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TITOLO TESI

New associations between native parasitoids and exotic insects introduced in Italy and in Europe

(Nuove associazioni tra parassitoidi indigeni e insetti esotici neointrodotti in Italia ed in Europa)

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Photographs were made by myself

Introduction

The introduction of exotic insect species is one of the most important threats to biodiversity (Colombo and Juker, 2004). This phenomenon may cause both economic and environmental damage, like production losses, increases of pesticide treatments, and modification of native flora and fauna. To prevent these invasions, besides international regulations, there are institutions like the European and Mediterranean Plant Protection Organization (EPPO), with the aim to control imported goods or determine and study and harmful pests. Nevertheless, the introduction of exotic pests is an increasing issue, difficult to control.

Classic insect biological control, based on importation of natural enemies from the country of origin of the exotic pest (Caltagirone, 1981), has been successfully used for over 120 years, and use of more than 2000 species of natural enemies has resulted in the control of at least 165 pest species worldwide (van Lenteren et al, 2006). This strategy has provided good results, even in recent time, in the world (Mineo et al, 1999; Hanks et al, 2000; Viggianiet al, 2000; Lambkin, 2004; Quacchia et al, 2008); but has also raised some criticism (Michaud, 2002), as well as concern about the potential negative effects induced by exotic natural enemies on indigenous and, in general, on non-target insects (Howarth, 1997; van Lenteren et al, 2003). The introduction of non-native natural enemies to new landscapes creates novel interactions with native communities; these interactions can adversely affect the demographics of native species, which may in turn prevent non-native species from establishing in a new community (Sakai, et al, 2001). The ecologists are interested in the evolving interactions, and their implications, between indigenous and non-native antagonist species. Following a lag time of variable duration, non-native antagonist species may in turn become invasive (Blossey and Notzold, 1995; Sakai et al, 2001).

It is however important to note that, after the 1992 Rio Convention on Biodiversity, international organizations and national governments published general guidelines for the import and release of invertebrate biological control agents (e.g. The Food and Agriculture Organization of the United Nation code of conduct for the import and release of exotic biological control agents; [Greathead, 1997]). In Europe, a methodological guide on how to measure the environmental impact of using invertebrates for biological control of arthropods was published as a result of the Regulation of Biological Control Agents (REBECA) project, funded by the EU-Commission (Ehlers, 2011). Prior to the release of a beneficial insect into the environment, all of the risks and benefits should always be weighed (van Lenteren et al., 2003; Bigler et al, 2006; van Lenteren and Loomans, 2006; De Clercq et al, 2011).

Beyond the limits of classical biological control it is useful to improve knowledge on the role played by indigenous antagonists, including parasitoids, in the control of introduced pests. It has to be pointed out that, in many cases, in biological control, a better effectiveness occurred resulted more effective compared to the previous ones (Hokkanen and Pimentel, 1984); in Italy there are cases in which the activity of indigenous parasitoids was of support in the control of introduced exotic insects (Giorgini et al, 1998; Viggiani, 2000).

My research work focused on the study of the new associations occurring between indigenous parasitoids and three exotic pests introduced in Italy and Europe and was performed within a project “PRIN 2008” (Programmi di Ricerca di Rilevante Interesse Nazionale - Research Programs of National Interest) especially devoted to this topics. The three insects considered were the Geranium Bronze *Cacyreus marshalli* Butler (Lepidoptera: Lycaenidae), a pest of *Geranium* plants (chapter 2, page 5); the Oriental Chestnut Gall Wasp *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae), a plague of *Castanea* sp. (chapter 3, page 16) and the Multicolored Asian Ladybug *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae); this ladybug was introduced into Europe and Italy as a biological control agent, but for since some years it has considered as an invasive species, dangerous for local enthomophagous insects (chapter 3, page 31). The studies were performed with field samplings and laboratory tests on the three species, as described in the following text.

Cacyreus marshalli Butler

Introduction

Cacyreus marshalli Butler is a Lepidoptera species from Lycaenidae family. The genus *Cacyreus* includes nine species, eight of them exclusive of Africa, one found in the Arabic area (Ackery et al, 1995). Except two species (*C. audeoudi* Stempffer and *C. ethiopicus* Tite) all these butterflies were known as pests of Lamiacee and Geraniacee; in particular *C. palemon* (Stoll), *C. dicksoni* Pennington, *C. neibuhri* Larsen and *C.marshalli* feed on *Geranium* and *Pelargonium* plants (Clark and Dickson, 1971).

The Geranium Bronze *C. marshalli*, native of southern Africa, can live in different habitats, from sea coast to mountain (Clark and Dickson, 1971) its life cycle is influenced by environmental conditions, in particular temperature: at 20°C the cycle is complete in 60 days, at 30°C in a month (Sarto i Monteys, 1994). In the Balearic island this species has probably up to six overlapping generations per year (Sarto i Monteys, 1994). Eggs, whitish to light-yellow or brown in colour, 0.5 mm in diameter x 0.3 mm in height, are oviposited near flower buds or rarely on leaves (EPPO, 1993) (Fig. 1). The first instar larva is basicaly green in colour but sometimes yellowish with whitish tufts of setae. It has an average lenght of 1 mm which increases to 2 mm within 8 days (Clark and Dickson, 1971). Second, third and fourth instar grow to 3, 6 and 13 mm typically in 8, 8 and 9 days, respectively; the colour may be greenish and/or yellowish with or without pink markings, tufts of setae are always present (Clark and Dickson, 1971). The larvae produce galleries in the stems and flower peduncles, which become packed with frass. The damage becomes most visible during the hot season when the larvae are most active; flowers can be totally eaten by the larvae. Damage is often associated with secondary damage by microorganisms which can also colonize the tissue around the entrance hole of the larvae into the peduncles (Sarto i Monteys and Maso, 1991). Leaves may be partially eaten by the larva but this symptom is less frequent and can be confused with feeding by snails (Sarto i Monteys and Maso, 1991). Seriously affected plants may die as a

Figure 1: Adult, egg laid on stem, four instar larva with damage and pupa of *Cacyreus marshalli*

result of the infestation. Fourth (last) instar larvae emerge from stems, making circular holes and feed on leaves and flowers (Fig. 1). The larvae turn into pupae, which have no cocoon, mainly at the base of the plant near the soil surface (Favilli and Manganelli, 2006); usually they are very hairy; greenish, pale-yellowish or brownish, with brown mottling and an average size of 9 mm (Clark and Dickson, 1971) (Fig. 1). The development time varies depending on temperature (17 days at 20°C, 8 days at 30°C) (Sarto i Monteys, 1994). Female adults have a wingspan of 18-27 mm while male adults have a wingspan of 15-23 mm. *Cacyreus marshalli* has a bronze brown colouring of the upper wing surface with white spots on the fringe (Fig. 1).

The first record in Europe of this butterfly was in United Kingdom, at Cheshunt in Hertfordshire, in 1978; when two larvae were accidentaly found on plants of the genus *Pelargonium* originated from the Republic of South Africa. Fortunately these larvae were identificated by the British Ministry of Agriculture, Fisheries and Food (MAFF) in Harpenden, Hertfordshire, and destroyed (Sarto i Monteys, 1992). But in the early 1990s some samples of *C. marshalli* were found in the Balearic Archipelago (Spain) (Eitschberger and Stamer, 1990); the high number of samples, five males and eight females, suggests that the introduction had happened some years before (Eitschberger and Stamer, 1990). Nowadays *C. marshalli* is estabilished in various European countries (Favilli and Manganelli, 2006; Pignataro et al, 2007; Verovnik et al, 2011). In Italy it was first recorded in 1996 in Latium (Trematerra et al, 1997) and later spread to most other regions (Jucker et al, 2009).

The Geranium Bronze mainly attacks *Pelargonium* and *Geranium* plants and has become one of the most harmful insect pest of cultivated geraniums in Italy and other European countries where it occurs. As a consequence it has been included in the EPPO A2 quarantine list (EPPO, 2002). However *C. marshalli* shows some preferences among different cultivars of *Pelargonium*; in particular it has shown preference for the varieties *zonal* and *peltatum*, over the varieties *grandiflorum* and *capitatum* (Sarto i Monteys, 1994; Lupi and Jucker, 2005). In laboratory tests larvae also ate wild species like *Geranium pyrenaicum* Burm, *G. robertianum* L, *G. rotundifolium* L (Sarto i Monteys, 1994; Lupi and Jucker, 2005). Therefore this species is not only dangerous to cultivated geraniums, but also rapresents a potential threat for Italian (and European) biodiversity, for wild-growing native *Geranium* species and indigenous lycaenid butterflies through of competition (Quacchia et al, 2008). The fast spreading and estabilishment of *C. marshalli* in introduced areas is probably due to various factors including polyvoltine cycle, lack of diapause requirement (in Italy larvae don't show a diapause period [Trematerra et al, 1997]), difficult detection of the larvae in the stems and also the scarcity of effective natural enemies compared with the native range (EPPO, 2002; Quacchia et al, 2008).

Difficulties in controlling this pest are due to the life cycle of the larvae, which live inside the plants for long time. Initially in the aeras of introduction chemical control (Herrero et al, 2002: Sacco et al, 2004) or the use of different varieties of *Pelargonium* and *Geranium* were utilized (Alonso et al, 2004; Lupi and Jucker, 2005). In South Africa *C. marshalli* is controlled by a complex of predators and parasitoids like Diptera Tachinidae and Hymenoptera Braconidae of the genus *Apanteles*, parasitoids of third instar larvae (Clark and Dickson, 1971). In Italy there were a few records of predation by spiders of the family *Thomisidae* and lizards of the genus *Podarcis* (Favilli and Manganelli, 2006). In Europe recorded natural enemies of the Geranium Bronze include the oophagous parasitoid *Trichogramma evanescens* Westwood in Spain (Sarto i Monteys and Gabarra, 1998) and the tachinid larval parasitoid *Aplomya confinis* (Fallen) in Italy (Vicidomini and Dindo, 2007), but their impact on the target species has not been remarkable so far. Moreover the predatory activity of *Macrolophus caliginosus* Wagner toward the eggs and young larvae of *C. marshalli* has been shown and the possible use of this mirid as biocontrol agent of the lycaenid has been suggested (Sacco et al, 2009). In this framework, studies aimed at increasing the knowlwdge on the possible role of other native natural enemies in the control of *C. marshalli* are justified. In this part of my research the acceptance and suitability of the Geranium Bronze by two indigenous parasitoids, *Exorista larvarum* (L.) (Diptera: Tachinidae) and *Brachimeri tibialisi* (Walker) (Hymenoptera: Chalcididae), was thus investigated in the laboratory, so as to evaluate the possibility that a new association between these two beneficial insects and the aliene lycaenid butterfly also occurs in the field. The two parasitoids are wildspread in Italy and most other European countries where *C. marshalli* has been introduced (www.faunaeur.org). *Exorista larvarum* and *B. tibialis* are polyphagous parasitoid of Lepidoptera, including *Lymantria dispar* (L.) and other defoliators (Luciano and Protta, 1984) and are currently maintained in a continuous colture in the laboratory of Entomology at the University of Bologna (Italy) using *Galleria mellonella* (L.) as factitious host. *Exorista larvarum* (Fig. 2) is a gregarious larval parasitoid which lays eggs on the host body. The newly hatched larvae penetrate the host intergument and continuosly grow until pupation which generally occurs outside the host carcass (Michalkova et al, 2009). In the laboratory of the University of Bologna an *E. larvarum* colony was estabished in 1992 and augmented in 2004 with adult emerged from *Lymantria dispar* (L.) and *Hyphantria cunea* (Drury) larvae that were field-collected in the province of Bologna (Italy, 44°30’27’’N, 11°21’5’’E).

Bachymeria tibialis (Fig. 2) is a solitary parasitoid which develops from egg to adult in the host pupa. *Brahymeria tibialis* colony was established in the early 2000s with adults emerged from *L. dispar* pupae field-collected at Tempio Pausania (Sardinia, Italy, 40°54’0’’N, 9°6’0’’E).

Figure 2: Females of *Exorista larvarum* on lump of sugar, female of *Brachymeria tibialis* on cage wall and larva of *Cacyreus marshalli* fed with leaf of *Pelargonium* spp. after exposure to *E. larvarum* (the eggs of the parasitoid are visible on the body)

Aims of research

The present work was aimed at the studying the acceptance and suitability of *C. marshalli* by *E. larvarum* and *B. tibialis*, compared to the factitious host *G. mellonella*, maintained as control.

Materials and Methods

The experiments concerning *E. larvarum* and *B. tibialis* were performed in the laboratory of Entomology at the University of Bologna, Italy; the colonies of the parasitoids and the experiments were all conducted in a rearing chamber at 26±1°C, 65±5 RH and L16:D8 photoperiod.

Insect rearing

The trials were carried out using *C. marshalli* larvae that were collected in the field from *Pelargonium* sp. in Bologna area (northern Italy, 44°48’39’’N, 11°37’84’’E). The larvae were individually mantained in 10-cm diameter plastic Petri dishes inside the rearing chamber and fed with leaves of *P. zonale* (Fig. 2) until they reached for the four (final) instar, identified according to Clark and Dixon (1971) and Favilli and Manganelli (2006) (at least 7 mm in length).

Exorista larvarum and *B. tibialis* were reared on *G. mellonella* as described by Dindo et al. (2001; 2007).The adults of both parasitoids were kept in plexiglass cages (40x30x30 cm).

Exorista larvarum adult were fed on cotton balls soaked in a honey and water solution (20% honey) and lump sugar (Fig. 2). One day per week 80 larvae of the factitious host *G. mellonella* were placed inside *E. larvarum* cages and removed after the parasitoid had laid about 4-5 eggs per larva; subsequently the host larvae were removed and mantained in a plastic box (10x20x10 cm) in the rearing chamber waiting for the exit of the parasitoid larvae, which subsequently turned into puparia. The puparia obtained were used for creating new cages of adults.

Brachymeria tibialis adults were fed on cotton balls soaked in a honey and water solution (20% honey) and drops of honey. One day per week some 2-4 days old pupae of *G. mellonella* were placed inside *B. tibilalis* cage and removed after one hour; the pupae were mantained in the rearing chamber waiting for the parasitoidadult emergence.

The lepidopterous larvae were reared on an artificial diet (Campadelli, 1987), at 30±1°C, 65±5% RH and in complete darkness. The larvae were fed every two days until shortly before maturity; these mature larvae were used to maintain the parasitoid colony, for the experiments or to maintain the *G. mellonella* colony.

Acceptance and suitability of *Cacyreus marshalli* and *Galleria mellonella* by *Exorista larvarum*

The experiment was performed under no-choice conditions. I tested sixty larvae of *C. marshalli* and sixty larvae of the control species *G. mellonella*. All larvae were of the last instar, which proved to be the most suitable to *E. larvarum* in the case of *G. mellonella* (Mellini et al, 1994). A preliminary experiment showed that no *E. larvarum* eggs were laid on *C. marshalli* larvae unless the latter were exposed to flies for 2 hours at least. For the experiment, therefore, *C. marshalli* or *G. mellonella* larvae were placed individually in a plexiglass cage (40 x 30 x 30 cm) which contained about 25 adult females and 25 adult males of *E. larvarum*; all flies had emerged 5-6 days before (Dindo et al, 2007) and had already oviposited on *G. mellonella* larvae. The larvae were removed after 2 hours and the eggs laid on their body were counted. The larvae with eggs were individually kept in 10 cm diameter Petri dishes with food until death, parasitoid puparium formation or host emergence (Fig. 2). The newly formed puparia were weighed and individually kept in glass tubes until parasitoid emergence. The sex of the newly-emerged adults was recorded. The experiment was carried out at 26±1°C, 65±5% RH, and a L16:D8 photoperiod.

Each host larva was considered as a replicate. The larvae were considered as “accepted” when at least one *E. larvarum* egg was found on their body. As a measure of host acceptance, I considered 1) the number and percentage of larvae with at least one *E. larvarum* eggs (over the total number of larvae exposed to flies = 60) and 2) the mean number of eggs per accepted larva (the highest the eggs per larva, the highest the acceptance of the host larvae by the parasitoid flies). The number and percentage of suitable larvae (*i.e.* accepted larvae from which puparia formed) were also calculated. The weights of the newly-formed puparia, the development times from egg to puparium and from puparium to adult emergence and sex ratio were recorded. The number and percentage (based on puparia) of emerged flies was also calculated. The number and percentage (based on puparia) of emerged flies was also calculated. All these parameters were also considered as criteria for host suitability. Host mortality was evaluated as number and percentage of dead larvae based on accepted larvae.

Acceptance and suitability of *Cacyreus marshalli* and *Galleria mellonella* by *Brachymeria tibialis*

Also this experiment was performed under no-choice conditions. *Cacyreus marshalli* or *G. mellonella* 2-day old pupae were individually exposed to about 10 *B. intermedia* females and 10 males in a plexiglas cage. Due to low availability of parasitoids in the laboratory colony, adults of mixed ages were used. The parasitoid females had already oviposited in *G. mellonella* pupae before the experiment. In this trial, the host pupae were removed from the cage as soon as a female pierced their body with the ovipositor. The pupa was considered as not accepted if no ovipositor insertion was detected following 2 hours after its placement in the cage. Upon removal from the cage, all pupae (accepted and not accepted) were individually kept in 5-cm diameter plastic Petri dishes until death, parasitoid or host emergence. The newly emerged parasitoid adults were sexed and weighed.

Twenty-five pupae of each species were tested, each of which was considered as a replicate. Host acceptance and suitability were respectively evaluated as number and percentage of accepted pupae and number and percentage of accepted pupae which let a parasitoid adult emerge. Successful parasitism (= number of host pupae which let a parasitoid adult emerge/original number of pupae x 100) was also calculated. The newly-emerged adults were sexed and their weights and development times from egg to adult were separately recorded for males and females

Statistical analysis

The Kruskall-Wallis non-parametric test was used to analyze the mean number of eggs/number of accepted larvae, the puparium weights and development time from egg to puparium of *E. larvarum* and the overall development time of *B. tibialis*, due to heteroscedasticity. One-way ANOVA was used to analyze the effects of *C. marshalli* and *G. mellonella* on weights of adult *B. tibialis* and *E. larvarum*. The data for other parameters were analyzed by 2x2 contingency tables using Yates correction for small numbers (< 100). All statistical tests were done with STATISTICA 6.0 (StatSoft, 2001).

Results

Acceptance and suitability of *Cacyreus marshalli* and *Galleria mellonella* by *Exorista larvarum*

The mean length of all larvae tested was 0.93±0.06 cm (mean±SE); instead the mean length of *G. mellonella* was 2.4±0.03 cm (mean±SE). *Exorista larvarum* females *a*ccepted *C. marshalli* larvae, though at a significantly lower rate than *G. mellonella* larvae (χ^2=27.55, df=1, P=0.00001) (Fig. 3-a). The mean number of eggs per accepted larva was also significantly lower for *C. marshalli* than for *G. mellonella* (Tab. 1). Following acceptance, parasitoid puparia were obtained from both hosts, but at a significantly lower rate in the case of the Geranium Bronze (χ^2=13.96, df=1, P=0.0002) (Fig 3-b). *Galleria mellonella* larvae produced a minimum of 1 and a maximum of 3 puparia each, whereas successfully parasitized *C. marshalli* larvae produced only one puparium. Only in a case I obtained a puparium of *E. larvarum* from a pupa of *C. marshalli*, meaning that the parasitoid had continued its development after the host larval stage, an unusual event. Independently of puparium formation, most of the accepted *C. marshalli* larvae died, at a not significantly different level compared to *G. mellonella*, whereas the non-accepted ones pupated and emerged as adults. Compared to *G. mellonella*, total mortality was however significantly lower for *C. marshalli* than for *G. mellonella* (χ^2=3.64, df=1, P=0.006) (Fig. 3-c).

The weight of the puparia formed in *G. mellonella* was significantly higher compared to that of the puparia obtained from *C. marshalli* (Tab. 1), but the parasitoid development times, from egg to puparium and from puparium to adult, were not significantly different between the two host species (Tab. 1). Fly emergence was significantly lower (χ^2=4.03, df=1; P=0.045) for the puparia obtained from *C. marshalli*

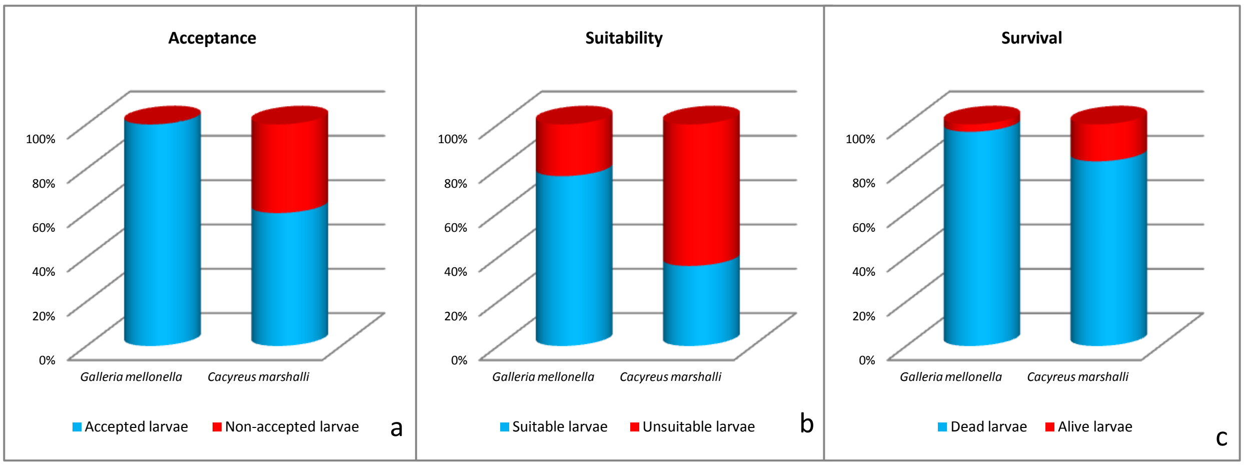

Figure 3: Acceptance and suitability of *Cacyreus marshalli* and *Galleria mellonella* by *Exorista larvarum*: a) accepted larvae (%), b) suitable larvae (=accepted larvae form which puparia formed) (%), c) dead larvae (on accepted larvae) (%). Original number of larvae = 60 per species

(=15.2%) compared to those formed in *G. mellonella* (=50%) (only two of the puparia formed in Geranium Bronze larvae let an adult emerge); it should be noted that in a few cases *E. larvarum* tried to emerge by puparium but were not successful. Only two adult parasitoids were obtained from *C. marshalli*, 1 male and 1 female; as a result the parasitoid sex ratio was female biased (2.6:1) when compared to *G. mellonella* results. Due to the low number of flies obtained from the latter host, no statistics was done either for this parameter or for the development time from puparium to adults. These time were (means±SE) 8.9±0.2 and 9.5±0.5 days for the flies respectively obtained from *G. mellonella* (n=36) and *C. marshalli* (n=2), thus slightly longer in the latter host species. Mating capacity and female fecundity were not checked in this study, due to the very low number of flies obtained from *C. marshalli*.

Table 1: Acceptance and suitability of *Cacyreus marshalli* and *Galleria mellonella* by *Exorista larvarum*: parasitoid eggs per acceptate larva, puparium weight and development time from eggs to puparium. Means±SE. Number of replicates (n) is given in parentheses over the means. Means in a column followed by the same letter are not significantly different, P>0.05; Kruskall-Wallis test.

Host species	Eggs/accepted larva (no)	Puparial weight	Time from eggs to puparium (days)
Galleria mellonella	(60) 38.6±3.6a	(72) 30.2±1.6a	(72) 8.1±0.1a
Cacyreus marshalli	(36) 2.4±0.3b	(13) 9.1±0.9b	(13) 8.2±0.6a
H	64.9	28.4	0.15
N	96	85	85
P	0.00001	0.0001	0.96

Acceptance and suitability of *Cacyreus marshalli* and *Galleria mellonella* by *Brachymeria tibialis*

The mean (±SE) pupal lenght was 0.8±0.02 cm for *C. marshalli* and 1.5±0.2 cm for *G. mellonella*. 100% *G. mellonella* and 52% *C. marshalli* pupae were accepted by *B. tibialis* females (fig. 4-a); the difference in host acceptance was significant (χ^2=13.27, df=1; P=0.0003). There was no significant difference between the two host species with regard to suitability, indicated by the percentage of accepted pupae which let a parasitoid adult emerge (χ^2=2.61, df=1; P=0.11) (Fig. 4-b). The percentage for *C. marshalli* was considerably lower compared to that recorded for *G. mellonella* and, at the end of the study, successful parasitism was significantly lower for *C. marshalli* than for *G. mellonella* (χ^2=13.72, df=1; P=0.002) (Fig. 4-c). All the accepted pupae of both the host species died, whether successfully parasitized or not, whereas the non-accepted ones (only *C. marshalli*) emerged as adults.

From exposed *C. marshalli* seven parasitoids emerged, all of them were males; some females emerged from *G. mellonella*, but for this host species the sex ratio was male biased (15 males: 6 females). The result concerning the mean weights and development time are shown in the Table 2. The mean weights and development times of the six *B. tibialis* females emerged from *G. mellonella* pupae were respectively 13.8±0.1 mg and 15.7±0.2 days. Since no females were obtained from *C. marshalli* pupae, no statistical analysis was possible for this parameter.

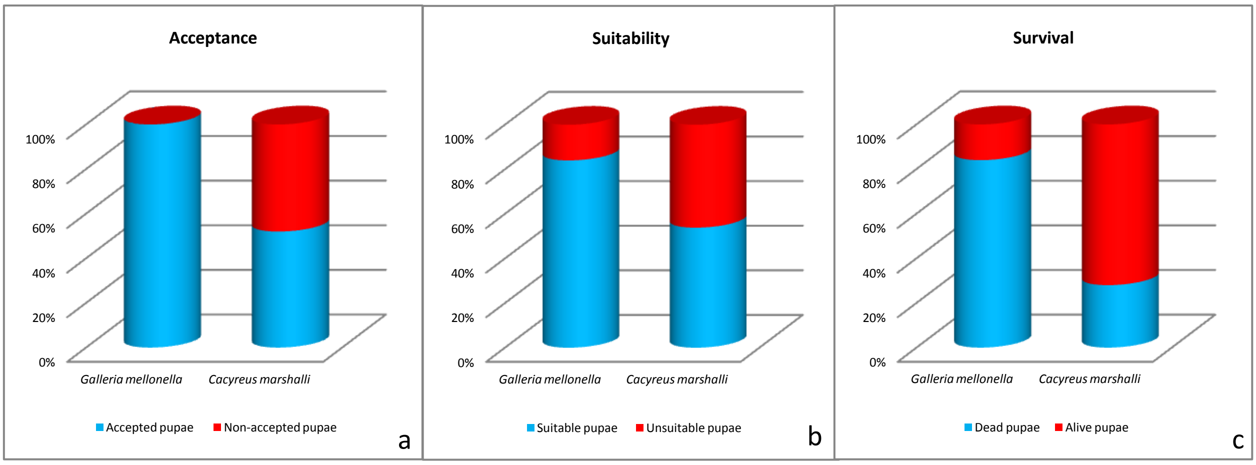

Figure 4: Acceptance and suitability of *Cacyreus marshalli* and *Galleria mellonella* by *Brachimeria tibialis*: a) accepted pupae (%), b) suitable pupae (accepted pupae from which parasitoid adults emerged) (%), c) successfully parasitized pupae (%), calculated on the original number of pupae (=25). The unparasitized pupae included both the non-accepted pupae and unsuitable ones. Yates corrected χ^2 values are presented (sample size <100).

Table 2: Acceptance and suitability of *Cacyreus marshalli* and. *Galleria mellonella* by *Brachymeria tibialis*:, parasitoid male adult weight and development time from eggs to adult. Means±SE. Number of replicates (n) is given in parentheses over the means. Means in a column followed by the same letter are not significantly different, P>0.05; one-way ANOVA or Kruskall-Wallis test.

Host species	Weight (mg)	Time from eggs to adult (days)
Galleria mellonella	(15) 8.4±0.1 a	(15) 15.7±0.3a
Cacyreus marshalli	(7) 3.8±0.3 b	(7) 14.9±0.4 a
H(N)		1.46(22)
F (df)	340.96 (1,20)	
P	0.0000001	0.23

Discussion

As expected, no successful parasitism of *C. marshalli* by *E. larvarum* was found, because the mature larvae of this butterfly are considerably undersized compared to the recorded host species of the tachinid fly (Carretti and Tschorsnig, 2010). Successful parasitism occurred in a previous laboratory trial, but at very low rates (Depalo et al, 2010; 2012). As hypothesized by some authors, similarly to most tachinids which lay eggs on the host body (Stireman, 2002; Dindo, 2011) at close range (such as cage environment) *E. larvarum* primarily uses visual cues and motion signal in host location and acceptance. Besides being undersized compared to *G. mellonella* larve, *C. marshalli* larvae are also not very mobile. Their low acceptance by *E. larvarum* females was therefore probably related to these two physical factors, besides chemical cues, which are, however, less important in the process of host location and acceptance for Tachinidae, compared to Hymenoptera parasitoids (Mellini, 1991). Suitability to *E. larvarum* was also lower for *C. marshalli* compared to *G. mellonella*. Similarly to acceptance, this parameter was apparently affected by host size, which also influenced the parasitoid size. Whit this regard, Baronio et al. (2002) showed that *E. larvarum* development on artificial substrate was not only affected by the amount of food, but also by vital space available to larvae (*i.e.* the volume of the rearing containers). In particular, at equal amounts of medium, the number of individuals attaining larvael maturity and pupation was significantly lower in small containers, compared to standard ones. Otherwise, the puparium size was found not to be affected by vital space. Based on these findings, in *C. marshalli–E. larvarum* association, it may be hypothesized that parasitoid development was negatively affected by host size, whereas the puparium size was primarily influenced by the low amount of food resources available.

For *B. tibialis*, host location and acceptance are guided by chemical and physical cues (Drost and Cardé, 1992). Either of these stimuli could influence the lower acceptance of *C. marshalli* pupae by *B. tibialis* compared to *G. mellonella* pupae; but the effect of pupal size on this parameter is doubtful, because the recorded hosts of *B. tibialis* also include species of similar size as *C. marshalli* (Noyes, 2012). More research is therefore needed to clarify this matter. Moreover, no significant difference was found in the suitability of *C. marshalli* and *G. mellonella*, despite the different pupal size, but, as expected, *B. tibialis* adults (all males) emerged from *C. marshalli* were much smaller compared to those obtained from *G. mellonella*. As it is well known, the size of insects, including parasitoids, is often linked to their quality (Godfray, 1994). It may therefore be argued that the fitness of *E. larvarum* and *B. tibialis* obtained from *C. marshalli* is lower than that of the parasitoids formed from *G. mellonella*; due to be low number of adults obtained. This hypothesis was not tested in this study, and could be the subject of further research. It would be even more interesting to test to what extent previous experience with *G. mellonella* may influenced the acceptance and suitability of *C. marshalli* by the female wasps and flies.

In conclusion, the results obtained with *E. larvarum* and *B. tibialis* suggest that both these native parasitoids have, the potential to create a new association with *C. marshalli* in nature. However in the short run their possible contribution to biological control of this pests seems especially related to host mortality due to incomplete parasitoid development; in fact most *C. marshalli* larvae accepted by *E. larvarum* and all *C. marshalli* pupae accepted by *B. tibialis* died, despite the low successful parasitism. These results indicate that, similarly to native parasitoids antagonists of other alien insect pest in Italy, *E. larvarum* and *B. tibialis* could effectively contribute to lowering the population of *C. marshalli*, even if parasitism is successfully completed in only a few cases; but additional studies are required to better evaluate this issue.

Figure 5: Larva of *Cacyreus marshalli* killed by *Exorista larvarum* larvae; larva of *Exorista larvarum* emerged from larva of *Cacyreus marshalli* and puparium of *Exorista larvarum* emerged from *Cacyreus marshalli* larva

Dryocosmus kuriphilus Yasumatsu

Introduction

Dryocosmus kuriphilus Yasumatsu is a hymenopteran species belonging to the family Cynipidae, subfamily of Cynipinae, tribe of Cynipini. Its native area is in China, where it lives at the expense of chestnut trees (*Castanea* sp.); *D. kuriphilus* is one of the two species in this tribe (and the only palearctic species) to induce galls on *Castanea* (Felt, 1940; Stone et al, 2002; Ács et al, 2007). From China *D. kuriphilus* has spread to many chestnut wood areas of the world, so that it is wordwilde considered one of the major pests of chestnut trees (Moriya et al, 1990; Brussino et al, 2002). It is also known with its common English name: oriental chestnut gall wasp.

Dryocosmus kuriphilus reproduces by thelytokous parthenogenesis; no males are known, and it has one generation per year (Moriya et al, 2003) (Fig. 6). The adult females live 2-10 days (Yasumatsu, 1961). They are 2.5-3 mm long on average; body is black, legs, scapulus and pedicels of antennae, apex of clypeus and mandibles are yellow-brown; head is finely sculptured; scutum, mesopleuron and gaster are highly polished, smooth; propodeum has 3 distinct longitudinal carinae; propodeum, pronotum (especially above) are strongly sculptured; scutum has 2 uniformly impressed and pitted grooves (notaulices) that converge posteriorly; the radial cell of forewing is opened; the antennae are 14-segmented with apical segments not expanded into a club (EPPO, 2005). The females emerge in early summer, from the end of May until the end of July, depending on locality and chestnut (Bosio et al, 2009), and immediately lay eggs inside chestnut buds that will develop the following spring. Each female may lay more than 100 eggs, with 23-30 eggs found in one bud (Otake, 1980; Otake, 1989; EPPO, 2005).

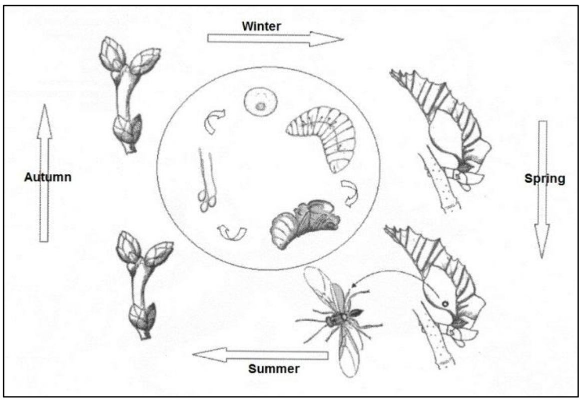

Figure 6: Life cycle of *Dryocosmu kuriphilus* (from Alma and Quacchia, 2012; modified)

Eggs are oval, milky white, 0.1-0.2 mm long with a long stalk. They hatch in 30-40 days and first instar larvae remain within the eggs and overwinter in the plant buds.

The larvae are 0.2-0.6 mm in length in the first instar, 0.8-1.5 mm in the second, 2.3-2.5 mm when fully grown (Viggiani and Nughes, 2010), milky white, without eyes and legs. At bud burst in spring, larval feeding induces the formation of green or rose-colored galls. These are are unilocular or multilocular, 5-20 mm in diameter on *Castanea crenata* Siebold and Zucc. (Tamura, 1960; Otake, 1980), 8-15 mm on *C. sativa* Miller or *C. sativaXC. crenata* in Europe (Breisch and Streito, 2004). Each gall can contain 1-5 gall-cells and can host 1-16 larvae (Zhang, 2009). The larvae live in the gall for 30-70 days, generally for 50 days (Ding et al, 2004). Pupation occurs within the gall from mid-May to mid-July. The pupae are 2.5 mm long, black or dark brown.

Dryocosmus kuriphilus emerged as a pest in the the mid twentieth century and is now one of the most important global insect pests of *Castanea* trees (Aebi et al, 2006). The first record out of China of this pest was in Japan in 1941 (Moriya et al, 2003). Subsequently it was found in 1958 in Korea (Murakami et al, 1995) and in Nepal in 1999 (Abe et al, 2007). The first record out of Asia was in North America, in 1974 in Georgia, USA (Cooper and Rieske, 2007); after this finding *D. kuriphilus* spread quickly throughout the USA. In Europe the first record was in Italy (in Piedmont) in 2002 (Brussino et al, 2002); after this first record *D. kuriphilus* was reported in other European countries (tab. 3).

Table 3: Spread of *Dryocosmus kuriphilus* in Europe (from EPPO, 2012)

Country	Year of first record	Status	Reference (First record)
Croatia	2010	Present, under official control	Matosevic et al, 2010
Czechia	2012	Transient, under eradication	EPPO, 2012
France	2005	Present, restricted distribution	Aebi et al., 2006
France (Corse)	2010	Present, restricted distribution	Aebi et al., 2006
Hungary	2008	Absent, considered eradicated	EPPO, 2009
Italy	2002	Present	Bosio et al, 2003
Italy (Sardinia)	2008	Found	http://www.sardegnaagricoltura.it/index.php?xsl=44
Italy (Sicily)	2010	Found (around Catania)	EPPO, 2011
Netherlands	2008	Transient, under eradication	EPPO, 2010
Slovenia	2004	Absent, pest eradicated	Knapic et al, 2009
Switzerland	2009	Transient, actionable under eradication	Foster et al., 2009

In its native areas *D. kuriphilus* feeds on *C. mollissima* Blume (Chinese chestnut) (Zhu et al, 2007). In the countries of introduction it has adapted to live on different species of genus *Castanea* such *C. crenata* Sieb. et Zucc. (Japanese chestnut) (Kato and Hijii, 1993), *C. dentata* (Marsh.) (American chestnut) (Anagnostakis, 2001), *C.*

sativa Mill. (European chestnut) (Brussino et al, 2002) and their hybrids. It also infests *C. seguinii* Dode in China, but is not known to attack the wild North American species *C. pumila* Mill. (Rieske, 2007) and *C. pumila* Miller (ex C. *alnifolia* Nuttal) (EPPO, 2005), which are very often grown near infested chesnuts. Also, the numerous cultivars show different rates of infestation: for example the varieties "Marsol" and "Madonna", in Italy, were highly susceptible to be attack by the *D. kuriphilus*, while conversely the French "Bouche de Bétizac" seems to be immune (Ministero delle politiche agricole, 2010).

The introduction is thought to be associated with the import of infested Chinese chestnut trees (infected cultivars and/or material for grafting) (Aebi et al, 2006). Local spread occurs throug the movement of infested planting material (EFSA, 2010), or by flight of the adult females during the period of flights.

The damages were caused by the larvae which induce the formation of galls on developing leaves and twigs. After adult emergence, the galls dry and become wood-like, and remain attached to the tree for up to two years. While new galls are readily detected on plants, eggs or first instar larvae inside the buds cannot be detected by simple visual inspections. *Dryocosmus kuriphilus* disrupts development of twigs and leafs and it reduce fruiting. Severe infestation can result in mortality of young trees (Payne et al, 1975; Dixon et al, 1986; Anagnostakis and Payne, 1993), probably in combination with other detrimental factors such as fungal infection, drought or severe attack by other herbivores (Moriya et al, 2003). The reduction of fruit yield can be high, up to 50-80% (Payne et al, 1983; EPPO, 2005; EFSA, 2010).

Figure 7: Galls of *Dryocosmus kuriphilus*

Chestnut cultivation fulfils several important commercial, socio-cultural, and ecological roles in southern Europe. For example in Piedmont, chestnut orchards are traditionally planted for fruit and wood production, representing a significant additional long-term income for many farmers. From an ecological point of view, chestnut forests are the major woodland type in Northern Italy (Bounous, 2002). Due to the threats for chestnut-growing, *D. kuriphilus* has been included in 2003 to the EPPO A2 action list (no. 317), and EPPO member countries are thus recommended to regulate it as a quarantine pest. Due to its rapid spread, in Italy a specific Ministerial Decree has been issued in 2008 (Ministero delle politiche agricole, Decreto 30 ottobre 2007, 2008), in order to prevent its propagation.

The effectiveness of chemical control is limited by the cryptic nature of this insect, which is in dormant buds for the majority of its life cycle, and by the difficulty of precisely detecting of adult emergence (Torii, 1959; Murakami, 1981; EFSA, 2010). Moreover, chemical treatments often are impractical due to the natural distribution of chestnut trees on steep terrain. Mechanical removal of infested twigs (pruning) and the protection of immature twigs with nets, although effective, do not represent practical solutions because of labour intensiveness. Breeding of resistat chestnut varieties was successful for about 20 years in Japan, but these were eventually rapidly overcome by novel aggressive strains of *D. kuriphilus* (Murakami, 1981). Within some parts of their native area in China *D. kuriphilus* populations are kept at low densities, presumably by natural enemies like hymenopteran parasitoids, although little is known of alternative sources of mortality of the pest in this region (EFSA, 2010). In galls of *D. kuriphilus* collected in China, Korea and Japan several chalcid parasitoids have been found (Tab. 4); futhermore other parasitoids has been found in other countries of introduction, Italy included (Stone et al, 2002; Aebi et al, 2006) (Tab. 4). All these parasitoids are known as antagonists of oak gall wasps, showing that, when *D. kuriphilus* becomes established in new environments, local gall parasitoids are rapidly recruited as enemies of this wasp.

Generally, the attack rates of indigenous parasitoid species are low (Stone et al, 2002; Aebi et al, 2007), but some of these appeared to be very effective, especially *Torymus sinensis* Kamijio (Hymenoptera: Torymidae). *Torymus sinensis* was described as a specific parasitoid of *D. kuriphilus* (Murakami et al, 1977) and some research showed that it is highly species-specific (Quacchia et al, 2008) presumably due to the highly similar biological cycles of the parasitoid and the chestnut gall wasp (Moriya et al, 2003). Programs of biological control, based on the introduction of *T. sinensis*, have been started in some countries; the first was made in Japan, where this parasitoid has shown encouraging results (Quacchia et al, 2008; Moriya et al, 2003). After this first program, other were conducted in North America in the 1970s, and in Europe (Italy) in the 2000s (Payne, 1978; Aebi et al, 2006; Cooper and Rieske, 2007; Quacchia et al, 2010).

Table 4: Hymenoptera Chalcidoidea parasitoids emerged from galls of *Dryocosmus kuriphilus* in Asia (from: EPPO, 2005; Yasumatsu and Kamijio, 1979), North America (from: Payne, 1978; Cooper and Rieske, 2007) and Europe (from: Speranza et al, 2009; Aebi et al, 2007; Graziosi and Santi, 2008).

	Name	**FamilyandSubfamily**
Asia	*Torymus sinensis* Kamijio	Torymidae:Toryminae
	Torymus beneficus Yasumatsu et Kamijo	
	Torymus geranii (Walker)	
	Megastigmus nipponicus Yasumatsu et Kamijo	Torymidae:Megastigminae
	Megastigmus maculipennis Yasumatsu et Kamijo	
	Ormyrus punctiger Westwood	Ormyridae
	Ormyrus flavitibialis Yasumatsu et Kamijo	
	Eurytoma brunniventris Ratzeburg	Eurytomidae:Eurytominae
	Eurytoma setigera Mayr	
	Eurytoma schaeferi Yasumatsu et Kamijo	
	Sycophila variegata (Curtis)	
	Eupelmus urozonus Dalman	Eupelmidae:Eupelminae
	Cynipencyrtus flavus Ishii	Tanaostigmatidae
North America	*Torymus tubicola* (Osten-Sacken)	Torymidae:Toryminae
	Torymus advenus (Osten-Sacken)	
	Ormyrus labotus Walker	Ormyridae
	Sycophyla mellea (Curtis)	Eurytomidae:Eurytominae
Europe	*Torymus flavipes* (Walker)	Torymidae:Toryminae
	Torymus erucarum (Schrank)	
	Megastigmus dorsalis (Fabricius)	Torymidae:Megastigminae
	Ormyrus pomaceus (Geoffroy)	Ormyridae
	Eurytoma brunniventris Ratzeburg	Eurytomidae:Eurytominae
	Sycophyla bigutta (Swederus)	
	Eupelmus urozonus Dalman	Eupelmidae:Eupelminae
	Mesopolobus sericeus (Forster)	Pteromalidae:Pteromalinae

The introduction of non-native natural enemies to different countries as biological control agents that are self-perpetuating, self-dispersing and potentially permanent is a way of controlling pest insects, but this system can create new interaction with native communities (Sakai, et al, 2001). These interactions can adversely affect the native species, which may in turn prevent non-native species from establishing in a new community (Sakai, et al, 2001). Although *T. sinensis* is known as a specific parasitoid of *D. kuriphilus*, it should be noted that such supposed specificity is exceptional among parasitoid species attacking cynipid galls (Bailey et al, 2009). The host range of *T. sinensis* has never been studied or tested in detail in its native or introduced areas, whether in the laboratory or field (EFSA, 2010; Aebi et al, 2011). To date, only a few experiments have been conduced (Quacchia et al, 2008) and there are uncertainties about the experimental design and the choice of non-target host species, above all for the limited range of alternative host galls (EFSA, 2010). However, this range of alternative hosts is limited, and other alternative host galls on other plants (such as *Diplolepis rosea* (L.) or *Dryocosmus cerriphilus* Giraud, the only species of genus *Dryocosmus* present in Europe) were not considered. The parasitoid communities associated with cynipid communities on oak, rose and chestnut galls are species-rich and generally regarded as closed; for example cynipids on each host plant taxon are attacked by a relatively few (but slightly overlapping) sets of natural enemies (Csoka et al, 2005). Moreover, as it has been seen in oak gall parasitoids (Nicholls et al, 2010), a shift from galls on chestnut to those on alternative hosts would allow *T. sinensis* to disperse beyond chestnut trees. Another risk associated with the introduction of *T. sinensi* is the possibility of hybridization with native species of *Torymus* (EFSA, 2010; Aebi et al, 2011). Hybridization of a biological control agent with native species is considered as an environmental risk to non-target species, and is a general threat to native biodiversity from related introduced or invading species (e.g. Perry et al, 2002). A case of hybridization was reported between *T. sinensis* and Japanese *T. beneficus* Yasumatsu et Kamijio (Moriya et al, 1992); hybrids were also detected in the field and molecular markers proved their hybrid origin (Yano et al, 200).

Also due to the above mentioned potential risk related to the introduction of exotic natural enemies (which, at present, however seem to be limited for *T.* sinensis), it is worth to deepen our knowledge on the new association between this wasp and indigenous parasitoids.

Aims of research

My research was aimed at improving the knowledge on the association occurring in the field between *D. kuriphilus* and native parasitoids and, possibly, at increasing the list of parasitoids that have adapted to develop on this new host, in Emilia Romagna region. An other aim was to gain some preliminary laboratory data about the timing of emergence of the native parasitoids compared to *D. kuriphilus*.

Materials and Methods

This research was conduced in collaboration with the DIVAPRA (University of Turin).

For this research I collected galls from infested chestnuts. The selected area was located in Castel del Rio, a municipality in the Bologna Province (44°12’50’’ N, 11°30’15’’ E), on the Tuscan-Emilian Appenines. Historically, the town's countryside is well known as an important chestnut area; the tipical chesnut of this area, "il Marrone di Castel del Rio", has been cultivated for over 500 years and has received the European

Protected Geographic Status (PGI-Protected Geographical Indicatio) (http://www.marronedicasteldelrio.it/index.htm).

The sampling occurred in the locality “Sestetto”, at an altitude of 460 m.a.s.l. The area appears as a mixed forest dominated by *C. sativa*, but there were also other tree species either spontaneous (eg. *Quercus pubescens* Willd and *Fagus sylvatica* L.) or planted (eg. *Prunus avium* L. and *Ficus carica* L.). The reason of this mixture is probably related to change in management landscape occurred in the second half of the XX century, when the local population fled the area and cultivated fields have gradually been invaded by wild species. In the mid 1980 there was instead a gradual increase in the area used for the cultivation of the chestnut. The first record of *D. kuriphilus* in this area was in 2009 (A. Pollini, personal communication), one year after the first record of this gall wasp in Emilia Romagna (Graziosi and Santi, 2008).

Figure 8: Up: the municipality of Castel del Rio and the area of research (in yellow); down: the locality "Sestetto"

The sampling was performed in 2010, 2011 and 2012, in cooperation with the local chesnut growers and Dr. Aldo Pollini. Galls were selected randomly from chestnut trees. Each year, sampling was conducted in late spring (Tab. 5), when the trees of *C. sativa* were in the vegetative stage and the galls were evident on the plants. All the galls collected were transferred into laboratory; they were counted, put in a plastic boxes (30x18x10 cm,150 per box) with perforated covers closed by a very narrow wire netting and placed at standard conditions in a rearing chamber at 26±1°C, 65±5 RH and L16:D8 photoperiod. The boxes were daily checked and all the insects found inside the boxes (*D. kuriphilus* or others) were captured by an insect aspirator, killed by freezing and subsequently observed under a microscope. The samples were classified with the aid of guides (Chinery, 2004; de Vere Graham and Gjswijt, 1998), dubious samples were sent to external researchers (A. Quacchia and R. Askew) to confirm the identification. In November 2011 some winter galls were also collected in order to assess possible presence of overwintering parasitoids.

Table 5: Dates of sampling, number of galls collected from trees of *Castanea sativa* in collection sites (I or II) in the three years of research.

Date of sampling	Number of galls	Site
01 June 2010	649	I
16 June 2010	2061	
25 May 2011	2050	I
25 May 2011	237	II
09 June 2011	2000	I
12 July 2011	218	I
20 November 2011	560	I
28 May 2012	3500	I
21 June 2012	1300	

The specific aim of the first year of research was to find if any native parasitoid emerged from to *D. kuriphilus* galls collected in the area of study. Since new associations were found, in the second year I enlarged the research: during the first date of sampling I collected galls also in a second site (II), placed at a lower altitude (430 m.a.s.l.) than the first and south-facing, and I also made a third sampling in the first site in early summer (Tab. 5). Due to the results obtained in the second year, in the third year of research I decided to perform only two sampling in a single site, the first (I).

The variables considered for this research were: the species (or genus) richness of parasitoids found, the relative abundance of each insects species/genus calculated on the overall number of insects emerged from galls (in total and in different year of sampling), the time from the day of sampling to insect emergence (in days) and percentage of parasitism. The latter, following the DIVAPRA indications, was calculated assuming the presence of 3.5 gall-cells per gall; therefore the percentage of parasitism was calculated as: number of parasitoids emerged from galls/(number of galls x 3.5). Since all galls were maintained in the standard conditions descrived above, the time from sampling to insect emergence was measured to have a preliminary information about the timing of insect emergence (either *D. kuriphilus* or parasitoids)

Statistical analysis

The analysis was performed for the second and third year of research. The data concerning the insect species, the time and the site of sampling were analyzed by 2x2 contingency tables. Statistical tests were done with STATISTICA 6.0 (StatSoft, 2001).

Results

Parasitoids found

All parasitoids found are known as antagonists of oak gall wasps. The most common genus was *Torymus* Dalman and almost all the individuals of this genus were *T. flavipes* (Walker). In all three years the other genera/species found were a small percentage of the parasitoids emerged (Fig. 10). Since there are still some doubts about the classification of a number of specimens, in the text I generally refer to samples with the genus name. Below I report notes on the most common parasitoid found, *T. flavipes*, with particular reference to external morphology.

***Torymus flavipes* (Walker)**

This ectoparasitoid species presents two generation per year; the first generation usually emerges in late spring, the second in early autumn (Askew, 1965). The adults emerge from the oak galls where they have overwintered. Usually only one egg is laid on a host, which is paralysed, but in some case more eggs can be found in a gall (Askew, 1965).

The female wasp measures 1.7-3.3 mm, *i.e* 2.5 mm on average, excluding the ovipositor. The head is coriaceous, metallic green with red/brown tints. The eyes are large and bright red, however the ocelli are almost colourless. The antennae are dark brown with a yellow marked scape and pale sensillae running along the segments. There is one ring and seven funicular segments. The thorax is metallic gold green and bronze. The wings are clear and hairy, with pale brown hairs. The veins are yellow brown and although the stigmal vein is short the stigma and uncus are both well formed. The legs have metallic green coxae with only afew long, pale hairs on the top of the rear pair. The rest of the legs are coloured pale yellow with 5 tarsal segments and darker claws. The gaster (abdomen) is a shiny brilliant metallic gold green with visible segments that are lightly sculptured and punctured. It is thin from above and from the side view it appears triangular. The ovipositor sheaths are hairy, dark above and yellow below.

The male measures 1.6-2.9 mm with an average of 2.4 mm. The head is slightly different from the female, being coriaceous, bright metallic green and hairy. The antennae are grey brown in colour, with a glossy pedicel and yellow markings on the scape and longitudinal sensillae. The thorax is a bright metallic green with some gold or bronze tinges and it is hairy. The stigmal vein is short but the stigma is sizable. The legs have metallic green coxae with a few long pale hairs on the top of the rear pair. The gaster is bright metallic green at the front, changing to bronze in the rear half with some hairs. It is slender from above but appears to be quite full from the side.

There are differences in colour and form between individuals of the two generations of *T. flavipes*. Insects of the first generation are darker and more bronzed than those of the second generations, and tipically all the femora and the hind tibiae are marked with metallic green. Second generation pasasitoids are a much brighter green, with generally only the hind femora marked with metallic green. There is also a marked difference in length of the ovipositor sheathes: insects of the autumn generation always have short ovipositor sheaths; whilst those of the spring generation are dimorphic, sometimes having short ovipositor sheaths similar to the autumn generation, but most often having considerably longer ovipositors (Askew, 1965).

Torymus flavipes (Walker) ♀ *Mesopolobus tibialis* (Westwood) ♀ *Ormyrus pomaceus* (Geoffroy) ♀ *Megastigmus dorsalis* (Fabricius) ♀ *Sycophila iracemae* Nives-Aldrey ♂

Figure 9: Parasitoids emerged from *Dryocosmus kuriphilus* galls in 2011 (identified by R. Askew)

Parasitoids emerged (quantitative and qualitative data)

In 2010 (01/16 June) I collected 1356 galls; from these 1627 insects emerged, most of which were *D. kuriphilus* (Fig. 10-a). Most parasitoids were *Torymus* (70.14%) (Fig. 10-b); the other species found were *Mesopolobus* (20.14%), *Ormyrus* (7.91%), *Eurytoma* (1.08%) and *Megastigmus* (0.72%).

In 2011 (25 May, 9 June, 12 July) I collected 4505 galls; from these 3209 insects emerged, in this year the percentage of parasitoids and of *D. kuriphilus* were almost equaled (Fig. 10-a). Most parasitoids (92.38%) were *Torymus* (Fig. 10-c), whereas fewer than 10% belonged to other species.

In 2012 (28 May, 21 June) I collected 4800 galls; from these 2224 insects emerged, mainly *D. kuriphilus* (Fig. 10-a). The few parasitoids found (0.68%) belonged to only three species (Fig. 10-d).

In total, in the 3 years, I collected 12015 galls and captured 7060 insects, mainly *D. kuriphilus* (Fig. 10-a). Among the parasitoids found, 88.89% were *Torymus* (Fig. 10-e), the others were mainly *Mesopolobus* and *Ormyrus*.

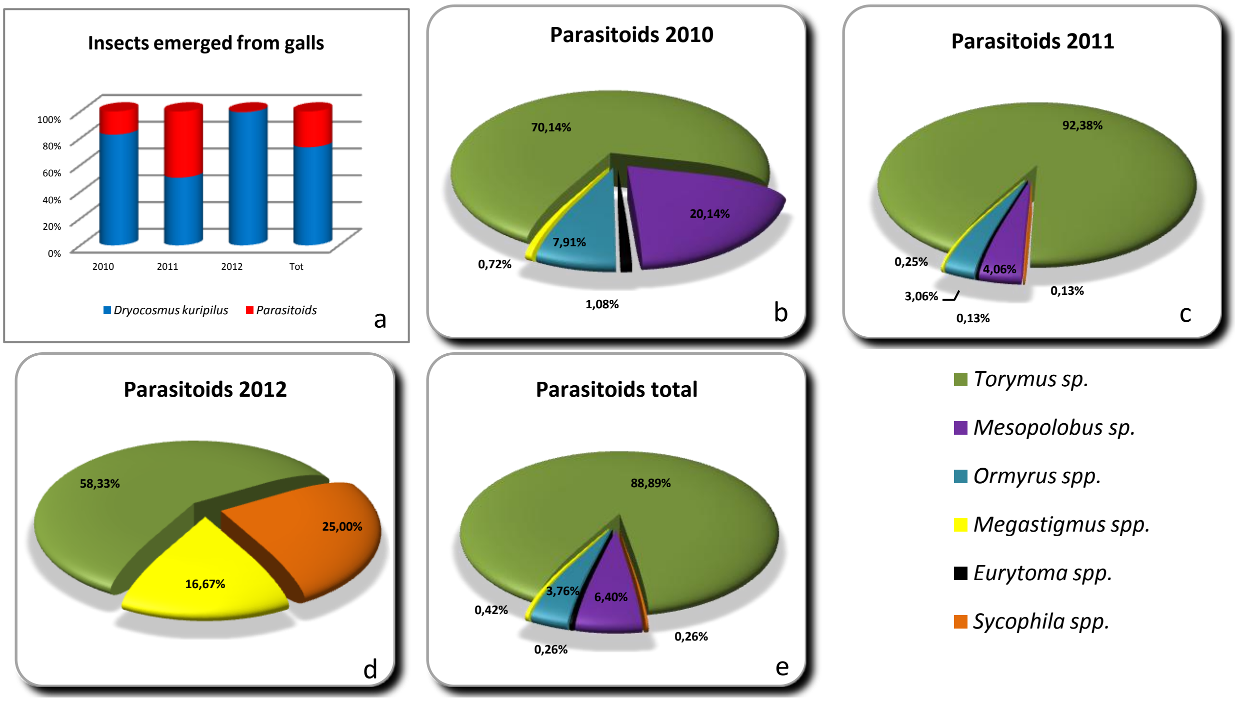

Figure 10: Percentage of *Dryocosmus kuriphilus* and parasitoids emerged from the galls collected in the 3-year research and in total (a) and percentages of parasitoid genera or species emerged in the 3-years research (b-d) and in total (e). The percentages were calculate considering all insects emerged from galls (a) or all parasitoid found (b-e).

Flight time (time from gall collection to insect emergence)

These laboratory data gave preliminary information concerning the timing of insect emergence (either *D. kuriphilus* or parasitoids); they cannot, however, be considered as representative of the field situation, since the galls were all maintained at the same standard conditions when they were transported to the laboratory. Due to the low number of parasitoids found in 2012 I decided to perform statistical anlyses only on the time of emergence (detected in the laboratory, from the data of field collection) of the insects found in 2011.

The largest number of *Torymus* emerged a few weeks after the first sampling and increased steadily until the first half of June; after this period the number of emerging specimens decreased rapidly. Conversely, the largest number of *D. kuriphilus* emerged almost one month after the second field sampling (Fig. 11-a) and increased quickly. Among the other species, *Mesopolobus* and *Ormyrus* were constantly found, whereas the other species were found sporadically. Despite the low number of individuals found, *Mesopolobus* showed two different peaks of emergence (Fig 11-b): in early June (like *Torymus*) and in late June. Also *Ormyrus* showed two peaks of emergence, both in mid

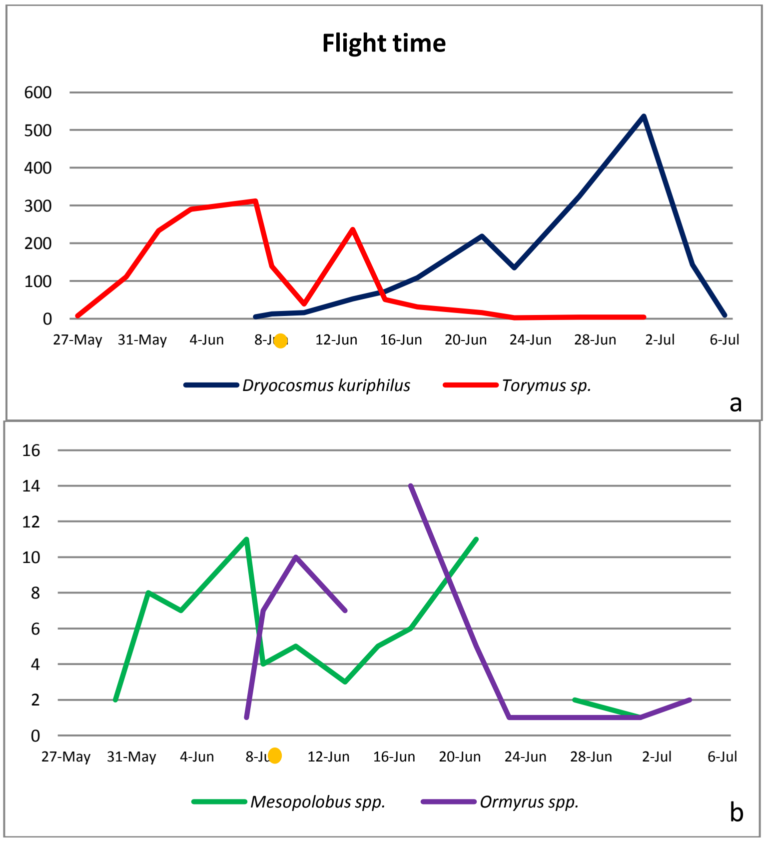

Figure 11: Times of emergence of the most abundant insect species found in 2011. The adults emerged in the laboratory from field-collected galls. The graphs show the number of parasitoids found daily in the boxes containing the galls collected (in all sites) in the first (25 May) and second sampling (9 June, yellow point) until 6 July. In (a) I reported the data concerning the two most abundant insects, *D. kuriphilus* and *Torymus* spp; in (b) the other two relatively abundant parasitoids, *Mesopolobus* spp. and *Ormyrus* spp.

June (Fig. 11-b). All parasitoids species (including Torymus) were found in increasingly small numbers, until their complete disappearance, from the end of June.

No statistical difference was found in the relative abundance of *D. kuriphilus* and parasitoids (of all species) emerged from the galls collected on May 25 in the two site placed at different altitudes (460 and 430 m.a.s.l.); in both sites the relative abundance of parasitoids was considerably higher compared to *D. kuriphilus* (Tab. 6). Conversely, a statistical difference was found in the relative abundance of parasitoids and *D. kuriphilus* between the samples collected on the different dates on site I (Tab. 6). In particular, from the galls collected on June 9, the number of parasitoids compared to *D. kuriphilus* dramatically decreased comared to May 25.

Table 6: Relative abundance (= number and percentages, calculated on the total number of insects emerged), of *Dryocosmus kuripilus* and all parasitoids found: a) in two sites of field-sampling (I and II) and b) at the same site (I) on different dates (May 25 and June 9 2011)

Date/Site	*D.kuriphilus*	Parasitoids	% *D.kuriphilus*	% parasitoids	χ^2	P
May 25/I	254	1059	19.3	80.7	1.83	0.18
May 25/II	62	208	22.9	77.1		
May 25/I	254	1059	19.3	80.7	1004.38	0.00001
June 9/I	1220	329	78.8	21.2		

Statistical differences were found in the relative abundance of *Torymus* and other parasitoids emerged both on the same date between the two sites, and on different dates at the same site (Tab. 7).

Table 7: Relative abundance (= number and percentages, calculated on total number of parasitoids emerged), of *Torymus* and other parasitoids found a) in two sites of sampling (I and II) and b) at the same site (I) on different dates (May 25 and June 9 2011)

Date/Site	*Torymus* sp.	Other parasitoids	% *Torymus* sp.	% other parasitoids	χ^2	P
May 25/I	1025	34	96.79	3.21	118.06	0.00001
May 25/II	163	50	76.53	23.47		
May 25/I	1025	34	96.79	3.21	39.61	0.00001
June 9/I	209	40	83.94	16.06		

Percentage of parasitism

The total percentage of parasitism (calculated on the gall number, see materials and methods), across all three years of research, was 4.47%. The higher percentage was found in 2011 (Tab. 8). As mentioned before, in the year 2012 only few parasitoids were found (Tab. 8).

Table 8: Percentages of parasitism calculated for each date of sampling and in total for each year of research (2010, 2011, 2012). All samplings were performed in the site I except (*) which was made at site II at altitude 430 m.a.l.s.

Date of sampling	N° galls	Parasitoids	Parasitization (%)	Total parasitization per year (%)
01 June 2010	649	37	1.63	3.07
16 June 2010	2061	254	3.52	
25 May 2011	2050	1025	14.29	9.79
25 May 2011*	237	213	25.68	
09 June 2011	2000	330	4.71	
12 July 2011	218	4	0.52	
28 May 2012	3500	6	0.05	0.09
21 June 2012	1300	9	0.20	

Discussion

The control strategy of *D. kuriphilus* in Italy and other countries of introduction is mainly focused on the importation and release of *Torymus sinensis*. Considering the high specificity of interactions occurring between the two wasps, this classical biological control approach seems to be the best possibility. The introduction of an alien parasitoid in a new area of new species is however always hard for different reasons, including the difficulties of adaptation of the new species to the new habitat. *Torymus sinensis* has shown high capacity to adapt itself to new habitat in Japan and in North America, but also in these countries the contribution of indigenous parasitoids has proved important (Cooper and Rieske, 2007).

Moreover in Italy, where *T. sinensis* was introduced in some areas as biological control agent, some indigenous parasitoids were found on *D. kuriphilus* galls (Quacchia, et al, 2012). A new association between *D. kuriphilus* and *T. flavipes* was found in different chestnut areas in Bologna district (Emilia Romagna) by Santi and Maini (2011). My research has shown that also other sites of Emilia Romagna this association is occurring. The data of 2010 and 2011 showed that a range of native parasitoids can attack *D. kuriphilus*. Most species were found in a low number, but *Torymus* spp. (probably *T. flavipes*), has shown high capacity of adaptation to this new host, thus confirming the finding of Santi and Maini (2011). From 2010 to 2011 the number of parasitoids and the percentage of parasitism increased rapidly (Tab. 8), contrary to 2012. The dramatic reduction of parasitoids found in 2012 is presently unexplained. It can be hypothesized that temperature had an influence: in fact in spring 2012 in Emilia Romagna weather conditions were highly variable with temperatures which rapidly (even in 2-3 days) dropped from 20 ° C to 8 ° C. My hypothesis is that *Torymus* spp. adults emerged from their overwintering sites, oak galls, when the temperature was higher but they were killed by the subsequent drop in temperature. It is also possible that the parasitoids were killed by fungi present on galls (A. Pollini, personal communication). It should be noted that also the number of *D. kuriphilus* found was lower in 2012 that in 2011, despite the number of galls collected was similar (Tab. 8).

The low number of other parasitoids found in the 3-year research may be due to their biology and lower suitability of the new host to them. Given the large number of *Torymus* spp. present in that area (at least in 2011), competition may have occurred among the different species.

An objective problem in this research was the little information existing on the range and biology of the indigenous parasitoids of gall wasps. Scant information is in general available on these parasitoids: *Torymus sinensis* itself was described relatively recently (in 1982) and since then its biological cycle (highly synchronized with that of D. kuriphilus) has been studied (Alma and Quacchia, 2012).

The area of my research was a mixed forest dominated by *C. sativa*, but other plants present in the same area (trees but also shrubs and herbaceous plants) could be a source of other gall wasps, hosts of the parasitoids found; in fact all the parasitoids found present a large range of hosts (Noyes, 2012). The parasitoids found in my research (expecially *Torymus* flavipes) have shown the capacity to develop on *D. kupiphilus* larvae when the galls are fresh; but their destiny after the emergence is unknown. Probably they have a second generation on oak galls and overwinter in other oak galls; in spring time they emerge and attack other gall-wasps, includeding *D. kuriphilus* galls. It has to be noted that no parasitoid emerged from overwintering chestnut galls collected in the same area; at the moment, it seems therefore unlikely that the native parasitoids may overwinter in these galls (Francati, unpublished data).

The complex of parasitoids found by me include some species which were also found in other regions of Italy (Quacchia et al, 2012). It is also possible that in other areas of Italy other parasitoids could adapt to this host. More research on the parasitoids involved in the new association with the chestnut gall wasp is required, either concerning the species, quantitative data ant their biology especially on their life cycle and the other hosts present in the area. The final purpose is not only to improve the knowledge on these insects but also to exploit them as biological control agents of *D. kuriphilus* in support of *T. sinensis*.

Harmonia axyridis (Pallas)

Introduction

Harmonia axyridis (Pallas) is a species of Coleoptera of the family of Coccinellidae. The presumed native distribution extends from the Altai mountains (Siberia) to the Pacific Coast, and from southern Siberia to southern China (Korschefsky, 1932; Dobzhansky, 1933; Chapin, 1956; Sasaji, 1977; Kuznetsov, 1997). However this ladybug could be found also in Japan, Korea, Taiwan and in Bonin and Ryukyu archipelago (Dobzhansky, 1933; Chapin, 1956; Iablokoff-Khnozorian, 1982). Numerous subspecies and aberrationes have been described for this polymorphic species (Korschefsky, 1932) so that *H. axyridis* is often referedd to by its English common name, the multicolored Asian ladybugs (Kock, 2003). It is well known as an active predator of aphids, scales and other insects.

Harmonia axyridis usualy has two generations per year in Asia (Sakurai et al, 1992; Asawa, 2000), in North America (LaMana and Miller, 1996; Koch and Hutchison, 2003) and Europe (Ongagna et al, 1993); however, four or five generations per year were also observed (Wang, 1986; Katsoyannos et al, 1997). Typically, females can lay 20/30 eggs per day (Takahaschi, 1987), but in laboratory conditions they can lay more than 3000 eggs with a mean of 25.1 eggs per day (Hukusima and Kamei, 1970). LaMana and Miller (1998) showed that, at 26°C on a diet of *Acyrthopiphon pisum* Harris, the mean duration of each stage was: egg 2.8 days, first instar larva 2.5 day, second instar 1.5 days, third instar 1.8 days, four instar 4.4 days, pupa 4.5 days (Fig. 13). Eggs are oval-shaped and about 1.2 mm long (El-Sabaey and El-Gantiry, 1999); freshly oviposited eggs are pale yellow in color and with time turn to a darker yellow and become grey-black before hatching (He et al, 1994; El-Sabaey and El-Gantiry, 1999). The larval size ranges from 1.9 to 2.1 mm in the first instar to 7.5 to 10.7 mm in the fourth instar (Sasaji, 1977). Larvae are covered with many tubercles (“scoli”) (Savoiskaya and Klausnitzer, 1973). The various instars can be relatively easily distinguished from one another based on coloration; first instars generally have a dark blackish coloration (Sasaji, 1977; Rhoades, 1996), second instars are similar except for coloration of the dorsal-lateral areas of the first (Rhoades, 1996) or first and second abdominal segments (Sasaji, 1977). In the third instar the orange coloration covers the dorsal and dorsal lateral areas of the first abdominal segment and dorsal lateral area of the second to fifth abdominanl segment (Sasaji, 1977; El-Sabaey and El-Gantiry, 1999); the fourth instar is similar to the third, but the scoli of the dorsal area of the fourth and fifth abdominal segment are also orange (Sasaji, 1977). Like other members of the subfamily Coccinellinae pupae are exposed and the fourth instar exuvium remains attached to the posterior end of the pupa, where the pupa is attached to the substrate (Savoiskaya and Klausnitzer, 1973). Cannibalism appears to play an important role in the population dynamics of *H. axyridis* (Osawa, 1993). The intensity of cannibalism seems to be inversely related to aphid density (Hironori and Katsuhiro, 1997; Burgio et al, 2002). The intensity of sibling cannibalism on eggs was density independent, while non-sibling cannibalism on eggs was density dependent (Osawa, 1993) and most intense near aphid colonies (Osawa, 1989). Larval cannibalism increased as a function of conspecific larval density (Michaud, 2003).

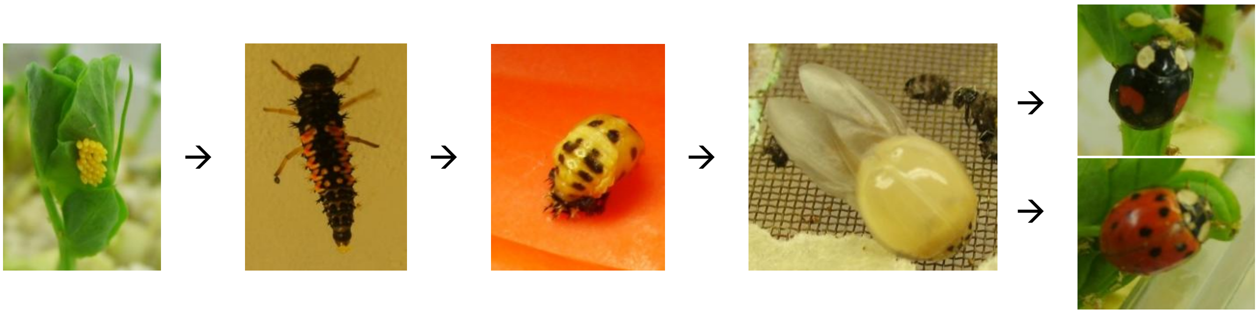

Figure 12: Life cycle of *Harmonia axyridis*= eggs, four instar larva, pupa, adult just emerged, two different adult morphotypes (*spectabilis* and *succinea*)

Adults are 4.9-8.2 mm in length and 4.0-6.6 mm in width (Kuznetsov, 1997). Coloration and maculation is highly variable (Korschefsky, 1932). The head can be black, yellow or black with yellow marking (Sasaji, 1977; Kuznetsov, 1997); the pronotum is yellowish with black markings in center, these markings can be four black spots, two curved lines, a black M-shaped or may be black trapezoid (Chapin and Brou, 1991). The ventral surface can be yellow-orange to black (Chapin and Brou, 1991; Kuznetsov, 1997). Adults of *H. axyridis* are highly polimorphic for the colour and pattern of their elytra. The ground colour may be orange, red or black. Orange and red form may be patterned with 0-21 black spots (the *succinea* form complex), or may show a grid-like black pattern (*axyridis* form); black or melanic forms usualy have two (*conspicua* form) or four (*spectabilis* form) large orange or red spots; other forms are possible, especially in areas of origin, such as the rare form *nigra* (Tan, 1946). This variation has been shown to have a genetic basis, controlled by a multi-allelic gene, with melanic forms generally genetically dominant to non-melanic forms (Hosino, 1933; Tan and Li, 1934; Komai, 1656; Sasaji, 1977). Larval diet (Grill and Moore, 1998) and temperature to which pupae are exposed (Sakai et al, 1974) may also influence the adult coloration and maculation. Colour polymorphism was also shown to vary seasonally within a year (Komai, 1656; Osawa and Nishida, 1992). Spatial variation in the frequency of the various colour morphs has also been documented (Dobzhansky, 1933).

Adults typically live 30 to 90 days depending on temperature (He et al, 1994; El-Sabaey and El-Gantiry, 1999; Soares et al, 2001). Pre-mating and pre-oviposition periods were shown to decrease with increasing temperature (He et al, 1994; Stathas et al, 2001). In Asia the start of migratory flights ranges from mid-October (Liu and Qui, 1989) to late November (Sakurai et al, 1993); *H. axyridis* expresses a hypostactic behavior, meaning that it migrates towards prominent, isolated objects on the horizon (Obata, 1986). Overwinter aggregations may be formed on buildings, often on walls with a southern or western exposure (Kidd et al, 1995). Most of the winter is spent by the adults in diapause that appears to be regulated by corpora allata (Sakurai et al, 1992); this period is entered with an empty digestive tract, enlarged fat body (Iperti and Bertand, 2001). In later winter or early spring ladybugs switch from diapause to quiescent state (Iperti and Bertand, 2001) and upon arrival of warm temperature they mate and disperse from overwinter sites (LaMana and Miller, 1996). During the fall migrations, they preferentially choose to land on white or light-coloured objects (Tanagishi, 1976; Obata, 1986). During the summer beetles may become quiescent (Sakurai et al, 1992).

Althought it is mainly known as a tree species (Hodek, 1973) both in its native area and in the other countries, *H. axyridis* was found in numerous habitats like as meadows, heathland, riparian areas (Adriaens et al, 2008), reeds (Ware et al, 2005; Brown et al, 2008) and cultivated areas (Colunga-Garcia and Gage, 1998). *Harmonia axyridis* appears to have a high ability to track aphid population in space and time (Osawa, 2000; With et al, 2002). Peak arrival and oviposition generally occurs before or at the peak of the aphid population (Hironori and Katsuhiro, 1997; Osawa, 2000). If a coccinellid oviposits when an aphid colony is waning, it is possible that the coccinellid dies due to starvation (Dixon, 2000). Han and Chen (2002) showed that *H. axyridis* could respond to volatiles from aphids and aphid-damaged tea shoots. This coccinellid is a predator of numerous aphid species (Hodek, 1996). They also feed on Tetranichidae, Psyllidae, Coccoidea, immature stages of Chysomelidae, Curculionidae and Lepidoptera (Kock, 2003), and also on pollen and nectar (LaMana and Miller, 1996; Berkvens et al, 2008). The total numeber of aphids consumed through the larva stages can vary from 90 to 370 aphids, depending on the aphid species (Hukusima and Kamei, 1970), with increasing aphid consumption for each successive instar (Hukusima and Kamei, 1970).

Despite having aposematic coloration and reflex bleeding (Grill and Moore, 1998) of alkaloid secretion (Alam et al, 2002), *H. axyridis* does have natural enemies and may fall victim of predation. Eight species of birds preyed on *H. axyridis* in Russia (Nechayev and Kuznetsov, 1973). Dutcher et al. (1999) reported hight densities of *H. axyridis* on tree without ants compared to trees with ants. Howerer, *H. axyridis* was more successful than *C. septempunctata* at avoiding attack red imported fire ants, *Solenopsis invicta* Buren (Dutcher et al, 1999). Male-killing bacteria can influence the demography of coccinellids; the male killing bacterium infecting some populations of *H. axyridis* was identified as a member of the genus *Spiroplasma* (Majerus, 1999). Several parasitoids attack *H. axyridis*. A phorid *Phalacrotophora* sp. was reported to parasitize *H. axyridis* pupae in its native Asian range (Maeta, 1969; Osawa, 1992). Dysney (1997) described this phorid as *Phalacrotophora philaxyridis* Disney. In Korea two parasitoids parasitized *H. axyrids* adults: the tachinid fly *Deigeria lutuosa* (Park et al, 1996) and the braconid wasp *Dinocampus coccinellae* (Schrank) (Park et al, 1996).

Harmonia axyridis is a well-known aphid predator in its native range (Hukusima and Kamei, 1970), for thir reason it has been utilized in augmentative biological control in Asia (Seo and Youn, 2000), Europe (Trouve et al, 1997) and North America (LaRock and Ellington, 1996). The relative ease of *H. axyridis* rearing makes it particularly attractive for biological control. The mass rearing can be made on a variety of aphid species (Hodek, 1996), but it is also possible on many non-aphid foods, such as the eggs of various Lepidoptera (Abdel-Salam and Abdel-Baky, 2001), pulverized drone bee brood (Okada and Matsuka, 1973), pollen and fruit (Berkvens et al, 2008) and some artificial diets (Niijima et al, 1986; Dong et al, 2001; Sighinolfi, et al., 2008). However the number of commercial insectaries rearing *H. axyridis* is decreasing in the word, due to its potential pest status; nowadays, in Europe, this coccinellid (a flighless strain) is produced and commercialized only by BIOTOP (Valbonne, France). In fact adverse effects of this ladybug on insects, humans and crops have been reported (BioControl, 2011). For example in a nine-years-study of the abundance of various Coccinellidae in an agricultural landscape a decrease in the abundance of native coccinellids after the establishment of *H. axyridis* was shown (Colunga-Garcia and Gage, 1998); intraguild predation has been examined as a mechanism leading to displacement of native species since *H. axyridis* appeared to be a top predator in the guild of aphidophagous insect (Dixon, 2000). Numerous studies indicated that *H. axyridis* can effectively utilize other members of the aphidophagous guild, as well as other non-pest insects, as a food source (Tab. 9).

Table 9: Non-pest insect prey of *Hamonia axyridis* (from Kock [2003] modified)

Order	Famiy	Species	Staged consumed*	Reference
Coleoptera	Coccinellidae	*Adalia bipunctata*	PP,P	Sakuratami et al., 2000
			L	Kajita et al, 2000
			E	Lynch et al., 2001
			E	Burgio et al, 2002
		Adonia variegata	E	Lynch et al., 2001
		Coleomegilla maculata	E,L	Cottrell and Yeargan, 1998
		Coccinella septempunctata	L	Hironori and Katsuhiro, 1997
			L	Yasuda and Ohnuma, 1999
			L	Yasuda et al, 2001
		C. septempunctata brucki	P	Takahashi, 1989
			L	Dixon, 2000
		Cycloneda sanguinea	E, L	Michaud, 2002
		Propylea japonica	L	Dixon, 2000
		P. quatuordecimpunctata	E	Lynch et al, 2001
Lepidoptera	Nymphalidae	*Danaus plexippus*	E, L	Koch et al, 2003
Neuroptera	Chysopidae	*Chrysoperla carnea*	E	Phoofo and Obrycki, 1998
Diptera	Syrphidae	*Episyrphus balteatus*	L	Ingels and de Clercq, 2011

*E= eggs, L= larva, PP= prepupa, P= pupa

In many cases *H. axyridis* had a greater ability than the other guild member to utilize heterospecifics for food. The intensity of predation on other guild members appeared to be inversely related to aphid density (Hironori and Katsuhiro, 1997; Burgio et al, 2002). It was also suggested that high predation rates of *H. axyridis* on its own eggs compared to eggs of other coccinellid species may mitigate the displacement of native coccinellids (Lynch et al, 2001; Burgioet al, 2002). The displacement of native coccinellids might also be driven by indirect mechanisms, one of them is the resource competition; Michaud (2002) showed that, in citrus groves, *H. axyridis* was a more voracious predator and had higher fecundity and fertility than *Cycloneda sanguinea* L. Alternatively Hoogendoom and Heimpel (2002) obseved an indirect interaction between *H. axyridis* and *Coleomegilla maculata* De Geer mediated by the parasitoid *D. coccinellae*: the presence of *H. axyridis* actually benefited *C. maculata* by diverting some of parasitoid eggs away from the *C. maculata* population.

Harmonia axyridis is the prime example of a biological control agent reported to have become a nuisance of humans; in late autumn, when swarms of this ladybug fly to the overwintering sites like buildings (Kidd et al, 1995), many people become vexed. Some people have even developed an allergic rhinoconjunctivitis to *H. axyridis* (Huelsman et al, 2002). Surprisingly *H. axyridis* has also been reported to bite humans (Huelsman et al, 2002). Furthermore, *H. axyridis* has attained the status of a potential pest of fruit production and processing; in autumn adults of these ladybugs have been reported aggregating on, and in some case feeding on, different fruits. This is a particularly important problem in vineyards that grow grapes for vine production (Radcliffe and Flanders, 1998; Ejbich, 2003); *H. axyridis* is apparently difficult to remove from clusters of grapes during harvest, so some get crushed with the grapes and their alkaloids taint the flavour of the resulting wine (Ejbich, 2003).

However, the species has also a long history of use as a classical biological control agent of aphids and coccids in North America, where it was first introduced in 1916 in California (Gordon, 1985); it has been widely used for pest control in crops as diverse as pecans (Tedders and Schaefer, 1994) and red pines (McClure, 1987). Despite releases in fourteen US states between 1964 and 1982 (Gordon, 1985) *H. axyridis* was not reported as established in the country until 1988 (Chapin and Brou, 1991); after this initial detection it spread rapidly across North America (Tedders and Schaefer, 1994; Colunga-Garcia and Gage, 1998). There is uncertainty over the source of this established population, which might have originated from eigther a single intentional release or accidental introduction (Krafsur et al, 1997), but Day et al (1994) suggested that the source could have been accidental seaport introductions. In Europe, early introductions of *H. axyridis* occurred in the east, including Ukraine from 1964 (for the contol of aphids on fruit trees) (Katsoyannos et al, 1997) and Belarus from 1968 (Sidlyarevich and Voronin, 1973). In western Europe, *H. axyrids* was first used as a biological control agent in 1982 in France, introduced by Institut National de la Recherche Agronomique (INRA) (Iperti and Bertand, 2001), but it was in quarantine until 1990 (Kabiri, 2006). After this first introduction *H. axyridis* was largely used in many countries in Europe as an agent for biological control (Tab. 10). In Italy, it was used in greenhouses in the north from 1995 to 1999 (Burgio et al, 2008), but in 2000 the commercialization was been interrupted due the concern aroused by its invasiveness and its impacts on the indigenous coccinellids and other predators (Burgio et al, 2002). The first known record of *H. axyridis* in the wild was in Turin in 2006 (Burgio et al, 2008); after this initial detection the ladybug rapidly spread across all northern Italy (Burgio et al, 2008). At present only two records of *H. axyridis* in central Italy are known, the first in vineyards of Tuscany (Canovai and Lucchi, 2011) and the second near a building in Abruzzo (Olivieri, 2011). No records are presently known for the Southem Italy, Sicily or Sardinia (EPPO, 2012). *Harmonia axyrisid* has never been imported as a biocontrol agent in UK, but in September 2004 some specimens were found in the field (Majerus et al, 2006; Roy et al, 2006); the species has been recorded and mapped and its rapid spread to north and west from southeast is clear (Roy et al, 2005; Brown et al, 2007). Since mid 2007 *H. axyridis* was recored in 88% of English and 38% of Welsh vice-countries. *Harmonia axyridis* was also found in South America (de Almeida and da Silva, 2002) and in Africa (Brown et al, 2011).

Table 10: Summary of *Harmonia axyridis* occurrence in Europe until 2010 (from Brown et al. [2011], modified)

Country	**Year of first record in the wild** (not necessarily established)	**Deliberately introducted?** (earliest year of introduction)	**Evidedence of establishment?**	**Reference**
Austria	2006	no	yes	Rabitsh and Schuuh, 2006
Belarus	Unknown	Yes (1968)	Unknown	Sidlyarevich and Voronin, 1973
Belgium	2001	Yes (1997)	Yes	Adriaens et al, 2003
Bosnia and Herzegovina	2010	No	No	Kulijer, 2010
Bulgaria	2009	No	Yes	Tomov et al, 2009
Croatia	2008	No	Yes	Stanković et al, 2001
Czech Republic	2006	Yes (2003)	Yes	Brown et al, 2008
Denmark	2006	Yes (2000s)	Yes	Steenberg and Harding, 2009
England	2003	No	Yes	Majerus et al, 2006
France	1991	Yes (1982)	Yes	Coutanceau, 2006
Germany	1999	Yes (1997)	Yes	Tolasch, 2002
Greece	1998	Yes (1994)	Limited	Kontodimas et al, 2008
Hungary	2008	No	Yes	Merkl, 2008
Ireland	2010	No	No	http://invasivespeciesireland.com/
Italy	2006	Yes (1990s)	Yes	Burgio et al, 2008
Latvia	2009	No	Yes	Barsevskis, 2009
Liechtenstein	2007	No	Yes	Brown et al, 2008
Luxembourg	2004	No	Tes	Schneider and Loomans, 2006
Netherlands	2004	Yes (1996)	Yes	Cuppen et al, 2004

Northern Ireland	2007	No	No	Murchie et al, 2008
Norway	2006	No	Yes	Staverlokk et al, 2007
Poland	2006	No	Yes	Przewozny et al, 2007
Portugal	None	Yes (1984)	No	Brown et al, 2011
Romania	2009	No	Yes	Marko and Pozsgai, 2009
Scotland	2007	No	Yes	Holroyd et al, 2008
Serbia	2008	No	Yes	Thalji and Stojanovic, 2008
Slovakia	2008	No	Yes	Brown et al, 2011
Slovenia	2008	No	Yes	Bravnicar et al, 2009
Spain	2007	Yes (1995)	No	Goldarazena and Calvo, 2007
Sweden	2007	No	Yes	Brown et al, 2008
Switzerland	2004	Yes (1996)	Yes	Klausnitzer, 2004
Ukraine	2009	Yes (1964)	Yes	Marko and Pozsgai, 2009
Wales	2006	No	Yes	Brown et al., 2008

In a risk assessment of 31 exotic natural ememies of pests in biological control in Europe, *H. axyridis* had the second highest environmental risk index (Brown et al, 2008); search of new associations between this coccinellid and native parasitoids is therefore justified. Shea and Cresson (2002) showed that the impact of native parasitoids on introduced exotic insects is often not remarkable in the first phases of invasion, but becomes more and more effective in controlling the exotic pest populations over time. Some of the parasitoids of *H. axyridis* in the areas of origin are present in also other countries; in these countries, indigenous parasitoids, with a biology similar to those present in the native areas, were also recorded; for example in Canada *H. axyridis* was parasitized by the tachind fly *Strongygaster triangulifera* (Loew) (Katsoyannos and Aliniazee, 1998) and in North America and Europe by the wasp *Dinocampus coccinellae* (Firlej et al, 2005; Berkvens et al, 2010). *Harmonia axyridis* has spread in Europe at a very fast rate; it is not possible to fully explain the mechanisms of spread leading to the current distribution because of the spatial and temporal aspects of the deliberate releases (Brown et al, 2008). Researches indicate a variable time lag between initial establishment and major explansion in the country where *H. axyridis* was deliberately introduced (Brown et al, 2008); in France this time was approximately 13 years, in Netherlands 6 and in Belgium 4 (Brown et al, 2008). In countries where the species has not been intentionally introduced there seems to be very little time lag between the first record and major expansion (e.g. in England). The reasons for the time lag between establishment of *H.axyridis* and major expansion in Europe are as yet unknown; it is possible that it is related to the genetic makeup of releases and to differences between environmental conditions in the locations from where releases stocks originated and locations where they were released. In some cases, releases did not lead to establishment such as in Greece (Kontodimas et al, 2008) and Azores islands (Soares and Serpa, 2007).

Aims of research

The aim of this part of the research was to find new field association between *H. axyridis* and native coccinellid species and indigenous parasitodis in Italy (Emilia Romagna) or other Europian countries (Ascot, Berkshire, UK). I chose to capture eggs, larvae, pupae and adults with the aim to find some parasitoids of the different stages of the coccinellids (Riddick et al, 2009). The purposes was to gain information on the range of the parasitoids of coccinellids existing in the study areas and their impact on *H. axyridis* compared to the native species. Should any parasitoid have been detected, the following thesis part were intended to be focused on the investigation on the biology, host-parasitoid interaction and rearing technique of the species found. This part of the research was preliminary to the others.

Part one: searching parasitoids in the field

Materials and Methods

Samples of coccinellids larvea, pupae and adults were collected using the sight insect catch system in Emilia-Romagna (Italy) and Berkshire (UK). Eggs were collected togheter with the supporting leaf. The captures were made from 2010 to 2012 in Italy, mostly from early Spring to late Autumn, and in Autumn 2011 (from September to December) in the UK. The areas of research in Emilia-Romagna were: Bologna (city, suburbs and campus University) (44°48’39’’N, 11°37’84’’E), Granarolo dell’Emilia (Bologna, 44°33’15’’N, 11°26’38’’E), Crevalcore (Bologna, 44°43’0’’N, 11°9’0’’E), Nonantola (Modena, 44°41’0’’N, 11°2’0’’E) and Ravenna province (44°25’4’’N, 12°11’58’’E). In UK the area of research was the campus of Imperial College London at Silwood Park (Ascot, Berkshire, 51°24’0’’N, 00°40’0’’E).

The samples were collected on different trees (peach and apple treees, oaks and plane trees) but also bushes (roses and bay laurel) and grasses (sunflowers, wheat, nettle, etc.), and on buildings walls; they were transferred in a Petri dish into the laboratory and stored in plastic boxes (25x20x10 cm) in rearing chambers at 26±1°C, 65±5 RH and L16:D8 photoperiod. Frozen eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Phyralidae) provided by BIOTOP (Valbonne, France) were used as food. When necessary (*e.g.* food shortage) eggs were integrated/replaced with other food sources like cotton balls soaked in a honey and water solution (20% honey) or fresh eggs of lepidopterous species *Galleria mellonella* (L.). The samples were maintained until death; in detail, the larvae and pupae were maintained under observation until the adults emerged and then for until death; the ladybugs were observed daily to detect potential emergences of parasitoids, to add new food, to remove dead individuals and to clean the boxes. When a parasitoid was found, it was isolated in a plexiglass cylinder (10 cm height, 9 cm diameter) and maintained under observation for all its life. The Coccinellidae were classified with the aid of a guide (Chinery, 2004)

The following measurements were made: 1) number of *H. axyridis* and other coccinellids found; 2) number and species of parasitoids found (as it well be mentioned below, only one species, *Dinocampus coccinellae*, was found); 3) percentage of parasitism (= number of parasitoid emerged/total number of ladybug of each species); 4) time from the collection of ladybugs to parasitoid coccon finding (mean±SE); 5) time from the finding of parasitoid cocoon to adults emergence (mean±SE). With the aim to create a colony of *D. coccinellae* for subsequent studies all adults of the parasitoid were put in a plexiglass cage with some adults of *H. axyridis*; for this reason, at this stages of the research, data on parasitoid adult lifespan were not collected.

Statistical analysis

The effects of variables 2), 3) and 5) (descrive above) in number of *H. axyridis* and other coccinellids found were analysed by one-way ANOVA. No statistical analysis was made for the variable 4) because of the uncertainty of real day of parasitization occurrence. The statistical tests were done with STATISTICA 6.0 (StatSoft, 2001).

Results

In 2010 (February-November) in Italy I collected 1583 ladybugs (Fig. 13-a), most of which were *H. axyridis*; the majority were adults (79.47%) but also larvae and pupae (5.69%) were found; most of other coccinellids were *C. septempunctata* (5.94% adults, 1.20% larvae) or *Hippodamia variegata* (Goeze) (2.53%, only adults). The other Coccinellidae reached a total percentage of 5.18% (4.23% adutls, 0.95% larvae and pupae) this group, called “Others” in the graph, includes the species *Adalia bipunctata* (L.) and different genera among which *Calvia* spp., *Propylea* spp., *Halyzia* spp., which were grouped all together due to their low number.

In 2011 (February-July) I collected 178 ladybugs (Fig. 13-b), almost all of which were *H. axyridis* adults (74.72%), but also larvae and pupae were found (16.85%). The other samples were *C. septempunctata* (5.62%) and “Others” (2.81%), all adults.

In the 2012 (March-June) I collected 1380 ladybugs (Fig. 13-c) most of these were overwinteing adults of *H. variegata* (71.30%) (almost all these individuals were collected in Ravenna province). The other were *H. axyridis* (8.48% adults, 7.17% larvae and pupae), *C. septempunctata* (5.87% adults, 1.16% larvae and pupae) and “Others” (3.12% adults, 1.90% larvae and pupae). The Figure 13-d shows the coccinellids collected only in the Bologna area (the same of the year before). In this area, I collected 401 ladybugs, most of which were *H. axyridis* (29.18% adults, 24.96% larvae and pupae), *C. septempunctata* (20.20% adults, 3.99% larvae and pupae) and “Others” (10.72% adults, 9.98% larvae and pupae); only a few were *H. variegata* (1.25% only adults).

Overall, in three years in Italy I collected 3141 ladybugs (Fig. 13-e). The majority represented taxon was *H. axyridis* (48.01% adults, 6.97% larvae and pupae), followed by *H. variegata* (32.60%), the latter found mostly in early March 2012 near Ravenna; the other coccinellids included *C. septempunctata* (5.890% adults, 1.11% larvae or pupae), and other species all comprised in the “Others” group (3.66% adults, 1.75% larvae and pupae). *Harmonia axyridis* was more abundant in fall, because in Italy it was collected in overwintering sites were big number of ladybugs use to aggregate.

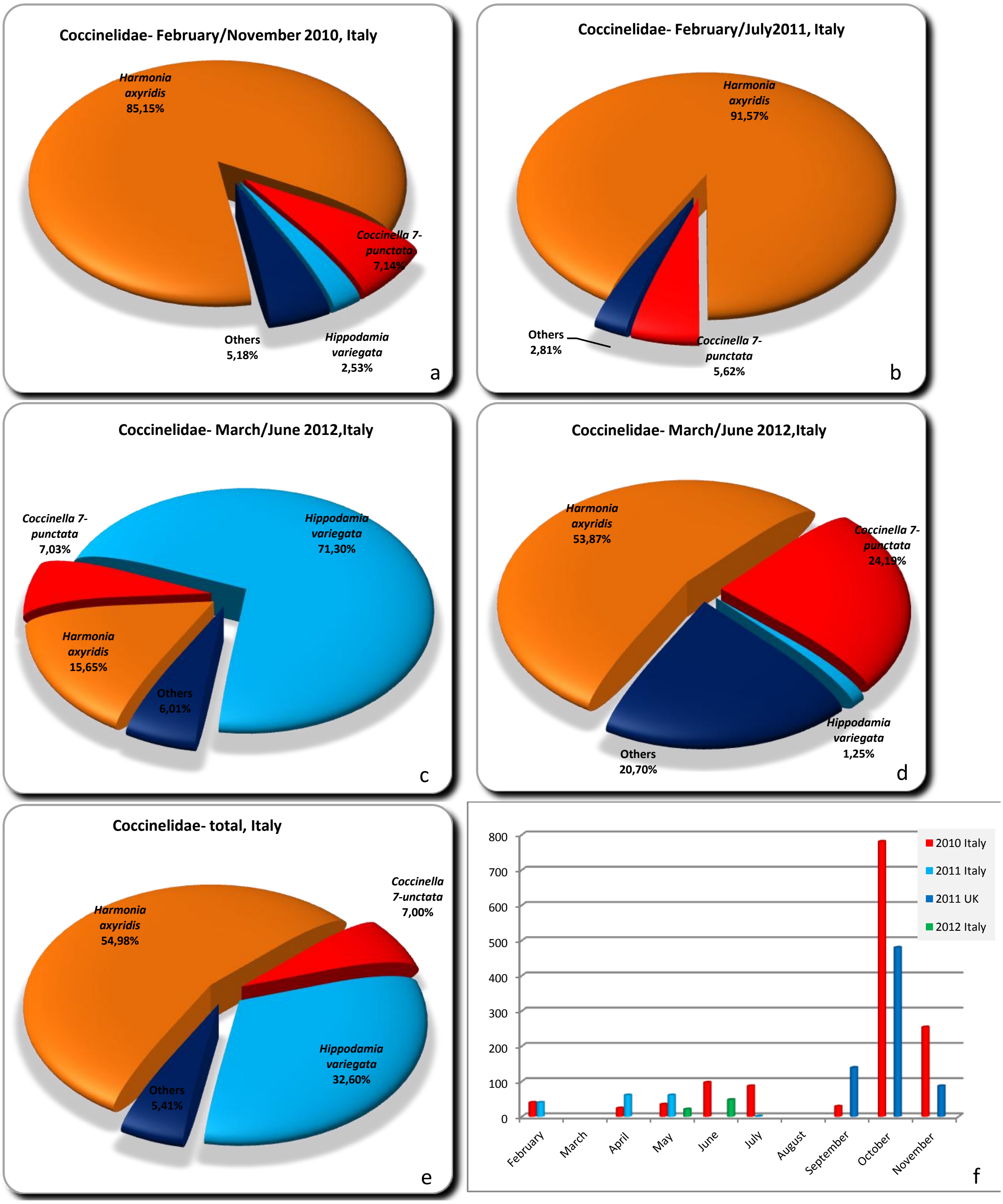

Figure 13: Percentages of ladybugs species found, in Italy, in the three years of research (a-d) and in total (e). The percentage were calculated considering all the stages collected (adults, larvae and pupae) per group. For the percentages considering the adults and immature stages separately, refer to the text. In f) are reported the numbers of *H. axyridis* captured in the several months in Italy and UK during the entire research

In the UK, from September to December 2011, I collected 717 ladybugs (Fig. 14); almost all were *H. axyridis* (89.40% adults, 2.65% larvae or pupae). Most of the others were *C. septempunctata* (4.88%, all adults), the remaining were *A. bipunctata*, *Calvia* spp., *Halyzia* spp., all including in “Others” group, (2.65% adults, 0.42% larvae and pupae).

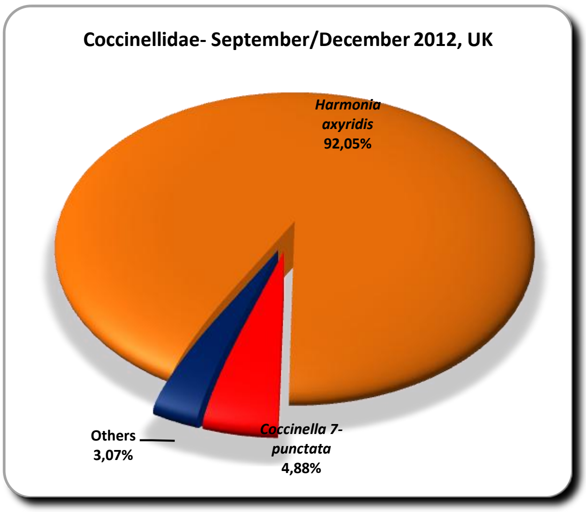

Figure 14: Percentages of ladybugs species found in the UK from September to December 2011. The percentages were calculated considering all stages collected (adults, larvae and pupae) per group. For the percentages considering the adults and immature stages separately, refer to the text

The only parasitoid found on *H. axyridis*, in these three years, in Italy and in UK, was *Dinocampus coccinellae* (Schrank). In Italy, from one pupa of *A. bipunctata* three larvae of *Phalacrotohora fasciata* (Fallén) (Diptera: Phoridae) emerged (classidied by R.H.L. Disney). During field research I also observed some Diptera on the remains of pupae of *H. axyridis*; unfortunately I could not capture those samples and establish whether they were parasitoids, but the behavior shown by them highlighted an interest to pupae (the flies carefully examined the pupae as if they were willing to oviposit). It is therefore possible to speculate that those Diptera were parasitoids of *H. axyridis*.

In Italy *D. coccinellae* emerged from only three coccinellid species: *H. axyridis*, *C. septempunctata* and *H. variegata*. In 2010 (Fig. 15-a) almost all parasitoids emerged from *H. axyridis* (86.89%), fewer from *C. septempunctata* (11.48%), a very few from *H. variegata* (1.64%) (the percentages were calculated as number of *D. coccinellae* emerged from one coccinellid species/ total number of parasitoids emerged from all species). Except for one case, a larva of *H. axyridis,* all samples of *D. coccinellae* emerged from adult ladybugs. In 2011 (Fig. 15-b) the parasitoids emerged only from the adults of two species: *H. axyridis* (80.00%) and *C.septempunctata* (20.00%). Conversely, in 2012 (Fig. 15-c) a few parasitoids emerged from *H. axyridis* (11.76%) and higher percentages were obtained from *C. septempunctata* (47.06%) and *H. variegata* (41.18%). Over the three years (Fig. 15-d), however, I found most parasitoids on *H. axyridis* (72.04%), fewer in *C. septempunctata* (19.35%), an fewer still in *H. variegata* (8.60%).

Conversely, the highest percentages of parasitism per species (= numbero of *D. coccinellae* emerged from one coccinellid species/number of coccinellids of that species) were always found in *C. septempunctata*. In 2010 this percentage was 3.93% for *H. axyridis* (4.21% if we consider only adult *H. axyridis*), 6.19% for *C. septempunctata* (7.45% of which only on adults) and 2.50% for *H. variegata*. In 2011 it was 7.36% for *H. axyridis* (11.76% only on adults) and 30.00% for *C. septempunctata*. In 2012 was 0.93% for *H. axyridis* (1.71% only on adults), 8.25% for *C. septempunctata* (9.88% only on adults) and 0.71% for *H. variegata*. Overall, for all three years, the percentages of parasitism found were 3.88% for *H. axyridis* (4.54% only on adults), 8.18% for *C. septempunctata* (9.73% only on adults) and 0.78% for *H. variegata* (no parasitized immature *H. variegata* were found).

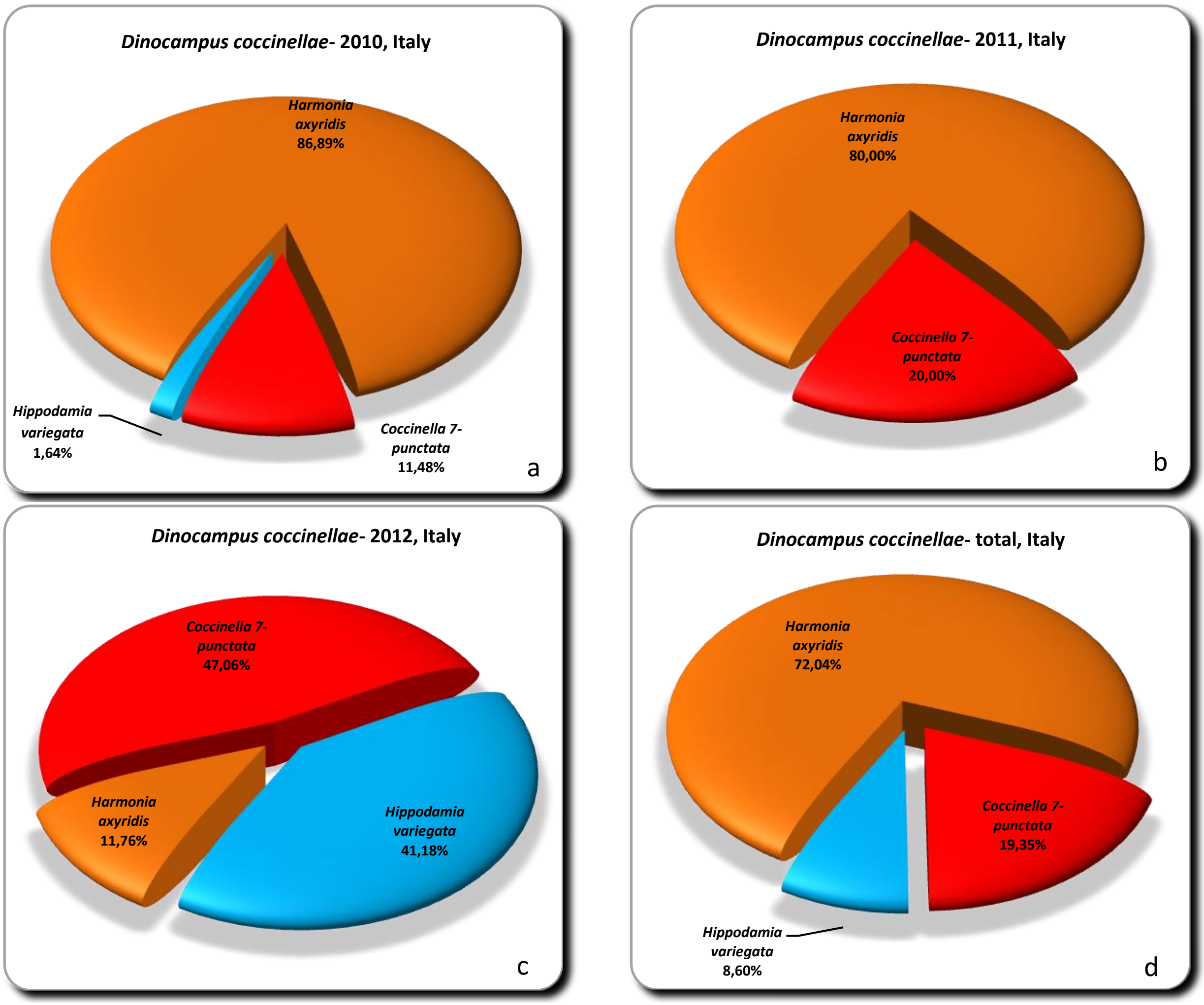

Figure 15: Percentages of *Dinocampus coccinellae* found, in Italy, in the three years of research (a-c) and in total (d). The percentages were calculated separately for each species.

In UK *D. coccinellae* emerged only from adults of two species: *H. axyridis* and *C. septempunctata (Fig. 16)*; most parasitoids emerged from *C. septempunctata* (87.50%) and a few from *H. axyridis* (12.50%). For *C. septempunctata* the percentage of parasitism was very high (40.00%); while it was very low for *H. axyridis* (0.30%).

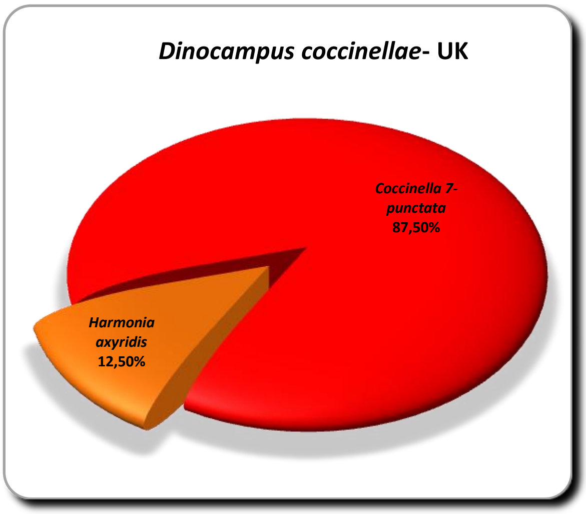

Figure 16: Percentages of *Dinocampus coccinellae* found in UK from September to December 2011. The percentages were calculated separately for each species.

The mean times (±SE) of development of *D. coccinellae* from the discovery of parasitoid cocoon to adult emergence were, in Italy, 7.75(±0.33) days in *H. axyridis*, 6.57(±0.57) days in *H. variegata*, 7.71(±0.47) days in *C. septempunctata* (Tab. 11). In this species, in UK, the mean time from cocoon to adults was 10.18(±0.60) days. No statistical difference was found among the three species of ladybugs in Italy (F=1.16, df=2.39, P=0.32); instead a significant difference was found between *C. septempunctata* in Italy and in England (F=8.49, df=1.16, P=0.010); no statistical analysis was done between *H. axyridis* in Italy and England due to the low number of samples found in the UK.

Table 11: Mean time (±SE) of development of *Dinocampus coccinellae* from discovery of cocoon to adult emergence in Italy and UK. Number of samples (n) is given in parentheses over the means. Means of Italian ladybugs in the column followed by the same letter are not significantly different; means of Italian and UK *Coccinella septempunctata* in square brackets are significantly different. Values are significant at P>0.05 or lower.

	Species	From cocoon to adult
Italy	*Harmonia axyridis*	(n=28) 7.75(±0.33) a
	Coccinela septempunctata	(n=7) 7.71(±0.47) a [a]
	Hippodamia sp.	(n=7) 6.57(±0.57) a
		F(df)= 1.16 (2,39) P= 0.32
UK	*Harmonia axyridis*	
	Coccinela septempunctata	(n=11) 10.18(±0.60) [b]
		F(df)= 8.49 (1,16) P= 0.010

Part two: *Dinocampus coccinellae* (Schrank)

Introduction

Dinocampus coccinellae (Schrank) is a hymenopteran species belonging to the subfamily of Euphorinae, family Braconidae. This species has a cosmopolitan distribution covering all continents except Antartica. The natural geographic range is difficult to reconstruct (Balduf, 1926); the wasp might either have been introduced to North America from Europe or may be native to both North America and Eurasia. It is thought that this parasitoid arrived in some non-native countries together with ladybugs released for biological control purposes. It was probably transferred to the Hawaiian islands with the introduced *Olla v-nigrum* (Mulsant) (Timberlake, 1918), and to New Zealand with another biocontrol agent, *Coccinella undecimpunctata* (L.) (Gourlay, 1930).

Biology

Dinocampus coccinellae usually reproduces by thelytokous parthenogenesis (Ceryngier and Hodek, 1996). However, males can sporadically appear (Muesebeck, 1936; Hudon, 1959; Wright, 1978; Geoghgan et al, 1998; Shawet al, 1999), and at least some of its strains are actually deuterotokous. In some experiments *D. coccinellae* males showed courtship behaviour, involving wing vibration (Wright, 1978; Geoghgan et al, 1998; Shaw et al, 1999) and walking in tight circles (Wright, 1978). All males tried to mount females but only one completed the mating. This male mated with four females for about 20 minutes per female; only 24 of the 74 ladybugs offered to those four mated females were parasitized and from 11 of them adults wasps developed, all of them being females (Wright, 1978).

Dinocampus coccinellae mostly oviposits into adult ladybug beetles but, especially when adult hosts are scarce, larvae and pupae can also be parasitized (Smith, 1960; Maeta, 1969; Filatova, 1974). The wasp most frequntly parasitizes its hosts when they are mobile (Bryden and Bishop, 1945; Walker, 1961; Richerson and DeLoach, 1972; Orr et al, 1992). Pre-oviposition and oviposition behaviour may be divided in three stages: pursuit and investigation of the host without extending the ovipositor, ovipositional stance with the ovipositor extended ventrally and forwards between the legs and ovipositional attack (Richerson and DeLoach, 1972); these activities are stimulated both by visual (movement, colour and size of a potential host) and olfactory (host derived substance) cues (Ceryngier et al, 2012); odour is an important stimulus for host recognition but not for host acceptance (Richerson and DeLoach, 1972). The movement of the host not only facilitates its location but also makes oviposition easier; when ladybugs are walking their elytra are slightly raised and the head is extended forwards, which gives the female parasitoid better access to the soft membranes between the posterior abdominal segments or between the head and torax, the host areas into which *D. coccinellae* most often oviposits (Balduf, 1926; Iperti, 1964; Sluss, 1968). If a potential host is motionless, the wasp stimulates such a host to walk by drumming it with the antennae, encircling it and probing with the ovipositor (Balduf, 1926; Walker, 1961; Richerson and DeLoach, 1972). The host, in response to examination, may display some defensive behaviour: immobilization, rapid escape, attacks on the parasitoid with mandibles and attempts to kick the parasitoid ovipositor with hind legs (Firlej et al, 2010). *Dinocampus coccinellae* oviposition has to be very rapid because of host mobility and defensive reactions (Balduf, 1926).

Dinocampus coccinellae is generally known as a parasitoid of ladybugs belonging to the subfamily Coccinellinae. It can successfully parasitize a wide array of species belonging to this subfamily, but some evidence indicates that representatives of other subfamilies or even families may also serve as occasional hosts (Ceryngier et al, 2012); there are also cases of successful laboratory parasitism on non-Coccinellinae host (Ceryngier et al, 2012). The rate of parasitism of ladybugs by *D. coccinellae* may fluctuate considerably depending on localion, season and host. Ladybugs are often more heavily attacked when aggregating at overwintering sites rather than when they are active (Iperti, 1964; Parker et al, 1977; Anderson et al, 1986); parasitism rates of such aggregating ladybugs may differ remarkably between overwintering sites in the same region. Due to parasitoid preferences, differential parasitism rates in relation to the species, sex, age and developmental stage of the host have been recorded. For example it was noted (Maeta, 1969; Parker et al, 1977) that females ladybugs are parasitized to more frequently than males or that adults are preferred to juvenile hosts (Geoghegan et al, 1998) and older larvae are preferred to younger ones (Obrycki et al, 1985).

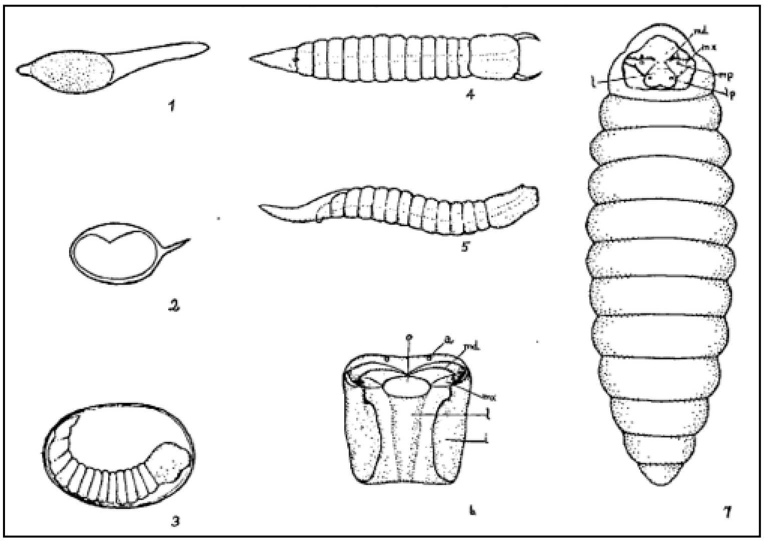

Figure 17: 1) Egg from oviduct, or newly deposited in host, x 100; 2) Same egg aged about four days , x 40; 3) Egg shortly before hatching, x 40; 4) first instar larva, dorsal wiew, showing mandibles extended, squarish head, tail-like appendage, and alimentary tract in outline x 25; 5) First instar larva, lateral view, x 25; 6) Head capsule of firs instar larva, ventral view, x 69; 7) Mature larva, ventral view, showing mouthparts, x 17.5 [a – antennae, md – mandibles, mx – maxillae, o – oral cavity, l – labium, i – inflexed portion of tergum of head, m.p. – maxillary palpus, l.p. – labial palus] (from Balduf, 1926)

Eggs of *D. coccinellae* are elongate and measure about 250 µm by 30µm. the hatched larvae undergo three instars (Ogloblin, 1924); the first instar larva is the only equipped with grasping mandibles used to kill other parasitoids (Ogloblin, 1924). Although it is a solitary parasitoid more than one egg and/or first instar larva, sometime as many as a few dozen, can be found in a single host (Oglobin, 1913; Balduf, 1926; Maeta, 1969). Only one larva survives to the second instar (Balduf, 1926; Sluss, 1968); however, two cocoons emerged from one single ladybug have sporadically been observed (M. I. Scheider, personal comunication). Also the rate of superparasitism (proportion of parasitized hosts harbouring more that one parasitoid individual) in field samples may be quite hight (Ceryngier et al, 2012). The frequent superparasitism of host by *D. coccinellae* may indicate a weak ability of this species to discriminate between unparasitized and already parasitized host (Okuda and Ceryngier, 2000). At 20-26°C the egg-larva development of *D. coccinellae* lasts 2-3 weeks (Sluss, 1968; Obrycki et al, 1985; Kadono-Okuda et al, 1995; Firlej et al, 2007), of which 5-10 days are occupied with embryonic development (Balduf, 1926; Sluss, 1968; Kadono-Okuda et al, 1995). When the parasitized host stage is a larva or pupa the parasitoid development is arrested at the first instar larva until the eclosion of the host to adult (Kadono-Okuda et al, 1995). *Dinocampus coccinellae* usually diapauses as a first instar larva, or occasionally as an egg (Balduf, 1926; Wright and Laing, 1982). In diapausing hosts the parasitoid will not develop beyond the first larval instar (Kadono-Okuda et al, 1995) but causes a decrease in the duration of its host's diapause (Ceryngier et al, 2004).

In the early phase of *D. coccinellae* development a stimulatory effect of the parasitoid on the maturation of the female host's gonads may be observed. Later vitellin accumulated in the host oocytes is reabsorbed (Kadono-Okuda et al, 1995) and ovarian maturation is inhibited (Balduf, 1926; Walker, 1961; Maeta, 1969). The gonads of male hosts also seem to be affected by the development of this parasitoid since inhibition of spermatogenesis activity was found in some experiments (Ceryngier et al, 2004). During most of its development, the *D. coccinellae* larva does not feed directly on the host tissues. As observed by Sluss (1968) only the first instar larva may directly consum the host's fat body. Afterwards, larval nutrition is mediated by teratocytes, cells derived from the serosa of the parasitoid egg, known to occur in some parasitic wasps (Salt, 1968; Tremblay, 1966; Dahlman et al, 2003). When the egg hatches, its serosal membrane dissociates into individual teratocytes which are released into the host haemocoel. In *D. coccinellae* the major function of teratocytes is to provide food for the developing parasitoid larvae. These teratocytes grow due to synthesis and accumulation of a specific polypeptide (Kadono-Okuda et al, 1995) and their numbers decreases as a result of larval feeding (Sluss, 1968; Kadono-Okuda et al, 1995). During developmental arrest of the parasitoid larva in a juvenil or diapausing host, teratocyte development is also halted.

The mature *D. coccinellae* larva emerge from the host ladybug through the membrane between the fifth and sixth or between the sixth and seventh abdominal tergites and then spins its cocoon between the legs of the host before pupation (Balduf, 1926; Sluss, 1968; Maeta, 1969). About 30 minutes before the emergence of the larva, the ladybug becomes immobile and usually remains in this condition until its death. The legs of the immobilized beetle can hold the cocoon and it is assumed that their extensor muscles are paralyzed (Balduf, 1926; Bryden and Bishop, 1945; Walker, 1961). The duration of the pupal stage of *D. coccinellae* at 22-26°C is about 7-10 days (Obrycki et al, 1985; Firlej et al, 2007); after completing its pupal development the wasp leaves the cocoon by biting through it all the cephalic end (Balduf, 1926). As a consequence of the indirect feeding of *D. coccinellae* larva most of the host organs remain intact during the development of the parasitoid larva. The only organs found to be strongly affected are the gonads, the development of which is inhibited, and the fat body that degenerates and considerably decreases in size (Sluss, 1968). Due to the relatively little damage inflicted, the ladybugs usually survive the emergence of the parasitoid larva; Triltsch (1996) found that some laboratory parasitized *C. septempunctata* females not only survived parasitism but also started laying eggs about 12 days after the emergence of the parasitoid larva (Berkvens et al, 2010).

Aims of research

This part of research was focused on the investigartion of the biology, host-parasitoid interaction and rearing technique of *D. coccinellae*. In particular the aims were to study 1) host acceptance and development time of *D. coccinellae* on *H. axyridis*, 2) fecundity and longevity of *H. axyridis* females parasitized by *D. coccinellae* and 3) development of *D. coccinellae* on *H. axyridis* reared with artificial diet.

Materials and Methods

Breeding of insects

To have insects available for the experiments, it was necessary to set up a laboratory colony of *H.* axyridis. The colony was established with adults which were collected early February 2010 at Crevalcore (Modena, Italy, 44°43’0’’ N,11°9’0’’ E) and augmented with other adults which were found in differen locations of Bologna area in all 2010. These adults were maintained in plastics boxes (25x20x10 cm) and fed with frozen eggs of *E. kuehniella* with the aim to start the laboratory colony and also to find native parasitoids possibly emerging from the wild ladybugs.

The eggs laid from the wild ladybugs were trasferred to plastic boxes of similar size. The larvae were fed *ad libitum* with frozen eggs of *E. kuehniella* until pupation. Adults then were moved into a plexiglass cage (40x30x30 cm) and fed with frozen eggs of *E. kuehniella*. The cage was checked every two days to find new eggs for the colony maintenance and for the experiments. In the second half of 2010 I noticed a decrease of fecundity and consequentely a decrease in the number of the larvae which developedt to adults. Therefore, for colony mantainance, I decided to switch to the natural food of the coccinellid such as alive aphids; as espected, this food enhanced female fecundity. More eggs and larvae were thus obtained with the advantage to decrease production costs.

Figure 18: Larvae and adults of *Harmonia axyridis* which feed with *Myzus persicae*; adult of *Dinocampus coccinellae* which try to parasitize a ladybug; mature third instar larva of *D. coccinellae* which emerged from *H. axyridis* and (up) a cocoon

To have aphids, I started a colony of *Myzus persicae* Sulzer on plants of *Pisum sativum* L. This aphid species was chosen because it is easy maintain in continuous rearing. Every week, seeds of *P. sativum* were put in bowls (7x19x12 cm) filled with perlite, Agrilit®3 (Agriperlite Italiana, Alzaia Trento, Italy). Bowls were put in plastic boxes (13x36x24 cm), with perforated covers inside a rearing chamber (21±1°C, 65±5 RH and L16:D8 photoperiod). After 2/3 days plants infested with aphids were put in bowls containing uninfested plants. The bowls with *M. persicae* were put inside plexiglass cages with *H. axyridis* adults; periodically the plants were checked to find eggs, which were in turn placed in plastic boxes, similar to those used for aphids. The new larvae were fed *ad libitum* until adult emergence (Fig. 18).

The first *D. coccinellae* adult emerged in the laboratory from a field-collected specimens of *H. axyridis* collected in Bologna in the second half of 2010. *Dinocampus coccinellae* adults were used to start a laboratory colony The colony was maintained by keeping some *D. coccinellae* and *H. axyridis* adults in a plexiglass cage until death or parasitoid cocoon finding. Honey drops and frozen *E. kuehniella* were used as food for the parasitoids and the ladybugs, respectively. The eggs were preferred to aphids because their use facilitated to check the cage to find new cocoons on *H. axyridis*. The cages was maintained in a another rearing chamber with the same conditions to avoid contamination between two colonies. To try to increase the parasitoid colony, in 2011 I decided to remove the ladybugs from the parasitoid cage after one week and to keep them in plastic boxes until death or parasitoid cocoon emergence.

The experiments concerning *H. axyridis* and *D. coccinellae* were performed in the laboratory of Entomology at the University of Bologna, Italy. The colonies of the ladybug and the parasitoid were mantained in rearing chamber at 26±1°C, 65±5 RH and L16:D8 photoperiod. The experiments were all conducted in the same condictions.

Host acceptance, suitability and development time of *Dinocampus coccinellae* in *Harmonia axyridis*

The experiments were performed under no-choice conditions. I tested three different time of exposition of *D. coccinellae* to *H. axyridis*, resultimg in the following three treatments: 5 minutes (A) (Berkvens et al, 2010), 1 hour (B), 24 hours (C). for each treatments, 100 *H. axyridis* adults 3-4 days old, of both sexes, were used; each adult corresponded to a replicate. *Dinocampus coccinellae* females were obtained from the culture maintained on *H. axyridis* and were 2-3 days old. For the experiments five ladybugs per treatment were exposed to one parasitoid; the arenas used for the different times of exposition were: a 9 cm diameter Petri dish (A), a plastic cylinder (20 cm height, 9 cm diameter) (B) and a plexiglass cage (20x20x20 cm) (C). I decided to provide parasitoids with more space to fly in long expositions, and reduce risk of super pasitizatism; in the 24 hours treatment (C), inside the cage, more than one *D. coccinellae* at each time was used with the proportion 5 ladybugs/1 parasitoid. Specimens were fed with drops of honey (*D. coccinellae*) and frozen *E. kuehniella* eggs (*H. axyridis*). After the exposition *H. axyridis* were removed from the arenas, placed in plastic boxes (11x30x19 cm) and fed with frozen *E. kuehniella* eggs until cocoon appearance or ladybug death; the new cocoons found were placed individually in a plastic cylinder (20 cm height, 9 cm diameter) and checked daily until adult emergence.

The ladybug was considered as suitable when a cocoon of *D. coccinellae* was found under its body. The percentage of parasitism (= number of cocoons found/number of ladybugs exposed), the percentage of emergence (= number of parasitoids/number of cocoons) and the percentage of adult yield (= number of adult parasitoids/number of ladybugs exposed) were calculated. The development times (in days) (larval: from exposure to cocoons detection; pupal: from cocoon detection to adult emergence; and total: from exposure to adult emergence) were also calculated. The time of larval development was calculated from the end of exposure, when *H. axyridis* were removed from arena and placed inside the plastic boxes.

Fecundity and longevity of *Harmonia axyridis* parasitized by *Dinocampus coccinellae*

For this experiment, 20 couples of *H. axyridis* were used, together with 20 control couples (*i.e.* non-parasitized specimens); all adults were 2-4 days old. All ladybugs, taken from the colony as pupae, were placed individually in a plastic cylinder (2.5 cm height, 4.5 cm diameter) until adult emergence; they were then fed with *M. persicae* on *P. sativum* plants until the beginning of the experiment. *Dinocampus coccinellae* were taken from the stock colony as cocoon that were separated from the host; these cocoons were placed individually in a plastic cylinder (10 cm height, 9 cm diameter) until adult emergence and fed with drops of honey. For the experiments one female of *H. axyridis* was placed in the cylinder of *D. coccinellae* (1-2 days old) for half hour (based on the results obtained in the previous experiments). After this time each ladybug was placed with an unparasitized male in another cylinder (8 cm height, 6 cm diameter), lined with bubble wrap as an oviposition substrate (Sighinolfi et al., 2008); the couple was fed *ad libitum* with *M. persicae*. The female was observed for 25 days, which is more than the mean time of parasitoid development observed in the previous experiment. The samples were checked daily and when a cocoon was found its was removed from the host ladybug which was then fed until its death. The control couples were maintained under similar conditions.

Each female of *H. axyridis* was considered as a replicate. Time from exposure to first oviposition (= time of pre-oviposition), time of oviposition (= time from the first to last oviposition), adult lifespan of the ladybugs were recorded. A couple was considered fecund when the female has laid one egg at least. The percentage of fecund couples (based on the original number of couples, n = 20), the eggs/female laid and the percentage of first instar larvae emerged from the eggs were also calculated.

Development of *Dinocampus coccinellae* on *Harmonia axyridis* reared with artificial diet

For this experiment, 20 adults of *D. coccinellae* were used; all samples were 1-2 days old. The parasitoids, taken from the colony as only cocoons that were separated from the host, were placed individually in a plastic cylinder (20 cm height, 9 cm diameter) until adult emergence and fed with drops of honey. *Harmonia axyridis* eggs were taken from the colony and fed with *M. persicae* on *P. sativum* palnts until 2-4 days of adult life, when the experiment started. For the experiment,20 replicates were performed. Each replicate consisted of 10 *H. axyridis* which were exposed to *D. coccinellae* inside the above-mentioned cylinder for one hour. After exposure, the ladybugs were removed from the cylinder, and divided in two groups (A and B) of 5 individuals. Each group was placed in a pastic box (5x10x15 cm). Group A was fed with an artificial diet (Sighinolfi et al, 2008) integrated with a cotton ball soaked in water. Group B was fed with frozen *E. kuehniella* eggs and maintained as control. The experiment was repeated with the first two generations of the parasitoid (so that the parental, the F1 and F2 generation were tested). For the F1 and F2 only 10 replicates were used. The ladybugs were observed daily for 30 days in order to check cocoon emergence or ladybug death.

The results were evaluated in terms of percentage of cocoons (based on number of ladybugs exposed), percentage of adult emergence (= number of emerged adults parasitoids/ number of cocoons) and percentages of adult yields (= number of adult parasitoids/number of ladybugs exposed). Times (in days) from exposure to cocoon detaction, time from cocoon to adults emergence, time of total development and adult *D. coccinellae* life span were also calculated.

Statistical analysis

For the first experiment the number of cocoons, of emerged adults (over cocoons) and total adult yields (over original number of ladybugs exposed) were analyzed by 2x2 contingency tables. The development times (B) from cocoon to parasitoid emergence were analyzed by one-way ANOVA; due to heteroscedasticity, the Kruskall-Wallis non-parametric test (followed by a non-parametric multiple range test) was used to analyze (A) the times from the ladybugs exposure to *D. coccinellae* to cocoon detection and (C) the total development time.

For the second experiment the number of females which oviposited at least one egg (fecund female) and number of females from which eggs at least one larva hatched (fertile females) were analyzed by 2x2 contingency tables. The pre-oviposition times were analyzed by One-way ANOVA and mean separation was performed by applyng Turkey test. The Kruskall-Wallis non-parametric test was used to analyze the adult lifespan and the length of the oviposition period (data not homogeneous). To obtain indexes of fecundity I used the number of eggs laid in the first 10 days after oviposition started (Ferran et al, 1998; Evans et al, 1999) and up to 25 days from the ladybug exposure (*e.g.* the total time devoted to the observation of the adult couples). The percentages of hatched larvae over the number of eggs oviposited by each female were also calculated. The A-index of fecundity was analyzed by One-way ANOVA, whereas the Kruskall-Wallis non-parametric test was used to analyze the B-index and the percentages of hatched larvae (data not homogeneous). The percentages values were transformed from the analyses by the ARCSIN transformation (Zar, 1984).

In the third experiment the number of cocoons found, of emerged adults (over cocoons), total adults yields (over original number of ladybugs exposed) and survived *H. axyridis* were analyzed by 2x2 contingency tables. The development times (for all treatment) ad adult lifespan were analyzed by a factorial analysis of variance (Zar, 1984) (2x3 factors tested for the effects of food [*Ephestia* eggs or Artificial diet] and generation [Parental, F1 and F2]).

All statistical tests were done with STATISTICA 6.0 (StatSoft, 2001)

Results

Host acceptance, suitability and development time of *Dinocampus coccinellae* in *Harmonia axyridis*

The percentages of parasitism (*i.e.* the percentages of cocoons found) (Fig. 19-a) were: 18% for the treatement “5 minutes”, 36% for the treatement “1 hour”, 29% for the treatement “24 hours”. Significant differences were found between “5 minutes” and “1 hour” (χ^2= 8.22, df=1, P=0.0041) but not between “5 minutes” and “24 hours” (χ^2= 3.37, df=1, P=0.0666) and between “1 hour” and “24 hours” (χ^2= 1.12, df=1, P=0.2906).

The percentages of parasitoid emergence (Fig. 19-b) found were: 89% for the treatement “5 minutes”, 89% for the treatement “1 hour”, 79% for the treatement “24 hours”. No statistical differences were found between the treatments “5 minutes”/”1 hour” (χ^2= 0.21, df=1, P=0.6466), treatments “5 minute”/”24 hours” (χ^2= 0.20, df=1, P=0.6526) and treatments “1 hour”/”24 hours” (χ^2= 0.52, df=1, P=0.4727).

The percentages of total yields (Fig. 19-c) found were: 16% for the treatment “5 minutes”, 32% for the treatement “1 hour”, 23% for the treatement “24 hours”. A statistical difference was found between treatments “5 minutes”/”1 hour” (χ^2= 7.02, df=1, P=0.0081) but not between treatments “5 minutes”/”24 hours” (χ^2= 1.56, df=1, P=0.2116) and treatments “1 hour”/”24 hours” (χ^2= 2.03, df=1, P=0.1541).

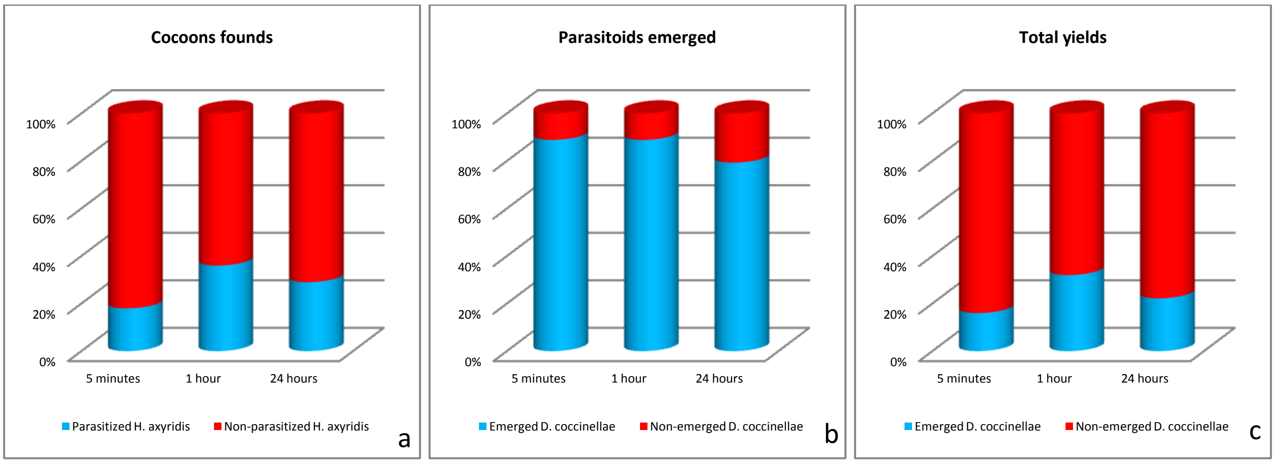

Figure 19: Acceptance and suitability of *Harmonia axyridis* by *Dinocampus coccinellae*: a) percentage of parasitism (*e.g.* percentage of cocoons found) (on all ladybugs exposed), b) percentage of adult emergence (on all cocoons found), c) percentage of adult yields (on all ladybugs exposed). Original number of *Harmonia axyridis*= 100 per treatment

The results concerning the time of development are all shown in Tabel 12.

Table 12: Mean times of development of *Dinocampus coccinellae* in *Harmonia axyridis*: a) from ladybug exposure to parasitoid cocoon detection, b) from parasitoid cocoon detection to adult emergence, c) from ladybug exposition to parasitoid adult emergence. Means in a column followed by the same letter are not significantly different, P>0.05.

Treatments	**Time from exposition to cocoon found**	**Time from cocoon to adult emergence**	**Time from exposure to adult emergence**
5 minutes	17.44±1.34 a	7.44±0.96	24.68±1.20 a
1 hour	17.08±0.77 a	7.25±0.76	24.38±0.87 a
24 hours	19.41±3.21 b	7.48±1.2	26.70±3.59 b
H (N)=	23.66 (83)		11.34 (71)
F(df)=		0.43 (2,64)	
P=	0.00001	0.65	0.035

Fecundity and longevity of *Harmonia axyridis* females parasitized by *Dinocampus coccinellae*

It has to be emphasised that the percentage of successful parasitism of the exposed *H. axyridis* females (based on the number of females on which a parasitoid cocoon was detected over the number of females exposed to *D.* coccinellae) was low (15%). Of the females exposed, 85% laid eggs; two successfully parasitized ladybugs laid eggs before and after the cocoon emerged.

For fecundity, no statistical difference was found between “exposed” and “non-exposed” (= control) females (χ^2=1.44, df=1, P=0.23) (Tab. 13) (the “exposed” group includes both successfully parasitized females and females on which cocoon were never detected). Of these females, 70.6% were fertile (at least one larva hatched from eggs), whereas all the control females were fertile; the difference was significant (χ^2=4.52, df=1, P=0.03) (Tab. 13).

Table 13: Difference between the number and the percentages of *Harmonia axytidis* exposed and not-exposed to *Dinocampus coccinellae* relative to a) females which oviposited at least one eggs (= fecund females) and b) females from which eggs at least one larva harched (=fertile females) (2x2 contigency tables).

Treatment	**Ovipositing females**		**Non-ovipositing females**		χ^2	**df**	**P**
	Number	**Percentage**	**Number**	**Percentage**			
Exposed	17	85%	3	15%	1.44	1	0.23
Not-exposed	20	100%	0	0%			
	Fertile females		**Non-fertile females**				
	Number	**Percentage**	**Number**	**Percentage**			
Exopsed	12	70.6%	5	29.4%	4.52	1	0.03
Not-exposed	20	100%	0	0%			

The exposure had no significant effect either on *H. axyridis* lifespan (Tab. 14) or on pre-ovipostition time (Tab. 14), but its influence was significant for the oviposition time (Tab. 14).

Table 14: Means duration (±SE) in days of a) adult lifespan, b) pre-oviposition time and c) oviposition time of female of *Harmonia axyridis* females exposed or not-exposed to *Dinocampus coccinellae*. Number of samples (n= number of replicates) is given in parentheses over the means.

Treatment	Adult lifespan	Pre-oviposition time	Oviposition time
Exposed	(20) 23.5±1.09	(17) 6.82±0.94	(17) 11.29±1.16
Not-exposed	(20) 25.3±1.6	(20) 7.70±0.45	(20) 15.30±0.52
F(dg)=		1.04 (1,35)	
H(N)=	1.67 (40)		7.17 (37)
P=	0.2	0.31	0.0074

The majority of the eggs were laid in the first 10 days of oviposition; the not-exposed females laid more eggs on average than the exposed ones both in the first 10 days and in total (Tab. 15). Also the eggs hatched (eggs from which larvae emerged) were more in the not-exposed *H. axyridis* both in the first 10 days and in total (Tab. 15). Highly significant differences were found between the two groups relative to eggs laid and fertility (Tab. 15).

Table 15: Difference between *Harmonia axyridis* females exposed and not-exposed to *Dinocampus coccinellae* relative to: a) number of eggs laid in 10 days, b) number of eggs laid in 25 days (total time of the experiment, from adult exposure to *D.* coccinellae), c)percentage of larvae hatched from the eggs laid in 10 days (fertility) and d) percentages of larvae hatched from eggs laid in 25 days (total time of experiment, from adult exposure to *Dinocampus. coccinellae*). Number of samples (n) (= number of females which laid at least one egg) is given in parentheses over the means

Treatment	Eggs laid (10 days)	Eggs laid (total)
Exposed	(17) 160.77±29.84	(17) 226.36±52.39
Not-exposed	(20) 359.7±21.33	(20) 529.08±24.05
F(df)=	30.82 (1,35)	
P=	0.000003	0.0002
H(N)=		17.71 (37)
	Eggs hatched (10 days)	**Eggs hatched (total)**
Exposed	(17) 20.63±4.83	(17) 18.94±4.67
Not-exposed	(20) 49.92±3.11	(20) 51.22±3.40
F(df)=	28.05 (1,35)	30.97 (1,35)
P=	0.000007	0.000003

Development of *Dinocampus coccinellae* in *Harmonia axyridis* reared with artificial diet

The data concerning percentages (±SE) of cocoons detected of emerged adults, of the adult yields and survived *H. axyridis* are summarized in Tabel 16. According to the result, the host food did not significantly influence the parasitoid development, either the larva (from host exposure to cocoon) or the pupal (from cocoon to adult emergence). The adult yields and *H. axyridis* survival were also unaffected by host food. Also the generation effect was not significant, as well as the interaction between the two factors.

Table 16: Development of *Dinocampus coccinellae* in *Harmonia axyridis* reared on two different types of food (frozen *Ephestia kuehniella* eggs or artificial diet): a) cocoons founds (%), b) parasitoid adult emergence (%), c) adult yields (%), d) alive *Harmonia axyridis* (%), as related to the combination of the factors “generation” and “food”. Number of replicates (n) is given in parentheses over the means (±SE).

Parameters (%)	**Food**	**Generation**			**ANOVA results**		
		Parental	F1	F2	Food Effect	Generation Effect	Interation
Cocoon	*Ephestia* Eggs	(20) 44±4.7	(10) 18±6.3	(10) 42±8.7	F=0.06 df=1,73 P=0.81	F=2.01 df=2,73 P=0.14	F=1.83 df=2,73 P=0.16
	Artificial diet	(20) 35±7.1	(10) 32±8.5	(10) 31.1±6.7			
Emerged Adults	*Ephestia* Eggs	(18) 75.9±8.3	(6) 100±0.0	(10) 87.5±10.0	F=0.34 df=1,58 P=0.56	F=0.35 df=2,58 P=0.71	F=0.07 df=2,58 P=0.93
	Artificial diet	(14) 76.2±9.6	(9) 83.3±11.8	(7) 81±9.2			
Adult yields	*Ephestia* Eggs	(20) 23.9±5.3	(6) 16.7±6.8	(10) 27.4±8.7	F=0.90 df=1,66 P=0.35	F=0.60 df=2,66 P=0.55	F=0.16 df=2,66 P=0.85
	Artificial diet	(20) 30.7±6.9	(9) 28.5±9.5	(7) 10.69±4.0			
Alive *H. axyridis*	*Ephestia* Eggs	(20) 30.1±6.7	(10) 31.6±10.0	(10) 34.6±10.9	F=0.19 df=1,73 P=0.66	F=0.65 df=2,73 P=0.53	F=0.43 df=2,73 P=0.64
	Artificial diet	(20) 34.6±7.7	(10) 16.9±5.3	(10) 24.0±8			

The data concerning the mean time (±SE) from host exposure to *D. coccinellae* cocoon detection, from cocoon to parasitoid adult emergence, from exposure to parasitoid adult emergence (= total development) and adult *D. coccinellae* lifespan are summarized in Tabel 17. The total development were significantly influence both the food effect (time were longer for the parasitoids reared on diet-fed *H. axyridis*) and by the generation effect (time were shorter for the F2 parasitoids). The parasitoid lifespan was also significantly affected by the generation effect (the F2 parasitoids lived less) and the interaction between food and generation was also significant.

Table 17: Mean times (±SE) of development of *Dinocampus coccinellae* in *Harmonia axyridis* reared on two different types of food (frozen *Ephestia kuehniella* eggs or artificial diet): a) from ladybug exposure to parasitoid cocoon detection, b) from parasitoid cocoon to adult emergence, c) from ladybug exposure to parasitoid adult emergence, d) adult parasitoid lifespan as related to the combination of the factors “food” and “generation”. The number of replicates is given in parenthesis over the means.

Parameters (time- in days)	**Food**	**Generation**			**ANOVA results**		
		Parental	F1	F2	Food Effect	Generation Effect	Interation
From exposure to cocoon	*Ephestia* Eggs	(18) 16.92±0.3	(6) 17.47±0.78	(10) 16.80±0.23	F=0.25 df=1,59 P=0.62	F=0.57 df=2,59 P=0.95	F=0.75 df=2,59 P=0.48
	Artificial diet	(15) 17.55±0.25	(9) 17.06±0.85	(7) 17.30±0.31			
From cocoon to adults (1)	*Ephestia* Eggs	(16) 6.77±0.19	(6) 8.13±0.46	(9) 6.7±0.37	F=0.26 df=1,52 P=0.61	F=4.86 df=2,52 P=0.01	F=1.69 df=2,52 P=0.19
	Artificial diet	(12) 7.44±0.3	(8) 7.64±0.33	(7) 6.93±0.34			
From exposure to adults (2)	*Ephestia* Eggs	(16) 23.96±0.19	(6) 25.62±1.02	(9) 22.41±0.93	F=4.27 df=1,52 P=0.04	F=6.36 df=2,52 P=0.003	F=0.98 df=2,52 P=0.38
	Artificial diet	(12) 25.08±0.36	(8) 25.64±0.46	(7) 24.14±0.45			
Parasitoid adults lifespan (3)	*Ephestia* Eggs	(17) 7.19±0.25A	(6) 6.83±0.54A	(9) 4.7±0.41A	F=0.72 df=1,51 P=0.40	F=15.07 df=2,51 P=0.00001	F=3.33 df=2,51 P=0.04
	Artificial diet	(12) 6.15±0.32AB	(8) 9.63±2.25A	(7) 5.36±0.34B			

(1) The grand means dor the generation effect are shown in table 18;

(2) The grand means for the food and generation effect are respectively shown in tabels 19 and 18;

(3) Means in a raw followed by same capital letters were not significantly different (one-way ANOVA).

The table 18 and 19 respectively show the grand means for the generation and food effect on development time

Table 18: Grand means for the generation effect on *Dinocampus coccinellae* development time from cocoon detection to adult emergence and from host exposure to adult (total development). Means in a column followed by the same letters are not significantly different.

Generation	Time (in days)	
	From cocoon to adults	From host exposure to adults
Parental	(28) 7.06±0.18 ab	(28) 24.44±0.22 ab
F1	(14) 7.85±0.27 a	(14) 25.63±0.49 a
F2	(16) 6.80±0.25 b	(16) 23.17±0.59 b
F=	4.73	6.69
df=	2,55	2,55
P=	0.012	0.025

Table 19: Grand means for the food effect on *Dinocampus coccinellae* development time from host exposure to adult (total development). Means in a column followed by the same letters are not significantly different.

Food	Time (in days)
	From exposure to adult
Ephestia Eggs	(31) 23.83±0.39 a
Artificial diet	(27) 25.00±0.26 b
F=	5.49
df=	1,56
P=	0.02

Discussion

Developing control methods against *H. axyridis* remains a challenge, for several reasons. It is the first time that a predatory ladybug, or any other predatory beetles, requires control. Thus, there is no previous experience on which to base new management strategies and research on this topic is to date limited. While the control methods presently used or under development may solve the problems in buildings, orchards or vineyards, it is very unlikely that any of these methods will limit the impact of *H. axyridis* on native biodiversity. From its appearance in the field in many countries of Europe, *H. axyridis* has shown to have a strong impact on native species (Brown et al, 2001). The first part of my research was devoted to searching new association between native parasitoids and *H. axyridis*. Only the one between *H. axyridis* and *D. coccinellae* was found and it was somewhat expected. In fact *D. coccinellae* is already know as a parasitoid of *H. axyridis* in some area of introduction of this coccinellid, like Canada (Firlej et al, 2005), Belgium (Berkvens et al, 2010) or UK (Aldeburgh, Suffolk [Browdrey and Mabbott, 2005] and Colchester, Essex [Fremlin, 2007]); I confirmed that this adaptation has happened aso in Italy. As reported by Hoogendoorn and Heimpel (2002) the abundance of *H. axyridis* could have a positive effect on the population dynamic involving the interaction between the coccinellid complex and *D. coccinellae*, as this parasitoid could prefer the new host to the native ones.

In my research areas I always found that *H. axyridis* was the most abundant coccinellid species, both as adult and as immature stages (Figure 13, page 42). The percentages of parasitism by *D. coccinellae* found for this ladybug were hower lower than for the others (3.88% in Italy, 0.30% in UK). Conversely, *C. septempunctata* showed higher percentages of parasitism in Italy despite the low number of samples collected (Figure 15, page 44). Other field-collected ladybugs were parasitised by *D. coccinellae*, but at low rate. This was expected, because, usually, *D. coccinellae* prefers bigger host (Richerson and Deloach, 1972) and all the other coccinellids found were smaller than *H. axyridis* or *C. septempunctata*. Also Koyama and Majerus (2008) found that *H. axyridis* had lower susceptibility than *C. septempunctata* to *D. coccinellae*.

The differences that I found between the parasitism rates of *H. axyridis* in Italy and UK could be also due to the different periods devoted to field samplings (three years in Italy, three months in UK). However, even if only the samples collected in the same period of the year (September/December), although in two different years (2010 and 2011) in the two countries, are considered, the difference is still high (Tab. 20). Unfortunaly, I do not have data from the same period in the same year for the two countries.

Table 20: Number of *Harmonia axyridis* and *Dinocampus coccinella*e and percentage of parasitism found in Italy and UK in the same period (September/December) in two different years (2010 in Italy and 2011 in UK)

	Italy **September/December 2010**	**UK** **September/December 2011**
Harmonia axyridis	1070	712
Dinocampus coccinellae	40	2
Parasitism	3.7%	0.3%

The low rate of parasitism in the field could be due to the low capacity of *D. coccinellae* to locate and accept *H. axyridis.* Accoding to Richerson and Deloach (1972) host location and recognition are mediate by odours, but odours have less importance for acceptance. The high spread capacity and the wide range of preys of *H. axyridis* (higher than for other ladybugs) could make more difficult the encounter between these two species. Moreover, usually the field data relative to *H. axyridis* and its parasitoids (in native or introduced areas) report the rate of parasitism, but usually this rate was calculated in different way by different authors (Ceryngier et al, 2012).

My field data confirm the observations of other authors (including Berkvens et al., 2010) above the low percentage of parasitism of *H. axyridis* by *D. coccinellae*. The laboratory tests however gave more encouraging results. In my laboratory tests *D. coccinellae* showed a better possibility of adaptation to *H. axyridis* compared to the field.

In the laboratory experiment aimed at evaluating the more appropiate time of host exposure the rate of parasitism was higher than in the field. The best results, in parasitoid yields and time of development, were obtained with the treatment "1 hour". In the "24 hours" treatment, the yield was lower and the development time increased, possibly due to superparasitism and the consequent cocurrence for food. The treatment "5 minutes", time used by other authors in experiments concerning this parasitoid (e.g. Berkvens et al, 2010), gave poor results.

Insect fecundity may be affected by different factors (Leather, 1988; Honek, 1993). In the present study, in spite of the low successful parasitism (the parasitoid larval development was apparently not complete) the exposure to *D. coccinellae* had an important effect on the reproductive capacity of the female ladybugs. In fact, while *H. axyridis* females exposed to parasitoid could oviposit, the eggs laid and the larvae hatched were significantly fewer compared to the control ladybugs. This result suggest that, despite the low succesful parasitism detected in the field, *D. coccinellae* may play a role in controlling *H. axyridis* population by decreasing female fecundity and fertility. It might be also considered a field release of parasitized *H. axyridis* (which behave similarly to unparasitized ones) for implementing a sort of concurrence for food and males between parasitized and unparasitized *H. axyridis*. In fact, the parasitized females remain attractive to males (in one case I even observed a male which tried to mate with a female with cocoon); the lower number of eggs laid by parasitized females and low fertility could reduce over time the number of *H. axyridis* present in the area.

The experiment concerning the rearing of *D. coccinellae* on *H. axyridis* fed on two different foods was aimed at improving and simplifying the parasitoid maintenance. The artificial diet tested (Sighinolfi er al , 2008) is easy to prepare and cheap compared to *E. kuehniella* eggs. The latter, though expensive, are commonly used as standard food for coccinellids due to easiness of handling (Signinolfi et al, 2008). The results of my experiments showed that it is possible to rear *D. coccinellae* on diet-fed *H. axyridis* adults and this may allow to speed and facilitate the parasitoid rearing. The fitness of the parasitoid was not influenced by the food supplied to *H. axyridis*. *Dinocampus* *coccinellae* could develop in *H. axyridis* fed with artificial diet, for more generations without major impact on the yields of adults. Only the parasitoids development from exposure to adult emergence was negatively influenced by food (it was longer in diet-fed *H. axyridis*). In my experiment, I did not make a comparison with aphid-fed *H. axyridis* (something that my be done in future tests). However, the diet preparation is simpler and reguires less labour comparted to the maintenance of aphid colony. Its used to feed parasitized *H. axyridis* adults seems therefore to be convenient.

Probably *D. coccinelle* alone cannot reduce the population of *H. axyridis* in Europe, due to the problem of host recognition mentioned above and the problems associated to its biology (Berkvens et al, 2010); but to date, apart from tachinids belonging to the genus *Medina*, sporadically found in Korea, it is the only known natural enemy of this ladybug (Kenis et al, 2008). Morever, it has not to be neglected the effect of some superior intraguild predator over *H. axyridis*, including *Anatis ocellata* (L.) and *Chrysoperla carnea* (Stephens) (Nedved et al, 2013). However it is the aggressive *H. axyridis* that acts as a superior intraguild predator.

The combined use of ladybug parasitoids, *D. coccinellae* and other (still to be detected), and females with reduced reproductive capacity following parasitization could have effects on the field populations of *H. axyridis*.

Further research is needed to improve knowledge about the association between *H. axyridis* and *D. coccinellae* in field and semi-field conditions and to find other parasitoids species of this ladybug, in Italy and Europe.

General conclusions

Classical biological control has raised some concern in the last years. Michaud (2002) reported that there are several problems with the way it is often applied. Firstly, classical introductions are increasingly used as a first line of defense against invasive pests, regardless of need or the availability of suitable candidate species for introduction and no time frame is allocated for assessing the responses of indigenous natural enemies before the introduction of exotic species. Secondly, due to the large investments spent on classical biological control, there is pressure in demonstrating its success which may in biased assessment and oversimplistic communication of its efficacy; at the same time, the contributions of native parasitoids and predators to suppression of the target pest may go unreported and unrecognized. Thirdly, the application of the classical approach to every new pest may lead to underestimate the potential resilience of native ecosystems to invasions. Moreover, the indiscriminate application of the classical approach may be environmentally irresponsible as it exposes native ecosystems to a risk of non-target effects (Messing and Wright, 2006; De Clercq et al, 2011; Katsanis et al, 2013). *Harmonia axyridis* is a good example of an exotic beneficial insect which has become a problem in the countries of introduction. Finally, many countries have enacted laws which are rigid in regulating the introduction of exotic species, including those useful (e.g. in Italy DPR n. 120/2003 art. 12, 3). For all these reasons, researches aimed at exploiting the role of indigenous natural enemies for the control of alien insect pest need to be encouraged. Moreover, the exploitation of such a strategy may be complementary to the classical biologic control, which, despite the concerns raised, when well applied has also produced excellent results (DeBach, 1964; Greathead, 1986; Gould et al, 2008)

My research work has shown that indigenous parasitoids may have an effect in the containment of introduced insects. *Cacyreus marshalli* and *D. kuriphilus* were both parasitized by native parasitoids, either in the laboratory or in the field. Specifically, for *C. marshalli* the laboratory tests have demonstrated that native polyphagous parasitoids of Lepidoptera have the potential to adapt to this non-permissive host, as also shown with other native parasitoid species in some field research (Sarto i Monteys and Gabarra, 1998; Vicidomini and Dindo, 2007); for *D. kuriphilus* the low specificity of gall parasitoids has enabled them to adapt to this new host (as also shown by other authors, including Santi and Maini, 2011, and Quacchia, et al, 2012). It is however unlikely that the parasitoids that I have tested in the laboratory or found in the field can provide, by themselves, an efficient control of the alien pests; but their effects could be useful in combination with other indigenous parasitoids or as a support to classical biological control strategies.

Dinocampus coccinellae, the antagonist of *H. axyridis* that I have found in nature, is also present in the native area of this ladybugs, as well as in other areas of introduction (Firlej et al, 2005); therefore its adaptation to this "new" host was not unexpected. The results of my experiments showed that *D. coccinellae* could have an impact, direct or indirect, on *H. axyridis* populations, besides having an indirect beneficial effect on other coccinellid populations, due to the control exerted over the concurrent exotic ladybug (Koyama and Majerus, 2008). Other parasitoids of ladybugs, which I did not find in my samplings campaigns, may also be adapting to this host, as it happened in other countries of introduction (Riddick et al, 2009).

In general, the exploitation of native natural enemies of alien insect pests can be complementary to classical biological control also because the latter may require some years to produce result. This requires studies on the suitable natural enemies of introduced pest in the native country, with a period of time sufficient to verify the establishment of the introduced insects in the new area. These two aspects are not always easy to manage; e.g. natural enemies of *C. marshalli* in its native country are little known, and *T. sinensis* has been discovered and studied only after *D. kuriphilus* has spread in Japan from China (Yasumatsu and Kamijio, 1979)

Hokkanen and Pimentel (1984) suggested that new association between exotic insect species and native antagonists, expecially parasitoids, may provide, over time, higher successful parasitism compared to former associations. An example of an efficient native parasitoid for the control of an alien insect pest in Italy is represented by the eulophid parasitoid *Diglyphus isaea* (Walker), an efficient antagonist of two exotic leafminers, which is also commercialized for inoculative release (Bazzocchi et al, 2003). In conlusion I think it is desirable to improve knowledges on the interaction between indigenous parasitoids (and predators) and alien insects pests so as to plan biological control strategies that combine the exploitation of indigenous antagonists and classical biological control in order to minimize impact of the new species on the envirenment.

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