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Semiochemical exploitation by two egg parasitoids, *Trissolcus basalis* and *Trissolcus brochymenae* (Hymenoptera: Scelionidae)

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Dedication

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To my amazing **husband Mouadh** whose sacrificial care made it possible for me to complete this thesis. Thanks for supporting and encouraging me to believe in myself.

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and the happiness of my life.

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With all my best wishes

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Chapter 1

Chapter 1

Chemo-orientation in insect egg parasitoids mediated by semiochemicals

Abstract

Chemo-orientation in egg parasitoids, a self-controlled movement in time and space, is led by volatile or substrate-borne chemicals distribution in the environment for purposes of locating host, food, mates and other resources. The perception of these chemical stimuli can be either from a distance by exploiting long-range infochemicals in the case of volatile chemo-reception and/or upon contact with the cues when wasps are relatively close to the host in the case of contact chemo-reception by perceiving short-range infochemicals. In the present thesis, two different examples of chemo-orientation were treated. The first is an olfactory chemo-reception concerning *Trissolcus basalis* (Wollaston) and its perception of buckwheat plant volatiles whereas the second is a contact chemo-reception in *Trissolcus brochymenae* (Ashmead), which exploits its host footprints upon contact with the substrate.

Key words: Volatile cues, substrate-borne cues, contact, olfaction, sensilla, *Trissolcus basalis*, *Trissolcus brochymenae*.

1. Introduction

Insect parasitoids leave in a complex environment where they need to identify and locate food, mate, suitable hosts and ovipositional sites (Städler, 1984; Giunti *et al.,* 2015). Therefore in order to optimize their searching process, parasitoids adopt different searching modalities, random or systematic, in response to different needs (Colazza *et al.,* 2014). Actually, parasitoids' orientation is guided mainly by chemical and physical cues that can be integrated at the same time (Jander, 1963; Godfray, 1994). Although physical cues such as visual, acoustic, mechanical signals play an important role in mediating parasitoid orientation, semiochemicals appear to play the major role in long-range and short-range orientation (Vinson, 1976; Vet and Dicke, 1992; Meyhöfer *et al.,* 1994; Battaglia e*t al.*, 2000; Steidle and Van Loon, 2003; Kroder *et al.,* 2007).

Semiochemicals are defined as chemical signals used to mediate interaction between two organisms (Nordlund and Lewis, 1976). The importance of these cues in mate finding and parasitoids foraging behaviour has been well documented (Lewis and Martin, 1990; Hou and Yan, 1997). Semiochemicals are divided into two main categories, pheromones and allelochemicals, based on their effect (Vet and Dicke, 1992). Pheromones are chemical signals used by parasitic wasps for intra-specific communication (Godfray, 1994). Most of these chemical signals are used for finding and recognizing a mate (sexual pheromones) but some other groups of compounds are also playing an important role like alarm, aggregation and marking pheromones (reviewed by Ruther 2013). However, parasitoids may use pheromones of their hosts as kairomones in their host location process. Hence, the term used depends on whether the considered interaction is analysed at the intra- or inter-specific level (Dicke and Sabelis, 1988). Regarding allelochemicals, they are semiochemicals that mediate interaction between individuals belonging to different species, inter-specific interaction. Depending on whether the benefit is for the emitter or receiver, these chemicals are classified into different groups: allomones, kairomones and synomones. Allomones are a class of components that benefit the emitter but not the receiver. For instance, when disrupted, individuals of stink bugs (Heteroptera: Pentatomidae) discharge a large quantity of defensive secretions, strong-smelling and irritating chemicals acting as defensive tools, which are produced in dorsal abdominal and metathoracic glands respectively for immature instars and adults (Aldrich, 1998). Kairomones, on the other hand, are advantageous compounds just for the receiver (Nordlund and Lewis, 1976).

For instance, host derived allelochemicals such as feces, saliva, egg glue, silk from the pupal cocoon, or, as previously reported, pheromones serve as kairomones for parasitoid' host location. For example, females of the endophagous parasitoid *Aphidius ervi* (Haliday) (Hymenoptera: Braconidae) respond positively to a secretion from the cornicle of the pea aphid *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae) (Battaglia *et al.*, 1993; Quicke, 1997), while sex pheromones of host adults evoke attraction in many species of parasitic Hymenoptera, including Aphelinidae, Encyrtidae, Eulophidae, Pteromalidae, Scelionidae, Trichogrammatidae and Braconidae (Benelli *et al.*, 2014). Regarding synomones, they are beneficial cues for both the receiver and the emitter, such as the chemical cues emitted from the plants in response to herbivory, called herbivore induced plant volatiles (HIPVs). In tritrophic interactions, these plant volatiles are known to attract natural enemies (De Moraes *et al.,* 1998). For instance, when the elm leaf beetle *Xanthogaleruca luteola* (Muller) (Coleoptera: Chrysomelidae) feeds upon elm leaves, it scratches them by its mouth then glues an egg in the scratch using an oviduct secretion. Applying this secretion to an artificial scratch induces the secretion of synomones by the elm plant, which attract and guide the egg parasitoid *Oomyzus gallerucae* (Fonscolombe) (Hymenoptera: Eulophidae) to its specific host (Meiners and Hilker, 2000; Meiners *et al.*, 2000).

In a complex chemical environment, female parasitoids need to correctly identify reliable and detectable cues that lead them to their hosts, explained as the "reliability-detectability" problem by Vet and Dicke (1992). Actually, to face this difficult situation, females may resort to cues belonging to different categories. First, insect parasitoids may rely on detectable and reliable cues coming from the habitat, the host microhabitat or the food plant. Specifically, HIPVs are known as detectable and reliable cues and they are exploited by many parasitoids (De Moraes *et al.,* 1998). In addition, parasitoids may exploit detectable cues coming from non-target host stages called indirect host-related cues. These cues are indirectly associated with the host presence and they guide the parasitoid to get into the proximity of the attacked stage (Meiners and Peri, 2013). For example, egg parasitoids have developed their ability to eavesdrop in adult host pheromones as kairomones to overcome the low host eggs detectability. In fact, they associate these pheromones with microhabitats where eggs are likely to be present (Huigens and Fatouros, 2013). Moreover, egg parasitoids may rely on more reliable cues coming from the host growth stage that is directly attacked called direct host-related cues, such as host egg masses volatiles and contact kairomones. However, the detection of such cues might be hard except in the very close nearness of the host.

During the process of host and food location, if the insect orientation is directed by chemical cues it is called chemo-orientation which was defined by Bell and Tobin (1982) as a “self - controlled movement of an organism in time and space guided by spatial and temporal distribution of chemicals in the environment”. Chemo-orientation can also be defined as antennal searching because the parasitoids antennae are mainly used to explore these chemicals (Ruther *et al.,* 2002). Chemo-orientation is adopted by the insects when they reach an active chemical space, where the volume of stimulus is strong enough to activate a searching pattern (Bell et *al.,* 1995).

According to the nature of that stimulus leading the orientation, chemo-orientation can be classified into volatile chemo-orientation if the cues are volatile stimuli or contact chemo-orientation in the case of substrate-borne chemicals (Vinson, 1984). In the beginning of the host location process, parasitoids exploit mainly airborne volatile cues since they can be perceived at a relatively long distance while substrate-borne cues become progressively successful when parasitoids are relatively near to the host (Vinson, 1998; Vinson, 2010). These chemical cues can be acquired through two different modalities, which are temporal comparison and instantaneous comparison and the insect parasitoid can switch between them in acquiring information (Bell and Tobin, 1982). In temporal comparison, the information is compared in two different temporal moments because it arrives from only a sensor or from two/more sensors too close each other for detecting a difference in stimulus gradient. In instantaneous comparison, also known as spatial comparison, chemicals are perceived at the same time in two different spatial points by two or more organ receptors spaced at sufficient distance far from each other.

Chemo-orientation in insect parasitoids follows the general mechanisms of chemical orientation in insects. Two main orientation mechanisms increasing the insects’ probability to explore and find resources have been identified when chemicals are elaborated, taxis and kinesis (Bernays, 1993).

Taxis are orientation mechanisms triggered by stimuli providing directional information (Kennedy, 1977; Dusenbery, 1992; Wyatt, 2003). The distinction between tropotaxis (bilateral simultaneous comparison), telotaxis (unilateral spatial resolution) or klinotaxis (successive comparison) is based on the means by which the directionality of a stimulus is determined by a given sensory system (Klowden, 2007).

Kinesis are orientation mechanisms based on the modulation of the activity by the intensity of the stimulus regardless of its directionality (Fraenkel and Gunn, 1961). They may involve modulation of the speed (orthokinesis) or the turning rate (klinokinesis) of an individual in response to the intensity changes of a stimulus (Dethier *et al.*, 1960; Crespo *et al.*, 2011).

Responses to chemicals cues leading the chemo-orientation are expected to be under a genetic control and they are expected to be fixed, nevertheless learning behavior in insects, defined as the relatively long-term and reversible behavioral changes due to the influence of experience, can modify parasitoids behaviors, particularly their responses to stimuli that elicit weak innate response (Vet and Dicke, 1992; Vet *et al.,* 1995). Learning behaviour is of great significance in biocontrol and it has received increasing attention since experienced parasitic wasps are more sensitive to specific chemical signals and more accurate in searching the target host (Liu *et al.,* 2003). Learning in host foraging behavior has been demonstrated in many species of Hymenopteran parasitoids. For example, experienced females of *Trichogramma brassicae* (Bezdenko) (Hymenoptera: Trichogrammatidae) on tomato plants, search longer than naive females (Bjorksten and Hoffmann, 1998). Investigations on the association between the egg parasitoid *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae) and its host *Nezara viridula* L. (Heteroptera: Pentatomidae) revealed the ability of female wasps to change their foraging strategy in accordance with past experience with the host kairomones (Peri *et al.*, 2006; Dauphin *et al.,* 2009).

1.1. Morphological approach

Different sensorial modalities can be adopted by insects to reduce their distance from stimuli and maximize their distance from a stress source (Jander, 1975). Host location and selection process is mediated by integration, within the insect central nervous system (CNS), of numerous sensory inputs including olfactory and gustatory semiochemical cues. During a given searching behavior and depending on the host habitat that the insect parasitoid exploits, chemical stimuli can be perceived from a distance using olfactory sensilla (volatile chemo-reception) and/or upon contact with the cues using gustatory sensilla (contact chemo-reception) (Visser, 1988; Vet and Dicke, 1992; Turlings *et al.,* 1995; Isidoro *et al.,* 1996; Roux *et al.*, 2005; Rocha *et al.*, 2007; Dweck, 2009; Das *et al.*, 2011). Studies about insect parasitoids chemical ecology addressed mostly more attention to volatiles perception (Kaissling, 1971; Guillot and Vinson, 1972; Kaissling *et al.*, 1987; Höller *et al.*, 1993; Gullan and Cranston, 1994; Quicke, 1997).

On the contrary, few studies practically evaluated the role of contact chemo-reception in host location and/or recognition in various parasitoid species (Vinson, 1991; Bénédet *et al.,* 1999; Bénédet *et al.,* 2002; Van Lenteren *et al.*, 2007; Colazza *et al.,* 2014).

1.1.1. Olfactory chemo-reception

The sensitivity of insects' olfactory systems is remarkable. It allows to organisms the detection and the distinction between thousands of odors, which are low molecular masses. Olfaction is a critical sense to execute crucial biological behaviors for insect parasitoids such as habitat searching, host location and recognition, mating and oviposition. Antennae are the primary olfactory organs in insects. They play a major role in odors perception and host location for parasitic wasps (Hays and Vinson, 1971; Weselow, 1972).

The typical insect antenna is divided into three main parts: a scape, a pedicel and flagellum. The flagellum is often divided into several parts called flagellomeres (Schneider, 1964; Chapman, 1998; Groba *et al.*, 2014). To recognize chemical cues in such an olfactory landscape of diverse semiochemicals, insect possess a highly sophisticated olfactory system equipped with many olfactory receptor neurons (ORNs) housed in arrays of innervated sensory hair structures called sensilla (Clyne *et al.*, 1999; Gao and Chess, 1999; Keil, 1999). These ORNs are bipolar. They extend their branched projections called dendrites into the sensillar lumen and send their axons into the olfactory lobe, which is considered as the first olfactory information processing center in the insect brain (Leal, 2013; Nakamoto, 2016). Olfactory sensilla are mainly located on the insect antennae and the flagellum bears most of them (McAlpine, 1981; Chapman, 1998; Hu *et al.*, 2010; Hansson and Stensmyr, 2011). However, few olfactory sensilla are located on other organs such as mouthparts or external genitalia (McIver, 1971; Crespo *et al.*, 2011; Ali *et al.,* 2015).

Odorants enter through pores located on the sensilla surface then they get in an aqueous lymph, where they are transported by odorant binding proteins (OBPs) to reach a spectrum of molecular receptors present on the dendrites of ORNs (Steinbrecht, 1996). These OBPs are secreted by support cells surrounding the ORNS.

They bind odorant messages to the odours receptors creating ORNs depolarisation. Thus, a neuronal signal is decoded by the insect brain which is giving information about behavioural response decisions (Pelosi, 1998; Swarup *et al.*, 2011; Leal, 2013; Carraher *et al.*, 2015).

Insect sensilla have been classified in aporous, uniporous and multiporous, depending on the number of the pores reached by dendrites (Fig. 1). Several studies showed that olfaction is associated with multiporous plate sensilla, which is the more general term adopted for many different structures and it is sometimes misused or ambiguous because of the diverse outer features of these sensilla (Zacharuk, 1980; Chapman, 1982; Steinbrecht, 1996; Basibuyuk and Quicke, 1999).

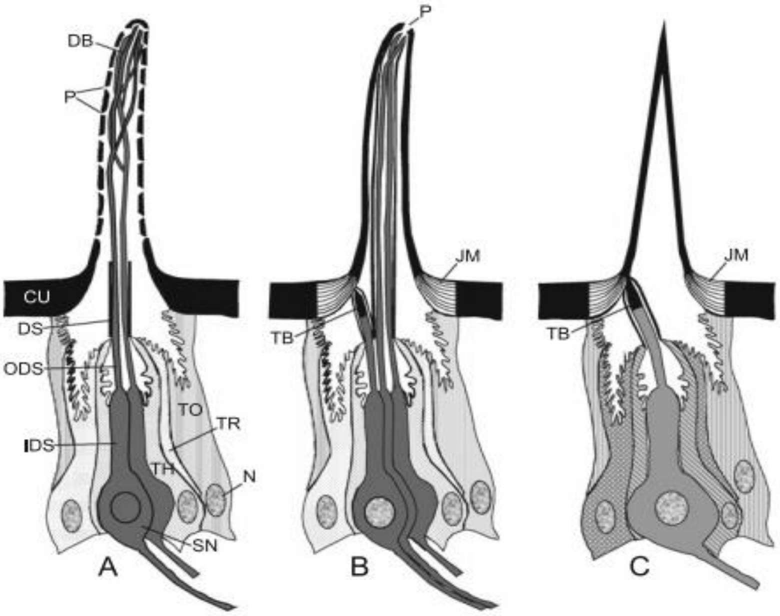

Figure 1. Schematic drawing of different types of sensilla. (a) Multiporous sensillum; (b) uniporous sensillum; (c) aporous sensillum. CU- cuticule, DB-dendritic branches, DS-dendrite sheath, IDS-inner dendritic segment, JM-joint membrane, N-nucleus, ODS- outer dendritic segment, P- pores, SN- sensory neurons, TB tubular body, TH-thecogencell, TO-tormogen cell, TR- trichogen cell (source: Romani *et al.,* 2010).

Pores can be covered by special structures such as movable lobes and spherical projections (Isidoro *et al.,* 2001). According to pores structures two kinds of olfactory sensilla with highly different wall pores are distinguished; single-walled sensilla, if the wall pore is pore-tubule type, and double-walled sensilla, if the wall is spoke-channel type (Steinbrecht, 1996; Chapman, 1998). There are usually several types of olfactory sensilla on the antenna of a given species (Steinbrecht, 1969; Altner and Prillinger, 1980; Steinbrecht, 1996).

In Hymenoptera parasitoids, antennal sensilla seem to be the most involved structure in detecting chemical stimuli (Vinson, 1984). For a more advantageous understanding of their chemical communication, the antennal sensilla ultrastructure has been largely investigated in various parasitic Hymenoptera species using electron microscopy techniques (Norton and Vinson, 1974; Dweck, 2009; Onagbola *et al.*, 2009; Wang *et al.*, 2010; Li *et al.*, 2011). In egg parasitoids, for example, scanning electron microscopy (SEM) studies of antennal sensilla were carried out for *Anaphes listronoti* and *Anaphes victus* (Hymenoptera: Mymaridae), two egg parasitoids of the carrot weevil *Listronotus oregonensis* (LeConte) (Coleoptera: Curculionidae). The female antennal sensilla of both parasitoid species were compared on the basis of SEM investigations. Seven different types of sensilla were present on female antennae with a morphological similarity in the two species: sensilla chaetica types 1, 2, 3 and 4, trichoid sensilla, basiconic sensilla and placoid sensilla. Among these, only sensilla chaetica type 2, basiconic and placoid sensilla were presumed to be associated with olfaction (Van Baaren *et al.,* 1999).

The external morphology of female antennal sensilla of *Trichogramma nubilale* (Hymenoptera: Trichogrammatidae), a parasitoid of the European corn borer *Ostrinia nubilalis* (Htibner) (Lepidoptera: Pyralidae) showed six sensilla differing in the morphology and the structure plus one seta and one campaniform-like structure. According to sensilla positions and pore numbers, three sensilla with an olfactory function were distinguished: multiporous pitted trichodea type A, multiporous grooved basiconica type C and multiporous pitted placodea type A. The latter two were suggested to be respectively receptors of airborne chemicals cues and receptors of long-range chemical cues emanating from the host or host habitat (Olson and Andow, 1993).

For the egg parasitic wasp of butterflies and moths *Trichogramma evanescens* (Westwood) (Hymenoptera: Trichogrammatidae), antennal sensilla description was carried out based on the number and location of pores on the sensilla surface. Several types of sensilla were present from clava to radicle such as aporous trichoid sensilla, uniporous trichoid sensilla, multiporous gustatory sensilla. More interest and a deeper description were assigned to olfactory sensilla which were multiporous sensilla trichoid type A, multiporous placoid sensilla and bulb-shaped basiconic sensilla (Van Der Woude and Smid 2015).

In the egg parasitoid *Trissolcus basalis* antennal chemosensilla have been mapped and described. The Sickle shaped sensilla (SK) are the sole olfactory organs in either sex in host finding process according to their structure with numerous thin wall pores and numerous neurons (Fig. 2). Antennae of *T. basalis* female present about 36 SK altogether on the dorsal side of the flagellomeres (from the 3rd to 11th antennomers) while in male antennae these sensilla are about 170 with an irregular distribution on the dorsal side of the flagellomeres, from the 3rd to the 12th antennomers. Their cuticular component consists of an outstanding, rather rigid hair-shaft about 15-17µm, notably expanded at the base to closely fit the round and rigid socket, sharply bent anteriorly just above the base and then running, slightly diverging from antennal surface to an acutely pointed tip. There are no pore tubules but some electrodense material connecting the pore outlets to the dendritic branches (Bin *et al.,* 1989).

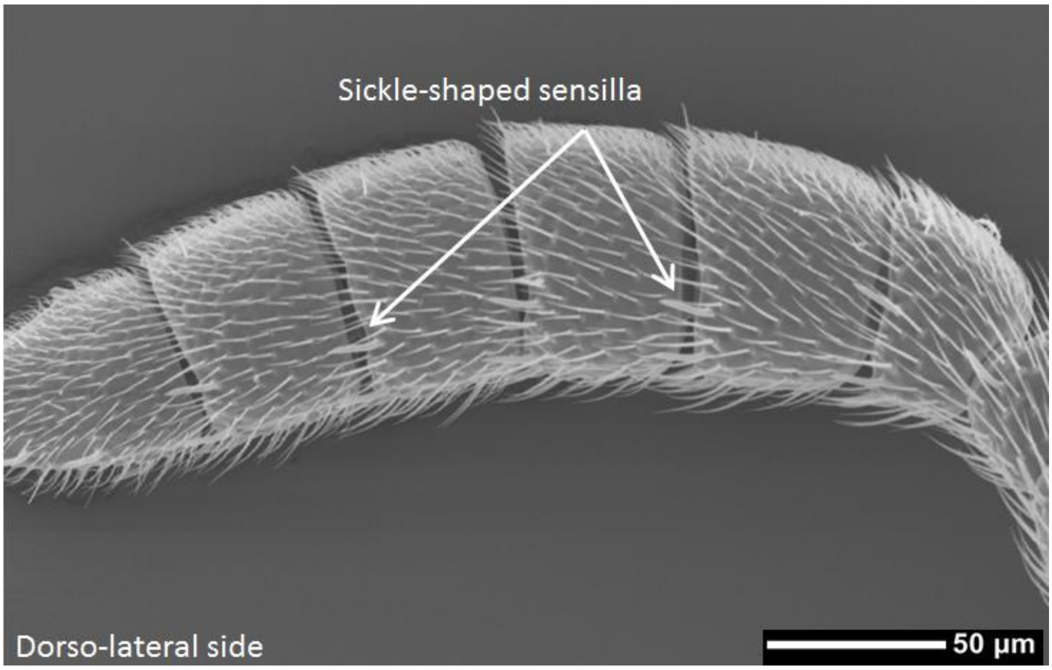

Figure 2. Antennal sickle shaped sensilla (SK) of *Trissolcus basalis*.

1.1.2. Contact chemo-reception

For contact chemo-reception, cues are percept by specific gustatory receptors, which are located in several parts of the insect body. These receptors are mostly abundant on insect mouthparts, but may also be found on their antennae, tarsi, and genitalia (Isidoro *et al.,* 1996; Ali *et al.,* 2015). Taste sensilla were examined in various insect species. For Hymenoptera parasitoids, antennal receptors seem to have the main role also in detecting contact chemical cues (Vinson, 1984; Chapman, 2003).

Taste is usually attributed to uniporous sensilla and the perception of chemical cues is through the contact with the substrate (Romani *et al.,* 2010). However, in females of the same species, "multiporous sensilla" were described as gustatory because they bear numerous pores on the truncate flattened tip and they are generally located in a restricted ventrally or/and apically area of wasps' antennae where they can easily touch the potential host (Bin *et al.,* 1989; Isidoro *et al.*, 1996; Isidoro *et al.,* 2001). In order taste the contact chemicals, this area of the antennae has to be maintained in touch with the surface since the insect parasitoid perceives substrate-born cues thought antennal drumming or stroking (Lewis and Jones, 1971; Norton and Vinson, 1974; Lokey and Ashley, 1984; Romani *et al.,* 2002). For Scelionidae egg parasitoid, gustatory sensilla are related with exocrine glands (Bin *et al.,* 1989; Isidoro *et al.,* 2001). It was suggested that these glands might be implicated in the dissolution or degradation of mucopolisaccharides and adhesive substances used in attaching the host eggs to the plant (Isidoro *et al*, 1996).

Taste transduction begins when a contact chemical encounters the pore at the tip of a taste sensillum, diffuses through this pore and then dissolves into the fluid surrounding the dendrites (Shields, 1996). Once the chemical stimulus reaches the distal dendritic membrane, it interacts with specific receptor proteins and/or ionic channels leading to changes in membrane conductance (Chapman, 1982; Glendinning, 2008). Then, chemical signals are transformed in nervous activity and transmitted to the central nervous system, where differences in impulse rate decode according to the differences in stimulus intensity (Bell and Tobin, 1982; Bell *et al.,* 1995).

In egg parasitoids associated with Heteropteran hosts and in particular in *T. basalis,* four types of gustatory sensilla were found (Bin *et al.,* 1989). Two of these, sensilla chaetica (CH) and grooved peg (GP), are present in both sexes, and they are probably involved in the detection of general tastants. Two other types are specific to one sex: females present papillary sensilla (PS) called also "multiporous gustatory sensilla", while males present sensilla conica (SCO). These sensilla are likely to be involved in sex-specific activities, such as mating or egg-laying (Bin *et al.,* 1989; Iacovone *et al.,* 2015; Iacovone *et al.*, 2016). Recently, sensilla chaetica has got more interest in gustatory perception and contact chemo-reception researches. Because of their morphology and position, these sensilla are considered as the most suitable gustatory sensilla for electrophysiological studies using the tip recording technique (Hodgson *et al.,* 1955; Iacovone *et al.,* 2016).

Also Bin (1981) suggested that chaetica sensilla are more rigid than the others gustatory sensilla. They touch the substrate first and therefore may detect information directly from the host. This hypothesis was supported by a combination of ultrastructural and behavioral investigations carried out on several parasitoid families including Scelionidae (Bin *et al.,* 1989; Isidoro *et al.,* 1996; Romani *et al.,* 2010).

Gustatory sensilla chaetica are uniporous and they can be recognized easily based on their external morphology (Bin *et al.,* 1989; Isidoro *et al.,* 1996; Romani *et al.,* 2010). They have an outstanding hair-shaft. They are almost perpendicular to the ventral side of the antennal surface and ranging about 11 to 13.5µm in length and 0.8 to 1 µm in diameter at the base. They are thick walled and gradually tapering from the base to a roundish, uniporous tip, which is inserted in a specialized flexible socket. They are located on the ventral-subventral side of the distal half of antennomers 6th to 11th. Specifically in *T. basalis* females, they are about 27 altogether; one group of 3 is located on antennomer 11, four groups of 4 are located on each antennomer from 7 to 10 and eight are irregularly distributed on the antennomer 11 (Bin *et al.,* 1989). In *Trissolcus brochymenae* (Ashmead) (Hymenoptera: Scelionidae) females, antennal sensilla chaetica have a very similar morphology and distribution than those described for *T. basalis* (Iacovone *et al.,* 2015). Antennal sensilla chaetica were found also in other egg parasitoids associated with heteropteran host, which are *Trissolcus japonicus* (Ashmead) and *Trissolcus plautiae* (Watanabe) (Hymenoptera: Scelionidae), two solitary endoparasitoids of the brown winged green bug *Plautia stali* (Scott) (Heteroptera: Pentatomidae). They are setiform with longitudinal grooves on their surface. These sensilla are sparsely distributed on the flagellum of females (A6-A11) and males (A6-A12) of both species. The number of these sensilla was not significantly different between sexes for either *T. japonicus* or *T. plautiae*. It was assumed that they are seemingly involved in contact chemoreception (Isidoro *et al.,* 1996; Shi-Yong *et al.,* 2016).

1.2. Chemical approach

Insect parasitoids rely on a variety of chemical cues regulating their orientation. These chemicals have been well studied in Hymenoptera parasitoids. Several volatile and contact cues have been chemically identified (Karlson and Butenandt, 1959; Quicke 1997; Kainoh, 1999; Keeling *et al.,* 2004).

Eavesdropping on volatile pheromonal cues released by the non-target stages is efficient for parasitoids only when denoting the presence of the preferred host stage. In some species of egg parasitoids, the low detectability of host egg cues is overcome by relying on cues originating from the host adult stage such as sex and aggregation pheromones (Jones *et al.*, 1973; Vinson, 1976; Weseloh, 1981; Godfray; 1994; Nordlund, 1994; Hilker and Meiners, 1999; Steidle and Van Loon, 2002). One of the pioneering studies was conducted by Lewis *et al.*, (1982). The authors demonstrated for the first time the exploitation of adult host pheromones by egg parasitoids in a study regarding *Trichogramma pretiosum* (Riley) (Hymenoptera: Trichogrammatidae) and its host *Heliothis zea* (Lepidoptera: Noctuidae). The female moth sex pheromone was identified as a mixture of (Z)-11-hexadecenal, (Z)-9-hexadecenal, (Z)-7-hexadecenal and hexadecanal (Klun *et al.*, 1980). A synthetic sex pheromone and volatiles from abdominal tips of *H. zea* females increased egg parasitism rates by *T. pretiosum* in the field (Lewis *et al.*, 1982). Furthermore, Noldus (1988) confirmed that the moth sex pheromone serves as a kairomone for the wasp females.

Host aggregation pheromone can also be used by some egg parasitoids as an alternative solution to the low detectability of host egg cues faced. *Riptortus clavatus* (Thunberg) (Heteroptera: Alydidae) males produce an aggregation pheromone consisting on a blend of three components in a ratio of 1:5:1 ((E)-2-hexenyl (E)-2-hexenoate, (E)-2-hexenyl (Z)-3-hexenoate (E2HZ3H), and myristyl isobutyrate). Therefore, the use of traps baited with the synthetic version of this insect pheromone showed the capture of its egg parasitoid *Ooencyrtus nezarae* (Ishii) (Hymenoptera: Encyrtidae) in soybean fields (Leal *et al.*, 1995). Later on, field experiments proved that one of the three components alone, (E)-2-hexenyl (Z)-3-hexenoate, attracted the wasp females and resulted in a higher parasitism rate in treated plots compared to the untreated ones (Mizutani *et al.*, 1997; Mizutani, 2006).

Host adult secretions are also benefic volatile cues for egg parasitoids in host location. They are classified as long-range infochemicals. For instance, volatiles deriving from *N. viridula* adults were found to attract *T. basalis* (Bin *et al.*, 1988). The (E)-2-decenal, a short-chain α,β-unsaturated aldehyde and a compound of the defensive secretion from metathoracic gland of *N. viridula*, appeared responsible for this attraction (Mattiacci *et al.*, 1993).

This finding was confirmed by a recent study proving a positive wasp response as well as an increased searching behavior when exposed to defensive compounds from *N. viridula* metathoracic scent gland secretion. The wasps exhibited a clear preference for 4-oxo-(E)-2-hexenal and (E)-2-decenal (Laumann *et al.*, 2009).

Other species of egg parasitoids developed the ability to use host-induced plant synomones as reliable cues when looking for their hosts. These synomones are previously described as HIPVs. They are released in response to herbivore feeding or oviposition (Vet and Dicke, 1992; Fatouros *et al.*, 2008; Bruce *et al.*, 2010; Tamiru *et al.*, 2011; Tamiru *et al.*, 2012). Higher synomone levels are likely induced by feeding activity than by egg deposition (Conti and Colazza, 2012).

In some plant species, egg deposition combined with adults feeding is necessary to induce volatiles, which attract egg parasitoids (Meiners and Hilker, 1997, Meiners and Hilker, 2000, Hilker and Meiners, 2002, Hilker *et al.*, 2002). On bean plants, *Vicia faba* L. and bean *Phaseolus vulgaris* L., the combination of *N. viridula* oviposition and feeding activities induced a significant increase in the level of terpenoids like linalool, (E)-β-caryophyllene, (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and (3E)-4,8-dimethyl-1,3,7-nonatriene. Extracts from feeding-damaged plants with *N. viridula* egg masses were characterized by increased rates of (E)-β-caryophyllene in comparison with extracts from feeding-damaged plants without egg masses. The extracts contained two fractions but only the one containing (E)-β-caryophyllene attracted *T. basalis* (Colazza *et al.*, 2004).

Volatiles from undamaged plants are considered as chemical cues for egg parasitoids providing little reliable information regarding the potential host presence (Romeis *et al.*, 2005; Wäckers, 2005; Fatouros *et al.*, 2008). Exploiting such infochemicals can optimize the parasitoid searching efficacy by concentrating its efforts on sites where host are most likely present (Boo and Yang, 1998). Various studies have shown that volatiles deriving from undamaged plants or plant extracts elicited behavioral responses in *Trichogramma* spp. (Bar *et al.*, 1979; Altieri *et al.*, 1982; Nordlund *et al.*, 1985; Kaiser *et al.*, 1989; Reddy *et al.*, 2002; Alsaedi *et al.*, 2016). *Trichogramma chilonis* (Ishii) (Hymenoptera: Trichogrammatidae), an egg parasitoid of the polyphagous noctuid moth *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), presented an olfactory attraction to the emitted volatiles by undamaged sorghum plants, *Sorghum bicolor* L.

The parasitoid females were arrested by perceiving volatile cues released during sorghum reproductive stages, which is the preferred period for *H. armigera* oviposition and attraction (Romeis *et al.,* 1997). Another study showed that the same parasitoid *T. chilonis* presented behavioral responses to volatiles emanating from undamaged hot pepper plant *Capsicum annuum*, its host *Helicoverpa assulta* (Guenée) (Lepidoptera: Noctuidae) host plant (Boo and Yang, 1998).

In host location process, egg parasitoids can employ short-range cues, which are deposited by their hosts on the substrate and perceived upon landing on the plant (Van Alphen and Jervis 1996). These chemical cues are a result of the host activity. For female parasitoids, they represent reliable information in the host presence and detection (Vet and Dicke 1992).

Host egg volatiles and contact kairomones are short range cues that play a role in host egg searching and recognition (Strand and Vinson, 1982; Leonard *et al.,* 1987; Bin *et al.,* 1993; Conti *et al.,* 2003). This semiochemical level has been studied for the egg parasitoid *Telenomus podisi* (Hymenoptera: Scelionidae). The influence of volatiles released by eggs and egg extracts of the stink bug *Euschistus heros* has been evaluated in Y-tube olfactometer. The wasp has shown an attraction to the volatiles emitted from one egg cluster and its acetone extracts. The major components of egg clusters acetone extracts were saturated and unsaturated fatty acids, including hexadecanoic acid, linoleic acid, and (Z)-9-octadecenoic acid. It has been suggested that the existing volatile components in one egg cluster and its acetone extract are able to change *T. podisi* foraging behavior (Michereff *et al.,* 2016).

Egg contact kairomones are much more exploited by insect parasitoids for host recognition than are host egg volatiles for host location (Conti and Colazza, 2012). Bin *et al.,* (1993) discovered that *Trissolcus spp.* use the existing chemicals over heteropteran host egg mass as cues in host acceptance process. The origin of the recognition kairomones are follicular secretion and egg extracts of heteropteran hosts but their chemical nature has not been defined yet (Sales *et al.,* 1978; Bin *et al.,* 1993; Conti *et al.,* 2003, Conti and Colazza, 2012).

Indirect host-related cues originating from the juveniles or adult stages produce arrestment and searching behaviors in egg parasitoids (Godfray, 1994; Fatouros *et al.,* 2008). The responses of Trichogrammatidae and Scelionidae species to lepidopteran moth scales were previously studied (reviewed by Colazza *et al.,* 2010). Egg parasitoids belonging to these families use wing scales of Lepidoptera adults to find host eggs.

Chemical analysis of Lepidoptera scales revealed the presence of linear hydrocarbons (Jones *et al.,* 1973) and organic acids (Gueldner *et al.,* 1984). Similarly, the present kairomones from *Ostrinia nubilalis* adults' scales affected host-seeking behavior of its parasitoid *Trichogramma nubilale,* which was attracted and kept within the kairomone-treated area (Shu and Jones, 1989). The kairomone was identified as a mixture of three dimethylnonatriacontanes. The 13,17 isomer, the most active one, induced klinokinetic and a retention effect in *T. nubilale* while the other two isomers, 11-15 and 15-19, had klinokinetic effect but failed to retain wasps in the treated area (Shu *et al.,* 1990).

Chemical traces left on plants by insects when moving over and around a substrate, called footprints, are also indirect host-related cues perceived as kairomones by egg parasitoids in host finding process (Wäschke *et al.,* 2013). Many heteropteran egg parasitoids such as *Trissolcus spp.* (Colazza *et al.,* 1999; Conti *et al.,* 2004; Salerno *et al.,* 2006), *Telenomus podisi* (Borges *et al.,* 2003) and *Gryon boselli* (Mineo and Szabo) (Hymenoptera: Scelionidae) respond to chemical footprints of their pentatomid host bugs (Conti and Colazza, 2012). To date, a handful of studies have tried to identify the footprint chemicals used by parasitoids in host finding and recognition. In this context, footprint chemistry of *N. viridula* was examined regarding its parasitoid *T. basalis*. The normal alkanes presence, with qualitative and quantitative differences among the sexes, was reported by extracts analysis of the pentatomid cuticular lipids. Extracts were composed mainly of linear alkanes from nC19 to nC34. The compounds nC23, nC24, and nC25 were present more in female extracts of *N. viridula* than in male hexane extracts. The n-nonadecane compound (nC19) was recovered only from the cuticle and footprints of males but was missing in the bug females footprints. By adding this compound to *N. viridula* females' cuticular extracts, *T. basalis* females reduced their arena residence time. This response is similar to the behavior of female wasps when exposed to *N. viridula* male hexane extracts (Colazza *et al.,* 2007).

1.3. Behavioral approach

The effectiveness of insect parasitoids as biological control agents depends on their host location behavior. Analysis of the searching behavior of a parasitoid species is indispensable to evaluate this effectiveness. Manipulating parasitoids’ behavior using semiochemicals, which can improve the ability of mass-reared parasitoids to locate the host, has been suggested by many authors (Lewis *et al.,* 1982; Vet *et al.,* 1995).

A parasitoid female searching for host encounters an enormous variety of existing stimuli in the environment. At the beginning of the host location process, it perceives and responds mainly to airborne volatile cues, toward which it flies, seeking out the preferred habitat. Upon landing, the female parasitoid starts walking and perceiving substrate-borne cues, and by their mean it locates the host and makes a physical contact. This switch from one type of cue to another is generally correlated with a variation in the parasitoids searching pattern (Isidoro *et al.,* 2001; Colazza *et al.,* 2014).

Generally the perception of substrate-borne chemical cues by parasitoid female within a profitable patch leads to a flight suppression and an arrestment behavior of that female. In addition to arrestment, other behaviors like an intense antennation can be noticed (Vinson, 1998; Colazza *et al.,* 2014). Simultaneous arrestment periods, drumming, tapping or immobilization of antennae on the substrate, have been registered in many wasp species (Klomp, 1981; Gazit *et al.,* 1996). For example, *T. basalis* arrested facing cues left by adult and immature instars of *N. viridula* and this arrestment response was characterized by an initial prolonged motionless period with the antennae kept in contact with the substrate (Colazza *et al.,* 1999). If oviposition by the parasitoids in their host eggs is not occurring within a certain amount of time, their sensitivity to host footprints register a progressive decrease. They ultimately leave the contaminated area, showing a less intense arrestment response on the next patch they encounter (Peri *et al.,* 2006).

Regardless of the perceived stimulus, whether is volatile or substrate borne cue, two different searching modalities, random searching and trail following behavior, can be adopted by insect parasitoids during host location process in response to specific needs. Trail-following behavior seems to be activated by stimuli directly emitted by target organisms while random searching behavior is more likely triggered by cues indirectly associated with the target organism (Godfray, 1994; Colazza *et al.,* 2014).

1.3.1. Random searching behavior pattern

Random searching, called “motivated searching” by Vinson 1998, is a searching behavior in and around the vicinity of a contaminated patch by turning in random directions with respect to the stimulus.

In general, it is associated with non-directional cues which are, as mentioned previously, cues indirectly associated with the target organism that inform wasps about closeness of nearby hosts but do not provide exact and precise information about host location (Godfray, 1994; Vinson, 1998). Random searching is characterized by a flight inhibition and a calibration of the turning rate (klino-kinesis) and the locomotion rate (ortho-kinesis) on the base of the stimulation allowing the insect to stick around resource patch (Vinson and Lewis, 1965; Shorey, 1977; Kennedy, 1978; Waage, 1978; Colazza *et al.,* 2014).

When *T. brochymenae* females are in contact with a contaminated area by adult footprints of *M. histrionica*, they acquire a motivated searching behavior marked by a variation in locomotion activity. They show an intense and a longer searching activity on patches where footprints are present with an increased residence time. Their walking pattern was affected as well (Conti *et al.,* 2003; Conti et *al.,* 2004). The wasp females presented an intense substrate examination and search on the chemical footprint left on substrate by host mated females that had not yet laid eggs than on the traces left by parous or virgin host females (Salerno *et al.,* 2009).

During a random searching, the speed of the parasitoids movement is correlated with the concentration of chemical cues and the stimulation intensity. Indeed, parasitoids present fast movements with a low turning rate when exposed to a low concentration of a chemical but they slow down their movements and increase the turning rate when exposed to a higher concentration of cues (Shorey, 1977; Waage 1978). The total searching time and local searching intensity, are two other parameters that can be affected during this type of searching, either by the emitter densities or by the cue qualities (Bouchard and Cloutier, 1984; Colazza *et al.,*1999). A qualitatively superior patch retains the insect to remain longer that a poor patch, reflecting an efficient searching modality (Bell, 1984; Bell, 1991).

The searching time and intensity are higher in patches where the density of the cue is higher. The arrestment response of *Telenomus remus* (Hymenoptera: Scelionidae) on hexane extract of eggs of its host *Spodoptera frugiperda* (Lepidoptera: Noctuidae) was dose dependent and the search was more intense in a patch where host presence is higher (Gazit *et al.,* 1996).

Many parasitoids display turning angles beyond 90° when they move from a profitable to an unfavorable stimulus (Bell and Tobin, 1982). This happen at patch edges, when parasitoids pass from an area contaminated with the stimulus to a surrounding area where they lose contact with the cues (Waage, 1978; Peri *et al.,* 2006; González *et al.,* 2011).

The measurement of turning angle at patch edges may be specified by internally-stored information (Bell, 1991). *Trichogramma evanescens* responds to a kairomone from its host *Pieris brassicae* (L.) (Lepidoptera: Pieridae) by exhibiting a random searching behavior, which is characterized by a reduction in walking speed and increased turning. By leaving kairomone area the egg parasitoid exhibits an increase in turning angles followed by an increased activity and walking speed (Gardner and Van Lenteren, 1986). After going out from the patch, some parasitoids resume the research activity and enter again into the patch once or more times but for a shorter period (Vinson and Lewis, 1965).

Learning affects the random searching behavior of insect parasitoids and changes their innate response to a contact stimulus (Lewis and Jones, 1971; Grasswitz, 1998; Steidle, 1998; Papaj and Vet, 1990). Previous studies have investigated the role of experience on substrate-borne chemical cues in *T. basalis* during the search of *N. viridula* eggs. Wasps responded innately to chemical traces left by *N. viridula* walking over the substrate. However, the previously exposed individuals to the host kairomone reduced their searching time. The effect was not durable in time, wasps restored previous behavior after a period of 72 hours (Peri *et al.,* 2006).

1.3.2. Trail following behavior pattern

Trail-following is a searching behavior, led by air-borne or substrate-borne cues providing directional information. It is activated by chemicals cues released directly by the target organism, which act as the “Ariadne’s thread guide” directing insect parasitoids toward the chemicals’ source (Colazza *et al.,* 2014).

In air-borne trails, up-wind flights or walking toward air currents represent examples of trail following responses. Evidence, largely from wind tunnel studies, indicates that upwind anemotaxis in insect parasitoids is odor induced (Vet *et al.,* 1995; Vinson, 1998). Plant odors are carried downwind from the host plant and their dispersion is dominated by turbulent diffusion in a spatially complex manner (Murlis *et al.,* 1992; Murlis *et al.,* 2000).

Once they perceive the odors, insects present an oriented zigzag flight upwind towards the source (Kennedy, 1977; Cardé, 1996). They may be directed to the source from at least certain meters away or until that source is apparent enough for a visual response (Drost and Cardé, 1992; Bernays and Chapman, 1994; Finch and Collier, 2000).

Loss of contact with the plume triggers 'casting' or wide lateral excursions cross-wind with counterturns and loops but without upwind progress until the insect renews the contact with the plume (Kennedy, 1983; Cardé, 1996). The flight direction of natural populations of *Platygaster subuliformis* (Kieffer) (Hymenoptera: Scelionidae), an egg-larval parasitoid of the Brassica pod midge *Dasineura brassicae* (Winnertz) (Diptera: Cecidomyiidae), from and toward their hosts' host plant *Brassica napus* L. (Brassicaceae) was investigated. Traps surrounding a plot of host plants were hanged to monitor the parasitoids flight activity while flying towards and away from the plot. The study revealed that *P. subuliformis* locates the plot by upwind anemotaxis responding to odor cues that are released from Brassica plants and carried downwind from the crop (Williams *et al.,* 2007).

In substrate-borne trails, the chemicals left by one insect on a substrate are consistently followed by another one that is keeping contact with the substrate as it moves (Shorey, 1977; Quicke, 1997). Parasitoids, by means of stimulus-intensity comparisons series over the chemical trail, they maintain their bodies near the central axis of the trail along an array of non-random turns and walks in straight connecting lines (Shorey, 1977; Kapranas *et al.,* 2013). This zigzag pattern is adopted in response on losing contact with the chemical cues at the trail edges. *Ascogaster reticulate* Watanabe (Hymenoptera: Braconidae), an egg-larval parasitoid of *Adoxophyes honmai* (Yasuda) (Lepidoptera: Tortricidae), exploits plant chemicals in locating host eggs (Seino *et al.,*2010). In a tritrophic context, investigations of the response of the parasitoid to cues from host egg-laden tea leaves showed that wasp females presented a zigzag walking behavior while this behavior was not shown on intact leaves (Piyasaengthong *et al.,* 2016).

In other species, if at the end of the trail the parasitoid do not find the desired host immediately, it can change its behavior to another one termed Area-restricted searching, which serves to keep parasitoids within the stimulus area (Klomp, 1981; Rogers and Potter, 2002). This Area-restricted searching has a biological value analogous to the turning-back behavior at the patch edge in the random searching (Colazza *et al.,* 2014).

2. Research objectives and outline of the thesis

In the present thesis, two different examples of chemo-orientation were treated. The first is contact chemo-orientation of *Trissolcus brochymenae* Ashmead (Hymenoptera: Scelionidae), which perceives its host footprints upon contact with the substrate, whereas the second is volatile chemo-reception concerning *Trissolcus basalis* Wollaston (Hymenoptera: Scelionidae) and its perception of buckwheat plant volatiles. The aim of this research project was to investigate the mechanisms used by *T. brochymenae* and *T. basalis* in host and food location process respectively.

A consistent bibliography elucidated the ability of parasitoids to learn by association (Vet and Dicke, 1992; Turlings *et al.,* 1993; Vet *et al.*, 1995; Steidle and van Loon, 2003). Parasitoids can learn to associate a specific olfactory cue with a successful oviposition in a host (Hoedjes *et al.,* 2011). In a first study case, we investigated the role of parasitoid experience on indirect-host related contact cues using the tritrophic system *B. oleracea – M. histrionica – T. brochymenae*. The purpose was to understand how unrewarding (lack of oviposition) and rewarding experiences (successful oviposition) change the innate response of *T. brochymenae* towards host walking traces in the context of the parasitoids' host location process.

Host location is important for adult parasitoids for reproductive purposes but they are not the only needed resources to achieve a successful reproduction. The location of food, such as nectar and honeydew, is also contributing in the reproductive success. Parasitoids have to balance host and food needs to advance their efficiency as biocontrol agents (Lewis *et al.,* 1998). Several egg parasitoid use companion plant volatiles as long-range infochemicals to locate food. For *T. basalis*, previous behavioral experiments tested the parasitoid's response to extract of buckwheat flowers headspace and a synthetic blend of buckwheat volatiles indicated that *T. basalis* females have sensitive and selective olfactory responses to buckwheat plant, *Fagopyrum esculentum,* a companion plant and a source of nectar. Also electroantennography showed that most of six tested synthetic buckwheat plant compounds evoked significant responses on *T. basalis* antennae (Foti *et al.,* 2016). This suggests that antennal olfactory sensilla play an important role in buckwheat volatile compounds perception.

In this study, the responses of *T. basalis* females to individual compounds of buckwheat plant volatiles was assessed using coupled Gas Chromatography-ElectroAntennographic Detection (GC-EAD) and Single Sensillum Recording technique (SSR). The aim of these experiments was to identify the key active buckwheat volatile compounds and to explore the olfactory activities of buckwheat volatiles on the ORNs of the wasp females with the hypothesis that *T. basalis* has a set of specialized ORNs for buckwheat plant volatile compounds.

2.1. Crops

- Broccoli, *Brassica oleracea,* is a cool-season crop that performs poorly in hot weather. As a member of the crucifer family, broccoli is closely related to other crops such as cabbage, cauliflower, and brussels sprouts but it is believed to be the first of the cole crops to evolve from wild cabbage. Broccoli has originated from the eastern Mediterranean, from where it was introduced into Italy (Gray, 1982). Previous studies have showed that epicuticular wax of *B. oleracea* absorb the chemical footprints of *N. viridula* and allows *T. basalis* females to discriminate between hosts of different sex, with an extended searching behavior of the egg parasitoids in females traces than traces of males (Lo Giudice *et al.,* 2011). It has been demonstrated also that *B. oleracea* releases host-induced plant synomones as a consequence of *M. histrionica* activity on leaves. These synomones are adsorbed by the leaves epicuticular waxes and perceived by the egg parasitoid *T. brochymenae* at a very short distance and on contact (Conti *et al*., 2010; Frati *et al.,* 2013).

- Buckwheat, *Fagopyrum esculentum,* is a broadleaf plant native to northern Asia, China. It was introduced into Europe in the middle ages, reaching Germany early in the 15th century (Hughes and Hensen, 1934). It has a fast growth and a short sowing-to-flower period (Bowie *et al.,* 1995). Buckwheat grain is grown mainly for human consumption but can also be used as animal feed, green manure crop, smother crop to crowd out weeds and as a source of buckwheat honey (Campbell, 1997). It has accessible nectar for wasps (Patt *et al.,* 1997). Previous studies showed that buckwheat has beneficial effects on parasitoid species. Buckwheat floral resources increased the longevity of the parasitoid *Aphidius ervi* (Araj *et al.,* 2006; Araj and Wratten, 2013) and that the presence of inflorescences in buckwheat plants increased the longevity of *T. basalis* adult females (Rahat *et al.,* 2005).

Later on, Foti *et al.*, (2016) have proved that access to buckwheat flowers heightened the offspring of female wasps and that buckwheat floral scent, headspace extracts of buckwheat flowers and a blend of six buckwheat plant volatiles showed attractiveness in olfactometer bioassays. Furthermore, electroantennography showed that most of the six tested buckwheat plant compounds evoked significant responses in *T. basalis* antennae.

2.2. Insects

- *Nezara viridula* commonly named the southern green stink bug (Fig. 3) is a highly polyphagous pest in many temperate and tropical regions in the world on many important food and fiber crops (Todd, 1989; McPherson and McPherson, 2000). Its origin is a matter of debate but it is believed to have originated in Ethiopia from where it has spread around the world thanks to its capacity of flying and human trade routes. It has a cosmopolitan distribution and it is present in tropical and subtropical regions of America, Africa, Asia, Australia and Europe (Hokkanen, 1986; Todd, 1989; Panizzi, 2008). This pest presents piercing-sucking mouthparts, which destroy only few cells leaving a minimal mechanical damage and the main damage is caused by the insect saliva, which lead to a reduced quality of the crop (Miles, 1972). All the plant parts can be attacked by *N. viridula* including stems, leaf veins, fruiting structures and growing shoots (Panizzi and Slansky, 1991).

Figure. 3. *Nezara viridula* adult.

- *Murgantia histrionica*, commonly named the harlequin bug, is an exotic pest ranging from the Atlantic to the Pacific coasts, which is native to Central America (Walsh, 1866; McPherson, 1982). It has been reported to feed on over 50 species of plants, including plants in the Brassicaceae family such as cabbage, broccoli, kale, turnip, radish, horseradish, mustard, rape (McPherson, 1982). The bug individuals are large, colored with black and orange or red markings (Fig. 4), slow moving, resistant to flying, and often clumped, making them easy to find and capture (Aliabadi *et al.*, 2002). Harlequin bugs feed on the leaves and stems of plants, provoking blotching at the feeding area by sucking the plant's sap. The attacked seedling plants by *M. histrionic* bugs wilt, turn brown, and eventually die (White and Brannon, 1933).

Figure 4. *Murgantia histrionica* adult.

2.3. Parasitoids

- *Trissolcus basalis* is probably an African naïve species (Fig. 5). It is a solitary egg parasitoid, which has been used as an effective biological control agent against the southern green stink bug *N. viridula*. After a first reputedly successful control in Australia in 1933 (Wilson, 1960; Bin *et al.,* 1989; Clarke, 1990), numerous attempts have been made in several countries (Egypt, Antilles, south Africa, Hawaii, Brazil and USA *etc.*) with a long history of research as a natural enemy of *N. viridula* (Crouzel and Saini, 1983; Hoffmann *et al.*, 1991; Clarke and Walter, 1995; Colazza and Bin, 1995; Ehler, 2002).

Host location strategies adopted by this wasp have been extensively explored. Plant volatiles coming from the food plant, food of the host, or food odors have been shown to have an important role in host location.

Figure 5. Adult female of *Trissolcus basalis* over *Nezara viridula* egg mass.

- *Trissolcus brochymenae* is closely related species of *T. basalis* (Fig. 6). It is an egg parasitoid measuring about 1.1–1.3 mm long (Johnson, 1984). It is attacking at least 11 Nearctic pentatomid bug species (Salerno, 2000). It is considered as a potential biological control agent of *M. histrionica* and host selection process of this egg parasitoid has been investigated (Conti *et al.,* 2003, Conti *et al.*, 2004, Conti *et al.,* 2010; Frati *et al.,* 2013). Recently, antennal gustatory perception and behavioral responses in these wasp females were carried out since antennal contact chemoreception plays a prominent role in *T. brochymenae* ecology (Iacovone *et al.,* 2016).

Figure 6. *Trissolcus brochymenae* adult over *Murgantia histrionica* eggs.

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Chapter 2

Chapter 2

Effect of previous experience on the response of an egg parasitoid to substrate-borne semiochemicals

Abstract

Chemical traces deposited by walking adults of the harlequin bug (*Murgantia histrionica*) on cabbage leaves and adsorbed by leaf surfaces induce an innate arrestment behavior in the egg parasitoid *Trissolcus brochymenae* (Ashmead) (Hymenoptera: Scelionidae) leading to an intense searching behavior on host-contaminated areas. In this study, It was examined how previous experience, which plays a key role in the parasitoid host location, can influence *T. brochymenae* response to host walking traces left on leaf surface. Results showed that: 1) an unrewarded experience (repeated encounters with host-contaminated areas not followed by oviposition experience) decreased the intensity of the parasitoid response; 2) wasp responses became stronger after a rewarded experience (successful oviposition); 3) responses of wasps varied according to the elapsed time between two consecutive unrewarded events in a host-gender specific manner. The potential significance of these results to the host location behavior of egg parasitoids is discussed.

Key words

Trissolcus brochymenae, *Murgantia histrionica*, host location, substrate-borne infochemical, learning.

This chapter is based on the work contained in the following paper:

Peri, E., Salerno, G., Slimani, T., Frati, F., Conti, E., Colazza, S., & Cusumano, A. (2016). The response of an egg parasitoid to substrate-borne semiochemicals is affected by previous experience. *Scientific reports*, 6, 27098.

1. Introduction

Chemical cues, air-borne and substrate-borne chemicals, play an important role in insect parasitoids foraging behavior. Such cues are directly or indirectly related to the host (Godfray, 1994) and used by parasitoids during host habitat and host location (Vinson, 1985; Lewis and Martin, 1990; Tumlinson *et al.,* 1992; Vet and Dicke, 1992; Fatouros *et al.*, 2008, Colazza *et al.,* 2014). Female parasitoids must cope with complicated, heterogeneous and chemically dynamic environments in the process of host location. The "reliability-detectability" problem in the responses of parasitoid to semiochemicals has been hypothesized to be faced by them since cues emanating from the host are often with low detectability but high reliability whereas plant volatiles are very detectable but quite low reliable and do not represent a guaranty of the host presence (Dicke *et al.,* 1990; Turlings *et al.*, 1990; Du *et al.*, 1996). Parasitoids have developed efficient and various sophisticated searching strategies involving exploitation of numerous environmental cues to overcome the challenge of hosts finding and to perform optimally in changing environments (Tamò *et al.*, 2006; Rasmann and Turlings, 2007; Gols *et al.*, 2012; Moujahed *et al.*, 2014).

During the foraging process, the capacity of parasitoids to adjust (increase or change) their responses to semiochemical cues based on experience and their learning ability of profitable cues have been demonstrated in a wide variety of parasitoid species in either preadult or adult stages (Turlings *et al.*, 1993). Associative learning, defined as “the establishment through experience of an association between two stimuli or between a stimulus and a response” has been well documented for several parasitoid species over years (Godfray and Waage, 1988; Lewis and Tumlinson, 1988; Turlings *et al.*, 1989; Vet, 1988; Vet and Groenewold, 1990; Kester and Barbosa, 1991; Lewis and Takasu, 1990; Petitt *et al*, 1992; Poolman-Simons *et al.*, 1992; Tumlinson *et al.*, 1992; Vet and Dicke, 1992; Dukas and Duan, 2000; Meiners *et al.*, 2003; Kruidhof *et al.,* 2013; Canale *et al.,* 2014). It seems to be a common circumstance in the Hymenoptera parasitoids. That means that wasps learn to respond to new cues to which they originally show no or limited responses (conditional stimuli) when they are present and associated with innately recognized host or host by product (unconditional stimuli) through that process of associative learning.

Therefore, they become reactive to the newly learned cues and exploit them in their hosts searching (Hoedjes *et al.,* 2011; Turlings *et al.,* 1993). Cues that are unreliable indicators of the host presence over the evolutionary time but that became associated with the host presence can be used by the parasitoids (Vet *et al.,* 1990; Hoedjes *et al.,* 2011).

Air-borne semiochemicals have attracted the main attention in considerable studies assessing the response of parasitoid foraging behavior to experience and learning (Lewis and Martin, 1990; Papaj and Vet, 1990; Steidle, 1998; Steidle and Van Loon, 2003). When investigating learning and experience in parasitoids, which are controlling herbivore hosts, it is crucial to examine the role that the plant plays (Mumm and Hilker, 2006). Host-finding ability of parasitoids is affected by numerous plant characteristics such as plant attractants, plant organs, plant structural refuges and plant morphological features (Price *et al.,* 1980). When exploiting substrate-borne cues by walking on the plant, wasps have to deal with plant surface structures such as trichomes or veins. In fact, trichrome and their exudates can decrease the walking speed of parasitoids or even trap them inhibiting their searching behavior and their ability in locating a host (Rabb and Bradley, 1968; Obrycki, 1986; Treacy *et al.,* 1986; Keller, 1987; Kauffman and Kennedy, 1989; Kashyap *et al.,* 1991; Romeis *et al.,* 2005). Moreover, parasitoid discrimination between contact kairomone components from the similar existing components in plant cuticle is mandatory because some chemical compounds such as linear and branched-hydrocarbons can be present in both components (Müller and Riederer, 2005; Lo Giudice *et al.,* 2011; Colazza *et al.,* 2014).

Wasp responses to traces of host adults while locating a host are generally observed in scelionid egg parasitoids (Hymenoptera: Scelionidae) that are attacking pentatomid bugs (Heteroptera: Pentatomidae). The kairomonal effect of chemical traces which are left by harlequin bug adults *Murgantia histrionica* Hahn while walking over cabbage leaves has been observed in the wasp *Trissolcus brochymenae* Ashmead (Peri *et al.,* 2013). As responses to traces of host adults, an innately generated arrestment response of parasitoid females, characterized by an initial prolonged motionless period and by keeping the antennae in contact with the substrate, has been observed. This arrestment behavior was followed by an intense searching behavior characterized by klinokinesis and negative orthokinesis as mentioned previously in the introduction (reviewed by Colazza *et al.,* 2014).

Host gender and physiological status are important in determining adult traces hierarchical value. Indeed, female parasitoids are able to discriminate between the left contact kairomones by female and male of bugs and they remain more time on the contaminated patches by the bug gravid females (Conti *et al.,* 2003; Salerno *et al.,* 2009).

It was proved in many researches that the parasitism success by some egg parasitoids is correlated with the eggs age. It decreases through the time since the host quality declines as eggs develop (Lewis and Redlinger, 1969; Marston and Ertle, 1969; Leibee *et al.,* 1979; Houseweart *et al.,* 1982; Reznik and Umarova, 1990; Beserra *et al.,* 2002). *Trissolcus brochymenae* adopts a particularly adaptive fine-tuned foraging strategy based on indirect-host related contact chemicals since it emphasizes the probability of wasps in locating freshly laid *M. histrionica* eggs which are known to be more suitable for parasitoid offspring (Vinson, 2010). However, the adjustment mechanism of *T. brochymenae* females foraging behavior in response to previous experience is still not known

The response on filter paper arenas contaminated with host residues becomes weaker by wasps re-encountering of the patches without successful oviposition occurrences, but the females response was stronger on kairomone patches where oviposition occurred (Peri *et al.,* 2006). The time window that *T. basalis* wasps need to “forget” unrewarded experiences on female residues was approximated to be 3 days, but under stressful temperature conditions this time can be lengthened (Abram *et al.,* 2015). By forgetting an unrewarded experience, the parasitoid females that encountered a contaminated filter paper arena behave as they were naïve (Peri *et al.,* 2006). However, the role of the plant surface as the real interface for host-parasitoid interactions in nature was not taken into account by these studies since they were carried out on filter paper rather than using plant surface as a natural substrate.

In this chapter, the ability of parasitoid to associate indirect-host related contact cues with experience was studied in the tritrophic system *Brassica oleracea* L. (Brassicaceae) *Murgantia histrionica* (Heteroptera: Pentatomidae) –*Trissolcus brochymenae* (Hymenoptera: Scelionidae). The contribution of unrewarding (lack of oviposition) and rewarding experiences (successful oviposition) in changing the innate response of *brochymenae* towards host footprint traces in the parasitoids’ host location process was investigated.

Since parasitoids present innate responses to host walking residues, the variable-response model proposed by Vet *et al.,* (1990) is used to produce predictions rather than classical conditioning theory in which an unconditioned stimulus is paired with a conditioned stimulus (Hammer and Menzel, 1995). The variable-response model aims to predict how and when the wasp behavior is influenced by learning. In accordance with the model, there is a possible hypothesis that the parasitoid females that successfully located and parasitized host eggs when exploring a leaf surface contaminated with host walking traces should reinforce their response the intensity of to the same stimulus in the near future, while the response of those that were not able to locate host eggs should be decreased. The response of female parasitoids was hypothesized to be affected by time elapsed between two successive unrewarding visits to the contaminated areas in a host-gender specific manner as result of the different hierarchical value of residues from host adults.

2. Materials and methods

2.2. Insect colonies

In 2000 the colony of harlequin bugs (*M. histrionica*) were established from cabbage fields situated in Beltsville (USA), however, in the same year, the *T. brochymenae* colony was originally obtained from wasps emerging from *M. histrionica* eggs found in fields located in San Diego (USA). Both insects colonies were maintained under quarantine conditions, in a growth chamber (25 ± 1 °C, 60 ± 5% RH, 16 h:8 h light: dark) at the Entomology laboratories of the University of Perugia (Italy).

Murgantia histrionica colony was reared in plastic cages where they were fed with cabbage leaves changed every 2-3 days. In order to have individuals of known ages continuously available, every 2-3 days, the last instar nymphs were individually isolated into a single plastic pot (Ø = 40 mm, height = 65 mm) and observed daily until adult emergence (Fig. 1a). Bugs designed for bioassay were mated male and female adults approximately 10–14 days post-emergence, with females in pre-ovipositional state. Mated adults were obtained from pairs separated after mating and isolated individually for 24 hours before experiments.

Concerning *T. brochymenae* colony, it was reared on *M. histrionica* eggs masses that were glued on paper strips. Wasp adults were kept in 85 ml glass tubes fed with honey-water solution kept in controlled environment. After emergence, male and female parasitoids were kept together to mate (Fig. 1b). In all experiments, female wasps were 2–3 days old, mated and naïve (i.e., they had not previously encountered adult host chemical cues). Before 24 h from the bioassays, female wasps were individually isolated in small vials then at least 30 min before testing they were allowed to acclimatize to the conditions of the bioassay room.

Figure 1: Insect colonies rearing. (a) plastic cages containing *M. histrionica individuals* and (b) *T. basalis* adults in glass tubes.

Seeds of *Brassica oleracea* plants (var Italica cv Marathon) were planted in polystyrene pots filled with peat and were grown under controlled greenhouse conditions (25 ± 3 °C, 50 ± 10% relative humidity) (Fig. 2). After one week, seedlings were transplanted individually in 14 cm diameter plastic pots, fertilized with commercial soil (Trflor—HOCHMOOR), and watered as needed. The used plants in all conducted experiments were 5–6 week old.

Figure 2: *Brassica oleracea* plants grown under controlled conditions.

2.2. General bioassay procedure

In order to assess parasitoids' response to host walking traces adsorbed on the leaf surface, a protocol similar to that of Peri *et al.,* (2013) was used. Briefly, bioassays were conducted in an open arena consisting of the adaxial surface of a leaf disk (5 cm in diameter) exposed to a single female or male bug for 30 min to contaminate it with the stink bug's walking residues (Fig. 3). As described by Peri *et al.,* 2013, the adults used were with excised stylets in order to prevent bugs from feeding and thus to obtain leaf disks contaminated only with chemical traces. For stylet excision, bugs were previously anaesthetized inside a glass tube with CO_2 for 4–5 s in order to immobilize their labium.

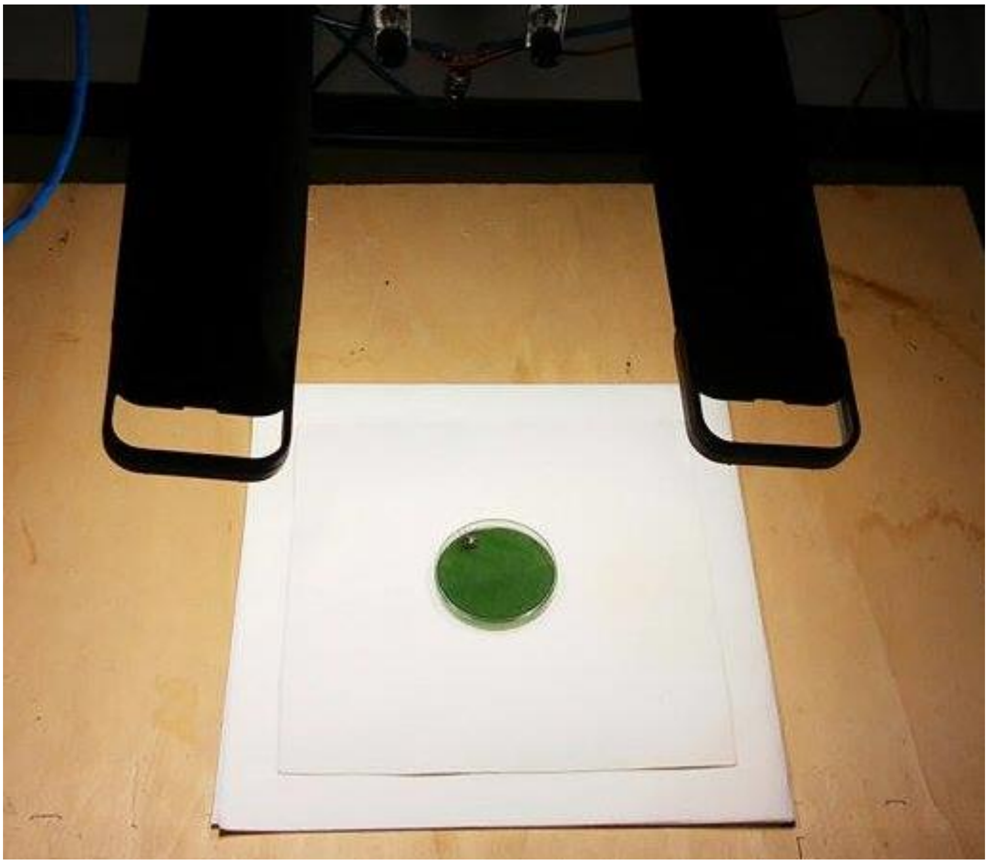


Figure 3: Adaxial surface of cabbage leaf disk exposed to a single individual of *M. histrionica* to be contaminated with the bug walking residues in open arena.

Stylets were drawn from labium using an entomological pin and were amputated half their length using precision microscissors under a stereomicroscope with optical fiber illumination. Bugs were then placed inside a plastic dish (12 cm diameter) for 24 h allowing them to recover, and were subsequently used to contaminate the leaf disk as described above. Trails started by realizing a single parasitoid female into the center of the leaf and observed until the wasp flew away or reached the leaf disk borders. Wasp response was measured in terms of residence time, i.e., the time spent by the wasps on the leaf disk, which in several past studies (Conti *et al.*, 2003, Peri *et al.*, 2006; Abram *et al.*, 2015) was strongly correlated with increased turning frequency and slower walking speed, therefore it was recorded as a measure of the intensity of each parasitoid's response towards host traces. The residence time of naïve wasps on untreated leaf disks is about 5–10 seconds. Experiments were carried out from 9:00 to 13:00 in an isolated room maintained at 25 ± 1 °C, with lighting on the arena provided by two 19 cm long fluorescent tubes.

2.3. Experiment 1: unrewarded experience

The first experiment was aimed to examine the parasitoid behavioral response when encountering successive leaf areas contaminated with male or female host traces without successful oviposition (unrewarding experience). First, naïve wasps were tested on host-contaminated areas. These wasps were considered experienced after displaying a typical arrestment behavior and leaving the patch. They were then recaptured, isolated in small vials and re-tested 1 h later on newly treated arenas according to the four following treatments: (1) naïve and experienced wasps were both tested on female traces (Female-Female, FF), (2) naïve wasps were tested on female traces but experienced wasps were tested on male traces (Female-Male, FM), (3) Both, naïve and experienced wasps were tested on male traces (Male-Male, MM), or (4) naïve wasps were tested on male traces but experienced ones were tested on female traces (Male-Females, MF). For each treatment 26–31 successful replicates were performed.

2.4. Experiment 2: rewarded experience

In the second experiment an investigation about the influence of a successful (i.e., rewarded) oviposition experience on the wasps’ behavioral responses was carried out. Therefore, single, naïve *T. brochymenae* wasps were released onto a leaf disk contaminated with traces of host males or females and a host egg mass (five eggs) in the middle of the leaf disk. This training phase was conducted to obtain experienced wasp, thus the residence time of the wasp was not recorded. Then, experienced wasps (i.e. those that had located the egg mass and parasitized one egg) were recaptured and kept isolated in a small vial for 1 hour. After that they were tested on cabbage leaf surfaces treated with chemical traces from host females or males as previously described, and their residence time was recorded. First two treatment combinations were used in running these experiments: (1) oviposition on female traces and tested on female traces (Female_oviposition_Female, F_o_F) or (2) oviposition on male traces and tested on male traces (Male_oviposition_Male, M_o_M).

It is well known that a successful oviposition among egg parasitoids of stink bugs generally increases turning frequency, decreases walking speed and increase residence time in a manner analogous to contact with host chemical residues (Bin *et al.*, 1993).

Thus, to separate the effect of oviposition experience and host chemical residues, experienced wasps tested on uncontaminated leaf disks were used as controls, leading to two additional treatments: (3) oviposition on female residues and tested on uncontaminated arena (Female_oviposition_Blank leaf, F_o_B), or (4) oviposition on male residues and tested on uncontaminated arena (Male_oviposition_Blank leaf, M_o_B). For each treatment 31–35 successful replicates were performed.

2.5. Experiment 3: hierarchical value of host traces

The third experiment was conducted to evaluate the possible hierarchical value of areas contaminated by chemical traces left by host females or males on the parasitoids' behavioral response, as a function of the elapsed time between two consecutive unrewarded events. First, to obtain experienced wasps, naïve females were exposed to a leaf disk area treated with female or male walking residues of *M. histrionica*. The residence time of the wasp was not recorded during this training phase. Then, the experienced parasitoid (*i.e.*, those that have left the patch after showing an arrestment response) were captured and kept in a small vial and fed with a drop of honey-water solution for 24, 48 or 72 h. These experienced wasps were then re-tested on another leaf disk treated with female or male walking traces. These tests were carried out using two host gender combinations: (1) testing naïve wasps on female traces and re-testing them on female traces after 24, 48 or 72 hours (F_24 h_F, F_48 h_F, F_72 h_F); (2) testing naïve wasps on male traces and re-testing them on male traces after 24, 48 or 72 hours (M_24 h_M, M_48 h_M, M_72 h_M). It has been demonstrated in several parasitoid species that extended periods without host contact can affect their host-searching behavior (for a review, see Jervis *et al.*, 2014). Therefore, in order to distinguish the effect of unrewarded experience and increased duration without successful oviposition on wasps' behavioral response, naïve wasps used as control, were kept in a vial for the same amount of time as experienced ones. Control wasps were tested alternately with experienced females. For each treatment 27–30 successful replicates were performed.

2.6. Statistical analyses

Regarding the Experiment 1, a Generalized Linear Mixed Model (GLMM) was used with treatment combination, experience status, and treatment × experience interaction as fixed terms and parasitoid identity as a random term, with parasitoid residence time as the response variable.

To further investigate the role of the experience status, its effect in each pairwise treatment separately was tested when a significant interaction was found. Concerning Experiment 2, a Generalized Linear Model (GLM) was fitted, testing the dependence of parasitoid residence time on oviposition treatment. If the model detected significant differences amongst factor levels, it was proceeded to pairwise comparisons to determine which differed using the *glht* function found in the *multcomp* package of the R software (Bretz *et al.*, 2010). For experiment 3, a GLM was first fitted with time interval, host gender and the time interval × host gender interaction as explanatory factors using parasitoid residence time of experienced wasps as response variable. Then the data were analyzed separately for each of the two combinations (Female-Female, Male-Male) as a significant effect of host gender was found (see results) and the interest was given to the dependence of parasitoid response on the host gender over time. Thus for each combination, a GLM was fitted with time interval, experience status, and the time interval × experience interaction as explanatory factors and parasitoid residence time as the response variable. To more study the role of the experience status, its effect in each time interval treatment (24, 48 or 72 hours) was tested when a significant time interval × experience interaction was found. For all of the GLMM and GLM analyses above, it was assumed a gamma error distribution with a reciprocal link function, since residence time data were not normally distributed (typical for time-to-event data), and the variance changed faster than linearly with the mean. Significance of the fixed terms in the model was determined using Likelihood Ratio Tests (LRTs) comparing the full model with and without the factor in question (Crawley *et al.*, 2007) Model fit was assessed with residual plots. All statistical analyses were performed with R software version 3.1.3. (R Core Team, 2015).

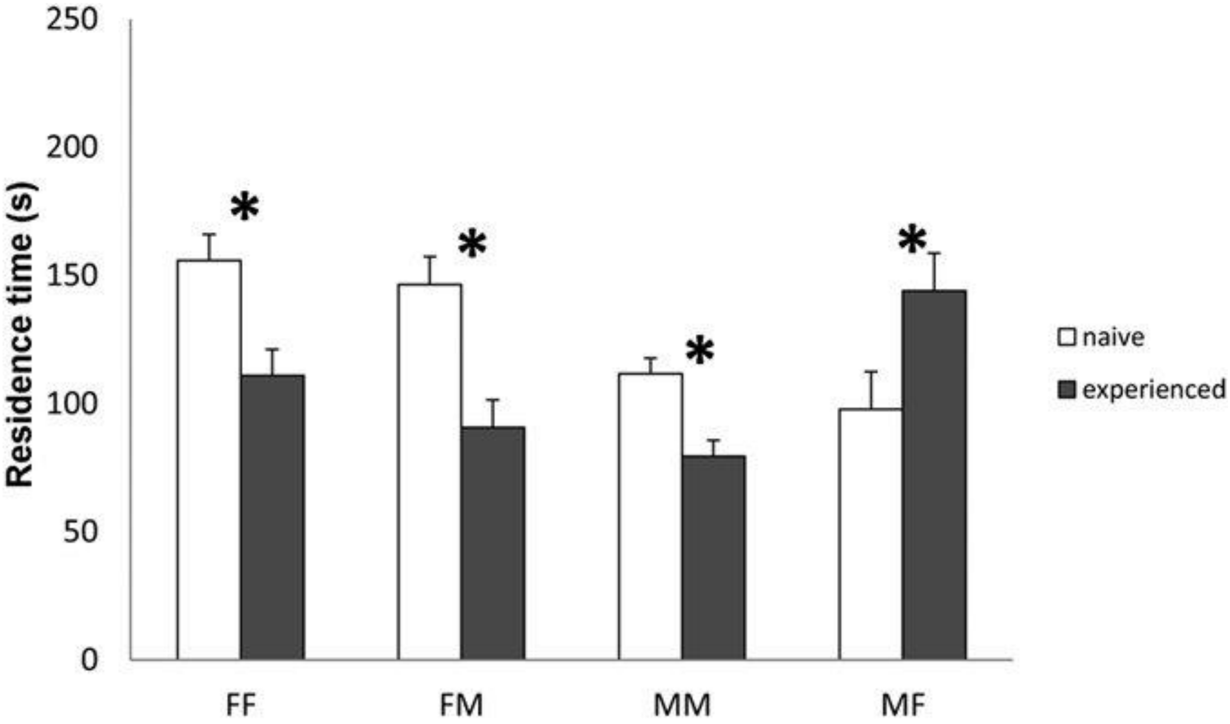
3. Results

3.3. Experiment 1: unrewarded experience

Trissolcus brochymenae wasps' residence time was significantly affected by the treatment × experience interaction ($\chi^2 = 7.46$; df = 3; $P < 0.001$). A large and statistically significant effect of experience status on the wasps' residence time was observed in each of the four treatments (Fig. 4). The residence time of females wasps experienced with host female chemical residues was significantly reduced upon re-encountering the same type of chemical cue (group FF: $\chi^2 = 13.88$; df = 1; $P < 0.001$).

Parasitoids that were experienced on female walking traces and re-tested on male walking traces also spent less time on the arena when re-encountering host male residues (group FM: $\chi^2 = 16.24$; df = 1; $P < 0.001$).

Figure 4: Behavioral response of *Trissolcus brochymenae* with unrewarded experience.



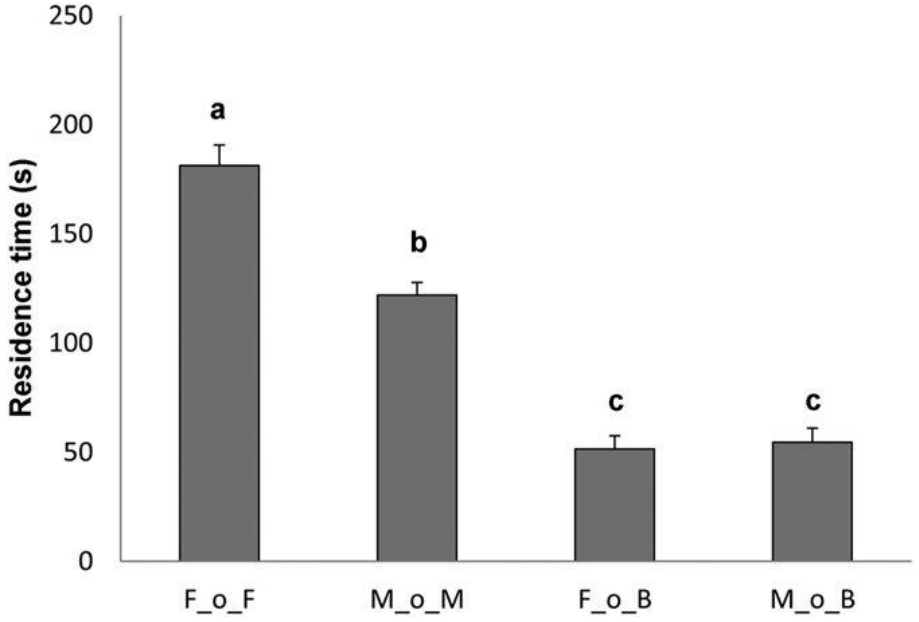
Mean (±SE) residence time of *Trissolcus brochymenae* females encountering for the first time (naïve, white bars) or re-encountering (experienced, grey bars) *Murgantia histrionica* adult walking traces adsorbed on cabbage leaf disks. MM=experienced and tested on male traces, N=31; MF=experienced on male traces and tested on female traces, N=29; FM=experienced on female traces and tested on male traces, N=27; FF=experienced and tested on female traces, N=26. Asterisks indicate significantly different means within each pairwise combination (GLMM, $*P<0.05$).

A similar effect was observed on naïve parasitoids that were tested on host male traces when re-encountering the same type of chemical cue (group MM: $\chi^2 = 13.31$; df = 1; $P < 0.001$). By contrast, wasps with experience on male residues increased their residence time by re-testing them on host female residues (group MF: $\chi^2 = 11.90$; df = 1; $P < 0.001$) (Fig. 4).

3.4. Experiment 2: Rewarded experience

T. brochymenae females residence time was influenced by previous successful oviposition experience when tested in arenas contaminated by host chemical residues (GLM, $\chi^2 = 38.14$; df = 3; $P < 0.001$) (Fig. 5).When compared to rewarded females encountering host male residues (group M_ko_M), rewarded females re-encountering host female residues (group F_ko_F) showed longer arena residence time (GLM, z = 2.83, $P < 0.05$).

Figure 5: Behavioral response of *Trissolcus brochymenae* with rewarded experience.



Mean (±SE) residence time of *Trissolcus brochymenae* females allowed to oviposit on a host egg mass in the presence of either host female or male traces and then tested according to different conditions: F_o_F=oviposition on female traces and tested on female traces, N=32; M_o_M=oviposition on male traces and tested on male traces, N=31; F_o_B=oviposition on female traces and tested on uncontaminated cabbage leaf disks, N=35; M_o_B=oviposition on male traces and tested on uncontaminated cabbage leaf disks, N=34. Different letters above bars indicate significantly different means (GLM, $*P<0.05$).

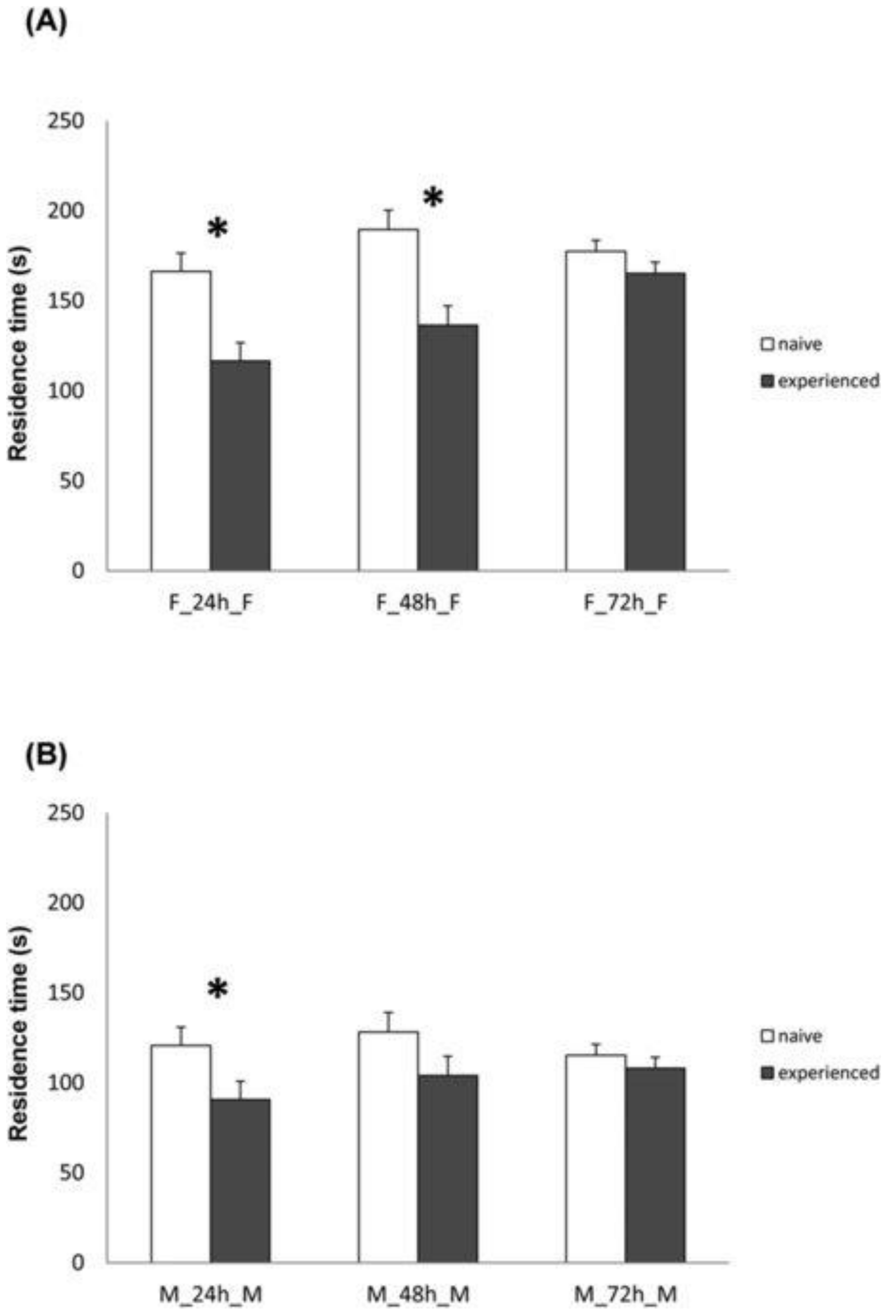
The effect of oviposition experience *per se* on residence time could be excluded because *T. brochymenae* females with successful oviposition experience did not remain longer when re-encountered the untreated arenas (group F_o_B) (Fig. 5). In fact, the arrestment response of rewarded wasps changed significantly in comparison to the control in in the presence of host female residues ($z = -7.56$, $P < 0.001$). Similarly, the residence time of rewarded wasps on host male chemical cues was significantly different from controls (group M_o_B) GLM, $z = -5.43$, $P < 0.001$).

3.5. Experiment 3: hierarchical value of host traces

Experienced *T. brochymenae* wasps' residence time of was significantly affected by both the time interval (χ^2=3.32; df=2;P<0.05) and host gender (χ^2=5.42; df=1;P<0.01), nevertheless the host gender × time interval interaction was not significant (χ^2=0.15; df=2; P=0.705). The time interval × experience interaction influenced significantly the residence time of wasps when experienced on female residues and then re-tested on female residues (χ^2=6.08; df=2;P<0.01) (Fig. 6a).Within each time interval treatment, the residence time in the arena was significantly different between naïve and experienced wasps provided that the time interval was not longer than 48hours (group F_24_F, χ^2=9.46; df=1;P<0.01; group F_48_F, χ^2=9.01; df=1;P<0.01; group F_72_F, χ^2=0.27; df=1;P=0.601) (Fig. 6a).

The wasp residence time was influenced by the time interval × experience interaction when *T. brochymenae* females were experienced on male residues and re-encountered male residues (χ^2=3.27; df=2;P<0.05) (Fig. 6b). However, a significant difference between naïve and experienced wasps' arena residence time was registered only when the time interval within each treatment did not exceed 24h (group M_24_M, χ^2=8.46; df=1;P<0.01; group M_48_M, χ^2=2.01; df=1;P=0.106; group M_72_M, χ^2=0.55; df=1;P=0.462) (Fig. 6b).

Figure 6: Behavioral response of *Trissolcus brochymenae* for evaluation of hierarchical value of host traces.



Mean (±SE) residence time of *Trissolcus brochymenae* females trained and tested on 2 consecutive leaf disk arenas contaminated with traces of *Murgantia histrionica* adults (combination “female-female, F_F” in figure A or “male-male, M_M” in figure B) at intervals of 24, 48, or 72 h (experienced, grey bar), F_24 h_F, N = 30, F_48 h_F, N = 28, F_72 h_F, N = 29; M_24 h_M, N = 27, M_48 h_M, N = 28, M_72 h_M, N = 30. As controls, *T. brochymenae* females tested at the same times as experienced females were used (white bars; naïve females). Asterisks indicate significantly different means within each pairwise combination (GLM, *P < 0.05).

4. Discussion

In this chapter it has been shown that the females of *T. brochymenae* parasitoids adjust their instinctive behavioral response to *M. histrionica* chemical residues over natural substrates on the basis of previous experience suggesting that experience play a relevant role in host location process regarding egg parasitoids of heteropteran hosts once they land on an infested plant. Experience on chemical cues related to the host may also have an important role in host location because the left chemical traces by many herbivores during their movements on the plant can be detected as host location kairomones by their biocontrol agents (Müller and Riederer, 2005; Lo Giudice *et al.*, 2011; Quicke, 1997; Rostás and Wölfling, 2009).

For insect parasitoids which exploiting substrate-borne infochemicals, the adoption of various searching patterns is possible based on the information that they receive. If a directional information regarding the host location is received, the parasitoid adopt trail-following search while receiving non-directional information which indicate that a host is near provoke the adoption of biased-random search (Godfray, 1994; Colazza *et al.*, 2014). *T. brochymenae* females adopt the latter searching pattern as result of an innate response that the left walking residues by *M. histrionica* adults induce (Conti *et al.*, 2003; Salerno *et al.*, 2009). Thus, once landing on an infested plant, the possibility of finding host eggs increases because of delayed flight tendency, prolonged stay on the plant, and extensive walking exploration. The obtained results suggest that the patch time allocation of *T. brochymenae* females could be modified by the reproductive success accumulated while foraging on plant surfaces contaminated with contact kairomones left by *M. histrionica* (Fig. 5). An oviposition reward increased the intensity of response of *T. brochymenae* to host female traces, a phenomenon that seems in agreement with the type of experience defined as “α-conditioning” (Carew, 1984; Vinson, 1998) in which rewards enhance innate responses towards a given stimulus. In contrast, *T. brochymenae* females displayed a decrease in the intensity of their behavioral responses when unrewarded individuals were re-tested on leaf disks contaminated with the same type of indirect host-related cues (Fig. 4). In studies testing associative learning, unrewarding experiences induce wasps to cease responding to the learned cues (Papaj, *et al.*, 1994; Takasu and Lewis, 1996). In the parasitoid *Microplitis croceipes* Cresson, a negative experience (i.e. oviposition in an unsuitable host) significantly decreases the learned response to vanilla induced by antennating host frass with the odor (Takasu and Lewis, 2013).

For *T. brochymenae*, the adaptive significance of unrewarded experience may be that chemical traces left by *M. histrionica* adults cannot guarantee the presence of host eggs or provide directional information (Vet and Dicke 1992). Hence, even in patches contaminated by 'promising host cues', it could be adaptive for parasitoids to lose their searching motivation and leave the area if hosts are not found after a certain amount of time (Vet and Dicke, 1992). In fact, in insect parasitoids, patch leaving tendency is assumed to increase over time but it can be adjusted by successful oviposition events (Waage, 1978, Haccou *et al.,* 1991; Hemerik *et al.,* 1993; Wajnberg, 2006; Thiel and Hoffmeister, 2009). Rewarded and unrewarded experiences in *T. brochymenae* affect the subsequent level of response towards host traces in agreement with the prediction that the response potential of a stimulus can change as consequence of learning (Vet *et al.,* 1990).

Results of the experiments show that over a period of some days, the behavioral response of *T. brochymenae* was influenced by the time elapsed between two consecutive unrewarded events. As expected, residence time of wasps with a previous unrewarded experience tended to increase relative to naïve controls, suggesting that parasitoids "forgot" an encounter with host walking traces adsorbed on leaf disks without successfully locating a host egg mass. Interestingly it was found a host gender-specific effect when comparing the results of the residence time between naïve and experienced parasitoids as wasps' behavioral responses differed over time depending on whether experience occurred on male- or female host traces (Fig. 6). A first possible hypothesis to explain our results is that parasitoids forgot unrewarded experiences on male-associated cues faster than experiences with female-associated cues. This appears to support the theory that the temporal window of previous experience is affected by the rank of how well-correlated stimuli are with their associated resources (Hoedjes *et al.,* 2011; Menzel, 1999) since female host traces, which should be more strongly associated with host egg presence, elicit a more intense response. A second possible explanation is that the host-gender effect shown in our experiments may be simply due to differences in strength of reaction between parasitoid responses to female and male host traces as different stimuli used in the foraging process can elicit different levels of response (Vet *et al.,* 1990).

Under such hypothesis, the group of parasitoids tested on male traces may fail to reach significance when the time interval was longer than 24 h due to a weaker level of response, and not due to a higher tendency to forget.

In fact, an aspect that limits our current understanding of the results is the inability to link the behavioral responses adjusted by experience with the memory structure possessed by *T. brochymenae*. Depending on the variability of the environment and the reliability of host-related cues, insect parasitoids have been shown to retain information in different memory phases, e.g. short-term memory (STM), medium-term memory (MTM) or long-term memory (LTM), which differ in terms of stability, duration and energy consumption (Collatz *et al.,* 2006; Smid *et al.,* 2007; Schurmann *et al.,* 2015). To discriminate between the two hypotheses, further studies should investigate whether *T. brochymenae* females use different forms of memory to store previous foraging experiences on traces left by male and female *M. Histrionica* hosts even if our results may suggest that unrewarded experience is stored in medium-term memory.

The role of experience on substrate-borne chemical cues has been previously investigated in the egg parasitoid *T. basalis* when searching for *N. Viridula* host eggs (Peri *et al.,* 2006; Abram *et al.,* 2015). Overall, strong similarities were found between *T. basalis* and *T. brochymenae as i*n both species the behavioral response decreases after one hour in unrewarded females and increases in rewarded females. However, while the innate *T. basalis* response to host male traces was not modified by experience gained during foraging, an opposite outcome was found for *T. brochymenae* found suggesting that experience is more relevant for the latter species. It is possible that such differences can be related to the oviposition strategies evolved by the herbivore species, which consequentially affect the spatial distribution of the host resources for the associated egg parasitoids. While the oviposition strategy of *N. viridula* (the main host of *T. basalis*) consists of laying 2–3 egg masses each made up of about 100–120 eggs, *M. histrionica* (the main host of *T. brochymenae*) produces about 8–9 egg masses which consist only of up to 12 eggs each (Todd, 1989; Zahn *et al.,* 2008). Consequently, a *T. brochymenae* female should be expected to encounter a higher number of egg masses during their lifetime than *T. basalis,* and so a finely-tuned response to experience could be particularly adaptive in the former species. In fact, it has been suggested that the number of lifetime learning events can influence the value of experience, leading to the prediction that when hosts are encountered once or only a few times, experience is less likely to be relevant (Roitberg *et al.,* 1993). Indeed, in the parasitoid *Melittobia digitate* Dahms, the innate response to contact kairomones was not affected by previous host exposure experience (Gonzalez *et al.,* 2011).

This could be because *M. digitate* females usually locate and parasitize only a single host and thus, prior host experience would not be favored by selection in this parasitoid species. Alternatively, the differences found between *T. brochymenae* and *T. basalis* could be explained by a difference in the chemistry of walking traces between females and males of their respective hosts, under the hypothesis that this difference is larger between females and males in *N. viridula* (the host of *T. basalis*) as compared to *M. histrionica* (the host of *T. brochymenae*). In the case of *T. basalis*, host gender discrimination is based on *n*-nonadecane, a cuticular hydrocarbon that is present only in walking traces of *N. viridula* males but not on those left by females (Colazza *et al.*, 2007) whereas the chemical nature of host gender discrimination by *T. brochymenae* is still unknown. To test this alternative hypothesis, further studies are required to identify the chemical differences between walking traces of males and females of *M. histrionica* in order to clarify which compound(s) is involved in host sex discrimination by *T. brochymenae*. Finally, it is not possible to exclude that methodological differences in the substrate used in the two studies (filter paper arenas for *T. basalis* and leaf disks for *T. brochymenae*) can be responsible for the different outcome found in terms of experience on male host traces. However, as detection of host kairomones should be facilitated in simplified experimental conditions in which parasitoids do not have to cope with potentially confounding leaf features, it is unlikely that the innate *T. Basalis* response to host male traces would be modified by previous experience even if more natural substrates would have been employed.

In conclusion, the results of the present chapter contribute to a better understanding of the role played by experience in the behavioral response to indirect host-related cues by egg parasitoids. It was shown that parasitoid experience can be expressed in a host gender-specific manner, and that this phenomenon is observed in a tritrophic context where natural substrates are present.

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Chapter 3

Chapter 3

Antennal olfactory responses of *Trissolcus basalis* (Hymenoptera: Scelionidae) to buckwheat plant volatile compounds

Abstract

In insect parasitoid females, antennae, bearing chemosensory, mechanosensory and thermo-hygroreceptive sensilla, are involved in the location and selection. In particular, perception of plant volatiles plays a basic role in recognition of nutritional resources. For *Trissolcus basalis*, an egg parasitoid of the green stink bug *Nezara viridula*, previous behavioral experiments, testing the parasitoid's responses to the headspace extracts of buckwheat flowers and a synthetic blend of buckwheat flower volatiles indicated that *T. basalis* females have sensitive and selective olfactory responses to buckwheat plant *Fagopyrum esculentum*. Electroantennography also showed that most of the six synthetic compounds, identified from the headspace of buckwheat plants, evoked significant responses on *T. basalis* antennae. This suggests that antennal olfactory sensilla play an important role in buckwheat volatile compounds perception of *T. basalis*. Subsequent electrophysiological experiments using gas chromatograph-linked electroantennographic detection (GC-EAD) showed that two buckwheat flower volatiles, 3-methylbutanoic and 2-methylbutanoic acids, elicited consistent responses at antennae level. Then, we examined the sensitivity of antennal olfactory sensilla to seven buckwheat plant volatile compounds using single sensillum recording technique (SSR) to determine the response profiles of individual olfactory receptor neurons (ORNs) to these compounds. All seven buckwheat volatiles elicited responses from some ORNs, and 3-methylbutanoic acid and *p*-benzoquinone were the most active compounds showing consistent responses. These results represent a first step toward the identification of specialized ORN's for *T. basalis,* which could help in developing a semiochemical-based strategy to enhance the activity of the parasitoid in the field.

Keywords

Buckwheat, Chemoreception, Egg parasitoid, Electrophysiology, GC-EAD, Olfaction, Olfactory receptor neuron, Sensilla, Single sensillum recording, Volatile compound.

1. Introduction

Phytophagous insects have species-specific range of host plants for oviposition and feeding (Dyer *et al.,* 2007). Although they rely on diverse signals such as visual, tactile and chemical cues, olfaction is often considered the most important sense in their host and food-source location (Bernays and Chapman, 1994; Hildebrand and Shepherd, 1997; Quicke, 1997; Schoonhoven *et al.,* 2005; Andersson, 2007). Location and selection of food resources is an arduous task for insects because they live in a quite complex odor environment and olfactory landscape. Therefore, insects have developed highly sensitive and selective chemo-sensory system not only to locate and recognize their host plants but also to assess the food quality (Renwick, 1989; Gripenberg *et al.,* 2010; Bruce and Pickett, 2011). The composition of the perceived chemicals is a key factor in the host plant specificity (Bengtsson *et al.,* 2006; Dyer *et al.,* 2007; Bruce and Pickett, 2011).

Phytophagous insects present a highly efficient and sophisticated olfactory system characterized by species-specific odor recognition based on the specificity of their olfactory receptor neurons (ORNs) in discriminating between single perceived compounds, the ratio of constituent in the blend and the blends when perceived as a whole (Bruce *et al.,* 2005; McCormick et a*l.,* 2012). Food and host location in insect parasitoid follows the same mechanism used by phytophagous insects. Most of the parasitoids host location analyses have focused on parasitic wasps where olfactory mechanisms have been dealt with the most attention and may be the main means of finding hosts in many parasitoid species (Van Alphen and Vet, 1986; Godfray, 1994).

Wasps use volatile compounds from the plant or host/plant complex to locate a suitable host habitat and for host location, recognition and acceptance (Peri *et al.,* 2013). Parasitoid species feed on hosts of different nature but most of them rely on plant-derived sugar-rich food such as nectar for their survival and energetic needs (Jervis *et al.,* 1996; Wäckers, 2005; Vollhardt *et al.,* 2010). Studies showed that parasitoids could learn to associate food-based cues with nectar location (Lewis and Takasu, 1990; Wäckers and Swaans, 1993; Jervis *et al.,* 1996; Takasu and Lewis, 1996). Two parasitoid species, *Edovum puttleri* (Hymenoptera: Eulophidae) and *Pediobius foveolatus* (Hymenoptera: Eulophidae), were able to associate nectar location with the odors emitted by both artificial nectars and real nectaries of dill flowers and snap bean stipules (Patt *et al.,* 1999). Previous studies also have found that the parasitoid *Microplitis croceipes* (Hymenoptera: Braconidae) can locate the extra-floral nectar of cotton as food resources from short distances (Röse *et al.,* 2006).

Electrophysiology is a useful tool for the assessement of the olfactory system responses to odor stimuli (Van Der Pers and Minks, 1996; Stensmyr *et al.,* 2001; Rostelien *et al.,* 2005; Ulland *et al.,* 2008: Olsson and Hansson, 2013). For the measurement of the insect antennal responses, two basic techniques exist, electroantennography and single sensillum recording (SSR). The latter is more precise and reliable method allowing the identification of the functional specialization of ORNs in many insect species and the linking of odorant-induced responses to olfactory neuron activities (Den Otter *et al.,* 1980; Hansson, 2002; Mustaparta, 2002). It has been extensively used in testing the responses of ORNs in many insects such as *Drosophila melanogaster*, *Anopheles gambiae*, *Aedes aegypt*, *Sitona lepidus* and *Helicoverpa species* (Park *et al.,* 2013; Lin and Potter, 2015; Chang *et al.,* 2016).

For parasitic wasps, electrophysiological studies have not been active, probably because of technical difficulty due to the small size of the insects and antennae (Pettersson *et al.,* 2001; Van Baaren *et al.,* 2007). Experiments at sensilla scale of an extremely tiny hymenopteran parasitic wasp has been done recently on *Trissolcus brochymenae* Ashmead (Hymenoptera: Scelionidae) measuring about 1.1–1.3 mm long (Johnson, 1984), by exanimating the sensitivity of antennal gustatory sensilla chaetica to several classical tastants. The study showed that *T. brochymenae* sensilla chaetica respond to serial concentrations of sucrose, salts, and quinine indicating that corresponding gustatory receptors are expressed in these sensilla. The study showed also that the tip sensilla respond differently to sucrose and KCl versus the proximal sensilla (Iacovone *et al.,* 2015).

Trissolcus basalis Wollaston (Hymenoptera: Scelionidae) is a closely related species to *T. brochymenae*; it is an egg parasitoid and an important biological control agent of the green stink bug *Nezara viridula* (Heteroptera: Pentatomidae), a serious pest of more than 30 different crops (Todd, 1989). The antennae play a major role in host finding for this species and previous research presumed that sickle shaped sensilla are the sole olfactory organs in either sex and they are the most likely involved in plants and host location (Vinson, 1985; Bin *et al.,* 1989).

Recently, a study has revealed that access to buckwheat flowers (*Fagopyrum esculentum*) increased *T. basalis* offspring. Also combined behavioral experiment and electroanntenography testing the parasitoid’s responses to headspace volatile extracts of buckwheat and its synthetic mimic have shown that females of *T. basalis* have sensitive and selective olfactory responses to some major buckwheat volatiles (Foti *et al.,* 2016).

Based on these findings we investigated the electrophysiological response profiles of antennal ORNs in female *T. basalis* to identify the functional types of ORNs and the corresponding active plant volatile compounds, and to examine the response spectra of these ORNs, using SSR. Our study aims to explore the olfactory activities of buckwheat volatiles on antennae and on the ORNs in the olfactory sensilla of the wasp females with the hypothesis that *T. basalis* has a set of specialized ORNs for buckwheat plant volatile compounds.

2. Materials and methods

2.1. Insects

Females of *T. basalis* were used in the experiment. They were reared in glass tubes (16 ml) on the egg masses of *N. viridula* under controlled conditions (25 ± 1°C, 60 – 65% RH and L16: D8 photoperiod) and fed with drops of honey-water solution (80:20 v/v). Host eggs were obtained from *N. viridula* reared in a climatic chamber (25 ± 1°C, 60 – 65 % RH and L16: D8 photoperiod), inside insect rearing cages (47.5 x 47.5 x 47.5 cm) (BugDorm-44545, MegaView Science Co. Ltd., Taichung, Taiwan) and fed with seasonal organic vegetables.

Host eggs were exposed to the parasitoids for 3 to 4 days then left for incubation. Male and female *T. basalis* emerging from the host eggs after 12 to 14 days were kept together in the same tube to allow mating. Females used for the electrophysiology experiments were 3 days old for GC-EAD recordings and 2 to 15 days old for SSR studies. *Trissolcus basalis* females were distinguished from males by the shape and size of their antennae. Female antennae are composed of 11 antennomers ending by a relatively large “antennal club” while male antennae are composed of 12 antennomers with a moniliform terminal segment (sensu Bin, 1981; Bin *et al.,* 1989).

2.2. Test compounds and buckwheat extracts

Headspace extracts of buckwheat plant were used in our experiments. Seeds of buckwheat, *Fagopyrum esculentum* (Moench) cv kaitowase, were sown in 10-cell plug trays filled with standard potting mix containing slow-release fertilizer. After germination, 1-week-old seedlings were transplanted into 1-l plastic pots, filled with the same kind of soil used for sowing and watered three times weekly or more often as deemed necessary. All plants were covered by an anti-insect net and grown in a greenhouse. In all experiments, plants were used when in full bloom. Volatile organic compounds (VOCs) were collected from flowering buckwheat plants, and they contained both vegetative and floral compounds.

Excised shoots were singly placed in glass vial (volume: 520 ml). The cut end was immediately submerged in 25 ml of water to reduce the emission of green leaf volatiles and desiccation during volatile collection.

A push-pull VOC collection system was used as described earlier (Foti *et al.* 2016; Rostás and Eggert, 2008). Air from a compressed air cylinder was filtered with 400 ml of activated charcoal (Alltech, USA), and was then pushed into the headspace collection vessel through a teflon tube at a rate of 300 ml min-1. With a vacuum pump (ILMVAC GmbH, Germany) 300 ml min-1 of air was pulled out through a trapping filter containing 30 mg SuperQ (ARS Inc., USA).The adsorbed compounds were eluted from the trapping filter with 150 µl of methylene chloride (Sigma-Aldrich, Australia) and concentrated under nitrogen prior to GC injection.

Eighteen synthetic compounds were tested in our SSR study (Table 1). Among them, seven compounds are present in buckwheat volatiles, and eleven other compounds are common plant volatile compounds. Each test compound was dissolved in hexane to 1 µg/µl. The test compounds were divided into three different mixture groups (Table 1 A, B and C) that were also prepared in hexane at 1 µg/µl concentration. Hexane was used as a solvent control stimulus (Table 1). Glass Pasteur pipettes (146 mm, Fisher Scientific, USA) were used as stimulus odor cartridge for our SSR study. A piece of filter paper (5 × 30 mm, Whatman No 1, USA), loaded with 10 µl of a test solution, was inserted into a glass Pasteur pipette (Fig.1).

Figure 1. Glass Pasteur pipettes used to deliver stimulus volatiles to the antennae, each containing a piece of filter paper strip impregnated with a test compound or a mixture.

The tip of the pipette was inserted into a small hole in the main glass tube where a continuous charcoal-filtered and humidified airflow (500 - 600 ml/min) was blown over antennal preparation. In order to stimulate the antenna, a 0.1-s pulse of airflow (10 ml/s) was injected through the wide end of Pasteur pipette using an electronic airflow controller (C5-55, Syntech, The Netherlands). The test pipette was renewed after being used five times. The wide end of the pipette was wrapped with aluminum foil to reduce the evaporation.

Table 1. Test compounds for the SSR study of *Trissolcus basalis* and their purity.

Mixture groups	**Compound**	**Chemical purity (%)**
Mixture A	2-Methylbutanoic acid	98
Buckwheat plant volatiles	3-Methylbutanoic acid	99
	Hexanoic acid	≥99
	α-Farnasene	≥96
	p-Benzoquinone	≥98
	(*Z*)-3–Hexen-1-yl acetate	98
	Butanoic acid	≥99
Mixture B	1-Nonanol	98
Common plant volatiles	Geraniol	98
	(±)-Linalool	97
	2-Phenylethanol	99
	Benzldehyde	99.5
	Citral (geranial + neral)	96
Mixture C	(*E*)-β-Caryophyllene	98.5
Common plant volatiles	Germacrene-D	40
	Geranyl acetate	98
	(*Z*)-3-Hexen-1-ol	98
	(*E*)-2-Hexenal	98

2.3. Gas chromatograph-linked electroantennographic detection

GC-EAD recordings were carried out using female *T. basalis* and headspace extracts of buckwheat plants. After anaesthetizing a female wasp with CO_2, the head was isolated from the body. A glass capillary filled with electroconductive gel (Spectra 360, Parker Laboratories Inc., Orange, USA) was used as the reference electrode, and another gel-filled glass capillary was used as the recording electrode after drawing to a fine point using a microelectrode puller (PP-830; Narishige, Japan).

The electroconductive gel of the reference electrode was brought into contact with the neck of the isolated head, while the recording electrode was connected to the distal end of the antenna after a small terminal part of the last flagellomere was severed off using microscissors. The antenna was positioned in the middle of airstream blown from a glass tube delivering the GC effluent with the airstream to the antenna. The position of the electrodes was controlled by using micromanipulators (Leitz, Leica, Germany and P-225, Sutter Instruments, USA). The electrophysiological responses of *T. basalis* antennae were monitored using a high input-impedance AC/DC probe, a data acquisition controller (Type IDAC-4) and software Autospike 32 (Syntech, Hilversum, The Netherlands). One µl of the buckwheat headspace extract was injected into a GC (Agilent 7890A, Agilent Technologies, USA) with a flame ionization detector (FID) and a split/splitless injector. A capillary column (30 m x 0.25 mm i.d. x 0.25 µm DB-5, Agilent Technologies, USA) was used for the analyses.

The GC oven temperature was programmed from 60°C (held for 1 min) to 240°C at 10°C min-1. Helium was used as the carrier gas. The column effluent was split 1:1 with one part going to the FID of the GC and the other through a heated transfer line into a humidified and charcoal-filtered airstream (600 ml min-1) directed at the antennal preparation. The volatile chemical compounds in the headspace extracts of buckwheat plant were identified using a gas chromatograph-mass spectrometer (GC-MS) (GCMS-QP2010, Shimadzu Corporation, Japan) as described in Rostás *et al.,* (2015). The compounds in the extracts were tentatively identified using GCMS solution v. 2.72 software (Shimadzu Corporation, Japan) with NIST 11 and Wiley 10 mass spectral libraries, and the software MassFinder4/Terpenoids library (Hochmuth Scientific Software, Germany). Standards that were commercially available (Sigma-Aldrich, Australia) were used to confirm the identities of EAD-active compounds.

2.4. Single sensillum recording

To prepare a whole-body preparation for SSR study, a female *T. basalis* was mounted on a Plasticine block with U-shaped thin copper wire restrains. The antennae were further fixed gently using finer copper wires to immobilize them. Then, the insect preparation was positioned in the middle of the charcoal-filtered and humidified main air stream. Two kinds of electrodes were used. The reference electrode which was inserted into the abdomen was a fine tip (tip diameter < 10 µm) glass electrode (0.86 mm ID, A-M systems Inc., USA) filled with 0.1 M saline solution while the recording electrode was an electrochemically sharpened tungsten electrode, inserted in the sensillum (tip diameter < 0.1 µm) (Fig. 2).

The position of the electrodes was controlled with micromanipulators (Leitz, Germany and MP-225, Sutter Instrument, USA). The electrical signals were amplified through a preamplifier, digitized at 12,000/s sampling rate, processed with a PC-based signal processing system (IDAC-4, Syntech, The Netherlands) and analyzed using software (Autospike 32, Syntech, The Netherlands).

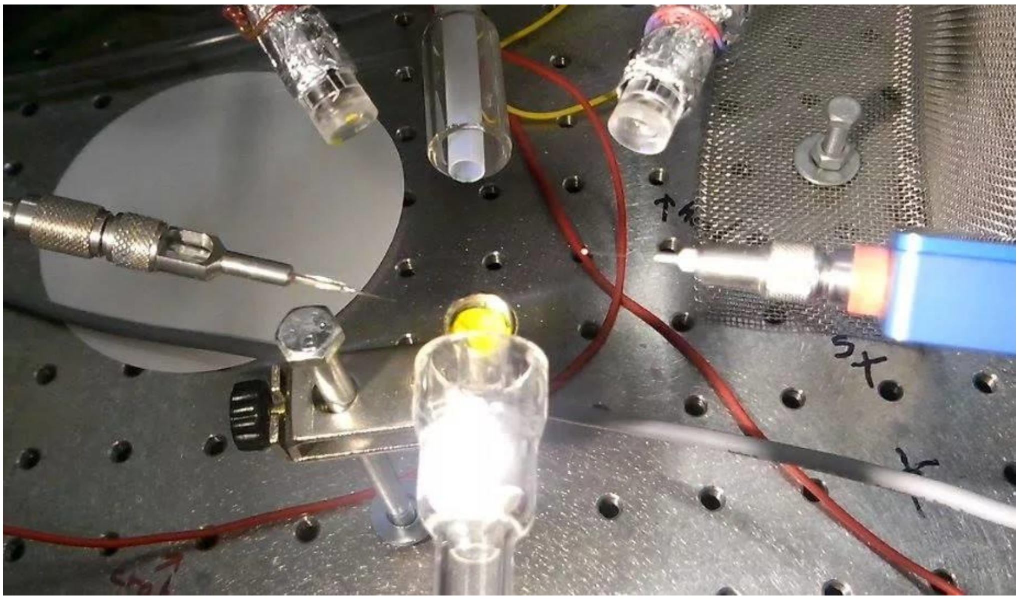


Figure 2. The reference and recording electrodes used in SSR recordings.

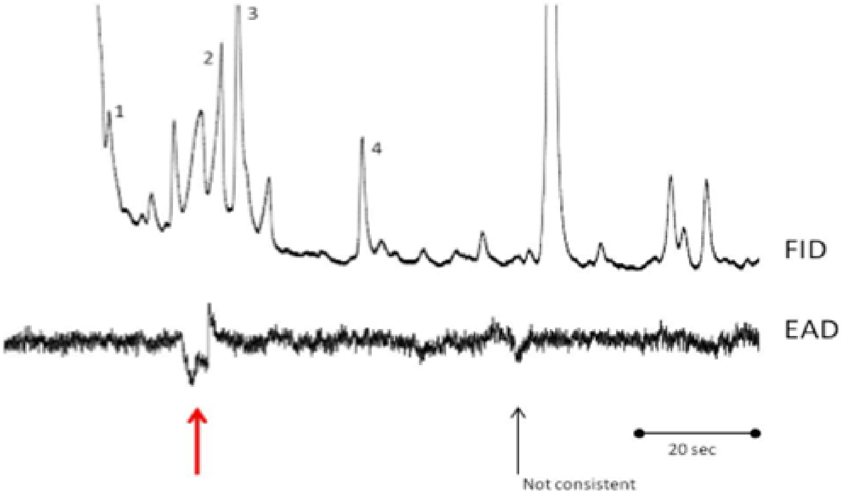
Once a stable electrical contact was made between the electrodes and a sensillum and a spontaneous firing of action potentials was detected, the antenna was stimulated with a series of three mixtures of test compounds (Table 1). If any mixture elicited an electrophysiological response after the stimulation, the compounds contained in the mixture were tested individually and randomly.

Time interval between two successive stimulations was approximately 30 seconds. In this study, the response profiles of 43 sensilla present in different parts of the antennae of female *T. basalis* were investigated from 30 individuals. The responses of the ORNs in these sensilla were analyzed by comparing the number of the action potentials between before and after the odor stimulations. The number of spikes generated for 1 s after stimulation was subtracted by the average number of spikes for 1 s before the stimulation. Then, the sensilla and ORNs were classified into different types, according to their response profiles.

3. Results

3.1. Identification of EAD-active compounds

Although several overlapping GC peaks were present in a narrow time band, GC-EAD responses to buckwheat plant headspace extracts appeared to indicate that two compounds, corresponding to 3-methylbutanoic acid and 2-methylbutanoic acid, respectively, elicited consistent responses from *T. basalis* antennae (Fig. 3 red arrow). The tentative assignment of these two EAD-active compounds was based on their retention index and the slow increasing slope of these two aliphatic acids which is typical FID signal in GC analysis of aliphatic acids using DB-5 capillary column.



Peak	Retention time	Compound
1	1.412	Butanoic acid
2	1.742	3-Methylbutanoic acid
3	1.784	2-Methylbutanoic acid
4	2.145	Pentanoic acid

Figure 3. Flame ionization detector (FID) and electroantennographic detector (EAD; *Trissolcus basalis* antenna) responses to headspace volatiles from buckwheat plants. Two distinct EAD responses, corresponding to the compounds #2 (3-methylbutanoic acid) and #3 (2-methylbutanoic acid), respectively, are indicated in a red arrow.

3.2. Overall profiles of sensilla and ORNs

The ORNs examined in the antennae of *T. basalis* females showed average spontaneous firing of action potentials at 1,554 ± 0,614 spikes/s (mean ± SE, *n* = 110, minimum 28, maximum 30). The solvent control showed no activities on any of the ORNs tested.

The plant volatiles mixtures were tested on the 67 sensilla exhibiting spontaneous firing of action potentials in the female *T. basalis* antennae examined. Among the 67 sensilla, 56 sensilla were found to contain ORNs responsive to at least one mixture whereas 11 other sensilla were not responsive (NR) to these compounds (Table 2). The 67 sensilla could be classified into six different classes, depending on their response profiles to three mixture groups tested (Table 2).

Among the three mixtures tested, mixture A (mixture of buckwheat plant volatiles) elicited significant responses from 55 sensilla, comprising > 98% (55 out of 56) of the entire responsive sensilla population.

Among them, 44 sensilla exhibited exclusive responses to Mixture A. No sensilla were responsive exclusively to Mixture B, and only one sensillum was exclusively responsive to Mixture C (Table 2, Class C sensilla). Eleven other sensilla showing responses to Mixture B or Mixture C also responded to the Mixture A.

Table 2. Sensillum classes identified in *Trissolcus basalis* females according to their responsiveness to three mixtures of plant volatile compounds: A, B and C. *Hexane was used as control solvent.

Mixtures	**Sensillum class and number observed**							**Total**
	NR	**A**	B	C	AB	AC	ABC	
	11	44	0	1	4	3	4	67
Hexane*								0
Mixture A		●			●	●	●	55
Mixture B			●		●		●	8
Mixture C				●		●	●	8

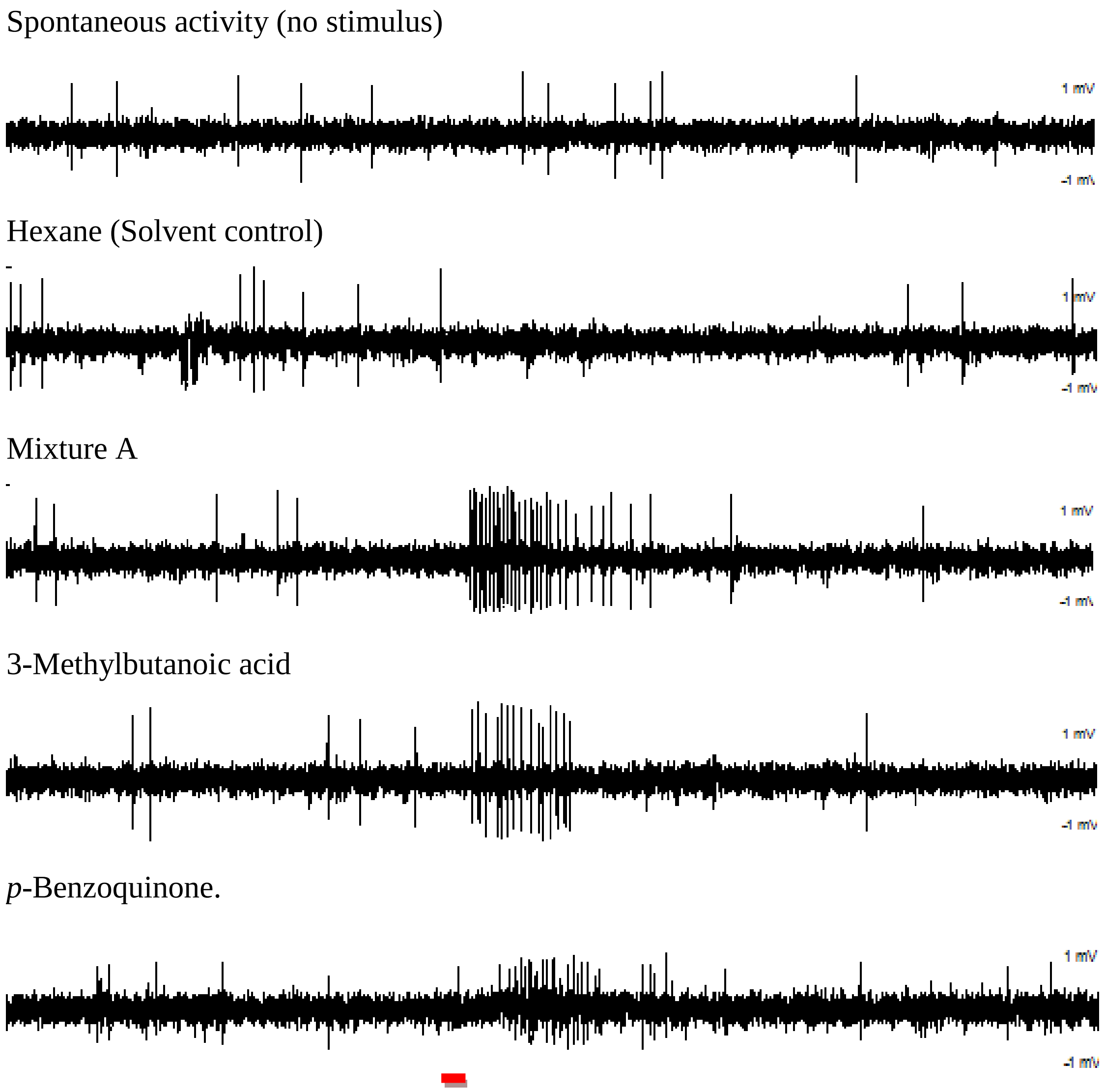
When the responses of ORNs in the 44 sensilla, exclusively responsive to Mixture A to the individual compounds contained in the mixture A, were further tested, clear responses of ORNs to some of the individual compounds could be observed from 32 sensilla.

The other 12 sensilla were either non-responsive (NR) to the individual compounds or did not last responses long enough to test all the individual compounds. The 32 sensilla containing ORNs responsive to the individual buckwheat volatile compounds could be classified into 13 class (A1 to A13), based on their response spectra to these compounds (Table 3).

Among the seven buckwheat volatiles compounds tested, some of four compounds (*p*-benzoquinone, 2-methylbutanoic acid, 3-methylbutanoic acid and butanoic acid) elicited significant responses from the ORNs present in 30 sensilla, comprising 93.8% (30 out of 32) of the sensilla examined (Table 3).

In contrast, three other buckwheat volatile compounds (α-farnesene, (*Z*)-3-hexenyl acetate and hexanoic acid) elicited mild responses from three sensilla: α-farnesene and (Z)-3-hexenyl acetate from only one sensillum, respectively, and hexanoic acid from two sensilla. These ORNs exhibited varying degree of responsiveness to the corresponding active compounds.

Two compounds, 3-methylbutanoic acid and *p*-benzoquinone, were the most active compounds, eliciting consistent and significant responses from the largest proportion of the ORNs (Table 3, Fig. 4). The ORNs present in the most abundant class of olfactory sensilla, A6, were responsive to both *p*-benzoqinone and 3-methylbutanoic acid, whereas the ORNs present in the next two most abundant classes of sensilla (A3 and A7) were responsive only to *p*-benzoquinone or 2-methylbutanoic acid and 3-methylbutanoic acid, respectively (Table 3). In our experiment, all the active compounds on *T. basalis* ORNs were excitatory, increasing the number of action potentials after stimulation in their corresponding ORNs.



Spontaneous activity (no stimulus)

Hexane (Solvent control)

Mixture A

3-Methylbutanoic acid

p-Benzoquinone.

Figure 4. Examples of the responses of ORNs present in a Class A6 sensillum of female *T. basalis* antenna, indicating the number of action potentials were increased after stimulation with Mixture A, 3-methylbutanoic acid or *p*-benzoquinone. Each trace shows an SSR trace recorded for 5 s. Red bar below the trace indicates the timing of stimulation with corresponding compound for 0.1 s.

Table 3. Sensillum classes identified in *Trissolcus basalis* females according to their responsiveness to individual compounds of mixture A; Buckwheat volatile compounds. *Hexane was used as control solvent.

Compounds	**Sensillum class and the number observed**													Total number of responsive sensilla per compound
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	
	1	1	5	2	1	**7**	5	3	2	2	1	1	1	
hexane														0
α-farnasene	•											•		2
(Z)-3–hexen-1-yl acetate		•												1
hexanoic acid												•		1
***p*-benzoquinone**			•			•			•	•	•		•	**18**
2-methylbutanoic acid				•			•		•		•		•	11
3-methylbutanoic acid					•	•	•	•	•	•		•	•	**22**
butanoic acid								•		•	•	•	•	8

In our study, all ORNs showed narrow response spectra, exhibiting responses only to one or a few compounds among the buckwheat compounds tested.

4. Discussion

Current GC-EAD study indicates that the antennae of female *T. basalis* can detect two volatile compounds, 2-methylbutanoic acid and 3-methylbutanoic acid, with relatively high sensitivity. Although poor separation between these two compounds on the GC column (DB-5) used and the presence of several compounds co-eluting within a narrow time band made it difficult to designate the two EAD-active peaks precisely as these two compounds, our subsequent SSR study showing the presence of major population of ORNs sensitive to 2-methylbutanoic acid and 3-methylbutanoic acid support this idea. These two compounds are main components of volatile emanations of buckwheat flowers (Foti *et al.*, 2016).

This study clearly indicates the presence of antennal ORNs in female *T. basalis* for detecting plant volatile compounds. These ORNs appear to be highly specialized for detecting a limited range of plant volatile compounds such as 2-methylbutanoic acid, 3-methylbutanoic acid, p-benzoquinone and butanoic acid, and the majority of ORNs of female *T. basalis* examined in this study appeared to be specialized for these compounds. It is interesting that only small proportion of olfactory sensilla in female *T. basalis* contained ORNs responsive to general plant volatiles that are present in common across many plant species, which may suggest that *T. basalis* have relative poor sense of smell for common plant volatiles whereas they develop highly sensitive olfactory sensory system for a limited range of volatiles produced by specific groups of plants including buckwheat. This kind of specialization may be efficient for insects like *T. basalis* having limited spatial capacity for harboring different types of olfactory sensilla. The presence of different classes of ORNs, each of which has distinct response spectra across these four volatile compounds, suggests that female *T. basalis* are able to distinguish the compounds from each other, and this may be useful for *T. basalis* to locate buckwheat plants at the right stage and discriminate them from other plants. We have only investigated the responsiveness of ORNs in female *T. basalis*, and it would be interesting to examine the profiles of ORNs in male *T. basalis* to see if there is any sexual dimorphism in the olfactory sensory system regarding plant-volatile detection.

This work represents the first exploration of olfactory perception at sensilla level for a tiny egg parasitoid. The single sensillum recording technique demonstrated clearly that *T. basalis* have particularly strong and consistent responses to buckwheat flower volatiles.

Based on these results, flowers can be considered as the critical part in buckwheat plant, responsible for the olfactory attraction of female parasitoids. This is consistent with previous olfactometer bioassays that revealed the *T. basalis* female preference for the buckwheat flowers over the stem (Foti *et al.,* 2016).

Buckwheat plant proved a great potential as “companion plant" and management option in orchards by enhancing the effectiveness of insect parasitoid for pest biocontrol (Stephens *et al.,* 1998; Austin and Dowton, 2000). It has been commonly used in manipulation programs (Heimpel and Jervis, 2005). Previous studies showed that planting buckwheat generates a better abundance of parasitoids (Platt *et al.,* 1999), improved sex ratios (Berndt *et al.,* 2002) and parasitism rates (English-Loeb *et al.,* 2003, Al-Doghairi and Cranshaw, 2004; Tylianakis *et al.,* 2004), and, therefore, it leads to lower pest abundance (Nicholls *et al.,* 2000). Rahat *et al.,* (2005) explored the potential of buckwheat nectars provision in maximizing *T. basalis* effectiveness. In laboratory experiments, the longevity of adult wasp females provided with excised buckwheat plant shoots bearing inflorescences was increased compared to the longevity of *T. basalis* on inflorescence-free shoots. Taken together, the current study suggests that the egg parasitoid’s exploitation of buckwheat plants can be enhanced by the presence of the olfactory sensory system specialized for buckwheat flower volatiles. For nectar source location, plant volatile compounds are detected by the olfactory system, which helps the insect parasitoids locate the source. The behavioral mechanism of *T. basalis* relating to nectar and host plant location is not well understood although their host location behavior is relatively well known.

Understanding the antennal olfactory perception and the identification of buckwheat plant active compounds stimulating ORN’s in *T. basalis* species have been the major outcome of this work, which could lead to new chances in improving the biological control of *N. viridula* population since it might be possible to exploit the active volatile compounds of buckwheat plant to attract *T. basalis* and to enhance its activity in the field. The olfactory-active compounds eliciting significant electrophysiological responses on the parasitoid antennae were identified, and the specificity of *T. basalis* ORNs to different of individual volatile compounds produced by buckwheat was demonstrated in GC-EAD and SSR experiments. Additional detailed study at single sensillum level such as dose response recordings and examining male parasitoids’ ORN responses would be useful.

Studies on the behavioral activities of the ORN-active compounds would also be interesting since this will help advance our knowledge about the mechanisms underlying the wasp females' sensory physiology and chemical ecology. Further trials of habitat management using buckwheat plant rows is required to understand whether extra-floral nectaries and buckwheat flowers are located and how buckwheat plant volatile compounds are exploited by the parasitoid under semi-field and field conditions. The knowledge gained from these experiments will ultimately benefit the development of the green stink bug *N. viridula* management strategies and its conservative biological control by attracting and retaining populations of *T. basalis*.

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Chapter 4

Chapter 4

Concluding remarks and future perspectives

Understanding the mechanisms governing host and food-foraging by insect parasitoids is essential to manage their environment for an enhanced biological control program where they can be effective as biological control agents. It is well-known that, during host and food resources location process, parasitoids rely heavily on chemical cues, existing in the complex olfactory landscape in which they live (Lewis and Takasu 1990; Wyatt 2003). In this respect, the present thesis studied the chemo-reception of two egg parasitoid species perceiving infochemicals in a context of host and food location process. Throughout three years of research, experiments were conducted for two main purposes; the first one was to investigate the learning abilities of *Trissolcus brochymenae* (Ashmead) (Hymenoptera: Scelionidae) to substrate-borne semiochemicals during host location process, while the second aimed to understand the role of the flowering plants as food resources on foraging behavior of *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae).

This thesis is divided in three main chapters. The first chapter represents a bibliographic research about the chemo-orientation of insect parasitoids by focusing mainly on egg parasitoids. Indeed, morphological, chemical and behavioral approaches leading their foraging strategies were elucidated.

In the second chapter, it has been shown that the females of *T. brochymenae* parasitoids adjusted their innate behavioral responses to their host *M. histrionica* footprints over natural substrates as result of previous experience; this implies that their learning ability contributes in host location process upon landing on host-infested plants. Moreover, it was clarified how rewarded and unrewarded experience affected the instinctive behavior of the parasitoid females and how the wasp females responded to the elapsed time between two successive unrewarded events according the host-gender. In the future it is very interesting to conduct chemical investigations in order to identify the chemical differences between *M. histrionica* males and females footprints over the substrate and to recognize the involved compound(s) in host gender discrimination by the egg parasitoid.

The third chapter has contributed to understand plant volatiles perception in *T. basalis* females. The major active compounds at sensilla level and the existence of a major group of ORNs specialized for buckwheat volatile compounds were determined. These results confirm the wasp ability to respond to the plant volatiles, as a blend or as individuals, and to exploit these olfactory cues in locating the available food sources. Further experiments on electrophysiological and behavioral activities of the ORN-active compounds would be useful for future work, such as dose responses experiments and evaluation of the behavioral significance on these compounds under semi-field and field conditions.

In foraging strategies (food and host), the perception and the use of chemical cues by parasitoids should be well understood because this knowledge is important and would improve the natural enemy effectiveness in the field and contribute to implement successful biological control strategies using semiochemicals.

In general, the present findings have led to a better understanding of the perception of indirect host-related cues by the egg parasitoids *T. brochymenae* under a tritrophic context. A particular knowledge has been gained in relation to the role of experience in the behavioral response of the wasp to these cues. In agro-ecosystem learning may reinforce the behavioural responses of parasitoids' females to find their hosts, as consequence it could be relevant in pest control process and it may bring significant adjustments in the associated tactics within pest management programs.

In order to ensure their reproduction and survival, insect parasitoids rely not only on their host volatiles but also on floral plant volatiles for plant nectar location. In fact, it has been proven that a sustainable and efficient biological pest control is based on habitat management through providing or/and improving floral resources within a primary crop. Foraging behaviour in the parasitoids attraction to the resources offered by flowering plant is known to be guided upon the detection of some specific key volatile compounds, therefore detection of EAD and ORN-active buckwheat volatile compounds in the electrophysiological experiments represent a basic knowledge that can support the manipulation of *T. basalis* foraging behavior of in the future.

Finally, understanding how buckwheat plants provision enhances the wasp fitness, abundance and recruitment in the field, with an assessment of the pest risk, may represent a further step in the biological control of the green sting bug. In addition, field evaluation of “Attract and reward” biological control strategy by combing buckwheat as nectar plant rewards with a long range infochemical attractant can achieve promising results.

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