Olfactory information use for foraging in *Microplitis mediator*, a parasitoid of the cabbage moth *Mamestra brassicae*

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SUMMARY

My PhD project was at the interface between behavioural ecology and biological control, two disciplines that do not easily intertwine because of their divergent aims. On one hand, behavioural ecology is a fundamental science that seeks to understand animal behaviour from an evolutionary perspective and generally works from the point of view of the individual. On the other hand, as an applied science, biological control seeks to elaborate concrete strategies to improve pest control and works from population and community perspectives. However, the success of biological control methods depends on the behaviour of biological control agents, *i.e.* natural enemies of target pests, which creates a perfect opportunity for behavioural ecology and biological control to work hand in hand. In this work, I studied the foraging behaviour of the larval parasitoid *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae) to improve the control of its host the cabbage moth *Mamestra brassicae* (Linnaeus) (Lepidoptera: Noctuidae), which is an important cabbage pest distributed throughout Europe and Asia. Information about the biology, the life cycle and the rearing method of *M. mediator* and *M. brassicae* is detailed in chapter 1.

Parasitoids that complete their development inside or on the body of their host, eventually killing it, are the most important group of natural enemies in the context of biological control of insect pests. Most parasitoid species feed on (extra-) floral nectar as adults to ensure their survival. However, nectar is usually lacking in crops due to the scarcity or absence of flowering plants, causing a major problem to parasitoids that must travel outside the crops (*i.e.* far from the target pests) to find food sources. To palliate this problem, floral subsidies can be added as wildflower strips along field margins or as companion plants inside the crops to attract the parasitoids that are present in the vicinity and to increase their lifespan and fecundity (*i.e.* their pest suppression potential). The selection of these floral subsidies must be based on different criteria, such as their suitability as a food source for the parasitoids, their attractiveness and their selectivity in favouring the parasitoids but not the pests. Flower attractiveness is critical for food foraging parasitoids and is predicted to have an impact on parasitoid population dynamics. Olfactory attractiveness is especially important to attract parasitoids from the neighbourhood because floral scents can travel, contrary to visual cues, and therefore constitute long-range cues.

In a first study (chapter 2), I tested the olfactory attractiveness of flowers/inflorescences of five wildflowers species (bishop's weed, cornflower, buckwheat, candytuft, and oregano) to naive female *M. mediator*. I conducted choice tests in a Y-tube olfactometer to test the olfactory attractiveness of flowers/inflorescences against air and the relative attractiveness of the flower species offered in paired choice. I showed that all the flower species were highly attractive to female *M. mediator* when tested against air, but that in paired choice tests cornflower and candytuft were as attractive as each other and both more attractive than buckwheat. This indicates that *M. mediator* is able to use olfactory cues to identify potential food sources and has evolved preferences that could be exploited in biological control. In particular, this study has shown that cornflower is a very promising floral subsidy.

However, in a patchy and unpredictable environment, not all food sources are equally abundant and/or near, and parasitoids are expected to have evolved capacities to detect the most promising food sources in terms of proximity and/or abundance. In a second study (chapter 3), I tested whether female *Microplitis mediator* foraging for food sources, *i.e.* flowers of cornflower and

inflorescences of buckwheat, were able to use quantitative olfactory information to orient themselves towards the most promising (*i.e.* most abundant and/or closest) food sources. I conducted behavioural assays in a 6-arm olfactometer where groups of six wasps were released and faced with a gradient of volatile concentration created by using different numbers of flowers/inflorescences as odour sources. I also collected and analyzed the volatiles emitted by different numbers of flowers/inflorescences of the two flower species. The results showed that female *M. mediator* were able to use quantitative olfactory information. In general, they were most attracted to the highest numbers of flowers/inflorescences, which also emitted the highest volatile quantities. However, the response of the wasps towards the two flower species differed. The contrast between the different numbers of flowers/inflorescences was important for the wasps to be able to discriminate and had to be higher with cornflower than with buckwheat. With cornflower, the flower species emitting both substantially higher absolute volatile quantities and more potentially attractive types of volatile compounds (*e.g.* benzenoïds), the response of the wasps to single flowers was very strong already and showed saturation with increasing numbers of flowers. Conversely, with buckwheat, the flower species emitting low volatile quantities, the response of the wasps to few inflorescences was weak but accelerated with increasing numbers of inflorescences. This suggests that a higher sensitivity at low volatile quantities than at high volatile quantities could have been selected in *M. mediator*, which would be adaptive. These results highlight the importance of taking flower density into account to optimize the use of floral subsidies for biological control purposes. In particular, these results suggest that cornflower should be attractive at low densities whereas higher densities of buckwheat could be needed to attract *M. mediator* in the field.

To conclude, my work has shown that *M. mediator* is well adapted for food foraging, because it can use both the quality and the quantity of olfactory information to localize potentially rewarding food sources. I also demonstrated that studying the foraging behaviour of a parasitoid provides relevant information that can be exploited to improve its use for biological control.

TABLE OF CONTENTS

GENERAL INTRODUCTION

At first glance, behavioural ecology and biological control appear to be two very distant fields. On one hand, behavioural ecology is a fundamental science that seeks to explore the relationships between behaviour, ecology and evolution (Danchin et al., 2008). Behavioural ecology provides an evolutionary perspective on behaviour and generally works from the point of view of the individual (Roitberg, 2004). On the other hand, biological control is traditionally defined as "the action of parasites, predators, or pathogens in maintaining another organism's population density at a lower average than would occur in their absence" (DeBach, 1964). As an applied science, it seeks to elaborate strategies to solve concrete pest management problems and works from population and community perspectives (Roitberg, 2004). However, the success of biological control depends on the behaviour of the so-called “biological control agents”, which are natural enemies of the target pest. Given the wide range of behaviours that natural enemies can express, behavioural ecology is needed to predict when behavioural variations will occur and how to exploit them (Roitberg, 2007). Conversely, insect pest management offers behavioural ecologists a perfect research framework because many fitness-related parameters can be readily measured for insects in the laboratory and in the field, contrary to mammals and birds (Roitberg, 2007).

Natural enemies of insect pests include predators that must consume several preys to complete their development, pathogens (bacteria, fungi and viruses), parasites (entomopathogenic nematods living in the soil) and antagonists (competitors) (Mills and Wajnberg, 2008). But the most important group of natural enemies in the context of biological control of insect pests are the parasitoids, whose adult females lay their eggs in or on other insects (Mills and Wajnberg, 2008). The parasitoid larvae develop by feeding on the host bodies, resulting in the death of the host, which confers parasitoids a certain degree of host specifity (Murray et al., 2010). The behaviour of female parasitoids is a key determinant of their effectiveness as biological control agents (Lewis et al., 1990). In fact, female parasitoids have to complete several foraging tasks during their adult life to maximize their lifetime reproductive success, which include searching for suitable food sources, for a mating partner, and even more importantly for suitable hosts (Hilker and McNeil, 2008). Their efficiency in localizing and parasitizing hosts and their retention in target areas will have direct consequences on host-parasitoid population dynamics, and hence on the biological control of insect pests (Mills and Wajnberg, 2008; Van Lenteren, 1986). This is why parasitoids constitute an ideal biological model for conducting research in the context of both behavioural ecology and biological control.

My PhD project stemmed from a larger applied project aiming at enhancing the biological control of cabbage pests through the use of functional biodiversity, *i.e.* the part of the naturally occurring biodiversity providing desired ecosystem services (Moonen and Bàrberi, 2008). The use of functional biodiversity for insect pest management purposes can be achieved through conservation biological control. This particular type of biological control aims at favouring natural enemies by providing them with limiting resources or mitigating adverse factors through habitat manipulations or alteration of crop production practices (Ehler, 1998; Landis et al., 2000; Naranjo, 2001). Parasitoids in particular are often limited in agricultural systems by the availability of essential resources such as (extra-) floral nectar, which is a food source for most of them (Hogg et al., 2011). The idea behind the project in which my work was included was to add floral subsidies that would constitute additional food sources for the parasitoids in cultures of white cabbage *Brassica oleracea* var. *capitata* L. Although cabbage cultures are under the pressure of a variety of lepidopteran pests (Ahuja et al., 2010; Poelman et al., 2008) that are all attacked by specific parasitoids, I chose to restrain my work to the parasitoid *Microplitis mediator* (Haliday). My choice was driven by the scarcity of publications available on this parasitoid species compared to other parasitoids of cabbage pests, such as *Diadegma semiclausum* (Hellen), a parasitoid of the diamondback moth *Plutella xylostella* (Linnaeus) that has to date already been intensively studied (Abbas, 1988; Gichini et al., 2008; Huang et al., 2009; Kwon et al., 2003; Momanyi et al., 2006; Ohara et al., 2003; Winkler et al., 2006). Moreover, *M. mediator* is a generalist parasitoid that has been reported on 40 different hosts within the Noctuidae superfamily (Mir Khan, 1999) and has therefore the potential to be used to control different pest species.

Floral subsidies can be added as wildflower strips planted along the margin of cabbage fields and/or as companion plant planted inside the field near the cabbage plants (Pfiffner et al., 2003; Ponti et al., 2007). The goal is to increase parasitoid density by attracting and retaining parasitoids that are present in the vicinity of the field and to increase the performance of the parasitoids (*i.e.* their pest-suppression potential) by enhancing their longevity and fecundity (Mills and Wajnberg, 2008). The suitability of floral subsidies for conservation biological purposes is based on several criteria like nectar accessibility in relation to parasitoid mouthpart morphology, nectar suitability (*i.e.* absence of toxicity), nectar quality (*i.e.* nutritional value), flower attractiveness (*i.e.* detectability in terms of olfactory and visual cues) (Wäckers, 2004) and selectivity in favouring parasitoids rather than pests (Lavandero et al., 2006). While Geneau et al. (2012) investigated the selective effects of several wildflower species on the longevity and fecundity of *M. mediator* and its host the cabbage moth *Mamestra brassicae* (Linnaeus), I focused on the attractiveness of the wildflower species to *M. mediator*. Flower attractiveness is crucial in determining the encounter rate of a parasitoid with a flower (Wäckers, 2004) and is predicted to have an impact on parasitoid population dynamics

(Bianchi and Wäckers, 2008). It was therefore an ideal opportunity to conduct research in behavioural ecology with an applied perspective.

In my first chapter, I summarized the information available about the biology of *M. mediator* and its host *M. brassicae* and presented the laboratory rearing protocols used to produce the insects for my experiments. While the *M. brassicae* rearing already existed at the start of my PhD project, I had to develop the rearing of *M. mediator* and to optimize the protocol in order to produce maximum numbers of insects with a minimum time investment. I collected data on parasitoid pupation rates under different host densities and discussed further possibilities for increasing parasitoid harvest rates. I also checked that parasitoid eclosion rates were high and that the sex ratio in the rearing was not male-biased, which could lead to the collapse of the rearing on the long-term (Zhou et al., 2007). Finally, I discussed the advantages of rearing the parasitoids on cabbage versus on an artificial diet in the light of potential behavioural alteration of parasitoids reared in artificial conditions (Gandolfi et al., 2003).

In my second chapter, I investigated the attractiveness of several wildflower species that were suitable candidates for conservation biological purposes. I focused on the olfactory attractiveness of the flowering plants because volatile cues can guide parasitoids to their target over long distances (Hilker and McNeil, 2008), which is required to attract parasitoids that are present in the vicinity of cabbage fields. Parasitoids are also known to use visual cues to locate flowers (Begum et al., 2004). Visual cues may be more reliable than olfactory cues, which are directly affected by abiotic factors such as wind speed, temperature or air pressure (Farré-Armengol et al., 2013; Hilker and McNeil, 2008). But visual cues can be blocked by physical barriers (Turlings et al., 1993), and like contact cues, they act as a guide over shorter distances (Fellowes et al., 2005). I therefore focused on olfactory cues and investigated the attractiveness of different floral scents to food-deprived female *M. mediator* in choice tests in a Y-tube olfactometer. I first tested the olfactory attractiveness of flowers/inflorescences against an air control and then conducted pair-wise comparisons between the different attractive floral scents. I could identify the most attractive flower species and tied my results to the findings of the parallel study of Géneau et al. (2012) about the effects of these flower species on the survival and fecundity of *M. mediator*. This allowed me to discuss the behavioural response of the wasps from an evolutionary perspective and to highlight the potential importance of my findings for conservation biological control.

In my third chapter, I used two flower species that differed in attractiveness in my second chapter to test whether food-deprived female *M. mediator* were able to use quantitative olfactory information in order to orient themselves towards the most promising food sources. I conducted behavioural assays in a 6-arm olfactometer where groups of six wasps were released and faced with a gradient of volatile concentration. I created the gradient of volatile concentration by using different

numbers of flowers/inflorescences as odour sources. I tested whether the contrast between the different numbers of flowers/inflorescences was important for the wasps to be able to discriminate between them and whether the response of the wasp was the same for the two flower species. In another experiment, I collected and analyzed the volatiles emitted by different numbers of flowers/inflorescences of the two flower species. I searched for qualitative and/or quantitative differences between the two floral scents and tested whether volatile quantity correlated with the number of flowers/inflorescences. I proposed hypotheses based on the floral volatile analyses to explain the behavioural responses of the wasps towards the two flower species and their difference in attractiveness. My reflexion was mostly focused on the point of view of the female parasitoids and how they should use quantitative olfactory information to forage for food efficiently. I nevertheless highlighted how this individual behaviour could have an impact on the use of floral subsidies for conservation biological control purposes.

To conclude, I summarized all my findings and discussed them again in the context of behavioural ecology and biological control.

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CHAPTER 1

Rearing of *Microplitis mediator* (Hymenoptera: Braconidae) and its host *Mamestra brassicae* (Lepidoptera: Noctuidae)

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Abstract

Establishing continuous and reliable laboratory colonies of pest-parasitoid systems is a base to perform manipulated experiments for biological control. Here we present the rearing protocols that we developed for an efficient rearing of the cabbage moth *Mamestra brassicae* and its key parasitoid *Microplitis mediator*.

1. Introduction

The cabbage moth *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae) is one of the major cabbage pests in Europe (Finch and Thompson, 1992). Its larvae are attacked by braconid wasps (Johansen, 1997), among which the larval parasitoid *Microplitis mediator* (Haliday, 1834) (Hymenoptera: Braconidae) is the most important one (Lauro et al., 2005). Establishing continuous and reliable laboratory colonies of these two species is important for biological control purposes. Firstly, regular supply of *M. brassicae* and *M. mediator* is necessary to conduct experiments on these species, which should provide more insight into how to promote *M. mediator* while deterring *M. brassicae* (Belz et al., 2013; Bianchi et al., 2005; Géneau et al., in press; Géneau et al., 2012; Lauro et al., 2005; Luo et al., 2010; Pfiffner et al., 2003). Secondly, mass rearing of *M. mediator* is a prerequisite for inundative releases. Here, we present rearing protocols for this pest-parasitoid system.

2. *Mamestra brassicae* rearing

2.1. Biology of *M. brassicae*

The cabbage moth *M. brassicae* is widely distributed throughout Asia and Europe (Johansen, 1997). Its polyphagous larvae feed mainly on cabbage (*Brassica oleracea* L., Brassicaceae), but other plants in the *Brassica* genus, as well as tomato, beet, onion, and some flowers or forest trees, are known as alternative host plants (Hill, 1987). The moths fly only at twilight and during the night, and hide under the leaves during the day. In central Europe, *M. brassicae* can have two generations per year, with the second one being the most damaging for the crops. The adult moths of the first generation emerge in May and June, and those of the second generation in July and August. The larval stages (and thus the damage) follow in June and from August until October (Hill, 1987). The females lay their eggs in clusters on the underside of the cabbage leaves. Larvae emerge after approximately seven to ten days (up to two weeks at lower temperatures). In instars I to IV, they are found on the outer leaves in which they make holes and eat the veins. They become photophobic in instar V and hide in the core of the cabbage head (Johansen, 1997) where they cause the economically most severe damage through the accumulation of faeces and the development of mould around the feeding holes (Fortmann, 2000). Pupation takes place in the soil at 3-5 cm depth, and diapausing pupae are the overwintering stage (Johansen, 1997). The egg and larval stages of *M. brassicae* both have their natural enemies. The egg stage suffers from predation by carabids and syrphids and is also parasitised by parasitoids from the *Trichogramma* and *Telenomus* genera

(Pfiffner et al., 2003). The larval stages are mainly parasitised by endoparasitoids from the Braconidae family like *M. mediator* and *Aleiodes sp.* (Johansen, 1997; Pfiffner et al., 2003), but parasitism by some Ichneumonidae (Pfiffner et al., 2003) and even by an ectoparasitoid from the Eulophidae family (Van de Veire, 1993) have also been reported. There is to our knowledge no information available about pupal predation or parasitisation, which does not mean that it does not occur.

2.2. Rearing protocol

The *M. brassicae* laboratory colony was established at FiBL (Frick, Switzerland) in 2004 with larvae collected from organic cabbage fields in the Bielersee region, Switzerland. It is annually supplemented with 200 new field-caught larvae to reduce potential inbreeding effects. The rearing is maintained in a climate chamber at 23±2 ◦C, 60±10% r.h. and a L16:D8 photoperiod.

Adults of *M. brassicae* are kept in a transparent acrylic cylinder (24 cm diameter, 23.5 cm height) for egg-laying (hereafter called "egg-laying cylinder") (Fig. 1 and 2a). The bottom of an egg-laying cylinder is made of a cardboard disc (24 cm diameter) fixed to the cylinder with a one-sided aluminium ring (24.5 cm diameter, 1 cm width, 1 cm height) that fits exactly around the base of the cylinder. A plastic sheet (27.5 cm x 29 cm) covered by two or three paper towels are placed over the cardboard to ensure impermeability and absorb humidity, respectively. The cylinder is closed by a sheet of green paper fixed to the top of the cylinder by another one-sided aluminium ring. Two green paper sheets (A3, 29.7 cm x 42 cm) cover the inside of the cylinder so that the moths can lay their eggs on them. The moths are provided with honey and water as a diet: filter papers (9 cm diameter, Macherey-Nagel GmbH & Co. KG, Düren, Germany) are rolled up and put through 1 cm holes pierced into the lid of a Petri dish (8 cm diameter, 3 cm height) filled with water. A drop of honey is added at the top of each filter paper with a brush. Two Petri dishes with five filter papers each are placed in each cylinder. Since the moths lose a lot of scales and lay eggs on the bottom of the egg-laying cylinders as well as on the Petri dishes and the filter papers, they are transferred to a new egg-laying cylinder every two to three days. To do so, the old egg-laying cylinder (without bottom and top covers) is placed on top of a new one and the moths are gently pushed down with a brush. The moths resting on the bottom of the old egg-laying cylinder and on the old Petri-dishes are transferred to the new egg-laying cylinder the same way.

To start a new cycle (*i.e.* a complete generation from eggs to adult eclosion), four clutches of fresh (*i.e.* less than 24 hours-old) *M. brassicae* eggs (approximately 200 to 400 eggs) are cut from a paper covering the inside of an egg-laying cylinder and placed in a smaller tube (hereafter called "rearing tube") (Fig. 1 and 2b).

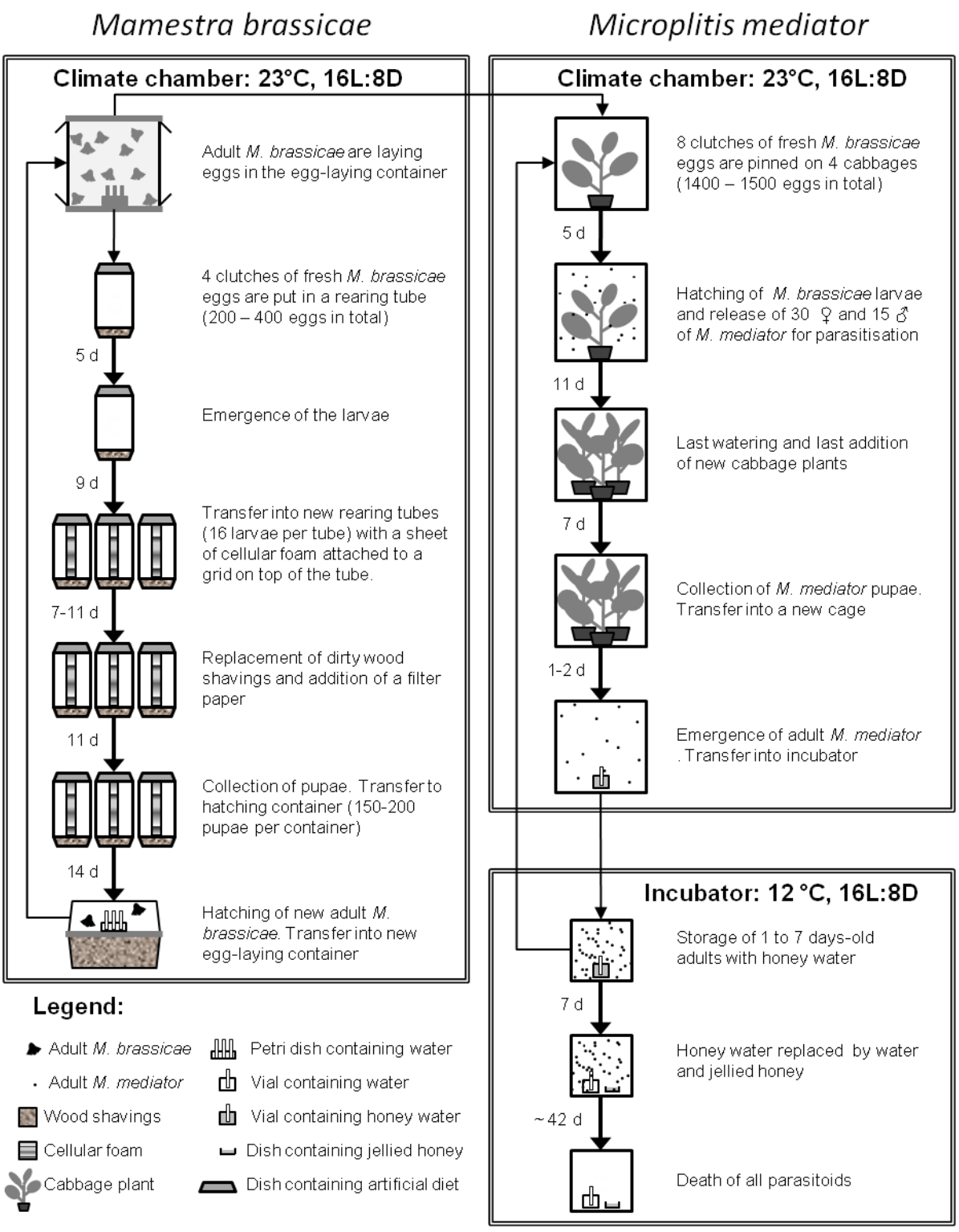

Fig. 1 – Chronological steps of the *Mamestra brassicae* (left) and the *Microplitis mediator* (right) rearings. Times in days between each step are indicated next to the bold arrows.

The rearing tube is composed of a transparent plastic tube (9 cm diameter, 17 cm height) placed on top of a transparent plastic dish (10 cm diameter at the top, 8 cm diameter at the bottom, 5 cm height) filled with wood shavings that absorb humidity as well as the faeces of the larvae. The wood shavings are sterilized by heating them for two hours at 90°C. The larvae are fed on an artificial diet (Beet Armyworm Diet, Bio Serv, Frenchtown, NJ, USA), supplemented with 0.2% Aurofac 100 (Selectchemie, Zürich, Switzerland) placed in a second inverted plastic dish on top of the rearing tube. Placing the artificial diet at the top prevents the diet from being contaminated by the faeces. After the egg clutches have been placed on the wood shavings, the junctions between the tube and the dishes are sealed with Parafilm (Bemis Company, Inc., Neenah, WI, U.S.A.) and fixed with tape, to avoid escape of the young *M. brassicae* larvae.

The larvae of *M. brassicae* emerge four to six days after egg laying. To check for the hatching success of the *M. brassicae* eggs, 30 clutches collected on six different dates (5 clutches per date) were photographed with a camera (Somikon USB Digital-Mikroskop-Kamera, PEARL.GmbH, Germany), and the number of eggs of each clutch was counted before and after hatching using UTHSCSA ImageTool 3.00. The mean hatching success was 98.49 ± 0.58%. Approximately 14 days after egg laying, 16 larvae each are transferred to new rearing tubes to avoid competition as the larvae grow (Fig. 1). These new rearing tubes do not need to be sealed, as the larvae are too big to escape at this stage. A metal grid (4 cm x 8 cm) is put on top of the opening of the rearing tube, under the dish containing the artificial diet, and a sheet of cellular foam (approximately 23 cm x 5 cm) is fixed to the grid so that it touches the wood shavings. The cellular foam helps the larvae climb to the top of the rearing tube onto the grid and access the diet, which is particularly important from instar III onward as they become too heavy to climb the walls of the rearing tubes. The number of new rearing tubes depends on the desired yield. Twenty-one to 25 days after egg laying, the dirty top layer of the wood shavings is removed. A filter paper with a triangle (approximately 4.5 cm x 4.5 cm x 3.5 cm) cut out is placed on top of the wood shavings, and approximately 1 cm of fresh wood shavings is spread over it. This enables the larvae ready to pupate to dig into the wood shavings under the filter paper by passing through the triangle hole while being protected from the faeces of the larvae that are still feeding.

The pupae are collected from all of the rearing tubes 32 to 36 days after egg laying and placed in a new container in which they eclose (hereafter called “eclosion container”) (Fig. 1 and 2c). The eclosion container is made of two transparent plastic dishes (24 cm diameter x 8 cm height) held together by a two-sided aluminium ring (24.5 cm diameter x 1 cm width x 2 cm height). The top dish serves as a lid. The pupae are placed in the bottom plastic dish in 3 layers of 50 to 60 pupae alternated with wood shavings (maximum 150 to 200 pupae in total per container), so that all the pupae are properly buried. The larvae that have not pupated are left in the rearing tubes for one

more week. Pupae were counted after collection and the mean number of pupae collected for 20 tubes (*i.e.* 320 larvae) was 233.80 ± 18.10, which represents a pupation rate of 73.06 ± 5.66 % (n = 10). Adult moths start to eclose approximately 14 days after pupation. The number of newly eclosed adults was counted and the eclosion success of *M. brassicae* pupae was 72.14 ± 2.40 % (n = 39 cycles for a total of 7927 pupae) (Fig. 3). The eclosion container is then checked every two to three days, and newly eclosed moths are transferred to an egg-laying cylinder. Water is provided in a Petri dish with filter papers as described before, and renewed as needed. As the newly eclosed moths can lay eggs in the eclosion containers, the lid and Petri dish are checked and changed for new ones if there are many eggs, or cleaned with a paper towel to remove the eggs if there are just a few. The eclosion containers are placed in 2 cm of soap water to prevent the entry of mites.

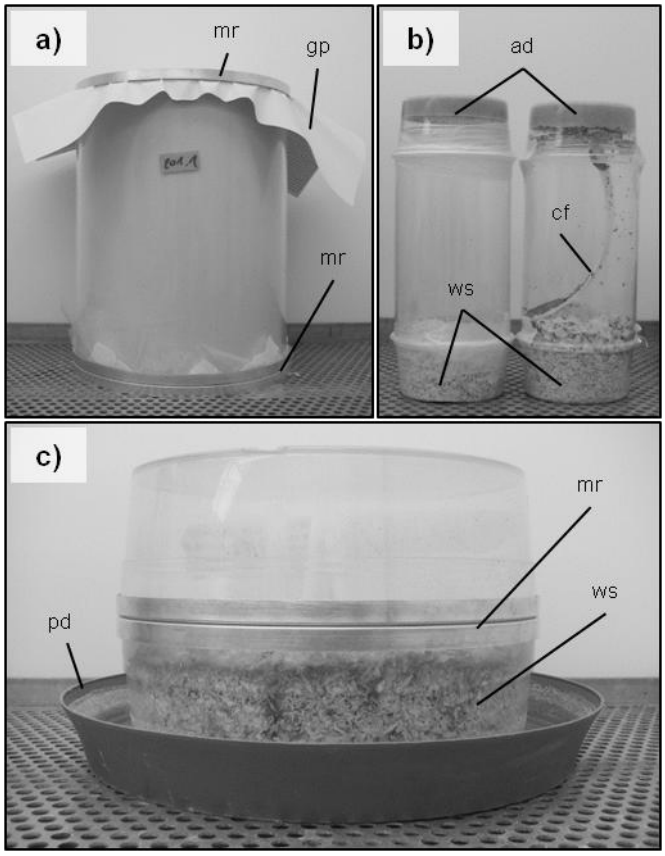

Fig. 2 – Pictures of a) the egg-laying cylinder, b) the sealed rearing tube without cellular foam (left) and the unsealed rearing tube with cellular foam (right), and c) the eclosion container used in the *Mamestra brassicae* rearing. ad, artificial diet, cf, cellular foam, gp, green paper, mr, metal ring, pd, plastic dish, ws, wood shavings.

The pupae of *M. brassicae* can be stored at 4°C for many months either in sterilised soil or in wood shavings covered with a filter paper. In the second case the container must be sealed with Parafilm to keep the humidity inside so that the pupae do not desiccate. After four to six months of storage, 70.03 ± 3.06 % of the pupae were still alive, *i.e.* exhibited some abdominal movements when

gently squeezed with insect handling tweezers (n = 17 cycles for a total of 2005 pupae). Adult moths eclosed from 84.98 ± 0.62 % of the surviving pupae and from 60.06 ± 6.87 % of the total number of stored pupae (n = 5 cycles for a total of 544 pupae). Since the eclosion success after cold storage was not measured during the same years as the eclosion success without cold storage, we cannot directly compare them. But the most important point is that the eclosion success after cold storage is still very high.

All materials are washed after use. The plastic cylinders and dishes are either hand washed with hot soap water and rinsed thoroughly with clear water afterwards, or put in the dishwasher (60°C, without drying). The rearing tubes are rinsed with hot water before being put into the dishwasher to remove the faeces left by the larvae. Tubes and dishes are then sprayed with ethanol (70%), except for the egg-laying cylinders as ethanol causes cracks in the acrylic glass. The metal grids are rinsed with hot water and soaked in water with detergent (Deconex 11 Universal, Borer Chemie AG, Zuchwil, Switzerland) during one week, after which they are thoroughly rinsed and decalcified by rubbing them with a sponge.

Transferring the larvae into new rearing tubes, adding the filter papers after removal of the dirty wood shavings, and collecting the pupae represent the highest workloads. For 20 new rearing tubes per week, they take approximately 45, 60 and 60 minutes, respectively.

3. *Microplitis mediator* rearing

3.1. Biology of *M. mediator*

Microplitis mediator is the major larval parasitoid of *M. brassicae* (Bianchi et al., 2005; Lauro et al., 2005). It is a generalist endoparasitoid, that has been reported on approximately 40 different noctuid hosts (Mir Khan, 1999). Its geographical distribution ranges from Central Europe to China (Foerster and Doetzer, 2003). It was introduced to Canada in the early 1990's to control populations of the bertha armyworm *Mamestra configurata* (Walker, 1856) (Lepidoptera: Noctuidae) (Foerster and Doetzer, 2003). *Microplitis mediator* females parasitise the first three instars of *M. brassicae* larvae, although the third instar is suboptimal because approximately half of the parasitisation attempts are unsuccessful, and because immature parasitoids then often fail to complete their development (Lauro et al., 2005). During parasitisation, female *M. mediator* inject, along with one egg, some calyx fluid and a bracovirus that protects the egg from encapsulation (Tanaka, 1986; Tanaka, 1987) and delays the development of the host larva (Kadash et al., 2003). Superparasitism can occur, but only one parasitoid larva is able to complete its development. The parasitoid larva

emerges from the instars IV and V of the host 8 to 30 days after parasitisation, depending on temperature (Foerster and Doetzer, 2003). The pupation of the *M. mediator* larva takes place outside the host larva, which dies after parasitoid emergence. Adult *M. mediator* emerge from the pupae between 5 to 25 days after pupation, depending again on temperature (Foerster and Doetzer, 2003). As many parasitoid species, adult *M. mediator* require sugar feeding to survive and produce the maximal possible number of offspring (Géneau et al., 2012; Luo et al., 2010). Females are longer-lived than males (Géneau et al., 2012; Luo et al., 2010) and their mean lifespan ranges between 15 and 30 days after emergence, depending on food quality and oviposition activity (Foerster and Doetzer, 2003; Géneau et al., 2012; Luo et al., 2010). They start to parasitise larvae on the day of emergence and remain reproductively active until the end of their life (Foerster and Doetzer, 2003; Géneau et al., 2012).

3.2. Rearing protocol

3.2.1. Rearing on cabbage

The parasitoid rearing at FiBL (Frick, Switzerland) was established in a climate chamber at 23 ±1°C, 50 ±15% r.h. and 16L:8D photoperiod in May 2009. The rearing was started with *M. mediator* pupae from a laboratory population reared at Wageningen University (The Netherlands) initiated by individuals collected on *M. brassicae* larvae in Brussels sprouts fields near Wageningen. Parasitoids are reared on *M. brassicae* larvae fed on white cabbage *Brassica oleracea* L. var. *capitata* L. (Brassicaceae). Cabbage plants are grown from seeds in GroBanks (CLF Plant Climatics, Germany) at 21±2 °C, 50±15% r.h. and 12L:12D photoperiod in trays (33 cm x 51 cm) of soil (Einheitserde Classic, Gebrüder Patzer GmbH & Co.KG, Germany) fertilized with 3 g/L of Tardit 3M (Hauert HBG Dünger AG, Switzerland). Plants are transplanted into pots (12 cm diameter, 10 cm height) after three to four weeks with the same amount of fertilizer and transferred into a greenhouse. They are watered as needed and used as food for the *M. brassicae* larvae between the 11-leaf and the 15-leaf stages (i.e between three to four weeks after transplantation). The youngest cabbage plants (about three weeks after transplantation) are used to feed the young *M. brassicae* larvae (instars 1 to 3), as the leaves must be thin for the larvae to be able to easily feed on them. As the larvae grow, they become able to feed easily on older cabbage plants. The pot size is optimal, because with smaller pots the cabbage plants tend to fall as they grow heavier and the ground dries faster, but bigger pots take too much space. Approximately 15 cabbages plants are needed per week to start one new cycle (*i.e.* a complete generation from parasitisation to adult eclosion) per week.

To start a cycle (*i.e.* a complete generation from parasitisation to adult eclosion), eight clutches of fresh *M. brassicae* eggs are cut and pinned onto the underside of the leaves of four cabbage plants (1448.20 ± 64.97 eggs per cage, n = 10 cages). The pots are placed on a plastic dish

(32 cm diameter, 4 cm height) to allow easy watering from below, in an insect rearing cage (47.5 cm x 47.5 cm x 47.5 cm, BugDorm-44545F, MegaView Science Co., Taichung, Taiwan). Two pieces of paper (A3, 29.7 cm x 42 cm) are placed under the plastic dish to absorb humidity or water that could splatter during watering.

Mamestra brassicae larvae hatch five days after the eggs are put on the cabbage plants. Approximately 45 adults of *M. mediator* (30 females and 15 males, two-day to one week-old) are introduced into the cage on the day of emergence of the *M. brassicae* larvae, or one day before, so that they can parasitise the larvae in their first and second instar stages. On the day of introduction into the cage, the parasitoids are provided with a paper towel on which some honey is spread and which is then sprayed with water, to ensure that they survive long enough to parasitise the larvae. The paper towel is suspended from the top of the cage and removed from the cage 2 to 3 days later.

The cages are checked regularly while the *M. brassicae* larvae are developing, and new cabbage plants are added as needed until 11 days after parasitisation. A maximum of five cabbage plants can be placed on the plastic dish, after which the new cabbage plants are added on smaller plastic dishes (12 cm diameter, 3 cm height) instead, next to the big plastic dish. The total number of cabbage plants at the end of the cycle is usually seven to eight per cage. From 12 days after parasitisation, new cabbage plants are not provided anymore, because the parasitoid larvae are getting ready to pupate, and new cabbage plants would just feed the biggest unparasitised larvae. Cabbage plants are watered as needed by pouring water into the plastic dishes only until 11 days after parasitisation, so that the material in the cage is not too humid when the pupation of the parasitoids takes place.

The pupae of *M. mediator* are collected 18 days after parasitisation, by checking systematically all the material in the cage (cabbage leaves, pots, paper, as well as nylon mesh, plastic panels and bottom of the cage). They are carefully removed from their substrate by hand or with insect handling tweezers, and transferred to a Petri dish which is then placed in a new rearing cage with honey water *ad libitum*. The honey water is prepared in a small vial (4.5 cm diameter, 4 cm height), by mixing approximately three grams of honey with 25 ml of water. A filter paper is rolled up and put through a 0.5 cm hole pierced in the lid of the vial so that the lower part is soaked in the honey water. Fresh honey water is provided every two to three days to prevent fermentation and the formation of mould. The mean number of pupae collected per cage was 332.80 ± 40.63, which represented a pupation rate of 22.85 ± 2.64 % (n = 10 cages) (Fig. 3).

We conducted a small experiment to test whether the pupation rate could be increased by decreasing the number of *M. brassicae* eggs initially provided in the cage. We provided either 900 ± 5 or 600 ± 5 *M. brassicae* eggs per cage at the beginning of the cycle and counted the number of pupae collected at the end (n = 10 cages per initial number of eggs). The mean numbers of pupae collected

were 199.20 ± 27.34 for an initial number of 600 eggs, which represents a pupation rate of 33.20 ± 4.57 %, and 342.40 ± 31.71 for an initial number of 900 eggs, which represents a pupation rate of 38.05 ± 3.52 % (Fig. 3).

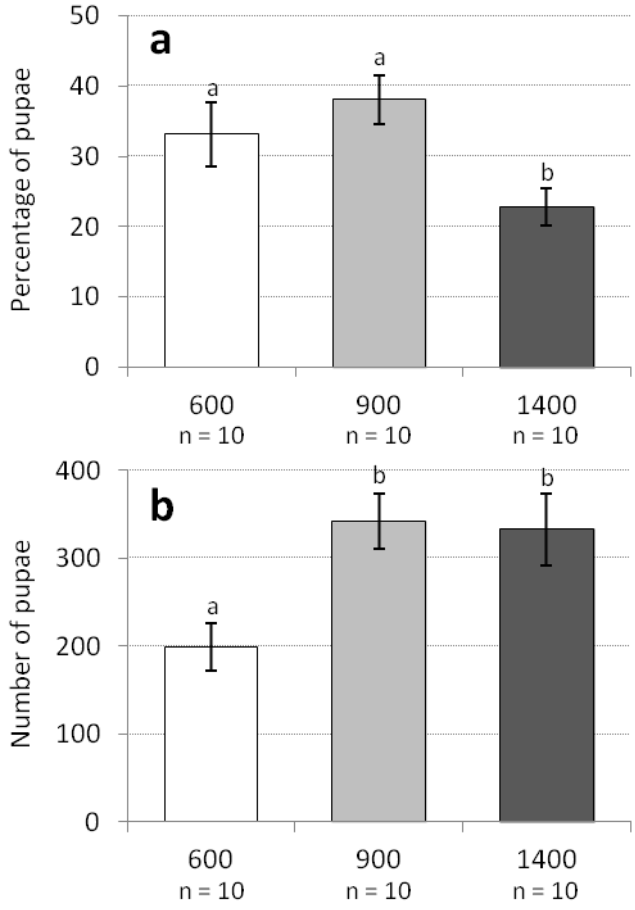

Fig. 3 – Comparison of a) the mean (± SE) pupation rate and b) the mean (± SE) number of pupae of *Microplitis mediator* collected from cages with initial numbers of 1448.20 ± 64.97 (1400), 900 ± 5 (900) and 600 ± 5 (600) *Mamestra brassicae* eggs. Bars with different letters are significantly different from each other (linear model, $p < 0.05$).

Statistics were conducted on these data using R 2.13.2 (R Development Core Team, 2011). We performed linear models to compare the mean pupation rate and the mean number of pupae collected at the end of the cycle between the treatments with different initial numbers of *M. brassicae* eggs, *i.e.* 1400 eggs (referring to the regular mean initial number of eggs used in the rearing), 900 eggs and 600 eggs. The mean pupation rate and the mean number of pupae were both significantly affected by the treatment ($F = 4.506$, $df = 27$, $p = 0.0205$ and $F = 5.648$, $df = 27$, $p = 0.0089$, respectively). The mean pupation rate was significantly different between the 1400 and the

900 eggs treatments (contrast; t = 2.939, df = 27, p = 0.0067) and borderline significantly different between the 1400 eggs and the 600 eggs treatments (contrast; t = 2.001, df = 27, p = 0.0555) but did not significantly differ between the 900 and 600 eggs treatments (contrast; t = 0.937, df = 27, p = 0.3569) (Fig 3.a). Conversely, the mean number of pupae collected at the end of the cycle was significantly different between the 600 eggs treatments and the two other treatments (contrasts; 900 eggs: t = 3.006, df = 27, p = 0.0057 and 1400 eggs: t = 2.805, df = 27, p = 0.0092), but did not differ between the 900 and 1400 eggs treatments (contrast; t = 0.202, df = 27, p = 0.8418) (Fig. 3b).

The pupae of *M. mediator* can be stored for a few days at 10 to 12°C to delay the eclosion of the adults. At 23 ±1°C, adult parasitoids start to eclose one to two days after the collection of the pupae. To check for the eclosion success of the *M. mediator* pupae and the sex-ratio, a total of 200 pupae were collected on 20 different dates (10 pupae per date). The number of eclosed males and females was counted, and pupae that did not eclose within ten days were considered dead. The mean eclosion success was 96.50 ± 1.67 % and the mean sex-ratio was balanced at 50.31 ± 3.13 % of females. Newly eclosed parasitoids are aspirated regularly using a D-cell vacuum insect aspirator (MX-991/U, Hausherr's Machine Works, Tom Rivers, NJ, U.S.A.) and put in a smaller rearing cage (24.5 x 24.5 x 24.5, Bugdorm-42222F, MegaView Science Co., Taichung, Taiwan) in an incubator at 12 ±1°C, 60 ±2% r.h. and 16L:8D to decrease their metabolic activity and consequently increase their lifespan. Some of these individuals are used for parasitisation during the first week after eclosion. During this time, they are provided with honey water *ad libitum* to ensure that they are well fed before parasitisation. The remaining parasitoids are kept as a stock in the incubator until they die. From the second week after eclosion, they are provided with water (using the same kind of vial and filter paper as for the honey water) and with approximately 3 to 5 ml of jellied honey in a small plastic dish. Jellied honey is denser than honey water, and thus supposedly less easy to ingest for the parasitoids, but has the advantage to last for weeks (*i.e.* until the death of the parasitoids) without needing to be replaced. The jellied honey is prepared by mixing 3 g of white gelatine (Dr Oetker AG/SA, Obergösgen, Switzerland) with 100 ml of distilled water. The solution is slightly heated and 200 g of honey are added. The mixture is then poured into syringes and kept in the fridge at 4°C until use.

All materials are washed after use. The vials containing water or honey water, as well as the dishes containing jellied honey, are washed thoroughly using hot water. They are then sprayed with ethanol (70%) and rinsed with demineralised water. The rearing cages and the plastic dishes used to water the cabbage plants are washed in hot soapy water, rinsed thoroughly with clean water, sprayed with ethanol (70%) and rinsed again with demineralised water.

The transplantation of the cabbage plants into pots and the collection of the pupae in the cages represent the highest workload. At a production rate of 15 cabbages per week, the transplantation of

the cabbage plants lasts about one hour. The time needed to collect the pupae is 1.5 hours per cage on average.

3.2.2. Rearing on artificial diet

Alternatively to the rearing on cabbage, we developed a rearing protocol of *M. mediator* on the same artificial diet as used in the *M. brassicae* rearing. However, this rearing was not maintained over a prolonged time, and we can therefore not judge if maintaining a cabbage free rearing would change the behaviour of adult *M. mediator*, as was shown in another parasitoid species (Bautista and Harris, 1997; Gandolfi et al., 2003).

This rearing is maintained in the same climate chamber as the rearing on cabbage. One clutch of fresh *M. brassicae* (reduced to exactly 30 eggs) is put in a small vial (4.5 cm diameter, 4 cm height) (hereafter called "parasitisation vial") with a 1.5 cm diameter hole in the lid that allows air exchange. Another 1.5 cm diameter hole is pierced on the side of the parasitisation vial and a little tube (1.5 cm diameter, 1.5 cm length) made of flexible plastic is inserted and glued into the hole with hot glue, so that 1 cm of the tube emerges on the outside of the vial. This opening can be closed by sticking an Eppendorf tube (1 cm diameter, 4 cm height) into it. A layer of artificial diet (approximately 1 cm) is poured into the parasitisation vial, which is then put upside down so that the artificial diet is at the top. A piece of paper towel is placed between the vial and the lid to prevent the larvae from escaping and to absorb humidity.

The larvae of *M. brassicae* hatch in the parasitisation vial after five days. On the day of hatching of the larvae, four two-day to one-week old adults of *M. mediator* (two females and two males) are introduced into the vial through the plastic tube emerging from the side of the parasitisation vial. The opening is closed again after all the parasitoids have entered the parasitisation vial. The parasitisation is done in a small vial to ensure that the female parasitoids find all the larvae without any difficulty and maximize the parasitisation rate, a technique that was established in a previous study (Géneau et al., 2012).

Two days after parasitisation, the growing *M. brassicae* larvae are transferred into a modified rearing tube (see *Mamestra brassicae* rearing protocol), the bottom of which is made of a paper towel fixed to the tube with a rubber band instead of a plastic dish filled with wood shavings. The wood shavings are not needed because the *M. brassicae* larvae will not reach pupation. The larvae develop until the pupation of the parasitoids 16 days after transfer into the rearing tube. The pupae are then collected as described for the rearing on cabbage. The mean number of pupae collected per tube was 7.65 ± 1.62, which represents a yield of 25.51 ± 5.41 %.

The parasitisation vials and rearing tubes are hand washed or put into the dishwasher, as described for the *M. brassicae* rearing.

4. Discussion

Our rearing of *M. brassicae* seems to be optimal because the hatching success of the eggs, the pupation rate and the eclosion success of the adults are all very high. The use of the rearing tubes allows high numbers of individuals to be produced in a relatively limited space. The fact that the larvae have limited contact with the faeces decreases the risk that they get contaminated by mold developing on the faeces, and the separation of the larvae into several rearing tubes also limit the spread of diseases because contaminated tubes can be kept apart and/or discarded. The use of the artificial diet constitutes a tremendous time saver, as the larvae in the fifth instar stage consume high amounts of food, so maintaining the same production of moths on cabbage would require an incredible production of cabbage plants. One downside of this approach could be that growing on an artificial diet could influence the behavior of adult moths. In fact, it has been shown in *Spodoptera littoralis* (Boisduval, 1833) (Lepidoptera: Noctuidae) that the choice of the oviposition site is influenced by the larval food plant (Shikano and Isman, 2009). Experiments should be conducted in *M. brassicae* to test whether growing on the artificial diet results in behavioural alterations if rearing on artificial medium is used for behavioural studies. In our case the rearing was kept on the artificial diet for the sake of efficiency, as we only needed a high egg production to expose eggs in the field (Balmer et al., submitted) and to measure the fecundity of female moths fed on different wildflower plants (Géneau et al., 2012).

Contrary to the *M. brassicae* rearing, the *M. mediator* rearing was kept on cabbage because we used the adult parasitoids for behavioural assays (Belz et al., 2013) and wanted their behaviour to stay as close to natural as possible. It has been shown in some parasitoid species that the host searching ability of adult females was compromised when the parasitoids were raised on an artificial diet (Bautista and Harris, 1997; Gandolfi et al., 2003). The cabbage production stays reasonable because we do not raise the unparasitised *M. brassicae* larvae until the end of their development and stop adding new cabbage when parasitoid larvae start to emerge from parasitised caterpillar that stay small. We however showed that it is possible to rear the parasitoids on the artificial diet, which could be a good starting point for developing a mass rearing of *M. mediator*. But like in *M. brassicae*, the behaviour of adult *M. mediator* reared on the artificial diet should be investigated, because decreased host searching capacities in female parasitoids are very undesirable if the aim is to use them for biological control.

Efficiency is more difficult to achieve in the parasitoid rearing than in the *M. brassicae* rearing. Pupation rates are quite low, whatever the diet and protocol used. We found that the pupation rate was almost equal with initial numbers of 600 and 900 eggs and 10% higher than with an initial number of 1400 eggs. However, the number of pupae collected in the end was almost equal

with an initial number of 900 and 1400 eggs and significantly higher than with 600 eggs. This shows that the initial number of *M. brassicae* eggs should be around 900, as both the pupation rate and the number of pupae collected are maximized. The decreased pupation rate from 900 to 1400 eggs could mean that the 30 female *M. mediator* have reached their maximum parasitisation capacity with 900 eggs or that the mortality of the *M. brassicae* larvae is higher with 1400 eggs. The parasitisation rate might be increased by introducing more female *M. mediator* into the cages, but it would not necessarily be an advantage because females that are used for the rearing are lost for experiments. It could also lead to increased intraspecific competition between females (Greenberg et al., 1995), increased mortality of the *M. brassicae* larvae and decreased emergence of parasitoid adults (Shepard and Gale, 1977). Despite a low pupation rate, the number of pupae collected at the end of the cycle is high and was always sufficient to provide enough insects for our experiments, which is the most important parameter.

To conclude, our rearing of *M. brassicae* and *M. mediator* proved to be reliable over the years, and the production was easy to regulate to match our needs by changing the number of cycles started every week. These protocols should be useful to anyone wanting to rear these two species, and could also be an inspiration on how to start a rearing of closely related species.

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CHAPTER 2

Olfactory attractiveness of flowering plants to the parasitoid *Microplitis mediator*: potential implications for biological control

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Abstract

In agricultural landscapes, the lack of floral nectar can be a major difficulty for nectar feeding parasitoids. This problem can be reduced by the addition of suitable wildflowers. To date, flowers have mainly been studied in terms of effects on parasitoid fitness, not taking into account the essential role of flower attractiveness for foraging parasitoids. This study experimentally tested the olfactory attractiveness of five wildflowers (bishop's weed, cornflower, buckwheat, candytuft, and oregano) to the parasitoid *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae). We conducted choice experiments in a Y-tube olfactometer to test the attractiveness of flowers against air, and relative attractiveness in paired choice tests. Our results showed that all the flowers were highly attractive and that in paired choice tests cornflower and candytuft were equally attractive and more attractive than buckwheat. These results indicate that *M. mediator* has evolved innate preferences that could be effectively exploited in biological control.

1. Introduction

Many agricultural landscapes offer unfavourable conditions to natural enemies such as arthropod predators and parasitoids. Frequent and intense disturbances, *e.g.* pesticide applications or harvest, can influence insect diversity and - in the case of parasitoids - reduce species richness, abundance and effectiveness (Landis and Menalled, 1998; Naranjo and Ellsworth, 2009). The scarcity or absence of flowers in crop fields can also be a critical issue for the survival of natural enemies, because floral nectar is a major food source for many of them (Hogg et al., 2011; Letourneau and Altieri, 1999). These unfavourable conditions are accentuated in annual monocultures which represent temporary habitats that are less stable, and thus exposed to higher levels of disturbance, than perennial cultures (Ferro and McNeil, 1998; Landis et al., 2000). This can result in lower establishment rates of natural enemies and consequently lower success in controlling the pests (Landis et al., 2000).

Conservation biological control can mitigate these problems through habitat management, *i.e.* by modifying the habitat to enhance the abundance and activity of natural enemies and consequently improve the control of pest populations (Jonsson et al., 2008; Landis et al., 2000; Naranjo and Ellsworth, 2009). For example, wildflower strips (*i.e.* wildflowers planted along the field margin) or companion plants (*i.e.* non-crop plants inserted within the field and alternating with the crop plants) can be added as alternative food sources (Lee and Heimpel, 2005; Pfiffner et al., 2009; Ponti et al., 2007). Like other intercrops and ground covers, they also represent shelter habitats that offer protection to the natural enemies during pesticide applications, harvest, overwintering or in case of unfavourable weather (Griffiths et al., 2008; Landis et al., 2000; Pfiffner and Luka, 2000).

Wildflower strips and companion plants have already been used in the field to enhance predators and parasitoids, but the results are mixed. Some studies show that the provision of floral resources increases parasitism rates (Ellis et al., 2005; Lavandero et al., 2005; Ponti et al., 2007), while others show no effect (Berndt et al., 2002), negative effects (Bone et al., 2009) or effects that vary depending on the year (Lee and Heimpel, 2005) or the field location (Pfiffner et al., 2009). This inconsistent pattern of effects of wildflower strips and companion plants stresses the importance of studying in detail the decision making and preferences of predators and parasitoids for food sources. In particular, conducting flower screening to identify suitable flowering plants that will effectively 1) attract natural enemies into the field and closer to the pest, and 2) offer the natural enemies accessible nectar as food resource to increase their lifespan and/or the fecundity (Wäckers, 2004) is important to improve the effects of wildflowers on parasitoid performance.

In the case of parasitoids, flower screening has so far focused on the identification of wildflower species that enhance the survival and/or the fecundity of the studied species (Baggen and

Gurr, 1998; Géneau et al., 2012; Lavandero et al., 2006; Nafziger and Fadamiro, 2011; Winkler et al., 2006; Witting-Bissinger et al., 2008). But before being able to feed on flowers, foraging parasitoids have to locate them by using for example olfactory (Desouhant et al., 2005; Leius, 1960; Patt et al., 1999; Siekmann et al., 2004; Takasu and Lewis, 1996) and/or visual cues (Begum et al., 2004; Kugimiya et al., 2010). Food sources that are highly attractive are thus more likely to be visited than food sources that are poorly detectable (Wäckers, 2004). Bianchi and Wäckers (2008) used a spatially explicit simulation model to explore how the attractiveness and the nectar availability of flowering field margins affect parasitoid and ultimately pest populations. This model predicts that the attractiveness of flowers is an important aspect of parasitoid population dynamics that should be taken into account when selecting flowering plants used for habitat management. Despite its predicted importance for effective conservation biological control of pest species, the attractiveness of flowering plants to parasitoids remains poorly studied (Kugimiya et al., 2010; Leius, 1960; Wäckers, 2004).

The aim of this study was to investigate the olfactory attractiveness of five different flowering plants to females of the parasitoid *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae). *Microplitis mediator* is an important parasitoid of *Mamestra brassicae* (Linnaeus) (Lepidoptera: Noctuidae) (Lauro et al., 2005), a major cabbage pest in Europe (Finch and Thompson, 1992). It is also a generalist parasitoid that has been reported on about 40 different Noctuidae hosts (Mir Khan, 1999) and has already been used for biological control (Li et al., 2006). Mass releases of *M. mediator* led to successful control of cotton bollworm *Helicorverpa armigera* (Hübner) (Lepidoptera: Noctuidae) populations in Northwestern China (Li et al., 2006). Furthermore the longevity and fecundity of *M. mediator* are greatly enhanced by the provision of suitable sugar (Luo et al., 2010) or nectar food sources (Géneau et al., 2012), but flower attractiveness to this parasitoid has not been investigated so far. To test the olfactory attractiveness of the flowering plants, we used choice experiments in a Y-tube olfactometer. We first tested whether the different wildflowers were attractive to female *M. mediator* by comparing the flower odours to two control odour sources (air and a piece of stem of the same plant), and then tested the flowers against each other in paired choice experiments to assess olfactory preferences of *M. mediator* among these flowers.

2. Materials and methods

2.1. Plants and parasitoids

We tested the attractiveness of bishop's weed, *Ammi majus* L. (Apiaceae); cornflower, *Centaurea cyanus* L. (Asteraceae); buckwheat, *Fagopyrum esculentum* Moench (Polygonaceae);

candytuft, *Iberis amara* L. (Brassicaceae); and oregano, *Origanum vulgare* L. (Lamiaceae). These flowering plants have properties that make them promising candidates for habitat management, *e.g.* beneficial effects on insects' longevity and/or fecundity (Géneau et al., 2012; Lavandero et al., 2006; Lee and Heimpel, 2008; Nafziger and Fadamiro, 2011; Winkler et al., 2006; Witting-Bissinger et al., 2008), attractiveness to some parasitoid species (other than *M. mediator*) (Wäckers, 2004), the production of easily accessible extrafloral nectar (Koptur, 2005; Winkler et al., 2009), or subsidiary uses like medicinal use (Ammon et al., 2006; Fabre et al., 2000) that could be of interest for farmers.

Flowering plants were grown from seeds in GroBanks (CLF Plant Climatics, Germany) at 21±2 °C, 40±10% r.h. and 12L:12D photoperiod. Seeds were planted in trays in soil (Einheitserde Classic, Gebrüder Patzer GmbH & Co.KG, Germany) fertilized with 3 g/L of Tardit 3M (Hauert HBG Dünger AG, Switzerland) and transplanted into pots (12 cm diameter, 10 cm height) after three to four weeks with the same amount of fertilizer. Plants were checked on a daily basis and watered as needed until they bloomed.

Microplitis mediator pupae came from a laboratory population initiated by individuals collected on *Mamestra brassicae* in Brussels sprout fields near Wageningen (the Netherlands) and reared at Wageningen University. The parasitoid rearing was then established in a climate chamber at 23 ±1°C, 60 ±10% r.h. and 16L:8D photoperiod. Parasitoids were reared on *M. brassicae* larvae fed on white cabbage *Brassica oleracea* L. var. *capitata* L. (Brassicaceae). Adults of *M. mediator* hatched in the room where the experiments took place, at 16L:8D photoperiod. All the females used for the experiments were food deprived (*i.e.* provided with only water from emergence to the behavioural test), naive (*i.e.* no contact with the tested odour sources previous to the experiments) and between 24 and 72 hours old. Females were used only once and discarded after the behavioural test.

2.2. Experimental setups

2.2.1. Y-tube olfactometer

The attractiveness of the different flowers was assessed over three experiments where the olfactory preference of the parasitoid was tested in olfactory choice tests thanks to a Y-tube olfactometer (Fig.1). The Y-tubes had an internal diameter of 1 cm, and the two arms of the Y-tube were connected through a connector to a bent tube, which in turn was connected to a container containing an odour source (Fig.1.a). As odour sources, one flower (for *C. cyanus*) or one inflorescence (for *A. majus*, *F. esculentum*, *I. amara* and *O. vulgare*) was cut and the stem was wrapped in a wet piece of cotton to prevent wilting. The flowers were used within one hour and a half after cutting. The inflorescences of *A. majus* were sometimes too big for the container, so 5 to 8 flowers of the same inflorescence were cut and put together like a small bouquet in the container. To ensure the right functioning of the set-up, we used honey (Waldhonig, Migros, Switzerland) as a

positive control odour source, as preliminary experiments had shown that it was attractive to female *M. mediator*. To prepare the honey odour source, one to two drops of honey were spread on a piece of filter paper (Whatman, GE Healthcare, United Kingdom) previously humidified with water so that the honey covered approximately 2 cm². A white cardboard shield was placed between the Y-tube and the odour source containers to prevent the insects from having any visual information about the odour sources and thus avoid potential confounding effects due to visual attractiveness of the flowers. A metal mesh at the end of each arm prevented the insects from entering the containers and contacting the odour sources.

For behavioural scoring, the Y-tube was divided by thin lines into different zones which represented the proximity to the odour (Fig. 1.b). The zone between the opening of the central tube and the junction lines (8.5 cm before the end of each arm) was referred to as the "base" of the Y-tube. Each arm was divided into the "proximal zone" between the junction line and the finish line (3 cm before the end of the arm), and the "distal zone" between the finish line and the metal mesh at the end of the arm.

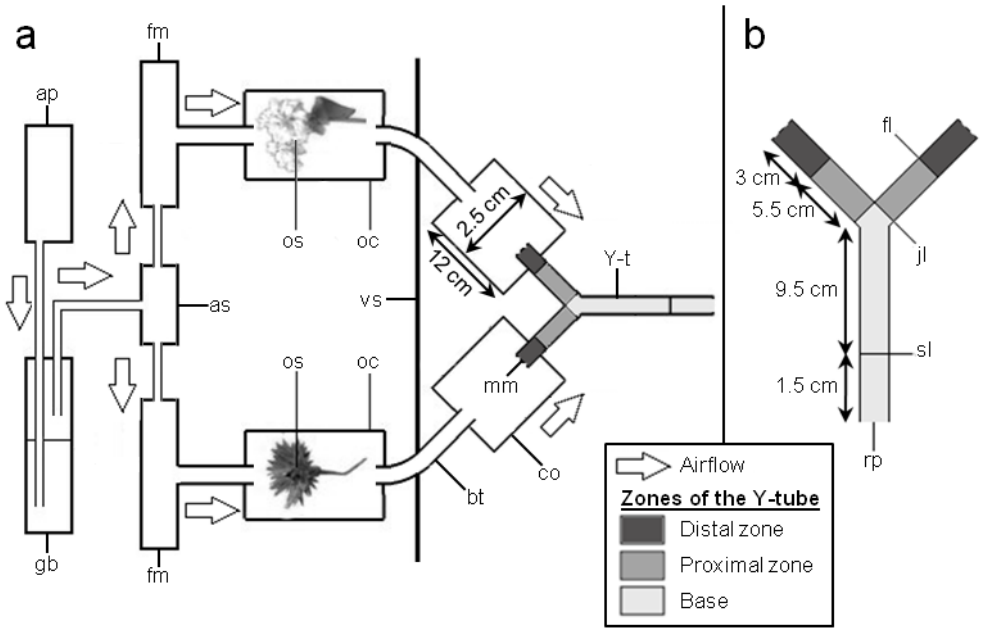

Fig. 1 – Experimental setup. Top view of a) the setup and b) the Y-tube. Two odour sources are placed in the odour source containers. A parasitoid is released in the Y-tube at the release point and has to make a choice between the two odours at the junction line. ap, air pump, as, air splitter, bt, bent tube, gb, gas washing bottle, co, connector, fl, finish line, fm, flow meter, jl, junction line, mm, metal mesh, oc, odour source container, os, odour source, sl, start line, vs, visual shield, Y-t, Y-tube.

Experiments took place in a dark room to avoid directional light, and a light bulb (375 lux) was placed centrally approximately 50 cm above the setup. Air was pushed through the olfactometer

by a vacuum pump, filtered on glass cotton and activated charcoal to limit impurities in ambient air, humidified in a gas washing bottle containing demineralised water, and led into each arm of the Y-tube at 0.433 l/min, so that the airflow at the release point of the insects was 0.866 l/min. Flow meters (Teflonrotameter 150 mm, Analyt-MTC, Germany) were used to control the airflow.

The Y-tubes, connectors and bent tubes were made of glass, and all the other tubes used to connect parts of the setup or transfer the insects in the Y-tube were made of Teflon (PTFE) to allow the cleaning with solvents. The cleaning was done by rinsing the parts with hexane (Roth AG, Switzerland), acetone (Roth AG, Switzerland) and dichloromethane (Roth AG, Switzerland) (once each, in this order). Y-tubes were cleaned at the end of each day (*i.e.* after one to five replicates). On a given day, one Y-tube was used per odour source, and odour sources were always connected to the same arm of the Y-tube to prevent odours from mixing with each other. Every container was connected to the same bent tube and connector during the entire duration of an experiment and all these parts were cleaned after each experiment.

2.2.2. Behavioural tests

To start a measurement, one female parasitoid was transferred from the cage to the Y-tube in a 2.5 cm Teflon tube closed on one end by a metal mesh. The tube was fixed to the base of the Y-tube, and the test began when the female crossed the start line (9.5 cm before the bifurcation). The time the female spent in each zone was recorded in real time during 5 minutes using JWatcher V1.0 (Blumstein and Daniel, 2007). The small fraction of females that did not cross the start line within five minutes were considered as non-responders and removed from the experiment.

In experiment 1, we tested the absolute attractiveness of the flowers and of honey to female *M. mediator*, *i.e.* whether the odour sources were attractive. An odour source was considered attractive when the parasitoids exhibited a preference for it. We considered two measurements of female preference. The first choice on one hand was defined as the arm in which females crossed the junction line for the first time, to test whether a preference could be detected as soon as the females entered an arm or whether the females needed some exploration before displaying a preference. The residence time on the other hand was obtained by the time females spent in the distal zones of the two arms. Females were considered to have a lasting preference for an odour source when they spent significantly more time in the distal zone of its arm than in the distal zone of the other arm. The flowers tested in experiment 1 were *A. majus, C. cyanus*, *F. esculentum*, *I. amara,* and *O. vulgare*. The odour sources were compared to a piece of filter paper humidified with tap water as a negative control. The filter paper was used to add humidity in the arm to balance the extra humidity produced by the flower. In a control experiment, the humid filter paper did not significantly affect the

preferences of the female parasitoids in the olfactometer compared to air (Wilcoxon signed-rank test, n = 50, V = 691.5, p = 0.099) and, hence, was considered a neutral control.

In experiment 2, we tested whether the results obtained in experiment 1 reflected a specific response to the volatiles from the flowers and not just due to volatiles emitted by the cut stem. The experiment was set up exactly like experiment 1, except that a flower or inflorescence was compared to a piece of stem of the same flowering plant. The piece of stem was taken directly under the flower or inflorescence used as an odour source and was approximately the same length as the stem left under the flower or inflorescence, so that the flower or inflorescence was the only difference between the two arms. For time and practical reasons, we left aside *A. majus* and *O. vulgare*, which are difficult to rear, limiting their usefulness for application in the field, and focused on *C. cyanus*, *F. esculentum*, and *I. amara*.

In experiment 3, we tested the relative attractiveness of the different odour sources to females *M. mediator* using paired-choice tests in order to assess which floral scent was most attractive. A flower was considered more attractive than another when the parasitoids exhibited a preference for it, and the short-term and long-term preferences were measured like in the previous experiments. The setup was like in experiment 2, except that odour sources were compared against each other in pairs.

All the odour sources (experiments 1 and 2) or pairs of odour sources (experiment 3) were tested in a randomized order for each replicate, and one individual female was used for each odour source or pair of odour sources. Odour sources were renewed for each replicate and their positions (*i.e.*, left or right in the Y-tube) alternated between replicates to randomize possible side-effects on the preferences of *M. mediator*. One to five replicates were performed on a given experimental day.

2.3. Statistics

Statistical analyses were conducted using R 2.13.2 (R Development Core Team, 2011). We treated as non-responders the females that never entered any distal zone for the analysis of the residence time, and those that never entered any proximal zone for the analysis of the first choice, and removed them from the analysis. For the residence time, the total time spent in the distal zone varied between females, so we defined a preference index by calculating the ratio of the total time spent in the distal zone of one arm over the total time spent in the distal zones of both arms. The data were not normally distributed, even after transformation, so we performed a Wilcoxon signed-rank test to test whether there was a significant preference for one arm or the other (*i.e.* whether the preference index was significantly different from 0.5). For experiments 1 and 2, we used a generalized linear model with quasibinomial data distribution to test for significant differences among all the odour sources. We performed two Pearson's Chi-squared tests for the analysis of the

first choice, first to test whether the numbers of females that first chose one arm or the other were significantly different, and then, for experiments 1 and 2, to test whether there was a significant difference in the first choice among the odour sources.

3. Results

In experiment 1 where we tested the absolute attractiveness of flowers compared to air as a neutral control, the parasitoids spent significantly more time in the arm connected to the odour source over air for all the tested odour sources (flowers and honey) (Fig. 2.a). There was no significant difference in the residence time between the odour sources (χ^2 = 10.092, df = 5, p = 0.073). Except when offered *C. cyanus* and *O. vulgare*, significantly more parasitoids showed a first choice towards the odour over air (Fig. 2.b), and there was no significant difference in the first choice among the odour sources (χ^2 = 2.807, df = 5, p = 0.730).

In experiment 2, where we tested the attractiveness of flowers in comparison with the stem, the parasitoids spent significantly more time in the distal zone of the arm connected to the flowers over the stem for the three flowers tested (Fig. 3.a). Like in experiment 1, there was no significant difference in the residence time between the flowers (χ^2 = 3.083, df = 2, p = 0.214). Except when offered *F. esculentum*, significantly more parasitoids showed a first choice for the flower over the stem (Fig.3.b), and there was no significant difference in the first choice among the three flowers (χ^2 = 4.656, df = 2, p = 0.098).

In experiment 3 where we tested the relative attractiveness of *C. cyanus*, *I. amara* and *F. esculentum* in paired-choice tests, distinct differences in olfactory attractiveness emerged between flowers. Parasitoids significantly preferred *C. cyanus* and *I. amara* over *F. esculentum*, whereas they showed no significant preference between *C. cyanus* and *I. amara* (Fig. 4.a). This pattern was mirrored in terms of the first choice (Fig. 4.b).

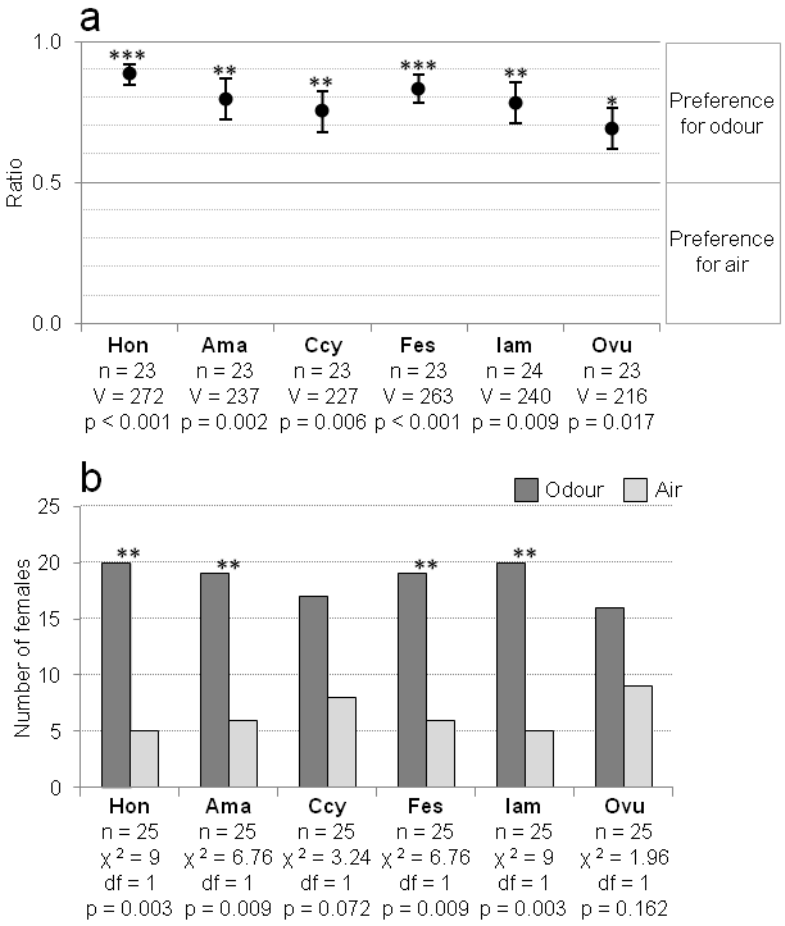

Fig. 2 – a) Number of females that first chose the odour arm (dark grey) or the air arm (light grey). Asterisks indicate significant differences (Chi-squared test, * $p < 0.05$, ** $p < 0.01$). b) Mean (± standard error) ratio of the total time spent in the distal zone of the odour arm over the total time spent in the distal zones of both arms (odour and air). A ratio of 0.5 indicates no preference, whereas a ratio significantly superior or inferior to 0.5 indicates a preference for the odour or the air (*i.e.* a repellence of the odour), respectively. Asterisks indicate a significant deviation from 0.5 (Wilcoxon signed rank test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Ama, *Ammi majus*, Ccy, *Centaurea cyanus*, Fes, *Fagopyrum esculentum*, Hon, Honey, Iam, *Iberis amara*, Ovu, *Origanum vulgare*.

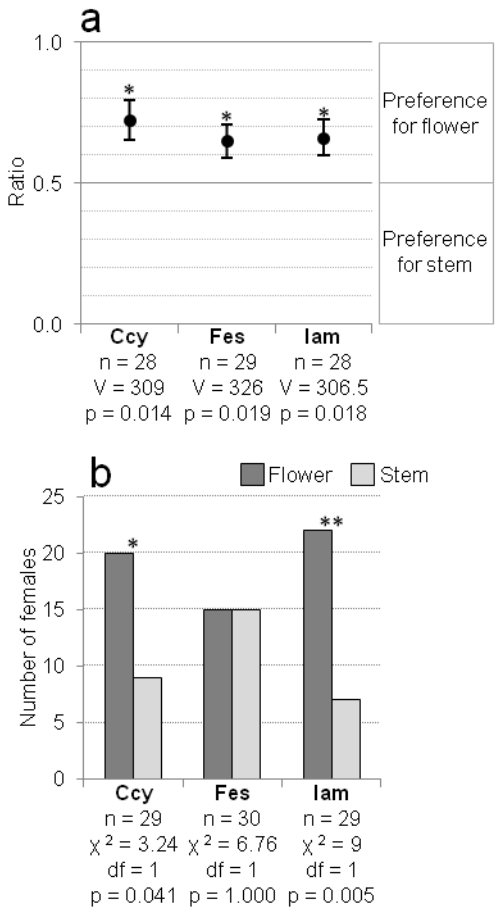

Fig. 3 – a) Number of females that first chose the flower arm (dark grey) or the stem arm (light grey). b) Mean (± standard error) ratio of the total time spent in the distal zone of the flower arm over the total time spent in the distal zones of both arms (flower and stem). See Fig. 2 for abbreviations and plot details.

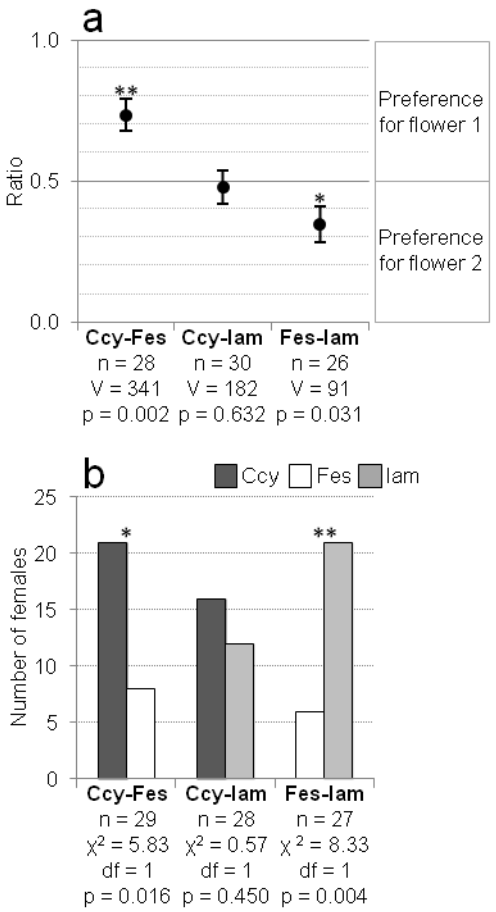

Fig. 4 – a) Number of females that first chose the *Centaurea cyanus* (Ccy, dark grey) arm, the *Fagopyrum esculentum* (Fes, white) arm, and the *Iberis amara* (Iam, light grey) arm. b) Mean (± standard error) ratio of the total time spent in the distal zone of one of the flower arm over the total time spent in the distal zones of both flower arms. Pair names give the two flowers tested against each other separated by a dash. Flower 1 and flower 2 refer to the first and second flower in the pair name, respectively. See Fig. 2 for plot details.

4. Discussion

The aim of this study was to investigate the olfactory attractiveness of five different flowering plants to female *M. mediator* to assess their suitability for conservation biological control in cabbage fields. The flower species we tested were all highly attractive when compared to air, both in terms of first choices and residence time, implying strong innate preferences by naive female *M. mediator*. This result is in line with a previous study which showed that naive females of the parasitoids *Pimpla turionellae* (Linnaeus) (Hymenoptera: Ichneumonidae), *Heterospilus prosopidis* (Viereck) (Hymenoptera: Braconidae) and *Cotesia glomerata* (Linnaeus) (Hymenoptera: Braconidae) exhibited innate preferences for flower odours tested against air (Wäckers, 2004). In our case, the attraction was maintained when *C. cyanus*, *F. esculentum* and *I. amara* flowers or inflorescences were compared to a piece of stem of the same plant, which demonstrates that female *M. mediator* were really attracted by the flower odours and not by volatiles emitted by other parts of the plant such as the stem. Kugimiya et al. (2010) showed a similar specific response to odours of mustard flowers *Brassica rapa* L. (Brassicaceae) in the parasitoid *Cotesia vestalis* (Haliday) (Hymenoptera: Braconidae). The results of experiment 3 showed that although all the flowers were similarly attractive when compared to air, some of them were more attractive than others when tested by pairs. *Centaurea cyanus* and *I. amara* were equally attractive when tested in paired choice, and they were both more attractive than *F. esculentum* which was the least attractive flower. This highlights the fact that absolute attractiveness is certainly important to identify flowers that are neither neutral nor repulsive to the parasitoids. But how attractive a flower is in the end and, hence, how easily it may be located by a foraging parasitoid, also depends on other available flowers.

The low attractiveness of *F. esculentum* compared to the two other plants can be explained by different mechanisms that remain to be elucidated in further experiments. The results of experiments 1 and 2 show that *F. esculentum* inflorescences produce attractive volatile molecules. A first hypothesis is that the molecules produced by *F. esculentum* are qualitatively less attractive or detectable to *M. mediator* than those produced by *C. cyanus* and *I. amara*. Second, the molecules produced by the three plants may be qualitatively equally attractive or detectable, but flowers of *F. esculentum* may produce them in lower quantities. Evidence supporting the importance of volatile quantity on parasitoid attraction was provided in a study about *Cotesia marginiventris* (Cresson) (Braconidae) (Turlings et al., 2004). Females of this parasitoid in fact respond in a dose-related manner to host induced green leaf volatiles and are more attracted in a 6-arms olfactometer to the arm connected to the highest number of plants (Turlings et al., 2004) If females *M. mediator* exhibited the same kind of dose-related response to find their food, they would then be more attracted by the flower emitting the highest amounts of chemical attractants when given the choice

between two flowers producing equally attractive volatiles. In the current setup, we could not distinguish if *F. esculentum* inflorescences were less attractive than the flowers of *C. cyanus* and the inflorescences of *I. amara* because they emitted volatiles that were qualitatively less attractive or because they emitted quantitatively less volatiles. Further tests are needed to collect, identify and quantify the volatiles emitted by the different flowering plants and to test whether female *M. mediator* respond in a dose related manner. It has to be noted that cutting the flowers could also have an influence, if one flower species were more stressed by the cutting than others. Although we cannot completely exclude this hypothesis, it seems unlikely since we used the flowers shortly after cutting, as has previously been done in another study investigating flower attractiveness to parasitoids (Wäckers, 2004). The analysis of flower volatiles has also often been done on cut flowers or inflorescences (Borg-Karlson et al., 1993; Raguso et al., 2006; Verdonk et al., 2005), or even on separate flower parts (Flamini et al., 2003), and it has been shown in *Lysimachia* species that cutting did not influence floral scent emission immediately after cutting (Schäffler et al., 2012).

Our results have implications for biological control, as we have shown that five different flowering plants that could be or are already used in wildflower strips or as companion plants are also attractive to *M. mediator* when offered as the sole wildflower (*i.e.* none of them is neutral or repulsive). However, some of them seem to be better suited than others. The attractiveness of the flowering plants did not correlate fully with parasitoids' performance on these flowers, a finding that is also in line with previous results on other parasitoid species (Wäckers, 2004). In a parallel study, Géneau et al. (2012) found that *C. cyanus* and *F. esculentum* greatly enhanced the longevity and fecundity of *M. mediator* compared to water, whereas *I. amara* had no detectable beneficial effect. Our expectation for adaptive olfactory attractiveness of plants that enhance parasitoid fitness was confirmed for *C. cyanus*. However, we did not expect *I. amara* to be attractive, let alone more attractive than *F. esculentum*. The lack of correlation between attractiveness and benefit to the parasitoids for these two flowering plants is hard to explain at first sight. It could result from the fact that in the field parasitoids are likely to encounter a broad range of different flowers, depending on the place where they emerge, so it would not be advantageous to develop a specific attraction to one or a few floral scents (Wäckers, 2004). Conversely, to maximize the chances of finding flowers (and thus food) in an unknown environment, they may respond to floral volatile compounds that many different flowering plants have in common, even at the risk that some of these plants do not provide suitable nectar. Parasitoids should then refine their ability to recognize suitable flowers through learning an ability that was shown in females of the related species *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae), which are able to associate odours with the presence of food and became more and more accurate in their choice with increasing number of odour experiences

(Takasu and Lewis, 1996). It is likely that *M. mediator* have evolved similar mechanisms of associative learning.

Flower attractiveness could of course be affected by other factors like visual cues (Begum et al., 2004; Kugimiya et al., 2010). But given the innate preferences of female *M. mediator* for the different flower odours shown in our study, it seems that this parasitoid evolved mechanisms to respond to distinct plant volatiles and use these olfactory cues to locate available food sources efficiently. Based on that, our results provide a panel of interesting flowers attractive to *M. mediator* that could be tested for their attractiveness and beneficial effects in the field. *Centaurea cyanus* appears to be the most promising one, because of its high absolute and relative attractiveness to the parasitoid and beneficial effect on its longevity and fecundity (Géneau et al., 2012). Although *I. amara* was as attractive as *C. cyanus*, it would clearly be less efficient in enhancing *M. mediator* in the field because it is not a suitable food source (Géneau et al., 2012). *Fagopyrum esculentum* in contrast is a very good food source for *M. mediator*, and numerous laboratory studies (Araj et al., 2011; Géneau et al., 2012; Lavandero et al., 2006; Nafziger and Fadamiro, 2011; Winkler et al., 2006; Witting-Bissinger et al., 2008) and field studies (Hogg et al., 2011; Lavandero et al., 2005; Lee and Heimpel, 2005; Lee and Heimpel, 2008; Platt et al., 1999; Scarratt et al., 2008) have demonstrated its value for enhancing natural enemies. However it is less attractive than both *C. cyanus* and *I. amara*, so it seems less efficient at attracting *M. mediator* in an environment where it is not the only flower available – which would be the case in a wildflower strip composed of multiple flowers or in/around cabbage fields where other weeds are growing. Additionally, *C. cyanus* is the only plant among those we tested that produces easily accessible extrafloral nectar (Winkler et al., 2009), which is generally a much longer-lived potential resource for beneficial insects than floral nectar (Koptur, 2005). There are therefore good reasons to think that *C. cyanus* has the best potential among the different flowers tested in this study to promote *M. mediator* in the field. This highlights the importance of taking flower attractiveness into account in the choice of suitable wildflowers for conservation biological control.

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CHAPTER 3

Quantitative olfactory information use for food foraging in the parasitoid *Microplitis mediator* (Hymenoptera: Braconidae)

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Abstract

In parasitoids, larvae feed on the body of their hosts whereas adults have to seek other sugar-rich food sources, primarily (extra-) floral nectar, to ensure their survival. As not all food sources are equally abundant and/or near, adult parasitoids are expected to have evolved capacities to detect the most promising food sources in terms of proximity and/or abundance. In this study, we tested whether female *Microplitis mediator* foraging for food sources, *i.e.* flowers of cornflower *Centaurea cyanus* and inflorescences of buckwheat *Fagopyrum esculentum*, were able to use quantitative olfactory information to orient themselves towards the most promising (*i.e.* most abundant and/or nearest) food sources. We used a 6-arm olfactometer where the wasps could choose between the scents emitted by different numbers of flowers/inflorescences. Additionally, we identified and quantified the volatiles emitted by the flowers/inflorescences of the two flower species in order to better explain the observed behavioural responses of the wasps. We show that female *M. mediator* were able to use quantitative olfactory information, as we predicted. In general, they were most attracted to the highest numbers of flowers/inflorescences, which also emitted the highest volatile quantities. However, the discrimination ability of the wasps was limited and depended on the flower species. Their response to single flowers was very strong already and showed saturation with increasing numbers of flowers in *C. cyanus*, the flower species emitting both substantially higher absolute volatile quantities and more potentially attractive types of volatile compounds (*e.g.* benzenoïds). Conversely, the response of the wasps to few inflorescences (inferior to four) was very weak but accelerating with increasing numbers of inflorescences in *F. esculentum*, the flower species emitting low volatile quantities. The wasps also needed a higher contrast to discriminate between numbers of flowers/inflorescences in *C. cyanus* than in *F. esculentum*. This suggests that a higher sensitivity at low volatile quantities could have been selected in *M. mediator* and that the wasps are less choosy at high volatile quantities, which would be adaptive. These results highlight the importance of taking flower density into account to optimize the use of floral subsidies for biological control purposes.

1. Introduction

In parasitoids, larvae feed on the body of their hosts whereas adults have to seek other food sources, such as honeydew produced by aphids and (extra-)floral nectar (Wäckers, 2005). Some adult female parasitoids can also feed by imbibing blood from their hosts. These host-feeding parasitoids are generally ectoparasitoids that lay large yolky eggs on the body of their hosts and need host blood primarily for egg production (Harvey et al., 2012). Honeydew and (extra-)floral nectar on the other hand are sources of carbohydrates that are primarily utilized for maintenance, both in host-feeding and non host-feeding parasitoid species (Harvey et al., 2012; Zhang et al., 2011). Honeydew has generally proved to be a less suitable food source than (extra-)floral nectar (Wäckers et al., 2008), and finding (extra-)floral nectar is thus crucial for the survival of most adult parasitoids (Wäckers, 2005).

The time spent by female parasitoids on foraging for food (hereafter referred as "food foraging") cannot be devoted to finding hosts (hereafter referred as "host seeking"), which comes at a high cost because host encounters are directly correlated with offspring production (Gols et al., 2005). Female parasitoids thus face a trade-off between searching for hosts to enhance their fecundity and searching for food to enhance their survival (Desouhant et al., 2005; Lewis et al., 1998; Siekmann et al., 2004; Sirot and Bernstein, 1996). As they should tend to minimize the time they devote to food foraging, the selective pressure acting on traits that enhance food foraging efficiency of female parasitoids, for example by using olfactory cues to locate suitable food sources, is expected to be high.

It has previously been shown that female parasitoids are able to use olfactory and visual cues to successfully locate floral food sources (Begum et al., 2004; Belz et al., 2013; Géneau et al., in press; Kugimiya et al., 2010; Wäckers, 2004). However, these studies only investigated the quality of the cue, be it a colour or an odour, which conveys information about the presence of a certain type of resource. But in a patchy and unpredictable environment, not all food sources are equally abundant and/or near, and female parasitoids should also be able to use quantitative information to orient themselves towards the most promising resources in terms of proximity and/or abundance. Usage of such information should allow the wasps to increase their chances of getting a full meal and minimize the foraging costs in terms of energy usage and predation risk during food search and movement between patches. One study has shown that host seeking female *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) were able to use quantitative olfactory information. When tested in a 6-arm olfactometer, they were more attracted to the air blown over the highest numbers of maize plants attacked by their hosts, and the highest numbers of plants also emitted the highest quantities

of volatiles (Turlings et al., 2004). However, such kind of dose-dependent response has to our knowledge never been investigated in food foraging parasitoids.

In a previous study, we have shown that female *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae), which are generalist non-host feeding parasitoids of noctuid pests, are differentially attracted to different floral scents. They were attracted to the scent of cornflower *Centaurea cyanus* L. (Asteraceae) and buckwheat *Fagopyrum esculentum* Moench (Polygonaceae), demonstrating that they used qualitative olfactory information to localize food sources whose quality potentially differs (Belz et al., 2013). These two flower species also both increased the longevity and lifetime fecundity of *M. mediator* (Géneau et al., 2012), but cornflower was more attractive than buckwheat (Belz et al., 2013). However, the study could not distinguish whether the differential olfactory attractiveness of cornflower and buckwheat was due to qualitative differences of the flower species or quantitative differences between the odour blends emitted by them.

Here, we tested whether *M. mediator* is able to use quantitative olfactory information. We expect that selection should not only have favoured choice mechanisms in wasps to distinguish between food qualities, but also use of quantitative information indicative of proximity and/or abundance of a food source. This information could also be relevant for biological control purposes. *Centaurea cyanus* and *F. esculentum* have both been used as floral subsidies, either in wildflower strips planted along field margins or as companion plants inside the crops (Balmer et al., submitted; Pfiffner et al., 2003). They have been shown to specifically enhance the longevity and fecundity of *M. mediator* but not of *M. brassicae* (Géneau et al., 2012). The purpose of the floral subsidies is to attract parasitoids that are present in the vicinity of the field and to provide them with food sources that are otherwise lacking right next to the pest larvae to minimize parasitoid movements away from the pests. The ultimate goal of this approach is to increase pest parasitism rates and crop yield (Landis et al., 2000). However, several studies have failed to demonstrate that the presence of floral subsidies lead to increased parasitism rates (Berndt et al., 2002; Pfiffner et al., 2009) and increased parasitism rates do not necessarily translate into reduced crop damage or increased yield (Balmer et al., submitted). There is thus room for methodological improvement, and the effect of different flower densities on pest parasitism rates is one parameter to optimize. We therefore assessed in the laboratory whether flower density does have an influence on the attraction of parasitoids.

Specifically, we performed behavioural experiments in a 6-arm olfactometer to test (1) whether female *M. mediator* responded in a dose-dependent manner to the odour emitted by different numbers of flowers of cornflower and of inflorescences of buckwheat, and (2) whether their response differed for these two flower species. We further conducted qualitative and quantitative analyses of the volatiles emitted by different numbers of flowers of cornflower and of inflorescences of buckwheat to test (3) whether they were producing different types of volatile compounds and/or

different quantities of volatile compounds. Finally, we tested (4) whether the quantities of emitted volatiles correlated with the numbers of flowers. These experiments allowed us to associate the behavioural responses of the parasitoids not only with the numbers of flower/inflorescences, but also with volatile quantities.

2. Materials and methods

2.1. Plants and parasitoids

Flowering plants were grown from seeds in GroBanks (CLF Plant Climatics, Germany) at 21±2 °C, 40±10% r.h. and 12L:12D photoperiod. The parasitoid rearing was established with *M. brassicae* as a host in a climate chamber at 23 ±1°C, 60 ±10% r.h. and 16L:8D photoperiod. The rearing conditions of the plants and the parasitoids are described in Belz et al. (2013). All the females used for the experiments were provided with only water from emergence to the behavioural test and had no contact with the tested odour sources previous to the experiments. They were aged between 24 and 72 hours and used only once.

2.2. Behavioural experiments

2.2.1. The 6-arm olfactometer

To test the response of female *M. mediator* to different numbers of flowers of *C. cyanus* and inflorescences of *F. esculentum*, we conducted behavioural experiments in a 6-arm olfactometer built based on Turlings et al. (2004). The 6-arm olfactometer that we used in this study only had a few differences to the one described in Turlings et al. (2004). The major difference was that our olfactometer was not equipped for conducting volatile collections directly from the odour sources used during the bioassays. Some of the glass parts were also simplified, but distances and tube diameters were maintained to ensure compatibility of results.

As odour sources, we used flowers of *C. cyanus* or inflorescences of *F. esculentum.* Flowers and inflorescences were cut and the stem was first wrapped in a wet piece of cotton and then in a piece of aluminium to prevent wilting and limit the emission of volatiles by the cut part. The odour sources were used immediately after cut and put inside glass vials, either size A or size B depending on the number of flowers/inflorescences. The size A vials were 2.5 cm diameter x 12 cm long tubes with a small 1 cm diameter opening on one end. The size B vials were made of a top part (8.5 cm diameter x 10 cm height) screwed onto a bottom part (7.5 cm diameter x 9.5 height) through a ground-glass joint (73-80 mm) and had three 1 cm diameter connections (a horizontal one on the bottom part and two vertical ones on the top part). One of the two vertical connections was sealed

with a Teflon cap during all the experiments. Control vials contained wet cotton and a piece of aluminium.

The air was first filtered by glass cotton and activated charcoal to limit impurities present in ambient air and pushed into the system by a vacuum pump. The air passed through a gas washing bottle containing demineralised water to be humidified before being split and lead through six flow meters (Teflonrotameter 65 mm, Analyt-MTC, Germany) regulating the airflow. The air was pushed through the vials containing the flowers/inflorescences inside each arm of the central part of the olfactometer at 0.433 l/min.

2.2.2. Behavioural assays

To test whether female *M. mediator* use quantitative olfactory information to locate the most promising patches of *C. cyanus* flowers and *F. esculentum* inflorescences, we conducted a first experiment in which we offered the wasps the choice between air blown over one, two and four flowers/inflorescences in three of the six arms of the olfactometer while the three other arms remained as controls. Since the females responded differently to *C. cyanus* and *F. esculentum*, we then conducted different additional experiments for the two flower species to further test how the wasps respond to odours emitted by different numbers of flowers/inflorescences. In *C. cyanus*, we tested (1) whether an increase of the contrast between the numbers of flowers would enable the wasps to differentiate between them and (2) whether the response observed with a higher contrast between the numbers of flowers would hold for increased numbers of flowers. We thus increased the numbers of flowers to 1, 6, 11 and to 11, 16, 21, respectively. In *F. esculentum*, we tested (1) whether the response observed in the first experiment would hold with higher numbers of inflorescences and (2) whether the females would distinguish between the different numbers of inflorescences if the contrast between them was decreased. We thus offered them 4, 6, 8 and 5, 6, 7 inflorescences, respectively.

Female *M. mediator* were aspirated from their cage with a D-cell vacuum insect aspirator (MX-991/U, Hausherr's Machine Works, Tom Rivers, NJ, U.S.A.) into an insect-release vial. They were released in groups of six in the central chamber of the olfactometer. Most of the females walked up towards the light placed above the central part of the olfactometer and arrived inside the chamber where they could make a choice for one of the arms. Thirty minutes after release, the number of females that had made a choice, *i.e.* that were inside a trapping bulb or inside the glass part supporting a trapping bulb connected to the end of each arm, was counted for each one of the six arms. All the females were removed before a new group was released.

If not mentioned otherwise, experiments consisted of six blocks, with each block consisting of six releases of six wasps (36 wasps per block). For the experiment with 11, 16 and 21 *C. cyanus*

flowers, we could conduct only five blocks due to a flower shortage: four blocks with six releases of six wasps each, and one block with only five releases of six wasps each. The six releases of a block were usually conducted on the same day. Only in a few cases the number of wasps available on a given day was not sufficient to conduct the six releases which were then conducted over two or three consecutive days. The wasps were tested with the same odour sources on a given day and the positions of the odour sources remained the same for an entire block. The position of the odour sources was randomized between blocks. All the glass parts of the olfactometer, as well as the Teflon tubes located after the vials containing the flowers, were thoroughly washed after each block with acetone (Technical grade, Axon Lab AG, Switzerland) and pentane (≥ 99.0 %, Fluka Analytical, Sigma-Aldrich) and then placed in an oven at 250°C for six hours. The vials containing the odour sources were pre-washed with water if necessary to eliminate nectar and pollen remains.

2.3. Floral volatile analysis

2.3.1. Headspace volatile collections

In a separate experiment, we collected the volatiles emitted by *C. cyanus* flowers and *F. esculentum* inflorescences. Flowers and inflorescences were prepared the same way as described in "2) a – The 6-arm olfactometer" and were put inside the same types of vials for the headspace volatile collections. The air inside the vials was sucked through a volatile collection trap (VCT) containing 30 mg of Super-Q (ARS Inc., Gainesville, FL, USA) by a vacuum pump. The VCT was inserted directly into the screw cap of the small opening of size A vials, and in the screw cap of one of the top connections of size B vials. The other end of the VCT was connected via a Teflon connector to a Teflon tube leading to a flow meter (Teflonrotameter 150 mm, Analyt-MTC, Germany) that kept a constant airflow of 1.732 l/min through the VCT. A filter made of glass cotton and activated charcoal was connected before the vial containing the flowers to limit the collection of impurities contained in ambient air. Each collection lasted two hours and the collected volatiles were eluted from the Super-Q with 200 µl of acetone (ROTISOLV ≥ 99.9% UV/IR-Grade, Carl Roth GmbH + Co. KG) immediately after. The eluted solution was kept at -30°C until analysis in a gas chromatogram-mass spectrometer (GC-MS). The VCTs were washed twice each with dichloromethane (ROTISOLV ≥ 99.9% GC Ultra Grade, Carl Roth GmbH + Co. KG), acetone and heptane (ROTISOLV ≥ 99.9% UV/IR-Grade, Carl Roth GmbH + Co. KG) and put in an oven at 70°C to dry over night before being used again. The glass vials were washed once with the same solvents between each collection. If necessary, they were pre-washed with demineralised water to eliminate nectar or pollen remains.

For the purpose of compound identification we made a volatile collection based on a larger number of flowers/inflorescences to obtain adequate compound quantities for reliable mass-spectral identification. To this end, we put between 25 and 50 *C. cyanus* flowers and between 50 and 100 *F.*

esculentum inflorescences inside a size B vial. The collections for the two flower species were done in parallel and volatiles from an empty vial were also collected at the same time to serve as a control. The stems were not wrapped in cotton and aluminium because there were too many flowers. This collection, hereafter referred as the "reference collection", was done only once and only used for mass-spectral analysis for compound identification.

To compare the quantities of volatiles emitted by flowers of *C. cyanus* and inflorescences of *F. esculentum*, we put one flower of *C. cyanus* and one inflorescence of *F. esculentum* inside separate size A vials. We always collected in parallel the air from a control container in which we put a piece of wet cotton and a piece of aluminium, and we did 10 collections per flower species.

Finally, to compare the quantities of volatiles emitted by different numbers of flowers of *C. cyanus* and inflorescences of *F. esculentum*, we used the same procedure as described for the collection of volatiles emitted by single flowers and inflorescences but put the required number of flowers or inflorescences inside the containers instead of one. We did 10 collections per number of flowers for each of the two flower species.

2.3.2. Gas chromatography-mass spectrometry

We injected 2 µl of the extract from the VCT elution into a GC-MS (Agilent Technologies 7890A GC coupled to a Agilent 5975 MSD) equipped with a polar capillary column (VF-WAXms, 30 m (L) x 0.25 mm (ID) x 0.39 mm (OD), Agilent Technologies AG, Switzerland), and with a helium flow rate of 1 ml/min. The injector temperature was set to 250° and the MS source temperature to 230°C. The runs were operated in splitless mode. After a solvent delay of 4.5 minutes, the oven was held at 40°C for 6 minutes and the temperature then steadily increased by 10°C/min until it reached 230°C where it was maintained for 5 minutes.

2.3.3. Volatile identification and quantification

Chromatograms were analysed using ChemStation software (Hewlett-Packard, Agilent Technologies). Peaks were identified by using the NIST/EPA/NIH Mass Spectral Library version 2008 (NIST 08, National Institute of Standards and Technology, Gaithersburg, MD), based on their retention time and mass spectrum. When the mass spectrum of a peak showed a high match factor with the mass spectrum of a compound in the library, it was further checked in Pherobase (http://www.pherobase.com/) and in literature references whether it had already been identified as a floral compound in other flower species. The identity of individual major peaks was also confirmed by comparison of both mass spectra and retention times with those of synthetic compounds. The identity of a peak was considered as confirmed when the match factor with a compound in the library was superior to 900 (which is an excellent match, a perfect match resulting in a value of 999)

and this compound had already been identified in at least one other floral scent or when the mass spectrum and retention time of the peak were exactly identical to the mass spectrum and retention time of a pure synthetic compound. For each flower species, the identity of the peaks that were not present in the control sample (*i.e.* the sample collected from the vial with only aluminium and wet cotton) was determined from the reference collection. Peaks identified as floral compounds in the reference collection were then integrated in the chromatograms resulting from the collection of the volatiles emitted by one or more flowers or inflorescences of the corresponding flower species.

2.4. Statistical analyses

Statistical analyses were conducted using R 2.13.2 (R Development Core Team, 2011) and JMP Pro 10.0.1 Version 2 (SAS Institute Inc., 2012).

For the behavioural analyses, we performed generalized linear mixed effect models (glmmPQL) in R with the number of wasp per olfactometer-arm as dependent variable, the number of flowers as a fixed factor, the date and the release as random factors and a poisson error distribution to test for significant differences between the numbers of wasps attracted by the different numbers of flowers. Since the glmmPQL does not report overall p-value, we transformed the number of flowers into an ordered factor (which is sensible because we have a clear prediction that the number of wasps recruited should increase with increasing numbers of flowers) and ran the glmmPQL again. We took the p-value of the linear term (which indicates whether the numbers of wasps recruited is linearly ascending) as a p-value for an overall test.

For the floral volatile analyses, we conducted linear models in R (lm), first to test whether the mean total quantity of volatiles emitted per one flower/inflorescence differed between *C. cyanus* and *F. esculentum*, and second to test whether there were differences between the quantities of volatiles emitted by the different numbers of flowers within each flower species. For all the linear models that were conducted, the residues were normally distributed. Finally, for two floral compounds in *C. cyanus* (acetophenone and benzaldehyde), we performed a student t-test in JMP to compare whether four flowers emitted significantly different amounts of the compounds than two flowers.

3. Results

3.1. Behavioural assays

In all the experiments, there was a significant positive overall effect of the number of flowers on the number of attracted wasps (see Fig. 1 and 2 for statistical values).

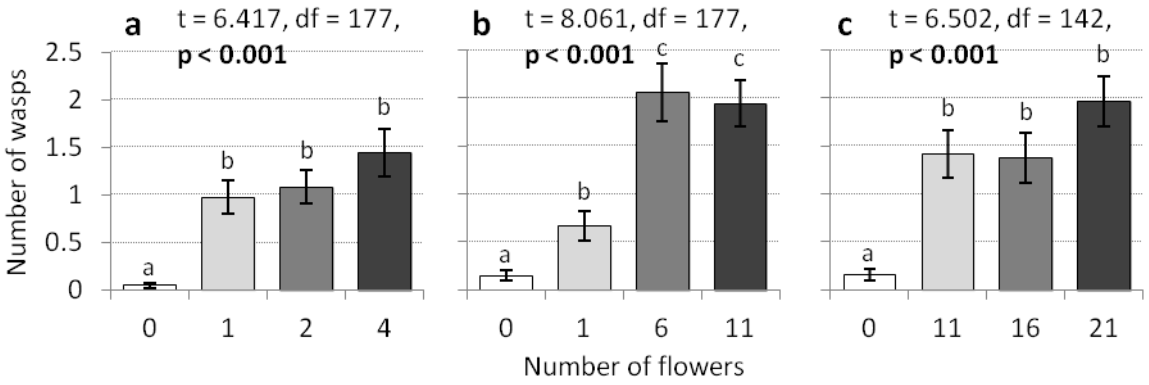

Fig. 1 – Mean (± SE) number of female *M. mediator* counted in the arms of the 6-arm olfactometer when offered (a) 1, 2 and 4, (b) 1, 6 and 11 and (c) 11, 16 and 21 flowers of *C. cyanus*. The numbers of wasps counted in the three control arms (0 flowers) are summed. Bars with different letters differ significantly (generelized linear mixed model, $p < 0.05$).

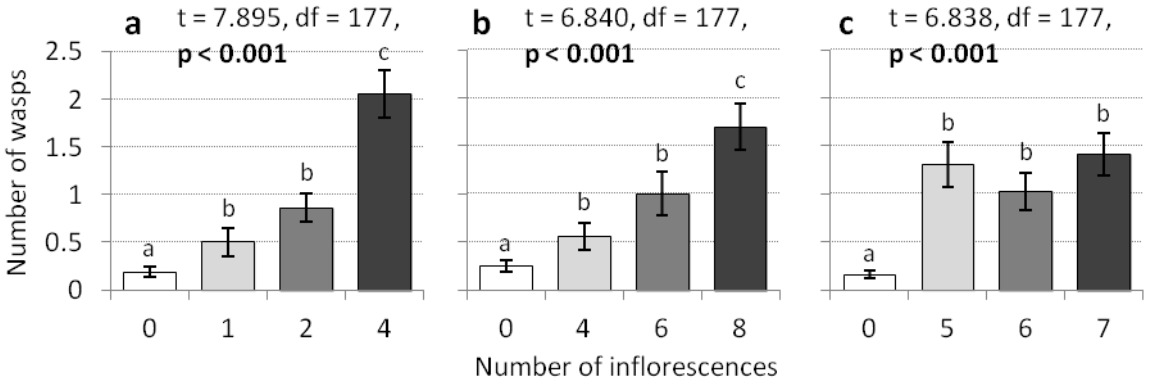

Fig. 2 – Mean (± SE) number of female *M. mediator* counted in the arms of the 6-arm olfactometer when offered (a) 1, 2 and 4, (b) 4, 6 and 8 and (c) 5, 6 and 7 inflorescences of *F. esculentum*. See Fig. 1 for plot details.

In *C. cyanus*, the first experiment showed that significantly more wasps were attracted in the arms with 1, 2 and 4 flowers than in the control arms (contrast; $t = 5.579$, $df = 177$, $p < 0.001$; $t = 5.815$, $df = 177$, $p < 0.001$; $t = 6.437$, $df = 177$, $p < 0.001$; respectively). The number of attracted wasps tended to increase with the number of flowers, but the difference between the numbers of wasps attracted by the different numbers of flowers was not significant (contrasts; 1 versus 2 flowers: $t = 0.407$, $df = 177$, $p = 0.684$; 1 versus 4 flowers: $t = 1.586$, $df = 177$, $p = 0.114$; 2 versus 4 flowers: $t = 1.190$, $df = 177$, $p = 0.236$) (Fig. 1a). In the second experiment, there were significantly more wasps attracted by 1, 6 and 11 flowers than by the controls (contrasts; $t = 3.667$, $df = 177$, $p < 0.001$; $t = 7.507$, $df = 177$, $p < 0.001$; $t = 7.311$, $df = 177$, $p < 0.001$; respectively). There were also significantly more wasps attracted by 6 and 11 flowers than by 1 flower (contrasts; $t = 3.772$, $df = 177$, $p < 0.001$

and t = 3.561, df = 177, p < 0.001, respectively), but the numbers of wasps attracted by 6 and 11 flowers were not significantly different from each other (contrast; t = 0.262 , df = 177, p = 0.793) (Fig. 1b). In the third experiment there were again significantly more wasps attracted by 11, 16 and 21 flowers than by the controls (contrasts; t = 5.625, df = 142, p < 0.001; t = 5.543, df = 142, p < 0.001; t = 6.722, df = 142, p < 0.001; respectively), but no significant differences between the numbers of wasps attracted by the different numbers of flowers (contrasts; 11 versus 16 flowers: t = 0.089, df = 142, p = 0.929; 11 versus 21 flowers: t = 1.289, df = 142, p = 0.200; 16 versus 21 flowers: t = 1.376, df = 142, p = 0.171) (Fig. 1c).

In *F. esculentum*, the first experiment showed that there were significantly more wasps attracted by 1, 2 and 4 inflorescences than by the controls (contrasts; t = 2.524, df = 177, p = 0.013; t = 4.425, df = 177, p < 0.001; t = 7.886, df = 177, p < 0.001; respectively). There were also significantly more wasps attracted by 4 inflorescences than by 1 (contrast; t = 4.442, df = 177, p < 0.001) and 2 inflorescences (contrast; t = 3.358, df = 177, p = 0.001) (Fig. 2a). In experiment 2, the response was similar. There were significantly more wasps attracted by 4, 6 and 8 inflorescences than by the controls (contrasts; t = 2.186, df = 177, p = 0.030; t = 4.399, df = 177, p < 0.001; t = 6.688, df = 177, p < 0.001; respectively). There were also significantly more wasps attracted by 8 inflorescences than by 4 (contrast; t = 3.496, df = 177, p = 0.001) and 6 inflorescences (contrast; t = 2.027, df = 177, p = 0.044) (Fig. 2b). In the third experiment, there were significantly more wasps attracted by 5, 6 and 7 inflorescences than by the controls (contrasts; t = 6.988, df = 177, p < 0.001; t = 5.987, df = 177, p < 0.001; t = 7.335, df = 177, p < 0.001; respectively). However, there were no significant differences between the numbers of wasps attracted by the different numbers of flowers (contrasts; 5 versus 6 flowers: t = 1.0176, df = 177, p = 0.310; 5 versus 7 flowers: t = 0.378, df = 177, p = 0.706; 6 versus 7 flowers: t = 1.389, df = 177, p = 0.167) (Fig. 2c).

3.2. Floral volatile analysis

3.2.1. Volatile identification

In *C. cyanus*, we found 19 floral peaks in the reference collection, among which 11 were also present on the chromatograms of the treatments with one, two or four flowers. Eight of the 11 compounds could be identified (Table 1). The compounds identified in the floral scent of *C. cyanus* were one alcohol (1-phenylethanol), three benzenoids (acetophenone, benzaldehyde, benzyl alcohol), and four sesquiterpenoids (alpha- and beta-caryophyllene, beta-cubebene and (Z)-beta-farnesene). Benzyl alcohol is also an alcohol but nevertheless belongs to the benzenoids because it possesses a benzene ring. For the subsequent quantification, the peak of acetophenone had to be pooled with the smaller peak of (Z)-beta-farnesene because they constituted a double peak and

could not be integrated separately. The scent of *C. cyanus* flowers was dominated by benzaldehyde and by acetophenone coupled with (Z)-beta-farnesene (Fig. 3a).

In *F. esculentum* we found nine floral peaks in the reference collection, which could all be subsequently quantified on the chromatograms of the treatments with one, two and four flowers. Three of the nine compounds could be identified (Table 1), which represents a lower diversity of compounds than in *C. cyanus*. These compounds were one acid (isovaleric acid), one monoterpenoid (beta-ocimene) and one nitrogen-containing compound (indole). The major compounds emitted by *F. esculentum* inflorescences were isovaleric acid and one unidentified compound, hereafter referred as "unidentified x", that was also present in the scent of *C. cyanus* (Fig. 3b). "Unidentified x" was the only compound shared by both flower species.

Table 1 – Compounds identified in the reference collection for *C. cyanus* and *F. esculentum*. The identification of compounds marked with an asterisk was confirmed through comparison with the pure synthetic compound. [a]Chemical Abstracts Service (CAS) registry number. [b]Retention time in minutes.

Flower species Compounds	Formula	CAS[a]	RT[b]
C. cyanus			
Alcohol:			
1-phenylethanol	$C_8H_{10}O$	13323-81-4	19.125
Benzenoids:			
Acetophenone*	C_8H_8O	98-86-2	17.313
Benzaldehyde*	C_7H_6O	100-52-7	15.693
Benzyl alcohol	C_7H_8O	100-51-6	19.840
Sesquiterpenoids:			
alpha-caryophyllene*	$C_{15}H_{24}$	6753-98-6	17.520
beta-caryophyllene*	$C_{15}H_{24}$	87-44-5	16.637
beta-cubebene	$C_{15}H_{24}$	13744-15-5	17.999
(Z)-beta-farnesene	$C_{15}H_{24}$	28973-97-9	17.376
F. esculentum			
Acid:			
Isovaleric acid	$C_5H_{10}O_2$	503-74-2	17.530
Monoterpenoid:			
beta-ocimene	$C_{10}H_{16}$	13877-91-3	11.455
Nitrogen containing compound:			
Indole	C_8H_7N	120-72-9	25.310

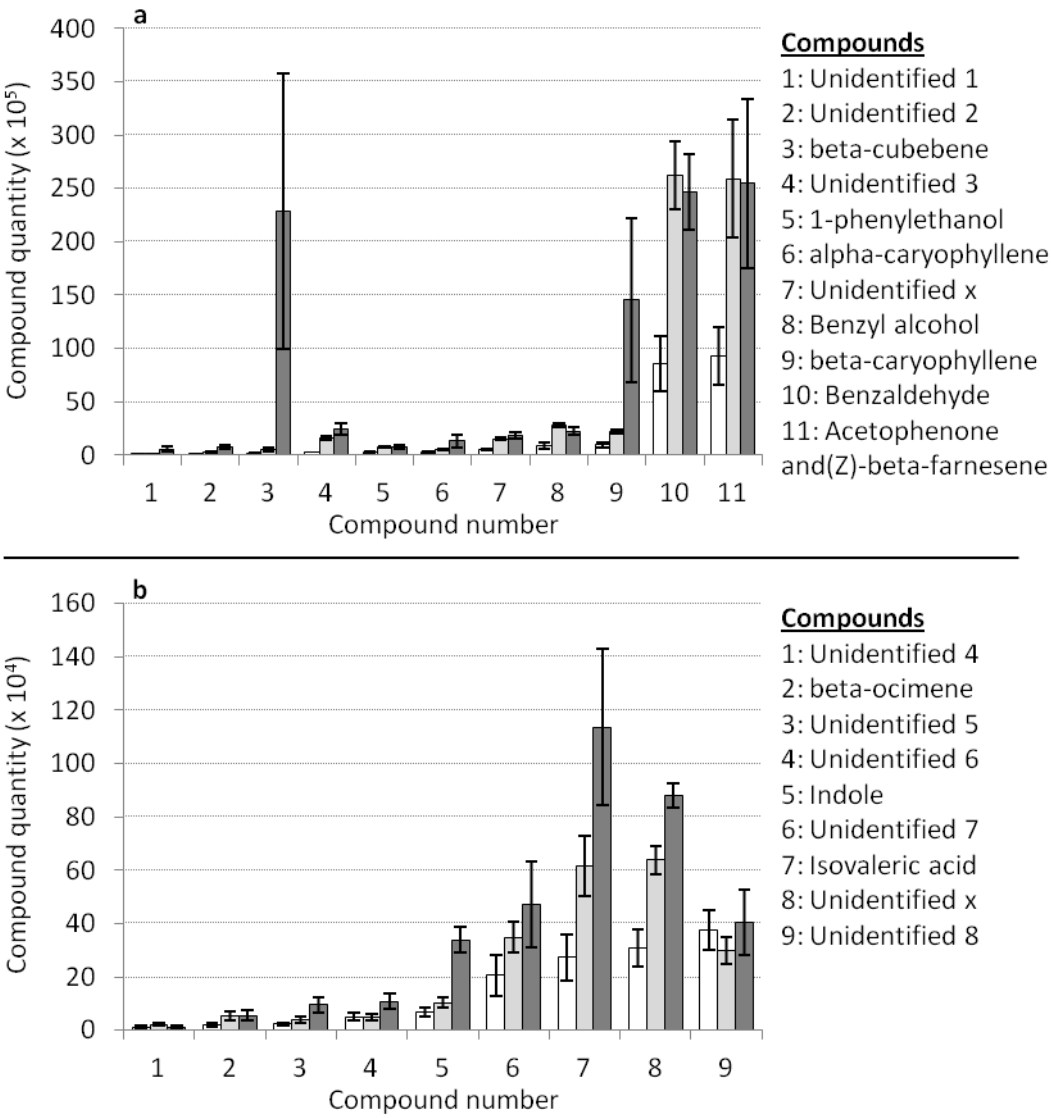

Fig. 3 – Mean (± SE) quantity of the different compounds (expressed as the mean (± SE) peak area in arbitrary unit) produced by one (white), two (light grey) and four (dark grey) (a) flowers of *C. cyanus* and (b) inflorescences of *F. esculentum*. Acetophenone and (Z)-beta-farnesene were integrated together because they constituted a double peak and could not be integrated separately. "Unidentified x" is an unidentified compound shared by both species.

3.2.2. Volatile quantities

One flower of *C. cyanus* emitted a 12-fold higher total quantity of volatiles than one inflorescence of *F. esculentum* (F = 13.115, df = 18, p = 0.002) (Fig. 4).

In both flower species, there was a significant effect of the number of flowers on the total quantity of emitted volatiles (Fig. 4). In *C. cyanus*, 4 flowers emitted significantly more volatiles than one (contrast; t = 3.716, df = 27, p = 0.001) and marginally significantly more volatiles than 2 (contrast; t = 1.990, df = 27, p = 0.057). The quantity of volatiles emitted by one and 2 flowers did not

differ significantly (contrast; t = 1.727, df = 27, p = 0.096) (Fig. 5a). In *F. esculentum*, 2 inflorescences emitted significantly more volatiles than one (contrast; t = 3.204, df = 27, p = 0.035) and 4 inflorescences emitted significantly more volatiles than 2 (contrast; t = 2.715, df = 27, p = 0.011) and one (contrast; t = 5.919, df = 27, p < 0.001) (Fig. 5b).

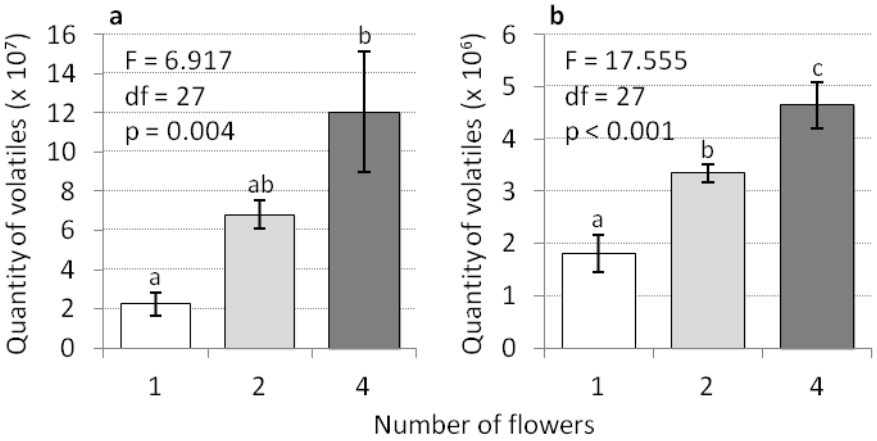

Fig 4 – Comparison between the mean (± SE) total quantities of volatiles (expressed as the mean (± SE) sum of all peak areas in arbitrary units) emitted by 1, 2 and 4 (a) flowers of C. cyanus and (b) inflorescences of F. esculentum. Bars with different letters are significantly different from each other (linear model, p < 0.05).

In both flower species, patterns of increase were similar for most of the different flower compounds. However, in both species, the quantity of some compounds did not change in the same manner between the flowers numbers (Fig. 3). In *C. cyanus* especially, a few replicates of the four flowers treatment produced unexpected high amounts of beta-cubebene and beta-caryophyllene while the amounts of acetophenone coupled with (Z)-beta-farnesene and the amounts of benzaldehyde did not significantly increase from two to four flowers, contrary to what would be expected (t = 2.070, df = 9, p = 0.068 and t = 1.564, df = 9, p = 0.152, respectively) (Fig. 3a).

3.3. Association between volatile quantities and wasps behaviour

To compare wasp preferences with volatile quantities rather than numbers of flower/inflorescences, we substituted the numbers of flowers/inflorescences by the corresponding volatile quantities that we obtained from the volatile collection experiment. This comparison has to be interpreted cautiously because we sampled the volatile quantities in a different experiment, but it allows us to qualitatively assess how the preference is related to volatile quantities, which is of interest to understand dose-response relationships. We assumed that the total quantities of volatiles emitted by the flowers of *C. cyanus* and by the inflorescences of *F. esculentum* during the volatile collections were representative of the total quantities of volatiles emitted by the flowers of *C. cyanus*

and by the inflorescences of *F. esculentum* in the behavioural assays. We plotted the mean numbers of attracted wasps against the quantity of volatiles per flower-number treatment (Fig. 5). The relationship between the number of attracted wasps and the quantity of volatiles differed between the two flower species: there seems to be saturation of the wasps' response with increasing volatile quantities in *C. cyanus* whereas wasps' attraction seems to accelerate with increasing volatile quantities in *F. esculentum*.

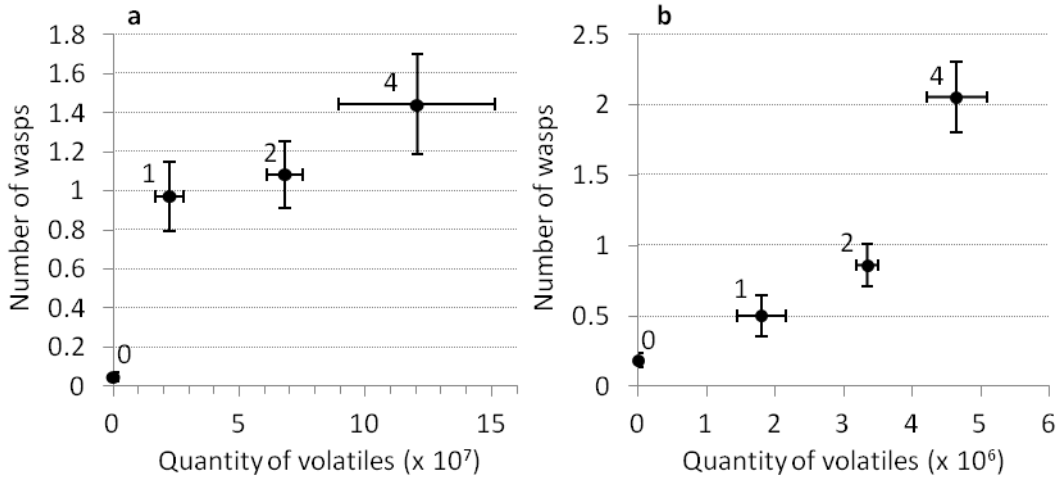

Fig. 5 - Mean (± SE) number of wasps attracted by 0, 1, 2 and 4 (numbers next to the dots) (a) flowers of *C. cyanus* and (b) the inflorescences of *F. esculentum* plotted against the mean (± SE) quantity of volatiles (expressed as the mean (± SE) sum of all peak areas in arbitrary units) emitted by 1, 2 and 4 flowers/inflorescences.

4. Discussion

In a previous study we had shown that female *M. mediator* used qualitative olfactory information to detect potential food sources. They were attracted by the scent of *C. cyanus* flowers and *F. esculentum* inflorescences, and when given the choice between the two species they preferred *C. cyanu*s over *F. esculentum* (Belz et al., 2013). In the present study, we demonstrated that the female wasps were able to discern quantitative olfactory information, which is likely to be of importance to them in order to localize and orient themselves towards the most abundant and/or the nearest food sources. In both flower species, they were able to differentiate between the different numbers of flowers/inflorescences when the contrast between them was high enough (Fig. 1b, 2a and 2b) and they were most strongly attracted to the highest numbers of flowers/inflorescences. The fact that volatile quantities correlated with the numbers of

flowers/inflorescences (Fig. 4) implies that the wasps responded to volatile quantities in a dose-dependent manner.

However, the wasps responded differently towards the two flower species. The dose-dependent response was clearer in *F. esculentum*, as the wasps seemed not to discriminate between the numbers of flowers/inflorescences as well in *C. cyanus* as they did in *F. esculentum*. One flower of *C. cyanus* was enough to recruit almost as many wasps as four flowers (Fig. 1a), and the contrast between the numbers of flowers/inflorescences that the wasps needed to differentiate between the odour sources was higher in *C. cyanus* than in *F. esculentum* (five versus two flowers/inflorescences, respectively). The response of the wasps quickly levelled off for increasing numbers of flowers in *C. cyanus* (Fif. 1b), while it increased more strongly with increasing numbers of inflorescences in *F. esculentum* (Fig. 2b). The two different types of the wasps' response were even clearer when the numbers of wasps recruited by one, two and four flowers/inflorescences were plotted against the quantity of volatiles emitted by the same numbers of flowers/inflorescences. The response saturated in *C. cyanus* while it seemed to accelarate in *F. esculentum* (Fig. 5).

Different non-mutually exclusive hypotheses relying on the floral volatile analyses could explain the dissimilar behavioural responses of the wasps towards the two flower species. First, we showed that *C. cyanus* and *F. esculentum* released very different kinds of compounds, and the sensitivity of the wasps to these compounds may differ. On one hand, the scent of *C. cyanus* was dominated by two benzenoïds (benzaldehyde and acetophenone) previously found in other *Centaurea* species (Andersson et al., 2002). Benzenoïds, in particular benzaldehyde, are the most widespread floral scent compounds and are known to be important attractants for pollinators such as flies, butterflies and hymenopteran species (Dötterl et al., 2005; Jürgens, 2004; Jürgens et al., 2009). It has even been hypothesized that benzaldehyde produced in nectar of *Dianthus inoxianus* Gallego could act as a "nectar guide" and/or be an honest signal indicating rewarding flowers (Balao et al., 2011). On the other hand, inflorescences of *F. esculentum* emitted mostly isovaleric acid and an unidentified compound, whereas compounds that are found in moth pollinated species, like beta-ocimene and indole, were minor (Jürgens et al., 2003; Levin et al., 2001). Benzenoïds were surprisingly absent from the scent of *F. esculentum* that seem to emit less potentially attractive compounds than *C. cyanus* flowers. If the female wasps identify the compounds contained in the scent of *C. cyanus* as reliable indicators of very rewarding flowers, this could explain why *C. cyanus* was more attractive than *F. esculentum* inflorescences in our previous study (Belz et al., 2013). It could also explain why in the present study the scent emitted by single flowers of *C. cyanus* was enough to elicit a strong response from the wasps.

The difference in the behavioural responses of the wasps towards *C. cyanus* and *F. esculentum* could also be explained by the quantitative differences between their floral scents. Single

C. cyanus flowers emitted much higher total quantities of volatiles than single *F. esculentum* inflorescences, which could contribute to signal them as a very rewarding food source, *i.e.* one abundant enough to provide the wasps with a full meal and/or close enough to be reached without a long travelling time. Information about the distance to the food source may however vary with the pattern of odour diffusion (advection or turbulence) (Farré-Armengol et al., 2013). Nevertheless, the fact that the response of the wasps to increasing numbers of *C. cyanus* flowers quickly levelled off could be due to the high amounts of volatiles that they emitted, either because the wasps had reached a sensory threshold above which they did not have the capacity to discriminate between the different quantities of volatiles anymore, or because they made the choice not to discriminate. In this experiment, we cannot distinguish between these two hypotheses. But whatever the mechanism involved, it would make sense from the point of view of the wasps to not be too sensitive at high volatile quantities.

At high volatile quantities, small increases of volatile quantities should not have a strong impact on the probability of getting a full meal since this probability is already very high, so wasps that are very sensitive to small increases of volatile quantities would not perform better than less sensitive wasps. Conversely, at low volatile quantities, the wasps should be more sensitive to each small increase of volatile quantities, as it would have a strong impact on the probability of getting a full meal. Selection should thus favour high discrimination abilities at low volatile quantities. This would explain the saturated response of the wasps towards increasing numbers of *C. cyanus* flowers that emit high volatile quantities as well as the accelerating response of the wasps towards increasing numbers of *F. esculentum* inflorescences that emit low volatile quantities. To confirm this hypothesis, it would be interesting to test the response of the wasps towards diluted scent of single *C. cyanus* flowers and towards very high numbers of *F. esculentum* inflorescences.

As we predicted, *M. mediator* has evolved foraging capacities to not only detect qualitative information but also quantitative information about potential food sources. It is also adapting its foraging behaviour in response to the specificities of the perceived floral scent, *i.e.* the type of compounds and/or the quantities of compounds that are produced, and seems to discriminate between food sources only when it is advantageous. This type of flexibility should help the wasps to localize the most rewarding and/or the nearest food sources efficiently. These innate preferences are however not perfectly adapted to the environment, as *F. esculentum* appears to be identified as a low rewarding food source by the wasps, because it elicits a weak response at low numbers of inflorescences, while it is a very suitable food source (Géneau et al., 2012). The discrimination abilities of *M. mediator*, and hence its food foraging efficiency, should be further refined by learning (Vet et al., 1995). It has been shown in the related species *Microplitis croceipes* Cresson that females are able to associate an odour with the presence of food (Takasu and Lewis, 1996). Moreover,

parasitoids should be able to learn not only a type of odour (*i.e.* certain types of volatile compounds) but also a specific quantitative blend of volatiles (*i.e.* relative quantities of the different compounds within the blend as well as total quantity of the whole blend) to improve their response to a certain type of food source that is present in the environment at a particular time. It has previously been shown that host seeking female parasitoids can be guided by specific volatile blends released from sites where hosts are present (Hilker and McNeil, 2008). They are able to use the quantitative ratio of a host-indicating key compound to quantities of background volatiles to successfully localize their hosts (Beyaert et al., 2010). Such high sensitivity to quantitative ratios is also expected to have evolved in female parasitoids to enhance the efficiency of food detection and localization.

The fact that the attraction of *M. mediator* by flowering plants was mediated by volatile quantities, which depended on flower densities, could be of importance for biological control. There is a need to improve the use of *C. cyanus* and *F. esculentum* as floral subsidies, *i.e.* floral resources added in wildflower strips planted along field margins or as companion plants inside the crops to provide parasitoids with food sources that would otherwise be lacking and increase pest parasitim rates. Here we show that attention should be paid to flower densities and optimal flower densities should be assessed for each flower species. *Fagopyrum esculentum* has become something of a model plant for floral subsidy studies (Mills and Wajnberg, 2008). Our study however suggests that the density of flowering plants needed to attract a certain number of *M. mediator* individuals would probably be higher in *F. esculentum* than in *C. cyanus*. The final decision on which floral subsidy to use should also be based on the costs that would be required to plant the adequate flower density to recruit a sufficient number of wasps to significantly reduce pest damage and increase yield.

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GENERAL DISCUSSION

Summary of results

In the present work focusing on the foraging behaviour of *Microplitis mediator* (Haliday), I first demonstrated that females of this parasitoid species use olfactory cues to locate potential food sources, *i.e.* flowers or inflorescences providing nectar (Chapter 2). Although I did not directly work on food intake, I assumed that female *M. mediator* were foraging for food because they were food deprived, and it has been shown in the related species *Microplitis croceipes* (Cresson) that unfed females search for food while well-fed females search for hosts (Takasu and Lewis, 1993). I identified five wildflower species that were attractive to naive female *M. mediator*, which was more than expected. In fact, Wäckers (2004) tested the attractiveness of 11 flower species to three different parasitoid species, and only four of the flower species tested in his study were attractive. Among the attractive wildflower species that I identified, the cornflower, *Centaurea cyanus*, was the most promising one, as it combined a high attractiveness with a positive effect on the longevity and fecundity of *M. mediator*. Buckwheat, *Fagopyrum esculentum*, was less attractive than *C. cyanus*, although it was as suitable as a food source. Finally, candytuft, *Iberis amara*, was as attractive as *C. cyanus* but did not increase the longevity and fecundity of *M. mediator* (Géneau et al., 2012).

In chapter 3, I showed that female *M. mediato*r were not only able to use qualitative olfactory information but also quantitative information to orient themselves toward the food sources with the potential to offer them the most abundant reward and/or to be located at the shortest distance. In the 6-arm olfactometer, they were generally more attracted by the scent emitted by the highest numbers of flowers/inflorescences of *C. cyanus* and *F. esculentum*. However, the response of the wasps towards the two flower species differed. The contrast between the different numbers of flowers/inflorescences was important for the wasps to be able to discriminate and had to be higher in *C. cyanus* than in *F. esculentum*. In *C. cyanus*, the response of the wasps to single flowers was very strong already and showed saturation with increasing numbers of flowers. Conversely, in *F. esculentum*, the response of the wasps to few inflorescences (inferior to four) was weak but accelerated with increasing numbers of inflorescences.

The qualitative analysis of the floral volatiles emitted by *C. cyanus* and *F. esculentum* that was conducted in chapter 3 revealed that these two flower species produce very different types of volatile compounds. *Centaurea cyanus* emits a higher diversity of compounds than *F. esculentum*. Some of the compounds found in the scent of *C. cyanus* (benzenoids) are well-known potential insect attractants (Jürgens, 2004; Jürgens et al., 2009). I also analysed the scent emitted by *Iberis amara*

inflorescences using the protocol described in chapter 3, and found that it emitted three acids/esters (3-hexen-1-ol acetate, (E-), beta-phenethyl acetate, isovaleric acid), two alcohols (3-hexen-1-ol, phenylethyl alcohol), two benzenoids (2-phenylethanol, phenylacetaldehyde) and one nitrogen bearing compound (benzyl nitrile) (Appendix, Table x1). These results were not presented in chapter 3 because they could not be connected to any behavioural assays in the 6-arm olfactometer, but they will be of importance for further discussion here. The quantitative analysis of the floral scents showed that volatile quantities correlated with the numbers of flowers in both *C. cyanus* and *F. esculentum*, but that single *C. cyanus* flowers emitted a 12-fold higher quantity of volatiles than single *F. esculentum* inflorescences.

Below, I would like to further discuss these findings in the context of behavioural ecology on one hand, and in the context of biological control on the other hand, and to propose potentially interesting future research questions.

Interpreting wasp foraging behaviour from an evolutionary perspective

According to the present work, female *M. mediator* seem well adapted for food foraging. They are able to use olfactory cues to identify potential food sources and are attracted by floral scents emitted by very different flowers, which should increase their likelihood of finding a food source compared to random searching (Röse et al., 2006). The fact that they are attracted by the scent of flowers that do not constitute a suitable food source, like *I. amara*, seems maladaptive at first glance. It however makes sense considering that *M. mediator* has a wide distribution range from Central Europe to China (Foerster and Doetzer, 2003) and is likely to have experienced very different environments during its evolution. Even locally, the flower species that will be blooming at the time a wasp emerges are hardly predictable. The reliability of cues from flowers in indicating a suitable food source are thus likely to be low over evolutionary time. Being attracted only by very specific floral odour blends that do not have a high likelihood of being encountered should not be selected because it would strongly decrease the likelihood of the wasps to find food sources and place them under a high risk of dying from starvation. On the contrary, *M. mediator* is expected to have evolved preferences for volatile compounds that are common to the scent of many flower species, even at the risk that some of them do not provide any (suitable) nectar reward (Wäckers, 2004).

The fact that the floral scents of *C. cyanus*, *F. esculentum* and *I. amara* all contained very ubiquitous floral scent compounds known for attracting pollinators supports this hypothesis. In particular, benzenoids found in the scents of *C. cyanus* and *I. amara* could be possible attractants (Jürgens, 2004; Jürgens et al., 2009). The scent of *F. esculentum* lacked these benzenoid compounds. But *F. esculentum* inflorescences emitted some beta-ocimene, a monoterpenoid that is commonly

found in floral scents (Andersson et al., 2002; Levin et al., 2001). Although beta-ocimene was not a major compound in the scent of *F. esculentum*, it does not indicate that the wasps do not use it. It was shown in the egg parasitoid *Closterocerus ruforum* (Krausse) that the attraction of host seeking females by volatiles emitted by the host plant was triggered by a slight increase in the quantity of one minor compound of the blend, which was sufficient to signal the presence of suitable hosts on the plant (Beyaert et al., 2010). The major compounds in a floral scent may thus not necessarily be the ones that are used by the wasps to localize the flower/inflorescence. The results of the behavioural tests in the Y-tube olfactometer where *F. esculentum* inflorescences were compared to a piece of stem demonstrated that the floral scent of *F. esculentum* does contain some attractive compounds and beta-ocimene could be one possible attractants, as it is very ubiquitous in floral scent (Andersson et al., 2002; Levin et al., 2001). Finally, it has been shown that *Origanum vulgare* emits three benzenoids (2-phenylacetaldehyde, 2-phenylethanol and benzaldehyde) as well as beta-ocimene (Andersson et al., 2002), which also support the idea that *M. mediator* could be attracted by very common floral scent compounds.

The present study was however conducted with naive wasps that had no feeding experience. But it has been well described that adult parasitoids are able to learn and to associate cues that they encounter in the environment with the presence of a resource after very few experiences (Bleeker et al., 2006; Segura et al., 2007; Van Nouhuys and Kaartinen, 2008; Wäckers and Lewis, 1994; Wardle, 1990). Learning can even take place during the larval stage of the parasitoid and influence adult behaviour, which is called “pre-imaginal learning” (Gandolfi et al., 2003; Gutierrez-Ibanez et al., 2007). The learning abilities of parasitoids have mostly been studied and reviewed in host seeking female parasitoids (Turlings et al., 1993; Vet et al., 1995), but were also shown in food foraging parasitoids (Olson et al., 2003; Röse et al., 2006; Takasu and Lewis, 1996). It is thus likely that *M. mediator* has evolved such learning abilities to refine its foraging behaviour and adapt it to the resources that are currently available in its environment. This would enable adult *M. mediator* to correct for mismatches between floral attractiveness and floral reward and to increase their foraging efficiency. After a few encounters with an attractive but unrewarding flower species like *I. amara*, the wasps should cease to be attracted by it. Conversely, a few encounters with a mildly attractive but very rewarding flower species like *F. esculentum* should increase the attraction of the wasps by this flower species. Studying the learning abilities of *M. mediator* could be one possible area of future research.

In chapter 3, I showed that food foraging female *M. mediator* are not only well adapted to identify potential food sources, but also to localize the ones that are likely to be the most profitable for them. In a patchy and unpredictable environment, not all the food sources are equally abundant and/or near, and quantities of volatiles give information about abundance and distance to the food

sources. Female *M. mediator* were able to compare the quantity of volatiles emitted by different numbers of flowers/inflorescences and to use that information to orient themselves towards the highest numbers of flowers. This should increase their likelihood of finding very rewarding food sources efficiently and to get full meals in a minimum amount of time compared to if they would be attracted by the quality of floral scents only. Moreover, the response of the wasps to volatile quantities was adapted to the situation. In the case of *F. esculentum*, which emits low volatile quantities, they needed a small contrast between the numbers of inflorescences to be able to discriminate between them. Their response to low numbers of inflorescences (inferior to four) was weak but was accelerating with increasing numbers of inflorescences. This type of response seems adaptive because low volatile quantities are likely to indicate scattered or faraway food sources, so the wasps should not be strongly attracted by them. However, at low volatile quantities, each slight increase of volatile quantity is expected to have a substantial impact on the probability of getting a full meal. A high sensitivity should therefore be selected in the wasps at low volatile quantities because wasps that are highly sensitive to slight differences in volatile quantities should perform better than wasps that are less sensitive. Conversely, high volatile quantities are likely to indicate very abundant or close food sources and the wasps should be very attracted to them. Above a certain threshold of volatile quantity, the probability for the wasps to get satiated without having to travel over a long distance is not expected to be dramatically influenced by slight increases of volatile quantities. A high sensitivity should therefore not be selected in the wasps at high volatile quantities because wasps that are very sensitive to slight differences in volatile quantities should not perform better than wasps that are less sensitive. This would explain the response exhibited by female *M. mediator* towards *C. cyanus* flowers, which emit high volatile quantities.

To conclude, the results of the present work indicate that female *M. mediator* are able to perceive and use qualitative and quantitative volatile information to forage for food efficiently. To deepen the understanding of the foraging behaviour of *M. mediator*, electroantennograms could be conducted to identify which compounds from the floral scents of *C. cyanus* and *F. esculentum* elicit an antennal response, *i.e.* are physiologically perceived by *M. mediator*. After that, it would be possible to conduct bioassays in an olfactometer to test the attractiveness of the compounds that elicit an antennal response by offering them singly or as a blend to *M. mediator*. After identifying attractive compounds/blend of compounds, it would be possible to vary volatile concentration to study the dose-response of the wasps very finely and to test whether their sensitivity decreases with increasing volatile quantities, as hypothesised earlier. It would also be possible to disentangle the effects of scent quality and scent quantity. By offering the wasps the choice between equal concentrations of the compound/blend corresponding to *C. cyanus* and the compound/blend corresponding to *F. esculentum*, it would be possible to test if the low attractiveness of *F. esculentum*

compared to *C. cyanus* is due to the emission of less attractive types of compounds and/or lower total volatile quantities. A choice could also be given to the wasps between a low concentration of a highly attractive compound/blend and a high concentration of a weakly attractive compound/blend to test which parameter between volatile quality and volatile quantity is the most important for foraging wasps. If quality is more important than quantity, the wasps would choose the most attractive compound, whereas if quantity is more important than quality, the wasps would choose the least attractive compound. This kind of work would require very modern equipment (especially to conduct the electroantennograms) and was not the scope of my PhD, but could be an interesting topic for future research. It would give insights into the mechanisms involved in olfactory information use in *M. mediator*.

Potential for exploiting flower attractiveness and wasp foraging behaviour for biological control

In crops where flowering plants are scarce, floral subsidies can be added to attract nectar feeding parasitoids that are present in the vicinity and increase their survival and/or fecundity by providing them with food sources that would otherwise be lacking (Landis and Menalled, 1998). The ultimate goal of this approach is to increase pest parasitism rates and crop yield (Mills and Wajnberg, 2008). Floral subsidies can be planted as wildflower strips along field margins or as companion plants inside the crops (Landis et al., 2000). They must be selected according to certain criteria, such as their attractiveness, their effects on the longevity and fecundity of the parasitoids (Wäckers, 2004) and their selectivity (*i.e.* they must benefit the parasitoids but not the pests) (Lavandero et al., 2006). Flower attractiveness to parasitoids is expected to have an impact on parasitoid population dynamics and hence, on pest control (Bianchi and Wäckers, 2008). The identification of attractive flower species presented in chapter 2 is thus important for the selection of floral subsidies that have the potential to attract *M. mediator* in the field. To date, *F. esculentum* has somewhat become a reference for floral subsidy studies (Mills and Wajnberg, 2008), but I showed that *C. cyanus* seems more promising because of its higher attractiveness to *M. mediator*.

Parallel to my project, the effect of *C. cyanus* as a companion plant on the control of *M. brassicae* has been tested in a large-scale field experiment conducted over two years (Balmer et al., submitted). It showed that the presence of *C. cyanus* significantly increased the parasitism of *M. brassicae* larvae by *M. mediator* in one of the two years of the study, which demonstrates that the use of selected companion plants can lead to an increase of parasitism rates. However, the increased parasitism rate did not translate into reduced crop damage or yield increase. Contrary to what was expected, a significant yield increase due to the presence of *C. cyanus* was detected in the year where there was no significant increase of the parasitism of *M. brassicae* larvae (Balmer et al.,

submitted). One possible explanation is that the yield increase was due to increased parasitism of other important cabbage pests, such as *Pieris brassica*e (Linnaeus) (Ahuja et al., 2010), by their parasitoids, although they were initially not targeted. The attractiveness and the suitability of *C. cyanus* as a food source should be tested for other important parasitoids of cabbage pests, particularly egg parasitoids. Egg parasitoids indeed have a higher potential in reducing crop damage than larval parasitoid, because the development of larval parasitoids requires the host larva to stay alive and keep feeding whereas each egg parasitized results in the death of one pest individual before it can cause any damage to the crop (Balmer et al., submitted).

Another possible explanation for the mitigated effects of *C. cyanus* as a companion plant could be that the flowers also attract more pest species. The study of Géneau et al. (2012) showed that *C. cyanus* selectively benefit *M. mediator* because it does not increase the longevity and fecundity of *M. brassicae*. However, if *C. cyanus* attracts a higher density of *M. brassicae* in the field, pest damage will also increase, which could counteract the beneficial effects of increased densities of *M. mediator*. The fact that *C. cyanus* flowers emit volatiles that are known to be moth and butterfly attractants (Dötterl et al., 2005; Jürgens, 2004; Jürgens et al., 2009) suggests that they could be rather unselective and attract *M. brassicae*. However, the floral volatile analysis and the behavioural assays presented in chapter 3 were conducted in daylight, as we were testing the attractiveness to *M. mediator*, which is diurnal. But *M. brassicae* is nocturnal and scent emission by *C. cyanus* flowers could differ between day and night, as shown in other flower species (Balao et al., 2011). It would thus be important to conduct behavioural assays to test the attractiveness of *C. cyanus* to *M. brassicae* in night conditions. As suggested above, conducting electroantennograms on *M. mediator* would allow to identify compounds that are physiologically perceived by this parasitoid species. The same could be done for *M. brassicae*, followed by behavioural assays to test the attractiveness of single compounds or blends of compounds. In theory, it would then be possible to identify compounds/blends that are attractive to *M. mediator* and not to *M. brassicae* and to search in databases like Pherobase (http://www.pherobase.com/) for flower species that emit these selective compounds/blends. This could be a valuable tool for the selection of potential floral subsidies.

Finally, another reason why the effects of *C. cyanus* as a companion plant were limited could be due to the density of cornflowers planted in the field. The results presented in chapter 3 suggest that *C. cyanus* flowers should be very attractive, even at low densities. However, the behavioural assays were conducted in very controlled laboratory conditions. In the field, floral scents are immediately diluted (Farré-Armengol et al., 2013) and mixed with background odours (Hilker and McNeil, 2008). Parallel to my work, a study was conducted to collect directly in the field volatiles from wildflower strips containing *C. cyanus* and from plots where *C. cyanus* was added as a companion plant (Barloggio, unplublished data). The volatile collection and the volatile analyses were

done using the same volatile collections traps, the same elution method and the same GC-MS temperature program as presented in chapter 3. These field volatile collections did not contain any of the volatile compounds that I identified in the scent of *C. cyanus*, suggesting that the scent of *C. cyanus* is much diluted in the field. This could indicate that the density of flowers planted in the field was too low to attract sufficient numbers of *M. mediator* to have a substantial effect on cabbage yield. The density of flowers planted in the field should be optimized so that the quantity of floral volatiles produced reaches the same range as the quantity of volatiles produced by single flowers in the volatile analyses presented in chapter 3. The behavioural assays in the 6-arm olfactometer indeed showed that the quantity of volatiles emitted by single *C. cyanus* flowers was sufficient to efficiently recruit *M. mediator*. However, the flower densities needed to achieve these volatile quantities could be too high to be an economically viable solution.

Conclusion

To conclude, it is quite clear to me that it is much easier to conduct experiments in controlled conditions in the laboratory than to put the results into practice in the field where sources of variation are infinite. My PhD project yielded some answers about the adaptive functions of *M. mediator* foraging behaviour, but more work is needed until these findings really benefit the biological control of *M. brassicae* in the field. I believe that models should be of help to translate the behavioural data that are collected in the laboratory into useful predictions on parasitoids and pests population dynamics (Mills and Kean, 2010; Roitberg, 2007). However, I hope that I have been able to show, at least to some extent, how behavioural ecology and biological control can work hand in hand and benefit each other.

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APPENDIX

Table x1 – Compounds identified (8 over a total of 14 floral peaks) in the reference collection for *I. amara*. [a]Chemical Abstracts Service (CAS) registry number. [b]Retention time in minutes.

Compounds	Formula	CAS[a]	RT[b]
Acid / Ester:			
3-hexen-1-ol acetate, (E-)	$C_8H_{14}O_2$	3681-82-1	12.590
beta-phenethyl acetate	$C_{10}H_{12}O_2$	103-45-7	19.160
Isovaleric acid	$C_5H_{10}O_2$	503-74-2	17.530
Alcohol:			
3-hexen-1-ol	$C_6H_{12}O$	544-12-7	13.650
Phenylethyl alcohol	$C_8H_{10}O$	60-12-8	20.210
Benzenoids:			
2-phenylethanol	$C_8H_{10}O$	60-12-8	20.210
Phenylacetaldehyde	C_8H_8O	122-78-1	17.215
Nitrogen containing compound:			
Benzyl nitrile	C_8H_7N	140-29-4	20.430

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