Promoting *Cotesia rubecula* Marshall, 1885 (Hymenoptera: Braconidae) against the cabbage pest *Pieris rapae* Linnaeus, 1758 (Lepidoptera: Pieridae) through flowering plants

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SUMMARY

Conservation biological control is an approach to control pests on an environment-based manner that can contribute to a reduction in pesticide use. Providing food sources and protection for natural enemies and aesthetically enriching production landscapes in form of flower strips, promotes biodiversity and ecosystem conservation. FiBL has developed a tailored flower strip mixture for cabbage cultivation that contains the flower species *Fagopyrum esculentum* Moench (Polygonaceae), *Vicia sativa* L. (Fabaceae) and *Centaurea cyanus* L. (Asteraceae). It was shown to have positive effects on various antagonists of cabbage pests. In this thesis we investigated the potential of the flower strip mixture to control the cabbage pest *Pieris rapae* (Linnaeus 1758) (Lepidoptera: Pieridae), by promoting its antagonist *Cotesia rubecula* (Marshall 1885) (Hymenoptera: Braconidae).

To conduct these investigations, a successful rearing of both *C. rubecula* and *P. rapae* had to be established. Because a complete knowledge transfer from publications is not always possible, experimental conditions were evaluated prior to testing the suitability of the flower species to attract *C. rubecula* and their potential to enhance both insect species in longevity and fecundity trials.

Besides exhibiting exploitable nectar, flowers should preferably be olfactorily attractive, as highly attractive flowers are easily located, reducing the time spent searching for food and subsequently increasing the *per capita* host searching efficiency. With a Y-tube olfactometer we found that *C. cyanus* and to a lesser extent *V. sativa* successfully attract *C. rubecula*. Also *F. esculentum* attracts *C. rubecula*, but only after a rewarding feeding experience. Even though not every flower offering accessible nectar is also innately attractive, it can still be suitable for conservation biological control purposes as feeding experience can change this attraction. Moreover, the application of mixtures containing attractive and rewarding flowers could help increase the success of such programs. These results support the application of the flowers in the field.

Many times flowers have been deployed for conservation biological control purposes. But rarely has it been checked whether these flowers were selective plants, enhancing beneficial insects but not the pests. The three flowering plants were suggested as selective plants for conservation biological control purposes against the cabbage moth *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae). Here, we tested their effects on the fitness of *P. rapae* and its antagonist *C. rubecula*. We performed survival and fecundity assays in the laboratory for each insect. The longevity of the wasps was enhanced by all flower treatments and their fecundity by *F. esculentum* and *C. cyanus* as a function of longevity. But also the butterflies' longevity and fecundity were enhanced by *F. esculentum* and *C. cyanus*. Selective plants for conservation biological control, which are suitable for a certain pest-beneficial insect complex, are not necessarily suitable for another insect complex present in the same crop. In this case the flowers should not be supplemented to the fields as a conservation biological control measure against *P. rapae*.

To reach desired pest control effects, flowers need to be carefully selected to promote the beneficial insect of interest, but not the pest. Although laboratory results may support or oppose the application of flowers in the field, only field trials can reveal the actual effects. In this thesis, field trials were conducted over two consecutive years to evaluate the potential of the FiBL developed flower strip mixture to control *P. rapae*. Because it is known that parasitism rates decrease with increasing distance from flower strips, cabbage plants were additionally intercropped with *C. cyanus.* The aim was to draw parasitoids into the field and retain them there, consequently increasing parasitism rates. The supplemented flowers did not lower cabbage yields nor increased *P. rapae* pest densities in insecticide-free plots. This indicates, that insecticide applications could possibly be spared. In fact, we found a positive correlation of parasitism rates and wild *P. rapae* pest densities in control fields and a negative correlation in flower supplemented fields. The selected flowers seem to be suitable for conservation biological control purposes only when pest densities are low, as they seemingly ignore hosts for nectar when pest densities are high.

GENERAL INTRODUCTION

Over the last 100 years, Europe has suffered a strong decline of biodiversity in agricultural landscapes (Donald *et al.* 2006; Ekroos *et al.* 2010; Ollerton *et al.* 2014), due to habitat loss or fragmentation, changes in landscape structure and agriculture intensification as leading causes (Robinson & Sutherland 2002; Fischer & Lindenmayer 2007; Tsiafouli *et al.* 2015). A seasonal decline of 76% and a mid-summer decline of 82% in flying insect biomass was estimated by Hallmann *et al.* (2017) over 27 years of study and they claimed that the loss of insect abundance and diversity is expected to provoke cascading effects on food webs and to endanger ecosystem services.

To counteract these trends, farmers can positively influence biodiversity through in-field options and ecological compensation (Stoeckli *et al.* 2017). Organic farming aims to increase species richness, averaging 30% higher species richness than conventional farming systems (Bengtsson *et al.* 2005). Other potential benefits include higher biodiversity and improved soil and water quality per unit area, higher nutritional value, and enhanced profitability (Seufert & Ramankutty 2017). In contrast to integrated production (IP) and conventional practices, no chemical-synthetic insecticides are permitted in organic agriculture. Therefore, allowed pesticides are massively restricted to a few products. Accordingly, the potential to protect beneficial insect populations is higher in organic than in IP and conventional production. However, there is a number of organic broad-acting insecticides that are permitted in organic agriculture. They contain the active substances spinosyn A and B, two fermentation products of the bacteria *Saccharopolyspora spinosa* (Actinomycetales: Pseudonocardiaceae) (Kirst *et al.* 1991), which are very effective against multiple pests. Unfortunately, they affect not only pest insects, but beneficial insects too (Mason *et al.* 2002; Schneider *et al.* 2003; Schneider *et al.* 2004; Xu *et al.* 2004; Biondi *et al.* 2012; Liu & Zhang 2012; Firake *et al.* 2017). Furthermore, their widespread and repeated use increases the risk of resistance development by the targeted species (Zhao *et al.* 2002). For these reasons among many, insecticides

should only be used as a last resort and not as a common measure against pest insects.

In 2018 the federal popular initiative "For clean drinking water and healthy food - no subsidies for the use of pesticides and prophylactic antibiotics" has been launched in Switzerland (Initiative-sauberes-Trinkwasser 2019). When such initiatives find acceptance, the need for alternatives to insecticides rises even further.

Conservation biological control as part of an Integrated Pest Management strategy, is a sustainable approach to pest control that can contribute to a reduction in pesticide use. It is a complex strategy based on a number of environmental and behavioral processes, functioning at numerous scales, and mediated by management actions that are, potentially targeting a broad range of pest organisms (Begg *et al.* 2017). Sowing functional flower strips at field margins can be part of a conservation biological control approach.

In industrialized countries, flower strips are a common measure to promote biodiversity and ecosystem service conservation, as they not only provide additional food sources and protection for natural enemies and pollinators, but also aesthetically enrich production landscapes (Westphal *et al.* 2015). These management systems support biological control through a combination of effects of increased immigration, retention, longevity and fecundity on natural enemies (Altieri & Letourneau 1982).

In Switzerland, farmers have to manage part of their land as biodiversity promotion areas (3.5% for special crops such as cabbage and 7% for others), for which they receive subsidies (BLW 2019). These areas are important because they enhance biodiversity, potentially increasing pest regulation. Furthermore, they also shape the landscape serving as recreation areas, benefiting human health. Since ecological compensation areas need to be implemented anyway in Swiss agriculture, it makes sense to pay attention to the functional diversity as it does not only lead to more biodiversity, but is also expected to deliver more ecosystem services (Uyttenbroeck *et al.* 2015). Functionality may be improved through selecting specific flower species, which to a greater extent enhance beneficial insects and not pests.

The brassica family is one of the ten economically most valuable crop families worldwide (Fahey 2003). Apart from their nutritional value, brassica crops produce glucosinolates, which are associated with anti-carcinogenic properties (Devlieghere *et al.* 2003). In Switzerland, cabbages and other brassicas were cultivated on an area of 1137 ha, producing 39295 tons in 2017 (FAOSTAT 2017).

The small white *Pieris rapae* (Linnaeus 1758) (Lepidoptera: Pieridae) is one of the major lepidopteran pests in cabbage cultivations together with the large white *Pieris brassicae* (Linnaeus 1758) (Lepidoptera: Pieridae), the cabbage moth *Mamestra brassicae* (Linnaeus 1758) (Lepidoptera: Noctuidae) and the diamondback moth *Plutella xylostella* (Linnaeus 1758) (Lepidoptera: Plutellidae) (Patriche *et al.* 2005). Other lepidopteran pests found in cabbage fields are *Autographa gamma* (Linneaus 1758) (Lepidoptera: Noctuidae) and *Helicoverpa armigera* (Hübner 1808) (Lepidoptera: Noctuidae) (Patriche *et al.* 2005).

Larvae of the cabbage white butterflies are worldwide considered as major pests in several economically important brassica crops (Harvey *et al.* 2010). While young larvae superficially feed on cabbage leaves leaving the upper leaf surface intact, older larvae make holes in the leaves and are more likely to eat through small veins (CABI 2019). More than 80 % of feeding damage is caused by the fifth instar (Wei *et al.* 1983). Often they eat their way through to the center of the head, causing yield loss. Moreover their excrement can make the cabbage unmarketable when present in masses. The chewing damage together with the moist excrements open gates for secondary pests such as fungi, viruses and bacteria. If not controlled, damage from *P. rapae* larvae can result in total crop loss (Hely *et al.* 1982).

As a conservation biological control measure, FiBL has developed a tailored flower strip mixture for cabbage cultivation which was recognized and provisionally approved as a biodiversity promotion area by the Federal Office for Agriculture (FOAG) in September 2015. This enabled farmers to purchase the seed mixture and register the strip as a biodiversity promotion area for which they receive subsidies. Contributing to biodiversity in Switzerland in 2019 with "flower strips for pollinators and other beneficial insects" is rewarded with 2500 CHF/ha (BLW 2019). The five

commercially available flower strip mixtures which are eligible for subsidies are "pollinator basic version", "pollinator full version", “beneficial insects summer culture”, “beneficial insects winter culture” and "beneficial insects cabbage cultivation" (AGRIDEA 2019).

The last mentioned mixture contains the flower species *Fagopyrum esculentum* Moench (Polygonaceae), *Vicia sativa* L. (Fabaceae) and *Centaurea cyanus* L. (Asteraceae). The cornflower *C. cyanus* is an annual plant often found adjacent to crop fields (Kremer 2005). In addition to floral nectar, it also produces nectar in extrafloral nectaries that are located at the flower buds (Lauber & Wagner 2007). It blooms from June until October (Binz & Heitz 1990), during which it attracts many insects (Kühne *et al.* 2006). The common vetch *V. sativa* is a nitrogen fixing annual plant. Just as *C. cyanus*, it produces both floral and extrafloral nectar (EFN). Whereas the EFN is found at the stipules. As a cultivated and ruderal species, it is also found among crop fields (Lauber *et al.* 2012). It blooms between June and October (Landolt *et al.* 2010). Buckwheat *F. esculentum* is an annual cultivated crop plant, that only produces nectar in floral nectaries (Lauber *et al.* 2012). It blooms from July until September (Lauber & Wagner 2007).

Centaurea cyanus and *F. esculentum* were shown to increase fitness in terms of longevity or fecundity of *Cotesia glomerata* (Linnaeus, 1758) (Hymenoptera: Braconidae) (host: *P. rapae* and *Pieris brassicae* (Linnaeus, 1758) (Lepidoptera: Pieridae)) (Lee & Heimpel 2007a; Winkler *et al.* 2009b), whereas *F. esculentum* was also shown to enhance fitness of many other parasitoids of diverse cabbage pests such as *Diadegma semiclausum* Hellen (Hymenoptera: Ichneumonidae) (Wratten *et al.* 2003; Winkler *et al.* 2009b) (host: *P. xylostella*), *Aphidius ervi* Haliday, 1834 (Hymenoptera: Braconidae) (Wade & Wratten 2007) and *Diaeretiella rapae* (M'Intosh, 1855) (Hymenoptera: Ichneumonidae) (Araj & Wratten 2015) (hosts: aphids) and *Trichogramma exiguum* Pinto & Platner, 1978 (Hymenoptera: Trichogrammatidae) (host: *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae)) (Witting-Bissinger *et al.* 2008).

Further, house own studies have shown the positive effects of the strip mixture on biodiversity and beneficial insects such as *Microplitis mediator* (Haliday 1834) (Hymenoptera: Braconidae) and *Telenomus laeviceps* Förster, 1861 (Hymenoptera: Scelionidae), both antagonists of the cabbage pest species *Mamestra brassicae* (Linnaeus 1758) (Lepidoptera: Noctuidae) (Géneau *et al.* 2012; Balmer *et al.* 2013; Belz *et al.* 2013; Géneau *et al.* 2013; Balmer *et al.* 2014; Barloggio *et al.* 2018). Also *Diadegma fenestrale* (Holmgren, 1860) (Hymenoptera: Ichneumonidae), an antagonists of *Plutella xylostella* (Linnaeus 1758) (Lepidoptera: Plutellidae) was shown to profit from these flowers (Géneau *et al.* 2012). Information on the control of *P. rapae* however, was still missing. Since *P. rapae* poses a greater threat than *P. brassicae* in Switzerland, we focused our research on this pest and its main larval antagonist *Cotesia rubecula* (Marshall 1885) (Hymenoptera: Braconidae), aiming to add further information on the effects of the FiBL developed flower strip mixture.

Cotesia rubecula is a solitary endoparasitic koinobiont and the main larval antagonist of *P. rapae* (Harvey *et al.* 1999). This means that the host larva continues to live during parasitoid development but dies shortly after the parasitoid larva has left the host's body. It lays single eggs into first, second and third instars of *P. rapae.* The developmental time of *C. rubecula* (9 to 14 days) depends on the temperature, the quality and age of its host and the plant the host is feeding on (Parker & Pinnell 1970; Harvey *et al.* 1999; Harvey & Wagenaar 2006; Talaei 2009). These wasps have a high potential fecundity and their life expectancy is greatly influenced by sugar consumption (Nealis 1990; Sengonca & Peters 1993; Wäckers & Swaans 1993). In fact, *C. rubecula* wasps need to locate food at least once a day to avoid starvation (Siekmann *et al.* 2001). Without feeding, females usually die after 2–3 days at 25 °C (Wäckers & Swaans 1993). A female wasp can store up to 80 – 90 eggs in her ovaries (Nealis 1990) and within their first three days of life they mature about 100 eggs (Siekmann *et al.* 2004). Female wasps can parasitize up to ten larvae a day under semifield conditions (Nealis 1990) and have a mean longevity of 25.1 days at 22-24 °C (Parker & Pinnell 1970).

To address the objectives of this thesis, it was necessary to build a successful insect rearing of the pest and its antagonist and to choose reliable experimental setups for fecundity and longevity trials for both insect species. In CHAPTER 1 we provide insight on the method selection and development.

Flowers used in conservation biological control programs should preferably be olfactorily attractive, as highly attractive flowers are easily located and thereby reduce the time spent for food search. Subsequently the *per capita* host searching efficiency should increase. In CHAPTER 2 we thus focused on the olfactory attractiveness of *F. esculentum*, *C. cyanus* and *V. sativa* to *C. rubecula.* With a Y-tube olfactometer we tested whether these flowers have the potential to attract *C. rubecula* and whether innate attraction changes after a rewarding feeding experience. This would mean that innately unattractive flowers could still be suitable for conservation biological control purposes, as parasitoids are able to learn to exploit their nectar. We evaluated whether the tested flowers are olfactorily suitable to be exploited in conservation biological control programs to control *P. rapae* in brassica fields.

Flowers implemented in conservation biological control programs have rarely been checked whether they are selective plants, enhancing beneficial insects but not the pests. The three flowers *F. esculentum*, *C. cyanus* and *V. sativa* were proposed as selective plants for conservation biological control purposes against the cabbage moth *M. brassicae*, only enhancing its parasitoid *M. mediator.* As it would be useful if these flowers selectively enhanced many parasitoids of cabbage pests, we tested their effects on the fitness of *P. rapae* and its antagonist *C. rubecula* in CHAPTER 3. Survival and fecundity assays were performed for each insect in the laboratory. We evaluated whether the tested flowers can be recommended to control *P. rapae* in brassica fields.

To check whether results from our laboratory experiments allow to make predictions about parasitism rates in the field and whether the flower strip mixture is also suitable for the control of *P. rapae*, we conducted field trials over two consecutive years in 2016 and 2017 (CHAPTER 4). To evaluate the potential of this flower strip and *C. cyanus* as companion plants, treatments included fields supplemented with flower strip and companion plants, with flower strip only and without flowers.

Parasitism rates were determined with qPCR from *P. rapae* larvae that were exposed and recollect on exposure sets. The development of supplemented flowers was monitored and the amount of wild *P. rapae* was recorded in insecticide-free and -treated plots as well as cabbage yield and damage, to assess whether insecticide sprays against lepidopteran larvae could be spared.

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CHAPTER 1

Fundamentals and method development

Abstract

In conservation biological control, flowers should be carefully selected to promote the beneficial insects of interest, but not the pests. The procedure to find such plants includes a thorough literature research and laboratory experiments in controlled conditions before determining their performance in the field. After building a successful rearing of *Cotesia rubecula* (Marshall 1885) (Hymenoptera: Braconidae) and its host *Pieris rapae* (Linnaeus 1758) (Lepidoptera: Pieridae), optimal experimental conditions were chosen to test the suitability of *Centaurea cyanus* L. (Asteraceae), *Fagopyrum esculentum* Moench (Polygonaceae) and *Vicia sativa* L. (Fabaceae) to attract *C. rubecula* and to test their potential to enhance both insect species in longevity and fecundity trials. Insight on the method selection and development is provided.

Introduction

The success of conservation biological control programs relies on the careful selection of nectar providing flowers (Tooker & Hanks 2000; Vattala *et al.* 2006). The procedure to find such plants is to first conduct a literature research, then to test the plants of interest in the laboratory and finally to determine their performance in the field. To test their potential to control pest species densities in the field, by enhancing the fitness of the beneficial insect and not of the pest, experiments with the target insects need to be conducted. In order to do that, insect rearing should be developed to conduct experiments the whole year round. Descriptions of different rearing of insects can be found in the literature, but often published studies are difficult to reproduce (Pusztai *et al.* 2013; Baker 2016), as location, material and other factors such as climate may vary. The same is true for experimental setups to test the influence of flowers on different life parameters of insects.

Here, after a thorough literature research, we developed a rearing of the cabbage pest *Pieris rapae* (Linnaeus 1758) (Lepidoptera: Pieridae) and its main antagonist *Cotesia rubecula* (Marshall 1885) (Hymenoptera: Braconidae). Two main critical points characterized the rearing of the pest insect for which we had to find an optimal food source and a solution to the production of sterile eggs by adults hatching in the rearing room.

Methods to evaluate the effects of flowers on parasitoids and lepidopteran pests have been described in Géneau *et al.* (2012) and Belz *et al.* (2013). Once the rearing of insects was established, we used these insects to test the olfactory attractiveness of *Centaurea cyanus* L. (Asteraceae), *Fagopyrum esculentum* Moench (Polygonaceae) and *Vicia sativa* L. (Fabaceae) to *C. rubecula* and their effect on longevity and fecundity of both the parasitoid and its host *P. rapae*. Soon, however, we noticed that a complete knowledge transfer from the publications to our system was not always possible, as test insects died earlier than expected.

Therefore, preliminary trials were conducted to find the best experimental setup and conditions possible, enabling us to collect reliable data in the laboratory.

In this chapter, we a) built a functioning rearing of *P. rapae* and *C. rubecula*, b) performed pre-experiments to determine whether the olfactory attraction of *C. rubecula* to flowers can be tested with an olfactometer, c) tested different caging systems and climatic conditions to perform longevity and fecundity studies with *C. rubecula,* d) determined the optimal dissection time point of parasitized larvae for fecundity trials with *C. rubecula*, e) found a solution to handle the factor weight in fecundity and longevity trials with *P. rapae*, and f) figured out how to get female butterflies that are mated only once, since multiple mating influences fecundity. The problems we faced and the solutions we found are provided in this chapter.

Rearing of insects

Breeding of P. rapae food plants

The plant on which host larvae feed is an important factor in parasitoid development (Talaei 2009). Because *C. rubecula* completed its development most rapidly on *Brassica oleracea* (Harvey & Wagenaar 2006) and *P. rapae* was found to lay significantly more eggs on cabbage than wallflowers (Hopkins & Van Loon 2001), we chose to keep our rearings on Cabbage F-1 Hybrid Stonehead Precision (*Brassica oleracea* var. capitate), which seeds were kindly provided by Max Schwarz AG.

To ensure enough nutritional material for larvae of the small white *P. rapae*, these seeds were sown weekly into compressed soil pots (Max Schwarz AG) and were kept at 24±2 °C, 55±15% r.h. and a 16L:8D photoperiod in Grow-Banks (CLF Plant Climatics, Germany). After three weeks they were transplanted to 12 cm diameter (10 cm height) pots with 3 g slow-release formulation fertilizer (Tardit 3M, Hauert HBG Dünger AG, Switzerland 15-8-12 N-P-K) per liter of soil (Einheitserde Classic, Gebrüder Patzer GmbH & Co. KG, Germany). To promote growth of thin

leaves and therefore ensure a good development of *P. rapae* larvae at young age, the plants were regularly watered.

Pieris rapae

A laboratory colony of *P. rapae* was started in June 2015 with adult individuals collected from organic cabbage fields in Wynau and Madiswil, Switzerland.

Field collected butterflies were allowed to oviposit on potted cabbage plants. These plants with eggs were then transferred to a climate chamber with cool white lamps (Philips 36 W) at 23±2 °C (Gossard & Jones 1977; Harvey *et al.* 1999; Harvey & Wagenaar 2006; Yildizhan *et al.* 2009), 50±10% r.h. (Harvey *et al.* 1999; Yildizhan *et al.* 2009), 16L:8D (Gossard & Jones 1977; Harvey *et al.* 1999; Harvey & Wagenaar 2006; Talaei 2009; Yildizhan *et al.* 2009) and kept in 47.5 cm x 47.5 cm x 47.5 cm BugDorm-4090 cages with fine mesh (0.68 mm) (Megaview Science Co., Taichung, Taiwan). Once larvae hatched, fresh cabbage was provided *ad libitum* until pupation. When pupae hardened up, they were harvested, put in a vessel lined with a napkin and transferred to 47.5 cm x 47.5 cm x 93.0 cm BugDorm-4180 cages made of fine mesh (0.68 mm) with two transparent plastic foils at the front and back side (Megaview Science Co., Taichung, Taiwan). Adults were fed with a 10% honey solution (Gossard & Jones 1977; Thoms & Philogène 1979; Jones *et al.* 1982) offered with help of a yellow sponge, which prevented them from drowning.

Two main critical points characterized this rearing method: i) dependency on cabbage plants and ii) production of sterile eggs by adults hatching in the rearing room. At the end, the efficiency of the new rearing system was evaluated.

Dependency on cabbage plants

The production of cabbage plants is resource and time consuming. To eliminate this step, we tried to rear *P. rapae* larvae on an artificial diet (Imported cabbageworm diet from Frontier Scientific Services formerly ASDI; see Appendix). This would allow to store the diet in a space saving manner and to use it when needed, thereby reducing

the workload which comes with fresh unprocessed diet. We followed the instructions by Webb and Shelton (1988), who reared *P. rapae* on an artificial diet and offered oviposition sites other than intact plants. Butterflies oviposited on parafilm-wrapped beakers that were covered with brassica plant leaves, to stimulate oviposition. This step worked very well. However, the artificial diet was not accepted by our *P. rapae* larvae. Instead they preferred to eat the styropor cups containing the diet and parafilm strips. A possible explanation why a supposedly accepted cabbageworm diet was not eaten by our larvae is that the larvae from abroad which were able to develop on that diet, were likely adjusted to it over several generations. Also, Jones *et al.* (1982) noticed marked differences between Australian and Canadian individuals in the timing of egg production, suggesting that varying food preferences between populations from different (bio-)geographic regions are possible too. Therefore, we were forced to continue with the cabbage production, in order to ensure a good quality food for *P. rapae*.

Production of sterile eggs by adults hatching in the rearing room

Young cabbage plants, as egg deposition sites, were offered to the first generation of laboratory reared adults in the rearing chamber. After we noticed that no larvae hatched from the laid eggs, suggesting that the eggs were not fertilized, we went back to literature research and came across the importance of daylight for mating success. In fact, natural daylight is essential for mate recognition and accordingly for mating to take place (Makino *et al.* 1952; Obara & Majerus 2000). Hence, cages containing butterflies and pupae were placed in front of a window in a laboratory at 26±2 °C, 40±10% r.h.. To further enhance their activity, especially during cold and cloudy days, halogen lamps with 400 W light bulbs were installed above the cages with a 16L:8D photoperiod, as Webb and Shelton (1988) stated that during winter, natural sunlight alone is not strong enough to allow normal activity. These changes led to a constant production of fertile eggs.

Evaluation of the optimized rearing system

The sex of 1006 butterflies was determined from which 498 were males and 508 were females, which is in line with Richards (1940) who found that adult *P. rapae* populations usually had a 1:1 sex ratio. In this rearing system, female butterflies were able to lay 16±7 eggs a day (N = 8). After five days larvae hatch and go through the five instar stages during approximately 15 days. Once pupated, they rest in this phase for roughly nine days until butterfly emergence.

We evaluated the rearing success based on the number of eggs in a rearing unit and the resulting number of adults. We formed three groups with 50 to 200, 201 to 400 and 401 to 600 eggs for rearing success evaluation. The highest rearing success was reached when we used 50 to 200 eggs per rearing unit with a significant influence of the amount of eggs on the rearing success (One-way ANOVA with log10 transformed data, $F_{2, 34} = 10.07$, $p < 0.001$; Tukey post-hoc test $p < 0.05$) (Figure 1-1).

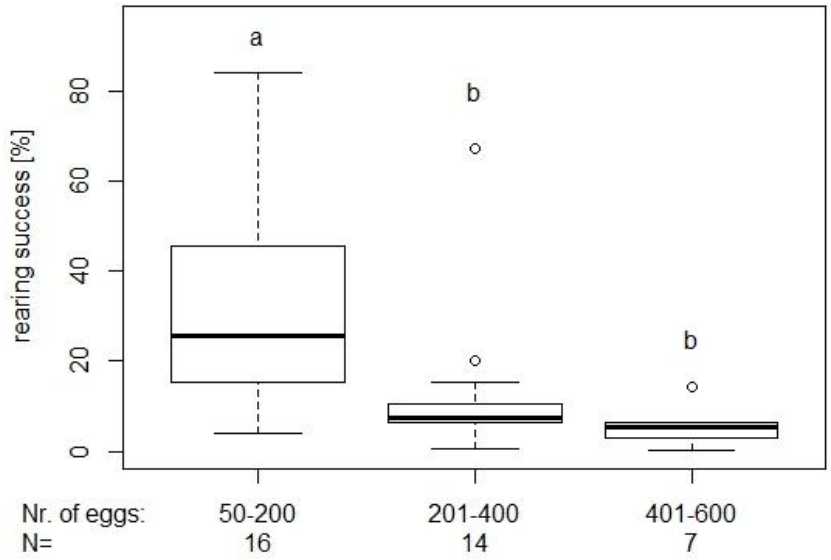

Figure 1-1: Boxplot of the *P. rapae* rearing success (egg to adult development). The rearing series were divided into three groups with 50 to 200 eggs, 201 to 400 eggs and 401 to 600 eggs. Significant differences are indicated by different lower case letters.

While a maximum amount of 75 butterflies per series can be achieved within 50 to 200 eggs, 80 can be reached with 201 to 400 eggs and only 46 with 401 to 600 eggs.

Based on the collected data, the maximum amount of eggs per series should not exceed 400 in order to reduce workload and optimize the rearing success. Keeping less individuals in a single cage, prevents overcrowding, reduces stress and pathogens from building up in these cages.

Cotesia rubecula

A laboratory colony was started with *C. rubecula* cocoons and *C. rubecula* infested *P. rapae* larvae collected in summer 2015 from organic cabbage fields in Wynau and Madiswil (Switzerland).

Wasps were reared on *P. rapae* larvae feeding on cabbage and kept in 47.5 cm x 47.5 cm x 47.5 cm BugDorm-4090 cages with fine mesh (0.68 mm) (Megaview Science Co., Taichung, Taiwan). The rearing chamber had continuous air circulation, which is advantageous for mating occurrence (Parker & Pinnell 1970), 22±2 °C (Parker & Pinnell 1970), 55±5% r.h. (Harvey *et al.* 1999), L16:D8 photoperiod (Harvey *et al.* 1999) and was illuminated by four 58 W neon tubes per m^2 located approximately 65 cm above the cages (Philips Master LD-D 58W/840 Reflex).

Cotesia rubecula females lay their eggs in first to third instar larvae of *P. rapae* and virgin females produce only male (haploid) progeny (Harvey *et al.* 1999). To ensure mating before parasitism, we placed a cabbage plant with freshly laid *P. rapae* eggs in a cage and added three female and three male wasps that have recently emerged from their cocoons. These were able to mate before host larvae hatched. We evaluated the rearing success based on the number of host eggs invested in a rearing unit and the resulting number of parasitoid cocoons. We formed three groups with 80 to 300, 301 to 500 and 501 to 1000 host eggs for rearing success evaluation. After parasitism, parasitoid larvae emerged 10 to 12 days later and immediately started to spin a cocoon, leaving behind a lethal hole in the host larvae. These cocoons were collected and placed singly in plastic containers to easily determine the sex under a binocular, after emergence of the wasp. The wasps were fed with honey gelatine (3 g

gelatine, 100 ml dest. water and 200 g honey) smeared on the outside of the cages. Daily drops of water on top of these cages were provided for them to drink.

Evaluation of the rearing system

In this rearing system adults had a mean longevity of 25±10 days (N = 6) which is in good correlation to Parker and Pinnell (1970). The duration from parasitism until imaginal egression was 17-19 days compared to the reported development time of *C. rubecula* ranging from 9 to 15 days, which depended on host age, host diet and rearing temperature (Parker & Pinnell 1970; Harvey *et al.* 1999; Harvey & Wagenaar 2006; Talaei 2009).

The rearing success was highest, when 80 to 300 *P. rapae* eggs were provided compared to 301 to 500 or 501 to 1000 eggs (One-way ANOVA, $F_{2, 27} = 5.028$, $p = 0.014$, Tukey post-hoc test $p < 0.05$) (Figure 1-2).

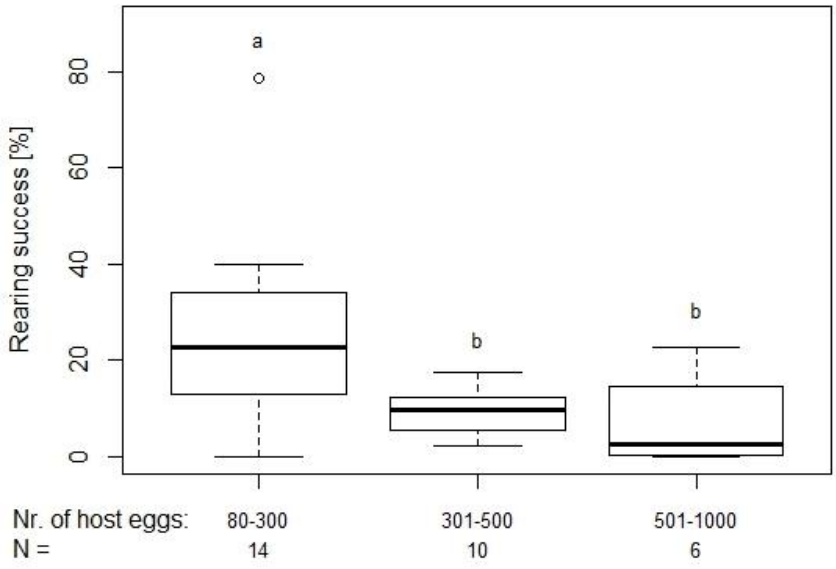

Figure 1-2: Boxplot of the *C. rubecula* rearing success (percentage of cocoons resulting from the amount of host eggs provided). The rearing series are divided into three groups with 80 to 300 host eggs, 301 to 500 host eggs and 501 to 1000 host eggs. Significant differences are indicated by different lower case letters.

Considering the collected data, the maximum amount of invested host eggs per series should not exceed 300, as workload can be reduced and the rearing success optimized. Increasing the number of parasitoids to supposedly get more cocoons,

will not improve the rearing success as Talaei (2009) explained that a large number of *P. rapae* that had been parasitized by *C. rubecula* died, presumably due to mutilation of the host at the time of oviposition.

Lab-experiments

Pre-trials of longevity experiments with C. rubecula

The goal of these pre-trials was to check whether the experimental conditions implemented by Géneau *et al.* (2012) to investigate the effect of different flower species on the longevity of the parasitoid *Microplitis mediator* (Haliday, 1834) (Hymenoptera: Braconidae), would also be suitable for *C. rubecula*. To this end, newly hatched female wasps were placed singly inside 47.5 cm x 47.5 cm x 93.0 cm BugDorm-4180 cages made of fine mesh (0.68 mm) with two transparent plastic foils at the front and back side (Megaview Science Co., Taichung, Taiwan) with a flowering plant and a water source accordingly (Figure 1-3 a). The flowers tested were common vetch *V. sativa*, cornflower *C. cyanus* and buckwheat *F. esculentum*. In the negative control treatment only water was offered and in the positive control treatment honey was additionally provided. These cages were placed in the lab and the survival of the wasps was recorded on a daily basis. Both relative humidity and temperature were measured.

Survival in Bugdorms in the laboratory

In the very first replicate performed within large cages in the laboratory (25±1 °C and 33±4 % r.h.) (Figure 1-3 a), female wasps survived longest (14 days) when provided with *F. esculentum*, followed by *V. sativa* (7 days) and by the two cornflower types and the positive (honey) and negative (water) control (all between 1 and 3 days). The fact that the wasp in the positive control did not survive longer than two days, made us suspicious, which is why we have replaced the cages after the first trials with a

newly developed caging system (Figure 1-3 b, c). The goal was to increase the relative humidity, as it might play a role in the wasp's survival.

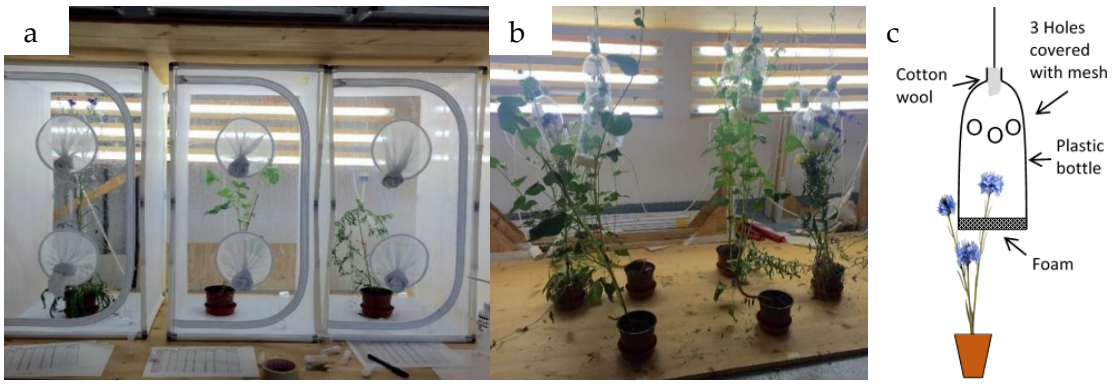

Figure 1-3: a) Cages with flowering plants to assess the effect of different nectar sources on the longevity of *C. rubecula*. b) Survival setup with plastic bottles. c) Schematic illustration.

Survival in plastic bottles in the laboratory

Each treatment was replicated ten times. In the new caging system only buckwheat (F. es; 13.6±2.3 days (Mean±SE)) significantly prolonged the life of *C. rubecula*, when compared to the control treatment water (Ctrl.-; 1.7±0.15 days) and the other treatments cornflower (C. cy; 4.6±0.93 days), honey (Ctrl.+; 5.4±1.26 days) and common vetch (V. sa; 2.9±0.32 days) (pairwise t-test, $p < 0.001$). All other treatments were not significantly different from each other (pairwise t-test, $p > 0.05$) (Figure 1-4). Within both control treatments honey and water, we measured 25 °C and r.h. 44±3%, while 24±1 °C and r.h. 74±5 % were measured within the bottles containing flowers.

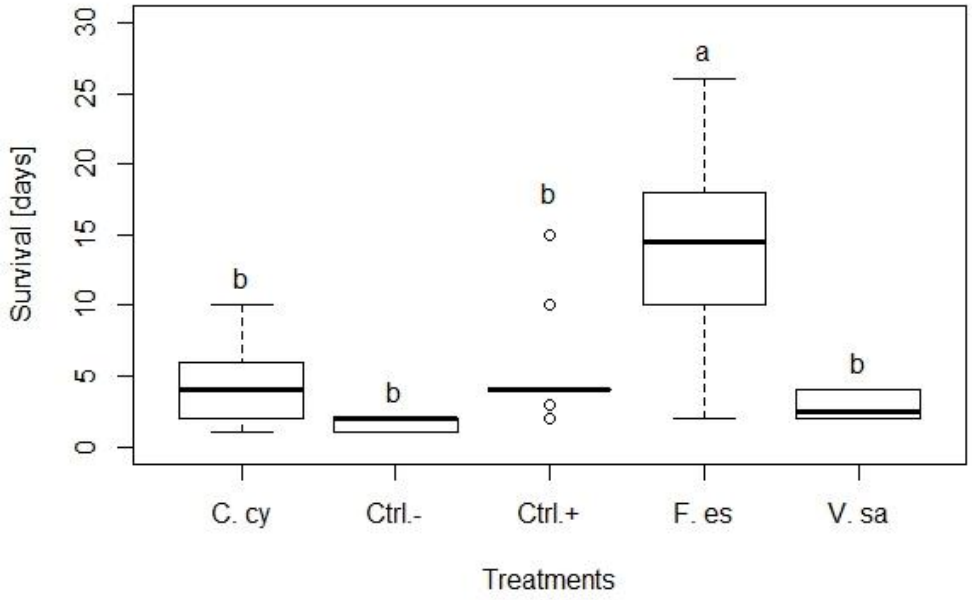

Figure 1-4: Survival of *C. rubecula* females in plastic bottles in the lab. Different lower case letters indicate significant differences. C. cy = cornflower, Ctrl.- = water, Ctrl.+ = honey, F. es = buckwheat, V. sa = common vetch. N = 10 for all treatments.

Regarding the results presented in Figure 1-4, the situation did not improve much, as the wasps still died at an early age in the honey treatment (Ctrl.+) 5.4±1.3 days, although we know from personal experience and literature (Parker & Pinnell 1970), that they have a mean longevity of 25 days when provided with honey. We did however increase the relative humidity by 10 % in the control treatments, but maybe still not enough and certainly not as stable as it would be in a climate chamber. Therefore we repeated the trial with the new caging system in the rearing chamber with 22±2 °C and 55±5 % r.h..

Survival in plastic bottles in the rearing chamber

In the climate chamber the relative humidity was further increased by another 10 %. Replicate numbers varied for each treatment. Honey (Ctrl.+), cornflower (C. cy) and buckwheat (F. es) significantly increased longevity compared to the water treatment (Ctrl.-) with a mean survival of 25±4.2 days (N=6), 10.3±3 days (N=3) and 20.5±1.3 days (N=4), respectively. Mean survival in the control treatment was 2.6±0.5 days

(N=7) and in the common vetch treatment (V. sa) 5.2±1.2 days (N=5) (One-way ANOVA with log10 transformed data, $F_{4, 20} = 22.61$, $p < 0.001$, Tukey post hoc $p < 0.05$) (Figure 1-5).

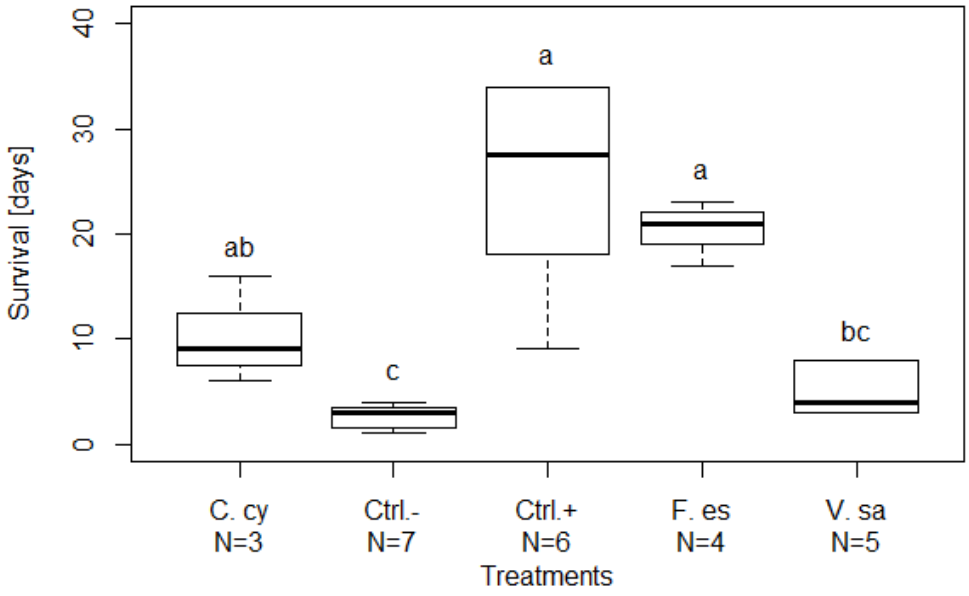

Figure 1-5: Survival of *C. rubecula* females in plastic bottles in the climate chamber. Different letters indicate significant differences. C. cy = cornflower, Ctrl.- = water, Ctrl.+ = honey, F. es = buckwheat, V. sa = common vetch.

We finally produced results we could trust and had a suitable infrastructure, system and knowledge ready to conduct survival experiments with *C. rubecula*.

Pre-trials of fecundity experiments with C. rubecula

Pre-trials were conducted to determine the most suitable dissection time point and method to evaluate the fecundity potential of wasps offered different flowers.

Determination of the optimal dissection time point

To address the optimal dissection time point, a group of larvae was simultaneously offered for parasitism and were continuously dissected at a later date to follow the developmental stage of the parasitoid larvae. This was done under a binocular with needles.

Depending on temperature, the duration of the parasitoids development varies. When the temperature increases, the development is faster. In Figure 1-6 a depicted, are the different stages (without the latest larval stage) of *C. rubecula*, which can be found within the host larvae *P. rapae*. When parasitized larvae get dissected a day after parasitism, parasitoid eggs are found, which can be distinguished through trained eyes from divers larval tissues. Although Harvey *et al.* (1999) reported that *C. rubecula* eggs hatched approximately three days after oviposition in *P. rapae* at 25±2 °C, parasitic larvae already hatched two days after parasitism at the same temperature. In this stage it is easier to distinguish the larvae from host tissues and the older they get, the easier it becomes to detect them. Until day three, we may find more than one egg or larva within a single host (Figure 1-6 b).

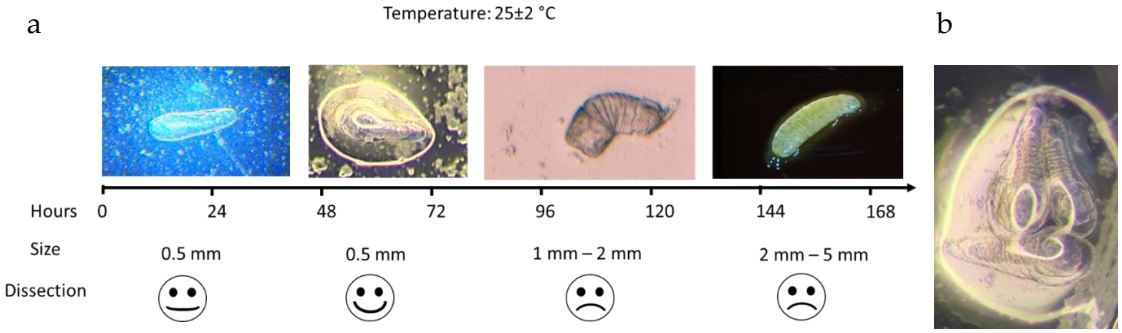

Figure 1-6: a) Approximate scale of *C. rubecula* larvae developmental stages at 25±2 °C. Optimal dissection time point is between 48 and 72 hours after parasitism. b) Three newly hatched *C. rubecula* larvae approximately 48 hours old found within a single host larvae.

Multi-parasitism is a consequence of the experimental setup

Host larvae should not be dissected later than three days after parasitism. When dissected between zero and three days after parasitism, we often found multiple eggs or larvae within the host, whereas four days after parasitism, we usually found only one larva. This because once the parasitoid larvae hatch and reach a certain developmental stage, they start to attack and eat each other till only one survives. Optionally, the parasitized larvae can be kept in the fridge at 4 °C to postpone the dissection time point. Keeping the larvae cool, slows down the development of the

host and the parasitoid. Although Sengonca and Peters (1993) reported that *C. rubecula* is expected to lay eggs singly into host larvae, a female wasp was able to lay several eggs in one single host larva (Figure 1-6 b). This led us to the question whether the host was parasitized several times or whether more than one egg were deposited at once. In fact, in the field *P. rapae* larvae are usually well distributed and not found in high densities on a single host plant. Therefore, the number of host encounters is limited and single eggs should be laid into host larvae to maximize the return after investment, as only one parasitoid larva will survive. To test this hypothesis, female wasps were allowed to pierce into host larvae only once and these were subsequently checked for the number of eggs laid. In one of ten larvae, two instead of one egg were found. We therefore conclude, that this was an unfortunate event, not intended by the wasp and that one parasitism attempt corresponds to one egg laid and that multi-parasitism is a consequence of the experimental setup.

Optimal host developmental stage, number of hosts and exposure period

Even though mortality of *C. rubecula* is lowest in L1 larvae (Harvey *et al.* 1999), we decided to use L2 larvae since they have a greater chance of surviving multi-parasitism than L1 larvae. L3 larvae would even have a greater chance to survive than L2 but they are also able to defend themselves better against the parasitoids (Nealis 1990) and therefore could lower the parasitoids possible egg deposition potential. We decided to expose ten larvae for one hour because in semi field trials, Nealis (1990) reported that *C. rubecula* deposit a maximum number of ten eggs per day at high host densities under semi-field conditions. We considered one hour as sufficient since parasitism activity decreased with time and after one hour no activity could be observed.

Successive changes of the experimental setup

The search for the optimal caging system was solved with the pre-trials for longevity experiments, but these pre-experiments were performed simultaneously, which is why the experimental setup and successive changes were the same as for the

survival experiments described above with the difference that female wasps were not kept singly but paired with a male wasp. Although female wasps are able to lay fertile eggs without mating, we decided to keep them with a male wasp, as unmated females produce haploid and therefore only male progeny. The ultimate goal in conservation biological control, however, is to keep pest densities under control with as many female progeny as possible, since they are the ones who parasitize the pest larvae.

Daily, female wasps were taken out of their cages containing flowering plants (*C. cyanus*, *F. esculentum* and *V. sativa*) and controls (honey and water) and were presented to ten *P. rapae* larvae (L2) on a piece of cabbage leaf within a plastic container for one hour and then placed back into their cage. The parasitized larvae were kept in plastic containers with cabbage leaf material until they were sacrificed through dissection the following days.

In trials conducted with large cages (N = 2 for each treatment), wasps survived between one and three days in every replicate except for one female that survived for nine days in the buckwheat treatment and managed to lay 45 eggs during its lifetime. Also, a maximum amount of larvae found within a single host larva was 17 when provided with buckwheat.

As we changed to the setup in the bottles in the laboratory (N = 5 for each treatment), the maximum amount of larvae found within a host larva was 34 in the water treatment and a total amount of 116 eggs was laid in a buckwheat treatment, in which a female wasp survived for 14 days.

Females provided with buckwheat (F. es; 6.8±2 days (Mean±SE)) survived significantly longer compared to common vetch (V. sa; 1.6±0.25 days) and water (Ctrl.-; 1.2±0.2 days). The treatments honey (Ctrl.+; 4.2±0.5 days) and cornflower (C. cy; 3.8±0.74 days) were not significantly different from the other treatments and each other (One way ANOVA, $F_{4, 20} = 5.072$, $p = 0.006$, Tukey post hoc, $p < 0.05$) (Figure 1-7).

All male wasps died latest after five days with one exception in the buckwheat treatment, which managed to survive for 24 days. There was no significant difference between the treatments (One way ANOVA, $F_{4, 20} = 1.786$, $p = 0.171$) (Figure 1-7).

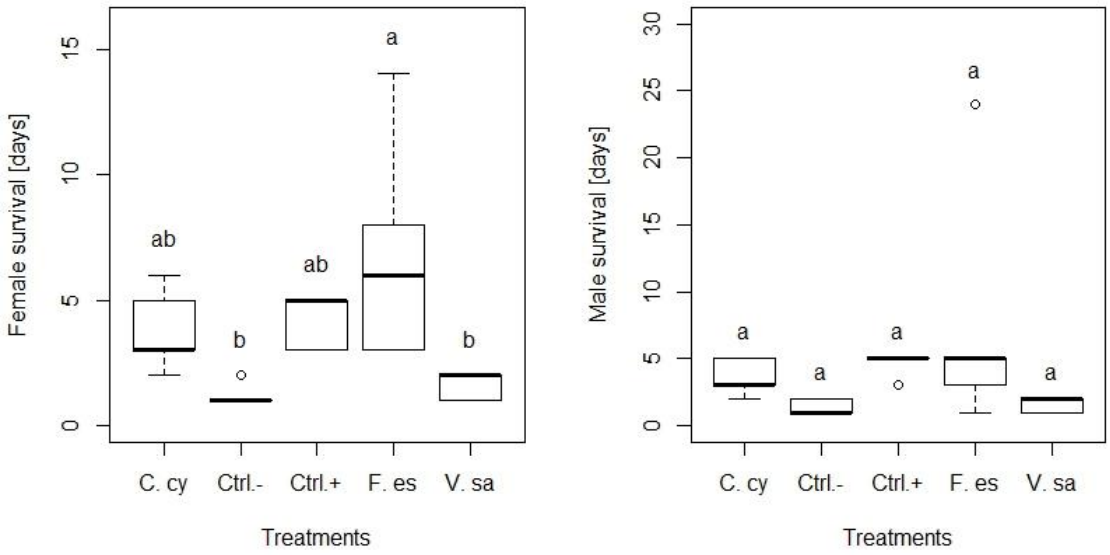

Figure 1-7: *Cotesia rubecula* female and male survival in fecundity trials within plastic bottles in the lab. N = 5 for each treatment. Different lower case letters indicate significant differences.

When provided with buckwheat, the highest number of potential offspring was reached (One way ANOVA, F 4, 20 = 3.632, p = 0.022, Tukey post hoc, $p < 0.05$) (Figure 1-8). The amount of potential offspring correlates with the days of female survival.

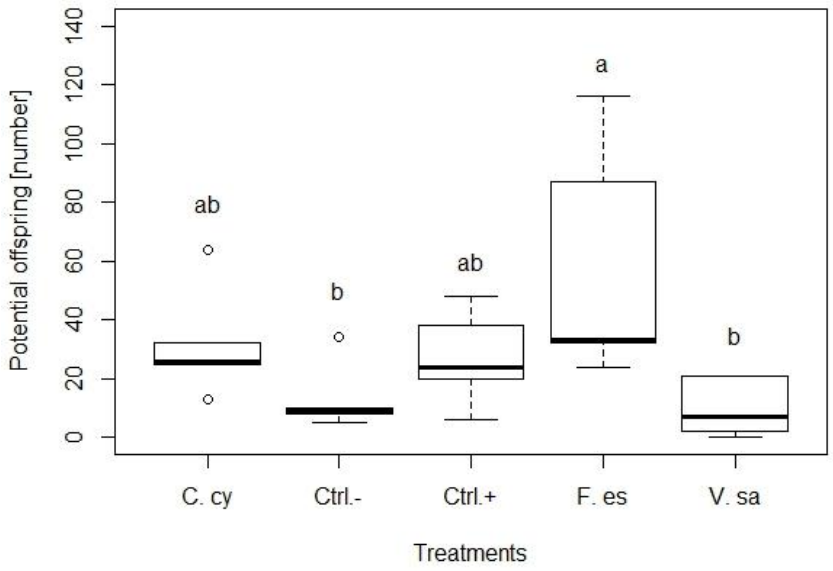

Figure 1-8: Potential offspring (sum of parasitoid eggs and larvae found within host larvae) in fecundity trials within bottle cages in the lab. N = 5 for each treatment. Different lower case letters indicate significant difference.

The survival and fecundity experiments were performed simultaneously and the experimental set up was almost identical for both. Also here we concluded that the most reliable results are produced when the wasps are kept in plastic bottles in a regulated climate chamber, because there, the effect of flowers on the fitness of parasitoids is improved (see page 21).

Pre-trials olfactometer

We tested whether an olfactometer (Figure 1-9 a, b), which was used for attractiveness studies with *M. mediator* by Belz *et al.* (2013), would also be suitable for attractiveness studies with *C. rubecula*. For these pre-experiments, 30 virgin female wasps, less than 24 hours old and unfed were used. The experiment began, when a single wasp passed the start line and finished, when it reached one of the finish lines. If the wasp did not respond nor reach one of the both finish lines within five minutes, it was replaced. A detailed description of the olfactometer is provided in Barloggio *et al.* (2018).

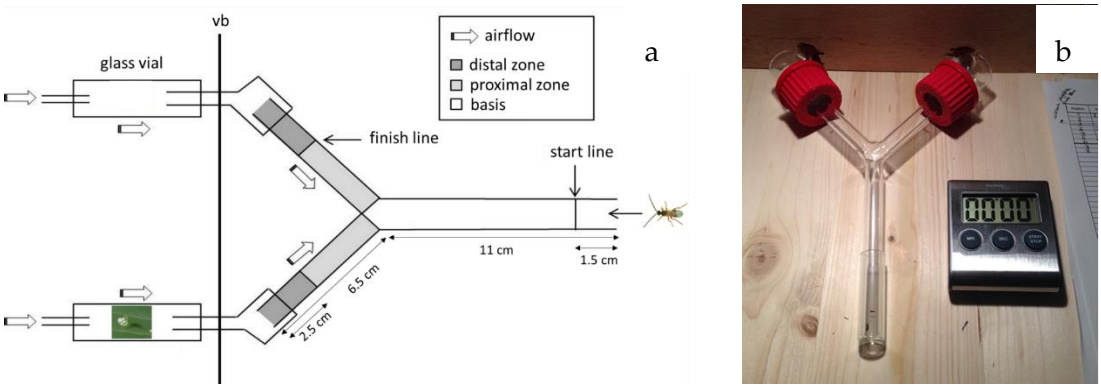

Figure 1-9: a) Schematic illustration of the Y-tube of the olfactometer. The parasitoid is released at the base of the Y-tube and the experiment starts, as soon as the parasitoid passes the start line and ends when it reaches one of the finish lines within five minutes. vb: visual barrier (adapted from Belz *et al.* (2013)). b) Y-tube and timer.

The experimental design and the olfactometer itself were tested using cabbage leaves with host larvae feeding on them against air. Since we know that glucosinolates released from cabbage plants through herbivory or mechanical damage, are a highly attractive source for *C. rubecula* wasps (personal observation), we expected that the arm with the glucosinolate cues will be chosen over the arm with air. The position of these arms was switched every now and then to avoid false results due to positional effects and the airflow was set to 757 ml/min. Temperature and humidity were recorded inside the glass vials containing the olfactory cues, to get a better picture of the experimental conditions.

From the 30 newly hatched unfed female virgin wasps, 18 reached the finish line in the arm with cabbage and host larvae cues, while only three chose the arm with air. Nine wasps did not make a valid decision within five minutes (Figure 1-10).

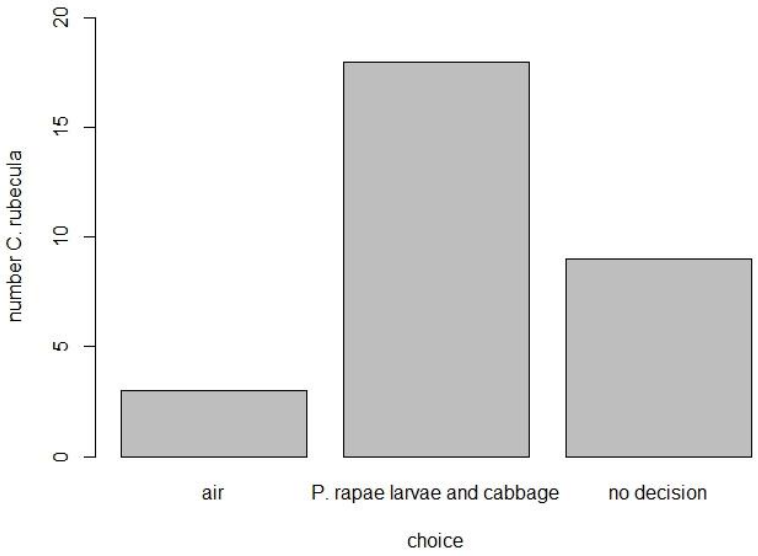

Figure 1-10: Olfactometer attractiveness test. 30 unfed female *C. rubecula* wasps younger than 24 hours were given the choice between cabbage with *P. rapae* larvae and air. Wasps which did not reach one of the finish lines within five minutes were dismissed.

The measured temperature and relative humidity in the arm with the target cue *P. rapae* larvae and cabbage was 27±1 °C and 80±9 %, respectively. The same temperature was found in the arm with only air, while the relative humidity was 70±9 %.

The majority of our wasps (60%) chose the arm containing cabbage and host larvae, which led us to the conclusion, that the experimental setup is appropriate to answer our research questions on flower attractiveness to *C. rubecula*. While the temperature remained on the same level in both vials, the relative humidity was constantly higher in the vial containing cabbage and *P. rapae* larvae, compared to the vial containing only air. However, both values lie in a rather high range of 70 – 80 % r.h. and should therefore not have any significant influence on the outcome of results.

Pre-trials of longevity experiments with P. rapae

Because we experienced, that conducting experiments in regulated chambers produced most reliable results with *C. rubecula*, we wanted to perform the longevity experiments with *P. rapae* in these chambers as well. Since *P. rapae* survives better when kept in large cages (Gossard & Jones 1977), we used BugDorms (47.5 cm x 47.5 cm x 93.0 cm) for the experiments. Unfortunately not enough space was available for the cages in the rearing chambers, which is why we had to conduct the experiments in the lab.

To examine whether we can produce meaningful results with the given possibilities, materials and methods, we performed longevity trials with the flowers *V. sativa*, *C. cyanus*, *F. esculentum* and the two controls water (negative) and 10 % honey solution (positive). Because larger individuals are expected to survive longer (Gilbert 1984), each individual's weight was assessed by weighing *P. rapae* pupae and subsequently placing them singly into containers (5.5 cm diameter, 6.3 cm height) until butterfly emergence. Adding a piece of napkin and a stick to climb on after hatching, reduced the number of adults with crippled wings. Once emerged, they were marked with a number on their wings for identification, in order to assign the corresponding pupal weight to each individual.

Newly hatched male and female butterflies were separately placed in BugDorms (47.5 cm x 47.5 cm x 93.0 cm) containing a potted flowering plant and water or in the control treatments either water only or 10% honey solution. A maximum of three individuals were placed inside a cage and the amount of flowering plants equalized to these. Placing more than one butterfly of the same sex into a cage, enabled us to produce more replicates, as space was a limiting factor. The maximum amount of three was chosen, because overcrowding could induce stress, which could lead to shorter longevities. The experimental units were placed on a rack in the lab with room temperature 25±1 °C and 33±4 % r.h. and extra lights placed at the back of the cages for a L16:D8 photoperiod. The survival was recorded on a daily basis at 11.30 am. Treatments were continuously replicated according to

the availability of insects and flowering plants until we reached 4 – 7 replicates for each treatment. To ensure that the butterflies do not die from drought, plenty of water was added regularly to each plant and water station (yellow sponge).

In the negative control treatment water (Ctrl.-), females survived for 3.33±0.4 days (Mean±SE), with 10% honey solution (Ctrl.+) 5.17±1.2 days, buckwheat (F. es) 8.6±2.7 days, vetch (V. sa) 4.7±0.9 and cornflower (C. cy) 4.8±0.9 days. The treatments were not significantly different (ANOVA with log10 transformed data, $F_{4, 22} = 1.728$, $p = 0.179$, Tukey post hoc $p < 0.05$) (Figure 1-11).

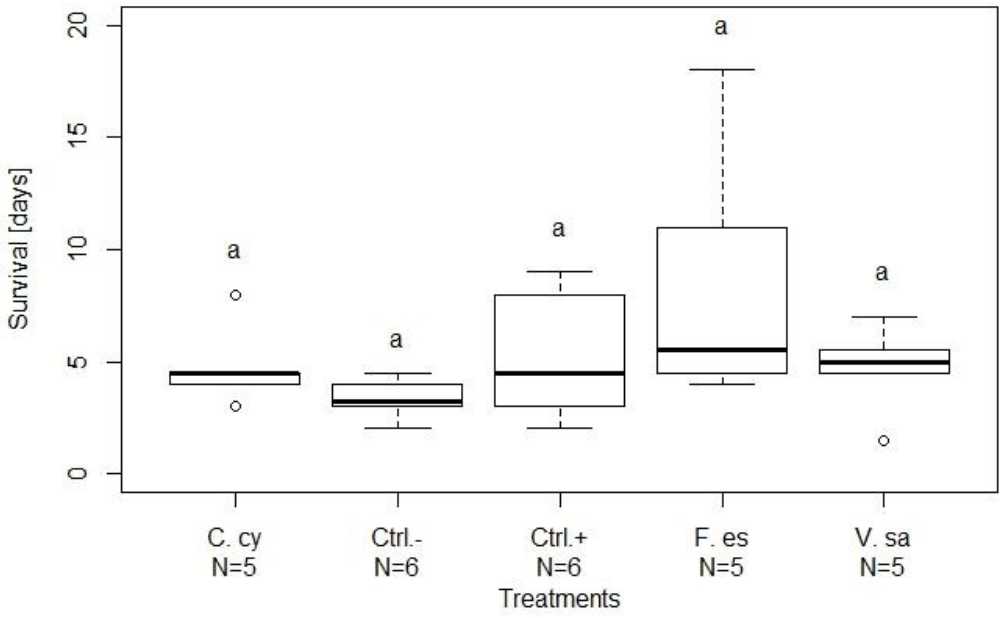

Figure 1-11: Survival of *P. rapae* females in flower and control treatments. Different lower case letters indicate significant differences. N = number of replicates.

In the negative control treatment water (Ctrl.-), males survived for 3.75±0.5 days, with 10% honey solution (Ctrl.+) 4.43±0.5 days, buckwheat (F. es) 8.08±2 days, vetch (V. sa) 3.07±0.3 days and cornflower (C. cy) 5.3±0.7 days. Only buckwheat and common vetch were significantly different (ANOVA with log10 transformed data, $F_{4, 27} = 4.039$, $p = 0.011$, Tukey post hoc $p < 0.05$) (Figure 1-12).

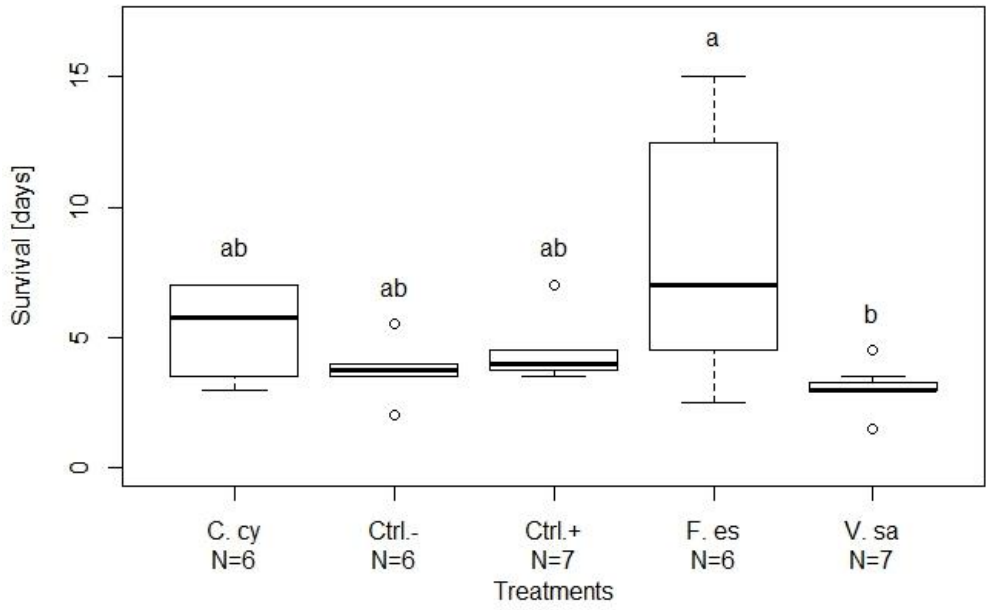

Figure 1-12: Survival of *P. rapae* males in flower and control treatments. Different lower case letters indicate significant differences. N = number of replicates.

Indeed we were able to confirm that bigger individuals have a tendency to live longer, when plotting the pupal weights against the survival days from all treatments (Figure 1-13).

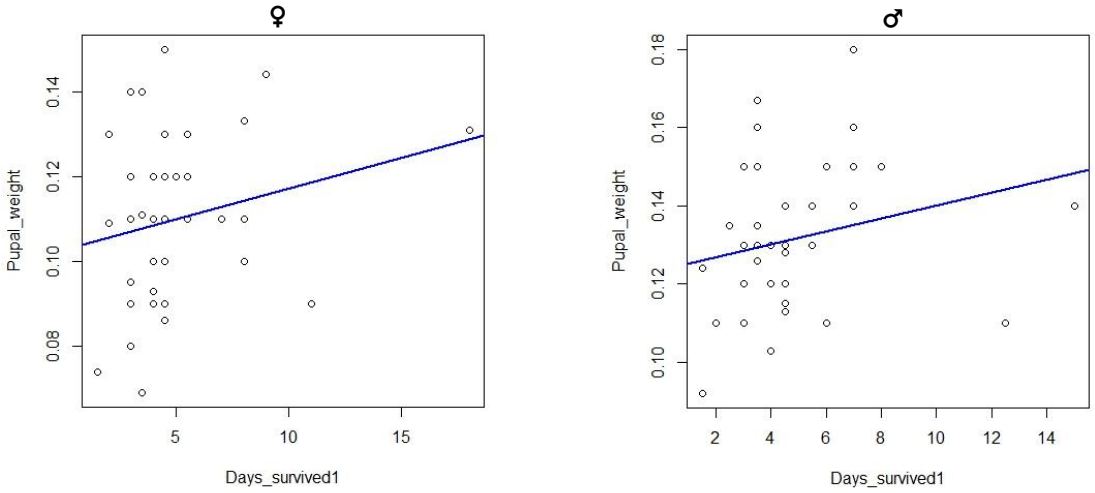

Figure 1-13: Pupal weights plotted against the number of days adult female (left) and male (right) *P. rapae* survived. The blue trend line indicates that heavier individuals tend to live longer.

Because unexpectedly the 10% honey solution offered with the same sponge as in the *P. rapae* rearing did not significantly increase longevity compared to the water treatment, we assumed that single *P. rapae* struggle to find the source compared to multiple *P. rapae* in the rearing, which probably learn from each other where to find it. A possible solution would be to implement artificial flowers to offer the honey solution (Yildizhan *et al.* 2009), however, water as only control is sufficient to make a statement about the potential of the flowers to increase longevity.

Although Winkler *et al.* (2009b) has shown that *P. rapae* survival increases with higher humidity, we consider our experimental setup good enough to detect differences in *P. rapae* enhancement among our study plants. Winkler *et al.* (2009b) have also performed survival experiments with 45±5 % r.h., which they described as humidity met in the field during summer. This humidity was similar to the one in our lab and the outcome of survival was comparable for both treatments buckwheat and water control.

Yet we had to question whether our possibilities to perform these experiments were sufficient or not, especially as we could not find any significant differences among the treatments for female longevity. However, the trend lines in Figure 1-13 confirm that pupal weight had an influence on *P. rapae* longevity. The reason why there were significant differences detected among the male treatments and none for the females was that for one part we had less replicates for the females and for the other part that the different pupal weights were not evenly distributed among the female treatments. By chance the majority of male pupal weights were very similar. To exclude that individual size influenced the outcome of our main trials, we had to distribute the weights equally among the treatments. Also the number of replicates was increased.

Pre-trials of fecundity experiments with P. rapae

To determine the influence of the flowering plants on fecundity of *P. rapae*, we intended to use the same set up as for the survival experiments. Here however, we had to solve crucial points concerning *P. rapae*s' mating behavior.

Because larger individuals are expected to survive longer and produce more offspring (Jones *et al.* 1982; Gilbert 1984), each individual's weight was assessed. This problem was solved as above by weighing *P. rapae* pupae and singly placing them into containers until butterfly emergence. This ensured that individual size could be included in the analysis.

Since natural daylight is required for mate recognition (Makino *et al.* 1952; Obara & Majerus 2000), we placed several cages, each containing one *P. rapae* couple (< 24 h), in front of the lab windows. Soon we had to realize, that none of the assembled couples mated. To solve this problem, we kept multiple individuals in one cage enabling them to choose their own mates.

Once emerged, butterflies were marked with a number for identification, placed in a collective cage exposed to daylight with a water source and were allowed to mate in the morning until lunch time. Fortunately, their numbered wings did not hinder them from mating. As soon as a couple formed, they were separated from the rest and kept together in a cage until copulation ended voluntarily.

Once copulation ended, the male butterfly was removed. This step was necessary to ensure that mating happens only once since the spermatophore, which is transferred from male to female during copulation, is a male nutrient investment and hence influences the survival and fecundity of females positively (Oberhauser 1989; Cahenzli & Erhardt 2012). If multiple mating was allowed, detected differences in egg production could not be assigned to the flowering plants.

Unmated butterflies were put back into their containers with some drops of water to keep them alive for the time being. These same butterflies were given a last chance to mate the next morning together with newly emerged ones. We decided to

not let them mate more than two days after emergence, as we were not allowed to feed them in order to determine the effect of the flowers on their egg laying performance. Also, we wanted to avoid big age differences as this could have an effect on the quantity of laid eggs (Hopkins & Van Loon 2001).

However, despite the number of butterflies in the cage and the halogen lamps to increase activity, mating success was very low on cloudy or rainy days. Whereas on sunny days up to 80 % of the butterflies copulated. To navigate the amount of butterflies which were able to mate, we synchronized their emergence by keeping some of the pupae close to emergence at 4 °C, until other pupae reached the same stage.

For egg deposition in the main trials, mated females were brought to the rack into the testing cages with the flower and control treatments and a cabbage plant for egg deposition. Eggs were counted and removed on a daily basis until female death.

Acknowledgements

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Appendix

Imported cabbageworm diet

PRODUCT #F9299B
IMPORTED CABBAGEWORM DIET
(BULK PACKAGING)

Agar 20.0 gm/L

Dry Mix 167.5 gmL

- Sucrose
- Cellulose
- Potassium Sorbate
- Vitamin Mix, Vanderzant
- Aureomycin (5.5% Active)
- Cabbage Flour
- Salt Mix, Wesson
- Methyl Paraben
- Casein
- Wheat Germ, Stabilized
- Linseed Oil, Raw
- Potassium Hydroxide Solution (18.3% w/w solution) **not provided**; Optional (=KOH Solution 4.8 gm/L)

DIRECTIONS FOR PREPARING 1 LITER OF DIET

1. Set up blender or mixer and proper containers.
2. Add 20.0 gm Agar to 800 ml. of cold water.
3. Bring to a full boil for one minute while stirring constantly.
4. Transfer agar solution to blender container and add Dry Mix (167.5 gm).
5. Add Potassium Hydroxide Solution at 4.8 grams per liter if used.
6. Blend for 30 seconds or until mixed thoroughly.
7. Dispense immediately

STORE UNUSED DIET IN REFRIGERATOR

CHAPTER 2

Innate and learned olfactory attraction to flowering plants by the parasitoid *Cotesia rubecula* (Marshall, 1885) (Hymenoptera: Braconidae): potential impacts on conservation biological control

Fataar S., Kahmen A. and Luka H., Biological Control (2019)

Abstract

In conservation biological control, flowers can be used to increase the biological control potential of parasitoids, which benefit from the offered food sources. Besides exhibiting exploitable nectar, flowers should preferably be olfactorily attractive, as highly attractive flowers are easily located, reducing the time spent searching for food and subsequently increasing the *per capita* host searching efficiency. In this study we thus focused on the olfactory attractiveness of *Fagopyrum esculentum* Moench (Polygonaceae), *Centaurea cyanus* L. (Asteraceae) and *Vicia sativa* L. (Fabaceae) to *Cotesia rubecula* (Marshall, 1885) (Hymenoptera: Braconidae), a larval parasitoid of the cabbage pest *Pieris rapae* (Linnaeus, 1758) (Lepidoptera: Pieridae). With a Y-tube olfactometer we found that *C. cyanus* and to a lesser extent *V. sativa* successfully attract *C. rubecula*. Also *F. esculentum* attracts *C. rubecula*, but only after a rewarding feeding experience. All three tested flowers seem to be suitable to be exploited in conservation biological control programs to control *P. rapae* in brassica fields. Even though not every flower offering accessible nectar is also innately attractive, it can still be suitable for conservation biological control purposes as feeding experience can change this attraction. In fact, the application of mixtures containing attractive and rewarding flowers could help increase the success of such programs.

Keywords

Braconidae; Olfactometer; Parasitoids; Attraction; Flower Odor; Associative Learning

Introduction

Agricultural landscapes dominated by monocultures often offer good conditions for pest insects to develop. To control these, organic farming focuses on environmental friendly strategies such as conservation biological control. It is a strategy to increase pest control through modification of the environment near crop fields. Usually, this includes supplementing nectar providing flowers to monocultures to increase the fitness of natural enemies such as parasitoids, and simultaneously providing them with shelter and overwintering sites (Balmer *et al.* 2014; Balzan *et al.* 2014). Sugar rich food sources are important for most insects to fulfill their energy needs (Bianchi & Wäckers, 2008), enhancing their life expectancy, realized fecundity and dispersal capacity (Wäckers 2004; Romeis *et al.* 2005; Wäckers *et al.* 2006; Wäckers *et al.* 2007; Bernstein & Jervis 2008; Géneau *et al.* 2012). Strengthened through nectar uptake, parasitoids can emphasize on host searching and keep pest densities under control.

The success of conservation biological control programs depends on the right choice of flower sources. The goal is to select flowers that enhance beneficial insects such as parasitoids or predators, but not pest insects (Winkler *et al.* 2010). In the flower selection process, different aspects need to be considered such as nectar accessibility (Patt *et al.* 1997; van Rijn & Wäckers 2016) and quality (Vattala *et al.* 2006) as well as visual and olfactory attractiveness (Kugimiya *et al.* 2010). Optimal parasitoid food sources would combine attractiveness with accessible nectar.

Parasitoids are exposed to different stimuli in the environment and have therefore evolved innate senses to find nutrition and hosts, enabling them to survive and reproduce (Zuk & Kolluru 1999). To locate host or food sources, parasitoids follow olfactory, visual and vibrational cues (Desouhant *et al.* 2005; Kroder *et al.* 2007; Ichiki *et al.* 2011; Pérez *et al.* 2011; Belz *et al*. 2013). In fact, the attraction to and reliable detection of targets is increased when multiple cues are combined (Laubertie *et al.* 2006). Floral scent can play a major role in flower location by parasitoids (Leius 1960; Wäckers 1994; Patt *et al.* 1997). When it comes to olfactory cues, different factors such as volatile quantity (Castelo *et al.* 2003; Turlings *et al.* 2004), reproductive (Shahjahan

1974) and hunger state of the parasitoids (Wäckers 1994; Luo *et al.* 2013) influence their behavior.

Here we focused on olfactory cues, because insects can locate these from long distances (Muhlemann *et al.* 2014), as opposed to visual cues, which are detected from shorter ranges. In fact, as shown by Hempel de Ibarra *et al.* (2015) insects' resolution of chromatic vision is limited, allowing them to locate and discriminate targets only in close proximity. In conservation biological control programs, natural enemies are ideally attracted from overwintering sites early in the season, enabling them to build up stable populations ready to suppress pest densities. However, overwintering sites of natural enemies such as forests are not always immediately adjacent to crop fields. Therefore, flowers in such programs should preferably be detectable from long distances. Simulations by Bianchi and Wäckers (2008) suggested that the attractiveness of flowers is an important feature that should be taken into account when selecting flowering plants, implying that adapting nectar supply to the needs of parasitoids has the potential to increase their effectiveness as biological control agents.

Cotesia rubecula (Marshall 1885) (Hymenoptera: Braconidae) is the main larval endoparasitoid of the small white *Pieris rapae* (Linnaeus 1758) (Lepidoptera: Pieridae), a pest of the brassica family. This pest is also attacked by *Cotesia glomerata* (Linnaeus 1758) (Hymenoptera: Braconidae), another larval endoparasitoid. However, the solitary *C. rubecula* regularly outcompetes the gregarious *C. glomerata* in this host (Geervliet *et al.* 2000), which was also confirmed in a field study conducted in Switzerland by Pfiffner *et al.* (2009). Therefore, the enhancement of *C. rubecula* may reduce *P. rapae* population densities, resulting in less crop damage (Herlihy & van Driesche 2013) and possibly higher yields (Balmer *et al.* 2014). *Cotesia rubecula* feeds on the host during larval stages, while adult wasps feed on nectar sources, which usually are not associated with host habitats (Wäckers 1994). At least once a day, adult *C. rubecula* wasps have to locate food to avoid starvation (Siekmann *et al.* 2001) and therefore their ability to innately follow or to learn relevant cues to find food sources, is crucial. When exposed to flower odors from ground-elder

(*Aegopodium podagraria* L.; Apiaceae) or rapeseed (*Brassica napus* L.; Brassicaceae), *C. rubecula* wasps are innately attracted to these, regardless of their state of hunger (Wäckers & Swaans 1993). This suggests that the behavior of *C. rubecula* could be manipulated by the provision of the right source of olfactory cues and exploited in conservation biological control programs.

In this study, we investigated the olfactory attractiveness of buckwheat (*Fagopyrum esculentum* Moench; Polygonaceae); cornflower (*Centaurea cyanus* L.; Asteracea) and common vetch (*Vicia sativa* L.; Fabaceae) to *C. rubecula*. These flowers are the main components of an existing flower strip used in Switzerland to promote pest control in brassica fields. This tailored flower strip was developed to selectively enhance *Microplitis mediator* (Haliday, 1834) (Hymenoptera: Braconidae) a parasitoid of the cabbage moth *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae) (Géneau *et al.* 2012). Further, the odor of *F. esculentum* was shown to be attractive to the egg parasitoids *Trissolcus basalis* (Wollaston, 1858) (Hymenoptera: Platygastridae) (Foti *et al.* 2016) and *Telenomus laeviceps* Förster, 1861 (Hymenoptera: Scelionidae) (Barloggio *et al.* 2018), both natural enemies of brassica pests. Also the odor of *C. cyanus* has been proven to be attractive to *T. laeviceps* (Barloggio *et al.* 2018) and *M. mediator* (Belz *et al.* 2013) and for the latter, even an increase in field biocontrol activity through supplementing *C. cyanus* has been demonstrated (Balmer et al. 2014). Hence the innate attractiveness of these flowers to *C. rubecula* was studied too, as it would be useful if they were attractive to many parasitoids of cabbage pests and specifically to understand if they can be exploited in the control of *P. rapae*.

Besides the innate attraction to an olfactory cue, learned attraction may also play an important role. To cope with variation in olfactory cues, parasitoids can use flexible behavior as a strategy to locate food sources (Wilson & Woods 2016). Accordingly, they should learn to follow new cues when exposed to environmental changes. While (associative) learning in parasitoids can be investigated with hosts for oviposition as reward (Mölck *et al.* 2000; Langley *et al.* 2006; Smid *et al.* 2007; Kruidhof *et al.* 2015; Segura *et al.* 2016), parasitoids can also be conditioned to respond to new odors associated with food as reward (Takasu & Lewis 1996; Luo *et*

al. 2013; Giunti *et al.* 2016). As demonstrated by Goyret *et al.* (2008), learning abilities could allow insects to forage more efficiently by concentrating their foraging efforts on specific flower species with reliable nectar rewards. Taking this into consideration, we investigated the learning ability of *C. rubecula* after exposure to a rewarding, but olfactorily unattractive flower.

Specifically, we a) investigated the absolute olfactory attractiveness of the three flowers against air as a neutral control, b) tested the relative olfactory attractiveness among the flowers in paired-choice tests and c) examined if innate attraction of *C. rubecula* can be altered through associative learning.

Material and Methods

Flowering plants

Centaurea cyanus, V. sativa and *F. esculentum* were grown from seeds in GroBanks (CLF Plant Climatics, Germany) at 24±2 °C (day), 18±2 °C (night), 55±5 % relative humidity and L16:D8 photoperiod. Seeds were sown in compressed soil cubes (Max Schwarz AG, Villingen Switzerland). After three weeks, plantlets were transplanted into pots (12 cm diameter, 10 cm height) containing soil (Einheitserde Classic, Gebrüder der Patzer GmbH & Co. KG, Germany) fertilized with 3 g/1 l slow-release formulated fertilizer (Tardit 3M, Hauert HBG Dünger AG, Switzerland). Plants were checked on a daily basis and watered as needed until they bloomed.

Parasitoids

Cotesia rubecula individuals had been collected in 2015 from parasitized *P. rapae* larvae in Swiss organic cabbage fields and had since then been reared on *P. rapae* larvae feeding on white cabbage plants (*Brassica oleracea* var. *capitata*). Wasps were fed *ad libitum* with honey gelatine (200 g honey (Switzerland), 100 ml demineralized water and 3 g gelatine (Dr. Oetker)).The rearing was kept in BugDorm units (47.5 cm x 47.5 cm x 47.5 cm) in a climate chamber with 22±2 °C, 55±5 % relative humidity and

a 16L:8D photoperiod. As post-emergence experience was shown to affect parasitoid searching responses (Sheehan & Shelton 1989; Kester & Barbosa 1991), cocoons were regularly harvested and packed singly in plastic containers until emergence, preventing any post-emergence experience with plant odors before the experiments.

Y-tube olfactometer – Test procedure

To test the attractiveness of the selected flowers we used the Y-tube olfactometer described by Barloggio *et al.* (2018). The olfactometer was placed under a construction with an opaque curtain to block out all light sources which might influence the decision making, except for the table lamp placed above the bifurcation of the Y-tube (approximately 30 cm above (20W)), ensuring the same light conditions for both arms. Further, a wooden plate served as visual barrier (vb) to block out visual cues from flowers (Figure 2-1). Before entering the olfactometer at a speed of 757 ml/min, air was purified through a charcoal filter and humidified. This air then reached two glass containers, each containing an odor source. Flower heads were cut just before the testing session. Because it was demonstrated that there is no preference for odors resulting from the cut stem over flowers (Belz *et al.* 2013) and cutting did not influence floral scent emission immediately after cutting *Lysimachia* species (Schäffler *et al.* 2012), flower stems were not wrapped in wet cotton wool sealed with parafilm as it was done by Wäckers (2004). Mean temperature and relative humidity were 27±1.3 °C and 66±12 % in each glass vial containing flowers or air (data logger DS1923 Hygrochron, Thermodata). The trials were performed during the period of main parasitoid activity, between 10 am and 12 pm. When insects are most active, the production of floral scent is higher, increasing flower detectability (Muhlemann *et al.* 2014). Unfed virgin female parasitoids (< 24 h) were introduced in the central part of the Y-tube olfactometer, 1.5 cm from the start line (Figure 2-1). After passing this start line, the parasitoids had to reach one of the finish lines (14 cm apart from the start line) within five minutes (Figure 2-1). Individuals which have not made a choice in the time given, were replaced. The position of the arms containing

odor sources was swapped after three tested parasitoids and the odor sources were renewed after six tested parasitoids. Once 30 replicates per treatment have been reached, all the elements described in Figure 2-1 were cleansed following the procedure in Belz *et al.* (2013).

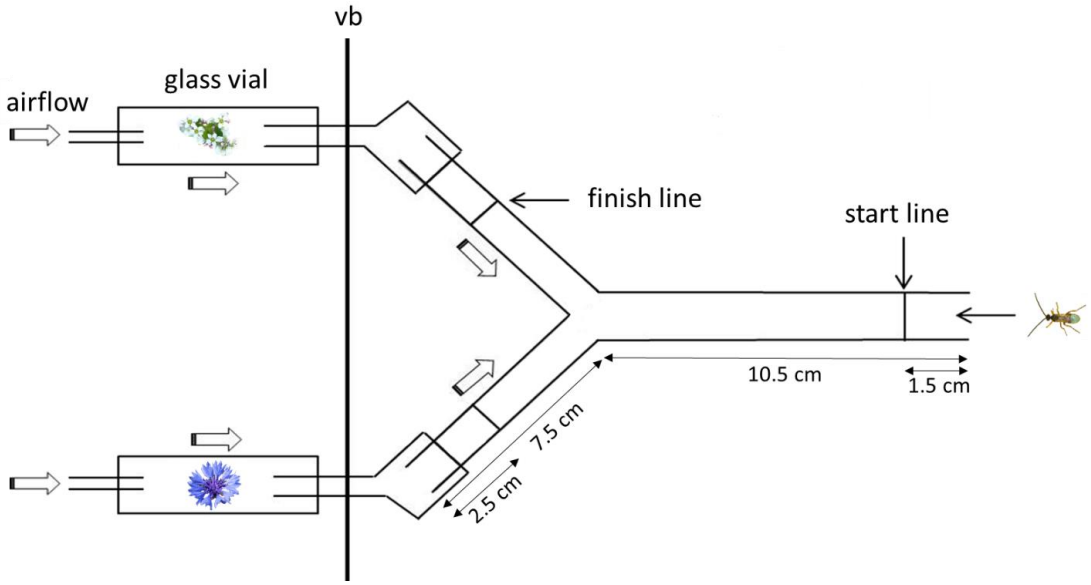

Figure 2-1: Schematic illustration of the Y-tube of the olfactometer. *C. rubecula* is released at the base of the Y-tube and the experiment starts, as soon as the parasitoid passes the start line and ends when it reaches one of the finish lines within five minutes. vb: visual barrier (adapted from (Belz *et al.* (2013))).

Absolute and relative olfactory attractiveness

The absolute olfactory attractiveness of *C. cyanus*, *F. esculentum* and *V. sativa* was tested by providing the choice of flower odors and clean air in a Y-tube olfactometer. This determined whether they are attractive, neutral or even repellent to *C. rubecula*.

In paired-choice tests the relative olfactory attractiveness between *C. cyanus*, *F. esculentum* and *V. sativa* was tested to find the most attractive flower to *C. rubecula* among them.

Associative learning potential of C. rubecula

To test if the innate attraction of *C. rubecula* can be altered through associative learning, newly hatched female wasps were first fed with *F. esculentum* for one day,

starved the following day and then tested for the absolute olfactory attractiveness of *F. esculentum* against air and the relative olfactory attractiveness between *F. esculentum* and *C. cyanus*. These two plant species were chosen because the first two trials revealed that *F. esculentum* was innately unattractive and *C. cyanus* innately attractive to *C. rubecula*.

Statistics

Data analyses were conducted with R version 3.3.0 (R Core Team 2016). Females that never passed a finish line within five minutes were treated as non-responders and were therefore omitted from the analysis. Count data from all the trials were analyzed with the Pearson's Chi-squared test by comparing the observed proportions against 0.5 (expected proportions of the zero hypothesis: no preference) (Belz *et al.* 2013; Barloggio *et al.* 2018). The mean ratios and standard errors were determined for each odor couple tested. After six replicates, the odor sources were renewed and the number of responsive females used to calculate the proportion of females choosing odor 1 over odor 2. This was repeated until we reached 30 responsive females per treatment.

$$proportion\,\mathrm{n} = \frac{\#\ females\ which\ chose\ odor\ 1}{\#\ responsive\ females}$$

The calculated mean ratios result from the sum of proportions over the number of coupled odor sources used for each treatment.

$$Ratio = \frac{proportion\,1 + proportion\,2 \ldots . + proportion\,\mathrm{n}}{N\ odor\ couple}$$

Results

In all trials a total of 333 female wasps were tested, of which 240 (72.1 %) made a decision within five minutes and 93 (27.9 %) did not.

Absolute and relative olfactory attractiveness

The first trial, in which we tested the absolute attractiveness of *C. cyanus*, *F. esculentum* and *V. sativa* compared to air as a neutral control, parasitoids chose the arm containing a flower source significantly more often than air for *C. cyanus* (Pearson's Chi-squared test against 0.5,p = 0.001) and marginal more for *V. sativa* (Pearson's Chi-squared test against 0.5, p = 0.068). *Fagopyrum esculentum* was not preferred over air (Pearson's Chi-squared test against 0.5, p = 0.715) (Figure 2-2).

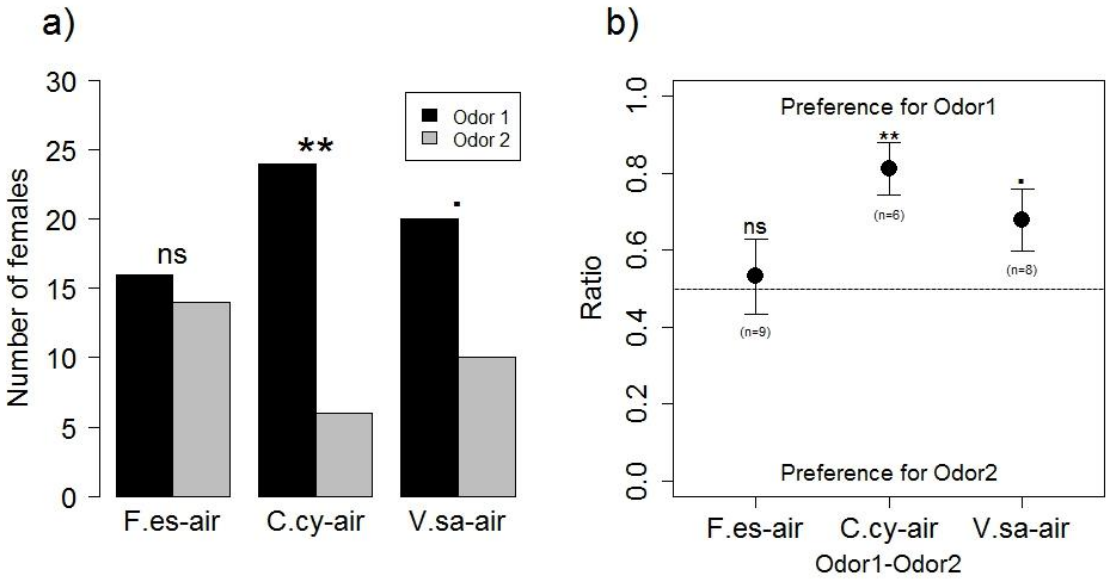

Figure 2-2: Absolute attractiveness of flowers to female *C. rubecula* wasps with air as a neutral control. a) Total number of female wasps per odor source. b) Proportion of odor preferences (mean±se). Values above the dotted line (expected proportion = 0.5) indicate a preference for odor 1 and below for odor 2. (n=) corresponds to the amount of flower sources used to reach 30 replicates. F.es = *Fagopyrum esculentum*, C.cy = *Centaurea cyanus*, V.sa = *Vicia sativa*. Pearson's Chi-square test, ** = $p < 0.01$; • = marginal significant (p = 0.068); ns = not significant, N = 30 per treatment.

The relative attractiveness of *C. cyanus*, *F. esculentum* and *V. sativa* was tested in paired-choice tests. Results showed that only *C. cyanus* was significantly preferred over *F. esculentum* (Pearson's Chi-squared test against 0.5, p = 0.004) (Figure 2-3).

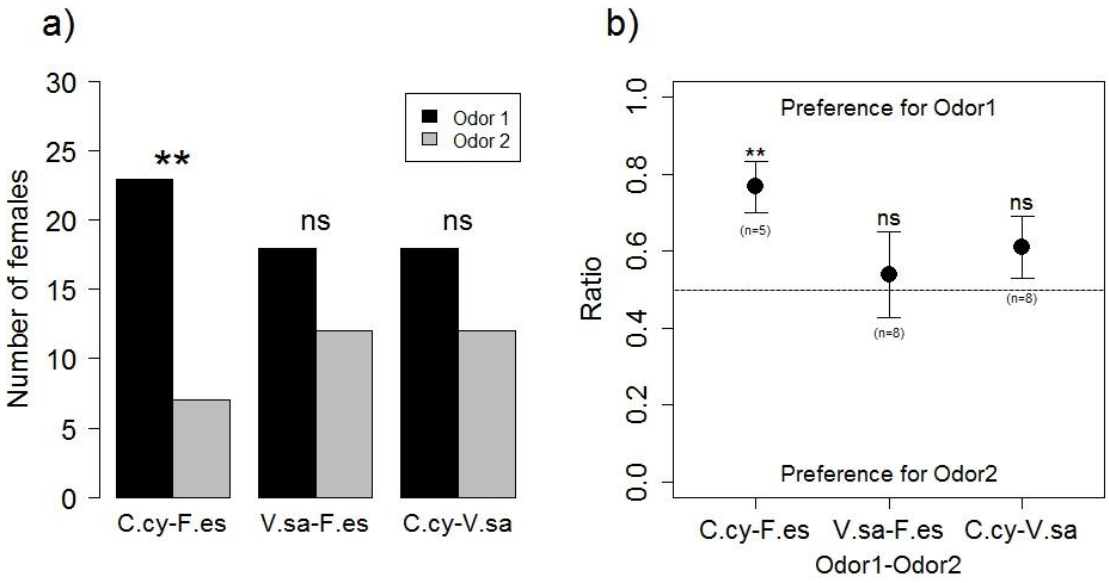

Figure 2-3: Relative attractiveness of *C. cyanus* (C.cy), *F. esculentum* (F.es) and *V. sativa* (V.sa) in paired-choice tests. a) Total number of female wasps per odor source. b) Proportion of odor preferences (mean±se). Values above the dotted line (expected proportion = 0.5) indicate a preference for odor 1 and below for odor 2. (n=) corresponds to the amount of flower sources used to reach 30 replicates. Pearson's Chi-square test, ** = $p < 0.01$; ns = not significant, N = 30 per treatment.

Associative learning potential of C. rubecula

We investigated whether *C. rubecula* can develop a preference for a previously unattractive odor through associative learning. We showed that *F. esculentum* was significantly preferred over air when the wasps previously fed on this plant (Pearson's Chi-squared test against 0.5, $p < 0.001$) (Figure 2-4). In the paired-choice test, the innately attractive *C. cyanus* was only marginally preferred over *F. esculentum* when insects had previously fed on this plant (Pearson's Chi-squared test against 0.5, $p = 0.068$) (Figure 2-4).

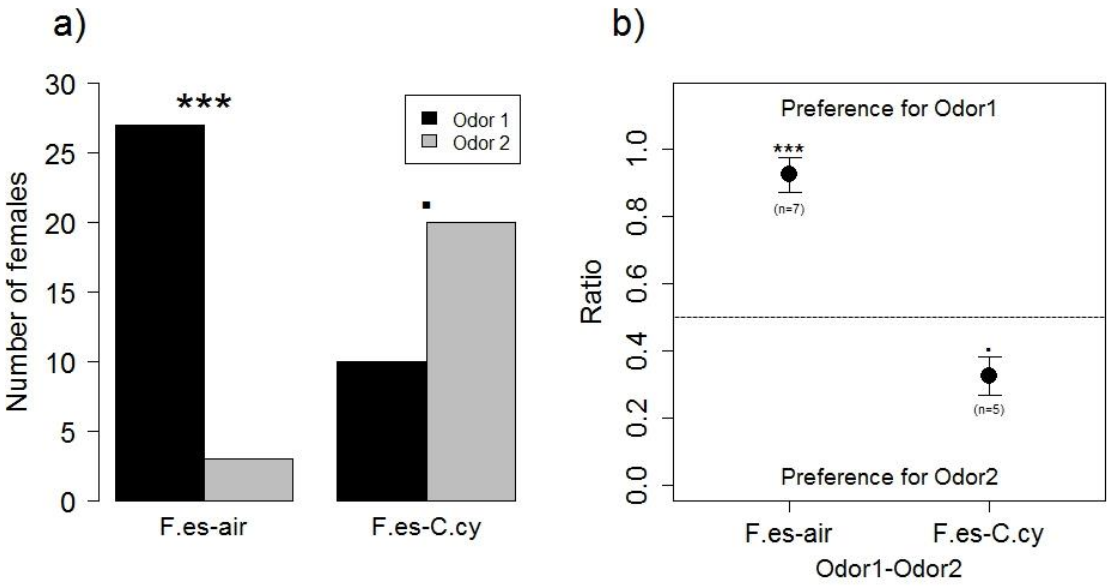

Figure 2-4: The influence of *F. esculentum* feeding experience on attractiveness of *F. esculentum* to female *C. rubecula* wasps with air as a neutral control and *C. cyanus*. a) Total number of female wasps per odor source. b) Proportion of odor preferences (mean±se). Values above the dotted line (expected proportion = 0.5) indicate a preference for odor 1 and below for odor 2. (n=) corresponds to the amount of flower sources used to reach 30 replicates. F.es = *Fagopyrum esculentum*, C.cy = *Centaurea cyanus*. Pearson's Chi-square test, *** = $p < 0.001$; • = marginal significant ($p = 0.068$); N = 30 per treatment.

Discussion

The aim of this work was to test the absolute and relative olfactory attractiveness of *C. cyanus*, *F. esculentum* and *V. sativa* to *C. rubecula*, the main larval antagonist of the cabbage pest *P. rapae*. Further, we investigated whether *C. rubecula* can be drawn to an unattractive odor, through associating it with rewarding nectar, after feeding experience. The results of these trials are discussed to assess the suitability of the selected flowers to control *P. rapae* in brassica fields through conservation biological control.

Depending on the combination of the tested odors, the wasps' responsiveness varied. Overall 27.9 % remained unresponsive, meaning that they had not made a choice within five minutes and were therefore omitted from the analyses. The reasons for the unresponsiveness remain unclear but it is known that responsiveness

can be linked to the number of eggs in the parasitoids ovaries (Shahjahan 1974). Steinberg *et al.* (1992) showed that for *C. glomerata* wasps at the age of one to two days, only 48 % responded within 10 minutes, when tested for the response to volatile info-chemicals emitted by the plant-host complex in the Y-tube olfactometer. We therefore assume that the overall unresponsiveness of 27.9 % within five minutes is acceptable and the received results through the Y-tube olfactometer should thus be reliable.

Regarding the results of the absolute and relative olfactory attractiveness trials, it is evident that among the tested flowers, *C. rubecula* shows the strongest innate attraction to *C. cyanus*, followed by a marginal attraction to *V. sativa* and no attraction to *F. esculentum*. Although being food-deprived, only *C. cyanus* out of the three tested flowers was significantly attractive when compared to air and other flowers in first choice experiments, indicating olfactory innate preferences for this plant species by female *C. rubecula*. This is in line with Wäckers (2004), who reported that despite being food-deprived, parasitoids exhibited a positive innate response to flowers of only four out of the 11 plants tested. This points out, that not every flower offering accessible nectar is also innately attractive.

Besides *C. rubecula* and *M. mediator*, which display an innate attraction to olfactory cues of open *C. cyanus* flowers (Belz *et al.* 2013), a wide spectrum of beneficial insects also visit this flower. Most of which belong to the families of Syrphidae, Ichneumonidae, Formicidae, Chrysopidae, Vespidae and Coccinellidae (Stettmer 1993; Winkler *et al.* 2005a). This broad range of visiting beneficial insects emphasizes the important role that *C. cyanus* could play in conservation biological control.

To date, no studies have reported the olfactory attractiveness of *V. sativa* to parasitoids. We demonstrated here that the odor of *V. sativa* attracts *C. rubecula* only marginally. However, in a study conducted by Bugg *et al.* (1989) 60 individuals of Ichneumonidae (Hymenoptera) were found feeding on the closely related species *Vicia faba* L. (Fabaceae). If these individuals were visually or olfactorily attracted remains, yet, unclear. This suggests that *V. sativa* could also be exploited in

conservation biological control programs as *C. rubecula* is likely attracted by more than one cue. But further experiments need to be conducted to confirm this.

Surprisingly, *F. esculentum* was not attractive to *C. rubecula* although it was shown to be olfactorily attractive to *M. mediator* a parasitoid of the same family (Belz *et al.* 2013) and to both the egg parasitoids *T. basalis* (Foti *et al.* 2016) and *T. laeviceps* (Barloggio *et al.* 2018). Further, it is also known to positively influence longevity of different parasitoids (Wratten *et al.* 2003; Lavandero *et al.* 2006; Winkler *et al.* 2006; Irvin *et al.* 2007; Witting-Bissinger *et al.* 2008; Nafziger & Fadamiro 2011; Géneau *et al.* 2012; Araj & Wratten 2015; Russell 2015). We thus expected that it would also be attractive to *C. rubecula*. Nevertheless, many studies have proven that the value of *F. esculentum* for enhancing natural enemies is enormous and therefore its detectability is all the more important.

The lack of attraction to *F. esculentum* shows that innate odor responses alone would markedly restrict the exploitation of potential nectar sources of supplementary plants in conservation biological control systems. In addition to volatiles, primary food foraging might be initially guided by innate responses to specific visual cues such as flower colors (Wäckers 1994; Kugimiya *et al.* 2010). This could be the reason why *F. esculentum* increased parasitism rates by *C. rubecula* on *P. rapae* larvae over four years, when planted as 3 m wide flowering borders along-side cabbage fields (Lee & Heimpel 2005). The fact that *C. rubecula* does express innate attraction to colors was shown by Wäckers (1994), who discovered that *C. rubecula* is innately attracted to yellow, irrespective of its hunger state. In addition to these studies, investigations of the attractiveness and usage of *F. esculentum* by *C. rubecula* in the field are clearly needed.

In this study, *C. rubecula* was drawn to the innately unattractive odor of *F. esculentum* after feeding experience. While inexperienced wasps did not show an attraction to the nectar source, feeding experienced wasps learned to associate the odor with a food reward. When *F. esculentum* experienced wasps were given the choice between *F. esculentum* and *C. cyanus*, the innately attractive *C. cyanus* was, however, still preferred. Yet, this attraction became only marginal compared to

unexperienced wasps, which clearly preferred *C. cyanus*. It is evident, that the innate preference for *C. cyanus* is strongly conserved and cannot be easily altered. This was also shown for *Venturia canescens* (Gravenhorst, 1829) (Hymenoptera: Ichneumonidae), which is able to associate food to a visual cue through training without modifying its innate preference to the preferred color yellow (Lucchetta *et al.* 2008).

In a memory consolidation study, Smid *et al.* (2007) demonstrated that after a single-trial with a plant odor as conditioned stimulus and an oviposition event as reward, *C. rubecula* formed memory that waned before 24 h. Three trials spaced in time were required for long-term memory to form and a complete memory consolidation took two to three days. In our case, the wasps were able to feed multiple times during 24 h, were starved the following day and subsequently tested. The fact that they clearly were drawn to the associated odor of *F. esculentum*, suggests that they have formed memory lasting longer than 24 h. This finding points out that even though a flower is olfactorily unattractive at first, it can still be suitable for conservation biological control purposes.

From a more applied point of view, these results can be exploited to better understand the potential of the selected flowers in the control of the cabbage pest *P. rapae*. The selection of highly attractive flowers helps to reduce the time invested by parasitoids in food searching, which in turn increases the *per capita* host searching efficiency. We showed that *C. cyanus* and to a lesser extent *V. sativa* successfully attract *C. rubecula*. On the other hand, the odor of *F. esculentum* attracts *C. rubecula* only after a rewarding feeding experience. These results point out the importance of using more than just one flower species to attract and retain beneficial insects in a conservation biological control programs. In fact, the application of mixtures containing attractive and rewarding flowers could help increase the success of such programs. Even though olfactometer experiments only deliver restricted information regarding the real behavior of insects on the plant itself (Ballhorn & Kautz 2013), our experiments support the suggestions from Géneau *et al.* (2012), that *C. cyanus*, *V. sativa*, and to a lesser extent *F. esculentum* are suitable flowers for conservation

biological control, regarding their olfactory attractiveness. However, whether our tested flowers can improve the fecundity and longevity of *C. rubecula*, as well as its biological control efficiency, still needs to be tested in the laboratory and the field, respectively.

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CHAPTER 3

Higher promotion of *Cotesia rubecula* (Hymenoptera: Braconidae) than its host *Pieris rapae* (Lepidoptera: Pieridae) through flowering plants: perspectives for conservation biological control

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Abstract

Multiple times flowers have been deployed for conservation biological control purposes. But rarely has it been checked whether these flowers were selective plants, enhancing beneficial insects but not the pests. The three flowering plants *Fagopyrum esculentum* Moench (Polygonaceae), *Centaurea cyanus* L. (Asteraceae) and *Vicia sativa* L. (Fabaceae) were suggested as selective plants for conservation biological control purposes against the cabbage moth *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae). Here, we tested their effects on the fitness of *Pieris rapae* (Linnaeus, 1758) (Lepidoptera: Pieridae) another cabbage pest and its antagonist *Cotesia rubecula* (Marshall, 1885) (Hymenoptera: Braconidae). We performed survival and fecundity assays in the laboratory for each insect. The longevity of the wasps was enhanced by all flower treatments and their lifetime fecundity by *F. esculentum* and *C. cyanus* as a function of longevity. But also the butterflies' longevity and fecundity were enhanced by *F. esculentum* and *C. cyanus*. Therefore, based on these results of laboratory tests, we cannot recommend any of the tested flowers as selective flowering plants against the cabbage pest *P. rapae*.

Keywords

Selective flowering plants; Conservation biological control; *Pieris rapae*; *Cotesia rubecula*; Fecundity; Longevity

Introduction

In pest management, conservation biological control is an approach, which aims to reduce pesticide use as part of an integrated plant protection strategy (Begg *et al.* 2017). As a branch of conservation biological control, habitat manipulation aims to increase vegetation diversity and complexity in managed landscapes, to provide food and shelter for natural enemies of arthropods (Landis *et al.* 2000; Frank *et al.* 2008) and thereby enhancing their biological control potential (Begum *et al.* 2006). For instance, flower strips are sown adjacent to fields or flowers can be interplanted in the crop. Thereby the time spent for food localization by parasitoids can be reduced and more time can be invested towards host searching, ultimately leading to higher parasitism rates (Takasu & Lewis 1995; Siekmann *et al.* 2004).

Studies reported higher parasitism rates of insect pests in fields with flower strips (Pfiffner & Wyss 2004; Lavandero *et al.* 2005; Ponti *et al.* 2007; Haaland *et al.* 2011) and accordingly a higher effectiveness of reducing pests and crop damage (Balmer *et al.* 2014; Tschumi *et al.* 2015). In some cases, however, no effects could be shown (Berndt *et al.* 2002; Vollhardt *et al.* 2010) and even negative effects, such as unintended pest conservation, have been demonstrated (Bone *et al.* 2009).

Instead of diversity *per se*, targeted diversification programs could provide an appropriate form of diversity in which beneficial insects are conserved and pests not (Tschumi *et al.* 2015). By studying the interaction between pests and their antagonists in different habitats, tailored flower mixtures can be developed to increase the effectivity of natural enemies and to avoid unintended pest conservation (Diekötter & Crist 2013; Ekroos *et al.* 2014; Lu *et al.* 2014; Mitchell *et al.* 2014; Tscharntke *et al.* 2016). While targeted approaches identifying and providing preferred floral food resources have proven to be potentially successful for a range of predatory and parasitic natural enemies (Olson & Wäckers 2007; Bianchi & Wäckers 2008; Tschumi *et al.* 2015), there are still many beneficial insects for which preferred food sources have not yet been identified.

Nectar and pollen are important to enhance the fitness of most adult Hymenoptera by increasing parasitoid longevity and fecundity (Siekmann *et al.* 2001; Wäckers 2001; Tylianaiks *et al.* 2004; Winkler *et al.* 2006; Géneau *et al.* 2012; van Rijn & Wäckers 2016). But not all nectars are equally beneficial to parasitoids, as they consist of a mixture of naturally occurring sugars of which rhamnose for instance was shown to reduce parasitoid survival and even suppressed the nutritional benefit of glucose when mixed together (Wäckers 2001). Therefore, flowers should be carefully selected in the lab before their application in the field. But even if longevity and fecundity are promoted by flowers in the field, it does not automatically translate into increased parasitism (Lee & Heimpel 2008).

Properties of flowering plants, which lead to selective enhancement of beneficial insects and not their pests are nectar composition and quality (Vattala *et al.* 2006), flower morphology (e.g. nectar accessibility) (Patt *et al.* 1997; Winkler *et al.* 2009b; van Rijn & Wäckers 2016), differentiated preferences of insects towards these flowers (Bianchi & Wäckers 2008) and differentiated enhancement (Winkler *et al.* 2009b). Since mouthpart structures of parasitoids are rather short and unspecialized (Jervis 1998; Baggen *et al.* 1999; Wäckers 2004; Heil 2011), flower morphology predominantly influences parasitoids' access to nectar (Patt *et al.* 1997; Wäckers 2004; Vattala *et al.* 2006), restricting nectar exploitation to flowers with short, open corollas and extrafloral nectaries (EFN). Lepidopterans by contrast have specialized mouthparts that allow them to reach hidden nectar, but the morphology of these mouth parts restricts nectar exploitation to flowers with diluted nectar (Watt *et al.* 1974; Daniel *et al.* 1989; Winkler *et al.* 2009b). Hence, it is not surprising that most butterfly-pollinated plants produce dilute nectars of 15-30% sucrose (Baker & Baker 1982).

Extrafloral nectar can be quite different in composition to the floral nectar of the same plant (Lundgren 2009). In addition, EFN can be usually found over a long period of time and well before the plant blooms, because the production of EFN does not depend on flowers, which may be available only for short periods (Heil *et al.* 2004). Nectar can thus be provided to parasitoids early in the growing season before

the onset of flowering, rendering the parasitoids ready to suppress pest population growth in the case of habitat manipulation (Lu *et al.* 2014). Extra floral nectars are easily accessible for a wide range of parasitoids and since they are usually more concentrated than floral nectars, they can be a very valuable food source for beneficial insects (Koptur 2005), but not for lepidopteran pests, since they rely on less concentrated food sources. Indeed, Azzouz *et al.* (2004) have shown that the lifetime of a braconid increased significantly with increasing sugar concentration and that longevity primarily depended on the concentration of the sugar solution, rather than the quantity of sugar solution ingested.

Ideally plants selected for conservation biological control should enhance beneficial insects for a specific crop but not the pests. Because Géneau *et al.* (2012) recommended *Centaurea cyanus* L. (Asteraceae) (floral and EFN), *Vicia sativa* L. (Fabaceae) (floral and EFN) and *Fagopyrum esculentum* Moench (Polygonaceae) (floral nectar) as selective flowering plants for conservation biological control against the cabbage pest *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae), we investigated the effects of these plants on another relevant cabbage pest and its antagonist, namely *Pieris rapae* (Linnaeus, 1758) (Lepidoptera: Pieridae) and *Cotesia rubecula* (Marshall, 1885) (Hymenoptera: Braconidae).

We a) offered these plants to both insect species and recorded how long they survive when feeding on the different nectars, b) assessed how many offspring they can potentially produce through nectar uptake of the different flower species, by counting the laid eggs. We discussed whether our study flowers with their different morphologies and nectar supply can be recommended for conservation biological control against *P. rapae*.

Material and Methods

Plant and insect rearing

Brassica oleracea convar. capitata var. alba for rearing maintenance and *F. esculentum*, *C. cyanus* and *V. sativa* for experiments were grown from seeds in grow banks at 24±2 °C, 55±15 % relative humidity and 16L:8D photocycle. Because only a small amount of *V. sativa* plants produced flowers in our rearing, we had to use plants without flowers (floral nectar), but with EFN at the stipules, for the experiments.

Pieris rapae larvae were reared in a climate chamber on cabbage plants at 23±2 °C, 55±5 % r.h. and 16L:8D. Pupae were collected and transferred to cages in the lab, until emergence. Adults were fed with diluted honey (10 %) and a cabbage plant was provided for oviposition. *C. rubecula* were reared on *P. rapae* larvae feeding on *B. oleracea* in a climate chamber at 22±2 °C, 55±5 % r.h. and 16L:8D. Parasitoid cocoons were collected and singly placed in plastic containers until emergence. Adults were fed *ad libitum* with honey-gelatine (200 g honey (Switzerland), 100 ml demineralized water and 3 g gelatin (Dr Oetker, Germany)).

Cotesia rubecula longevity assay

To assess the effect of nectars from different species on male and female longevity of *C. rubecula*, parasitoids (<24h old) were kept singly in plastic bottles containing *C. cyanus*, *V. sativa* and *F. esculentum*, offering floral and/or EFN. Bottle openings were sealed with a water soaked cotton wick and the cut bottoms with an incised sponge disc around the plant stems. Three holes in the bottle sealed with close-meshed nets provided ventilation. A visual description is provided in Barloggio *et al.* (2018). Parasitoids were either kept with water only (control) or provided with water and one of the flower species. This experiment took place in a climate chamber with 22±2 °C, 55±5 % r.h. and 16L:8D. Parasitoid survival was recorded daily between 11:00 a.m. and 12:00 p.m. and a total of 30 replicates per treatment and sex were performed.

Cotesia rubecula fecundity assay

To examine how nectar of the different flower species affects *C. rubeculas*' fecundity, parasitoids were kept just as described in the longevity experiments, but in pairs of one male and one female. Daily, parasitoids were transferred for one hour to a plastic vessel (4.4 cm Ø, 4.2 cm height) containing ten second instar host larvae on a piece of cabbage. This procedure was repeated until female death. Parasitism took place between 10:00 a.m. and 12 p.m.. Host larvae were dissected under a binocular one or two days after parasitism and the number of parasitoid offspring (egg or hatched larvae) was counted. The number of hosts which died before dissection was recorded. Because *C. rubecula* as a solitary endoparasitoid lays its eggs singly into its host larvae, each egg or larvae was counted as a potential offspring even when multiparasitism occurred as a consequence of the experimental setup. We performed 20 replicates per treatment. Lifetime parasitism, which is the total amount of eggs laid during an individual's lifetime and average daily parasitism, which corresponds to the total amount of eggs laid during an individual's lifetime divided by the days it survived, were statistically analyzed.

Pieris rapae longevity assay

To assess how nectars from different flower species affect male and female *P. rapae* longevity, butterflies (<24h old) were kept individually or in a maximum of three of the same sex in a cage (47.5 cm x 47.5 cm x 93.0 cm) with the same number of plant species as butterflies per cage unit. Each unit contained either *C. cyanus*, *F. esculentum* or *V. sativa* and a water soaked sponge. Instead of a potted plant, a pot filled with soil was placed in the control treatment. Plants were watered daily and the sponges kept soaked. At every stage of a butterfly's life cycle, larger individuals survive rather better than smaller ones (Gilbert 1984). To account for individual size, pupae were weighed, given an ID and kept singly in plastic containers (5.5 cm Ø, 6.3 cm height) until emergence. The different weight classes were evenly distributed among the treatments. For identification (in the case of three individuals per cage unit), adults

were numbered with a marker on the lower side of their wings. Experiments took place in the lab with 25±5 °C and 39±14 % r.h.. To ensure a 16L:8D cycle, neon tubes were installed in front of the cages. Survival was recorded daily between 11:00 a.m. and 12:00 p.m. and 20 replicates per treatment and sex were performed.

Pieris rapae fecundity assay

To evaluate how *P. rapae* fecundity is influenced by nectars of the different flower species, factors such as body size and nuptial gifts had to be regarded. A nuptial gift is a nutritional gift given by the males during sexual intercourse, which improves the fitness of the recipient females. Because pupal weight is linearly related to lifetime egg production and therefore larger individuals lay more eggs than small ones (Jones *et al.* 1982; Gilbert 1984), pupae were weighed just as in the longevity experiment and once mated, the different female weight classes were evenly distributed among the treatments. Multiple weighed and numbered butterflies (<24 h and <48 h old) of both sexes were kept together in the morning in a cage (47.5 cm x 47.5 cm x 93.0 cm) to allow mating until noon. Unmated butterflies were put back singly into their emerging vessels and were given another chance to mate the following day. Drops of water kept them alive for the time being. Once mated, the couple was transferred to another cage and the male was removed as soon as copulation ended. This step ensured, that butterflies mated only once, because monarch butterflies which were allowed to mate several times laid more eggs than singly-mated females (Oberhauser 1989). Also, higher sugar concentrations in male nutrition lead to more eggs being laid by the female partner, indicating the influence of nuptial gifts on the fitness of females (Cahenzli & Erhardt 2012). The mated females were kept singly as described in the longevity experiment, but with an additional cabbage plant for egg deposition. Laid eggs were counted daily and subsequently removed until female death. These trials took place in the lab at 25±5 °C and 39±14 % r.h. and 15 replicates per treatment were completed. Lifetime fecundity, which is the total amount of eggs laid during an individual's lifetime and average fecundity per day, which corresponds to the total

amount of eggs laid during an individual's lifetime divided by the days it survived, were statistically analyzed.

Statistical analysis

All statistical analyses were performed with R Statistical Software version 3.3.1 (R Core Team 2016) and all treatment means are indicated with standard errors. Longevity of male and female *C. rubecula* was analyzed through a generalized linear model (glmer function from the package lme4) with Poisson errors, "treatment" as fixed factor (four levels: control and the three flower species), and "calendar week of replicate onset" as random factor. The lifetime parasitism was analyzed using a generalized linear model with Poisson errors, "treatment", "female age" and "male age" as fixed factors and "calendar week of replicate onset" as random factor, corrected for overdispersion. A stepwise model reduction was employed, with the least significant interaction always being removed first (Crawley 2007), in this case "male age". To control the varying survival rate of the larvae until dissection and its possible effect on *C. rubecula* fecundity outcome, the log-transformed proportion of surviving hosts was integrated as an offset. Average parasitism was fitted to a generalized linear model with quasi-Poisson errors and with "treatment" and "male age" as fixed factors, but without the fixed factor "female age" since lifetime fecundity was divided by female age to receive the average parasitism per day and was therefore already integrated. Also here, "male age" was removed from the model because of non-significance.

Longevity of female and male *P. rapae* was analyzed through a generalized linear model with Poisson errors, "treatment" and "weight" as fixed factors and "calendar week of replicate onset" and "level (position of the cage during the trial)" as random factors. Weight of female and male butterflies was not significant and removed from the model. Data concerning the lifetime fecundity of *P. rapae* were fitted to a generalized linear model with Poisson errors, "treatment", "female weight", "male weight" and "egg deposition days" as fixed factors and "calendar

week of replicate onset" and "level" as random factors, corrected for overdispersion. Egg deposition days is the number of days on which butterflies were able to lay eggs and is a replacement for female age as the age of females partly differed by one day at the onset of the experiment. "Male weight" was removed from the model due to non-significance. Similarly, average fecundity was fitted to a generalized linear model with quasi-Poisson errors, but without "egg deposition days" since lifetime fecundity was divided by egg deposition days to receive the average fecundity per day and was therefore already integrated. Also in this case "male weight" was not significant and removed from the model.

As post-hoc test, for all the models above, we used multiple pairwise comparisons to determine significant differences between the treatments (glht function from the package multcomp).

Results

Cotesia rubecula longevity assay

In the control treatment mean longevity for females and males was 1.9±0.1 and 2±0.1 days, respectively. Following numbers for each treatment refer to females first and then to males, if not stated differently. Highest mean longevity was reached in the *F. esculentum* treatment for both sexes 15.3±1 and 13.4±0.8 days (8 and 7 fold more compared to the control), followed by the *C. cyanus* treatment 6.6±0.7 and 5.3±0.5 days (3.5 and 2.7 fold increased longevity) and the *V. sativa* treatment 3.6±0.2 and 3.8±0.3 days (1.9 fold increased longevity for both sexes). Within each sex all treatments differed significantly from each other (Generalized linear model, all $p < 0.05$) (Figure 3-1, Table 3-1).

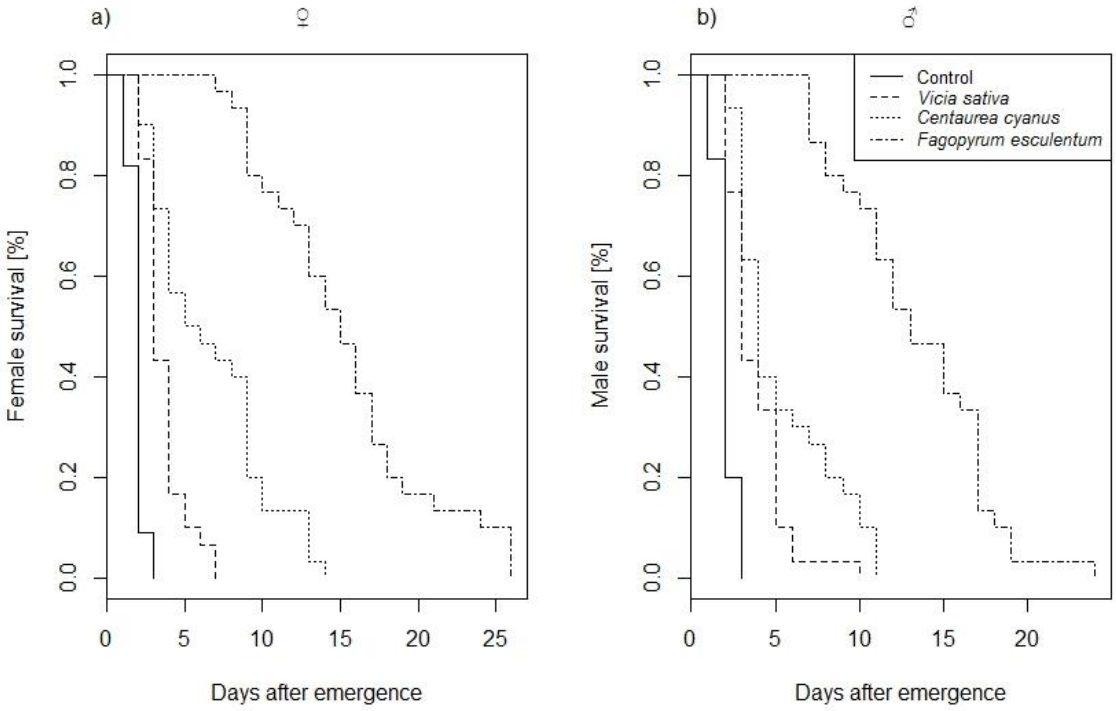

Figure 3-1: a) Survival curves of female and b) male *C. rubecula* wasps provided with either water (Control), *V. sativa* (EFN only), *C. cyanus* or *F. esculentum*. For females and males all treatments differ significantly from each other (Generalized linear model, all $p < 0.05$).

Table 3-1: Table of the GLM results of *C. rubecula* female and male survival when provided with either water (Ctrl), *V. sativa* (EFN only) (*V. sa*), *C. cyanus* (*C. cy*) or *F. esculentum* (*F. es*).

Female survival					**Male survival**				
GLM Family: poisson					GLM Family: poisson				
Fixed effects	**Estimate**	**S. E.**	**z value**	**P value**	**Fixed effects**	**Estimate**	**S. E.**	**z value**	**P value**
Ctrl-*C. cy*	-1.246	0.153	-8.156	**< 0.001**	Ctrl-*C. cy*	-0.964	0.151	-6.408	**< 0.001**
Ctrl-*F. es*	2.082	0.141	14.8	**< 0.001**	Ctrl-*F. es*	1.883	0.137	13.7	**< 0.001**
Ctrl-*V. sa*	0.64	0.164	3.904	**< 0.001**	Ctrl-*V. sa*	0.617	0.159	3.88	**< 0.001**
V. sa-F. es	-1.442	0.107	-13.48	**< 0.001**	*V. sa-F. es*	-1.267	0.107	-11.89	**< 0.001**
V. sa-C. cy	-0.606	0.124	-4.908	**< 0.001**	*V. sa-C. cy*	-0.348	0.123	-2.83	**0.005**
F. es-C.cy	0.836	0.090	9.267	**< 0.001**	*F. es-C.cy*	0.919	0.094	9.826	**< 0.001**

Cotesia rubecula fecundity assay

The mean parasitism per day did not differ significantly between the treatments for water 6.7±4.4 eggs, *F. esculentum* 6.3±1.1 eggs, *C. cyanus* 7.4±2.6 eggs and *V. sativa* 5.4±3.5 eggs (Generalized linear model, all $p > 0.05$) (Figure 3-2 a, Table 3-2). Influenced by the survival of the female wasps (Generalized linear model, z = 6.035, $p < 0.0001$), significant higher lifetime parasitism was found in *C. cyanus* (26.5±22.8 eggs, mean female longevity 4.5±0.6 days) and *F. esculentum* treatments (116.3±40.7 eggs, mean female longevity 19.4±1.3 days) compared to the water control (6.7±4.4 eggs, mean female longevity 2±0 days) (Generalized linear model, both $p < 0.05$). But they did not significantly differ from each other (Generalized linear model, z = 0.169, $p = 0.866$). Also, no significant difference to the water control was found in the *V. sativa* treatment (10.1±9.1 eggs, mean female longevity 2.8±0.2 days) (Generalized linear model, z = 1.266, $p = 0.205$) (Figure 3-2 b, Table 3-2). The highest realized lifetime fecundity was observed in the *F. esculentum* treatment with a total of 171 eggs of a 25-day-old female wasp, which had 24 occasions to parasitize.

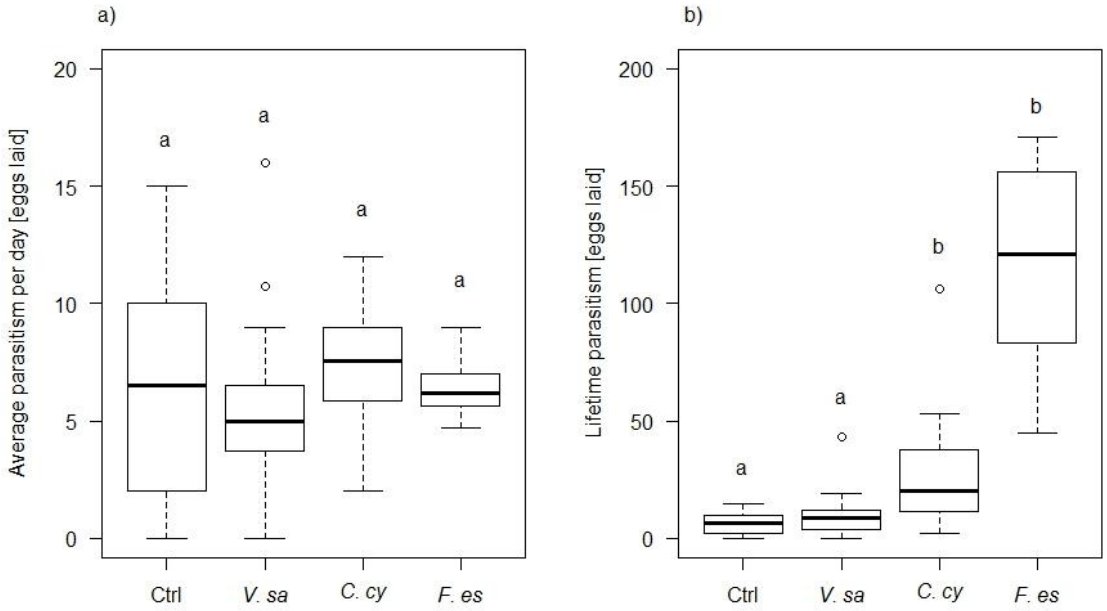

Figure 3-2: a) *Cotesia rubecula* average parasitism per day and b) realized lifetime parasitism when provided with either water (Ctrl), *V. sativa* (EFN only) (*V. sa*), *C. cyanus* (*C. cy*) or *F. esculentum* (*F. es*). Different letters indicate significant differences (Generalized linear model, $p < 0.05$).

Table 3-2: Table of the GLM results of *C. rubecula* average parasitism per day and realized lifetime parasitism when provided with either water (Ctrl), *V. sativa* (EFN only) (*V. sa*), *C. cyanus* (*C. cy*) or *F. esculentum* (*F. es*).

Average parasitism per day					**Lifetime parasitism**				
GLM Family: quasipoisson					GLM Family: poisson				
Fixed effects	**Estimate**	**S. E.**	**t value**	**P value**	**Fixed effects**	**Estimate**	**S. E.**	**z value**	**P value**
Ctrl-*C. cy*	0.091	0.153	0.593	0.555	Ctrl-*C. cy*	0.965	0.189	5.112	**< 0.001**
Ctrl-*F. es*	-0.057	0.159	-0.36	0.720	Ctrl-*F. es*	1.017	0.358	2.836	**0.005**
Ctrl-*V. sa*	-0.212	0.165	-1.287	0.202	Ctrl-*V. sa*	0.243	0.192	1.266	0.206
			z value		Age (f)	0.108	0.018	6.035	**< 0.001**
V. sa-F. es	-0.155	0.167	-0.929	0.353	*V. sa-F. es*	-0.774	0.341	-2.268	**0.023**
V. sa-C. cy	-0.303	0.162	-1.873	0.061	*V. sa-C. cy*	-0.722	0.179	-4.022	**< 0.001**
F. es-C.cy	-0.148	0.155	-0.952	0.341	*F. es-C.cy*	0.052	0.308	0.169	0.866
					Correlation of Fixed Effects				
						C. cy	*V. sa*	*F. es*	
					Age (f)	-0.258	-0.087	-0.869	

Pieris rapae longevity assay

The mean longevity of female and male butterflies in the water control was 3.9±0.3 and 3±0.2 days, respectively. For females and males the treatments *C. cyanus* (6.3±0.5 and 5.9±1 days, respectively increased by a factor of 1.6 and 2) and *F. esculentum* (7.2±0.9 and 8.1±1 days, respectively 1.9 and 2.7 fold increased) differ significantly from the water control and *V. sativa* (4±0.2 and 3±0.2 days, respectively) (Generalized linear model, all $p < 0.05$). They also differed within each other for males (Generalized linear model, $z = 3.27$, $p = 0.001$), but not within each other for females (Generalized linear model, $z = 1.035$, $p = 0.301$). There was also no significant difference between water and *V. sativa* (Generalized linear model, $p > 0.05$) for both, males and females (Figure 3-3, Table 3-3).

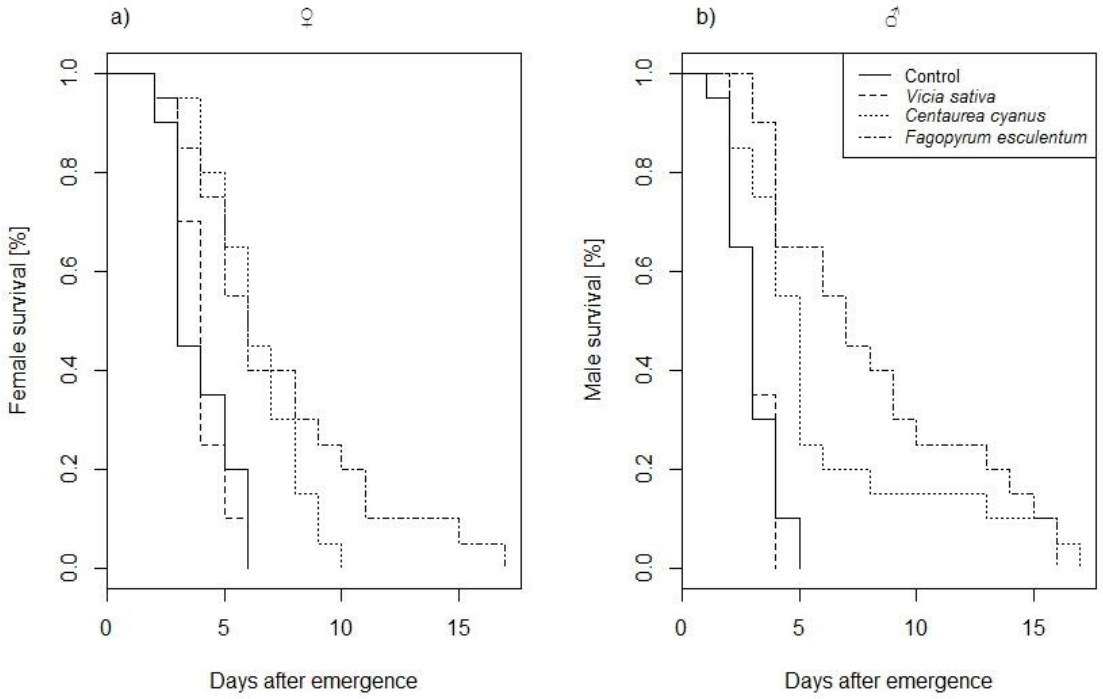

Figure 3-3: a) Survival curves of female and b) male *P. rapae* butterflies provided with either water (Control), *V. sativa* (EFN only), *C. cyanus* or *F. esculentum*.

Table 3-3: Table of the GLM results of *P. rapae* female and male survival when provided with either water (Ctrl), *V. sativa* (EFN only) (*V. sa*), *C. cyanus* (*C. cy*) or *F. esculentum* (*F. es*).

Female survival					**Male survival**				
GLM Family: poisson					GLM Family: poisson				
Fixed effects	**Estimate**	**S. E.**	**z value**	**P value**	**Fixed effects**	**Estimate**	**S. E.**	**z value**	**P value**
Ctrl-*C. cy*	0.461	0.149	3.093	**0.002**	Ctrl-*C. cy*	0.62	0.162	3.817	**< 0.001**
Ctrl-*F. es*	0.59	0.144	4.097	**< 0.001**	Ctrl-*F. es*	1.043	0.152	6.851	**< 0.001**
Ctrl-*V. sa*	-0.015	0.173	-0.084	0.933	Ctrl-*V. sa*	0.079	0.193	0.408	0.683
V. sa-F. es	-0.604	0.146	-4.13	**< 0.001**	*V. sa-F. es*	-0.964	0.165	-5.838	**< 0.001**
V. sa-C. cy	-0.475	0.147	-3.228	**0.001**	*V. sa-C. cy*	-0.541	0.170	-3.177	**0.002**
F. es-C.cy	0.129	0.124	1.035	0.301	*F. es-C.cy*	0.423	0.129	3.27	**0.001**

Pieris rapae fecundity assay

The mean number of laid eggs per day differed significantly between the water control 4±5 eggs, *F. esculentum* 15±10 eggs and *C. cyanus* 23±14 eggs (Generalized linear model, all $p < 0.05$). No significant difference was measured between the control and *V. sativa* 2±4 eggs (Generalized linear model, t = -0.769, $p = 0.445$) (Figure 3-4 a, Table 3-4). Weight of females significantly influenced the mean number of eggs laid (Generalized linear model, t = 2.473, $p = 0.017$), whereas heavier females laid on average more eggs per day than lighter females ($R^2 = 0.055$, $F_{1,58} = 4.428$, $p = 0.04$). The different female weights were equally distributed among the treatments (ANOVA, $F_{3,56} = 0.371$, $p = 0.774$) (Table 3-4).

Influenced by the survival of the butterflies (Generalized linear model, z = 3.417, $p < 0.0001$), a significant higher lifetime fecundity was found in *C. cyanus* (127±164 eggs, mean female longevity 9.2±1.2 days) and *F. esculentum* (194±153 eggs, mean female longevity 12.9±2.5 days), compared to the water control (19±25 eggs, mean female longevity 4.6±0.4 days) and *V. sativa* (11±17 eggs, mean female longevity 4.5±0.3 days) (Generalized linear model, all $p < 0.05$). No significant difference was found between the control and *V. sativa* and between *C. cyanus* and *F. esculentum* (Generalized linear model, all $p > 0.05$) (Figure 3-4 b, Table 3-4)

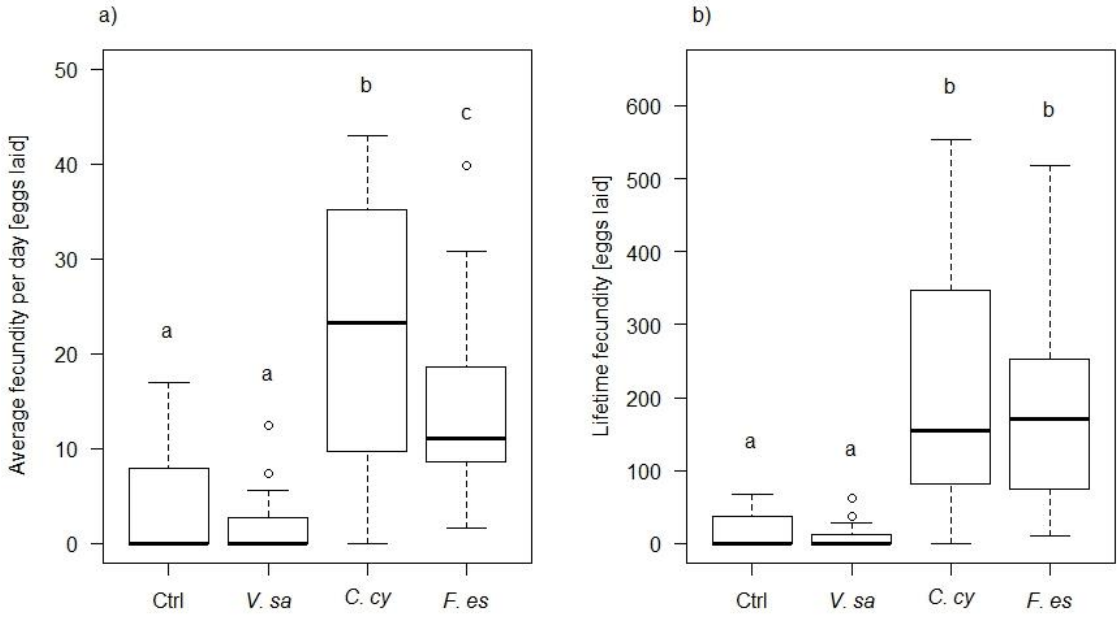

Figure 3-4: a) ***Pieris rapae*** **average eggs laid per day and b) realized lifetime fecundity when provided with either water (Ctrl),** ***V. sativa*** **(EFN only) (*V. sa*),** ***C. cyanus*** **(*C. cy*) or** ***F. esculentum*** **(*F. es*). Different letters indicate significant differences (Generalized linear model, $p < 0.05$).**

Table 3-4: Table of the GLM results of *P. rapae* average parasitism per day and realized lifetime parasitism when provided with either water (Ctrl), *V. sativa* (EFN only) (*V. sa*), *C. cyanus* (*C. cy*) or *F. esculentum* (*F. es*).

Average fecundity per day					**Lifetime fecundity**				
GLM Family: quasipoisson					GLM Family: poisson				
Fixed effects	**Estimate**	**S. E.**	**t value**	**P value**	**Fixed effects**	**Estimate**	**S. E.**	**z value**	**P value**
Ctrl-*C. cy*	1.772	0.356	4.982	**< 0.001**	Ctrl-*C. cy*	2.979	0.789	3.778	**< 0.001**
Ctrl-*F. es*	1.33	0.371	3.587	**< 0.001**	Ctrl-*F. es*	2.189	0.936	2.339	**0.019**
Ctrl-*V. sa*	-0.417	0.543	-0.769	0.445	Ctrl-*V. sa*	0.122	0.732	0.166	0.868
Weight (f)	0.012	0.005	2.473	**0.017**	Weight (f)	0.033	0.011	2.984	**0.003**
			z value		Eggdep. days	0.202	0.06	3.365	**< 0.001**
V. sa-F. es	-1.747	0.453	-3.777	**< 0.001**	*V. sa-F. es*	-2.067	0.903	-2.289	**0.022**
V. sa-C. cy	-2.189	0.451	-4.857	**< 0.001**	*V. sa-C. cy*	-2.857	0.76	-3.759	**< 0.001**
F. es-C.cy	-0.442	0.219	-20.18	**0.044**	*F. es-C.cy*	-0.79	0.67	-1.181	0.238

Correlation of female weight on the mean nr. of eggs laid per day (LM)

R^2	**$F_{1,58}$**	**P value**
0.055	4.428	**0.040**

Correlation of Fixed Effects

	Weight (f)	*C. cy*	*F. es*	*V. sa*
Weight (f)	-	0.056	0.058	0.151
Eggdep. days	0.062	-0.404	-0.612	-0.009

Distribution of female weight classes among the treatments (ANOVA)

$F_{3,56}$	**P value**
0.371	0.774

Discussion and Conclusions

The aim of this study was to find out if an already existing flower strip mixture recommended for conservation biological control of *M. brassicae* in cabbage cultivations can also be recommended for the conservation biological control of *P. rapae*, another pest of brassicas.

Cotesia rubecula longevity and fecundity

Although benefits of higher sugar concentrations for parasitoids was shown (Azzouz *et al.* 2004), *F. esculentum* with only floral nectar promoted longevity most in comparison to *C. cyanus* and *V. sativa*, which both have EFN that is expected to be higher concentrated. A possible explanation for this finding is that an upper limit of viscosity exists, when it comes to parasitoid food intake. This was demonstrated by Faria *et al.* (2008) in feeding experiments with diluted honeydew, where the nutritional value of pure honeydew was primarily restricted by its high viscosity.

The nutritional status of female insects has an impact on the egg production process (Cicero *et al.* 2012), which is why the provision of food sources is all the more important. Especially for *C. rubecula,* a synovigenic endoparasitoid of *P. rapae* that continues to mature eggs after adult emergence. These females can store up to 80 – 90 eggs in their ovaries (Nealis 1990) and within their first three days of life they mature about 100 eggs (Siekmann *et al.* 2004). This would require them to live for at least three days to potentially parasitize 100 hosts, a requirement which is enabled by *C. cyanus*, *V. sativa* and *F. esculentum*. Therefore, these flowers seem to be good candidates for conservation biological control purposes. However, under semifield conditions, female wasps were shown to parasitize up to ten larvae a day (Nealis 1990) and increased fecundity was rather a result of increased longevity than an effect of the nectar offered in this study. These findings relativize the potential of the flowers increasing fecundity in conservation biological control.

Also in both koinobiont endoparasitoids *Meteorus pulchricornis* Wesmael, 1835 (Hymenoptera: Braconidae) (Harvey *et al.* 2017) and *Microplitis mediator* (Haliday, 1834) (Hymenoptera: Braconidae) (Géneau *et al.* 2012), reproductive success did not vary significantly with diet. A koinobiont is a parasitoid whose larval growth is on or within a host that continues to develop after oviposition. The studies above indicate that koinobionts are not very sensitive to diet contents as long as sugar is involved and this might be a possible explanation why no difference was found in the average daily fecundity of *C. rubecula*. Sources derived from larval stages such as proteins and

lipids might play a greater role. Indeed, protein and lipid reserves acquired during the immature stage are used to produce eggs, while adult-acquired sugars are used for somatic maintenance and locomotion (Casas *et al.* 2005; Jervis *et al.* 2008).

Pieris rapae longevity and fecundity

Two out of the three tested flowers, *C. cyanus* and *F. esculentum*, increased both the fecundity and longevity of *P. rapae*. The higher lifetime fecundity in *F. esculentum* and *C. cyanus* seems to be a result of enhanced longevity. Whereas the higher average fecundity in these two plant treatments is likely a results of nectar composition. Even though *P. rapae* generally lived shorter with *C. cyanus* than *F. esculentum,* indicating that *F. esculentum* nectar is more nutritious, it reached a higher average fecundity in *C. cyanus* that cannot be fully explained. In fact, a maximum lifetime egg deposition of 554 eggs by a female *P. rapae* aged 14 days within the *C. cyanus* treatment, was recorded in this study. A similar amount of eggs was found in a 21-day-old individual who laid 586 eggs when fed with 10 % sucrose (Hopkins & Van Loon 2001). The enhanced egg production in *P. rapae* after feeding on *C. cyanus* could be due to the content of amino acids in its nectar. In particular the amino acid proline might play a role since it was shown to enhance fecundity of the egg parasitoid *Trissolcus grandis* (Thompson, 1861) (Hymenoptera: Scelionidae) (Hajirajabi *et al.* 2016). Indeed, high amounts of the amino acid proline were measured in *C. cyanus* (Gardener, personal communication in Barloggio *et al.* (2018)). Also the attraction to amino acids by female *P. rapae* but not by males suggests that amino acids in nectars might be relevant for egg production in this species (Alm *et al.* 1990).

Pieris rapae matures eggs throughout its adult life (Jones *et al.* 1982) and has a mean maximum egg load of around 50 mature eggs (Hopkins & Van Loon 2001). These eggs, however, are only fertile after mating (personal observation). In this study, oviposition commenced mostly after two to four days with a rapid increase and following tailing just as described in Hopkins and Van Loon (2001). This implies that flowers which do not support longevity of *P. rapae* or even shorten it so that

none or only few eggs can be laid, should be preferably used for conservation biological control purposes. In this study, however, *P. rapae* survived on average by at least two to four days in each treatment. Long enough to find a mating partner and lay eggs without additional uptake of nutrients as adults. To not further increase the chance of finding a mate, optimal flowers for conservation biological control would deny access to their nectar for various brassica pests.

Differentiated enhancement of the cabbage pest and its antagonist

The herbivore *P. rapae* is less prone to starvation than its parasitoid *C. rubecula*, as it survived longer in the water control treatments due to resources obtained during the larval stage. But depending on the flower species and sex of the insects, the impact of food supply on the average life span was up to 6.2 fold higher for the parasitoid compared to the herbivore. Winkler *et al.* (2005b) found similar patterns, in which the life span of the parasitoid *Diadegma semiclausum* (Hellen, 1949) (Hymenoptera: Ichneumonidae) was about 6 fold higher than for its herbivore host *Plutella xylostella* L. (Lepidoptera: Plutellidae).

Although the average daily fecundity of *P. rapae,* but not that of *C. rubecula*, was enhanced after feeding on *F. esculentum,* suggesting that this flower species increases *P. rapae* densities in the field, Lee and Heimpel (2005) found no increase in *P. rapae* abundance on cabbage crops bordered by *F. esculentum*, but higher parasitism rates near this flowering plant as compared to the control. This finding, which is in contrast to the increased fecundity effects of *F. esculentum* on *P. rapae* in this study, might be explained through the 6 fold higher increased life expectancy in the wasp compared to the butterfly. Nevertheless, it should be questioned, whether these laboratory results can reflect what is going on in the field.

Winkler *et al.* (2009b) stated that *P. rapae* is mainly active during sunny periods with corresponding low relative humidity, during which flowers have an increased nectar viscosity caused by evaporation. They have reported that longevity of female *P. rapae* increased from 9.6±2.2 days at 45±5 % r.h. to 16.5±0.6 days at 90±5 %

r.h. after feeding on *F. esculentum*. Due to its short and open corollas, nectar viscosity of *F. esculentum* is very prone to relative humidity. Flowering plants with exposed nectaries, which are expected to be more affected by evaporation, are more likely to effectively exclude *P. rapae* feeding on these plant species in the field. Also, parasitoids are mainly active during sunny periods of the day and are able to deal with highly concentrated sugar sources (Siekmann *et al.*, 2001, Wäckers 2000), rendering them less restricted to nectar intake at low relative humidity. This suggests that plants with exposed nectaries can be good candidates for conservation biological control purposes.

Further, it is advantageous if the selected flowers also promote other beneficial insects in the same habitat. Indeed, *F. esculentum* increased fitness in terms of longevity or fecundity of many parasitoids of diverse cabbage pests such as *Cotesia glomerata* (Linnaeus, 1758) (Hymenoptera: Braconidae) (host: *P. rapae* and *Pieris brassicae* (Linnaeus, 1758) (Lepidoptera: Pieridae)) (Lee & Heimpel 2007a; Winkler *et al.* 2009b), *M. mediator* (Géneau *et al.* 2012) and *Telenomus laeviceps* Förster, 1861 (Hymenoptera: Scelionidae) (Barloggio *et al.* 2018) (host: *M. brassicae*), *D. semiclausum* (Wratten *et al.* 2003; Winkler *et al.* 2009b) and *Diadegma fenestrale* (Holmgren, 1860) (Hymenoptera: Ichneumonidae) (host: *P. xylostella*) (Géneau *et al.* 2012), *Aphidius ervi* Haliday, 1834 (Hymenoptera: Braconidae) (Wade & Wratten 2007) and *Diaeretiella rapae* (M'Intosh, 1855) (Hymenoptera: Ichneumonidae) (Araj & Wratten 2015) (hosts: aphids) and *Trichogramma exiguum* Pinto & Platner, 1978 (Hymenoptera: Trichogrammatidae) (host: *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae)) (Witting-Bissinger *et al.* 2008).

Also *C. cyanus* increased fitness of the cabbage pest antagonists such as *M. mediator*, *D. fenestrale* (Géneau *et al.* 2012), *T. laeviceps* (Barloggio *et al.* 2018) and *C. glomerata* (Winkler *et al.* 2009b). But unlike Winkler *et al.* (2009b), *P. rapae* longevity was significantly increased after feeding on *C. cyanus*. In addition, the average daily fecundity was enhanced for *P. rapae* too but not for *C. rubecula*. Therefore it seems as if *C. cyanus* is not a good candidate for the control of *P. rapae* through conservation

biological control when tested in no choice trials even though the increase in survival was 3 fold higher for female *C. rubecula* than female *P. rapae*.

However, simulations done by Bianchi and Wäckers (2008) show that the attractiveness of flowers is an important feature that should be taken into account when selecting flowering plants. Their study implies that adapting nectar supply to the needs of parasitoids has the potential to increase their effectiveness as biological control agents. Direct visual and sweep net sampling on eight plant species (among them *C. cyanus* and *F. esculentum*) were used to identify flowers that are selectively visited by parasitoids and not by their hosts and apparently neither *C. cyanus* nor *F. esculentum* were visited by *P. rapae* (Winkler *et al.* 2005a). This suggests that *P. rapae* is not or only to a small extent attracted to these flowers.

V. sativa (EFN) supported *M. mediator* fecundity (Géneau *et al.* 2012) but not *T. laeviceps* (Barloggio *et al.* 2018) nor *C. rubecula* in this study. As *Vicia sepium* L. floral nectar is expected to be accessible to *P. rapae* (Winkler *et al.* 2009b), same may be true for *V. sativa*. This however could not be confirmed due to missing flowers. Just as for *M. brassicae (Géneau et al. 2012)*, we found no increased longevity or fecundity in *P. rapae*. It seems to be likely that *P. rapae* was not able to feed on the EFN sources provided, due to the lack of detectability and or high EFN viscosity. With floral nectar, however, an increase might be possible as, *Vicia americana* scored 6th highest visitation index through *Colias alexandra* W.H. Edwards, 1863 (Lepidoptera: Pieridae) from 22 flowering plants observed (Watt *et al.* 1974). But because *V. sativa* only produces a few single flower heads, which are open for a very short time (roughly 24 hours, personal observation in growbanks), the risk that butterflies benefit more from *V. sativa* than their respective parasitoids from the EFN is lowered.

We anyway need to be cautious on drawing conclusions about wild insects with results obtained from lab insects. In laboratory strains, traits tended to change in the direction of increased fitness for Hymenoptera, but changes in Lepidoptera were often in the opposite direction (Hoffmann & Ross 2018). This means that the differences in fitness enhancement might not be so far apart as detected in this and possibly many other studies.

None of the examined flowers are fully selective for the pest-antagonist complex *P. rapae* and *C. rubecula* except for *V. sativa* without flowers but the enhancement of the parasitoids was very low. Whether these plants can be used in a conservation biological control program remains yet unclear. At this point we cannot recommend any of these plants as selective plant species against *P. rapae*. As a future perspective, flowers could be offered in mixtures rather than singly in laboratory experiments. This would be an approximation to the situation in the field. In addition, results could be very different if the test species is given the possibility to choose which food source it wants to consume. However, field tests will reveal whether our results are confirmed or not, as several influencing factors such as timing, weather, distribution in the field, attractiveness, competition etc. play a role in the successful exploitation of nectar in the field by the insects.

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CHAPTER 4

Pest density dependent success of conservation biological control measures: The incentive to parasitize

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Abstract

In conservation biological control, the pest control potential of beneficial insects can be increased by implementing flowers that provide food and shelter for them. However, to reach desired pest control effects, flowers need to be carefully selected to promote the beneficial insect of interest, but not the pest. A flower strip consisting of *Centaurea cyanus* L. (Asteraceae), *Vicia sativa* L. (Fabaceae) and *Fagopyrum esculentum* Moench (Polygonaceae) had been developed to selectively enhance *Microplitis mediator* (Haliday, 1834) (Hymenoptera: Braconidae), a parasitoid of the cabbage moth *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae). In this study, field trials were conducted over two consecutive years to evaluate the potential of this flower strip and *C. cyanus* as companion plant, to control *Pieris rapae* (Linnaeus, 1758) (Lepidoptera: Pieridae), another cabbage pest, by enhancing its main larval parasitoid *Cotesia rubecula* (Marshall 1885) (Hymenoptera: Braconidae). We found a positive correlation of parasitism rates by *C. rubecula* and wild *P. rapae* pest densities in control fields and a negative correlation in flower supplemented fields. The selected flowers appear to be suitable for conservation biological control purposes only when pest densities are low, as *C. rubecula* seemingly ignores hosts for nectar when pest densities are high.

Keywords: Tailored flower strip; Insect herbivores; Parasitoids; Companion plants; *Cotesia rubecula*; *Pieris rapae*

Introduction

In many parts of the world, larvae of the cabbage white butterflies, *Pieris rapae* L. (Lepidoptera: Pieridae) and *P. brassicae* L. (Lepidoptera: Pieridae), are considered major pests in several economically important brassica crops such as various cultivars of cabbage (Harvey *et al.* 2010). In particular, the widespread *P. rapae* is a greater problem than *P. brassicae* in Switzerland. Due to high fecundity of pierids and a low market tolerance of pest damaged cabbage plants, the use of pesticides is essential to increase marketable crop yields (Firake *et al.* 2017).

In organic production, the use of synthetic pesticides is prohibited. However, allowed insecticides containing spinosad and Bt are commonly used in organic cabbage cultivation against lepidopteran pests. While Bt products kill insects through ingestion and act in alkaline gut conditions, which is mainly found in lepidopteran insects (Kwa *et al.* 1998; Firake *et al.* 2017), spinosad products additionally act through contact and are effective against a broader range of insects. The mechanism of action involves the disruption of nicotinic acetylcholine receptors and g-aminobutyric acid- gated ion channels of insect nervous systems (Kirst 2010). Unfortunately, beneficial organisms such as parasitoids are also negatively affected by spinosad products (Mason *et al.* 2002; Schneider *et al.* 2003; Schneider *et al.* 2004; Xu *et al.* 2004; Biondi *et al.* 2012; Liu & Zhang 2012; Firake *et al.* 2017) and frequently repeated use may lead to resistance development in pest species (Zhao *et al.* 2002). Although it has been classified as a reduced-risk product due to its low environmental persistence and very low toxicity to most vertebrates (Thompson *et al.* 2000), spinosad products should only be used as a last resort and not as a common measure against pest insects.

Conservation biological control is an approach aiming to reduce pesticide use and increase biodiversity (Begg *et al*. 2017). It is defined as the "Modification of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests" (Eilenberg *et al*. 2001). As a branch of conservation biological control, habitat manipulation increases the diversity and

complexity of vegetation in cultivated landscapes to provide food and other resources for the natural enemies of arthropods (Landis *et al.* 2000; Frank *et al.* 2008). This can be in form of flower strips and companion plants.

When a parasitoids lifetime is short and resources are scarce, the decision to search for food or hosts may have a great impact on its fitness (Siekmann *et al.* 2004). In early summer host availability is low, which is why parasitoids are under more time pressure to find suitable hosts (Phillips & Kean 2017). This can be helped by planting a flower strip early in the season that attracts parasitoids from the environment though floral scent and color (Leius 1960; Wäckers 1994), enabling them to find alternative hosts, shelter and food in form of nectar and pollen (Griffiths *et al.* 2008; Manandhar & Wright 2015; Westphal *et al.* 2015; Gurr *et al.* 2017), which support survival and fecundity (Vollhardt *et al.* 2010).

The scarcity of parasitoid food sources in large monocultures can also be fought by interplanting flowering herbaceous plants within the cropping system (Syme 1975; Altieri & Letourneau 1982; Andow 1991; Balmer *et al.* 2014). And since various studies have shown that parasitism rates are lower with increasing distance to flower strips (Pfiffner *et al.* 2003; Tylianakis *et al.* 2004; Lavandero *et al.* 2005; Begg *et al.* 2017) and fed parasitoids are less likely to enter the patch where hosts are (Lee & Heimpel 2007b), combining a flower strip with companion plants could offer a solution to draw parasitoids from the flower strip into the field and retain them there, enabling them to spend more energy and time on host instead of food search (Takasu & Lewis 1995).

Although neither the conservation of natural enemies nor their success in delivering biological control can be guaranteed (Begg *et al.* 2017), several studies have reported higher parasitism rates of insect pest in fields with flowering strips (Pfiffner & Wyss 2004; Lavandero *et al.* 2005; Ponti *et al.* 2007; Haaland *et al.* 2011) and accordingly high effectiveness of reducing pests and crop damage (Balmer *et al.* 2014; Tschumi *et al.* 2015).

Flower strips may improve parasitism of certain lepidopteran cabbage pests in adjacent fields by increasing diversity and abundance of parasitic wasps (Pfiffner *et al.* 2003). Pfiffner *et al.* (2009) found that the ability of flower strips to positively enhance parasitism rates of *P. rapae* larvae, was site dependent and Maguire (1984) discovered that cabbage fields became more attractive to *P. rapae* when tomato plants were grown around the crop, as a result of flowering tomato plants attracting the butterflies through provision of nectar. These butterflies then oviposited on many of the neighboring cabbage plants. To reach desired pest control effects, flowers need to be carefully selected to promote the beneficial insect of interest, but not the pest (Winkler et al. 2010).

In Switzerland, a tailored flower strip seed mixture for brassica crops is commercially available and contains the flowers buckwheat (*Fagopyrum esculentum* Moench; Polygonaceae) cornflower (*Centaurea cyanus* L.; Asteraceae) and common vetch (*Vicia sativa* L.; Fabaceae) as main nectar providers and corn poppy (*Papaver rhoeas* L.; Papaveraceae) as a pollen provider. Lab and field studies have revealed that these plants attract and enhance natural occurring antagonists of brassica pests such as *Diadegma fenestrale* (Holmgren, 1860) (Hymenoptera: Ichneumonidae), *D. semiclausum* Hellen (Hymenoptera: Ichneumonidae) and *Microplitis mediator* (Haliday 1834) (Hymenoptera: Braconidae), important antagonists of *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae) and *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae) (Géneau *et al.* 2012; Balmer *et al.* 2013; Belz *et al.* 2013; Balmer *et al.* 2014). Further, *F. esculentum* strips increased parasitism rates of *P. rapae* larvae (Lee & Heimpel 2005) and *P. rapae* adults were not observed or captured in substantial numbers on *C. cyanus* nor *F. esculentum* in flowering field margins (Winkler *et al.* 2005a).

In this study, knowledge to this tailored flower strip is added by investigating its potential in the control of *P. rapae* through promotion of its main larval parasitoid *Cotesia rubecula* (Marshall 1885) (Hymenoptera: Braconidae). Moreover we combined this strip with intercropped *C. cyanus* plants since they display easily accessible extra-floral nectar in addition to their floral nectar, have an extended flowering

period, are olfactorily attractive to *C. rubecula* (Fataar *et al.* 2019) and do not negatively affect cabbage growth (Balmer *et al.* 2014).

Specifically, we conducted trials in organic cabbage fields over two consecutive years in Switzerland. Treatments included fields supplemented with flower strip and companion plants, with flower strip only and without flowers. To a) check whether the supplemented flowers established successfully, we monitored their development in the field. The number of flowering buds from the intercropped plants was compared to mean parasitism rates per field to assess the importance of the successful establishment of companion plants. To b) evaluate the effect of the treatments on *P. rapae* parasitism rates by *C. rubecula*, lab reared *P. rapae* larvae were exposed in the trial fields and recollect to subsequently analyze parasitism rates with qPCR. Further, we c) assessed whether insecticide sprays against *P. rapae* larvae could be spared, by recording the amount of wild *P. rapae* larvae and determining the yield and weight loss due to herbivory of cabbage plants at harvest in insecticide-free and treated plots.

Material and Methods

Floral provision

To test the influence of the tailored flower strip mixture (Table 4-1) and *C. cyanus* companion plants on *C. rubecula* parasitism rates, trials were conducted in 2016 and 2017 in organic cabbage fields located in the two regions of Oberaargau and Seeland in Western Switzerland.

Table 4-1: Composition of the tailored flower strip seed mixture.

Flower species	***Amount (kg/ha)***
Main nectar providers	
Buckwheat (*Fagopyrum esculentum*)	11
Common vetch (*Vicia sativa*)	44.8
Cornflower (*Centaurea cyanus*)	4.1
Accompanying species	
Corn poppy (*Papaver rhoeas*)	0.1

Treatments included fields supplemented with flower strip and companion plants, with flower strip only and without flowers in 2016. Whereas in 2017 we dropped the treatment with flower strip only, due to a shortage of organic cabbage fields (from eleven to seven). Treatment associated, a 3 m wide flower strip (UFA Samen, Switzerland) consisting of the flower species *C. cyanus*, *F. esculentum*, *V. sativa* and *P. rhoeas*, was sown along field margins around mid of April and approximately four-week-old *C. cyanus* plants were intercropped in a density of one cornflower per square meter, two weeks after cabbage had been planted. In each field a 16 x 21 m insecticide free area was defined, in which depending on the treatment, cornflowers were intercropped (Figure 4-1). This area was defined 46 m away from the field margin. A 6 x 6 m data collection plot was defined within this area, 10 m away from another, depending on the treatment, field margin or flower strip.

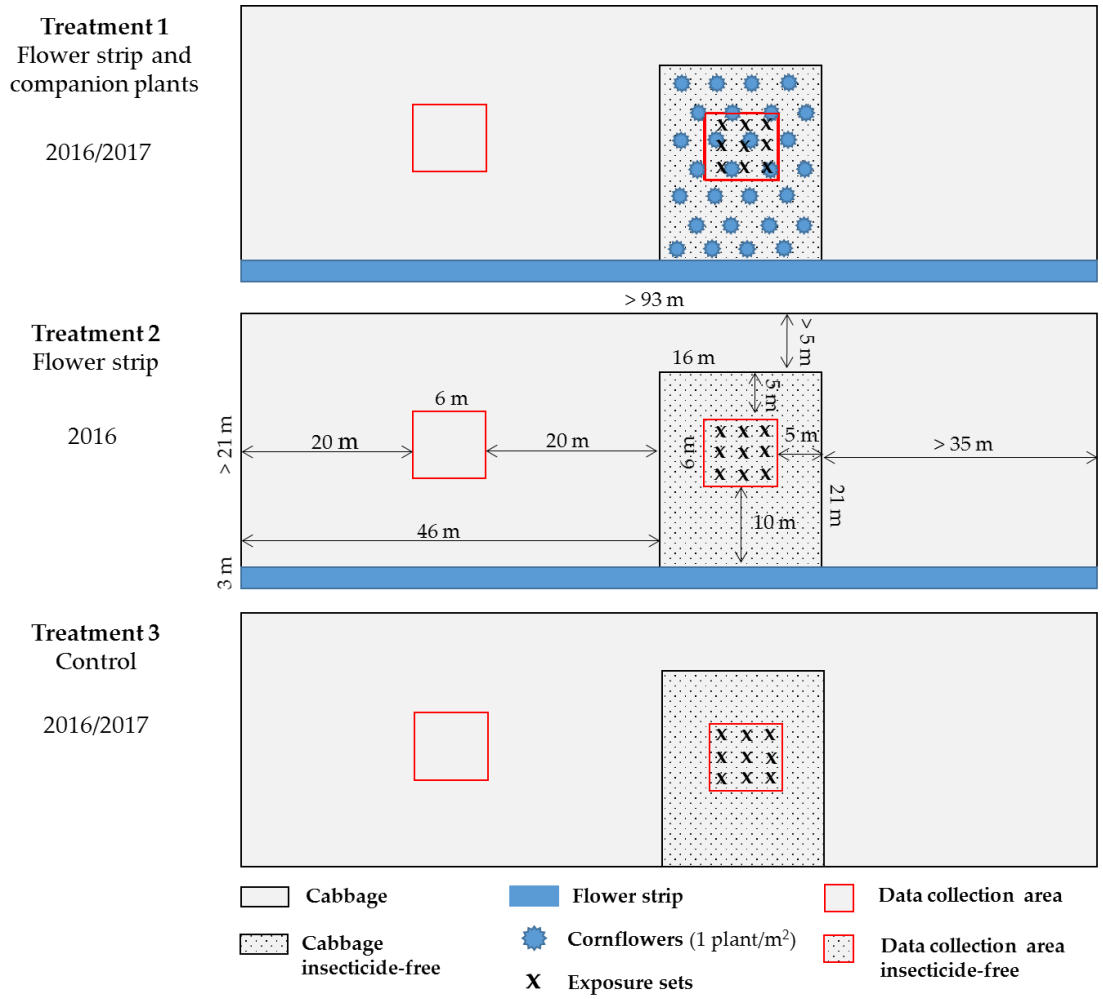

Figure 4-1: Field trial experimental design.

Botanical recordings

To monitor how well the field supplemented flower species *C. cyanus*, *F. esculentum* and *V. sativa* developed, percentage soil coverage was recorded in week 26 in 2016 and week 27 and 33 in 2017. Also the percentage soil coverage of spontaneous flora in the control strips at the field margin within cabbage fields, was recorded. The registration of the plant species in the field and in the flower strip was done by the expert Heiner Lenzin, who followed a modified abundance dominance scale according to Braun-Blanquet (1964). To assess the availability of the intercropped nectar plants, the survival rate of cornflowers was recorded and the number of flowering buds counted for both years in July week 30.

Measurement of P. rapae pest densities and damage threshold

Pieris rapae has two generations (third generation possible) in Switzerland, beginning in May and July and a flight peak in July (Balmelli *et al.* 2012). According to the Swiss farmers (personal communication), the cabbage cupping stage is the most susceptible one, since cabbage pests can cause great damage in the closing head and are simultaneously protected from non-systemic insecticides. To determine whether our conservation biological control measures were able to keep wild *P. rapae* populations under the damage threshold of one larvae per plant in the pre-heading and cupping to early heading stage (Liu *et al.* 2005), wild *P. rapae* individuals were monitored twice on 24 defined cabbage plants per field, 12 in the insecticide-free area and 12 in the insecticide-treated area (Figure 4-1). This was done in the pre-heading stage in June 2016 calendar week 29 and in the early heading stage in August 2016 calendar week 33 and according to the analogous cabbage growth stages 2017 in June calendar week 26 and July calendar week 29 in 2017.

Measurement of parasitism rates

To assess the importance of the quantity of available food sources on parasitism rates, we compared the number of recorded flowering buds (see page 97) and the respective mean parasitism in each field for both years. Further, the effect of the treatments "control", "flower strip" and "flower strip and companion plants" on parasitism rates, was investigated in each year. Because *C. rubecula* population densities depend strongly on the available densities of their hosts, we compared the number of hosts per field (see page 98) with the mean parasitism for each field.

Plant and host rearing

Brassica oleracea convar. *capitata* var. *alba* were grown from seeds in grow banks at 24±2 °C, 55±15 % r.h. and 16L: 8D for *P. rapae* rearing maintenance and as exposure plants for field trials.

Pieris rapae larvae were reared on cabbage plants in a climate chamber at 23±2 °C, 55±5 % r.h. and 16L:8D. Adults were fed with 10 % diluted honey.

Exposure set

To ensure a sufficient amount of larvae for analysis, an exposure set was developed on which we exposed and recollected laboratory reared *P. rapae* larvae within data collection plots (Figure 4-1). In a plant bowl (Naxos Ø 30 cm/ height 11 cm terracotta, Coop Bau & Hobby) a layer of fleece (grey bars) was placed and covered with soil to retain humidity as long as possible (Figure 4-2 a, b). A potted 4-week-old cabbage plant (Ø 12 cm/ height 10 cm pot) was placed inside this plant bowl and a water filled saucer kept the plant watered. Beforehand, plants were acclimatized to outdoor conditions under a fine insect net for two days. To prevent ground dwelling predators from reaching host larvae on the exposure plants, insect glue strips were applied to the outer and inner upper edge of the plant bowl (yellow suns) (Figure 4-2 a). Host larvae were put in plastic tubes in groups of six and stored overnight at 4 °C, before bringing them out into the field. Cooling them overnight was essential to prevent cannibalistic behaviors between the larvae in the absence of food. The next day these tubes were opened and placed under exposure plants in a way that larvae could crawl out directly onto these (Figure 4-2 c). To monitor *C. rubecula* parasitism rates, 54 *P. rapae* larvae (if available) were distributed among nine exposure sets (six larvae per set) in each data collection plot and were carefully recollected with a paintbrush a week later. This was done from June until September in 2016 and until August in 2017 for a total of 9 weeks (2 weeks earlier in 2017, due to plant growth stage). Recollected larvae were kept in 2 ml plastic tubes at -20 °C until we assessed whether they were parasitized, with the molecular method qPCR.

Figure 4-2: a) Schematic cross-section illustration of the exposure set. b) Exposure set in a cabbage field. c) Open plastic tube containing six *P. rapae* larvae (L1-L2) leaned on an exposure plant.

DNA extraction

To prepare the crude DNA extract, larvae were placed in 2 ml screw cap vials (Sarstedt Cat. 72.609.001) together with 3 ceramic beads (SiLibeads Typ ZY, 3.3 mm, Sigmund Linder GmbH, Warmensteinach, Germany) and 500 µl of extraction buffer consisting of 10 mM Tris-HCl pH 8.0, 1 mM Na_2EDTA, 0.5% (w/v) Tween 20 and 50 µg/ml Proteinase K (Kawasaki 1990; Dilworth & Frey 2000). A TissueLyser II (Qiagen) homogenized larvae at a frequency of 30/s for 10 minutes. Subsequently screw cap vials were incubated in a heating block at 95°C for 10 min and stored until further processing at -20°C. Defrosted vials were centrifuged at 20'000 rcf for 1 min and the clear supernatant was 10x diluted with TE (10 mM Tris, 1 mM Na_2EDTA, pH 8.0) fo qPCR analysis.

qPCR setup and conditions

The reaction volume of 10 µl consisted of 1 µl 10x diluted DNA extract and of 9 µl Master Mix (5 µl KAPA PROBE FAST qPCR Kit Master Mix 2x Universal (Sigma-Aldrich)), 2 µl ultrapure water, 2 µl of pre-diluted primer and probes at a final concentration of respectively 0.4 µM and 0.1 µM for *C. rubecula* and of 0.1 µM for both *P. rapae* primer and probe (Table 4-2).

Table 4-2: Primer pairs (F/R) for TaqMan qPCR to detect *C. rubecula* DNA and confirm *P. rapae* host DNA in a duplex qPCR reaction. FAM and ROX = specific fluorescent dyes, BHQ = "black hole quencher". Probe bases typed in capitals and marked with "+" are LNA ("locked nucleic acids") modified to increase specificity.

Species	Primer	Sequence (5′-3′)	Product length
Pieris rapae	*P. rapae*_F	GCCTTCCCACGAATAAATAATATAAG	144 bp
	*P. rapae*_R	ACCTCTGTGAGCAATATTAGAAG	
	*P. rapae*_P	ROX-cctgt+Tcc+T+G+Ctccatt-BHQ2	
Cotesia rubecula	*C. rubecula*_F	TGGTACTGGTTGAACTGTATATC	107 bp
	*C. rubecula*_R	TAGAAGATGCTCCAGCTAAATG	
	*C. rubecula*_P	FAM-cagat+Attc+C+A+Ccatgacct-BHQ1	

To increase specificity of the fluorogenic hydrolysis probes, species specific bases were modified with locked nucleic acids (LNA, see Table 4-2) and synthesized by Eurogentec (Belgium). The primer and their corresponding fluorogenic hydrolysis probes were developed and selected with Beacon Designer based on mitochondrial cytochrome oxidase I (COI) sequences retrieved from NCBI: KJ165179.1 for *Pieris rapae*, and DQ411830 for *Cotesia rubecula*. Specificity of the primers and probes was assessed with extracts of lab reared *P. rapae* and *C. rubecula* (FiBL Switzerland) and absence of cross-reactivity with extracts of lab reared *M. brassicae* and field collected *C. glomerata* (Linnaeus, 1758) (Hymenoptera: Braconidae) and *C. vestalis* (Haliday, 1834) (Hymenoptera: Braconidae) (FiBL Switzerland). For the positive and negative control 1 µl of reference solution and ultrapure water, respectively, were added to the Master Mix instead of the diluted DNA extract. Following cycling conditions were set for the TaqMan qPCR amplification in a Rotorgene Q (Qiagen), employing the 72 well rotor: 3 min at 95°C for the activation of hot start DNA polymerase, followed by 45 cycles of 3 s at 95°C and 20 s at 60°C. After each cycle the fluorescence was recorded for both colors FAM (green) and ROX (orange).

Measurement of crop yield

A direct comparison of the cabbage head weights between the fields was not possible, since the cabbage head mass depends strongly on the cabbage variety, the location characteristics such as soil and climatic factors (Shelton *et al.* 1982) and the cultivation measures, which were different among the fields. However, to find possible differences between insecticide-treated and insecticide-free cabbage heads within treatments, 24 cabbage heads were harvested per field, 12 within the insecticide-free data collection plot and 12 within an insecticide-treated plot (Figure 4-1). Three weight measurements were taken and the weight loss due to herbivory was calculated (Table 4-3). Cabbage plants were cut off directly below the base of the first stem leaves and then weighed as the initial weight (measurement 1). Then the stem with its leaves were removed and the remaining cabbage head weighed as the head weight (measurement 2). All damaged wrapper leaves were removed to produce a marketable head. The cabbage was then weighed a third time and the weight recorded as the final weight (measurement 3).We subtracted the final weight from the head weight and called it weight loss due to herbivory (measurement 4).

Table 4-3: Harvest measurements and parameters.

Measurements		Measured parameters
1	Cabbage head, incl. stalk + stalk leaves	Initial weight (Kg)
2	Cabbage head, excl. stalk + stalk leaves	Head weight (Kg)
3	Marketable cabbage head	Final weight (Kg)
4	Head weight minus Final weight	Weight loss (Kg)

Statistical analysis

Pest densities

We assessed the differences in pest numbers between insecticide-free and -treated plots within treatments for each year with one-way Anova and Paired t-tests with the

p-value adjustment bonferroni. One control field in 2017 was not included in the analysis as wild *P. rapae* larvae were only recorded once due to early harvest.

Parasitism rates

All data analyses were conducted with R Statistical Software version 3.3.1 (R Core Team 2016). *Pieris rapae* parasitism rates were analysed through a generalized linear model (glmer function from the package lme4) with binomial errors because of the binomial distribution of the response variable "parasitized" or "non-parasitized" larva. The fixed factor "treatment" had three levels in 2016: flower strip plus companion plants, flower strip and control and two levels in 2017: flower strip plus companion plants and control. Weather data "temperature", "precipitation", and "sunshine duration" served as co-factors, and "field" and "week" as random factors. The models were corrected for overdispersion. In the model building process, a stepwise model reduction was employed, with the least significant interaction always being removed first (Crawley 2007). To deal with the varying amount of recollected host larvae, we used the function cbind to produce the parasitism rates, e.g. cbind(parasitized larvae, recollected larvae – parasitized larvae) to include in the function. Meteorological data such as temperature, precipitation and sunshine duration were received from Meteoswiss stations located nearest to our study sites for both years to include in our statistical analysis, as these could have an effect on *C. rubecula* parasitism performance.

To assess how the wild population of *P. rapae* influenced *C. rubecula* parasitism rates we performed a Pearson correlation analysis. The correlation was performed with the mean parasitism rates recorded per field and with the sum of wild *P. rapae* larvae recorded per field, for each treatment, including both years. Mean parasitism per week is the percentage of parasitized larvae from the number of recollected larvae. The mean parasitism per field is the mean of the mean parasitism per week, recorded over 9 weeks. The sum of wild *P. rapae* larvae per field is the sum of larvae recorded on two dates in both insecticide-free and –treated data collection plots per field (see page 98). One control field in 2017 was not included in the

correlation analysis as wild *P. rapae* larvae were only recorded once due to early harvest.

Crop yield

A generalized linear model (glm function from the package lme4) with "plot" as explanatory variable (levels: insecticide-free and insecticide-treated), and a poisson data distribution was used to find differences within the three weight measurements (Initial weight, final weight and weight loss) in insecticide-free and –treated plots for each treatment and year. For overdispersed data, a quasipoisson data distribution was used.

Results

Floral provision

Flower strip

In 2016, the four sown plant species developed very differently depending on the location (Table 4-4). Two locations, with very low cover of *V. sativa* and high proportions of *F. esculentum* stood out. At the remaining sites, *V. sativa* and *C. cyanus* were dominant with similar high coverage ratios. Spontaneous occurring species dominated the strip compared to the sown species.

At the record day in July 2017, *C. cyanus* had the largest cover assets compared to the other sown species, *F. esculentum* and *V. sativa* (Table 4-4). Although not intended, the seed mixtures used included the species *Vicia villosa* (Fabaceae), which explains the low number of *V. sativa*. The picture then changed considerably within one month. *C. cyanus* was often only found dead and dry in August. In contrast, *V. villosa*'s cover values in most places had increased, growing over the other living and dead plants. However, this was not observed everywhere. The spontaneous flora in the cabbage fields increased in August but was not conspicuously different to July.

Table 4-4: Mean soil coverage rates of the sown and spontaneously occurring plant species.

			In strip/field margin soil coverage [%] per treatment (mean±SE)			
Year	**Calendar Week**	**Treatment**	***Centaurea cyanus***	***Fesculentum esculentum***	***Vicia sativa***	**Spontaneous species**
2016	**26**	**Flower strip + cp**	48±14	5±5	18±0	99±28
		Flower strip	20±7	3±2	23±6	115±26
		control	0	0	0	26±14
2017	**27**	**Flower strip + cp**	58±10	1±1	4±2	46±6
		control	0	0	0	10± 6
	33	**Flower strip + cp**	16± 11	0.4±0.1	13±13	69±25
		control	0	0	0	19±12

Companion plants

The survival rates of intercropped cornflowers in 2016 were in the range of 70 % and 80 % for three fields and 40 % in a field which has been extensively weeded. The overall survival rate of cornflowers planted between cabbage plants was accordingly 65±17 % for the four fields and the mean amount of flowering buds per field was 2319±795 in week 30.

In 2017, the survival rates of cornflowers planted in the field were in the range of 50 % for two fields and 10% in a field which has been extensively weeded. Accordingly the overall survival rate of cornflowers planted between cabbage plants was 36±18 % for the three fields and the mean amount of flowering buds per field in week 30 was 519±437.

Measurement of P. rapae pest densities and damage threshold

At none of the recorded dates in both years was the damage threshold of one larva per plant reached for *P. rapae* larvae only, with densities ranging from 0 to 0.75 larvae per plant.

In 2016, cabbage fields were on average treated 0.9±1.5 times with spinosad and 1.7±1.3 times with Bt products, whereas in 2017 these products were used 1±0.7 and 2.4±1.5 times, respectively (personal communication with the farmers).

Pest densities did not differ significantly in the insecticide-free and -treated plots within treatments in each year (Generalized linear model, all $p > 0.05$) (Table 4-5).

Table 4-5: Comparisons of the number of wild *P. rapae* larvae found on two collection dates in insecticide-free and –treated plots within each treatment and year. For the control treatment in 2017 we performed an Anova due to normal distribution. All other treatments were compared with pairwise comparisons using t test with pooled SD and P value adjustment method: bonferroni.

		Control				Flower strip			Flower strip + c. p.		
		mean	**S.E**	**P value**		**mean**	**S.E**	**P value**	**mean**	**S.E**	**P value**
2016	**Insecticide-free**	1.67	0.49	0.18		1.5	0.76	0.92	1	0.5	1
	Insecticide-treated	0.67	0.49			1.63	0.98		1	0.63	
					$F_{1,10}$						
2017	**Insecticide-free**	3.17	0.87	0.83	0.05				2.5	1.38	0.56
	Insecticide-treated	2.83	1.25						1.5	0.92	

Measurement of parasitism rates

Recollection rates

In 2016, a total of 3211 larvae were exposed of which 1631 could be recollected, corresponding to a total recollection rate of 51 %. The recollection rates in the different treatments was 52 % for both "control" and "flower strip" and 49 % for "flower strip and companion plants". Whereas in 2017, 3090 *P. rapae* larvae were exposed and 1497 recollected. The recollection rate in the flower treatments was 43 % and in the control treatments 53 %, leading to a total recollection rate of 48 %.

Parasitism rates

The newly developed duplex qPCR detected both *P. rapae* and *C. rubecula* with an amplification efficiency of 0.92 and 0.94, respectively. No cross-reactivity was shown

with *M. brassicae*, *C. glomerata and C. vestalis*. *Pieris rapae* and *C. rubecula* were readily detected with 91 % of Cq values between 15 and 22 and with 88 % of Cq values between 24 and 31, respectively. A total of 3128 collected larvae was confirmed as *P. rapae* and a total of 1037 larvae produced the second signal specific for *C. rubecula*, i.e. parasitized host.

There was no correlation between the amount of available food sources (flowering buds) and the mean parasitism rates per field (Table 4-6).

Table 4-6: Table of the number of flowering buds recorded in week 30 in both years 2016 and 2017 and the respective measured mean parasitism rates.

Year	**2016**				**2017**		
Mean parasitism [%]	40.3	58.4	47.6	37.4	18.1	40.9	22.5
Flowering buds [Nr.]	1470	1581	3068	3155	82	358	1116

Mean parasitism rates per treatment in 2016 were 32.7±8, 33.4±6.3 and 46.5±6.4 for the three treatments "control", "flower strip" and "flower strip plus companion plants", respectively. Whereas in 2017 we recorded of mean parasitism rate of 41±5.9 in the control treatment and 27.2±5.2 in the flower treatment with companion plants (Table 4-7). In both years, a marginal significant difference was found between the control treatment and the flower strip treatment plus companion plants (2016: generalized linear model, $z = 1.832$, $p = 0.067$, 2017: generalized linear model, $z = -1.729$, $p = 0.084$). All other comparisons were not significant (generalized linear model, $p > 0.05$) (Table 4-7).

Table 4-7: Mean field parasitism rates and standard error per treatment and year. And the GLM results of the parasitism comparisons between the treatments control (Ctrl.), flower strip (FS) and flower strip + companion plants (FS + CP).

		Parasitism rates (Mean±S.E.)			
	Year	**Ctrl.**	**FS**	**FS + CP**	
	2016	32.7±8.0	33.4±6.3	46.5±6.4	
	2017	41±5.9		27.2±5.2	
Year	**Factors**	**Estimate**	**S. E.**	**z value**	**P value**
2016	**Ctrl. - FS**	0.381	0.820	0.465	0.642
	Ctrl. - FS + CP	1.481	0.809	1.832	0.067
	FS - FS + CP	-1.100	0.735	-1.495	0.135
	precipitation	0.068	0.054	1.252	0.210
2017	**Ctrl. - FS + CP**	-0.873	0.505	-1.729	0.084
	precipitation	1.338	2.403	0.557	0.578
AIC 2016: 438.1, df = 81, AIC 2017: 362.3, df = 52					

We found an effect of supplementing flowers in dependency of available hosts per field. As no significant differences between the numbers of wild larvae collected from insecticide-free and -treated plots were found, we accumulated these numbers from both collection dates per field. When the recorded number of wild *P. rapae* larvae was low in control fields without supplemented flowers, mean parasitism rates measured over 9 weeks per field and year, were low too. Whereas in fields with higher numbers of *P. rapae* larvae, higher parasitism rates were recorded (t = 3.75, df = 4, p = 0.02, 95% conf.int = [0.25, 0.99], R = 0.88) (Figure 4-3 a). Mean parasitism rates in control fields ranged from 18.5 % to 51 %, whereas mean parasitism rates in flower strip supplemented fields ranged from 23.4 % to 44.2 % and in fields with flower strip and companion plants 18.1 % to 58.4 %. In fields with flower strips, mean parasitism rates are seemingly independent of the number of wild *P. rapae* larvae, although a slight negative correlation could be observed (t = -1.13, df = 2, p = 0.38, 95% conf.int = [-0.99,0.84], R = -0.62) (Figure 4-3 b). Supplementing fields with flower strips and companion plants lead to higher

parasitism rates, when wild *P. rapae* numbers were low, and to lower parasitism rates, when these were high (t = -3.47, df = 5, p = 0.018, 95% conf.int = [-0.98,-0.24], R = -0.84) (Figure 4-3 c).

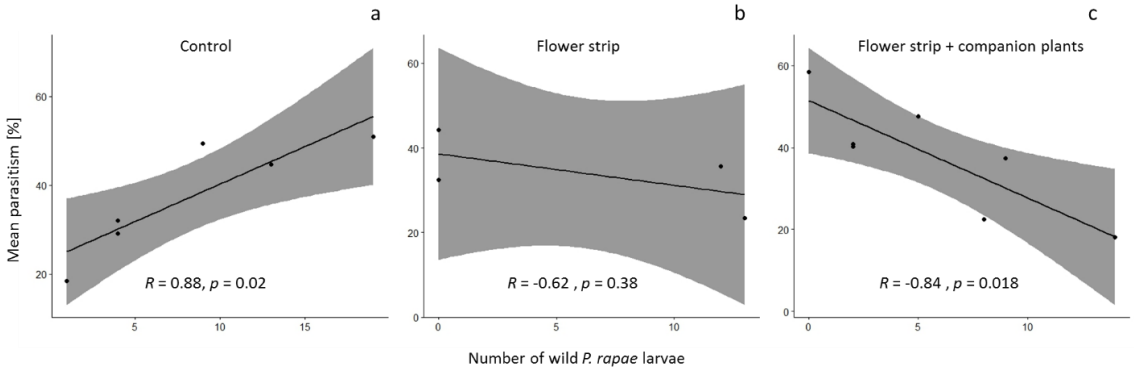

Figure 4-3: Pearson correlations of the mean parasitism rates per field and the number of wild *P. rapae* larvae per field, for the treatments control, flower strip and flower strip + companion plants. R = sample estimate. p = significance level of the t-test. The grey areas designate the confidence interval of the correlation coefficient at 95 %.

Measurement of crop yield

The initial weights (measurement 1), final weights (measurement 3) and weight loss (measurement 4) between the insecticide-free and -treated plots within the same treatment were not significantly different in both years (generalized linear, all $p > 0.05$) (Table 4-8).

Table 4-8: Table of GLM results of the comparisons of the initial and final weight of cabbage heads and the weight loss, between insecticide-free and –treated plots, within each treatment in 2016 and 2017. The z value refers to poisson and the t value to quasipoisson (quasip.).

		Control			Flower strip			Flower strip + companion plants		
Year		Initial weight	Final weight	Weight loss	Initial weight	Final weight	Weight loss	Initial weight	Final weight	Weight loss
2016	GLM family	quasipoisson		poisson	quasip		poisson		poisson	
	Fixed effects	insecticide-free - insecticide-treated								
	Estimate	0.083	0.067	0.268	0.244	0.035	-0.018	-0.027	-0.083	-0.010
	S. E.	0.218	0.234	0.683	0.238	0.162	0.488	0.116	0.141	0.441
	z or t value	0.379	0.288	0.392	1.025	0.218	-0.037	-0.234	-0.587	-0.023
	P value	0.707	0.775	0.695	0.308	0.828	0.971	0.815	0.557	0.981
2017	GLM family	quasip.		poisson				quasipoisson		poisson
	Fixed effects	insecticide-free - insecticide-treated								
	Estimate	-0.099	-0.112	-0.244				0.031	0.023	0.421
	S. E.	0.098	0.122	0.325				0.178	0.195	0.521
	z or t value	-1.012	-0.913	-0.749				0.174	0.120	0.809
	P value	0.314	0.361	0.454				0.862	0.905	0.419

Discussion

Floral provision

Flower strips

Flower strips that promote naturally occurring beneficial insects and thus lead to natural pest control, can be annual or perennial sown strips. As a welcomed side

effect, other beneficial insects such as hover flies or bees (pollinators) and ground beetles, rove beetles and spiders (predators) are promoted (Luka *et al.* 2015).

The average degree of sown species coverage in 2016 is to be classified as too low. Only four out of eight sites, with high cover percentages of sown plants, were successful. In this respect, the remaining sites are to be assessed as insufficient, due to varying causes.

In 2017, the sowing of *V. villosa* had a great impact on the appearance of the flower strips and their development until mid-August. The observed different occurrence of this flower species probably depended on the amount of seeds introduced.

The cover of spontaneously occurring species in the control strips (field margin within the field) increased from early July to mid-August as a result of not maintaining them with the same caution as the interior of the fields.

The occurrence of the spontaneous flora in flower strips, where no herbicides are permitted, depends not only on the species sown and their sowing density, but also on the existing seed bank. While within the fields (control strips), the occurrence of spontaneous flora is mainly influenced by weeding and cultivation measures. In addition, regional differences such as mesoclimate, always have an influence on the development of plant species.

In general, the biodiversity of flower strips is welcome from a nature conservation point of view, but from an agronomic point of view, weed growth in the flower strips was too excessive in both years.

Companion plants

Once intercropped cornflowers established themselves, they persisted in the field till the end of the cabbage season, producing a lot of flowering buds. We consider a survival rate of roughly 70% as sufficient to provide enough nutritional resources for beneficial insects throughout the cabbage season, which was the case in 2016 only. Once cornflowers fully bloom, they keep on producing new buds and the amount of flowering buds in the field remains relatively stable, at least until mid of September.

The survival rate is not only dependent on biotic, but also on abiotic factors such as mechanic weeding. Which is why we found satisfying to rather low survival rates 2017 in all three fields. The difference between these two years cannot be due to the fact that the season started two weeks earlier in 2017, as experience has shown us, that once cornflowers were established, the amount of flowering buds remained relatively stable until mid of September.

Measurement of P. rapae pest densities and damage threshold

There is no clear consensus on damage thresholds for lepidopteran larvae in cabbage cultivations. In Switzerland, Balmelli *et al.* (2012) suggest 10-30 small larvae or 1-4 large larvae on ten cabbage plants (five at the field margin and five in the field). Whereas dynamic intervention thresholds based on crop growth stages were suggested by Liu *et al.* (2005) and Andaloro *et al.* (1983). Last published a detailed description not only including cabbage growth stages but also differentiated between small and large larvae and took into account whether cabbage is processed into sauerkraut or is grown as storage and fresh market cabbage. We stuck to the simplified dynamic threshold suggested by Liu *et al.* (2005), which is more strict than the one suggested by Andaloro *et al.* (1983) and did not find any *P. rapae* densities that reached that threshold in either study year. Unlike Pfiffner *et al.* (2009), who found mean *P. rapae* caterpillar densities (±S.E.M.) of 1.4±0.09 and 3.2±0.15 per plant, at two different sites in Switzerland, emphasizing that population densities are dynamic and may vary from year to year.

We did not find any significant differences in pest densities among insecticide-free and -treated plots, very likely due to low pest population densities at the time of record. Although brassica crops suffered higher infestation levels by *P. rapae* when bordered with plants suitable for this herbivore (Zhao *et al.* 1992; Winkler *et al.* 2010), we did not find any negative influence of the intercropped cornflowers, suggesting that they are indeed suitable for conservation biological control measures against *P. rapae*.

Measurement of parasitism rates

Accordingly to our expectations, we found that parasitism rates increased with the addition of flower sources in 2016. However, contradictory was true for 2017. This suggested, that another factor which was not included in the analysis influenced parasitism rates. Indeed we found a dependency on the available host population. In fields without supplemented flowers and few recorded wild larvae, parasitism rates were lower compared to fields in which more wild larvae were found. This is likely due to the fact that the population size of parasitoids is dependent on the number of available hosts, e.g. more hosts equals more parasitoids. And although a high density of hosts produces a high concentration of volatiles attractive to wasps (Geervliet *et al.* 1998), we did not find higher parasitism rates due to aggregation in fields with low pest numbers. We therefore conclude that higher numbers of parasitoids were present in the control fields, in which we found greater parasitism rates.

Because only well-fed *C. rubecula* wasps exhibit a preference for hosts (Siekmann *et al.* 2004), we expected to find higher parasitism rates in flower supplemented fields. Clearly the feeding state of the wasps alone, does not explain their incentive to parasitize. If flowers are supplemented to a field in which pest densities are low, the few parasitoids present will be drawn to attractive intercropped flowers placed near the hosts (aggregation). Once they realize that hosts are nearby too, their incentive to parasitize is high as they do not have many chances to lay their eggs when only few hosts are available. The recorded parasitism rates in this study were accordingly high. However, if many host larvae are available, the motivation to parasitize seems to be lower in the presences of flowers. Having a meal appears to be preferred over laying eggs, e.g. ignoring hosts for nectar. Consequently we recorded lower parasitism rates.

This raises the question whether parasitism is *per se* lower in fields with companion plants and high host densities, or whether this finding was a consequence of the experimental design. *Centaurea cyanus* was shown to be olfactorily attractive to *C. rubecula* (Fataar *et al.* 2019). Therefore it is likely that, parasitoids would fly into the

C. cyanus intercropped plot to have a meal and leave that plot again once satiated, when the incentive to parasitize is low due to the many occasions they have to parasitize. When pest densities are generally low, parasitoids are likely attracted by a volatile mixture of flowering *C. cyanus* and herbivore damaged cabbage plants. The incentive to parasitize is accordingly high. Further clarifications are clearly needed.

In a study conducted by Zhao *et al.* (1992), relative parasitism rates of *P. rapae* by *C. rubecula* were 39.9 %, 22.2 % and 26.2 % in broccoli interplanted with nectar-producing plants, nearby nectar-producing plants and broccoli monocultures, respectively. In their study they worked with plots in which the general pest density level assumingly was same for all. Besides higher parasitism rates in flower intercropped broccoli, they also found higher pest numbers, which is in contrast to our study. Clearly the choice of companion plants plays a critical role and the observed higher parasitism rates in their study are likely a consequence of an aggregation reaction of the pest and its antagonist caused by the flowers.

As discussed by Siekmann *et al.* (2004), *C. rubecula* indeed seems to follow qualitative and not quantitative cues as the varying amounts of flowers in the fields of this study, did not influence parasitism rates. This means that it would not be a problem if flower strips and companion plants do not grow as desired, as seemingly a lower number of nectar providing plants is enough to provide a sufficient amount of food sources.

Various studies reported parasitism rates of *P. rapae* by *C. rubecula* from 47±3 % up to 75 % (Van Driesche 2008; Pfiffner *et al.* 2009; Herlihy & van Driesche 2013). In our study we found average parasitism rates in the same range but also clearly below. A possible explanation apart from (Zhao *et al.* 1992) for the seemingly only study with low rates, is that our analyzed larvae are lab animals that were exposed for a week only. Whereas in other studies, either wild larvae were collected or lab animals were exposed for a longer period, giving the parasitoids more time to parasitize these hosts. Another bias of results could be due to the fact that parasitized larvae are easier to find compared to unparasitized larvae, as they become less active after parasitism.

In order to determine whether parasitism rates actually make a significant contribution to pest control, an experimental setup as used in Herlihy and van Driesche (2013) in which they tested the effects of natural enemy exclusion on survival of cohorts of *P. rapae* larvae, is required for detailed information. Another less informative option is to investigate the effective damage and influence on crop weights.

Measurement of crop yield

Several cultivars of common cabbage can endure some defoliation without reduction of head weight at harvest and are even able to over-compensate for defoliation at the pre-heading stage. At the cupping stage, however, plants are more sensitive to defoliation (Liu *et al.* 2005).

Furlong *et al.* (2008) observed that Bt is safe for natural enemies of *P. rapae* and also found a synergistic effect of Bt plus natural enemies on the yield of brassica crops. When comparing cabbage head weights and the weight loss due to herbivory in the insecticide-free and -treated plots within treatments in this study, no significant differences were found, which suggests that one or the other insecticide application could have been spared. It also suggests, that adding tailored flowers into the field does not lead to higher damage due to herbivory and accordingly also not to higher yield loss.

Conclusions

To our knowledge, we are the first to demonstrate that the influence of flowers on *P. rapae* parasitism rates through *C. rubecula* is dependent on *P. rapae* population densities in the field. Our results also reveal that it is very important to study the behavior of insects not only in the lab but also in the field. The varying success of conservation biological control studies may be due to the fact that pest densities have been overlooked or not included in the analysis.

Further, the flowers used in this study did not increase *P. rapae* pest population densities nor decrease cabbage yields. Also the implemented flower strips and companion plants do not need to flourish perfectly as *C. rubecula* appears to follow qualitative and not quantitative cues when hosts and food are available. If *P. rapae* was the only present pest species in cabbage cultivations, applied insecticide sprays could be reduced. But also, because conservation biological control measures in cabbage crops tolerate a specific amount of pest pressure, especially when cabbage plants are processed into sauerkraut (Andaloro *et al.* 1983).

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GENERAL DISCUSSION

The aim of this PhD thesis was to assess with lab and field trials, whether a commercially available flower strip mixture for cabbage cultivations that contains the species *Vicia sativa*, *Fagopyrum esculentum* and *Centaurea cyanus,* is suitable to control the cabbage pest *Pieris rapae* by enhancing its main larval parasitoid *Cotesia rubecula*.

The main findings and achievements of this thesis are:

- Establishment of a stable and reliable rearing of *Pieris rapae* and *Cotesia rubecula*, allowing a constant production of individuals for trials.
- Establishment of experimental designs to receive reliable results in longevity and fecundity trials with *Pieris rapae* and *Cotesia rubecula*.
- *Centaurea cyanus* and *Vicia sativa* are suitable to olfactorily attract naive *Cotesia rubecula* in the lab.
- *Fagopyrum esculentum* olfactorily attracts *Cotesia rubecula* after feeding experience in the lab.
- *Centaurea cyanus*, *Vicia sativa* and *Fagopyrum esculentum* increase longevity and fecundity of *Cotesia rubecula* in the lab.
- *Centaurea cyanus* and *Fagopyrum esculentum* increase longevity and fecundity of *Pieris rapae* in the lab.
- Development of a suitable exposure set to expose and recollect *Pieris rapae* host larvae in the field.
- Development of qPCR markers for the detection of parasitism by *Cotesia rubecula* in *Pieris rapae*.
- The flower strip mixture and intercropped *Centaurea cyanus* do not enhance *Pieris rapae* pest densities in the field.
- The flower strip mixture and intercropped *Centaurea cyanus* increase parasitism rates when pest densities are low in the field.
- *Cotesia rubecula* ignores hosts for nectar when *Pieris rapae* host densities are high in the field.

How well laboratory results reflect the actual situation in the field

Before testing flowers in the field without knowing how they may affect beneficial and pest insects, it is advisable to conduct a thorough literature research and laboratory trials. However, often published studies are difficult to reproduce (Pusztai *et al.* 2013; Baker 2016). This was confirmed in the survival trials in CHAPTER 3 in which we found a significant positive influence on the survival of *Pieris rapae* through *Centaurea cyanus*, unlike Winkler *et al.* (2009b).

Further, conducting laboratory trials with lab reared insects can also lead to false conclusions about wild insects. Hoffmann and Ross (2018) have shown that with time, properties tended to change towards increased fitness for lab reared hymenopteran insects and the changes in lab reared lepidopteran insects were often in the opposite direction. Indeed we have noticed that lab reared *Pieris rapae* could not fly as fast as wild individuals, which is why once in a while rearing of insects should be refreshed with wild individuals.

Laboratory tests cannot always be transferred to the actual situation in the field and sometimes other tests are needed to explain field results. In CHAPTER 2 we have shown that the odor of *Fagopyrum esculentum* flowers is not innately attractive to *Cotesia rubecula* suggesting that innate odor responses alone would markedly restrict the exploitation of *Fagopyrum esculentum* in the field. However, a flower strip consisting of *Fagopyrum esculentum* was shown to increase parasitism of *Pieris rapae* by *Cotesia rubecula* (Lee & Heimpel 2005). Further investigations of the attractiveness and usage of *Fagopyrum esculentum* by *Cotesia rubecula* in the field are clearly needed.

When not chosen wisely, flower species can increase pest densities in brassica fields (Maguire 1984; Zhao *et al.* 1992). In CHAPTER 3 we have shown that *Centaurea cyanus* increases longevity and fecundity of *Pieris rapae* in the lab which suggests that higher levels of *Pieris rapae* should be found in field plots intercropped with *Centaurea cyanus* (CHAPTER 4). This, however, was not the case. Clearly the attractiveness of flowers in an important feature that influences the exploitation of

flowers in the field (Bianchi & Wäckers 2008). Indeed, Winkler *et al.* (2005a) did not observe any *Pieris rapae* visiting *Centaurea cyanus* in their field study. Also Winkler *et al.* (2009a) have shown that laboratory studies establishing nectar exploitation under controlled conditions cannot always be extrapolated to the actual exploitation under field conditions.

Undoubtedly it is indispensable to conduct field trials. Nevertheless, lab trials can sometimes give good indices and explain unexpected field results. In CHAPTER 2 the odor of *Centaurea cyanus* flowers was shown to be innately attractive to *Cotesia rubecula*, suggesting an aggregation reaction of these wasps in field plots intercropped with *Centaurea cyanus* and consequently higher parasitism rates. Indeed in CHAPTER 4, we were able to demonstrate that higher parasitism rates were found in fields with intercropped *Centaurea cyanus*. This finding however applies only to fields with low host densities. In fields with higher host densities contrary was true. This finding however could be explained with the laboratory experiments conducted by Siekmann *et al.* (2004) who foresaw that the number of available hosts in the field would somehow influence parasitism rates. They have shown that only well fed *Cotesia rubecula* prefer hosts over nectar and that hungry wasps are attracted to food and host stimuli in equal proportions. Their lab results suggest that *Cotesia rubecula* ignores hosts for food in fields with high host densities.

Important aspects of the behavior of *Cotesia rubecula*

The solitary endoparasitoid *Cotesia rubecula* lays its eggs singly into first second and third instar larvae of *Pieris rapae* (Harvey *et al.* 1999). To find these hosts, on which their own existence depends, they follow visual information from herbivore damage (Wäckers 1992) and volatiles from frass and regurgitate of the host (Agelopoulos & Keller 1994a). The blend of volatiles emitted by frass is herbivore-species specific (Agelopoulos & Keller 1994b). Once they find hosts they can discriminate whether the host is parasitized or not by using host-induced plant volatiles (Fatouros *et al.*

2005). Further they were shown to forage more efficiently in diculture than monoculture with experience having no effect (Perfecto & Vet 2003).

Adult *Cotesia rubecula* are not only dependent on hosts for reproduction but also on sugar sources for their own survival. In fact, they have to locate food sources at least once a day to avoid starvation (Siekmann *et al.* 2001). Feeding on nectar increases their fitness in terms of longevity and fecundity (CHAPTER 3). To find food sources they follow visual cues such as the color yellow (Wäckers 1994) and olfactory cues from flowering plants (Fataar *et al.* 2019).

Cotesia rubecula can form long term memory (Smid *et al.* 2007) and are able to associate an innately unattractive flower odor with rewarding nectar, after feeding experience (Fataar *et al.* 2019).

As energy and time are limited, *Cotesia rubecula* wasps need to choose whether they go searching for food or hosts. Especially in monocultures (host habitat) where food sources are not abundant, their decision has a strong impact on fitness.

Hungry parasitoids landed more often and spent more time searching on yellow targets (food cue), while sugar-fed individuals displayed a higher overall foraging activity (Wäckers 1994). Given a choice between flower odors and odors from host-infested leaves, food-deprived individuals chose flower odors, while sugar-fed individuals preferred host associated odors (Wäckers 1994). Therefore, and because only well-fed wasps exhibited a preference for hosts (Siekmann *et al.* 2004), we expected that intercropping monocultures with flowers (e.g. bringing hosts and food together) would lead to higher parasitism rates.

However, in reality the incentive to parasitize seems not to only depend on the hunger state of *Cotesia rubecula,* but also on the number of available hosts in a field. *Cotesia rubecula* appears to ignore hosts for nectar when host numbers are high (CHAPTER 4). When a limited number of hosts are present, parasitoids should be more apt to attack hosts to secure their existence, especially as supernumerary *Cotesia rubecula* larvae compete to the extent that only a single parasitoid can survive (Salt 1961). Older larvae have an advantage over younger larvae, which means that adults

probably race to be the first to parasitize hosts when they are scarce. Accordingly higher parasitism rates were found in the presence of flowers and low host availability (CHAPTER 4).

Conservation biological control in cabbage cultivations

The success or failure of conservation biological control measures can have many causes. Besides a huge labor investment that comes with field trials, results are often influenced by various factors such as field site (Pfiffner *et al*. 2009), insecticide application (Furlong *et al*. 2008), interaction of plant and insect communities at larger regional scales (Tscharntke & Brandl 2004), complexity of landscapes (Bianchi *et al.* 2006; Kleijn *et al*. 2011; Tscharntke *et al*. 2012; Midega *et al*. 2015; Banks & Gagic 2016), the year (Lee & Heimpel 2005), the spatio-temporal synchrony or asynchrony of pests and natural enemies (Bianchi *et al.* 2009; Schellhorn *et al.* 2014; Neuville *et al.* 2016; Begg *et al*. 2017), the frequent non-linear character of population dynamics (Andow & Risch 1985; Poveda *et al*. 2008), competition between species using the same sources (Caballero-López *et al*. 2012) and the increasing abundance of predators (Rosenheim *et al*. 1995; Xue *et al*. 2012; Messelink *et al*. 2013) and hyperparasitoids (Araj *et al.* 2009) due to flowers. In CHAPTER 4 we found that parasitism rates of *Pieris rapae* by *Cotesia rubecula* depend strongly on the number of available hosts in the field. But even if the abundance of natural enemies is increased, it does not necessarily translate into stronger pest suppression or reduced crop damage (Balzan *et al.* 2016). Accordingly we have not found any influence positive or negative of the conservation biological control measures on *Pieris rapae* pest numbers (CHAPTER 4). This raises the question to whether conservation biological control measures are even supportable in cabbage crops.

Depending on the growth stage of the plant, several pest species (insects and diseases) that attack cabbage may or may not cause economic injury (Andaloro *et al.* 1983). Andaloro *et al.* (1983) reported that an economic loss in cabbage marketability can be reflected in either weight or grade. Whereas for fresh market cabbage the

grade could be reduced if even slight damage occurs on the wrapper leaves or head. When the wrapper leaves are not yet present in the developmental stage of the cabbage, the risk of damage leading to a loss in head weight (yield) is greater. Conservation biological control measures in cabbage crops tolerate a certain amount of pest pressure. It comes in handy that the grade of processed cabbage (sauerkraut) is rarely affected by defoliating insect pests because regardless of herbivory, all green leaves must be removed from the head, before being processed into sauerkraut (Shelton & Andaloro 1982). This suggests, that these conservation biological control measures can indeed be suitable when a certain grade of damage is allowed.

Conclusive remarks on the potential of the flower strip mixture for cabbage cultivation

Flowers for conservation biological control should preferably be attractive and offer accessible food sources that lead to an aggregation of beneficial insects and enhance their fitness, ultimately leading to higher parasitism rates of pests and accordingly reduced crop damage. The flower species *Centaurea cyanus*, *Fagopyrum esculentum* and *Vicia sativa* of the flower strip mixture for cabbage cultivations, have been shown to positively influence a variety of cabbage pest antagonists in laboratory and field trials.

More specifically, *Centaurea cyanus* increased longevity and/or fecundity of *Cotesia rubecula* (CHAPTER 3), *Cotesia glomerata* (Winkler *et al.* 2009b), *Microplitis mediator* (Géneau *et al.* 2012), *Diadegma fenestrale* (Géneau *et al.* 2012) and *Telenomus laeviceps* (Barloggio *et al.* 2018) in the lab and is also olfactorily attractive to *Cotesia rubecula* (Fataar *et al.* 2019), *Microplitis mediator* (Belz *et al.* 2013; Géneau *et al.* 2013) and *Telenomus laeviceps* (Barloggio *et al.* 2018). In field trials, it was shown to increase parasitism rates of *Mamestra brassicae* by *Microplitis mediator* (Balmer *et al.* 2013; Balmer *et al.* 2014), but not of *Plutella xylostella* by *Diadegma semiclausum* nor *Pieris rapae* by *Cotesia rubecula* (Balmer *et al.* 2013). However, increased parasitism rates for *Pieris rapae* by *Cotesia rubecula,* mediated by *Centaurea cyanus,* were found in fields

with low host availability (CHAPTER 4). The abundance of *Mamestra brassicae* and *Plutella xylostella* larvae was not significantly affected by the presence of *Centaurea cyanus* in the field (Balmer *et al.* 2013; Balmer *et al.* 2014) and neither longevity nor fecundity were increased of *Mamestra brassicae* by *Centaurea cyanus* in the lab (Géneau *et al.* 2012). Contradictory to Winkler *et al.* (2009b), survival and fecundity were enhanced in *Pieris rapae* (CHAPTER 3), but no nectar exploitation in the field was observed (Winkler *et al.* 2005a), nor was the abundance increased ((Balmer *et al.* 2013); CHAPTER 4).

Longevity and/or fecundity is increased by *Fagopyrum esculentum* in beneficials relevant in brassica crops such as *Cotesia glomerata* (Lee & Heimpel 2007a; Winkler *et al.* 2009b), *Microplitis mediator* (Géneau *et al.* 2012), *Diadegma fenestrale* (Géneau *et al.* 2012), *Telenomus laeviceps* (Barloggio *et al.* 2018), *Aphidius ervi* (Wade & Wratten 2007), *Diaeretiella rapae* (Araj & Wratten 2015), *Trichogramma exiguum* (Witting-Bissinger *et al.* 2008), *Diadegma semiclausum* (Wratten *et al.* 2003; Winkler *et al.* 2009b) and *Cotesia rubecula* (CHAPTER 3) in laboratory trials. No increase in fitness was found for *Mamestra brassicae* (Géneau *et al.* 2012) and no nectar exploitation was observed in the field by *Pieris rapae* (Winkler *et al.* 2005a). *Fagopyrum esculentum* is olfactorily attractive to *Microplitis mediator* (Belz *et al.* 2013) and *Telenomus laeviceps* (Barloggio *et al.* 2018) and after feeding experience to *Cotesia rubecula* too (Fataar *et al.* 2019). Lee and Heimpel (2005) did not find higher densities of *Plutella xylostella* or *Pieris rapae* in Brassica production plots bordered by *Fagopyrum esculentum* compared to control plots and accordingly no elevated sugar levels were found in this species collected from *Fagopyrum esculentum* bordered fields, but also not for its parasitoid *Diadegma semiclausum* (Winkler *et al.* 2009a).

Vicia sativa extrafloral nectar enhances fitness in term of longevity and/or fecundity of *Microplitis mediator*, *Diadegma fenestrale* (Géneau *et al.* 2012), *Telenomus laeviceps* (Barloggio *et al.* 2018) and *Cotesia rubecula* (CHAPTER 3). Whereas no fitness increase was recorded for *Mamestra brassicae* (Géneau *et al.* 2012) and *Pieris rapae* (CHAPTER 3).

These studies indicate that the interaction between nectar-producing plants, pests and parasitoids are complex and may be different for each species. Finding a strip mixture, that enhances as much beneficial insects as possible without increasing the fitness of pests is extremely difficult. Nevertheless, this strip mixture has been shown to enhance antagonists of the relevant pest species *Mamestra brassicae*, *Plutella xylostella* and *Pieris rapae* without increasing their pest densities in the field, suggesting that it is indeed suitable for conservation biological control purposes in cabbage cultivations.

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