LUCREZIA GIOVANNINI[a*] - GIUSEPPE MAZZA[a] - GIUSEPPINO SABBATINI PEVERIERI[a] - IMMACOLATA IOVINELLA[a] - BRYAN NAQQI MANCO[b] - DODLY PROSPER[b] - JUNEL BLAISE[b] - CHRISTOPHER MAY[b] - NATALIA VANDENBERG[c] - LEONARDO MARIANELLI[a] - PIO FEDERICO ROVERSI[a]

CLASSICAL BIOLOGICAL CONTROL OF *TOUMEYELLA PARVICORNIS*: CHALLENGES AND OPPORTUNITIES FOR A POTENTIAL CANDIDATE

[a]*CREA–Research Centre for Plant Protection and Certification, I-50125 Florence, Italy*
[b]*Department of Environmental and Coastal Resources, National Environmental Centre, Providenciales, Turks and Caicos Islands*
[c]*Systematic Entomology Laboratory, ARS, USDA c/o National Museum of Natural History, Smithsonian Institution, Washington, DC 20560, USA*
[*]Corresponding author: lucrezia.giovannini@crea.gov.it

Giovannini L., Mazza G., Sabbatini Peverieri G., Iovinella I., Manco B.N., Prosper D., Blaise J., May C., Vandenberg N., Marianelli L., Roversi P.F. – Classical biological control of *Toumeyella parvicornis*: challenges and opportunities for a potential candidate.

Toumeyella parvicornis, native to the Nearctic regions, represents an increasing threat to pine forests worldwide. Severe infestations of this invasive scale have long been present in the Caribbean Islands of Turks and Caicos (TCI), and recently in Europe (Italy and France). Classical biological control could represent the most promising method for the long-term management of *To. parvicornis* in newly invaded areas. This study, based on an entomological expedition to Turks and Caicos Islands, allowed the discovery and selection of the coccinellid predator *Thalassa montezumae* as a potential biological control agent. This predator was never recorded before on TCI and was probably introduced from the area of origin of *To. parvicornis*. Here, the rate of infestation was evaluated and the pest suppression impact of *Th. montezumae* was investigated, both in the field and under laboratory conditions. Given the promising results of field studies and predation tests, the predator was imported into Italy, under quarantine conditions, to carry out a preliminary risk assessment to evaluate its potential impact on non-target species. Predation tests were conducted on different developmental stages of various scales and other Hemiptera (e.g., Aphididae, Aleyrodidae) common in Europe. The results reveal that *Th. montezumae* predates only scales, mainly at juvenile stages, but with a rate of predation significantly higher for *To. parvicornis*. Further studies are needed to mitigate the risk of underestimating environmental impacts in the use of *Th. montezumae* as a biological control agent for *To. parvicornis*.

Key Words: Coccinellidae; pine tortoise scale; predator; risk assessment; *Thalassa montezumae*

INTRODUCTION

The pine tortoise scale *Toumeyella parvicornis* (Cockerell) (Hemiptera: Coccidae) is a scale insect noxious to pines in the Nearctic region, mainly in the central and eastern USA, but it has also been reported from eastern and central Canada, as well as Mexico (Dueñas-López, 2022). In the native range, infestations of this scale generally occur on young trees where the most common impact consists of reduced growth as a result of insect feeding and reduced photosynthetic activity due to sooty mold on the needles. Seedlings and saplings suffer the greatest damages, and in the worst-case scenario, the infestation is followed by plant death (Clarke, 2013). In the northern part of its natural range*, To. parvicornis* has one generation per year, but the number of generations increases in warmer climates (Clarke, 2013).

In its native range, a variety of natural enemies (predators and parasitoids) have been documented for *To. parvicornis*, including mainly lady beetles. Moreover, larvae of entomophagous Lepidoptera and Diptera, and hymenopteran parasitoid species belonging to the families Encyrtidae and Aphelinidae were reported. Such natural enemies usually maintain *To. parvicornis* populations at low levels, but scanty data are available in the native range. Therefore, this scale is generally considered a secondary pest that flares up occasionally due to the disruption of natural enemies through insecticide use or climatic conditions (Clarke, 2013).

Scale insects are one of the most commonly transported insects in the plant trade and one of the most successful invasive groups of insects (Inghilesi *et al.*, 2013). *Toumeyella parvicornis* represents a recent example having been found for the first time outside its native range, in the Caribbean Islands of Turks and Caicos (TCI) in 2005 and in Puerto Rico in 2009 (Hamilton, 2006; Segarra-Carmona and Cabrera-Asencio, 2010). In Europe, the first finding of this scale insect occurred in Italy in 2014, and it is now present in several sites in the central and southern regions of the country (Garonna *et al.*, 2015; SFR, 2023). More recently it was found in France (EPPO, 2021).

Favorable climatic conditions in the TCI permit *To. parvicornis* to complete multiple generations per year, and the absence of local natural enemies has contributed

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to an impressive population growth (Malumphy *et al.*, 2012), as was also observed in Italy (Garonna *et al*., 2018). In the TCI, where the accidental introduction of this pest was probably due to the importation of cut pine trees as Christmas trees from the USA (Malumphy *et al*., 2012), *To. parvicornis* has led to near extinction of the endemic Caribbean pine *Pinus caribaea* var. *bahamensis* (the unique native pine tree in the Lucayan Archipelago), with a decline of 98% of the pine trees (Malumphy *et al*., 2012; Sanchez *et al*., 2019). In response to the alarming decline of TCIs' wild Caicos pine populations, a team of local and international scientists has developed the Caicos Pine Recovery Project (CPRP) to rescue this pine species from local extinction (Hamilton *et al*., 2006; Sanchez, 2019).

In Italy, *To. parvicornis* has spread rapidly in several locations (EPPO, 2022), and phytosanitary measures were promptly applied to contain or eradicate local infestations (MIPAAF, 2021). In the Italian-invaded areas, the main damage occurred to stone pines (or umbrella pine) *Pinus pinea,* mainly in urban environments, but *P. nigra* and *P. pinaster* are also attacked and threatened by this pest (Garonna *et al.*, 2018). Italy has more than 620,000 hectares of pine forests (Gasparini *et al*., 2022) that are potentially threatened by *To. parvicornis.* For this reason, management programs for this pest are urgently needed. In the area of origin, limited and localized infestations are sometimes treated with aerial applications of insecticides, but treatments are often detrimental to natural enemies (Sheffer and Williams, 1987). In the Italian-invaded areas, endotherapic treatments are applied to single plants in urban areas and seem to be effective against this pest (Bertin *et al*., 2022; Di Sora *et al*., 2022), but this approach is tricky in forest ecosystems due to the cost, constraints in the technical application of the method and, above all, the potential side effects on the ecosystem. Thus, biological control is considered a promising method for long-term management of this pest in newly invaded areas. In Italy, the generalist parasitoid *Metaphycus flavus* Howard (Hymenoptera, Encyrtidae) will exploit *To. parvicornis*, but is unable to successfully control it (Garonna *et al*., 2018).

The current work focuses on an entomological survey conducted on known sites where *To. parvicornis* occurs in high densities in forest stands. The goal was to assess the impact of the scale pest and to search for natural enemies of potential use in a biological control program. Candidate species collected during the survey were then tested under laboratory conditions to determine their response both to the scale pest and to non-target species (preliminary risk analyses). The results obtained in this work could play a pivotal role in providing an effective tool for the long-term management of *To. parvicornis* in the native and invaded areas.

MATERIAL AND METHOD

The present study was performed in a three step workplan: 1) conducting an entomological survey in known infested areas to collect field data about *To. parvicornis* and detect potential natural enemies to be evaluated as biological control agent (BCA) candidates; 2) performing laboratory tests *in situ* with potential BCAs recovered during the surveys; 3) importing selected potential BCAs into Italy, to perform for preliminary risk assessment under laboratory quarantine conditions.

Turks and Caicos Islands infested areas as study sites and field investigation

Turks and Caicos Islands (TCI), an overseas territory of the United Kingdom in the Caribbean, were selected as study sites due to the severe *To. parvicornis* infestations in their pine forests over the last two decades. The Caribbean pine is the only native *Pinus* species present in the TCI and Bahaman archipelago. In TCI the Caicos Pine occurs exclusively on the islands of Pine Cay, Middle Caicos, and North Caicos, where it is the key species of the pineyard ecosystem (Hamilton, 2006; Sanchez *et al.*, 2014; Sanchez *et al*., 2019). Recently, frequent signs of one or more unidentified natural enemies of *To. parvicornis* (either parasites or predators) have been reported at these sites (authors pers. obs.). The collecting expedition to TCI was conducted by personnel of CREA (Research Centre for Plant Protection and Certification) in May 2023 thanks to a scientific research permit of the local authorities (Department of Environment and Coastal Resources 2023-02-22-16) and within the Italian national research program on *To. parvicornis* control strategies (MASAF, DM 0023205_17/01/2023). Based on information from previous studies (Hamilton, 2006; Malumphy *et al*., 2012), the state of *To. parvicornis* infestations in TCI was investigated, and the presence of natural enemies in pine tree forests was explored. To investigate *To. parvicornis* populations and the presence of potential natural enemies, 20 pine trees were randomly chosen from each pine yard of the three islands (North Caicos NC: 21°54'40"N 72°00'06"W; Middle Caicos MC: 21°48'56"N 71°47'19"W; Pine Cay PC: 21°52'42"N 72°05'25"W; the coordinates refer to the central point of each pine forest investigated). Contrary to most endemic populations, where the bark-infesting form of *To. parvicornis* is the dominant morph, on TCI it is exclusively present under the smaller, more slender needle-infesting form (Clarke, 2013). Thus, from each of the 20 pine trees, 36 needles from the canopy were randomly handpicked (n=720 needles per island), and all insects associated with *To. parvicornis* were collected. The collection of pine needles and insects was repeated on three different days (a day per island), at the same time of the day, and with the same weather conditions. Moreover, in addition to the 2.160 needles collected for the evaluation of the rate of infestation and predation, about 5.000 infested needles were collected during the entire survey for parasitoid emergence. All the material was labeled and transferred to the laboratory for further investigations.

Laboratory studies at the Turks and Caicos Islands

A rough-and-ready field laboratory was established in a facility room on Kew settlement on North Caicos Island. For each of the pine needles collected during the field surveys on the three different islands (see above), the number of *To. parvicornis* were counted and their developmental stage (adults *vs.* juveniles) noted along with any signs of parasitism or predation. After that, needles infested with *To. parvicornis* were put inside insect cages (Vermandel®, Kweekkooi 30x30x30cm) separated according to the three different study sites and maintained under local environmental conditions (25.9 °C and RH 80%; 10:14 LD) to detect emerging parasitoids or other natural enemies.

Insects collected in the field during the survey were separated as predators or others, and the predators (both adult and juvenile stages) were reared at environmental conditions (as described above) for successive identification and further studies. Among them, only predators of scales (coccids and mealybugs) were selected, resulting in two Coccinellidae subsequently identified as *Thalassa montezumae* Mulsant and *Cycloneda sanguinea* (Linnaeus) (see details on species identification in the Results), and both species were tested to assess feeding ability under laboratory conditions.

Predation tests were performed under laboratory conditions (as described above) to evaluate the predatory capability of the two coccinellid species against the prey *To. parvicornis*. The sexes of the adult beetles were determined using differences in the shape of the abdominal ventrites and color pattern dimorphism of the head and pronotum.

Individuals of both predator species were singularly isolated in plastic Petri dishes (90 mm of diameter) and starved for 24h, as performed in Mazza *et al.* (2021). In total, 35 individuals of Coccinellidae were tested: 20 (n=10 females, n=10 males) for *Th. montezumae* and 15 individuals for *C. sanguinea* (n=8 females, n=7 males). Afterward, a pine needle infested by 10 *To. parvicornis* adult females was offered to each single predator to evaluate the extent of their predatory response. After 24h, each needle was checked, and the number of scales predated was counted to define the predation rate for each species of Coccinellidae. The same procedure was performed with the immature stages of both coccinellid species. In this case, 10 larvae of *Th. montezumae* and only three of *C. sanguinea* were tested. No other juvenile specimens of *C. sanguinea* were found in the field during the survey (see Results). Coccinellid species were identified using the keys to the North and South American coccinellid fauna as well as digital images from internet resources such as iNaturalist.org and BugGuide.Net (Gordon, 1985; Milleo *et al.,* 2004; Gordon and Canepari, 2008).

Laboratory studies in Italy

Based on the rate of predation of *To. parvicornis* observed in the field on all the pine yards investigated on Turks and Caicos Islands, the population level of the detected natural enemies, and the results obtained from predatory tests (see Results), *Th. montezumae* was selected for importation into Italy. Approximately 200 adult-live specimens (males and females in equal numbers) of *Th. montezumae* (maximum number of individuals allowed to be exported) were transferred to Italy under quarantine conditions in the laboratories of CREA. Imported adults were naïve specimens obtained from pupae collected in TCI. Export from the TCI and import to Italy was performed under the authorization of DECR (n. TCI 2022 55-56-57, 17-May-2023) and the Italian National Phytosanitary Organization (n. IT-0255655/2023 01/01, 17-May-2023).

Since no information about genus and species assignment, survival rate, longevity, and rearing method was available at the time of field collection at TCI and in the first part of laboratory studies, the lady beetles were placed into insect cages (Vermandel®, Kweekkooi 45x45x60cm) and reared on a mixed diet based on different species of scales, aphids, and honeydew *ad libitum*.

The individuals of *Th. montezumae* were promptly used to perform predation tests, like those performed in the TCI where the target species *To. parvicornis,* collected in infested sites of Italy, and a pool of non-target species belonging to the local Hemiptera fauna, were tested as potential prey. These species are commonly known in the same environment as *To. parvicornis*. In addition to *To. parvicornis* collected on *P. pinea*, 12 Hemiptera species were tested as prey (see Results for the list of the species, Fig. II). Details on species identification through morphological identification and DNA sequencing of the 5' region of mitochondrial cytochrome oxidase I gene, are provided in Supplementary Materials (SM1). Where available, both adults and juvenile stages (nymphs and crawlers) of each scale species (n=10 for each stage) were offered to a single individual of *Th. montezumae* (n=10 females, n=10 males) as prey for 24h. After each replicate, the individuals of *Th. montezumae* tested were transferred to a second cage and maintained with the mixed diet. To evaluate a larger set of non-target species, it was necessary to re-use adults of *Th. montezumae*. Such specimens were tested again after two weeks of feeding on mixed diet. Tests were performed under laboratory conditions in a climatic chamber (26°C; RH 65%; 16:8 LD).

Statistics

The rate of infestation and the rate of predation among the sites of the three Turks and Caicos Islands investigated, were analyzed by Kruskall–Wallis and Dunn's multiple comparison post hoc test, using each plant as a replicate. The predatory ability of the two coccinellids found in the field, *Th. montezumae* and *C. sanguinea*, and the percentage of individuals of *To. parvicornis* predated under laboratory conditions by each species were compared using Mann-Whitney; the same statistic was also used to compare the predation rate of larval and adult stages

of each species and to compare the rate of predation of males and females of *Th. montezumae* in TCI.

Regarding the data carried out in Italy, the number of individuals predated and not predated (both target and non-target species) by females and males was analyzed using contingency table and the Chi-square test with Yates' correction. The same Chi-square test was used to compare the number of individuals predated and not predated between each non-target species and the target species *To. parvicornis*. Statistics were performed using Graphpad Prism version 8.4.0 for MacOS X (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

RESULTS

Field investigations at Turks and Caicos Islands

All the pine trees randomly chosen in each site were infested by *To. parvicornis*. In total 8,104 individuals of *To. parvicornis* were counted on the 2,160 needles collected with a total of 2,565, 2,357, and 3,182 individuals for NC, MC, and PC, respectively. The rate of infestation was not significantly different among the three islands (H = 2.26; P = 0.323) (Fig. I).

Of the total number of individuals of *To. parvicornis* collected on the three islands, 55.35 ± 2.48% belong to juvenile stages and 44.92 ± 2.75% to the adult stage. The rate of predation by natural enemies on the pest was not significantly different among the three islands (H = 3.358; P = 0.1866) with 47.33%, 58.55%, and 56.73% of scales (both adults and juvenile stages) that presented signs of predation for NC, MC, and PC, respectively.

Only two species of Coccinellidae (both adults and larval stages) were identified as potential predators of *To. parvicornis* among the insects collected in the field and associated with the *To. parvicornis* presence on pine trees. One of the two species of Coccinellidae was easily morphologically identified with keys of Gordon (1985) as a native species of TCI *Cycloneda sanguinea* (Linnaeus) and then verified by molecular analysis (see SM1). The second species, belonging to the genus *Thalassa,* was initially misidentified as a *Hyperaspis* sp. of the "signata group". Only more recently, in laboratory studies in Italy, and thanks to the cooperation with skilled co-authors, taxonomic analysis permitted the identification of the species collected as *Th. montezumae* (Gordon, 1985; Milleo *et al*., 2004; Gordon and Canepari, 2008). This species was never recorded before in TCI and it was presumably introduced from the same native area of *To. parvicornis*.

For each of the three islands investigated, *Th. montezumae* was the predominant species associated with *To. parvicornis*, with a total of 172, 73, and 86 individuals collected in NC, MC, and PC, respectively on the 60 sampled pine trees. Also, the larval stages of *Th. montezumae* proved to be the predominant form in all of the three islands, with a mean of 64.79 ± 3.17% of larvae. Adult and larvae of *C. sanguinea* have been found sporadically on pine trees with 22 (19 adults and 3 larvae) and 9 individuals (adults) collected in NC and MC respectively; *C. sanguinea* was never found on PC. From all the needles collected during the entire field survey (approximately 7160 needles infested) and reared in the insect cages, no additional predators or parasitoids emerged.

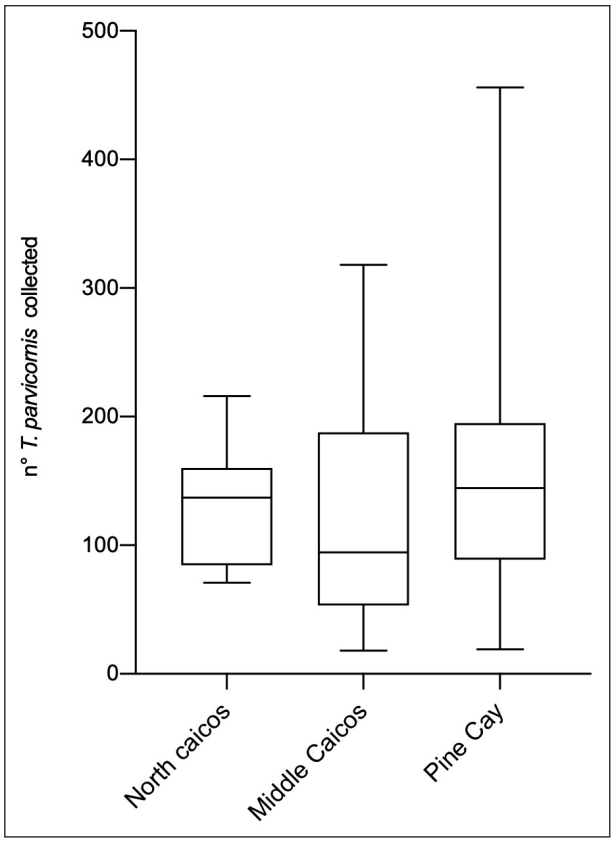

Fig. I - Rate of infestation among the three sites investigated. In the boxplots the boxes indicate the first and third quartile, the line in between shows the median, whiskers indicate 1.5× the interquartile range.

Laboratory studies at the Turks and Caicos Islands

The results of the predation tests conducted in the laboratory at the TCI, evidenced that the rate of predation on *To. parvicornis* by the adults of *Th. montezumae* was on average 82.00 % ± 0.51 and 87.00 % ± 0.47 by females and males respectively without significant difference between sexes (U=36.50; P=0.3281). The percentage of individuals predated by *Th. montezumae* is significantly higher than that predated by *C. sanguinea* (U=0; P<0.0001, female and male pooled data) for which only 7.50% ± 0.31 and 11.43% ± 0.32 of preys were consumed on average by adults females and males, respectively. Additionally, the rate of predation proved significantly different between the adult and immature stages of *Th. montezumae*: the mean percentage of individuals predated was 84.50% ± 3.44 and 50.00% ± 1.01 by adults (female and male pooled data) and larvae respectively (U=34; P=0.0021). The mean percentage of *To. parvicornis* predated by the larvae of *C. sanguinea* was only 1.33 %± 0.41 of the total number of individuals offered as prey.

Laboratory studies in Italy

The number of individuals predated and not predated was not significantly different between the females

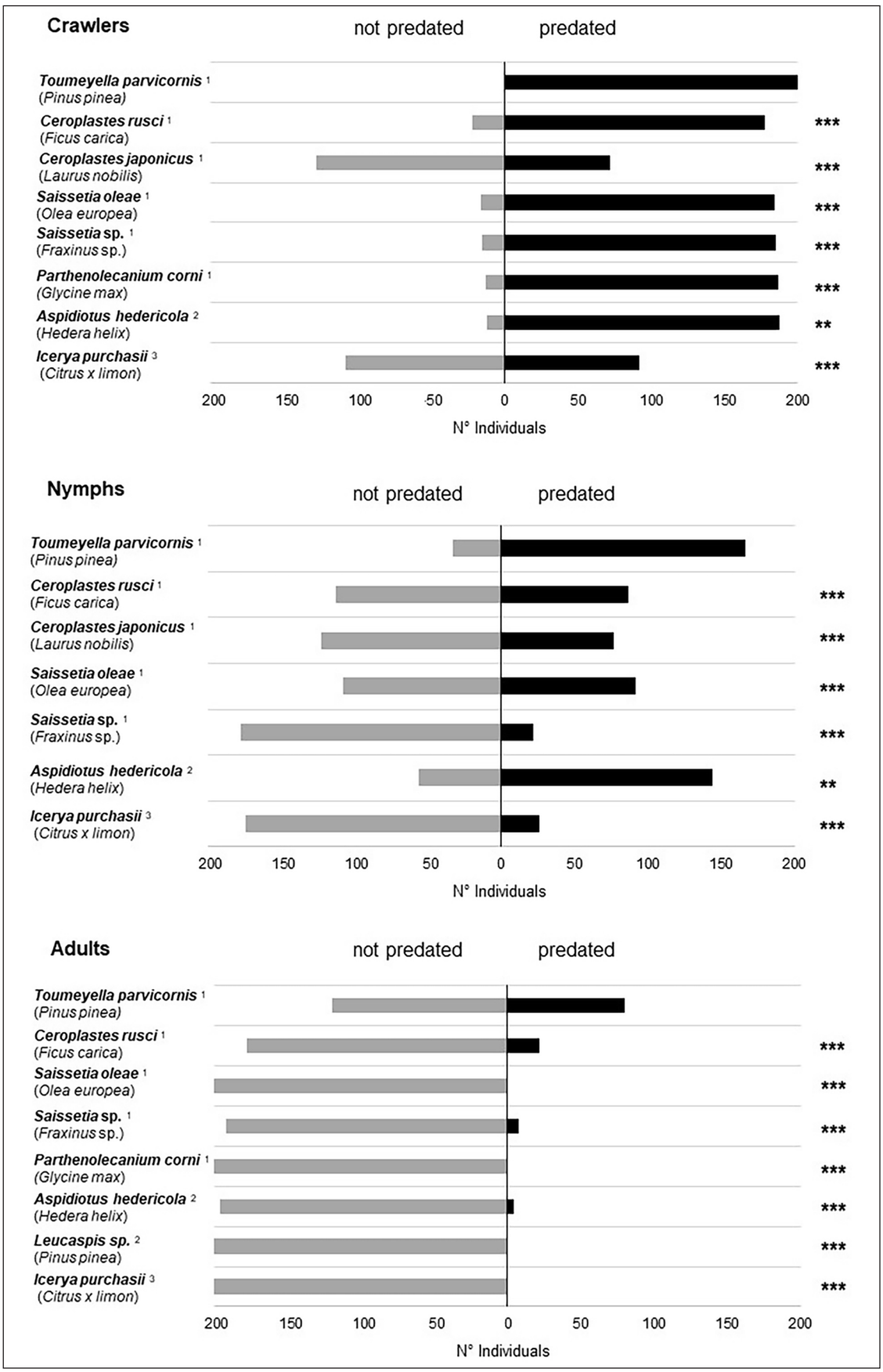

Fig. II - Results of predation tests by *Thalassa montezumae* (females and males pooled data) for each developmental stage of target and non-target species under laboratory condition (16:8 L:D, RH 65% and 24h of time exposure). Number to the superscript associated to the species tested refers to family: (1) Coccidae, (2) Diaspididae, (3) Margarodidae. Names among parentheses refer to host plant. Asterisks indicate significant differences between the non-target species and *Toumeyella parvicornis* (Chi-square test with Yates' correction, *P<0.05; **P<0.01; ***P<0.001). For the aphids and aleurodid species, see details in the Results.

and males of *Th. montezumae* tested ($\chi2$=0.049, df=1, P=0.8244). The number of individuals predated differs among the species tested and developmental stages provided as prey when compared to *To. parvicornis*. The results of predation tests with scale species are summarized in Fig. II and Supplementary material (SM2). None of the aphid species [*Aphis cytisorum* Hartig collected on *Cytisus scoparius*, *Aphis nerii* Boyer de Fonscolombe on *Nerium oleander*, and *Uroleucon aeneum* (Hille Ris Lambers) on *Cynara cardunculus*] were predated nor were juveniles of the aleyrodid species *Aleurocanthus spiniferus* Quaintance collected on *Citrus* × *limon*.

DISCUSSION

Toumeyella parvicornis is a significant pest of *Pinus* species and represents a severe threat to pine forests worldwide. The decline of the Turks and Caicos Islands' native pine forests of *P. caribaea* var. *bahamensis* represents a global warning for pine forests worldwide. Despite the effectiveness of some chemical treatments, such as endotherapy, classical biological control may be the only viable long-term management method for this pest in newly invaded areas.

Although some authors (e.g., Clarke, 2013; Dueñas-López, 2022) report a long list of potential natural enemies, none of these are considered specific for *To. parvicornis* (Dueñas-López, 2022) and studies about their relationships are scarce. The infestations on TCI represent a unique opportunity to explore new potential prey-predator interactions and the observed effectiveness of predation in these adventive ranges can contribute to advancing the knowledge in this field of pest control (Avila *et al.*, 2023). Prior to the present study, no natural enemies of *To. parvicornis* were recorded on the TCI, although large congregations of the coccinellid *C. sanguinea* were observed on infested pine trees (Malumphy *et al*., 2012). The possibility of this lady beetle being a predator of *To. parvicornis* was hypothesized at that time, but never successively investigated. *Cycloneda sanguinea* is an opportunistic predator known for its preference for aphids (e.g., Gordon, 1985). The predation tests conducted in the present study at the TCI confirm this aspect, reporting the very low predation ability on *To. parvicornis,* both by adult and larval stages of *C. sanguinea*. In contrast, the second species of Coccinellidae, *Th. montezumae,* was found in very large numbers on infested pine trees and is likely the main species responsible for the high number of predated individuals of *To. parvicornis* observed in the field. To the best of our knowledge, this species of Coccinellidae has never been documented in the TCI before and thus was presumably introduced as the pine tortoise scale.

Based on the available literature, *Thalassa* is a New World genus containing six described species ranging from southern USA and Cuba to South America (Millléo *et al.*, 2004). *Thalassa montezumae* was reported by Gordon (1985) to occur in north Mexico and southern U.S.A. (Arizona and Texas). It was recently observed also in Florida (Francis *et al.*, 2022). The genus *Thalassa* is poorly investigated and, so far, the only known associated prey are *Toumeyella mirabilis* (Cockerell) and *Phalacrococcus howertoni* Hodges & Hodgson (Hemiptera: Coccidae) (Gordon, 1985; Francis *et al.,* 2022).

Since the field studies conducted in 2011 in TCI did not reveal the presence of *Th. montezumae* (Malumphy *et al.,* 2012), it is possible that this species may have been accidentally introduced only recently. It's reasonable to assume that the accidental introduction of *Th. montezumae* on TCI is connected to commercial trade mainly with the U.S.A. (e.g., Florida like its prey), which represents the most common commercial partner (Observatory of Economic Complexity, 2021). Future phylogeographic reconstruction studies could help to solve this aspect. Since the distribution of this species overlaps with the *To. parvicornis* ones, *Th. montezumae* could be considered a natural enemy of this scale in the area of origin. However, on TCI, the year-round reproductive activity of its prey may have led the *Th. montezumae* population to rapidly spread and multiply, explaining the large number of individuals found in the field.

Understanding predator foraging strategies is of primary importance for the development of successful biological control programs. In this case, the high predation rate displayed during laboratory tests conducted in TCI, both by the adult and larval stages (84.5% predation ability for adults and 50.0% for juveniles), and the rate of *To. parvicornis* predation observed in the field (50% on average of predated specimens) together suggest that *Th. montezumae* would be a promising classical biological control agent.

Biological control is an environmentally sound and effective method for reducing pest populations, and coccinellids are linked to biological control more often than any other taxa of predatory organism (Obrycki and Kring, 1998). However, beneficial organisms considered for classical biological control programs might also feed on non-target species and affect either directly or indirectly associated species, causing adverse effects on the ecosystem. For this reason, the non-target risk analysis represents a mandatory step before introducing an exotic species into a new area, even if considered useful (Van Lenteren *et al.,* 2006). Non-target risk analysis is a complex of procedures that need to be performed at different levels of complexity using a multidisciplinary approach aimed to simulate environmental factors as accurately as possible (Van Lenteren *et al*., 2006).

The use of field-collected adult specimens in this study allowed us to quickly test different species of scales (native and introduced) and other hemipteran species present in Italy. Therefore, this study represents a first attempt to analyze the risks of the introduction of *Th. montezumae* for classical biological control of *To. parvicornis* in European countries.

In the present study, *Th. montezumae* displayed the ability to feed on most of the species tested as potential food, even if significant differences were observed when compared to the target host *To. parvicornis*. In all cases,

the adults of *Th. montezumae* exhibited a high rate of feeding behavior on crawlers of the tested species, with the only exception being the margarodid *Icerya purchasi* Maskell. The unique waxy-cotton secretions of the latter species may be responsible for its lack of appeal. *Leucaspis* sp. is also waxy, but it could not be tested in the crawler stage. Wax production is a common defensive strategy used by plants (Gorb and Gorb, 2017), herbivores (Schoeller *et al*., 2018; Yamazaki, 2017), and predators (Agarwala and Yasuda, 2001) to reduce their susceptibility and may even prove toxic to some predators. *Thalassa montezumae* may not have adapted to this kind of prey. In contrast, another coccinellid, *Novius cardinalis* (Mulsant), appears to have specialized as a predator of *I. purchasi* and related species (family Margarodidae) (Causton *et al.,* 2004).

As for crawlers, highly significant differences were observed for the sessile nymphal stages of non-target species tested. The lowest number of individuals predated by *Th. montezumae* was obtained by testing adult specimens. Here a significantly lower predation rate was observed in all of the seven non-target prey species tested. None of the aphid species tested were predated by the adults of *Th. montezumae*, nor were the juvenile stages of the mealybug *A. spiniferus.* Thus, the results of this study suggest the lady beetle *Th. montezumae* is a predator of scales.

In conclusion, the present study provides key information about the potential use of a BCA against *To. parvicornis* and a potential feeding range of *Th. montezumae* within the frame of non-target risk assessment. The rate of infestation of *To. parvicornis* evidenced in TCI and the discovery of a new natural predator of this pest, the coccinellid *Th. montezumae*, native from the same area of origin of the pest, can in turn help to improve the management of this pest in newly invaded areas.

Laboratory tests have their own limitations in that it is extremely difficult to accurately reproduce the cues, stimuli, and other ecological filters that could affect searching and feeding behaviours of biological control agents in a natural environment (Kuhlmann *et al.,* 1999; Sands and Van Driesche, 1999). Therefore, the results of the laboratory experiment need to be carefully evaluated. Studies on the biology, physiology, and behavior of *Th. montezumae, vs* the target prey *To. parvicornis,* and non-target species as well, are ongoing to gather more information about its potential use as a biological control agent and predict the risk of negative environmental impacts.

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AUTHORS CONTRIBUTION:

LG, GM, GSP, and PFR conceptualized research; LG and GM performed field work and laboratory tests at the TCI. BNM, DP, JB, and CM locally organized and supported field survey; LG, GM, and NV performed taxonomical identification; LG, GM, GSP, and LM performed tests in the laboratory in Italy; LG performed statistical analyses; II performed molecular analyses, LG and GSP prepared the draft of the manuscript; GSP managed project and PFR provided fundings. All the authors contributed to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL 1

Molecular identification of collected species

Species identification was confirmed by DNA sequencing using the 5' region of mitochondrial cytochrome oxidase I gene as follows: DNA was extracted from whole individuals using QIAmp DNA extraction Kit (QIAGEN) following manufacturer instruction, the final elution step was performed in 50 µl of AE Buffer supplied with the kit. Amplification was obtained using LCO1490 and HCO2198 (Folmer et al., 1994) primers for aphids and Coccinellidae, while for the non-target scales PCOF1 and LepR1 (Park et al., 2010) primers were used. The PCR reaction was performed in 50.0 µl total volume containing 25.0 µl of DreamTaq Hot Start PCR Master Mix (2X) (ThermoFisher Scientific), 0.6 µM of each primer, and 50 ng of DNA. The resulting amplicons were purified and sequenced using SeqStudio Genetic Analyzer (Applied Biosystems) following the suggested protocol. Sequences were submitted in GenBank. Species attribution was defined with a BLAST similarity search.

Morphological identification of collected species

For *Leucaspis* sp., a clear sequence could not be obtained with standard primers for scales to assess the species. Consequently, the identification was limited to the genus level using the morphological keys of Kosztarab & Kozár (1988).

Table S1 - GenBank accession number of 5' region of mitochondrial cytochrome oxidase I gene sequences of species tested.

Species tested	**GenBank Accession number**
Ceroplastes rusci	PP646221
Ceroplastes japonicus	PP646222
Saissetia oleae	PP646231
Parthenolecanium corni	PP646224
Aspidiotus hedericola	PP646230
Aphis cytisorum	PP646226
Aphis nerii	PP646227
Uroleucon aeneum	PP646228
Saissetia sp.	PP646223
Icerya purchasi	PP646225
Aleurocanthus spiniferus	PP646229
Cycloneda sanguinea	PP680712

SUPPLEMENTARY MATERIAL 2

Table S2 - Results of Chi-square test with Yates' correction between the target species *To. parvicornis* and each non-target species.
The number of superscript associated with the species tested refers to the family: (1) Coccidae, (2) Diaspididae, (3) Margarodidae. Asterisks indicate significant differences between the non-target species and *Toumeyella parvicornis* (Chi-square test with Yates' correction, *P<0.05; **P<0.01; ***P<0.001). For the aphids and aleurodid species, see details in the Results.

Species	Crawlers					Nymphs					Adults				
	predated	not predated	CHI-square	Df	P	predated	not predated	CHI-square	Df	P	predated	not predated	CHI-square	Df	P
Toumeyella parvicornis[1]	200	0				167	33				80	120			
Ceroplastes rusci[1]	178	22	21.21	1	<0.0001	87	113	67.32	1	<0.0001	22	178	42.76	1	<0.0001
Ceroplastes japonicus[1]	186	14	12.51	1	0.0004	77	123	83.24	1	<0.0001					
Saissetia oleae[1]	184	16	14.65	1	0.0001	92	108	59.98		<0.0001	0	200	97.52	1	<0.0001
Saissetia sp.[1]	185	15	13.58	1	0.0002	22	178	208.0	1	<0.0001	8	192	73.44	1	<0.0001
Parthenolecanium corni[1]	187	13	11.45	1	0.0007						0	200	97.52	1	<0.0001
Aspidiotus hedericola[2]	188	12	10.40	1	0.0013	144	56	6.994	1	0.0082	4	196	84.76	1	<0.0001
Leucaspis sp.[2]											0	200	97.52	1	<0.0001
Icerya purchasii[3]	92	108	145.2	1	<0.0001	26	174	196.2	1	<0.0001	0	200	97.52	1	<0.0001