



# New host-parasitoid association and the Trojan horse strategy adopted by the cuckoo wasp *Elampus bidens* with two vectors of Flavescence Dorée: *Scaphoideus titanus* and *Orientus ishidae*

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With 4 figures

**Abstract:** Chrysidid wasps adopt different strategies to enter host nests whilst evading detection. However, the specific oviposition strategies of most species are still largely unknown, and it has only become evident in recent years that some brood parasitic species are actually endoparasitoids in intermediate hosts that serve as the prey of their final host. Here we report for the first time the biology of *Elampus bidens* and describe a novel association between this cuckoo wasp and two invasive leafhopper species: *Scaphoideus titanus* and *Orientus ishidae* (Cicadellidae). Both leafhoppers are prey of the cuckoo wasp's principal host and are common invasive pests of European vineyard agro-ecosystems that are vectors of 16SrV phytoplasma agents of grapevine Flavescence Dorée, an economically important disease. This association is characterised by the “Trojan horse” strategy, in that the chrysidid lays an egg in the body of 3<sup>rd</sup> to 5<sup>th</sup> instar nymphs of *O. ishidae* or *S. titanus*, which are later captured by an apoid wasp and carried into the wasp's nest and placed in a brood cell. Biological control of these two vectors has not been effective so far, because the use of entomophagous insects has had little impact on their population growth in both their native range and Europe. Here we present the results of a survey of the parasitisation rate of *E. bidens* in north-western Italy, finding that the rate varies according to the sampling region and can reach an average of over 25% in areas characterised by less-intensive agriculture. A similar parasitisation result has never been reported for the two invasive leafhoppers until now, thus has potentially valuable economic implications. We did not discover any other Hemipteran host for *E. bidens*, therefore the original host of this cuckoo wasp is still unknown.

**Keywords:** parasitisation rate, invasive species, larvae development, integrated pest management, biological control

## 1 Introduction

Cuckoo wasps (Hymenoptera: Chrysididae) are ancestrally parasitoids and secondarily cleptoparasites (Pennacchio & Strand 2006; Pauli et al. 2019). Cleptoparasitic Hymenoptera lay their eggs into provisioned brood cells of host nests, and then the offspring feeds on prey originally collected by the host for its own offspring (Cardinal et al. 2010). However, the specific oviposition strategies of most cuckoo wasp species are still largely unknown, and only in recent years it has become evident that some cuckoo wasp species show a combination of endoparasitic and cleptoparasitic behaviour: they

develop during their first larval instar stage in the prey of their predatory host before this prey is subsequently caught by the host (José et al. 1970; Veenendaal 2012; Winterhagen 2015). To date, only a few species are known to exhibit this remarkable behaviour.

Chrysidid species use mason wasps, honey wasps, apoid wasps, mason bees, slug moths, sawflies, and stick insects as their hosts (Kimsey & Bohart 1991; Pauli et al. 2019). Despite a long history of taxonomic study, the reproductive behaviour of some European genera in the tribe Elampini (e.g., *Holopyga*, *Omalus*, *Pseudolopyga*, and *Pseudomalus*) has only recently been described (José et al. 1970; Venedal

2012; Pärn et al. 2015; Paukkinen et al. 2015; Winterhagen 2015). Specifically, some species of the tribe utilise the “Trojan horse” strategy (Strohm & Liebig 2008), laying an egg on a herbivorous insect, such as an aphid, which is then hunted by an apoid wasp and deposited with additional prey in the host’s nest for the cuckoo wasp to be able to complete its development. Once in the nest, the Elampini larvae consume the collected paralysed insects which are thus unavailable for consumption by the larvae of the apoid wasp (Tsuneki 1952). The oviposition strategy of species in the genus *Elampus* has remained unknown, even though some species in this genus are known to be brood parasites of species in at least two genera of Psenidae: *Mimesa* and *Mimumesa*. Species of *Mimesa* and *Mimumesa* predate adult and subadult leafhoppers (Hemiptera: Auchenorrhyncha: Cicadellidae) and planthoppers (Hemiptera: Auchenorrhyncha: Delphacidae) (Mocsáry 1889; Spooner 1948; Rosenheim & Grace 1987).

In the present study, we describe for the first time the biology of the Palaearctic species *Elampus bidens* (Förster, 1853) (Hymenoptera: Chrysididae: Elampini) and report a novel parasitic association between this cuckoo wasp species and two Cicadellid leafhoppers introduced to Europe and of major agricultural economic importance: *Scaphoideus titanus* Ball, 1932 and *Orientus ishidae* (Matsumura, 1902). *Scaphoideus titanus* is a Nearctic species and was first reported in Italy in 1963 (Vidano 1964). The leafhopper is known to be the main vector of Flavescence dorée phytoplasmas (FDp), a serious disease of the common grape vine in temperate Europe caused by 16Sr-V group phytoplasmas (Caudwell et al. 1970; Malembic-Maher et al. 2011; Alma et al. 2019; Bocca et al. 2020a). Indeed, during 2005 in Italy, the Italian government and the European Union reimbursed Italian wine growers 34 million euros to compensate for yield losses and replanting (Belli et al. 2010). *Orientus ishidae* is an East Palaearctic species recently recorded in Italy (Guglielmino 2005) and which has been found to be a vector of FDp (Lessio et al. 2016). *Scaphoideus titanus* and *Orientus ishidae* are both very common pests in Italian vineyard agroecosystems, and *O. ishidae* has been shown to not only feed on grape vine, but on additional plant species (Lessio et al. 2019).

Biological control of *O. ishidae* and *S. titanus* has been ineffective so far, because the selected entomophagous insects released for this purpose in the field to date have had little impact on leafhopper population development in their native range as well as in Europe (Schvester et al. 1962; Vidano 1966; Nusillard et al. 2003; Chuche & Thiéry 2014; Bocca et al. 2020b). The highest population density of these two species can be found in the ecological corridors formed by uncultivated areas or woods with wild grapevine, which represent a source of re-infestation (Lessio et al. 2019; Pavan et al. 2012). In such environments, no chemical control is allowed (Camerano & Terzuolo 2015). The absence of effective control strategies against FDp vector species spread raises the need to consider such environments as an oppor-

tunity rather than a pest control problem. In fact, they can represent habitat for native predators or parasitoids that have the potential to limit leafhopper populations. If native insects are to provide a level of pest control, it is known that natural enemies in newly invaded areas require time to adapt to novel exotic species (Cornell & Hawkins 1993).

Given the potential of *E. bidens* as biocontrol agent of *O. ishidae* and *S. titanus*, we investigated the biological interaction of *E. bidens* and the two invasive leafhoppers in more detail, years after their introduction to Italy. We (i) describe the lifecycle of *E. bidens* and its relationship with the two leafhopper species, (ii) report the rate of parasitisation of *O. ishidae* and *S. titanus* by the cuckoo wasp in different areas of Piedmont, Italy; (iii) discuss possible native leafhopper hosts of *E. bidens* and apoid wasp predators of the two leafhoppers, and (iv) observe the oviposition behaviour of *E. bidens* in semi-field conditions and its subsequent larval development.

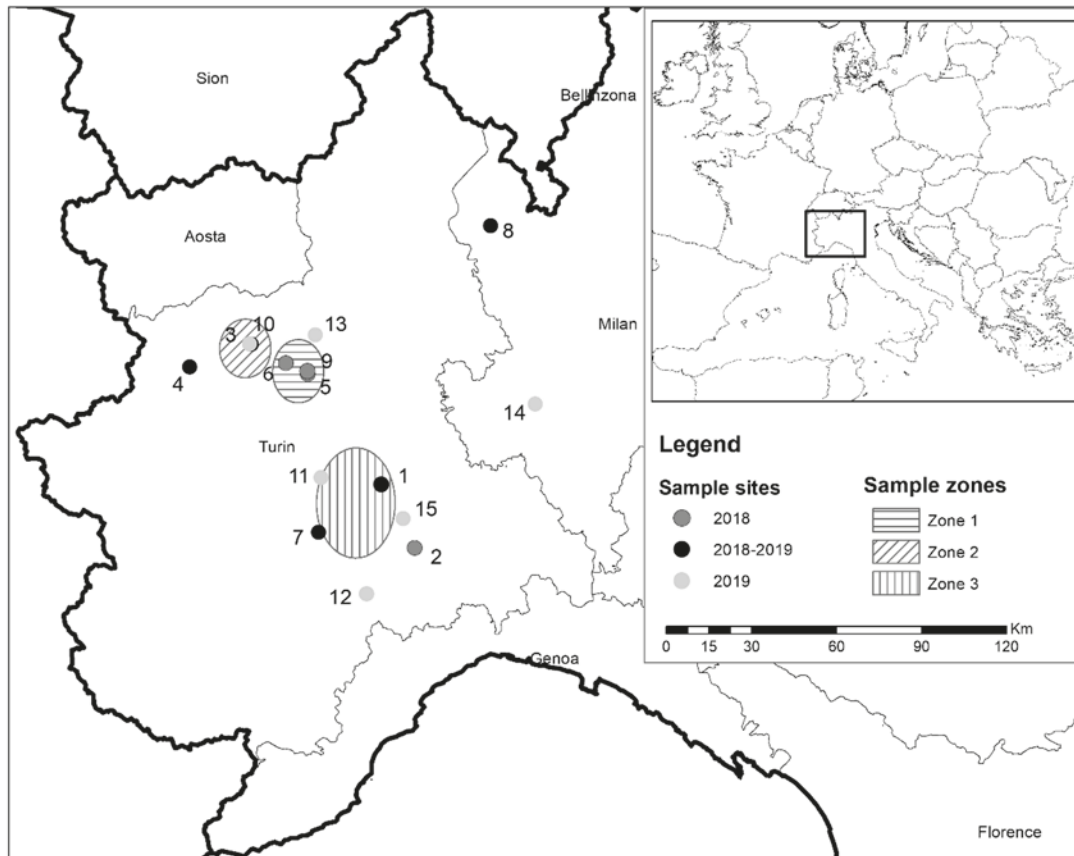
## 2 Material and methods

This research was carried out over six years (2015–2020). During the first three years (2015–2017) the survey was performed to assess the possible adaptation of *E. bidens* to *S. titanus*. The identity of *Elampus* larvae was confirmed using COI barcoding. In 2016 and 2017, the parasitisation rate was quantified. Based on these results, in the following two years (2018, 2019), the survey was continued to evaluate the host range of *E. bidens* and also to identify the apoid wasps that hunt these two leafhopper species. During the year 2020, we conducted a preliminary study upon the larval development and the parasitic behaviour of *E. bidens*.

### 2.1 Study area

During 2015, preliminary surveys were conducted in Canavese (in the province of Turin) and Monferrato Astigiano (in the province of Asti) in the region of Piedmont, north-western Italy. Based on the preliminary results of these surveys, a new sampling design was set-up to study the variation in the parasitisation rate between areas and years. In 2016 and 2017, three zones were surveyed, zones 1 and 2 in Canavese and zone 3 in Monferrato Astigiano (Fig. 1). Canavese is a rural region with vineyards covering 2% of the territory (Regione Piemonte 2010). Other main land uses include forests (37%), arable lands (31%), grasslands and pastures (3%) and urban areas (8%) (Regione Piemonte 2010). The Monferrato zone represents a rural region with higher density of conventional vineyards, covering 11% of the territory (Regione Piemonte 2010). Other main land uses include oak forests (22%), hazelnut orchards and other fruit crops (5%), arable lands (37%), grasslands and pastures (7%), and urban areas (7%) (Regione Piemonte 2010).

In 2018 and 2019, the study area was extended and included 15 additional sampling sites across the provinces of



**Fig. 1.** Study area and location of sample zones (filled areas) in 2016 and 2017 and sites (dots) sampled in 2018 (dark grey), 2019 (light grey), and both in 2018 and 2019 (black).

Turin, Piedmont (Asti, and Cuneo), and Lombardy (Varese and Pavia) (see Tables S1, S2 in the supplementary material). These areas were characterised by agricultural lands, with a high density of vineyards, uncultivated areas with wild grapevine and wood patches, mixed oak-forest, and riparian forest.

## 2.2 *Scaphoideus titanus* parasitisation rate in 2016 and 2017

Five sites per zone were chosen in uncultivated areas or woods where wild grapevine was present. At each site, an area of ca. 1500–2000 m<sup>2</sup> was sampled. Nymphs of *S. titanus* were collected by sweep netting every ten days, between mid-July (July 20) and mid-October (October 20), for three minutes per plant per sampling site in each zone (N = 10 plants per site). This resulted in a total of nine sampling periods. At one sampling site in zone 1, no *S. titanus* nymphs were found during 2016. This site was consequently excluded from subsequent analyses, leaving a total of 14 sites in 2016 and 15 sites in 2017.

In order to assess the rate of parasitisation of leafhoppers by *E. bidens*, all field collected cicadellid specimens were reared in small cages (insect-proof polyethylene and nylon

mesh 20 cm × 20 cm × 40 cm high) with a grapevine plant inside. Emergence of first instar *E. bidens* larvae (endoparasitic larvae) was recorded. If no larvae emerged, leafhoppers were observed until their death and subsequently dissected with micropins in the laboratory to check for the presence of *Elampus* larvae.

## 2.3 Survey on *Elampus bidens* host associations in the field in 2018 and 2019

Across the expanded range of sites, areas of wild grape vine were selected. Leafhopper nymphs and adults were collected by sweep netting in 15-day intervals over a circular area ca. 20 m in diameter for 1 hour at each location. In 2018, Malaise traps were used to screen for apoid wasps that possibly preying on leafhoppers. Five traps were placed at four localities (Tab. S2), using 70% ethanol as killing agent. The traps were checked every 15 days, starting from the first of June, and stopping at the end of October. In 2018 and 2019, we additionally hand-collected with the aid of a butterfly net apoid wasps that possibly prey on leafhopper. Ten collecting spots per locality were chosen in a linear transect, each separated by 15 m. These were sampled for 30 minutes. Surveys for principal hosts were *a priori* restricted to apoid wasps

based on the published literature for other Elampini species (e.g. Mocsáry 1889; Spooner 1948; Rosenheim & Grace 1987; Winterhagen 2015).

## 2.4 COI DNA barcoding of chrysidid larvae

We inferred the identity of chrysidid larvae found in *S. titanus* and *O. ishidae* using a standard COI DNA barcoding approach (Hebert et al. 2003). DNA was extracted from the tissues of the chrysidid larvae as described by Pauli et al. (2019). PCRs were performed as outlined by Pauli et al. (2019), applying the oligonucleotides LCO1490-JJ (5'-CHA CWA AYC ATA AAG ATA TYG G-3') and HCO2198-JJ (5'-AWA CTT CVG GRT GVC CAA ARA ATC A-3') (Astrin & Stüben 2008) or *Omalus*-F (equimolar mix of 5'-TTC GAC AAA CCA TAA GGA TAT TGG-3', 5'-TTC TAC AAA TCA TAA AGA TAT TGG-3', 5'-TTC AAC AAA TCA CAA GGA TAT TGG-3', 5'-TTC GAG TAA TCA CAA AGA TAT TGG-3', 5'-TTC AAC TAA TCA TAA AGA TAT TGG-3') and *Omalus*-R (equimolar mix of 5'-AGA AAT TAT TCC AAA ACC AG-3', 5'-CGA AAT TAT CCC AAA CCC AG-3', 5'-AGAAAT TAT TCC AAA TCC AG-3', 5'-AGAAAT TAT ACC AAA ACC AG-3') (Niehuis, present study) to prime the reactions. PCR products were cleaned with the Illustra ExoProStar kit (GE Healthcare Life Sciences, Garching, Germany) and then sent for bidirectional Sanger sequencing to Macrogen (Amsterdam, the Netherlands). Forward and reverse stands were assembled with the software Geneious version 10.2.3 (Kearse et al. 2012). The same software was used to compare the COI DNA sequences of the chrysidid larvae with COI DNA barcode sequences of various species of the genus *Elampus* (i.e., *E. bidens* [KY430834.1], *E. panzeri* [AJ514367.1], *E. konowi* [KY430833.1], *E. constrictus* [KY430835.1], and *E. spina* [transcriptome shotgun assembly GBWX00000000.1] published by Niehuis & Wägele (2004) and Pauli et al. (2019). We sequenced the DNA of a total of 13 larvae. We additionally sequenced COI DNA barcodes of two adult *E. sanzii* to rule out the remote possibility that the barcoded chrysidid wasp larvae belong to this species (Tab. S3).

## 2.5 Larval development experiment

During 2020, the interaction among leafhoppers and *E. bidens* was investigated using a large rearing cage (insect proof polyethylene and nylon mesh 40 cm × 40 cm × 100 cm high: in semi-field out-door conditions with one potted healthy wild grapevine plant (Kober 5BB) and one potted hazelnut tree (*Corylus avellana* L.). Female *E. bidens* (N = 12) were collected during the first ten days of July from localities where both leafhopper species were present, and transferred to the above rearing cage. Third instar nymphs and adults of *S. titanus* and of *O. ishidae* (N = 15 nymphs and N = 10 adults of each species) were also placed into the cage. A diet consisting of honey and pollen was given the cuckoo wasps in addition to a solution of water-honey sprayed onto the leaves daily.

Parasitisation behaviour was monitored daily for 3 hours in the morning and 2 hours in the afternoon. In order to assess differences in larval development between the two leafhopper species and whether there was a parasitisation preference by *E. bidens*, records of successful host detection were kept. A host detection was recorded as successful when the searching female approached and examined the leafhopper, then attacked it by clamping around the prey like a ball for at least 2-3 seconds. The successful parasitisation can be easily detected after dissection by the presence of an *E. bidens* egg. After parasitisation events, leafhoppers were individually stored in cylindrical plexiglass cages (diameter 15 cm, height 25 cm) with a leaf of grapevine (*S. titanus*) or hazelnut (*O. ishidae*) for feeding. To determine larval development of the leafhoppers, three nymphs of each species were dissected every two days starting 24 h after parasitisation until the death of the last specimen (a period of ca. 30 days). Leafhoppers were killed with ethyl acetate in the lab before dissection. The specimens were dissected into a phosphate buffered saline (PBS) solution onto a microscope slide (25 mm × 75 mm × 1 mm). Larval presence of *E. bidens* was assessed by dissecting each leafhopper specimen under a stereomicroscope. The abdomen was separated from the rest of the body using a pair of entomological needles.

## 2.6 Statistical analysis

The relationship between sampling zone and parasitisation rate of *S. titanus* nymphs collected in 2016 and 2017 was assessed using generalised linear mixed effect models (GLMMs, package *lme4*, Bates et al. 2015). As the analysed data proved to be zero-inflated, we applied a negative binomial error structure. Zone was treated as a fixed factor and the sampling date nested within sampling year were used as random factors. The final model was compared to a null model by ANOVA. Turkey HSD post-hoc tests were conducted using the *glht* function from the *multcomp* package (Hothorn et al. 2008).

The possible preference for *E. bidens* for one of the two leafhopper species collected in 2018 and in 2019 was tested using Chi-squared tests. In order to ensure that samples were drawn from the same populations, the analysis was restricted to sites where both leafhopper species were found together with at least one species parasitised (N = 7 over 2018 and 2019, site 1 and 3 in both years).

To compare the proportion of parasitized *S. titanus* with the proportion of parasitized *O. ishidae* reared in semi-natural conditions, a Chi-squared test was also used. All analysis was conducted in R version 4.0.3 (R Core Team 2020).

## 3 Results

### 3.1 *Scaphoideus titanus* parasitisation rate in 2016 and 2017

During the preliminary survey carried out in 2015, a total of 364 *S. titanus* specimens were collected, 19% of which

carried an endoparasitoid larva inside. The larvae were identified as *Elampus bidens* (Förster, 1853) (Hymenoptera, Chrysididae) by means of a DNA comparison between the adults and the larvae (see below).

During 2016, a total of 1,175 *S. titanus* specimens were collected and the parasitisation rates recorded were:  $21.4\% \pm 2.5\%$  (mean  $\pm$  standard error of the mean) in zone 1,  $27.8\% \pm 2.0\%$  in zone 2, and  $8.2\% \pm 2.0\%$  in zone 3. In zone 1, the highest observed parasitisation was  $28.8\% \pm 5.1\%$  (September 20), in zone 2 it was  $36.9\% \pm 5.3\%$  (August 10), and in zone 3 it was  $11.3\% \pm 7.0\%$  (July 20). In zone 1 and zone 2, endoparasitoid larvae were found until the beginning of October, in zone 3 until the end of September.

In 2017, a total of 1,305 *S. titanus* were collected. The parasitisation rates were:  $3.9\% \pm 1.0\%$  in zone 1,  $24.9\% \pm 2.7\%$  in zone 2, and  $6.3\% \pm 1.5\%$  in zone 3. In zone 1, the highest observed parasitisation rate was  $7.9\% \pm 5.1\%$  (September 10), in zone 2 it was  $35.9\% \pm 8.1\%$  (July 20), and in zone 3 it was  $10.3\% \pm 6.4\%$  (September 30). As in 2016, endoparasitoid larvae were found until the beginning of October both in zone 1 and in zone 3 and until the end of September in zone 2.

Zone was found to have a significant effect on parasitisation rate (GLMM,  $\chi^2 = 9.509$ ,  $p = 0.009$ ). Over both years, a parasitisation rate of  $25.4\% \pm 1.8\%$  was observed in zone 2, consistently higher than that observed in zone 1 (i.e.,  $11.6\% \pm 3.5\%$ ) and that observed in zone 3 (i.e.,  $7.2\% \pm 3.5\%$ ). However, post-hoc Tukey tests found that only zone 2 had a significantly higher observed parasitisation rate than zone 3 ( $z = 2.910$ ,  $p = 0.010$ ), with a marginal result for the difference between zone 2 and zone 1 ( $z = 2.104$ ,  $p = 0.088$ ). There were no differences between zone 1 and zone 3 ( $z = -1.037$ ,  $p = 0.552$ ).

For *S. titanus* specimens that were observed to be parasitised in 2016 and 2017, about 30% survived for more than 30 days. However, none of the cuckoo wasp larvae were observed to complete their development cycle and emerge from the leafhoppers.

### 3.2 Survey on *Elampus bidens* host association in the field in 2018 and 2019

#### 3.2.1 Leafhopper hosts

A total of 4,662 planthoppers and leafhoppers belonging to 13 species (Tab. S1) were collected during 2018 and 2019 to detect the potential hosts of *E. bidens*. Endoparasitoid larvae of *E. bidens* were found only in *S. titanus* and *O. ishidae* (Fig. 2), at 3<sup>rd</sup> instar nymphs and adults for both species.

In 2018, a total of 920 *S. titanus* and 850 *O. ishidae* were collected, and 217 larvae were found inside *S. titanus* and 224 were found in *O. ishidae* (Tab. S1). In 2019, a total of 413 *S. titanus* and 949 *O. ishidae* were collected, and 72 endoparasitoid larvae were found inside *S. titanus* specimens and 252 larvae in *O. ishidae*.

We found both leafhoppers to occur in sympatry across the two sampling years (2018 and 2019) at a total of eight

locations (i.e., sites 1, 2, 3, 6, 7, 10, 13, 15). However, at three of these locations (i.e., 2, 7 and 13), no larvae of *E. bidens* were recorded. At sites where both *S. titanus* and *O. ishidae* were found together and at least one species was parasitised (i.e., sites 1, 3, 6, 10, 15), 187 parasitised and 422 non-parasitised *S. titanus* were collected (= 30.7% parasitisation rate), and 224 parasitised and 463 non-parasitised *O. ishidae* were collected (= 32.6% parasitisation rate). The difference in the rate of parasitisation was not statistically significant (Chi-squared,  $\chi^2 = 0.538$ ,  $p = 0.463$ ).

#### 3.2.2 Search for the principal host of *Elampus bidens*

In 2018, using the Malaise trap, a total of three Hymenoptera specimens belonging to the family Bembicidae and nine belonging to Psenidae were found (*sensu* Sann et al. 2018). The Bembicidae were collected at the beginning of July and comprised two *Gorytes quadrifasciatus* (Fabricius, 1804) and one *Argogorytes* spp. Ashmead, 1899. All specimens of the family Psenidae were identified as *Psen ater* (Oliver, 1792) and were collected from the middle of August until the end of September. No *E. bidens* specimens were captured. Using hand nets, a total of nine adult specimens of *E. bidens* were collected at the end of June and the first ten days of July, and 14 *P. ater* specimens were collected in the second half of July until the end of September. *Psen ater* collection occurred at 80% of the sites where larvae or adults of *E. bidens* were previously found (Tab. S2).

In 2019, using hand nets, 23 adult specimens of *E. bidens* were found at six localities exclusively in the first week of July. Two specimens of *Gorytes quadrifasciatus* were collected in two localities, whilst 19 specimens of *Psen ater* were captured in six localities in the equivalent period of the previous year. Psenid wasps were found at 70% of sampled sites where larvae or adults of *E. bidens* were previously collected (Tab. S2).

#### 3.3 COI DNA barcoding of chrysidid larvae

We inferred the identity of 13 larvae collected at a total of six locations COI DNA barcoding. The obtained COI DNA barcode nucleotide sequences were identical among each other and to a reference barcode nucleotide sequence of *E. bidens* (i.e., KY430834). The nucleotide sequences differed consistently from those of other species of the genus *Elampus* that are known to or that could theoretically occur at the study sites (i.e., *E. constrictus*, *E. konowi*, *E. panzeri*, *E. sanzii*, *E. spina*).

#### 3.4 Larval development experiment

Of the twelve adult *E. bidens* females kept in flight cages in 2020, eight females of *E. bidens* were able to survive for an average of  $24.3 \text{ days} \pm 3.2 \text{ days}$  under rearing conditions, while the other four specimens quickly died. After a few days inside the cages, females began to scour the plants for leafhopper nymphs. The seeking behaviour was mainly focused on the lower part of the leaves where the leafhopper nymphs usually feed. It was often observed that the host did



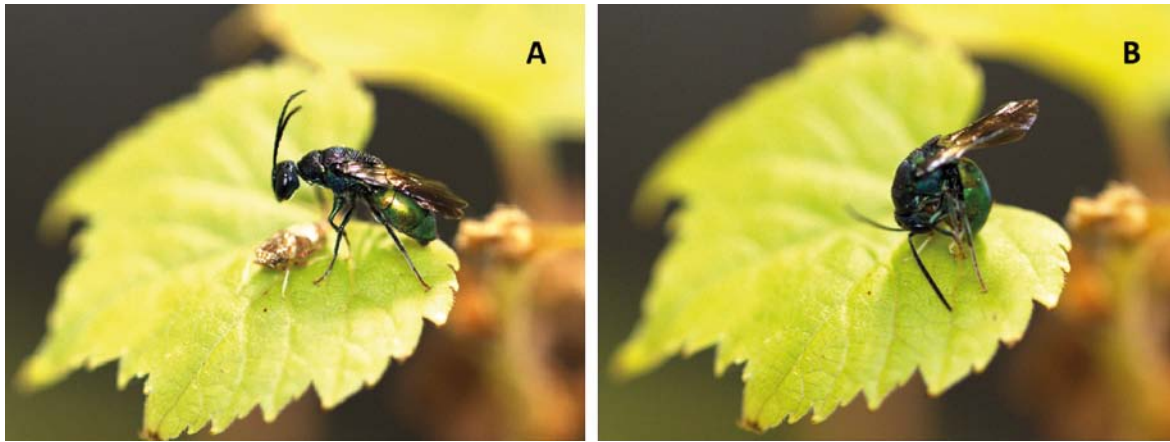
**Fig. 2.** Larvae of *Elampus bidens* inside specimens of *Scaphoideus titanus* (A, B) and *Orientus ishidae* (C, D).

not appear to be immediately detected even if close to the wasp. Once detected, the oviposition phase was always preceded by a short host examination period that has never been observed before for chrysidids. During this phase, the female wasp approaches and examines the host with its antennae erect, vibrating, pointing straight upwards, and not touching the leafhopper nymph. Subsequently, the females attack their hosts, clamping their body around them before releasing them at the end of oviposition after a few seconds (Fig. 3; Video S1). This behaviour is unusual, as all previously reported oviposition observations of chrysidid have involved physical inspection of the host with the antennae (José et al. 1970; Veenendal 2012; Winterhagen 2015). Cuckoo wasp eggs or larvae were found inside the third and following nymphal instars of their hosts. As for adult leafhoppers, the female wasps were clearly hesitant during the examination phase, often allowing the leafhoppers to leave. Only one adult of *S. titanus* was successfully parasitised, all other attempts failed before the *E. bidens* female could commence oviposition.

A total of 87 (47 *O. ishidae* and 40 *S. titanus*) leafhoppers were found being parasitised during this experimental survey and no statistical differences were found between the parasitised proportion of leafhopper species ( $\chi^2 = 0.64$ ,

$p = 0.42$ ,  $df = 1$ ). All parasitised nymphs were able to moult and reach the adult stage. The egg of *E. bidens* found in *S. titanus* is oval and hyaline and measures 470–540  $\mu\text{m}$  in length. We observed different development phases of the first larval stage of *E. bidens* inside *S. titanus* and *O. ishidae*. The first instar emerged from the egg two days after the oviposition and measured 560–750  $\mu\text{m}$  in length. It was hymenopteriform and semi-transparent, with 13 segments and unsclerotised head. Larval morphology showed no differences in body shape through the two different instars observed (1<sup>st</sup> and 2<sup>nd</sup>), except (a) the size which ranged from about 0.5 mm to nearly 3.0 mm in length (Fig. 4) and (b) the coloration which became darker internally due to food and waste that accumulated during the development. The larva remains at the end of the first instar in a form of quiescence.

The first instar appeared about 8–10 days after parasitisation, and even after 25 days it was possible to find it alive but static. Parasitised individuals of the two leafhopper species survived for more than twenty days and maintained their ability to fly and move, but they appeared to be less reactive and more static than the unparasitised individuals. The larvae of the second stage were only observed on three specimens collected from the field; this stage was never observed



**Fig. 3.** Parasitisation of a *Orientus ishidae* nymph by a female of *Elampus bidens*: **(A)** host examination phase; **(B)** attack phase with host clamping.

under laboratory conditions. In their morphology, they were similar to the 1<sup>st</sup> instar larvae, but displayed sickle-shaped mandibles.

#### 4 Discussion

We here report the first documented host-parasite association that involves the two exotic leafhoppers *Scaphoideus titanus* and *Orientus ishidae*, the cuckoo wasp *Elampus bidens* and an apoid wasp, likely *Psen ater*. The “Trojan horse” strategy of laying an egg into the body of the leafhopper intermediate host that is subsequently captured and carried into the brood cell by a predatory wasp has not previously been documented for members of the genus *Elampus*. This behaviour has been hypothesised to have arisen in order to elude the defence strategies used by the host wasps to prevent the brood parasite from entering its nest (Strohm & Liebig, 2008; Wurdack et al. 2015).

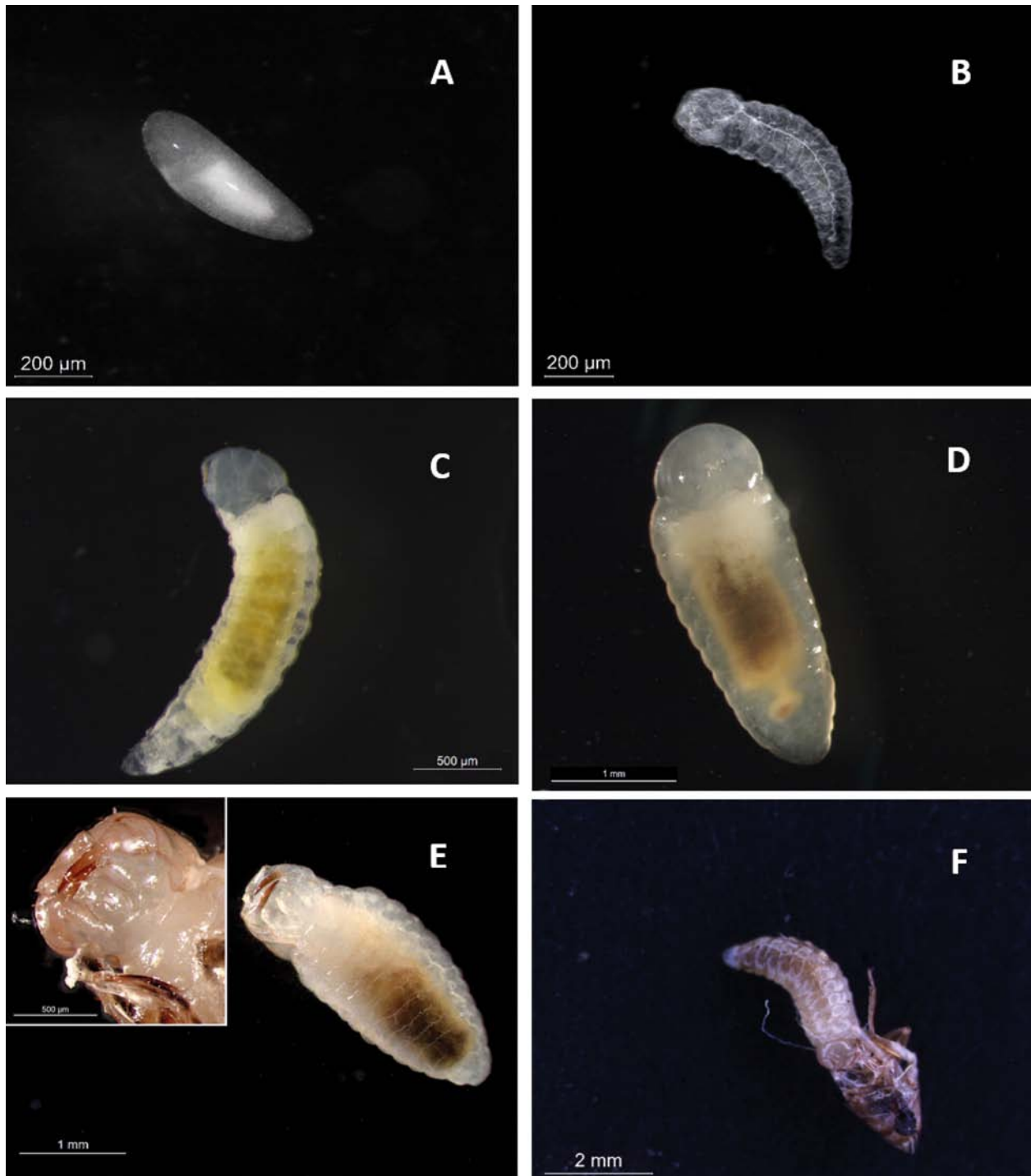
An endoparasitoid behaviour provides an evolutionary advantage to a brood parasitic wasp, avoiding the risk that the egg is detected and removed by the principal host. Species of the genus *Elampus* are known to be brood parasites of at least two genera of the apoid wasp family Psenidae: *Mimesa* and *Mimumesa* (Mocsáry 1889; Spooner 1948; Rosenheim & Grace 1987), both known to be predators of nymphal and adult cicadellids and delphacids. Nevertheless, these field observations were based on finding *Elampus* adults in psenid wasp nests, without any note on the parasitic strategy used by the cuckoo wasps. The putative principal host of *E. bidens* identified here, *Psen ater*, is known to hunt leafhoppers of the family Cicadellidae (Bitsch et al. 2007). However, we do not know whether the cicadellids amassed in the nest or the progeny of the wasp itself are the final target of the chrysidid wasp, as in the case of the chrysidid wasp *Chryselampus sculpticollis* (Abeille de Perrin, 1877) and its psenid host

*Psenulus fuscipennis* (Dahlbom, 1843) (Martynova & Fateryga 2014). One common trait in all studied *Elampus* larvae is the presence of large toothed mandibles (Grandi 1959; Tormos et al. 1999; Martynova & Fateryga 2014). These are similar to the mandibles found in larvae of brood parasitic bees within the Nomadinae which are used to destroy host eggs or early instar larvae (Rozen 1977). These mandibles are unlikely to be used exclusively to consume paralysed larvae given their presence in *Chryselampus* that feeds on soft-bodied aphids and psenid larvae (Martynova & Fateryga 2014).

Though strongly suggested, this study does not unambiguously demonstrate this host-brood parasite relationship between *E. bidens* and *P. ater*. In the field, *E. bidens* was consistently observed to be active before *P. ater*, raising the possibility that an alternative wasp species serves as a host. If *E. bidens* is to be used as a conservative biological control agent against exotic leafhopper pests in uncultivated areas, the here hypothesized host-brood parasite association must be confirmed, as both chrysidid and the second host would be necessary for a captive breeding to deepen our understanding of their biology.

##### 4.1 Native and alien leafhopper hosts

Despite extensive searches, no native leafhoppers were found to be attacked by *E. bidens*. Thus, *S. titanus* and *O. ishidae* represent the only known intermediate hosts attacked by *E. bidens* so far. This result is possibly explained by the high abundance of these two invasive leafhoppers at our study sites. The two exotic cicadellids rapidly increased their population sizes and spread their range in Italian vineyard agroecosystem since their introduction to Europe (Lessio et al. 2016, 2019; Alma et al. 2019). From these preliminary results it seems that a host preference by *E. bidens* does not emerge as there is not a clear association between the parasitization rate and the leafhopper species. It is likely that



**Fig. 4.** *Elampus bidens* larval development from egg to 2<sup>nd</sup> instar larva: (A) Egg. (B–D) 1<sup>st</sup> instar larva. (E) 2<sup>nd</sup> instar larva. (F) 2<sup>nd</sup> instar larva inside *Scaphoideus titanus* body.

*E. bidens* tend to parasitize the most abundant species close to the Psenid nests. Despite the lack of other parasitised leafhoppers, it is reasonable to assume that *E. bidens* used and likely still uses native leafhoppers as intermediate hosts. Shifts to novel hosts by other parasitic Hymenoptera (Ichneumonidae) have been found to occur within two gen-

erations without a measurable survival cost for the parasitoid, and this rapid adaptation can even result in a measurable decrease of fitness for the parasitoid when developing in its original host (Jones et al. 2015). This behaviour might explain the absence of observed chrysidid larva inside native leafhopper species.



The exotic origin of the leafhoppers used by this Palaearctic chrysidid suggests a recent evolution of this association, as previously all three taxa showed allopatric distributions. No studies carried out on the biology and biological control of *S. titanus* since its first record in Europe have reported any possible presence of an endoparasitoid; only ectoparasitoids belonging to the family Dryinidae (Hymenoptera) have been reported (Vidano 1964; Cravedi 1993; Alma & Arzone 1994; Nusillard et al. 2003).

#### 4.2 Importance of parasitisation of alien pest

We found the average rate of parasitisation of *S. titanus* by *E. bidens* to be close to 30% in Canavese sites, across the duration of our investigation. This is the first report in which such a high average annual parasitisation rate on *S. titanus* is documented. This raises the hope that *E. bidens* could possibly serve as a conservative biological control agent against this invasive leafhopper species in uncultivated areas near vineyards as part of integrated pest management. The uncultivated areas can be considered not as a mere issue but as a resource in those portions of the vineyard agroecosystem whereby the control of leafhoppers is difficult by other means. So far, biological control of *S. titanus* has not been effective because of the low impact of entomophagous insects on the population development of this species (Schvester et al. 1962; Vidano 1966; Nusillard et al. 2003; Chuche & Thiery 2014; Bocca et al. 2020b). In France, tests on biological control of *S. titanus* by natural enemies from the Nearctic and by indigenous antagonists proved to be ineffective (showing a low percentage of parasitised individuals, < 1%; Nusillard et al. 2003). Likewise, release of the Dryinidae *Gonatopus sepsoides* Westwood and *Gonatopus lunatus* resulted in a low rate of parasitisation of *S. titanus* in Italy (i.e., < 1% of leafhoppers; Arzone & Alma 1994).

#### 4.3 Endoparasitic development

From rearing of the parasitised leafhoppers it came to light that many leafhopper specimens were able to survive for about a month, but the *E. bidens* larvae never emerged from them and ultimately died along with the hosts. Furthermore, we observed that *E. bidens* larvae primarily feed on the leafhoppers' gonads. In fact, none of the parasitised males had intact testis and none of the parasitised females had intact ovaries. It has been reported that the larvae of other koinobiont parasitoids keep their host alive for an extended period of time by feeding on non-viable tissues (Harvey & Strand 2002; Harvey et al. 2011). *Elampus bidens* seems to apply a similar strategy, which intuitively makes sense if the cuckoo wasp requires the leafhopper to serve as a vector and thus has to be captured by the primary host. We noticed that *E. bidens* larvae found in the field-collected subadult leafhoppers had a smaller body size than larvae found in adult leafhoppers (data not shown). This could suggest that *E. bidens*

deposits its eggs primarily in subadult leafhoppers. Indeed, we found *E. bidens* to oviposit into an adult leafhopper in our semi-natural rearing experiments only a single time, while in the same experiments, we found *E. bidens* to oviposit into more than 80 subadult leafhoppers, a result consistent with this idea. Why *E. bidens* likely prefers subadult leafhoppers is unclear, though it is likely related to the hunting strategy of *P. ater*, which principally collects leafhoppers in their nymphal stage.

We never observed *E. bidens* to continue development beyond the 2<sup>nd</sup> instar stage and/or to hatch from the parasitised leafhoppers. We assume that the larvae lack a cue required to continue their development. As reasonable cue could be the venom or components of it injected by the principal host when paralyzing the leafhopper as hypothesised by José et al. (1970) in the comparable case of *Pseudolopyga carrilloi* (Bohart & Brumley, 1967).

## 5 Conclusions and future directions

Our research has shed light on novel interactions between herbivorous, brood parasitic, and predatory insect species, with potentially important economic implications. Given the pest and disease pressure created by the two invasive leafhoppers *S. titanus* and *O. ishidae* and the absence of effective control strategies against the spread of FDp vectors, the discovery of an indigenous antagonist of these two species is remarkable and important, as it opens up new prospects for conservative biological control in uncultivated areas near vineyards as part of an integrated pest management. The fact that we found the highest rate of parasitisation by the antagonist in largely uncultivated areas underpins the importance of natural environments – and hence their protection – as a resource for indigenous biological control agents. As this association is very complex to evaluate the effectiveness of potential conservative biological control, additional research questions need to be answered, though, specifically, it is needed confirmation of the secondary apoid wasp host, a precise phenological investigation of the synchronicity between *E. bidens*, the primary, and secondary hosts, and to investigate if landscape-level characters influence or determine the distribution of *E. bidens* and the secondary host.

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**Supplement Tables S1–S3**