Telenomus laeviceps Förster, 1861 (Hymenoptera: Scelionidae), a potential biocontrol agent against the cabbage pest *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae)

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel
von

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Basel, 2018

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

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Basel, den 27. Februar 2018

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ACKNOWLEDGEMENTS

Firstly, I would like to express my gratitude to my thesis advisor Dr. Henryk Luka for giving me the opportunity to work at FiBL and to conduct this interesting project. I am also thankful to the faculty representative Prof. em. Dr. Peter Nagel for the time dedicated to evaluate this thesis. Besides my advisors, I would like to thank Dr. Lucius Tamm for trusting me and giving me the chance to coordinate a big project like Biocomes.

I am grateful to the co-referee Prof. Dr. Ansgar Kahmen for taking the time to read and evaluate this thesis and to Prof. Dr. Christine Alewell for taking the chair of the doctoral examination in a very professional way.

Thank you to the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 612713 for the financial support. My sincere thanks also go to all the people involved in the Biocomes project, which are too many to be mentioned individually, but your contribution was very well appreciated. A special thanks also to all the farmers that during these four years kindly made their fields available for the field trials.

I thank all the FiBL employees, trainees and master students that in one way or another contributed to this work by helping in the lab or during the field trials. In particular, I would like to thank Perrine Girard for the huge work conducted during her master thesis and for her contagious good mood.

A very special thank you goes to my fellow PhD student Shakira Fataar for the stimulating discussions, for the long days spent on the road during the field trials and for the long evenings spent at FiBL working together before important deadlines. Among all the ups and downs, you made these three years fly, it was really fun working with you! And not to forget how you pushed me towards basketball, I never thought it would be possible ;)

Finally, but by no means least, thanks go to my mum and stepdad for almost unbelievable support. You are very important to me and I dedicate this thesis to you.

SUMMARY

In agriculture, the widespread use of insecticides with ecotoxicological side effects has become an increasing problem, stressing the importance of alternative solutions. Invertebrate biocontrol agents are becoming increasingly important in the attempt to reduce or even replace the use of these insecticides. Different families of egg parasitoids were taken into consideration and their potential as biocontrol agents evaluated. The greatest emphasis has been placed on the genus *Trichogramma*. However, of the known 200 species of *Trichogramma*, only 19 species have been mass reared and used in augmentative biological control programs. These numbers point out the difficulties of developing new biocontrol agents and the importance of accurate feasibility studies to evaluate the potential of the new candidates, increasing their chance to land on the market.

This thesis investigated the biology of the egg parasitoid *Telenomus laeviceps* Förster, 1861 (Hymenoptera: Scelionidae) to build a stable rearing in order to conduct field efficacy trials, evaluating its performance in the control of the cabbage moth *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae). Laboratory trials were conducted to evaluate the influence on the parasitation performance of *T. laeviceps* of (I) the number of females parasitizing the same egg clutch, (II) temperature and (III) the egg deprivation time (the time until mated females come into contact with host eggs). This last point had the greatest effect on the parasitation rate and the proportion of female offspring produced, with egg deprived females performing better then newborn females.

An important aspect that determines the performance of this parasitoid, in the laboratory and in the field, is the provision of an exploitable food source. The presence of nectar near the release point in the field was shown to increase the effectiveness and persistence of released biocontrol agents, such as diverse *Trichogramma* spp.. We conducted laboratory experiments to test the influence of cornflower, *Centaurea cyanus* L.

(Asteraceae); buckwheat, *Fagopyrum esculentum* Moench (Polygonaceae) and common vetch, *Vicia sativa* L. (Fabaceae) on fecundity and longevity of *T. laeviceps*. Furthermore, the olfactory attractiveness of these flowers was evaluated in olfactometer trials. These flowers are the main components of a tailored flower strip implemented in Switzerland in the production of cabbage crops. In the presence of these three nectar providers, *T. laeviceps* survived significantly longer then when provided with water only. In addition, its fecundity was enhanced by *C. cyanus* and *F. esculentum.* These two flowers were further proved to be olfactory attractive for *T. laeviceps*.

The geographic distribution of a biocontrol agent is extremely important to determine its potential market. In fact, it is easier to obtain permits to release a biocontrol agent if it is native for the country of interest. To this end, we conducted field trials in three European countries to collect egg parasitoids. The sampled egg parasitoids were determined at the genus level to discriminate between *Trichogramma* spp. and *Telenomus* spp. (morphological species determination) and at the species level to identify *T. laeviceps* (molecular species determination). For the molecular species determination, specific qPCR markers were developed during the project.

Together with our commercial partner, a field delivery system was developed to effectively release *T. laeviceps* in the field. Preliminary field trials were conducted in 2015 to test the release method and to determine the control potential of two densities (120'000 and 240'000 parasitoids/ha) of the biocontrol agent. Results were very promising, with a mean parasitation rate for both densities of 36 % and maximal values of 70 %. However, the production of such a high density was, with the former production system, economically not feasible. Therefore, a density of 65'000 parasitoids/ha was calculated based on the costs of one application of the insecticide spinosad. Efficacy field trials were conducted in 2016 and 2017 to test the plant protection potential of this density compared to standard insecticides applied in organic agriculture. The results showed that the parasitation performance of the released parasitoids was not enough to efficiently control the cabbage moth.

Finally, over the same two years, separate field trials took place to test the potential of the provision of flowering plants in the promotion of *T. laeviceps*. Conservation biocontrol includes measures applied at the field level, aiming to promote different ecosystem services such as pest control or pollination. Released *Trichogramma* spp. benefit from conservation biocontrol, showing an increased parasitation performance and persistence in the field. Here, we tested the influence on the parasitation performance of *T. laeviceps* of flower strip and cornflowers as companion plants (2016 and 2017), flower strip only (2016) and control without provision of flowers (2016 and 2017). In 2016, released *T. laeviceps* and natural occurring *T. laeviceps* and *Trichogramma* spp. took advantage from the provided flowering plants. Further, the total control of cabbage moth eggs, due to egg parasitoids and predators, was 15 % higher in field with flowers than in control fields.

GENERAL INTRODUCTION

After the Second World War, as a result of improved living conditions, mortality rates decreased (Tilman *et al.* 2001). Increasing growth rates caused the world population to grow by more than four billion since 1950 (Bongaarts 2009). To cope with this phenomenon, financial investments have focused on crop research, infrastructure and market development. These, together with the appropriate policy support, have facilitated the development of improved crop genetics, fertilizers, irrigation techniques and pesticides. This, has led to increased yield per hectare cultivated land (Pingali 2012). During this period, the world was witnessing the so-called green revolution (Pingali 2012). The green revolution had severe consequences for the environment, causing the loss of organic matter (Matson *et al.* 1997), water pollution (Tilman *et al.* 2001), decline of biodiversity (Naranjo & Ellsworth 2009; Bommarco *et al.* 2011; Winqvist *et al.* 2012) and resistance of pests to pesticides (Parra 2010a). Alternatively, integrated pest management became more important, including biological control programs (Parra 2010a). For instance, the increased awareness of the harmful effects of broad-spectrum insecticides has shifted the efforts of universities and research institutes towards the search for new biocontrol agents. The gained knowledge was then transferred to companies, which were responsible for rearing and making natural enemies available to end users. This PhD thesis is an example of such collaboration. The presented work was supported financially by the EU-project Biocomes, where research facilities met with industry partners in order to develop new solutions against major pests in agriculture and forestry. The goal of our work package within the Biocomes project was to provide a solution to control the cabbage moth *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae) in different brassica crops using an egg parasitoid as released biocontrol agent.

The brassica family belongs to one of the ten economically most important crop families (Fahey 2003). In addition to their nutritional value, *Brassicaceae* are an important source of glucosinolates. Glucosinolates give them their characteristic aroma and taste and were shown to have anti-carcinogenic properties (Devlieghere *et al.* 2003). Cabbage (*Brassica oleracea* var. capitata) is a member of the *Brassicaceae* and native to Southern and Western Europe. It is cultivated on a surface of 2.6 Mio hectares, producing more than 55 Mio tons fresh heads per year (FAOSTAT 2017). Various insect pests, among others Lepidopteran pests like *M. brassicae*, *Pieris rapae* (Linnaeus, 1758) (Lepidoptera: Pieridae) and *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae), attack this crop family and cause severe yield losses (Cartea *et al.* 2009; Ahuja *et al.* 2010). In organic agriculture, larvae of the cabbage moth can be controlled with specific, organic insecticides based on the bacterium *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae). However, only small instars are susceptible to *B. thuringiensis* (Kwa *et al.* 1998). Large *M. brassicae* larvae cause most of the damages, either by feeding on leaves or by contaminating the crop with faeces. These larvae can only be controlled by the application of broad-spectrum insecticides, based on the active substances spinosyn A and D. Spinosyns are fermentation products of the bacterium *Saccharopolyspora spinosa* (Actinomycetales: Pseudonocardiaceae) (Kirst *et al.* 1991; Kirst 2010). Although Kirst (2010) has shown fewer side effects of spinosad on non-target insects than older insecticides, such as pyrethroids, other studies have found a decrease in species richness, abundance and effectiveness of natural enemies (Naranjo & Ellsworth 2009; Bommarco *et al.* 2011; Winqvist *et al.* 2012). In addition to these ecotoxicological side effects, resistance can be developed, leaving farmers without any alternative solution (Zhao *et al.* 2002). Therefore, new products such as invertebrate biocontrol agents could provide valid alternatives.

The first step in the development of a biocontrol agent is selecting the right species to control the pest of interest. *M. brassicae* larvae and eggs are attacked by several natural enemies, such as the larval parasitoid *Microplitis mediator* (Haliday, 1834)

(Hymenoptera: Braconidae) or the egg parasitoids *Trichogramma brassicae* (Bezdenko, 1968) (Hymenoptera: Trichogrammatidae), *Tr. evanescens* Westwood, 1833 (Hymenoptera: Trichogrammatidae) and *Telenomus laeviceps* Förster, 1861 (Hymenoptera: Scelionidae) (Bianchi *et al.* 2005; Pfiffner *et al.* 2009; Harvey & Gols 2011; Johnson & Cora 2011). In contrast to larval parasitoids, egg parasitoids kill their hosts before they develop into crop damaging larvae. Therefore, egg parasitoids should be preferred as potential biocontrol agents. In field trials in Switzerland, natural occurring *Telenomus* sp. (later to be known as *T. laeviceps*) parasitized more pests than mass released *Tr. brassicae* (Balmer et al. 2013). Therefore, the aim of this thesis was to develop a new biocontrol agent based on the egg parasitoid *T. laeviceps* against the cabbage pest *M. brassicae*.

T. laeviceps is a small parasitic wasp distributed across Europe and North Africa, able to parasitize eggs of different insect pests belonging to the Noctuidae, Geometridae and Nolidae (Mexia *et al.* 2004; Klemola *et al.* 2009; Bayle 2012; Petrov 2012). We performed basic studies in order to better understand the biology of *T. laeviceps*, facilitating the development of a stable rearing (CHAPTER 1). The parasitation rate and the proportion of female offspring can be good predictors for the success of a parasitoid rearing (Roitberg *et al.* 2001; Parra 2010b). As shown for other *Telenomus* spp., these traits may be affected by i) the number of females parasitizing the same egg clutch (Carneiro *et al.* 2009), ii) the temperature (Legault *et al.* 2012; Pomari *et al.* 2012) and iii) the egg deprivation time (the time until mated females come into contact with host eggs) (Charnov & Skinner 1985; Bruce *et al.* 2009). Furthermore, effects of prolonged rearing on the body size of *T. laeviceps* were investigated.

Parallel to the development of a rearing, it is important to clarify the application potential of the new biocontrol agent, which is strongly dependent on its geographic distribution. For instance, *T. laeviceps* is native in Switzerland. Therefore, permits to release this biocontrol agent were obtained from the authorities of interest. In contrast, in other European countries, where *T. laeviceps* has not yet been recorded, releases are

not authorised. This strict regulation is due to the increasing introduction of exotic insect pests in Europe, caused by the augmentation of tourism and international trade (Bigler *et al.* 2005). In an attempt to control these exotic pests, insecticides are applied, rising the need for alternative solutions, such as classical biological control (Waage 1997; Sheppard *et al.* 2006). This led to a drastic increase in the number of exotic invertebrate biological control agents (IBCA) introduced (van Lenteren 1997). Several publications have drawn attention to the risks of exotic IBCA species (Howarth 1991). Consequently, the European Commission has strengthened the regulation of products based on non-native organisms. Therefore, in order to expand the application potential of *T. laeviceps*, we sampled egg parasitoids in three European countries (CHAPTER 2). The parasitoids collected were determined at the genus level to discriminate between *Trichogramma* spp. and *Telenomus* spp. (morphological determination). *Telenomus* spp. wasps were additionally determined at the species level using the developed molecular marker, in order to specifically identify *T. laeviceps* (CHAPTER 1).

The next step in the development of a biocontrol agent is the identification of the right approach to release the parasitoid into the field, as well as the right density to achieve good pest control (Parra 2010a). In this project, I collaborated with a commercial partner, who already has a lot of expertise in the production and commercialization of *Trichogramma* spp.. Thus, in order to exploit the same machinery used for the production of the end formulation of *Trichogramma* spp., the same field delivery system was also used for *T. laeviceps* and tested in the preliminary trial conducted in 2015 (CHAPTER 3). In this trial, the biocontrol agent was released in two densities: 120'000 and 240'000 parasitoids/ha. The lower density of 120'000 parasitoids/ha was described by Oztemiz (2008) as the most suitable for releasing *T. evanescens* against eggs of *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae). Since little is known about the dispersal capacity of *T. laeviceps*, a higher density has also been tested. The mass-production of *T. laeviceps* and its host *M. brassicae* was still under development and therefore the production costs still high. Thus, for the 2016 and 2017 field trials, an economically

feasible density was calculated, matching the costs of an application with the insecticide spinosad, which allowed us to release 65'000 parasitoids/ha. I tested this density in efficacy trials conducted over two consecutive years. I compared the plant protection potential of *T. laeviceps* to standard insecticides used in organic agriculture (CHAPTER 3).

The field performance of a released biocontrol agent could be improved by the availability of easily exploitable food sources. Under field conditions, released adult *Trichogramma* spp. were observed to feed on several flowers, such as buckwheat, *Fagopyrum esculentum* Moench (Caryophyllales: Polygonaceae); mustard, *Brassica juncea* L. (Brassicales: Brassicaceae); dill, *Anethum graveolens* L. (Apiales: Apiaceae) or avocado flowers, *Persea americana* Mill (Laurales: Lauraceae) planted near the crop field (Wellinga & Wysoki 1989; Begum *et al.* 2004; Begum *et al.* 2006; Manandhar & Wright 2015). The use of selected flowers sown or planted near or into crop fields is a practice known as conservation biocontrol. Conservation biocontrol describes measures applied at field level to increase the overall biodiversity in the field and surrounding areas, in order to promote different ecosystem services such as pest control or pollination (Moonen & Bàrberi 2008; Plecas *et al.* 2014; Gaigher *et al.* 2015; Inclan *et al.* 2015; Inclán *et al.* 2015). If the released biocontrol agent benefits from selected flowers, the combination of augmentative and conservation biocontrol could increase its effectiveness and persistence. These could in turn reduce the amount of released biocontrol agents and therefore decrease the costs for the end users. In Switzerland, a tailored flower strip for brassica crops is already commercially available for farmers. The seed mixture contains, as main nectar providers, cornflower, *Centaurea cyanus* L. (Asterales: Asteraceae); buckwheat, *F. esculentum* and common vetch, *Vicia sativa* L. (Fabales: Fabaceae). Field trials showed that this tailored flower strip attracts and enhances the natural occurring antagonists *M. mediator, Diadegma fenestrale* (Förster, 1869) (Hymenoptera: Ichneumonidae) and *D. semiclausum* (Förster, 1869) (Hymenoptera: Ichneumonidae), important antagonists of *M. brassicae* and *P. xylostella* (Géneau *et al.* 2012; Balmer *et al.*

2013; Belz *et al.* 2013; Balmer *et al.* 2014). Cornflowers, beside floral nectar, also display easily accessible extra-floral nectar. This feature, together with a prolonged blossom, makes them good candidates for intercropping, since they provide nectar over a long period of time. In CHAPTER 4, I tested the olfactory attractiveness and effects on longevity and fecundity of *C. cyanus, F. esculentum* and *V. sativa* on *T. laeviceps* in the laboratory. Furthermore, I tested the influence of different habitat managements on the performance of *T. laeviceps* in the field (CHAPTER 5). For that, parasitoids were released into fields with flower strip and companion plant (2016 and 2017), flower strip only (2016) or without flowers (control) (2016 and 2017).

NOTE

on the taxonomy and nomenclature of the study insect as used throughout the present thesis:

Scelionidae Haliday, 1839[1]

Telenominae Thomson, 1860

Telenomus Haliday, 1833

Telenomus laeviceps Förster, 1861: XL[2]

Förster, A., 1861. Ein Tag in den den Hoch-Alpen. Pp I-XLIV in: Anonymous, Programm der Realschule zu Aachen für das Schuljahr 1860-61. Aachen.

Locus typicus: Switzerland, Canton of Grisons, Upper Engadine, Val Roseg (ca 1800 – 2500 m asl)

[1] The family group classification of Platygastroidea and the subsequent nomenclature is debated. Throughout the present thesis we use "Scelionidae", for pragmatic reasons only: When starting the studies of the thesis *Telenomus* was mainly assigned to Scelionidae and still today, the following two concepts are in concurrent use: *Telenomus* is regarded as part of Scelionidae Haliday, 1839 (e.g. WaspWeb [van Noort (2018)];

Masner (1993); Talamas and Buffington (2015)) as well as of Platygastridae Haliday, 1833 (e.g. Hymenoptera Online [Johnson *et al.* (2018)]; Sharkey (2007); Nesheim *et al.* (2017)).

[2] The identification of the specimens of *Telenomus* used for the present study was carried out in 2011 for Forschungsinstitut für biologischen Landbau (FiBL), Frick, Switzerland by the expert Norman F. Johnson, Ohio State University, Columbus, U.S.A., which is gratefully acknowledged.

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CHAPTER 1

Perspectives for a new biocontrol agent: molecular determination and rearing of *Telenomus laeviceps*, an egg parasitoid of the cabbage moth *Mamestra brassicae*

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Abstract

In agriculture, the widespread use of insecticides with ecotoxicological side effects has become an increasing problem, stressing the importance of alternative solutions. The release of biocontrol agents (e.g. parasitoids) can provide such an alternative. Cabbage, an important and worldwide-distributed vegetable, is a host plant of the cabbage moth *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae). However, so far, for organic agriculture no pest control without adverse side effects is available for this crop. In this study we thus investigated the biology of the parasitic wasp *Telenomus laeviceps* Förster, 1861 (Hymenoptera: Scelionidae), a potential biocontrol agent against *M. brassicae*. We developed specific primers for the molecular determination of field sampled *T. laeviceps* and additionally tested rearing parameters such as parental female density, temperature and egg deprivation period. We successfully developed a tool for the determination of *T. laeviceps* among field-sampled parasitoids and established a lab-scale rearing based on the identified optimal rearing conditions. Here, we provide the tools for the development of a large-scale rearing of *T. laeviceps*, which are relevant for implementing this species as a biocontrol agent.

Keywords: augmentative biological control, body size, Noctuidae, qPCR, rearing conditions, Scelionidae, *Telenomus laeviceps*

Introduction

The cabbage moth *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae) is an insect pest distributed throughout Europe and Asia (Johansen 1997). Its polyphagous larvae feed on leaves of many brassica plants, causing damage and high economic losses (Pfiffner *et al.* 2009). Besides the mechanical damage, contamination of the crop with faeces further diminish its quality and market value, or even makes the crop no longer marketable (Omino *et al.* 1973; Devetak *et al.* 2010). In organic farming, to date, the only

efficient way to prevent this pest is through the application of broad-spectrum insecticides containing the active substances spinosyn A and D, two fermentation products of the actinomycete *Saccharopolyspora spinosa* (Kirst 2010). Although Kirst (2010) indicated less side-effects of spinosad on non-target insects compared to earlier insecticides like pyrethroids, other studies found a decrease in species richness, abundance and effectiveness of natural enemies (Naranjo & Ellsworth 2009; Geiger *et al.* 2010; Bommarco *et al.* 2011; Winqvist *et al.* 2012). Biological control methods can provide a successful alternative to the usage of broad-spectrum insecticides and preserve and promote natural enemy diversity in crop fields at the same time (Parra 2010a). For the control of *M. brassicae* however, there is no biocontrol agent available so far.

The first two steps in designing a new biocontrol agent are the selection of the appropriate natural enemy of the pest of interest and the development of a small scale rearing of this natural enemy for basic studies (Parra 2010a). Larvae and eggs of the cabbage moth are attacked by a number of natural enemies, such as the larval parasitoid *Microplitis mediator* (Haliday, 1834) (Hymenoptera: Braconidae) or the egg parasitoids *Trichogramma evanescens* Westwood, 1833 (Hymenoptera: Trichogrammatidae), *Trichogramma brassicae* (Bezdenko, 1968) (Hymenoptera: Trichogrammatidae) and *Telenomus* sp. (Bianchi *et al.* 2005; Pfiffner *et al.* 2009; Harvey & Gols 2011). The effectiveness of *Tr. brassicae* was tested in previous field trials in Switzerland (Balmer *et al.* 2013; Balmer *et al.* 2014). Parasitation rates through released and naturally occurring *Trichogramma* spp. were lower however, than those of naturally occurring *Telenomus* sp. (Pfiffner *et al.* 2009; Balmer *et al.* 2013). The genus *Telenomus* is featured through egg parasitation. In contrast to larval parasitation, they kill their hosts before they develop into crop damaging larvae. Thus, *Telenomus* sp. represents an optimal candidate for the development of a new biocontrol agent against the cabbage moth.

In 2011, several individuals of the genus *Telenomus* were collected through exposition of trap eggs (cabbage moth) in brassica fields (Swiss Plateau) (Leg. FiBL). The

collected parasitoids were identified based on morphological traits as *Telenomus laeviceps* Förster, 1861 (Hymenoptera: Scelionidae) (Johnson & Cora 2011) and used to establish a rearing. *Telenomus laeviceps* is a small parasitic wasp (own data: females: 0.67±0.06 mm, males: 0.66±0.05 mm) distributed across Europe and North Africa, able to parasitize eggs of different insect pests belonging to the Noctuidae, Geometridae and Nolidae (Mexia *et al.* 2004; Klemola *et al.* 2009; Bayle 2012; Petrov 2012) (Table 1-1). The parasitation rate and the proportion of female offspring can be good predictors for the success of a parasitoid rearing (Roitberg *et al.* 2001; Parra 2010b). As shown for other *Telenomus* spp., these traits can be influenced by (I) the number of females parasitizing the same egg clutch (Carneiro *et al.* 2009), (II) temperature (Legault *et al.* 2012; Pomari *et al.* 2012) as well as (III) the egg deprivation time (the time until mated females come in contact with host eggs) (Charnov & Skinner 1985; Bruce *et al.* 2009). Furthermore, the field performance of a biocontrol agent may be positively correlated with its body size (Kazmer & Luck 1995; Bennett & Hoffmann 1998).

In order to use *T. laeviceps* as a biocontrol agent, an establishment of a large-scale rearing is necessary. In this wasp family, however, species determination based on morphological traits is uncertain and restricted to a hand full of expert taxonomists. In addition, rearing conditions have not been investigated so far. Thus, the aim of this study was a) developing specific qPCR markers for species determination, b) investigating the effect of parasitoid density, temperature and egg deprivation time on rearing success, and c) measuring possible changes in body size due to breeding (performance estimate for field conditions).

Table 1-1 Distribution and hosts of the egg parasitoid *Telenomus laeviceps*.
n/a: not available.

Hosts	Family	Host plant	Distribution	Reference
Mamestra brassicae (Linnaeus, 1758)	Noctuidae	Brassicaceae	Switzerland	This paper
Abrostola asclepiadis (Denis & Schiffermüller, 1775)	Noctuidae		France	Bayle (2012)
Operophtera brumata (Linnaeus, 1758)	Geometridae		Finland	Klemola et al. 2009
Epirrita autumnata (Borkhausen, 1794)	Geometridae		Finland	Klemola et al. 2009
Helicoverpa armigera (Hübner, 1808)	Noctuidae	Tomato	Portugal	Figueiredo and Mexia (2000) Mexia et al. (2004)
Anarta myrtilli (Linnaeus, 1761)	Noctuidae	Fruit tree	Bulgaria	Petrov (2012)
Nycteola asiatica (Krulikovsky, 1904)	Nolidae	Fruit tree	Bulgaria	Petrov (2012)
n/a	n/a	n/a	Estonia Latvia Russia Sardinia Ukraine Moldova	de Jong (2014)
n/a	n/a	n/a	Russia, far east Poland Moldova Ukraine Georgia Japan	Personal communication of Dr. Alex Gumovsky based on the collection of Dr. Svetlana Kononova (Institute of zoology NAS Kiev, Ukraine)
Mamestra oleracea (Linnaeus, 1758)	Noctuidae	n/a	Morocco	Berthet (2002)

Material and methods

Rearing and experimental conditions

The parasitoids used in these trials descend from *Telenomus laeviceps,* which emerged from cabbage moth eggs exposed in organic cabbage fields in the Swiss Plateau (47th parallel north) in 2012 and were since then reared at the Research Institute of Organic Agriculture (FiBL), Switzerland. The small scale rearing of *T. laeviceps* was conducted in glass tubes (14.5 cm, ø 3 cm) (rearing unit) on cabbage moth eggs in a climate chamber at 22 ± 2 °C and 55 ± 5 % RH, with a photoperiod of 16:8 (L:D). To ensure a sufficient number of adult wasps for experiments, three rearing units were started weekly. A rearing unit consisted of 1500-2000 cabbage moth eggs (< 24 h old) and approximately 100 wasps (70 % females and 30 % males). Based on preliminary experiments, in which wasps of different ages were able to parasitize host eggs, ten-day-old wasps were used for optimal parasitation results. Females were allowed to parasitize the provided batch of eggs for seven days. Afterwards, the parasitized eggs were placed in an empty rearing unit until wasp emergence. Parasitoids were fed with honey-gelatine *ad libitum* (200 g flower honey (Switzerland), 100 ml demineralized water and 3 g gelatine (Dr. Oetker)), provided on a piece of white paper placed in each rearing unit. With these rearing conditions, progeny emerged 14 days after parasitation onset.

Experiments were conducted in a climate chamber under the same conditions as in the rearing. Cabbage moth eggs younger than 24 hours were used for the experiments, similarly to Chabi Olaye *et al.* (1997). Number of parasitized eggs, emergence rate and number of female progeny were measured as fitness predictors. In each trial, the number of parasitized eggs was determined by taking pictures just before and seven days after parasitation. The initial number of eggs, as well as the number of parasitized eggs, was counted using the computer program Mouse Clickr (Dejco). To

assess the sex ratio of the progeny, wasps were first numbed with carbon dioxide and then counted under a stereo microscope (Olympus SZ-ET, Japan).

Development of species-specific qPCR marker for T. laeviceps

To facilitate species determination of field collected *T. laeviceps*, molecular primers were developed. To prepare the crude DNA extract, ten adult *T. laeviceps* were placed in 2 ml screw cap tubes (Sarstedt Cat. 72.609.001) together with 0.25 ml of zirconia/silica beads (ø 0.5 mm, BioSpec Products, Bartlesville, OK, USA) and 300 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). Parasitoids were crushed using a FastPrep-24® (MP Biomedicals) for 40 s at a speed of 6 m s^{-1}. The tubes were then incubated in a heating block at 95 °C for 10 min, subsequently centrifuged at 20,000 g for 1 min and stored at -20 °C. Using universal primers LCO1490 and HCO2198 (Folmer *et al.* 1994), we amplified the mitochondrial cytochrome oxidase subunit I (COI) region with 2.5 µl of undiluted supernatant of the extracts in a total of 25 µl reaction volume containing 5 µl of 5x PCR buffer, 1.25 µl of each primer prediluted at 3 µM, 13.75 µl ultrapure water, 0.75 µl dNTP 10 mM and 0.5 µl of DNA polymerase (Hot Taq DNA Polymerase, Peqlab 01-8130). Following PCR cycling conditions were used: activation of hot start Taq DNA Polymerase for 2 min at 95 °C, followed by 33 cycles of denaturation at 98 °C for 20 s, annealing at 55 °C for 15 s, extension at 72 °C for 45 s and a final extension step at 72 °C for 3 min.

Amplified DNA fragments were purified using a QIAquick PCR Purification kit (QIAGEN, 28104) following the kit's protocol. The purified DNA was sent to Microsynth AG (Switzerland) for Sanger sequencing in both directions with the above mentioned primers, LCO1490 and HCO2198, respectively, resulting in a contig of 643 bp, which was submitted at NCBI Genbank under the accession number KY308192.

Table 1-2 Mitochondrial cytochrome oxidase subunit I (COI) sequences of pests and parasitoids used to develop the species-specific primers.
Ethanol-stored insect samples are highlighted grey. [a] NCBI: retrieved from NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/), ABC: ethanol-stored insects provided by Andermatt Biocontrol AG (Switzerland), FiBL: insects provided by the Research Institute for Organic Agriculture (Switzerland). Cq: quantification cycles, * Cq < 30, ** Cq = 30-39 and * Cq > 40. n/a: not available.**

Species	Origin	Source[a]	Accession	Cq
Telenomus laeviceps Förster, 1861	Switzerland	FiBL	KY308192	***
Telenomus dalmanni (Ratzeburg, 1844)	USA	NCBI	KC778451	n/a
Telenomus goniopis Crawford, 1913	USA	NCBI	KC778478	n/a
Telenomus busseolae Gahan, 1922	Australia	NCBI	DQ888418	n/a
Trichogramma brassicae (Bezdenko, 1968)	China	NCBI	DQ177916	n/a
Trichogramma evanescens Westwood, 1833	Iran	NCBI	JX131626	n/a
Microplitis mediator (Haliday, 1834)	Canada	NCBI	HQ941791	n/a
Plutella xylostella (Linnaeus, 1758)	Korea	NCBI	DQ076411	n/a
Pieris rapae (Linnaeus, 1758)	China	NCBI	JQ996397	n/a
Mamestra brassicae (Linnaeus, 1758)	China	NCBI	JQ235745	n/a
Spodoptera littoralis (Boisduval, 1833)	United Kingdom	NCBI	FN908019	n/a
Sitophilus granarius (Linnaeus, 1758)	Switzerland	ABC	n/a	*
Agrotis ipsilon (Hufnagel, 1766)	Switzerland	ABC	n/a	**
Helicoverpa armigera (Hübner, 1808)	Switzerland	ABC	n/a	**
Adalia bipunctata (Linnaeus, 1758)	Switzerland	ABC	n/a	*
Spodoptera littoralis (Boisduval, 1833)	Switzerland	ABC	n/a	*
Spodoptera exigua (Hübner, 1808)	Switzerland	ABC	n/a	*

Table 1-2: continuation

Species	Origin	Source[a]	Accession	Cq
Anisopteromalus calandrae (Howard, 1881)	Switzerland	ABC	n/a	*
Cryptophlebia leucotreta (Meyrick, 1913)	Switzerland	ABC	n/a	*
Cydia pomonella (Linnaeus, 1758)	Switzerland	ABC	n/a	*
Mamestra brassicae (Linnaeus, 1758)	Switzerland	FiBL	n/a	*
Telenomus spp. (9 species)	USA	Norman Johnson	n/a	**(all samples)

We compared this COI sequence of *T. laeviceps in silico* to homologous sequences (mainly from other *Telenomus* species) and COI sequences of other insect species (e.g. possible hosts) retrieved from NCBI GenBank (Table 1-2). The sequences were aligned with MEGA 6.0 (Tamura *et al.* 2013) and specific primers and probes were developed using Beacon Designer (PREMIER Biosoft International). To increase the specificity of the marker, some bases at and adjacent to polymorphic target specific regions of the fluorogenic TaqMan probe were LNA (=locked nucleic acid) modified (Table 1-3).

The specificity of the developed qPCR-TaqMan-marker was additionally evaluated by running qPCR reactions with DNA of different insect species (pests and parasitoids) (Table 1-2). Furthermore, the marker was tested against nine other *Telenomus* spp., which were previously determined morphologically as non-*T. laeviceps* (Dr. Norman Johnson, Ohio State University, USA).

Table 1-3 Primer pairs (F/R) for TaqMan qPCR to detect *Telenomus laeviceps* DNA. FAM = specific fluorescent dye, BHQ = "black hole quencher". LNA modified bases of the probes (P) are marked with bold capital letters.

Species	Primer	Sequence (5'-3')	Length
Telenomus laeviceps	*T. laeviceps*_F	CAGGAACAGGATGAACTATTTATC	102 bp
	*T. laeviceps*_R	AAATTGATGAAATTCCTGCAATATG	
	*T. laeviceps*_P	FAM-tcaac**AC**aattaaatcc**TTC**aa-BHQ1	

Influence of parental female density on parasitation performance of T. laeviceps

When the rearing of *T. laeviceps* was started back in 2012, mated females were placed singly in glass tubes containing *M. brassicae* eggs, avoiding intraspecific competition between parasitizing females. The reached rearing efficiency was satisfying, with a constant production of progeny. However, the workload due to the selection of females was very high. Therefore, with this trial we aim to evaluate whether a group of parental females parasitizing the same egg clutch interfere with each other, negatively affecting the rearing efficiency. To this end, 150 eggs were placed in a glass tube (14.5 cm, ø 3 cm) with two parental female densities: three females per 150 eggs and one female per 150 eggs (control). Carneiro *et al.* (2009) showed that in *Telenomus remus* Nixon, 1937 (Hymenoptera: Scelionidae), the highest total parasitation performance was reached when three parental females parasitized the same egg clutch. In this trial, females were allowed to mate during ten days and were subsequently offered host eggs for three days. During the experiment, wasps were fed *ad libitum* with honey-gelatine and a total of 30 replicates for each density was tested. The parasitation rate and proportion of female progeny were recorded, both important predictors for a successful rearing.

Influence of different temperature regimes on developmental rate and parasitation performance of T. laeviceps

The impact of temperature on developmental time (from parasitation to adult emergence) and on the parasitation performance of the parental females was evaluated. The experiment was carried out in a climatic chamber (Percival, Model I-36LLVL, CLF Laborgeräte GmbH, Germany) with a photoperiod of 16:8 (L:D) and 50 ± 10 % RH. The following temperatures were tested: 30 °C, 28 °C, 26 °C, 24 °C, 22 °C and 20 °C. This range represents temperatures measured in Swiss cabbage fields during summer 2015 (based on self-collected weather data with Funk-Wetterstation WS 0101 Professional USB, Conrad Switzerland). A replicate consisted of one female and a clutch of *M.*

brassicae eggs (200 ± 50 eggs) placed in a glass tube (14.5 cm, ø 3 cm). This amount of eggs was based on previous pre-trials, which showed that the realised lifetime fecundity of a *T. laeviceps's* female is maximum 150 parasitized eggs. Females were allowed to mate for ten days, followed by a three-day-period of parasitizing host eggs. During the experiment, wasps were fed *ad libitum* with honey-gelatine. Replicates were checked daily for emerging progeny. For each temperature, 30 replicates were tested.

Influence of three distinct egg deprivation periods on longevity and fecundity of T. laeviceps

In this experiment, we investigated the egg deprivation time (the time until females have access to host eggs) on the longevity and fecundity of *T. laeviceps.* During the egg deprivation times, females were allowed to mate (mating period). Three egg deprivation periods were tested: < 24 hours, six days and eleven days. One female and one male were placed in a glass tube (5 cm length, 1.5 cm diameter) for the given time spans. Afterwards, males were removed and an egg clutch of 150 ± 50 eggs was placed in each tube. The eggs were replaced daily at 1 p.m. and the parasitation and emerging rates assessed per day.

Since not only egg deprivation time but also the corresponding mating period could potentially affect female longevity (Hardy *et al.* 2007), we further integrated control treatments to distinguish between the effects resulting from egg deprivation and those from mating period on the longevity of females. Therefore, *T. laeviceps* pairs were allowed to mate for either < 24 hours, six days or eleven days and were subsequently separated. From there on, the wasps' longevity was recorded without providing the female host eggs for egg deposition. During the experiment, wasps were fed *ad libitum* with honey-gelatine. In total, we tested 20 females per deprivation period and per control treatment.

Influence of long-term rearing on the body size of T. laeviceps

The parasitoids used in the above presented experiments were reared under laboratory conditions during several generations. Since long-term rearing can affect the body size of parasitoids (Grenier & De Clercq 2003), we measured 40 wasps (20 females and 20 males) for each of the following conditions: individuals after one and after five years of rearing and wild *T. laeviceps* collected in nature. These wild individuals were collected from cabbage moth eggs exposed in Swiss organic cabbage fields during summer 2017. Parasitoids were placed singly on a cover glass with a 2 mm scale (1 interval 0.01 mm, Leitz Wetzlar, Germany), photographed using a binocular (Leica M205C) connected to a camera (Progress Gryphax, Jenoptik Optical Systems GmbH) and afterwards measured using ImageJ (Version 1.47).

Statistical analysis

Data analyses were conducted with R version 3.3.0 (R development core team, 2016). Statistical analysis of the data from the two female densities experiment were conducted through a generalized linear model with binomial errors for the proportion data (parasitation rate, emergence rate and proportion of female offspring) and poisson errors for the count data (total progeny), with fixed factor parental female density (two levels: one or three females).

To compare the effect of the distinct temperature regimes on the developmental time, number of parasitized eggs, emergence rate and number of females and males produced, different regression curves (linear, quadratic or polynomial) were fitted to the measured variables. The models based on different regression curves were compared using ANOVA and the best fitting model chosen (Crawley 2007).

Survival curves of egg deprived females and of males were drawn using the Kaplan-Meier survival estimation and compared by a Mantel-Cox model. The number of parasitized eggs and the number of females produced were analysed with generalized

linear models with Poisson errors (glmer function from the package lme4), corrected for overdispersion. The models used the fixed factor treatment (three levels: 0, 6 or 11 days), the covariate female age and the random effect replicate. Emergence rate was analysed with a generalized linear model with binomial errors, using the explaining variable treatment (three levels: < 24 h, six or eleven days).

Data from the comparison of the body size of *T. laeviceps* were first checked for normal distribution and afterwards analysed with an analysis of variance with Tukey HSD as post-hoc test. The body size of females and males was compared using a linear mixed-effects model (lmer function from the package lme4) with sex as fixed factor (two levels: females and males) and rearing status as random factor.

Results

Development of species-specific primers for T. laeviceps

The newly developed TaqMan qPCR marker for *T. laeviceps* (Table 1-3) was proven to be highly specific to *T. laeviceps*, producing a specific signal with quantification cycles (Cq) values between 20 and 30. When tested for other parasitoids and potential hosts, the marker produced no (Cq values > 40) or weak signals (30 > Cq values < 40) (Table 1-2).

Influence of female density on parasitation performance of T. laeviceps

The proportion of female offspring was not significantly influenced by the number of females parasitizing the same egg clutch (Table 1-4). On the other hand, the total parasitation rate, emergence rate as well as the amount of progeny produced were significantly higher with three compared to one parental female per rearing unit (Table 1-4).

Table 1-4 Impact of females' density on important life history parameters. Generalized linear model, with binomial (parasitation rate, emergence rate and proportion of females) and poisson (total progeny) errors.

	Number of females/150 eggs			
	1	3	Z	p
Parasitation rate (± sd)	63.87 ± 11.92	90.66 ± 10.41	2.321	0.02
Emergence rate (%) (± sd)	62.67 ± 29.87	89.95 ± 11.46	2.284	0.02
Prop. of females (%) (± sd)	68.34 ± 29.84	52.92 ± 28.96	- 1.034	0.3
Total progeny (± sd)	58.8 ± 29	121.5 ± 30	25.05	< 0.0001

Influence of different temperature regimes on developmental rate and parasitation performance of T. laeviceps

The developmental time of *T. laeviceps* increased significantly with decreasing temperature (linear regression, $R^2 = 0.925$, $t_{1,167} = -45.41$, $p < 0.0001$) (Figure 1-1A), while the number of parasitized eggs was not significantly influenced by temperature (linear regression, $R^2 = 0.014$, $t_{1,178} = -1.906$, $p = 0.058$) (Figure 1-1B). The amount of females produced was temperature dependent (polynomial regression, $R^2 = 0.137$, $t_{29,177} = 3.726$, $p < 0.0001$), being negatively influenced from 20 °C to 26 °C and positively from 26 °C to 30 °C (Figure 1-1C). A similar pattern was observed for the emergence rate (polynomial regression, $R^2 = 0.03$, $t_{30,178} = 2.575$, $p = 0.01$) (Figure 1-1D). There was no significant relation between the number of male progeny produced and temperature (polynomial regression, $R^2 = 0.026$, $t_{30,177} = 0.359$, $p = 0.72$) (Figure 1-1E).

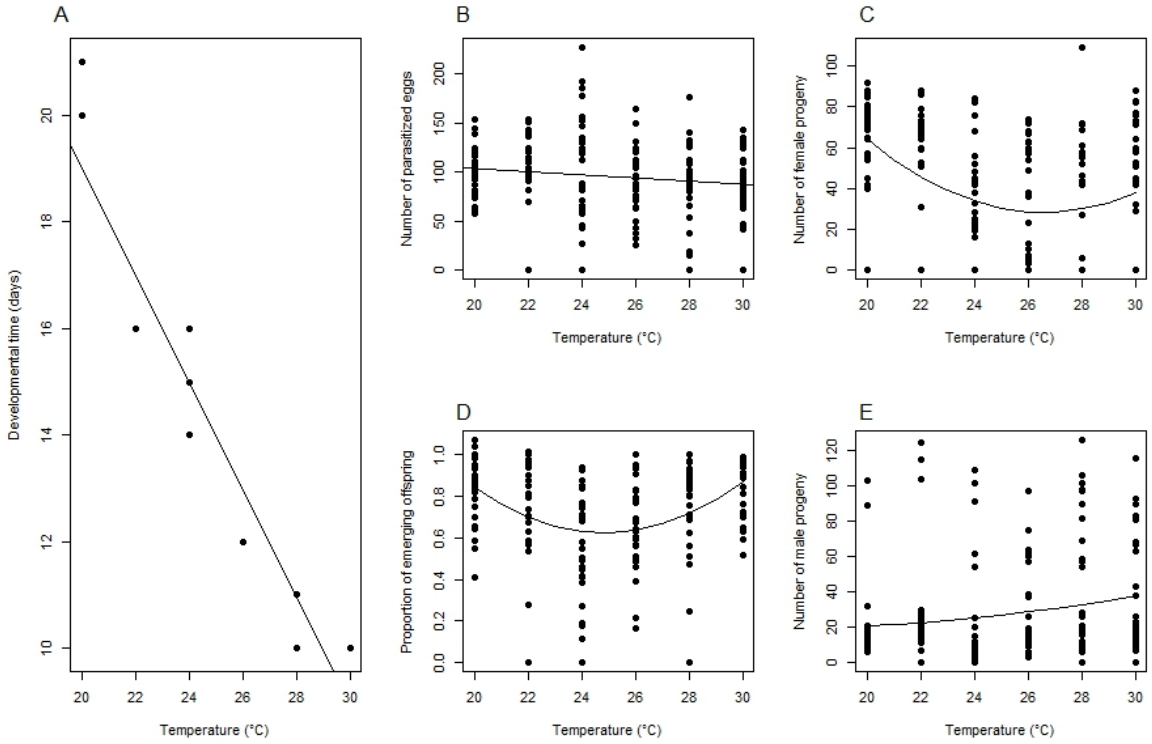

Figure 1-1 Influence of different temperature regimes on main parameters determining the reproductive performance of *T. laeviceps*.
A: developmental time (Linear regression, R^2 = 0.925, $t_{1,167}$ = -45.41, p < 0.0001), B: number of parasitized eggs (Linear regression, R^2 = 0.014, $t_{1,178}$ = -1.906, p = 0.058), C: number of female progeny (Polynomial regression, R^2 = 0.137, $t_{29,177}$ = 3.726, p = 0.0003), D: emergence rate (Polynomial regression, R^2 = 0.03, $t_{30,178}$ = 2.575, p = 0.01) and E: number of male progeny (Polynomial regression, R^2 = 0.026, $t_{30,177}$ = 0.359, p = 0.72).

Influence of three distinct egg deprivation periods on longevity and fecundity of T. laeviceps

Females that were egg deprived for six or eleven days parasitized significantly more eggs throughout their lifetime than females provided with eggs immediately after emerging (generalized linear model, both p < 0.0001), on average 79.3 ± 17.29 (< 24 h old), 149.84 ± 14.56 (six days old) and 142.65 ± 12.01 (eleven days old). The emergence rate of progeny was not significantly different between the distinct egg deprivation

periods (Generalized linear model, all $p > 0.2$). However, females that were not egg deprived, produced significantly less females compared to six and eleven day-egg-deprived females (generalized linear model, respectively $z = 2.011$, $p = 0.04$ and $z = 2.496$, $p = 0.01$), on average 7.9 ± 3.06 (0 days old), 45.68 ± 7.2 (six days old) and 56.2 ± 7.55 (eleven days old) throughout their lifetime. Survival of females which had the chance to lay eggs upon emergence, was significantly lower compared to egg deprived females (Mantel-Cox, both $p < 0.005$) (Figure 1-2). No difference was found between the longevity of females in the three control treatments.

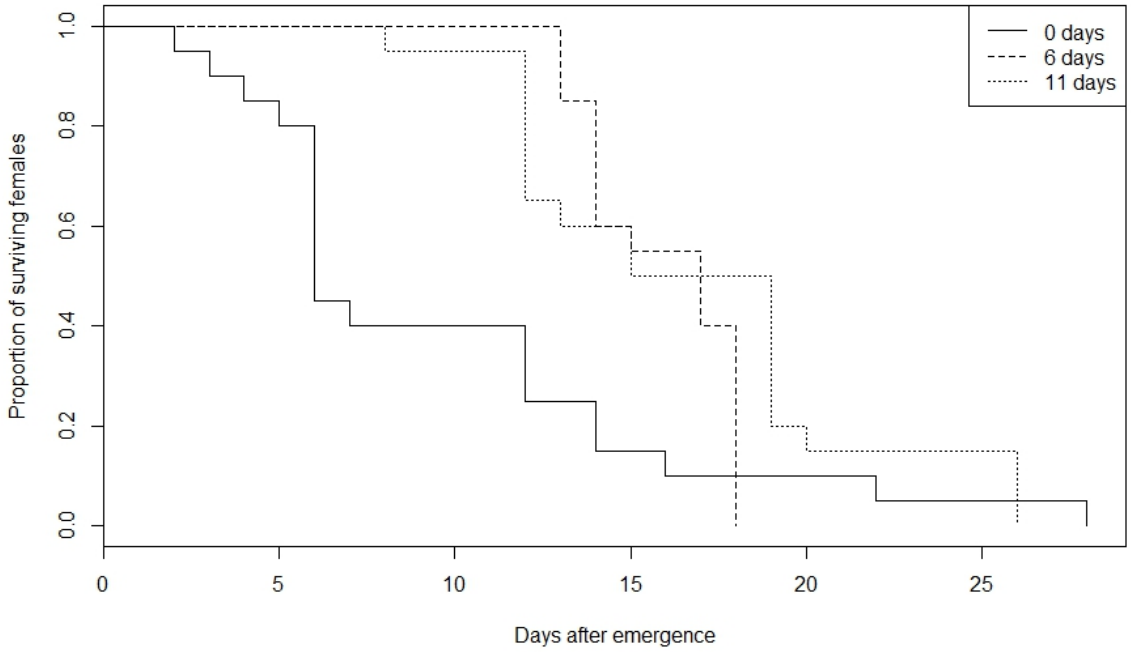

Figure 1-2 Kaplan-Meier estimates of survival functions of females belonging to the three egg deprivation periods.

Influence of long-term rearing on the body size of T. laeviceps

The body size of male and female *T. laeviceps* was significantly influenced by their rearing status (Analysis of variance, $F_{2,57} = 38.45$, $p < 0.0001$) (Figure 1-3). After one year

of rearing, the body size of *T. laeviceps* was significantly increased compared to field sampled individuals (Analysis of variance with Tukey HSD post-hoc test, $p < 0.0001$ for both females and males), with females measuring 0.782 ± 0.06 mm and 0.665 ± 0.06 mm and males 0.798 ± 0.04 mm and 0.656 ± 0.05 mm, respectively. No difference was found between parasitoids reared during one or five years (Analysis of variance with Tukey HSD post-hoc test, females: $p = 0.653$ and males: $p = 0.237$). The body size did not significantly differ between females and males within each rearing status (Linear mixed-effects model, $t_{3,117} = -0.554$, $p = 0.709$).

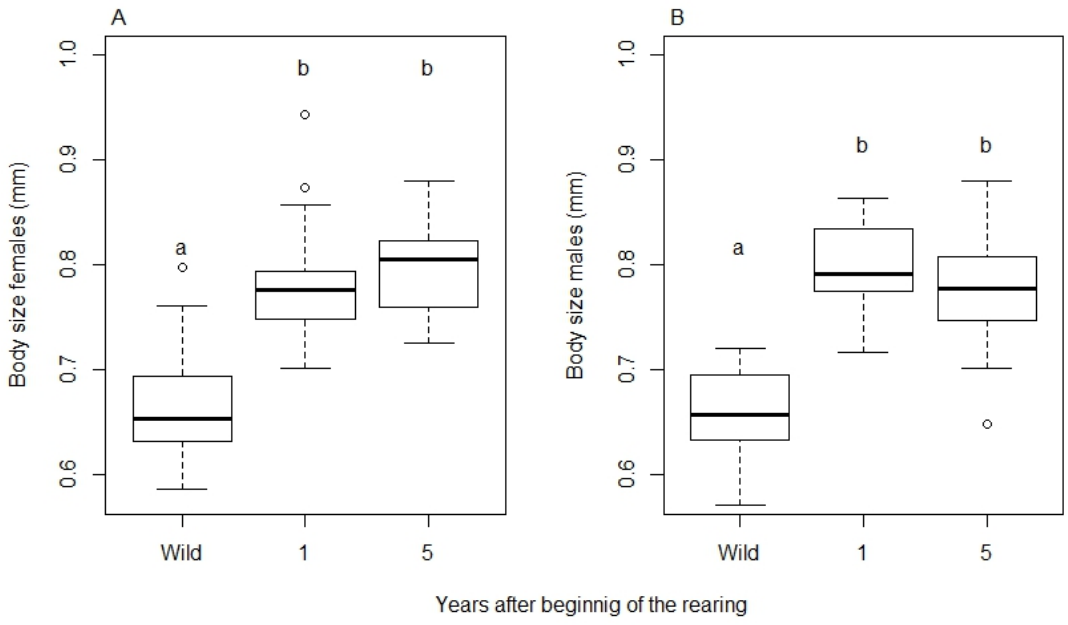

Figure 1-3 Body size of *Telenomus laeviceps* depending on rearing status.
(A) females and (B) males, wild: field sampled individuals (summer 2017). Different letters indicate significant differences (Analysis of variance, p<0.05, N=20).

Discussion

To ensure a biocontrol product of high quality, it is important to regularly check if the reared individuals belong to the correct species, especially after refreshing the reared

population with field-sampled individuals to avoid the rise of detrimental genetic conditions due to inbreeding. Furthermore, the risk of introducing competitive parasitoids in the rearing could have detrimental effects on *T. laeviceps* reproduction, emphasizing the importance of species determination before refreshing the reared population. To this end, we developed a species-specific qPCR TaqMan assay for *T. laeviceps*, which can be used to determine the presence of *T. laeviceps* in field-collected noctuid eggs, either at an early stage (as parasitized eggs) or at the imaginal stage (wasps).

After the collection and species identification of the biocontrol agent of interest, a rearing for basic studies should be established. A first attempt to rearing *T. laeviceps,* was done by placing females singly together with host eggs in rearing units and thereby avoiding intraspecific competition. However, as this is very time consuming, several adults (males and females) should be kept together in the same rearing unit to reduce workload, despite the risk of intraspecific competition. In this study, we showed that the parasitation performance of three parental females kept together was greater than the one of the control without intraspecific competition. Based on these two tested densities, we concluded that, intraspecific competition did not severely influence the parasitation performance of *T. laeviceps*. Since this trial was conducted at a lab-scale level (with just few individuals/rearing unit), further studies are needed to rule out negative effects of intraspecific competition in commercial mass rearing (several thousand individuals/rearing unit).

Among all the rearing conditions, temperature is one of the most important, influencing different aspect of a parasitoid's parasitation performance (Bueno *et al.* 2008; Legault *et al.* 2012; Pomari *et al.* 2012). The developmental time of *T. laeviceps* is inversely proportional to temperature, as shown by Bueno *et al.* (2008) for *Telenomus remus*, ranging from ten days at 30 °C to 20 days at 20 °C (Figure 1-1A). Temperature did not influence the number of parasitized eggs (Figure 1-1B), but the successful emergence of

the progeny (Figure 1-1C), indicating that regulatory processes like the production of specific immunosuppressive proteins or the injection of venoms (Beckage & Gelman 2004; Burke & Strand 2014) responsible for the deactivation of the host's immune system, were impaired, affecting the development of the parasitoid egg inside the host egg. Host-parasitoid interactions can be very complex and depend on various factors such as temperature or host hormone levels (Beckage & Gelman 2004). Thereby temperature can play an important role between the ability of a parasitoid to successfully develop and the ability of the host to defend itself (Thomas & Blanford 2003). If both the host and the parasitoid are insensitive to temperature changes, the resulting number of progeny produced will also be unaffected by temperature, but in general, host-parasitoid interactions are more complex (Thomas & Blanford 2003). For *T. laeviceps* we found an initial decrease in the number of emerging progeny with increasing temperature from 20 °C to 24 °C. The lowest number of progeny was found at 24 °C, followed by an increase with higher temperatures towards 30 °C (Figure 1-1D). This pattern can also be observed in the interaction between *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae) and larval parasitoids (Fellowes *et al.* 1999). Egg immune responses to a parasitation attempt can include encapsulation or melanization of the parasitoid egg (Reed *et al.* 2007). On the other hand, egg parasitoids have evolved strategies to counteract the host immune system. In Scelionidae, cell masses can be released during parasitoid development from the embryonic membrane and are later on referred as teratocytes, if they persist in the host's body after hatching of the parasitoid (Strand 2014). Teratocytes can synthesize proteins that inhibit the synthesis of host proteins linked to larval development (Rana *et al.* 2002; Reed *et al.* 2007). To date very little is known about how these processes operate in both *T. laeviceps* and *M. brassicae*. However, our results indicate that at 24 °C, the performance of the host immune system is highly active and/or the synthesis of proteins by teratocytes is inhibited. However,

further research is needed to better understand the interaction between *T. laeviceps-M. brassicae* under different temperature regimes.

Among insects, females can emerge with a complete stock of mature eggs, or produce them throughout their life. These two strategies are commonly known as pro-ovigeny and synovigeny (Jervis *et al.* 2007; Jervis *et al.* 2008). However, most hymenopteran parasitoids emerge with a limited amount of mature eggs and produce more throughout their life in dependency of energy, acquired through food intake (Jervis *et al.* 2001; Boivin 2010). Thus, depending on the parasitoid species, a certain amount of time is required, before they reach their full parasitation potential. To test which egg production scheme *T. laeviceps* follows, host eggs were offered at three distinct time points for parasitation. Upon emergence, females immediately parasitized an average of 20 ± 15 host eggs, just a few the following day and again an average of 20 ± 15 eggs a day later, indicating that they need a day to rebuild a stock of eggs (Figure 1-4). Hence, the average of 20 ± 15 eggs seems to be the limiting amount of eggs that *T. laeviceps* can produce per day when provided with a suitable food source (honey). On the other hand, six and eleven-day-egg deprived females parasitized 72 % of eggs (80-100 eggs) during the first day of provision, with the number of parasitized eggs per day constantly decreasing to zero within five days (Figure 1-4). Furthermore, their parasitation performance was significantly higher than the one of females parasitizing immediately after emergence, indicating that *T. laeviceps*, just like other egg parasitoids (Boivin 2010), is moderately synovigenic. If we look at the results from a more applied point of view, we need to distinguish between the performance of the parasitoid in the rearing unit and in the field as a biocontrol agent. In the first scenario, the parasitoid should be able to build a self-sustaining population, implying a high parasitation rate and a sex ratio shifted towards females.

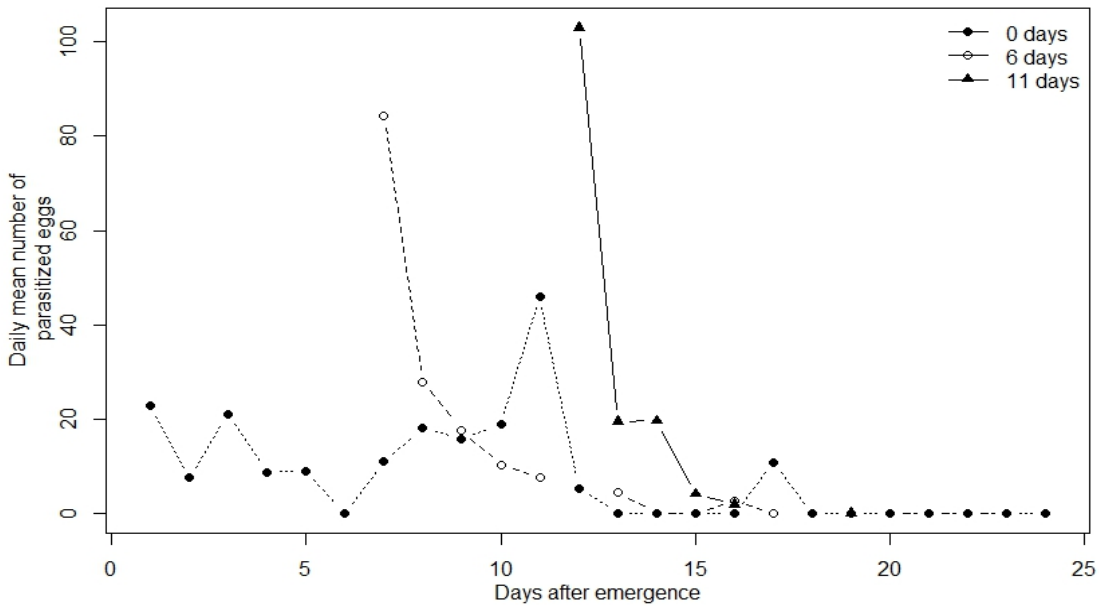

Figure 1-4 Daily mean number of parasitized eggs through females of the three egg deprivation periods, from experiment start until death of female wasps.

This is relevant to decrease production costs and provide a competitive and sustainable product. In the laboratory, it is therefore important to give females the time to build a complete stock of eggs, ultimately increasing their parasitation performance. In the field however, where food sources for beneficial insects may be low, like e.g. nectar, an ideal biocontrol agent should be able to parasitize as many hosts as possible directly after emergence. *Mamestra brassicae* egg clutches occurring in nature are smaller than the ones produced under rearing conditions, ranging from ten to 100 eggs (Injac & Krnjajic 1989). This implies that just one to five females are sufficient to parasitize such egg clutches. Our study showed that females of *T. laeviceps* constantly produce eggs during the first eight days of their life and are able to parasitize up to 20 eggs every couple of days. In an inundative biocontrol agent release, several hundred females are released in the field,

meaning that they already could potentially parasitize several thousand eggs on their emergence day.

Once a stable and efficient rearing is available, it is important to monitor the effects of prolonged rearing conditions on the biocontrol agent. As body size may be linked with longevity, fecundity and flight ability, it seems to be a good indicator of a biocontrol agents' field performance (Vet *et al.* 1994; Roitberg *et al.* 2001; Grenier & De Clercq 2003). In *Trichogramma pretiosum* Riley, 1879 (Hymenoptera: Trichogrammatidae) and *Tr. carverae* (Oatman and Pinto, 1987) (Hymenoptera: Trichogrammatidae) increased body size was linked to an increase in their flight capacity and field performance (Kazmer & Luck 1995; Bennett & Hoffmann 1998). We found an increase in the body size of *T. laeviceps* after only one year of rearing. This size remained constant even after five years and did not increase further. The increase in body size is most likely limited by the size of host eggs. Parasitoids with a specific body size are linked to hosts laying eggs of the right size to allow parasitoid development, as described for *Telenomus nitidulus* (Thomson, 1844) (Hymenoptera: Scelionidae) (Grijpmal *et al.* 1991). The cabbage moth eggs provided to *T. laeviceps*, were produced by a long-term rearing. Since this could have had an influence on the size of *M. brassicae* eggs, we collected wild eggs in Swiss cabbage fields during summer 2017 and compared their size to the one of the rearing population. We did not find any difference between wild and reared eggs (data not shown), meaning that, although reared *T. laeviceps* are bigger than the wild ones, they are still able to parasitize wild cabbage moth eggs. This further supports the idea to use *T. laeviceps* as a biocontrol agent against the cabbage moth.

The presented knowledge on *T. laeviceps* can be useful to develop a lab-scale rearing. However, the implementation of the results to achieve a mass rearing is possible, but requires further optimization.

Acknowledgments

The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 612713. We want to thank Andermatt Biocontrol AG, in particular Marcel Tanner and Monika Häberli, for providing us insect material to test the specificity of the developed marker and Dr. Norman Johnson (Ohio State University), for morphological species determination, as well as for the provision of diverse *Telenomus* spp. wasps. A big thank you also to Frederik Nagel for helping with the measurements of the body size of *Telenomus laeviceps*. Special thanks goes to Shakira Fataar, Fabian Cahenzli and Anina Knauer for valuable comments on the manuscript. We would also like to thank Pius Andermatt and Oliver Kindler (Syngenta, Stein, Switzerland) for the provision of the artificial diet used to rear the cabbage moth.

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CHAPTER 2

Sampling of *Telenomus laeviceps* in European brassica vegetable production areas and molecular species determination

Abstract

For arthropod biocontrol agents to be safely released and tested under field conditions, they need to be native for the region of interest, reducing potential side effects on non-target species. *Telenomus laeviceps* Förster, 1861 (Hymenoptera: Scelionidae) was reported to be present in some European countries, but data are very scarce. Based on its known geographic distribution, samplings of egg parasitoids were conducted in three European countries where *T. laeviceps* was expected to be present. To this end, research partners were identified in Spain, Italy and Sweden. Samplings were conducted in 2015 and 2017 by exposing trap eggs (cabbage moth) in commercial organic brassica fields (2015) or hobby gardens (2017). Parasitized eggs were incubated until parasitoid emergence and their species determined, either morphologically (at the genus level) or molecularly (at the species level).

After molecular species determination, *T. laeviceps* was detected in different samples collected in Sweden. Validation of the results by an expert taxonomist are currently on-going and if confirmed, this first record for Sweden will be submitted to the Fauna Europaea database.

Introduction

In the last decades, due to a massive boost in tourism and international trade, Europe is witnessing an increase in the number of introduced exotic plant and invertebrate pest species (Bigler *et al.* 2005). In the attempt to control these exotic pests, insecticides with ecotoxicological side effects are applied, rising the need for alternative solutions, such as classical biological control (Waage 1997; Sheppard *et al.* 2006), defined as "the intentional introduction of an exotic, usually co-evolved, biological control agent for permanent establishment and long-term pest control" (Eilenberg *et al.* 2001). This led to a drastic increase in the number of exotic invertebrate biological control agents (IBCA) introduced in different environments (van Lenteren 1997), with different publications drawing

attention on the associated risks (Howarth 1991). Most European countries have signed the convention on biological diversity, which expects the endorsing countries to "prevent the introduction of alien species and, when prevention fails, to control those exotic species, which threatened indigenous ecosystem, habitats or species, as far as possible" (UN 1992). In order to fulfil these requirements, regulatory procedures evaluating the risks of IBCA before release are essential throughout Europe. Unfortunately, depending on each country, these procedures are applied differently, with some being stricter than others. The precautionary principle is the basis of the European risk management and encourage measures that protect human health and the environment from uncertain risks (Kriebel *et al.* 2001). To regulate the registration of IBCA, as happens for synthetic plant protection products, is customary the application of the precautionary principle (Ehlers 2011). The EU Commission states that "recourse of the precautionary principle presuppose that potentially dangerous effects have been identified and that scientific evaluation does not allow the risk to be determined with sufficient certainty". It follows that, to be able to release an exotic IBCA, a complete and detailed risk assessment should be conducted and if the results are not unambiguously, the precautionary principle will be applied and releases forbidden.

In the process of developing a new IBCA, its natural geographic distribution should be taken into account, in order to first, evaluate eventual associated risks and second to enable the estimation of its field of application and thus, its market potential. To date, *Telenomus laeviceps* Förster, 1861 (Hymenoptera: Scelionidae) has been registered in the Fauna Europaea database, only for few countries (Figure 2-1, Table 1-1). The latest distribution map of *T. laeviceps* reveals that this parasitoid is present in the north-east, south, west and central of Europe. This suggests that it should be present throughout Europe and possibly also in the bordering countries. Based on that, we looked for *T. laeviceps* in Sweden, Spain and Italy, which have not reported the presence of this parasitoid yet. These countries have been chosen because *T. laeviceps* was found to

be present in neighbouring countries such as Portugal, Switzerland and Latvia. Further, the main host of this parasitoid, the cabbage moth *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae), is present in all three countries, either in high densities in the country itself or in bordering areas (EPPO 2017).

To prove the presence of *T. laeviceps* in the above mentioned countries, host eggs of the cabbage moth were exposed in commercial organic brassica fields (2015) and hobby gardens (2017). Sampled parasitoids were determined at the species level using the developed qPCR marker (CHAPTER 1) and the results were validated through morphological species determination.

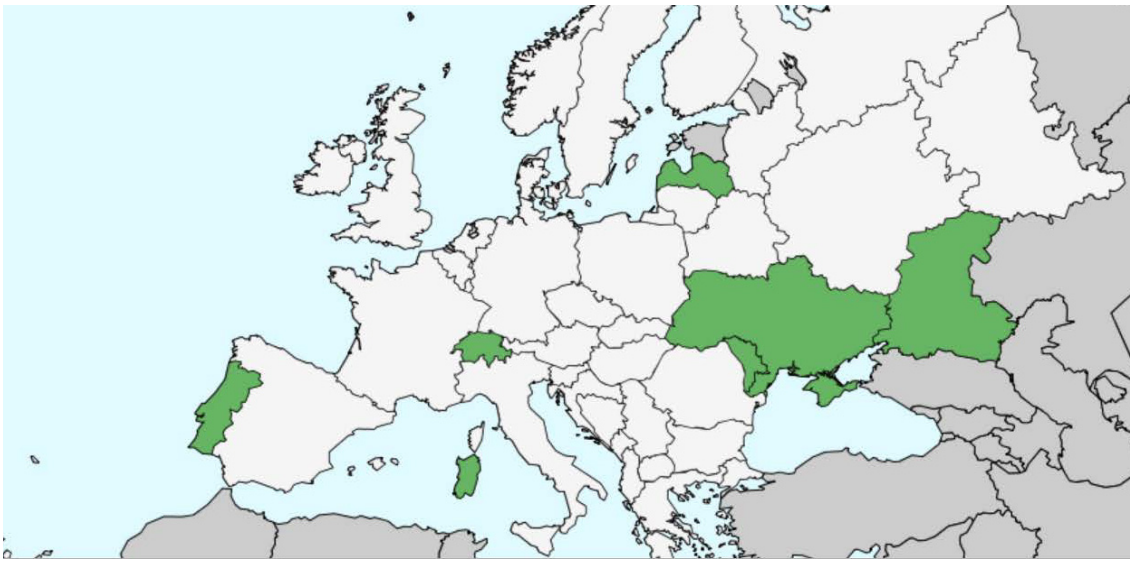

Figure 2-1 Distribution of *Telenomus laeviceps* in Europe as described in the Fauna Europaea database.

Material and methods

Sampling of T. laeviceps in brassica fields

To assess the presence of *T. laeviceps* in Spain, Italy and Sweden, egg parasitoids were collected in 2015 and 2017 through expositions of cabbage moth eggs in different regions of each country (Figure 2-2).

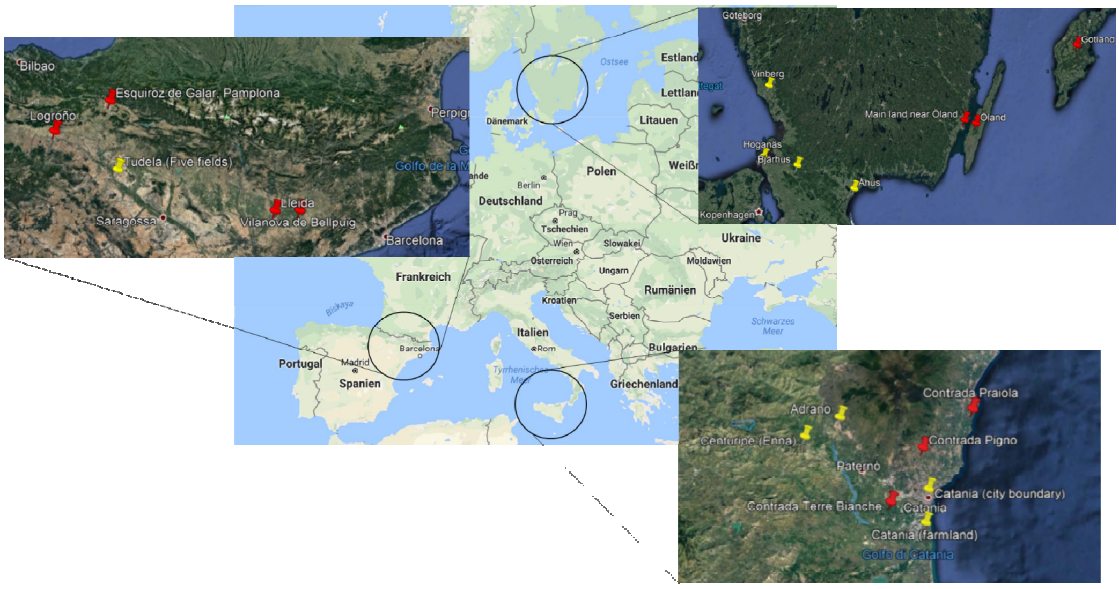

Figure 2-2 Locations of the cabbage moth egg expositions during the field trials 2015 (yellow) and 2017 (red).

In 2015, eggs were weekly shipped to the three involved partners, to contain the workload and costs associated with an in-house rearing of the cabbage moth in each partner facility. Eggs were exposed in commercial organic brassica fields. The number of fields varied between countries, with five fields located in Italy, five in Sweden and six in Spain. Expositions were planed based on the crop phenology for each country, starting at the beginning of June (Sweden and Spain) or August (Italy) and ending,

respectively in September or end of October. The sampling procedure was standardized for each partner over both years. Cabbage moth eggs were exposed during two consecutive days on the lower side of the selected plant, recollected and put in a box until larvae hatched from the unparasitized eggs. To reduce the risk of larvae feeding on the parasitized eggs, this box was placed in soapy water, allowing the dispersal of the larvae and at the same time killing them. The remaining parasitized eggs were put in a small glass tube until parasitoid emergence. Afterwards, 70 % ethanol was added in the tubes to preserve the parasitoids until molecular species determination. In 2015, each partner conducted six expositions of 20 egg clutches (150 ± 50 eggs) per exposition, for a total of 120 egg clutches exposed in each field.

During the field trials 2015 some limitations were identified and improvements undertaken for the new sampling campaign conducted in 2017. The first limitation was represented by the shipment of cabbage moth eggs. In fact, through the shipment, the eggs aged until arrival and subsequent exposition in the field, limiting the exposition time in the field. Further, the shipment of eggs drastically reduced the flexibility of the partners, because they had to adjust the expositions according to the scheduled shipment and not to the weather conditions, which are important factors influencing the field performance of many small bodied parasitoids. For these reasons, we changed the approach in 2017. Every week, each partner was provided with approx. 130 host pupae so that they could have a small cabbage moth rearing in their own facilities. That way, they were able to autonomously produce cabbage moth eggs and expose them when the conditions to find egg parasitoids were optimal. A further limitation was due to the crop protection measures applied at the field level, which included, depending on the field, the application of broad-spectrum insecticides or the use of nets to cover the crop. For these reasons in 2017 eggs were exposed in hobby gardens growing brassicas or in the proximity of organic fields. The number of expositions was not standardised, ranging

around 200 egg clutches for each country, depending on the work capacities of each partner.

DNA extraction and molecular species identification

To prepare the crude DNA extract, five adult parasitoids were placed in 2 ml screw cap tubes (Sarstedt Cat. 72.609.001) together with 0.25 ml of zirconia/silica beads (ø 0.5 mm, BioSpec Products, Bartlesville, OK, USA) and 300 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). Parasitoids were crushed using a FastPrep-24® (MP Biomedicals) for 40 s at a speed of 6 m s^{-1}. The tubes were then incubated in a heating block at 95 °C for 10 min, subsequently centrifuged at 20,000 g for 1 min and stored at -20 °C. The extracted DNA was later analysed through qPCR. The qPCR reactions were performed with Rotor-Gene Q (Model 5-Plex HRM, QIAGEN) and the 10 µl reaction volume consisted of 1 µl undiluted crude extract and 9 µl Mastermix (5 µl SsoAdvancedTM Universal Probes Supermix 2x (Bio-Rad Laboratories Inc., USA), 2 µl ultrapure water and 2 µl of pre-diluted primer and probes at a final concentration of 0.3 µM and 0.1 µM for primers and probes, respectively). The TaqMan qPCR for the amplification had following cycling conditions: 3 min at 95°C for the activation of hot start DNA polymerase, followed by 45 cycles of 5 s at 95 °C and 30 s at 60 °C. After each cycle the fluorescence was recorded for both colors FAM (green) and ROX (orange). The qPCR analysis included the DNA of the collected parasitoids, a reference sample containing *Telenomus laeviceps* DNA (positive control) and a water control (negative control).

Results

Sampling of T. laeviceps in brassica fields

Only four egg clutches were parasitized during the field trials conducted in 2015, all of them originating from Italy. After incubation, the emerging wasps were determined as individuals from the genus *Trichogramma*. In 2017, a total of 15 egg clutches, out of the several hundred exposed, were parasitized. Five egg clutches were parasitized in Spain and four in Italy, from which only *Trichogramma* spp. emerged. In Sweden, one clutch was parasitized by *Trichogramma* spp., while four by *Telenomus* sp.. While the water control in the qPCR analysis produced Cq-values > 37, the collected parasitoids produced Cq-values between 29 - 30, which lie in the range of the positive control values containing *T. laeviceps* DNA (Cq = 28 - 30). For the first time, *T. laeviceps* was identified in Sweden.

Discussion

The developed molecular markers were successfully used to determine the species of field sampled parasitoids. During the sampling campaign 2015 we failed to find *T. laeviceps* in our partner's countries, motivating us to repeat the trials in 2017. In contrast to 2015, a higher number of egg clutches were parasitized in 2017. This indicates that changing the exposition areas was crucial and that, as expected, the plant protection measures applied at the field level strongly influenced the diversity and abundance of parasitoids (Geiger *et al.* 2010; Biondi *et al.* 2012; Dudley *et al.* 2017). Through molecular species determination we confirmed that the parasitoids sampled in Sweden belong to *T. laeviceps*, enabling release field trials with this species in this country. After the final validation of the results through morphological species determination (currently on-going) this first record for Sweden will be submitted to the Fauna Europaea database. Interestingly, *T. laeviceps* was found on the islands of Gotland and Öland in Sweden

(2017, Leg. Mariann Wikström), where brassica crops were almost absent. This lead to the question: how did *T. laeviceps* reproduce, if its host host-plant and therefore its main host are absent? Carefully looking at the areas were *T. laeviceps* was collected in Sweden, we found a relatively high density of scots pines. In scots pine forests in Poland a parasitoid belonging to the genus *Telenomus* was found to parasitize eggs of the European pine moth *Dendrolimus pini* (Linnaeus, 1758) (Lepidoptera: Lasiocampinae) (A. Sierpinoka, personal communication). *D. pini* was reported to be present in many European countries, including Sweden (USDA 2012). In Germany the European pine moth is becoming an increasing problem and field surveys showed that the eggs were parasitized by *Telenomus laeviusculus* (Ratzeburg, 1844) (Hymenoptera: Scelionidae), a major antagonist of this pest (Möller & Engelmann 2008). Together with this parasitoid, three additional egg parasitoids of the genus *Telenomus,* namely *Telenomus verticillatus* (Kiefer, 1917) (Hymenoptera: Scelionidae) (Sierpińska 1998), *T. phalaenarum* (Nees, 1834) (Hymenoptera: Scelionidae) (Melis 1940) and *T. tetratomus* (Kiefer, 1917) (Hymenoptera: Scelionidae) (Malyshev 1996) were described to parasitize eggs of *D. pini* in Poland, Italy and Russia, respectively. Our finding demonstrates that *D. pini* is susceptible to distinct *Telenomus* spp. and suggests that *T. laeviceps* is also a potential antagonist of the European pine moth, but laboratory trials testing the parasitation performance of *T. laeviceps* on eggs of *D. pini* are needed. Because *T. laeviceps* has been confirmed in another country and may be able to fight another problematic pest, its market value increases.

Acknowledgments

The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 612713. The work presented in this chapter would not have been possible without the precious collaboration of our partners: ARA testing facility (Italy), Agro Plantarum (Sweden) and OpenNatur (Spain). In particular, I would like to thank, Anna Renda, Antonino Azzaro,

Mariann Wikström, Ann-Sofie Birch-Jensen, Tânia Marques and Victor Perdrix Sapiña for the conduction of the field trials in their respective countries. A special thank you to our industry partner Andermatt Biocontrol AG (Switzerland), represented by Marcel Tanner, for the provision to the partners of cabbage moth pupae during the field trials. To conclude, a huge thank you to Shakira Fataar for valuable comments on this chapter.

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CHAPTER 3

Efficacy field trials evaluating the performance of *Telenomus laeviceps* Förster, 1861 (Hymenoptera: Scelionidae) in cabbage fields

Abstract

The intensification of agriculture negatively impacts the existence of arthropod natural enemies, creating the need for alternatives, such as classical or augmentative biological control. Under laboratory conditions, the parasitoid *Telenomus laeviceps* Förster, 1861 (Hymenoptera: Scelionidae) was able to effectively parasitize eggs of the cabbage moth *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae). In organic farming, larvae of this pest are difficult to control. To date, the only efficient method against these is the application of broad-spectrum insecticides based on the active substance spinosyn. Thus, *T. laeviceps* represents a good candidate to control this pest in a more sustainable way. Field trials were conducted over three years to test the field performance of this parasitoid and to understand its plant protection potential compared to standard phytosanitary measures (spinosad applications). Results have shown that the parasitation performance of this parasitoid is highly dependent on the density of the released biocontrol agent, with the highest parasitation rate achieved after a release of 240'000 parasitoids/ha.

Introduction

Over the last 50 years, the agricultural system in developing countries has changed dramatically, witnessing a so-called green revolution, in which food crop productivity has grown enormously (Pingali 2012). This has been allowed by a combination of high rates of investment in crop research, infrastructure and market development and adequate policy support, resulting in improved crop genetic, fertilizers, irrigation and pesticides and ultimately increased crop yield per hectare cultivated land (Pingali 2012). The resulting increase in the use of agrochemicals has caused several problems, such as resistance to insecticides or environmental contamination (Parra 2010a). Furthermore, the intensification of agriculture has led to a drastic reduction in natural habitat for many insect species and the widespread use of broad-spectrum insecticides has further

exacerbated the situation (Tilman *et al.* 2001; Fuller *et al.* 2005; Geiger *et al.* 2010). This loss of natural habitat negatively affects the existence of many arthropod predators and parasitoids, decreasing their species richness, diversity and effectiveness (Naranjo & Ellsworth 2009; Bommarco *et al.* 2011; Winqvist *et al.* 2012). As a result of the green revolution, integrated pest management programs have gained importance, including biological control programs (Parra 2010a). The research objectives of universities and research institutes shifted towards the search for new biocontrol agent candidates and the acquired knowledge is transferred to companies responsible for rearing and making candidates available to end users. Biocontrol agents belonging to the families *Trichogrammatidae*, *Mymaridae*, *Scelionidae* and *Platygastridae* are already implemented in different crops (Peña *et al.* 2010). Worldwide, the most commonly used biocontrol agents belong to the genus *Trichogramma*, which are released against various lepidopteran pests, such as *Ostrinia nubilalis* (Hübner, 1796) (Lepidoptera: Crambidae) or *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae) (Oztemiz 2008; Bai *et al.* 2011). However, for other crops, such alternatives are still missing or not fully efficient.

Following the post green revolution trend, an initiative was launched in 2017 in Switzerland to direct the Swiss Confederation's subsidies to farmers who completely abandon pesticide use. If this initiative is accepted by the Swiss population, those farmers will urgently need alternatives to insecticides. In this project, we focused on the control of the cabbage moth *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae), an important pest of cabbage crops that causes high economic losses (Pfiffner *et al.* 2009). In organic farming, to date, the only effective way to prevent this pest is through the application of broad-spectrum insecticides containing the active substances spinosyn A and D, two fermentation products of the bacterium *Saccharopolyspora spinosa* (Actinomycetales: Pseudonocardiaceae) (Kirst 2010).

Here, we investigated the potentials behind the use of the egg parasitoid *Telenomus laeviceps* Förster, 1861 (Hymenoptera: Scelionidae), as a biocontrol agent of *M.*

brassicae. This egg parasitoid is distributed across Europe, able to parasitize eggs of various insect pests belonging to the Noctuidae, Geometridae and Nolidae (Mexia *et al.* 2004; Klemola *et al.* 2009; Bayle 2012; Petrov 2012). According to Parra (2010a), the following steps are essential for the development of a biocontrol agent: i) collection, identification and maintenance of a laboratory population, ii) selection of a suitable host for mass rearing, iii) biological and behavioural studies, iv) identification of an appropriate form of release and number of insects released and v) performance assessment. These last two points are investigated and discussed in this chapter. The parasitation performance of this parasitoid was investigated through laboratory studies, demonstrating that one female can parasitize around 150 host eggs (CHAPTER 1). Notably, these experiments were conducted under optimal and protected laboratory conditions. In order to test the performance of *T. laeviceps* under uncontrolled conditions, field trials were carried out over three years in white cabbage fields in Switzerland. The objectives of the first field trial were to identify the appropriate release quantity of parasitoids and to test the efficiency of the chosen field delivery system. In 2016 and 2017, the number of released parasitoids was adjusted to the equal costs of a single application of the widely used insecticide spinosad. This because of the high production costs of the biocontrol agent, which did to allow to release the more effective density found in 2015. In the 2016 and 2017 efficacy trials, the parasitation performance of *T. laeviceps* and its plant protection potential were compared to standard phytosanitary measures.

Material and methods

Proof of concept: release of two Telenomus laeviceps densities

The preliminary field trial to evaluate the parasitation efficiency of two *T. laeviceps* densities was carried out in Western Switzerland during summer 2015 in an organically

managed white cabbage field. The parasitoids were released in the following densities: 120'000 and 240'000 parasitoids/ha, and tested against a release-free control. The lower density of 120'000 parasitoids/ha was described by Oztemiz (2008) as the most suitable for releasing *Trichogramma evanescens* Westwood, 1833 (Hymenoptera: Trichogrammatidae) against eggs of *H. armigera*. Since the parasitation performance of *T. laeviceps* was not known, a higher density was also tested. The parasitoids were released into defined plots within a white cabbage field (354 x 70 m). One field was used for the trial and each treatment was repeated three times within it, resulting in a total of nine plots. The plots were 10 x 10 m wide and 20 m apart. *T. laeviceps* was released in the two tested densities four times during the cabbage growing season, in calendar weeks 24, 27, 30 and 33. The first release matched the cabbage plantation, while the following three were conducted every three weeks. The field delivery system consisted of a folded cardboard containing parasitized eggs (Figure 3-1, left). Honey-gelatine was added to the field delivery system as food for emerging adults. In fact, as shown for *Trichogramma minutum* Riley, 1871 (Hymenoptera: Trichogrammatidae), the provision of a sugar-rich food source after emergence help increase their parasitation performance (Leatemia *et al.* 1995). The adult parasitoids emerged one to two days after exposition in the field. After two releases, the predation of the parasitized eggs exposed in the delivery system was evaluated and a predation rate of about 40 % was measured. We have therefore decided to equip the field delivery system with a protective net (Figure 3-1, right), eliminating the predation problem. The parasitation rate was monitored by providing host eggs for *T. laeviceps* in the field. The cabbage moth host eggs (100 ± 50 eggs/clutch) were exposed during two days on nine selected cabbage plants within each plot, resulting in a total of 27 egg clutches per treatment and exposition. Expositions were done three times a week for a total of 28 egg expositions. This prolonged exposition period covered the whole cabbage growing season. To estimate the parasitation rate due to natural occurring *T. laeviceps*, eggs were exposed once before the first release of the parasitoids. Pictures

before and after exposure were taken to determine the parasitation rate and the predation rate of the released parasitized eggs. The parasitation rate was defined as the proportion of parasitized eggs over the recollected eggs. Wasps of 130 randomly selected samples (parasitized between June and July) were counted and the sex ratio determined.

Figure 3-1 Field delivery system without (left) and with (right) protective net against predators.

Efficacy trials comparing Telenomus laeviceps releases to standard plant protection measures

After the first field trial, it was clear that *T. laeviceps* was able to cover at least 20 m, allowing them to move between plots. In order to avoid biases due to parasitoids moving between treatments, the number of plots per field was reduced to three and the distance between them was increased from 20 to 30 m. Since the size of the fields was a limiting factor, 30 m was the maximal distance allowed by the smallest field. Trials were conducted in Switzerland for two consecutive years. In both years fields were located in Eastern Switzerland with four and six fields in 2016 and 2017, respectively. In each field, three treatments were tested. We defined a plot with *T. laeviceps* (BCA) releases, an insecticide-treated plot and an untreated control plot. Compared to 2015 (120'000 and

240’000 parasitoids/ha), the amount of parasitoids per release has been reduced to an economically feasible density of 65’000 parasitoids/ha, matching the costs of a single application of the insecticide spinosad (217 CHF/ha/application). *T. laeviceps* was released into the field with the same field delivery system as in 2015 (Figure 3-1, left), but this time without honey-gelatine and protective net. This was done to reduce the predation of the parasitized eggs due to the provision of honey-gelatine and to keep the production costs of the parasitoids as low as possible by removing the net. Two weeks after cabbage plantation in the field, the parasitoids were released for the first time. Depending on the variety of cabbage, this happened between week 22 and 28. The second release was conducted five weeks after the first release. In 2017, after a first analysis of the collected data, *T. laeviceps* was released a third time in the week 35, with an addition of honey-gelatine. In 2016, due to unknown production problems by Andermatt Biocontrol Ag (commercial partner in the project), parasitoids from the second release emerged with an emergence rate below 20 %. Therefore, a third release was conducted one week after the second. To monitor the parasitation rate, cabbage moth eggs (100 ± 50 eggs/clutch) were exposed during two days, on a weekly basis, on nine selected cabbage plants per plot. The recollected eggs were placed in a plastic box floating in soapy water and incubated until the host larvae hatched from the unparasitized eggs. The hatching larvae were allowed to disperse, falling in the water and subsequently dying. The remaining parasitized eggs were further incubated until parasitoid emergence. To reduce the number of weekly visits to the fields, UV-sterile eggs were used in 2017, reducing travel frequency from twice to once a week. The possible influence of UV-sterile eggs on the parasitation performance of *T. laeviceps* has been clarified through laboratory trials presented in chapter 5. Recollected eggs were incubated until parasitoid emergence. In both years, pictures of the exposed host eggs were taken before and after exposure. The predation rate was assessed by calculating the difference between exposed and recollected eggs. The parasitation rate was defined as

the proportion of parasitized eggs and the recollected eggs. In order to facilitate the interpretation of the results, accompanying data such as meteorological data (temperature, air humidity and precipitation) (Meteoswiss), plant protection measures applied to the field (list provided by the farmers) and the amount of pest-beneficial insects were recorded. For the last point, 12 cabbage plants per plot were selected and the number of insects recorded twice, once in June and once in July.

To help quantify the impact of *T. laeviceps* on the yield compared to the other two treatments, 12 cabbage heads per plot were harvested. We selected the plants to be harvested based of the pest-beneficial monitoring conducted during the growing season. The following variables were recorded: weight of the cabbage without stem leaves (cabbage head with damaged leaves) and final weight.

Statistical analysis

Data analyses were conducted with R version 3.3.0 (R development core team, 2016). Data from the first field trial were analysed with a generalized linear model (glmer function from the package lme4) with binomial errors, dependent variable parasitation rate, fixed factor treatment (three levels: control and two *T. laeviceps* densities) and random effect week. To determine the influence that each biocontrol agent release had on parasitation rate, different regression curves (linear, quadratic or polynomial) were fitted. The models based on different regression curves were compared using ANOVA and the best fitting model chosen (Crawley 2007).

Since no parasitation was measured during the field trials 2016, these data concerning the parasitation performance were not statistically analysed. But, the predation rate was analysed with a generalized linear model with binomial errors, fixed factor treatment (three levels: BCA, control and insecticide), weather data (temperature, precipitation and humidity) as co-variables and random factors field and week.

Data from the efficacy field trials 2017 measuring the predation rate of the exposed eggs between the three treatments were analysed with a generalized linear model with binomial errors, fixed factor treatment (two levels: BCA, insecticide and control), weather data (temperature, precipitation and humidity) as co-variables and field and week as random factors. Data of the total parasitation rate and of *T. laeviceps* parasitation alone, were analysed with the same model as the one used for the predation rate. Data collected after the third release with provision of honey-gelatine were not integrated in the analysis.

The weight of a cabbage head is strongly dependent on the cabbage variety. Since it is extremely difficult to find fields growing the same variety, the comparison of the yields becomes difficult and constrained to one single field. To compare the influence of treatments on the cabbage weight between different fields, a standardized value is needed. At this end, we calculated the proportion of weight loss, a standardized value to compare different cabbage varieties:

$$Proportion\ of\ weight\ loss = \frac{weight\ with\ damaged\ leaves - \ final\ weight}{weight\ with\ damaged\ leaves}$$

These data were afterwards analysed using a linear mixed effects model, with treatment (three levels: BCA, control and insecticide) as fixed factor and field as random factor.

Results

Proof of concept: release of two Telenomus laeviceps densities

No difference was found in the parasitation rate of *T. laeviceps* between the control plot and the two plots with the distinct *T. laeviceps* densities (generalized linear model, all $p > 0.75$), respectively 36 ± 2 %, 36 ± 2 % (1200 wasps/100 m^2) and 35 ± 2 % (2400 wasps/100

m^2). The mean weekly parasitation rates ranged between 0 and 70 % (Figure 3-2) and the overall mean proportion of females was 66.53 %.

The mean parasitation rate after each *T. laeviceps* release was significantly dependent on the release number, showing a polynomial fitting of the model to the data, with maximal values reached after the second release (polynomial regression, $F_{2,25}$ = 6.545, p = 0.005) (Figure 3-3).

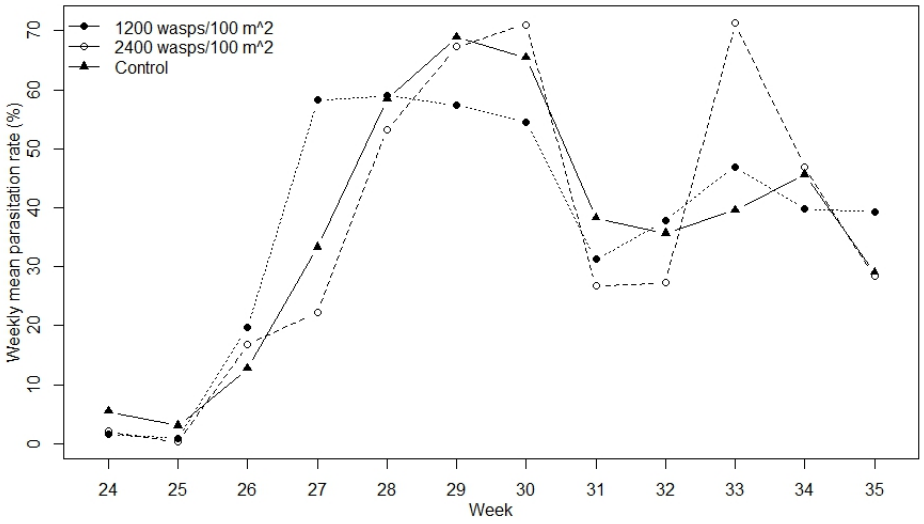

Figure 3-2 Weekly mean parasitation rate for the three treatments during the preliminary field trial 2015.

Efficacy trials comparing Telenomus laeviceps releases to standard plant protection measures

Due to unknown production problems in 2016, the emergence rate of *T. laeviceps* in the field was lower than 20 %. Consequently, no parasitation was measured in any of the fields. The predation rate of the exposed eggs was significantly lower in the insecticide treated plot, than in the untreated (Generalized linear model, z = -2.429, p = 0.015) and BCA treated plots (Generalized linear model, z = 2.721, p = 0.007). No difference was

found between the other two plots (Generalized linear model, $p > 0.05$). In the insecticide treated plot the mean predation rate was 1.03 ± 6.36 %, while in the BCA and untreated plots it was 8.56 ± 2.95 % and 7.66 ± 3.29 %, respectively. Weather variables did not influence the predation rate.

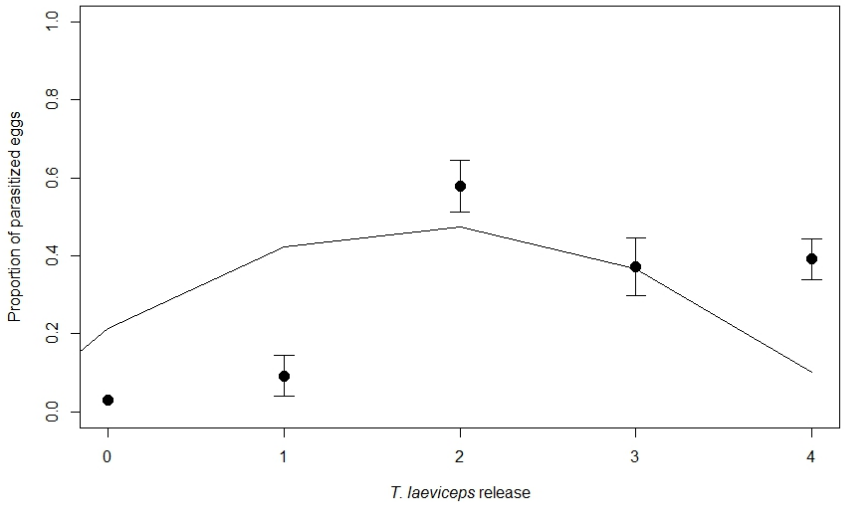

Figure 3-3 Mean parasitation rate measured in 2015 after each *T. laeviceps* release. 0: parasitation rate due to the wild population measured before parasitoid releases (Polynomial regression, $F_{2,25} = 6.545$, $p = 0.005$).

In contrast to what was found in 2016, the predation rate of the exposed eggs was not influenced by treatment (Generalized linear model, all $p > 0.05$) in 2017. Total parasitation rate and parasitation rate of *T. laeviceps* were also not influenced by the three treatments (generalized linear model, all $p > 0.05$), with an average parasitation rate of 0.75 ± 0.41 % due to *T. laeviceps* in BCA treatment, 0.45 ± 0.35 % in the insecticide treatment and 0.1 ± 0.07 % in the control. The proportion of female offspring was significantly higher in the insecticide treated plots (87.79 ± 7.49 %) compared to the BCA plots (47.15 ± 8.88 %) (Generalized linear model, $z = 2.99$, $p = 0.003$) and marginal significantly higher compared to the control plots (36.67 ± 4.79 %) (Generalized linear

model, z = - 1.827, p = 0.067). Weather variables did not influence neither the predation nor the parasitation rate.

In both years, no significant difference in the proportion of weight loss was measured (linear mixed effect model, all $p > 0.05$).

Discussion

In 2015 we conducted the first releases of *T. laeviceps* with the density corresponding to the one applied for *Trichogramma* spp. of 120'000 parasitoids/ha (Oztemiz 2008). As at the time little was known about the parasitation potential of *T. laeviceps*, a higher density of 240'000 parasitoids/ha was also tested. The results were very promising, with a maximum parasitation rate of almost 70 %. Despite this success, the production of the biocontrol agent was too expensive and unmarketable. Therefore, the amount of parasitoids released per hectare was reduced, matching the costs of one application of the insecticide spinosad. The economically feasible number of 65'000 parasitoids/ha has been calculated on the basis of the current production system. This was the density released in the efficacy trials conducted in 2016 and 2017. Based on the results of the trials performed in 2015, the number of releases was decreased to two. In fact, in 2015 the parasitation rate markedly raised after the second release and remained constant after the third and the fourth.

As already mentioned above, in 2016 some unknown production problems compromised the quality of the released biocontrol agent, which emerged at an insufficient rate in the field. As a result, the outcome of the trials did not meet the expectations of detecting parasitation rates other than zero. The absence of parasitation due to natural occurring *T. laeviceps* could be due to the different plant protection measures applied to the 2015 and 2016 fields. In fact, in 2015 the field was extensively managed, helping natural populations to better survive and establish. The incompatibility of biocontrol agents with insecticides and fungicides is a known

problem for several systems (Thomson *et al.* 2000; Cônsoli *et al.* 2001; Carvalho *et al.* 2003; Manzoni *et al.* 2006; Giolo *et al.* 2007). An interesting result of this trial lays in the predation rate of the exposed cabbage moth eggs, which was significantly lower in insecticide-treated plots, confirming the negative effects these compounds have on the overall biodiversity of a crop field (Naranjo & Ellsworth 2009; Bommarco *et al.* 2011; Winqvist *et al.* 2012).

In 2017 the emergence rates of the released parasitoids was optimal, but not the measured parasitation rates, as these were far below our expectations. A mean parasitation rate around 0.1-0.75 % is insufficient to control the cabbage moth. It also indicates that, taking into account the absent parasitation rate of 2016, obtained without sufficient release of biocontrol agents, by releasing 65'000 parasitoids/ha the parasitation rate achieved was still insufficient. Under laboratory conditions, a well-nourished female can parasitize up to 150 host eggs (CHAPTER 1). 65'000 released parasitoids with a proportion of 70 % females (45'500 females) should be able to parasitize more than 6 Mio. eggs. Therefore, this density should be enough to control the cabbage moth effectively. As in 2016, these fields were intensively managed. Although *T. laeviceps* was released in an insecticide-free plot, the surrounding areas were treated, making the plot a sort of island from which the parasitoids could not escape. Considering that *T. laeviceps* move primarily by walking between plants, the chance to come into contact with insecticides is high, reducing the effective number of surviving wasps.

The role that a sugar-rich food source plays in the parasitation performance of *T. laeviceps* in the field is not certain. In the laboratory trials showed that *T. laeviceps* is moderately synovigenic (CHAPTER 1), which means that they emerge with a limited number of mature eggs, and that a complete stock is built throughout a female's life. This implies that adults must be provided with an adequate food source to reach their optimal parasitation performance (Boivin 2010). This point has already been discussed for *T. minutum* where the authors stressed the importance of feeding the adult

parasitoids with a sugar-rich food source to increase their parasitation performance in the field (Leatemia *et al.* 1995). On the other hand, laboratory trials showed that *T. laeviceps* can parasitize on average 20 ± 15 host eggs on their emergence day (CHAPTER 1), meaning that 45'500 females should be able to parasitize almost 1 Mio. eggs right after emergence. This, together with the increased predation of the released parasitized eggs due to the provision of honey-gelatine, led us to test in 2016 and 2017 releases of *T. laeviceps* without food provision. The third release conducted in 2017 with provision of honey-gelatine did not increased the parasitation in the field. This further suggested that honey-gelatine is not mandatory for *T. laeviceps*. Although the importance for *T. laeviceps* of sugar provision in the field is not certain, we tested, first under laboratory conditions and then in the field, the role of flowers in the promotion of survival and fecundity of this parasitoid. The trials and the results are presented in the chapters 4 and 5 of this thesis.

In 2016, we were not able to evaluate the effects of the lower density, because the lack of parasitation was led back to the insufficient emergence rate. The limitations of the conducted field trials were not visible and therefore it was not possible to correct them in 2017. A possibility to increase the effectiveness of *T. laeviceps*, without an increase in the density, is to release mated adult parasitoids. As showed in chapter 1, females reach their optimal parasitation performance after a period of egg deprivation, during which they are allowed to mate and feed. However, this would imply the development of a new field delivery system suitable for the adult *T. laeviceps*. Further, it would be necessary to maintain and store the adults until they are released in the field, which could lead to higher production costs. To reduce the production costs, one possibility could be to find an alternative host cheaper to produce and maintain, allowing costs to be reallocated to the maintenance of the adult parasitoids. The use of factitious host is widely implemented in the production of several biocontrol agents (Parra 2010b; Sivinski 2013). However, it is known that the genus *Telenomus* has a

narrow host-range, making difficult to find alternative hosts (Mills 2010). Therefore, this genus is to consider costly to mass rear and difficult to use as biocontrol agent.

These field trials add further evidences in the difficulties encountered when testing the effectiveness of biocontrol agents. In fact, the benefits of released biocontrol agents have been demonstrated only in 20 % of the studies (Gurr & Kvedaras 2010; Sivinski 2013). Based on what discussed above, there is still a lot of work to be done before the product is ready for the market. The proof of concept and several laboratory experiments have confirmed the potential of *T. laeviceps* as biocontrol agent to control the cabbage moth. Further works should be done to optimize the production and identify the most suitable formulation to release *T. laeviceps* in the field.

Acknowledgments

This study was funded by the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 612713. Thank you to Andermatt Biocontrol for the provision of *T. laeviceps* used in the field trials 2016 and to Pius Andermatt and Oliver Kindler (Syngenta, Stein, Switzerland) for the provision of the artificial diet used to rear the cabbage moths. A huge thank you to all the involved farmers for the appreciated collaboration. Special thanks go to Shakira Fataar for valuable comments and discussions about the manuscript, as well as for the help in the field.

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CHAPTER 4

Selective flowers to attract and enhance *Telenomus laeviceps* (Hymenoptera: Scelionidae): a released biocontrol agent of *Mamestra brassicae* (Lepidoptera: Noctuidae)

Barloggio G., Tamm L., Nagel P. and Luka H. (2018), Bulletin of Entomological Research

Abstract

The importance of the right food source for the survival and reproduction of certain insect species is well documented. In the case of biocontrol agents, this is even more important in order to reach a high predation or parasitation performance. The egg parasitoid *Telenomus laeviceps* Förster, 1861 (Hymenoptera: Scelionidae) is a promising candidate for mass release as biological control agent of the cabbage moth *Mamestra brassicae* (Linnaeus 1758) (Lepidoptera: Noctuidae). However, adult *T. laeviceps* need a sugar rich food source to increase their parasitation performance and produce a good amount of female offspring. For example, honey added in the field delivery system or nectar provided by sown flower strips could be suitable food sources. In Y-tube olfactometer experiments, we first tested whether the three nectar providing plant species *Centaurea cyanus* L. (Asteraceae), *Fagopyrum esculentum* Moench (Polygonaceae) and *Vicia sativa* L. (Fabaceae) are attractive to *T. laeviceps*. Furthermore, we assessed their effects on survival and parasitation performance of adult *T. laeviceps*. We found that flowers of *F. esculentum* and *C. cyanus* were attractive in contrast to *V. sativa.* Also fecundity and the number of female offspring produced was higher for females kept on *F. esculentum* and *C. cyanus* than on *V. sativa.* In contrast, survival was similar on all treatments. Our findings present a further key step towards the implementation of *T. laeviceps* as biocontrol agent.

Introduction

In the life-history of organisms, resources to invest in reproduction can be gained following two main strategies (Stephens *et al.* 2009). Capital breeders are born with an accumulation of resources in their bodies that can be directly invested into reproduction, while income breeders acquire the needed resources immediately before reproduction (Stephens *et al.* 2009). Among insects, these two strategies are often referred as pro-ovigeny and synovigeny, where the first one indicates females born with a complete

stock of mature eggs, while in the second one these stock is absent or limited and eggs are produced throughout adult life (Jervis *et al.* 2007; Jervis *et al.* 2008). Most hymenopteran parasitoids are however between these two extremes and are considered to be moderately synovigenic, usually emerging with very limited larval reserves and die within few days without access to a suitable adult food source (Jervis *et al.* 2001; Boivin 2010). Thus, adults require suitable non-host food sources to satisfy 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). Also small egg parasitoids from the genus Trichogramma and Telenomus benefit from sugar rich food sources, as shown for *Trichogramma pretiosum* Riley, 1879 (Hymenoptera: Trichogrammatidae), *Trichogramma platneri* Nagarkatti, 1975 (Hymenoptera: Trichogrammatidae) and *Telenomus podisi* Ashmead, 1893 (Hymenoptera: Scelionidae) (Ashley & Gonzalez 1974; McDougall & Mills 1997; Lahiri et al. 2017). *Trichogramma* spp. are widely used in agriculture as biocontrol agents (Parra 2010), but because *Trichogramma* spp. are clearly synovigenic, it can be challenging to apply them efficiently for pest control and keep the maintenance costs as low as possible (Mills *et al.* 2000). Indeed, studies in agricultural fields conducted on *Trichogramma* spp. demonstrated the importance of a constant food provision throughout the parasitoid life and not just before their release in the field to ensure high parasitation rates (Ashley & Gonzalez 1974; Leatemia *et al.* 1995; Díaz *et al.* 2012). Under field conditions, *Trichogramma* spp. was observed to consume nectar of different flowers near crop fields, like e.g. red clover (*Trifolium pratense* L.), buckwheat (*Fagopyrum esculentum* Moench), mustard (*Brassica juncea* L.), dill (*Anethum graveolens* L.) or avocado flowers (*Persea americana* Mill.) (Wellinga & Wysoki 1989; Begum *et al.* 2004; Begum *et al.* 2006; Witting-Bissinger *et al.* 2008; Díaz *et al.* 2012). Therefore, herbs in flower strips, planted next to the crop plants threatened by pest insects, could serve as nectar sources providing sugar and other nutrients (Witting-Bissinger *et al.* 2008; Balzan

& Wäckers 2013; Balzan *et al.* 2016). However, apart from nectar quality and quantity, nectar accessibility, determined by flower morphology and the presence of extra-floral nectar, is decisive for small bodied parasitoids. This emphasizes the importance to select the right plant species for flower strips depending on the parasitoid of interest (Patt *et al.* 1997; Tooker & Hanks 2000; Vattala *et al.* 2006).

The present study is focused on *Telenomus laeviceps* Förster, 1861 (Hymenoptera: Scelionidae), an egg parasitoid distributed across Europe, able to parasitize eggs of different insect pests belonging to the Noctuidae, Geometridae and Nolidae (Mexia *et al.* 2004; Klemola *et al.* 2009; Bayle 2012; Petrov 2012). This parasitoid can be used in brassica fields as biocontrol agent against the cabbage moth *Mamestra brassicae* L. (Lepidoptera: Noctuidae) (CHAPTER 1). Studies have been conducted on its biology and the results clearly showed that *T. laeviceps* emerges with limited larval reserves (CHAPTER 1). This implies that, in order to reach a maximum parasitation performance and proportion of female offspring, sugar rich food sources are needed directly after adult emergence (CHAPTER 1). Since *T. laeviceps* is released via field delivery systems as parasitized eggs, similar to the one commercially used for different *Trichogramma* species, adult wasps emerge directly in the field, benefiting from an easily exploitable food source near the release point. A possible solution would be the addition of honey to the field delivery systems. However, preliminary field trials revealed that the provided honey indirectly increased predation of the exposed parasitized eggs, reducing the effective number of released parasitoids. Furthermore, honey should be provided only shortly before the exposition in the field of the field delivery systems, causing additional efforts and costs for end users. Besides honey, a further solution can be the provision of nectar sources near the crop field, for example as planted flower strips, as already implemented for some *Trichogramma* spp. that are used as biocontrol agents (Wellinga & Wysoki 1989; Begum *et al.* 2004; Begum *et al.* 2006; Díaz *et al.* 2012).

In the present work, we focused on the promotion of *Telenomus laeviceps* through the provision of nectar sources. We conducted laboratory trials with cornflower, *Centaurea cyanus* L. (Asteraceae); buckwheat, *Fagopyrum esculentum* Moench (Polygonaceae) and common vetch, *Vicia sativa* L. (Fabaceae) to test their influence on the parasitation performance of *T. laeviceps*. These flowers are already commercialized as main components of a tailored flower strip for the promotion of beneficial insects of cabbage pests (Balmer *et al.* 2013; Balmer *et al.* 2014). Besides being part of the tailored flower strip, these flowers were already implemented to promote other parasitoid species, like *Microplitis mediator* (Haliday, 1834) (Hymenoptera: Braconidae), *Dolichogenidea tasmanica* (Cameron, 1912) (Hymenoptera: Braconidae), *Trichogramma* spp. or *Anagyrus pseudococci* (Girault, 1915) (Hymenotpera: Encyrtidae) (Berndt *et al.* 2002; Witting-Bissinger *et al.* 2008; Géneau *et al.* 2012; Géneau *et al.* 2013; Irvin & Hoddle 2015). Furthermore, these flowers did not increase the survival nor the fecundity of *M. brassicae*, the most important host of *T. laeviceps* (Géneau *et al.* 2012). Since the olfactory attractiveness can help reducing the time spent searching for food and therefore increase the *per capita* host searching efficiency (Wäckers & Swaans 1993; Hegazi *et al.* 2000; Jervis & Heimpel 2007; Jervis *et al.* 2008), olfactometer trials were conducted to determine the attractiveness potential of these flowers towards *T. laeviceps*. However, attractiveness alone is not enough to reach the desired pest control. Hence, we conducted laboratory experiments testing the influence of the selected flowers on survival and parasitation performance of *T. laeviceps*.

Material and methods

Parasitoid

Rearing of the egg parasitoid *T. laeviceps* started in 2012 at the Research Institute of Organic Agriculture (FiBL), Switzerland, from individuals collected with trap eggs

(cabbage moth *M. brassicae*) from organic cabbage fields in the Swiss Plateau (47th parallel north). *T. laeviceps* was reared in glass tubes (14.5 cm, ø 3 cm) on cabbage moth eggs in a climatic chamber at 22±2 °C, 16:8 (L:D) photoperiod and 55±5 % RH. To ensure the supply of a sufficient number of adult wasps for the experiments, three new rearing units were started weekly. A rearing unit consisted of 1500-2000 cabbage moth eggs (<24h old) and approximately 100 ten days old wasps (70% females and 30% males). Females were allowed to parasitize the provided batch of eggs during seven days. Afterwards, the parasitized eggs were placed in an empty rearing unit until wasp emergence. Parasitoids were fed with honey-gelatine *ad libitum* (200 g flower honey (Switzerland), 100 ml demineralized water and 3 g gelatine (Dr. Oetker, Germany)), provided on a piece of white paper placed in each rearing unit. With these rearing conditions, following generations of parasitoids emerge 14 days after parasitation started.

Plants

The flowering plants used in these experiments were grown in climatic chambers (GroBanks (CLF Plant Climatics, Germany)) at 24 °C (day) and 18 °C (night), 55±5 % RH and with a 16:8 (L:D) photoperiod. To ensure a constant supply of flowers, weekly 14 seeds per flower species were sown in 4x4 cm pressed soil blocks (Schwarz AG, Villigen, Switzerland). After three weeks, seedlings were transplanted to 12 cm diameter pots (10 cm height) in soil (Einheitserde Classic, Gebrüder Patzer GmbH & Co.KG, Germany) fertilized with slow-release formulation fertilizer (3 g/l of Tardit 3M (Haubert HBG Dünger AG, Switzerland)). Plants were regularly checked and watered as needed.

Olfactory attractiveness of different flowers for Telenomus laeviceps

The attractiveness of the flowering plants was tested in a Y-tube olfactometer as described by Belz *et al.* (2013). The experiments were conducted in a dark room, during

the period of main parasitoid activity, between 10 and 12 am. An artificial light source (20 W) was placed 28 cm above the Y-tube glass. The humidified charcoal-filtered air passed at a speed of 757 ml/min through two glass containers, each containing one odour. A visual barrier was placed between the Y-tube and the two odour containers (Figure 4-1).

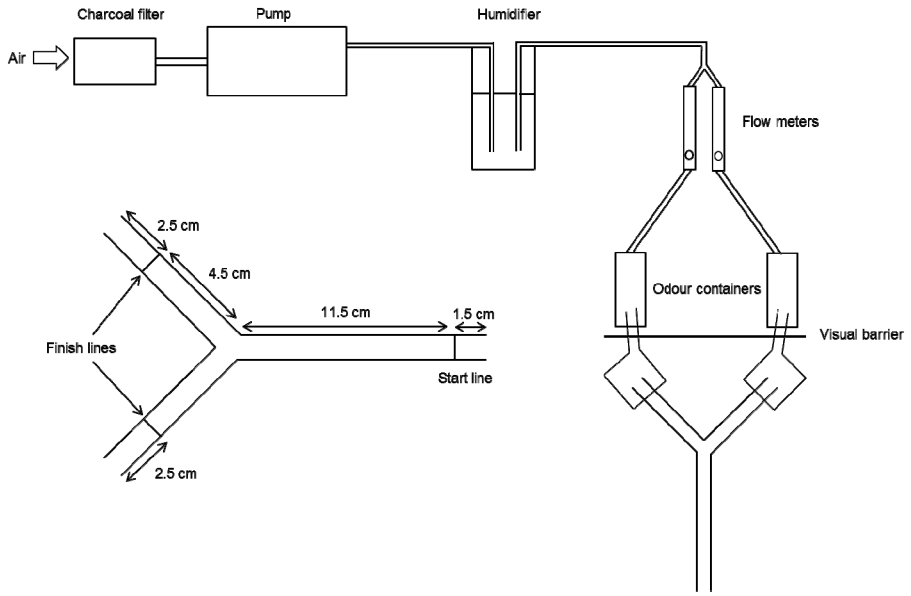

Figure 4-1 Set up of the Y-tube olfactometer.
Air passes through two odour containers and enters the Y-tube. Parasitoids were inserted at the base of the Y-tube and the assessments were started when the wasps crossed the start line.

Newly hatched (<24 h old) and unfed *T. laeviceps* females were used for the experiments. Females were inserted at the base of the Y-tube and the assessment started when they crossed the start line (Figure 4-1). They had 5 min time to take a decision by crossing one of the two finish lines (Figure 4-1). If they did not chose within the given time, they were discarded from the experiment.

We tested the attractiveness of the three flower species *C. cyanus, V. sativa* and *F. esculentum* against ambient air only. *C. cyanus* and *F. esculentum* were, in addition, tested one against the other. During the experiments *V. sativa* flowers were absent, therefore

only the attractiveness of plants displaying extra-floral nectar was tested. Thirty parasitoids were tested per comparison. Flower buds for *V. sativa* or inflorescences for *C. cyanus* and *F. esculentum* were freshly cut and placed in the odour containers. For *V. sativa*, the presence of extra-floral nectar was confirmed with the help of a binocular. After six tested wasps, the odour sources were renewed. The position of the odour sources was switched after three wasps had been tested to avoid biases due to positional effects.

Survival and parasitation performance of Telenomus laeviceps in the presence of different nectar resources

We tested the influence of nectar availability of *C. cyanus*, *F. esculentum*, *V. sativa* and a water control on the survival and parasitation performance of *T. laeviceps*. During the experiments, flowers of *V. sativa* were absent, therefore only plants presenting extra-floral nectar were used.

Parasitation performance and survival experiments were conducted in plastic cages (Figure 4-2) in a laboratory at 23 ± 2 °C and 90 ± 9 % RH in the presence of flowers and at 23 ± 1 °C and 46 ± 6 % RH in the negative control. Temperature and relative humidity were measured inside the cages with small data loggers (DS1923 Hygrochron, Thermodata). In contrast to temperature, relative humidity in the plastic cages differed between the control and the three flowers. To exclude biases in the results due to this difference, a small trial was conducted in a climatic chamber with higher RH values compared to the laboratory (55±5 %). Under higher RH the parasitoids died within one or two days, as in the control with lower RH (data not shown). Plastic cages were designed to allow air circulation and at the same time to prevent the small parasitoids from escaping. 1.5 l plastic bottles opened at the bottom and closed with a sponge cut in the middle were used as cages. This opening allowed the insertion of the flowers in the

cages (Figure 4-2). On the top, bottles were closed with wet cotton, which was daily watered to ensure water provision during the whole experiment.

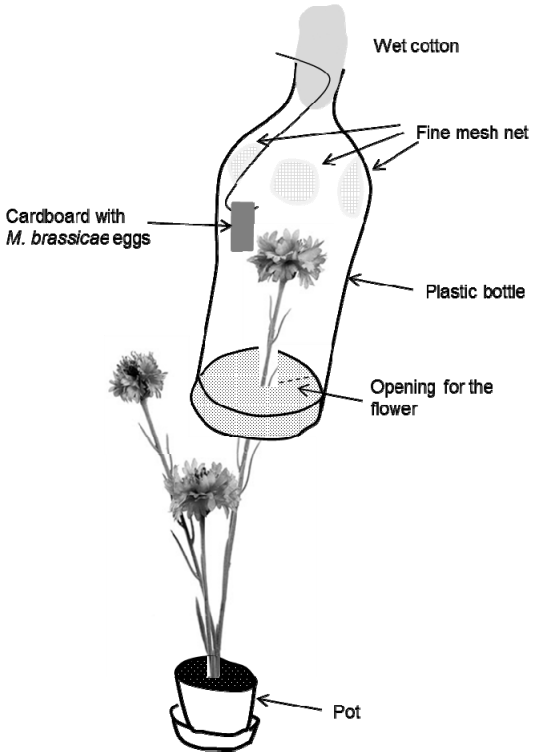

Figure 4-2 Set up of the cage used for the fecundity and survival experiments.
A plastic bottle was opened at the bottom and closed with a sponge, cut in the middle to allow the positioning of the flower. For the fecundity experiments, additional to the flower, *M. brassicae* eggs were provided.

Plants in pots were used for the trials. To assess the survival of *T. laeviceps*, one female and one male (both <24h old) were placed in a plastic cage in the presence of one of the four treatments. For the fecundity experiment, cabbage moth eggs were additionally provided at the top of the bottle, on a daily basis, until female death (Figure 4-2). In both trials, mortality was assessed daily at 9.30 am. For both trials, ten replicates per treatment were tested. One replicate per treatment was started weekly. The parasitized

eggs were counted, as well as the number of offspring hatched. The sex of the offspring was also determined.

Statistical analysis

Data analyses were conducted with R version 3.3.0 (R development core team, 2016). The count data from the olfactometer experiment were analysed with a Pearson's Chi-square test by comparing the observed frequencies against 0.5 (expected frequency of the zero hypotheses: no preference).

Data from the survival experiment were interval censored and therefore plotted following the non-parametric maximum likelihood estimate for the distribution of interval censored data. The overall influence of treatments on survival was tested by an asymptotic Logrank k-sample test. Since this kind of analysis does not allow a post-hoc test for the pairwise comparison of the different treatments, single analyses were conducted through the asymptotic Logrank two-sample test. The resulting p-values were adjusted with the Bonferroni correction for multiple comparisons. The survival data were fitted to the model with the icfit and ictest function included in the interval package.

The number of parasitized eggs and the number of females produced were analyzed with generalized linear models with Poisson errors (glmer function from the package lme4) and the fixed factor treatment (four levels: water and the three flower species), corrected for overdispersion.

Results

Olfactory attractiveness of different flowers for Telenomus laeviceps

Out of the three flowers tested, only *C. cyanus* and *F. esculentum* were significantly attractive for *T. laeviceps* females compared to the control. We found no significant

difference between *V. sativa* (extra-floral nectar only) and the control. *C. cyanus* and *F. esculentum* were found to be equally attractive (Figure 4-3A and B).

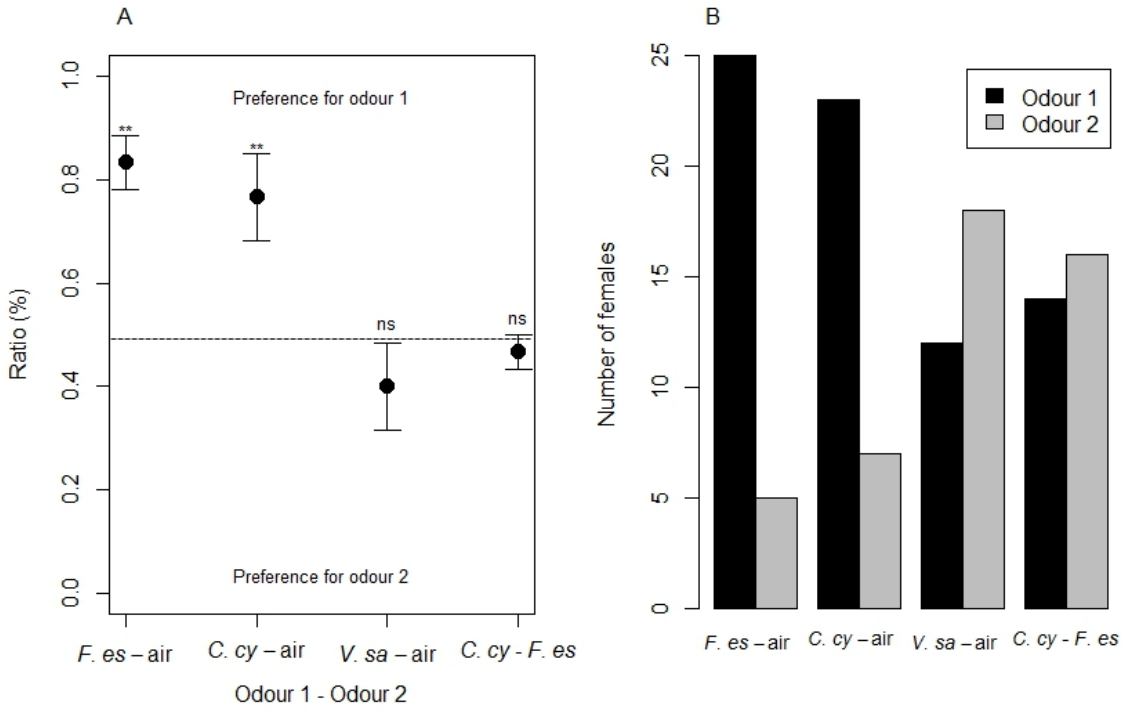

Figure 4-3 Results of the Y-tube olfactometer trials.
A) proportion of females choosing odour 1. Values above the dotted line (expected frequency = 0.5) indicate a preference for odour 1 and below for odour 2. (B) Number of females choosing either odour 1 or odour 2. F. es: *F. esculentum*; C. cy: *C. cyanus* and V. sa: *V. sativa*. Pearson's Chi-square test, ** p < 0.01; ns : not significant (p > 0.05), N = 30 per treatment.

Survival and parasitation performance of Telenomus laeviceps in the presence of different nectar resources

The presence of eggs during the survival experiment did not influence the survival of both females and males (asymptotic Logrank two-sample test, all p<0.3). We therefore

decided to not discriminate between parasitizing or not parasitizing females by pooling the data for each treatment. We found a significantly higher survival rate of *T. laeviceps* females for all three flowers tested compared to the control (asymptotic Logrank k-sample test, N=20, all $p<0.0001$). In the presence of *V. sativa* (extra-floral nectar only), *F. esculentum* and *C. cyanus* (floral and extra-floral nectar), females survived significantly longer compared to water (Figure 4-4A). No difference was found in the survival of females between the three flowers (asymptotic Logrank two-sample test, N=20, all $p>0.8$). Similar results were found for males (Figure 4-4B).

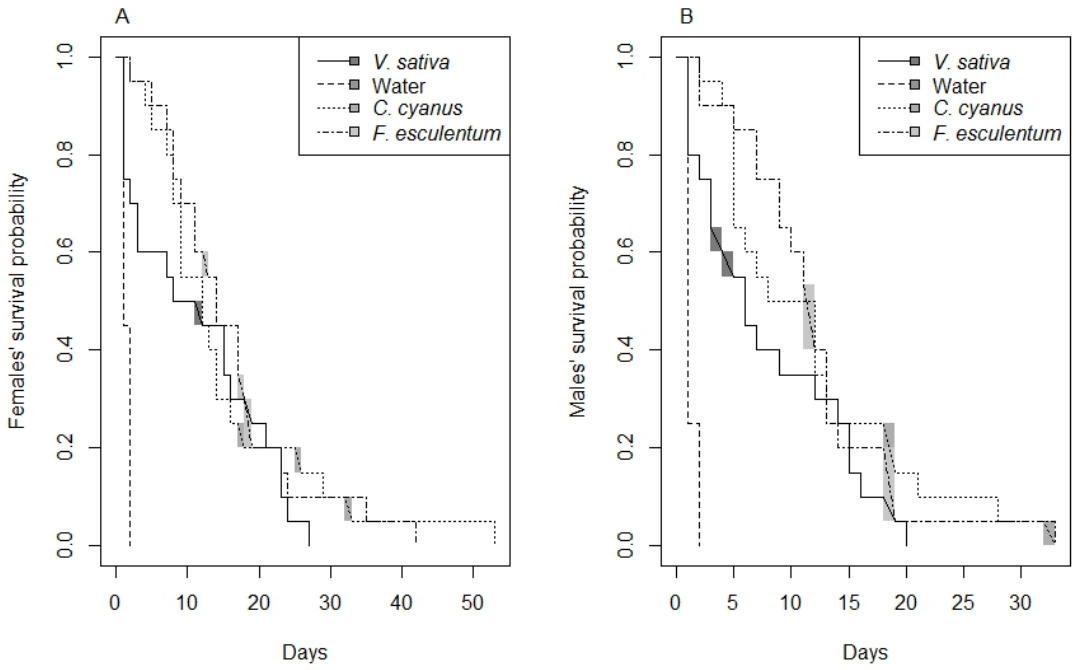

Figure 4-4 Survival of *T. laeviceps* in the presence of three nectar providers.
(A) Survival of *T. laeviceps* females in presence of *V. sativa*, *C. cyanus*, *F. esculentum* and water. The three flowers significantly increased their longevity (asymptotic Logrank k-sample test, N=20 per treatment, $\chi^2 = 17.87$, $p = 0.0005$). (B) Survival of *T. laeviceps* males in presence of *V. sativa*, *C. cyanus*, *F. esculentum* and water. The three flowers significantly increased their longevity (asymptotic Logrank k-sample test, N=20 per treatment, $\chi^2 = 20.517$, $p = 0.0001$).

Similar to survival, the number of parasitized eggs in the water control (19.1 ± 11.2 eggs) was significantly lower than in the *F. esculentum* (204.7 ± 42 eggs; generalized linear model, z = 3.542, p < 0.0001) and *C. cyanus* (202.5 ± 42.8 eggs; generalized linear model, z = 3.531, p < 0.0001) treatment.

No significant difference was found between the water control and *V. sativa* (144.8 ± 67.91) (generalized linear model, z = 1.666, p = 0.09). *C. cyanus* and *F. esculentum* equally enhanced the fecundity of *T. laeviceps* females (generalized linear model, z = -0.013, p = 0.989), but significantly differed to *V. sativa* (generalized linear model, both p<0.03) (Figure 4-5A).

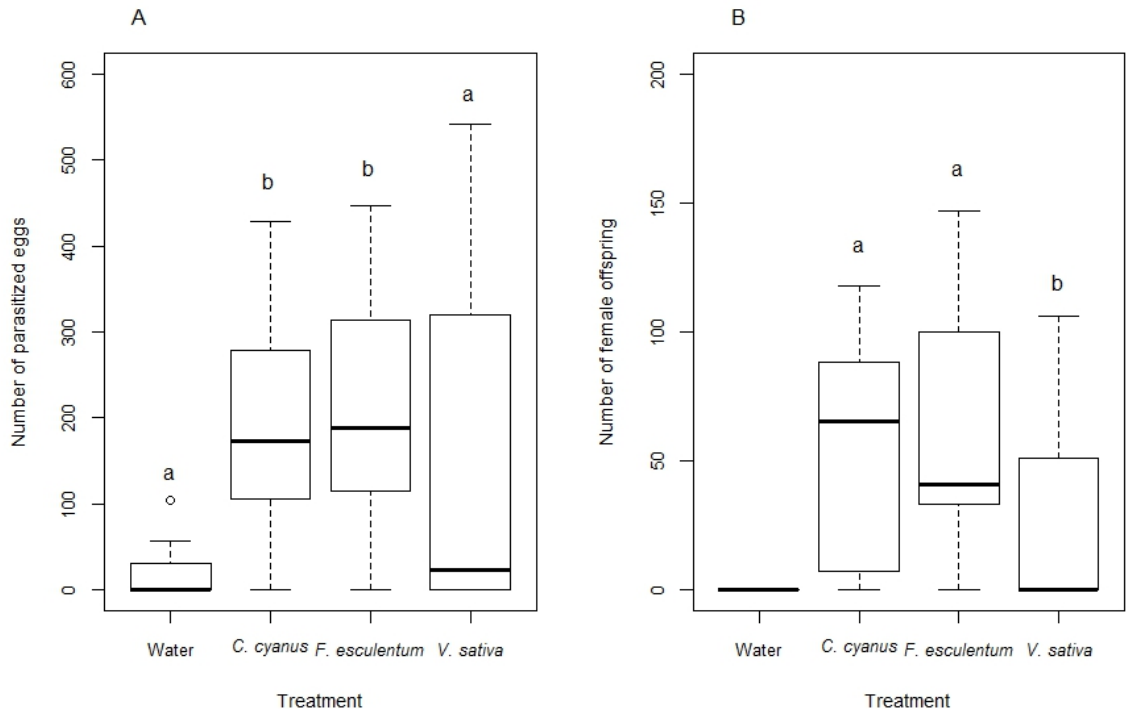

Figure 4-5 Parasitation performance of *T. laeviceps* in the presence of three flowers. (A) Number of parasitized eggs and (B) number of female offspring produced in the presence of the four treatments. Different letters indicate significant differences (generalized linear model, p < 0.05, N = 10 per treatment).

The number of female offspring produced depended also significantly on the specific food source. Compared to *V. sativa* (25.5 ± 13.72 female offspring), parasitizing females produced significantly more female offspring in the presence of *C. cyanus* (generalized linear model, z = -2.4, p = 0.016) and *F. esculentum* (generalized linear model, z = -2.42, p = 0.016), respectively 57.2 ± 13.94 and 55.2 ± 15.22 female offspring. The number of female offspring did not significantly differ between *C. cyanus* and *F. esculentum* (generalized linear model, z = 0.024, p = 0.98). No females were produced in the water control (Figure 4-5B).

The daily fecundity of *T. laeviceps* females was approximately the same in all treatments (Figure 4-6A). Females were able to parasitize right after hatching on average 9.6 ± 2.9 eggs, with a rapid increase up to 26.1 ± 8 eggs on the second day. Female offspring was produced from the third to the fourth experimental day (Figure 4-6B), while the production of males started from the first day and stayed constant until death of the parasitizing female (Figure 4-6C).

Discussion

The aim of the present study was to clarify if selected flowers can increase the longevity and parasitation performance of the biocontrol agent *T. laeviceps*, as well as attract them through volatile cues. Results clearly showed that *C. cyanus* and *F. esculentum* enhance the performance of *T. laeviceps* and further, successfully attract them.

The olfactory attractiveness of the selected flowers is important in some biological control programs, like conservation biocontrol, where natural enemies have to be attracted into the crop field (Jervis & Heimpel 2007; Balmer *et al.* 2013; Balmer *et al.* 2014). In an augmentative biological control program, natural enemies are released into the crop field, reducing the need to select highly attractive food sources. On the other hand, flowering strips are usually sown at the field margin and in big crop fields several hundred meters should be covered by parasitoids to reach them.

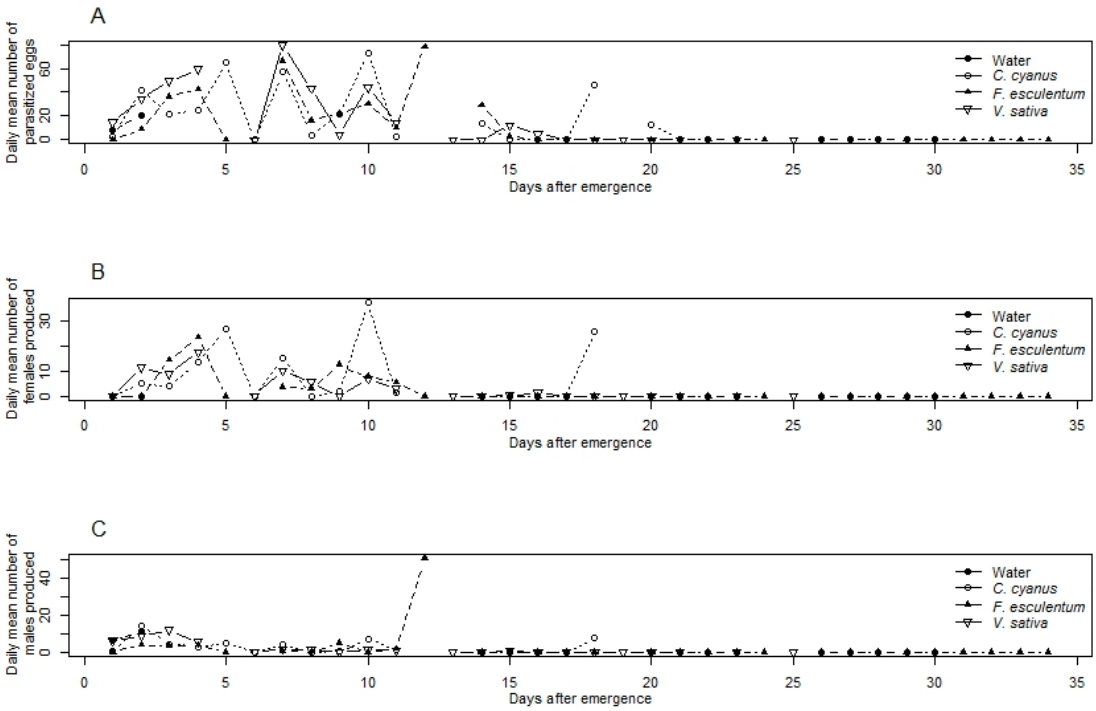

Figure 4-6 Daily mean parasitation performance of *T. laeviceps*.
(A) Daily mean number of parasitized *M. brassicae* eggs and (B) female and (C) male offspring produced in the presence of different nectar resources or water only.

If the sown flowers are not only beneficial, but also attractive, the food searching time can be considerably reduced and the *per capita* host searching efficiency increased (Jervis & Heimpel 2007). Two of the three tested flower species, cornflowers and buckwheat, equally attract *T.* laeviceps and can successfully be applied to decrease their food searching time. As already pointed out, we were able to test only the extra-floral nectar of the common vetch. The lack of attractiveness of the volatiles released by the extra-floral nectar is in line to what was shown by Géneau *et al.* (2013) and Rose *et al.* (2006), for respectively cornflower and cotton. Further experiments should be conducted to assess the olfactory attractiveness of common vetch floral nectar.

Once the parasitoid located the food source it should be able to take advantage of it. Carbohydrates represent an important source of energy for many adult parasitoids

(Leatemia *et al.* 1995; Steppuhn & Wäckers 2004). A minor source of carbohydrate is represented by host-feeding, which is found to take place in some egg parasitoids (Rivero & West 2005; Ferracini *et al.* 2006), but was never been described for *T. laeviceps*. Despite that, in Lepidopteran eggs carbohydrates are present as glycogen and most parasitoids, lacking the specific debranching enzyme, could not utilize it (Leatemia *et al.* 1995; Romeis *et al.* 2005). An important sugar source is represented by floral nectar, but not every flower is equally suitable for a particular insect. In fact, factors determining nectar accessibility, like floral morphology, are of crucial importance (Jervis 1998; Jervis & Heimpel 2007). Small bodied parasitoids, such as *Telenomus laeviceps* or *Trichogramma* spp., have difficulties exploiting floral nectar, because petals and stamen filaments can act as barriers (Patt *et al.* 1997). Therefore, the presence of easily accessible extra-floral nectar and other exposed nectaries can sensibly enhance the fitness of these small parasitoids (Patt *et al.* 1997; Jervis 1998).

Our results clearly showed that *T. laeviceps* longevity is significantly enhanced by *C. cyanus*, *F. esculentum* and *V. sativa*. Both *C. cyanus* and *V. sativa* display extra-floral nectar, explaining the increased longevity of *T. laeviceps*. Therefore, also if extra-floral nectar of *V. sativa* does not attract *T. laeviceps*, combined with highly attractive flowers, like *C. cyanus*, can be successfully used to enhance this parasitoid. On the other hand, *F. esculentum* did not present any extra-floral nectar, but its simple floral structure allows *T. laeviceps* to easily reach the nectar, as already demonstrated for *Trichogramma* spp. (Witting-Bissinger *et al.* 2008). Besides floral structure, nectar composition also plays an important role determining the suitability of a nectar source for the target parasitoid. The two main components of nectar are sugars and amino acids (Gardener & Gillman 2002), the first being important for somatic maintenance and locomotion, while the second for egg manufacture (Bernstein & Jervis 2008). The presented results indicate that the three flowers tested display an exploitable sugar composition, allowing the processing into blood sugar and glycogen, both important fuel of somatic functions

(Bernstein & Jervis 2008). Results about fecundity of *T. laeviceps* reveal something interesting. Two out of the three tested flowers, *C. cyanus* and *F. esculentum*, equally increased the parasitation performance of the parasitoid, but *V. sativa* did not, although it increased their survival. This suggest that the nectar of *V. sativa* lack some kind of component important for egg manufacture. As a moderate synovigenic parasitoid, egg-limitation is a major constrain for *T. laeviceps*, which emerge with a limited stock of mature eggs and need a suitable food source throughout their life to continuously produce those (Barloggio *et al.* Submitted). An important component of the dietary intake of many insects responsible for egg manufacture is represented by amino acids (Mevi-Schütz & Erhardt 2005; Bernstein & Jervis 2008). Nectar is the most relevant amino acid source for insects and can significantly vary between flower species, as well as within the same family (Gardener & Gillman 2002). The nectar amino acid composition of *V. sativa* was analysed by Gardener and Gillman (2002) and revealed a total amount of amino acids of 4581 ± 1928.1 pmol/µl of nectar. The total amount of amino acids present in the nectar of *C. cyanus* is similar to the one of *V. sativa*, namely 5496 ± 1627 pmol/µl of nectar (Gardener, personal communication). Looking at the data more carefully shows that the major difference in the nectar amino acid composition of this two flowers lays in the absence of proline in *V. sativa*, against the 1937 ± 360 pmol/µl of nectar present in *C. cyanus*. Proline is a rapidly metabolized amino acid, resulting in high levels of ATP (adenosine triphosphate) (Hajirajabi *et al.* 2016). In the egg parasitoid *Trissolcus grandis* (Thomson, 1861) (Hymenoptera: Scelionidae), proline added to a normal sugar-rich diet was shown to enhance fecundity (Hajirajabi *et al.* 2016). With these results, we additionally confirm the importance of proline for the egg manufacture in egg parasitoids. Amino acid analysis of *F. esculentum* nectar could further confirm this point, by potentially showing a similar amount of proline as in *C. cyanus*.

The back-up of a released biocontrol agent through food provision could downsize the necessary number of parasitoid releases and ultimately reduce the costs

for the end user. Our results clearly showed that *T. laeviceps* longevity and fecundity are significantly increased by *C. cyanus* and *F. esculentum*. These two flower species, together with *V. sativa*, are the main components of an already existing tailored flowering strip for brassica crops (Balmer *et al.* 2013; Balmer *et al.* 2014). This flowering strip was already proved to be beneficial for other natural enemies of different cabbage pests, like the parasitoids *Microplitis mediator* or predators like carabid beetles or spiders (Balmer *et al.* 2013; Ditner *et al.* 2013; Balmer *et al.* 2014). Furthermore, there is evidence that the presence of a non-target habitats like flowering strips, gives the chance to the released parasitoids to overwinter in the proximity of the field, being already present at the beginning of the next growing season (Babendreier *et al.* 2003; Kuske *et al.* 2003). With the present work, we showed that *T. laeviceps* can take advantage of nectar sources and therefore that the combination of augmentative biological control with habitat management could lead to an even more efficient pest control in brassica fields, potentially reducing, or even replacing, the use of insecticides applied against the cabbage moth.

Acknowledgment

This study was funded by the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 612713. We are thankful to Schwarz AG, Villigen (Switzerland) and UFA Samen (Switzerland) for the provision of the material needed for flower propagation. Special thanks go to Shakira Fataar for valuable discussions about the manuscript and Fabian Cahenzli for helping with the statistical analysis and for comments on the manuscript. We would also like to thank Pius Andermatt and Oliver Kindler (Syngenta, Stein, Switzerland) for the provision of the artificial diet used to rear the cabbage moths.

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CHAPTER 5

Promotion of the biocontrol agent *Telenomus laeviceps* through flowering plants: interplay of conservation and augmentative biological control

Barloggio G., Tamm L., Girard P., Nagel P. and Luka H. (in preparation)

Abstract

The intensification of agriculture negatively affects the existence of arthropod natural enemies, such as parasitoids or predators. In the last decades, efforts of universities and research institutes have shifted towards identifying alternative solutions to ecotoxicological insecticides. Augmentative biological control represents such an alternative. *Telenomus laeviceps* Förster, 1861 (Hymenoptera: Scelionidae), can be released into brassica fields to control the cabbage moth *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae). Laboratory experiments showed that the parasitation performance of this parasitoid can be markedly increased by the availability of sugar rich food sources. As a plant protection strategy, conservation biological control methods can use nectar-providing flowers to attract and enhance natural enemies. In this study, we investigated the potential of selected flowers to promote released *T. laeviceps* under field conditions. Depending on the year, positive or no effects on the total parasitation of cabbage moth eggs were achieved. However, the overall reduction of cabbage moth eggs, due to parasitoids and predators, was in both years 15 % higher in fields with implemented flowers.

Introduction

In the last 15 years, augmentative and inundative releases of biocontrol agents, in particular egg parasitoids, have gained increasing economic importance (Peña *et al.* 2010). The commercially available egg parasitoids do not only belong to the widely used *Trichogramma* spp. (Trichogrammatidae), but are also members of the families Mymaridae, Scelionidae and Platygastridae (Peña *et al.* 2010).

Telenomus laeviceps Förster, 1861 (Hymenoptera: Scelionidae) is an egg parasitoid distributed throughout Europe and North Africa, parasitizing eggs of different insect pests belonging to the Noctuidae, Geometridae and Nolidae (Mexia *et al.* 2004; Klemola *et al.* 2009; Bayle 2012; Petrov 2012). This parasitoid can be used as a biocontrol agent

against the cabbage moth *Mamestra brassicae* (Linnaeus, 1758) (Lepidoptera: Noctuidae). Basic studies were conducted to better understand its biology and the resulting information was applied to build a stable rearing (CHAPTER 1). In order to increase their parasitation performance and to mature a complete batch of eggs, adult *T. laeviceps* depend on non-host food sources, such as floral nectar. (CHAPTER 1). In the laboratory, honey-gelatine *ad libitum* was provided to adult parasitoids for survival and reproduction. The parasitation performance of *T. laeviceps* in the field could benefits from honey-gelatine added to the field delivery system used to release them. However, preliminary field trials have shown that the presence of honey-gelatine significantly increases the predation of exposed parasitized eggs, reducing the number of emerging *T. laeviceps* (CHAPTER 3). Conservation biological control can remedy this situation as it represents an alternative to provide sugar-rich food sources through flowering plants.

Applying flowers to the field increases its general biodiversity and thus promotes different ecosystem services such as pest control or pollination (Moonen & Bàrberi 2008; Plecas *et al.* 2014; Gaigher *et al.* 2015; Inclan *et al.* 2015; Inclán *et al.* 2015). In the field, survival and fecundity of released *Trichogramma* spp. can be enhanced by different flowers, like e.g. *Fagopyrum esculentum* Moench (Polygonaceae), *Vigna unguiculata* (L.) (Faboideae), *Crotalaria juncea* L. (Crotalarieae), Anethum graveolens L. (Apiaceae) or *Persea americana* Miller (Lauraceae), planted near the crop field (Wellinga & Wysoki 1989; Begum *et al.* 2004; Begum *et al.* 2006; Manandhar & Wright 2015). This indicates that the provision of exploitable nectar resources could increase the efficacy and persistence of the released biocontrol agents.

In Switzerland, a tailored flower strip for brassica crops is already present on the market and available for farmers. The seed mixture contains cornflower, *Centaurea cyanus* L. (Asteraceae), buckwheat, *F. esculentum* and common vetch, *Vicia sativa* L. (Fabaceae). Field trials have shown that this tailored flower strip attracts and enhances natural occurring antagonists of different brassica insect pests, like *Microplitis mediator*

(Haliday, 1834) (Hymenoptera: Braconidae), *Diadegma fenestrale* (Förster, 1869) (Hymenoptera: Ichneumonidae) and *D. semiclausum* (Förster, 1869) (Hymenoptera: Ichneumonidae), important antagonists of *M. brassicae* and *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae) (Géneau *et al.* 2012; Balmer *et al.* 2013; Belz *et al.* 2013; Balmer *et al.* 2014). In addition to floral nectar, cornflower displays easily accessible extra-floral nectar. This, together with its extended flowering period, makes the cornflower a good candidate for intercropping. Laboratory trials have shown that flowering cornflower, buckwheat and common vetch (extra-floral nectar only) increased the longevity of *T. laeviceps* (CHAPTER 4). Further, cornflower and buckwheat enhance the fecundity of *T. laeviceps* and are olfactory attractive to them. The aim of this study was to test the parasitation performance of released *T. laeviceps* in the presence of these flowers.

Field trials were conducted in organic cabbage fields in Switzerland over two consecutive years. *T. laeviceps* were released in fields with or without provision of flowers and their parasitation performance monitored through expositions of cabbage moth eggs. Treatments included fields with flower strip and companion plants, control fields without flowers and flower strip without companion plants (only in 2016). In 2017, experimental conditions were upgraded by using UV-sterile eggs to monitor the parasitation performance of *T. laeviceps*. Beforehand, their suitability to be parasitized by *T. laeviceps* was tested in laboratory trials.

Material and methods

Influence of UV-treated M. brassicae eggs on the parasitation performance of T. laeviceps

Laboratory trials were conducted to test the parasitation performance of *T. laeviceps* when provided with UV-sterile cabbage moth eggs. The parasitoids used in these trials

were reared as described in chapter 1. Experiments were conducted in a climatic chamber at 22±2 °C, 16:8 (L:D) photoperiod and 55±5 % RH. Fresh cabbage moth eggs (< 24 h old) were exposed to UV-light for 45 minutes. This period was determined through the evaluation of the hatching rate of *M. brassicae* eggs exposed to UV-light during different periods. We started with 5 minutes and increased the exposition period by 5 minutes after every trial until no larvae hatched from the treated eggs. A batch of 150 UV-sterile eggs was provided for 24 hours to one mated *T. laeviceps* female (seven days old). After that, eggs were removed and incubated until wasp emergence. Untreated eggs were used as a control. For each treatment, 35 replicates were tested.

To exclude an influence of the parasitation period on the number of parasitized eggs and females produced, we conducted a laboratory trial testing two parasitation periods. As for the previous trial, a batch of 150 UV-sterile eggs was provided to one mated *T. laeviceps* female (seven days old) for either two or seven days. 15 replicates for both parasitation periods were tested. For both trials, we compared the parasitation rate and the number of female in the progeny. During the experiment, wasps were fed *ad libitum* with honey-gelatine (200 g flower honey (Switzerland), 100 ml demineralized water and 3 g gelatine (Dr. Oetker)).

Promotion of released T. laeviceps through flowering plants

To test the influence of our conservation biocontrol measures on released *T. laeviceps*, field trials were conducted in 2016 and 2017 in organic cabbage fields in Switzerland. A 3 m wide flower strip (UFA Samen, Switzerland), containing *C. cyanus*, *F. esculentum, V. sativa* and *Papaver rhoeas* L. (Papaveraceae), was sown along one field margin around mid of April, while companion plants (cornflowers) were planted by hand two weeks after cabbage planting (1 cornflower/m^2). *T. laeviceps* was released twice in a density of 65'000 parasitoids/ha during the cabbage growing season. This is an economically feasible density, matching the costs of one spinosad (insecticide)

application. The first release was done two weeks after cabbage planting and the second five to six weeks after the first release. The following treatments were tested: i) flower strip and companion plants (2016 and 2017), ii) flower strip (2016) and iii) control without addition of flowers (2016 and 2017). One field was defined as a true replicate, with just one treatment tested per field. This is of advantage in the performance evaluation of released biocontrol agents, which could move between treatments defined within the same field. The trial set up was equal in both years. In each field a 16 x 21 m insecticide free area was defined, in which cornflowers were planted or not, depending on the treatment. This area was defined 46 m apart from the field margin (Figure 5-1). Within this area, a 6 x 6 m data collection plot was defined, 10 m distant from the flower strip, if present (Figure 5-1).

In 2016, the trials were conducted on 11 white cabbage fields, four of which were implemented with flower strip and companion plants, four with a flower strip and three were used as control. To monitor the parasitation rate, cabbage moth eggs (100 ± 50 eggs/clutch) were exposed during two days, on a weekly basis, on nine selected cabbage plants within the data collection plot. The recollected eggs were placed in a plastic box floating in soapy water and incubated until the host larvae hatched from the unparasitized eggs. The hatched larvae were allowed to disperse, thereby fell into the water and subsequently died. The remaining parasitized eggs were further incubated until parasitoid emergence.

In 2017, only seven fields were make available by the farmers. In order to have at least three fields for each treatment we decided not to include the treatment with only the flower strip. This was done based on the results of the trials conducted in 2016. We defined four control fields and three fields with flower strip and companion plants. To monitor the parasitation performance of *T. laeviceps* UV-sterile eggs were exposed instead of untreated eggs, reducing travel frequency from twice to once a week. Recollected eggs were incubated until parasitoid emergence. In both years, pictures of

the exposed host eggs were taken before and after exposure. The predation rate was assessed by calculating the difference between exposed and recollected eggs, while the parasitation rate as the proportion of parasitized eggs and recollected eggs.

In order to facilitate the interpretation of the results, accompanying data such as meteorological data (temperature, air humidity and precipitation) (Meteoswiss), plant protection measures applied to the field (list provided by the farmers) and the amount of pest-beneficial insects were recorded. For the last point, 12 cabbage plants per plot were selected and the number of insects recorded twice, once in June and once in July.

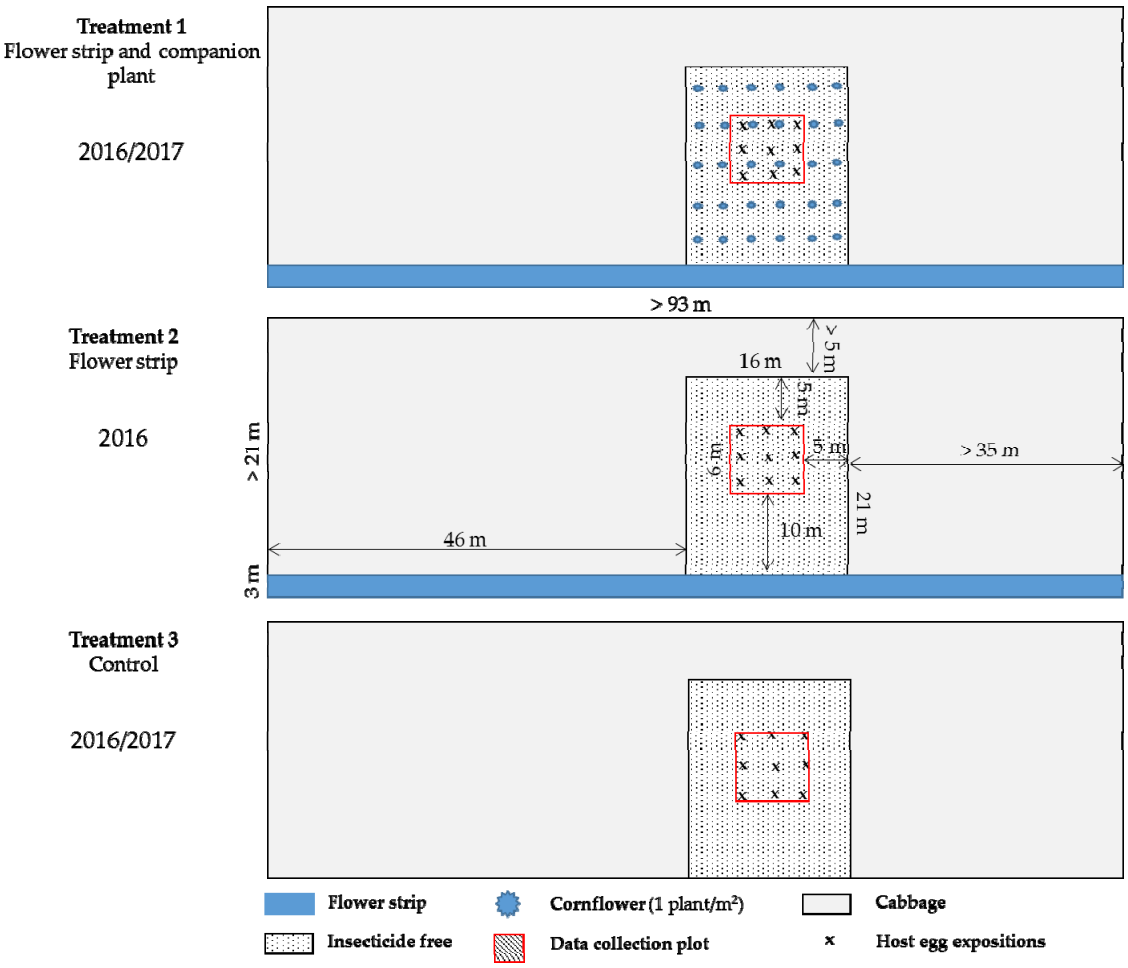

Figure 5-1 Set up of the field trials 2016 and 2017.

Statistical analysis

Data analyses were conducted with R version 3.3.0 (R development core team, 2016). Data from the two laboratory experiments were analysed through a generalized linear model (glmer function from the package lme4) with parasitized eggs and number of female offspring as dependent variable, Poisson errors and treatment (two levels: treated and untreated eggs (trials 1) or two and seven days (trials 2)) as fixed factor.

In 2016, field 1 and 7 were excluded from the statistical analysis. Field 7 experienced different experimental conditions, with Brussel sprouts planted instead of white cabbage. In field 1 no parasitation was measured during the whole season. This makes difficult the fit of the data into a model. The total parasitation rate (*T. laeviceps* and *Trichogramma* spp.) and the rate of *T. laeviceps* alone as well as the predation rate were analysed with a generalized linear model with binomial errors, fixed factor treatment (three levels: flower strip plus companion plants, flower strip and control), weather data (temperature, precipitation and humidity) as co-factors, and field and week as random factors. The model was corrected for overdispersion. The proportion of female offspring was analysed in the same way as the parasitation and predation rate, but without correction for overdispersion, as this was not necessary. In the model building process, not significant co-factors were removed from the model.

Data from the field trials 2017 measuring the total parasitation rate (*T. laeviceps* and *Trichogramma* spp.), of *T. laeviceps* alone and the proportion of female offspring were analysed with a generalized linear model with binomial errors, fixed factor treatment (two levels: control and flowers), weather data (temperature, precipitation and humidity) as co-factors, and field and week as random factors. The two models describing the parasitation rates were additionally corrected for overdispersion. Data about the predation rate of the exposed eggs between the two treatments were analysed with a generalized linear model with binomial errors, fixed factor treatment (two levels: control and flowers), weather data (temperature, precipitation and humidity) as co-

factors, and field and week as random factors. In the model building process, not significant co-factors were removed from the model.

Results

Influence of UV-treated M. brassicae eggs on the parasitation performance of T. laeviceps

We found no difference in the measured parameters between untreated and treated eggs: number of parasitized eggs (Generalized linear model, z = -1.316, p = 0.188) and number of female progeny (Generalized linear model, N = 35, z = -0.815, p = 0.415). The parasitation period (two or seven days) did not significantly influenced the number of parasitized eggs (Generalized linear model, z = -0.28, p = 0.779), as well as the number of female progeny produced (Generalized linear model, z = -1.152, p = 0.249).

Promotion of released T. laeviceps through flowering plants

In 2016, a problem in the production of *T. laeviceps* occurred, impairing their emergence in the field, with emergence rates around 10-20 %. The parasitation of *M. brassicae* eggs was monitored through the exposition of 66'309 eggs. The total parasitation rate (*T. laeviceps* and *Trichogramma* spp.) measured in 2016 was higher in fields with flower strip and companion plants, compared to control fields without addition of flowers (Generalized linear model, z = -2.649, p = 0.008) (Figure 5-2, left), with mean values of 11.71 ± 2.77 % and 3.67 ± 1.19 %, respectively. No difference was found between the other treatments (Figure 5-2, left). The proportion of parasitized eggs by *T. laeviceps* was, however, not significantly influenced by the treatments (Figure 5-3, left), with values of 9.98 ± 2.68 %, 5.76 ± 1.81 % and 3.05 ± 1.15 %, for, flower strip plus companion plants, flower strip and control, respectively. The predation rate of the exposed cabbage moth

eggs was not influenced by treatment (Generalized linear model, all $p > 0.05$) (Figure 5-4, left).

In contrast to 2016, the emergence rate during the 2017 trials was nearly 100 %. During this trial, we exposed 34'257 *M. brassicae* eggs to monitor the parasitation rate. Here, no difference was found between the two treatments, for both the total parasitation rate and the rate of parasitized eggs by *T. laeviceps* (Figure 5-2 and Figure 5-3, right). The mean total parasitation rate measured in the flower treatment was 9.99 ± 1.95 %, while in the control 11.69 ± 1.62 %. The predation rate was enhanced in fields with flower strip and companion plants compared to control fields without flowers (Generalized linear model, $z = 2.140$, $p = 0.032$) (Figure 5-4, right).

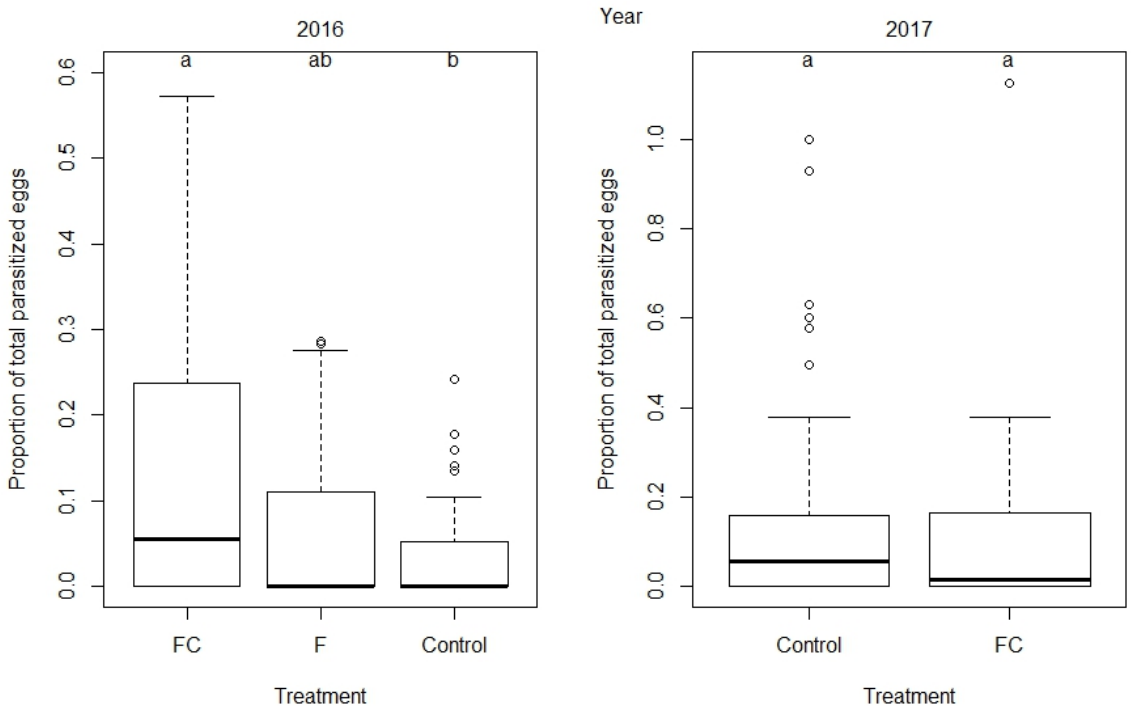

Figure 5-2 Total parasitation rate (*T. laeviceps* and *Trichogramma* spp.) measured in 2016 (left) and 2017 (right). FC: flower strip and companion plants; F: flower strip; and control: without flowers. Different letters indicate significant differences (Generalized linear model, $p < 0.05$).

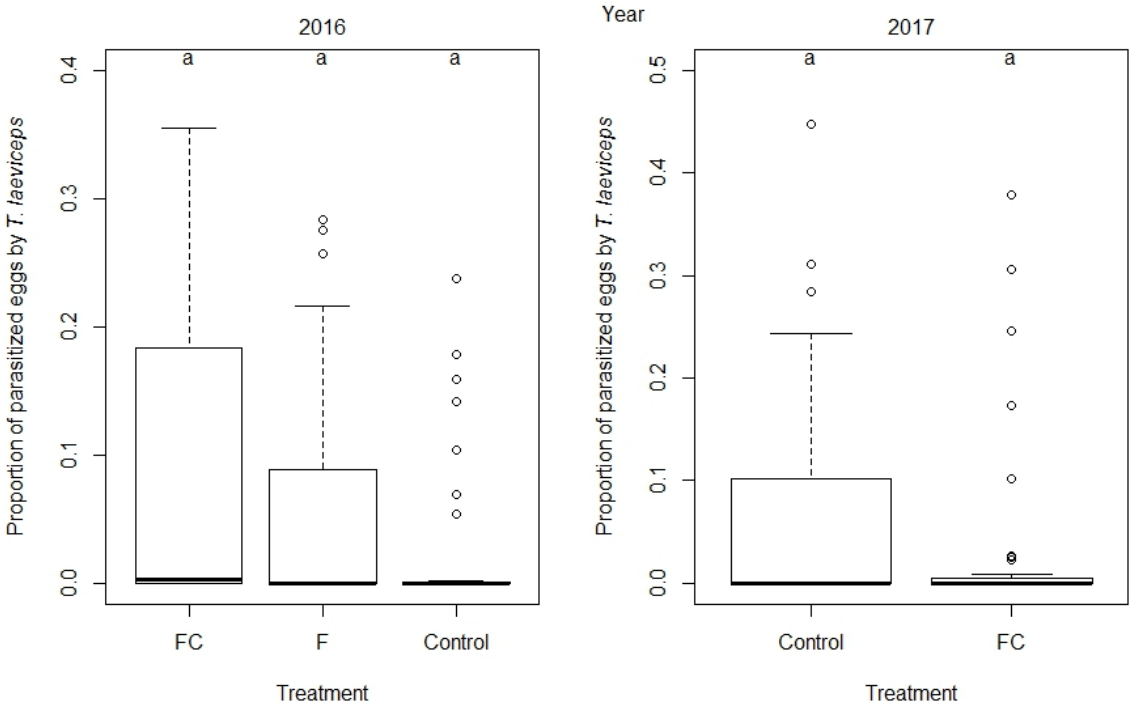

Figure 5-3 Parasitation rate of *T. laeviceps* measured in 2016 (left) and 2017 (right). FC: flower strip and companion plants; F: flower strip; and control: without flowers. Different letters indicate significant differences (Generalized linear model, $p < 0.05$).

Table 5-1 Summary of the results of the field trials 2016 and 2017.
FC: treatment with flower strip and companion plants, Control: treatment without provision of flowers.

	2016		2017	
	FC	Control	FC	Control
Mean parasitation rate (%)	11.71 ± 2.77a	3.67 ± 1.19b	9.99 ± 1.95A	11.69 ± 1.62A
Mean predation rate (%)	20.1 ± 3.43a	12.62 ± 2.11a	41.02 ± 4.73A	24.76 ± 3.17B
Total reduction of eggs (%)	31.81 ± 6.2	16.29 ± 3.3	51.01 ± 6.68	36.45 ± 4.79
Control potential of flowers (%)	15.52 ± 2.9		14.56 ± 1.89	

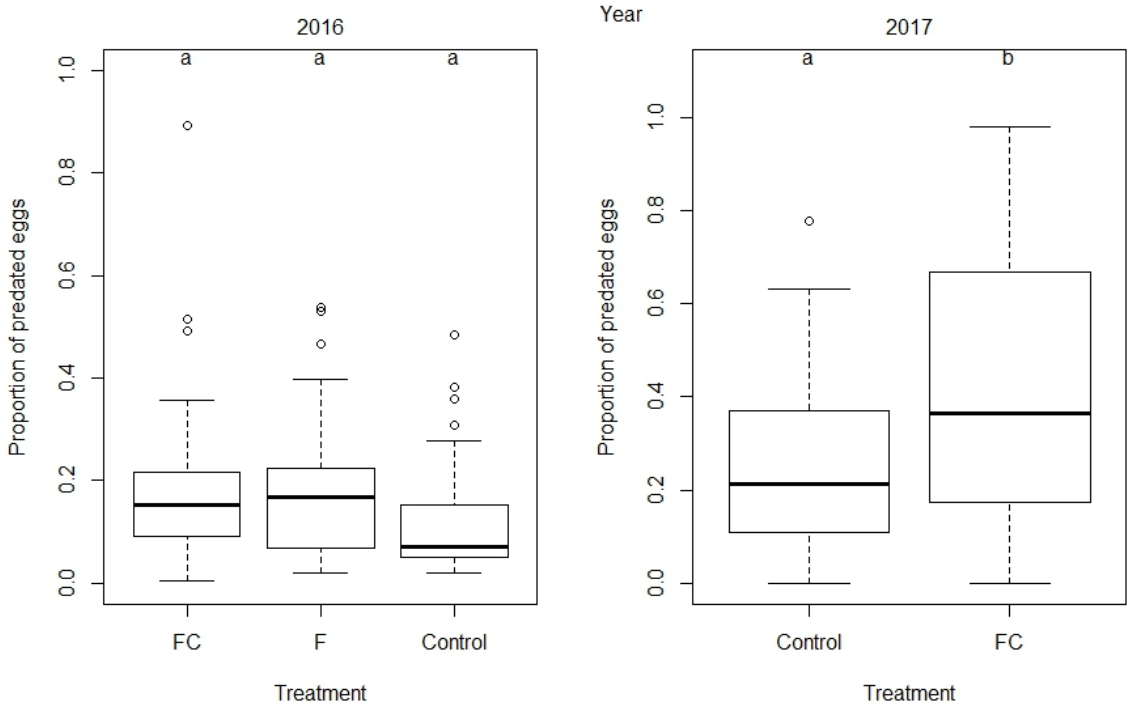

Figure 5-4 Predation rate of the exposed cabbage moth eggs measured in 2016 (left) and 2017 (right). FC: flower strip and companion plants; F: flower strip; and control: without flowers. Different letters indicate significant differences (Generalized linear model, $p < 0.05$).

Discussion

The goal of this project was to test the combination of augmentative and conservation biocontrol to increase the control of the cabbage moth by enhancing the released parasitoids *T. laeviceps*. Further, the parasitation through *T. laeviceps* of UV-sterile cabbage moth eggs was evaluated in laboratory trials.

In 2016, the total parasitation rate was significantly higher in fields with flower strips and companion plants than in fields without flowers (Table 5-1). Considering the low emergence of the released *T. laeviceps* during the trials, we can assume that the parasitation measured was mainly due to the wild population. This would mean that

natural occurring *T. laeviceps* and *Trichogramma* spp. profit of habitat management. However, in 2017, although an emergence rate of 100 %, we could not confirm these findings. In fact, we did not find any difference in the parasitation performance of *T. laeviceps* between treatments (Table 5-1). Furthermore, there is no difference in the parasitation rate in the presence of flowers measured in 2016 and 2017, in both years ranging around 10 % (Table 5-1). This despite the successful emergence of the parasitoids in 2017. The major difference between these trials was the exposition of UV-treated eggs in 2017. To exclude negative effects of the use of these eggs on the parasitation performance of *T. laeviceps*, laboratory trials were performed testing whether UV-treated *M. brassicae* eggs were as suitable as untreated eggs for parasitation by *T. laeviceps.* We showed that *T. laeviceps* parasitized the UV-treated eggs equally good as the untreated eggs. The use of UV-sterile host eggs in parasitoid mass rearing is not new and is already applied to rear *Trichogramma* spp. on sterile *Ephestia kuehniella* Zeller, 1879 (Lepidoptera: Pyralidae) eggs (Voegele *et al.* 1974; Parra 2010). This has several advantages, such as the increased hygiene of the rearing, safe exposition of the eggs in the field or the prevention of hatching host larvae from the unparasitized eggs (Parra 2010; Sivinski 2013). In fact, hatched host larvae feed on parasitized eggs, decreasing the efficiency of the rearing. Here, we primarily used the sterile eggs in the field trials. These eggs can be safely exposed to monitor the parasitation performance of *T. laeviceps*, meaning the risk of host larvae hatching in the field is prevented. Further, UV-sterile eggs allowed us to extend the exposition period in the field from two days (2016) to seven days (2017), reducing the logistic labour without trade-offs. In our laboratory trials, a prolonged parasitation period had no influence on the number of parasitized eggs by *T. laeviceps*. Thus, the length of the parasitation attempt should not bias the parasitation rates measured in the field. However, the predation rate is likely to be higher due to the prolonged exposition in the field and this might negatively influence the parasitation rate too, as parasitized eggs are longer exposed to predation. Laboratory

studies compared the susceptibility to predators, such as *Macrolophus pygmaeus* (Rambur, 1839) (Heteroptera: Miridae), *Nesidiocoris tenuis* (Reuter, 1895) (Heteroptera: Miridae) and *Orius niger* (Wolff, 1811) (Heteroptera: Anthocoridae), of two developmental stages (three and seven days after parasitation) of the egg parasitoids *Trichogramma evanescens* Westwood, 1833 (Hymenoptera: Trichogrammatidae) and *Trichogramma achaea* Nagaraja and Nagarkatti, 1970 (Hymenoptera, Trichogrammatidae) to unparasitized eggs of E. *kuehniella* and *Tuta absoluta* (Meyrick, 1917) (Hymenoptera: Gelechiidae) (Chailleux *et al.* 2013; Cabello *et al.* 2015; Pehlivan *et al.* 2017). Results clearly showed that predators prefer feeding on unparasitized eggs. In fact, during the pupation process, the parasitoid deposit melanin within the host egg (Pehlivan *et al.* 2017), increasing the mechanical resistance of the egg shell. The development of *Trichogramma* spp. proceeds rapidly. Larval development starts 25 hours after parasitation occurs and prepupae are formed already after 66 hours (Wu *et al.* 2005). This indicates that the eggs are susceptible to predators the first three days after parasitation and that an increase in the predation of the parasitized eggs due to prolonged exposition in the field is unlikely.

Interestingly, in the presented field trials, the predation rate followed an inverse pattern compared to the parasitation rate. In 2016, no significant difference was measured between treatments (Table 5-1). On the other hand, in 2017, the predation rate in fields with flowers was twice as high as in the control fields (Table 5-1). Furthermore, when the level of predation was high, no difference in the parasitation rate between treatments was measured. This suggests that, high density of predators negatively affected the performance of *T. laeviceps*. In a study testing the same flower strip as in this work, the gut content of predators collected in fields with and without flower strip was analysed. They found that the overall abundance and diversity of predators was higher in fields with flower provision. Further, DNA of released *Trichogramma brassicae* (Bezdenko, 1968) (Hymenoptera: Trichogrammatidae) was detected in the gut of different carabids, staphylinids and spiders (Balmer *et al.* 2013). This is a phenomenon

known as intraguild predation, where different trophic levels interact and compete for the same resources (host and prey) (Polis *et al.* 1989; Rosenheim *et al.* 1995). Of all the factors determining a biocontrol agent's success, intraguild predation seems to have the greatest impact. Released parasitoids like *Tr. evanescens, Tr. achaea, Tr. pretiosum* or *Blaesoxipha* spp. were negatively impacted by the presence of predators (Ruberson & Kring 1991; Chailleux *et al.* 2013; Cabello *et al.* 2015; Pehlivan *et al.* 2017). This work adds further evidence about the difficulties encountered when testing the effectiveness of augmentative biocontrol agents. Indeed, the benefits of released biocontrol agents have been demonstrated only in 20 % of the studies (Gurr & Kvedaras 2010; Sivinski 2013).

Here, we tested the combination of augmentative and conservation biocontrol to reduce cabbage moth densities. As we saw, in 2016, the parasitation performance of *T. laeviceps* and *Trichogramma* spp. was enhanced by nectar sources. However, this is only true if intraguild predation is not interfering with the parasitoids. On the other hand, high levels of predations also contribute to decreased pest populations. Therefore, we now discuss the results considering the control of the cabbage moth as a whole and not just due to *T. laeviceps*. Pest-beneficial monitorings conducted during the field trials showed that the number of cabbage moth larvae per plant was higher in fields without flowers, in both years 2016 and 2017. In 2016, the mean number of cabbage moth larvae per cabbage plant was 0.04 in the flower and 0.26 in the control treatment. In 2017, the abundance of larvae was overall higher, with 0.11 and 0.48 larvae per plant, in the flower and control treatment, respectively. The abundance of cabbage moth larvae is linked to the abundance of eggs. The total reduction of cabbage moth eggs, calculated as the sum of the mean parasitation and predation rate, was higher in the flower treatment compared to the control (Table 5-1). The impact of predation and parasitation differed between years, but the control potential of the flowers, measured as the difference between the total reduction of eggs in the flowers and control treatments, remained constant (Table 5-1). In both years, the implementation of flower strip and companion

plants increased the control of the cabbage moth of approximately 15 % compared to the control (Table 5-1). Studies already proved the potential of the combination of released biocontrol agents with conservation biocontrol, but the increase in the pest control was attributed to direct advantages on the biocontrol agents (Lee & Heimpel 2005; Begum *et al.* 2006; Sivinski 2013). Here, we demonstrated that conservation biocontrol can both, increase the predators abundance and the effectiveness of released *T. laeviceps* and natural occurring *T. laeviceps* and *Trichogramma* spp., contributing to the control of the cabbage moth.

Acknowledgments

This study was funded by the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 612713. We are thankful to UFA Samen (Switzerland) for the provision of the cornflower seeds and to Schwarz AG, Villigen (Switzerland) for their propagation. Thank you to Pius Andermatt and Oliver Kindler (Syngenta, Stein, Switzerland) for the provision of the artificial diet used to rear the cabbage moths. A huge thank you to all the involved farmers for the appreciated collaboration. Special thanks go to Shakira Fataar for valuable comments and discussions about the manuscript and Fabian Cahenzli for helping with the statistical analysis.

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GENERAL DISCUSSION

Aim of this PhD thesis was to provide a valid alternative to the use of broad-spectrum insecticides used in organic agriculture against the cabbage moth *M. brassicae*, by developing a biocontrol agent based on the egg parasitoid *T. laeviceps*. Further, the potential behind the combination of augmentative and conservative biocontrol to promote and retain released *T. laeviceps* was investigated.

The main findings and achievements of this thesis:

- Establishment of a stable and reliable rearing of *T. laeviceps*, allowing a constant production of individuals with an excellent proportion of female offspring (60 - 70 %).
- Development of qPCR markers for molecular identification of *T. laeviceps*.
- Successful application of the developed markers to identify collected *T. laeviceps* in Sweden.
- Successful sampling in Sweden of *T. laeviceps* during summer 2017, representing a first record for this country.
- Development of a suitable field delivery system to release *T. laeviceps* in the field.
- Increase of the parasitation rate of exposed *M. brassicae* eggs after releases of high densities of *T. laeviceps* in 2015 (successful proof of concept).
- *C. cyanus, F. esculentum* and *V. sativa* were identified as suitable to enhance longevity of *T. laeviceps*.
- *C. cyanus* and *F. esculentum* were proved to increase fecundity of *T. laeviceps*.
- Females of *T. laeviceps* showed innate preference towards olfactory cues of *C. cyanus* and *F. esculentum*.

Appendix 1 Limitations of *Telenomus laeviceps* as biocontrol agent and suggested improvements.

	Achieved	Limitations	Improvements
Lab-scale rearing	Yes	None	None
Mass rearing (commercial partner)	Yes	1. Low proportion of females (30-40 %) 2. Reduced emergence rate after delivery	1. This point is for me difficult to discuss since the rearing method used by the partner is unknown to me 2. Simulate the delivery conditions in the lab (temperature, humidity, mechanic disturbance due to transport, etc.)
Field delivery system	Yes	Suitable only for the release of parasitized eggs	Development of an adequate system to release adults, such as the small plastic container used to release the parasitoid *Aphidius colemani* Viereck, 1912 (Hymenoptera: Braconidae)
Provision of honey-gelatine in the field	–	Increased parasitation rate of the exposed eggs	1. Protective net 2. Replace honey-gelatine with nectar providing flowers (conservation biocontrol)
Release of *T. laeviceps* 120'000-240'000 parasitoids/ha	–	Good efficacy, but too high production costs	Improve the cost-efficiency of the rearing, e.g. by using a factitious host or store the host eggs after treating them with UV-light
Release of *T. laeviceps* 65'000 parasitoids/ha	–	Too low efficacy	Release adult parasitoids to increase their parasitation performance without an increase in the density

Important aspects of the biology of *Telenomus laeviceps*

Before this project started, several attempts to rear *T. laeviceps* at FiBL have been conducted, without any success. The field sampled wasps were brought into the laboratory in 2012 as parasitized eggs and incubated until adult emergence. The sex ratio of the F_0 population was shifted towards females, as usually found in other egg parasitoids (Boivin 2010). However, the sex ratio of the F_1 population (first laboratory generation) was strongly shifted towards males, with just a few to none females emerging. Therefore, the first objective of this thesis was to the better understand the biology of *T. laeviceps*, in order to build a stable rearing. Results summarized in chapter 1 show that intraspecific competition does not impair the success of the rearing. This in contrast to what was found by Carneiro *et al.* (2009) in *Telenomus remus* Nixon, 1937 (Hymenoptera: Scelionidae). The proportion of females in the progeny of this parasitoid decreased of 39 % when three females parasitized the same egg clutch instead of one female alone. The parasitation performance of *T. laeviceps* increased after a period of time in absence of host eggs (egg deprivation), during which females were allowed to mate and feed. In *T. laeviceps*, as in most Hymenopteran parasitoids, the sex of the progeny is determined through arrhenotokous parthenogenesis. In this context, environmental factors plays an important role in the decision making of the females (Heimpel & de Boer 2008). In other *Telenomus* species such as *T. nawai* (Ashmead, 1904) (Hymenoptera: Scelionidae), *T. remus* or *T. coloradensis* Crawford, 1910 (Hymenoptera: Scelionidae), temperature (Bueno *et al.* 2008; Pomari *et al.* 2012), intraspecific competition (Carneiro *et al.* 2009), humidity (Pomari-Fernandes *et al.* 2014) and time before first parasitation take place (egg deprivation time) (Carneiro *et al.* 2010) were described as important sex determinants. However, in *T. laeviceps*, of all the tested parameters (CHAPTER 1), the egg deprivation time had the strongest influence on the amount of female offspring produced. This suggests that *T. laeviceps* emerge with limited larval

resources, needing an appropriate food source right after emergence to produce a complete stock of mature eggs. Laboratory trials showed that, without a sugar rich food source, *T. laeviceps* can still parasitize some eggs (CHAPTER 1), but the progeny will consists only of males. Looking at *T. laeviceps* as a biocontrol agent, this finding represents an exploitable feature. In fact, since it is the intention to release *T. laeviceps*, there is no need to build up self-sustaining populations in the field. Consequently, the production of females in the progeny is not mandatory. On the other hand, this indicates that the control potential of the released females is not completely exploited, opening up the possibility to increase their parasitation performance. This can be done by providing females an exploitable food source near the release point in the field.

Augmentative and conservation biocontrol

The effectiveness and persistence of released *Trichogramma* spp. can be enhanced by flowers such as buckwheat, mustard, dill or avocado flowers planted near crop fields (Wellinga & Wysoki 1989; Begum et al. 2004; Begum et al. 2006; Manandhar & Wright 2015). A tailored flower strip for brassica crops is commercially available in Switzerland. This strip attracts and enhances natural enemies like *M. mediator* or *D. semiclausum*, but has no positive effects on pests (Géneau et al. 2012; Belz et al. 2013; Géneau et al. 2013). Its main components, *C. cyanus, F. esculentum* and *V. sativa* (extra-floral nectar only) enhanced the longevity of *T. laeviceps* under laboratory conditions (CHAPTER 4). Further, *T. laeviceps* showed an innate preference for olfactory cues produced by *C. cyanus* and *F. esculentum*. *C. cyanus* and *F. esculentum* were observed to enhance the parasitation performance of *T. laeviceps* females (CHAPTER 4). The fact that *V. sativa* increased the longevity but not the fecundity of *T. laeviceps* could be due to the nectar composition of these flowers. Aside from water, the two main nutritional components of nectar are sugars and amino acids (Gardener & Gillman 2002). Sugars are important for somatic maintenance and locomotion, while amino acids are needed in egg production

(Bernstein & Jervis 2008). The total amount of amino acids present in the nectar of *C. cyanus* is similar to the one of *V. sativa* (Gardener, personal communication). However, in *V. sativa* the amino acid proline is absent, in contrast to the 1937 ± 360 pmol/µl-of-nectar present in *C. cyanus*. In the egg parasitoid *Trissolcus grandis* (Thomson, 1861) (Hymenoptera: Scelionidae), proline added to a normal sugar-rich diet was shown to enhance fecundity (Hajirajabi *et al.* 2016). Our finding add further evidence about the importance of proline for egg production in egg parasitoids. From a more applied point of view, this emphasizes the importance to develop a flower mixture, composed of flowers displaying complementary features. Some flowers should enhance the fecundity of the parasitoids, whereas others should increase longevity or produce exploitable olfactory cues in order attract parasitoids. Results about the effectiveness of the combination of augmentative and conservation biocontrol in the field were not as clear as the results gained in the laboratory trials. In 2016, flower strips, combined with companion plants increased the parasitation performance of *T. laeviceps* compared to control fields without flowers. However, this finding was not confirmed in 2017 (CHAPTER 5). In contrast, in 2017, predation rate was significantly higher in fields provided with flower strips and companion plants than in control fields. Increased predation, but not parasitation, was previously shown by Balmer *et al.* (2013). The high level of predation could have had an influence on the amount of parasitized eggs predated and thus mask the effects of flowers on the parasitation rate. Balmer *et al.* (2013) investigated the insect community of cabbage fields in the presence of different amount of flowers. They also molecularly analysed the gut content of field collected predators. DNA of *Trichogramma brassicae* (Bezdenko, 1968) (Hymenoptera: Trichogrammatidae) was detected in the gut of different carabids, staphylinids and spiders, suggesting that an increase in the density of predators could indirectly impact the parasitation rate of egg parasitoids. Intraguild predation can negatively affect the performance of released parasitoids (Rosenheim *et al.* 1995). This could explain why the

benefits of these biocontrol agents have been demonstrated only in 20 % of the studies (Gurr & Kvedaras 2010; Sivinski 2013). However, here we need to consider the increased predation as an improvement in the pest control due to conservation biocontrol. In fact, the parasitation and predation of the cabbage moth eggs both contribute to the control of this pest. Considering this, in both years the implementation of flower strips and companion plants increased the control of the cabbage moth of approximately 15 % compared to the control.

Telenomus laeviceps as a biocontrol agent: constraints and suggested improvements

Trichogramma species have drawn most attention as biocontrol agents. These species have dominated the market of egg parasitoids based biocontrol agents, mainly because of their ease of rearing on factitious hosts (Parra 2010). However, of the known 200 species of *Trichogramma*, only 19 species have been mass reared and used in augmentative biological control programs (Li 1994). Besides rearing, the main factors determining the success of biocontrol agents are i) the technical effectiveness, such as the ability to suppress the pest or the predictability of a release's success, ii) the benefits for human health (public good), iii) the ease of use, determined by the shelf life, formulation or delivery, iv) the commercial viability and v) the impacts on non-target organisms and environment (safety) (Mills 2010). *T. laeviceps* satisfies the safety and public good criteria, allowing releases safe for the environment. However, the technical effectiveness is the main constrain, limiting the implementation of *T. laeviceps* on a large scale.

The ability of *T. laeviceps* to suppress the pest in the field was tested over three years. In the first field trial conducted in 2015 we measured an increased parasitation rate of cabbage moth eggs after releases of *T. laeviceps*. This was the first evidence of the effectiveness of released *T. laeviceps* under field conditions. However, the production costs of the tested densities were too high. In the attempt to reduce the costs associated

with the rearing, the parasitation performance of *T. laeviceps* on eggs of several alternative hosts was tested in laboratory trials (data not shown). These hosts were chosen based on the Noctuid pests reared by our commercial partner. The production of these pests was already automated and therefore cost efficient. Unfortunately, *T. laeviceps* turned out to be very selective. This was shown for several *Telenomus* species, which are effective egg parasitoids against different Lepidopteran pests, but the lack of an alternative host, due to their narrow host spectrum, is a major constrain in their mass production (Mills 2010). On the other hand, the narrow host spectrum of a biocontrol agent is not always a limitation, reducing the risks for vulnerable non-targets insects (Sivinski 2013).

To face the high production costs, an economic feasible density of 65'000 parasitoids/ha was calculated and tested in efficacy field trials. In 2016, the emergence of *T. laeviceps* in the field was compromised by some problems in the production. Therefore, the evaluation of the economic feasible density failed. The trials were repeated in 2017 using the same density. However, the parasitation rate measured was way below our expectations, with mean values of 0.75 %. As pointed out in chapter 3, 65'000 parasitoids with a proportion of females of 70 % should be able to parasitize up to 6 Mio. eggs. This should be enough to keep the *M. brassicae* population below the damaging threshold.

In the field, the success of a biocontrol agent is determined by a combination of several factors such as i) the dispersion of the released parasitoids, ii) the incompatibility of *T. laeviceps* releases with applied plant protection measures or iii) the lack of an exploitable food source near the release point.

Released biocontrol agents can be forced to disperse if the host is present at low densities (Mills & Wajnberg 2008). In this case the application of attractive volatiles such as herbivore induced plant volatiles, could retain the released parasitoids in the crop field (Khan *et al.* 2008).

A biocontrol agent alone can hardly be enough to suppress the pest of interest, thus, the compatibility of biocontrol agents and standard phytosanitary measures is an important aspect to determine the success of an augmentative biocontrol program (Geiger *et al.* 2010; Moens *et al.* 2012). The field trials presented in chapters 3 and 5 were performed under two distinct plant protection regimes. The efficacy trials (CHAPTER 3) were conducted in organic white cabbage fields intensively managed over the last years. In contrast, the impact of conservation biocontrol on the parasitation performance of *T. laeviceps* was tested under more extensive plant protection schemes (CHAPTER 5). This led to a generally higher parasitation rate in the extensively managed fields, with mean values up to 12 %, while ranging around 0.75 % in the intensively managed fields. Field trials conducted with different egg parasitoids, belonging to the genus *Trichogramma,* demonstrated the incompatibility of simultaneous releases of parasitoids and fungicide (Thomson *et al.* 2000; Manzoni *et al.* 2006) and insecticide use (Cônsoli *et al.* 2001; Carvalho *et al.* 2003; Giolo *et al.* 2007). The only known successful integrated pest management program was achieved by combining *Trichogramma* spp. releases with applications of *Bacillus thuringiensis*, reaching a greater suppression of stem borers in corn (Wang *et al.* 2005; Jalali & Singh 2006).

To increase the compatibility of biocontrol agents and integrated pest management programs, it is necessary to reduce the amount of insecticides applied. To this end, releases can be combined with other techniques to reach a higher pest control. In this work, we already discussed the implementation of conservation biocontrol to increase the effectiveness of augmentative biocontrol programs. Here, parasitoids are enhanced and retained in the crop field through provision of nectar providing flowers. Alternatively, biocontrol agents can be withhold in the crop field by the application of infochemicals. For example, *Trichogramma* spp. releases combined with behavior-modifying infochemicals, such as kairomones released by lepidopteran scales, attract the parasitoids were they are most needed (Beevers *et al.* 1981; Lewis *et al.* 1985). The

suitability of this technique to increase the parasitation performance of *T. laeviceps* should be investigated under laboratory conditions. Olfactometer trials could be conducted to test the olfactory attractiveness of volatiles released by *M. brassicae* scales.

Based on the data collected during this project, improvements are needed before *T. laeviceps* could be placed on the market. The main constraints and the suggested improvements are summarized in Appendix 1.

Conclusive remarks on the potential of *T. laeviceps* as biocontrol agent

This thesis has investigated the potential of *T. laeviceps* as released biocontrol agent against the cabbage moth. This parasitoid can be easily reared and its parasitation performance under laboratory conditions is optimal to achieve a good control of *M. brassicae*. However, at the moment, the costs associated with the production of *T. laeviceps* did not allow releases with a sufficient number of parasitoids to reach the desired biocontrol effects. As in other systems, the use of one distinct plant protection measure against a pest is not always sufficient. A combination of several strategies often achieves better pest control. Thus, releases of *T. laeviceps* should be combined with phytosanitary measures such as conservation biocontrol or application of specific insecticides like *B. thuringiensis*. The provision of flowering plants combined to releases of *T. laeviceps* achieved a 15 % decrease of cabbage moth eggs compared to control fields. The implementation of additional measures could further increase the control of *M. brassicae*.

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