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Acceptance and suitability of *Nezara viridula* nymphs as hosts for *Trichopoda pennipes*

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Abstract

Trichopoda pennipes (F.) is a parasitoid species introduced in various countries as a biological control agent against the southern green stink bug *Nezara viridula* (L.). Several studies were performed to investigate the interactions between this parasitoid and the host, but they were focused above all on the adults. Our research was focused on the effects of exposure and parasitization by *T. pennipes* on the five-instar nymphs of *N. viridula*. We observed that the exposure to the parasitoid had effects on the development time of the five-instar nymphs and on the lifespan of the nymphs and the adults that emerged from them.

Key words: southern green stink bug, biological control, tachinid parasitoid, *Trichopoda pennipes*.

Introduction

Trichopoda pennipes (F.) (Diptera Tachinidae) is a parasitoid species originating from North America (Salerno *et al.*, 2002). Its host range, in its natural geographic area, includes several species of Pentatomidae, Coreidae, Pyrrhocoridae and Alydidae (McPherson *et al.*, 1982; Panizzi and Slanky, 1985; Jones, 1988); but it has probably co-evolved with the squash bug *Anasa tristis* DeGeer (Rhynchota Coreidae) (Jones, 1988). However, since its arrival in North America in the 1700s (Jones, 1988), the southern green stink bug *Nezara viridula* (L.) (Rhynchota Pentatomidae) became suitable as host of this parasitoid species (Salerno *et al.*, 2002). Due its affinity with the cosmopolitan pest *N. viridula*, *T. pennipes* was imported into various countries with differing results (Davis, 1964; Jones, 1988). In 1983, this parasitoid was recorded in central Italy for the first time due to a fortuitous introduction (Tschorsnig *et al.*, 2012). After this first record, *T. pennipes* spread quickly into other countries of Europe and the Mediterranean area (Tschorsnig *et al.*, 2012), where it was found on *N. viridula* and *Graphosoma lineatum* (L.) (Rhynchota Pentatomidae) (Tschorsnig, 2017). Different factors could have contributed to the spread of *T. pennipes* in Italy and Europe, such as the tendency of its host to aggregate, the possibility that the parasitoid larva could overwinter inside its host and the suitability of an Italian population of *N. viridula* as host (Colazza *et al.*, 1996).

T. pennipes is an imaginal, and occasionally a nymphal, parasitoid (Harris and Todd, 1981; Ruberson *et al.*, 2010). Like other tachinid flies, it lays macrotype eggs which it glues onto the host's body (Harris and Todd, 1981); the eggs are laid usually on the side of the abdomen and thorax, but sometimes they can be found on the upper surface of the body and the head, rarely on the antennas or legs (Worthley, 1924a). Eggs which have hatched show a circular hole on the flattened side pressed against the body surface, so it is impossible to state if an egg has hatched without removing it (Worthley, 1924b). The newly hatched larva penetrates the victim's body, drilling directly the body-wall of the host, and it grows at the expense of bug's tissues (Worthley, 1924a). The larvae develop through three instars; the fully developed last instar leaves the host through the anal opening or through the intersegmental membrane nearby and drops to the soil to pupate (Worthley, 1924a; Salerno *et al.*, 2002). Although several larvae often enter the host's body, only one is able to complete its development (Shahjahan, 1968a).

Several studies were performed on the biological interactions between *T. pennipes* and adults of *N. viridula*, both in the laboratory (Giangiuliani and Farinelli, 1995; Pilkay *et al.*, 2014) and in the field (Salerno *et al.*, 2002; Tillman, 2008). However, studies on the interactions between this parasitoid and nymphs are often limited to the rate of parasitism in the field (Salerno *et al.*, 2002). The aim of this research was to investigate the effect that the parasitization of *T. pennipes* has on the five-instar nymphs of *N. viridula* in the laboratory.

Materials and methods

Rearing of *Nezara viridula*

The colony of *N. viridula* was started from eggs laid by individuals collected in the garden of the Department of Agricultural and Food Sciences (DISTAL) of the University of Bologna (44°30'51"N 11°24'19"E) and from Crevalcore (Bo) (44°43'21"N 11°09'32"E) from October 2015 and implemented by successive captures from the same localities. The rearing was conducted under controlled conditions (25 ± 1 °C, 70% RH and photoperiod 16:8 L:D), inside plastic boxes (13 × 36 × 24 cm) with a perforated top closed with a fine metallic mesh, to allow ventilation. The colony was reared with fresh vegetables: celery (*Apium graveolens* L.), soybean (*Glycine max* [L.] Merrill) and sunflower seeds (*Helianthus annuus* L.) (Bin *et al.*, 1993; Salerno *et al.*, 2006). Two times a week the colony was checked to change the vegetables, clean the box and collect the eggs laid, usually on sheets of paper inserted in the adult's box. The eggs collected were transferred to other boxes; the nymphs obtained from the eggs were maintained in the same conditions as the adults for all their development, the newly emerged adults were collected and put into new boxes for breeding.

Rearing of *Trichopoda pennipes*

The colony of *T. pennipes* was started from puparia that emerged from wild individuals of *N. viridula*, collected in the same areas described above, from November 2015. The adults were maintained in a Plexiglas cage (30 × 40 × 30 cm) under controlled conditions (25 ± 1 °C, 70% RH and photoperiod 16:8 L:D). They were fed with sugar cubes, cotton balls soaked in a solution of water and honey (~25%) and distilled water (Shahjahan, 1968b; Giangiuliani and Farinelli, 1995). Weekly, for the oviposition, about 40 adults of *N. viridula* were exposed to a parasitoid colony; the individuals were taken from the cage when they had at least 3-4 parasitoid eggs on their body (Shahjahan, 1968b), and were transferred into plastic boxes and maintained in the same rearing conditions as the *N. viridula* colonies. The fresh puparia obtained were collected and transferred into a new cage, on a Petri dish with a layer of wet vermiculite, in order to maintain the high humidity necessary for the puparia to be able to emerge as adults (Giangiuliani and Farinelli, 1995; Dindo and Grenier, 2014).

Nymph's exposure

In order to prepare the experiment, a preliminary test was performed. Twelve five-instar nymphs, of unknown age, were taken from the standard colony of *N. viridula* and were exposed to *T. pennipes*. They were removed after one hour, transferred in a box, reared and observed for one month.

The experiment was refined following the results obtained in the preliminary test and was performed under no-choice conditions. In order to obtain a high number of five-instar nymphs of the same age, 100 individuals of four instar nymphs were taken from the colony of *N. viridula* and checked daily. When the five-instar nymphs were found, they were randomly separated into two groups: the first group (n = 42; Exposed) was exposed to the parasitoids, the second group (n = 42; Not-Exposed) was maintained as control. The experiment began 1 day after the emergence of the five-instar nymphs, and one replicate was performed. The bugs were all placed inside the cage, as in the standard exposure, and removed after the parasitoids had laid at least 2-3 eggs on their bodies (Shahjahan, 1968b). The Exposed and Not-Exposed individuals were reared as in the standard conditions until the death of the nymphs or adults emergence. The new adults, of both experimental groups, were transferred to other boxes, divided according to the time of emergence and rearing. All samples were kept under observation for 40 days, more than the average time required for larvae of *T. pennipes* to develop inside adults of *N. viridula* in our standard colony, and were checked daily in order to detect dead bugs or parasitoid puparia.

All dead insects (nymphs or adults) were examined under a microscope and dissected, in order to evaluate the presence or absence of parasitoid larvae inside the host and its instar of development.

Anatomical examination

In order to evaluate the presence or absence of parasitoid larvae inside the host, its stage of development and the immune reaction, all dead insects (nymphs or adults) were preserved in a freezer (−18 °C) and subsequently examined anatomically. First, the specimen was dissected under saline (Ringer's solution) under a stereomicroscope; after that the soft tissue and the haemocyte capsules were dissolved with a saturated solution of potassium hydroxide (KOH) followed by washing with distilled water. The larvae found were mounted on a slide with Faure liquid and observed with a Carl Zeiss Axioskop light microscope equipped with AxioCam digital camera.

Parameters

The parameters considered for the nymphs were: 1a) mortality (%) = percentage of Exposed or Not-Exposed nymphs which died before the moult; 2a) time of development (in days) = time from when the five instar was found to adult; 3a) fate of the eggs (for Exposed only): penetrated (**P** - egg with entrance hole of the larva), died in penetration (**DP** - the larva pierced the host but died before penetrating), host reaction (**HR** - the larva pierced the host but died inside the egg due to the some kind of host reaction), not hatched (**NH**); 4a) condition of penetrated larvae (for Exposed only): encapsulated (**En** - death as a result of the host's immune reaction, *i.e.* a large number of haemocyte cover the larva), not encapsulated (**N-En** - death without suffering the host's immune reaction), no larvae (**NL** - no larva was found inside the nymph).

The parameters considered for the adults obtained were: 1b) mortality (%) = percentage of Exposed or Not-Exposed adults which died before 40 days of observation; 2b) longevity (in days) = adults longevity from the moult to the 40 days of observation; in this case, for the insects which were still alive at the end of the time of observation, the parameters were assessed at 40 days; 3b) presence of larvae and their instars (for Exposed only); 4b) condition of larvae (for Exposed only): encapsulated (**En** - death as a result of the host's immune reaction), not encapsulated (**N-En** - death without suffering the host's immune reaction), no larvae (**NL** - no larva was found inside the adult).

Also considered was the successful parasitization (%) = percentage of Exposed nymphs which produced at least one puparium. The result of parasitization and the parameters 1b and 2b were compared with same parameters obtained on *N. viridula* adults used to maintain the *T. pennipes* colony (n = 42) (Exposed adults).

The mortality and the rate of parasitism were analysed by 2 × 2 contingency tables using Yates correction for small numbers (<100); the times were analysed using Student's *t-test*. All statistical tests were done with STATISTICA software for Windows (StatSoft, Inc., Version 10. 2011).

Results

Preliminary test

In the preliminary test we considered only the conditions of the parasitoid larvae and the dissection was done the same day as the death. Of the twelve Exposed nymphs, only three died before the moult.

Figure 1. Ventral view of an exposed *N. viridula* nymph that dead during the ecdysis.

Two nymphs died during the ecdysis, while after the dissection we found respectively 2 L_1 alive N-En and 3 L_1 dead N-En. Of the nine adults obtained from the nymphs, two had no larva inside and three had respectively 1 L_2 dead N-En, 1 L_1 En + 2 L_2 dead N-En, 3 L_1 En + 1 L_2 En +1 L_2 alive N-En. Two stink bugs were alive at the end of the observation period; they were killed and, after the dissection, we do not found any larva inside. As a consequence of these preliminary tests, we decided to check also the fate of the eggs present on the nymphs that died before the moult and to compare the developmental data (duration, moult, survival) of Exposed nymphs with a control group.

Results for nymphs

Some of the Exposed nymphs died during the ecdysis, they appeared small and deformed (figure 1) and were considered as "dead before the moult". The mortality between the Exposed and the Not-Exposed was extremely significant (table 1). The individuals of the Exposed (n = 23) became adults after 7.04 ± 0.04 days, the Not-Exposed (n = 39) after 7.59 ± 0.21 days; the difference between these two groups was significant (t = 2.01; df = 60; $P = 0.05$).

On the 19 nymphs that died before the moult, 60 eggs were counted; most of which were laid on the venter of the thorax, as in adults (Shahjahan and Beardsley, 1975). The number of eggs, their fate and the condition of the larvae (when present) are reported in table 2.

Results for adults

After the moult, the adults obtained from Exposed lived on average 8.74 ± 1.69 days, whereas the adults from Not-Exposed lived 22.00 ± 2.11 days (t = 4.35; df = 60; $P = 0.0001$). The mortality between the Exposed and the Not-Exposed, recorded before the end of the observation period (40 days), was not significant (table 3). Comparing the results of adults obtained from the Exposed nymphs with those of the Exposed adults, in both cases all bugs died; but the Exposed adults lived on average 17.24 ± 1.31 days, with an extremely high statistical difference between the two groups (t = 3.91; df = 63; $P = 0.0002$). The number and the condition of the larvae (when present) are reported in table 2.

Results for parasitoid obtained

We obtained only four puparia from the Exposed nymphs, with a rate of 9.52% of successful parasitization; instead, the *N. viridula* Exposed adults showed a parasitism rate of 40.48%. The difference between these two groups was statistically significant (table 4).

The developmental time of the pupae of *T. pennipes* (from the eggs laid to pupae detection) into nymphs was 19.00 ±1.73 days, and in the adults 15.94 ± 0.52 days. The difference between these two times was statistically significant (t = 2.30; df = 19; $P = 0.033$).

Discussion

N. viridula is a worldwide pest of agricultural and horticultural crops (Todd, 1989; Panizzi *et al.*, 2000); it is highly polyphagous and feeds on more than 30 families of plants (Todd, 1989). Various researches have demonstrated the ability of *T. pennipes* to contain the adults of this pest (McPherson *et al.*, 1982, Jones, 1988;

Table 1. Number and percentage of mortality of *N. viridula* five instar nymphs Exposed or Not-Exposed to *T. pennipes* (df = 1).

	Exposed		Not-Exposed		χ^2	P
	n	%	n	%		
Alive (moult)	23	54.76	39	92.86	13.86	0.0002
Dead (not moult)	19	45.24	3	7.14		

Table 2. Results of microscope observations and dissections of *N. viridula* nymphs Exposed and *N. viridula* adults obtained from nymphs Exposed to *T. pennipes*. (a) days of life of the nymphs from exposure to death; (b) fate of eggs found on the nymph's body and, in parentheses, condition of larvae found inside the nymph's body; (c) days of life of the adults obtained from nymphs Exposed from moult to death; (d) condition of larvae found inside the adult's body.

Singular specimen	Days[(a)]	Status of eggs[(b)]	Singular specimen	Days[(c)]	Status of larvae[(d)]
Nymph	4	4 NH	Adult	3	1 L_2 N-En
Nymph	4	2 NH + 1 DP	Adult	3	1 L_2 N-En
Nymph	4	1 DP	Adult	3	1 L_2 N-En + 1 L_1 En
Nymph	4	3 DP	Adult	3	1 L_1 En
Nymph	5	1 NH	Adult	3	1 L_2 N-En
Nymph	5	1 NH+2 HR	Adult	3	2 L_1 En
Nymph	5	1 P (1 L_1 En)	Adult	4	1 L_1 En + 1 L_2 N-En
Nymph	7	1 NH +1 HR	Adult	4	NL
Nymph	7	1 HR	Adult	4	1 L_2 N-En
Nymph	7	1 NH +2 HR +1 DP	Adult	4	1 L_1 N-En + 1 L_2 N-En
Nymph	7	5 P (1 L_1 En)	Adult	5	1 L_1 En
Nymph	7	1 NH + 1 P (1 L_1 En)	Adult	8	NL
Nymph	7	1 P + 3 HR (NL)	Adult	8	1 L_3 N-En
Nymph	7	1 NH + 4 DP + 4 P (4 L_1 En)	Adult	8	1 L_3 N-En
Nymph	9	1 NH + 3 HR	Adult	9	1 L_2 N-En
Nymph	9	4 NH + 1 P + 1 HR (1 L_1 En)	Adult	9	1 L_3 N-En
Nymph	9	1 NH + 3 HR	Adult	10	1 L_2 N-En
Nymph	9	1 P (1 L_1 N-En)	Adult	10	NL
Nymph	9	3 NH	Adult	12	NL
			Adult	16	NL
			Adult	16	NL
			Adult	16	NL
			Adult	43	1 L_1 En

Condition of the eggs on nymphs body: penetrate (P - egg with entrance hole of the larva), died in penetration (DP - the larva pierced the host but died before penetrating), host reaction (HR - the larva pierced the host but died inside the egg due to the immune reaction), not hatched (NH). Condition of penetrated larvae: encapsulate (En - death as a result of the host's immune reaction, *i.e.* a large number of haemocyte cover the larva), not encapsulate (N-En - death without suffering the host's immune reaction), no larvae (NL - no larva was found inside the host).

Table 3. Number and percentage of mortality, recorded before the end of the observation period (40 days), of *N. viridula* adults obtained from five instar nymphs Exposed or Not-Exposed to *T. pennipes* (df = 1).

	Exposed		Not-Exposed		χ^2	*P*
	n	%	n	%		
Alive	1	4.35	7	17.95	1.33	0.249
Dead	22	95.65	32	82.05		

Table 4. Number of *T. pennipes* puparia and percentage parasitized of *N. viridula* adults obtained from five instar nymphs Exposed and adults of *N. viridula* Exposed to the parasitoid (df = 1).

	Nymphs		Adults		χ^2	df	*P*
	n	%	n	%			
Parasitized	4	9.52	11	40.48	9.143	1	0.0025
Not parasitized	38	90.48	25	59.52			

Giangiuliani and Farinelli, 1995; Pilkay *et al.*, 2014); our study showed the potentialities, in the laboratory, of this parasitoid against the nymphs.

The nymphs exposed to the parasitoids died earlier and more frequently compared to those not exposed; the first nymphs died when the parasitoid's larvae emerged from the eggs. Several larvae pierced the host's body, as normally occurs in nature (Shahjahan, 1968a.), but although sometimes we did not find a host reaction of bugs, these penetrations seem to have influenced the survival of many of these nymphs. All the parasitoid larvae found in the nymphs were located in the area of the wing muscles, as is normal in the adults of this species (Martini, unpublished data). It is interesting to note how some individuals of *N. viridula* died during the moult. The ecdysis was interrupted in different moments and the nymphs were more or less free of the old exuviae. We can suppose that, in those cases, the *T. pennipes* larvae caused also some hormonal imbalance in the bug. Therefore, the time of development of the exposed nymphs was shorter and they showed malformation after the moult. On the other hand, in *Galleria mellonella* (L.) (Lepidoptera Pyralidae) parasitized by the tachinid *Gonia cinerascens* Rondani, an increase of incomplete metamorphosis (% of pharate pupae) was related to superparasitism (Dindo, 1983).

All parasitoid larvae found in the nymphs were in L_1 instar (supplemental material figure S1), probably at that age the larva can pass from nymph to adult (Mellini, 1991). This could have influenced the development time of the larvae, which stay longer in the L_1 instar to make the passage to adulthood; as a consequence the developmental time in the Exposed nymphs was longer than the Exposed adult. The number of puparia obtained from the Exposed nymphs was very low, if compared to puparia obtained from Exposed adults; but this probably is due to the higher mortality found in Exposed nymph group.

Although the mortality of the adults is not statistically different, the adults obtained from Exposed nymphs died earlier than the adults obtained from Not-Exposed nymphs and also the Exposed adults. Most of *N. viridula* had at least one larva inside them (16 out of 23), in most cases they were not encapsulated. All the encapsulated larvae were at the first instar; this could be due to the greater susceptibility of this instar to the immune response of the host. In the adult hosts, the first instar larvae of *T. pennipes* try to avoid this immune response by hiding in wing muscles (Martini, unpublished data) and similar behaviour was also observed in some other Tachinid species (Mellini, 1991); however, in the nymphs these muscles are not completely developed. All larvae that reached the second and third instar died with their hosts. Their presence probably had a negative effect not only on the longevity of the insects, but also on their fitness. In fact, although our study did not focus on fertility, we observed that the adults obtained from Exposed nymphs did not lay any eggs; conversely, in the Exposed adults we found fertile couples at the end of experimentation.

In nature, the females of *T. pennipes* are attracted by the male aggregation pheromone of *N. viridula* (Mitchell and Mau, 1971; Harris and Todd, 1980; Aldrich *et al.*, 1987; 1993), and the same pheromone causes the aggregation of both the females and the five instar nymphs (Harris and Todd, 1981). Although the nymphal stage seems to be less preferred by *T. pennipes* in the field (Salerno *et al.*, 2002), studies performed on another Tachinid species, *Trichopoda giacomelli* (Blanchard), showed that the genus *Trichopoda* can have a great role in the natural control of *N. viridula* populations, also affecting fourth and fifth instar nymphs (Liljesthröm and Rabinovich, 2004). Our laboratory study showed a high potentiality of this parasitoid against the five nymphal instars of *N. viridula* and these potentialities could be improved, due to the tendency of aggregation of different susceptible stages of *N. viridula*. Some aspects observed during this study remain to be clarified, such as the possible effects on the endocrine system of nymphs and on the fertility of the adults. Aspects little known mainly because the interactions between host and parasitoid, especially in the Tachinidae, are not easily classified (Dindo, 2011). Further studies would be desirable to better understand the potential of *T. pennipes* to contain this or other species of pentatomid pests.

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