ALINE RODRIGUES PÔRTO PEDROSA

EXPERIENCE WITH THE ASSOCIATION OF VOLATILES WITH FOOD DOES NOT AFFECT RESPONSE IN THREE INSECT PREDATORS

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

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APROVADA: 30 de julho de 2012.

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ABSTRACT

PEDROSA, Aline Rodrigues Pôrto, M.Sc., Universidade Federal de Viçosa, July, 2012. **Experience with the association of volatiles with food does not affect response in three insect predators.** Adviser: Angelo Pallini Filho. Co-advisers: Arnoldus Rudolf Maria Janssen and Madelaine Venzon.

Predators and parasitoids of herbivorous arthropods use various odours when searching for prey, such as the sex and aggregation pheromones of their victims, as well as the plant volatiles, which production is induced upon herbivory. Insects can detect a wide range of volatile organic compounds, and blends are perceived differently than a compilation of individual compounds. Predators have to deal with a variety of volatile blends associated with their prey since volatile blends vary in their composition among host plant and herbivore species. Because generalist predators face a large variation in volatile cues associated with the presence of prey and prey availability can vary during the lifetime of predators, it is unlike that predators have an innate response to all volatile mixtures. Learning to associate different odour blends with the occurrence of prey could decrease the amount of energy and time spent in searching for a suitable prey. This work aimed to study the olfactory learning ability of predators. I studied whether the response of *Ceraeochrysa cubana* larvae, *Podisus nigrispinus* and *Cycloneda sanguinea* adults to a volatile blend with which they were unfamiliar increased after previous experience in which this blend was associated with food availability.The conditioned stimulus (volatile blends) and unconditioned stimulus (food) were paired either daily or continuously. The response of predators to volatiles of mint oil or clean air was tested using an Y-tube olfactometer. *Ceraeochrysa cubana* were repelled by mint oil volatiles after previous experience with this blend. However, mint oil odours were nor attractive neither repellent for *C. cubana*, *C. sanguinea* and *P. nigrispinus* after continuous experience. Sequential choices, with reinforcements after each test, did not change the response of the predators. My results suggest that none of these predators change their response to mint oil volatiles after experience with this blend and *Diatraea saccharalis* eggs within three days of sequential training. More extensive experiments are needed to conclude if *P. nigrispinus*, *C. sanguinea* and *C. cubana* are capable of associative learning.

RESUMO

PEDROSA, Aline Rodrigues Pôrto, M.Sc., Universidade Federal de Viçosa, julho de 2012. **Experiência com voláteis associados ao alimento não afeta a resposta de três insetos predadores.** Orientador: Angelo Pallini Filho. Coorientadores: Arnoldus Rudolf Maria Janssen e Madelaine Venzon.

Predadores e parasitóides de artrópodes herbívoros utilizam vários odores durante a busca por presas, tal como feromônios sexuais e de agregação de suas vítimas, assim como voláteis de plantas cuja produção é induzida pela herbivoria. Insetos são capazes de detectar uma vasta gama de compostos orgânicos voláteis e as misturas são percebidas diferentemente da compilação dos compostos individuais. Predadores têm que lidar com uma variedade de misturas de voláteis associados as suas presas, pois as misturas de voláteis variam em sua composição dentre as espécies de plantas hospedeiras e de herbívoros. Como predadores generalistas enfrentam uma grande variação nas pistas voláteis associadas à presença de presa e a disponibilidade de presas pode variar durante a vida dos predadores, é improvável que predadores possuam uma resposta inata para todas as misturas de voláteis. Aprender a associar diferentes misturas de odores com a ocorrência de presas pode reduzir o tempo e a energia despendidos na busca por presas adequadas. Esse trabalho teve como objetivo estudar a capacidade de aprendizado olfativo de predadores. Foi investigado se a resposta de *Podisus nigrispinus*, *Cycloneda sanguinea* e larvas de *Ceraeochrysa cubana*; a uma mistura de voláteis, inicialmente desconhecida por eles, aumentava após a experiência prévia com essa mistura de voláteis associada à disponibilidade de alimento. O estímulo condicionado (mistura de voláteis) e o estímulo não-condicionado (alimento) foram pareados ou diariamente ou continuamente. A resposta dos predadores aos voláteis do óleo de menta ou do ar limpo foi testada em um olfatômetro de tubo-Y. *Ceraeochrysa cubana* foi repelido pelos voláteis do óleo de menta quando entrou em contato com essa mistura previamente. No entanto, os odores do óleo de menta não foram nem atrativos nem repelentes para *C. cubana*, *C. sanguinea* e *P. nigrispinus* após treinamentos contínuos. Escolhas sequenciais, com reforços após cada teste, não modificaram a resposta dos predadores. Os resultados sugerem que nenhum dos predadores testados mudou sua resposta a voláteis do óleo de menta após experiência com essa mistura e ovos de *Diatraea saccharalis* durante os três dias de treinamento

seqüencial. Experimentos mais extensos são necessários para concluir se *P. nigrispinus*, *C. sanguinea* e *C. cubana* são capazes de aprender associações.

INTRODUCTION

Most insect predators are generalist (Sabelis, 1992), i.e. they prey on various arthropods that can feed on different plant species. Carnivores may use chemical stimuli like odors emitted by plant as well as volatiles released by their prey to locate the general prey habitat (Vet & Dicke, 1992; Zhu et al., 1999). When in the patch in which prey are usually found, predators can use vision, movement and volatiles to find their prey (Hajek, 2004).

Herbivore arthropods are under strong selection to release as little information as possible about their presence (De Boer & Dicke, 2004). However, several natural enemies are known to exploid pheromones of their prey as kairomones during foraging (Lewis et al., 1982; Wiskerke et al., 1993). Natural enemies may use the aggregation pheromones (Payne et al., 1984; Dwumfour, 1992), sex pheromones (Zhu et al., 1999) and alarm pheromones (Zhu et al., 1999) of their prey to locate it. Predators and parasitoids can also use hydrocarbons from herbivore cuticle as short-range cues or contact semiochemicals (Han & Chen, 2002).

Moreover, herbivores by-products can also betray the herbivore presence (Godfray, 1994; Borges et al., 2003). Host-derived chemicals (i.g. chemical footprints) can be uncharged lipophilic compounds of low volatility which can be sorbed onto the leaf surface and stay there for a long time (Müller & Riederer, 2005) or even aldehydes that rapidly oxidizing to fatty acids and last only few minutes at the plant surface (Collatz & Steidle, 2008). Honeydew excreted by herbivores has weak volatility and are a searching stimulant for natural enemies that used it as a contact kairomone (Han & Chen, 2002). Several predators were observed trail-following in response to lepidopteran frass (van Loon et al., 2000) during short-range searching.

Additionally to the prey chemicals, predators can exploit information from the prey's environment to locate their victim. Plants emit several organic compounds throughout their lives, many of which are volatiles. Volatile organic compounds (VOCs) are functionally related with the physiology and ecology of the plant (Kant et al., 2009). Over evolutionary time, some of these compounds obtained a signaling function in plant protection and communication (Holopainen, 2004). For this evolution to occur, the variation among VOC blends must be heritable and should correlate positively with lifetime reproductive success (fitness) of individual plants. The positive correlation between plant fitness and VOC mixture variation may occur when herbivorous natural enemies respond innately to specific VOCs or learn to prefer VOC blends (Allison & Hare, 2009).

Insect attacks induce the emission of a group of VOCs called herbivore-induced plant volatiles (HIPVs). These mixtures are mostly composed of fatty acid derivatives, terpenes and indole (Farmer, 2001) and usually vary in their composition among plant genotypes (Rapusas et al., 1996), species and other taxonomic levels (Knudsen et al., 2006). Commonly systemic (Röse et al., 1996), the production of HIPVs is induced by herbivore damage and chemicals released by the herbivore (Mattiacci et al., 1995), in a way that their spectra differ from constitutive VOCs and those induced by mechanical damage (Brilli et al., 2009).

HIPVs can serve as an indirect defence of plants against herbivores as it can attract parasitoids and predators (Dicke & Sabelis, 1988), which use the HIPVs as cues to find their herbivorous prey (McCormick et al., 2012). An important question is whether predators can recognize one or few induced compounds in the mixture or the mixture is perceived as a synthetic whole. Research suggests that individual HIPV compounds have no a priori meaning for predators since the attraction to individual compounds was weaker than to full HIPV blend (van Wijk et al., 2008). Some mixtures may elicit a very different response from that caused by their compounds. A mixture can be attractive but its individual elements can be repellent if offered separately (Webster et al., 2010). Or even, compounds that do not elicit any response may become attractive when mixed (Riffell et al., 2009). Moreover, compounds that are not associated with prey can affect the response of predators to mixtures. This all supports the idea that odour blends are perceived as a synthetic whole rather than as individual compounds (van Wijk et al., 2010). Contrasting, researchers have demonstrated that natural enemies are attract to prey's pheromones components, individually or as blends. One or all pheromone components can attract parasitoids and predators with different or similar magnitude of the attraction by the pheromone blend (Reddy et al., 2002).

Moreover, the interaction between the kairomones from the herbivores and the synomones from the host-plants can reinforce the attractive effect of plant-herbivore complexes on natural enemies (Vet & Dicke, 1992). Herbivore-induced plant volatiles and pheromones from the herbivore act as long-range cues while herbivore by-product chemicals act as short-range signals (Han & Chen, 2002). It is likely that natural enemies benefit from exploiring all available volatile cues to locate their prey (Reddy et al., 2002).

Because volatile blends that are produced by plants in response to herbivory vary greatly in their composition and concentration (Van Den Boom et al., 2004) and prey availability can also vary (e.g. prey can be available on a specific host plant, for a limited period or patchily distributed in space, Drukker et al., 2000), predators with a wide range of host-plant and prey species face a large variation in volatile cues associated with the presence of prey (Krips, 1999). Polyphagous predators may have to switch prey and host plants many times along their lives. The ability to learn new associations with prey availability and volatile blends would be more useful than innate and immutable responses when facing variability in prey-host-plant information (Glinwood et al., 2011). An innate preference to an odour is inherent to individuals, heritable and independent of previous experience (Drukker et al., 2000). It is unlikely that generalist predators can have an innate response to all volatile blends they face during their lifetime. Learning, on the other hand, allow predators to deal with this variation in volatile cues. Learning may be defined as any relatively permanent change in an organism behaviour which occurs as a result of practice (Kimble, 1961), so that its subsequent behaviour is better adapted to its environment (Rescorla, 1988).

Innate and learned responses to chemical stimuli may differ according to the level of dietary specialization of natural enemies (Vet & Dicke, 1992). Natural selection is expected to favor innate responses in specialist natural enemies, which may feed on only one or few prey species hosted by one or a small number of plants. This leads to a small range of volatiles, to which natural enemies are likely to respond inherently (Allison & Hare, 2009). Natural enemies with a vast range of prey or with prey occurring on a number of host plant species, on the other hand, face a varying information content of volatiles through ecological and evolutionary time and evolved learned responses following the dynamic nature of their diet (Allison & Hare, 2009). Learning has been studied in many arthropod taxa, in particular social insects (Farina et al., 2005) and parasitoids (Stireman, 2002) and, to a lesser extent, predators (Guillette et al., 2009; Rahmani et al., 2009). Learning plays an important role in the decisions made by many insects (Papaj & Lewis, 1993), but these animals may learn in different ways (Dukas, 2008).

There are many ways of acquiring odour preference or aversion: imprinting, habituation, sensitization, associative learning and operant conditioning (Rescorla, 1988; Drukker et al., 2000). Imprinting occurs rapidly in an early period in life and the stimulus is learned independent of reinforcement (Gould, 1993; Hall & Halliday, 1998); habituation is a gradual decrease of a response to a stimulus after some long exposure to this stimulus, without paired unconditioned stimulus (e.g. shock) (Groves & Thompson, 1970; Carlsson et al., 1999; Rankin et al., 2009); sensitization is also a gradual learning independent of pairing with another stimulus, however it is an increase in the response to a stimulus along with the exposure to that stimulus (Papaj & Prokopy, 1989; Hall & Halliday, 1998); associative learning occurs when two stimuli are paired, the conditioned (e.g. odour) and the unconditioned (reward or punishment), and a preference or aversion response is linked to one of them (Lewis & Tumlinson, 1988; Hall & Halliday, 1998); finally, operant conditioning may be explain as the learning of impact that an organisms actions have on the world, its occurs when the organism can associate its own behaviour (e.g. side choice) to a reinforcer rewarding (e.g. food) or punishing (e.g. shock) (Rescorla, 1988).

Sensitization and habituation predict that exposure always lead to an increase or decrease, respectively, in the response, independent of the context (positive or negative stimulus) (Rescorla, 1988). Associative learning, on the other hand, may result in different outcomes according to the context, preference in a positive context and aversion in a negative context. Associative and gradual learning are not mutually exclusive, two stimuli may be repetitively paired or temporally closely paired for a long period of time, then stimulating a gradual change (increase or decrease) in the response (Drukker et al., 2000).

Associative learning has been demonstrated for many parasitoids, which can link an HIPV blend with the presence of hosts after a short exposure, for example during oviposition in a host (e.g., Lewis & Takasu, 1990). Studies with predators show that the foraging behaviour of these natural enemies is affected by experience with HIPVs when immature (imprinting) (e.g., Dicke et al., 1990a; Takabayashi & Dicke, 1992; Krips et al., 1999). Other researchers have demonstrated associative learning (e.g. Drukker et al., 2000) and sensitization (e.g. Dicke et al., 1990b; Egas & Sabelis, 2001) of predators towards HIPVs.

Main Question

In this thesis I studied the olfactory learning capacity of three generalist predators. I investigated whether these predators were capable of associating unfamiliar volatile blends with the availability of food. I also studied whether learning occurs after only one experience, or whether several encounters with a volatile blend associated with a reward (food, etc.) are needed.

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CHAPTER 1

ASSOCIATIVE LEARNING OF VOLATILE CUES BY *Ceraeochrysa cubana* (Neuroptera: Chrysopidae) LARVAE

PEDROSA, Aline Rodrigues Porto, M.Sc., Universidade Federal de Viçosa, july 2012. **Associative learning of volatile cues by *Ceraeochrysa cubana* (Neuroptera: Chrysopidae) larvae.** Adviser: Angelo Pallini Filho. Co-adviser: Arne Janssen and Madelaine Venzon.

ABSTRACT

To find their herbivorous prey, natural enemies are known to use the sex and aggregation pheromones of their victims, herbivore-induced plant volatiles, and even constitutive plant volatiles as signals for the presence of their prey. The use of volatile cues enhances the foraging efficiency of natural enemies, decreasing time and energy costs. Natural enemies have to deal with a variety of volatile blends associated with their prey, and it has been suggested that the ability to learn the association of odours with the occurrence of prey is one way to cope with this variation. However, little is known about the skills of chrysopid larvae involved in prey location once on the host plant, despite these larvae being important predators for the control of several pest species. This work aimed to fill this gap by studying the learning ability of olfactory cues by lacewing larvae. Specifically, I tested whether *C. cubana* larvae are capable of associating mint oil volatiles with food availability after several days of experience and with continuous pairing of mint oil odours and food. The response of *C. cubana* to mint oil volatiles and clean air was tested in to an Y-tube olfactometer. The larvae were repelled by mint oil volatiles and showed no significant preference for volatiles of catnip oil, a substance that is claimed to be attractive for adult Chrysopidae. The number of training trials did not affect the response of *C. cubana* to mint oil odours. Likewise, continuous training did not change the response of *C. cubana* larvae. My results suggest that *C. cubana* larvae can perceive volatiles; however they showed no evidence for learning the association of an odour and a reward (e.g. food), irrespective of the number of previous experiences or the interval between experiences.

INTRODUCTION

Many animals spend most of their time and energy searching for food and mates (Bell, 1991). To decrease the time and energy needed for finding prey, insects often use several cues. Natural enemies may use volatiles as signal on the searching for prey. The use of volatile cues that are both reliable and easy detectable enhances the foraging efficiency of natural enemies (Vet & Dicke, 1992). Although herbivores were selected not to produce volatiles in order to avoid attracting natural enemies, they do emit sex and aggregation pheromones that can betray their location to the enemies (Boo et al., 1998; 2003). Constitutive volatiles from host plant are usually detectable but not always indicate prey presence. However, plants are known to emit volatile blends upon being attacked by herbivores, and these volatiles are therefore reliable indicators of the presence of herbivores (Dicke, 1999).

Moreover, volatile mixtures often elicit different responses in insects than their individual elements (e.g. Riffell et al., 2009; Webster et al., 2010). Therefore, odour blends are believed to be perceived as one synthetic whole by arthropods (van Wijk et al., 2010). Each combination of plant and herbivore results in a distinct blend of volatiles (Dicke et al., 1998) and plant species can release a different mixture for each herbivore attacking them (De Moraes et al., 1998; Dicke et al., 1998). Predators that use volatiles as searching cues may therefore assess the identity and quality of the host plant and prey (Glinwood et al., 2011). However, generalist predators may have to deal with different prey species on different host plant species during their successive generations (Boivin, 2010), and these prey and host plants are all associated with different volatile blends. This variation makes it unlikely that generalist predators innately recognize all cues associated with the presence of prey (Papaj & Lewis, 1993).

Generalist predators may have to switch prey and host plants many times along their lives, facing a large variation in volatile cues associated with the occurrence of prey (Krips, 1999). The ability to learn new associations with prey availability and volatile blends would therefore be well adaptive (Glinwood et al., 2011). Learning can be defined as any relatively permanent change in behaviour which occurs as a result of practice (Kimble, 1961). Learning is broadly documented for arthropods. The adaptive significance of learning was demonstrated for antlions (Guillette et al., 2009), grasshoppers (Dukas & Bernay, 2000), mites (Egas & Sabelis, 2001), bees (Gould, 1993), parasitoid wasps (Lewis & Tumlinson, 1988) and social insects (Farina et al., 2005). Most of the works concerns insect learning during foraging (Dukas, 2008), reporting more efficient searching behaviour (Ettifouri & Ferran, 1993), capture efficiency (Guillette et al., 2009), and prey recognition in adult predators (Guershon & Gerling, 2006) following a previous experience.

An environment that changes between generations but is predictable during a generation favors the evolution of learning (Stephens, 1991). Natural enemies can become more responsive to a blend of volatiles or adjust their preference in a choice situation after a short moment of exposure to plant volatiles and a suitable prey (De Boer & Dicke, 2004; Drukker et al., 2000). This type of learning allows carnivores to specialize temporarily on the host plants harbouring their prey at a determined place and time (De Boer et al., 2005). Experience with volatiles during development may influence adult (Dicke et al., 1990; Takabayashi & Dicke, 1992; Krips et al., 1999) and larvae (Neuser et al., 2005; Salloum et al., 2011) foraging behaviour. However, little is known about changes in immature behaviour in response to a previous experience. Immature insects should be able to search for suitable prey at patches where preys are irregularly distributed. Moreover, immatures capable of learn to associate volatile cues could enhance their foraging success.

Lacewings are generalist holometabolous predators of eggs and soft-bodied insects (New, 1975). Adult lacewings can feed on nectar, pollen, honeydew, other arthropods, but adults of some species do not require food. Lacewing larvae are all voracious predators with large pointed jaws (Reddy, 2002; Hajek, 2004). They are well known as natural enemies of aphids, but they also eat spider mites, scales, psylla, mealybugs, whiteflies, thrips, leafhoppers and Lepidoptera eggs (Canard & Principi, 1984; New, 1988). Hence, they are important natural enemies for the natural and biological control of various insect pests.

Lacewings (Neuroptera: Chrysopidae) respond positively to a variety of semiochemicals, such as host plant volatiles (Zhu et al., 1999; 2005), herbivore-induced plant volatile (James, 2003; Jones et al., 2011), aphid sex pheromone (Boo et al., 1998; 2003) and male-produced chrysopid aggregation pheromone (Zhang et al., 2004; Jones et al., 2011). Methyl salicylate, a volatile induced by the attack of whiteflies and aphids (Walling, 2000), attracted *Chrysopa nigricornis* and *Chrysoperla carnea* adults in field experiments (James, 2003; Zhu et al., 2005). Squalene, another HIPV, was found to be attractive only to male *C. nigricornis* (Jones et al., 2011). Traps baited with nepetalactone and nepetalactol isomers, components of aphid pheromone and present in catnip plants (*Nepeta cataria*, Laminaceae), captured males and females of *C. carnea*, *Chrysopa septempunctata* and *Chrysopa oculata* (Hyeon et al., 1968; Birkett & Pickett, 2003; Zhu et al., 2005). Iridodial isomers, components of the male-produced chrysopid aggregation pheromone, aphid sex pheromone and present in catnip plants, attracted *Chrysopa coloradensis*, *Chrysopa quandripunctanta*, *C. septempunctata*, *C. nigricornis* and *C.*

oculata in field experiments (Chauhan et al., 2004, 2007; Zhang et al., 2004, 2006; Jones et al., 2011). *Chrysopa nigricornis* and *C. oculata* were also attracted to the combination of methyl salicylate and iridodial (Jones et al., 2011). Therefore, green lacewings appear to be attracted to a large number of semiochemicals and HIPVs, which guide both female and male chrysopids to prey-infested plants (Zhu et al., 1999).

Ceraeochrysa cubana (Hagen) (Neuroptera: Chrysopidae) are important natural enemies in tropical and subtropical regions (López-Arroyo et al., 1999). This predator shows a great efficiency in crop pest regulation in many agroecosystems and is well recommended to introduction in biological control programs (Venzon & Carvalho, 1993; Freitas & Scaloppi, 1996; Santa-Cecília et al., 1997; Albuquerque et al., 2001; Souza & Carvalho, 2002). Chrysopid larvae are polyphagous, move throughout the plant and have a high searching capacity (Núñez, 1988). Larvae of *C. cubana* are voracious predators (Souza et al., 2008) with large pointed jaws and with many sensilas along their body (Pedrosa, A.R.P. personal observation).

To my knowledge, the majority of studies on foraging lacewings worked with adults. However, larvae of *C. cubana* are the only predacious stage (Venzon, 1991). Researchers argue that, even when adults are not predators, the ability to use volatiles to locate suitable patches for oviposition is highly adaptive (Reddy, 2002). Still, little is known about larva skills involved in prey location once on the host plant. This work aimed to study lacewing larvae olfactory learning ability. At first, the ability for olfactory perception of *C. cubana* larvae was tested. Then, I studied whether these lacewing larvae are capable of associating new volatiles with food availability after two and four days of experience. I also studied the occurrence of gradual learning with reinforcement after each choice.

MATERIAL AND METHODS

1. Prey rearing

Diatraea saccharalis (Lepidoptera: Pyralidae) was reared on an artificial diet based on yeast, soy flour and wheat germ (Macedo et al., 1983) under laboratory conditions (26 ± 1 °C; 60 ± 10% UR; 12:12 light : dark regime) following Parra (1999). Egg batches were placed in glass jars (500 ml) with artificial diet, which were closed with fine metal mesh. Larvae were allowed to hatch and feed *ad libitum* and they were transferred to Petri dishes (14 cm diameter) with artificial diet when nearing pupation. Pupae were incubated in cylindrical plastic tubes (20 cm height x 10 cm diameter) lined with paper, in which adults were allowed to emerge, mate and oviposit on the paper. The paper with the eggs was removed daily and pieces of paper with egg batches were cut for the rearing and for subsequent use in experiments.

2. Predator rearing

Second instar larvae of *Ceraeochrysa cubana* were obtained from the rearing of Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG), Viçosa. Predator larvae were reared in plastic tubes (7.5 x 3.5 cm) with eggs of *Anagasta kuehniella* (Zeller) (Lepidoptera: Pyralidae) as food. Adults were fed with an artificial diet based on honey and yeast and maintained in plastic cages for mating and oviposition. Predators were kept individually for 24 hours in plastic tubes with a piece of moist cotton wool as water source and closed with a fine mesh to increase motivation to forage before the tests.

3. Preparation of volatile dispensers

Mint oil (*Mentha piperita*, Oito Ervas, Marataízes, Brasil) was used in association with the availability of *D. saccharalis* eggs. This oil is repellent (Hori, 1999; Koschier & Sedy, 2003) and toxic to arthropods (Choi et al., 2004; Odeyemi et al., 2008). The major components of this oil are menthol, menthone, (+)-menthyl acetate, menthofuran and isomenthone (Yang et al., 2010), limonene and 1,8-cineole (Rohloff, 1999). The predators tested here had not been exposed previously to this volatile blend, enabling the study of learning a new association between food and an odour.

Catnip oil (*Nepeta cataria*, The Essencial Oil Company, Chicago, USA) was used to test the olfactory response of larvae. Catnip is an herbal plant in the mint family. Its oil contains volatile compounds (nepetalactone, nepetalactol and iridodial) similar to those present in aphid and chrysopid pheromones. These blends are known to attract lacewing adults in the field and laboratory (Birkett & Pickett, 2003; Chauhan et al., 2004; Zhang et al., 2004).

Volatile dispensers were made of Parafilm®, which was cut into strips of 5.2 cm^2, rolled up and tightly flattened in layers of ± 5 mm. Each roll was cut into 5 pieces of 7 mm long, and each piece was stuck on a separate pin. Some dispensers were incubated in a Petri dish (9 cm diameter) with mint oil; some were incubated with catnip oil and others were kept in a clean Petri dish. After 12 hours, the dispensers were taken from the Petri dishes and placed on a tissue paper to dry. Dispensers were then used in training trials and olfactometer tests.

4. Olfactometer experiments

The response of predators to odours was tested in an Y-tube olfactometer (Sabelis & van de Baan, 1983; Janssen et al., 1999). The olfactometer consisted of a glass tube (27 x 3.5 cm) in form of a Y, with a Y-shaped metal wire in the middle to guide the lacewing larvae. The base of the tube was connected to a pump that produced an airflow from the arms of the tube to the base (Janssen et al., 1999). Each arm was connected to a glass container (50 x 36 x 43 cm) and a polystyrene foam plate with dispensers with mint oil as above was placed inside one of the containers. The containers had an air inlet and outlet (2.5 cm diameter) in opposite walls. Two hot-wire anemometers were used to measure the wind speed in each arm of the olfactometer, which was calibrated (0.45m/s) with valves between the air outlet of the containers and the arm of the olfactometer (Sarmento et al., 2007). When wind speeds in both arms are equal, the air coming from the containers forms two separate fields in the base of the Y-tube with the interface coinciding with the metal wire (Sabelis & van de Baan, 1983).

Second instar larvae of *C. cubana* were introduced one at a time, by disconnecting the pump and placing the larva on the metal wire at the base of the Y-tube. After reconnecting the pump, the larvae started walking upwind to the junction of the wire, where it had to choose one arm. Each individual was observed until it had reached the end of one arm or for a maximum of 5 minutes and was subsequently removed. Predators that did not make a choice within 5 minutes were excluded from further analysis. Each predator larva was considered one replicate. The number of predators tested per treatment vary depending on the rearing supply. Odour sources were switched to the opposite arm of the olfactometer after each five predators that made a choice, to correct for any unforeseen asymmetry in the experimental set-up.

5. Olfactory response

The response of twenty second instar lacewing larvae to catnip oil volatiles was tested in the olfactometer as above to verify their capacity to respond to volatiles in this olfactometer.

6. Predator experience

6.1 Daily training

Second instar larvae of *C. cubana* were placed in two tent cages (BugDorm-2400 Insect Tent (BD2400)/MegaView Science Co., Ltd.) with moist cotton wool in a Petri dish (14 cm diameter) as water source and a polystyrene foam plate at the bottom of the cage, in which the dispensers with eggs batches of *D. saccharalis* were stuck. In one cage, the dispensers had mint oil while those in the control cage had not. The plate with eggs was offered at the beginning of the day and removed after the egg batches were completely consumed by the larvae. This procedure was repeated for two or four days. At the third or fifth day, respectively, the *C. cubana* larvae were removed from the cages and kept individually in plastic tubes. Because of cannibalism inside the cages, the supply of *C. cubana* for each treatment differed. Depending on this supply, 18 to 21 predators were tested per treatment at the Y-tube olfactometer.

6.2 Continuous training

Thirty-seven second instar lacewing larvae from the same culture as above, hence, without experience with mint oil, were tested for preference for mint oil or clean air in the olfactometer. Each arm of the Y-tube was connected to a glass tube (20 x 3.5 cm), closed with mesh on both sides. Either one dispenser with mint oil was put inside the tube, or the tube was left empty. The mesh served to produce a laminar air low, as well as to avoid the use of visual cues by the predators when choosing.

Based on the response of each larva, they were assigned to one of two groups: a) those that had selected the arm with mint oil received *D. saccharalis* eggs, while b) those that had chosen for clean air were kept without food. Larvae were kept individually in the plastic tubes and the tubes were placed in plastic containers (35 x 25 x 24 cm) with or without a dispenser with mint oil, according to the choice of the larvae. Hence, mint oil volatiles were paired with *D. saccharalis* eggs for one group of *C. cubana* larvae, while the other group experienced the association of clean air with the absence of food. The plastic tubes were closed with mesh, which avoided the predators to escape but allowed volatiles to enter the tubes. The containers had an air inlet (4 cm diameter) and outlet (2 cm diameter) in opposite walls and were connected to a pump that produced airflow preventing the formation of high concentrations of the volatiles. The larvae were kept overnight in this way and were tested again for preference for mint oil the next day. This procedure was repeated during three days, hence, each day, one group of predators were split into two further groups, depending on their choice.

7. Data analysis

Differences in numbers of predators choosing for volatiles or clean air were tested using log-linear models for contingency tables (Crawley, 2007), and when necessary, proportions of predators choosing for one odour source were compared with a binomial test with a probability of 0.5 at a 5% significance level.

RESULTS

1. Olfactory response

To assess the capacity of *C. cubana* larvae to respond to volatiles in an Y-tube olfactometer, their response to catnip oil volatiles was tested. Half of the *C. cubana* larvae tested choose catnip oil while the other half choose clean air (χ^2 = 2.22 x 10^{-15}, d.f = 1, P = 1.00).

1.1 Daily training

To investigate whether *C. cubana* larvae can learn to associate mint oil with food, the larvae were offered experience with oil paired with *D. saccharalis* eggs two or four times before testing the response of the larvae to mint oil volatiles. *Ceraeochrysa cubana* larvae with daily training experience were repelled by mint oil volatiles (χ^2 = 4.19, d.f = 1, P = 0.04, Figure 1). This repellence was even stronger for *C. cubana* larvae that had been feeding without exposure to mint oil volatiles for four days (binomial test, P = 0.02, Figure 1). The number of training trials showed no significant interaction with the response of *C. cubana* to clean air and mint oil odours (deviance = 0.05, d.f = 1, P = 0.82, Figure 1). *Ceraeochrysa cubana* that had previous experience with mint oil odours equally responded to mint oil volatiles and clean air (deviance = 2.89, d.f = 1, P = 0.09), although there was a trend to prefer clean air.

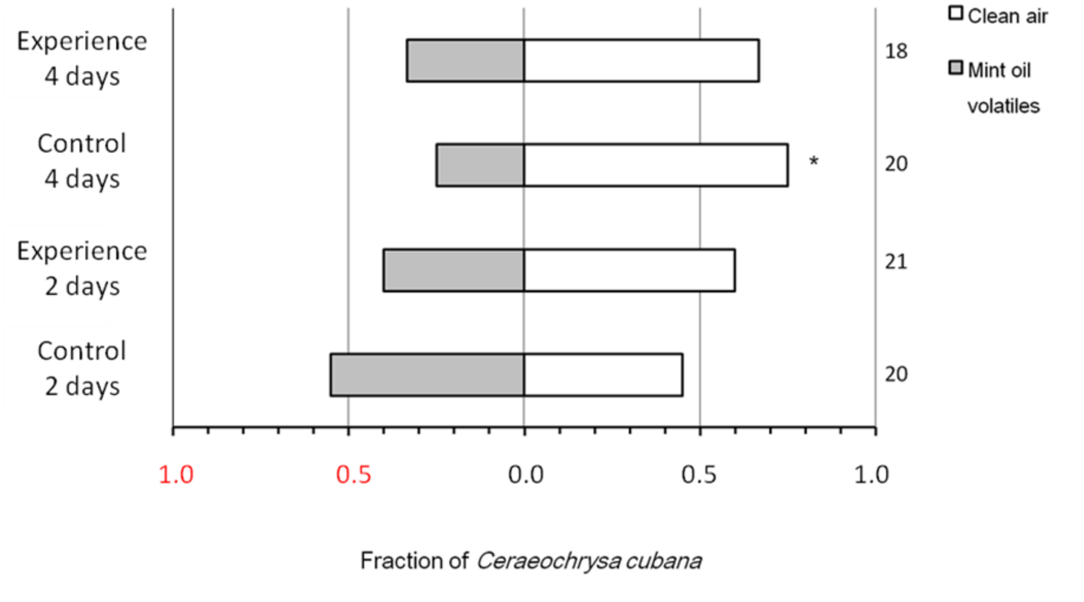

Figure 1 – Choice of *Ceraeochrysa cubana* larvae when offered mint oil odours vs. clean air after two or four days of previous experience with mint oil or with no previous experience (Control). The numbers of replicates are indicated on the left side of the graph. * indicates a significant difference at the 5% level.

1.2 Continuous training

During all continuous training, *C. cubana* larvae showed no preference for mint oil volatiles or clear air (binomial test, P = 0.10, Figure 2). The average proportion of larvae attracted to mint oil volatiles was 0.43 ± 0.04 (Figure 2). The response of *C. cubana* larvae with successive reinforcements after trials did not change with the number of trials (χ^2 = 1.49, d.f = 1, P = 0.22, Figure 2). A proportion of 0.50 of the *C. cubana* larvae with experience with mint oil and clean air respectively chose for mint oil on the third day (binomial test, P = 0.66). Larvae that chose mint oil twice changed their response in 38% of the cases at the last day (binomial test, P = 0.85). 20% of the larvae that choose clean air on the first two days changed their choice on the third test (binomial test = 0.05), and 71% of those with experience with clean air and mint oil respectively changed their choice again (binomial test, P = 0.23) (Figure 3).

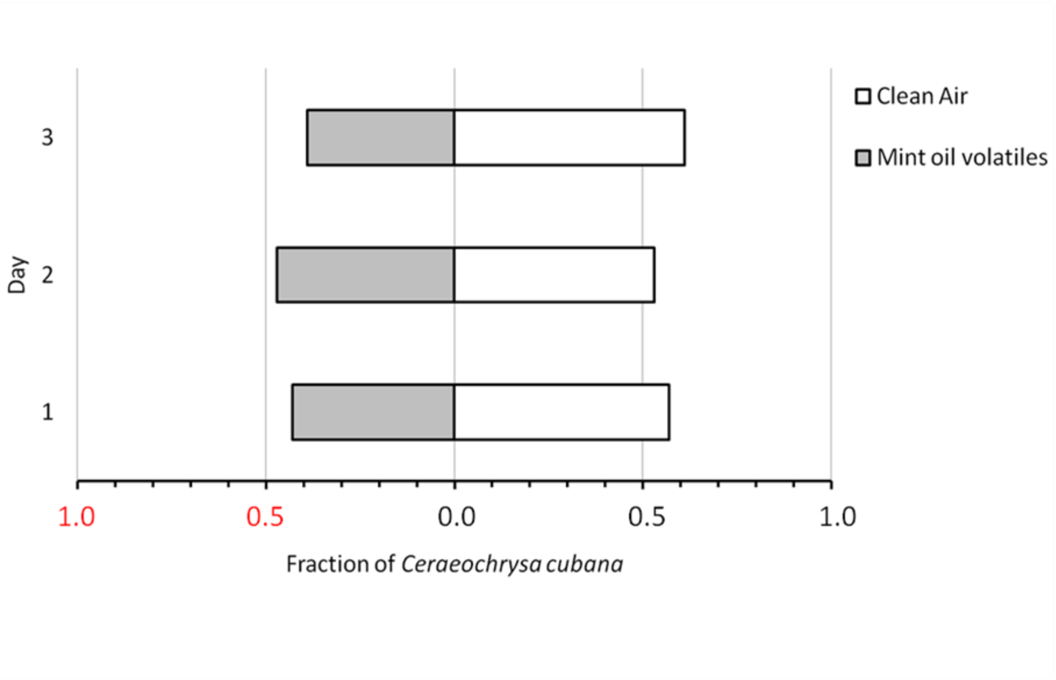

Figure 2 – Choice of *Ceraeochrysa cubana* larvae when offered mint oil odours vs. clean air at three continuous days with reinforcement training after each choice.

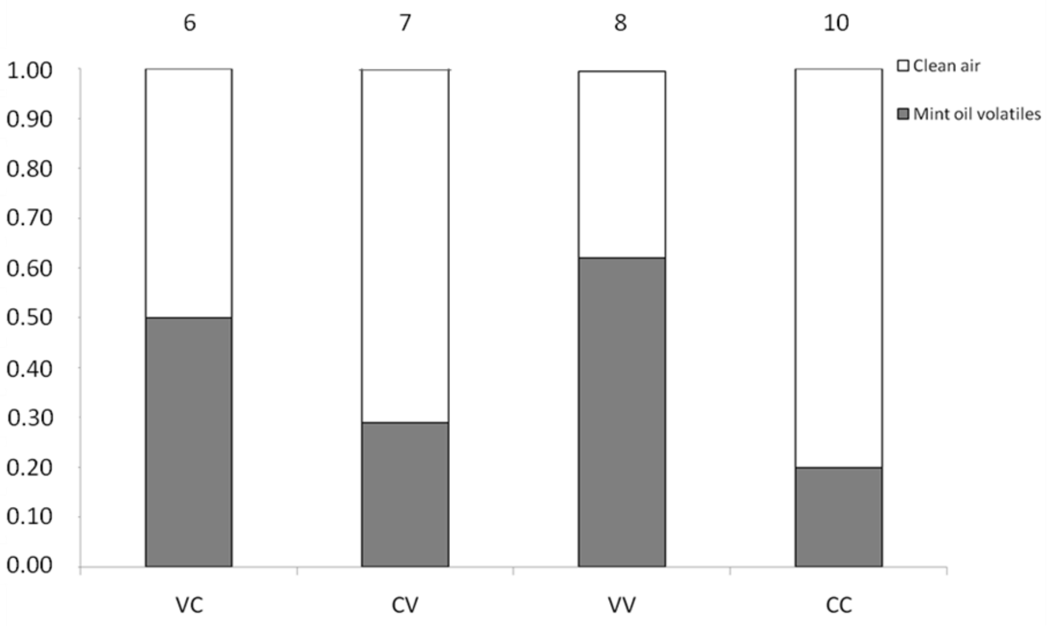

Figure 3 – Fractions of *Ceraeochrysa cubana* larvae choosing mint oil volatiles or clean air at the third day for all combinations of previous two choices (e.g. VV is the group that had chosen mint oil volatiles twice). Numbers at the top of the bars indicate the number of replicates.

DISCUSSION

Ceraeochrysa cubana larvae were expected to respond positively to catnip oil volatiles, because nepetalactone, nepetalactol and iridodial isomers (all components of catnip oil) are known to attract adults green lacewings of many species (Hyeon et al., 1968; Chauhan et al., 2004; Zhu et al., 2005; Zhang et al., 2006). These volatiles are present in aphid sex pheromones (Birkett & Pickett, 2003). It was therefore expected that chrysopid larvae could innately use these olfactory cues for locating their preferential prey. Alternatively, larvae with previous experience with aphids and their volatiles would be expected to learn to identify this prey. However, lacewing larvae are known to use tactile cues when searching for prey (Hutchins et al., 2003) and are believed to identify prey only after contacting it with their mouthparts (Hajek, 2004). Perhaps there was no selection for olfactory searching in chrysopid larvae, which could explain the lack of response to a blend similar to blends emitted by their prey. However, first and second instar larvae of the chrysopid *C. carnea* were attracted to the volatiles of the prey species (Sengonca et al., 1995). Therefore, even though no publication on antennae in larval chrysopids was found, we believe that lacewing larvae have functional receptors. Further investigations are needed to explore how volatiles can affect the behaviour of *C. cubana* larvae.

Larvae of *C. cubana* showed no response to mint oil odours after two or four previous experiences with this blend paired with food. This differs from what was found for *Drosophila* larvae, which enhance their response to amyl acetate after three paired trials with fructose (Neuser et al., 2005). However, my data are consistent with results with *Spodoptera littoralis* larvae, which were attracted to hexanol only after six paired trials with fructose and did not significantly respond to this volatile after only two or three training trials (Salloum et al, 2011). Hence, *C. cubana* larvae may need a higher number of training trials for associative learning to occur. However, if the period needed to learn were longer than the duration of larval instar and larvae forget experiences when changing instar, learning would be quite useless for the larvae. Each instar of *C. cubana* extends for a period of, approximately, four days when feeding on moth eggs (Santa-Cecília et al, 1997, López-Arrojo et al., 1999). However, the brain centers involved in olfactory associative learning are known to remain intact during metamorphosis of the fruit fly *Drosophila melanogaster* (Armstrong et al., 1999). Therefore, even though some of the *C. cubana* larvae changed instar during the training period if they had learned the associating between the mint oil blend and *D. saccharalis* eggs it would remain in the following stages.

Alternatively, *C. cubana* might learn the association between mint oil and food with fewer training trials, but with shorter intervals between training trials and between the last training trial and the olfactometer test. *Ceraeochrysa cubana* were allowed to feed on *D. saccharalis* eggs until consuming all food available, which took some hours. One training trial was run per day and the response to mint oil volatiles was tested only on the following day. *Drosophila* sp. and *S. littoralis,* on the other hand, were trained for a few minutes, with short time intervals and tested soon after training (Neuser et al., 2005; Salloum et al., 2011). This kind of training trial was not possible for *C. cubana* larvae, since this predator did not begin to eat immediately after food is offered (Pedrosa, A.R.P. personal observation).

The continuous training test was then performed to investigate the response of *C. cubana* larvae shortly after training. Moreover, if associative learning is not formed after continuous mint oil and food pairing, it would be expected that *C. cubana* were repelled by mint oil odours. *Ceraeochrysa cubana* larvae with a two-day experience with mint oil continuously paired with food showed no change in their response to mint oil. The absence of attraction and repellence of *C. cubana* to mint oil after continuous training could be due to olfactory habituation. Habituation is a simple form of learning, consisting of a gradual decrease of a response to a stimulus after at least 12-24h of exposure to this stimulus, without a paired unconditioned stimulus (e.g. food) (Groves & Thompson, 1970; Carlsson et al., 1999; Rankin et al., 2009). However, *C. cubana* larvae with a two-day experience with clean air and no food were also not repelled by mint oil volatiles on the third day. Therefore, more experiments are needed before any conclusion about the habituation to mint oil odours by *C. cubana*.

Similarly, *C. cubana* larvae with two different previous experiences, one positive (mint oil odours with food) and one negative (clean air without food), irrespective of the order; showed no difference in their response on the third day trial. This also indicates that associative learning did not occur after continuous training with one positive and one negative reinforcement.

Another possibly explanation is that although *C. cubana* can learn volatile blends associated with food, they did not change their response in the olfactometer because *D. saccharalis* is not a very rewarding food. However, *C. cubana* larvae develop faster and sometimes turn into larger adults when feeding on moth eggs than with a diet of aphids (López-Arrojo et al., 1999). Moreover, preliminary tests showed that *C. cubana* larvae feed on *D. saccharalis* eggs, confirming that it is a suitable prey.

Therefore, *C. cubana* larvae are believed not to learn with two or four previous experiences, with daily paired or continuously paired stimuli. My results suggest that, even though *C. cubana* larvae can perceive volatiles, they do not show behavioural changes that suggest learning of the association of an odour and a reward (e.g. food), irrespective of the number of previous experiences or the interval between experiences.

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Chapter 2

ASSOCIATIVE LEARNING WITH CONTINUOUS EXPERIENCE WITH VOLATILES ASSOCIATED WITH FOOD IN THREE PREDATOR SPECIES

PEDROSA, Aline Rodrigues Porto, M.Sc., Universidade Federal de Viçosa, july 2012. **Associative learning with continuous experience with volatiles associated with food in three predator species.** Adviser: Angelo Pallini Filho. Co-adviser: Arne Janssen and Madelaine Venzon.

ABSTRACT

Predators can use several volatile blends to locate their prey, but the large natural variability in the composition of volatile mixtures may render innate responses to specific volatiles useless. The ability to learn volatiles associated with prey may allow predators to deal with this variability. However, little is known about the ability of predators learning the association between a volatile blend and the presence of prey after successive experiences. The aim of this work was to study gradual learning of the association of a volatile with food by predators. I studied whether the response of *Ceraeochrysa cubana* larvae, *Podisus nigrispinus* and *Cycloneda sanguinea* to a new volatile blend associated with food availability (eggs of *Diatraea saccharalis*) increased with reinforcement after each choice. The response of predators to volatiles of mint oil or clean air was tested using an Y-tube olfactometer. Mint oil odours were neither attractive nor repellent for *C. cubana, C. sanguinea* and *P. nigrispinus* after several days of experience. Sequential choices, with reinforcements after each test, did not change the response of predators. My results show that none of these predators demonstrate learning of the association of mint oil volatiles and food within three days of sequential experiences. More extensive experiments are needed to conclude if *P. nigrispinus, C. sanguinea* and *C. cubana* are capable of associative learning.

INTRODUCTION

Plants are known to release various chemicals, many of which are volatiles (Farmer, 2001). Blends of volatile organic compounds (VOCs) are emitted from many plant organs and the composition of these blends varies considerably, with the principal molecules being fatty acid derivatives, terpenes and indole (Farmer, 2001); and also varying between plant species and other taxonomic levels (McCormick et al., 2012). Herbivores can use such plant volatiles to locate their host plants. However, plants do not undergo attacks passively; they evolved various strategies to reduce herbivore damage (Walling, 2000). One of the most remarkable of these strategies is the release of specific blends of volatiles after attack by herbivorous arthropods (Farmer, 2001). These herbivore-induced plant volatiles (HIPVs) can attract predators and parasitoids of the herbivores (Turlings et al., 1990; Dicke et al., 1990; Dicke et al., 1998), which is thought to increase plant fitness (Kessler & Balwin, 2001). Like VOCs, the herbivore-induced plant volatiles may vary with different herbivore species feeding on the same plant species (De Boer & Dicke, 2004), with the same herbivore species feeding on plants of different species (Takabayashi et al., 1991), among leaves of different age (Takabayashi et al., 1994) and with physical conditions of the environment.

The recruitment of natural enemies that feed on herbivores is an important indirect defence of plants (McCormick et al., 2012). The variability in the composition of HIPV mixtures may confuse the carnivores looking for suitable prey (Krips et al., 1999). However, many insects are capable of detecting a wide range of VOCs. Studies suggest that while the olfactory sensitivities of sister species is quite similar, their behaviour towards odours can be very different (Kant et al., 2009). Moreover, HIPV blends are perceived as a synthetic whole by predators rather than a compilation of individual compounds (van Wijk et al., 2010). Predators that leave patches after exterminating their prey search for familiar volatile blends that were associated with the presence of prey in the patch that they are leaving, yet there is a high probability of detecting a different mixture of HIPVs (Krips et al., 1999). Therefore, natural enemies with a fixed preference for one particular volatile blend may not be able to always find new plants with prey effectively, and phenotypic plasticity may often be more adaptive for these carnivores (De Boer & Dicke, 2004). Moreover, immature predators may forage in a different habitat than their parents if adults disperse to a different patch to search for suitable prey (Boivin et al., 2010).

With a high within-generation predictability and low between-generation predictability of HIPVs, predators may have evolved the ability to learn (Stephens, 1991). Learning can be understood as any change in the behaviour of an animal after experience (Papaj & Prokopy, 1989). An increase in the response to a stimulus along with the exposure to that stimulus is known as gradual learning (Papaj & Prokopy, 1989; Hall & Halliday, 1998) and associative learning occurs when a response is linked to a conditioned stimuli (e.g. odour, shock) paired with an unconditioned stimuli (e.g. food, starvation). Gradual and associative learning can occur simultaneously if two stimuli were repetitively paired (Drukker et al., 2000a).

However, little is known about the ability of predators to gradually learn the association of a volatile blend and the presence of prey after successive experiences. The aim of this work was to elucidate the ability of gradual learning of the association of volatiles with food in predators. I studied whether the response of three important tropical natural enemies to an unfamiliar volatile blend associated with food availability increased with reinforcement after each choice.

The three predator species used were larvae of the lacewing *Ceraeochrysa cubana* (Hagen) (Neuroptera: Chrysopidae), females adults of the stinkbug *Podisus nigrispinus* (Dallas) (Heteroptera: Pentatomidae) and females adults of the ladybird *Cycloneda sanguinea* (Linnaeus) (Coleoptera: Coccinelidae). Larvae of *C. cubana* (Hagen) (Neuroptera: Chrysopidae) are important natural enemies of crop pests in tropical and subtropical regions (López-Arroyo et al., 1999). Chrysopid larvae are polyphagous, move throughout the plant and have a high searching capacity (Núñez, 1988). Lacewings are known to use volatiles as cues to locate their prey (e.g. Zhu et al., 1999, 2005; James, 2003a, 2003b). *Cycloneda sanguinea* (Linnaeus) (Coleoptera: Coccinelidae) also uses volatiles during foraging (Sarmento et al., 2007) and is usually found in many crops close to prey colonies (Araujo-Siqueira & Almeida, 2006). Larvae and adults ladybirds are voracious predators of aphids, mites, coleoptera larvae, and lepidoptera eggs and larvae (Clausen, 1972; Hodek, 1973, Bueno & Berti Filho, 1991). Coccinelid larvae are known to learn after an experience with prey of different quality (Boivin et al., 2010) and ladybirds adults increase their response to aphid-infested plants volatiles of a specific cultivar after feeding on aphid on this cultivar for few days (Glinwood et al., 2011). *Podisus nigrispinus* (Dallas) (Heteroptera: Pentatomidae) is a generalist predator commonly found in native and crops areas of South America (Freitas et al., 1990). Predacious stinkbugs feed on caterpillars and larvae of coleoptera and hymenoptera (Medeiros et al., 2003). Associative learning was observed for heteropteran bugs after experience with HIPVs of psyllid-infested leaves in presence or absence of prey (Drukker et al., 2000a).

In this work I studied whether the response of *C. sanguinea*, *P. nigrispinus* and *C. cubana* larvae to volatiles of mint oil changed after sequential trials with a positive (*D. saccharalis* eggs) reinforcement after each choice. It was expected that predators would increase their preference for mint oil volatiles after positive experiences.

MATERIAL AND METHODS

1. Prey rearing

Diatraea saccharalis was reared under laboratory conditions (26 ± 1 °C; 60 ± 10% UR; 12:12 light : dark regime) (Parra, 1999) on an artificial diet with cane yeast, soy flour and wheat germ as main components (Macedo et al., 1983). Egg batches were placed in glass jars (500 ml) with artificial diet and the jars were closed with fine metal mesh. Larvae were allowed to hatch and feed *ad libitum* on the artificial diet. Last instar larvae were transferred to Petri dishes (14 cm diameter) with artificial diet. Pupae were incubated in cylindrical plastic tubes (20 x 10 cm) lined with paper, in which adults were allowed to emerge, mate and oviposit on the paper. The paper with eggs was removed daily and pieces of paper with egg batches were cut for the rearing and for subsequent use in experiments.

2. Predator rearing

Larvae of *Ceraeochrysa cubana* were obtained from the rearing of Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG), Viçosa. Predator larvae were reared on *Anagasta kuehniella* (Zeller) (Lepidoptera: Pyralidae) eggs and the adults were fed with an artificial diet based on honey and cane yeast. *Cycloneda sanguinea* female adults were obtained from the rearing of Laboratório de Acarologia of Federal University of Viçosa (UFV). *Cycloneda sanguinea* was reared on various species of aphids collected from lemon, milkweed and corn on the campus of the UFV, Minas Gerais, Brazil (20^{o}45’ S, 42^{o}51’ W). *Podisus nigrispinus* female adults were obtained from the mass rearing of Laboratório de Controle Biológico de Insetos of Instituto de Biotecnologia Aplicada à Agropecuária (BIOAGRO). *Podisus nigrispinus* were reared on *Tenebrio molitor* (Coleoptera: Tenebrionidae) pupae.

Prior to experiments, all predators were starved for 24 hours to increase their motivation to forage.

3. Preparation of volatile dispensers

Mint oil was used in association with *D. saccharalis* eggs availability. Commercially available mint oil (*Mentha piperita,* Oito Ervas, Marataízes, Brasil) is mainly composed of menthol, menthone, (+)-menthyl acetate, menthofuran and isomenthone (Yang et al., 2010), limonene and 1,8-cineole (Rohloff, 1999). Predators tested here had not been exposed previously to this volatile blend, enabling the study of learning a new association between food and an odour.

Parafilm® strips were cut into 5.2 cm^2, rolled up, tightly flattened and cut into 5 pieces (7 x 5 mm). Each dispenser consisted in a Parafilm® piece stuck on a pin. Half of these dispensers were incubated in a Petri dish (9 cm diameter) with mint oil; the other half was kept in a clean Petri dish. Dispensers were dried on a tissue paper after 12 hours. Dispensers were then used on training trials and olfactometer tests.

4. Sequencial trials

4.1 *Ceraeochrysa cubana* and *Cycloneda sanguinea*

Thirty-seven larvae of *C. cubana* and twenty-one female adults of *C. sanguinea* were kept individually in plastic tubes (7.5 x 3.5 cm), closed with a fine mesh and were supplied with a piece of moist cotton wool as water source. Predators were tested for preference for the volatiles of mint oil or clean air using a Y-tube olfactometer (Sabelis & van de Baan, 1983; Janssen, 1999).

The olfactometer consisted of a glass tube (27 x 3.5 cm) in form of a Y, with a Y-shaped metal wire in the middle to guide the predators. The base of the tube was connected to a pump that produced an airflow from the arms of the tube to the base (Janssen, 1999). Each arm was connected to a glass container (50 x 36 x 43 cm) with an air inlet and outlet (2.5 cm diameter) in opposite walls. Two hot-wire anemometers were used to measure the wind speed in each arm of the olfactometer, which was calibrated (0.45m/s) with valves between the air outlet of the containers and the arm of the olfactometer (Sarmento et al., 2007). When wind speeds in both arms are equal, the air coming from the containers forms two separate fields in the base of the Y-tube with the interface coinciding with the metal wire (Sabelis & van de Baan, 1983). Each arm of the Y-tube was connected to a glass tube (20 x 3.5 cm), closed with mesh on both sides. Either one dispenser with mint oil was put inside the tube, or the tube was left empty. The mesh served to produce a laminar air low, as well as to avoid the use of visual cues by the predators when choosing.

Predators were introduced one at a time on the metal wire at the base of the Y-tube. After connecting the pump, the predator started walking upwind to the junction of the wire, where it had to choose one arm. Each individual was removed at the end of 5 minutes or after it had reached the end of the arm. Predators that did not make a choice within 5 minutes were excluded from further analysis. Odour sources were switched to the opposite arm of the olfactometer after each five predators that made a choice, to correct for any unforeseen asymmetry in the experimental set-up.

After the trial, two groups were created for each species of predator, according to the choice of each individual. Those that had selected the arm with mint oil received *D. saccharalis* eggs in association with mint oil volatiles, while those that had chosen the clean air arm were kept without food and odours. *Ceraeochrysa cubana* and *C. sanguinea* were kept individually in plastic tubes inside plastic containers (35 x 25 x 24 cm) with or without a dispenser with mint oil, according to their previous choice. The plastic tubes were closed with mesh, which avoided the predators to escape but allowed volatiles to enter the tubes. The containers had an air inlet (4 cm diameter) and outlet (2 cm diameter) in opposite walls and were connected to a pump that produced airflow preventing high concentrations of the volatiles. Predators were kept overnight in this way and were tested again for preference for mint oil the next day. These procedures were repeated during three days, hence, each day, each group of predators were split into two further groups, depending on their choice.

4.2 *Podisus nigrispinus*

Thirty female adults stinkbugs were tested for preference for mint oil volatiles or clean air using a different Y-tube olfactometer, which consisted of a glass tube (27 x 2 cm) in the form of a Y, connected to two plastic containers (35 x 25 x 24 cm) with an air inlet (4 cm diameter) and outlet (2 cm diameter) in opposite walls. One container received one dispenser with mint oil, which was placed close to the air inlet, and several *D. saccharalis* egg batches; the other container was left empty. The pump was disconnected and predators were all introduced at the same time at the base of the Y-tube. To correct for any unforeseen asymmetry in the experimental set-up, odour sources were switched to the opposite arm of the olfactometer after half of the stinkbugs had entered one of the containers.

Based on the response of each predator, it was assigned to one of two groups: Those that had selected the arm with mint oil were kept in plastic cups (500 ml) with *D. saccharalis* eggs and mint oil volatiles, while those that had chosen the clean air arm remained without food and volatiles. The plastic cups were placed inside the plastic containers with or without a dispenser with mint oil, according to the choice of the predators. The covers of the plastic cups were cut and closed with mesh, which allowed volatiles to enter the cups but avoided the predators to escape. Predators were kept overnight in this way and were tested again for preference for mint oil the next day. These procedures were repeated during three days, hence, each day, one group of predators was split into two further groups, depending on their choice.

5. Data analysis

Each predator was considered one replicate. The number of predators tested per treatment vary depending on the rearing supply. Differences in numbers of predators choosing for mint oil or clean air were tested using log-linear models for contingency tables (Crawley, 2007). To investigate if previous experience affected the subsequent choice, the fractions of predators that chose mint oil odours and clean air from each test were calculated. Fractions were then compared with a binomial test with a probability of 0.5 at a 5% significance level.

RESULTS

Mint oil odours were neither attractive nor repellent for *Ceraeochrysa cubana* (deviance = 1.49, d.f = 1, P = 0.22), *Cycloneda sanguinea* (deviance = 2.79, d.f = 1, P = 0.09) and *Podisus nigrispinus* (deviance = 0.40, d.f = 1, P = 0.53). Sequential choices, with reinforcements after each test, did not changed the response of *C. cubana* (Figure 1), *C. sanguinea* (Figure 2) and *P. nigrispinus* (Figure 3).

The test on the first day showed that 43% of *C. cubana* larvae prefer mint oil (Figure 1, binomial test, P = 0.25). On the second day, half of the *C. cubana* larvae that had previously chosen mint oil volatiles responded to mint oil while the other half choose clean air (binomial test, P = 0.60). A proportion of 0.45 of those that had previously chosen clean air preferred mint (binomial test, P = 0.41). 50% and 71% of the larvae that had two different previous experiences, first mint oil and second clean air or first clean air and second mint oil, respectively; changed their choice for the third time (binomial test, P = 0.66 and 0.23, respectively). On the group that had chosen mint oil twice, 62% of *C. cubana* chose mint oil for the third time (binomial test, P = 0.85). Eightly percent of the predators that preferred clean air on first and second day repeated their choice (binomial test, P = 0.05) (Figure 1).

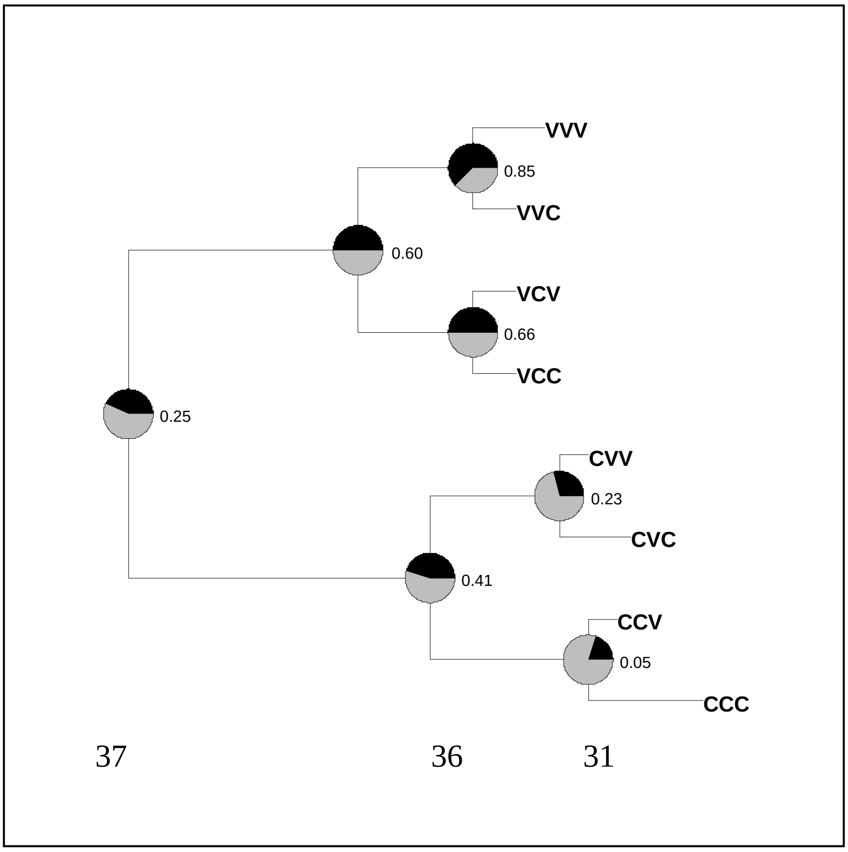

Figure 1 – Choice of *Ceraeochrysa cubana* larvae when offered mint oil odours vs. clean air. Pies represents the result of predators at each test, with “black” slices indicating the fraction of *C. cubana* that chose the mint oil arm and “gray” slices indicating the fraction of those that chose the clean air arm. Letters at the end of each line indicate the choice of the larvae along the days, with “V” indicating mint oil and “C” clean air. Numbers at each node indicate the p value of a binomial test with a probability of 0.5. Numbers at the bottom of the tree indicate the total number of predators tested at the first, second and third day, respectively.

At the first day, 57% of *C. sanguinea* preferred clean air (Figure 2, binomial test, P = 0.33). On the second day, 42% of those that previously responded to clean air changed their response (binomial test, P = 0.39) and 33% of the group that had chosen mint oil volatiles keep their choice (binomial test, P = 0.25). Half of the *C. sanguinea* with previous choices for mint oil and then clean air choose mint oil while the other half choose clean air on the third day (binomial test, P = 0.66). All predators that chose mint oil twice changed their response at the last day (binomial test, P = 0.12). Seventy-one percent of the ladybirds that chose clean air on the first two days repeated their choice for the third time (binomial test, P = 0.23) and 60% of those with a clean air-mint oil previous experience changed their choice again (binomial test, P = 0.50) (Figure 2).

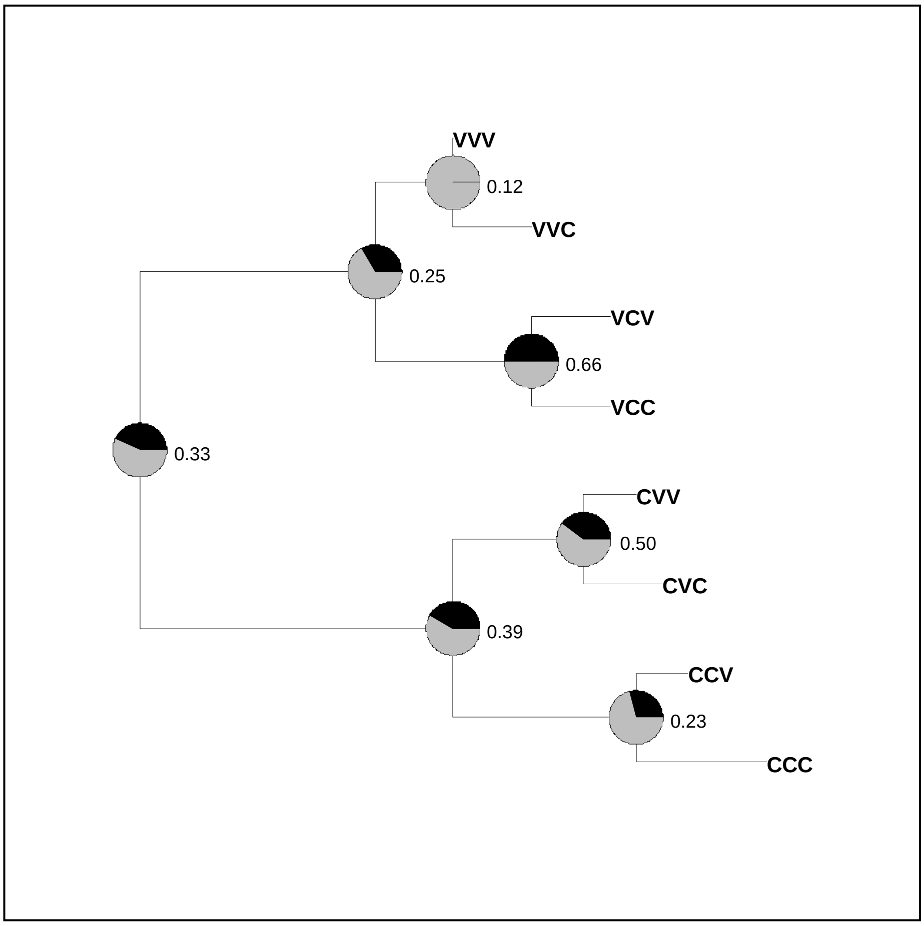

Figure 2 – Choice of *Cycloneda sanguinea* when offered mint oil odours vs. clean air. Pies represents the result of predators at each test, with "black" slices indicating the fraction of *C. cubana* that chose the mint oil arm and "gray" slices indicating the fraction of those that chose the clean air arm. Letters at the end of each line indicate the choice of the larvae along the days, with "V" indicating mint oil and "C" clean air. Numbers at each node indicate the p value of a binomial test with a probability of 0.5, n = 21.

Fourty-three percent of *Podisus nigrispinus* were attract to mint oil volatiles on the first day (Figure 3, binomial test, P = 0.29). Fifty-four percent of the group that had chosen mint oil maintained their choice (binomial test, P = 0.71), while 65% of those that chose clean air previously changed their response (binomial test, P = 0.93). On the third day, a proportion of 0.57 predators that had chosen mint oil twice and 0.33 of those that chose clean air twice maintained their response (binomial test, P = 0.77 and 0.89, respectively). Sixty-seven percent of the stinkbugs that switched from mint oil to clean air changed their choice on the third day (binomial test, P = 0.89). Finally, 55% of those that switched from clean air to mint oil on the first two days also changed their choice on the third day (binomial test, P = 0.50) (Figure 3).

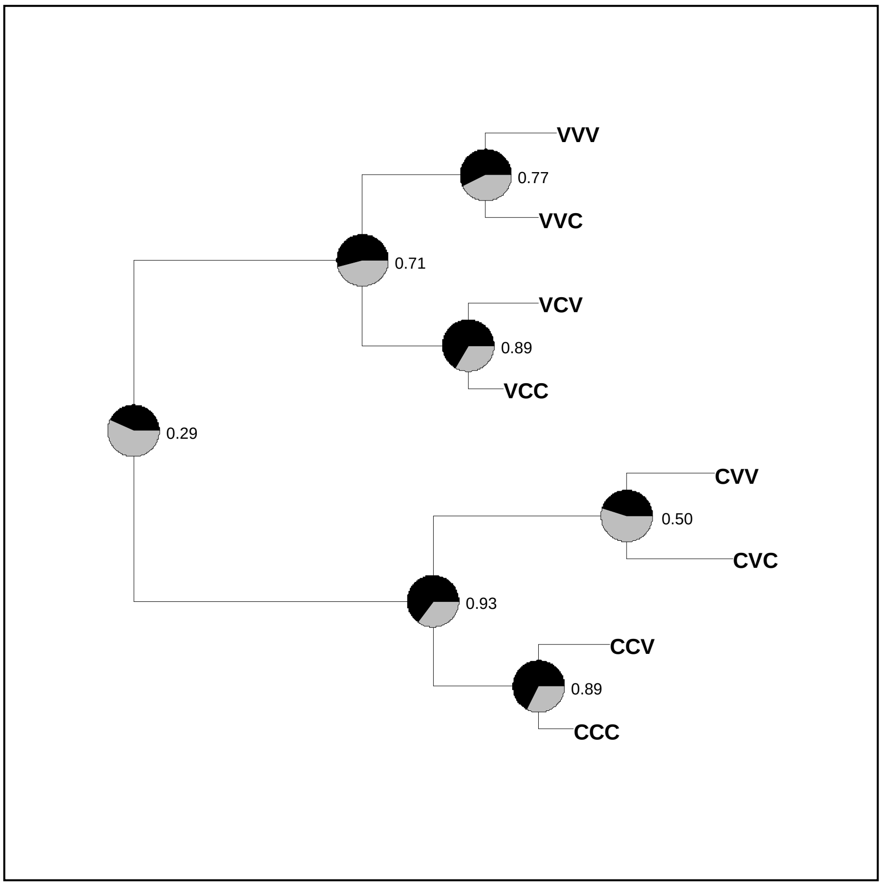

Figure 3 – Choice of *Podisus nigrispinus* when offered mint oil odours vs. clean air. Pies represents the result of predators at each test, with "black" slices indicating the fraction of *C. cubana* that chose the mint oil arm and "gray" slices indicating the fraction of those that chose the clean air arm. Letters at the end of each line indicate the choice of the larvae along the days, with "V" indicating mint oil and "C" clean air. Numbers at each node indicate the p value of a binomial test with a probability of 0.5, n = 30.

DISCUSSION

Cycloneda sanguinea, C. cubana and *P. nigrispinus* showed no modification in their response to mint oil volatiles during three days of sequential experiences with this blend when it was paired with *D. saccharalis* eggs. One could argue that *D. saccharalis* eggs are not a suitable prey and therefore no positive association was formed. However, preliminary tests confirmed that *C. sanguinea*, *P. nigrispinus* and *C. cubana* feed on *D. saccharalis* eggs. Although they are generalist predators, *C. cubana* and *C. sanguinea* feed mainly on aphids (Bueno & Berti Filho, 1991; Reddy, 2002) and *P. nigrispinus* most commonly prey on caterpillars (Medeiros et al., 2003). The defensive behaviour of aphids and caterpillars can interfere with the feeding success of natural enemies (Wyckhuys et al., 2008; Soares et al., 2009). In contrast, eggs are an inert prey and easy to handle. Moreover, while aphids and caterpillars are known to release chemicals that attract predators and parasitoids (Zhu et al., 1999; Rostás & Wölfling, 2009). And although oviposition of herbivores can induce plant to emitted volatiles that attract natural enemies by secreting an elicitor that is active when it contacts injured leaf surface (Meiners & Hilker, 2000). The *D. saccharalis* eggs offered to the three predators were oviposited on paper cheat and, therefore, are believed not to emit volatiles. This is important because volatiles emitted by prey could blend with the mint oil volatiles, which would preclude the association of mint oil blend with food. Therefore, *D. saccharalis* eggs were the most appropriate prey for pairing with mint oil odours.

Diatraea saccharalis eggs are probably an inferior prey for *C. cubana*, *P. nigrispinus* and *C. sanguinea*, which could result in an initial aversion to it. However, the other choice faced by predators was complete absence of food. Therefore, predators would be expected to choose an inferior prey rather than starvation. Patches usually contain prey of different values (Heller, 1980) and if the density of high quality prey decreases, it would be adaptive to accept low-quality prey (Sih & Christensen, 2001). *Ceraeochrysa cubana*, *P. nigrispinus* and *C. sanguinea* may need more time to change their response to an initially not attractive stimulus. These predators can survive several days without feeding. *Podisus nigrispinus* females can survive 15 days without prey by feeding on eucalyptus leaves (Holtz et al., 2009) and when plant material is also unavailable they survive for approximately 10 days (Molina-Rugama et al., 1998). Predators may maintain their preference for high-quality prey during the first period of starvation and only start foraging for inferior prey after a long period without feeding. Therefore, a longer period of starvation would perhaps be needed to change the response of the three predators to an inferior prey, as well as to learn the association of this prey with a volatile stimulus. The length of my experiments were consistent with Neuser et al. (2005), which showed that *Drosophila* larvae increase their response to amyl acetate associated with fructose on the third day of trials and no increment in learning was demonstrated with a larger number of training trials. However, larvae of the herbivore *Spodoptera littoralis* needed six sessions of hexanol paired with fructose to positive respond to this volatile (Salloum et al., 2011).

Foragers are expected to slowly or quickly change their behaviour according to the speed at which their environment changes. Different speeds of behavioural changes do not necessarily reflect differences in learning capacity of species (Drukker et al., 2000b). My results suggest that none of these predators are able to learn the association of mint oil volatiles and *D. saccharalis* eggs within three days of sequential experience. However, more extensive experiments are needed to conclude if *P. nigrispinus*, *C. sanguinea* and *C. cubana* are capable of associative learning.

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MAIN CONCLUSION

Larvae of *C. cubana* have functional receptors which can perceive volatiles. *Ceraeochrysa cubana* larvae are repelled by mint oil volatiles but continuous training resulted in no attraction or repellence of *C. cubana* larvae which could be due to habituation to mint oil odours. *Ceraeochrysa cubana* larvae are not able to learn the association of mint oil volatiles and *D. saccharalis* eggs with two or four previous experiences, with daily paired or continuously paired stimuli. Therefore, further investigations are needed to explore how volatiles can affect the behaviour of chrysopid larvae.

Podisus nigrispinus, *C. sanguinea* and larvae of *C. cubana* are not able to learn the association of mint oil volatiles and *D. saccharalis* eggs within three days of sequential training. More extensive experiments are needed to conclude if *P. nigrispinus*, *C. sanguinea* and *C. cubana* are capable of associative learning.