypothesized that parasitoids reared in the presence of an odor characteristic of apple orchards are more likely stay and parasitize the aphids living in this habitat. The odor extracted from apple tree leaves by hydrodistillation was used to condition the parasitoids. The parasitoids were either conditioned by soaking recently formed mummies briefly in a bath of hydrodistillate or by including hydrodistillate in the artificial diet used for rearing the larvae. The responses of the resultant female parasitoids to odors were determined in an olfactometer and/or by using a simple Petri dish test.

## Material and Methods

Colonies of *Aphidius matricariae* and *Ephedrus cerasicola* were established in the laboratory from specimens that emerged from mummified apple aphids collected from apple trees near Liège, Belgium, in spring 2005. Both species were reared on *Myzus persicae* fed on artificial diet at 20 ± 1.5 °C in dark boxes. The aphid *Dysaphis plantaginea* (Homoptera: Aphididae) was reared in cages on *Plantago lanceolata* L., in a controlled environment chamber at 20 ± 1.5 °C, 60% relative humidity and 16L : 8D photoperiod. In all experiments, 24 to 48 hours-old mated and naive females were used. The leaves used for the odor extraction were collected from Jonagold apple trees growing in the region of Sint-Truiden (Belgium) in October 2008. These leaves were stored at −18 °C until January 2009. Odor was extracted from 70 g leaves in 800 ml distilled water using a Clevenger apparatus. Normally, the hydro-distillation process separates water from essential oil containing low volatile compounds (Griffé 1998). As apple leaves are poor in essential oils, phase separation during the hydrodistillation does not occur and so the complete hydrodistillate containing mainly a mixture of terpenoids (monoterpenes and sesquiterpenes), was used (Bruneton 1999).

### Conditioning during the mummy stage

Once formed, the parasitoid mummies were soaked briefly in a bath of hydrodistillate and then placed on a filter paper and stored in a Petri dish at 20 ± 1.5 °C, 60% relative humidity and 16L : 8D photoperiod until the adults emerged. The percentage that emerged was recorded and the response of the females to odor tested in a Y-olfactometer. The internal diameter of the y-tube olfactometer glass tubing was 7 mm and the lengths of the arms 100 mm. Females were offered the choice of air drawn from a chamber containing a small apple plant infested by *D. plantaginea* and an empty chamber. The parasitoid's choice was recorded if it travelled more than 4 cm along either of the arms of the olfactometer. The time taken by the parasitoid to reach a decision was recorded or the parasitoid was removed after 10 min if no choice was made. There were between 30 and 40 replicates of each treatment (parasitoid species, conditioned or naïve). The contents of the chambers from which the air was drawn were reversed between replicates. The results were analyzed using $\chi^2$ tests to determine if a particular stimulus is preferred and $\chi^2$ homogeneity tests to compare naive and conditioned parasitoids. The results were considered significant for $\alpha = 0.05$ ($p < 0.05$) and highly significant for $\alpha = 0.01$ ($p < 0.01$).

### Conditioning during the larval stages: use of artificial diets

Apple leaf hydrodistillates at either a concentration 1 ml/100 ml, 1ml /200 ml or 1 ml/1000 ml were added to the artificial diet used for rearing the aphids host (*Myzus persicae*. These 3 dilutions were designated: D100, D200 and D1000, respectively. Parasitoids were placed with aphids when they reached 2nd larval stage. Two hours latter the parasitoids were removed and aphids kept on the artificial diet in a dark room at 20 ± 1.5 °C, 60% relative humidity, until mummification. Adults of *A. matricariae* and *E. cerasicola* were produced by rearing them for two successive generations on the modified artificial diet containing one of the 3 dilutions of hydrodistillate and on an unmodified artificial diet, which constituted the control. Percentage emergence of adults was recorded. In the tests, females fed a mixture of water and honey, which were naïve, unfertilized and aged 3 or 4 days were used.

#### *a) Searching behavior recorded in Petri dishes*

This experiment is based on the papers of Budenberg (1990) and Hagvar and Hofsvang (1991b). Five µl of the hydrodistillate were placed on a small piece of filter paper (1 cm × 1 cm) in the middle of a 90 mm diameter Petri dish. A circle of 40 mm diameter was drawn inside the perimeter of the dish. One female parasitoid was released randomly in the outdoor circle (Fig. 1). For each species and treatment (dilution and generation), 30 females were tested. The behavior of the parasitoid was recorded for 10 minutes using a video camera positioned above the

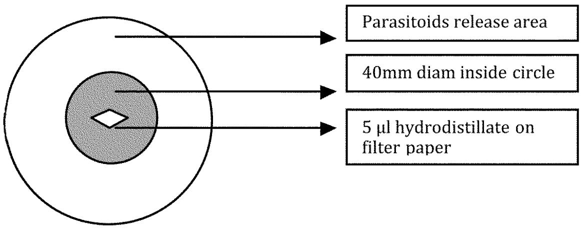

**Fig. 1** Illustration of the lay out of the Petri dishes used to observe the behavior of female parasitoids in the presence of apple leaf hydrodistillate.

Petri dish on an illuminating table in a dark room. Filter papers were used only once and a new Petri dish was used for each female. Images collected by the camera were analyzed using The Observer program. This program calculated the time spent by the parasitoids inside or outside the circle on the bottom of the Petri dish. The assumption was that as conditioning is likely to be positively correlated with the parasitoid's attraction for the hydrodistillate, the more time parasitoids spend inside the circle searching for a host, the more effective is the conditioning.

The results were statistically analyzed using a three-way cross ANOVA: hydrodistillate dilution, generation and parasitoid species.

#### *b) Searching behavior recorded in an olfactometer*

The parasitoid species that gave the best results in the tests in the Petri dish experiments was selected for the olfactometer tests: *E. cerasicola* (see the artificial diet results section). The protocol of this experiment was based on that of Chiu et al. 2009; Takemoto et al. 2009; Vos et al. 2001 and Du et al. 1996. The dimensions of the y-tube olfactometer were the same as in the previous experiment. In all experiments female parasitoids were given a choice between 50 µl distilled water and 50 µl of apple leaf hydrodistillate. A minimum of 30 females was used for each repetition. The choice was noted 5 minutes after the parasitoid was placed at the entrance of the central branch of the Y tube. The percentages of the females that entered each arm of the olfactometer were recorded and analyzed using logistic regression and contingency tables.

## Results

### The effect of conditioning during the mummy stage

The percentage emergence of adults from treated mummies was significantly lower than from untreated mummies (respectively, 52% versus 62% for *A.matricariae* and 51% versus 96% for *E. cerasicola*, $p < 0.001$), which indicates that this conditioning treatment reduced survival.

In general, conditioned parasitoids showed a more marked response than the control parasitoids (Fig. 2). Both species of parasitoids were clearly attracted to the odor from the apple sapling infested with aphids ($p = 0.0455$ for *A. matricariae*, $p = 0.0106$ for *E. cerasicola*). However, the control parasitoids also showed a preference for the odor from the aphid infested apple sapling and as consequence there were no significant differences in the responses of the control (parasitoids unconditioned) and conditioned parasitoids of both species to the odor coming from aphid infested apple saplings ($p = 0.3336$ for *A. matricariae*, $p = 0.2343$ for *E. cerasicola*), or between the behavior of adults resulting from the two types of conditioning ($p = 0.5237$ for *A. matricariae*, $p = 0.4121$ for *E. cerasicola*). There was also no significant difference between the responses shown by the two parasitoid species ($p = 0.5573$).

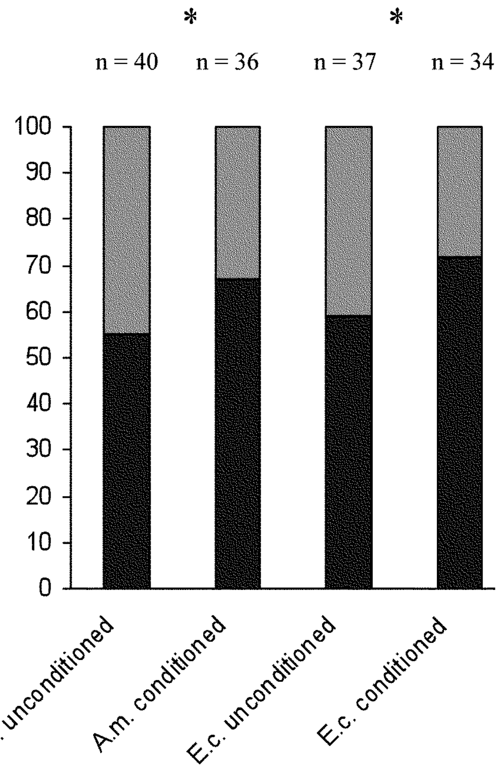

**Fig. 2** Percentages of conditioned and control parasitoids that were conditioned during the mummy stage, which chose the odor from aphid infested sapling of apple (grey) rather than the control (dark) (A.m. = *Aphidius matricariae*; E.c. = *Ephedrus cerasicola*).

### The effect of conditioning via the artificial diet

#### *a) Residence time in Petri dishes*

Fig. 3 shows the average time spent by 1st and 2nd generation conditioned and unconditioned individuals of *A.matricariae* and *E. cerasicola* in the central area of Petri dishes containing one of three dilutions of the hydrodistillate.

The statistical analysis shows a significant effect of generation ($p < 0.0001$), hydrodistillate dilution ($p = 0.0029$) and of the interaction species*generation ($p < 0.0001$). Comparison of the interaction species*generation using a Student t test reveals that the 2nd generation of *E. cerasicola* is significantly more attracted by the hydrodistil-

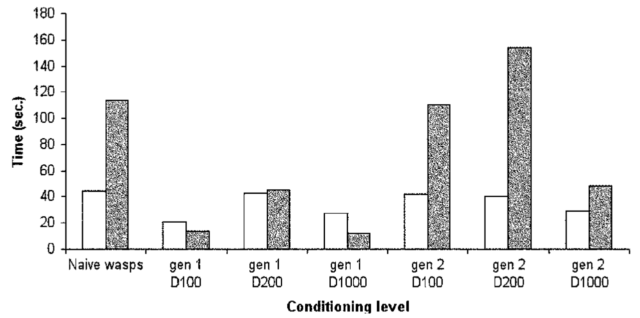

**Fig. 3** Time spent by the conditioned and unconditioned 1st and 2nd generation *A.matricariae* (white) and *E. cerasicola* (grey) in the central zone of Petri dishes treated with different concentrates of hydrodisitillate (Gen = generation; D = dilution).

late that 1st generation individuals. There is no significant difference between the generations of *A.matricariae* and this species is less responsive to the hydrodistillate than *E. cerasicola*. The analyses of the results for the conditioning revealed no significant response of the adults that were conditioned using dilutions 100 and 200×, or between those that were conditioned with a dilution of 200× of the hydrodistillate and those that were not conditioned. However, parasitoids conditioned with a dilution of 1000× were significantly less attracted by the odor of the hydrodistillate. The Scheffé contrasts indicate that *E. cerasicola* reared on an artificial diet that included hydrodistillate at dilutions of 100×, 200× et 1000× for one generation are significantly less attracted by the hydrodistillate than unconditioned *E. cerasicola* (respectively, $p = 0.0016$, $p = 0.023$ and $p = 0.000029$). After two generations of conditioning there was no difference in the response of conditioned et unconditioned *E. cerasicola* ($p = 0.85$, $p = 0.095$ and $p = 0.49$, respectively for dilutions 100×, 200× and 1000×), with a slight but insignificant indication that those reared on an artificial diet containing hydrodistillate at the dilution of 200× over two successive generations were more attracted by the hydrodistillate.

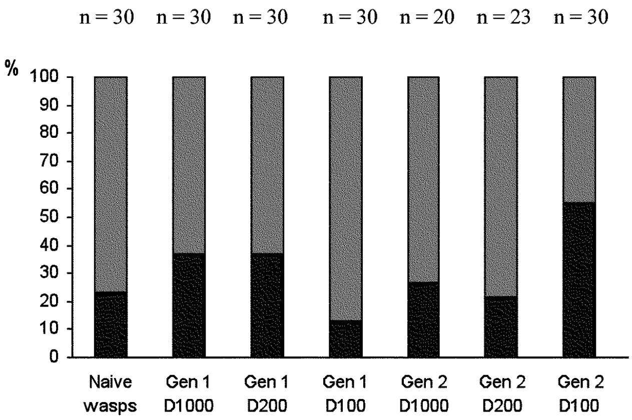

**Fig. 4** Percentage of conditioned and unconditioned parasitoids that chose the odor of distilled water (grey) and that of the hydrodisitllate (black). The parasitoid was conditioned by rearing it on an artificial diet including hydrodistillate at a concentration of 100, 200 or 1000× (D100, D200, D1000) over a period of either 1 or 2 generations (Gen1, Gen2).

### *b) Behavior recorded in the olfactometer*

Fig. 4 shows the choice made by conditioned and unconditioned parasitoids between distilled water and 100 or 200× dilutions of the hydrodistillate, after 1 or 2 generations of conditioning.

Logistic regression revealed a significant effect of the interaction generation*dilution ($p = 0.0083$). Parasitoids conditioned by using a dilution of hydrodistillate in the artificial of 100× were significantly more attracted to the odor of hydrodistillate than to that of distilled water ($p = 0.0106$). For the parasitoids conditioned to this dilution of hydrodistillate, Chi$^2$ tables revealed a significant difference between the response of the 1st and the 2nd generation of individuals of *E. cerasicola* ($p = 0.0026$).

## Discussion

It was known that some aphid parasitoids show preferences for particular plant cues, e.g. the cereal aphid *A. rhopalosiphi* can discriminate between different varieties of wheat (Wickremasinghe and van Emden 1992). When a parasitoid pupa is removed from the carcass of the aphid encasing it (the mummy) and left to emerge, the preference they previously showed for the wheat cultivar on which the aphid that hosted it was reared, disappears. This preference depend on the emerging females being exposed to certain chemicals in the mummy case (the cuticle of the dead host) (van Emden et al. 1996). For the purposes of this discussion this process will be referred to as "emergence conditioning". Corbet (1985) proposed that chemical information can be passed from larva to adult, either by means of information inside or outside the immature parasitoid, or neural changes induced in the larva that persist into the adult stage, the "chemical legacy hypothesis". Emergence conditioning is in accordance with this hypothesis as the behavioral responses of newly emerged adult parasitoid depend on exposure to chemical information carried over on the cuticle of dead host. The emerging parasitoid is apparently "conditioned" to respond to the first chemical information that it encounters after emergence, which are the chemicals in the case of its own mummy (Steinberg et al. 1993). However, even though emergence conditioning can establish the initial host preferences, these preferences can rapidly change as a result of subsequent foraging experiences (Storeck et al. 2000).

In the current experiments, *A.matricariae* and *E. cerasicola* emerged from mummies previously soaked in the hydrodistillate obtained from apple leaves showed a preference for the odor of the apple plant over that of distilled water in the olfactometer. But the conditioned and non-conditioned parasitoids did not show a significant difference in the attractiveness to the aphid infested plant relative to distilled water. However, the results indicate that the artificial diet used for rearing the parasitoids does not affect their host searching behavior and the attractiveness of aphid infested plants.

Over short distances (in Petri dishes), none of the dilutions of hydrodistillate used in the artificial diet significantly improve the parasitoids attraction to hydrodistillate compared to that of naïve wasps, either after 1 or 2 generations of conditioning. However, *E. cerasicola* have a significantly higher response after 2 generations than after 1generation of conditioning, witch is not the case for *A.matricariae*. It is probable that chemicals from the host plant present in the hemolymph of aphids are not detected by *A.matricariae* larvae or are not in the range of molecules to which this species can respond (Hoballah and Turlings 2005; Vet and Groenewold 1990). Possibly, *E. cerasicola* responds to these molecules after maternal marking. Although not statistically significant there is an indication that 1/200 dilution of the hydrodistillate included in the artificial diet results in conditioning. It is likely that a too low (100×) or to high (1000×) dilution is not suitable for conditioning *E. cerasicola*, either because they are toxic or the parasitoid is insensitive to them, but this needs to be verified.

In the olfactometer *E. cerasicola* shows a significantly more marked preference for the odor from plants infested with aphids after being reared for two successive generations on an artificial diet with added hydrodistillate at a dilution of 100×, compared to other dilutions. This indicates a maternal effect. Van Emden (2008) and Douloumpaka et al. (2003) report that during the marking process at the time of oviposition chemicals cues experienced by the mother are transmitted to their offspring. However, there was no significant difference in the response of conditioned and control (naïve) parasitoids.

## Conclusions

It is concluded that it may be possible to condition the parasitoids *E. cerasicola* to respond to apple, but the optimum concentration and the composition of the hydrodistillate used for the conditioning need to be determined "Emergence conditioning" by soaking mummies is easier to apply in the mass production of parasitoids for biological control in apple orchards. However, it would be interesting to investigate the preference of previously conditioned parasitoids for several host plants species. It should be noted that hydrodistillate contains only some the odors associated with apple. Also, a better application technique is needed to increase the percentage emergence of the parasitoids conditioned with hydrodistillate, as the high mortality recorded in these experiments may hamper mass production.

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