

**CLEBER MACEDO DE OLIVEIRA**

**ASSOCIATIVE LEARNING IN IMMATURES AND ADULTS OF  
GENERALIST PREDATORS**

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

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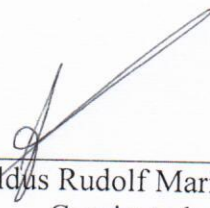
Maira Christina Marques Fonseca



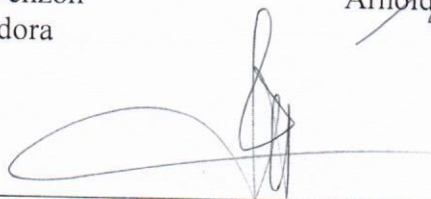
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Em agosto de 2010 iniciou o curso de Mestrado em Entomologia pela Universidade Federal de Viçosa, também sob orientação do professor Angelo Pallini. Em julho de 2012 obteve o título de *Magister Scientiae* em Entomologia pela Universidade Federal de Viçosa.

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defesa da tese tendo como membros da banca os pesquisadores já citados e Maira Christina Marques Fonseca e Fabrício Rainha Ribeiro.

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## ABSTRACT

OLIVEIRA, Cleber Macedo, D.Sc., Universidade Federal de Viçosa, setembro de 2012. **Associative learning in immatures and adults of generalist predators.** Adviser: Angelo Pallini Filho. Co-advisers: Madelaine Venzon e Arnoldus Rudolf Maria Janssen.

Plants produce organic volatiles continuously, however this blend of volatiles changes when they are infested by herbivores. These odours can be used by beneficial arthropods to locate prey. However, the blend of volatiles emitted by plants can vary with changing biotic and abiotic conditions. Due to variability of odours, it is expected that predators learn the association of odours and availability of food. Learning occurs when the response to an odour change due to an experience. Learning was demonstrated in many species of arthropods, however those studies assessed learning in adults. Those studies evaluated learning in the laboratory conditions and did not study the effect of the conditioned stimulus (i.e. odours) in the learning. Here, we aimed to study learning in immatures of the generalist predator *Ceraeochrysa cubana* and adults of *Orius insidiosus*. We assessed the effect of odours in the learning in two generalist predators and evaluated the effect of learning in the foraging behaviour of immature predators. We assessed the response of the predators to odours in the olfactometer and in a release-recapture experiment. The odours studied were methyl salicylate, rosemary oil liquid (*Rosmarinus officinalis*) and mint oil. Immatures of lacewings learned the association of methyl salicylate with availability and unavailability of food. This was the first study that assessed the learning in immatures predators. Learning changed the foraging behaviour of immature predators. Immatures of lacewings were able to learn two association with the same odour, association of volatile with food and after association of volatile without food, during the immature phase. Additionally, our results showed that the learning ability of *O. insidiosus* is dependent of the conditioned stimulus (odours).

## RESUMO

OLIVEIRA, Cleber Macedo, D.Sc., Universidade Federal de Viçosa, setembro de 2012.  
**Aprendizado associativo em imaturos e adultos de predadores generalistas.**  
Orientador: Angelo Pallini Filho. Coorientadores: Madelaine Venzon e Arnoldus Rudolf Maria Janssen.

As plantas produzem voláteis orgânicos continuamente, porém a mistura destes voláteis muda quando as plantas estão infestadas por herbívoros. Esses odores podem ser utilizados por artrópodes benéficos para localizarem presas/hospedeiros. Entretanto, a mistura dos voláteis emitidos pelas plantas podem variar devido a condições bióticas e abióticas. Devido à variabilidade dos odores, espera-se que os predadores possam aprender a associação de odores com a disponibilidade de presa. A aprendizagem ocorre quando a resposta a um estímulo muda devido a uma experiência. Aprendizado foi demonstrado em muitas espécies de artrópodes, porém estes estudos avaliaram aprendizado em adultos. Estes estudos avaliaram a aprendizagem em condições de laboratório e não estudaram o efeito do estímulo condicionante (por exemplo odores) na aprendizagem. Aqui foi estudado a aprendizagem em imaturos de *Ceraochrysa cubana*, um predador generalista e adultos de *Orius insidiosus*. Nós avaliamos o efeito dos odores na aprendizagem em dois predadores generalistas e avaliamos o efeito do aprendizado no comportamento de forrageamento dos imaturos. Nós avaliamos a resposta dos predadores aos odores em experimentos de olfatosmetria e em um experimento de liberação e recaptura de predadores. Os odores estudados foram metil salicilato, óleo de menta (*Mentha piperita*) e óleo de alecrim (*Rosmarinus officinalis*). Imaturos de crisopídeo aprenderam a associação de metil salicilato com a disponibilidade e indisponibilidade de alimento. Este foi o primeiro trabalho que avaliou aprendizado em imaturos de predador. Imaturos de crisopídeos são capazes de aprender duas associações com o mesmo odor, associação do odor com alimento e depois associação do mesmo odor sem alimento,

durante a fase imatura. Adicionalmente, nossos resultados demonstraram que a capacidade de aprendizado em *O. insidiosus* é dependente do estímulo condicionante (odor).

## **INTRODUCTION**

Learning has been defined as a change in behaviour as a result of experience (Papaj and Lewis 1993). This change can enable animals to adapt their behavior after changes in their environment (Dukas 2008). Learning has been demonstrated in many taxons of arthropods, such as social insects (Farina et al. 2005; Amdam et al. 2010) and parasitoids (Lewis and Tumlinson 1988; Lewis and Takasu 1990; Wackers and Lewis 1994; Stireman 2002). However, there are few studies investigating learning in predators (Drukker et al. 2000a; Drukker et al. 2000b). Learning allows individuals to explore the environmental resources that are unique to a particular time and place. The animals' learning ability enables them to respond to a type and amount of information that were not perceived or that does not elicit a response, and consequently increases their behavioral repertoire (Dukas et al. 2009).

Plants attacked by herbivores emit odours that are used by predators as cues to the location of herbivorous insects (Turlings et al. 1995). These odors are mixtures of compounds, which vary with the host plant species - even when attacked by the same herbivore, and the species of herbivore- even when they attack the same host plant species (De Moraes et al. 1998; Dicke et al. 1998). Due to variation in these blends of volatile emitted by plants, it has been suggested that animals can learn the association between blends of volatiles and the availability of food (Lewis and Tumlinson 1988; Turlings et al. 1993; Drukker et al. 2000; Takabayashi et al. 2006; Hilker and McNeil 2008; Baldwin 2010; Hare 2011; Riffell et al. 2013).

The preference for odours can be innate or acquired during an individual's lifetime. The innate preference of an odour is inherent to the individual, hereditary and independent of previous experience (Drukker et al. 2000). The preference for odours can be acquired in three different ways: a) imprinting, defined as learning that occurs early in

the individuals' life - with or without reinforcement (Gould 1993; Hall and Halliday 1998); b) sensitization, which is a gradual increase in response to a stimulus, along with exposure to the stimulus, even when it has not been combined with any other stimuli (Papaj and Prokopy 1989; Hall and Halliday 1998); and c) associative learning, where the pairing of a conditioned stimulus (i.e. odour) and unconditioned stimulus (i.e. food) results in a preference or aversion to a conditioned stimulus, depending on the learning context (Thorpe 1956; Lewis and Tumlinson 1988; Hall and Halliday 1998).

Entomophagous insects are known for detecting and using visual and olfactory cues to detect habitats with resources, they can learn how to associate these cues with the most profitable habitats containing appropriate food (Pérez-Maluf et al. 2008). Several species of predators use blend of odours emitted by plants to locate prey (Dicke and Sabelis 1988; Sabelis et al. 1999). Besides olfactory cues, predators can also use visual cues to locate prey (Michaud and Mackauer 1994; Udayagiri et al. 1997; Wäckers and Lewis 1999; Raguso and Willis 2005). The synergism of olfactory and visual cues can lead to high rate of encounter among predator and prey. It is expected that diurnal predators use more visual cues than nocturnal predators.

Generalist predators need to locate prey on more than one plant in their lifetime. Because of this, these predators need to learn the association of new blends of volatiles with the availability of food (Glinwood et al. 2011). Several studies have shown the ability of adult generalist predators to associate new odours with availability of prey (Drukker and Sabelis 1990; Drukker et al. 2000a; Drukker et al. 2000b; De Boer and Dicke 2004; De Boer et al. 2005). However, knowledge of the changes in immature behavior to a previous experience is still incipient and too little is known about the use of odours emitted by plants and the effect of learning on the biological control in field conditions.

In chapter one, we investigate if lacewing larvae are able to learn the association of methyl salicylate with the presence or absence of food. We also studied whether this learning affected the foraging behaviour of immature predators in the field. In Chapter two, I studied the ability of immatures of *Ceraeochrysa cubana* to learn two associations with the same odour during the immature phase. We investigated if the response of predators that learn the association of methyl salicylate with availability of food are affected by a new odour. In the last chapter, we investigate whether the predatory bugs *Orius insidiosus* are able to learn associations between two odours and the availability or absence of food.

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## CHAPTER 1

### **Associative learning in immature lacewings**

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## **ABSTRACT**

Plants attacked by herbivores are known to emit volatiles that are used by the predators of the herbivores to locate prey. The composition of these volatiles varies depending on the plant, the herbivore and abiotic factors. In this scenario, predators need to cope with this large variation of odours. It is suggested that they therefore learn the association between the presence of herbivores and the volatiles. Immatures of the lacewing *Ceraeochrysa cubana* (Hagen) (Neuroptera: Chrysopidae) are voracious polyphagous predators, which are important for biological control, whereas adults feed on plant-provided food and honeydew. Therefore, the study of the foraging behaviour and the effects of learning in immatures of this species is important. We therefore exposed immatures to methyl salicylate, which was either associated with eggs of *A. kuehniella* as food source or associated with the absence of food. Subsequently, their response to this volatile was tested in an olfactometer test. Immatures that had experienced the association of methyl salicylate with food were attracted to it, and immatures that were exposed to methyl salicylate during deprivation of food were repelled. Predator larvae that had experienced the association between methyl salicylate and food were released on a plant without food, and were found to use this volatile in locating patches with food. In contrast, larvae without such experience did not use these volatiles. We conclude that immature predators are capable of learning the association between volatiles and food or hunger and use this learning during foraging.

**Keywords:** Chrysopidae, Volatiles, Biological control, Methyl salicylate

## **INTRODUCTION**

Learning has been defined as a change in behaviour as a consequence of experience (Papaj and Prokopy 1989). It may help an animal to adapt its behaviour in response to changing environmental circumstances (Dukas 2008). Learning has been demonstrated in many arthropods, in particular in social insects (Farina et al. 2005; Amdam et al. 2010), parasitoids (Lewis and Tumlinson 1988; Lewis and Takasu 1990; Wackers and Lewis 1994; Stireman 2002) and, to a lesser extent, in predators (De Boer and Dicke 2004a; Guillette et al. 2009; Rahmani et al. 2009).

Learning can help individuals to exploit correlations in their environment that are particular for a certain time and place. An animal's ability to learn will depend on the amount of information they can respond to (Dukas et al. 2009). Entomophagous insects are known to use olfactory and visual cues to locate food and habitats. With experience, they can learn to associate these cues with the most profitable habitats or food (Pérez-Maluf et al. 2008). Arthropod predators and parasitoids use volatile organic compounds produced by plants to locate prey/host (Dicke and Sabelis 1988; Sabelis et al. 1999; Lewis and Takasu 1990), but they often use visual cues as well (Weseloh 1972; Michaud and Mackauer 1994; Wackers and Lewis 1994; Udayagiri et al. 1997). Odour preference may be innate or acquired during an individual's life. An innate response is heritable and does not require experience (Drukker et al. 2000a) but can be changed as a result of experience (Papaj and Prokopy 1989).

Odour preference may arise because of (1) imprinting, defined as learning rapidly during a sensitive period early in life - with or without new experience of the individuals with the cues (Gould 1993; Hall and Halliday 1998); (2) sensitization, when the response to a stimulus increases as a result of exposure to that stimulus (Papaj and Prokopy 1989; Hall and Halliday 1998); (3) associative learning, where a conditioned stimulus (i.e.

volatile) and an unconditioned stimulus (i.e. food) are paired, and the response (positive or negative) to the conditioned stimulus increases with increasing experience with the paired stimuli (Thorpe 1956; Lewis and Tumlinson 1988; Hall and Halliday 1998).

Plants attacked by herbivores are known to emit volatile compounds that are used by predators to locate prey (e.g. Turlings et al. 1995). These volatiles consist of a mixture of compounds that may vary with the species of host plant, even when attacked by the same herbivore, and with the species of herbivore, even when they attack the same species of host plant (Dicke et al. 1998; Moraes et al. 1998). Moreover, it can vary with plant genotype, plant age and abiotic conditions (Takabayashi et al. 1994). Because of this variation, natural enemies must cope with different signals that are associated with the presence of their prey (Takabayashi et al. 1991; Moraes et al. 1998; Sabelis et al. 1999a; Sabelis et al. 1999b; van den Boom et al. 2002; van den Boom et al. 2004; Sabelis et al. 2007). It has been suggested that animals can cope with this variation by learning the association between volatile blends and the presence of food (Lewis and Tumlinson 1988; Lewis and Takasu 1990; Turlings et al. 1993; Drukker et al. 2000; Takabayashi et al. 2006; Hilker and McNeil 2008; Baldwin 2010; Hare 2011; Riffell et al. 2013). Predators may have to switch among prey or host plants during their lives, so they need to associate new volatiles with prey availability (Dicke and Sabelis 1988). Many studies have demonstrated the ability of adult arthropod predators to associate new volatiles with prey (Drukker et al. 2000a; Drukker et al. 2000b; De Boer and Dicke 2004a; De Boer et al. 2005), but little is known about changes in immature behaviour as a result of a previous experience with volatiles.

Associative learning as studied in this paper requires that the conditioned (i.e. volatile) and the unconditioned stimulus (i.e. food or hunger) are paired, resulting in context-dependent preference or aversion depending on the unconditioned stimulus. This

kind of learning was demonstrated for predators, for example, predatory mites (Drukker et al. 2000a) and heteropteran bugs (Drukker et al. 2000b).

Immatures of lacewing, *Ceraeochrysa cubana* (Hagen) (Neuroptera: Chrysopidae), are important natural enemies in Neotropical America, with a potential for biological control (Lopez-Arroyo et al. 1999; Albuquerque et al. 2001). Lacewing larvae are predators of eggs of arthropods and soft-bodied insects, such as aphid (New 1975; Souza et al. 2008) and they are found in many crops, where they play an important role in the control of pests (Albuquerque et al. 1994; Albuquerque et al. 2001). Lacewing adults feed on plant-provided food and honeydew, so they are not important predators. Whereas these adults are known to respond to a variety of volatiles, such as aphid sex pheromones (Boo et al. 2003), aggregation pheromones of the conspecific (Chauhan et al. 2007; Jones et al. 2011) and herbivore-induced plant volatiles (James 2003a; Jones et al. 2011), nothing is known about the response of larvae, the most important predatory stage. The studies of learning are being performed with adult predator maybe because this phase are longer than immatures, and many studies with learning need much time to expose and training the individual. We studied the effect of experience with methyl salicylate, a component of herbivore-induced plant volatiles (Dicke et al. 1990), which was associated with the presence or absence of food to determine whether lacewing larvae were able to learn this association. We also investigated whether this learning affected the foraging behaviour of the larvae in an experiment where predators were exhibited to a blend of odours.

## **MATERIALS AND METHODS**

### **Rearing methods**

The culture of *Ceraeochrysa cubana* was established from insects collected from coffee and pepper crops around Viçosa (state of Minas Gerais, Brazil). Juveniles were fed with eggs of the Mediterranean flour moth, *Ephestia kuehniella* (Lepidoptera: Pyralidae), and adults received a yeast–honey solution (1:1) (Venzon and Carvalho 1992). Rearings were maintained at controlled temperature ( $25 \pm 2^\circ \text{C}$ ), relative humidity ( $75 \pm 5\%$ ), and photoperiod (12:12 L: D).

### **Odour sources**

Volatile dispensers were made of Parafilm®, which was cut into strips of  $5.2 \text{ cm}^2$ , rolled up and tightly flattened in layers of  $\pm 5 \text{ mm}$ . Each roll was cut into 5 pieces of 7 mm long (Janssen et al. 2014). One group of dispensers was incubated in 99% liquid synthetic methyl salicylate (Sigma-Aldrich, China) in a closed Petri dish and the other was kept in a clean Petri dish. After 24 hours, the dispensers were taken from the Petri dishes and placed on a tissue paper to dry. Dispensers with and without methyl salicylate were used in olfactometer tests and in training trials.

### **Y-tube olfactometer tests**

A Y-tube olfactometer (Sabelis and van de Baan 1983; Janssen et al. 1997) was used to test preference for or aversion to methyl salicylate compared to ambient air. The olfactometer consisted of a glass tube ( $\text{Ø} = 3.5 \text{ cm}$ ) in the form of a “Y”. Each of the arms of the Y-tube was connected with a plastic tube to a glass container (43 x 36 x 50 cm) in which three volatile dispensers were arranged. The base of the Y-tube was connected with a plastic tube to a vacuum pump, which was used to generate a constant air flow. The air



entered the glass containers and carried the odour out of it passing by the volatile dispensers to the arms of the Y-tube. The airflow in each arm of the olfactometer was calibrated to 0.50 m/s (VelociCalc® Air Velocity Meter 9545-A). One predator, twelve days old, was released at the downwind end of the Y-tube, and it was allowed to walk upwind along the base of the Y-tube and to choose one of the arms, connected to either the container with an odour source or the container with empty dispensers. A trial ended when the predator reached the end of one of the arms of the Y-tube or after 5 min, after which it was removed and the next predator was introduced. Each replicate consisted of twenty predators that had made a choice. After five animals had been tested and made a choice, the containers were connected to the opposite arm of the olfactometer and the experiment continued to correct for unforeseen asymmetries in the experimental set-up. Unless stated otherwise, predators were starved for 24 hours prior to testing. Four replicate experiments were carried out for the innate response and three replicate experiments were carried out for the training experiment each on a different day with a different group of predators and different volatile dispensers.

### **Associative learning**

To test the innate response, four groups of 30 individual third instar predators, ten days old, were taken from the rearing units. Individuals were incubated for 24 h in a plastic tube ( $\varnothing=3$  cm and 7.5 cm deep) without food, with a hole in the lid covered with thin mesh for ventilation. Subsequently, their response to methyl salicylate or ambient air was tested. Each group was tested on a different day.

The training started with first instar predators of 24 hours old that were taken from the rearing and randomly assigned to two groups. They were incubated in plastic tubes as above, and all plastic tubes were placed inside a plastic box (35 x 24 cm) with two

openings. One opening ( $\varnothing=3$  cm) was connected to a pump that produced an air flow from the room to the box at 0.45 m/s in the entry the box. The other side of the box had an air outlet. The volatile dispensers were put in Petri dishes below the input of the air flow (Figure 1). In a pilot experiment, cotton wool was put inside the tubes used in the training procedure and three volatile dispensers were put in the box and the pump was connected to the box. After 24 hours, the volatile could be perceived from the cotton wool that had been inside the tubes, confirming that odours were carried into the tubes in our set-up. During the first 96 h, the immatures of all groups received eggs of *E. kuehniella* in their tubes as food. Subsequently, they were incubated in a new plastic tube without food for 24 h. During the next four days, immatures were daily switched from tubes with food to tubes without food. During the period of feeding, one group was put inside a plastic box with three dispensers of methyl salicylate, whereas the control group was never exposed to it. After this period, the individuals of eleven days old of the two groups were tested for their response to methyl salicylate or ambient air.

To assess whether associative learning was involved (operant conditioning; Hall & Halliday, 1998), it was necessary to show a switch in response when methyl salicylate was associated with a negative stimulus. The same training as described above was given to two other groups, but one group was exposed to methyl salicylate when they were without food. The control group again was not exposed to methyl salicylate. The response of these groups was also assessed. The training experiment was repeated three times for each association. All data were analysed with a log-linear model for contingency tables with Generalized Linear Models (GLM) using a Poisson error distribution (Crawley 2007), with odour, side, training and replicate and all interaction as a factor. The analysis was performed with the statistical software R 2.15.1 (R-Development-Core-Team 2012). The minimal adequate model was obtained by removing non-significant interactions and

factors with deletion tests using the “anova” command in R (R Development Core Team 2012).

### **Release-recapture experiment**

A release-recapture experiment was done in an external area to assess the foraging behaviour of immatures of lacewings. Lacewings was trained and release to evaluate the effect of training in the behaviour of foraging in immature. This experiment is the first to evaluate the foraging behaviour in immatures and the effect of learning in this behaviour. In the experiment immature predators was exhibited to other volatile sources emitted by the plants that surround the area. A cage, consisting of a tray inside a wooden frame (1.60 × 1.60 × 1.70 m), covered with fine mesh was placed in the area with trees on one side and a building on the other side and grass in the other two sides. The tray was filled with soil and three black plastic discs (Ø=14 cm) with volatile dispensers of methyl salicylate and three plastic discs with dispensers without volatile were placed alternatingly in a hexagon (diam. 1 m) on top of the soil. Eggs of *E. kuehniella* were added to all discs in order to arrest arriving predators. Discs with dispensers with and without methyl salicylate occupied alternating positions to avoid any unforeseen directionality in predator dispersion (Janssen 1999). In two replicates, the three plastic discs with dispensers of methyl salicylate were put on positions 1, 3 and 5, in the other two replicates the discs with methyl salicylate were placed on positions 2, 4, and 6.

About 200 immature predators (24 hours old) were taken from the rearing. Each immature was incubated in a plastic tube as above and received the same experience as explained above, with methyl salicylate paired with the presence of eggs of *E. kuehniella* as food source, whereas the control group again had no experience with it. After the training period, the predators were carefully placed on a cabbage plant (*Brassica oleracea*

var. *capitata*, eight-leaf stage) in the middle of the hexagon and the predators were allowed to disperse from the plant to the discs. The cabbage plant was as a substrate to put the immatures and was used to confirm that the immatures would disperse from a plant to the discs and to simulate a more natural condition. Starting 1 hour after the release, all discs were sampled once per hour, during a total period of 6 h and again after 24 h of releasing. At each check, all predators found on the plastic discs were removed. The temperature inside the cage was between 25 and 30°C. For logistic reasons, the training group and the control group were released on different days with new dispensers of odour.

The total number of immatures that were recaptured on the discs with or without volatile were analysed with a Generalized Linear Models (GLM) using a Poisson distribution (Crawley 2007). Treatment (experience or control group) was used as factor. The analysis was performed with the statistical software R 2.15.1 (R Development Core Team 2012).

## **RESULTS**

### **Associative learning**

Naïve immatures of *C. cubana* did not show attraction or repulsion to methyl salicylate; 55 % of the predators chose methyl salicylate (Figure 2, 1<sup>st</sup> bar, d.f. = 1,  $\text{Chi}^2 = 0.80$ ,  $P = 0.37$ ). The individuals that were trained with the association between methyl salicylate and unavailability of food did not show attraction or repulsion to methyl salicylate (Figure 2, 3<sup>rd</sup> bar, d. f. = 1,  $\text{Chi}^2 = 2.41$ ,  $P = 0.1201$ ). Lacewings that had been exposed to methyl salicylate associated without food differed in the response in the olfactometer test compared with the control group (Figure 2, 2<sup>nd</sup> and 3<sup>rd</sup> bar, interaction of experience with odour: d. f. = 1,  $\text{Chi}^2 = 6.60$ ,  $P = 0.01$ ). The control group of the training of association

between methyl salicylate and unavailability of food did not show attraction or repulsion to methyl salicylate (Figure 2, 2<sup>nd</sup> bar, d. f. = 1,  $\text{Chi}^2 = 4.13$ ,  $P = 0.042$ ). There was no difference in the response of lacewing between the control group and the naïve group (Figure 2, 1<sup>st</sup> and 2<sup>nd</sup> bar, d. f. = 1,  $\text{Chi}^2 = 0.00$ ,  $P = 1$ ). In contrast, lacewings that were trained with the association between methyl salicylate and availability of food had an attraction to methyl salicylate (Figure 2, 5<sup>th</sup> bar, d. f. = 1,  $\text{Chi}^2 = 11.64$ ,  $P = 0.00064$ ). Compared with the control group, lacewings that were exposed to synthetic methyl salicylate associated with food were attracted to it (Figure 2, 4<sup>th</sup> and 5<sup>th</sup> bar, interaction of experience with odour: d. f. = 1,  $\text{Chi}^2 = 8.90$ ,  $P = 0.0029$ ). The individuals of the control group did not show attraction or repulsion to methyl salicylate (Figure 2, 4<sup>th</sup> bar, d. f. = 1,  $\text{Chi}^2 = 0.601$ ,  $P = 0.4382$ ). There was no difference in the response of lacewing between the control group of the association between methyl salicylate without food and the naïve group (Figure 2, 1<sup>st</sup> and 2<sup>nd</sup> bar, d. f. = 1,  $\text{Chi}^2 = 0.00$ ,  $P = 1$ ), and there was no difference in the response of lacewing between the control group of the association between methyl salicylate with food and the naïve group (Figure 2, 1<sup>st</sup> and 4<sup>th</sup> bar, d. f. = 1,  $\text{Chi}^2 < 0.00$ ,  $P = 1$ ).

### **Release-recapture experiment**

The release-recapture experiment revealed that a significant larger proportion of *C. cubana* with experience with methyl salicylate and food was recaptured on discs with methyl salicylate compared with predators that had not received such experience (Figure 3, d. f. = 1,  $\text{Chi}^2 = 24.3$ ,  $P < 0.0001$ ). Individuals of the control group were recaptured more on discs without methyl salicylate compared with discs with methyl salicylate (d. f. = 1,  $\text{Chi}^2 = 5.30$ ,  $P = 0.021$ ). The numbers of individuals from the training group that were recaptured on discs with methyl salicylate were higher than on discs without volatile (d.

f. = 1,  $\text{Chi}^2 = 30.64$ ,  $P < 0.0001$ ). On average, 49.5% of all released predators were recaptured. The other predators died, still walking in the area or escaped from the experiment area. Most predators were recaptured within 6 h after their release.

## DISCUSSION

It is known that predatory arthropods can use volatiles to explore their environment, but there is little knowledge of the ability of immatures to use these cues. However, immature predators are the most voracious stage in several groups of predators, such as in lacewings, some species of Syrphidae and Coccinelidae (Gilbert 1981; Lee and Kang 2004; Omkar and James 2004; Omkar and Pervez 2004; Cabral et al. 2009). It is therefore important to study the behaviour of these immature, more specifically, whether they also use volatiles to find plants with prey, and whether they are able to learn. To our knowledge, this is the first study that demonstrates that immature predators use volatiles to find prey and that they are able to learn the association of volatiles with food.

After having been exposed to methyl salicylate associated with food, immature *Ceraeochrysa cubana* were attracted to this compound. In contrast, immature lacewings that were exposed to methyl salicylate associated with the absence of food (hunger) were repelled by it (Figure 2); Hence, a change occurred in the behavior of the predator based on the association of a conditioned (odour of methyl salicylate) and unconditioned stimulus (presence or absence of food), confirming associative learning as defined by Papaj and Prokopy (1989). Associative learning was reported for adult predatory arthropods such as mites and bugs (Drukker et al. 2000a; Drukker et al. 2000b; De Boer and Dicke 2004a; De Boer and Dicke 2004b), and here we report it for immature lacewings, demonstrating that immature predators are also able to learn an association between volatile and the presence or absence of food.

How long the behavior of arthropods will remain affected by experience can vary extensively, from minutes to months, depending on the species, the age, the strength of the association of the rewarding or aversive stimulus and the number of experiences (Neuser et al. 2005; Blackiston et al. 2008; Tapia et al. 2015). Predators may need a few minutes to hours to locate new patches with prey, depending on the availability of food and the persistence of learning during foraging can increase the rate of finding of new prey patches. In this experiment we demonstrate that predators still arrive in patches with volatiles 24 hours after release.

Under natural conditions, predators are exhibited to a large variety of odours. The composition of odours emitted by plants varies due to herbivory, plant species, genotype, age and abiotic factors (Dicke and Sabelis 1988; Du et al. 1996; Dicke et al. 1998; Moraes et al. 1998; Gouinguene and Turlings 2002; van den Boom et al. 2004; Vallat et al. 2005; Glinwood et al. 2011). The role of plant-produced volatile compounds in the attraction of predators was extensively studied under laboratory conditions. Moreover, Janssen et al. (2014) showed that natural communities can learn to associate the presence of food with volatiles in the field. However, more experiments that evaluate the effect of learning in communities of predators in field conditions are necessary.

James (2003b; 2005) and James and Price (2004) showed that the use of herbivore-induced plant volatiles (HIPVs) (i. e. methyl salicylate) in a crop could increase establishment of certain beneficial insects and suggested that adding this compounds could improve biological pest control. Plants that are attacked by herbivores emitted the herbivore-induced plant volatiles that are responsible for recruit beneficial insects, so in this situation the application of HIPVs is unnecessary. In contrast, when plants are not attacked by herbivores, the volatiles emitted by the plants are not attractive for beneficial arthropods and application of HIPVs will attract those insects. However, we demonstrated

here that if this odour is not associated with a reward (i. e. food), predators may quickly learn to avoid it. Predators are being training every time in its lifetime in field. So application of odours in field conditions to attract predators need be paired with an alternative food. Due the association of odour and food the predators will persist in the area and will learn the association of the new odour with the reward.

In conclusion, our data show that juvenile lacewings have the ability to learn the association between odours and the presence or absence of food. Additionally, we demonstrated that learning affected the behaviour in immature lacewings and they can remember an association of a volatile with a positive stimulus for at least 24 hours. More studies are needed to investigate the importance of this leaning during foraging of predatory arthropods under more natural conditions.



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## FIGURE LEGENDS

Figure 1. Container used in the training of predators. It consisted of a transparent box (35 x 24 x 20 cm) with two openings to circulation of air. The air inlet was connected to a pump that produced an air flow at 0.45 m/s at the entry the box. All plastic tubes were put inside the box, closed with a fine mesh to prevent escape of predators from the tubes. Volatile dispensers were put in a Petri dish below the air inlet.

Figure 2. *Ceraeochrysa cubana* were offered a choice between the odours of methyl salicylate or ambient air. Shown are the proportions of individual *C. cubana*, that chose for odours of methyl salicylate (MeSa, right) and the proportions that preferred ambient air (left) in a Y-tube olfactometer. Shown are the innate response (N = 20 x 4), the response of predators that had experienced the association of MeSa with the absence of food together with the control group (N = 3) and predators that had experienced the association between MeSa and food and the other control group (N = 3). See text for further explanation. n.s.:  $P > 0.05$ ; \*:  $P < 0.05$ ; \*\*:  $P < 0.01$  (Generalized linear models with Poisson distribution.).

Figure 3. *Ceraeochrysa cubana*, either experienced with the association of MeSa with food or naïve, were offered a choice between discs with dispensers of odours with or without methyl salicylate. Shown are the cumulative numbers of predators recaptured on discs with odours of methyl salicylate (MeSa) (black bars) and without odour (white bars). The fraction of predators that chose the odours in the experienced and naïve group was tested with a generalized linear models with poisson distribution. See text for further explanation. n.s.:  $P > 0.05$ ; \*:  $P < 0.05$ ; \*\*:  $P < 0.01$ .

**Figure 1**

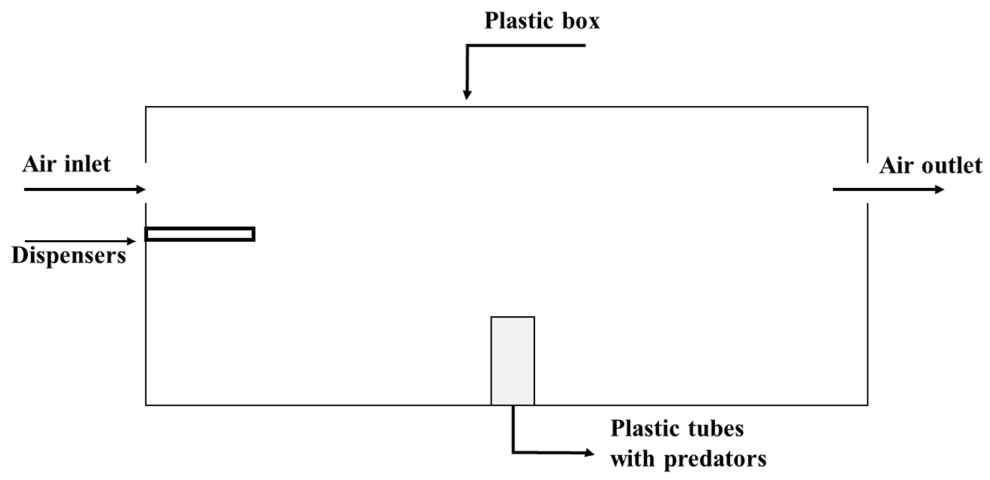
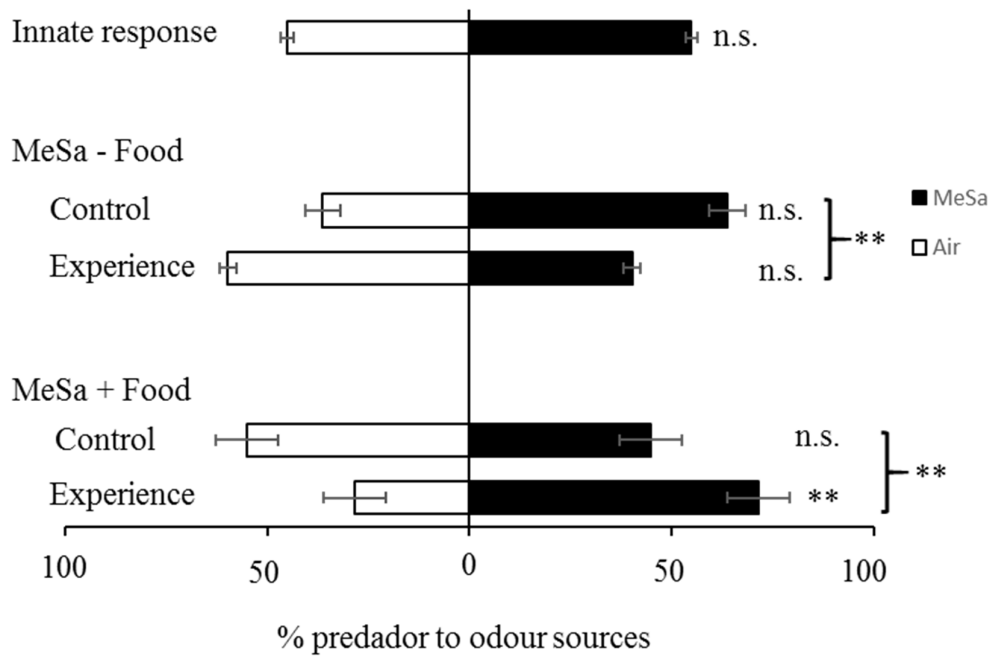
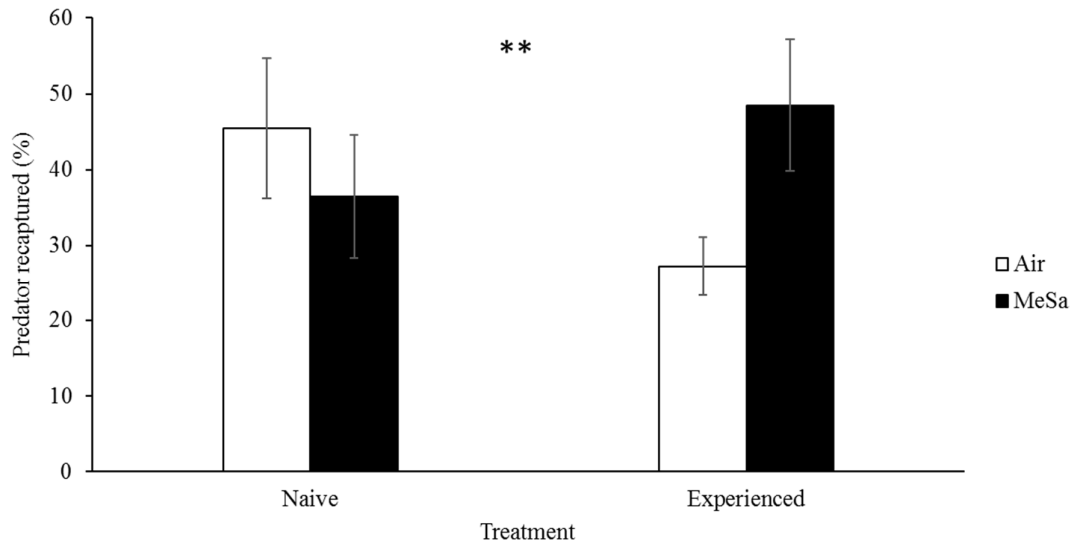




Figure 2.



**Figure 3.**



## CHAPTER 2

### **Sequential learning in a generalist predator**

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## ABSTRACT

Plants emit organic volatiles continuously, however, these volatiles change when they are attacked by herbivores. Arthropod predators and parasitoids use new volatile blends to locate their herbivorous prey/hosts. During their life span, these natural enemies need to cope with variations in blends of volatiles emitted by plants to locate prey/host and it is suggested that they cope with this variation by learning the association between the volatiles and the presence of food. Learning occurs when the response to a volatile changes due to an experience. Learning was reported for many species of arthropods, but to our knowledge, this is the second study demonstrating learning in immature arthropod predators (Chapter 1). We studied learning in immatures of the generalist predator *Ceraeochrysa cubana*, specifically whether they are able to learn two associations in their life and if attraction to a volatile is changed due to a new volatile. We used methyl salicylate and rosemary oil (*Rosmarinus officinalis*). Both volatiles are neither attractive nor repellent to naïve predators. After having been exposed to methyl salicylate with food, predators were attracted to this odour. However, predators that were trained the association between methyl salicylate and unavailability of food were repelled by this odour. This change in response to odour (attraction after repellence) occurred two times during the immature lifetime. Predators that had experienced the association between methyl salicylate with food when were exposed to methyl salicylate and ambient air have the same response of attraction to methyl salicylate then the group of experienced that were exposed to methyl salicylate and rosemary oil. This suggests that immatures are able to learn and they are able to locate an odour associated with food even in a test with a volatile to which they were never exposed. Our results show that immatures are capable of associative learning more than once during their immature life.

**Keywords:** Chrysopidae, Volatiles, Methyl salicylate, Rosemy oil, Associative learning.

## **INTRODUCTION**

Plants emit volatiles continuously (Kant et al. 2009), however when they are attacked by herbivores, the blend of volatiles changes (Turlings et al. 1990; De Moraes et al. 1998). These new volatile blends are used by arthropod predators and parasitoids to locate their herbivorous prey/host (Dicke and Sabelis 1988; Turlings et al. 1990; Sabelis et al. 1999; Ellingsen and Døving 1986; Bryant et al. 1991; Nevitt et al. 1995; Sabelis et al. 1999). In the field, the blend of volatiles emitted by plants can vary widely. The combination of a specie of herbivore and a plant results in a blend of volatiles that is specific for this combination (van den Boom et al. 2004). The same plant species attacked by another herbivore will elicit another blend of odours (De Moraes et al. 1998; Dicke et al. 1998). Besides biotic factors, abiotic conditions can influence the blend of odours (Takabayashi et al. 1994). Therefore, it is expected that predators continually encounter new associations of volatiles and the availability of food under field conditions.

Predators often show an innate response to volatiles emitted by plants (Turlings et al. 1995). An innate response is heritable but can change as a result of the animal's experience (Drukker et al. 2000). Learning has been defined as a change in behaviour (i.e. response) as a consequence of experience (Papaj and Prokopy 1989), and it was demonstrated in many arthropods (Lewis and Tumlinson 1988; Lewis and Takasu 1990; Wackers and Lewis 1994; De Boer and Dicke 2004). Arthropods can learn in three different ways: imprinting, sensitization and associative learning (Rescorla 1988; Drukker et al. 2000). Imprinting is a kind of learning that occurs quickly early in the life of individuals and does not depend on reinforcement (Gould 1993; Hall and Halliday 1998a). Sensitization is a type of learning when the response to a stimulus increases with the duration of the exposure to that stimulus (Papaj and Prokopy 1989; Hall and Halliday 1998) and associative learning is a kind of learning where a conditioned stimulus (i.e.

volatile, colour) and an unconditioned stimulus (i.e. reward or punishment) are paired, and the response (positive or negative) to the conditioned stimulus depends on the unconditioned stimulus (i.e. a reward or punishment) (Thorpe 1956; Lewis and Tumlinson 1988; Hall and Halliday 1998).

Associative learning was demonstrated for many different taxa of arthropods, for example, parasitoids that associate herbivore-induced plant volatiles with the presence of hosts after a brief experience (Lewis and Takasu 1990), and predators that learned the association of herbivore-induced plant volatiles with availability of food (Drukker et al. 2000). Animals cannot only learn associations of cues with the presence or absence of food, but can also learn associations of cues with food of different quality (Ardanuy et al. 2016) and risk of predation (Nomikou et al. 2003).

The majority of studies on the use of odours by arthropods to locate food concerns adults (Dicke and Sabelis 1988; Turlings et al. 1990; Sabelis et al. 1999). However, the immatures of many arthropods also attack prey and are sometimes more voracious than adults (Gilbert 1981; Lee and Kang 2004; Omkar and James 2004; Omkar and Pervez 2004; Cabral et al. 2006; Moura et al. 2006; Cabral et al. 2009). Some adults only feed on honey or other plant-provided food, and not on prey (Sheldon and MacLeod 1971; Villenave et al. 2005). Nevertheless, there is not much known of the capacity of immature predators to learn associations between food and volatiles. This study therefore specifically investigated learning behaviour in immature arthropod predators.

Immature lacewings of the species *Ceraeochrysa cubana* (Hagen) (Neuroptera: Chrysopidae) are predators of soft-bodied insects and eggs of arthropods (New 1975; Souza et al. 2008). They are important natural enemies in Neotropical America, and responsible for biological control of several pests (Lopez-Arroyo et al. 1999; Albuquerque et al. 2001). Lacewing adults are not important as predators; they mainly

feed on plant-provided food, such as pollen and nectar (Hagen 1987). The response of adult lacewings to volatiles was assessed in several studies, such as the response to aphid sex pheromones (Boo et al. 2003), aggregation pheromone (Chauhan et al. 2007; Jones et al. 2011) and herbivore-induced plant volatiles (James 2003; Jones et al. 2011), but knowledge of the immature response to odours is scarce. Understanding the occurrence and importance of learning the association between volatiles and prey in these immatures can help us understand how they cope with changing associations between volatiles and prey in the field. Hence, this study was performed to assess the response of immature *C. cubana* to different odours and to evaluate if it can learn several associations between odours and the presence or absence of food.

## **MATERIALS AND METHODS**

### **Rearing methods**

*Ceraeochrysa cubana* was collected from coffee and pepper crops around Viçosa (state of Minas Gerais, Brazil). Juveniles were fed with eggs of the Mediterranean flour moth *Ephestia kuehniella* (Lepidoptera: Pyralidae), and adults received a yeast–honey solution (1:1) (Venzon and Carvalho 1992). Rearings were maintained at a controlled temperature ( $25 \pm 2^\circ \text{C}$ ), relative humidity ( $75 \pm 5\%$ ), and photoperiod (12:12 L: D).

### **Volatile sources**

Volatile dispensers were made of Parafilm®, which was cut into strips of 5.2 cm wide, rolled up and flattened until they consisted of 10 tightly rolled layers. The rolls were cut into pieces of 7 mm long (Janssen et al. 2014). One group of dispensers was incubated in synthetic liquid methyl salicylate (Sigma-Aldrich, China) in a closed Petri dish, another group was incubated in rosemary pure essential oil (*Rosmarinus officinalis*) (WNF Ind. e



Com. Ltda), and a control group was kept in a clean Petri dish. After 24 hours, the dispensers were taken from the Petri dishes and placed on a tissue paper to dry. Dispensers with and without odour were used in the olfactometer tests and for giving predators experience. *Rosmarinus officinalis* oil are compound majority by  $\alpha$ -pinene, verbenone and camphor (Coelho 2009).

### **Y-tube olfactometer tests**

A Y-tube olfactometer (Sabelis and van de Baan 1983; Janssen et al. 1997) was used to test preference for or aversion to methyl salicylate compared to ambient air or to rosemary oil. The olfactometer consisted of a glass tube ( $\text{Ø} = 3.5 \text{ cm}$ ) in the form of a “Y”. Each arm of the Y-tube was connected with a plastic tube to a glass container (43 x 36 x 50 cm). The base of the Y-tube was connected to a vacuum pump that produced an airflow from the glass container that contained three volatile dispensers with and without volatiles through the arms of the tube to the base (Sabelis and van de Baan 1983). The airflow in each arm of the olfactometer was calibrated to 0.50 m/s (VelociCalc® Air Velocity Meter 9545-A). One predator was released at the downwind base of the Y-tube. It was allowed to walk upwind along the base of the Y-tube and to choose one of the arms, connected to either the container with an odour source or the container with empty dispensers. A trial ended when the predator reached the end of one of the arms of the Y-tube or after 5 min, when it was removed and the next predator was introduced. Predators were tested until twenty predators had made a choice in each replicate. After five animals had made a choice, the odour sources were connected to the opposite arm of the olfactometer and the experiment was continued. Unless stated otherwise, predators of the third instar were starved for 24 hours prior to being tested.

### **Response to a novel volatile after learning**

This experiment was designed to test whether the experience of association of a volatile with food resulted in increased attraction to volatiles in general or to the specific conditioned volatile. The training of individuals to associate a new odour with availability of food started with first instar predators of 24 hours old that were taken from the rearing and were haphazardly assigned to two groups (experienced and naïve). They were individually placed in plastic tubes and all plastic tubes were incubated inside a plastic box (35 x 24 x 20 cm) with two openings. One opening ( $\text{Ø}=3$  cm) was connected to a pump that produced an air flow from the laboratory to the box at 0.45 m/s at the entry of the box (Figure 1).

During the first 96 hours, immatures of all groups received eggs of *E. kuehniella* in their tubes as food. Subsequently, they were incubated in a new plastic tube without food for 24 hours. During the next five days, immatures were daily switched from tubes without food to tubes with food. During the feeding period, the tubes with individuals of one group were put inside a plastic box with three dispensers with methyl salicylate. The dispensers were placed inside the plastic box, and the air flow produced by the pump carried the volatiles to the individuals during the period of availability of food (Figure 1). The naïve group was submitted to the same changes in food availability inside a box with the same airflow, but they were exposed to ambient air. During the period of absence of food, all groups were put in a new plastic tube without food and they were put inside similar boxes and exposed to ambient air. After the period of training, the individuals of the experienced and the naïve group were tested for their response to methyl salicylate or ambient air, and another olfactometer test was done to assess the response of other individuals of the experienced and the naïve group to methyl salicylate or rosemary oil. This experiment with one or two odours in the olfactometer test was done to test if the

response of group with experience with the association between methyl salicylate and food persisted when they were exposed to a new odour together with the learned odour. The experiment was repeated four times on different days.

### **Ability of predator to learn two associations**

This experiment was designed to test if individuals could learn two associations (positive after a negative) with the same odour. The experiment started with first instar predators of 24 hours old that were taken from the rearing and randomly assigned to one of three groups (a naïve group, and two experienced groups). The experimental setup was the same as above. During the first 96 hours, immatures of all groups received eggs of *E. kuehniella* in their tubes as food. The naïve group was never exposed to methyl salicylate; the other two groups were exposed to methyl salicylate in association with food. Subsequently, they were switched to a new plastic tube without food for 24 hours. During the next two days, immatures were daily switched from tubes with food to tubes without food. After this period, the individuals were tested for their response to methyl salicylate relative to ambient air. This first test was done to confirm if the individuals learned the association of methyl salicylate with food.

Subsequently, the individuals of the two experienced groups were submitted to a new experience. One group was now exposed to the association between methyl salicylate without food, so they had a positive association first and a negative association with the same volatile subsequently. We refer to this group as the “double-experience” group. The other group was not exposed to methyl salicylate in this second experience. This served to investigate for how long the predators would remember the positive association of the first experience. We refer to this group as the “learning-and-forgetting” group. During the first 24 hours, the individuals were given food, and on the second day they were switch

to tubes without food, the “double-experience” group in the presence of methyl salicylate. This sequence was repeated on the next two days. On the fifth day, the response of all individuals to methyl salicylate compared to ambient air was assessed in the olfactometer test (Table 1).

Within each group, the preference for a volatile was tested with a log-linear model for contingency tables with Generalized Linear Models (GLM) using a Poisson error distribution (Crawley 2007) with the volatile, side and the replicate as fixed factors. The analysis was performed with the statistical software R 2.15.1 (R-Development-Core-Team 2012). The minimal adequate model was obtained by removing non-significant interactions and factors with deletion tests using the “anova” command in R (R Development Core Team 2012). The response of different groups was compared with a GLM with the proportion of predators that had chosen for a particular odour as dependent variable and the different groups as fixed factor.

## RESULTS

### Response to a novel volatile after learning

Naïve immatures of *C. cubana* were significantly repelled by methyl salicylate when it was offered together with ambient air (Figure 2, top bar, GLM, d.f. = 1,  $\text{Chi}^2 = 11.53$ ,  $P = 0.0007$ ). Immatures that had experienced the association of methyl salicylate with food were no longer repelled, nor attracted to methyl salicylate (Figure 2, 2<sup>nd</sup> bar from above, GLM, d.f. = 1,  $\text{Chi}^2 = 1.81$ ,  $P = 0.18$ ). The response of these two groups differed significantly (Figure 2, 1<sup>st</sup> and 2<sup>nd</sup> bars from above, GLM, d.f. = 1,  $\text{Chi}^2 = 11.3$ ,  $P = 0.0008$ ).

When immatures were given a choice between methyl salicylate and rosemary oil, naïve immatures of *C. cubana* had no significant preference or aversion to volatiles

(Figure 2, 4<sup>th</sup> bar from above, GLM, d.f. = 1,  $\text{Chi}^2 = 1.81$ ,  $P = 0.18$ ). Experienced immatures were attracted to methyl salicylate (Figure 3, 3<sup>rd</sup> bars from above, GLM, d.f. = 1,  $\text{Chi}^2 = 10.01$ ,  $P = 0.002$ ). The response of the experience and naïve group to methyl salicylate or rosemary oil differed significantly (Figure 2, 3<sup>rd</sup> and 4<sup>th</sup> bars from above, GLM, d.f. = 1,  $\text{Chi}^2 = 10.21$ ,  $P = 0.001$ ). The proportion of experienced immatures choosing methyl salicylate did not differ between the experiment in which they were offered a choice between methyl salicylate and air or between methyl salicylate and rosemary oil in the olfactometer (Figure 2, 2<sup>nd</sup> and 3<sup>rd</sup> bars from above, GLM, d.f. = 1,  $\text{Chi}^2 = 1.71$ ,  $P = 0.19$ ).

#### **Ability of predator to learn two associations**

Naïve immatures of *C. cubana* were again significantly repelled by methyl salicylate (Figure 3, top bar, GLM, d.f. = 1,  $\text{Chi}^2 = 6.79$ ,  $P = 0.009$ ). After the first training session, immatures of both groups that had experienced the association of methyl salicylate with food were attracted to methyl salicylate (Figure 3, 2<sup>nd</sup> and 3<sup>rd</sup> bar from above, GLM, d.f. = 1,  $\text{Chi}^2 < 13.59$ ,  $P > 0.002$ ). The response of the immatures that were trained was significantly differed from the response of naïve immatures (Figure 3, 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> bars from above, GLM, d.f. = 2,  $\text{Chi}^2 = 24.54$ ,  $P < 0.001$ ). The response of the two trained groups did not differ significantly (Figure 3, 2<sup>nd</sup> and 3<sup>rd</sup> bars from above, GLM, d.f. = 1,  $\text{Chi}^2 = 0.16$ ,  $P = 0.68$ ). In the second olfactometer test, immatures of *C. cubana* that were trained to the new association of methyl salicylate without food were repelled by methyl salicylate (Figure 3, 4<sup>th</sup> bar from above, GLM, d.f. = 1,  $\text{Chi}^2 = 6.80$ ,  $P = 0.009$ ). The immatures that were not exposed to methyl salicylate in the new training test showed neither attraction nor repulsion (Figure 3, 5<sup>th</sup> bars from above, GLM, d.f. = 1,  $\text{Chi}^2 = 2.42$ ,  $P = 0.12$ ). The response of the two groups differed significantly in this second test

(Figure 3, 4<sup>th</sup> and 5<sup>th</sup> bars from above, GLM, d.f. = 1,  $\text{Chi}^2 = 8.68$ ,  $P = 0.003$ ). The response of the individuals of the learning-and-forgetting group did not differ between the first and the second olfactometer test (Figure 3, 2<sup>nd</sup> and 5<sup>th</sup> bars from above, GLM, d.f. = 1,  $\text{Chi}^2 = 2.41$ ,  $P = 0.12$ ).

## DISCUSSION

We show here that the response of immatures of the lacewing *C. cubana* to methyl salicylate changed as a result of experience with the association of the volatile with and without food. We furthermore showed that this change in response could be reverted as a consequence of a new experience later in an individual's life. The response to a volatile associated with food was not modified when predators were test in an olfactometer test with an odour with which they had no prior contact, showing that the experience did not change the response to volatiles in general. The predators responded to a volatile associate with food even six days without contact with this volatile.

This change in response due to an experience is evidence of learning as defined by Papaj and Prokopy (1989) and was demonstrated in many other arthropods (Lewis and Tumlinson 1988; Lewis and Takasu 1990; Wackers and Lewis 1994; De Boer and Dicke 2004). The response to MeSa was dependent on the association of volatiles with food or absence of food, showing that the type of learning can be classified as associative learning (Thorpe 1956; Lewis and Tumlinson 1988; Hall and Halliday 1998).

Learning is a way in which arthropods can cope with the variation in volatiles that they encounter. When herbivorous prey are absent or present at low densities, beneficial arthropods need to disperse to a new plant with prey, and they can use herbivore-induced plant volatiles to locate these plants (De Moraes et al. 1998; Kessler and Baldwin 2001). Because volatiles of plants attacked by prey are very variable (De

Moraes et al. 1998; Dicke et al. 1998), associative learning can help individuals to locate a new plant with prey. When predators need to disperse more than once in their life, this exposure to new volatiles is recurrent and they need to cope with variability of volatiles more often. Under these circumstances, it is necessary that predators are capable of learning more than one association during their life. In this study, we demonstrated that immatures of lacewings are able to associate the same odour with the presence or absence of food during their lifetime.

In the field, arthropods are likely to be exhibited to odours continuously, and will use these odours to locate prey (Janssen et al. 2014). Whereas many arthropod predators have innate responses to various volatiles, these responses can be modified through learning. Arthropods have an innate response to volatiles that is genetically fixed (Turlings et al. 1995), however, individuals are submitted to encounters of conditioned and the unconditioned stimuli (i.e. availability or unavailability of food) and they will be trained, resulting in an associative learning (Thorpe 1964). The manipulation of ambient with extra odours with the goal to increasing the density of beneficial arthropods is not an efficient strategy. In the first moment, the beneficial arthropods will be attracted to volatile, but due the exposition of the individuals to the odour associated without food, they will learn the association and will disperse of the area. These individuals that learned that association will not be attracted to this volatile again. The attraction of predators to odour changes in the field due to learning, but the odours need to be paired with reward. Addition of methyl salicylate under field conditions results in an attraction of beneficial arthropods (James 2003), but these individuals that were attracted to the area probably will not persist in the area and will disperse due the unavailability of food. Plants emit blends of volatiles to attract predators when they are attacked by herbivores. These volatiles are used by beneficial arthropods as a conditioned stimulus, with prey being the

unconditioned stimulus. This association will result in learning and the number of individuals that respond to this volatile will increase. To be efficient, the odours added in agricultural fields to attract beneficial arthropods need be associated with some reward like alternative food.

In summary, based on our results we conclude that immatures of *C. cubana* can learn the association of methyl salicylate with food and subsequently learn the association of the same volatile with the absence of food. Thus, the response to methyl salicylate depends on the unconditioned stimulus with which it is associated. Immatures are able to learn two associations with the same odour. The response to volatile associate with food is not general but specific.

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**Table 1**

Immatures of *Ceraeochrysa cubana* were collected from the rearing and randomly assigned to different training groups. The diagram shows the procedure of the training, with all groups to test if the same individual could learn two associations (a positive after a negative) with the same volatile.

Day	Training		
	Naive	Learning-and-forgetting	Double-experience
1 - 4	Food	Food + MeSa	Food + MeSa
5	No food	No food - MeSa	No food - MeSa
6	Food	Food + MeSa	Food + MeSa
7	No food	No food - MeSa	No food - MeSa
8	Olfactometer test		
	-	Food - MeSa	Food - MeSa
9	-	No food - MeSa	No food + MeSa
10	-	Food - MeSa	Food - MeSa
11	-	No food - MeSa	No food + MeSa
12	-	Olfactometer test	

## FIGURE LEGENDS

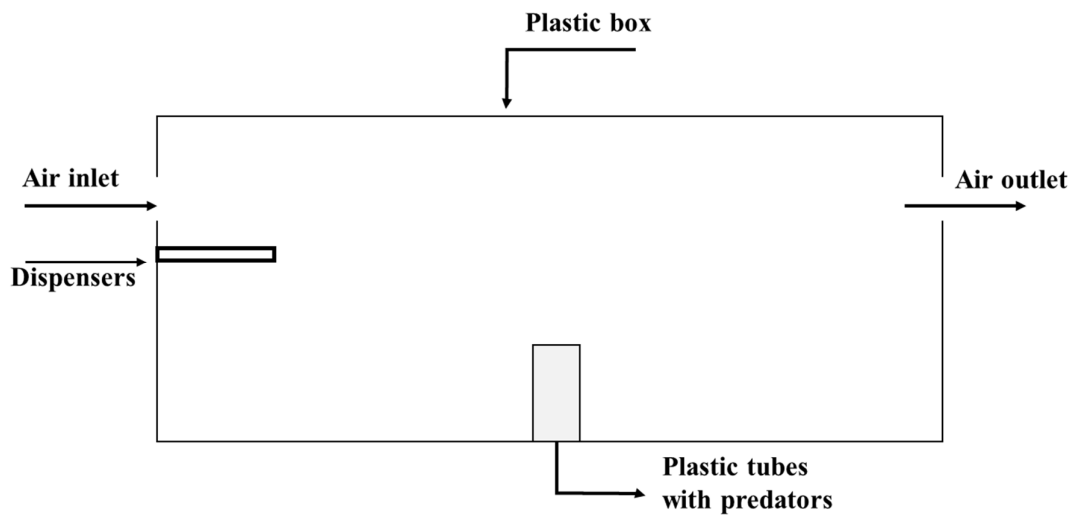
Figure 1. Container used for training of predators, consisting of a transparent box (35 x 24 x 20 cm) with two openings for the entrance and exit of air. The air inlet was connected to a pump that produced an air flow at 0.45 m/s at the entry of the box. All plastic tubes containing predators were put inside the box, closed with a fine mesh to prevent escapes. Volatile dispensers were put in a Petri dish below the air inlet. The volatile was carried out from the Petri dish to inside the plastic tubes.

Figure 2. Effect of a novel odour in the choice of training *Ceraeochrysa cubana* for availability of food associated with methyl salicylate (MeSa). Methyl salicylate was tested versus ambient air (1<sup>st</sup> and 2<sup>nd</sup> bar from above) or rosemary oil (3<sup>rd</sup> and 4<sup>th</sup> bar from above) in a Y-tube olfactometer. The naïve control group was never exposed to MeSa or rosemary oil. The experienced group was exposed to methyl salicylate associated with food and had never been exhibited to rosemary oil. Each bar represents the average response of four replicates compounds each of twenty individuals ( $\pm$  S.E.). Results of the generalized linear models with Poisson error distribution for the groups and comparison between the groups is given in the text. The black bar represents the fraction of predators that chose methyl salicylate, white bars represent the choice for ambient air and grey bars represent the choice for rosemary oil.

Figure 3. Choice of *Ceraeochrysa cubana* to methyl salicylate or ambient air in a Y-tube olfactometer. The response of the naïve group, predators that had no previous experience with methyl salicylate, the double-experience and learning-and-forgetting group, predators that had experienced the association of methyl salicylate with food, was assessed in an olfactometer test (First test). In the second training, predators of the double-experience group were exposed to methyl

salicylate associated without food and the learning-and-forgetting group was not exposed to methyl salicylate and the response of the predators was assessed in the olfactometer test (Second training). Black bars and white bars correspond to the average percentage of predators choosing for methyl salicylate (MeSa) and ambient air ( $\pm$  S.E.) respectively. Results of generalized linear models with a binomial error distribution for the response of predator or comparison of the choices between are given in the text.

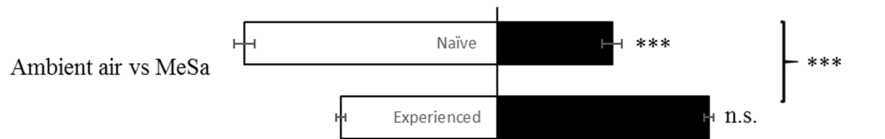
**Figure 1.**



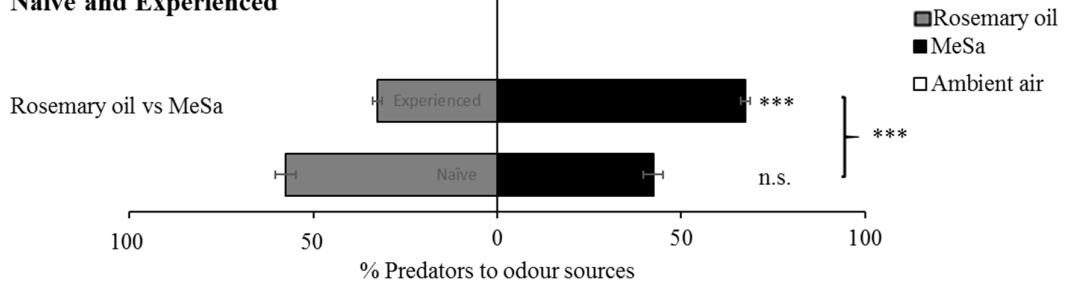


**Figure 2.**

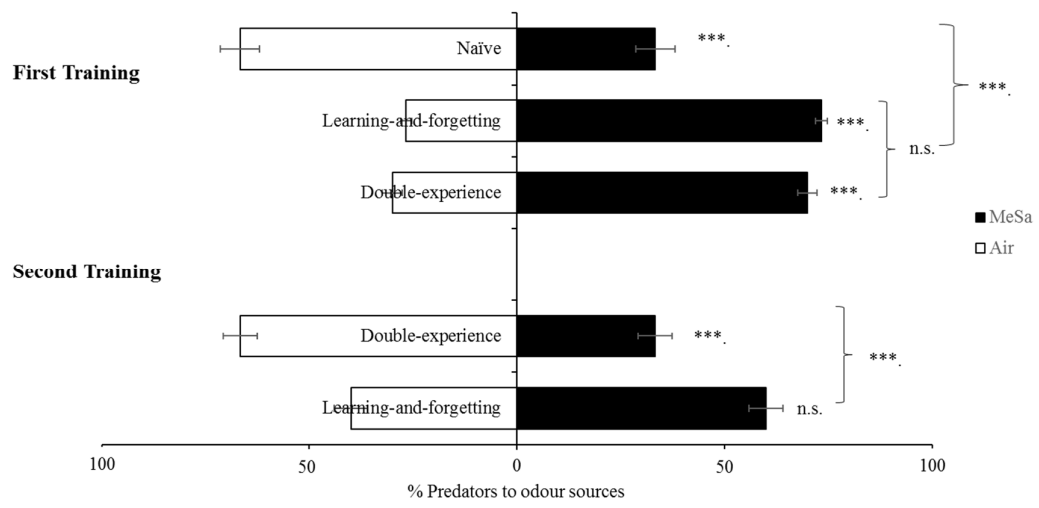
**Naïve and Experienced**



**Naïve and Experienced**



**Figure 3.**



## CHAPTER 3

### **Generalist predator cannot learn two associations**

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## ABSTRACT

Plants emit volatiles continuously; however, the composition of the blend of volatiles change when plants are attacked by herbivores. These herbivore-induced volatiles are used by arthropod predators to find their prey. However, these volatiles vary depending on the host plant species, the herbivore species and on abiotic conditions. It has been suggested that predators learn the association between volatiles and the presence of prey to cope with this variation. The use of volatiles in learning has indeed been proven for some arthropod predators, however it is not clear whether predators can learn various associations, which is what we investigated here. In this study, we investigated whether the predatory bug *Orius insidiosus* is able to learn associations between two odours and the availability or absence of food. One compound that is emitted by plants when they are attacked by herbivores and that is used by beneficial arthropods to locate prey is methyl salicylate. Depending on the concentration, this compound is attractive to a number of insects. Mint oil is a blend of compounds that is repellent and can be toxic for arthropods. Earlier experiments have shown that generalist predators could learn the association of methyl salicylate with food and the association of mint oil with the absence of food. Here, the predators were exposed to methyl salicylate or mint oil associated with food and to the other odour associated with the absence of food on the next day. This training was offered during four days. The response of the predators to the combination of odours (methyl salicylate versus ambient air, mint oil versus ambient air, and methyl salicylate versus mint oil) was assessed in an olfactometer test. The response of the predators to odours did not change with experience compared with a naïve group in any of the olfactometer tests. Hence, the predators cannot learn both associations at the same time.

**Keywords:** Volatiles, methyl salicylate, mint oil, *Orius*, herbivore-induced plant volatiles, foraging

## INTRODUCTION

Plants produce volatile organic compounds incessantly (Kant et al. 2009). However, when plants are attacked by herbivores, secondary metabolic pathways are elicited and the composition of the blend of volatiles changes (Turlings et al. 1990; Pare and Tumlinson 1997; De Moraes et al. 1998). These volatiles produced as a consequence of herbivory (herbivore-induced plant volatiles – HIPVs) are used by predators to locate their herbivorous prey (Dicke and Sabelis 1988; Turlings et al. 1990). In addition to abiotic factors, the blend of volatiles is variable depending on the plant species, the herbivore species and the combination of these factors (De Moraes et al. 1998; Dicke et al. 1998; Gouinguene and Turlings 2002; van den Boom et al. 2004).

Predatory arthropods may cope with the variability of the herbivore-induced plant volatiles by learning. Learning is a change in the response to a stimulus that occurs due an experience (Papaj and Prokopy 1989). This response can be innate and can change with experience. In associative learning, it is necessary that the conditioned stimulus (i.e. volatile) and unconditioned stimulus (i.e. reward or punishment) are paired, and through experiencing this association, the individual will be attracted to or repelled by the conditioned stimulus, depending on the unconditioned stimulus (Thorpe 1956). In nature, predators will experience an association between herbivore-induced plant volatiles as a conditioned stimulus and food as an unconditioned stimulus. Due to this experience, they can learn these associations and change their response towards the volatiles (Lewis and Takasu 1990). Herbivore-induced plant volatiles are cues that arthropod predators learn to associate with the presence of their herbivorous prey. However, it is not known if they can learn all associations between volatiles and unconditioned stimuli.

A group of predators that responds to herbivore-induced plant volatiles are *Orius* spp., which use these odours to locate prey (Ardanuy et al. 2016). Members of the genus

*Orius* are important natural enemies of several pests (van den Meiracker 1994; Bonte and De Clercq 2011, van Lenteren 2012; Pumariño et al. 2012; Calixto et al. 2013). It is known that anthocorid predators may also learn to associate herbivore-induced plant volatiles with the presence or absence of food (Drukker et al. 2000). *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) is known as a predator of small insects, such as aphids, thrips, whiteflies, scales, psyllids, caterpillars, eggs of insect and other arthropods (i.e. mites) (Lattin 1999; van Lenteren et al. 2003). In a previous experiment, we trained *Orius insidiosus* with methyl salicylate associated with food and unavailability of food with ambient air, and the opposite was trained too, methyl salicylate without food and ambient air with food, in this case the predator learn the association of methyl salicylate with food and was attracted to this volatile, but in the second experiment the predator was not repelled to this volatile. In another experiment we have the same trained procedure however with mint oil as an odour source. In this case the predator learns the association of mint oil with unavailability of food but did not learn the association of mint oil with food (Bernardo 2015). One supposition is that the predator was association the ambient air with availability and unavailability of food.

In this paper, we investigated whether the predatory bug *Orius insidiosus* is able to simultaneously learn associations between two odours and the availability or absence of food. To test this, we examined: (1) the innate response of *O. insidiosus* toward mint oil, methyl salicylate and to the two odours at the same time; (2) the response of predators that had experienced the association of a volatile with or without food.

## **MATERIALS AND METHODS**

### **Rearing methods**

A culture of *Orius insidiosus* was established from insects collected from tomato plants around Viçosa (state of Minas Gerais, Brazil). Cultures were supplemented with new individuals from the field during each laboratory generation. Adults and juveniles were fed with eggs of the Mediterranean flour moth *Ephestia kuehniella* (Lepidoptera: Pyralidae). Adults were kept in a glass jar (1 L) covered with thin mesh for ventilation. Inflorescences of *Bidens pilosa* L. (Asteraceae: Compositae) were supplied as oviposition substrate and a piece of moist cotton wool was used to supply water and to keep the inflorescences turgid. *Bidens pilosa* is a known oviposition substrate of *O. insidiosus* in the field (Silveira et al. 2003), and it is also used in mass rearing (Bueno et al. 2007). Cultures were maintained at controlled temperature ( $25 \pm 2^\circ\text{C}$ ), relative humidity ( $75 \pm 5\%$ ), and photoperiod (12:12 L: D). Predator nymphs were collected from the rearing units three times a week and were transferred to Petri dishes (9 cm diameter x 2 cm height) containing pieces of paper towel to reduce cannibalism. Nymphs were supplied with *E. kuehniella* eggs as food and a piece of moist cotton wool as water supply. The Petri dishes were covered with a plastic film (Guarufilme®, Guarufilme Ind. e Com. Ltda) with some holes in its surface for ventilation.

### **Odour sources**

Volatile dispensers were made from Parafilm® (Pechinery Plastic Packaging). Pieces of Parafilm® were rolled up and flattened until they consisted of 10 tightly rolled layers. The rolls were cut into pieces of 7 mm long (Janssen et al. 2014). Dispensers were incubated in synthetic liquid methyl salicylate (Sigma-Aldrich, China) or in liquid mint oil (World's Natural Fragrances, São Paulo, Brazil) in a closed Petri dish, and a control

group was kept in a clean Petri dish. After 24 hours, the dispensers were taken from the Petri dishes and placed on a tissue paper to dry. Dispensers with and without volatiles were used in the olfactometer test and in training trials.

Methyl salicylate is a compound of many herbivore-induced plant volatiles and is used by predatory arthropods to locate prey (Scutareanu et al. 1997). Depending on the concentration, this compound can be attractive for a large number of insects (James and Price 2004; Mallinger et al. 2011; Rodriguez-Saona et al. 2011; van Wijk et al. 2008). Mint oil (*Mentha piperita*) consists of a mixture of menthol, menthone, (+)-menthyl acetate, menthofuran, isomenthone (Yang et al., 2010), limonene and 1,8-cineole (Rohloff, 1999). It is repellent (Hori 1998; Koschier and Sedy 2003) and some compounds of this oil are toxic to arthropods (Choi et al. 2004; Odeyemi et al. 2008).

### **Y-tube olfactometer tests**

A Y-tube olfactometer (Sabelis and van de Baan 1983; Janssen et al. 1997) was used to test preference for or aversion to volatiles. The olfactometer consisted of a glass tube ( $\emptyset = 3.5$  cm) in the form of a “Y”. Each arm of the Y-tube was connected with a plastic tube to a glass container (43 x 36 x 50 cm) in which three volatile dispensers were arranged. The base of the Y-tube was connected to a vacuum pump that produced an airflow from the glass container that contained the volatile sources to the base of the “Y” tube (Sabelis and van de Baan 1983). The airflow in each arm of the olfactometer was calibrated to 0.35 m/s (VelociCalc® Air Velocity Meter 9545-A). One predator was released at the downwind base of the Y-tube. It was allowed to walk upwind along the base to the end of the Y-tube and to choose one of the arms, connected to a container with an odour source. A trial ended when the predator reached the end of the arm of the Y-tube or after 5 minutes, when it was removed and the next predator was introduced. Predators were



tested until twenty predators had made a choice in each replicate. After five animals had made a choice, the containers with the volatile sources were connected to the opposite arm of the olfactometer and the experiment was continued. Unless stated otherwise, predators were starved for 24 hours prior to being tested.

### **Innate response and associative learning of two odours**

The training started with adult predators of seven days old taken from the rearing units and randomly assigned to two groups (naïve and experienced group). Each predator was incubated in a plastic tube ( $\text{Ø}=3$  cm and 7.5 cm deep) covered with thin mesh for 24 hours, one individual per tube. All plastic tubes were placed inside a plastic box (35 x 24 cm) with two openings. One opening was connected to a pump that produced an airflow at the entrance of the box at 0.45 m/s (Figure 1). Three volatile dispensers were placed inside the plastic box in a Petri dish, 3 cm below the entrance of the airflow. In a pilot experiment, cotton wool was put inside the tubes used in the training procedure and three volatile dispensers were put in the box. After 24 hours, the volatile could be perceived from the cotton wool that had been inside the tubes, confirming that odours were carried into the tubes in our set-up.

During the first 24 hours, the individuals received eggs of *E. kuehniella* in their tubes as food. Enough eggs were added to ensure that they were not all consumed during the training period. Subsequently, the predators were incubated in a new plastic tube without food for 24 hours. During the next two days, each predator was daily switched from a tube with food to a tube without food. The experience given to one group in this experiment was the association of mint oil with food and methyl salicylate without food; the naïve group was never exposed to these volatiles. After the training period, half of the individuals of each group were offered a choice between methyl salicylate and ambient

air and the other half of the two groups was offered a choice between mint oil and ambient air. The training and choice test were repeated five times, including both groups in each replicate.

### **Discriminating between two volatiles**

This experiment aimed to evaluate if the predators changed the response in the olfactometer test when they were offered the choice between the two odours that were used in the training procedure. The training started with adult predators of seven days old taken from the rearing and haphazardly assigned to one of three groups. Two groups received an experience and one group not (naïve). The experiences given were: (1) association of mint oil with food and methyl salicylate associated without food (experience mint oil-food) as in the experiment above; (2) association of methyl salicylate with food and mint oil without food (experience MeSa-food), and the naïve group had no experience with these volatiles. The difference between the two groups that received an experience with a volatile was the identity of the volatile that was associated with food. The training procedure was the same as described above. Subsequently, individuals of all groups were tested for their response in an olfactometer test with methyl salicylate and mint oil as odour sources. The experiment was repeated in four blocks on different days.

The data were analysed with a log-linear model for contingency tables with Generalized Linear Models (GLM) using a Poisson error distribution (Crawley 2007). The analysis was performed with the statistical software R 2.15.1 (R Development Core Team 2012). The minimal adequate model was obtained by removing non-significant interactions and factors with deletion tests using the “anova” command in R (R Development Core Team 2012).

## RESULTS

### Innate response and associative learning

Naïve *O. insidiosus* did not show significant attraction or repulsion to MeSa (49.2 % chose MeSa) (Figure 2, top bar, d. f. = 1,  $\chi^2 = 0.03$ ,  $P = 0.86$ ). The response of naïve predators to mint oil and ambient air differed among replicates (Figure 2, 3<sup>rd</sup> bar,  $df=1$ ,  $\chi^2 = 13.87$ ,  $P = 0.02$ ). In two replicates, predators showed a preference for ambient air ( $P < 0.03$ ) and no significant preference was found in the other replicates (42.5 % chose mint oil) (Figure 2, 3<sup>rd</sup>.bar, d. f. = 1,  $\chi^2 = 2.71$ ,  $P > 0.18$ ). Hence, mint oil was not attractive for naïve predators.

The response of the predators that were trained with the association of mint oil with food and methyl salicylate without food also differed among replicates. In one replicate predators were attracted to ambient air ( $p=0.03$ ) and no significant preference was found in the other replicates. So experience with the association of mint oil with food did not result in attraction to mint oil (Figure 2, 2<sup>nd</sup> bar,  $P > 0.08$ ). Compared with the naïve group, predators that were exposed to the association of mint oil with food and methyl salicylate without food showed no significantly different response to methyl salicylate and ambient air (Figure 2, 1<sup>st</sup> and 2<sup>nd</sup> bar, d. f. = 1,  $\chi^2 = 0.00$ ,  $P = 1$ ). Likewise, the response of predators with experience with mint oil or ambient air did not differ from the naïve group (Figure 2, 3<sup>rd</sup> and 4<sup>th</sup> bar, d. f. = 1,  $\chi^2 = 1.21$ ,  $P = 0.27$ ). Based on these results we conclude that learning is dependent on the conditioned stimulus (i.e. volatile).

### Discriminating between two odours

Naïve *Orius insidiosus* did not show a preference for or aversion to mint oil and methyl salicylate (Figure 3, 1<sup>st</sup> bar, d. f. = 1,  $\chi^2 = 0.05$ ,  $P = 0.82$ ). The response of predators

that had experienced the association between mint oil with food and methyl salicylate without food differed significantly among replicates (Figure 3, 2<sup>nd</sup> bar, d. f. = 3,  $\text{Chi}^2 = 15.93$ ,  $P = 0.0011$ ), with one replicate where predators shown a preference for mint oil ( $P = 0.005$ ) and no preference in the other replicates ( $P > 0.08$ ). After experience with methyl salicylate associated with food and mint oil without food, *O. insidiosus* had no preference for methyl salicylate or mint oil (Figure 3, 3<sup>rd</sup> bar, d. f. = 1,  $\text{Chi}^2 = 3.22$ ,  $P = 0.07$ ). Additionally, there was no significant difference in preference among the three groups (Figure 3, d. f. = 2,  $\text{Chi}^2 = 1.92$ ,  $P = 0.38$ ).

## **DISCUSSION**

After having been exposed to the association of mint oil with food and methyl salicylate without food, the predators were neither repulsed by methyl salicylate nor attracted to mint oil (Figure 3). The response of predators with experience with the association of volatiles and food and volatiles without food did not differ significantly from predators without such experience (naïve) (Figure 2 and 3). The response of predators to odour sources was the same regardless of the association.

It is known that methyl salicylate is a compound of HIPVs (Scutareanu et al. 1997) and, depending on the concentration, this compound can be attractive or repellent to naïve predators (Dicke et al. 1990; Drukker et al. 2000; De Boer et al. 2004; van Wijk et al. 2011). Mint oil is a blend of volatiles that is repellent to arthropods (Hori 1998; Koschier and Sedy 2003) and can be toxic to them (Choi et al. 2004; Odeyemi et al. 2008). Bernardo (2015) showed that predators can associate methyl salicylate with the availability of food and mint oil with the absence of food, but she did not expose the predators to both volatiles. These results may be explained by the predators not learning the association of

volatiles with a reward or punishment, but instead learning the association of ambient air, which is probably not without volatiles, with the availability or unavailability of food. In our experiment, we therefore used two volatiles, one associated with food and the other associated with absence of food. We found that predators cannot learn these two associations simultaneously. Hence, we conclude that *Orius insidiosus* did not learn two associations in the experiment of Bernardo (2015), but only those of the single volatile present in her experiments. Based on our results and the results of Bernardo (2015), we conclude that learning an association in *Orius insidiosus* is affected by the volatile: a positive association could only be learned with methyl salicylate and a negative association only with mint oil.

It is known that arthropods can respond to colours, shapes, patterns and visual cues and they can also respond to more than one stimulus at the same time (Wäckers and Lewis 1994; Kelber 1996; Weiss 1997; Wäckers and Lewis 1999). In our study, we demonstrated that the generalist predator *Orius insidiosus* cannot learn the association of two different volatiles with two different unconditioned stimuli at the same time.

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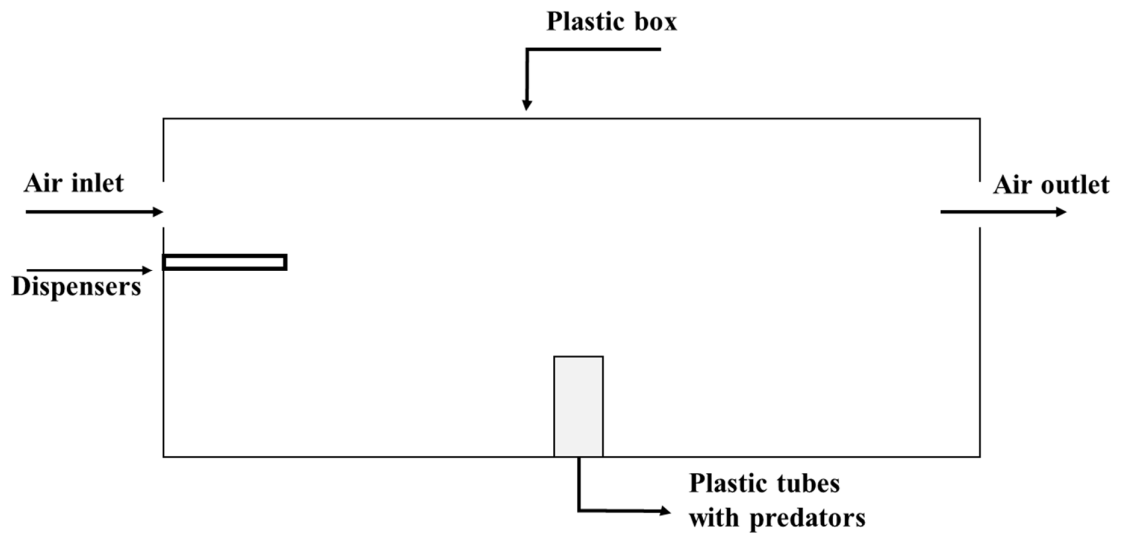
## FIGURE LEGENDS

Figure 1. Container used for training the predators. It consisted of a transparent box (35 x 24 x 20 cm) with two openings for air circulation. The air inlet was connected to a pump that produced an air flow at 0.45 m/s at the entry the box. All plastic tubes were put inside the box, closed with a fine mesh to prevent escape of predators from the tubes. Volatile dispensers were put in a Petri dish below the air inlet.

Figure 2. Choices, shown as the average percentage ( $\pm$  S.E., N = 5) of the response of the generalist predator *Orius insidiosus* to the odour of methyl salicylate (MeSa – black bars) and ambient air (white bars) (1<sup>st</sup> and 2<sup>nd</sup> bar) and the odour of mint oil (grey bars) or ambient air (white bars) (3<sup>rd</sup> and 4<sup>th</sup> bar). The first and the third bar from above show the choice of naïve predators to odours and the second and fourth bar show the choice of experienced predators. The experience corresponded to being exposed to the association of mint oil with food and methyl salicylate without food. There was no significant preference for any of the volatiles in all choices. The response of naïve and experienced individuals also did not differ significantly.

Figure 3. Choices, show as the average percentage of four replicates ( $\pm$  S.E) of the generalist predator *Orius insidiosus* for methyl salicylate (MeSa) (black bars) or mint oil (grey bars). The naïve group was never exposed to any of the odours (lowest bar). The middle bar shows the average choice of a group that was exposed to the association of mint oil with food and methyl salicylate without food. The top bar shows the average choice of a group that was exposed to the association of methyl salicylate with food and mint oil without food. The response of the three groups to odours did not differ significantly.

**Figure 1.**



**Figure 2**

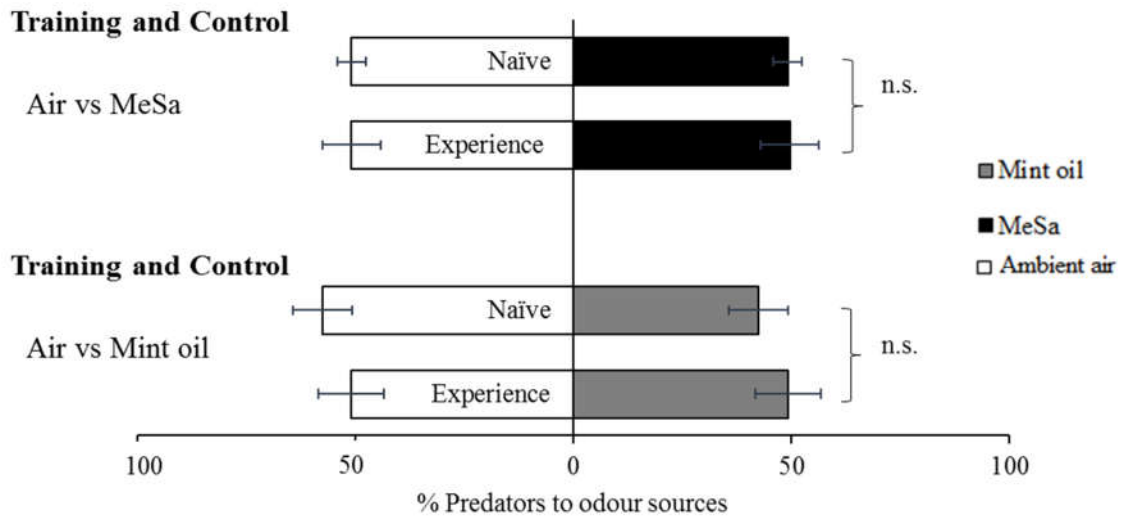
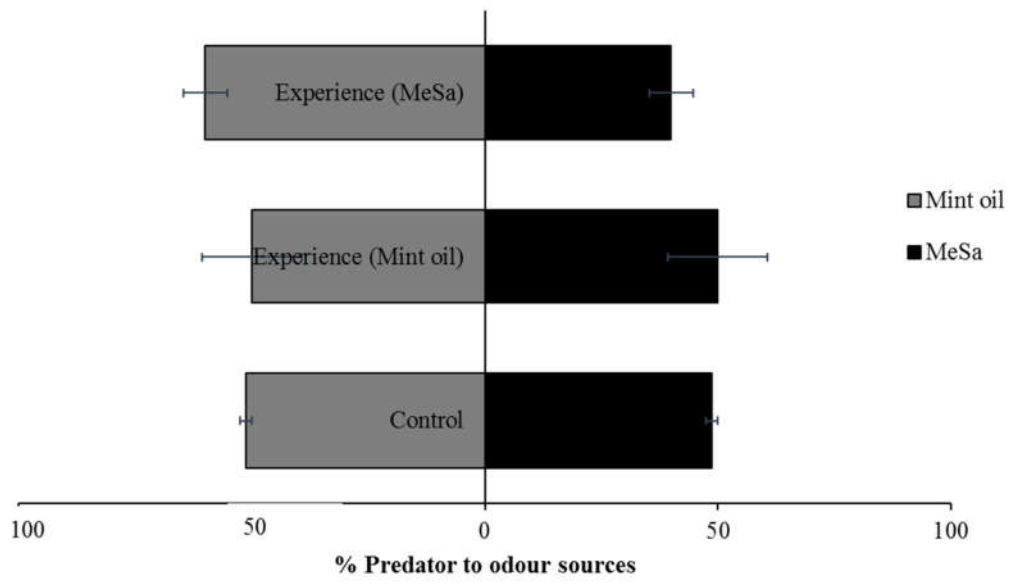


Figure 3.



## GENERAL CONCLUSIONS

Immatures of lacewings have the ability to learn the association between volatiles associated with the presence or absence of food. Juveniles have the same ability of learning as adults of other arthropods and they can sequentially learn the association of the same volatile with food or the absence of food during the immature phase. Learning affects the foraging of predators. After being exposed to the learning procedure during seven days, the learned response persisted for at least more six days. Immatures lacewings could disperse and find an odour associated with food even when were exposed to a choice with two odours, one that was used during training and one to which they were never exposed.

Adults of *Orius insidiosus* cannot learn the association of a herbivore-induced plant volatile (methyl salicylate) without food neither the association of mint oil with food. These results show that learning is dependent of the conditioned stimulus in this species.