Associated learning of odour and colour in the seven-spotted ladybird *Coccinella septempunctata* (L.) - an olfactometer experiment.

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Abstract

The ability of the seven-spotted ladybird *Coccinella septempunctata* (L.) to learn to associate a combined stimulus of colour and odour with food was investigated. Four odours (menthol, 1,8-cineole, methyl salicylate and perfume) and three colours (red, yellow and green) formed twelve treatments that were combined with aphids and sweetened butter. For testing the attraction of the given treatment, the ladybirds were tested in a two-way olfactometer in which the ladybirds chose between the treatment and a control.

The ladybirds were more attracted to three combinations (1,8-cineole and yellow, menthol and yellow and perfume and green) following the treatment. This could indicate conditioning or learning. They also showed a strong attraction to the combinations of 1,8-cineole and green and methyl salicylate and green even without prior treatment, which could indicate innate behaviour.

The results contribute to the understanding of ladybird foraging behaviour and learning abilities. The potential to increase the effectiveness of ladybirds as a biological control agent is discussed.

Sammanfattning **" Inlärning av färg och doft hos sjuprickiga nyckelpigor *Coccinella septempunctata* (L.) – ett olfaktormeter experiment"**

I denna undersökning studerades sjuprickiga nyckelpigors Coccinella septempunctata (L.) förmåga att associera en kombinerad stimuli av färg och doft med mat. Fyra dofter (mentol, 1,8-cineol, metyl salicylat, och parfym) och tre färger (rött, gult och grönt) formade tillsammans med mat tolv behandlingar. Nyckelpigornas attraktion till den givna behandlingen testades i tvåarmade olfactometrar där de valde mellan behandlingen och en kontroll.

Nyckelpigorna var mer attraherade till tre kombinationer (1,8-cineol och gult, mentol och gult och parfym och grönt) efter behandlingen. Detta kan indikera någon typ av betingat beteende eller inlärning. Nyckelpigorna var också starkt attraherade till kombinationen av 1,8-cineol och grönt och methyl salicylate och grönt även utan tidigare kontakt med behandlingen, vilket kan indikera någon typ av medfött beteende.

Resultaten bidrar till ökad kunskap om nyckelpigors födosök och inlärningsmöjligheter. I studien diskuteras även om resultaten kan bidra till förbättrad användning av nyckelpigor inom biologisk bekämpning.

Introduction

Insects, like other animals, respond to diverse cues while searching for shelter, food, oviposition sites or mates. The signals emitted by plants, prey or conspecifics are detected by vision, olfaction or by other sensory systems. Responses to many of these cues are innate, while some may be learned during the insect's lifetime.

Learning, especially associative learning, tends to adapt the insect to its current environment (Papaj, 1993). For a predator or parasitoid, the rate of encountering herbivores may be increased if learned information is integrated into the searching process. On the other hand, learning may have negative effects if the environment changes quickly, since the process of learning is not immediate. The speed, reversibility and mechanism of learning may be related to the frequency with which the individual has to switch resources in its lifetime (Vet & Dicke, 1992). The ability to learn differs among insect taxa, and some groups may be more 'prepared to learn' certain stimuli than others. For example, there is commonly believed to be a difference in learning between generalists and specialist species. The proposition is that generalist species will be more flexible in their host-selection behaviour than specialist species (Papaj & Prokopy, 1989). Host specialists may be more likely than host generalists to have innate responses to stimuli that are directly related to the host or to the host habitat (Waage, 1979).

The seven-spotted ladybird is a generalist predator that feeds on a diverse range of foods. Aphids constitute the principal food and coccids, adelgids, mites, honeydew, pollen, nectar and mildew are recorded as secondary foods. (Majerus, 1994). According to the theories mentioned above, ladybirds should have a good capacity to learn. However, the definition of generalist and specialist can sometimes be complicated, and the difference in learning between generalists and specialists may not be as easy to distinguish as previously thought. For instance, a species that is classified as a generalist over its entire range may consist of populations specialised on different host species, yet most species comparisons are made using insects collected from just one or a few localities (Fox & Morrow, 1981). Furthermore, a specialist with respect to host species may be a generalist with respect to individual plants within a host population or with respect to different parts of a particular plant (Bernays & Lee, 1988).

There are several definitions of learning, and many are broad and complex. Often they fail to exclude phenomena intuitively not considered to be learning. Papaj and Prokopy (1989) summarised some properties characteristic of learning as criteria by which it can be classified:

1. *The individual's behaviour changes in a repeatable way as a consequence of experience.*
2. *Behaviour changes gradually with continued experience, often following a so-called "learning-curve" to an asymptote.*
3. *The change in behaviour accompanying experience wanes in the absence of continued experience of the same type or as a consequence of a novel experience or trauma.*

An effect of experience on behaviour that meets all three criteria is almost certainly learning. But a change in behaviour due to experience may demonstrate just the first of these properties and still be considered learning. Similarly, effects of experience on behaviour not usually considered to be learning may exhibit one or more of these criteria. Learning is ultimately characterised by the exclusion of phenomena that are not learned. (Papaj & Prokopy, 1989)

There are also different types of learning. One of these is associative learning, which involves the establishment, through experience, of an association between two stimuli or between a stimulus and a response. Typically, the association is formed through the close temporal and spatial pairing of the stimuli or the stimulus and response (Papaj & Prokopy, 1989). Associated learning is mainly described by the first of the above criteria.

Much of the research on insect learning has been made on honeybees, mainly because of their experimental convenience (Gould, 1993). Most of the evidence comes from their food-foraging cycle, for example the study by Wei et al (2002). They showed that honeybees are guided by odours and local landmarks to find a food source, but the ability to return to these pinpoint locations relies critically upon their use of visual landmarks.

The ability to learn profitable cues has also been demonstrated for almost 20 different species of parasitoids. They appear to be particularly effective at using and learning a combination of olfactory and visual cues in host searching (Turlings et al 1993). It has also been suggested that parasitoids can learn to associate stimuli with non-host food such as nectar, which would minimise the disruption of eating from host finding (Lewis et al, 1997). Other insects have also been shown to posses the ability to learn, for example the cabbage butterfly, *Pieris rapae* (Lewis, 1999); moth *Manduca sexta* (Daly et al, 2001); anthocorid predator *Anthocoris nemoralis* (Drukker et al, 2000); locust nymph *Locusta migratoria* (Blaney & Simmonds, 1985) and cricket *Gryllus bimaculatus* (Matsumoto & Mizunami, 2002).

Coccinellids' ability to learn has not been studied extensively, although a few attempts have been made to shed light on this question. Ettifouri & Ferran (1993) reared larvae of *Harmonia axyridis* (Coleoptera*: Coccinellidae*) on two different types of diet (aphids or moth eggs) and showed that only larvae that ate the same prey as they were reared on adopted intensive searching movements after feeding. They claim that larvae reared on substitute prey may have a decreased efficiency in biological control systems. Mondor & Warren (2000) made a study that investigated ladybird learning. They determined whether ladybird beetles used colour to initially find suitable host plants and whether they were capable of associating characteristics such as colour with the presence of food. The authors claim that the beetles' foraging behaviour could be altered as a result of prior experience, because if the beetles were conditioned to only yellow or green they did not exhibit a preference for visiting or spending time on different colours.

The searching behaviour of predatory coccinellids was thought to be random, with detection of prey occurring only at actual contact, without the aid of previous visual or olfactory orientation to prey (Kehat, 1968; Storch, 1976). Evidence that visual cues may play a role comes from laboratory studies made by Harmon et. al. (1998). In their study, *Coccinella septempunctata* (Coleoptera*: Coccinellidae*) consumed more of one of the two colour morphs (red and green) of the pea aphid that contrasted most with the background colour. The study also showed that *H. axyridis* consumed more of the red morph irrespective of background colour.

According to a study by Udayagiri et al (1997), yellow was more attractive to *C. septempunctata* than red, green and white. Maredia et al. (1992) and Majerus (1994) also provide results that support this finding. The latter suggests that hungry ladybirds are more attracted to yellow and yellowish-green than other colours. This colour might resemble young plant shoots where aphids often are most prolific. The study by Mondor & Warren (2000) showed that naive ladybirds of both sexes made significantly more visits and spent more time on yellow compared with green. They therefore claim that *H. axyridis* is capable of responding to cues such as colour.

Studies suggest that semiochemicals are involved in the attraction of predators to prey (Vet & Dicke, 1992; Scutareanu et al, 1997; Royer & Boivin, 1999; Sabelis et al., 1999; Dicke et. al., 2003; Shimoda et al., 2002). These volatiles have also been shown to attract ladybirds. In olfactometer experiments (Ponsonby & Copland, 1995), *Chilocorus nigritus,* (Coleoptera*: Coccinellidae*) was able to orientate towards its host using chemical cues, but they could not distinguish between the attraction of host and host plant. The use of herbivore–derived stimuli is often limited by low detectability, especially at longer distances. Herbivores are small components of a complex environment, and if they produce odours at all, it will be in minute quantities. Stimuli from plants, on the other hand, are usually more readily available due to the plants relatively larger biomass, but are less reliable predictors of herbivore presence (Vet & Dicke, 1992). Rü & Makosso (2001) showed that *Exochomus flaviventris* (Coleoptera*: Coccinellidae*) females use herbivore-induced volatiles during foraging. More evidence comes from Ninkovic et al (2001), who showed that *C. septempunctata* responded positively to volatiles from aphid-infested plants and from previously aphid-infested plants, but not to volatiles from uninfested plants or from undisturbed aphids. They were also attracted to olfactory cues from diversified plant stands (Ninkovic & Pettersson, 2003). Stubbs (1980) and Obata

(1986) also confirm that coccinellids use visual and olfactory cues to detect prey.

The aim of the study

This study investigates the ability of the sevenspotted ladybird, *C. septempunctata,* to learn a combined stimulus of colour and odour. Their ability to locate prey by visual and olfactory cues is also studied, as well as their reaction to the semiochemicals used. The hypotheses are that ladybirds are able to associate a combination of colour and odour together with a positive stimulus. They may also find one or several of the colours or odours more attractive than another, which can be considered innate behaviour. The results will contribute to the understanding of ladybird foraging behaviour and learning abilities. It will also contribute to the improvement of using ladybirds as biological control agents, if predatory coccinellids alter their behaviour as a result of prior experience it could result in significant changes in predatory efficiency.

Material and Methods

Insects and plants

Adults of the seven spotted ladybird (*Coccinella septempunctata* (L.) were collected from an overwintering site in southern Uppsala, Sweden, at 59° 47’ 52”N, 17° 39’ 66” E. These were fed with the aphid species *Sitobium avenae, Metopolophium dirhodum* and the pea aphid *Acyrthosiphon pisum*. The reason for using different aphid species was simply that there were not enough of one species to feed all the ladybirds. Therefore most of the ladybirds received a mixture of *S. avenae* and *M. dirhodum*.

Barley plants (cv. Golf) were sown for use as a control odour in the olfactometry test. Seven pots were planted with five seeds in each. For the olfactometer test only five of these pots were used with three plants in each pot. More pots were planted than used because of uncertain germination of the seeds. The plants used in the olfactometer tests were infested with aphids three days before use (Gianoli & Niemeyer, 1997; Ninkovic et al., 2001). The same aphid species were used as the ladybirds had been fed with. Twenty aphids per plant were placed in the pots, a total of about sixty aphids per pot. This is the amount of aphids needed to trigger the induction of hydroxamic acid in wild wheat. Hydroxamic acids are plant secondary metabolites found in wild and cultivated Gramineae that play a major role in the resistance of cereals to insects (Gianoli & Niemeyer, 1997; Ninkovic et al., 2001).

Experimental treatments

Four different odours were tested, methyl salicylate, menthol, 1,8-cineole and perfume (Anais Anais). Perfume was used because it was considered to be a complex compound containing plant extracts. Many perfumes contain chemical compounds from citrus plants that are known to repel a number of insects. However Anais Anais had a flower like scent reminiscent of roses, which was assumed to be less likely to repel the beetles. Except for the perfume the compounds are constituents of essential oils, produced by plants. However, the perfume should also contain several plant chemicals. Methyl salicylate is a substance produced by wounded plants, released as a volatile (Pettersson et al, 1994; Shulaev et al 1997). Menthol is the chief monoterpene constituent of peppermint oil and 1,8-cineole is found in a number of oils e.g. oil from bay leaf and eucalyptus (Buckingham, 1994). To be able to use these chemicals in a practical way they were mixed with wax and formed as small pellets that were easy to use (Ninkovic et al., 2003). The pellets contained 10 % weight of active substance, and the mean weight of one pellet was 65 ± 5 mg. After two weeks the amount of active substance left in the pellet was 5,1% (Ninkovic et al, 2003). The perfume was trapped in micropipettes (2 microliter).

Three colours were used in the experiment: yellow, green and red. The colours used might have resembled different cues to the ladybirds. Young plants and stressed plants are more susceptible to aphids and they tend to look more yellowish than healthy green plants. However both green and yellow might resemble an infested plant since different plants, infested or uninfested, appear either green or yellow, with wavelengths between 500-600 nm. Red was the preferred colour of oviposition substrate for *Adalia bipunctata* (Coleoptera: *Coccinellidae*) (Iperti & Prudent, 1986). In other situations animals often associate red with distastefulness (Krebs & Davies, 1999).

Each colour and odour was combined to form a treatment, in total twelve combinations shown in table1:

Table 1, Treatments

Methyl salicylate and yellow
Methyl salicylate and green
Metyl salicylate and red
Cineol and yellow
Cineol and green
Cineol and red
Menthol and yellow
Menthol and green
Menthol and red
Perfume and yellow
Perfume and green
Perfume and red

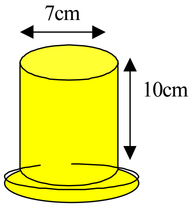
Picture 1, Cages with treatment

Experiments

Association of food with treatment

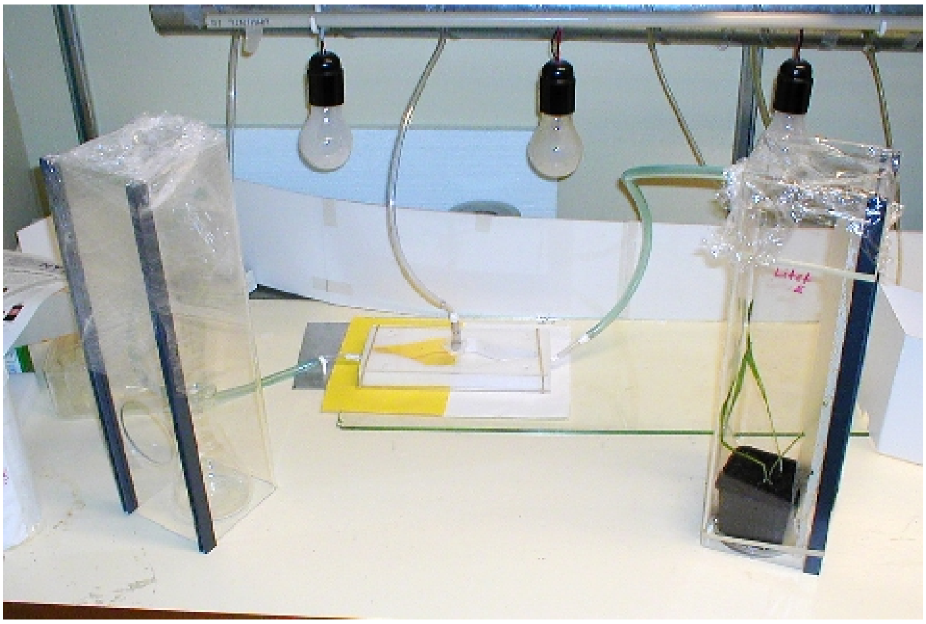
To test if the ladybirds could associate food with one of the above combinations the beetles were placed in cages together with food and treatment. Eight cages with five ladybirds in each were used, a total forty ladybirds per treatment. Cages (385 cm^3) were made of clear plastic tubes with net in the top and a Petri dish in the bottom. The buckets were covered with coloured plastic film (multi fix) and coloured paper was put under the Petri dish. Inside the cage was a bottle of water with cotton wool stuck to it, samples of odour, aphids, and a plate of Swedish sweetened butter (Fjällbrynt Messmör original, MILKO). The butter was a food-complement so that the ladybirds always had access to food (Al Abassi et al, 2001). The treatment continued for eight days. During that period about 100 aphids were brushed down to the ladybirds and the water and butter were refilled.

Fig 1, Size of the cage

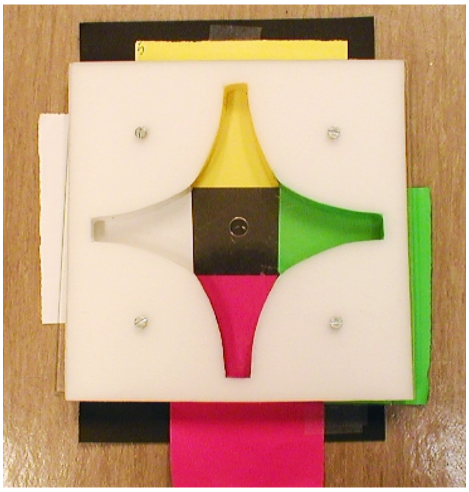


Olfactometer test

The behavioural effect of the treatment was tested with a two-armed version of the four-armed olfactometer (Pettersson, 1970; Ninkovic et. al., 2001). The apparatus consisted of an arena (6 x 6 cm) with a centre and with two conical, extended arms (7 cm) to which odours could be introduced from two connected plastic chambers. Air was drawn from the centre of the arena, creating two arm zones, one containing odour from the source attached to that specific arm, and a central zone in the middle of the arena. The arms were closed at the ends with a net, preventing the test animals from reaching the attached odour source in the arm. The arena was cut out in 2 cm Styrofoam with closed cells (Plastazote PZ940) and placed between two layers of 3 mm Perspex, held together with elastic rubber band. The walls of the arena were covered with white and coloured paper and the olfactometer was placed on half-coloured, half-white paper. The two arms represented choices for the insect, and the ladybirds chose between the odour from an aphid-infested plant together with white colour as a control, and the combined colour and odour from the treatment. The olfactometer was placed in a white observation box with light provided by two 40 W incandescent light bulbs. Prior to placing the ladybirds in the arena, the surface was washed with 80 % ethanol to remove substances emitted by organisms during the previous observations. One adult insect was placed in the olfactometer and its position in the arena recorded 10 times at 2-min intervals (long enough to permit an adult *Coccinellidae* to move from one end of the arena to the other). During an adaptation period of 10 min, a vacuum pump was started and air drawn through the tubes and arms toward the centre of the arena. The cumulative number of visits in the arm zones after 10 observations was regarded as one replicate. If the test individual did not move between 2 consecutive observations the series was interrupted and a new series of 10 observations started with a fresh insect (Ninkovic et al, 2001).



Picture 2, Olfactometer setup



Picture 3, Four-armed olfactometer colour test

Ladybirds were also tested in a four-armed olfactometer. Two separate tests were made to see if the ladybirds had any special preferences for the colours and chemicals used. The ladybirds chose between green, red, white and yellow on a black bottom in the first test, and between methyl salicylate, menthol, 1,8-cineol and an aphid-infested plant in the second test. The odour-test included 18 ladybirds versus 22 in the colour-test. The methodology was exactly the same as the above-described two-armed olfactometer. An olfactometer is mainly used to study olfactory cues, not visual. Nevertheless, since ladybird attraction to colours was to be tested in two-armed olfactometers, it was convenient to use a four-armed version when comparing the attraction of the four different colours.

Weight measurements

After testing in the olfactometer, ladybirds were weighed to see if there were any differences between the individuals, and how they reacted on the treatment and the food given. For weighing, the ladybirds were placed in a small Styrofoam container and placed individually on a scale.

Testing ladybird-memory

After staying in the treatment for eight days, olfactometer testing and weighing, the ladybirds were placed in new cages. These did not have any odour treatment and were not covered with colour. The food was also changed to dried catfood ("Kattens favorit, kött" Signum) that was crushed and mixed with water (Östman et al., 2000). The catfood was used because it was a new type of food, distinct to the previous diet to which ladybirds could have become conditioned. Ladybirds stayed in these cages for eight days and were then re-tested in the olfactometers to check for modified responses. Some combinations were further tested after another week, these ladybirds were kept in the cages for two weeks. The ladybirds were weighed after every olfactometer test.

Control

To be able to distinguish between innate behaviour and learning, the treated ladybirds were compared with non- treated ones. These ladybirds were also kept in cages for the same period of time, but in non-coloured cages and without odour. The food was the same and their performances in the olfactometer were compared with the treated ladybirds. Only combinations that had showed significant results with the treated ladybirds were tested with the non-treated.

Statistics

The results from the four-armed olfactometer were analysed with Friedman ANOVA and Kendall´s test for dependent samples. Wilcoxon matched pairs test were used to analyse the results from the tests with the two-armed olfactometer.

Results

Olfactometer tests

Ladybirds did not make significantly more visits to any of the four different colours in the four-armed olfactometer. The results were analysed with Friedman ANOVA and Kendall´s test for dependent samples (N=19, df = 3, p=0,35). In the test with four different odours the ladybirds were not more attracted to any of them (N= 18, df= 3, p= 0,092). The same statistical analysis was used as for the colours.

The results from the first two-armed olfactometer test with the ladybirds are presented in figures 2 and 3. The tests were performed directly after the ladybirds had spent one week in the cages and were analysed with Wilcoxon matched pairs tests. The ladybirds that had former experience of the treatment were significantly more attracted to six combinations of colour and odour than to white and the odour of an aphid-infested plant. Three of these combinations were not attractive to the non-experienced ladybirds. These combinations consisted of 1,8-cineole and yellow, menthol and yellow and perfume & green.

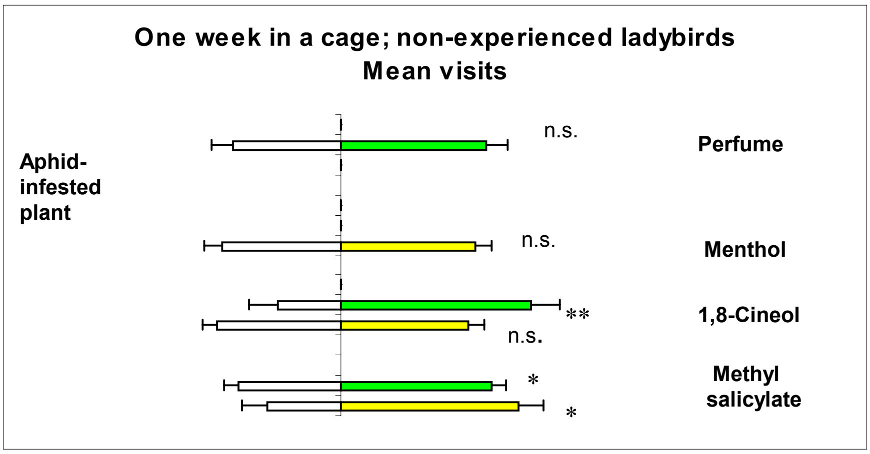


Figure 2. Mean visits of the non-experienced ladybirds in the arm with treatment and the arm with control, one week in a cage. *** p<0,005 , **p<0,01, * p<0,05. The non- experienced ladybirds were only tested for combinations that showed a significant result with the conditioned ladybirds.

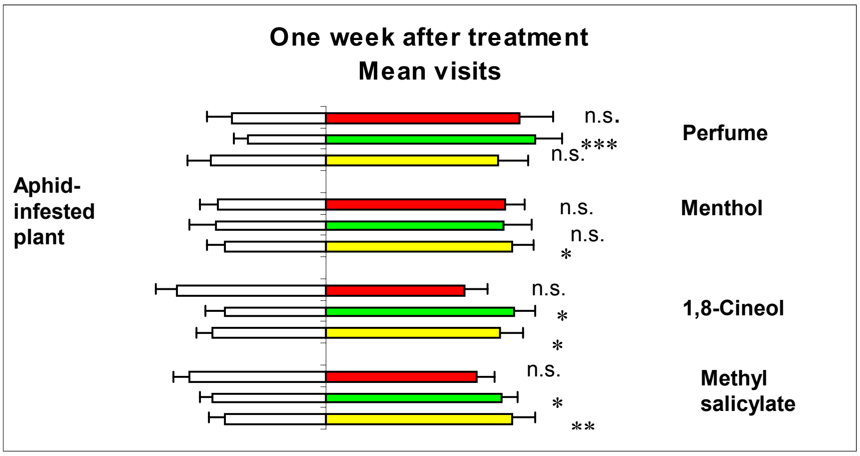


Figure 3. Mean visits of the conditioned ladybirds in the arm with treatment and the arm with the control, one week after treatment *** p<0,005 , **p<0,01, * p<0,05.

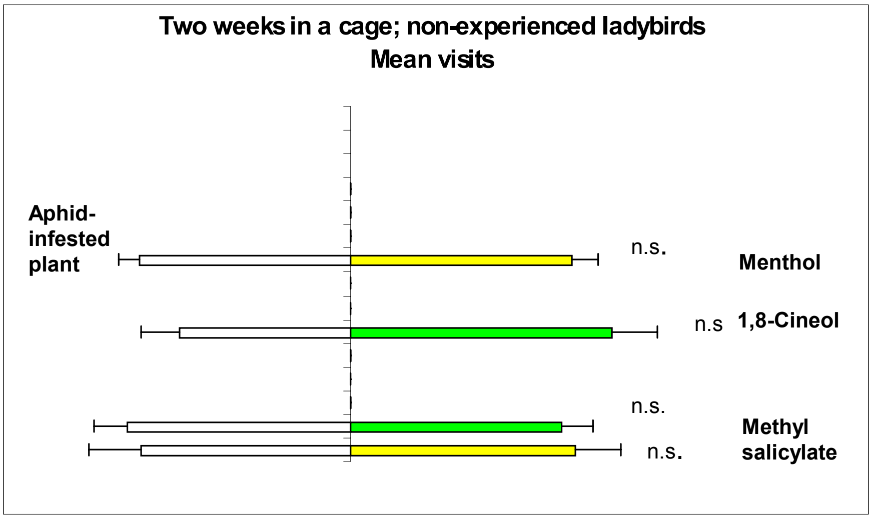


Figure 4. Mean visits of the non-experienced ladybirds in the arm with treatment or the arm with control, two weeks in a cage. *** p< 0,005 , **p<0,01, * p<0,05.

Figure 4 and 5 shows the results from the second olfactometer test. The ladybirds had spent one week in cages containing aphids and sweetened butter and one week in cages with catfood. The experimental treatments of colour and odour were only presented to the beetles together with the aphids and sweetened butter.

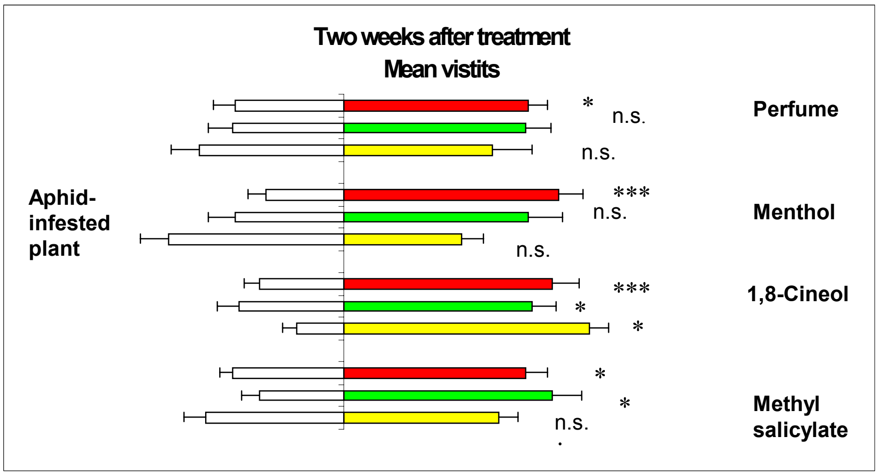


Figure 5. Mean visits of the conditioned ladybirds in the arm with treatment or the arm with control, two weeks after treatment ***p<0,005, **p<0,01, * p<0.05.

The results in figure 4 and 5 differ from the first olfactometer test, with some combinations being more attractive and some loosing the attraction they had in the first test. The combination of 1,8-cineole and yellow was still attractive to experienced ladybirds but not to non-experienced ones. The non-experienced ladybirds did not find any combination significantly attractive.

At the most, the experienced ladybirds spent three weeks in the cages, one week with experimental treatment and two weeks without it in cages with catfood. Not all combinations were tested after three weeks only the ones showing a significant attraction to the ladybirds two weeks after experiencing the treatment.

The results from the first and second olfactometer tests suggest that the combination of 1,8-cineole and yellow could show a similar trend to the combinations of 1,8-cineole and green and methyl salicylate and green. Unfortunately the cages with this combination could not be tested a third time because of technical problems.

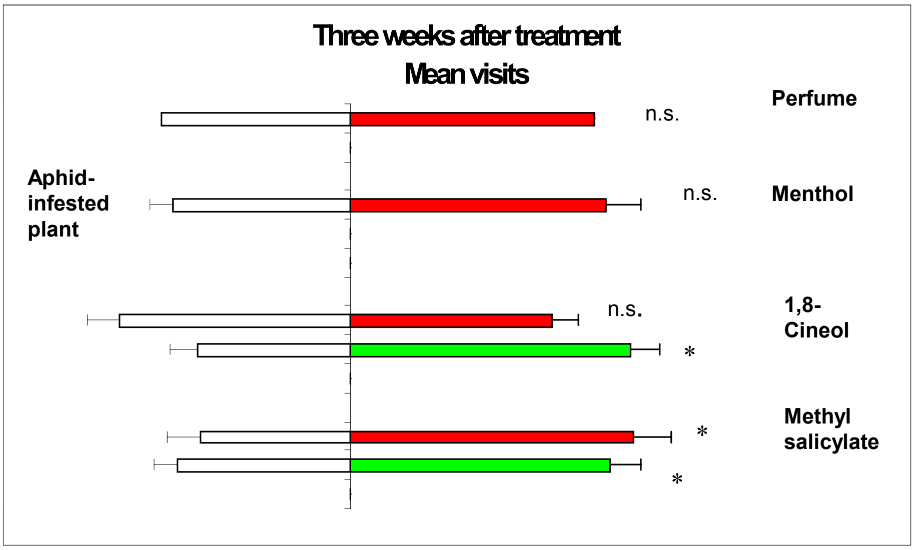


Figure 6. Mean visits of the conditioned ladybirds on the arm with treatment and the arm with control, three weeks after treatment ***p<0,005,**p<0,01,* p<0.05.

Weights

Ladybirds tended to loose weight in the cages, both when they received aphids and butter, and when they were fed catfood. The ladybirds might have been fed too little or with inappropriate food. Hungry or starved ladybirds can react differently to the stimuli given compared with non-hungry ones, and this may have affected the results.

Summary and discussion

Ladybirds that had spent one week with the treatment were significantly attracted to the combinations of 1,8-cineole and yellow, menthol and yellow and perfume and green, suggesting a conditioned effect. Another interesting finding was that both the ladybirds experienced to the treatment and the non-experienced ones were significantly attracted to the combination of methyl salicylate and green and 1,8-cineol and green, presumably an innate behaviour.

Attraction of experienced ladybirds to the combination of cineol and yellow, menthol and yellow and perfume and green may have been due to conditioning whereby they associated the combined odour and colour with the food given. The attraction disappeared when the ladybirds spent one week without the treatment. An exception from this was the combination of 1,8-cineole and yellow that still attracted the experienced ladybirds two weeks after the treatment. Yellow is known to be attractive to insects, and in studies by Udayagiri et al (1997) Majerus (1994), Mondor & Warren (2000) Maredia et al (1992) and Mensah (1997) ladybirds were more attracted to yellow than to other colours. Among the three combinations having a conditioning effect, there were two combinations that included yellow. The combination of 1,8-cineole and yellow remained attractive even two weeks after ladybirds experienced the treatment. Even though the ladybirds did not show any significantly higher attraction to yellow in the four-armed olfactometer, they may have a stronger capability to learn yellow than other colours. The learning process may be enhanced by an innate response to recognising wounded or stressed plants infested with aphids. Another explanation could be that yellow is the colour of their secondary food, pollen, and their learning abilities might be connected to secondary responses such as finding alternative food.

Ladybirds also showed a strong attraction to the combinations of methyl salicylate and green and 1,8-cineole and green. Every olfactometer test (except for the second test with the non-treated ladybirds) gave a significant result with more visits for these combinations. This may indicate an innate behaviour, where methyl salicylate and 1,8-cineole together with green symbolises an infested plant (James, 2003), that was more attractive than the actual aphid-infested plant on the other side of the olfactometer. As mentioned earlier odours from herbivore-infested plants are attractive to ladybirds (Ponsonby & Copland, 1995; Rü & Makosso, 2001; Ninkovic et al., 2001). The attraction may have been stronger because of a higher concentration of odour in the arm with the treatment, or because of the green visual stimulus. The control did only have the odour of an aphid-infested plant but not the colour. This might indicate that vision in combination with olfactory stimulus is important in ladybird searching behaviour. Responding to both stimuli would be an advantage compared to responding to an visual or olfactory stimulus separately. Attraction merely to green objects would not favour ladybirds since this would mean they would respond to most things in the natural environment. Green in combination with the odour of a wounded plant would therefore be a more accurate target for their prey and habitat search than a colour or odour alone.

The ladybirds did loose weight during the study. Whether this was due to lack of food or the nutritional status of the food given is hard to tell. Every week the beetles received 100 aphids, 50 at a time, and they also had sweetened butter as a complement when the aphids were eaten. It was interesting to see if the ladybirds made the same choice in the olfactometer after spending some time without the treatment. The aim was that ladybirds would associate the colour and odour with the presence of aphids, and possibly the sweetened butter. Therefore another food source was needed in the cages without treatment. Catfood was used as a completely new type of food that the ladybirds had never come into contact with. Catfood has been used to feed other types of generalist predators, e.g. Carabidae (Östman et al., 2001). However, the ladybirds still lost weight indicating that the food may not have been suitable. Hunger could therefore have affected their searching behaviour in the second and third olfactometer test.

If ladybirds were searching for prey in a completely random way, they would not have any use for the ability to associate stimuli with the presence of food. They are not blind but have three types of visual receptors: UV, blue and green receptors that allow them to respond to wavelengths from 310-600nm, as do most insects (Lin, 1993). Therefore it seems unlikely that they do not use vision in their search for prey or suitable habitats. Still it is not clear if visual or olfactory stimuli, or a combination of both, is the most important trigger for ladybird searching. Earlier mentioned studies by Obata (1986) suggest that coccinellids use visual and olfactory cues to detect prey. In that study beetles were attracted to small gauze or polyethylene bags by olfactory and visual cues suggesting the presence of aphids, including the odour of aphid-infested leaves, the odour of aphids, the odour of healthy leaves and the sight of leaves. A study by Hattingh & Samways (1995) also confirms that a combination of visual and olfactory stimuli is attractive to adult ladybirds. This study also showed that adults of *C. nigritus* were visually attracted to prominently silhouetted features, such as a horizon with a tree line and individual trees. Vertically oriented parallel lines were also more attractive to these predators than horizontal lines. The study also suggested that leaf shape, in addition to tree or habitat shape, were an important visual cue for fine aspects of habitat location. A study by Ninkovic & Pettersson (2003) also suggests that ladybirds are more attracted to a diversified plant stand than to a monoculture.

This study suggests that the combination of vision and odour are important when ladybirds search for prey or habitat. A random search for food must be much less efficient than if the ladybird recognises a good habitat by its shape, colour or odour. If the stimulus is recognised due to innate behaviour or whether the attraction changes depending on what the ladybird encounters is difficult to conclude from the current results. With the findings from this study one may conclude that ladybirds can be conditioned to certain combined stimuli, but the effect will disappear one week without re-enforcement. The ladybirds are not able to associate all treatments with positive rewards, only combinations with green or yellow. Nevertheless, the two combined stimuli of methyl salicylate and green and 1,8-cineol and green were attractive to the beetles even without prior treatment. The combinations both smelled alike and had the colour of wounded plants and it would be a good guidance to a habitat including aphids, or at least some other herbivore. If ladybirds were able to associate new stimuli with positive reward it could be used to improve their use as biological control agents. It would be interesting to study the behaviour of experienced and non-experienced ladybirds in natural conditions, in a field with the stimulus used in the treatment. But before doing this, further studies should be made on the combinations that showed a significant attraction to ladybirds.

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Appendix

Table 1 Results from olfactometer tests one week after treatment. One week after treatment means that the ladybirds were tested when they had spent one week in the cages together with the combined treatment

One week after treatment	**N**	**Visits on Control Mean ± S.E.**	**Visits on Treatment Mean ± S.E.**	**P-value n.s.=non significant**
Methyl salicylate & yellow	26	1,77 (0,27)	3,31 (0,40)	0,0078
Methyl salicylate &green	34	2,59 (0,31)	4,09 (0,37)	0,0306
Methyl salicylate & red	23	2,61 (0,31)	2,91 (0,34)	0,057 n.s.
Cineol & yellow	19	2,42 (0,34)	3,74 (0,49)	0,0162
Cineol & green	21	2,33 (0,46)	4,38 (0,53)	0,0370
Cineol & red	23	3,08 (0,44)	2,88 (0,48)	0,910 n.s.
Menthol & yellow	24	2 (0,35)	3,75 (0,41)	0,0192
Menthol & green	14	1,93 (0,46)	3,14 (0,49)	0,131 n.s.
Menthol & red	30	2,5 (0,41)	4,17 (0,46)	0,054 n.s.
Perfume & yellow	23	2,30 (0,44)	3,48 (0,59)	0,277 n.s.
Perfume & green	20	1,6 (0,28)	4,35 (0,55)	0,0018
Perfume & red	22	1,5 (0,40)	3,14 (0,53)	0,0674 n.s.

Table 2 Results from olfactometer tests two weeks after treatment. Two weeks after treatment means that the ladybirds were tested when they had spent one week in the cages with treatment, and one week in a non-coloured cage without odour.

Two weeks after treatment	**N**	**Visits on Control Mean ± S.E.**	**Visits on Treatment Mean ± S.E.**	**P-value n.s.=non significant**
Methyl salicylate & yellow	23	2,43 (0,39)	2,74 (0,34)	0,573 n.s.
Methyl salicylate &green	19	1,84 (0,39)	4,53 (0,64)	0,0204
Methyl salicylate & red	22	2,45 (0,269)	4,0 (0,49)	0,029
Cineol & yellow	14	1 (0,30)	5,14 (0,40)	0,016
Cineol & green	22	1,95 (0,40)	3,5 (0,44)	0,038
Cineol & red	22	1,64 (0,29)	4,05 (0,51)	0,0032
Menthol & yellow	21	2,76 (0,44)	1,86 (0,33)	0,198 n.s.
Menthol & green	9	2,11 (0,51)	3,56 (0,67)	0,16 n.s.
Menthol & red	20	1,45 (0,32)	3,95 (0,47)	0,003
Perfume & yellow	11	3 (0,60)	3,09 (0,83)	0,965 n.s.
Perfume & green	20	1,8 (0,40)	2,95 (0,41)	0,132 n.s.
Perfume & red	20	2,2 (0,43)	3,7 (0,39)	0,0283

Table 3 Results from olfactometer tests three weeks after treatment. Three weeks after treatment means that the ladybirds were tested when they had spent one week in the cages with treatment, and two weeks in a non- coloured cage without odour.

Three weeks after treatment	**N**	**Visits on Control Mean ± S.E.**	**Visits on Treatment Mean ± S.E.**	**P-value n.s.=non significant**
Methyl salicylate & yellow	-	-	-	-
Methyl salicylate &green	19	2,63 (0,36)	4 (0,45)	0,0395
Methyl salicylate & red	20	2,05 (0,46)	3,9 (0,53)	0,035
Cineol & yellow	-	sabotage	sabotage	sabotage
Cineol & green	22	2,27 (0,40)	4,18 (0,43)	0,025
Cineol & red	15	3,4 (0,47)	3 (0,38)	0,753 n.s.
Menthol & yellow	-	-	-	-
Menthol & green	-	-	-	-
Menthol & red	20	2,5 (0,34)	3,65 (0,48)	0,184 n.s.
Perfume & yellow	-	-	-	-
Perfume & green	-	-	-	-
Perfume & red	20	2,68 (0,32)	3,5 (0,39)	0,118 n.s.

Table 4 Results from olfactometer tests one week after placement in cage, non-treated beetles.

One week in a cage	**N**	**Visits on Control Mean ± S.E.**	**Visits on Treatment Mean ± S.E.**	**P-value n.s.=non significant**
Methyl salicylate & yellow	14	1,86 (0,38)	4,57 (0,63)	0,011
Methyl salicylate &green	30	3,17 (0,30)	4,73 (0,45)	0,025
Methyl salicylate & red	-	-	-	-
Cineol & yellow	25	3,36 (0,33)	3,52 (0,40)	0,78 n.s.
Cineol & green	20	1,20 (0,25)	3,70 (0,56)	0,004
Cineol & red	-	-	-	-
Menthol & yellow	26	2,62 (0,38)	3,00 (0,39)	0,32 n.s.
Menthol & green	-	-	-	-
Menthol & red	-	-	-	-
Perfume & yellow	-	-	-	-
Perfume & green	29	2,59 (0,39)	3,55 (0,54)	0,32 n.s.
Perfume & red	-	-	-	-

Table 5 Results from olfactometer tests two weeks after placement in cage, non-treated beetles.

Two weeks in a cage	**N**	**Visits on Control Mean ± S.E.**	**Visits on Treatment Mean ± S.E.**	**P-value n.s.=non significant**
Methyl salicylate & yellow	8	3,63 (0,89)	3,88 (0,79)	0,92 n.s.
Methyl salicylate &green	17	3,63 (0,56)	3,44 (0,52)	0,78 n.s.
Methyl salicylate & red	-	-	-	-
Cineol & yellow	-	-	-	-
Cineol & green	13	2,77 (0,61)	4,23 (0,72)	0,26 n.s.
Cineol & red	-	-	-	-
Menthol & yellow	16	3,36 (0,33)	3,52 (0,40)	0,07 n.s.
Menthol & green	-	-	-	-
Menthol & red	-	-	-	-
Perfume & yellow	-	-	-	-
Perfume & green	-	-	-	-
Perfume & red	-	-	-	-

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