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1 Adaptation of Indigenous Larval Parasitoids to *Tuta absoluta* in Italy

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13 Ferracini et al.: Indigenous parasitoids of *Tuta absoluta* in Italy

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16

## Abstract

17  
18 *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is a serious threat to tomato crops in South  
19 America. In Europe, after its first detection in Spain in 2006, it rapidly spread through the  
20 Mediterranean basin, reaching Italy two years later. The aim of our work was to find indigenous  
21 effective biological control agents and to evaluate their potential role in the control of larval  
22 populations of *T. absoluta* (tomato borer) in controlled conditions. Nine species of larval parasitoids  
23 emerged from field-collected tomato leaves infested by *T. absoluta*. The most abundant, *Necremnus*  
24 near *artynes* (Walker) and *N. near tidius* (Walker) (Hymenoptera: Eulophidae), were tested in  
25 laboratory parasitism trials. Furthermore, since the species *N. artynes* and *N. tidius* are each  
26 reported in literature as an ectoparasitoid of *Cosmopterix pulchrimella* Chambers (Lepidoptera:  
27 Cosmopterigidae) on upright pellitory plants, olfactometer bioassays were performed to assess the  
28 response of our parasitoids to the odors of tomato and pellitory leaves infested by *T. absoluta* and  
29 *C. pulchrimella*, respectively, compared with healthy ones. Both *Necremnus* species showed good  
30 adaptation to the invasive pest, and we observed a high larval mortality of *T. absoluta* due to host  
31 feeding and parasitism. Even olfactory responses highlighted a preference of both wasps for tomato  
32 plants infested by the exotic pest. These preliminary results demonstrated a high suitability of these  
33 indigenous natural enemies for controlling the tomato borer. Further investigations are therefore  
34 needed to confirm their role as potential biological agents in commercial tomato plantations.

## Keywords

36  
37 biological control, tomato borer, native natural enemy, exotic invasive pest, *Necremnus* spp.

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39 Native to Central America, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) (tomato  
40 borer) has been a pest of tomato crops in South American countries since 1970, and is distributed in  
41 Argentina, Bolivia, Brazil, Chile, Colombia, Ecuador, Paraguay, Peru, Uruguay and Venezuela  
42 (EPPO 2005). In 2006, it was accidentally introduced into Spain, and in the past few years has  
43 spread rapidly through many countries bordering the Mediterranean Sea, including Italy where it  
44 was first reported in 2008 (Urbaneja et al. 2009, Viggiani et al. 2009, CABI 2011). The species was  
45 added to the European and Mediterranean Plant Protection Organization (EPPO) A1 and A2 action  
46 lists for regulation as quarantine pests in 2004 and 2009, respectively, and is now considered one of  
47 the major pests on tomato crops. In fact, while its main host is tomato, infestation of potato,  
48 eggplant, pepper, tobacco, and common bean is also reported. Moreover, various solanaceous  
49 species such as *Solanum nigrum* L., *S. elaeagnifolium* Cav., *S. puberulum* Nutt, *Datura stramonium*  
50 L., *D. ferox* L. and *Nicotiana glauca* Graham are reported as wild hosts (EPPO 2005, EPPO 2009,  
51 CABI 2011).

52 *Tuta absoluta* is a multivoltine species; females lay eggs on epigeal parts of their host plants  
53 and a single female can lay up to 260 eggs during its lifetime (EPPO 2005). Through their feeding  
54 activity within the mesophyll of the leaves, the larvae produce large mines, thus affecting the  
55 plant's photosynthetic capacity; furthermore, they burrow into stalks, apical buds, and fruits. The  
56 pest affects tomatoes destined for the fresh market as well as for processing, with larvae causing  
57 losses in its area of origin of up to 80–100% (Desneux et al. 2010). Pupation may take place in the  
58 soil, on the leaf surface or within the mines, depending on environmental conditions (EPPO 2005).

59 The tomato borer is a very challenging pest to control. Chemical approaches are difficult  
60 because of the mine-feeding behavior of larvae, in addition to the resistance developed to many  
61 conventional insecticides and the side effects for useful organisms in integrated pest management  
62 (IPM) programs (Siqueira et al. 2000, Lietti et al. 2005, Cabello et al. 2009, IRAC 2011). So, as an  
63 alternative approach, biological control has been actively pursued. Several natural enemies  
64 occurring in its native area have been reported as fully documented by Desneux et al. (2010), and

65 the efficacy of entomopathogenic fungi, bacteria and nematodes have also been evaluated for the  
66 implementation of biological control strategies (Batalla-Carrera et al. 2010, González-Cabrera et al.  
67 2010, Pires et al. 2010, Hernández-Fernández et al. 2011). Natural enemies have been investigated  
68 in many South American countries with the aim of using them in biological control programs; in  
69 particular, research has been carried out on egg parasitoids such as *Trichogramma pretiosum*  
70 (Riley), *T. nerudai* (Pintureau & Gerding), *T. exiguum* Pinto & Platner and *Trichogrammatoidea*  
71 *bactrae* Nagaraja (Hymenoptera: Trichogrammatidae) (Pratissoli and Parra 2000, Zucchi and  
72 Querino 2000, Faria et al. 2008, Desneux et al. 2010, Virgala and Botto 2010), and larval  
73 parasitoids such as *Apanteles gelechiidivoris* Marsh, *Pseudapanteles dignus* (Muesebeck), *Bracon*  
74 spp. (Hymenoptera: Braconidae), *Dineulophus phthorimaeae* (de Santis) (Hymenoptera:  
75 Eulophidae), and *Diadegma* spp. (Hymenoptera: Ichneumonidae) (Colomo et al. 2002, Marchiori et  
76 al. 2004, Miranda et al. 2005, Bajonero et al. 2008, Sánchez et al. 2009, Luna et al. 2010).  
77 However, none of these beneficial organisms seem to have so far been decisive in controlling *T.*  
78 *absoluta* and the research is still ongoing.

79         Indigenous predators and parasitoids with the ability to control this exotic leafminer have also  
80 been investigated throughout the Mediterranean area. Larval parasitoids such as *Necremnus* spp.  
81 and *Stenomesus* spp. (Hymenoptera: Eulophidae) (Arnó and Gabarra 2010, Gabarra and Arnó  
82 2010, Rizzo et al. 2011, Zappalà et al. 2011a), and predators such as *Macrolophus pygmaeus*  
83 (Rambur), *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae), *Nabis pseudoferus* (Remane)  
84 (Hemiptera: Nabidae) (Urbaneja et al. 2009, Mollá et al. 2010, Fois et al. 2011, Zappalà et al.  
85 2011b), have been reported in Spain and more recently in Italy. Additionally, the shift of other  
86 indigenous entomophagous species, especially braconids and ichneumonids, onto the exotic pest  
87 has been documented by recent literature (Loni et al. 2011, Rizzo et al. 2011, Zappalà et al. 2011a),  
88 revealing a gradual adaptation of these generalist parasitoids to the new host. The egg parasitoid  
89 *Trichogramma achaeae* Nagaraja & Nagarkatti, which is however reported in the list of the  
90 hymenopteran species alien to Europe (Rasplus et al. 2010), was also tested in inundative biological

91 control programs, and has become commercially available following its promising potential  
92 (Cabello et al. 2009, Desneux et al. 2010).

93 Due to the huge potential of *T. absoluta* to expand its distribution range, a multidisciplinary  
94 approach exploiting natural enemies in several countries is gaining favor. With the aim of searching  
95 for indigenous parasitoids able to adapt to the new host, and which are suitable for mass-rearing and  
96 augmentation biological control programs, field surveys were firstly carried out in horticultural  
97 areas of Italy. The species that most frequently emerged from infested tomato, and that proved to be  
98 fit for mass-rearing, were therefore tested in the laboratory to evaluate their effectiveness in  
99 controlling *T. absoluta*; to contribute further to the implementation of effective and environmentally  
100 friendly control strategies, their ability to recognize and choose new host larvae was also assessed.

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## Materials and Methods

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**Field Collection and Rearing of Parasitoids.** In the two-year period 2009–2010, surveys  
were carried out in 24 IPM-protected tomato fields located in four Italian horticultural areas:  
Liguria (four fields), Campania (six fields), Sardinia (five fields), and Sicily (nine fields), where *T.*  
*absoluta* was established on tomato plants. In the surveyed sites, samples of 100 infested leaves  
were randomly collected from tomato crop once or twice in the mid-growing season, placed in  
sealed plastic bags, and transferred to the laboratory. All the leaves were carefully checked using a  
microscope for the presence of tomato borer larvae and pupae, in order to exclude any other insect  
infestation. The leaves were then placed in cages in climatic chambers at  $24 \pm 1^\circ\text{C}$ ,  $60 \pm 5\%$  RH,  
16:8 h (L:D) photoperiod to detect the emergence of possible larval parasitoids. Each cage consisted  
of a cardboard box ( $40 \times 60 \times 30$  cm) with a tight-fitting lid and two clear jars extending from two  
holes in its side. When the adults emerged, they were attracted to the light entering through the jars  
where they then accumulated and were easily collected with the aid of an insect aspirator. The  
emerged adult parasitoids were killed with ethyl acetate and stored in 70% ethanol in glass test  
tubes (60 mm high  $\times$  8 mm diameter) until identification. The parasitoids belonging to the

117 Eulophidae family were then identified at the DIVAPRA (Dipartimento di Valorizzazione e  
118 Protezione delle Risorse Agroforestali, University of Torino, Italy) (identifications by co-author P.  
119 N.), while all other parasitoid species were sent to the respective specialists. Voucher specimens  
120 were deposited at the DIVAPRA.

121 The most abundant species of the emerged parasitoids were selected on the basis of their  
122 suitability for mass-rearing, as tested at the Bioplanet laboratories (Bioplanet s.c.a., Cesena, Italy).  
123 Therefore, two species of the genus *Necremnus* that had proved to be fit for mass-rearing on *T.*  
124 *absoluta* on tomato plants at the Bioplanet laboratories, were tested at the DIVAPRA laboratories to  
125 assess their effectiveness in controlling the pest, and to study their behavior and host preference  
126 under laboratory conditions. Before using them in the experiments, the parasitoid adults from  
127 Bioplanet were sexed, fed every 48 h with drops of honey on cardboard, and kept in individual glass  
128 tubes (120 mm high × 18 mm diameter), in a climatic chamber at  $24 \pm 1^\circ\text{C}$ ,  $60 \pm 5\%$  RH, 16:8 h  
129 (L:D) photoperiod.

130 **Insect Colonies and Host Plant Management.** Colonies of *T. absoluta* were established  
131 starting from an initial culture collected in a tomato commercial plantation in Liguria (NW Italy). A  
132 continuous mass-rearing of all development stages was maintained on tomato plants in an open-  
133 sided greenhouse, in cages (150 × 150 × 110 cm) that had a stainless steel frame structure  
134 supporting an insect-proof net (mesh 0.23 × 0.23). Plants of *Lycopersicon esculentum* Mill.  
135 Montecarlo F1 variety were used for both mass-rearing and laboratory trials. In particular, plants  
136 used in the parasitism trials were approximately 25 cm high from the soil surface, in pots filled with  
137 a mixture of soil and covered with a layer of white sand in order to easily detect possible individuals  
138 dropped off during trials.

139 Moreover, for olfactometer bioassays, colonies of *Cosmopterix pulchrimella* Chambers  
140 (Lepidoptera: Cosmopterigidae), reported as a natural host of the related *Necremnus* species  
141 (Bernardo and Viggiani 2002, The Natural History Museum 2011), were also established, starting  
142 from an initial culture collected on upright pellitory [*Parietaria officinalis* L. (Urticaceae)] in



143 Piedmont (NW Italy), and maintained in the cages described above. Upright pellitory plants were  
144 collected in wastelands and cuttings were taken in order to obtain new plants for *C. pulchrimella*  
145 rearing.

146 All the plants were cultivated in plastic pots (diameter 20 cm), watered daily and fertilized,  
147 and kept in an open-sided greenhouse at  $27 \pm 3^\circ\text{C}$ ,  $55 \pm 23\%$  RH, 16:8 h (L:D) photoperiod.

148 **Parasitism Trials.** Before testing the selected parasitoids for their effectiveness in controlling  
149 tomato borer, laboratory trials were performed to assess if the parasitoid females showed any  
150 preference for different instar larvae. Potted tomato plants were infested with 20 larvae of *T.*  
151 *absoluta* of different instars, and encaged in Plexiglas cylinders (40 cm high  $\times$  18 cm diameter).  
152 Newly molted larvae (i.e., five for each of the four instars) were chosen and randomly distributed  
153 over the plant. Single five-day-old females ready to oviposit, after being mated and fed with honey,  
154 were introduced and maintained on the infested tomato plants for 48 h. Following this, the females  
155 were removed and the larvae were observed under a stereomicroscope to determine if they were  
156 dead or alive. Five replicates were performed for each parasitoid species.

157 To evaluate the effectiveness of the selected parasitoids as biocontrol agents, tomato leaves  
158 with *T. absoluta* larvae no older than 24 h were collected from mass-rearing cages, and the portion  
159 of the leaf which contained the larva inside the mine was cut off after checking that the mine was  
160 not empty using the stereomicroscope. This leaf portion was then fixed onto a leaf of healthy potted  
161 tomato plant with the aid of a drop of silicone. On each tomato plant consisting of four leaves, 20  
162 leaf sections were fixed homogeneously, so as to obtain a density of five larvae per leaf to ensure a  
163 consistent infestation while preventing plant collapse. The potted plant was placed separately inside  
164 a Plexiglas cage (20  $\times$  20  $\times$  30 cm) with two sides and a lid of fine gauze (30/10 net) and a mesh  
165 sleeve inserted in the middle of a side (diameter 11 cm) to allow access to the plant.

166 Larvae were left for 48 h to allow them to transfer from the leaf section to the plants and  
167 establish new galleries. One female parasitoid was then released into each cage and removed five  
168 days later. Five-day-old females ready to oviposit were used, after being mated and fed with honey

169 inside the rearing glass tubes. Ten replicates were performed for each parasitoid species and 10  
170 plants were also set up as control.

171 Cages were checked for parasitoid and moth emergence every day, and all the emerged  
172 individuals were aspirated off the cage through the sleeve and counted; the parasitoids were then  
173 sexed and sex ratio was provided. Collection continued for two weeks after initial emergence. After  
174 30 days, each experimental cage was dismantled and all the leaves observed under a  
175 stereomicroscope.

176 All the trials were performed in a climatic chamber at  $24 \pm 1^\circ\text{C}$ ,  $60 \pm 5\%$  RH, 16:8 h (L:D)  
177 photoperiod.

178 Moreover, single females of each parasitoid species were offered tomato leaves with five  
179 first-instar larvae, placed on a wet filter paper inside a Petri dish (diameter 10 cm), in order to  
180 obtain preliminary data on their behavior in relation to parasitism and host feeding. So, females,  
181 mated, fed with honey, and not experienced with a host were used for the experiments. Their  
182 behavioral sequence (e.g., host location and acceptance, oviposition, host-feeding) on larvae of *T.*  
183 *absoluta* was observed using a stereomicroscope for 30 min, and 10 replications were carried out for  
184 each parasitoid species.

185 **Olfactometer Bioassays.** In the olfactometer bioassays, five day-old females of the selected  
186 parasitoid species were used to assess their olfactory responses to the odors of tomato plants  
187 uninfested and infested by *T. absoluta*, and to the odors of *P. officinalis* uninfested and infested by  
188 *C. pulchrimella* as an alternative host. In particular, three comparisons were carried out for each  
189 parasitoid species: a) upright pellitory leaves infested by *C. pulchrimella* compared to tomato leaves  
190 infested by *T. absoluta*; b) healthy upright pellitory leaves compared to healthy tomato leaves; and  
191 c) healthy tomato leaves compared to tomato leaves infested by *T. absoluta*. Each odor source  
192 consisted of five tomato leaflets or pellitory leaves, uninfested or infested with a density of three  
193 larvae per leaf, for a total of 15 larvae. Before trials, insects were kept at  $15^\circ\text{C}$  without any host or  
194 plant in a glass tube for 18 h with a humid cotton cap and microdrops of honey. The bioassays were

195 carried out in a Y-shaped Pyrex tube (internal diameter 1.2 cm) formed by an entry arm and two  
196 side arms, each 10 cm long (70° angle), and positioned horizontally. Each side arm was connected  
197 to a round modified beaker (250 mL volume capacity, diameter 9 cm) as an odor-source container.  
198 Airflow was provided by an air pump (Air 275R, Sera, Germany), then filtered in an activated CO<sub>2</sub>  
199 filter, regulated with a flow meter at 2.5 L min<sup>-1</sup> (EK-2NRK, Comer, Italy) and humidified in a 1-L  
200 water bubbler half-filled with deionized water. After flow was established, a single parasitoid  
201 female was introduced into the entry arm. Each female was observed until she had moved at least 2  
202 cm up one of the side arms or until 10 min had elapsed. Females that did not choose a side arm  
203 within 10 min were considered as “no choice” and were not counted in the subsequent data analysis.  
204 For each test, a female was evaluated only once to prevent any behavior conditioned by experience.  
205 The odor sources chosen by females that responded were recorded. Thirty responses were recorded  
206 for each pair of odor sources. After testing five females, odor sources were switched between the  
207 left-hand and right-hand side arms to minimize any spatial effect on choices. The Y-tube and  
208 cameras were cleaned with mild soap and alcohol (70%<sub>v</sub>) and sterilized in an autoclave at 120°C for  
209 20 min. The olfactory bioassays were conducted at 24 ± 2°C, 50 ± 10% RH and 150 ± 10 lux.

210 **Statistical Analyses.** Significance of mean values of the data was analyzed by one-way  
211 analysis of variance (ANOVA), after ascertaining the homogeneity of variance (Levene’s test). In  
212 the olfactory bioassays, responses of parasitoid females were analyzed by a Chi-square test. The  
213 null hypothesis was that parasitoid females had a 50:50 distribution across the two odor sources. All  
214 analyses were performed using the software SPSS version 17.0 (SPSS, Chicago, IL, USA).

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## Results

217 Nine species of indigenous parasitoids emerged from tomato leaves infested by *T. absoluta*  
218 collected in Arma di Taggia (43°50'54" N - 07°50'27" E), Ceriale (44°06'0" N - 08°13'60" E), Pula  
219 (39°00'36" N - 09°00'6" E), Portopalo (36°40'24" N - 15°05'20" E), Sampieri (36°46'60" N -  
220 14°41'60" E), namely: *Necremnus* near *artynes* (Walker), *N.* near *tidius* (Walker), *Neochrysocharis*

221 *formosa* (Westwood), *Pnigalio* (= *Ratzeburgiola*) *cristatus* (Ratzeburg), *Pnigalio* sp. *soemius*  
222 complex (Hymenoptera: Eulophidae), *Diadegma ledicola* Horstmann (Hymenoptera:  
223 Ichneumonidae), *Bracon osculator* (Nees), *B. hebetor* Say, and *Agathis* sp. (Hymenoptera:  
224 Braconidae) (Table 1). The presence of these parasitoids varied in relation to the surveyed area;  
225 however the most abundant species were *N.* near *artynes* coming from Sardinia and Sicily, and *N.*  
226 near *tidius* and *D. ledicola* from Liguria (Table 1).

227 In preliminary multiplication trials performed at the Bioplanet, *D. ledicola* was found to be  
228 not suitable for wasp offspring and was subsequently discarded, whereas both *Necremnus* species  
229 proved to be fit for mass-rearing on *T. absoluta* on tomato plants (data not shown). Therefore,  
230 populations of *N.* near *artynes* and *N.* near *tidius*, obtained from tomato leaves collected in Pula and  
231 Arma di Taggia respectively, and mass-reared at the Bioplanet, were used for parasitism and  
232 olfactory bioassays.

233 **Parasitism Trials.** In the experiments, when different instar larvae were simultaneously  
234 offered, both *N.* near *artynes* and *N.* near *tidius* females were shown to prefer and set on larvae of  
235 the first two instars, which were well-accepted as hosts, both for feeding and oviposition ( $F = 87.55$ ;  
236  $df = 3$ ;  $P < 0.001$  for *N.* near *artynes*;  $F = 195.85$ ;  $df = 3$ ;  $P < 0.001$  for *N.* near *tidius*). In fact, high  
237 percentages of larvae of the first- and of the second-instar were killed by the wasps, whereas no  
238 larvae of the third- and fourth-instar were attacked (Table 2).

239 Therefore, the effectiveness of *N.* near *artynes* and *N.* near *tidius* as biocontrol agents was  
240 evaluated on larvae of the first two instars on potted tomato plants in cages. In these experiments, a  
241 high mortality of *T. absoluta* larvae was recorded when they were exposed to the parasitoids, with  
242 statistically significant differences between control and treated plants. The average number of adults  
243 of the emerged moths ( $\pm$  SE) was  $2.6 \pm 0.9$  and  $1.2 \pm 0.5$  in the presence of *N.* near *artynes* and *N.*  
244 near *tidius*, respectively, in contrast to  $17.2 \pm 0.6$  in the control ( $F = 180.28$ ;  $df = 2$ ;  $P < 0.001$ ).

245 Females of both *Necremnus* species were able to parasitize host larvae, and the average  
246 offspring ( $\pm$  SE) obtained was  $2.7 \pm 1.1$  and  $1.5 \pm 0.4$  with a sex ratio of 1:3.5 and 1:1♀:♂) for

247 *N. near artynes* and *N. near tidius*, respectively. The developmental time from the introduction of  
248 the wasps in the cage to the emergence of the progeny was shorter for *N. near artynes* at  $10.2\pm 0.1$   
249 days and longer for *N. near tidius* at  $14.3\pm 0.2$  days, while at least three weeks were needed for the  
250 adult moths to emerge. In the leaves observed with the stereomicroscope, 64.8% and 78.6% of the  
251 larvae exposed to *N. near artynes* and *N. near tidius*, respectively, were found dead inside the mines.  
252 Considering the death rate in control plants (14.0%), the previous mortalities can be ascribed to  
253 host-feeding or to failed larval development of the parasitoid.

254 Females of both *Necremnus* species showed the same behavior when they were introduced  
255 into the arena with tomato leaves infested by first-instar larvae of *T. absoluta*. First, the females  
256 walked and searched at random on the leaves until they located the mine. Then, they explored the  
257 mine inserting the ovipositor repeatedly at different points; they were guided by the host larva's  
258 movement until they could sting to inject venom and paralyze the larva. The ovipositing females  
259 laid one or more eggs per larva at different points of the mine, whenever reinserting the ovipositor.  
260 The location of eggs was related to the size and shape of the mine. Since the larva became  
261 paralyzed many hours after being injected by venom from the parasitoid female, the eggs were  
262 generally laid far from the host to be safe from its movement. After oviposition, the parasitoid  
263 females did not touch nor brush the point of insertion with the tip of the gaster. The females showed  
264 a more aggressive behavior when they killed the larva for host-feeding. In fact, they pierced the  
265 body of the larva with their ovipositor repeatedly, causing an irreversible paralysis followed by  
266 death. Drilling was accomplished by inserting the ovipositor, and swinging the valves up and down  
267 against each other. Once the ovipositor was completely withdrawn, females began to suck the  
268 haemolymph exuded at the puncture. This behavior lasted for up to 2–3 min.

269 **Olfactometer Bioassays.** Since the species *Necremnus artynes* (Walker) and *N. tidius*  
270 (Walker) are reported in literature as ectoparasitoids of *C. pulchrimella* on upright pellitory  
271 (Bernardo and Viggiani 2002, The Natural History Museum 2011), the olfactometer bioassays were  
272 performed to testing the attractiveness both of tomato and of pellitory plants for the wasp females.

273 Almost all the females tested in olfactometer bioassays responded by making a choice within the  
274 fixed time. In the experiment with *N. near artynes*, females proved to be more attracted by infested  
275 tomato compared to infested upright pellitory plants ( $\chi^2 = 19.20$ ;  $df = 1$ ;  $P < 0.001$ ), and by healthy  
276 tomato compared to healthy upright pellitory plants ( $\chi^2 = 4.80$ ;  $df = 1$ ;  $P = 0.028$ ). Significant  
277 differences in responses of *N. near artynes* females were also found when comparing infested with  
278 healthy tomato plants ( $\chi^2 = 10.80$ ;  $df = 1$ ;  $P = 0.001$ ) (Figure 1). In the experiment with *N. near*  
279 *tidius*, no significant preference of females was detected between healthy tomato and healthy  
280 upright pellitory plants ( $\chi^2 = 0.53$ ;  $df = 1$ ;  $P = 0.465$ ), and between infested tomato and infested  
281 upright pellitory plants ( $\chi^2 = 3.33$ ;  $df = 1$ ;  $P = 0.068$ ). By contrast, results for infested tomato plants  
282 showed them to be largely attractive in comparison with healthy plants ( $\chi^2 = 8.53$ ;  $df = 1$ ;  $P = 0.003$ )  
283 (Figure 2).

284

285

## Discussion

286 *Tuta absoluta* is considered one of the major pests on tomato and other solanaceous crops,  
287 both in the field and under protected conditions (EPPO 2005). Because of the ongoing spread of this  
288 exotic moth throughout Europe and the lack of totally satisfactory effective management options,  
289 most control attempts have moved towards a biological control approach. Several natural enemies  
290 have been reported, in particular Eulophidae, Braconidae and Trichogrammatidae as parasitoids,  
291 and Miridae, Nabidae and Pentatomidae as predators (Desneux et al. 2010). Nevertheless, some  
292 species that we obtained from field-collected samples are recorded here for the first time as larval  
293 parasitoids of *T. absoluta*. In fact, although species of the genera *Agathis*, *Bracon* (Hymenoptera:  
294 Braconidae), and *Diadegma* (Hymenoptera: Ichneumonidae) are already known as larval parasitoids  
295 of the tomato borer in South America (Colomo et al. 2002, Marchiori et al. 2004, Miranda et al.  
296 2005), *B. hebetor* and *D. ledicola* have not been previously reported, while *A. fuscipennis*  
297 (*Zetterstedt*) and *B. osculator* along with *D. pulchripes* (Kokujev) have been recently observed in

298 Tuscany (Loni et al. 2011) and Sicily (Zappalà et al. 2011a). However, these species are larval  
299 parasitoids of many Lepidoptera species (Milonas 2005).

300 At the same time, several species belonging to the Eulophidae family have been reported  
301 among larval parasitoids of *T. absoluta* in South America, and now also in the Mediterranean area  
302 (Desneux et al. 2010). These data are not surprising because this family includes several parasitoids  
303 of leafminers and gall-making larvae, often able to adapt to exotic hosts. Although records of  
304 *Neochrysocharis formosa*, *Pnigalio cristatus* and a species of the *P. soemius* complex have also  
305 been recently reported (Luna et al., 2011; Zappalà et al. 2011a), parasitoids of the genus *Necremnus*  
306 emerged from larvae of tomato borer in our studies as well as in others (Mollá et al. 2008, 2010;  
307 Gabarra and Arnó 2010, Fois et al. 2011, Rizzo et al 2011, Zappalà et al. 2011 a). Thus, when  
308 considering their suitability for mass-rearing as assessed at the Bioplanet, these species appear to be  
309 promising native biological control agents. In contrast, *D. ledicola* proved not to be fit for mass-  
310 rearing in spite of its abundance on *T. absoluta* in one of the surveyed tomato fields.

311 The genus *Necremnus* includes solitary and gregarious ectoparasitoids of larvae of  
312 coleopteran, lepidopteran and dipteran species (Coudron et al. 2000, Bernardo and Viggiani 2002,  
313 Dodsall et al. 2007), but most species have been poorly investigated so far and very little  
314 information, not always reliable, is available in the literature on their life history, behavior, and  
315 above all their hosts. For example, *N. tidius* is a solitary ectoparasitoid of coleopteran larvae with a  
316 Holarctic distribution (Gibson et al. 2005); Diptera and Lepidoptera have also been listed among its  
317 hosts but the non-coleopteran host association probably resulted from incorrect identification of the  
318 parasitoid (Dodsall et al. 2007). This misidentification would seem to be further confirmed by the  
319 different behavior of females after oviposition. In fact, unlike what has been observed for females  
320 of *N. tidius* (Dodsall et al. 2007), in our experiments females did not touch and brush the mine after  
321 oviposition. Additionally, some characters of *N. artynes*, described in the keys of Boucek (1958),  
322 Graham (1959) and Askew (1968), did not match completely those of our individuals. Hence, we

323 chose to name the species emerged from *T. absoluta* and here studied as *N. near tidius* and *N. near*  
324 *artynes* to avoid any misidentification while awaiting their systematic clarification.

325         Despite their specific identification requiring further investigation, the results obtained from  
326 laboratory experiments demonstrated that both *Necremnus* species effectively recognized and  
327 parasitized *T. absoluta* in our caged experiments. Therefore, these parasitoids are able not only to  
328 accept this new host, as also observed in other studies (Gabarra and Arnó 2010, Mollá et al. 2010),  
329 but can also reduce significantly infestations of the tomato borer under laboratory conditions.  
330 Parasitoid females exhibited a preference for larvae of the first two instars, on which they could  
331 oviposit or feed. Moreover, females of both species proved to be able to recognize and choose  
332 tomato leaves infested by *T. absoluta* larvae in the olfactometer bioassays. In particular, females of  
333 *N. near artynes* showed a very strong response to tomato leaves, both uninfested and infested, which  
334 could explain the frequent record of this native parasitoid on the exotic pest. It now remains to be  
335 assessed what stimuli form the trigger for behavioral responses of these parasitoids in order to  
336 obtain a better understanding of their behavior.

337         In our experiments the impact of the parasitoids was calculated as all dead hosts resulting  
338 from the presence of the parasitoids, not only hosts utilized for parasitoid reproduction. Females of  
339 both *Necremnus* species were observed to kill *T. absoluta* larvae and feed on their haemolymph;  
340 they probably need to feed on the host for maintenance and/or egg production, as do other  
341 synovigenic parasitic wasps (Giron et al. 2004). Destructive host-feeders can be considered to be  
342 better biological control agents even if host-feeding must not be used as the sole selection criterion  
343 (Jervis et al. 1996). However, in our experiments the impact of host-feeding by both *Necremnus*  
344 species could be overestimated. In fact, the females were kept in contact with the same larvae in the  
345 cages for five days, and consequently the probability that they could feed on the larvae in the mines,  
346 where they had previously laid eggs, was increased. When new larvae were offered, on *C.*  
347 *pulchrimella* approx 20% and 80% of host larvae were killed by females of *N. near tidius* for  
348 feeding and oviposition respectively, whereas host-stinging behavior was very rarely observed (P.



349 N., unpublished data), unlike other eulophid parasitoids of leafminers such as *Pnigalio soemius*  
350 (Walker) (Bernardo et al. 2006). Therefore, the high larval mortality of *T. absoluta* could be due  
351 partly to host-feeding and partly to the failure of parasitoid larval development on this new host  
352 under laboratory conditions, and needs to be further investigated.

353         Since conditions in the open field are much more varied than those in the laboratory and,  
354 among other factors, temperature is observed to play an important role in the development of  
355 arthropods, further investigations are needed to assess the behavior of both *Necremnus* species at  
356 different thermal conditions. Moreover, our preliminary findings about the host location and  
357 acceptance suggest that both *Necremnus* species have several behavioral traits that positively  
358 influence their performance as biological control agents. Hence, studies should be continued to  
359 investigate the suitability of the selected host for the parasitoid immature development with the aim  
360 of improving the efficiency of mass production and implementing augmentation of biological  
361 control programs.

362         The ability of *Necremnus* species to find and parasitize *T. absoluta* larvae makes them  
363 potential candidates for mass production and biological control, adding to the list another example  
364 of the adaptation of an indigenous parasitoid to an exotic pest and highlighting the importance of a  
365 rich and variegated biodiversity in finding new associations with harmful pests, as already reported  
366 in Nicoli and Burgio (1997). In the light of these satisfactory results, additional studies on both  
367 *Necremnus* species will be required to clarify their systematic position, detect their primary hosts,  
368 and to evaluate them in biological control and IPM programs in commercial tomato plantations.

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370

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375

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521 Figure captions

522

523 **Fig. 1.** Responses of *N. near artynes* (no. of responding females in bars) in a Y-tube olfactometer  
524 and, when present, number of non-responding individuals (NS) to the odors of infested ( $\delta$ ), or  
525 uninfested ( $\pi$ ) tomato plants with *T. absoluta* and of infested ( $\alpha$ ), or uninfested ( $\beta$ ) upright pellitory  
526 plants with *C. pulchrimella* for each compared pair. Numbers in bars represent individuals that  
527 moved toward the volatiles.  $\chi^2$  statistics (\*P<0.10; \*\*P<0.05; \*\*\*P<0.01; df=1) tested the  
528 hypothesis that the distribution of side-arm choices deviated from a null model where odor sources  
529 were chosen with equal frequency.

530

531 **Fig. 2.** Responses of *N. near tidius* (no. of responding females in bars) in a Y-tube olfactometer and,  
532 when present, number of non-responding individuals (NS) to the odors of infested ( $\delta$ ), or uninfested  
533 ( $\pi$ ) tomato plants with *T. absoluta* and of infested ( $\alpha$ ), or uninfested ( $\beta$ ) upright pellitory plants with  
534 *C. pulchrimella* for each compared pair. Numbers in bars represent individuals that moved toward  
535 the volatiles.  $\chi^2$  statistics (\*P<0.10; \*\*P<0.05; \*\*\*P<0.01; df=1) tested the hypothesis that the  
536 distribution of side-arm choices deviated from a null model where odor sources were chosen with  
537 equal frequency.

538

**Table 1** Indigenous larval parasitoid species emerged from tomato leaves infested by *T. absoluta*, collected in Italian horticultural areas

Species	Site	No. of emerged individuals	Date
<i>Necremnus</i> near <i>artynes</i>	Portopalo (SR), Sicily	11	9 July 2009
(Walker)	Pula (CA), Sardinia	27	21 July 2009 22 April 2010
<i>Necremnus</i> near <i>tidius</i>	Arma di Taggia (IM), Liguria	30	7 Oct 2009
(Walker)			
<i>Neochrysocharis formosa</i>	Ceriale (SV), Liguria	4	26 Aug 2010
(Westwood)			
<i>Pnigalio</i> (=Ratzeburgiola)	Ceriale (SV), Liguria	3	26 Aug 2010 10 Oct 2010
<i>cristatus</i> (Ratzeburg)			
<i>Pnigalio</i> sp. <i>soemius</i> complex	Ceriale (SV), Liguria	3	10 Oct 2010
<i>Diadegma ledicola</i>	Arma di Taggia (IM), Liguria	25	7 Oct 2009
Horstmann			
<i>Agathis</i> sp.	Sampieri (RG), Sicily	3	10 July 2009
<i>Bracon osculator</i> (Nees)	Ceriale (SV), Liguria	6	26 Aug 2010 10 Oct 2010
<i>Bracon hebetor</i> Say	Pula (CA), Sardinia	2	21 July 2009

**Table 2** Mean number of *T. absoluta* larvae ( $\pm$ SE) killed by single females of *N. near artynes* and *N. near tidius*. Each female was simultaneously offered 20 larvae, five for each instar, for 48 h

Species	Mean no. of <i>T. absoluta</i> larvae killed by the parasitoid			
	1 <sup>st</sup> -instar larvae	2 <sup>nd</sup> -instar larvae	3 <sup>rd</sup> -instar larvae	4 <sup>th</sup> -instar larvae
<i>N. near artynes</i>	4.20 $\pm$ 0.37 a	3.80 $\pm$ 0.20 a	0.00 $\pm$ 0.00 b	0.00 $\pm$ 0.00 b
<i>N. near tidius</i>	4.80 $\pm$ 0.20 a	4.60 $\pm$ 0.24 a	0.00 $\pm$ 0.00 b	0.00 $\pm$ 0.00 b

Within the same line, data (mean $\pm$ SE) followed by a different letter are significantly different (P<0.001; ANOVA)



\*\*\*



\*\*



\*\*



$\alpha$



$\beta$



$\pi$



$\delta$



$\pi$



$\delta$



$\alpha$



$\beta$



$\pi$



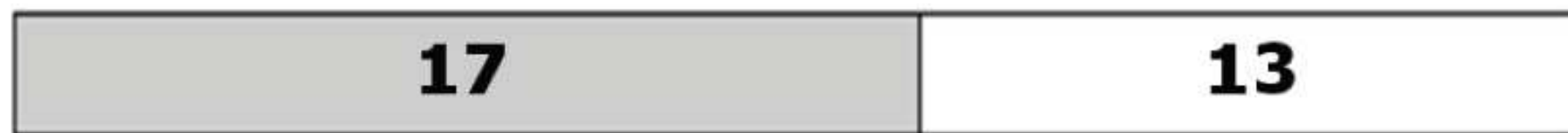
$\delta$



$\pi$



$\delta$



NS=1

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