Biological control of giant pine scale in Australia

Final report
Project No: PNC489-1819

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by

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IMPORTANT NOTICE

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Executive Summary

The main objective of this research was to assess risks associated with the introduction of a potential biological control agent of giant pine scale and determine the agent's suitability for long-term, sustainable management of giant pine scale in Australia. This was achieved by (1) importing quarantine cultures of the most promising giant pine scale biological control agent and testing its prey range in the native range of Greece, (2) collecting, identifying, and culturing giant pine scale, non-target test species and host plants for prey specificity studies, (3) assessing the risk of introduction to non-target species, and (4) preparing an application for release of the preferred agent *Neoleucopis* n. sp. B.

Three species of the predatory silver fly *Neoleucopis kartliana* sensu lato were found to occur in Greece. Results from surveys in Greece and evidence from the Italian island of Ischia demonstrated that when conditions are favourable, populations of *N. kartliana* sensu lato rapidly grow to levels that are damaging to giant pine scale populations. *Neoleucopis* larvae control giant pine scale populations by feeding on eggs and nymphs from spring through to autumn when insects are active. One of these species, *N.* n. sp. B, was found to be highly prey-specific in field and laboratory experiments. Further research is recommended to confirm the identity and efficacy of the three species, their interactions and prey-range, and to import, mass-rear, release and monitor one or more *Neoleucopis* spp. pending regulatory approval.

Biological control with the predator *N.* n. sp. B will, if approved for release, complement other management practices to prevent or slow the spread of giant pine scale into Australian softwood plantations. If successful, this project will provide a biological control option for the sustainable management of giant pine scale in Australia.

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Introduction

Giant pine scale (GPS) *Marchalina hellenica* Gennadius (Hemiptera: Coccoidea: Marchalinidae) is an exotic sap sucking scale insect that was detected feeding on *Pinus* in Adelaide, South Australia and Melbourne, Victoria in late-2014. GPS is native to the eastern Mediterranean region, particularly mainland Greece and Turkey where it is considered an economically important insect in the apiculture industry (Gounari, 2006). GPS is a concern in Australia because high population densities can lead to a decline in tree health and reduction in insect biodiversity (Petrakis et al. 2011). Importantly, GPS is a threat to Australia's soft wood industry (valued at $1.16 billion) as it attacks the dominant commercially grown species *P. radiata* and there are currently no effective control options for widespread infestations.
A feasibility study carried out by Agriculture Victoria identified *Neoleucopis kartliana* Tanasijtshuk (Diptera: Chamaemyiidae) as the most promising biological control agent of GPS. This was based on a literature review, field collections in northern Greece, and a case study of a successful GPS biological control programme carried out on the Italian island of Ischia (Lubanga et al., 2018). Research into the biological control of GPS was therefore proposed, including laboratory and field studies in Greece, and quarantine laboratory studies in Australia.

Declaration of giant pine scale as a target for biological control in Australia

A submission to nominate giant pine scale as a target for biological control was provided to the Victorian representative to the Plant Health Committee (PHC) (**Attachment 1 Nomination as target**). This is a mandatory regulatory step that is conducted prior to applying for release of a biocontrol agent. The submission will be considered by the PHC and a decision notified in due course. An important aim of this regulatory step is to identify potential conflicts that would preclude the use of biological control, such as beneficial uses of giant pine scale. The main beneficial use of giant pine scale in the native range is for production of pine honey. We contacted the main industry body for honey producers in Australia, the Australian Honey Bee Industry Council (AHBIC), and provided a briefing on giant pine scale, its impact, and the aims of the Australian biological control program. After consulting with State members, AHBIC provided a statement confirming that giant pine scale has no measurable value to honey producers in Australia. Furthermore, AHBIC supports non-chemical management of the pest. Feedback from AHBIC was included in the submission to PHC.

Neoleucopis risk assessment

GPS is native to Greece and Turkey, however native range studies in this project focused on Greece due to difficulties collecting and exporting insects from Turkey. Initial surveys carried out in Greece aimed to investigate the natural enemy complex of GPS (**Attachment 2 Avtzis et al. 2020**). While no parasitoids were found, GPS was attacked by several predators, most notably *Neoleucopis kartliana*. *Neoleucopis kartliana* was present at all sites investigated and was presumed to be specific to Marchalina, highlighting its potential as a biological control agent for GPS. The predatory fly had already been used to control GPS on the Italian island of Ischia. Molecular analyses of Greek samples revealed other Chamaemyiidae predatory flies with potential for an Australian classical biological control program.

Rearing Neoleucopis

The development of rearing protocols is essential for laboratory experiments and future mass-rearing and release programs for Neoleucopis. Rearing protocols for Neoleucopis were developed in Greece, where field-collected individuals were readily available. A modified protocol for newly imported pupae held in Australian quarantine required each pupa to be held separately in a capsule until emergence. The modified protocol aimed to prevent cross-contamination in case of undetected parasitism.

Neoleucopis pupae

Pupae were placed in ventilated emergence containers at 23°C, 60% RH, 18:6 (L:D), with a progressive transition from light to dark within an hour and vice versa (Table 1). Containers were checked daily for adult emergence.

Table 1. Light transition in the controlled environment cabinet for *Neoleucopis*.

Time	**% Light**
08:00	10
08:30	40
08:45	70
09:00	100
02:00	70
02:15	40
02:30	10
03:00	0

Neoleucopis adults

Adults were collected immediately after emergence, either with an aspirator or by individual collection using falcon tubes. Adults were then be transferred into insect rearing cages that are at least 60cm x 60cm x 180cm, along with GPS-infested potted *Pinus* trees. Suitable environmental conditions for cages are 21ºC, 60-65% RH, 13:11 (L:D) (natural lighting if possible).

A supplementary food source for adults was added to cages consisting:

- Water: provided by soaking cloth or cotton that remains wet (Figure 2(a)).
- Dry yeast & sugar droplets: a thick mixture of dry yeast and sugar (10:100) diluted with water, placed on cotton as droplets. The cotton may be placed in petri dishes (diameter 8.5cm) (Figure 2(b)).

The water container was replaced every 7 days or as necessary, and the petri dishes renewed every 2-3 days.

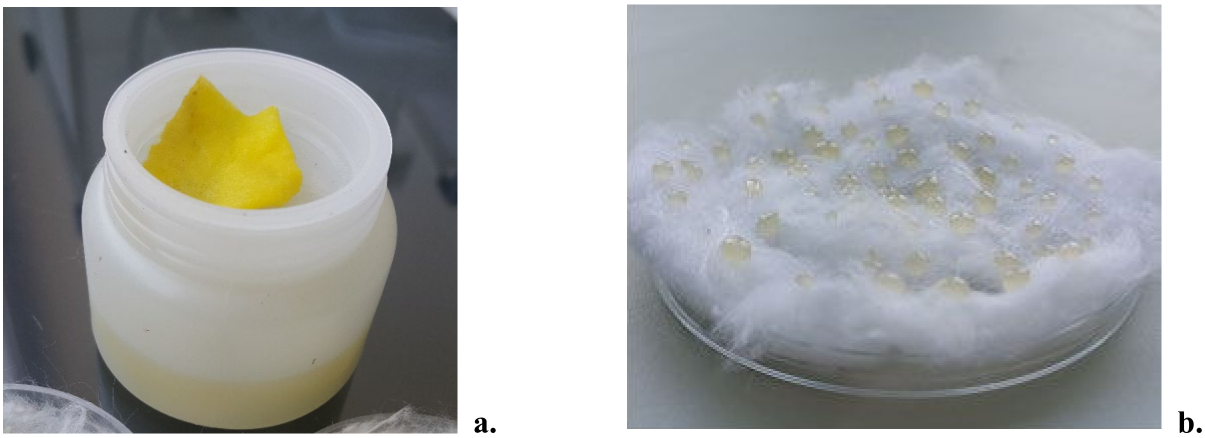

Figure 1. (a) container filled with water showing a cloth stripe protruding through the lid, and (b) dry yeast & sugar droplets placed on cotton.

Pupae recovered from rearing cages were placed into ventilated emergence containers and monitored for adult emergence.

Biology and ecology studies in the native range

The biology and ecology of both GPS (**Attachment 3 Eleftheriadou et al. 2023**) and Neoleucopis (**Attachment 4 Draft release application; Attachment 5 Eleftheriadou et al. 2022**) were studied in the native range of Greece. Surveys and molecular analyses in Greece revealed a *Neoleucopis* species complex of at least three morphologically similar species. *Neoleucopis kartliana* sensu lato comprises:

1. *Neoleucopis kartliana*, collected in northern Greece
2. *Neoleucopis* n. sp. A., collected in southern Greece
3. *Neoleucopis* n. sp. B., collected in northern Greece

Neoleucopis kartliana and *Neoleucopis* n. sp. B., possibly exhibit temporal niche compatibility by emerging at different times. *Neoleucopis hadzibeiliae* was previously assumed to co-occur with *N. kartliana* but was not recovered on *Pinus* spp. in Greece during these surveys.

Molecular delimitation of the three *Neoleucopis* species

Species delimitation and identification used mitochondrial DNA barcoding region for numerous (more than 400 in total) individuals. The length of the locus that was sequenced comprised 617 nucleotides (base pairs); Table 1. shows the pairwise distances, estimated as the number of nucleotides, between each of the *Neoleucopis* species are shown that occur in

Greece. Alignment of the sequences of the three *Neoleucopis* species is shown in Figure 1. In general, intraspecific (= among individuals that belong to the same species) divergence is lower than 2.5% for this region of mtDNA.

Table 2. Pairwise distances (nucleotide differences) between the three *Neoleucopis* species occurring in Greece. Above the shaded diagonal: the % percentage of nucleotide differences. Below the shaded diagonal: the number of SNP's (Single Nucleotide Polymorphisms) found among the 617 base pairs that were sequenced.

	N. kartliana	***Neoleucopis* n. sp. A**	***Neoleucopis* n. sp. B**
N. kartliana		9.8%	5.2%
***Neoleucopis* n. sp. A**	61/617		10.04%
***Neoleucopis* n. sp. B**	32/617	62/617	

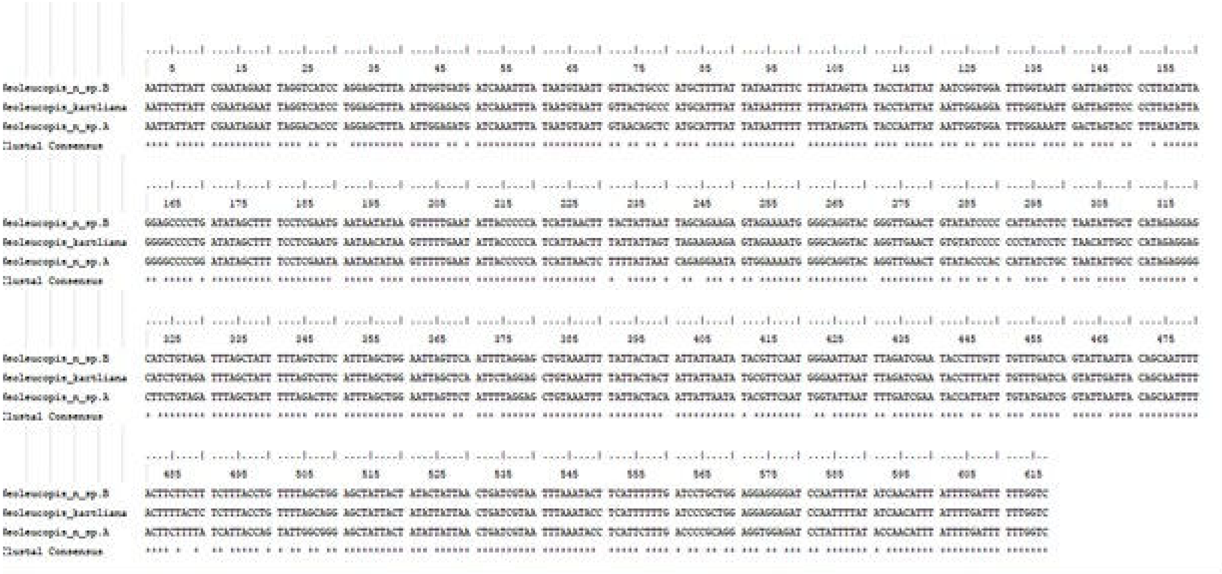

Figure 2. Alignment of the sequences from the three *Neoleucopis* species that occur in Greece.

Identification of native, non-target scale insects using morphology alone is similarly difficult, and there is a paucity of molecular data available. We used DNA sequencing of 128 individual specimens and molecular phylogenetics to assist with identification, and to help clarify the archaeococcoid family relationships amongst the Australian species, and the relationship between Australian archaeococcoids and giant pine scale (**Attachment 6 Mills et al. 2023, and Mills et al., in prep**). These studies make an important contribution to biological control of giant pine scale risk analysis and our knowledge of the identification and phylogenetic relationships of Australia's native scales.

Prey-specificity testing and field observations

Nb: Most available literature refers to *N. kartliana* sensu lato which we can assume includes *N.* n. sp. B. To avoid confusion, *N.* n. sp. B. is used to refer the species that has been tested while *N. kartliana* sensu lato is used when discussing literature referring to *Neoleucopis kartliana* species complex in general.

We carried out prey specificity studies of *N.* n. sp. B. to assess the risk of this species attacking non-target scale insects if released in Australia. The studies in Greece took advantage of an abundant Australian native scale insect *Icerya purchasi* (Hemiptera, Monophlebidae) to carry out laboratory and field prey specificity studies of *N.* n. sp. B (**Attachment 7 Eleftheriadou et al. 2024, Eleftheriadou et al., in prep.).** *Icerya purchasi* belongs to the scale insect family Monophlebidae which is the most closely related family to Marchalinidae to which GPS belongs (**Mills et al., in prep, Lubanga et al., in prep**). In addition, *I. purchasi* has similar morphology to GPS as both species produce cotton -like wax, occur in the same habitat, and have similar phenology. *Icerya purchasi* also ranks among the top 10 native scale insects prioritised for prey-specificity testing (**Lubanga et al. in prep**). This ranking was developed using an automated computer-based decision-support framework called PRONTI (Priority Ranking of Non-Target Invertebrates) (Todd, et al. 2015).
Prey specificity studies in Greece were carried out with mature *N. n. sp.* B larvae that had partially developed on GPS egg masses in the field in no-choice and choice laboratory studies. In a no-choice experiment *N. n. sp.* B. larvae confined on *I. purchasi* egg masses experienced high mortality (85%) while all larvae confined on GPS egg masses survived and completed development to the adult stage (n = 20). In the same trial, there was no significant change in *N. n. sp. B.* larval length on *I. purchasi* over a five-day period, indicating no development during this time, whereas N. n. sp. B. larvae on GPS showed a statistically significant increase in length over a three-day period.
In a choice laboratory experiment of 20 *N. n. sp. B*. larvae, all successfully pupated, with 13 choosing GPS egg masses and two choosing *I. purchasi* egg masses while 5 pupated on the petri dish, ignoring the egg masses.

Given that larvae were able to pupate in the absence of egg masses in the choice trial it is probable that the few larvae that completed development in *I. purchasi* egg masses in these laboratory studies obtained sufficient nutrition in the field while feeding on GPS egg masses to develop to adult stage, prior to being used in laboratory trials. The results above provide evidence that *I. purchasi* is not suitable prey for *N. n. sp.* B. as this species supported little development and was not preferred in choice trails.

Field surveys that involved collecting egg masses of both *I. purchasi* (from *Pittosporum tobira*) and GPS (collected from *Pinus halepensis*) at sites where these species co-occur found no *N. n. sp.* B. attacking *I. purchasi* at any of the sites surveyed. Additional observations in 2024 of *I. purchasi* on *Citrus* × *aurantium* in northern Greece supported the survey findings. Overall, results from laboratory and field studies in Greece indicate *N. n. sp.* B to be highly prey specific to *M. hellenica* in the overseas range. While choice and no-choice laboratory studies showed that a few *N. n. sp.* B. larvae completed development on a non-target Australian native scale insect (*I. purchasi*), field studies provided evidence that *N. n. sp.* B. does not attack *I. purchasi* even when it and GPS occur in sympatry. Native range surveys provide additional evidence that *N. kartliana* sensu lato is prey specific to GPS (Ülgentürk 2013; Lubanga 2018).

Further laboratory choice and no-choice experiments with Australian native scale/priority scale species would complement results from Greece.

Knowledge transfer

National workshop

A seminar titled "Impact and management of giant pine scale (GPS) in Australia" was held on Thursday 25 August 2022, at the Bunjil Place Function Centre, in Narre Warren, Victoria. The full-day seminar featured Australian and international speakers presenting information on the latest advances in research and management of giant pine scale. Input from attendees was sought on planning for long-term management of the pest. The afternoon included a visit to a nearby field site which highlighted the challenges facing tree managers and the Australian forest industry.

Publications

The primary outputs of the project are the draft application for release of *Neoleucopis* n. sp. B (**Attachment 4**) and the nomination of GPS as a target for biological control in Australia (**Attachment 1**). The project has also produced eight research papers, five of which have been published in peer-reviewed journals (**Attachments 2, 3, 5, 6 and 7**), and three that have either been submitted to a journal or are in preparation (unpublished research papers are listed below and are available on request):

- Eleftheriadou, N., Seehausen, L., Kenis, M., Lefoe, G., Lubanga, U., Gaimari, S., Havill, N., Garonna, A., Kavallieratos, N. and Avtzis, D. *Genetic diversity among species of* Neoleucopis *(Chamaemyiidae) associated with* Marchalina hellenica *(Hemiptera: Marchalinidae) in Greece and Italy*. Submitted manuscript under review.
- Lubanga, U., Collinson, N., Mills, P., Lefoe, G., and Todd, J. *Prioritising non-target scale insect species for prey specificity testing of* Neoleucopis *spp. (Diptera, Chamaemyiidae) in Australia.* In prep.
- Mills, P., Lubanga, U., and Lefoe, G. *Phylogenetics of Australian archaeococccoid scale insects to assist decision making for classical biological control*. In prep.

The publication success of the project provides a solid foundation for an application to release *N.* sp. B and for future GPS research.

Conclusion

Results from laboratory and field studies in Greece demonstrated that *Neoleucopis* n. sp. B is highly prey-specific to *M. hellenica* in the overseas range. While choice and no-choice laboratory experiments showed that a small proportion of *N.* n. sp. B. larvae could complete development on the non-target Australian native scale insect *I. purchasi*, field studies provided evidence that *N. n. sp.* B. does not attack *I. purchasi* under natural conditions, including in locations in Greece where *I. purchasi* and *M. hellenica* co-occur. Several native range surveys provided additional evidence that *N. kartliana* sensu lato is prey-specific to giant pine scale. Laboratory choice experiments with priority Australian native scale species would complement these results, as would further ecology and efficacy studies in Greece. A draft application for release of *Neoleucopis* n. sp. B has been prepared (**Attachment 1.4. Draft release application**) pending further identification and testing. Additional research may be required to confirm the identity, efficacy (relative to *Neoleucopis kartliana* and N. n. sp. A), and prey-range of *N.* n. sp. B.

Recommendations

The following recommendations are made to progress biological control of GPS in Australia:

1. Complete research into the specificity and efficacy of *Neoleucopis kartliana*, *N*. n. sp. A and *N.* n. sp. B., in Australia and Greece.
2. Maintain Australian non-target insect species cultures pending completion of quarantine testing and a release decision,
3. Conduct pre-introduction monitoring of GPS in Australia to select the most suitable release sites, and to obtain baseline data for biological control impact assessment studies (e.g., BACI design).
4. Commence a biocontrol agent release program in Australia pending timely biocontrol agent approval.

Attachments

Attachment 1 Nomination as target
Attachment 2 Avtzis et al. 2020
Attachment 3 Eleftheriadou et al. 2023
Attachment 4 Draft release application
Attachment 5 Eleftheriadou et al. 2022
Attachment 6 Mills et al. 2023
Attachment 7 Eleftheriadou et al. 2024

References

Gounari S. 2006. Studies on the phenology of *Marchalina hellenica* (Gen.) (Hemiptera: Coccoidea, Margarodidae) in relation to honeydew flow. J. Apic. Res. 45: 8–12. https://doi.org/10.1080/00218839.2006.11101305

Lubanga, U., Lefoe, G., Weiss, J., Kenis, M., Avtzis, D. & Kwong, R. (2018). Feasibility of biological control of giant pine scale *Marchalina hellenica* (Gennadius) (Hemiptera: Marchalinidae) in Australia. Agriculture Victoria, Bundoora, 43.

Petrakis PV, Spanos K, Feest A. 2011. Insect biodiversity reduction of pinewoods in southern Greece caused by the pine scale (*Marchalina hellenica*). For. Syst. 20: 27–41. https://doi.org/10.5424/fs/2011201-8924

Ülgentürk S, Szentkirályi F, Uygun N, et al. 2013. Predators of *Marchalina hellenica* (Hemiptera: Marchalinidae) on pine forests in Turkey. Phytoparasitica 41: 529–537. 529–537 (2013). https://doi.org/10.1007/s12600-013-0313-1

Plant Health Committee

Out of Session Paper

OOS-xxxx-xx Due Date: xx/xx/xxxx

Approval of giant pine scale *Marchalina hellenica* as a target for biological control

Presented by Department of Energy, Environment & Climate Action

For Decision

Recommendation

That the committee:

1. **APPROVES** giant pine scale *Marchalina hellenica* as a target for biological control.

Background

1. The Victorian Government Department of Energy, Environment & Climate Action has prepared this nomination of the introduced insect giant pine scale as a target for biological control in Australia (Attachment 1).
2. The nomination outlines the biology and taxonomy of giant pine scale, its status as a pest of pine trees in Australia and other countries where it has been introduced, and Australian honey bee industry comment.
3. The nomination identifies a beneficial use of giant pine scale as a source of honeydew for honey production (known as pine honey) in its native range of Greece and Turkey. Consultation with the Australian Honey Bee Industry Council (AHBIC) in 2020 confirmed that pine honey has negligible value in Australia. Furthermore, the AHBIC stated its preference for non-chemical control of giant pine scale in Australia.
4. Declaration as a target for biological can occur at any time during a biological control research program but the target must be approved before permission to release a biological control agent is sought.
5. Agriculture Victoria and European collaborators have identified three promising biological control agent species in Greece. Two of these species have been studied in laboratory, glasshouse and field experiments in Greece and have demonstrated a high level of specificity to giant pine scale.
6. As a result of this promising research, Agriculture Victoria will progress applications to introduce one or more of the prospective biological control agents, hence approval of giant pine scale as a candidate for biological control is sought.

Resource Implications

Nil.

Attachments

Attachment 1: Nomination of giant pine scale *Marchalina hellenica* (Gennadius) (Hemiptera: Coccoidea, Marchalinidae) as a target for biological control.

Attachment 2: Revised guidelines for the introduction of exotic biological control agents for the control of weeds and plant pests.

DRAFT

Application to release *Neoleucopis* nov. sp. B (Diptera: Chamaemyiidae) for the biological control of giant pine scale *Marchalina hellenica* Gennadius 1883 (Hemiptera: Coccoidea: Marchalinidae) in Australia

Technical contacts: Dr Greg Lefoe and Dr Umar Kombo Lubanga
Agriculture Victoria, AgriBio, 5 Ring Rd, Bundoora, VIC 3083

DRAFT

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Citation: Lubanga, U.K., Collinson, N., & Lefoe, G. (2023). Application to release *Neoleucopis* nov. sp. B (Diptera: Chamaemyiidae) for the biological control of giant pine scale *Marchalina hellenica* Gennadius 1883 **(**Hemiptera: Coccoidea: Marchalinidae) in Australia. Agriculture Victoria, Department of Energy, Environment and Climate Action (DEECA), Bundoora, Australia.

Executive summary

Giant pine scale (GPS), *Marchalina hellenica* (Gennadius, 1883; Hemiptera: Coccoidea: Marchalinidae) is an exotic sap sucking scale insect that was detected feeding on the novel host *Pinus radiata* in Adelaide, South Australia and Melbourne, Victoria (Lubanga et al., 2018) in late 2014. This scale insect is native to the eastern Mediterranean region, particularly mainland Greece and Turkey where it is considered an economically important insect in the apiculture industry (Gounari, 2006). GPS is a concern in Australia because high population densities can lead to a decline in tree health and reduction in insect biodiversity (Petrakis et al. 2011, Yesil et al. 2005). In addition, GPS is a threat to Australia's soft wood industry (valued at $1.16 billion) as it attacks the dominant commercially grown species, *P. radiata*, and there are currently no effective control options.

A feasibility study carried out by Agriculture Victoria identified *Neoleucopis kartliana* Tanasijtshuk 1986 (Diptera: Chamaemyiidae) as the most promising biological control agent of GPS. This was based on a literature review, field collections in northern Greece, and a case study of a successful GPS biological control programme carried out on the Italian island of Ischia (Lubanga et al., 2018). A recent survey in Greece involving molecular analyses revealed that *N. kartliana* sensu lato is a species complex comprising three species; *N. kartliana* sensu stricto, *N.* n. sp. A. and *N.* n. sp. B., of which only *N. kartliana* sensu stricto has been described (supplementary material one. The three *Neoleucopis* species are morphologically very similar (only distinguishable by microscopic features on male genitalia) which may explain why previous studies have considered them a single species based on morphological identification (Gaimari, et al. 2007; Ülgentürk 2013; Avtzis 2020). *Neoleucopis kartliana* sensu stricto and *N*. n. sp. B. co-occur in northern Greece and exhibit temporal niche partitioning by emerging at different times of the year. *Neoleucopis n sp*. A. occurs in southern Greece.

Agriculture Victoria scientists in collaboration with scientists from Greece based at Hellenic Agricultural Organization Demeter (HOA) carried out prey specificity studies of *N.* n. sp. B. to assess the risk of this species attacking non-target scale insects if released in Australia. The studies in Greece took advantage of an abundant Australian native scale insect (*Icerya purchasi* (Hemiptera, Monophlebidae)) to carry out laboratory and field prey specificity studies of *N.* n. sp. B. *Icerya purchasi* belongs to the scale insect family Monophlebidae which is the most closely related family to Marchalinidae to which GPS belongs (Mills et al., in prep, supplementary material two. In addition, *I. purchasi* has similar morphology to GPS (both species produce cotton -like wax), ecology (occur in the same habitat), and phenology (occur at the same time of the year). *Icerya purchasi* also ranks among the top 10 native scale insects prioritised for prey-specificity testing (Lubanga et al. in prep). This ranking was developed using an automated computer-based decision-support framework called PRONTI (Priority Ranking of Non-Target Invertebrates) (Todd, et al. 2015).

Prey specificity studies were carried out with mature *N. n. sp.* B larvae that had partially developed on GPS egg masses in the field in no-choice and choice laboratory studies. In a no-choice experiment *N. n. sp.* B. larvae confined on *I. purchasi* egg masses experienced high mortality (85%) while all larvae confined on GPS egg masses survived and completed development to the adult stage (n = 20). In the same trial, there was no significant change in *N. n. sp. B.* larval length on *I. purchasi* over a five-day period, indicating no development during this time, whereas N. n. sp. B. larvae on GPS showed a statistically significant increase in length over a three-day period.

In a choice laboratory experiment of 20 *N. n. sp. B*. larvae, all successfully pupated, with 13 choosing GPS egg masses and two choosing *I. purchasi* egg masses while 5 pupated on the petri dish, ignoring the egg masses.

Given that larvae were able to pupate in the absence of egg masses in the choice trial it is probable that the few larvae that completed development in *I. purchasi* egg masses in these laboratory studies obtained sufficient nutrition in the field while feeding on GPS egg masses to develop to adult stage, prior to being used in laboratory trials. The results above provide evidence that *I. purchasi* is not suitable prey for *N. n. sp.* B. as this species supported little development and was not preferred in choice trails.

Field surveys that involved collecting egg masses of both *I. purchasi* (from *Pittosporum tobira*) and GPS (collected from *Pinus halepensis*) at sites where these species co-occur found no *N. n. sp.* B. attacking *I. purchasi* at any of the sites surveyed.

Overall*,* results from laboratory and field studies in Greece indicate *N. n. sp.* B to be highly prey specific to *M. hellenica* in the overseas range*.* While choice and no-choice laboratory studies showed that a few *N. n. sp.* B. larvae completed development on a non-target Australian native scale insect (*I. purchasi*), field studies provided strong evidence that *N. n. sp.* B. does not attack *I. purchasi* even when it and GPS occur in sympatry. Several native range surveys provide additional evidence that *N. kartliana* sensu lato is prey specific to GPS (Ülgentürk 2013; Lubanga 2018; Üglentürk 2019; Avtzis 2020; Oğuzoğlu, et al. 2021).

Laboratory choice and no-choice experiments with a suite of Australian native scale/priority scale species are underway in Australia and will complement results from Greece once completed.

1. Information about the target species

Taxonomy

Kingdom: Animalia
Phylum : Arthropoda
Class : Insecta
Order : Hemiptera
Family : Marchalinidae
Genus : *Marchalina*
Species : *Marchalina hellenica* (Gennadius, 1883)

Synonym

- *Marchalina hellenica* Schmutterer, 1957
- *Marchalina hellenica* Vayssière, 1923
- *Monophlebus hellenicus* Gennadius, 1883

Common names

- Giant pine scale (GPS)

Phylogeny

Giant pine scale (GPS) (*Marchalina hellenica*) is the type species of the monotypic genus *Marchalina* described by Vayssière (1923) and belongs to the monotypic family Marchalinidae described by Koteja (1996). The family Marchalinidae is one of 11 families previously grouped as Margarodidae sensu (Gullan and Cook 2007; Hodgson 2006a; Morrison 1928). A recent molecular phylogenetic study analysing *18S* and *COI* genes has shown that GPS and Australian archaeococcoids form a clade (albeit with no support) (Mills et al., in prep: supplementary material two) (Fig. 1). Mills at al., (in prep) has shown that GPS is more closely related to Monophlebidae and not Callipappidae (Fig. 1.) as shown in a previous study Vea and Grimaldi (2016).

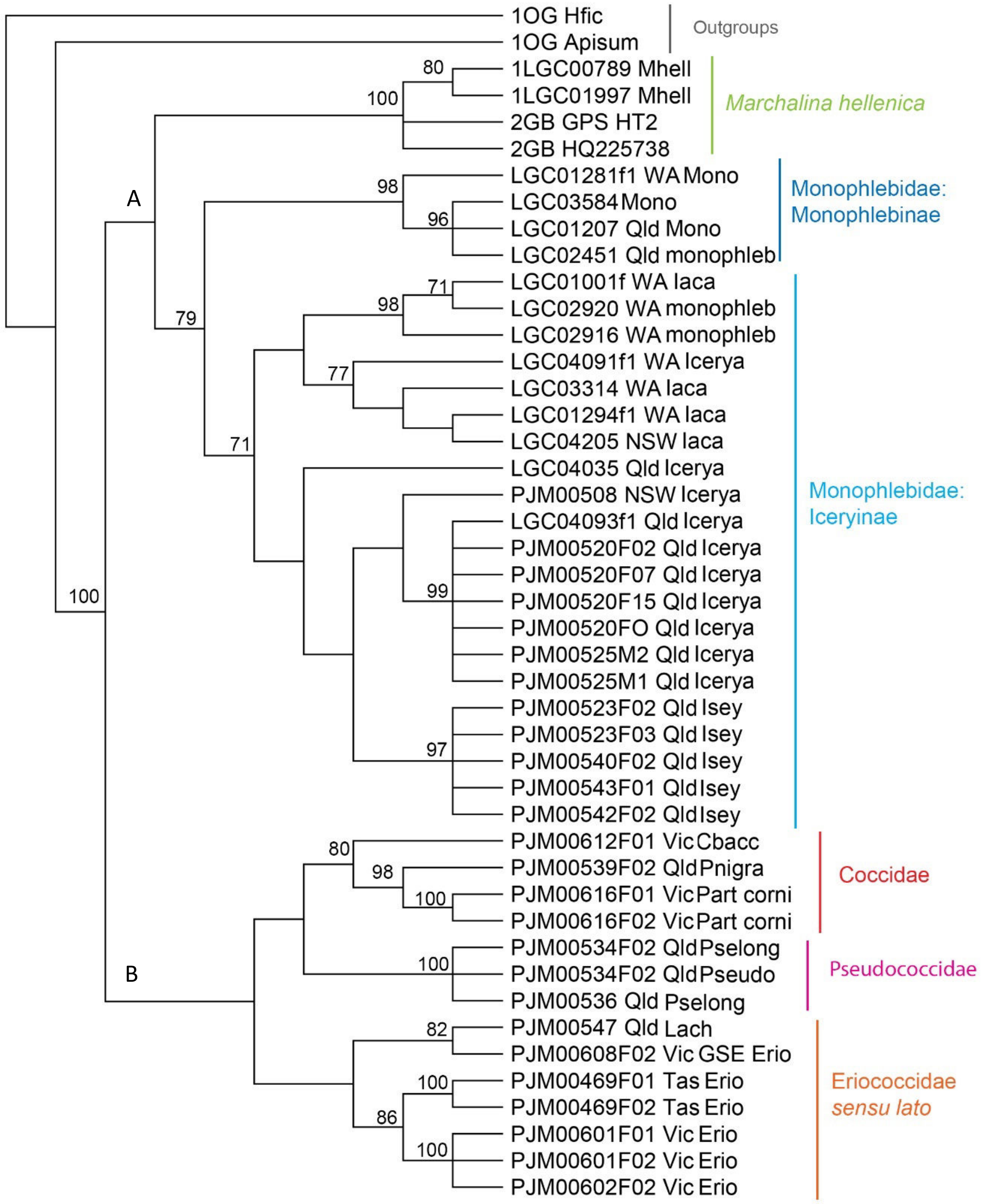

Figure 1. Strict consensus (SC) tree of 1st&2nd codons of partial *COI* constructed using Maximum Parsimony. Bootstrap (BS) values >70 shown on the nodes. The Australian archaeococcoids and giant pine scale (GPS) form a clade (A) separate from neococcoids (B) (albeit with no support) (Mills et al., in prep: supplementary material two).

2. A brief description of the biology of giant pine sale.

Giant pine scale is a sap sucking scale insect closely related to aphids (Aphidoidea), whiteflies (Aleyrodoidea) and jumping plant lice (Psylloidea), which make up the suborder Sternorrhyncha (Gullan and Martin 2009).

Giant pine scale is reportedly univoltine in its native and introduced ranges. Females are oval in shape and about 8-15 mm long and 4-6 mm wide. Males are smaller with elongated bodies and long dark-yellow legs. All life stages exude protective white sticky cotton-like substance known as flocculent. Females are apterous (wingless) and more commonly encountered in comparison to the rare alate (winged) males. For this reason, reproduction is thought to be mainly by parthenogenesis (without fertilization) (Hodgson 2006b). In Australia, male *M. hellenica* are yet to be reported from field populations (Jaroslow, et al. 2023) Females lay about 130–400 eggs in woolly ovisacs on the bark of host trees. The female lifecycle has three instars (developmental stages) plus the adult, whilst males have four instars plus the adult (Hodgson 2006b). Females overwinter as 3rd instars that moult into adults, while males can overwinter either as pre-pupa (3rd-instars) or pupa (4th-instar male) ((Hodgson 2006a; Hodgson 2006b).

Most life stages can be easily distinguished using the number of antennal segments, i.e., 6 for 1st and 2nd instars, 9 for 3rd instars and 11 segments for adult females. First instars are distinguished from 2nd instars by the reduced number of body setae, hairs and dermal spines. Mouth parts of 1st instars are also larger than antennae, whereas 2nd instar mouth parts are approximately the same or smaller than their antennae. In the northern hemisphere, crawlers (1st instars) usually emerge in late spring and settle in cracks and crevices under the tree bark where they feed and slowly grow until end of summer / start of autumn, when they moult into 2nd instars. This stage lasts for about 6–8 weeks before moulting into the overwintering 3rd instar. Adults are present from early-spring to late-spring (Table 1). The developmental phenology of GPS reported from the northern hemisphere is similarly seasonally linked in south-eastern Australia (Gounari 2006; Jaroslow, et al. 2023) (Table 1).

3. Native range and global distribution of giant pine sale

Giant pine scale is widely distributed along the coastal zones of Turkey and Greece around the Aegean Sea and Black Sea (up to 1200m above sea level) and on the island of Ischia in Italy where it was introduced in the 1960s (EPPO 2017; Petrakis 2011) (Fig. 2). According to Petrakis (2011), Turkey seems to be the native range of *Marchalina hellenica*, with Greece and other localities the introduced ranges. This contention is supported by the lack of any records of this species in Greece at prehistoric and classical times. Presumably, this scale insect was introduced to northern Greece by ancient beekeepers of the late Roman and Byzantine empires around the 3rd or 4th centuries (Jorro and Adrados 1985). The centre of evolution for *M. hellenica* is likely around Mount Carmel, northern Israel. This is the origin of *Pinus halepensis*, the primary host of *M. hellenica*. Analysis of ancient pollen revealed an invasion across the eastern Mediterranean by *P. halepensis* around 1100bce (Lev-Yadun 2002). *Marchalina hellenica* distributions may have shadowed *P. halepensis* during this time, moving into West Turkey and Greece over the following millennia. In the last two decades this species' range has expanded rapidly to include central and southern parts of Greece, Syria, and parts of Russia (García Morales 2016). This expansion was mainly driven by beekeepers spreading GPS to novel locations to boost production of pine honey associated with the honeydew excreted by giant pine scale (Bacandritsos 2004). It should be noted that a species with a similar description to GPS *(Marchalina caucasica* described by Hadzibeyli (1969)) is distributed in the Caucasus. This species was synonymised with GPS but species differences in morphological traits, life history (biennial), host genera *(Abies)* and geographic distribution (Caucuses) suggest that *M. caucasica* and *M. hellenica* are two separate species (Hodgson 2006b).

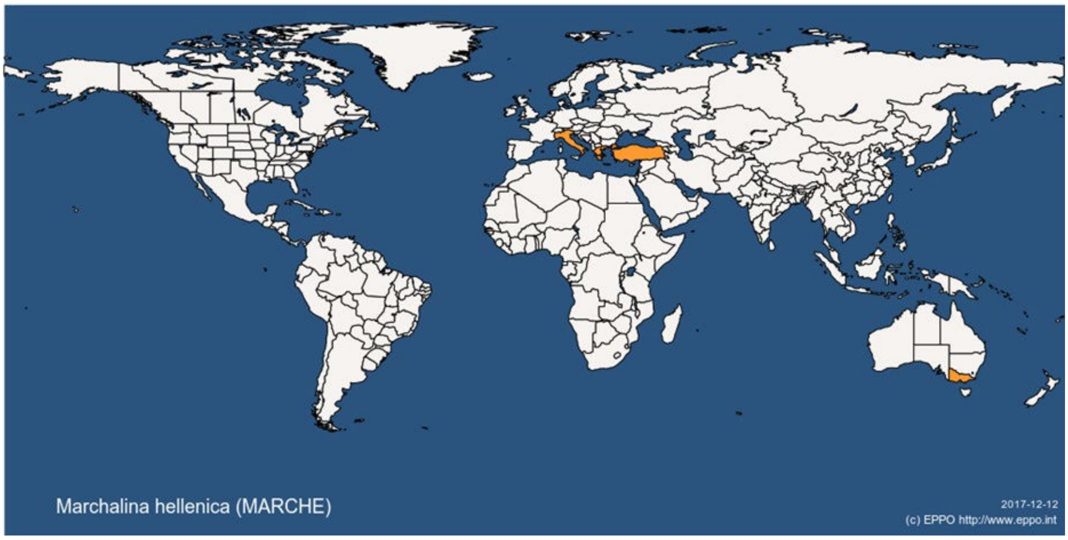

Figure 2. Global distribution of giant pine scale (GPS) (*Marchalina hellenica*) ((EPPO 2017).

4. Australian distribution of giant pine sale (GPS)

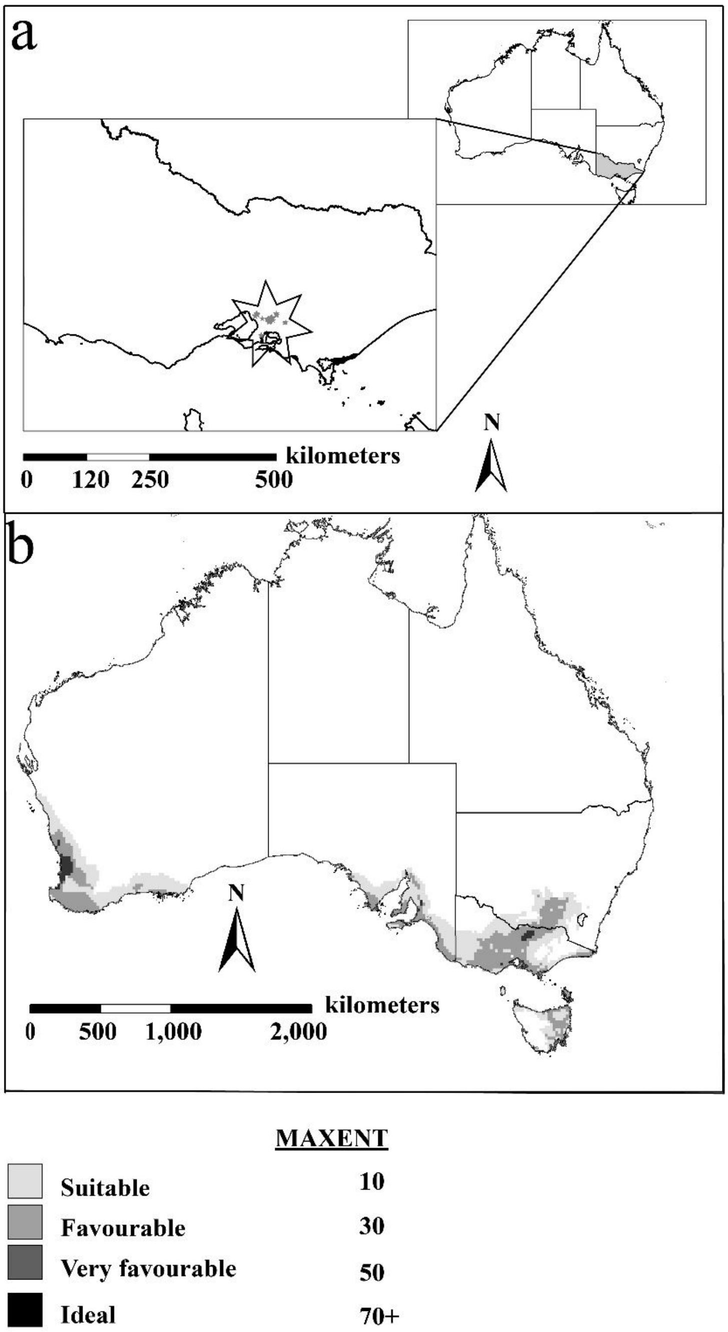

Figure 3. Distribution of giant pine scale (GPS) in Australia (a). The distribution is currently restricted to a few suburbs in Melbourne, Victoria. The predicted distribution of GPS in Australia based on climate matching with known locations from Europe (Eastern Mediterranean and Italy) is shown in (b). Different shades of grey represent suitability scale for MaxEnt (Lubanga et al. 2018).

5. The status of the giant pine sale in Australia, including a summary of the economic and environmental losses caused by the target

Economic status

In Greece and turkey, GPS is an economically important scale insect in the apiculture industry (Gounari 2006). Honeydew excreted by GPS is collected by honeybees, which convert it into pine honey that accounts for around 60-65% of the annual honey production in Greece (Thrasyvoulou 1995). Honeydew produced by hemipteran arthropods such as GPS is an important resource for beekeepers when floral nectar is not available. Although pine honey is an economically important non-wood forest product, its production is often not compatible with timber production (de-Miguel, et al. 2014). Feeding by large populations of the GPS can negatively affect tree hosts, anecdotally leading to severe dehydration and, in extreme cases, tree death (Gallis 2007). In Turkey, excessive feeding by GPS has been shown to have negative impacts on trees between the ages of 40 and 80 years (Yeşíl 2005). Reportedly, rate of volume increment for single trees aged 70 years old can be reduced by up to 2% and by up to 3.4% in entire stands of trees aged 40 years old (Yeşíl 2005), although the history of these infestations is unclear. Feeding by GPS may also indirectly affect hosts by predisposing weakened trees to attack by secondary, opportunistic pests such as such as bark beetles (Coleoptera: Scolytinae) and pathogens (especially fungi) that prefer to attack weakened and stressed trees (Mendel and Liphschitz 1988; Mita 2002).

Negative impacts associated with GPS have mainly been reported from locations where the scale has been introduced to boost honey production, for example in Attica, southern Greece (Gallis 2007; Petrakis 2010). In Attica, GPS associated alterations to pinewood habitat led to the local extinction of many insect species, either directly due to defoliation or indirectly through associated changes in host plant quality and humidity. The scale impacts the water potential of infested pines to such a degree that it can alter associated insect fauna assemblages (Petrakis 2011). GPS deteriorates ecosystem conditions by increasing crown transparency of the dominant pine species. For *Pinus radiata* forests, this would increase ground temperatures and likely deplete soil moisture content (Saremi 2014). The scale reportedly behaves as an invasive insect species in Attica due to the lack of natural enemies (Petrakis 2011).

Potential impacts to Australia's softwood Industry.

In Australia GPS attacks a novel host (*P. radiata*), the dominant species of Australia's soft wood industry. This makes it a threat to Australia's softwood industry (valued at $1.16 billion). Precise economic and environmental impacts of this insect remain unquantified. Although the impact on affected trees is immense, with timber growth on infested trees rendered practically unusable, due to GPS reducing the strength and density of timber growth (Arslan 2022a). Furthermore, non-timber products derived from *Pinus* may also be compromised due to radical changes in host plant phytochemistry associated with GPS (Arslan 2022b). It is highly likely that this insect will affect susceptible trees and plantations in Australia in ways similar to those reported in introduced ranges of Greece and Italy, given the absence of its natural enemies. Most of the impacts observed in Europe (such as reduction in tree volume increment) occur over a long time period (40-80 years), leaving land managers with few management options by the time of detection. As the population density of giant pine scale increases, the subsequent decline in tree heath may increase the impact of opportunistic invaders, such as insect pests (e.g., *Ips grandicollis* (Eichhoff, 1868) and pathogens (e.g., *Sphaeropsis sapinae* and *Botryoshaeria* sp) that are already associated with pine trees in Australia. Giant pine scale may also attack other hosts such as cedar, fir, and spruce, as has been reported in Europe (Tsagkarakis and (Tsagkarakis and Emmanouel 2016; Ünal 2017).

6. Current methods of control of giant pine scale

Currently there are no registered chemical products for controlling GPS in Australia. However, research is underway to identify effective chemicals that can be used to manage this scale. Recommended management practices include creating public awareness, observing strict hygiene practices in infested areas, and destruction of heavily infested trees. If control of potential vector animal species is available, this may assist with mitigating the chance of giant pine scale dispersal. For example, pine trees are popular scratching posts for feral deer, which may have crawlers stick to their fur and transporting them to the next scratching tree. As deer often travel many kilometres in one day, these animals present a reliable and largely undetectable vector opportunity for giant pine scale. Photographs have been captured of deer rubbing against infested trees D. Jaroslow (personal communication Aug 25 2022).

7. Approval of giant pine scale as target for biological control

An application to nominate GPS as a target for biological control was prepared and will be assessed by Plant Health Comittee.

8. Information on relevant legislative controls of the giant pine scale

Following the detection of GPS in south Australia (Adelaide) and Victoria (Melbourne) in 2014, the National Management Group (NMG) approved the National Giant Pine Scale Response Plan (V1.6) in 2015, which aimed to eradicate giant pine scale from Australia. Recommended responses included surveillance (to determine extent of spread and damage), chemical treatment of infested trees, containment of the scale within known infested sites, initiation of scientific studies to understand the biology of giant pine scale under Australian conditions, and engagement of affected stakeholders. In February 2016, a national committee reviewed the recommendations and agreed that eradication of GPS was no longer feasible because:

- destruction of all infested trees was not cost effective,
- chemical control was ineffective,
- there was low confidence in detecting the pest at very low densities, and
- most of the recommended control techniques were viewed as undesirable by the public.

Consequently, a new national response plan was agreed by the NMG in May 2017. The aim of this new response plan (V2.3, March 2017) is to enable an orderly transfer of management of GPS to land managers, landowners and industry. Specific objectives are to acquire, develop and transfer critical knowledge and information that will assist the community, landowners, industry, and other stakeholders limit the future impact of giant pine scale in urban, peri-urban and plantation environments. Activities outlined in this response plan fall under four major themes:

1) coordination and communication,
2) limiting impact on urban communities and minimizing further spread,
3) protecting the plant industry, and
4) complementary activities to acquire critical knowledge (research and analysis).

Subsequently, research and development projects have been initiated with the aim of preparing industry to actively and more effectively manage GPS in the future. Biological control is a cheap and sustainable option that can be used to manage giant pine scale in Australia.

9. History of control of giant pine scale by biological control

Giant pine scale was deliberately introduced to the Italian island of Ischia in the late 1980s. Biological control of GPS was subsequently attempted when the populations reached damaging levels on the island. The predatory fly *N. kartliana* sensu lato was introduced to Ischia in 2006, which led to a dramatic decline of giant pine scale populations (Garonna and Viggiani 2011).

10. Information on the potential biological control agent for giant pine scale

Neoleucopis kartliana sensu lato (see detailed description in section 13) have been reported from several studies as the most abundant prey specific natural enemies of GPS in Turkey and Greece where they keep the population of giant pine scale in check despite high rates of parasitism (Avtzis 2020; Gaimari, et al. 2007; Ülgentürk 2013). The predatory flies are multivoltine, an attribute that considerably increases their chances of adapting to novel environments and to impact several life stages of their univoltine host (Gaimari, et al. 2007; Eleftheriadou, et al. 2022).

Neoleucopis belong to the family Chamaemyiidae which are known to exhibit a high degree of trophic specialization at the generic or sub-generic level (Gaimari and Raspi 2002). Members of this family are commonly used in biological control programs of many other homopterous pests, including adelgids and aphids (Culliney, et al. 1988; Greathead 1995; Mills 1990).

11. Information about the agent species

Taxonomy

Kingdom:	Animalia
Phylum :	Arthropoda
Class :	Insecta
Order :	Diptera

Family : Chamaemyiidae
Genus : *Leucopis*
Subgenus: *Neoleucopis*
Species : n. sp. B.

Synonyms

NA

Common names

Silver flies

12. Agent biology and ecology

Neoleucopis n sp. B. belongs to a recently discovered species complex that comprises three known species: *Neoleucopis kartliana*, *Neoleucopis* n. sp. A, *Neoleucopis* n. sp. B. of which only *N. kartliana* has been described (supplementary material one). Preliminary surveys indicate spatial and temporal niche complementary between the three species. *Neoleucopis kartliana* sensu stricto and *Neoleucopis* n. sp. B are sympatric (both have been collected from northern Greece) while *Neoleucopis* n. sp. A has mainly been collected from southern Greece (supplementary material one). It is likely that *N. kartliana* sensu stricto and *Neoleucopis* n. sp. B., possibly exhibit temporal niche complementarity by emerging at different times of the year (supplementary material one). The three *Neoleucopis* species are morphologically very similar (only distinguishable by microscopic features on male genitalia) which may explain why previous studies have considered them a single species based on morphological identification (Gaimari, et al. 2007; Ülgentürk 2013; Avtzis 2020).

Laboratory and field prey specificity studies have been carried out for *N.* n. sp. B. both in Greece and Australia. However, the bulk of available literature refers to *N. kartliana* sensu lato which we can assume includes *N.* n. sp. B. To avoid confusion, *N.* n. sp. B. is used to refer the species that has been tested while *N. kartliana* sensu lato is used when discussing literature referring to *Neoleucopis kartliana* species complex in general (supplementary material one).

Neoleucopis kartliana sensu lato

Adults are approximately 2.2 -2.5 mm in length (Fig. 4). The flies complete 2-3 generations per year in Greece (Gaimari, et al. 2007) (Table 1). The first adults emerge in late spring (May-June), larvae are present from early spring to early autumn while pupae are present throughout summer and late Autumn and winter. Eggs are oviposited singly on the shoots near a colony of scales or on the ovisac of a GPS. Upon hatching the larvae penetrate GPS ovisacs to feed on the eggs (Fig. 4). The larvae can also feed on all GPS instar stages. *Neoleucopis kartliana* sensu lato are sensitive to temperature, humidity and lighting. Mating and oviposition can be affected if any of the above conditions is out of balance. Predation in the native range is heavily impacted by parasitism especially by parasitiods in the genus *Chartocerus* Motschulsky, 1859 (Hymenoptera: Signiphoridae) (Gaimari, et al. 2007).

Neoleucopis n sp. B.

So far, *Neoleucopis n. sp.* B is only known to occur in northern Greece, undergoes two generations per year with adults emerging from December to April and from August to October (supplementary material one).

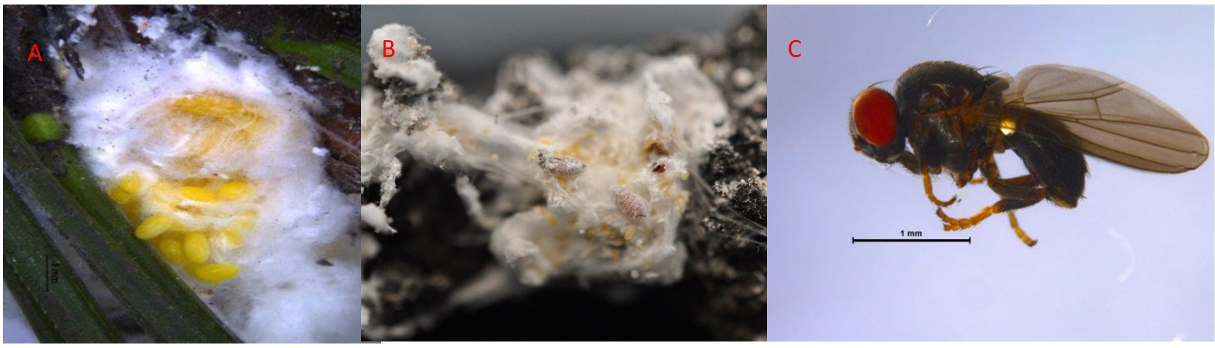

Figure 4 Giant pine scale egg mass (A) Females lay about 130–400 eggs in woolly ovisacs on the bark of host trees. *Neoleucopis* sp lay their eggs into giant pine scale ovisac sacs or next to the sac/feeding nymphs. The fly larvae feed on giant pine scale eggs/nymphs and may pupate inside the woolly ovisacs (B) or in the soil. The adult fly is shown in (C). Photos: Agriculture Victoria.

GPS in Australia	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Season	**summer**	**summer**	**autumn**	**autumn**	**autumn**	**winter**	**winter**	**winter**	**spring**	**spring**	**spring**	**summer**
Adults/ females								■	■	■	■	
Eggs									■	■	■	■
1st Instar crawlers	■	■	■								■	■
2nd instar crawlers	■	■	■	■	■	■						
3rd instar crawlers				■	■	■	■	■	■	■		
GPS in Greece												
Season	**winter**	**winter**	**winter**	**spring**	**spring**	**summer**	**summer**	**summer**	**summer**	**autumn**	**autumn**	**winter**
Adults/ females				🟥	🟥							
Eggs				🟥	🟥	🟥						
1st Instar crawlers				🟥	🟥	🟥	🟥	🟥	🟥			
2nd instar crawlers	🟥								🟥	🟥	🟥	🟥
3rd instar crawlers	🟥	🟥	🟥	🟥							🟥	🟥
***N. kartliana* in Greece**												
Adults					🟪	🟪		🟪	🟪			
Eggs												
Larvae				🟪	🟪	🟪	🟪	🟪	🟪	🟪		
Pupae	🟪	🟪	🟪			🟪	🟪				🟪	🟪

Table 1 Seasonal phenology of giant pine scale in Australia (shown in black) and Greece (shown in red), and *Neoleucopis spp.* In Greece (shown in purple). Adapted from (Eleftheriadou, et al. 2022; Gounari 2004; Gounari 2006; Jaroslow, et al. 2023).

13. Native range and host records

Neoleucopis kartliana sensu lato are reportedly native to the Mediterranean region of Turkey and Greece (Gaimari, et al. 2007). So far, these predatory flies have only been reported to attack giant pine scale in the native and introduced ranges (Avtzis 2020; Gaimari, et al. 2007; Oğuzoğlu, et al. 2021; Üglentürk 2019; Ülgentürk 2013).

14. Related Australia species and summary of their prey range

The Chamaemyiidae family (silver flies) has a worldwide distribution, and in Australia is represented by 38 species distributed between 8 genera. Australian chamaemyiids do not include any native species of the subgenus *Neoleucopis*, however, two *Leucopis* genera are present: *Leucopis (Leucopis) formosana* Hennig has been reported in Western Australia (ALA) and also occurs throughout Asia and the Afrotropics (Tanasijtshuk 1999); *Leucopis (Leucopis) argentata* Heeger was described from New South Wales (Tanasijtshuk 1996). Seven Chamaemyiid genera are native to Australia: *Leucochthiphila, Notochthiphila, Pseudoleucopis,* and *Chaetoleucopis*, are endemic to Australia, and a further three, *Acrometopia, Anochthiphila* and *Leucopis* are also found in other bioregions outside Australia.

Knowledge of the biology of Australian chamaemyiids is incomplete, and mostly indirect, being based on observations of related Northern Hemisphere species. However, while a handful of species can be generalist predators, the majority are prey specific larval predators of Hemipteran insects (Scott Ginn 2010)

15. Source of *Neoleucopis n. sp*. B for prey specificity testing in Australia

Neoleucopis n. sp. B. was sourced from a laboratory culture maintained at Hellenic Agricultural Organization Demeter (HOA) Thessaloniki Greece (Import permit:0006060764). The Greek culture was field collected and reared through one generation in the laboratory to eliminate occasional pupal parasitoids of the genus *Chartocerus* (Gaimari, et al. 2007).

16. Information on non-target organisms at risk from the agent

Rationale for prioritising native scale insects for *Neoleucopis sp.* prey-specificity tests

The Australian scale insect fauna comprises over 850 species (Austin, et al. 2004; García Morales M 2016). The majority of Australian scale insects belong to seven families: Asterolecaniidae, Coccidae, Diaspididae, Eriococcidae sensu lato, Lecanodiaspididae, Monophlebidae and Pseudococcidae ((Austin, et al. 2004; García Morales M 2016). Twelve other less abundant families are represented in Australia each comprising less than 20 described species (García Morales M 2016). Most of Australia’s native scale insect species are very poorly studied. Ecological, life-history and phylogenetic information that is critical to species prioritisation is in most cases unavailable. As such a wholistic approach involving a combination of the three approaches below was used. Details of the prioritisation process are provided (Lubanga et al., in prep).

1) traditional approach (Kuhlmann, et al. 2006)
2) an automated computer-based decision-support tool known as Priority Ranking of Non-Target Invertebrates (PRONTI) (Todd, et al. 2015) and
3) expert knowledge (Lubanga et al., in prep).

The initial prey specificity test list prepared using the traditional approach was expanded following review by six expert entomologists to include 127 native scale insects that were prioritised using PRONTI. PRONTI was developed by Plant and Food Research, New Zealand (Todd, et al. 2015). A comparison of the traditional approach of selecting non-target species (Kuhlmann et al. 2006) with PRONTI concluded that PRONTI could complement traditional approaches by providing transparent evidence to support the selection (or rejection) of non-target species for host/prey-range testing (Barratt, et al. 2016; Withers, et al. 2018). PRONTI ranked native scale insects using five criteria:

1) the potential hazard posed by *N. n. sp.* B. to each non-target species.
2) the potential degree of exposure of each non-target to *N. n. sp.* B.
3) the hypothetical ecological impact that may result from the exposure of the non-target to the hazard posed by *N. n. sp.* B.
4) the estimated economic, social and cultural value of each non-target; and
5) the assessed ability to source each non-target and to conduct tests.

Data on all 127 species of scale insects found in Australia were entered into a data base known as Eco Invertebase. The data included taxonomy, known food species, known predators, dry weight or size, rarity, density, ecology, anthropocentric value, ease of rearing, and status (endemic, native, introduced). Further data on the potential interaction between each non-target species and *N. n. sp.* B. if it were released in Australia were also added to the database. The top 29 species ranked by PRONTI (including the target species GPS) are shown in Table 2.

Table 2 Ranking of closely related scale species native to Australia or present as introduced species. Generated using Priority Ranking of Non-Target Invertebrates (PRONTI) computer model to rank species based on their relatedness and physical similarity to GPS, and their risk of exposure to the agent. * = occurs in Greece , ^ = occurs in Turkey.

Rank	Species Name	Family	Native	PRONTI score	% Uncertainty in ranking
1	***Marchalina hellenica***	**Marchalinidae**	**Introduced (target)**	**13568**	**17**
2	*Melanococcus viridis*	Pseudococcidae	Native	6299	33
3	*Poliaspis syringae*	Diaspididae	Native	6246	42
4	*Pseudoripersia brevipes*	Pseudococcidae	Native	6100	37
5	*Cryptes baccatus*	Coccidae	Native	5998	33
6	*Callococcus acaciae*	Eriococcidae	Native	5632	31
7	*Lobimargo* spp.	Eriococcidae	Native	5295	29
8	*Acanthococcus coriaceus*	Eriococcidae	Native	5171	17
9	*Icerya purchasi**^	Monophlebidae	Native	5121	12
10	*Nipaecoccus aurilanatus*	Pseudococcidae	Introduced	4471	23
11	*Fragorbis pseudopustulans*	Eriococcidae	Native	3943	27
12	*Parasaissetia nigra**^	Coccidae	Introduced	3787	19
13	*Icerya acaciae*	Monophlebidae	Native	3772	19
14	*Tanyscelis maculata*	Eriococcidae	Native	3763	33
15	*Icerya seychellarum*	Monophlebidae	Native	3341	13
16	*Opisthoscelis beardsleyi*	Eriococcidae	Native	3252	23
17	*Icerya* sp.	Monophlebidae	Native	3197	27
18	*Ceroplastes rubens*	Coccidae	Unknown	3178	23
19	*Pseudococcus calceolariae**	Pseudococcidae	Introduced	3048	12

20	*Poliaspis callitris*	Diaspididae	Native	3036	27
21	*Apiomorpha conica*	Eriococcidae	Native	3032	25
22	*Monophlebulus pilosior*	Monophlebidae	Native	3031	17
23	*Tanyscelis tripocula*	Eriococcidae	Native	3019	27
24	*Cylindrococcus spiniferus*	Eriococcidae	Native	2924	27
25	*Rastrococcus stolatus*	Pseudococcidae	Native	2906	33
26	*Parthenolecanium corni*^	Coccidae	Unknown	2903	25
27	*Alecanopsis casuarinae*	Coccidae	Native	2900	31
28	*Callipappus immanis*	Callipappidae	Native	2879	33
39	*Apiomorpha karschi*	Eriococcidae	Native	2826	29

18. Details on the quarantine facility and methods on containment

Neoleucopis n. sp. B was imported from Greece into DEECA's AgriBio quarantine insectary in 2022 according to DAWR quarantine approved directives (DAFF Import Permit: 0006060764; DAFF Testing Permit: PWS2022-AU-000843). All staff involved in the project were accredited and experienced quarantine operators.

19. The proposed source(s) of the agent

Insects were imported to Australia from a lab culture in Thessaloniki, Greece in 2022 and maintained under quarantine conditions at AgriBio, Bundoora, Victoria. A new import from the field would need to be collected and reared through a generation in Greece to establish a culture in Australia for mass rearing for release. Genetic markers established by x study will be used to verify the species identity of the imported agents.

20. Information on non-target organisms at risk from the agent

Native scale insects that are taxonomically closely related to giant pine scale, exhibit morphological and ecological similarities as well as seasonal phenology are most likely to be attacked (Kuhlmann, et al. 2006).

- morphologically similarity = production of white, cotton-like waxy flocculence.
- Ecological similarity = share a similar feeding niche to giant pine scale (I.e., free-living/under-bark stem feeders), and share a similar geographic range to giant pine scale in Australia (Melbourne south-eastern suburbs/near large populations of *Pinus radiata*).
- phenology similarities = when vulnerable life stages (eggs, nymphs occur) of the non-target insects occur when giant pine scale and the predatory fly are active.

There is current no evidence from literature and overseas field and laboratory prey specificity studies that *N. kartliana* sensu lato attacks any other scale insect other than giant pine scale*.*

21. Information and results on any other similar assessments, including environmental risk assessments, undertaken on the species both in Australia and overseas

Neoleucopis kartliana sensu lato was introduced to Ischia in 2006, as a biological control agent of GPS. The fly is now considered established in Ischia with no recorded attacks on any non-target species (Garonna and Viggiani 2011). The fly has tracked GPS to areas within Greece and Turkey where GPS has been deliberately introduced for pine honey production. There are no records of the fly attacking any scale insects other than GPS (Oğuzoğlu, et al. 2021; Üglentürk 2019).

22. Report of prey-specificity testing, including:

Prey specificity studies in the native range (Greece)

The prey-specificity of *N. n. sp.* B. in Greece was studied both in the laboratory and field by (Eleftheriadou et al., in prep, supplementary material three. The identity of *N. n. sp.* B was confirmed based on characteristic microscopic features located on male genitalia (Eleftheriadou et al., in prep, supplementary material three). A summary of these studies is provided here, with details provided in supplementary material three. An Australian native scale insect (*Icerya purchasi* Maskell (Hemiptera, Monophlebidae) that is abundant in Greece and sometimes sympatric with GPS was used to study the prey specificity of *N. n sp.* B in the laboratory and field. *Icerya purchasi* belongs to the scale insect family Monophlebidae which is the most closely related family to Marchalinidae to which GPS belongs (Mills et al., in prep, supplementary material two). *Icerya purchasi* also ranks among the top Australian native scale insects prioritized by PRONTI for prey specificity studies in Australia.

Laboratory trials were carried out studied in the laboratory at the Forest Research Institute of Thessaloniki (H.A.O. Demeter).

i. Laboratory studies: no-choice trials.

Development on *I. purchasi* or GPS

The ability of *N. n. sp.* B. larvae to feed and develop on eggs was studied in a petri dish no choice experiment. *Icerya purchasi* ovisacs were field collected on infested branches of *Pittosporum tobira*. The infested branches were thoroughly examined under a stereoscope to ensure that no other predators or contaminants were present. GPS-infested branches were collected from the suburban forest of Thessaloniki, Kedrinos Lofos, on 13th May 2022. The samples were transferred the laboratory and GPS ovisacs were collected from the samples. The ovisacs were examined under a stereoscope Zeiss Stemi 508 (Zeiss, Oberkochen, Germany, magnification range 6.3–50×). Early instar *N. n. sp.* B. larvae found inside the GPS ovisacs were collected and their length was measured using an AxioCam 208 stereoscope camera software (Zeiss, Oberkochen, Germany, 8.3 megapixels, 4K).

Each *Icerya purchasi* ovisac (n=17) and *N.* n. sp. B.-free GPS ovisacs (n=20) was placed inside a small petri dish (5.4cm diameter). A single N. n. sp. B larva (collected and measured as above) was added to each petri dish.

Ovisacs were monitored daily for potential predatory behaviour, or egg loss. Larval measurements were taken again three days later, on May 16th 2022, to examine whether the larvae fed on GPS eggs and developed, and two days after that, on May 18th to examine whether the larvae fed on *I. icerya purchase* eggs. *Neoleucopis.* n. sp. B larval development, pupation and adult emergence were quantified.

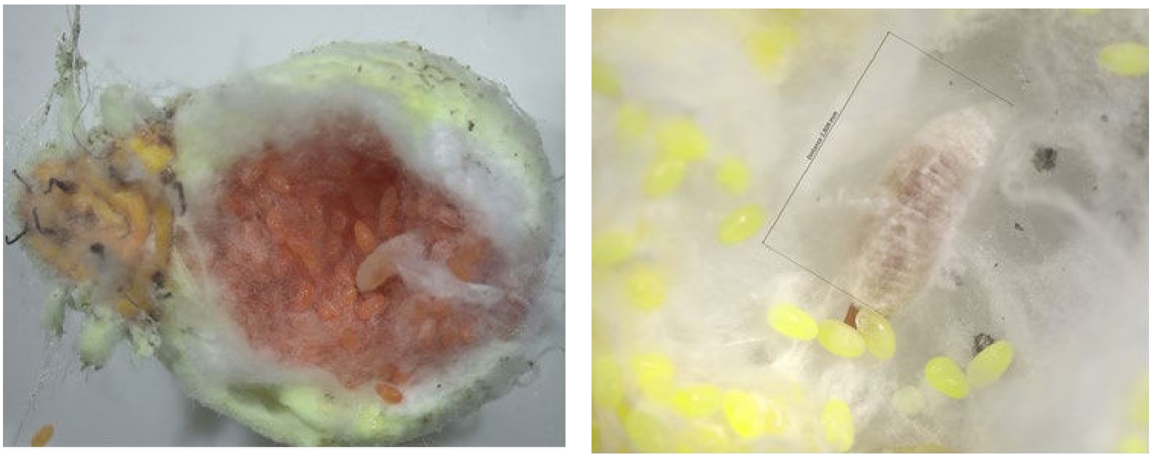

Figure 5. No-choice experiment showing *N. n. sp.* B placed inside an Icerya purchasi ovisac (above left) or a Marchalina hellenica ovisac (above right).

ii.Laboratory studies: choice trials.

The prey preference of late-stage (length ~ 3 mm, n = 20) *N. n. sp.* B. larval instars was investigated in a petri dish choice experiment. Egg masses of *I. purchasi* (n = 20) and GPS (n = 20) were collected as described in section 18.1.1 above. Both *I. purchasi* and GPS egg masses were placed alongside each other in a small petri dish (diameter = 5.4 cm) (Fig. 6). A single *N. n. sp.* B larva was introduced into each petri dish and allowed to choose between *I. purchasi* and GPS egg masses to complete feeding and pupate. *N. n. sp.* B late instar larvae were used in this trial to ensure mobility and choice which may not have been achieved with early instar larvae which are less mobile. Egg mass selection, feeding, pupation and adult emergence were quantified for both treatments.

Figure 6. *Neoleucopis n. sp.* B (middle) provided with a choice of a *M. hellinica* (GPS) ovisac (left) and an *I. purchasi* ovisac (right).

iii.Field trials

To investigate whether *N. n. sp.* B. attacks *I. purchasi* in the field, sites around Thessaloniki (Greece) where both *I. purchasi* and GPS as well as *N. n. sp.* B are known to occur were surveyed at a time of the year when egg masses of both *I. purchasi* and GPS are known to be present and *N. n. sp.* B flies are known to be active. The sites studied comprised of *Pittosporum tobira* plants infested with *I. purchasi* growing adjacent to GPS-infested pine trees. In some cases, *I. purchasi* individuals were recorded attacking adjacent pine trees due to their polyphagous nature. Samples of *P. tobira* branchlets infested with *I. purchasi* were collected and transferred to the laboratory and examined under a stereoscope for *N. n. sp.* B larvae. After inspection, the *P. tobira* samples were kept in small, well aerated cages (30×30×30 cm) and monitored for emergence of adult *N. n. sp.* B flies to detect any *N. n. sp.* B flies missed during sample inspection.

Prey specificity studies in Australia

A large tent choice experiment was set up at in the quarantine laboratory at AgriBio. Ten scale insect species including GPS (on their respective host plants) were presented to recently emerged adult *N. n. sp.* B in a large tent (300 cm x 300 cm x 200 cm) (Fig. 7). The different scale insect species (treatments) were arranged such that they were equi distant from the centre (87 cm) and from one another (53 cm) as shown in Fig. 7. All treatments were rotated one step in a clockwise direction three times a week to minimise position effects. Adult *N. n. sp.* B (n = 4) were introduced at the centre of the tent and allowed to choose between the scale insects for oviposition. The scale insects tested in this trial are shown in Table 3. All test plants were isolated into individual cages and monitored weekly for signs of *N. n. sp.* B larval predation and adult emergence.

Figure 7 Large tent experiment carried out in the quarantine facility at AgriBio containing host plants infested with test species (see Table 3). This set up allowed the *N. n. sp. B.* insects to fly around in the tent and choose between scale insects for oviposition. The insects were provided with sugary water, and an artificial diet that comprised of yeast and honey.

Species	Family	Host plant	Rank on PRONTI list	Justification for testing
Marchalina hellenica	**Marchalinidae**	***Pinus radiata***	1	Target species
Cryptes baccatus	Coccidae	*Acacia dealbata*	4	Distributed in GPS infested localities. Very abundant and easy to rear.
Callococcus acaciae	Eriococcidae	*Kunzea leptospermum*	5	Ecologically and morphological similar to GPS. Very abundant and easy to rear.
Icerya purchasi	Monophlebidae	*Citrus reticulata*	8	Closely related, ecologically and morphological similar to GPS. Very abundant and easy to rear.
Dactylopius coccus	Dactylopiidae	*Opuntia monacantha*	n/a	Safeguard species: biological control agent of Prickly pear.
Dactylopius opuntia	Dactylopiidae	*Opuntia robusta*	109	Safeguard species: biological control agent of *Opuntia robusta*.
unidentified scale	Eriococcidae	*Bursaria spinosa*	n/a	Ecologically similar to GPS. Very abundant and easy to rear.
unidentified scale	Eriococcidae	*Eucalyptus camaldulensis*	n/a	Ecologically and morphological similar to GPS. Very abundant and easy to rear.
unidentified scale	Pseudococcidae	*Acacia paradoxa*	n/a	Ecologically and morphological similar to GPS. Very abundant and easy to rear.
unidentified scale	Pseudococcidae	*Acacia verticillata*	n/a	Ecologically and morphological similar to GPS. Very abundant and easy to rear.

Table 3 Native and economically important scale insects included in the large tent choice experiment carried out in the quarantine facility at AgriBio. Note that most of the species prioritised by PRONTI for testing were not available for testing at testing time. The Justification for the species tested is provided in the first column on the right. Morphological similarity is used here to refer to scale insects that produce white cotton-like wax similar to GPS.

23. Results

Prey specificity studies in the native range (Greece)

i.Laboratory studies: no-choice trials

All *N. n. sp.* B. larvae (n = 20) survived the and completed development to reach the adult stage on GPS. Several larvae were observed preying on GPS eggs, indeed egg loss was notable in every GPS ovisac. In contrast, *N. n. sp.* B larvae feeding on *I. purchasi* experienced high mortality. Ten larvae died before pupation, while seven larvae pupated within *I. purchasi* ovisacs, only three completed developments to the adult stage. Average larval length increased over a three-day period with access to natural prey (GPS) (student t-test: *p* = 0.036, SD = 0.464; df = 47) (Fig. 8). There was no significant change in average larval length over a five-day period (student t-test: p = 0.6013; SD = 0.529; df = 37) (Fig. 9). This implies that these larvae did not grow during this time. In addition, *N. n. sp. B* larvae were not observed preying on *I. purchasi* eggs and they produced red-hued excrement, in contrast with larvae preying on GPS eggs, which produced transparent or yellow-hued excrement.

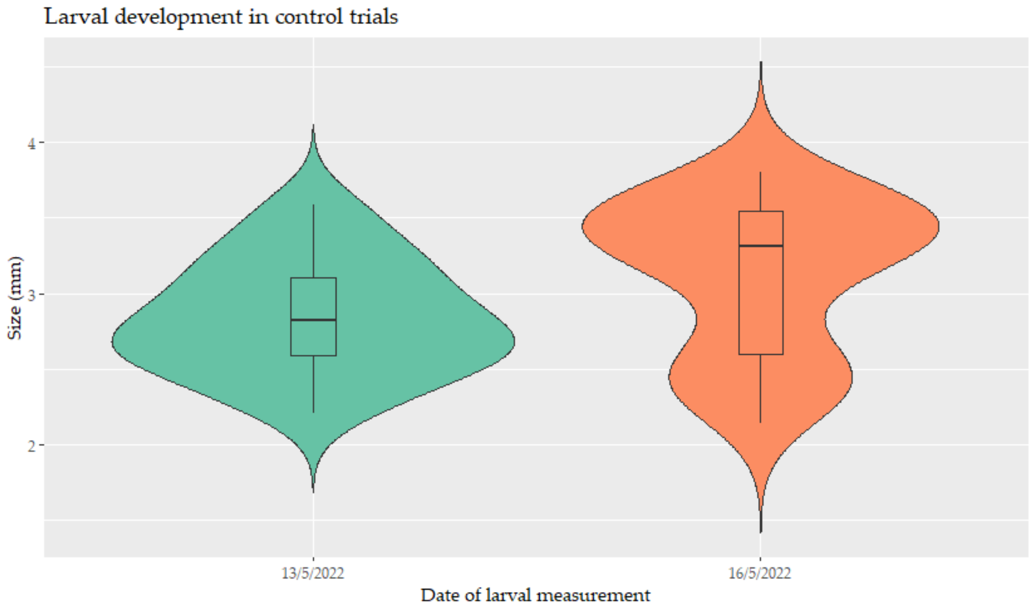

Figure 8. Violin boxplot of the initial size (left) and the final size (right) of *N. n. sp.* B. larvae across 3 days when provided only ovisacs of the target species (GPS) during no-choice trials.

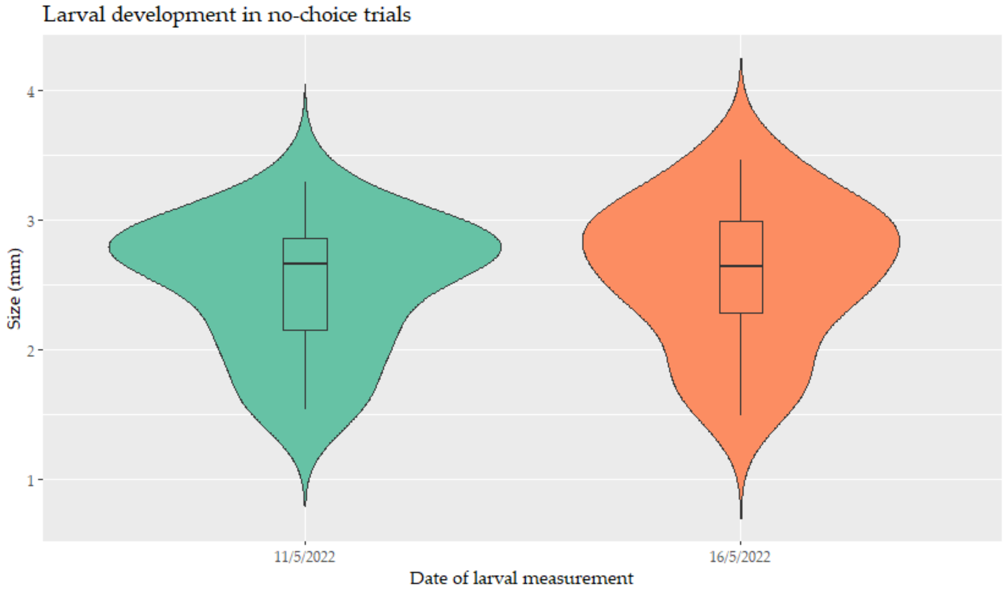

Figure 9. Violin boxplot of the initial size (left) and the final size (right) of *N. n. sp. B* larvae within 5 days when provided only ovisacs of the non-target species (*I. purchasi*) during no-choice trials.

ii. Laboratory studies: choice studies

In total, 13 larvae pupated inside GPS ovisacs while 5 pupated on the petri dish, ignoring the ovisacs, and 2 pupated inside *I. purchasi* ovisacs. All *N. n. sp. B*. larvae (n = 20) survived the choice trials and reached adult stage. Feeding (egg loss) was notable in several GPS ovisacs once the inspection of the larvae was completed.

iii. Field trials

No *N. n. sp.* B. individuals were detected during inspection of the *P. tobira* branches infested by *I. purchasi* that were collected from Thessaloniki on the 11th of May 2022. Accordingly, *P. tobira* branches infested by *I. purchasi* installed in small, well aerated containers did not yield any *N. n. sp. B*. adults.

b. Prey specificity studies in Australia

The life cycle of *N. n. sp. B.* is estimated to last six months (Eleftheriadou personal communication). Since the large tent experiment was set up in mid-January 2023, complete development/adult emergence, if any, is expected by around mid-July. So far, no evidence of *N. n sp.* B larval predation has been recorded on any test species during the weekly inspections, however the number of adults used in this trial was low, and the trial was not replicated.

24. Discussion

Overall*,* results from this study support the contention that *N. n. sp.* B is highly prey specific to GPS (*M. hellenica*). While choice and no-choice laboratory studies showed that a few *N. n. sp.* B. larvae completed development on a non-target Australian native scale insect (*I. purchasi*) in Greece, field studies provide strong evidence that *N n. sp.* B. does not attack *I. purchasi* even when the two species occur in sympatry.

It is important to note that mature *N. n. sp.* B larvae that had partially developed on GPS ovisacs in the field were used in choice and no-choice laboratory studies. It is probable that the few larvae that completed development on *I. purchasi* egg masses in laboratory studies could have obtained sufficient nutrition in the field while feeding on GPS egg masses prior to being used in laboratory trials. The abnormal colour of excrement recorded from *N. n. sp.* B confined on *I. purchasi* egg masses could indicate unfavourable nutrition which may also explain the lack of development recorded from these larvae.

There is currently no evidence of non-target attack from the large tent choice experiment being carried out in Australia. Given the small number of flies tested (n = 4) and lack of development or attack recorded on the target species (GPS), results from this experiment are inconclusive. Further testing involving exposing a larger number of flies to prioritised Australian native scale insects is required to confirm the prey specificity of *N. n. sp.* B. prior to submitting an application for its release.

Overseas host records, including literature and discussions with experts

Several native range surveys record *N. kartliana* sensu lato as an abundant, specific natural enemy of GPS (Avtzis 2020; Lubanga 2018; Oğuzoğlu, et al. 2021; Üglentürk 2019; Ülgentürk 2013). *Neoleucopis kartliana* sensu lato is now considered established on the Italian island of ischia but has not been reported to attack any of the 225 scale insects that occur in Italy (Garonna and Viggiani 2011). Greece is home to over 245 described scale insect species including 40 Coccidae, three Monophlebidae, 65 Pseudococcidae, and 19 Eriococcidae, while Turkey is home to over 450 scale known insects including 65 Coccidae. 3 Monophlebidae, 120 Pseudococcidae, 38 Eriococcidae and 5 Margarodidae (García Morales M 2016). GPS has been intentionally spread by humas to new locations in Greece and Turkey for pine honey production (Tsagkarakis and Emmanouel 2016). While *N. kartliana* sensu lato have managed to track their prey to new locations within the native range, there is currently no evidence of the flies attacking any other scale insect species (Oğuzoğlu, et al. 2021; Üglentürk 2019). Indeed, a survey for pests of scale insects in Turkey's forests did not find any evidence of *N. kartliana* sensu lato attacking any scale insect other than GPS (Üglentürk 2019).

Risk evaluation to non-target species

Giant pine scale is the only known prey of *N. Kartliana* sensu lato reported from several field surveys conducted in the native range of Greece and Turkey (Avtzis 2020; Lubanga 2018; Oğuzoğlu, et al. 2021; Ülgentürk 2013). In Australia, scale insect families that are closely related to GPS and have morphological, ecological and phenological similarities were predicted to be at the highest risk of attack by *N. kartliana* sensu lato (Lubanga et al., in prep). However, results from laboratory studies in Greece showed that *I. purchasi* (an Australian native scale and in the top 10 priority for testing) was not ideal prey for *N n.* sp B. (Eleftheriadou et al., in prep, supplementary material four). Three other scale species (*Parasaissetia nigra*, *Pseudococcus calceolariae* and

Parthenolecanium corni) that were prioritised for testing in Australia (at priorities 12, 19 and 26) also occur in Turkey and Greece but there are no reports of them being attacked by *N. kartliana* sensu lato (Oğuzoğlu, et al. 2021; Üglentürk 2019).

25. Possible interactions, including conflicts with existing biological control programs

Many ant species collect GPS honeydew (Üglentürk 2012), and may also go on to disrupt ecology, a common feature of honeydew exploitation by domineering Hymenoptera ((Gardner-Gee 2013; Helms 2002; O'Brien 2014). It is plausible that honeydew-exploiting Hymenoptera might aggressively defend these resources from introduced biological control agents, although this did not occur in Italy.

26. Information on where, when and how initial releases would be made

If approved for release, a population of *N. n. sp.* B will be field-collected in Greece, reared through a generation in the HAO-Greece laboratory and exported to Australian quarantine. Once in the quarantine laboratory at AgriBio the culture will be reared through a complete generation to ensure that no parasitoids or other contaminants are present. Pending satisfaction of this and other import permit conditions, the insects will be mass-reared at Agriculture Victoria's AgriBio facility. The insects will be initially released at selected sites in the eastern Melbourne suburbs where GPS infestations currently occur. Monitoring and evaluation of establishment, dispersal and impact assessments will be conducted by Agriculture Victoria. Information on whether the agent has established populations in other countries outside its native range, and if so, details of introduction, spread and any non-target impacts recorded.

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28. Supplementary material

a. Supplementary material one: The *Neoleucopis kartliana* species complex

b. Supplementary material two: Phylogenetics of Australian scale insects with respect to the introduced giant pine scale insect *Marchalina hellenica* and biocontrol implications

c. Supplementary material three: Revealing a promising biological control agent against the giant pine scale, *Marchalina hellenica*: Prey-specificity of a new *Neoleucopis* species (Diptera: Chamaemyiidae).

Attachment 3

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Prospects for classical biological control of *Marchalina hellenica* in Australia

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Abstract The giant pine scale, *Marchalina hellenica* Gennadius (Hemiptera: Marchalinidae), is a sap-sucking insect native to South-East Europe that was recently introduced in Australia where it threatens the pine forestry industry. To initiate a classical biological control programme, a literature review and field surveys were carried out in Greece to investigate the natural enemy complex of the scale. While no parasitoids were found, *M. hellenica* was attacked by several predators, most notably *Neoleucopis kartliana* Tanasijtshuk (Diptera: Chamaemyiidae). *N. kartliana* was present at all sites investigated and is presumably specific to *Marchalina.* This highlights its potential as a biological control agent for *M. hellenica*. This predatory fly has already been used to control *M. hellenica* on the Italian island of Ischia. Interestingly, molecular analyses of Greek samples revealed other Chamaemyiidae predatory flies that can be potentially included in the Australian classical biological control programme of *M. hellenica*.

Keywords Biological control · Chamaemyiidae · Marchalinidae · Predatory flies

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Introduction

In late 2014, an exotic scale insect, *Marchalina hellenica* Gennadius (Hemiptera: Coccomoprha, Marchalinidae), commonly known as giant pine scale, was detected in Australia on ornamental pine trees in Adelaide (South Australia) and Melbourne (Victoria) (Semeraro, pers. comm.). This scale insect is native to the eastern Mediterranean region, particularly mainland Greece and Turkey. In these two countries, *M. hellenica* feeds on pines *Pinus* spp., especially *P. brutia* and *P. halepensis*, but it also develops on firs *Abies cephalonica* (Gounari 2006; Bacandritsos et al. 2004; Tsagkarakis and Emmanouel 2016). It has been synonymised with *M. caucasica* Hadzibeyli (Jashenko 1999), which would enlarge its distribution to the Caucasus (Georgia, Russia, Armenia) and its host range to *Picea* spp. However, Hodgson and Gounari (2006) consider *M. hellenica* and *M. caucasica* to be two separate species. This assertion is based on differences in host trees, life history and morphological traits. While adult females and 3rd instar female crawlers of *M. caucasica* and *M. hellenica* appear to be identical, potential significant differences can be seen in the 2nd instars and apterous adult males of both species.

In Greece and Turkey, *M. hellenica* is an economically important insect in the apiculture industry because honeydew excreted by *M. hellenica* is collected by honey bees, which convert it into pine honey (Gounari 2006). This pine honey accounts for around 60–65% of the annual honey production in Greece (Thrasyvoulou and Manikis 1996). Because of its importance in apiculture, *M. hellenica* has been intentionally introduced to new areas, such as mountainous regions, several Greek islands and the Italian island of Ischia (Mendel et al. 2016). In some of its introduced range, *M. hellenica* has become a pest, building to high population densities which have been linked to declines in tree health and a reduction in insect biodiversity (Petrakis et al. 2011; Yeşil et al. 2005).

The biology of *M. hellenica* is described in Priore et al. (1996), Bacandritsos et al. (2004), Gounari (2006) and Hodgson and Gounari (2006). In Europe, *M. hellenica* is univoltine and overwinters as 3rd instars (last nymphal stage) on the bark of conifer stems and branches. Adults, mainly oval shaped females measuring about 8–13 mm long and 4–6 mm wide, occur in spring (March–June). Females predominantly reproduce by parthenogenesis which makes the smaller males, characterised by elongated bodies and dark yellow legs, extremely rare. Currently, little is known about the role of males in *M. hellenica*'s reproduction or conditions under which they are produced. Females, which do not feed, produce a woolly ovisac in which they place their unfertilized eggs for protection until they hatch. Females and males pass through three and four nymphal stages, respectively. The newly hatched nymphs (crawlers) wander in search of cracks and crevices in the bark of host trees to settle. They then cover their bodies with white cottony filaments, initiate feeding on tree sap and gradually develop into 2nd instar nymphs (Gounari 2006). Third instars appear in autumn (September–December) and overwinter (December–March) to become active again in early spring (March–June) when temperatures exceed 5 °C.

In Australia *M. hellenica* was found feeding on a novel host, the North American species *Pinus radiata*. *P. radiata* is the most valuable commercial softwood species in Australia, accounting for about 74.5% of the nation's softwood plantation estate (Downham and Gavran 2017). Softwood plantations are a significant component of Australia's commercial forests, making up 53% of the total plantation area of 1,974,770 ha (Downham and Gavran 2017). These plantations are managed mainly for saw log production. Precise economic and environmental impacts of giant pine *M. hellenica* in Australia remain unquantified. However, high population densities of the scale in Europe have been linked to decline in tree health and insect biodiversity (Yeşil et al. 2005; Petrakis et al. 2011). It is highly likely that this insect will affect susceptible trees and plantations in Australia in ways similar to those reported in Greece, Turkey and Italy given the absence of its natural enemies. Most of the impacts observed in Europe (such as reduction in tree volume increment) occurred over a long period (between 40 and 80 years), leaving land managers with few management options by the time of detection. Several opportunistic pests [e.g. *Ips grandicollis* (Coleoptera: Curculionidae)] and pathogens (e.g. *Sphaeropsis sapinea* and *Botryosphaeria* sp.) are already associated with pine trees in Australia (Wingfield et al. 2001). As the population density of *M. hellenica* increases, leading to decline in tree heath, the impact of these opportunistic invaders may increase. Giant pine scale may also attack other hosts such as fir trees, as has been reported in Greece (Tsagkarakis and Emmanouel 2016).

The distribution of *M. hellenica* in Australia is currently restricted to urban and peri-urban areas around Melbourne but the scale could easily spread to commercial softwood plantations located in suitable localities in south eastern and south western Australia if left un-checked (Fig. 1). The scale poses a significant threat to Australia's soft wood industry because there are currently no effective control options for this invasive pest. Eradication by destruction of infested trees was successful in South Australia but not in Victoria due to widespread infestations (over 4300 trees infested in the south east suburbs of Melbourne). Chemical control using imidacloprid was attempted but treatments were ineffective and live scale persist on treated trees. Research is underway to identify more effective insecticides. However, currently there are no registered chemical products for controlling this insect in Australia.

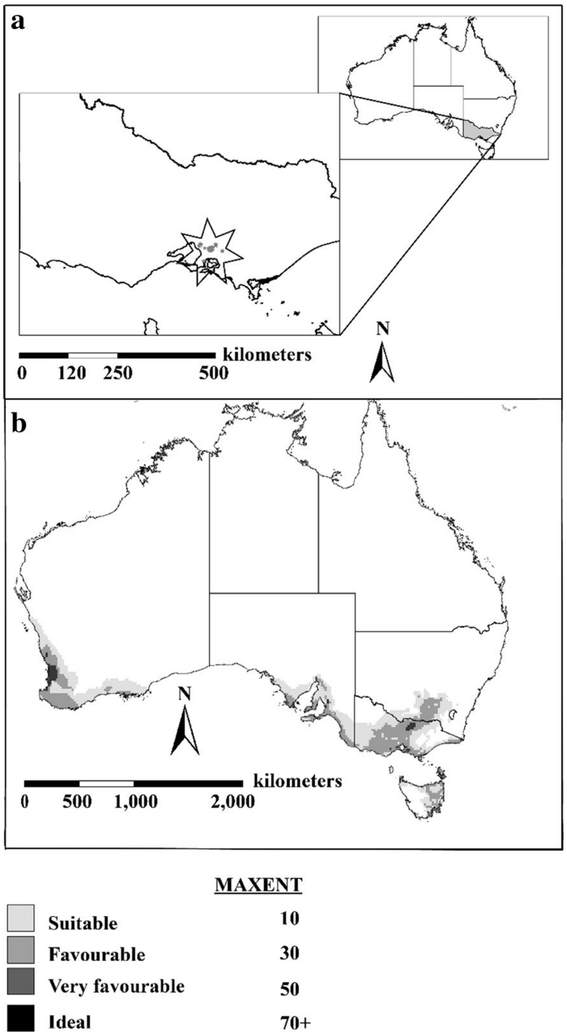

Fig. 1 Distribution of *Marchalina hellenica* in Australia (**a**). Note that the distribution is currently restricted to a few suburbs in Melbourne, Victoria. The predicted distribution of *M. hellenica* in Australia based on climate matching with known locations from Europe (Eastern Mediterranean and Italy) is shown in (**b**). Different shades of grey represent suitability scale for MaxEnt (Lubanga et al. 2018)

Classical biological control, i.e. the introduction of a specialised natural enemy from the region of origin of an invasive pest, can provide an effective, sustainable and long-term control of the pest, provided the biological control agents are carefully selected to prevent effects on non-target organisms in the region of introduction (Hajek et al. 2016). Insect pests of trees in general, and scale insects in particular, are especially suitable for classical biological control (Kenis et al. 2017). The first step of a classical biological control programme involves assessing the natural enemy complex of an invasive pest in its native region through literature and field surveys (Kenis et al. 2017). In this paper, we review published and unpublished work on natural enemies of *M. hellenica* in Europe. We present our novel field observations of natural enemies of the scale in its native range (Greece). We evaluate and prioritise candidate agents in terms of their potential efficacy and host specificity. Finally, we discuss prospects for the biological control of *M. hellenica* in the Australian context.

Material and methods

Literature review on natural enemies

A literature search for natural enemies of *M. hellenica* was carried out using CAB Direct and Google scholar, with the terms "*Marchalina hellenica*" AND ("parasitoid" OR "predator" OR "pathogen"). Grey literature was also searched, particularly from Greece but also from Italy and Turkey.

Survey study areas

To obtain an even more detailed insight into the duration of different development stages of *M. hellenica*, approximately 100 twig per bark samples from Kedrinos Lofos were regularly examined (every 2–3 weeks) at the Laboratory of Forest Entomology (Forest Research Institute, Hellenic Agricultural Organization Demeter) at Vassilika (Thessaloniki, Greece). Samples were then examined under a light microscope and life stage was determined based on descriptions provided by Hodgson and Gounari (2006).

In addition to this regular phenology sampling, two more surveys were carried out in Northern Greece (Central Macedonia Region) starting during autumn (October 2017) and spring (May 2018). These additional surveys aimed not only to gain more information on the abundance and seasonal phenology of *M. hellenica* but mostly to identify the spectrum of its natural enemy species complex before and after the overwintering (3rd) nymphal stage at different sites. *M. hellenica*'s preferred hosts, *Pinus brutia* and *P. halepensis*, were prioritised for sampling at six sites in total during two sampling periods, namely at Serres (autumn 2017), at Kedrinos Lofos and Metamorfosi (autumn 2017 and spring 2018) and at Sani, Edessa and Rizari (spring 2018) (Fig. 2). Site characteristics including altitude, latitude and longitude are summarised in Supplementary Table S1. A predatory fly, *Neoleucopis kartliana* Tanasijtshuk (Diptera: Chamaemyiidae), has already been used to successfully control *M. hellenica* on the Italian island of Ischia (Garonna and Viggiani 2011). As such, the survey prioritised Chamaemyiidae predatory flies to confirm their identity, quantify their abundance and estimate predation rates. Additionally, other potential natural enemies that were found in association with *M. hellenica* were also collected, identified and their abundance was quantified. Approximately 100 samples (twigs, branches and bark pieces infested with *M. hellenica*) were collected from each site. Samples were then transported to the Laboratory of Forest Entomology (Forest Research Institute, Hellenic Agricultural Organization Demeter) at Vassilika (Thessaloniki, Greece) and examined as described before. To estimate the abundance and predation rate of Chamaemyiidae predatory flies, the number of eggs, larvae and puparia found in *M. hellenica* egg masses was counted and expressed as count per 100 nymphs (in autumn) or 100 females (in spring) to draw the comparison between three (Kedrinos Lofos, Serres and Metamorfosi in autumn 2017) and five sites (Kedrinos Lofos, Metamorfosi, Sani, Edessa and Rizari in spring 2018).

Scale insects, and predatory fly larvae and pupae that showed signs of potential parasitism (mummified bodies) were isolated into covered Petri dishes contained in rearing cages to capture emerged parasitoids for identification. All potential natural enemies (but not mites) found in samples were collected and placed in 100% ethanol for further morphological and molecular analyses. DNA was extracted individually from each sample, using the Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) following the manufacturer's instructions, with the only adaptation at the initial grinding phase, which was done with small grinding balls. DNA amplification was run in 25 µl volumes, with HCO-LCO primers pair (Folmer et al.1994) that amplifies a part of cytochrome oxidase I mitochondrial gene. PCR protocol was identical with the one employed by Martinez-Sañudo et al. (2018). Finally, purification was performed using the PureLink PCR purification kit (Invitrogen) following the manufacturer's protocol and sequencing took place at CEMIA SA (Larissa, Greece) using an ABI 3730XL sequencer. Sequences were then visually examined with Chromas Lite and then aligned using Clustal X (Thompson et al. 1997).

Results

Literature review

Generally, data pertaining to natural enemies of *M. hellenica* in its native range are scarce. No information was gathered on parasitoids and pathogens. Published articles on predators and other species associated with *M. hellenica* have been compiled by García Morales et al. (2016) (Supplementary Table S2). By the time of this study, only one extensive survey for predators had been carried out, in Turkey (Ülgentürk et al. 2013). This survey concluded that the most efficient and common predator of *M. hellenica* was *N. kartliana* which was found in sampling sites in Turkish provinces. All the other 12 predatory species (lacewings, ladybirds, anthocorid bugs and mites) were less abundant and known to be polyphagous species.

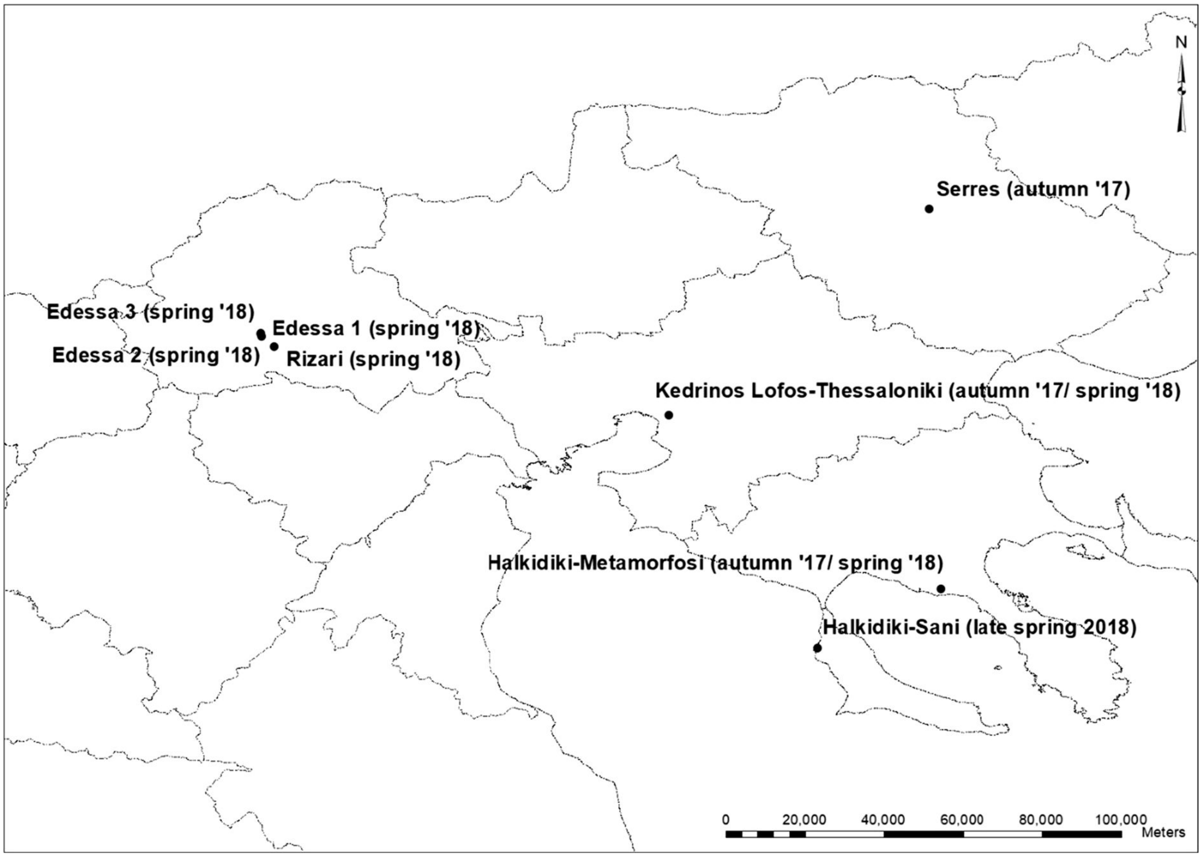

Fig. 2 Sites in Central Macedonia (Greece) surveyed for *M. hellenica* and its natural enemy complex in both sampling periods (autumn 2017 and spring 2018)

Gaimari et al. (2007) and Ülgentürk et al. (2013) provide useful data on predation of *M. hellenica* by *N. kartliana* in Greece and Turkey respectively. Additionally, Gaimari et al. (2007) provides a comprehensive illustration of all *N. kartliana* developmental stages, eggs, larvae and puparia, usually found among *M. hellenica* colonies on the bark of host trees. The larvae feed on all immature stages of *M. hellenica*. *N. kartliana* is presumed to go through two to three generations per year in the region of Athens (Gaimari et al. 2007). The predatory fly overwinters as puparia and adults are known to emerge in early spring (mid-March), and summer (June and August). Puparia are known to be parasitized by an unidentified parasitoid belonging to the genus *Chartocerus.* This parasitoid may negatively impact fly populations, causing *M. hellenica* populations to increase (Gaimari et al. 2007).

Neoleucopis kartliana is apparently specific to *M. hellenica* in Greece and Turkey. However, it was first described from adults reared from a puparium found on the bark of Oriental spruce, *Picea orientalis,* in Georgia, without prey affiliation. Considering that a potentially different species of *Marchalina* (*M. caucasica*) is known to feed on *P. orientalis* in the Caucasus (Hodgson and Gounari 2006, but see Jashenko 1999, who had synonymised the two species), it is possible that *N. kartliana* is genus-specific. In addition, another fly species, *Neoleucopis hadzibeiliae* Tanasijtshuk is known to prey on ovisacs of *M. caucasica* in Georgia (Tanasijtsiiuk 1986). In an older study (Nicolopoulos 1965), the chamaemyiid fly preying on *M. hellenica* in Greece had been identified as *Leucopis obscura* Haliday. However, the taxonomy of this species remains unresolved (Ravn et al. 2013). For example, *Leucopis obscura* may refer to *Neoleucopis obscura* or *N. atratula* (Ratzeburg), both species associated with woolly aphids of the family Adelgidae in Europe. Thus, a misidentification for *N. kartliana* or *N. hadzibeiliae* is likely.

Evidence that *N. kartliana* is an efficient biological control agent is provided by the biological control programme on the Italian island of Ischia, where the predatory fly was used to successfully control *M. hellenica* (Viggiani and Mustica 2008). *M. hellenica* was introduced to Ischia in the 1960s, where it reached very high populations that caused serious damage to susceptible pine trees (Viggiani and Mustica 2008). The predatory fly was released on Ischia in 2006 as a biological control agent, and scale populations apparently declined dramatically following its release (Garonna and Viggiani 2011). However, there are no detailed scientific studies documenting this biological control programme.

Survey results

Abundance and seasonal phenology of M. hellenica

Generally, *M. hellenica* was easy to find in Central Macedonia but, in most pine stands, populations were rather low with mainly single adults or nymphs on the trunks and branches. However, at certain stands (e.g. individual trees in Kedrinos Lofos) some tree trunks and branches were found fully covered with white cotton-like wax secreted by the scale, and dead branches were commonly observed.

The life cycle was rather similar at all investigated sites, albeit slightly delayed at Edessa sites which were situated at higher elevations (400–500 m vs. $<$ 200 m). During the first surveys in autumn (October 2017), 2nd and 3rd instar nymphs were found, mainly on branches. From November 2017 to March 2018, only overwintering 3rd instars were found (Supplementary Figure S1). Early in spring (April 2018), more than 95% of the scales were adult females that had moved to oviposition sites on tree trunks and branches. At Kedrinos Lofos and Metamorfosi 30% and 94% of the females found had started laying eggs, respectively. Late in spring (May 2018), many eggs had already hatched, and first instars were crawling to feeding sites.

Natural enemy complex of M. hellenica in Central Macedonia (Greece)

No parasitized nymphs were found during our surveys. By far the most abundant predators were chamaemyiid flies of the genus *Neoleucopis*, which were found at all sites and all dates. However, molecular analyses of 38 *Neoleucopis* larvae and puparia, all collected at Kedrinos Lofos, revealed that two chamaemyiid fly species coexist, differing by about 10% of their bases. None of them were comparable to sequences in existing international barcode datasets, which do not contain any *Neoleucopis* sp.

Few other predators were found during our surveys, and always in low numbers, even in May (spring 2018) when generalist predators such as ladybirds and lacewings are supposed to be abundant (Supplementary Table S3). Most of these predators were generalist feeders and although they can be identified via morphological and molecular techniques to species level, no additional efforts were made to identify these species since none of them would be considered suitable classical biological control agents (Supplementary Table S3). Caterpillars of at least two Lepidopteran species were commonly found in the cottony secretion, on which they probably feed. The most common species, found at all sites, was a species of the genus *Cadra* (*Ephestia*) (Pyralidae). *Cadra* spp. have been occasionally associated with *M. hellenica* (Nicolopoulos 1965, in García Morales et al. 2016). The second lepidopteran species, *Nemapogon variatella* (Clemens) (Tineidae), is a well-known species that feeds on bracket fungus or dead wood. In addition, less than ten lacewing larvae (Neuroptera) belonging to at least three unidentified species were collected from the samples. Finally, thrips of the subfamily Phlaeothripinae were found at various sites.

Abundance and life cycle of Neoleucopis spp.

Neoleucopis kartliana was present at all sites in the two sampling seasons (autumn 2017 and spring 2018) during which the surveys were carried out. Regular observations between October and May provided insights into the phenology and life cycle of *N. kartliana* during this period (Supplementary Figure S1). In autumn (October 2017), *N. kartliana* life stages were dominated by larvae of various stages feeding on the 3rd instar *M. hellenica*, a few eggs and puparia (Supplementary Figure S1). In winter time (November 2017 to February 2018), many puparia of *N. kartliana* were observed in the vicinity of 3rd instar *M. hellenica* nymphs. In spring time (April 2018), no *N. kartliana* life stages were found. Only empty puparia on branches where *M. hellenica* nymphs had overwintered, suggesting that, at this date, adults were flying and about to oviposit. Later in spring (April–May 2018), eggs and larvae were found in high numbers in *M. hellenica* ovisacs. In late spring (May 2018), mature larvae, full puparia and empty puparia were found in various proportions at all sites. In Edessa, the majority were larvae, whereas, at lower elevation sites, most larvae had already pupated and even emerged into adults.

In autumn (October 2017), the abundance of *N. kartliana* larvae (presented as larvae per 100 *M. hellenica* ovisacs) varied considerably between sites. *N. kartliana* was most abundant at Metamorfosi and Kedrinos Lofos (with around 20 and ten fly individuals per 100 *M. hellenica* nymphs, respectively) and least abundant at Serres, where less than one fly larva per 100 *M. hellenica* nymphs was recorded. Predation was much higher at Kedrinos Lofos and the two other sites. In Metamorfosi, at least one *N. kartliana* was found per five nymphs and, in Kedrinos Lofos, it was about one per ten nymphs. In spring (May 2018), the abundance of the fly was calculated as the number of fly eggs, larvae and pupae per 100 *M. hellenica* females with ovisacs, which reached 125 in Kedrinos Lofos and 73 in Sani (Fig. 3). In Kedrinos Lofos, up to eight *N. kartliana* were found per ovisac and 62% of the ovisacs were attacked by at least one *N. kartliana*. These numbers are very close to those of Gaimari et al. (2007) who, in Athens, found between one and five larvae preying per ovisac and the percentage of ovisacs with at least one larva reached 54%. Later in spring (May 2018), the relative abundance of *N. kartliana* was lower at three other sites, but this is likely due to larvae having already pupated and a proportion of the adults emerging. Indeed, in Metamorfosi, most ovisacs looked heavily damaged by predation in May 2018.

Parasitism of N. kartliana

Puparia of *N. kartliana* were found attacked by at least two parasitoids. Puparia with parasitoid holes were frequently found at different sites in spring (May 2018). We found two Signiphoridae adults walking among *M. hellenica* females and eggs in the cottony secretions (Metamorfosi) suggesting that puparia occurring in summer may contain Signiphoridae, as described by Gaimari et al. (2007). However, the barcoding of 15 overwintering puparia from Kedrinos Lofos collected earlier (winter 2017–2018) yielded three parasitoids of an unidentified species from the Figitidae family, suggesting a parasitism rate of overwintering puparia of about 20% which is in line with the number of empty puparia found with a parasitoid hole in April. Figitidae, in particular of the genus *Melanips*, are known to be larval–pupal parasitoids of *Leucopis* spp. (e.g. Kohler et al. 2008; Pujade-Villar and Vanegas-Rico 2015).

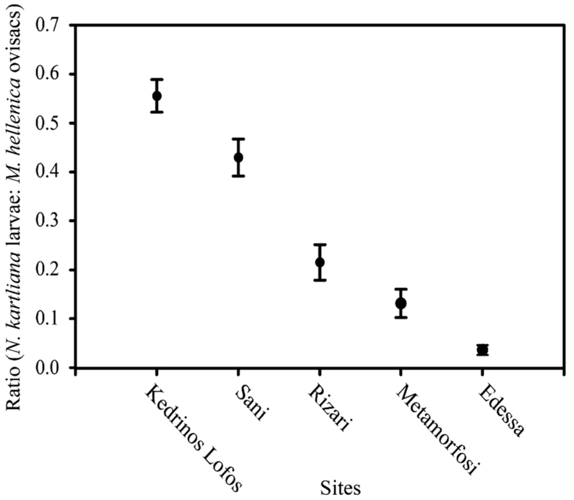

Fig. 3 Total number of *Neoleucopis kartliana* life stages (eggs plus larvae plus puparia) per 100 females as indicated by ovisacs at five sites in Central Macedonia at various dates in spring 2018. Bars represent SE

In Ischia, the fly was introduced without its parasitoids, which would explain the success of the introduction as *N. kartliana* populations are not limited by specialised natural enemies (Viggiani and Mustica 2008). In contrast, population levels of *M. hellenica* in Greece and Turkey are higher because *N. kartliana* population growth is limited by parasitoids (Supplementary Figure S2). Quarantine measures would eliminate parasitoids of *N. kartliana* prior to introduction to Australia.

Discussion

Results from this study suggest that classical biological control is a feasible management option that can be used in an integrated pest management programme to minimize the impact and spread of giant pine scale in Australia. The predatory fly *N. kartliana* was identified as the most suitable agent for introduction in Australia based on its previous success on the Italian island of Ischia and strong climate match between known native range occurrences of both *M. hellenica* and *N. kartliana* and areas of south eastern Australia where susceptible hosts occur. It is likely that other unidentified *Neoleucopis* spp. complement *N. kartliana* to manage *M. hellenica* populations in the native range. Once identified, such species will provide valuable alternatives to *N. kartliana* in the Australian giant pine scale biological control programme. Understanding the biology and complex ecological interactions between *Neoleucopis* spp. as well as their natural enemies (higher order predators and parasitoids) is critical to the success of this biological control programme.

Abundance and seasonal phenology of *M. hellenica*

As expected, *M. hellenica* was commonly encountered in the regular surveys in Thessaloniki, often occurring in what appeared to be patchy spatial distribution patterns similar to those reported by Gounari (2006). Similar *M. hellenica* distribution patterns have also been reported in Australian pine forests (Stephen Elms, pers. comm.). Insect distribution patterns arise as a consequence of complex ecological interactions (biotic and abiotic) that require rigorous ecological studies to tease apart. Nevertheless, it is reasonable to speculate that the patchy distribution of *M. hellenica* observed in this study could be attributed to poor dispersal capabilities of crawlers and natural enemy activity among other factors. The abundance and distribution of *M. hellenica* is also most likely temperature-dependant. Indeed, the development of the scale is delayed at higher altitudes (lower temperatures) e.g. at Edessa with an elevation of 400–500 m, compared to lower altitudes (higher temperatures) vs. at Kedrinos Lofos and Metamorfosi, with average elevations of 200 m. Clearly, further studies are required to understand the ecology, spatial and temporal distribution (i.e. regular, random or patchy) of *M. hellenica* to provide the information required to design effective sampling regimes. Such information will also increase the effectiveness of monitoring scale populations during evaluation of control practices including biological control.

Natural enemy complex of *M. hellenica* in central Macedonia

Marchalina hellenica is attacked by predators belonging to various insect taxa such as Diptera, Coleoptera, Neuroptera and Phlaeothripinae but not by parasitoids. Similar results were reported by Ülgentürk et al. (2013) who showed that, in Turkey, *M. hellenica* is attacked by up to 13 predators distributed in five orders (Acarina, Diptera, Coleoptera, Neuroptera and Hemiptera) but not by parasitoids. With the exception of *Neoleucopis* spp. which were the most abundant predators at all sites surveyed, other predators identified from both studies are not prey-specific and therefore unsuitable for biological control. For example, the lacewing larvae (Neuroptera) and thrips (Phlaeothripinae) we found are known to be generalist predators. Other insects such as the coleopteran adults and larvae of the family Laemophloeidae found during our surveys are predominantly saprophagous insects. While the Acarina can be predators, they occurred in numbers too low to warrant consideration as potential biological control agents.

Abundance and seasonal phenology of *Neoleucopis* spp.

Although this study initially intended to focus on *N. kartliana*, our target was expanded to include other *Neoleucopis* spp. detected via molecular analyses of samples thought to be *N. kartliana. Neoleucopis* spp. were present at all sites surveyed, albeit at varying abundances. Presumably, *N. kartliana* is the more abundant of two *Neoleucopis* spp. The other species remains unidentified but it is likely to be *N. hadzibeiliae* (Tanasijtshuk), a species known to attack *Marchalina* spp. in the Caucasus (Gaimari et al. 2007) or potentially a novel chamaemyiid species. Resolving the identity, ecology, biology and abundance of these two predatory species is critical to future biological control projects targeting *M. hellenica*.

Neoleucopis spp. overwinter as puparia which emerge into adults during early spring. The presence of empty puparia in *M. hellenica* ovisacs was used as a surrogate to indicate presence of adults in the field since adults were not sampled directly. Oviposition by *Neoleucopis* spp. most likely commences in mid-spring which is when eggs and larvae were easily found in *M. hellenica* ovisacs. By late spring, a few empty puparia were found suggesting emergence of a second generation during this time of the year. Although surveys were only carried out during late winter and spring, the phenologies observed during these times are consistent with the seasonal phenology of *N. kartliana* reported by Gaimari et al. (2007). Gaimari et al. (2007) also suggested that *N. kartliana* may go through 2–3 generations per year. Considering that we observed completion of a generation within one month in spring, it is highly likely that the *Neoleucopis* spp. has more than three generations per year. Gaimari et al. (2007) observed larvae preying on young nymphs in early to mid-summer and adults flying in late summer. In Australia, *M. hellenica* eggs can be found in late spring to mid-summer with newly emerged crawlers appearing from summer to mid-autumn. This implies that *M. hellenica* eggs and crawlers will be exposed to *Neoleucopis* larvae from spring to autumn should the predatory flies be introduced in Australia. This prey-predator temporal synchrony in addition to the multivoltine predator vs. univoltine prey scenario is likely to set the stage for suppression of the scale populations by the predatory flies.

Parasitism of *Neoleucopis* spp.

Results from field surveys in Greece indicate that *Neoleucopis* spp. are attacked by a suite of parasitoids belonging to the Signiphoridae and Figitidae families. Most of these parasitoids are yet to be properly identified but the Figitidae, in particular of the genus *Melanips*, are known to be larval–pupal parasitoids of *Leucopis* spp. (e.g. Kohler et al. 2008; Pujade-Villar and Vanegas-Rico 2015). It is common for natural enemies of herbivorous insects not to be at the top of their food chain. As a consequence, their ability to regulate populations of their prey or hosts is often constrained by the action of higher trophic levels (higher order predators and parasitoids) (Harvey et al. 2003). This may explain why *N. kartliana*, when introduced to the Italian island of Ischia where its natural enemies are absent, was able to effectively regulate the population of *M. hellenica*, unlike in Greece and Turkey where its natural enemies are abundant. Quarantine measures to eliminate parasitoids prior to introduction of *Neoleucopis* spp. to Australia (pending regulatory approval) would ensure freedom from any higher-level parasitoids of the predatory fly.

Invasive scale insects of trees are particularly suitable for control using classical biological control (Kenis et al. 2017). Several examples where scale insect pests of trees have been controlled using insect predators exist worldwide. For example, *Icerya purchasi* (Monophlebidae) was successfully controlled in many parts of the world using the vedalia beetle *Rodolia cardinalis* (Coccinellidae) (Hoddle et al. 2013). Another Monophlebidae, *Palaeococcus fuscipennis*, was controlled in Israel through the introduction of a ladybird and a parasitoid (Mendel et al. 1998). Flies of the family Chamaemyiidae have never been used as classical biological control agents against scale insects (with the exception of the poorly documented example in Ischia) but have been successfully released against woolly aphids of the family Adelgidae. For example, pine adelgids (*Pineus* spp.) were controlled successfully in New Zealand, Chile and Hawaii by various species of *Leucopis* (See Mills 1990 and Ravn et al. 2013 for review). Furthermore, there is good evidence that *Neoleucopis* spp. are at least specific to the genus *Marchalina*, increasing the likelihood of meeting Australia's stringent regulatory requirements. Therefore, we consider classical biological control (especially as part of an IPM strategy) to be a feasible option for control of *M. hellenica* in Australia.

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Attachment 3

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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Article

Phenology and Potential Fecundity of *Neoleucopis kartliana* in Greece

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Simple Summary: The silver fly *Neoleucopis kartliana* Tanasijtshuk (Diptera, Chamaemyiidae) is the most abundant predator of the giant pine scale (GPS), *Marchalina hellenica* (Hemiptera, Margarodidae), and is considered a major factor in controlling GPS populations in Greece and Turkey. GPS has recently been detected in Australia. While generally not harmful to trees in its area of origin, GPS has a detrimental impact on pine trees in Australia and, therefore, needs to be controlled. As part of an evaluation of the silver fly for importation to Australia where it may be used as a biological control agent against GPS, we studied several aspects of the fly's life history, namely its seasonal occurrence and number of generations per year (phenology), its acceptance of artificial food sources as adult flies, and the number of eggs females produce over their lifetime. We found that the fly has three generations per year and feeds on all life stages of GPS (eggs, nymphs, and adults). Adults readily feed on a mixture of sugar and dry yeast, and females emerge with no or few eggs and develop more as they age.

Abstract: *Neoleucopis kartliana* Tanasijtshuk (Diptera, Chamaemyiidae) is the most abundant predator of the giant pine scale (GPS), *Marchalina hellenica* (Hemiptera, Margarodidae) in Greece. GPS is native to Greece and Turkey, where it is not considered a pest of *Pinus* spp., but a valuable resource for pine honey production. However, its introduction to new areas leads to high population densities of the scale, linked to declines in tree health and insect biodiversity. To assess the potential use of *N. kartliana* for a classical biological control program in Australia, we studied selected life-history traits of the silver fly, namely its phenology in northern Greece, feeding preferences of adult flies on artificial food sources, and potential fecundity of female flies. The silver fly was present in every site in northern Greece studied and was found to have at least three generations per year in this area. The fly's overall sex ratio was 1:1, and adult females emerged with no or few mature eggs in their ovaries, but egg production was exponential until at least the eighth day after emergence. These findings increase our knowledge about the biology of *N. kartliana* and aided in the evaluation of the silver fly as a classical biological control agent against invasive GPS in Australia.

Keywords: Chamaemyiidae; Margarodidae; voltinism; egg development

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1. Introduction

The family Chamaemyiidae (Diptera) is a group of small flies, commonly known as silver flies, whose larvae prey on sternorrhynchous Hemiptera, particularly adelgids, aphids, mealybugs, scales [1,2], and psyllids [3]. The majority of chamaemyiids have one to three generations [4] and inhabit grassland, lowland, and montane habitats [5]. Larvae feed on adult soft-bodied hemipteran species, as well as on their nymphs and eggs [6], and they either pupariate on twigs and branches where their prey is found [7] or drop from the tree and pupariate in the soil [8]. Regarding the oviposition in Diptera, factors such as environmental temperature [9], quality and quantity of larval [10] and adult diet [11–13], mating [14], adult population density [9,12], age [13,15], photoperiod [16], and relative humidity [17] affect female egg production. Several Chamaemyiidae species have been successfully utilized in classical biological control programs throughout the world, e.g., Hawaii [18], New Zealand [19], and Chile [20,21]. Despite the potential of Chamaemyiidae as biological control agents against soft-bodied hemipteran species, the family has been understudied, and the biology and morphology of many species are not adequately described [1].

Neoleucopis kartliana Tanasijtshuk (Diptera, Chamaemyiidae) has been successfully used as a biological control agent against giant pine scale (GPS), *Marchalina hellenica* (Gennadius) (Hemiptera, Margarodidae) on the island of Ischia (Italy) [22]. GPS is a univoltine sap-sucking insect native to the eastern Mediterranean region, particularly Greece and Turkey. The scale feeds on *Pinus* spp., especially *P. brutia* and *P. halepensis*, but it can also infest *Abies cephalonica* Loudon (Pinales: Pinaceae) [23]. In its native range, it is considered an economically important insect for the apiculture industry rather than a major pest of *Pinus* spp., since it rarely causes tree mortality [24,25]. GPS excretes a sweet, glutinous substance called honeydew, which is collected and converted by bees into pine honey and represents 60–65% of the annual honey production in Greece [23,26]. Due to its importance to apiculture, GPS has been deliberately introduced to new areas of Greece and to the Italian island of Ischia [27], where, on several occasions, it became a pest, reaching high population densities associated with the decline in tree health and reduction in insect biodiversity on pines [25]. In late 2014, GPS was detected in Australia (Melbourne and Adelaide) on a novel host: the North American species *Pinus radiata* D. Don (Pinaceae), which represents 74.5% of the nation's softwood plantation estate [28]. Since its discovery, GPS population densities have increased dramatically, causing significant damage to untreated *P. radiata* in urban and peri-urban settings and threatening the pine forest industry of Australia [29]. The combination of GPS invading a novel environment without its natural enemies and the availability of suitable host trees increases the likelihood of GPS damaging susceptible trees and plantations if not controlled.

Recent research on the scale's natural enemy complex has shown that the silver fly *N. kartliana* is the most abundant predator among the natural enemies of GPS in its native range [29,30], suggesting the potential of *N. kartliana* as a classical biological control agent in Australia [29,31]. The species was previously studied by Gaimari et al. [32], who presented an extensive description of the morphology and biology of the silver fly in southern Greece. Here, we add to the knowledge about the species by (1) investigating the phenology of *N. kartliana* in northern Greece and (2) presenting novel data on the egg development in female flies (egg load or potential fecundity).

2. Materials and Methods

To study the phenology and occurrence of *N. kartliana* in northern Greece, we collected GPS-infested pine tree twigs and branches every 7–10 days between 6 November 2019 and 21 October 2021 from Kedrinos Lofos in Thessaloniki (57 sampling repetitions). No sampling took place between 4 March and 20 May 2020 because of the closure of the laboratory due to the COVID-19 pandemic. Additionally, to investigate the presence of the fly in different regions, we collected infested twigs and branches from eight sites in northern Greece: Stratoni-Stratoniki, Parthenonas, Katerini-Makriyalos, Pyrghetos-Tempi, Edessa (2–3 sampling repetitions each), Arnea, Alexandroupoli, and Thassos (1 sampling repetition each) (Figure 1). The samples were then transferred to the Laboratory of Forest Entomology (Forest Research Institute, Hellenic Agricultural Organization Demeter) at Vassilika

(Thessaloniki, Greece), where random samples of GPS-infested twigs were examined under a stereoscope Zeiss Stemi 508 (Zeiss, Oberkochen, Germany, magnification range 6.3–50×) to determine and count all silver fly and GPS stages present on the twig. The silver fly's developmental stages were evaluated according to the descriptions of Gaimari et al. [32] and the life stage of GPS according to those of Hodgson and Gounari [33]. Additionally, any species found on the infested branches were collected and kept in ethanol for future identification to further contribute to the description of the natural enemy complex of GPS. For a graphical analysis of GPS and *N. kartliana* phenology, the number of individuals per developmental stage and species was calculated as a percentage relative to other stages (n = minimum 100 for GPS).

Figure 1. Sampling locations for *Neoleucopis kartliana* in northern Greece.

For the investigation of selected life-history traits of adult *N. kartliana*, all remaining GPS-infested branches potentially containing *N. kartliana* were transferred to well-ventilated cages (60 × 60 × 60 cm) that were placed inside a climate chamber Termaks KB8400F (Termaks, Bergen, Norway) set to 23 °C and 60% relative humidity. In order to resemble the conditions from dusk to dawn, the climate chamber had a 16:8 h light:dark photoperiod with a gradual transition (lasting one hour) from 0% light to 100% light, and vice versa. The cages were inspected every 1–2 days in search of any *N. kartliana* adults.

To determine the overall sex ratio, emerging *N. kartliana* adults were individually collected in small falcon tubes (5.5 cm length and 1.5 cm diameter), and their sex was identified by visual inspection of their genitalia according to the descriptions provided by Gaimari et. al. [32] using a stereoscope (Zeiss Stemi 508, magnification range 6.3–50×).

To study the acceptance of artificial food sources as substitutes for GPS honeydew, which was presumed to be their natural food source [30] in the manner of other Chamaemyiid species [34], adults (n = 270) were gradually transferred to smaller cages (30 × 30 × 30 cm, mean number of adults per cage 15 ± 5) between 12 August and 5 October 2020. These individuals were provided with water (through a constantly soaked cloth strip laid loosely on a vial) and five different media simultaneously: (1) pine honey; (2) pine honey mixed with dry yeast diluted in water (2 mL:1 gr:100 mL); (3) water-diluted pasteurized milk (50:50) provided through soaked cotton on a petri dish (8.5 cm diameter); (4) dry yeast diluted with sugar; and (5) raw, moist yeast mixed with sugar. Artificial food sources (4) and (5) were both provided in different rates (5–50%) and different liquidity states on cotton laid over petri dishes. All food sources were renewed every 2–3 days, and the behavior of the flies was observed twice per day (morning and noon) every 1–2 days. Cotton was used as a substrate for all artificial food sources to resemble the cotton-like wax excreted by GPS under which the honeydew is naturally produced.

To investigate the development of eggs in the ovaries of adult *N. kartliana* females over time (often called egg load or potential fecundity), branch samples from Kedrinos Lofos (Thessaloniki) were placed in cages and positioned near a natural light source for at least two hours. Thus, emerging flies were attracted to the light source, promptly collected in small falcon tubes (5.5 cm length and 1.5 cm diameter), and isolated in small containers (7 cm height and 5 cm diameter) in which they were provided with water and artificial food source (4) (see paragraph above for more details on the artificial food sources). The containers were placed in a climate chamber with the conditions as described above for infested branches. After 3, 6, or 8 days of rearing, females were killed by placing them into 99% ethanol for several minutes. Flies were then dissected under a microscope, and the eggs were counted either immediately after emergence (n = 25), or 3 (n = 35), 6 (n = 34), and 8 (n = 38) days after emergence. To be considered mature, eggs had to carry the stripe pattern typically visible on oviposited eggs [32], which was visible at 40× magnification, confirming that the eggshell was fully developed.

The influence of female age on the number of mature eggs in the ovary was analyzed using a negative binomial generalized linear model fitted with the *nb.glm* function of the MASS package [35] in R [36]. Female age was taken as a continuous independent variable and the number of eggs as the dependent variable. The Poisson distribution was not used because the residuals were overdispersed, as indicated by Pearson's chi-squared test, which was resolved by using a negative binomial distribution.

3. Results

Neoleucopis kartliana was present at all sites (Figure 1) in this study; however, its abundances varied widely between sites and over the season.

The fly was observed in every subadult developmental stage on the branches (eggs, larvae, puparia). Eggs were usually located inside or close to the cotton-like wax produced by GPS. Larvae were spotted either inside the ovisacs of GPS or close to other developmental stages (first, second, and third instar nymphs and adults). Puparia were found either inside the wax of GPS or in bark crevices, without the presence of GPS being necessary.

The data from Kedrinos Lofos (Thessaloniki) (n = 1124 individuals) suggested that, unlike its univoltine prey, the silver fly has three generations per year in northern Greece (Figure 2). The fly's eggs were found during all developmental stages of GPS. However, the graphical analysis of *N. kartliana*'s generations was based on the relative abundance of larvae and puparia only, as they are greater in size and could therefore be more easily detected compared to the eggs. *Neoleucopis kartliana* larvae were observed feeding on all developmental stages of GPS. In the first *N. kartliana* generation, larvae (young and mature) preyed mostly on GPS eggs and adults; in the second fly generation, larvae preyed on the first-instar nymphs of GPS; while in the third fly generation, larvae preyed on the second- and third-instar nymphs of GPS (Figure 2). Although *N. kartliana* larvae did not extensively prey on the third instar nymphs of GPS due to overwintering as puparia, early emerging larvae of the subsequent fly generation (first) were found preying on third instar nymphs of GPS and remained attached to their prey during the scale's ecdysis.

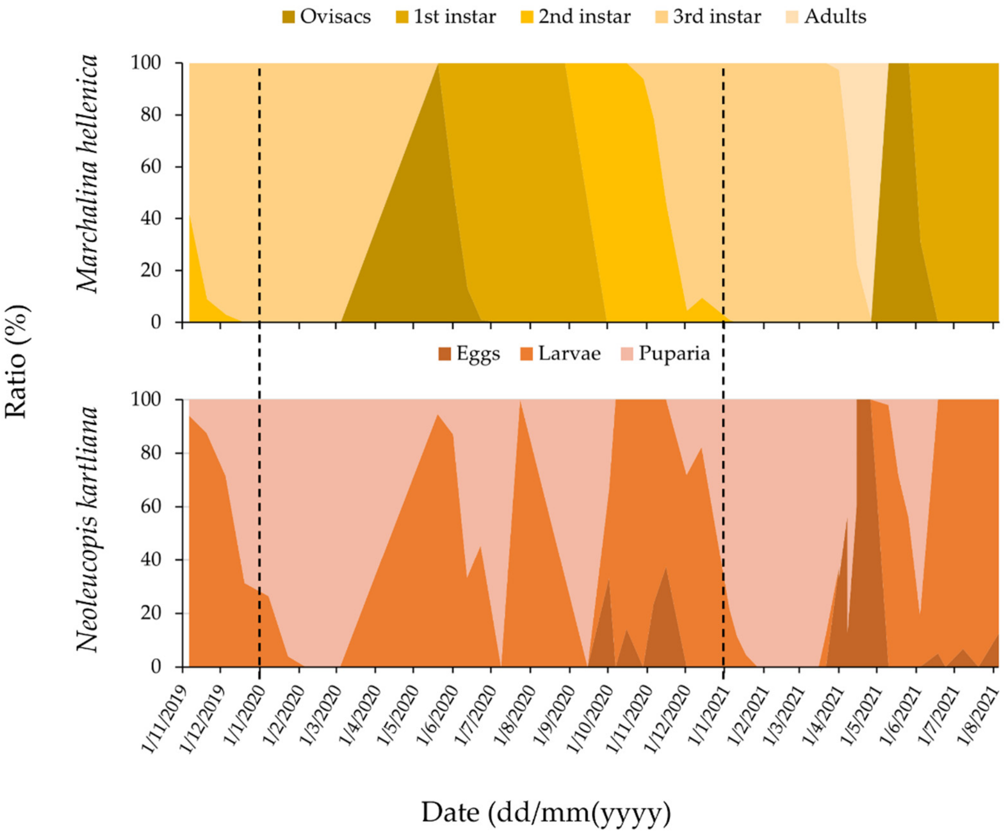

Figure 2. Ratio (%) of different developmental life stages of (upper panel) *Marchalina hellenica* and (lower panel) *Neoleucopis kartliana* in Kedrinos Lofos (Thessaloniki) between November 2019 and August 2021. The area between the two dashed lines is one full year (2020), in which *M. hellenica* underwent one full generation and *N. kartliana* underwent three.

A total of 6031 *N. kartliana* adults were sexed to estimate the overall sex ratio. With 50.8%:49.2% males:females, the sex ratio was almost 1:1, and no apparent difference in the sequence of emergence was observed between the sexes.

Data on the artificial food preference of adults could not be retrieved from this test, because the flies tended to frequently roam inside the cage. However, whenever flies were observed feeding, they were found on only two of the media provided. Adults introduced into the cage containing the different artificial food sources were observed to mainly feed on dry yeast diluted with sugar and, to a lesser extent, on the mixture of honey and yeast. When introduced into the cage, the flies roamed, inspecting the various artificial food sources. However, most flies soon gathered, attached their mouthparts, and fed only on the food sources mentioned above. Adults survived approximately two weeks in captivity with a sole providence of artificial food sources; however, this should not be considered as the fly's lifespan, as it was not estimated individually, but rather in groups of 15 ± 5 adults.

There was a significant effect of female age on the number of mature eggs found in the ovaries ($\chi^2 = 112.77$; df = 1; $p < 0.0001$). Dissections showed that within 24 h of emergence, females had either zero ($n = 21$) or one to two ($n = 4$) eggs in their ovaries. However, until the eighth day after emergence, eggs matured in an exponential manner (Figure 3), and a mean of 25.7 eggs was found, with a maximum of 79 mature eggs found in one female.

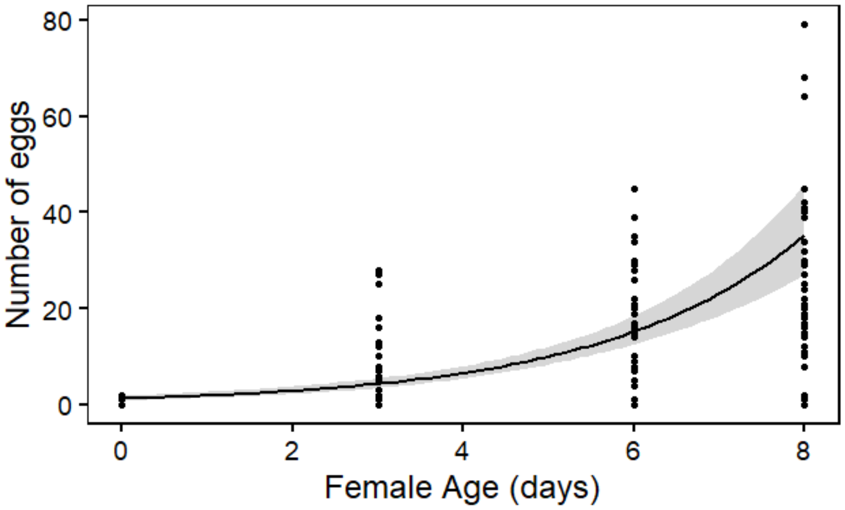

Figure 3. Number of mature eggs in ovaries of *Neoleucopis kartliana* at different ages. The regression line indicates the predictions of the negative binomial generalized linear model that are back-transformed from the log scale. The gray area around the line shows 95% confidence intervals.

4. Discussion

Results from this study support the proposed use of the predatory fly *N. kartliana* as a classical biological control agent to minimize the impact and spread of *M. hellenica* in Australia [17]. The silver fly has a high intrinsic growth rate, allowing it to undergo three generations per year in northern Greece, while its host is univoltine. It seems to prey indiscriminately on every developmental stage of the scale and was found to be present in all sites studied in northern Greece. Furthermore, potential fecundity of the fly was found to increase exponentially in the first eight days after emergence with an average of 25.7 eggs and females holding up to 79 eggs in their ovaries.

N. kartliana appears to have at least three generations per year, confirming the observations of Gaimari et al. [32], who suggested that *N. kartliana* has two to three generations annually. Additionally, other species of the genus *Neoleucopis* have been described to be at least bivoltine, e.g., *N. pinicola* [37] and *N. atratula* [38,39]. Multivoltinism is an attribute that may considerably increase the chances of adaptation to novel environments because it imparts the capability of surviving and reproducing under various environmental conditions [40]. In a study on introduced biological control agents, Crawley et al. [41] found that insects with the highest intrinsic growth rates that typically also had other characteristics of r-selected species (smaller body size and faster maturity resulting in several generations per year) were more likely to establish successfully than those with a slower growth rate. Accordingly, Hokkanen and Sailer [42] suggested that there is a positive correlation between success in biological control and the agent's power of increase over that of the prey, supporting that, in general, successful natural enemies have two generations for every host generation. An example supporting this theory is the parasitoid *Aphytis melinus* (DeBach) (Hymenoptera, Aphelinidae), which was successfully used as a biological control agent against the California red scale, *Aonidiella aurantii* (Maskell) (Hemiptera, Diaspididae), a worldwide pest of citrus [43]. According to Murdoch et al. [43], apart from prey specificity, another key feature leading to the success with this species appears to be the rapid development of *A. melinus* compared to the development of the pest, since the parasitoid has three generations for each scale generation. However, recent research suggests that, when considering the multivoltinism of a biological control agent in a more holistic context of biological control programs, agent-related life-history traits play a rather minor role, compared to those related to how and when agents are released [44]. Nevertheless, we show here that the three generations of *N. kartliana* allow this predator to feed on all life stages of the scale, which would maximize its impact on *M. hellenica* populations. This

Attachment 4

finding confirms those of previous studies on the feeding habits of Chamaemyiidae. For example, Satar et al. listed six *Leucopis* species that were observed preying on several developmental stages of aphids in Turkey [45].

We found that adult *N. kartliana* males and females emerged simultaneously, similar to *Leucopis argenticollis* and *L. piniperda* [46] (later both assigned to a new genus, *Leucotaraxis* [47]). Additionally, our data suggest that *N. kartliana* adults follow the Fisherian sex ratio (1:1) [48], which was also found for *N. pinicola* following laboratory rearing of field-collected puparia [37].

Neoleucopis kartliana adults survived for two weeks in captivity, successfully feeding solely on artificial food sources that consisted of water and a mixture dry yeast and sugar, as was also offered successfully to other silver flies [45]. The adults rejected or showed little interest in the alternative artificial food sources (pine honey, pine honey with dry yeast, milk, and a mixture of raw yeast and sugar). Flies generally require both sugar and protein to fully develop their reproductive systems and produce eggs, and different sources of protein can have various effects on longevity and fertility [12,17]. Although the traditional protein source used for dipteran species is milk powder, yeast could replace milk powder without a considerable loss of viability or egg production [12]. Chamaemyiidae flies are known to feed on honeydew produced by their host [49], which is a sugar-rich secretion of aphids and scale insects [50]. Because it is difficult and laborious to keep honeydew-producing scale insects alive on their host plants (especially when they are trees), replacing the natural food source with artificial ones can increase the efficiency of adult rearing. Gaimari and Turner [1] suggested the use of a mixture of honey and yeast as an artificial diet for adult *Leucopis* spp. While a mixture of sugar and yeast was used as an artificial diet for the *N. kartliana* adults in this study, our results do not contradict those of Gaimari and Turner, since *N. kartliana* adults did feed on the diet suggested by the authors but ultimately preferred the mixture of yeast and sugar. The comparative impact of the different food sources on the longevity and fecundity of *N. kartliana* remains to be studied.

Neoleucopis kartliana is oviparous, corresponding with most dipteran species [51]. Our results showed that females emerge with no or very few (one to two) mature eggs in their ovaries. The few eggs that were found in some freshly emerged females may have been developed in the few hours between emergence and dissection. Clearly, most of *N. kartliana*'s eggs mature after its emergence, making it a strongly synovigenic species (i.e., producing eggs throughout its adult life), which are typically relatively long-lived and dependent on external food sources, as shown for parasitoids [52]. The presence of mature eggs in an ovary is considered as the definitive characteristic for female sexual maturity [13], suggesting that there should only be a very short or no pre-mating period after emergence of females. This study showed that, over the span of 8 days, egg load increased exponentially to a mean of 25.7 eggs and a maximum of 79 mature eggs per female. Possibly, the eighth day after the emergence of *N. kartliana* is the transition point between sexually immature and mature female flies, similar to another Chamaemyiid species, *Leucopis palumbii* Rodani, that reaches sexual maturity at 8–10 days after emergence [53]. While this illustrates a promising intergenerational population growth rate, the silver fly's fecundity in nature has not yet been estimated, and it remains unclear if more eggs can be produced over a female's lifetime and if factors such as mating, food source, or environmental temperature affect egg production and, ultimately, realize the fertility of *N. kartliana*. Nevertheless, the maximum of 79 eggs produced by female flies in captivity signifies prospects for an increase in egg production above the current mean of 25.7 eggs if adjusted methods for rearing are practiced.

5. Conclusions

Phenological observations revealed that *N. kartliana* has at least three generations per year in northern Greece and is preying indiscriminately on every developmental stage of the univoltine GPS. According to laboratory observations, the silver fly is oviparous and produces eggs without mating occurrence, and can survive for about two weeks in captivity when provided with artificial food sources consisting of water, sugar, and dry yeast. Adult females emerge with no or very few mature eggs in their ovaries, after which egg production increases exponentially until at least the eighth day after emergence. This investigation of the silver fly's life-history traits helps to better understand its biology and contribute to its evaluation as a classical biological control agent of the invasive GPS in Australia. However, research on the fly's prey specificity, mating behavior, rearing, as well as definite lifespan and egg load throughout its lifespan remains to be conducted in order to further understand its behavioral ecology and safe use as a biological control agent and to optimize its chances of establishment, as other Chamaemyiid biological control agents have failed to establish in Australia [54].

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Article

Assessing the Prey Specificity of *Neoleucopis* spp. against *Marchalina hellenica*

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Abstract: *Marchalina hellenica* Gennadius (Hemiptera: Marchalinidae) is a scale insect native to Greece and Turkey and presently invasive in Australia, where it damages pine plantations. The silver fly, *Neoleucopis kartliana* Tanasijtshuk (Diptera: Chamaemyiidae), is the most abundant predator of *M. hellenica* in Greece and is presently being investigated as a potential biological control agent following the scale's introduction in Australia. This study, conducted in Northern Greece, revealed the presence of a second lineage, closely related to *N. kartliana*, referred to as *Neoleucopis* n. sp. *B*. Field surveys and laboratory experiments were conducted on *M. hellenica* and a taxonomically related scale insect, *Icerya purchasi* Maskell (Hemiptera: Monophlebidae), to test the larval growth and survival of the flies on the two prey species and assess their specificity for *M. hellenica*. The results suggest that both *Neoleucopis* spp. exhibit a high preference for *M. hellenica* when compared to *I. purchasi*. Larval growth was higher on *M. hellenica* than on *I. purchasi* but the difference was significant for *N. kartliana* only. Survival was significantly higher for both predators when provided *M. hellenica* compared to *I. purchasi*. Field surveys showed that both predators are abundant on *M. hellenica* colonies, whereas none of the two *Neoleucopis* lineages was found to have preyed on *I. purchasi*.

Keywords: silver flies; Marchalinidae; biocontrol; prey selectivity; predators

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1. Introduction

In the pursuit of a sustainable future, the imperative to align human activities with the preservation of ecological integrity has become increasingly prominent [1]. Sustainable development serves as a guiding principle, directing efforts toward meeting current societal needs while also safeguarding the prospects of future generations [2]. This strategic approach entails a delicate equilibrium between economic advancement, societal well-being, and environmental conservation [3]. Sustainable development acknowledges the intricate interplay between ecological health and human prosperity, advocating for a conscientious and responsible utilization of resources [4]. As global challenges, ranging from invasive species to environmental degradation, underscore the need for comprehensive solutions, the commitment to sustainable practices, including biocontrol, remains integral in ensuring the preservation of biodiversity and fostering a resilient, equitable, and enduring future [5,6].

The giant pine scale, *Marchalina hellenica* Gennadius (Hemiptera: Marchalinidae) is a univoltine sap-sucking scale insect native to the eastern Mediterranean region

(Greece and Turkey). *Marchalina hellenica* feeds on the sap of *Pinus* spp. and excretes honeydew, a sweet, glutinous, honey-like substance which is collected by bees and converted into pine honey. Pine honey production represents 60–65% of the annual honey production in Greece [7]. The importance of *M. hellenica* to apiculture, and the fact that it is rarely considered a primary factor in tree mortality [8], has led to its intentional introduction to new regions of Greece, and the island of Ischia (Italy) [9]. In these expanded ranges, *M. hellenica* has occasionally reached high population densities and has been associated with a decline in tree health and a reduction in insect biodiversity in pinewoods [8]. More recently, *M. hellenica* has invaded Croatia [10] and Australia [11]. In these countries, the impacts on tree health can be even more harmful, especially if new host associations are formed. For instance, *M. hellenica* was detected in Melbourne and Adelaide (Australia) in 2014 on a novel and highly susceptible host, *Pinus radiata* [11]. The scale's population rapidly increased and caused notable damage to *P. radiata* and other pine trees in urban and peri-urban settings [12]. Damage to *P. radiata* health is a particular concern, as it is a major component of Australia's softwood plantation estate [11,13]. The repeated invasions of *M. hellenica* underscore the urgent need for a sustainable biological control strategy. Implementing effective measures is crucial in order to mitigate its impact, preserve tree health, and maintain the equilibrium of insect biodiversity within affected ecosystems.

Research on the natural enemy complex of *M. hellenica* suggests that the silver fly *Neoleucopis kartliana* Tanasijtshuk (Diptera: Chamaemyiidae) is the most abundant predator among the scale's natural enemies in its native range (e.g., Greece and Turkey) [11,14]. Chamaemyiidae is a group of small flies that prey as larvae on soft-bodied hemipteran species, particularly aphids, mealybugs, and scales [15]. Nicolopoulos [16] reported that *Neoleucopis obscura* (Haliday) (Diptera: Chamaemyiidae) also attacked *M. hellenica* in Greece. However, it was later suggested that the *N. obscura* recorded in Greece [16] was in fact *Neoleucopis hadzibeiliae* Tanasijtshuk (Diptera: Chamaemyiidae) [17]. Based on the current knowledge, more than one *Neoleucopis* spp. prey on *M. hellenica* in Greece [11]. However, aside from *N. kartliana*, the identity of other *Neoleucopis* species in Greece remains unresolved.

Neoleucopis kartliana was purportedly introduced to the island of Ischia (Italy) for the control of *M. hellenica* [18] and has been proposed for the biological control of *M. hellenica* in Australia [11]. However, to our knowledge, no research has been conducted on the level of specificity of *N. kartliana* or any other *Neoleucopis* spp. preying on the genus *Marchalina*, which includes two known described species, *M. hellenica* and *M. caucasica* Hadzibeyli (Hemiptera: Marchalinidae) [19]. Our study was designed to assess the interaction between *Neoleucopis* spp. and an Australian scale insect species, *Icerya purchasi* Maskell (Hemiptera: Monophlebidae), closely related to *M. hellenica*, as a potential non-target species. Further research on *Neoleucopis* spp. that prey on *M. hellenica* in its native range along with prey specificity testing and risk assessment in both Greece and regions of introduction is necessary before considering *Neoleucopis* spp. for the biological control of *M. hellenica* in Australia or elsewhere.

Icerya purchasi, a native Australian scale, stands out among the Monophlebidae species prioritized for prey specificity testing [20]. *Icerya purchasi* was first recorded in Greece in 1927 and subsequently spread throughout continental Greece, where it is sympatric with *M. hellenica* [11,21]. *Icerya purchasi* belongs to the same superfamily as *M. hellenica* (Coccoidea). The two species also exhibit shared physiological characteristics, including a soft body structure, production of cottony secretions, and similarities in the morphology of their ovisac [19,22]. These attributes, and the presence of *I. purchasi* in areas of Greece where both *M. hellenica* and *Neoleucopis* naturally occur, provide an opportunity to assess its potential non-target impacts on an Australian scale present in the native range of the target pest in both laboratory and field studies. *Icerya purchasi* is notorious for being the target of the first successful classical biological control, when its predator, *Novius cardinalis* (Mulsant) (=*Rodolia cardinalis*) (Coleoptera: Coccinellidae), was introduced in California and successfully controlled its invasive scale in citrus groves [23,24]. The ladybird was later introduced in other parts of the world, including Greece, against *I. purchasi* [25,26].

This investigation carries substantial implications for assessing the risks associated with biological control agents in the context of managing *M. hellenica* in invaded regions. It aligns with the principles of sustainable development by seeking to address the present needs without jeopardizing the ability of future generations to meet their own requirements [2]. Within the scope of our research hypotheses, we examine potential distinctions in (1) the development and survival of *Neoleucopis* spp. when exposed to either *M. hellenica* or *I. purchasi*, and (2) the occurrence of *Neoleucopis* spp. on their natural hosts within the predator's native range.

2. Materials and Methods

2.1. Prey Specificity Experiments

To study the prey specificity of *Neoleucopis* spp. larvae, the host specificity protocol of van Lenteren et al. [27] (small-arena no-choice black-box test) was followed with slight modifications so that it applied to these predatory species. The co-occurrence of the target pest (*M. hellenica*), proposed biocontrol agents (*Neoleucopis* spp.), and a priority non-target Australian scale, *I. purchasi* [20], provided an opportunity to conduct laboratory and field prey range studies in the pest's native range in Greece. *Icerya purchasi* was therefore selected for prey specificity studies in Greece. Exercising the required host plant substrate maintenance and conducting observations on live plants, as stipulated by the established protocol, was not considered essential, given that the selected developmental stage for both the target and non-target species is the egg stage, in which fitness does not depend on feeding. The larval stage of *Neoleucopis* spp. was selected, as it feeds on the eggs of suitable prey during this stage [11,17].

In May 2022 and April 2023, months selected due to the documented presence of *N. kartliana* larvae in the field, as previously reported by Eleftheriadou et al. [28], *M. hellenica*-infested *Pinus brutia* Ten. (Pinales: Pinaceae) branches were collected from the suburban forest of Thessaloniki (Greece) (40°37′58″ N, 22°58′35″ E) and *I. purchasi*-infested *Pittosporum tobira* (Thunb.) W.T. Aiton (Apiales: Pittosporaceae) branches were collected from the city of Thessaloniki, Greece (40°37′34″ N, 22°57′06″ E). The branches were subsequently transferred to the Forest Research Institute of Thessaloniki, Greece (H.A.O. Demeter). *Marchalina hellenica* and *I. purchasi* ovisacs were carefully removed from the branches using soft forceps and inspected under a stereomicroscope to remove any present predators. *Neoleucopis* spp. larvae found inside the *M. hellenica* ovisacs were counted, collected, and individually placed back onto predator-free ovisacs inside Petri dishes (5.4 cm diameter). In 2022, twenty predators were individually assigned to *M. hellenica* to serve as controls (20 replications), and an additional twenty predators were designated for *I. purchasi* (20 replications). The dishes were then placed inside a climate chamber (Termaks KB8400F, Norway) at 23 °C, 60% RH, and a 16 h light/8 h dark photoperiod [28]. The above procedure was replicated once more in 2023, with the implementation of new dishes and *Neoleucopis* spp. larvae and the use of fresh *M. hellenica* and *I. purchasi* eggs. Ovisacs were visually inspected each day to observe predation on *M. hellenica* and *I. purchasi* eggs. Before exposure to prey, the length of *Neoleucopis* spp. larvae was measured using an AxioCam 208 stereoscope camera software Zen core 3.5 (Zeiss, Oberkochen, Germany, 8.3 megapixels, 4K). This process involved gently opening the ovisacs with soft forceps and allowing the larvae to extend their bodies fully before recording the measurements. In 2022, measurements were taken again three days post installation for larvae preying on *M. hellenica* and five days post installation for those preying on *I. purchasi*, and in 2023, three days post installation for all larvae to examine whether the *Neoleucopis* spp. larvae had successfully preyed on eggs and continued their development. In addition to the size increase of larvae, the number of individuals that pupariated and the number that were emerging as adults were recorded. Following the completion of the prey specificity experiments, puparia that did not produce adult specimens were dissected under a Zeiss Stemi 508 stereomicroscope (Zeiss, Oberkochen, Germany) using a scalpel to examine the presence of parasitoids. This examination sought to provide a comprehensive understanding of the factors influencing adult emergence, distinguishing between instances of unfavorable development and instances of parasitism, which could have affected the recorded mortality results. Subsequently, *Neoleucopis* spp. specimens that reached the adult stage were morphologically identified. For *Neoleucopis* spp. individuals that did not reach the adult stage, DNA barcoding was employed for identification, which is described in detail below (Section 2.3—Identification of the Chamaemyiid Species).

2.2. Field Surveys

To investigate whether *Neoleucopis* spp. attack the non-target species when both the target and non-target species are present in their natural habitat, branches of *P. tobira* infested with *I. purchasi* and *P. brutia* branches infested with *M. hellenica* were collected on two occasions, in May and April 2022, in Thessaloniki, Greece. The sampled *P. tobira* and *P. brutia* plants were less than 5 m apart. In addition, lightly infested *P. brutia* and *P. tobira* branches were sampled from several plants in the same area (~5 branches per plant species). The infested branches were transferred to the Forest Research Institute of Thessaloniki, Greece, and were then examined under a stereomicroscope in search of *Neoleucopis* spp. larvae. After inspection, the branches infested with *I. purchasi* were stored in small, ventilated cages (30 cm × 30 cm × 30 cm) inside a climate chamber at the aforementioned conditions to allow sufficient time for *Neoleucopis* spp. to develop to the adult stage and identify potentially undetected specimens. This was not done for branches infested with *M. hellenica*, as the presence of the fly has already been established.

2.3. Identification of the Chamaemyiid Species

Upon the conclusion of the prey specificity experiments, DNA was individually extracted from the *Neoleucopis* spp. specimens that did not reach the adult stage and remained sufficiently intact postmortem to yield viable results using PureLink™ Genomic DNA Mini Kit (ThermoFisher Scientific, Life Sciences Solutions, Carlsbad, CA, USA) following the manufacturer's protocol. DNA amplification was then performed in 25 µL volumes with HCO/LCO primers that amplify a fragment of the Cytochrome Oxidase One (COI) mitochondrial gene and with MyTaq™ Red Mix (BioLine GmbH, Luckenwalde, Germany). The thermal cycling conditions consisted of an initial denaturation step of 5 min at 96 °C, followed by 4 cycles of 60 s at 96 °C (denaturation), 60 s at 47 °C (annealing), and 60 s at 72 °C (extension). This loop was then followed by 35 additional cycles of 60 s at 96 °C, 60 s at 50 °C (annealing), and 60 s at 72 °C (extension). The final extension period was performed at 72 °C for 5 min [11]. The purification of PCR products was performed with PureLink™ PCR Purification Kit (ThermoFisher Scientific, Life Sciences Solutions, Carlsbad, CA, USA) following the manufacturer's protocol. Sequencing took place at CEMIA SA (Larissa, Greece) using an ABI 3730XL sequencer (ThermoFisher, Waltham, MA, USA). The obtained sequences were manually analyzed using Chromas Lite software version 2.01, aligned using Clustal X, and then blasted in NCBI GenBank. The morphological identification of *N. kartliana* adults and its distinction from different species was based on distinct characters of the male genitalia according to Gaimari et al. [17]. The molecular analyses revealed the presence of two *Neoleucopis* spp., *N. kartliana* and *Neoleucopis* n. sp. *B* (see Results).

2.4. Data Analysis

Analysis was conducted using R Statistical Software 4.2.2. [29]. Using the *glm* function of the stats package in R, two separate logistic regressions with binomial distribution were performed to test the influence of the two explanatory variables of prey (*M. hellenica* and *I. purchasi*) and predator (*N. kartliana* and *Neoleucopis* n. sp. *B*) on the predator's survival (live, dead) and development (increase in size or no increase). Tukey's HSD test at $p = 0.05$ was employed to compare multiple means.

Additionally, a regression-type approach was employed to explore the survival and growth dynamics of *Neoleucopis* n. sp. *B* and *N. kartliana*, examining their relationship with the potential explanatory variables of prey source (*M. hellenica* and *I. purchasi*). Differences in survival and growth between *Neoleucopis* n. sp. *B* and *N. kartliana* were assessed by incorporating the categorical variable of *Neoleucopis* lineage into the regression models. Binomial logistic regression models were utilized to link the dichotomous response variables of "survival" and "growth" to the explanatory variables of interest. Covariate selection was conducted via a backward stepwise approach to identify the best-fitting models that explain variations in survival and growth. Model selection was guided by the Akaike information criterion (AIC), with the preferred model demonstrating the lowest AIC value.

3. Results

3.1. Identification of the Chamaemyiid Species

Genetic analysis of *Neoleucopis* spp. individuals involved in the prey specificity experiments suggested the presence of two *Neoleucopis* spp., *N. kartliana* (n = 43) and possibly a different species, hereafter named *Neoleucopis* n. sp. *B* (n = 37) (intraspecific genetic distance = 5.2%). Additionally, these two *Neoleucopis* spp. display distinct morphological differences in their male terminalia, with the most notable distinctions observed in the epandrium and surstylus (Figure 1). Both DNA barcoding and morphological identification of the specimens used for the prey specificity experiments in 2022 showed that three individuals were *N. kartliana* and the remaining thirty-seven belonged to *Neoleucopis* n. sp. *B*, while in 2023, all forty individuals were *N. kartliana*.

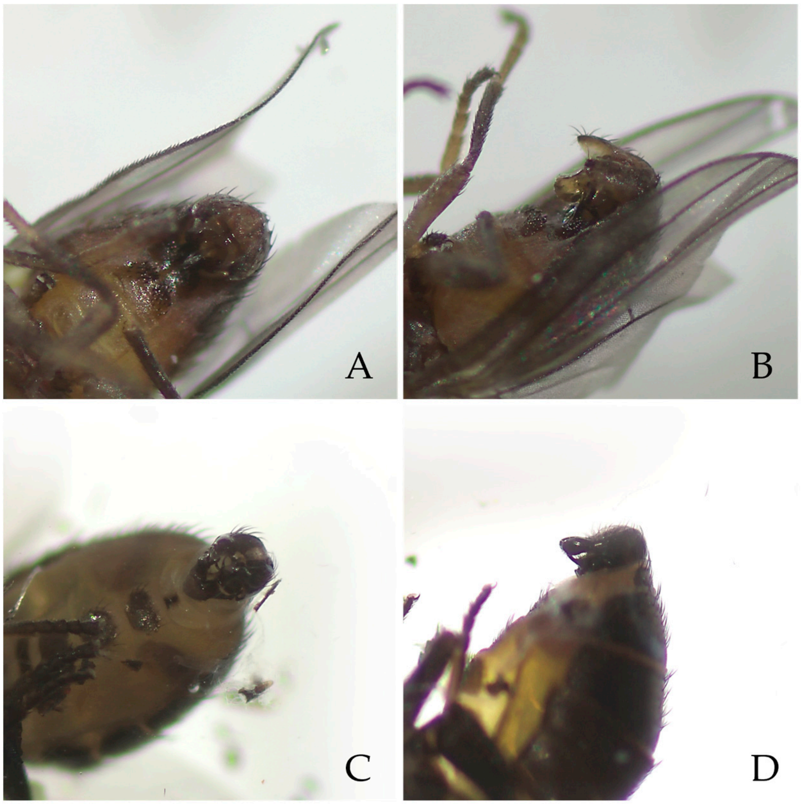

Figure 1. Anterior (left) and lateral (right) view of *N. kartliana* (**A**,**B**) and *Neoleucopis* n. sp. *B* (**C**,**D**) male terminalia.

Attachment 5

3.2. Prey Specificity Experiments

In the controls, several *Neoleucopis* spp. larvae were observed to prey on *M. hellenica* eggs. In both 2022 and 2023, egg loss was notable in every *M. hellenica* ovisac once the inspection of the larvae was completed. During prey specificity experiments in 2022, *Neoleucopis* spp. larvae were not witnessed preying on the eggs of *I. purchasi*; however, they produced red-hued excrements, in contrast to larvae preying on *M. hellenica* eggs, which produced transparent or yellow-hued excrements. It is important to note that the quantification of egg predation or direct observation of predation was not within the scope of the present study. No parasitoid was encountered during the inspection of the *Neoleucopis* spp. puparia after the completion of the experiments.

Regarding larval growth, a significant effect was demonstrated for the prey species, as well as for the predator × prey species interaction (Table 1). While *N. kartliana* exhibited significantly lower growth on the non-target species, *I. purchasi* (17.4%), compared to the target species, *M. hellenica* (95%), the difference was not significant for *Neoleucopis* n. sp. *B* (58.8% grew on the non-target vs. 80% on the target species) (Figure 2, Table S1).

Table 1. Analysis of deviance for the results of the logistic regressions analyzing the effect of predator, prey, and their interaction on larval growth and survival to the adult stage.

Explanatory Variable	χ^2	**df**	*p*
Larval growth			
Predator	1.966	1, 76	0.1609
Prey	24.463	1, 76	<0.0001 *
Predator × Prey	7.726	1, 76	0.0054 *
Survival to adult			
Predator	0.407	1, 76	0.5236
Prey	64.548	1, 76	<0.0001 *
Predator × Prey	<0.001	1, 76	1.0000

Asterisks declare significant difference.

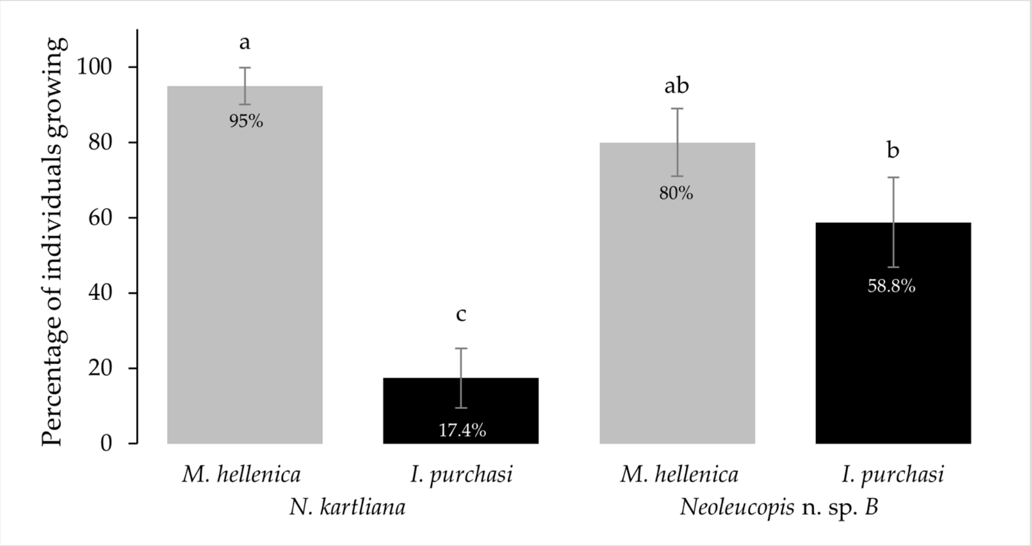

Figure 2. Bar plot of the percentage (means ± SE) of *N. kartliana* (left) and *Neoleucopis* n. sp. *B* individuals (right) growing when provided only the non-target scale insect (*I. purchasi*) (black bars) and only the target scale insect (*M. hellenica*) (grey bars) as food sources during prey specificity experiments. Error bars denoting standard error are incorporated, and significance levels are indicated. Means denoted by the same letter are not significantly different (Tukey's HSD test at $p = 0.05$).

Conversely, only the prey species demonstrated a significant effect on the survival of *Neoleucopis* spp., and no significant interaction effect was observed on survival between "predator" and "prey" (Table 1). Both predators displayed significantly higher survival on the target species (100%) compared to the non-target (*Neoleucopis* n. sp. *B*: 17.65%, *N. kartliana*: 26.09%) (Figure 3, Table S1).

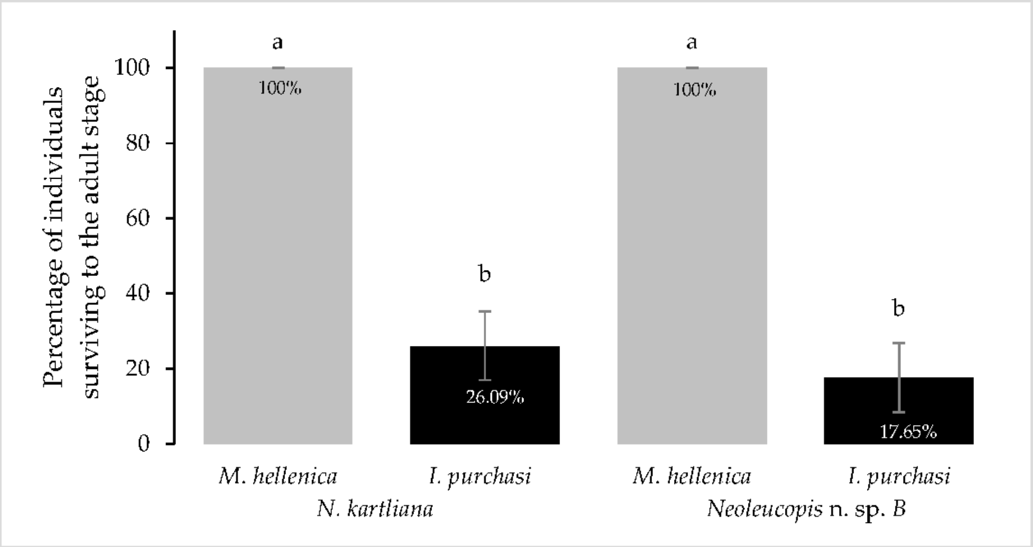

Figure 3. Bar plot of the percentage (means ± SE) of *N. kartliana* (left) and *Neoleucopis* n. sp. *B* individuals (right) that survived to the adult stage when provided only the non-target scale insect as a food source (*I. purchasi*) (black bars) and only the target scale insect (*M. hellenica*) (grey bars) during prey specificity experiments. Error bars denoting standard error are incorporated, and significance levels are indicated. Means denoted by the same letter are not significantly different (Tukey's HSD test at $p = 0.05$).

The parameter estimates of the two logistic regression models performed indicated that the food source significantly influences survival, with *M. hellenica* showing a notably positive effect compared to *I. purchasi*, enhancing the probability of survival for the *Neoleucopis* lineages tested. Specifically, the odds of survival were 6.166 times higher when provided with *M. hellenica* compared to *I. purchasi* (beta coefficient = 1.818; $p < 0.001$; odds ratio = 6.166; 95% confidence interval: 2.823–16.202) (Table 2).

Table 2. Parameter estimates of the binomial logistic regression models upon applying the backward elimination technique and retaining only the statistically significant independent variables for the responses of survival and growth of *Neoleucopis* spp.

Response	Covariate	Estimate	Significance	Odds Ratio	95% Confidence Interval of Odds Ratio
Survival	Intercept	−1.819	<0.001 *	0.162	(0.064, 0.328)
			Food source (ref. category: *I. purchasi*)		
	M. hellenica	1.818	<0.001 *	6.166	(2.823, 16.202)
Growth	Intercept	−0.752	0.003 *	0.471	(0.274, 0.760)
			Neoleucopis spp. (ref. category: *N. kartliana*)		
	Neoleucopis n. sp. *B*	0.450	0.082	1.568	(0.949, 2.635)
			Food source (ref. category: *I. purchasi*)		
	M. hellenica	0.501	0.058	1.615	(0.991, 2.825)

Asterisks declare significant differences.

In terms of larval growth, both the *Neoleucopis* lineage and the food source were found to have marginally significant effects. *Neoleucopis* n. sp. *B* exhibited a slightly higher probability of growth compared to the reference lineage, *N. kartliana*, with an odds ratio of 1.568 (beta coefficient = 0.450; $p < 0.1$; odds ratio = 1.568; 95% confidence interval: 0.949–2.635). Similarly, *M. hellenica* as a food source was associated with a greater likelihood of growth compared to *I. purchasi*, with an odds ratio of 1.615 (beta coefficient = 0.501; $p < 0.1$; odds ratio = 1.615; 95% confidence interval: 0.949–2.635) (Table 2).

3.3. Field Surveys

No *Neoleucopis* spp. were detected during the inspection of *P. tobira* branches infested with *I. purchasi* collected from the field containing 89 ovisacs. Furthermore, *P. tobira* branches, hosting *I. purchasi* and placed in small, well-aerated containers, failed to yield any *Neoleucopis* n. sp. *B* or *N. kartliana* adults upon examination. In stark contrast, branches infested with *M. hellenica*, sourced from the same location and time bearing 24 ovisacs, revealed a notable presence of 25 *Neoleucopis* spp. larvae upon visual sample inspection (Figure S1, Table S2).

4. Discussion

Although the integration of molecular tools has greatly contributed to the initial detection of cryptic speciation that may ultimately lead to the description of new species [30], conclusions should always be drawn with cautiousness for multiple reasons [31]. The wide range of average intraspecific pairwise nucleotide differences recovered for many species does not support the occurrence of universal numerical thresholds beyond which species could be delimited solely by DNA barcoding [32,33]. Additionally, inferences based only on a single marker, most commonly a mtDNA marker, can at times be misleading [34]. In the current study, pairwise nucleotide differences between *Neoleucopis kartliana* and *Neoleucopis* n. sp. *B* exhibited an average value of 5.2%. This, coupled with the distinct morphological differences observed in the male terminalia, raises questions on their taxonomic status. Nevertheless, the distinction between the two *Neoleucopis* lineages studied here and the identification of *Neoleucopis* n. sp. *B* fall beyond the scope of this research.

The prey preference exhibited by both *Neoleucopis* spp. (*N. kartliana* and *Neoleucopis* n. sp. *B*) in our experiments is evident in their marked preference towards *M. hellenica* eggs compared to the eggs of the non-target species, *I. purchasi*, revealing a selective feeding behavior. This pronounced preference is reflected across various aspects of the parameters that were studied. Firstly, during the prey specificity experiments, the larvae of *Neoleucopis* n. sp. *B* and *N. kartliana* were observed to prey exclusively upon *M. hellenica* eggs, demonstrating a preference for this target species. In contrast, a notable absence of feeding on *I. purchasi* eggs by either predator further underscores the probability of their prey selection. In previous research, *Leucopina bellula* (Williston) (Diptera: Chamaemyiidae) demonstrated similar results when tested for its predation behavior on both target (*Dactylopius opuntiae* (Cockerell) (Hemiptera: Dactylopiidae)) and non-target insect species (*Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae), *I. purchasi*, *Icerya seychellarum* (Westwood) (Hemiptera: Monophlebidae), *Phenacoccus solenopsis* Tinsley, *Planococcus citri* Risso, and *Pseudococcus viburni* Signoret (Hemiptera: Pseudococcidae)) [35]. The results suggested that no immature specimens preyed or developed on these non-target species. However, *L. bellula* larvae successfully preyed and developed successfully on the target insect *D. opuntiae* [35].

Secondly, *N. kartliana* exhibited a significantly higher probability of growth when feeding on the target species compared to when it was supplied with the non-target species. However, a higher probability of growth on the target species was not significant for *Neoleucopis* n. sp. *B*. The larvae of the latter predator produced red-hued excrements when provided with the non-target species, in contrast to the transparent or yellow-hued excrements produced when preying on the target species. This hue was likely due to the body pigmentation of *I. purchasi*, suggesting that the larvae had preyed upon the non-target species. These red-hued excrements were not produced by *N. kartliana*, suggesting that *N. kartliana* had not fed upon *I. purchasi.* The dietary preferences of insects encompass a wide array of food sources, leading to diverse fecal compositions [36,37]. It is expected that the form, texture, and color of fecal matter would vary in response to changes in an insect's diet, with successive pellets from the same individual potentially exhibiting alterations based on recent meals [37]. The assumption that excrements display the coloration of consumed prey after feeding has also been considered for *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) when feeding upon nymphs and adults of *Adelges tsugae* Annand (Hemiptera: Adelgidae) [38]. The fact that the probability of *Neoleucopis* n. sp. *B* growth was not significantly different between the target and non-target prey may also suggest a certain degree of feeding on the non-target species, underscoring the intricate dynamics of predator–prey interactions. Nevertheless, it has previously been noted that irrespective of whether growth manifests as continuous or discontinuous, the alignment between consumption rates and growth rates within an instar is typically not closely observed [39]. For example, Zheng et al. [40] subjected larvae of *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) to varying dietary regimes involving optimal or suboptimal quantities of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs across different larval instars. The results revealed that larvae with suboptimal food supplies during the first instar exhibited significantly prolonged developmental times, reduced weight gain, and a marginally lower efficiency of food conversion to body substance compared to those with optimal diets. Conversely, suboptimally fed second instar larvae experienced slightly prolonged development but demonstrated a similar efficiency of food conversion to body substance values to optimally fed counterparts [40].

Thirdly, when feeding on *M. hellenica*, the survival rate of both *Neoleucopis* spp. reached 100%. In contrast, the survival rate was significantly lower when larvae fed on the non-target species. The emergence of some *Neoleucopis* n. sp. *B* and *N. kartliana* adults when exclusively provided with *I. purchasi* raises intriguing considerations. This phenomenon could potentially be ascribed to the larvae being initially collected from *M. hellenica* ovisacs; therefore, they might have been supplied with enough of their natural food source (*M. hellenica*) before the start of the experiments to reach the minimal viable weight for reaching the adult stage [41], suggesting a carryover effect from their natural food source. Should *I. purchasi* be deemed an unsuitable food source for *Neoleucopis* spp., it is plausible that starvation could yield comparable outcomes. In early investigations regarding the dietary requirements for reaching critical and minimal viable weight, Beadle et al. [42] documented that *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) larvae, if deprived of food prior to 70 h after egg laying (AEL), exhibited stunted growth, failed to undergo metamorphosis, and eventually perished several days into the starvation period. Conversely, larvae subjected to starvation after the 70 h AEL mark remained stunted in growth but underwent metamorphosis, resulting in the emergence of diminutive adults. The demise of larvae starved before the 70 h AEL threshold was attributed to their failure to attain minimal viable weight, indicating insufficient body fat reserves necessary for survival through the metamorphic process [42]. Park et al. [43] investigated the effect of starvation on *Hermetia illucens* (L.) (Diptera: Stratiomyidae) larvae following 5- and 10-day feeding periods. They found that larvae subjected to different feeding durations exhibited distinct survival patterns during starvation. The group that was fed for 5 days and then starved showed sustained survival until approximately 20 days of starvation, followed by a rapid decline. Conversely, the group that was fed for 10 days and then starved experienced a sharp decrease in survival after 20 days of starvation, with a gradual decline thereafter over the 60-day observation period. The authors suggested that longer feeding periods may lead to larger energy reserves, extending survival duration. Nonetheless, the emergence rate for all groups exceeded 96%, indicating a successful completion of the life cycle regardless of starvation conditions [43]. This phenomenon prompts further exploration into the intricate ecological dynamics influencing the survival and developmental stages of *Neoleucopis* spp.

Numerous Chamaemyiidae species seek prey within confined spaces inaccessible to other predators, such as within densely wax-coated substrates, to find the housed aphidoid and coccoid colonies. In contrast, other chamaemyiids, such as those within *Leucopis* sensu stricto, exhibit broader feeding strategies [44]. The genera *Leucopis*, *Neoleucopis*, *Lipoleucopis*, *Cremifana*, and *Leucotaraxis* are adelgid specialists [45]. The native European *Neoleucopis atratula* (Ratzeburg) (Diptera: Chamaemyiidae) is at least genus-specific to *Adelges* spp. (Hemiptera: Adelgidae), particularly *Adelges piceae* (Ratzeburg), *A. merkeri* (Eichhorn), *A. nordmannianae* (Eckstein), and *A. tsugae* Annand [46,47]. *Neoleucopis atratula*, misidentified as *Leucopis obscura* Haliday, has already been introduced to control *A. piceae* in North America [47]. *Leucotaraxis* (=*Leucopis*) *argenticollis* (Zetterstedt) and *L. piniperda* (Malloch) are native adelgid-specific predators of *Adelges tsugae* Annand (Hemiptera: Adelgidae) in northwest USA and possible biological control agents of *A. tsugae* in the north and east USA [48]. Both *L. argenticollis* and *L. piniperda* exhibit a preference for feeding on *A. tsugae* [49]. The larvae of these flies are most abundant during the egg-laying stages of both generations of *A. tsugae* [50]. Although laboratory experiments under no-choice conditions have demonstrated that both flies can complete development on other adelgid species, their average lifespan and survival to adulthood are notably higher when reared on *A. tsugae* [50]. Similarly, in the current study, *Neoleucopis* n. sp. *B* exhibited non-significant differences in larval growth when preying on either the target or the non-target species, but survival was significantly affected, favoring *M. hellenica* as a food source. Considering the variation in the level of specificity within the Chamaemyiidae family, additional non-target species should be tested to further investigate the prey specificity of the here studied *Neoleucopis* spp. to *M. hellenica*, including through field surveys in Greece or Turkey. Of note, *M. caucasica*, the singular other species within the genus *Marchalina*, which infests *Abies nordmanniana* and *Picea orientalis* in Russia, Armenia, and Georgia [19,51], is known to be preyed on by *N. hadzibeiliae* [15,52]. Given the morphological similarities between *M. hellenica* and *M. caucasica* [19], as well as *N. kartliana* and its closely related species *N. hadzibeiliae* [17], combined with the general feeding patterns observed among chamaemyiids at the genus level, it can be assumed that *N. kartliana* is likely to prey on *M. caucasica* as well, should these two species come into contact. Further investigation of this matter is warranted. Moreover, considering the potential introduction of *Neoleucopis* spp. for the biological control of *M. hellenica* in invaded countries, it is crucial to investigate their prey specificity with multiple native species of the respective regions. This step is essential for the development of a successful biological control program tailored to the unique ecological context of each region.

So far, several chamaemyiids have been utilized as instrumental biological control agents in classical biological control programs throughout the world. Instances include the successful utilization of *N. obscura* against *Pineus boerneri* Annand (Hemiptera: Adelgidae) in Chile [53,54] and *P. pini* Goeze (Hemiptera: Adelgidae) in Hawaii [55] or *Neoleucopis tapiae* Blanchard (Diptera: Chamaemyiidae) against *P. pini* in New Zealand [45,53]. *Neoleucopis kartliana* was purportedly employed as a successful biological control agent against *M. hellenica* on the island of Ischia, where the scale became a pest after its introduction, highlighting the potential efficacy of chamaemyiids in managing invasive pests [18]. However, the absence of molecular analyses on the *Neoleucopis* species introduced in Italy underscores a critical knowledge gap. The lack of clarity regarding the precise identity of the introduced species in Italy, be it *N. kartliana*, *Neoleucopis* n. sp. *B*, or a combination of both, poses a challenge in identifying an optimal biological control agent for regions where *M. hellenica* has become invasive. Resolving this taxonomic ambiguity through comprehensive molecular analyses is indispensable for informed decision-making in devising effective and tailored biological control strategies.

The findings of this study indicate a discernible level of specificity exhibited by *Neoleucopis* n. sp. *B* and *N. kartliana* towards *Marchalina* sp. in Greece. This aligns with previous assumptions made for *N. kartliana*, recognized as a potential biological control agent against *M. hellenica* in Australia [11]. The co-occurrence of *N. kartliana* and *Neoleucopis* n. sp. *B* in northern Greece hints at a synergistic relationship, potentially enhancing the efficacy in suppressing *M. hellenica* population growth and maintaining ecological equilibrium within its natural habitat. Consequently, *Neoleucopis* n. sp. *B*, *N. kartliana*, or both, could be viable candidates for classical biological control against *M. hellenica* in Australia or other invaded regions. Such an approach holds promise for alleviating the impact of invasive species, aligning with broader goals of ecological sustainability.

The potential success of *Neoleucopis* spp. in managing *M. hellenica* underscores a crucial contribution to sustainable ecological practices. The efficient suppression of invasive species not only safeguards the health of local ecosystems but also mitigates potential cascading effects on biodiversity. The significance of our results extends beyond immediate pest management, pointing towards a potential paradigm for sustainable ecological preservation. Future prospects involve a comprehensive exploration of the biology, ecology, and prey range of *Neoleucopis* spp., offering a foundation for the development of ecologically sound and effective strategies against invasive species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su16072756/s1, Table S1: Larval growth, survival, and sex of each *Neoleucopis* lineage (*N. kartliana* and *Neoleucopis* n. sp. *B*) designated to the eggs of the target (*M. hellenica*) or the non-target (*I. purchasi*) species. Table S2: Total number of *Neoleucopis* spp. larvae encountered on branch samples infested with the non-target (*I. purchasi*) and target (*M. hellenica*) species collected on two occasions in 2022. Figure S1: *Neoleucopis* spp. larvae encountered in *Marchalina hellenica* ovisacs (A), observed to prey on the scale's eggs (B), and the absence of larvae in *Icerya purchasi* ovisacs (C).

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insects

Article

Uncovering the Male Presence in Parthenogenetic *Marchalina hellenica* (Hemiptera: Marchalinidae): Insights into Its mtDNA Divergence and Reproduction Strategy

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Simple Summary: *Marchalina hellenica* (Hemiptera: Marchalinidae) is a significant contributor to annual honey production in Greece and Turkey, where it is endemic. It was initially described as parthenogenetic, producing only females. The exact reproduction strategy of this species remains unknown. For this reason, we studied the emergence pattern of male individuals in Greece for two consecutive years (2021 and 2022). Furthermore, we examined the genetic variation among 15 geographically distant populations of *M. hellenica* in Greece using a mitochondrial DNA marker and compared the results with data from Turkey. This study documents the existence of an additional *M. hellenica* population in its native range that repeatedly produces males, suggesting a previously unknown role for males in the species' reproduction. The Greek and Turkish populations exhibited a strong genetic affinity, while the genetic pattern in Greece seems to have been obscured by human-aided dispersal.

Abstract: *Marchalina hellenica* (Hemiptera: Marchalinidae), an endemic species in Greece and Turkey, is a major contributor to the annual honey production in its native range. However, in the areas that it invades, lacking natural enemies, it has detrimental effects on pine trees and potentially contributes to tree mortality. Although it was originally reported as thelytokous, males were later reported in Turkey and on several of the islands of Greece. To further disambiguate the exact parthenogenetic reproduction strategy of *M. hellenica*, we studied the emergence pattern of male individuals in Greece for two consecutive years (2021 and 2022). Furthermore, we examined the genetic variation among 15 geographically distant populations of *M. hellenica* in Greece using a mitochondrial DNA marker and compared the results with data from Turkey. The findings of this study document the existence of an additional *M. hellenica* population in its native range that repeatedly produces males, apart from the areas of Greece and Turkey in which they were initially reported, suggesting that males play a major, so far unknown role in the reproduction of this species. The populations in Greece and Turkey exhibited a strong genetic affinity, while human-aided dispersal seems to have obscured the genetic pattern acquired.

Keywords: Marchalinidae; invasive species; parthenogenesis

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1. Introduction

Marchalina hellenica (Gennadius) (Hemiptera: Marchalinidae), a scale-insect species native to Greece and the coastline of Turkey [1–3], is the most significant honeydew-producing insect in Greece [1,4]. It feeds on the sap of pine trees (*Pinus* spp.), excreting a glutinous substance of slightly modified tree sap, called honeydew [1,2,5,6]. In its native range, *M. hellenica* is deemed a key insect for apiculture, since the honeydew produced by the scale is collected by bees, *Apis mellifera* Linnaeus (Hymenoptera: Apidae), and converted to pine honey, representing 60% of the honey production in Greece annually [5,6] and 50% in Turkey [6]. For this reason, there has been a significant concern among beekeepers in recent years, following the observation of a notable reduction in the amount of honeydew [2]. In Greece, *M. hellenica* primarily infests *Pinus brutia* and *Pinus halepensis*, but it has also been found on *Pinus pinea, Pinus nigra, Pinus maritima, Pinus sylvestris* [7,8], and *Abies cephalonica* [6]. Beyond its native range, *M. hellenica* has also been reported on *Pinus leucodermis* and *P. maritima* on the island of Ischia, in Italy [9], on *P. halepensis* and *P. pinea*, in Croatia [10], and on *Pinus radiata*, in Australia [11]. Although, in the past, *M. hellenica* was thought to infest *Picea* sp. in Russia, Armenia, and Georgia [12], it was later determined that the scale-insect species encountered in these countries was *Marchalina caucasica* Hadzibeyli (Hemiptera: Marchalinidae) [13]. In its native range, *M. hellenica* is not considered a serious pest and control measures are taken only sporadically, mainly for aesthetic reasons in urban areas [14]. Although *M. hellenica* is associated with detrimental effects on trees at high densities, such as branch and foliage desiccation, growth decline, and crown transparency [15,16], it only rarely causes tree mortality, and usually only in conjunction with other biotic and abiotic secondary stress factors [15,16]. In regions invaded by *M. hellenica*, similar or greater impacts on host trees have been observed [14]. The mild adverse effects of *M. hellenica* on pine trees in its native region have been attributed to the impact of its natural enemies [11]. In particular, *Neoleucopis kartliana* (Tanasijtshuk) (Diptera: Chamaemyiidae) is considered to be the most important natural enemy of *M. hellenica*, suppressing its populations in Greece [11], and it has been successfully used for the biological control of *M. hellenica* on the island of Ischia, Italy [17]. The recent invasion of *M. hellenica* in Australia triggered further studies on the biology of *N. kartliana* [18] and its prospects as a biological control agent against *M. hellenica* in that country [11].

Marchalina hellenica is univoltine and undergoes three female and four male nymphal instars [1,13]. Adult females, which bear 11-segmented antennae and lack mouthparts, usually appear on the branches of pine trees during April, where they oviposit a mean of 262 eggs in woolly ovisacs [1,2,5]. The 1st-instar nymphs, which bear 6-segmented antennae and have proportionately enlarged mouthparts, are encountered on trees between late April and early May, where they settle in groups inside bark crevices [1,2]. In early September, the 2nd-instar nymphs, which also bear 6-segment antennae and large mouthparts, appear on the trees [1,2]. In October, the nymphs molt into their 3rd instar, and overwinter until they molt again in April and give rise to adult females [1,2]. Third-instar female nymphs bear 9-segmented antennae and are significantly larger than 1st- and 2nd-instar nymphs. Although females are apterous [13], they can disperse to adjacent trees by walking and their ovisacs can be easily carried away by the wind [5].

There are three main insect genetic reproduction systems, diplodiploidy (with diploid males), haplodiploidy (with effective haploid males), and thelytoky (with no males) [19]. Based on the occurrence of parthenogenesis, parthenogenetic systems are categorized as either facultative, obligate, or cyclic [20]. Depending on the sexes produced by parthenogenesis, it is classified as arrhenotoky (producing only males), thelytoky (producing only females) and amphitoky or deuterotoky (producing both sexes) [20]. Mixed systems involving cyclic or facultative parthenogenesis can occur by switching between thelytoky and either diplodiploidy or haplodiploidy [19]. Most scale-insect families belong to a monophyletic clade that exhibits paternal genome elimination [21,22], and they exhibit a wide range of genetic systems [23], with parthenogenesis being either thelytokous, deuterotokous, or arrhenotokous [24]. Nur [25] described six parthenogenetic systems observed in scale insects based on (a) whether male individuals are absent or appear occasionally (obligate parthenogenesis and facultative parthenogenesis, respectively), (b) which sexes are produced by fertilized and non-fertilized eggs, and (c) how diploidy is restored in non-fertilized eggs [26]. There are only a few known obligatory thelytokous scale-insect species, e.g., *Protopulvinaria pyriformis* Cockerell (Hemiptera: Coccidae), and *Pulvinaria peregrina* (Borchsenius) (Hemiptera: Coccidae), which do not produce males in any geographic region [27]. Although many species were initially considered thelytokous [25], they were later observed to produce males amphimitically or parthenogenetically [24]. *Marchalina hellenica* was originally reported as obligatory thelytokous, since males were considered absent [25,28] and its females had no spermatheca [29]. Nikolopoulos [30] and Minachilis [31] first described males that were thought to belong to *M. hellenica*. However, it was later revealed that they belonged to a *Palaeococcus* (Hemiptera: Margarodidae) species [2,32]. In the early 2000s, Hodgson and Gounari [13] described apterous *M. hellenica* males, which have since been reported only on Greek Aegean islands (e.g., Rhodes, Crete, Samos, Ikaria) [2] and in Muğla province in Turkey [33]. Little is known about the exact role of males in the reproduction of *M. hellenica* and the circumstances under which they emerge.

Although studies have been conducted on the biology of *M. hellenica* in recent decades [1,4,5,13,33], the exact reproduction system of *M. hellenica* and its relation to genetic divergence remain largely unknown. Its population performance and reproduction system should be considered to estimate the evolution of a potential or ongoing invasion, since parthenogenetic species are commonly invasive [34]. Most of the genetic diversity seen in asexual arthropod populations could arise from multiple origins of clones from sexual ancestors rather than mutations within the asexual population [35,36]. Provided that *M. hellenica* is considered mainly parthenogenetic, an interesting question is whether different or geographically distant populations of *M. hellenica* are genetically divergent. This question has concerned the research community in the past. For instance, Bouga et al. [37] revealed a genetic population homogeneity of *M. hellenica* between Greece and Turkey, exhibiting only one haplotype in their mtDNA analysis. Thus, the objective of this research is to investigate the emergence pattern of male *M. hellenica* individuals and examine the genetic variation among geographically distant populations in Greece by using mtDNA markers, comparing them to already existing sequences deposited in GenBank. Through this approach, we intend to elucidate the intricate reproduction strategy of *M. hellenica* and gain a better understanding of its ecology in invaded areas.

2. Materials and Methods

2.1. Genetic Structure of Marchalina hellenica in Greece

To investigate the genetic variation among geographically distant populations of *M. hellenica* in Greece, samples of female individuals were collected from 13 populations of continental Greece (Katerini, Makriyalos, Alexandroupoli, Stratoni, Thessaloniki, Ioannina, Parga, Athens, Patra, Megalopoli, Korinthos, Larissa, and Kavala) and from two Greek islands (Samothraki and Lefkada). DNA was extracted from 113 *M. hellenica* individuals originating from the aforementioned populations using PureLink™ Genomic DNA Mini Kit (ThermoFisher Scientific, Life Sciences Solutions, Waltham, MA, USA) following the protocol suggested by the manufacturer. The DNA barcoding was then performed in volumes of 25 µL with HCO/LCO primers that amplify a fragment of mtDNA COI gene (654 bp) [38] and MyTaq™ Red Mix (BioLine GmbH, Luckenwalde, Germany). The PCR amplification consisted of an initial denaturation step of 5 min at 94 °C, followed by 5 cycles of 60 s at 94 °C (denaturation), 75 s at 47 °C (annealing), and 90 s at 72 °C (extension). This loop was then followed by 40 cycles of 60 s at 94 °C, 75 s at 52 °C (annealing), and 90 s at 72 °C (extension). The final extension period was performed at 72 °C for 7 min. Purification of PCR products was performed with PureLink™ PCR Purification Kit (ThermoFisher Scientific, Life Sciences Solutions, Waltham, MA, USA) following the protocol of the manufacturer. Sequencing was performed at CEMIA SA (Larissa, Greece) using a sequencer ABI 3730XL. Obtained sequences were examined manually using Chromas Lite software version 2.01 and then blasted in NCBI GenBank. To map the distribution of the obtained haplotypes, visualization was conducted using the QGIS 3.28.2 software based on the World Geodetic System 1984 (WGS 84) [39].

2.2. Biological Traits of Marchalina hellenica Males

For the study on the occurrence of male individuals of *M. hellenica*, branch samples of *P. brutia* infested by the scale were collected every 15 days for two consecutive years from the suburban forest of Thessaloniki (Kedrinos Lofos), in northern Greece. Branches with perimeters ranging from 2 cm to 13 cm and lengths ranging from 5.5 cm to 62.5 cm were selected using a measuring tape (DSOMHZ, length 150 cm, accuracy 1 mm), collected using extended pruners (Stanley Garden BDS6311), and individually placed in labeled plastic bags. Samples were transferred to the Laboratory of Forest Entomology (Forest Research Institute, HAO Demeter) at Thessaloniki (Greece), where they were studied under a stereomicroscope (Zeiss Stemi 508, Germany, 6.3–50× magnification range) to detect and isolate male *M. hellenica* adults. *Marchalina hellenica* individuals (min = 100) were also isolated on every collection day to estimate their developmental stage according to the descriptions of Hodgson and Gounari [13]. Since sex determination is not yet feasible in 1st and 2nd *M. hellenica* instar nymphs [13], the developmental stage of the early instars of the scale insect was estimated regardless of sex. The 3rd-instar female nymphs and adults of *M. hellenica* females, as well as the 4th instar and adults of *M. hellenica* males were recorded. The developmental-stage determination of females is considered crucial to estimate the emergence of male individuals in relation to females. Finally, the samples were transferred in ventilated cages (60 × 60 × 60 cm) in field conditions to record and collect any male adults that might have emerged. The cages were examined daily. The date and number of any emerging male *M. hellenica* individuals were recorded. Male adults were initially detected visually, since they have elongated bodies and dark legs and antennae [13], and then collected and kept in 98% ethanol. Subsequently, the identification of males was conducted based on the descriptions of Hodgson and Gounari [13] using a stereomicroscope.

Statistical Analysis

The association between the developmental stage of *M. hellenica* and the emergence of male adults was analyzed with a quasi-Poisson generalized linear model using the glm function in R [40]. A quasi-Poisson distribution was assumed because the Poisson distribution returned overdispersed residuals. The developmental stage of *M. hellenica* was considered as the independent variable and the count of emerging adults as the dependent variable. To determine which *M. hellenica* female instars are significantly associated with the male counts, a post hoc test with Tukey adjustments was performed.

3. Results

3.1. Genetic Structure of Marchalina hellenica in Greece

Out of the 113 *M. hellenica* sequences obtained, only two haplotypes were retrieved. These haplotypes differed only by a single nucleotide polymorphism (SNP), between cytosine (C) and thymine (T). The haplotype bearing cytosine (GPS-HT1, GenBank accession OQ506006) was identical to the GenBank accession HQ225738 that was identified by Bouga et al. [37] in four Turkish populations. Most of the individuals from the 15 Greek populations (94/113) exhibited the haplotype GPS-HT1, with only 19 out of the 113 individuals having the mutation that ranked them to the second haplotype (GPS-HT2, GenBank accession OQ506007). All the analyzed individuals from Thessaloniki, Makriyalos, and the island of Lefkada belonged to the rarer haplotype, GPS-HT2 (Table 1 and Figure 1), whereas all the remaining individuals from the other locations in Greece belonged to GPS-HT1 (Figure 1). The two haplotypes obtained in this study were not found simultaneously in any of the 15 sites studied. At each site, all the specimens exhibited a single haplotype (GPS-HT1 or GPS-HT2).

Attachment 6

Table 1. COI mtDNA sequence of two haplotypes revealed in *M. hellenica* (Giant Pine Scale (GPS)) populations from Greece and Turkey.

Source	COI mtDNA Sequence
Turkey (GenBank HQ225738)	ATTAATACATCATTTTTCAATCCAAGAAGAAATGGAAGTCCA
Greece (GPS-HT1 GenBank OQ506006)	C
Greece (GPS-HT2 GenBank OQ506007)	T

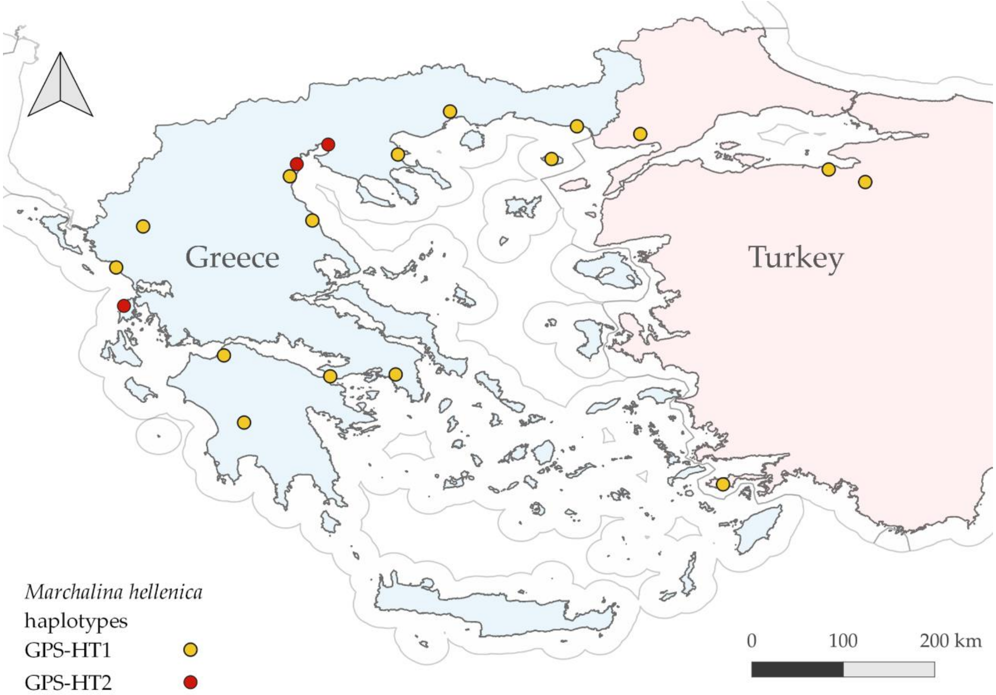

Figure 1. Haplotypes revealed by the mtDNA analysis in Greece (this study) and Turkey [37]. Haplotype 1 (GPS-HT1, yellow points) dominates Greece and four sites in Turkey, while haplotype 2 (GPS-HT2, red points) is exhibited only in three sites in Greece (Thessaloniki, Makriyalos, and Lefkada).

3.2. Biological Traits of Marchalina hellenica Males

Male *M. hellenica* individuals matching the descriptions of Hodgson and Gounari [13] were encountered in the samples from Thessaloniki both in 2021 and 2022. In 2021, a total of 70 *M. hellenica* males were found roaming inside the cages, while 2 additional adult males were found directly on the regularly collected *M. hellenica*-infested branches during the examination. Adult males were detected from early January to mid-April, when 3^{rd}-instar female nymphs and adult females were present (Figure 2). In 2022, male *M. hellenica* adults were again detected inside the cages in which the *M. hellenica*-infested branches were kept, in identical conditions to those in 2021, although in much lower numbers and with a shorter emergence duration. A total of 5 *M. hellenica* males were detected from late January to late March 2022 (Figure 2). It is worth noting that all the males encountered during this study were highly mobile inside the cages compared to the roaming females.

Attachment 6

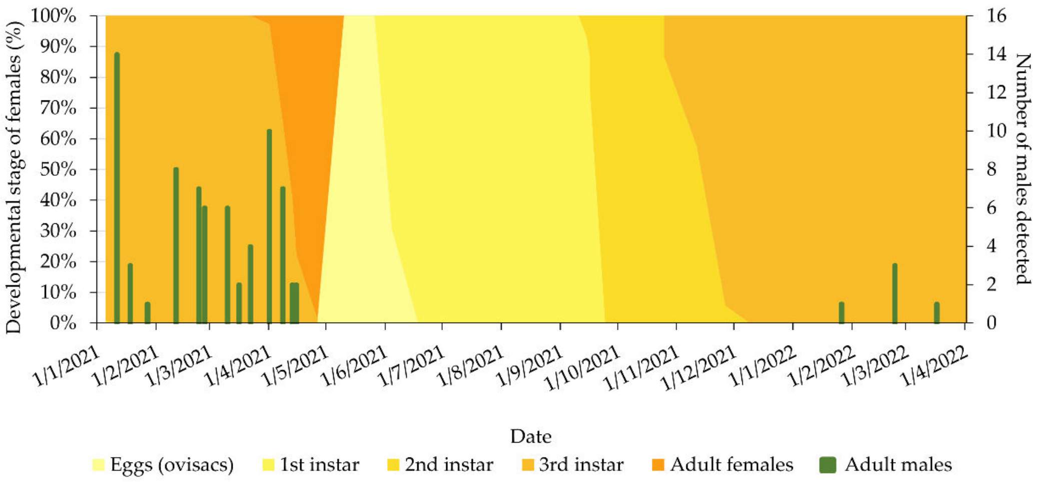

Figure 2. Percentage of the developmental stages of *M. hellenica* females (colored areas) and emergence of *M. hellenica* males (columns) in Kedrinos Lofos (Thessaloniki) between January 2021 and April 2022.

The emergence of males was significantly related to the female developmental stages (χ^2 = 16.251; df = 4,63; p =0.0027). In that, males only emerged concurrent with the 3^{rd}-instar nymphs (mean = 1.7 males per week) and adult females (mean = 3.7 males per week) and not during any of the other developmental stages (Figure 2).

4. Discussion

4.1. Genetic Structure of *Marchalina hellenica* in Greece

It is generally believed that parthenogenetic lineages are likely to suffer early extinction [41,42] because of the genetic bottlenecks that occur during the onset of parthenogenesis [43]. However, the ability to reproduce asexually facilitates the settlement of a species in a new area, because a single female individual can establish a new population [44–47]. Parthenogenesis is one of the most effective processes to overcome low population levels and low genetic diversity through uniparental propagation. This assists the expansion of a given species and the exploitation of resources [48]. Indeed, founder populations are typically restricted in size; in addition, parthenogenetic species do not need to find mates and, therefore, do not suffer from inbreeding in the manner of sexually reproducing species [49]. Additionally, parthenogenesis is likely to weaken the Allee effect and favor invasiveness [50]. The low migratory ability and the reproduction strategy of *M. hellenica* are the main characteristics that should be considered in population genetic studies. Both male and female *M. hellenica* adults are apterous [13]; therefore, their natural dispersal ability is considered low, and the main reproduction strategy of the species is parthenogenesis [5]. Due to these features, *M. hellenica* is not expected to exhibit high genetic variation [37]. Intraspecific variation in parthenogenetic organisms is attributed to different sources of parthenogenesis [51], through repeated hybridization and/or polyploidy [52,53], while many parthenogenetic species exhibit high genetic diversity, which can potentially compensate for the absence of DNA recombination [54]. Considering that the mitochondrial DNA (mtDNA) of eukaryote cells has a fast mutation rate, estimated to be 10–20× higher than that of nuclear DNA [55–57], leading to significant variation in mtDNA sequences, mtDNA markers have been extensively used to address evolutionary and population questions [37]. In asexual species, DNA recombination is usually insignificant, and such species are expected to have a low mutation rate due to the cost of replication fidelity and deleterious mutations [58]. Furthermore, it has been reported that asexual organisms accumulate deleterious mutations quicker than sexual organisms [59]. By contrast, the asexual and polyploid lineages of some tetrapods exhibit heteroplasmy and mtCOI changes more frequently than the sexual lineages [60,61]. Heteroplasmy (the occurrence of two or more mtDNA variants within a cell) is considered to rise through paternal leakage, implying that the paternal mitochondria are not always extinguished during egg fertilization [62]. For instance, in *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), heteroplasmy due to paternal leakage reaches up to 14% in its sexually reproducing populations [63]. Variation in the mtDNA of a parthenogenetic species could indicate multiple sources of parthenogenesis [64]. For evolutionary studies, cytochrome oxidase subunit I (COI) is considered the most appropriate molecular marker among mitochondrial protein-coding genes [65], and has been widely used in Hemiptera [66–68].

It is speculated that *M. hellenica* was introduced into northern Greece from Turkey by the Romans and Byzantines [69], who are considered responsible for the artificial geographical range of the two primary hosts of *M. hellenica*, *P. halepensis* and *P. brutia* [70], since there are no references to the presence of *M. hellenica* in Greece during the prehistoric and classical eras [69]. Bouga et al. [37], who performed a COI mtDNA screening of individuals from four populations in Turkey, revealed a single haplotype. All the Turkish populations exhibited the same haplotype as that which is the most abundant in Greece, while one other, more geographically confined haplotype occurred in Greece. This vividly demonstrates the need for a multi-marker approach in future research efforts, including both nDNA and mtDNA markers, to accurately depict the pattern of intraspecific divergence. The results of the current research exhibit a high genetic affinity level between the populations of Greece and Turkey. If *M. hellenica* had invaded Greece from Turkey through multiple introductions, the genetic diversity in Greece would have reached the levels of its region of origin [44,71]. Given the presence of mainly one COI mtDNA haplotype throughout the sampling sites in both Greece and Turkey, it is most probable that the *M. hellenica* populations in the two countries share a common genetic origin. This has been suggested for other species, such as the parthenogenetic species *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae), which exhibited a single COI mtDNA haplotype, attributed to a single introduction from China to Europe [72].

The 15 Greek populations of *M. hellenica* analyzed in this study belonged to two COI mtDNA haplotypes. The predominant haplotype in Greece is identical to the single haplotype from four sites in Turkey exhibited by Bouga et al. [37], while the second haplotype found in this study was only present in three sites of northern Greece (Thessaloniki, Makriyalos, and Lefkada). The sites where the second haplotype was present, although they all belonged to northern Greece, did not exhibit geographic continuity, failing to explain a natural spread of the species. This can be attributed to dispersal through human activities, considering that *M. hellenica* is a principal contributor to the annual honey production in both Greece and Turkey [1,4] and, for this reason, it has been deliberately introduced into new regions of Greece [16]. Unfortunately, the human dispersal of *M. hellenica* impedes the interpretation of our results, further complicating the search for its origin.

4.2. Biological Traits of Marchalina hellenica Males

The exact reproduction strategy of *M. hellenica* remains unknown. Parthenogenesis is frequently observed in Hemiptera; however, scales demonstrate the most abundant variety of reproduction strategies [73], and the identification of the reproduction system of parthenogenetic species is considered a challenging task [74], with reproductive parasites and endosymbiotic bacteria further complicating the reproduction system's identification [26]. For the first time in Greece, males, females, and 3rd-instar nymphs of *M. hellenica* were encountered at the same time of the year (January to late March) for two consecutive years (2021 and 2022), although males were found in low numbers compared to females, similarly to other coccids, which produce a sex ratio of 5%:95% (males:females) [75]. Male *M. hellenica* adults were encountered in Thessaloniki, where the second *M. hellenica* haplotype was present (GPS-HT2), indicating that males have a genetic effect on this population. The functionality of the male *M. hellenica* adults was not examined in this study through the inspection of mated females; however, the simultaneous emergence of 3rd-instar female nymphs, female adults, and male adults of *M. hellenica* is biologically sound, supporting the hypothesis of mating occurrence. The relatively high number of males during the two years indicates that some of the populations in northern Greece are facultatively parthenogenetic, whereas asexual lineages occur in southern Greece. Geographical parthenogenesis is observed in other insect species, such as *Clitarchus hookeri* (White) (Phasmatodea: Phasmatidae), in New Zealand [76], and *Coccus hesperidum* L. (Hemiptera: Coccidae), which all present one facultative parthenogenetic and one obligatory parthenogenetic lineage [77]. However, it is probable that *M. hellenica* reproduces sexually throughout its natural range, but has a low number of male individuals, as speculated recently [78].

In this regard, the Red Queen hypothesis, which has been applied to a wide range of organisms within Animalia [79–82], suggests that in coevolutionary struggles with natural enemies, the disproportionate attack of natural enemies on the most common phenotype could lead to the short-term coexistence of asexual and sexual populations [82–84]. Asexual reproduction would lead sexually reproduced natural enemies to become proficient at handling the defense mechanisms of a single clone, while their beneficiaries' own capabilities would be continuously improved [85]. Furthermore, some species exhibit both sexual and parthenogenetic lineages on different hosts or in different geographical regions [24,74,86], with parthenogenetic populations often living within distinct ranges, such as marginal habitats, or at a higher latitude or altitude than sexual lineages [87–89]. Jensen et al. [90] suggested that sexual populations, usually found at the central part of the range of the infestation, act as sources of populations choosing asexual reproduction, which are found in the marginal regions of infestations. Consequently, mainland populations can be considered more biologically adapted than marginal populations, since they face the stress of a more complex set of natural enemies [85]. In the case of *M. hellenica*, several studies have examined the effect of the stress of *N. kartliana* on the scale's populations, since it is the most abundant predator of *M. hellenica* [11,18]. Considering that *N. kartliana* has already been successfully used as a biocontrol agent against *M. hellenica* [17], it is most probable that it constitutes a major stress factor in the survival of *M. hellenica.* The sexual reproduction of *M. hellenica* and the abundance of *N. kartliana* in the same area [11] indicate that the reproductive strategy of *M. hellenica* can be explained by the Red Queen hypothesis, with mainland populations implementing sexual reproduction to counter the threat of *N. kartliana* to the survival of the population.

Asexual reproduction is a common feature among Hemipteran invaders, determining the success of invasions [91–94]. Considering that *M. hellenica* males exhibit a pattern of emergence, as suggested by this study, it is probable that an ongoing, or novel invasion of the species will be aided by the benefits of parthenogenesis, while the scale insect will also avoid the phenomenon of a genetic bottleneck due to facultative sexual reproduction, leading to DNA recombination. This emphasizes that *M. hellenica* constitutes a dangerous pest in the regions it has recently invaded.

5. Conclusions

In conclusion, the findings of this research provide new insights into the reproduction strategy of *M. hellenica* and its genetic affinity in Greece and Turkey. This contributes to the understanding of the establishment and ecology of this invasive species. However, this study also stresses the necessity for consistent investigation of the emergence of male *M. hellenica* individuals throughout not only its native habitat, but also the areas it has invaded, as described here, to better define the reproduction system of the species. Furthermore, additional research on the genetic variation throughout both Greece and Turkey, implementing a multi-marker approach, is needed to depict the pattern of intraspecific divergence of *M. hellenica* and determine its origin and genetic path.

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and D.N.A.; funding acquisition, N.E. and D.N.A. All authors have read and agreed to the published version of the manuscript.

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Attachment 6

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SCALE INSECT SURGERY: AN UNUSUAL TWIST TO STANDARD DNA EXTRACTIONS

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Abstract

Current methods for extracting DNA from scale insects typically involves killing specimens or using dead specimens for the first (lysis) step. This creates a quandary when studying species that are difficult to collect and rear in the laboratory. An example is the ongoing study into the phylogenetic relationships among Australia's native scale insects; part of an effort to identify a prey-specific biological control agent for the exotic pest giant pine scale *Marchalina hellenica* (Gennadius). Clarifying the phylogeny of native scales is important because it helps to determine which non-target species should be prioritised for prey-specificity testing of a prospective biological control agent. However, live insects are also needed to initiate laboratory cultures before prey-specificity testing can commence. Using specimens collected in Queensland, we tested whether it was possible to extract and sequence DNA from live specimens of *Monophlebulus* Cockerell by removing a single leg instead of sacrificing the whole individual. It was hoped these specimens could also be used to establish laboratory cultures. We successfully sequenced DNA from each leg that had been removed and specimens did survive at least several days post-leg removal, but no specimens survived to produce nymphs. Although this procedure could be used on other archaeococcoid scale insects such as *Callipappus* Guérin-Méneville, many neococcoid scale insects would unlikely survive the removal of a leg due to their smaller, sac-like bodies and reduced leg morphology. Our non-lethal DNA method for scale insects would be best used for other archaeococcoids that are difficult to collect in the field.

Keywords: *Angophora*, archaeococcoids, Barakula State Forest, digging, Geneious

Introduction

Specimens of scale insects preserved in high concentration ethanol can be used for molecular-based research and the cuticles after the DNA lysis step can be prepared as voucher specimens (Kondo and Watson 2022). However, sacrificing specimens for systematic studies has conservation and other research implications, especially if the specimens are rare (e.g., Lushai *et al.* 2000, Marschalek *et al.* 2013, Oi *et al.* 2013) or required for multiple experiments including behavioural (Oi *et al.* 2013) and host-range testing in biocontrol programs.

A biocontrol program is currently underway in Australia for the exotic pest *Marchalina hellenica* (Gennadius) or giant pine scale (GPS) (Avtzis *et al.* 2020). This program includes investigating the phylogenetics between Australia's native scale insects and *M. hellenica* to avoid non-target predation by the proposed biocontrol agent, *Neoleucopis kartliana* (Tanasijtshuk) (Lubanga *et al.* 2018). It is important to clarify the phylogeny of native species and species of special consideration (e.g., introduced species already being used in other biocontrol programs) to identify non-target species for prey-specificity testing

Attachment 7

Figs 1–3. Set-up for surgical removal of midleg from live specimens of *Monophlebulus*: (1) PJM00582F02 with arrow pointing to location of right midleg; (2) equipment and setup used during the removal of right midleg for DNA extraction; (3) PJM00582F02 post-surgery showing wound sealed with superglue.

experiments (Kuhlmann *et al.* 2005), however this usually requires dead specimens for DNA extractions and/or morphological examination.

Non-lethal DNA extractions using legs, wing tips, haemolymph and/or exuviae have been successfully undertaken on other arthropods including tarantulas (medial leg (Longhorn *et al.* 2007)), dragonflies (exuviae, midlegs and wing tips (Ožana *et al.* 2020)), butterflies (hindwing margins (Hamm *et al.* 2010); wing tips (Lushai *et al.* 2000); and prothoracic leg (Marschalek *et al.* 2013)) and bees (mid- and hindleg tarsi, wing tips (Châline *et al.* 2004); haemolymph, midleg tarsi (Holehouse *et al.* 2003); and partial antennae (Oi *et al.* 2013)) but to date, there are no reported attempts in scale insects. Our aim, therefore, was to determine whether it would be possible to extract and sequence DNA from legs removed from live scale insects from which specimens could still be used to establish laboratory cultures for biocontrol testing.

Materials and Methods

Three specimens of *Monophlebulus* Cockerell were collected on two plants of *Angophora* Cav. from Barakula State Forest in May 2021 (Table 1). It was unknown if the specimens had mated prior to collection. Additionally, no earlier-instar cuticles were found associated with the specimens that could also be used for non-lethal DNA extraction. The right midleg was chosen (Fig. 1) for DNA extraction for consistency with other studies for which amputated midlegs resulted in high rates of survival (e.g., Holehouse *et al.* 2003, Longhorn *et al.* 2007, Ožana *et al.* 2020). The leg was removed using a pair of sterilised forceps and fine pointed scissors (Fig. 2) and placed in DNA extraction lysis buffer solution (10 µL Proteinase K, 180 µL lysis buffer; Bioline). The wound was sealed with superglue (Fig. 3) and the specimen was left resting on its dorsum until the superglue had dried.

Each specimen was placed in a cylindrical, well-ventilated insect-collecting container (80 mm diameter) for at least 24 hours to recover before being moved

Attachment 7

Table 1. Details of specimens collected on 3.v.2021 from *Angophora leiocarpa*.

Species	Code	Location	Genbank #
Monophlebulus sp.	PJM00582F01	Dogwood Creek Campground, Barakula State Forest, Qld	*18S*: OR552975 *28S*: OR552972
Monophlebulus sp.	PJM00582F02	Dogwood Creek Campground, Barakula State Forest, Qld	*18S*: OR552976 *28S*: OR552973
Monophlebulus sp.	PJM00583	Track near Sideling Creek, Barakula State Forest, Qld	*18S*: OR552977 *28S*: OR552974

Table 2. Primers and amplification protocols used for this study.

Gene primer region name	Primer (5'–3')	PCR conditions	References
18S			
2880* B- (or Br)	F CTGGTTGATCCTGCCAGTAG R CCGCGGCTGCTGGCACCAGA	94°C (4 min), [94°C (30 s), 55°C (30 s), 72°C (1 min)] x 35, 72°C (1 min)	von Dohlen and Moran 1995
28S			
S3660* A335	F GAGAGTTMAASAGTACGTGAAAC R TCGGARGGAACCAGCTACTA	94°C (4 min), [94°C (30 s), 55°C (30 s), 72°C (1 min)] x 35, 72°C (1 min)	Dowton *et al.* 1998 Whiting *et al.* 1997
COI			
LCO1490 HCO2198	F GGTCAACAAATCATAAAGATATTGG R TAAACTTCAGGGTGACCAAAAAATCA	95°C (2 min), [94°C (40 s), 45°C (40 s), 72°C (1 min)] x 5, [94°C (40 s), 51°C (40 s) 72°C (1 min)] x 40, 72°C (5 min)	Folmer *et al.* 1994
Dyn			
3006F1.1* 3006R2.1	F CCGGAYATGGCGTTCGAAGCTA R TCTTCGTGGTTGGTGTTCATGTACGC	94°C (4 min), [94°C (30 s), 50°C (30 s), 72°C (1 min)] x 35, 72°C (1 min)	Hardy 2007
*= forward primer used with M13F-pUC tail (5'-GTTTTCCCAGTCACGAC-3'			

to a 140 mm diameter pot containing an *Angophora leiocarpa* (L.A.S. Johnson ex G.J. Leach) K.R. Thiele & Ladiges)) sapling or *A. floribunda* (Sm.) Sweet, a closely-related species to the host plant (Rutherford *et al.* 2021)) in Serles' native plant specialty mix potting soil. In some instances, individuals were moved into different pots due to wandering or death of their host plants. Saplings were monitored twice a week for signs of first-instar nymphs. When the saplings began to die in early January 2022 the topsoil was removed to check whether specimens were alive and had produced an ovisac.

DNA was extracted from each leg following the protocol from an Isolate II Genomic DNA Kit (Bioline cat. no. BIO-52067). Each leg was removed after the lysis step and stored in 70% ethanol. One mitochondrial (*COI*) and three nuclear (*18S, 28S* D2D3 and *Dynamin* (*Dyn*)) gene regions were tried using the primers and PCR conditions listed in Table 2. Each reaction contained 16 µL of reagents

(3 µL MangoTaq PCR buffer (5x), 1.2 µL dNTP (2 mM), 0.9 µL $MgCl_2$ (50 mM), 0.3 µL forward primer (10 µM), 0.3 µL reverse primer (10 µM), 0.6 unit of MangoTaq (cat. no. BIO-21083, Bioline, Australia) and 2–4 µL DNA template (c. 10 ng/µL). GIBCO ultrapure distilled water (Invitrogen) was used to bring the total volume of each reaction to 16 µL.

Successful amplicons were cleaned using 1 µL of a 1:1 ratio of Antarctic Phosphatase and Exonuclease I (New England Biolabs, Australia). Samples were incubated at 37°C for 30 min before degrading unwanted primers and nucleotides at 80°C for 20 min. Samples were sequenced using Sanger chemistry on an ABI 3730XL DNA Analyser located at Macrogen Inc. (Seoul, Republic of Korea). Trace files of sequences were edited in Geneious Prime® 2021.2.2. (Biomatters 2023). The edited sequences were checked against Genbank sequences labelled as '*Monophlebulus*' to confirm their validity.

Results

Once individuals had been transferred to a potted sapling they were observed slowly digging "head-first" into the soil. This behaviour has been observed for post-mated females of other monophlebulines (Bhatti 1989). No first-instar nymphs were observed emerging from the pots, and specimens were found dead in the soil six months after being transferred to the potted saplings. No ovisacs were found even though closely-related genera (e.g., *Melaleucococcus* Bhatti and *Nodulicoccus* Morrison) have been reported to produce a subterranean ovisac (Bhatti 1989). Although the removed specimens were placed in 100% ethanol for storage, their bodies had decomposed and their cuticles had sustained considerable damage whilst in the soil thus precluding future morphological species confirmation.

Sequences from two genes (*18S* and *28S* D2D3) for all three specimens were successfully amplified (Table 1); neither *COI* or *Dyn* amplified. Sequence length for *18S* was 594-bp long and 722-bp long for *28S*. Specimens were identical at *18S* and there was one nucleotide difference at *28S* between PJM00583 and the two individuals from the same tree (PJM00582F01–F02). Three nucleotide differences in *18S* (0.6%) were observed between the specimens collected in this study and the Genbank sequence labelled as '*Monophlebulus*' (EU087756; code CMU025) collected from Wattamolla, New South Wales (Unruh and Gullan 2008). The specimens from Table 1 differed from the *28S* Genbank sequence (EU087870; code CMU025) by 3.2–3.3 %. Genbank accession numbers are provided in Table 1.

Discussion

This is the first reported attempt that shows DNA can be obtained from scale insects using non-lethal methods. A limitation of this study was the low number of individuals tested; however, the difficulty in sourcing live specimens was one of the reasons for investigating non-lethal DNA extractions in the first place. Specimens were still able to crawl around and dig after the surgical removal of one of their legs, however, none of the specimens survived long enough to produce an ovisac to establish a second generation for prey-specificity testing. Sourcing additional specimens of *Monophlebulus* would be required to confirm that individuals that had undergone surgical removal of a leg can also be used for establishing laboratory cultures.

This procedure is likely to be successful for other specimens of archaeococcoids such as *Callipappus* Guérin-Méneville and other monophlebids because their legs tend to be large, articulated and sclerotised. Earlier-instar cuticles (exuviae) and exudate could also be used for non-lethal DNA extraction, yet studies using exuviae from dragonflies (Ožana *et al.* 2020) and haemolymph from bumblebees (Holehouse *et al.* 2003) found overall poorer amplification and lower-quality DNA compared with using midlegs. It is likely that this procedure on neococcoid scale insects would cause irreparable damage to specimens due to many species being smaller, sac-like, and exhibiting reduced leg morphology (Gullan and Martin 2009). In these cases, environmental DNA protocols that use discarded cuticles from earlier instars might be a better, non-lethal way of obtaining DNA from neococcoid scale insects. Additionally, many neococcoid scale insects and iceryine archaeococcoids can be found in large numbers, so sacrificing a few specimens for phylogenetic studies would not impact the ability to also rear from same population for biocontrol studies. Our non-lethal DNA extraction approach would be best used for archaeococcoid scale insects that are difficult to find and collect in the field.

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