

ENTOMOLOGY

Associations between the larval-pupal parasitoids *Erycia furibunda* and *E. festinans* (Diptera: Tachinidae) and respectively, the sympatric and syntopic butterflies *Euphydryas aurinia provincialis* and *Melitaea cinxia* (Lepidoptera: Nymphalidae)

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Abstract

Several studies on butterfly ecology and biology of Melitaeini butterflies have been carried out in the past, however the factors affecting butterfly mortality and the role of natural enemies on population dynamics are not yet fully known. Larval survival plays a key role in determining butterfly population size and distribution range; thus, knowing the sources and variation in larval mortality is essential understanding and predicting population dynamics. Butterfly larval mortality is generally ascribed to abiotic factors, predators and parasitoids (mainly Diptera and Hymenoptera). Among Diptera, tachinids parasitize primarily larval Lepidoptera. In this paper, we report the results of 5-year observations in the wild and captivity on the tachinids, *Erycia furibunda* and *E. festinans*, parasitoids of caterpillars of a population of *Euphydryas aurinia* spp. *provincialis* and *Melitaea cinxia* in Central Italy

revealing their host specificity. The hosts, *E. aurinia* and *M. cinxia*, and parasitoids, *E. furibunda* and *E. festinans*, inhabit the same habitat and their life cycles highly overlap, nevertheless, the parasitoids maintain their host specificity: *E. furibunda* as parasitoid of *E. aurinia*; *E. festinans* as parasitoid of *M. cinxia*. This was confirmed by our findings during the butterfly breeding activities carried out for over five years. Although the role of chemical cues in host finding requires further research, according to our observations the presence of only *E. furibunda* on larval webs of *E. aurinia* let us suppose that the mechanism by which *E. furibunda* locates its host could be based on olfactory cues emitted by feeding damage to host plants that act from afar. Similarly, the mechanism of host finding used by *E. festinans* could act to select its host, *M. cinxia*. Furthermore, we illustrate some diagnostic features of adults for the identification of the studied parasitoids.

Introduction

Parasitoids of butterflies fall into two insect orders, Diptera and Hymenoptera. They are of vast importance in all ecosystems as they control the population size of many of their hosts.

Several families of Diptera behave as parasitoids but only one, the large family Tachinidae, includes regular and important parasitoids of butterflies (Mellini, 1991; Belshaw, 1994; Cerretti & Tschornig, 2010). Despite their diversity and ecological impact, relatively little is known about the biology and basic ecology of tachinids and most species are still unidentified (Stireman *et al.*, 2006). Regarding the oviposition strategies, tachinids may adopt direct or indirect strategies to lay their eggs, depending on the species (Dindo, 2011). In direct strategies, oviposition may occur in diverse modalities, for example projecting or depositing eggs on the host integument (*i.e.* *Exorista larvarum* [Linnaeus 1758]) or inserting them into the host body (*i.e.* *Compsilura concinnata* [Meigen 1824] (Dindo & Nakamura, 2018)). In indirect strategies, the ovoviparous females may lay eggs close to a host, where the newly-hatched parasitoid larvae (of the planidium type) wait for a host to pass by. Moreover, some tachinids lay tiny microtype eggs on the host food (generally plant leaves) and these eggs hatch only after being ingested by a host. This strategy allows to parasitoids to reach hosts that live in concealed places (*i.e.* caterpillars inside their silken nest) that are inaccessible to females (Dindo & Grenier, 2014). The mechanisms of host selection in dipterans are related to

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their oviposition strategy. In dipteran parasitoids with direct strategies, females may use chemical cues to locate the host or the habitat of the host perceiving the stimuli derived from the interaction between the host and the plant it feeds on. Visual cues (*i.e.* host colors and movements) may also play a key role in host location in tachinids (Godfray, 1994; Dindo & Grenier, 2014; Depalo *et al.*, 2012). In fly species with indirect oviposition or larviposition strategy, females use physical and chemical cues for locating only the host habitat or microhabitat (Mellini *et al.*, 1980). Furthermore, associative learning through experience to locate a host has been documented in some tachinids species. Though superparasitism in the parasitic Diptera appears to be both widely distributed across species and common within populations, also the capacity to avoid superparasitism by recognizing previously parasitized hosts has been observed, but in a few cases (Feener & Brown, 1997; Dindo & Grenier, 2014). In the case of Tachinidae visual stimuli also play a role in host-searching behaviour by parasitoids (Stireman *et al.*, 2006). However, the mechanisms of host selection in Tachinidae, including the role of the host-plant, remain partially unknown, as few studies have been performed on these topics.

Tachinids parasitize an extensive range of plant-feeding insects and their hosts are primarily larval Lepidoptera belonging to many families. Many species are polyphagous and generalist, whereas others are extreme specialists exhibiting relatively narrow host ranges.

Among the butterflies belonging to the family Nymphalidae, the data concerning the dipteran parasitoids are really poor to the best of the authors' knowledge, especially for species in the Melitaeini tribe. Some host associations of tachinid taxa with Melitaeini species have been summarised in Van Nouhuys & Hanski (2004) and Shaw *et al.* (2009), but little is still known about the species of dipteran parasitoids and their biology.

The marsh fritillary butterfly, *Euphydryas aurinia* (Rottemburg 1775) (Nymphalidae, Melitaeini) is a butterfly species protected under the Habitats Directive (Annex II) and its subspecies *provincialis* (Boisduval 1828) was classified as "Least concern" in the IUCN Red List for Italian butterflies (Balletto *et al.*, 2015).

In this framework, in the context of an ongoing survey of the Lepidoptera fauna in Italy (Pinzari *et al.*, 2010, 2018a, 2018b, 2019a; Pinzari & Sbordoni, 2013; Pinzari, 2016a; Pinzari & Pinzari, 2019a, 2019b, 2019c), we extensively investigated many aspects of biology of a population of *E. aurinia provincialis* living in Central Italy: larval host plants preference, predators, adult population dynamics, mating behaviour and parasitoids (Pinzari, 2016b, 2019; Pinzari *et al.*, 2016, 2017, 2019a, 2019b).

E. a. provincialis is a monovoltine butterfly, which is limited to the Maritime Alps and the Apennine Mountains (Balletto *et al.*, 2014). It is characterized by six larval stages. The I-IV instar caterpillars are gregarious, then the V-VI-instar larvae are solitary (Pinzari *et al.*, 2016). Given the high number of individuals in a larval web, gregarious caterpillars represent an important food source for arthropods and small mammals (Van Nouhuys & Hanski, 2004; Pinzari, 2016b, 2019; Pinzari *et al.*, 2019a). The amount of larval survival plays a key role in establishing butterfly population size and distribution range; thus, knowing the sources and variation in larval mortality is essential to understand and predict population dynamics (Van Nouhuys & Hanski, 2004). In Europe, the known parasitoids of *E. aurinia aurinia* belong to Tachinidae and larval parasitoids are: *Chetogena rondaniana* (Villeneuve 1931), *Compsilura concinnata* (Meigen 1824), *Erycia furibunda* (Zetterstedt 1844) (Shaw *et al.*, 2009; Stefanescu *et al.*, 2009) and *E. fatua* (Megan 1824) (Porter, 1981; Van Nouhuys & Hanski, 2004). Our studies confirmed the host association between *E. aurinia* and *Erycia furibunda* and revealed some aspects of the parasitoid life cycle in Central Apennines (Latium,

Central Italy) (Pinzari *et al.*, 2017). Here, we also detected the co-occurrence of two species belonging to the genus *Erycia*: *E. furibunda* and *E. festinans*. Herting (1960) described *E. festinans* as an ovularviparous parasitoid of the first instar larvae of *Euphydryas* (Meigen 1824) *aurinia*, but recently Tschorsnig (2018) said that this information was likely to be wrong due to erroneous species determination. According to Herting (1960), *E. festinans* is also a larval pupal parasitoid of the Glanville fritillary, *Melitaea cinxia* (Linné 1758), that occurs together with *E. aurinia* in our study area (Pinzari *et al.*, 2010). *Melitaea cinxia* is a montane butterfly and is present throughout continental Italy and in Sicily (Balletto *et al.*, 2014).

The presence of populations of *E. a. provincialis* and *M. cinxia* from the area of Vallemare (Rieti, Lazio, Central Italy, Location WGS84: N42.4836°-E13.1148°) was reported by Pinzari *et al.* (2010). In this area, *E. a. provincialis* and *M. cinxia* inhabit the same habitat patches in montane grassland and slopes at 1000 m and the adult flight period (from the middle of May to the early days of July) and larval stages overlap. Females of *E. a. provincialis* oviposit on the plants *Gentiana cruciata* L., *Scabiosa columbaria* L. and *Cephalaria leucantha* (L.) Roem. & Schult., and caterpillars feed on these host plants and also *Lonicera caprifolium* L. (Pinzari *et al.*, 2016). Females of *M. cinxia* lay their eggs on the plants of *Plantago lanceolata* L. and *Veronica spicata* L. (Kuussaari *et al.*, 1998, Kuussaari *et al.*, 2004). In both species, the I-III instar larvae are gregarious and spin a communal "feeding" web on the host plants. They typically remain in groups until the beginning of the last molt larval instar in *M. cinxia* (Kuussaari *et al.*, 2004) and until the IV larval instar in *E. a. provincialis* (Pinzari *et al.*, 2016). Then, they become solitary and pupate within the vegetation close to the ground.

The aim of this paper is to shed some light on the host-parasitoid association between the two Diptera species, *Erycia furibunda* and *E. festinans*, and the two Melitaeini butterfly species, *E. a. provincialis* and *M. cinxia*. For this purpose, we carried out observations in the field on larval stages of the two butterfly species in the areas where the butterflies were found to coexist with both of the parasitoids. After that, we reared the caterpillars in a laboratory until the *Erycia* fly emerged from butterfly pupae in order to detect the host specificity of parasitoids.

Materials and Methods

Sampling of *M. cinxia* and of *E. a. provincialis* larvae

From 2015 to 2019, in April we collected larvae of *M. cinxia* and *E. a. provincialis* at their solitary stage (V-VI instar) across a wide area (ca. 2 ha) near the "crossroads Santa Maria del Monte", 1000 m (Pinzari *et al.*, 2010) at few kilometers from the locality Vallemare (Rieti, Italy). In 2019, we additionally collected larvae at IV-V instar to investigate other egg or larval parasitoids.

M. cinxia and *E. a. provincialis* are easily distinguishable at larval stage after the III instar on the basis of the colour of the cephalic capsule: red in *M. cinxia*, black in *E. a. provincialis*.

Rearing of *M. cinxia* and *E. a. provincialis* larvae

The larvae were reared individually inside small glass jars (diameter = 4 cm, height = 5 cm) with netted plugs in a laboratory in Rome at room conditions (ca. 20-22°C; 45-60 % relative humidity; natural photoperiod, 12-hour light-dark cycle) until pupation. The larvae of *M. cinxia* were fed mainly with leaves of *Plantago* sp. and *E. a. provincialis* with shoots of *Lonicera caprifolium* L. Every day, in the morning and in the evening, we provided fresh leaves to the larvae and cleaned the glass jars removing the faeces to avoid the formation of mold.

The species identification of parasitoids was performed after emergence by the methods described in the following paragraph.

Monitoring and sampling of *Erycia* adult parasitoids

In the context of previous field-work on all stages of the life cycle of *E. a. provincialis* (Pinzari *et al.*, 2016) we discovered the presence and identified the period during which *E. furibunda* females locate their hosts and parasitize the larvae of *E. a. provincialis*. The monitoring of butterfly life cycle was carried out over five years every week in Spring and Summer and every 15 days during the rest of the year. This monitoring focused on egg batches and larval webs as following: eggs (from May to the end of June); pre-diapause gregarious phase (I-III instar on larval webs) (from May to September); diapause phase (IV instar in larval webs) (from September to mid-February); post-diapause gregarious phase (IV instar) (from mid- February to mid-March); and solitary caterpillars (V-VI instar) (from March to mid-April) (Pinzari *et al.*, 2016). The appearance of adult parasitoids on the butterfly larval webs was conditioned by the hatching of *E. a. provincialis* eggs and also the development time of the caterpillars. A detailed article on this topic

is in preparation (Pinzari, unpublished data). Therefore, in Summer, in order to ascertain when and how long female parasitoids inhabit the butterfly larval webs for oviposition, from the second week of June up to the second week of September, we intensified the controls and every day we visited the butterfly host plants with and without larval webs in the study area and recorded presence/absence of *Erycia* flies. During five years of observations, 24th June was the earliest record and 29th August was the latest date of the occurrence of *Erycia furibunda* on the larval webs of *E. a. provincialis*.

The flies were photographed in the field and/ or collected on sight. They were collected in the daytime, when they rested on butterflies host plants or in the surrounding plants during feeding, by butterfly net.

Species identification of parasitoids

Adults of *E. furibunda* and *E. festinans* were identified by their habitus (Tschorsnig & Herting, 1994) and in particular on the basis of the colour of the *basicosta* (the distal plate in the axillary area of the wing). This is black and yellow in *E. festinans* (Figure 1A), completely yellow in *E. furibunda* (Figure 1B) and entirely black

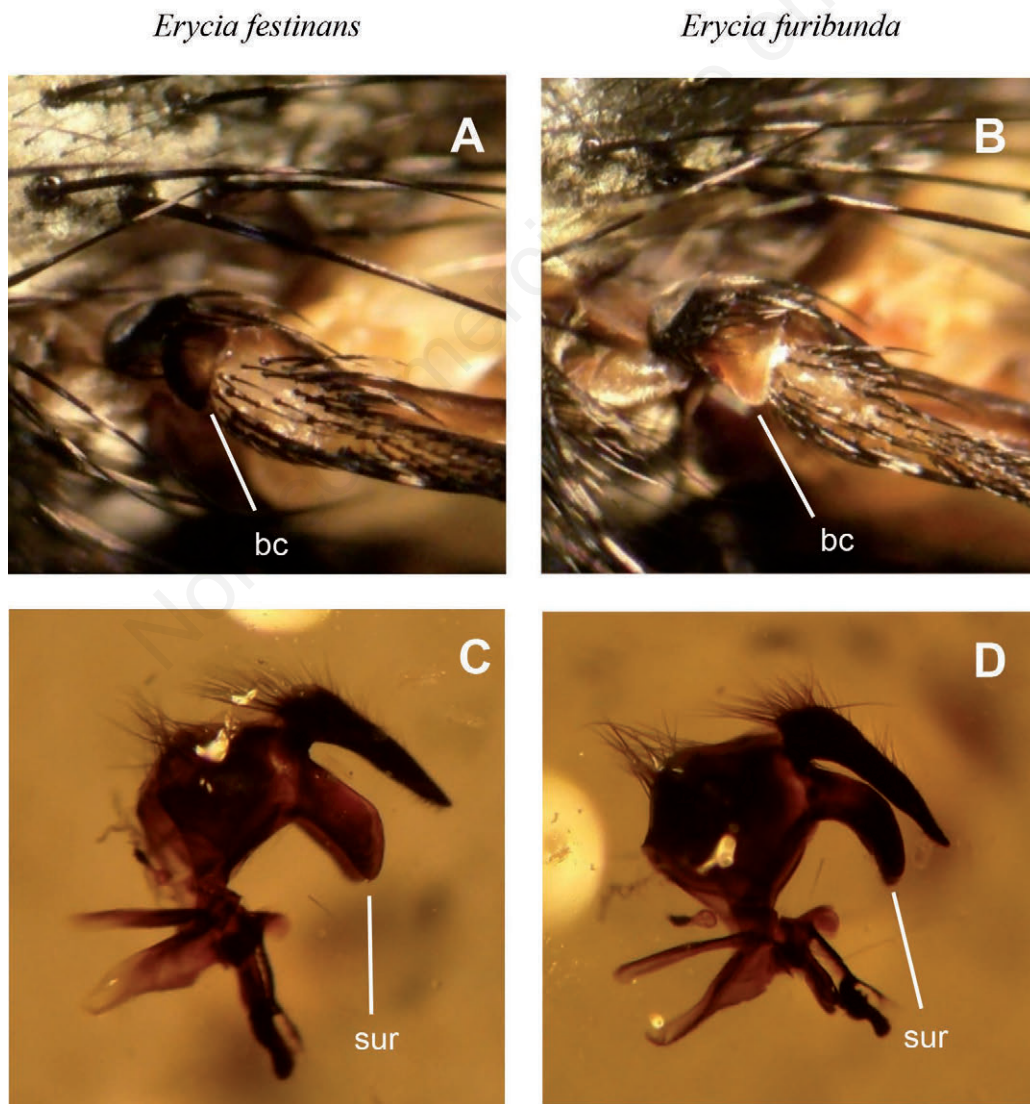


Figure 1. Diagnostic features for tachinid identification: A and B, *basicosta* in the axillary area of the wing along which the costa of the wing articulates; C and D, male *hypopygium* in lateral view. Abbreviations: bc = *basicosta*; sur = *surstylus*.

in *E. fatua*. *Erycia fatua* is morphologically very similar to *E. festinans* (Herting, 1960; Tschorsnig & Herting, 1994). Having the same hosts, there remains some doubt if they are really separate species (Tschorsnig, 2018). Specimens of *E. furibunda* and *E. festinans* were sexed after death. The species determination of males was confirmed by the examination of genitalia (Figures 1C and 1D).

P. Cerretti (Rome, Italy), H. Tschorsnig (Germany) and M.R. Shaw (Edinburgh, United Kingdom) confirmed identification of parasitoids and helped in sexing of individuals. These specimens are presently preserved in the Mario Pinzari's Collection in Rome (Italy).

Rearing of parasitoids

A parasitized pupa was identified by its change in colour from the typical whitish colour to reddish and later to dark brown; on the contrary, when the pupa is parasitoid-free it maintains its typical whitish colour and becomes transparent when the adults of *E. a. provincialis* emerge. The pupae were isolated and labelled. In the case of parasitized pupae, when the maggots were left to pupate and the puparia were individually kept in separate small glass jars (with one open netted side) at room conditions (ca. 20-22°C; 45-60 % relative humidity; natural photoperiod, 12-hour light-dark cycle) until the fly emergence. The pupae with no evidence of parasitization were also isolated and monitored until butterfly emergence or decease.

We additionally identified the species of the dead puparia by studying the content of puparia. The dead puparia were boiled in 10% potassium hydroxide solution for few minutes. The species determination was possible only when the flies completed the metamorphosis. The species determination of one of these specimens diverse from *Erycia* was committed to Dr Hans-Peter Tschorsnig (Staatliches Museum für Naturkunde Stuttgart, Germany).

Parasitized larvae by Hymenoptera were reared according to the rearing methods described above. Braconids and Ichneumonids emerged from butterfly larvae were preserved in dry condition or in alcohol 90%. They were identified by Dr. Mark R. Shaw (National Museums Scotland, Edinburgh, United Kingdom).

Both larvae and pupae were monitored at two fixed times, 7 AM and 7 PM, and also randomly at night and day.

The adults and all material from the rearing (parasitized butterfly pupae, puparia, etc.) are presently preserved in the Mario Pinzari's Collection in Rome (Italy).

Rate of dipteran parasitism

From pupae diverse species of Diptera, beside *Erycia* species emerged. Accordingly, for each butterfly species, we calculated: 1) the rate of dipteran parasitism (RPD), as the ratio between the number of emerging maggots (without distinguishing parasitoid dipteran species) from butterfly pupae and the total number of pupae; 2) the rate of *Erycia* parasitism (RPE), as the ratio between the number of emerging maggots of *Erycia* parasitoids from butterfly pupae and the total number of pupae (Table 1).

Rates of parasitism among years were compared and tested for significance using the Chi-Square test. All data analyses were performed in STATISTICA version 7.

Results

Sampling of *E. a. provincialis* and *M. cinxia*

We collected a total of 317 larvae of *E. a. provincialis* (in 2015, 28 individuals; in 2016, 50, Pinzari *et al.*, 2017; in 2017, 25; in 2018, 46 and in 2019, 168) and a total of 75 larvae of *M. cinxia* (in 2017, 28 larvae; in 2018, 22, and in 2019, 25).

Observations and records of *E. furibunda* and *E. festinans* adults in the wild

From 2015 to 2019, during July-August, we monitored *G. cruciata*, *S. columbaria* and *Plantago* sp. plants with and without larval webs in the study area to record *Erycia* parasitoids during the oviposition period. We collected twenty-three females of *E.*

Table 1. Rearing results and Ratio of Parasitism of *E. a. provincialis* - *E. furibunda* and *M. cinxia* - *E. festinans* systems.

Caterpillars	Total		Pupae			Puparia			Adult sex	
	Total	Total	Adult	Dead	Parasitized (RPD)	Adult	Dead	Parasitized (RPE)*	Males*	Females*
<i>Euphydryas aurinia provincialis</i>										
N	25	25	13	8	4 ⁽²⁾	3	0	3	2	1
%	-	100	52	32	16	100	0	12	67	33
N	46	44	32	3	9 ⁽⁴⁾	5	3 ⁽⁷⁾	8	5	3
%	-	96	73	7	20	63	37	18	62	38
N	168 ⁽⁵⁾	121	59	38	24 ⁽⁶⁾	18	2 ⁽⁷⁾	20	6	14
%	-	72	49	53	20	90	10	16	30	70
<i>Melitaea cinxia</i>										
N	28	27	13	7	7	6	1 ⁽¹⁾	7	3	4
%	-	96	48	26	26	86	14	26	43	57
N	22 ⁽³⁾	19	16	1	2	2	0	2	0	2
%	-	86	84	5	10	100	0	10	0	100
N	25	18	10	0	8	8	0	8	5	3
%	-	72	40	0	44	100	0	44	62	38
<i>Erycia furibunda</i>										
<i>Erycia festinans</i>										

RPD, rate of dipteran parasitism of the pupae, as the ratio between the number of emerging maggots (without distinguishing parasitoid dipteran species) from butterfly pupae and the total number of pupae; RPE, rate of *Erycia* parasitism of the pupae, as the ratio between the number of emerging maggots of *Erycia* parasitoids from butterfly pupae and the total number of pupae; as concerns puparia, the % of the dead and adults have been calculated on the total of parasitized pupae, while the % of fly sexes on the total of producing adults (including the sexed dead puparia).

⁽¹⁾it also included the dead puparia whose species was identified at this stage. ⁽²⁾it was identified as a female at puparium stage and then included in adult sex column. ⁽³⁾one puparium of these pupae died and was identified as tachinid by Tschorsnig. ⁽⁴⁾out of 22 caterpillars, 2 died due to the parasitism by *Hyposoter horticola* (Hymenoptera). ⁽⁵⁾out of 9 chrysalides, 1 was parasitized by a dipteran parasitoid which was unidentified due to absence. ⁽⁶⁾out of 168 caterpillars, 8 died due to the parasitism by *Cotesia* sp. and *Gelis* sp. (Hymenoptera). ⁽⁷⁾out of 24 puparia, 20 gave rise to *Erycia furibunda* adults, 4 died and did not complete the metamorphosis; these were identified as diptera. ⁽⁸⁾2 puparia were sexed after boiling of the puparia and then included in "Adult sex" column.

furibunda (Pinzari *et al.*, 2017) and only two females of *E. festinans*. These rested on grass and were far from the larval webs of both butterflies. We never recorded *Erycia* species on the larval webs of *M. cinxia* or *E. festinans* on the larval webs of *E. a. provincialis*.

E. festinans. Collected specimens: Borbona (RI), Italia, Fraz. Vallemare, 1 ♀, S. Maria Incrocio, 1000 m, 15.VII.2017, 1 ♀, idem, 5.VIII.2017, Manuela & Mario Pinzari leg.

E. furibunda. Collected specimens: Borbona (RI), Italia, Fraz. Vallemare, 2 ♀♀, S. Maria Incrocio, 1000 m, 2.VIII.2015, 2 ♀♀, idem 5.VIII.2015, 1 ♀, idem, 7.VIII.2015, 4 ♀♀, idem, 24.VII.2016, 1 ♀, idem, 30.VII.2016, 1 ♀, idem, 1.VIII.2016, 1 ♀, idem, 4.VIII.2016, 2 ♀♀, idem, 10.VIII.2016, 1 ♀, idem, 12.VIII.2016, 1 ♀, idem, 19.VIII.2016, 1 ♀, idem, 24.VIII.2016, 1 ♀, idem, 29.VIII.2016, 2 ♀♀, idem, 24.VI.2017, 1 ♀, idem, 8.VII.2017, 1 ♀, idem, 8.VIII.2017; 1 ♀, Bivio Brignola, 1062 m, 30.VII.2017; Manuela & Mario Pinzari leg. (Pinzari *et al.*, 2017).

Rate of parasitism

A first remarkable result was that the *Erycia* emerging from *E. a. provincialis* larvae was only *E. furibunda* and from *M. cinxia* was only *E. festinans* in our study locality.

From 2017 to 2019 years, out of 239 reared caterpillars of *E. a. provincialis*, 49 larvae died during observations for unknown causes and 190 pupated. Out of 190 pupae, 55% became butterflies, 26% died for unknown reasons, 19% died due to the presence of parasitoids (Table 1). For *M. cinxia*, from 2017 to 2019, out of 75 caterpillars, 64 formed the pupae and 11 died. Out of 64 pupae, 61% became butterflies, 12% died for unknown reasons, 26% died for parasitoids (Table 1).

Concerning *Erycia furibunda*, in 2015-2016, 31% of puparia died for unknown reasons, and the remaining 69% gave rise to 9 adults with a male-biased sex ratio (2:1) (Pinzari *et al.*, 2017). From 2017 to 2019 years, 16% of puparia died for unknown reasons, while 84% gave rise to 31 adults with a weakly female-biased sex ratio (1.4) (Table 1). In 5-years of investigation, we found a continuous incidence of the parasitoid *E. furibunda* on mortality of *E. a.*

provincialis caterpillars with the following RPE values: 2015 = 4%; 2016 = 24%, 2017 = 12%, 2018 = 18%, 2019 = 16% (Figure 2). There were not statistically significant differences among years when the rates of parasitism were compared. ($\chi^2=6.485$, $df=4$, $p=0.16$).

As regards *Erycia festinans*, in 3-years of investigation (2017-2019) 6% of puparia died and the remaining 94% gave rise to 16 adults with a sex ratio 1:1 (Table 1). In *M. cinxia*, the parasitoid *E. festinans* affected the survival of the caterpillars with a RPE equal to 26%, 10% and 44% respectively in 2017, 2018 and 2019 ($\chi^2=5.047$, $df=2$, $p=0.08$) (Table 1, Figure 2).

Rate of parasitism of other parasitoids of *E. aurinia provincialis*

Our observations in 2017 and 2019 years also revealed other larval pupal parasitoids of *E. a. provincialis*. In 2017, one fly (as a maggot), different from *Erycia festinans* and *Erycia furibunda*, emerged from a caterpillar of *E. a. provincialis*. It was determined at puparium stage as belonging to the family of Tachinidae by Tschorsnig (personal communication on Apr 12, 2019) and it is yet under study. In 2019, eight *E. a. provincialis* caterpillars showed some parasitic species of Hymenoptera, particularly braconid and ichneumonid species (possibly, *Cotesia* sp. and *Gelis* sp., Dr. Mark R. Shaw, personal communication on Jun 18, 2019).

Taking into account only the dipteran parasitoids, for *E. a. provincialis* the rates of parasitism (RPD) were comparable in 2017-2019 years (Table 1).

Discussion

Most efforts for butterfly conservation biology have been focused on getting the 'bottom-up' aspects of the habitat (host plant abundance, vegetation characteristics of host plant cover, thermal conditions of the sites, etc.) right for the dwindling butterfly population, on the contrary parasitoids and other 'top-down'

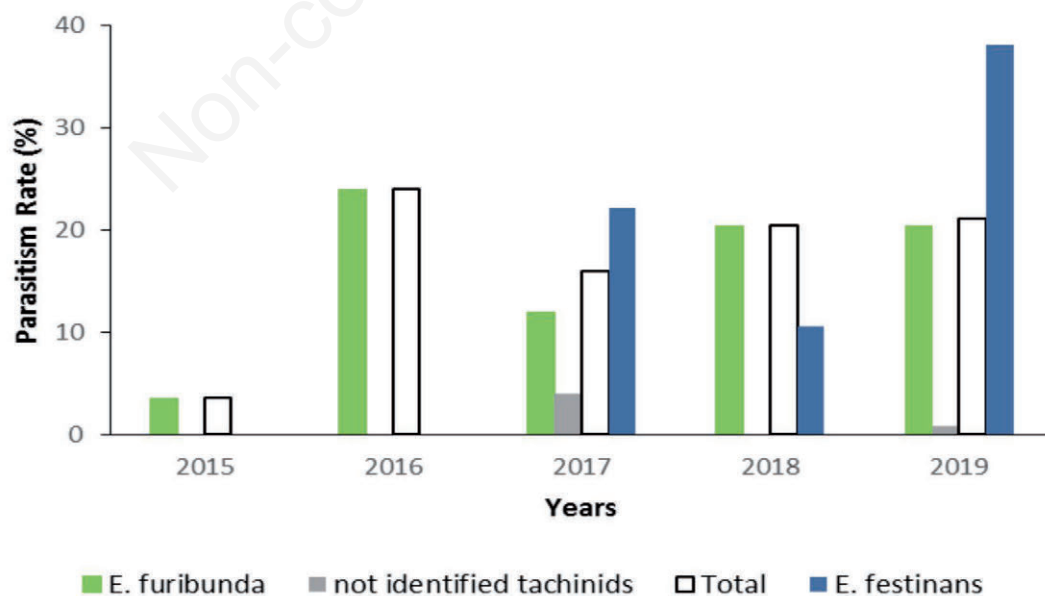


Figure 2. Parasitism Rates of pupae of *Euphydryas aurinia* (2015 – 2019) by *Erycia furibunda* (green), other non-identified tachinids (grey), non-identified Diptera (black) and total dipteran parasitoids (RPD)(white); *Melitaea cinxia* by *Erycia festinans*. Data collected in 2015 and 2016 years are from Pinzari *et al.*, (2017).

influences have generally been ignored (with some notable exceptions, see Shaw *et al.* 2009). Many works on butterfly parasitoids are mere compilations of the host-parasitoid records found in the literature, plagued with errors, ambiguity and non-quantitative data (Ford & Shaw, 1991; Shaw *et al.*, 2009; Cerretti & Tschorsnig, 2010). To prevent these problems, researchers need an assessment of recorded specimens by expert taxonomists and host data for parasitoids based on reared specimens. It is also important to establish a comprehensive knowledge of which species parasitize butterfly species and if a strict host-parasitoid association exists. However, it is hard to establish if a strict host-parasitoid association exists, especially in the case of tachinids. You may establish that a certain tachinid species successfully parasitize a host species as in the case of *Erycia furibunda* and *E. festinans*, but it is hard to exclude that other species may serve as suitable hosts. In fact, many tachinids are polyphagous and generalist and, often, the hosts are still unidentified (Dindo & Grenier, 2014).

Host association

In the study area, adults of the larval-pupal parasitoids, *Erycia furibunda* and *E. festinans*, emerged from puparia in the same area where the host butterflies, *M. cinxia* and *E. a. provincialis*, occurred.

It is known from the literature as *Erycia furibunda* behaved hilltopping for mating purposes: males emerged before females and moved to the nearby hill reliefs waiting for females to mate (Tschorsnig, 1996; Pinzari *et al.*, 2017). Throughout our study, we looked for the hill-topping behaviour of the species on the nearby reliefs in the study area, but unsuccessfully. We recorded only females of *Erycia furibunda* and *E. festinans* inhabiting their birth area for oviposition. *Erycia furibunda* oviposits on the larval web of *E. aurinia provincialis* and nearby eating caterpillars when the host larvae were at early larval stages before winter diapause (Pinzari *et al.*, 2017). *E. furibunda* and perhaps *E. festinans* might adopt an indirect oviposition strategy as witnessed even up to other tachinids (Dindo, 2011; Dindo & Grenier, 2014).

During the field phase, we were able to easily observe females of *E. furibunda* laying eggs on *E. a. provincialis* pre-hibernation larval webs on *G. cruciata* and *S. columbaria*. Indeed, we made multiple observations and even up to three females of *E. furibunda* were simultaneously seen on the same larval webs of *E. a. provincialis* (Pinzari *et al.*, 2017). On the contrary, it was more difficult to record females of *E. festinans* looking for their host *M. cinxia* on *Plantago* plants. *E. festinans* seems much less common than *E. furibunda* and we never recorded any parasitism event on *M. cinxia*.

In order to not disturb the balance between the butterfly, *E. a. provincialis*, and the studied parasitoids, we did not capture all *Erycia* flies observed on the butterfly larval webs on gentians or small scabious plants; thus, we cannot completely exclude that there could be individuals of both species, *furibunda* and *festinans*, on *E. a. provincialis* nests.

On the whole, in 3-years of investigation (2015-2017) we collected twenty-one females of *E. furibunda* on larval webs of *E. a. provincialis*, and no females of *E. festinans* or *E. furibunda* on the nests of *M. cinxia* on *Plantago* plants. The only two females of *E. festinans* were collected in the wild far from butterfly larval webs and also host plants.

In this study, the most important evidence of host association between butterfly and parasitoid species derived from the rearing observations, making it possible to associate *E. festinans* to *M. cinxia* and *E. furibunda* to *E. a. provincialis*, on the basis of the maggots emerging from butterfly species. In fact, only *E. furibunda* emerges from the pupae of *E. aurinia*, while *E. festinans* emerges only from pupae of *M. cinxia*. We cannot exclude that other butterflies may serve as suitable hosts for the studied *Erycia* species.

Host finding

The caterpillars of *M. cinxia* and *E. a. provincialis* have similar habitus in early stages; only after the III instar the colour of the cephalic capsule is different. Both butterflies share the larval lifestyle in a communal silken web in the same period and in the same places but they live on different host plants.

How do *Erycia* species locate and recognize host caterpillars? The information available indicates that tachinids use a wide diversity of cues (olfactory, visual, auditory, and tactile-chemosensory) to find their hosts. Many tachinids depend on chemical cues derived from the plants of their phytophagous insect hosts. Chemical cues may serve as attractants for tachinids to localize the habitats occupied by their hosts, and close-range cues can be used to locate the host. Close-range cues include odours associated with the host, such as host secretions, or excretions (particularly “frass”), and visual detection (Stireman *et al.*, 2006; Turling *et al.*, 1995; Hilker *et al.*, 2002; Tschornik & Hawkins, 2002; Dindo & Grenier, 2014). For example, in *Exorista larvarum*, the most important chemical cue in host localization by females is host-induced plant volatiles, while visual cues, natural maize volatiles and artificially damaged plants were less efficient for host localization (Depalo *et al.*, 2012). For *E. larvarum*, host motion is also a very important cue for host location, similarly to other tachinids displaying direct oviposition modes, including the congeneric *Exorista mella* Walker (Stireman, 2002).

As host plant secretions or excretions that could act as attractants for parasitoids, in northern Europe, *Lonicera implexa* Aiton leaves bearing egg clusters of *E. aurinia aurinia* have higher concentrations of iridoid glycosides than leaves on plants that bore no eggs. These foliar iridoid concentrations were likely the result of a plant response to egg deposition (Peñuelas *et al.*, 2006); *Succisa pratensis* Moench plants release a blend of volatile compounds in response to the attack by *E. aurinia* larvae (Peñuelas *et al.*, 2005). In Europe, *Succisa pratensis* releases methanol as a defence reaction to larvae of *E. a. aurinia* (Peñuelas *et al.*, 2005); *Veronica spicata* L., one of the host plants of *M. cinxia*, emits a blend of volatile organic compounds that are involved in the attraction of predators and parasitoids and act as an indirect defence of plants (Pinto-Zevallos *et al.*, 2013). In Finland, *Cotesia melitaearum* (Wilkinson 1937) (Hymenoptera: Braconidae) and *Hyposoter horticola* (Gravenhorst 1829) (Hymenoptera: Ichneumonidae) are two specialist parasitoids of *M. cinxia* that forage for different stages (larvae and eggs) and may rely on different herbivore-induced cues to locate the host. Herbivore-induced VOCs that are emitted by *V. spicata* L. for the presence of *M. cinxia* larvae may be responsible for the attraction of the parasitoid *C. melitaearum* *V. spicata* L. infested by *M. cinxia* larvae in the field (Van Nouhuys & Hanski, 1999). Therefore, there is evidence that *H. horticola* responds to volatiles emitted from oviposition-induced VOCs (Castelo *et al.*, 2010).

In our study area, the larvae of *E. a. provincialis* feed on *G. cruciata*, *S. columbaria*, *C. leucantha* and *L. caprifolium* (Pinzari *et al.*, 2017) and the larvae of *M. cinxia* feed on *Plantago* sp.

Although the role of volatile compounds in attracting parasitoids has not yet been investigated for our species, according to available literature on this topic, it is likely that the host plants of our studied butterflies emit volatile compounds which may have an attractive role. The discovery of *E. furibunda* specimens only on *E. a. provincialis* nests indicated that the butterfly attractants are very successful and perform well even from afar. On the other hand, *E. festinans* adults were never observed on the larval webs of *E. aurinia* and therefore we excluded that their choice could be based on some criteria “up close”. Unfortunately, the same does not apply to *E. furibunda*

and *E. festinans* on the larval webs of *M. cinxia*, since we have never seen any *Erycia* species on them.

Furthermore, various entomophagous insects are able to recognize the plants free of larvae, the plants that are devoured by caterpillars and the faeces of caterpillars on the basis of the different volatile compound released, as in *Spodoptera exigua* (Hübner 1808) (Lepidoptera, Noctuidae) (Turlings *et al.*, 2002). As stated before, also during our observations in the wild we recorded several arthropods on plants attacked by caterpillars and almost none on plants without caterpillars. Although the role of chemical cues in host finding requires further research, according to our observations the plants without feeding damage do not attract beneficial insects, except those for dietary reasons on flowers (bees, butterflies, etc.). Only when the eggs of *E. aurinia* hatch and caterpillars attack the host plants, predators and parasitoids make their appearance: the larval pupal parasitoid *Erycia furibunda* (Pinzari *et al.* 2017); hemipterans species like *Spilostethus saxatilis* (Scopoli 1763) and *Palomena prasina* (Linnaeus 1761) which feed on faeces (Pinzari, 2016) and the generalist predators of caterpillars *Picromerus bidens* (Linnaeus 1758) (Konvicka *et al.*, 2005; Pinzari *et al.*, 2019) and *Deraeocoris schach* (Fabricius 1781) (Pinzari, 2016). The spider *Phylloneta sisypbia* (Clerck 1757), which preys on all insects that frequent the plants including the caterpillars of *E. aurinia* and its parasitoids, can be found (Pinzari, 2019).

The impact of dipteran parasitoids on the studied butterflies

In this study we found a continuous incidence of the parasitoids *Erycia furibunda* and *E. festinans* respectively on *E. a. provincialis* and *M. cinxia* (Table 1). The recorded RPE values seem comparable to the values (11.1% and 4.5%) reported for *E. furibunda* by Stefanescu *et al.* (2009) in their 2-year study on the parasitoid complex attacking *Euphydryas aurinia* and *Euphydryas desfontainii* (Godart 1819) in Spain. The recorded RPE values have to be considered bearing in mind that they are calculated on the chrysalises, that is on the surviving caterpillars. *Erycia furibunda* and *E. festinans* are larval-pupal parasitoids (Shaw *et al.*, 2009), therefore, most maggots are likely to die together with the I-III pre-diapause caterpillars that did not survive due to summer droughts, winter and spring rigors and, parasitoid and predator attacks, probably more numerous than those known (Pinzari *et al.*, 2016; Pinzari, 2019; Pinzari *et al.*, 2019c). This fact is consistent with the reproductive strategy used both by butterflies and flies to oviposit numerous eggs.

The period of study was too short to have indications of the impact of natural enemies, such as parasitoids, on butterfly population size and further research on parasitism is needed (with a survey on abundance of adults) to draw any conclusions. However, the revealed difference in the rate of parasitism seems to be consistent with the adult population dynamics described in the past by Ford & Ford (1930) for *E. aurinia* and by Nieminen *et al.* (2004) for *M. cinxia*, typically affected by fluctuations, suggesting a possible role of *E. furibunda* and *E. festinans*, respectively, as a control agents of population size of *E. a. provincialis* and *M. cinxia* in Central Italy.

Conclusions

Our 5-year observations show that the two parasitoids are specific for the two butterflies: *Erycia furibunda* parasitizes only *Euphydryas aurinia* and *Erycia festinans* parasitizes only *Melitaea cinxia*.

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