ANA MARIA GUIMARÃES BERNARDO

ALTERNATIVE FOOD AND LEARNING AS A PROMISING STRATEGY FOR BIOLOGICAL CONTROL

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: 20 de julho de 2015.

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“Cada sonho que você deixa para trás, é um pedaço
do seu futuro que deixa de existir”

Steve Jobs

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BIOGRAFIA

Ana Maria Guimarães Bernardo, filha de Soeli Aparecida Guimarães Bernardo e Miguel Soares Bernardo, nasceu em Viçosa, Minas Gerais em 12 de fevereiro de 1990. Em março de 2008 ingresso no curso de Engenharia Agronômica na Universidade Federal de Viçosa (UFV).

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Em agosto de 2013 iniciou o curso de Mestrado em Entomologia pela Universidade Federal de Viçosa, sob orientação do Prof. Angelo Pallini e co-orientação da Prof.ª Madelaine Venzon e o Pesquisador Arne Janssen. Em julho de 2015 submeteu-se à defesa da dissertação.

SUMÁRIO

RESUMO

BERNARDO, Ana Maria Guimarães, M.Sc., Universidade Federal de Viçosa, julho de 2015. **Alimento alternativo e aprendizagem como uma estratégia promissora para o controle biológico.** Orientador: Angelo Pallini Filho. Coorientadores: Arnoldus Rudolf Maria Janssen e Madeleine Venzon.

Várias espécies de predadores onívoros são utilizadas no controle biológico. Orius são importantes inimigos naturais onívoros de pragas que afetam hortaliças e plantas ornamentais. A densidade destes predadores em campo pode aumentar com o fornecimento de alimentos alternativos, especialmente quando a presa é escassa. Além disso, é sugerido que predadores podem aprender associar fonte de odores com a presença de alimento, reduzindo o tempo de procura dos inimigos naturais e, consequentemente o número de pragas nas culturas. Com isso, o nosso objetivo foi encontrar alimentos alternativos de baixo custo e estudar a capacidade de aprendizagem do predador Orius insidiosus. Foi, portanto, avaliado a performance do predador O. insidiosus em quatro alimentos alternativos: pólen de Ricinus sp., pólen de abelha, ácaros detritívoros Tyrophagus putrescentiae e ovos de Anagasta kuehniella. Além disso, utilizou-se salicilato de metila sintético (MeSa) e óleo de menta como fontes de odores para estudar a capacidade de aprendizagem do predador. O predador apresentou uma melhor performance quando alimentava-se de A. kuehniella ou T. putrescentiae. Quando o predador associou alimento com a fonte de odor eles não mostraram preferência para óleo de menta, mas mostraram preferência para MeSa. No entanto, quando o odor era associado com ausência de alimento os predadores foram repelidos pelo óleo de menta e não houve atração ou repelência para MeSa. Estes resultados indicam que alimentos alternativos e de baixo custo (T. putrescentiae) podem ser utilizados com o objetivo de sustentar a população do predador em criações massal e, possivelmente, em culturas. Adicionalmente, nossos resultados mostram que O. insidiosus é capaz de aprender, mas essa capacidade de aprendizagem dos predadores varia com o composto volátil testado.

ABSTRACT

BERNARDO, Ana Maria Guimarães, M.Sc., Universidade Federal de Viçosa, July, 2015. **Alternative food and learning as a promising strategy for biological control**. Adviser: Angelo Pallini Filho. Co-advisers: Arnoldus Rudolf Maria Janssen and Madelaine Venzon.

Several species of omnivorous predators are used in the biological control. Omnivorous predatory bugs are important natural enemies of pest that affect crops in greenhouse. It has been suggested that the use of alternative foods promote the establishment of predators on crops. Moreover, some research propose that predators can learn to associate chemical compounds with presence of food, so reducing the time of searching of natural enemies and number of pest on crops. Our aim was to find cheaper alternative foods and investigate learning ability of the predator Orius insidiosus. We evaluated the performance of O. insidiosus on four alternative foods: Ricinus sp. pollen, bee pollen, the acarid prey Tyrophagus putrescentiae and eggs of Anagasta kuehniella. Furthermore, we used synthetic methyl salicylate (MeSa) and mint oil as odour sources to study the learning ability of this predator. The predatory bugs demonstrated a better performance when fed A. kuehniella or T. putrescentiae. Our results showed that when the predator had to associate odour sources with presence of food, they showed no preference to mint oil, but showed a preference to MeSa. In addition, when the predator had to associate odour sources without food, they showed a preference to mint oil, but did not show a preference to MeSa. These results reveal that alternative and cheaper foods (e.g. T. putrescentiae) can be used with aim to sustain the predator population on rearings and possibly in crops. In addition, our results showed that O. insidiosus is able to learn, but this learning ability of predators varies with volatile compounds.

GENERAL INTRODUCTION

It is known that omnivorous arthropods can utilize a variety of food sources, such as pollen, nectar and prey (Coll 1998; van Rijn and Tanigoshi 1999a; van Rijn and Tanigoshi 1999b; Nomikou et al. 2003). In addition, compared to specialist, omnivorous predators can persist longer in the field, which can be advantageous for biological control (Symondson et al. 2002). There are important omnivores predators for biological control, such as predatory mites (van Rijn and Tanigoshi 1999a; Bolckmans et al. 2005; Goleva and Zebitz 2013), lacewings (Albuquerque et al. 2001; Venzon et al. 2006; Morgado et al. 2014) and predatory bugs (van den Meiracker 1994; Bonte and De Clercq 2011; Pumariño et al. 2012; A.M. Calixto et al. 2013). Recently, the biological control of pest has resulted in an excessive introduction of natural enemies in agroecosystems. In some cases, there is no efficiency in the pest control due to the poor establishment of natural enemies (Messelink et al. 2014). The use of alternative food and volatile compounds has been suggested to better establishment of natural enemies on the crops (Drukker et al. 2000; van Rijn et al. 2002; Nomikou et al. 2010).

Plants have several ways to attract and arrest natural enemies in order to increase predation of herbivores, for example, by providing refuges (Walter and O’Dowd 1992; Walter 1996; Matos et al. 2006; Ferreira et al. 2008; Matos et al. 2011), alternative food such as nectar and pollen (Nomikou et al. 2002; van Rijn et al. 2002; Nomikou et al. 2010; Rezende et al. 2014) and by producing attractive volatile compounds (Sabelis and van de Baan 1983; Dicke and Sabelis 1988; Turlings et al. 1990; Drukker et al. 1995; Sabelis et al. 1999). Provision of alternative food and production of volatiles may result in an increase in the number of natural enemies on the plant, and consequently in a decrease of herbivores numbers (Drukker et al. 1995; Moraes and Pare 1998; Janssen 1999).

The production of volatile compounds by plants to attract predatory arthropods may increase predation on prey and reduce the damage caused by these herbivores (Heil 2008;

Dicke 2009). These volatile compounds are produced by damaged plants and may be attractive to natural enemies, such as parasitoids and predators of mites and insects (Dicke and Sabelis 1988; Turlings et al. 1990; Drukker et al. 1995; Sabelis et al. 1999). Predators can also learn to associate these volatile compounds with the presence of food (Vet and Dicke 1992; De Boer and Dicke 2006; Dukas 2008). Recently, some studies suggested that natural enemies could be able to learn associate different odour with the availability and unavailability of food in order to improve biocontrol, because this association may facilitate the encounter prey-predator (Drukker et al. 2000; Janssen et al. 2014; Giunti et al. 2015). Moreover, the natural enemies in foraging may use another type of chemical cues to locate their hosts or prey. They are attracted to the prey or host by presence of chemical signal, or "semiochemicals" called kairomones emitted by victims. That also is crucial to the success of foraging of the natural enemies (Vet and Dicke 1992; Stowe et al. 1995).

Predatory bugs are important natural enemies of many pests (van den Meiracker 1994; Bonte and De Clercq 2011; Van Lenteren 2012; Pumariño et al. 2012; Calixto et al. 2013; Wong and Frank 2013). The omnivore predatory bug Orius insidiosus (Say) (Hemiptera: Anthocoridae) is a generalist predator of small insects, such as thrips, scales, aphids, caterpillars, and eggs of various insects and other arthropods (e.g. mites) (Van Lenteren et al. 1997; Lattin 1999). Members of this genus are important natural enemies of western flower thrips, Frankliniella occidentalis (Pergande) (Thysanoptera: Thripidae) (Van den Meiracker and Ramakers 1991; Chambers et al. 1993; van den Meiracker 1994). It is known that eggs of the flour moths Anagasta kuehniella (Zeller) (Lepidoptera: Pyralidae) and Ephestia kuehniella (Zeller) (Lepidoptera: Pyralidae) have been extensively used for the mass production of Orius species because this diet has resulted in excellent rearing results for predators (Bueno et al. 2006; Bonte and De Clercq 2011). In addition, it has been suggested that these eggs could be added to crops for the improvement of biological control of pests by these predators (Cocuzza et al. 1997; Tommasini et al. 2004; Bonte 2008; Calixto et al. 2013). Nevertheless, these eggs are very expensive, precluding their commercial use. Thus, finding an alternative cheaper food could be a promising tool to biological control.

So, this research aimed to find a cheaper alternative food to the predatory bug O. insidiosus, which possibly could be used as food for commercial mass rearing and to establishment of predator population in the absence of the pest. Additionally, we aimed to know whether the predatory bugs could use chemical signal present in A. kueniella eggs to find this type of food. Furthermore, we investigated whether the predator is able to learn associating different odour sources with the availability of food. We expected that this association could reduce the time of natural enemies searching for food and reducing the number of herbivores on crops.

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CHAPTER 1

Performance of the predatory bug Orius insidiosus (Say) (Hemiptera: Anthocoridae) on alternative foods

Abstract

The anthocorid bug Orius insidiosus is a generalist predator of small insects and like other members of this genus it is economically important for biological control of pests that affect vegetable and ornamental crops. In order to improve pest control with this predator, its densities in the crops can be enhanced by the provision of alternative foods, especially when prey are scarce. Eggs of the flour moths have been extensively used as alternative food and have been suggested to improve the biological control of pests by Orius sp. However, these eggs are expensive, precluding their commercial use. Our aim was to find cheaper alternative foods and we therefore measured juvenile development and oviposition rate of O. insidiosus on four alternative foods: Ricinus sp. pollen, bee pollen, the acarid prey T. putrescentiae and eggs of Anagasta kuehniella. Juvenile development was significantly shorter on diets consisting of A. kuehniella, T. putrescentiae and Ricinus sp. pollen than on bee pollen and on the control (without food). Furthermore, female bugs had a higher oviposition rate when fed either A. kuehniella or T. putrescentiae, but produced fewer eggs when feeding on bee pollen and Ricinus sp. pollen. These results demonstrate that alternative and cheaper foods (e.g. T. putrescentiae) possibly can be used to sustain the predator population at periods of low prey densities in crops.

Keywords: Biocontrol, generalist predator, pollen, acarid, flour moth eggs

Introduction

Biological pest control in greenhouse crops is usually based on periodical releases of mass-produced natural enemies. However, in some cases there are shortcomings in pest control efficacy, which can often be attributed to poor establishment of natural enemies (Messelink et al. 2014). Compared to specialist predators, omnivorous natural enemies can persist longer in the field, which can be advantageous for biological control (Symondson et al. 2002). In some cases, predatory bugs can develop to the next instar or even complete development on plant-based diets (Naranjo and Gibson 1996; Lundgren et al. 2008). Besides plant-based diets, the addition of prey as alternative food to predators often benefits juvenile development time and survival, adult longevity and fecundity (Cocuzza et al. 1997). Several researchers have shown that the addition of alternative foods for predators can improve control of pests, resulting in lower pest densities (Nomikou et al. 2002; van Rijn et al. 2002; Nomikou et al. 2010; Rezende et al. 2014).

There are different methods to supply alternative food in crops. One way is the use of “banker plants”, which is based on the introduction of a non-crop plant that provide resources, such as food, prey or shelter to natural enemies (Avery et al. 2014). Another way is to supply pollen that can be used as food source for predators (van Rijn et al. 2002; Duso et al. 2004; González-Fernández et al. 2009). Plants may also provide extrafloral nectar, which can also enhance biological control by natural enemies (Wackers 2005; Rezende et al. 2014). Plants offering non-prey resources to natural enemies may be visited more often by natural enemies than plants that do not supply such resources, and they may thus gain protection from herbivores. Recent research has demonstrated another method for providing alternative prey species on mulch layers, which support the establishment of astigmatic mites and increase densities of soil predatory mites (Grosman et al. 2011).

Members of the genus Orius (Hemiptera: Anthocoridae) are omnivorous predators of small insects (e.g. thrips, scales, aphids, caterpillars), and eggs of various insects and other arthropods such as mites (Van Lenteren et al. 1997; Lattin 1999), but also they feed on plant materials (Salas-Aguilar and Ehler 1977; Coll 1998). Various species of Orius are economically important for biological control of pests that affect vegetable crops and ornamental plants in several countries in Europe, Asia, North and Latin America (van den Meiracker 1994; Van Lenteren et al. 1997; Van Lenteren 2012). Several species have received considerable attention as biological control agents, including Orius insidiosus (Say) that is recognized as a predator of many economically important pests in various cropping system (Reid 1991; Baez et al. 2004). There is much interest for Orius sp. as biocontrol agents, especially since it was observed that these bugs are capable of controlling the western flower thrips Frankliniella occidentalis (Pergande) (Thysanoptera: Thripidae) (Van den Meiracker and Ramakers 1991; Chambers et al. 1993). Silveira et al. (2004), Bueno and Poletti (2009) also showed that O. insidiosus could efficiently control thrips in greenhouse.

The study of diets for mass rearing and to establishment of O. insidiosus in crops is an important issue, because we have very limited information about the effects of different diets on development and oviposition of this predator bug (Calixto et al. 2013). Eggs of the flour moths Anagasta kuehniella (Zeller) and Ephestia kuehniella (Zeller) (Lepidoptera: Pyralidae) have been extensively used for the mass production of Orius species and it has been suggested that they could be added to crops to improve biological control of pests by these predators (Cocuzza et al. 1997; Tommasini et al. 2004; Bonte 2008; Calixto et al. 2013). Indeed, biological control companies sell moth eggs for this purpose. However, these eggs are relatively expensive. Husseini et al. (1993) showed the successful mass rearing of Anthocoridae on acarid mould mite, Tyrophagus putrescentiae Schr. This prey can easily be mass-produced throughout the year and its production needs a very simple and inexpensive technique.

Therefore, this study aimed to find a cheaper alternative food that can be used for commercial mass rearing and for establishment of the predator population in the absence of prey to boost biocontrol by O. insidiosus. We assessed juvenile development of O. insidiosus when fed eggs of the moth A. kuehniella, bee pollen, Ricinus sp. pollen and T. putrescentiae. Moreover, we determined the oviposition rate on the diets mentioned above.

Materials and methods

Rearing methods

Chrysanthemum cuttings were planted in pots (3 L / 21 cm diameter) filled with a commercial soil substrate (PlantMax, Eucatex Agro). Four cuttings were planted per pot and the apical bud was cut off using shears to encourage vegetative growth. The pots were placed in a cage covered with fine gauze in a greenhouse compartment (mean temperature: 25 ° C, r. h.: 70 % ± 10%, 11L:13D). The plants were fertilized weekly with NPK (5 - 15 - 15) and were watered twice a day in summer and once a day in winter. Plants with 8-10 completely developed leaves were used for experiments.

Orius insidiosus were collected from tomato plants at the campus of the Federal University of Viçosa, Minas Gerais, Brazil with the following geographic coordinates: 20 °46’ 9”S, 42°52’18” W. The specie was sent to Dr Luís Cláudio Paterno Silveira, Department of Entomology, Federal University of Lavras, Minas Gerais, for identification. They were reared in a room at 25 °C; 70% r.h. and 12L:12D inside glass jars (1L) with a hole in the lid, covered with nylon mesh for ventilation. Eggs of the flour moth, A. kuehniella, were provided as food three times per week. Wet cotton wool was used to supply water and inflorescences of Bidens pilosa L. (Asteraceae Compositae) were supplied as oviposition substrate. Bidens pilosa is an important host plant for Orius sp. in the field (Silveira et al. 2003) and they have often been used as oviposition substrate in rearings (Bueno et al. 2007). Predator nymphs were collected from this rearing unit and were placed in Petri dishes (9 cm diameter x 2 cm height) containing pieces of paper towel to reduce cannibalism of juveniles by adults and were also supplied with eggs of A. kuehniella as food, and wet cotton wool as water supply. The Petri dishes were sealed with plastic film with small holes on its surface for ventilation.

Alternative foods

Tyrophagus putrescentiae (Schrank) (Astigmata: Acaridae) was obtained from a rearing at the Agriculture and Livestock Research Enterprise of Minas Gerais (EPAMIG-Viçosa, MG) and were maintained on arenas consisting of PVC sheets (15x10 cm) on a block of polyethylene foam (4 cm high) inside a water-filled plastic tray (29 x 15 x 4 cm), surrounded by moistened cotton wool, which served as a source of water and as barrier to prevent escape of the mites. Cream crackers cookies were provided as food two times per week (Rodriguez Cruz, 2014). Eggs of A. kuehniella were provided by Bio-Insecta Agentes de Controle Biológicos (Lavras, Brazil). Bee pollen (Santa Bárbara® dehydrated pollen) is a commercial product consisting of a mix of various species of pollen, collected by honeybees from several flowering plants, such as Eucalyptus sp., B. pilosa L., Citrus sp., Angycus sp., and many other plants. Castor bean plant pollen, Ricinus sp. was collected at a farm in Viçosa-MG from flowers by shaking them over a Petri dish.

Juvenile development and survival

The experiments were carried out in an climatized room (25 ± 2ºC; 70 ± 10% R.H. and a photoperiod of 12L:12D). The juvenile development and survival of O. insidiosus was studied in small Petri dishes (Ø= 5.5 cm, h= 1.3 cm), which had one ventilation hole covered with a thin mesh. A chrysanthemum leaf disc (Ø= 2.5 cm) was placed inside each Petri dish containing a layer of 1.0 % agar, which serves to supply moisture to the predators. A control treatment consisted of chrysanthemum leaves without food. Newly hatched nymphs were removed from the stock culture and placed one on each Petri dish. Ample amounts of one of the alternative foods were supplied every two days. The juvenile development and survival were monitored every two days with a stereoscopic microscope. Twenty replicates were done for each diet. To analyse the effect of diets on developmental time, we used a time-to-event analysis (Cox proportional hazards regression model) using the function "coxph" of the library "survival" (R Development Core Team 2014; Therneau 2014). The contrasts among mean developmental times were assessed through model simplification (Crawley 2007). The analysis was performed with the statistical software R 2.15.1 (R-Development-Core-Team 2014).

Oviposition

To evaluate the effects of diet on fecundity, a pair of O. insidiosus adults, aged 24 hours, was placed in small Petri dishes (Ø= 5.5 cm, h= 1.3 cm) with one ventilation hole covered with a thin screen in a climate room as above. Each Petri dish contained a piece of moist cotton wool as a water supply, one of the diets (Ricinus sp. pollen, bee pollen, eggs of A. kuehniella or T. putrescentiae) and stems of B. pilosa plants, which were used as oviposition substrate. A control treatment consisted of small Petri dishes without food, but with moist cotton wool and stems of B. pilosa. Ample amounts of the diets were supplied every two days. The stems were replaced every two days and predator eggs were counted under a stereomicroscope. Oviposition was quantified for eight days. The effects of diet on the oviposition rates were assessed with Generalized Linear Models (GLM) with Poisson error distribution (Crawley 2007). The analysis was performed with the statistical software R 2.15.1 (R-Development-Core-Team 2014).

Results

Juvenile development and survival

The juvenile development rate was significantly affected by the predators' diet (Fig 1, Cox Proportional Hazards: Likelihood = 35.08, d.f.= 3, P< 0.0001). It was significantly shorter on diets consisting of A. kuehniella, T. putrescentiae and Ricinus sp. pollen than on bee pollen and in the control (without food). However, there was no significant effect of predators' diet on juvenile survival (Fig1, Proportional Hazards: Likelihood = 1.57, d.f.= 3, P= 0.6655). Predators in the control (without food) did not reach adulthood.

Oviposition

There was a significant effect of diet on oviposition rates of O. insidiosus (Fig. 2, GLM: Deviance = 962.2, df= 4, P<0.0001). The highest oviposition rate was found on a diet of eggs of A. kuehniella, the lowest on bee pollen and in the control (without food) (Fig. 2).

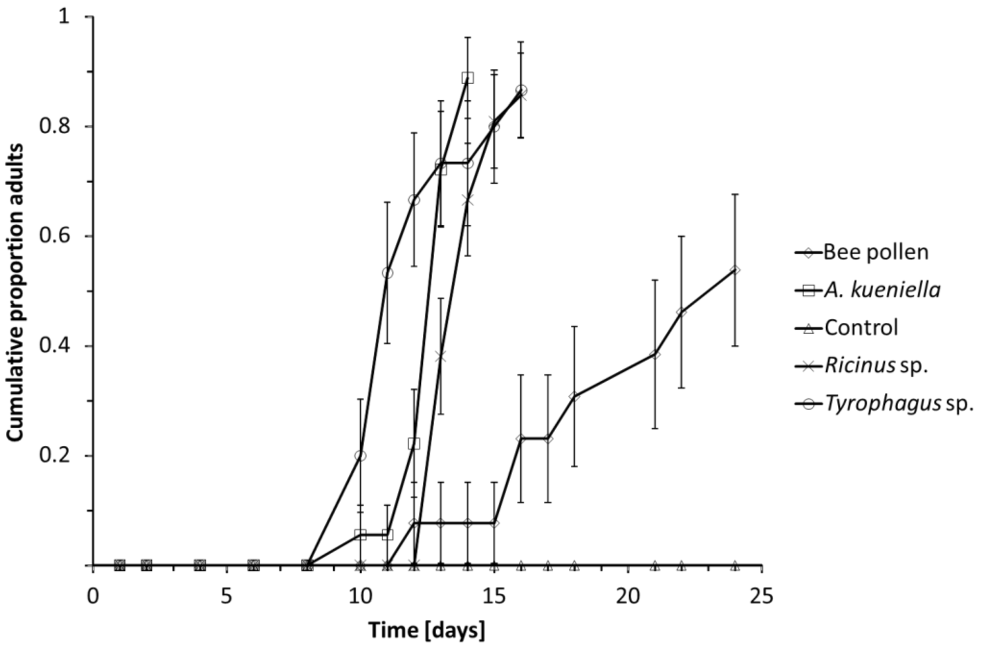

Figure 1. Development and survival of *O. insidiosus* fed on *A. kuehniella*, *T. putrescentiae, Ricinus* sp. pollen, bee pollen and in the control (without food). The horizontal axis represents time in days; the vertical axis shows the cumulative proportion of adults. The results are expressed as mean proportions of alive individuals that reached adulthood. Treatments not sharing the same letter were significantly different (P < 0.05).

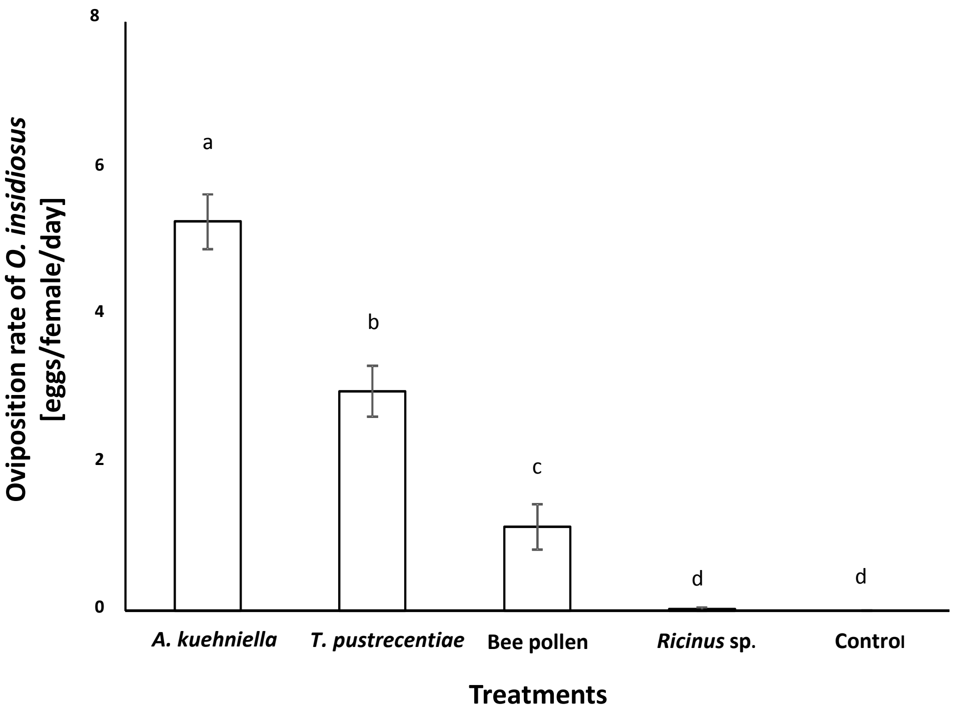

Figure 2. Average oviposition rate of female bugs on diets consisting of *A. kuehniella*, *T. putrescentiae*, bee pollen, *Ricinus* sp. pollen, and in the control (without food). Letters above the columns indicate significant differences among treatments (contrasts through model simplification after GLM, $P < 0.05$).

Discussion

Many studies show that addition of A. kuehniella and E. kuehniella eggs has resulted in excellent mass rearing performance of Orius spp.. Our results suggest that the mite T. putrescentiae and Ricinus sp. pollen are comparably high quality foods for juvenile development of O. insidiosus as are eggs of A. kuehniella (Fig. 1). In addition, our results also show that reproduction of the predator was high with T. putrescentiae; it was the second best diet after eggs of A. kuehniella (Fig. 2). Moreover, female bugs had a lower oviposition rate when fed diet of pollen, suggesting that pollen provides poor quality food compared with a diet of eggs of A. kuehniella (Fig. 2). Cocuzza et al. (1997) and Calixto et al. (2013) also found that the reproduction of Orius bugs was high on diets with E. kuehniella or A. kuehniella eggs and lower on a diet of pollen. Thus, the mite T. putrescentiae can be used as alternative food, because the predator showed a short juvenile development and high oviposition rate on this diet.

Plants that offer non-prey resources to natural enemies, such as pollen and nectar, can increase longevity, fecundity, dispersal capacity and host-finding efficacy of natural enemies compared with plants that not supply such resources (Jamont et al. 2013; Rezende et al. 2014). Pollen has been indicated as good alternative food for several Orius bugs, especially when prey is scarce (Coll 1998). Hence, the study of pollen as food sources is very important, because pollen from different plants species have distinct chemical-physical composition (Thompson 1999). However, our study shows that pollen provides limited nutrition value for the reproduction of O. insidiosus (figure 2). Kiman and Yeargan (1985) and Calixto et al. (2013) also found that pollen does not cause any increase in the oviposition of O. insidiosus. Therefore, the ability of these Orius bugs to utilize pollen as food allows their subsistence in the absence of prey, but according to our results, providing only pollen cannot be a good strategy to establish the population of O. insidious in mass rearing.

Some researchers have demonstrated that the addition of astigmatic mites as alternative food for predators can result in improved control of pests, resulting in lower pest densities. Grosman et al. (2011) demonstrated that astigmatic mite populations can be established in mulch layers and these populations can serve as alternative food for predators, resulting increase densities of predators. Messelink et al. (2009) also showed that application of yeast and sugar maintains populations of astigmatic mites, which are a good alternative food for establishment of predators. In addition, this alternative food can also supplement diets of predator in order to increase reproduction. Our results demonstrate that T. putrescentiae can be a good alternative food to improve establishment of the predators O. insidious. Therefore, this type of alternative food for supporting predators, especially when prey are scarce, can be a promising method that may increase the effectiveness of biological control and reduce the cost of predatory introductions.

Thus, our data support that T. putrescentiae could be used as alternative food. Moreover, it is necessary more research showing that the addition of this alternative food investigated here may reduce pest densities and promote better biological control. Furthermore, the effects of alternative food investigated here under laboratory conditions need to be tested on larger-scale experiments where they may differ. It is therefore necessary performing long-term experiments under field and greenhouse conditions. Adding T. putrescentiae is possibly a promising strategy for pest management and for mass rearing of predatory bugs.

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CHAPTER 2

Associative learning of the predatory bug Orius insidiosus (Say) (Hemiptera, Anthocoridae) varies with volatile compound

Abstract

Predatory arthropods use chemical information when searching for prey, particularly in the form of volatile cues released by plants in response to the feeding of their herbivores. It is known that these volatile cues may vary considerably with herbivore species and with the plant species. Predators have to deal with these variations by learning the association between the availability of food and volatile cues. Associative learning has been demonstrated in several groups of arthropods but there are few examples of learning in predators. We aimed to study the ability of learning of the predatory bug Orius insidiosus. We used synthetic methyl salicylate (MeSa) and commercially available mint oil as volatile sources. A Y-tube olfactometer was used to test preference or aversion to odours compared to clean air. After having been exposed to methyl salicylate together with food, female bug were attracted to this compound, but they were no repelled by this volatile when it had been associated with the absence of food (hunger). In contrast, after exposure to mint oil associated with food, female bugs were not attracted to this volatile, but were repelled by it when they had experienced it in association with hunger. Female bugs without previous experience with the two odours did not show attraction or repellence towards methyl salicylate or mint oil. Our results show that the ability of associative learning of predators is affected by the identity of the volatiles.

Keywords: Orius insidiosus, methyl salicylate, mint oil

Introduction

Many insect predators inhabiting plants are polyphagous (Sabelis 1992). They feed on prey that are patchily distributed on host plants and often persist for a short period. Therefore, predators need to switch to new prey, new host plants or even to a new habitat. It is well known that predators can use cues emitted by host plants and prey (alone or in association) to locate prey (Vet and Dicke 1992; De Moraes et al. 1998; van den Boom et al. 2004). Obviously, there is selection on herbivores to not produce cues that attract predators. Hence, to find prey in their natural habitat, predators use cues that are associated with the presence of prey (Heil 2008; Dicke 2009). Such associated cues may be odours produced by plants under herbivore attack. These herbivore-induced plant odours are known to attract the natural enemies of the herbivores, but it is also known that these odours vary considerably with herbivore and with plant species (Dicke et al. 1998; Moraes et al. 1998). Predators are thought to deal with this variation in cues by learning the association between the availability of food and their associated cues, which can be volatiles, but may also be visual cues (Lewis and Tumlinson 1988; Drukker et al. 2000; Hajek 2004).

Learning can be defined as any relatively permanent change in behaviour that occurs as a result of experience (Kimble 1961). Hence, the predator’s innate response to an odour can change as a result of the individual's experience (Drukker et al. 2000). Learning occurs in three essentially different ways: imprinting, sensitization and associative learning. Imprinting is defined as learning that occurs rapidly during an early period in life, with or without reinforcement (Gould 1993; Hall and Halliday 1998). Sensitization is a gradual increase in response to a stimulus along with repeated exposure to that stimulus, independent of this stimulus being paired with another stimulus (Papaj and Prokopy 1989; Hall and Halliday 1998). Associative learning occurs when two stimuli are paired, the conditioned (e.g. odour) and the unconditioned (e.g. rewarding or penalizing) stimulus, and results in preference in case of pairing with a rewarding stimulus or aversion, with a penalizing stimulus (Thorpe 1956; Lewis and Tumlinson 1988; Hall and Halliday 1998).

One of the first reports on learning shows that it occurs in man and other animals (Darwin, 1872). Later, Tinbergen (1932) demonstrated visual learning by a wasp. Additionally, Thorpe (1956) showed associative learning in different groups of animals. Learning now has been demonstrated in several arthropods, including social insects (Giurfa and Lehrer 2001; Farina et al. 2005; Amdam et al. 2010), herbivores (Egas and Sabelis 2001), parasitoids (Lewis and Takasu 1990; Wackers and Lewis 1994; Giunti et al. 2015) and predators (Drukker et al. 2000; Rahmani et al. 2009; Janssen et al. 2014). Nevertheless, there are only few examples of learning in predators.

Predatory arthropods use chemical information in prey searching, particularly in the form of volatile cues released by plants in response to feeding by herbivorous prey (Sabelis et al. 1999; Dicke and Sabelis 1988). However, these odours can vary, and natural enemies have to deal with this variation (Lewis and Tumlinson 1988; Turlings et al. 1993; Hilker and McNeil 2008). One solution is to learn to associate odours with the occurrence of prey (Drukker et al. 2000; De Boer et al. 2005; De Boer and Dicke 2006; Dukas 2008; Janssen et al. 2014; Giunti et al. 2015).

The omnivorous predator Orius insidiosus (Say) (Hemiptera: Anthocoridae) is a generalist predator of small insects (e.g. thrips, scales, aphids, caterpillars) and eggs of various insects and mites. Several species have received considerable attention as biological control agents, including O. insidiosus, which is recognized as a predator of many important pests in various cropping systems (Reid 1991; Baez et al. 2004). This predator is economically important for biological control of pests that affect vegetable crops and ornamental plants in several countries in Europe, Asia, North and Latin America (van den Meiracker 1994; Van

Lenteren et al. 1997; Van Lenteren 2012). Some research proposed that the addition of plant volatiles could improve the efficiency searching of natural enemies, reducing the time of search for prey. This fact allows the predators learn to associate plant volatiles with the presence of prey (Sabelis et al. 1999; Drukker et al. 2000). Therefore, the study of learning ability of predators is very important because it can be a promising tool to biological control. So, this research aimed to investigate whether the predatory bug O. insidiosus is able to learn and associate different odours with the availability of food in order to use this ability to improve its searching for prey and be more effective on biocontrol programs.

Materials and Methods

Predator rearing

Orius insidiosus were collected from plants at the campus of the Federal University of Viçosa. The specie was sent to Dr Luís Cláudio Paterno Silveira, Department of Entomology, Federal University of Lavras, Minas Gerais, for identification. The rearing of O. insidiosus was kept in a room at 25 °C; 70% r.h. and 12L:12D. Eggs of the flour moth, Anagasta kuehniella (Zeller) (Lepidoptera: Pyralidae), were provided as food three times a week. Wet cotton wool was used to supply water and inflorescences of Bidens pilosa L. (Asteraceae Compositae) were supplied as oviposition substrate. Predator nymphs from inflorescences were placed in Petri dishes (9 cm diameter x 2 cm height) containing pieces of towel paper to reduce cannibalism and they were supplied with eggs of A. kuehniella as food and wet cotton wool as water source. The Petri dishes were sealed with plastic film with small holes on its surface for ventilation. Adult predators were kept in glass jars (1L) with one hole in the lid, covered with nylon gauze for ventilation.

Volatile dispensers

We used synthetic methyl salicylate (MeSa) (Sigma-Aldrich, China), which has been identified as important component of herbivore-induced volatile blends (van Wijk et al. 2011; Dicke et al. 1990; Drukker et al. 2000; De Boer et al. 2004; De Boer and Dicke 2004a; De Boer and Dicke 2004b). We also used commercially available mint oil (Mentha piperita, World`s Natural Fragrances®, São Paulo, Brazil) as a volatile source. The latter is a blend of volatile compounds, predominantly menthol, menthone, (+)-menthyl acetate, menthofuran and isomenthone (Yang et al. 2010), limonene and 1, 8-cineole (Rohloff 1999). This compound can be toxic and repellent to arthropods (Hori 1999; Choi et al. 2004; Odeyemi et al. 2008), but Janssen et al. (2014) showed that predators may learn to associate it with the presence of food in the field. Volatile dispensers were made from Parafilm®, which was cut into strips of 5.2 cm wide, rolled up and flattened until it consisted of 10 tightly rolled layers. The rolls were cut into pieces of 7 mm long (Janssen et al. 2014). The dispensers were incubated for 12 hours in the liquid MeSa or mint oil. The next day, they were taken from the liquid and gently dried on the outside. The dispensers were used for training and for olfactometer tests.

Innate preference

Three groups of adult female bugs were collected from the rearing units to test their innate response to the volatiles. The adult females were starved individually for 24 h in plastic tubes (6.0 x 3.0 cm) with a hole in the lid covered with nylon gauze for ventilation, with wet cotton wool as a source of water. The female bugs were tested at an age of 1-3 weeks. The innate preference of these groups to synthetic methyl salicylate or mint oil compared to air was assessed in the Y-tube olfactometer. Three replicates were done per treatment.

Associating food with a volatile - training

Female bugs were introduced individually in plastic tubes (6.0 x 3.0 cm) with a hole in the lid covered with nylon gauze for ventilation, having wet cotton wool as a water source and eggs of A. kuehniella as food. Thirty plastic tubes were put inside a box with two openings. One of these openings was connected to a pump that produced an air flow inside the box. During the first day, three volatile (MeSa or mint oil) dispersers were placed in Petri dishes in the box. After this experience with food and the volatile, the female bugs were introduced in a new tube without food but with moist cotton wool. These tubes with female bugs were introduced in another, similar box without volatile dispersers, and kept there for another day. Subsequently, the bugs were put in new tubes with food and water and exposed to the volatile as on the first day. Finally, they were incubated another day without food and the volatile, as on the 2^{nd} day. Another group received the opposite experience, i.e. food without volatile and no food with a volatile, also during 4 days, alternating between food without odours and odours without food. A control group received the same treatment, receiving food on the 1st and 3rd day and no food on the 2nd and 4th day, but never with volatiles (female bugs without previous experience with volatile). After this training, the response of female bugs to the volatiles was tested in an olfactometer.

Olfactometer tests

A Y-tube olfactometer (Sabelis and van de Baan 1983; Janssen et al. 1997) was used to test preference or aversion to odours compared to air or to other odours. The olfactometer consisted of a glass tube (27 cm long, diameter 3.5 cm) in the form of a Y with the 3 arms of equal length, with a Y- shaped metal wire in the middle to guide the female bugs. The base of the tube was connected to an air suction pump that produced an air flow from the arms of the tube to the base (Janssen et al. 1999). Two anemometers were used to measure the wind speed in each arm, which was calibrated at 0.35 m/s. Each arm was connected to a glass container (50 x 36 x 43 cm) that had an odour source (a volatile dispenser) or clean air (a dispenser without volatiles).

The predatory bugs were starved for 24 h before the test. Each predator was tested individually in the olfactometer by disconnecting the pump and putting the predator on the metal wire at the base of the Y-tube. Subsequently, the pump was connected, forming an air flow from the containers through the olfactometer. The predator usually started walking upwind to the junction of the wire, where it had to choose for one of the two arms. The predator was observed for 5 minutes or until it reached the end of one of the arms of the olfactometer and was then removed. The female bugs that did not make a choice within 5 minutes were excluded from further analysis. After testing five female bugs that reached the end of one of the arms (hence, not counting the female bugs that did not make a choice), the metal wire in the middle of the tube was cleaned with a piece of cotton wool soaked in alcohol (70%) to remove any possible cues left behind by the predators. Subsequently, the wire was left to dry, the odour sources were connected to the opposite arm of the olfactometer to correct for eventual asymmetries in the set-up, and the wind speed was calibrated before the next group of female bugs was tested. Three or four replicates were done per treatment and each replicate consisted of 20 female bugs that had made a choice within 5 minutes. The treatments constituted of female bugs directly from rearing (innate preference), female bugs that had experienced the association of volatiles with food and female bugs that had experience with the association of volatiles and the absence of food.

The preference of the female bugs for volatiles or clean air was analyzed with a log-linear model for contingency tables with Generalized Linear Models (GLM) using a Binomial error distribution (Crawley 2007). It was used the statistical software R 2.15.1 (R-Development-Core-Team 2014) with experience of the predator bug and odour source as a factor. 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 2014; Therneau 2014). Subsequently, post hoc tests (Tukey's Honestly Significant Difference) were used to identify differences between treatments using the package “multcomp” of the library.

Results

Innate preference

Female bugs directly from the rearing unit did not show significant attraction or repellence to MeSa (60% chose MeSa) (GLM: Deviance = 2.41, df= 1, P=0.12) or mint oil (40% chose mint oil) (GLM, d.f. = 1, deviance = 10.35, P = 0.06).

Learning the association of food or absence of food with a volatile

The experience of the predator affected the choice in the group with MeSa (GLM: df= 4, Deviance = 12.94, P=0.01) and mint oil (GLM: Deviance = 22.73, df= 4, P<0.0001). After having been exposed to methyl salicylate together with food, female bug were attracted to this compound (80 % chose MeSa)(Figure 1), but they were no repelled by this volatile when it had been associated with the absence of food (63.3 % chose MeSa)(Figure 2). In contrast, after exposure to mint oil associated with food, female bugs were not attracted to this volatile (60 % chose MeSa)(Figure 3), but were repelled by it when they had experienced it in association with hunger (21.6 % chose MeSa)(Figure 4).

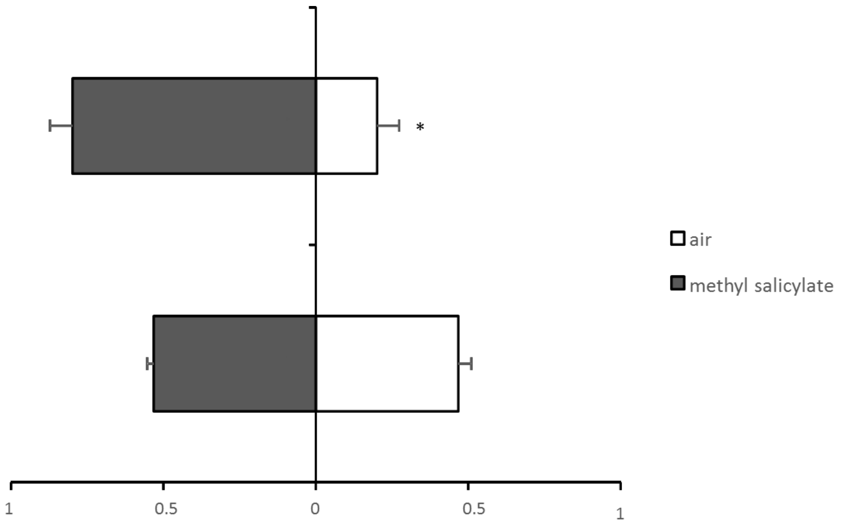

Fig. 1 Proportion of choice of female bugs with previous experience with methyl salicylate associate with food (bar above) or without previous experience with methyl salicylate (bar below). Shown are the fractions of female bugs that chose for odours of methyl salicylate (left) in a Y-tube olfactometer.

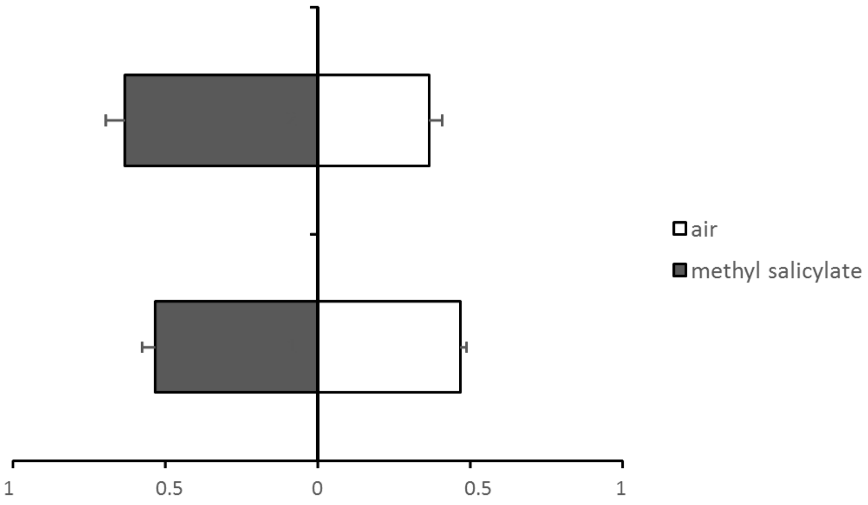

Fig. 2 Proportion of choice of female bugs with previous experience with methyl salicylate associate without food (bar above) or without previous experience with methyl salicylate (bar below).

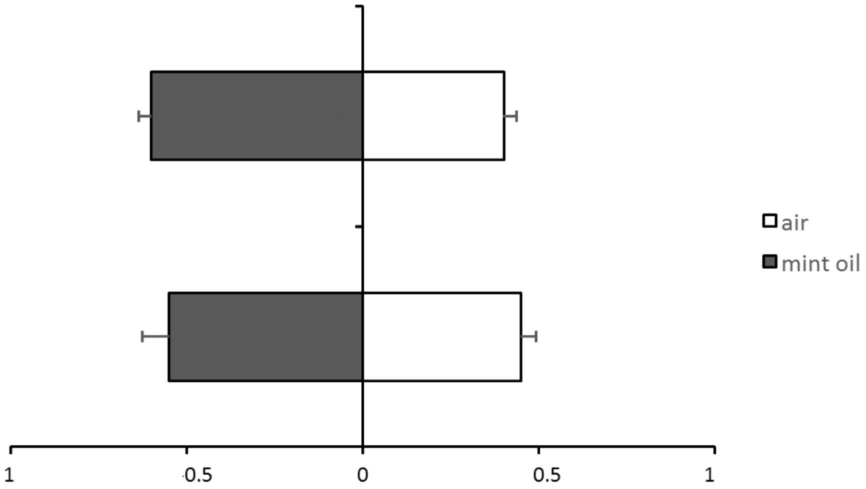

Fig. 3 Proportion of choice of female bugs with previous experience with mint oil associate with food (bar above) or without previous experience with mint oil (bar below).

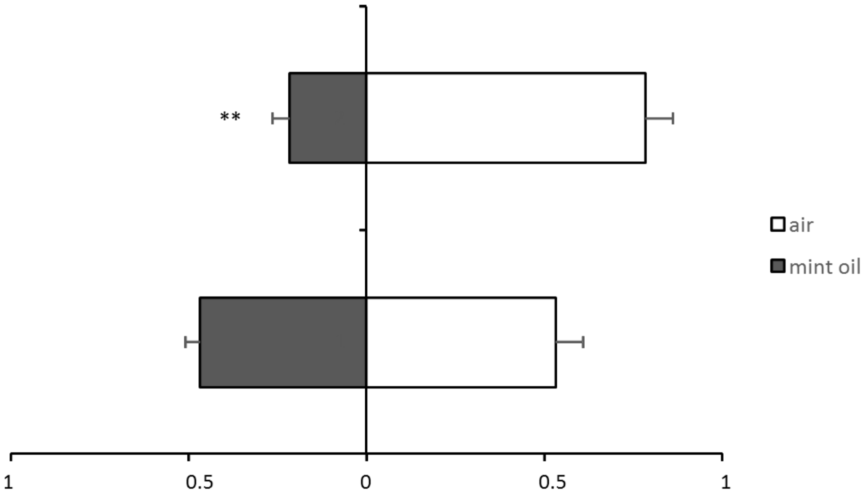

Fig. 4 Proportion of choice of female bugs with previous experience with mint oil associate without food (bar above) or without previous experience with mint oil (bar below) .

Discussion

After having been exposed to MeSa together with food, female bugs were attracted to this compound, but this no repelled them when it had been associated with the absence of food (Figure 1 e 2). MeSa has been identified in herbivore-induced volatile blends and this chemical compound is known to be attractive to naïve predators (van Wijk et al. 2011; Dicke et al. 1990; Drukker et al. 2000; De Boer et al. 2004; De Boer and Dicke 2004a; De Boer and Dicke 2004b). Possibly because of this, female bugs may not associate odour from MeSa with food absence. Moreover, the preference of female bugs to MeSa could be attributed to the presence of this compound in inflorescence of B. pilosa, which was supplied as substrate for predatory oviposition in the rearing.

However, after having been exposed to mint oil associated with food, the female bugs were not attracted to this volatile, but were repelled by it when they had experienced it in association with the absence of food (hunger) (Figure 2 e 3). Some researchers showed that mint oils can be toxic and repellent to arthropods (Hori 1999; Choi et al. 2004; Odeyemi et al. 2008). Possibly, female bugs may not be able to associate odours from mint oil with the presence of food. So, experiments are necessary to study the performance of these predatory bugs when fed A. kuehniella eggs with mint oil in order to verify whether this odour have some negatively effect in female bugs.

Our results show that experience with odour sources resulted in a change in the behavior of the predator bug in response of the conditioned (odour of MeSa or mint oil) and the unconditioned stimulus (availability or unavailability of food) (Thorpe 1956; Lewis and Tumlinson 1988; Hall and Halliday 1998). Drukker et al. (2000) also showed that associative learning occurs in predatory bugs, but here we demonstrated that this associative learning ability may vary with the volatile compound used.

In their natural habitat, natural enemies are exposed to a variety of odours. To find their prey, predators use volatiles cues that are associated with the presence of food, but it is known that these cues may vary with herbivore and plant species (Dicke et al. 1998; Moraes et al. 1998). Hence, predators have to deal with this variation by learning the association of these volatile cues with the presence or absence of food (Lewis and Tumlinson 1988; Drukker et al. 2000; Hajek 2004). Drukker et al. (2000) demonstrated that anthocorid predators learned to associate plant volatiles with the presence or absence of prey. De Boer and Dicke 2004 showed that when predatory mites were exposed to MeSa, they were attracted to this compound. Janssen et al. (2014) demonstrated that natural communities can learn to associate volatiles with the presence of food under natural conditions. In addition, it is suggested that the use of this learning ability can enhance the efficacy of biological control of pests by natural enemies (Drukker et al. 2000; Giunti et al. 2015). Because the retention and acquisition of information in memory may take minutes to months it is important to study how long the effects of learning persist in natural enemies (Neuser et al. 2005; Blackiston et al. 2008; Tapia et al. 2015). In order to know how much time the experience can contribute to the predatory find the prey, resulting in lower pest densities on crops. With this knowledge, we could estimate how often is necessary to release “experienced” natural enemies, for example, in greenhouse.

There are many studies on the learning ability of arthropod predators and parasitoids, but the majority was done under laboratory conditions. It is known that predators from a rearing may respond differently from predators that were taken directly from the field (Drukker et al. 2000). Additionally, De Boer and Dicke (2006) suggested that the response of predators to odours may depend on the rearing history; predators that were reared on cucumber plants with prey were attracted to the volatiles of infested cucumber plants, but were not attracted to infested lima bean plants. This shows the importance of more experiments under natural conditions, because the response of natural enemies may also vary with experimental conditions.

Here, we demonstrated that O. insidiosus have the ability to learn the association of volatiles with the presence or absence of food. We also showed that this learning ability may vary with the identity of the volatile compound. These findings indicate the importance of studying volatile compound in associative learning. Further studies are needed to investigate how this leaning ability can contribute to the foraging of predatory arthropods and how it can be used to improve biological control.

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GENERAL CONCLUSIONS

- Tyrophagus putrescentiae may be used as alternative food for commercial mass rearing and for establishment of Orius insidiosus population on rearings and possibly in crops.
- Orius insidiosus have the ability to learn the association of volatiles with availability of food.
- The learning ability may vary with the identity of the volatile compound.