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Host range testing of the nearctic beneficial parasitoid *Neodryinus typhlocybae*

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Abstract *Neodryinus typhlocybae* (Ashmead) (Hymenoptera: Dryinidae) is a natural enemy of the planthopper *Metcalfa pruinosa* (Say) (Hemiptera: Flatidae), introduced from North America into Europe and regionally established as a pest species. Prior to possible utilization of the parasitoid as a biocontrol agent in Austria, its potential negative impacts on eight native plant- and leaf-hopper species were examined in the laboratory. Non-target species were selected according to the following criteria (a) occurrence in Austria, (b) close phylogenetic relationship with *M. pruinosa*, (c) larvae free-living and surface-dwelling, (d) phenology, (e) larval size, (f) ecological similarity with *M. pruinosa* and (g) availability of sufficient numbers of individuals. The Auchenorrhyncha species *Issus coleoptratus* (Fabricius), *Chloriona smaragdula* (Stål), *Conomelus anceps* (Germar), *Alebra wahlbergi* (Boheman), *Empoasca* sp., *Idiocerus stigmaticalis* (Lewis), *Macrosteles septemnotatus* (Fallén) and *Japananus hyalinus* (Osborn) were chosen for testing. Larvae from both the target and the non-target species were offered separately to *N. typhlocybae* females in no-choice laboratory tests and all attacks, instances of host feeding and parasitizations were recorded. No non-target species was attacked, fed upon or parasitized by *N. typhlocybae*, whereas *M. pruinosa* was attacked frequently. This study supports the assumption that the host range of *N. typhlocybae* is restricted to Flatidae, of which only the introduced species occurs in Austria. Direct negative effects on other Auchenorryncha species in Austria are therefore unlikely to occur.

Keywords Classical biological control · Direct non-target effect · Dryinidae · Flatidae · Host specificity · Introduced natural enemy · Parasitoid · Pre-release evaluation · Risk assessment

Introduction

The nearctic plant-hopper *Metcalfa pruinosa* (Say, 1830) (Hemiptera: Flatidae) was accidentally introduced into Italy in 1979 (Zangheri and Donadini 1980). Different natural enemies successfully suppress populations of *M. pruinosa* in its area of origin, but the absence of specialized enemies in Europe allows the built-up of high population densities. This univoltine planthopper is generally introduced at the egg stage through plant imports. The current distribution of *M. pruinosa* comprises 15 European

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countries: Italy, France (Della Giustina 1986), Slovenia (Sivic 1991), Switzerland (Jermini et al. 1995), Croatia (Maceljski et al. 1995), Spain (Pons et al. 2002), Austria (Holzinger et al. 1996; Kahrer and Moosbeckhofer 2003), Serbia, Montenegro (Hrnčić 2003), Greece (Drosopoulos et al. 2004), Hungary (Orosz and Dér 2004), Turkey (Karsavuran and Güçlü 2004), Bulgaria (Tomov et al. 2006), the Netherlands (A. Sala, personal communication) and Bosnia-Herzegovina (Gotlin Čuljak et al. 2007). Introduced populations of *M. pruinosa* were eradicated in Great Britain and in the Czech Republic by insecticide applications (Malumphy et al. 1994; C. Malumphy and P. Lauterer, personal communication).

M. pruinosa is a polyphagous and gregarious species (Duso and Pavan 1987), that can feed on a wide variety of trees, shrubs and herbs (Bagnoli and Lucchi 2000). Mass occurrence of *M. pruinosa* can cause direct and indirect damage in orchards and vineyards (Stefanelli et al. 1994). Honeydew secretions are often colonized by black sooty mold resulting in a reduced fruit quality. Furthermore, single individuals of *M. pruinosa* were found to be infected with various phytoplasma groups, such as apple proliferation phytoplasma and stolbur phytoplasma, both posted on the European Plant Protection Organization (EPPO) A2 list of pests recommended for regulation and quarantine (Danielli et al. 1996; EPPO 2007). So far, transmission of phytopathogens by *M. pruinosa* has not been verified (Bressan et al. 2006). Chemical treatments and biological control have been used to combate this pest (Girolami and Conte 1999). *Neodryinus typhlocybae* (Ashmead 1893) (Hymenoptera: Dryinidae) is one natural enemy of *M. pruinosa* in North America (Dean and Bailey 1961; Wilson and McPherson 1981). Females parasitize and prey (host feeding) effectively on immature stages of the pest, and its larva develops as a koinobiont ectophagous parasitoid, killing *M. pruinosa* either in the third, fourth or fifth stage (Olmi 2000; A. Sala, personal communication). *N. typhlocybae* can reproduce either sexually or by facultative arrhenotokous parthenogenesis. Other hosts of *N. typhlocybae* in North America are *M. regularis* (Fowler), *Anormenis chloris* (Melichar) and *Anormenis septentrionalis* (Spinola) (Guglielmino and Olmi 1997), all flatid species not recorded in Europe (Fauna Europea 2004). The parasitoid has been released repeatedly for classical biological control of *M. pruinosa* and is successfully established in Italy, France, Slovenia, Switzerland and Croatia (Tommasini et al. 1998; Ciglar et al. 1998; Malausa 1999; Jermini et al. 2000; Žežlina et al. 2001). In 2007, *N. typhlocybae* has been released for the first time in Greece, the Netherlands and Spain (A. Sala, personal communication).

The first mass occurrence of *M. pruinosa* in Austria was detected in 2003 in a public park adjacent to a nursery in Vienna and subsequently led to the infestation of several sites by 2006. It is assumed that spreading will continue, facilitated by its polyphagy and by human activities and that adjacent important wine- and fruit-growing areas could be at risk. As the release of *N. typhlocybae* is being considered in Austria, and in view of the fact that no extensive parasitoid host range tests have yet been published, despite its release in eight European countries, the objective of the present study is to obtain information about the risk to indigenous non-target planthopper and leafhopper species. Results of the study will constitute a key factor in a risk analysis to be performed before this exotic beneficial arthropod can be safely utilized as a biological control agent in Austria.

Material and methods

Selection of non-target species

The non-target species for host range testing of *N. typhlocybae* were selected according to the following criteria: (a) occurrence in Austria, (b) close phylogenetic relationship with *M. pruinosa*, (c) larvae free-living and surface-dwelling, (d) phenology (phenological overlap of Auchenorrhyncha larvae and the potential parasitoid), (e) larval size, (f) ecological similarity to *M. pruinosa* (occurrence in deciduous forest) and (g) availability of sufficient numbers of individuals (Table 1).

Close phylogenetic relationship of non-target species to *M. pruinosa* was the basis for their selection, in accordance with Wapshere's centrifugal phylogenetic method (1974). As *M. pruinosa* is the only species of Flatidae in Austria, other families were considered according to their descending degree of relationship with Flatidae, based on the cladogram of Hemiptera after Bourgoin and Campbell (2002). The nearest related family is Tropiduchidae Stål, represented in Austria by only one rare species

Table 1 Auchenorrhyncha families occurring in Austria and their correspondence with the selection criteria for host range testing of *N. typhlocybae*

Infra order	Family	Phyl. rel. to Flatidae[a]	Larval habits[b]	Phenology	Larval size	Ecological similarity[c]	Availability[d]
Fulgoromorpha	Tettigometridae	?	−				
	Tropiduchidae	++	+	+	−	−	−
	Caliscelidae	+	+	+	−	−	?
	Issidae[e]	+	+	+	+	+	−/+
	Dictyopharidae	−	+	−/+	+	−	+
	Achilidae	−	−				
	Delphacidae[e]	−	+	+	+	−/+	−/+
	Cixiidae	−	−				
Cicadomorpha	Cicadidae	−	−				
	Cercopidae	−	−				
	Aphrophoridae	−	−				
	Membracidae	−	+	−/+	+	+	+
	Cicadellidae[e]	−	+	+	+	+	−/+

Species without the essential criterion of surface-dwelling and free-living larvae were not treated in the further selection procedure

[a] Phylogenetic relationship to Flatidae

[b] Surface-dwelling and free-living larvae

[c] Ecological similarity with *M. pruinosa*

[d] Availability of sufficient numbers of larvae

[e] Families from which species were tested in bold

? Status not clarified

++ Nearest related family to Flatidae

+ Criterion applies

− Criterion does not apply

−/+ Criterion does not apply to all species of the family

Trypetimorpha occidentalis (Huang and Bourgoin 1993). It had been planned to test this species even though it lives in a completely different habitat (dry grassland) but no larvae of *T. occidentalis* were found in the field. Therefore, it was removed from the initial test species list. The common species *Issus coleoptratus* (Fabricius 1781) of the family Issidae Spinola was tested, which occurs in deciduous forests. Two species from the family Delphacidae Leach, *Chloriona smaragdula* (Stål 1853) and *Conomelus anceps* (Germar 1821) were selected, despite that their host plants are completely different from those of the target species.

As close taxonomic relationship may not necessarily be a reliable enough criterion for host suitability, test species with ecological similarities to the target species were also selected (Babendreier et al. 2003; van Lenteren et al. 2006). The following species from Cicadellidae Latreille live in similar habitats as *M. pruinosa*: *Alebra wahlbergi* (Boheman 1845), *Idiocerus stigmaticalis* (Lewis 1834), *Macrosteles septemnotatus* (Fallén 1806), *Empoasca* sp. and *Japananus hyalinus* (Osborn 1900). The latter two species were also chosen with regard to possible positive non-target effects, as related species of both subfamilies (Typhlocybinae; Deltocephalinae) are known to damage economically important plants.

The larvae of the following families are not suitable test species because they develop endogenically or well-protected in spittle masses and are therefore not accessible to *N. typhlocybae*: Cercopidae Leach, Cicadidae Latreille, Cixiidae Spinola, Achilidae Stål, Tettigometridae Germar and Aphrophoridae Amyot and Serville. The larvae of Caliscelidae Amyot and Serville and Dictyopharidae Spinola live in habitats unsuitable for *M. pruinosa,*

which makes it unlikely that the parasitoid will come into contact with them.

Breeding of *Metcalfa pruinosa*, *Neodryinus typhlocybae* and non-target species

Various larval stages of *M. pruinosa* were collected from the first site of mass occurrence in Vienna with an aspirator from the end of May until the end of July (2005 and 2006) and at the beginning of September (2004). The larvae were kept on living plants (*Parthenocissus quinquefolia* (L.) Planchon, Vitaceae) for tests in an environmental chamber at 20°C, 65% relative humidity and 16:8 L:D photoperiod.

Specimens of *N. typhlocybae* used in this study were provided for three years by the firm Bioplanet s.c.a., Martorano di Cesena (FC), Italy. In 2004 females from both the overwintering (mid-June) and the first generation (July) were used for the host range tests. In 2005 and 2006 only the overwintering generation was used because a considerable part of the first generation went directly into diapause. Breeding took place in an environmental chamber at 25°C, 65% relative humidity, 18:6 L:D photoperiod. In 2004 cocoons containing pupae of females and males were kept in two different boxes (20 × 10 × 8 cm) until the adults emerged. All newly hatched wasps were then placed in a vented cylindrical box (height = 20 cm, diameter = 10 cm) with maple twigs (*Acer campestre* L., Aceraceae) for four days to allow for copulation. Subsequently, the females were placed singly in cylinders (height = 6 cm, diameter = 5 cm) marked with the date and an individual number. In 2005 and 2006 a different procedure was used as it was observed that males of *N. typhlocybae* usually emerged prior to females, indicating protandry. Both female and male cocoons were therefore placed in a box (60 × 40 × 52 cm) with maple twigs so that males had the opportunity to copulate with newly hatched females. After four days each female was removed singly to cylinders as described above. These cylinders were maintained at 20°C, 65% relative humidity, 18:6 L:D photoperiod. The wasps were provided with water (in wet cotton pads) and sugar-yeast pastry. Because *N. typhlocybae* is a larval parasitoid, only larvae of the selected test species were collected in Vienna and Lower Austria, together with their host plants (Table 2). For the taxonomic identification of the test species (Ossiannilsson 1981; Holzinger et al. 2003; Biedermann and Niedringhaus 2004) larvae were reared to the adult stage on host plants after the experiments, either in an environmental chamber at 25°C, 65% relative humidity, 18:6 L:D photoperiod (*A. wahlbergi, C. anceps, Empoasca* sp., *M. septemnotatus, J. hyalinus*) or in a branch cage in the field (*C. smaragdula*, *I. stigmaticalis*, *I. colepotratus)*.

Host range testing

Host range of *N. typhlocybae* was investigated from 2004 to 2006, following the guideline proposed by van Lenteren et al. (2003). The no-choice assay method was used to reveal if *N. typhlocybae* would attack a species not normally targeted. The non-target species were offered separately to *N. typhlocybae* females in no-choice tests in a small arena (Petri dishes, height = 2.9 cm, diameter = 8.5 cm) during the daytime and under controlled laboratory conditions. Leaves of their respective host plants were present in the Petri dishes. First, each non-target species was exposed to one unexperienced *N. typhlocybae* female with ten repetitions (negative control). To check the readiness for host feeding and oviposition, the *N. typhlocybae* females that had been previously tested in negative controls were then put together with *M. pruinosa* (positive control). In the positive control different larval stages of *M. pruinosa* were used depending on its phenology: L1 in May, L1, L2, L3, L4 in June, L3, L4, L5 in July and September. Different Petri dishes were used for negative and positive controls. Petri dishes were cleaned with water and alcohol after each experiment to ensure that no wax filaments or scent chemicals were present that could influence the parasitoids' behavior. New larvae and *N. typhlocybae* females were used for each experiment to avoid learning behavior. Depending on the abundance of the test species different numbers of larvae were used for the negative controls and corresponding numbers of *M. pruinosa* larvae for the positive controls (Table 2). The females were observed continuously for 1 h. The foraging behavior of *N. typhlocybae* consists of stereotypical behavioral sequences (searching, encounter, antennal examination, attack, host feeding, oviposition), similar to those described for *Anagyrus* sp. nov. nr. *sinope* (Noyes and Menezes) and *Leptomastix dactylopii* (Howard) (Chong

Table 2 Non-target Auchenorrhyncha species, their host plants and the numbers and larval stages used in the host range experiments with *N. typhlocybae*

Infra order	Taxa	Host plant	Larvae	
			Number	Stage
Fulgoromorpha	*Chlorions smaragdula*	*Phragmites australis*	20	L3, L4[a]
	Conomelus anceps	*Juncus* sp.	20	L4, L5
	Issus coleoptratus	*Hedera helix*	30	L3, L5
Cicadomorpha	*Alebra wahlbergi*	*Acer campestre*	40	L4, L5
	Empoasca sp.	*Sambucus nigra*	40	L4, L5
	Idiocerus stigmaticalis	*Salix alba*	40	L3, L4
	Macrosteles septemnotatus	*Filipendula ulmaria*	20	L5
	Japananus hyalinus	*Acer campestre*	40	L3, L4

[a] Both instars from the second generation

and Oetting 2007). Antennal examination of the larvae and the absolute number of attacks, instances of host feeding and parasitization were recorded. The behavior patterns were defined as follows: (i) attack: *N. typhlocybae* attempts to grab a larva with the forelegs (This attack can be either successful or not), (ii) host feeding: *N. typhlocybae* feeds on a larva; an injury is clearly visible and the larva does not survive, (iii) parasitization: *N. typhlocybae* inserts the tip of the abdomen in a larva, no feeding occurs and the larva survives. To test difference in host feeding and parasitization among the five larval stages of *M. pruinosa*, non-parametric statistics and a Mann–Whitney *U*-test were applied (significance level $P \leq 0.05$; SPSS 15.0, Chicago, Illinois, 2006).

Results

The results of the host range tests clearly indicate that none of the eight tested non-target species was attacked, parasitized or host fed by *N. typhlocybae*. Aggressive behavior of the dryinid wasp towards test larvae occurred in none of the 80 negative controls. *N. typhlocybae* examined all test species except *Empoasca* sp. In the positive controls with *M. pruinosa* 38% of the 250 offered larvae were attacked, 20.8% were parasitized and 12% were eaten (Table 3). About 88.8% of the tested *N. typhlocybae* females attacked *M. pruinosa*. The majority of the females (63.5%) attacked only once, 16.5% twice and 3.5% three times in the 1-h observation period. The maximum number of attacks per hour was four (1.2%). Nearly all of the females (86.2%) succeeded in overwhelming the larvae, leading to host feeding or parasitization. However, 13 attacks were not successful, due either to the wasp failing to catch its victim or to the almost equally large L5 being able to escape the wasp' clasp. The first instar of *M. pruinosa* ($n = 21$) was not eaten by *N. typhlocybae,* whereas the second and third larval stages were fed upon ($P < 0.0001$). The number of host feeding incidences among the second and third instars is not significantly different ($P = 0.799$). The average (±SE) larval stage fed upon by one female was 2.5 ± 0.5 ($n = 20$). Host feeding lasted for 4.3 ± 1.2 min on average and ranged between 2 and 6 min ($n = 23$). All females except one preyed only once during the experiment. *N. typhlocybae* used the third, fourth and fifth instars of *M. pruinosa* for oviposition. The fourth larval stage was significantly more often parasitized ($P = 0.015$) than the third and highly significantly more often parasitized ($P < 0.0001$) than the fifth larval stage. On average (±SE), one female parasitized the 3.8 ± 0.7 larval stage ($n = 38$). The mean oviposition process lasted for 4 ± 2.3 min ($n = 44$) with a minimum of 1 min and a maximum of 12 min. Parasitization occurred from 1 min after the trial's start to 1 min before its end. The maximum number of parasitizations was two ($n = 59$), but only 5% of the females ($n = 80$) parasitized twice in 1 h.

Discussion

Host range

There is an increasing consensus within the EU that assessment of both the host range of candidate invertebrate biological control agents (IBCA) and

Table 3 Total numbers of attacks, host feeding incidences and parasitizations of *N. typhlocybae* ($n = 80$) on *M. pruinosa* larvae ($n = 250$) and non-target plant- and leaf-hopper larvae ($n = 250$)

Behavioral elements			χ^2, *P*-value
N. typhlocybae	*M. pruinosa*	Non-target species	
Attacks	95	0	$\chi^2 = 108.75$, df = 4, $P < 0.0001$
Host feeding	30	0	$\chi^2 = 48.77$, df = 2, $P < 0.0001$
Parasitization	52	0	$\chi^2 = 31.6$, df = 2, $P < 0.0001$

environmental risks are required (Van Driesche et al. 2004). A growing number of host range tests for IBCA are conducted prior release but this was not the case for *N. typhlocybae*, which was successfully released to control *M. pruinosa* in some European countries. *N. typhlocybae* is on the list of biological control agents widely used in the EPPO region (EPPO 2002). Therefore, an investigation on the host range of *N. typhlocybae* was deemed to be the most important risk factor in an environmental risk assessment that will be performed in accordance with the guidelines proposed by van Lenteren et al. (2006), the EPPO Standard PM 6/2 (1) (2000) and Babendreier et al. (2005). The EPPO will be revising the "Positive List" of biological agents in the near future, and the results of this study could be used in the reviewing process.

The results presented above support the assumption that the host range of *N. typhlocybae* is restricted to the family Flatidae, as reported by Guglielmino and Olmi (1997). In the present study the parasitoid exclusively attacked, parasitized or preyed on *M. pruinosa* larvae. It may be assumed that in Austria *N. typhlocybae* will attack only *M. pruinosa*, as no other flatid species occurs. Since direct negative effects on non-target species are very unlikely to occur in the field, it can be concluded that no unacceptable risks for non-target species would arise from the release of the parasitoid. This conclusion can be applied to other countries only if *M. pruinosa* is the single flatid species present. As suggested in Kuhlmann et al. (2006), test lists should be revised if new information exists. Therefore, countries with native flatid species that wish to release *N. typhlocybae* should include them in host range testing. With respect to environmental conservation it is deemed necessary to subject the rare autochtonous flatid species *Phantia subquadrata* (Herrich-Schäffer 1838) to a host range test with *N. typhlocybae*. This species occurs in Greece and Italy, where *N. typhlocybae* has already been released (Girolami and Conte 1999), as well as in some other south-eastern European countries (Bulgaria, Montenegro, Serbia, Turkey; W. Holzinger, personal communication), where *M. pruinosa* is present, and *N. typhlocybae* might be released for biocontrol. The behavior of *N. typhlocybae* to this closely related species of the target would be valuable information for the overall risk assessment. As the host range of *N. typhlocybae* in the field in release areas has not yet been verified and in order to ensure that *N. typhlocybae* poses no threat to native flatid species, its host range should be examined in post-release monitoring similar to that used in the survey of Morrison and Porter (2006). M. Olmi (personal communication) tested *N. typhlocybae* with another flatid species, *Cyphopterum adscendens* (Herrich-Schäffer 1835). This flatid species was not attacked by the parasitoid.

In contrast to observations by Gervasini (2001), first instar larvae of *M. pruinosa* were not attacked by *N. typhlocybae* in the present study. Second and third instar larvae were preferred for host feeding over the first larval stage. A comparison of the seasonal occurrence of *M. pruinosa* larvae and *N. typhlocybae* adults shows that at the main eclosion time of the overwintering generation of *N. typhlocybae* (mid-June) only few *M. pruinosa* first instar larvae are still present and that second instars greatly prevail (Lucchi and Santini 1993; Girolami and Mazzon 2001). Thus, the female parasitoids of this generation hatch at a time when their host is available as more suitably sized prey.

Although *N. typhlocybae* did not attack non-target host species larvae for food or parasitization, it showed a chasing behavior in the negative controls with *I. coleoptratus* larvae, which was otherwise observed only with *M. pruinosa* larvae.

N. typhlocybae females ran after the larvae, especially in the experiments with younger *I. coleoptratus* larvae (L3). This particular behavior of *N. typhlocybae* towards larvae of a European issid species is of special interest. In its native North America *M. pruinosa* is frequently found in mixed species feeding assemblages with one issid species, *Acanalonia conica* (Say 1830) and two flatid species, *Ormenoides venusta* (Melichar 1902) and *Anormenis chloris* (Melichar 1902), whereby only the latter is also a host of *N. typhlocybae* (Wilson and McPherson 1980). *N. typhlocybae* may associate the occurrence of Issidae with that of host species and therefore as an indicator of prey/host microhabitat. This would be an example that cues derived from organisms interacting with the host in the feeding niche play an important role for the parasitoid in the host searching phase, as mentioned by Kuhlmann et al. (2006). It would be interesting to examine the behavior of *N. typhlocybae* towards its two non-host species, *A. conica* and *O. venusta*, and to determine if olfactory cues coming from them affect the orientation of *N. typhlocybae*.

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Gudrun Strauss is a research entomologist at the Plant Health Institute at the Austrian Agency for Health and Food Safety in Austria. The starting point for the research about the host range of *Neodryinus typhlocybae* was a faunistic research project about *Metcalfa pruinosa*. In the course of conducting a pest risk assessment for this invasive plant-hopper species, it was noticed that a general environmental risk assessment for its exotic biocontrol agent, *N. typhlocybae*, would be necessary. Currently she is involved in conducting a risk assessment for an invasive plant species. She uses computer modelling to predict regional establishment and spread of new species, based on the specific climatic requirements.