



Established *Paropsis charybdis* egg parasitoids are unlikely to control the newly invaded *Paropsisterna cloelia* (both Coleoptera: Chrysomelidae) in New Zealand

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Abstract The two egg parasitoids *Enoggera nassui* (Girault) and *Neopolycystus insectifurax* Girault (both Hymenoptera: Pteromalidae) are biological control agents of the eucalyptus-defoliating paropsine leaf beetle *Paropsis charybdis* Stål (Coleoptera: Chrysomelidae), an invasive species in New Zealand. Recently another leaf beetle, *Paropsisterna cloelia* (Stål) (Coleoptera: Chrysomelidae), has established in New Zealand, causing defoliation on select eucalypt hosts, e.g., *Eucalyptus bosistoana*. To determine if established egg parasitoids can control *Pst. cloelia*, the host suitability and host preferences of the two parasitoids were tested under laboratory conditions and compared with in-field egg parasitism at a site

where all parasitoid and beetle species were present. Laboratory and field assessments confirmed that both parasitoids can reproduce in *P. charybdis* and *Pst. cloelia* but have a strong preference for *P. charybdis*. Consequently, probabilities of unparasitized *Pst. cloelia* larvae hatching in the laboratory or field in the presence of either parasitoid was higher compared to *P. charybdis*, ranging between 32–79% versus 1–24%, respectively. Offspring of both parasitoids emerged 36% larger (wing length 1.40 mm versus 1.06 mm and 1.56 mm versus 1.07 mm for *E. nassui* and *N. insectifurax*, respectively) from *P. charybdis* eggs versus *Pst. cloelia* eggs, confirming *Pst. cloelia* eggs as lower quality hosts for these parasitoids. We conclude that *E. nassui* and *N. insectifurax* will not induce sufficient mortality in *Pst. cloelia* eggs to be effective biological control agents for this invasive beetle.

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Introduction

Eucalyptus-feeding insects from Australia continuously arrive and establish in New Zealand's exotic *Eucalyptus* plantings, likely exacerbated by New Zealand's geographic proximity to Australia (Weser et al. 2024). Eucalyptus specialists, such as paropsine leaf beetles (henceforth referred to as paropsines)

(Coleoptera: Chrysomelidae), are particularly successful and some species have become pests due to a lack of natural enemies (Withers 2001). *Paropsis charybdis* Stål was detected in New Zealand in 1916, has spread to all eucalyptus growing areas, and has been the most damaging eucalyptus defoliator of its most preferred hosts within the *Eucalyptus* subgenus *Symphyomyrtus*, including *Eucalyptus nitens* (H. Deane & Maiden) Maiden (Withers and Peters 2017). *Paropsisterna cloelia* (Stål) (*Paropsisterna* henceforth abbreviated to *Pst.* to distinguish from the genus *Paropsis*, in accordance with previous publications on these genera), first detected in New Zealand in 2016 in the Hawke's Bay, has since spread from Gisborne in the North Island to mid-Canterbury in the South Island (iNaturalist 2024b). Both paropsine species have similar distributions in Australia (iNaturalist 2024a, 2024b), suggesting similar climate tolerances, hence *Pst. cloelia* is likely to spread nationally. In New Zealand, *Pst. cloelia* adults have been observed on 15 eucalyptus species, some of which are unsuitable for larval development (Lin 2017; Murray and Kuwabara, unpublished work; H. Lin, pers. comm.). A few *Symphyomyrtus* species that are under investigation for a highly durable hardwood log production industry in New Zealand, e.g. *E. bosistoana* F. Muell., *E. quadrangulata* H. Deane & Maiden, *E. camaldulensis* Dehnh., and *E. tricarpa* (L.A.S. Johnson) L.A.S. Johnson & K.D. Hill, suffer the most damage (Millen et al. 2018).

Several biological control agents from Australia have been introduced to control *P. charybdis*. However, not all established, and impact has been variable (Murray et al. 2008). To date, two Australian solitary egg parasitoids, *Enoggera nassau* (Girault) (Hymenoptera: Pteromalidae) and *Neopolycystus insectifurax* Girault (Hymenoptera: Pteromalidae), have established widely in New Zealand (Murray et al. 2008). Both species are able to develop within several closely related paropsine genera present in Australia. *Enoggera nassau* has been reared from field-collected eggs of at least 14 species from the two closely related genera of eucalyptus-feeding paropsines, *Paropsis* Olivier, and *Paropsisterna* Motschulsky (Hayden et al. 2019; Mo and Farrow 1993; Murphy 2006; Naumann 1991), and *Neopolycystus insectifurax* from at least six species within the three genera *Paropsis*, *Paropsisterna*, and *Trachymela* (Mo and Farrow 1993; Murphy 2006; Tribe 2000).

Both have also been experimentally reared from *Dicranosterna* Motschulsky and *Trachymela* Weise (Murray 2010). In New Zealand, these parasitoids successfully control the second (late-summer) generation of *P. charybdis* with total parasitism rates up to 100%, but only reach 0-50% parasitism in the first (spring) generation (Jones and Withers 2003; Mansfield et al. 2011). The effectiveness of *E. nassau* is compromised by the self-introduced hyperparasitoid, *Baeoanusia albifunicle* Girault (Hymenoptera: Encyrtidae), with variable *E. nassau* hyperparasitism rates (Jones and Withers 2003). Both parasitoids have been reared from field-collected *P. charybdis* and *Pst. cloelia* eggs in New Zealand (T. Withers, pers. comm.). Results of laboratory trials with *E. nassau* performed by Murphy (2006) in Australia suggested that the parasitoid prefers *P. charybdis* over *Pst. cloelia*. Results of laboratory trials with *N. insectifurax* performed by Murray (2010) in New Zealand showed that the parasitoid prefers *P. charybdis* over *Trachymela* spp., but no *Paropsisterna* spp. were present in New Zealand then. The impact of both parasitoids on *Pst. cloelia* populations in New Zealand is therefore unknown.

This study aims to ascertain if either of the two egg parasitoids can effectively control *Pst. cloelia* populations. In this study, the host preferences and performance of both egg parasitoids *N. insectifurax* and *E. nassau* to *P. charybdis* and *Pst. cloelia* eggs were assessed in no-choice and choice laboratory trials. Subsequently, transferability of laboratory results was assessed by quantifying host-parasitoid interactions in a eucalyptus plantation containing all four species. Quantifying the effectiveness of established parasitoids in New Zealand will inform future integrated pest management of these paropsine pests and the need for additional classical or alternative biological control programs.

Material and methods

Laboratory trials

Insect colonies

A colony of *P. charybdis* was initiated from field-collected adults (TECT Park, Ngawaro, Western Bay of Plenty (37°56'48"S, 176°08'33"E) and Poronui Station, Central Plateau (38°59'59.6"S, 176°17'39.5"E),

November 2019). A colony of *Pst. cloelia* was established from field-collected larvae and adults (Taharua, Taupo (38°59'42"S, 176°17'43"E), December 2019 and Te Mata Peak, Hawke's Bay (39°41'45"S, 176°54'18"E), January 2020) under an Environmental Protection Agency development approval (NOC100191) at Scion's Invertebrate Transitional and Containment Facility. Beetle species were reared separately on *E. nitens* flush adult foliage collected twice per week from Atiamuri forest, Taupo (Oji Fibre Solutions Ltd) and stored in a $4 \pm 1^\circ\text{C}$ chill room with cut stem bases sitting in tap water. Approximately 40 adult *P. charybdis* beetles were maintained at $18 \pm 0.5^\circ\text{C}$ and a L:D 14:10 photoperiod in large, ventilated Perspex cages ($1.0 \times 0.8 \times 0.6$ m). The base of the cages was lined with paper towels, dead leaves and fresh foliage. Twice per week adult beetles were provided with freshly cut branches of *E. nitens* foliage bearing adult leaves and new flush (i.e., newly produced, tender leaves that are still expanding), with their cut bases sitting in tap water. Egg laying occurred on mature leaves and egg batches were removed each morning by cutting the leaf upon which they were laid, placed into individual labelled 90 mm Petri dishes and used daily for experiments. This ensured that egg batches were always less than 24 h old as both parasitoids show a preference for host eggs of that age (Murray 2010). The same rearing methods as for *P. charybdis* were used for *Pst. cloelia* with the only difference that *Pst. cloelia* were reared in medium sized Perspex cages ($0.8 \times 0.8 \times 0.6$ m) in a containment room at $20 \pm 0.5^\circ\text{C}$ and a L:D 14:10 h photoperiod. *Paropsisterna cloelia* preferentially lays eggs on flush leaves rather than mature leaves, so the cages contained a high proportion of branch ends bearing flush foliage.

Both parasitoid colonies of *N. insectifurax* and *E. nassau* were reared from field-collected parasitised *P. charybdis* eggs (Rotorua, 38°10'05"S, 176°19'11"E, December 2019 and January 2020). Continuous colony production followed the exact procedure developed by Murray (2010) and consisted of unsexed emerged adult parasitoids held along with the egg batch remains on which they emerged within plastic Petri dishes. A drop of honey was provided on a small square of tissue paper labelled with the date of emergence. Dishes were held on the bench in a temperature-controlled laboratory (20°C , 65% RH, and a L:D 14:10 photoperiod) under a mixture of

natural and fluorescent light. Parasitoids were either three (*E. nassau*) or seven (*N. insectifurax*) days old when used in assays.

No-choice tests

No-choice tests assessed host acceptance for and physiological suitability of the paropsine eggs for the two parasitoids. Experiments were conducted under both fluorescent (Philips Lighting TL-D De Luxe Triphosphor Colour 965 fluorescent tube) and natural (Velux Solar tube) lighting within a containment laboratory at $20 \pm 0.5^\circ\text{C}$, 65% RH and a L:D 14:10 photoperiod. Each assay consisted of one naïve female parasitoid transferred via a plastic Petri dish lid into another clean Petri dish containing an egg batch of either *P. charybdis* or *Pst. cloelia* presented on the leaf upon which it had been laid. Assays ran for 24 h (*E. nassau*) or 48 h (*N. insectifurax*) identical to the protocols previously developed by Murray (2010) to assess preference and performance of these two egg parasitoids towards paropsine species eggs. Different durations of assay were shown to be important to be able to compare results between the two parasitoids. *E. nassau* has a much faster rate of host searching and oviposition than *N. insectifurax* (Murray 2010). We aimed to complete a minimum of 20 replicates of each species. Prior to testing, adults of both species were observed for behaviour typical of females: slow walking on the base of the Petri dish (males tended to be more active on the lid of the Petri dish) and these individuals were prioritised for testing. The sex of each parasitoid was later confirmed via dissection of the abdomen under a microscope (Leica M165, Leica Microsystems, Wetzlar, Germany) under $20\times$ magnification. Data from any individuals subsequently identified as males were excluded from the dataset before analysis.

Choice tests

Two-choice tests assessed host preferences of the two parasitoid species. One hour before each trial, parasitoids were pre-screened for behaviour to identify females that were likely to be physiologically ready for oviposition, as per the method used by Murray (2010). This was undertaken by presenting them with one egg batch of the known host *P. charybdis* and carefully observing their behaviour.

Males tended to rapidly leave the leaf and walk quickly around the Petri dish, whereas females ready to oviposit would antennate egg batches carefully and stay on or near the leaf. Females were removed to an empty sterile Petri dish with a camel-hair brush just as they were observed to align their ovipositor for vertical insertion into the egg surface, but prior to having initiated insertion. Approximately one hour later, each female was independently presented with a paired two-choice test containing four freshly laid eggs of both *P. charybdis* and *Pst. cloelia*. Eggs were prepared an hour prior by cutting four eggs upon the leaf on which they are firmly glued from a larger egg batch using a sharp scalpel. The leaf was trimmed so that four eggs of each species were presented on a 4 × 4 mm section of *E. nitens* leaf, each arranged 20 mm apart and equidistant from the centre of a sterilized 55 mm Petri dish. The female was introduced into the base of the Petri dish containing the two leaf sections bearing eggs, by tapping the inverted lid on which the female parasitoid sat over the base, which resulted in the species of egg first encountered being haphazard (20 °C, 65% RH). If an egg was inadvertently damaged (eggs sometimes leaked their contents during subsequent rearing), sample size was reduced to three eggs. Paired two-choice tests lasted for exactly one hour for *E. nassau*i trials, and 6 h for *N. insectifurax* trials, an optimal duration to reveal host preference yet avoiding superparasitism in each species (Murray 2010). *N. insectifurax* has a tendency to guard the eggs they have just parasitised so requires the longer duration assay to ensure all eggs in the Petri dish have been encountered permitting choices being made on whether to oviposit or not.

In both no-choice and choice tests, the individual parasitoid was removed from the Petri dish at the end of the assays and placed immediately into 70% ethanol for later dissection. Egg batches were reared within individual Petri dishes (20 °C, 65% RH, and a L:D 14:10 photoperiod) and inspected daily. Point of hatch eggs (showing sclerotised structures in two parallel lines of three black dots along each side and red eyes that indicate hatching is imminent, Fig. S1 b and h) and newly emerged host beetle larvae were removed to avoid predation of neighbouring eggs. Individual egg fate was recorded after two weeks as either ‘larvae hatched’ (includes larvae removed at point of hatch), ‘parasitised’ if *N. insectifurax* and

*E. nassau*i embryos developed to point of identification or successfully emerged, or ‘dead’ if no development of either beetle or parasitoid was observed.

Field trial

Field site

A field site in Marlborough (41°38′35″S, 173°40′26″E) (a region of New Zealand located in the northeast of the South Island) with young *E. bosistoana* and *E. quadrangulata* (planted in 2018) was sampled for egg parasitism of *P. charybdis* and *Pst. cloelia* during the 2022/2023 eucalyptus growth season. Simultaneous phenology monitoring at two-week intervals showed that both beetles produced two generations there, with egg batches observed on the trees in November/December and January/February (Weser 2024). Sampling and hand collection of egg batches occurred on three dates during each beetle generation (generation 1: 09/11/2022, 23/11/2022, 07/12/2022; generation 2: 17/01/2023, 31/01/2023, 18/02/2023). Ten trees (1.6–3.0 m high) of the two eucalyptus hosts were randomly sampled without repetition on each date.

Egg batch abundance

Paropsis charybdis oviposit on mature leaves adjacent to new foliage, while *Pst. cloelia* oviposit directly on new flush foliage. Per tree, number of egg batches of each paropsine species were quantified on five branch sections with 50 (± 10%) new leaves and adjacent mature leaves, respectively. Sampling branches were chosen ad-hoc at heights up to 1.5 m around the entire tree circumference.

Parasitism

Following the egg batch abundance assessment, egg batches of *Pst. cloelia* and *P. charybdis* with more than eight eggs and no signs of predation damage were collected on their leaf from the entire crown. Sampling aimed to collect three egg batches per beetle species, per sampling tree, per date. However, variation in egg batch abundance largely prevented this ideal sampling effort. Where insufficient egg batches were available using this sampling strategy, a random selection of additional batches from sampled trees

where eggs were abundant was used to ensure that up to 30 intact egg batches per beetle species, eucalyptus species, and date were collected (Table S1). Egg batches at point of hatch were not collected. It was assumed that the collected egg batches had sufficient exposure to parasitism at the point of collection and, consequently, all collected egg batches were included in the analysis. Egg batches on their leaf were placed in individual Petri dishes (90 mm) and transported to the laboratory at 10–14 °C, where all intact eggs in each batch were counted under a stereo microscope (Leica EZ4 W, 8–35× magnification). Egg batches were reared under controlled conditions (20 °C, 70% RH, and a L:D 14:10 photoperiod) and assessed every 1–3 days until either beetle larvae or parasitoids emerged. Beetle larvae were removed after hatching or at point of hatch to prevent cannibalism of remaining eggs. Eggs that had not hatched or developed further after two weeks were declared dead. Individual egg fate was recorded per batch as ‘larvae hatched’ (includes larvae removed at point of hatch), ‘dead’, ‘*E. nassau*’ (includes counts of its emerged hyperparasitoids, *B. albifunicle* and *Tetrastichus* sp. (Hymenoptera: Eulophidae), Fig. S1 m, o, p), and ‘*N. insectifurax*’ (Fig. S1 n).

Parasitoid offspring size

As a proxy for body size, forewing length of *E. nassau* and *N. insectifurax* individuals reared in the laboratory from both paropsine beetle host eggs was measured from the tip of the wing to the tegulum (Leica M205 C microscope with a calibrated graticule). To test for any confounding effects of collection date or eucalyptus species, measurements were taken from three individual wasps of at least three, ideally four, batches collected from each of the two eucalyptus hosts on different dates.

Statistical analysis

Bayesian generalised linear mixed effects models (mixed effects GLMS) with count and categorical response variables and vague priors were run using WinBugs software (Lunn et al. 2000) and R package R2WinBugs (Sturtz et al. 2005) in R (R Core Team 2023). For the analysis of the choice data, paropsine species and parasitoid species were used as fixed effects, no random effects were used. Similarly for the

no-choice data, paropsine species was used as fixed effect, but additional random-effect adjustments needed to be made for the different assay dates, and the fact that multiple parasitoids have been used several times. Hence, parasitoid species and assay date were used as random effects. In the field trial, egg batch abundance and parasitism were both analysed using paropsine species and eucalyptus species as fixed effects. Date, plot, tree, and branch served as random effects in the analysis of egg batch abundance; date, plot, and tree were random effects in the analysis of parasitism. The analysis of parasitoid offspring size was carried out with paropsine species, parasitoid species, and eucalypt species as fixed effects, and date and egg batch as random effects. In each case, a burn-in of 1000 iterations were discarded, and the further 4000 iterations were thinned to obtain a posterior sample size of 1000. Convergence was assessed visually using trace and density plots for the model parameters (see Supplementary information for exact model formulation and WinBUGS code). In a GLM, the parameter of interest, such as the expected count or the expected probability of a certain response are modelled via a link function. The counts are typically modelled as a Poisson distribution with a log link function, and the multinomial responses are typically modelled via a multinomial logit link function. Due to the nature of the latter model, the effects and differences between groups are typically reported in terms of odds ratios (OR) and the corresponding 95% central credible intervals (CIs, in brackets). Posterior 95% CI containing 1.0 indicate insufficient statistical evidence for the difference between groups. The object of Bayesian inference, the posterior distribution, is commonly summarised via the posterior mean and the 95% CI, which contains 95% of the posterior probability distribution of the parameter in question. While Bayesian statistics does not have a concept of statistical significance, it can provide posterior probabilities for various statements, sometimes referred to as Bayesian P-values. The closer they are to 1.0, the more likely is the statement given the data. For example, when examining possible egg fates, we have calculated the posterior probabilities of the fate being the least likely and the most likely for various combinations of beetles/parasitoids, denoted $P_{\min}$ and $P_{\max}$, respectively.

Results

Laboratory trials

No-choice tests

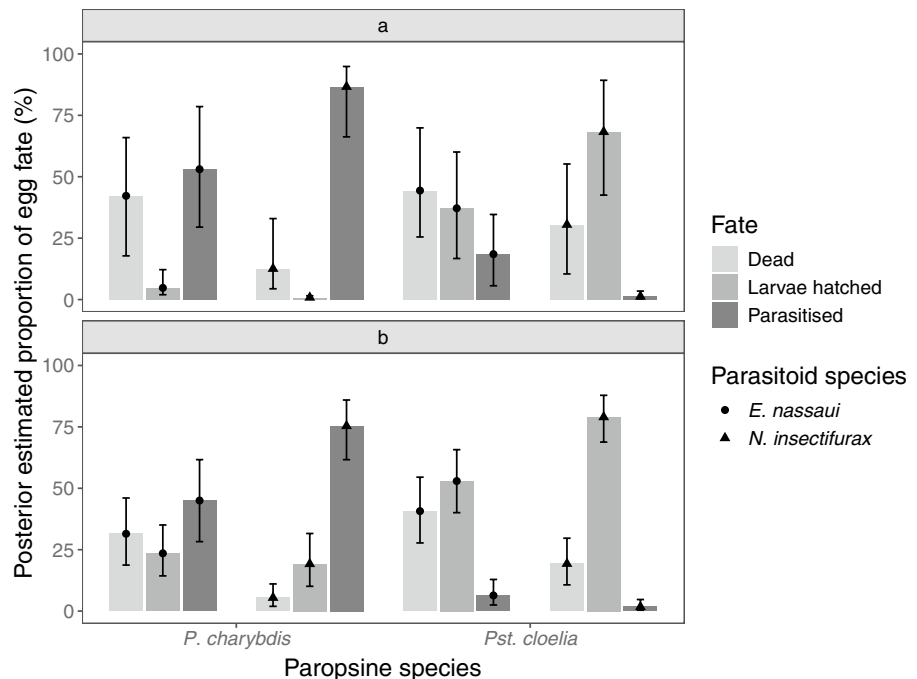
Predicted most and least likely egg fates varied between all beetle species/parasitoid species combinations. When *P. charybdis* eggs were the only choice for *E. nassaui* and *N. insectifurax*, the most likely egg fate was predicted to be ‘parasitised’ ($P_{\max}=0.61$ and $P_{\max}>0.99$, respectively) and the least likely fate was ‘larvae hatched’ ($P_{\min}>0.99$) (Fig. 1a, Table S2). When *Pst. cloelia* eggs were the only choice for *E. nassaui*, eggs were predicted to be most likely ‘dead’ ($P_{\max}=0.58$) and least likely ‘parasitised’ ($P_{\min}=0.84$). When paired with *N. insectifurax*, the most likely predicted fate of *Pst. cloelia* eggs was ‘larvae hatched’ ($P_{\max}=0.83$) and the least likely predicted fate was ‘parasitised’ ($P_{\min}>0.99$) (Fig. 1a, Table S2). Estimated probability of egg fates for each beetle species varied depending on the parasitoid they were paired with. *P. charybdis* egg fate was considerably more likely ‘parasitised’ (OR: 9.01 (95% CI: 1.10–92.18)) and less likely ‘dead’ (OR: 0.13 (0.013–0.96)) when paired with *N. insectifurax* than *E. nassaui* (Table S3). *Pst. cloelia* egg fate was

considerably less likely ‘parasitised’ (OR: 0.06 (0.02–0.25)) when paired with *N. insectifurax* than *E. nassaui* (Table S3). Moreover, probability of egg fates varied between beetles when paired with the same parasitoid. When paired with either parasitoid, *Pst. cloelia* egg fate was considerably more likely ‘larvae hatched’ (OR: 10.68 (2.41–40.37) and 238.56 (33.02–1532.89) for *E. nassaui* and *N. insectifurax*, respectively) and less likely ‘parasitised’ (OR: 0.21 (0.06–0.98) and <0.01 (<0.01 –0.01) for *E. nassaui* and *N. insectifurax*, respectively) compared to *P. charybdis* when paired with either parasitoid. When paired with *N. insectifurax*, egg fate ‘dead’ was also considerably more likely for *Pst. cloelia* than for *P. charybdis* (OR: 4.27 (1.52–17.28)) (Table S4).

Choice tests

Similar trends to the no-choice tests can be seen when a choice of beetle species eggs was presented to either egg parasitoid. Predicted most and least likely egg fates of *P. charybdis* and *Pst. cloelia* eggs varied dependent on the parasitoid species. When *E. nassaui* was given the choice between the two paropsine eggs, the most likely egg fate for *P. charybdis* eggs was ‘parasitised’ ($P_{\max}=0.79$) and the least likely ‘larvae hatched’ ($P_{\min}=0.80$), whereas it was the opposite

Fig. 1 Posterior mean estimated proportions of egg fates of *P. charybdis* and *Pst. cloelia* eggs and the respective 95% CIs shown as error bars for each parasitoid/host beetle egg combination following **a** no-choice tests where eggs of each species were separately exposed to one naïve female parasitoid of either *N. insectifurax* or *E. nassaui*, and **b** choice tests where four eggs of each species were simultaneously exposed to one naïve female parasitoid of either *N. insectifurax* or *E. nassaui*



for *Pst. cloelia* eggs, i.e., the most likely fate ‘larvae hatched’ ($P_{\max}=0.83$) and least likely ‘parasitised’ ($P_{\min}>0.99$). When *N. insectifurax* were given the choice between beetle eggs, *P. charybdis* eggs were most likely ‘parasitised’ ($P_{\max}>0.99$) and least likely ‘dead’ ($P_{\min}>0.99$), whereas *Pst. cloelia* egg fate was again most likely ‘larvae hatched’ ($P_{\max}>0.99$) and least likely ‘parasitised’ ($P_{\min}>0.99$) (Fig. 1b, Table S6). Fate of *P. charybdis* eggs was considerably more likely ‘parasitised’ (OR: 3.87 (1.40–10.25)) and less likely ‘dead’ (OR: 0.12 (0.04–0.33)) when paired with *N. insectifurax* than *E. nassau*. Fate of *Pst. cloelia* eggs was considerably more likely ‘larvae hatched’ (OR: 3.42 (1.55–8.12)) and less likely ‘dead’ (OR: 0.34 (0.13–0.83)) when paired with *N. insectifurax* than *E. nassau* (Fig. 1b, Table S3). Moreover, egg fates varied between beetle species when paired with the same parasitoid species in the same way as observed during no-choice tests. When paired up with either parasitoid, the fate of *Pst. cloelia* eggs was considerably more likely ‘larvae hatched’ (OR: 3.76 (2.05–6.93) and 16.84 (7.59–40.24) for *E. nassau* and *N. insectifurax*, respectively) and less likely ‘parasitised’ (OR: 0.08 (0.03–0.16) and <0.01 (<0.01 –0.01) for *E. nassau* and *N. insectifurax*, respectively) compared to *P. charybdis* eggs (Table S4). When paired with *N. insectifurax*, egg fate ‘dead’ was

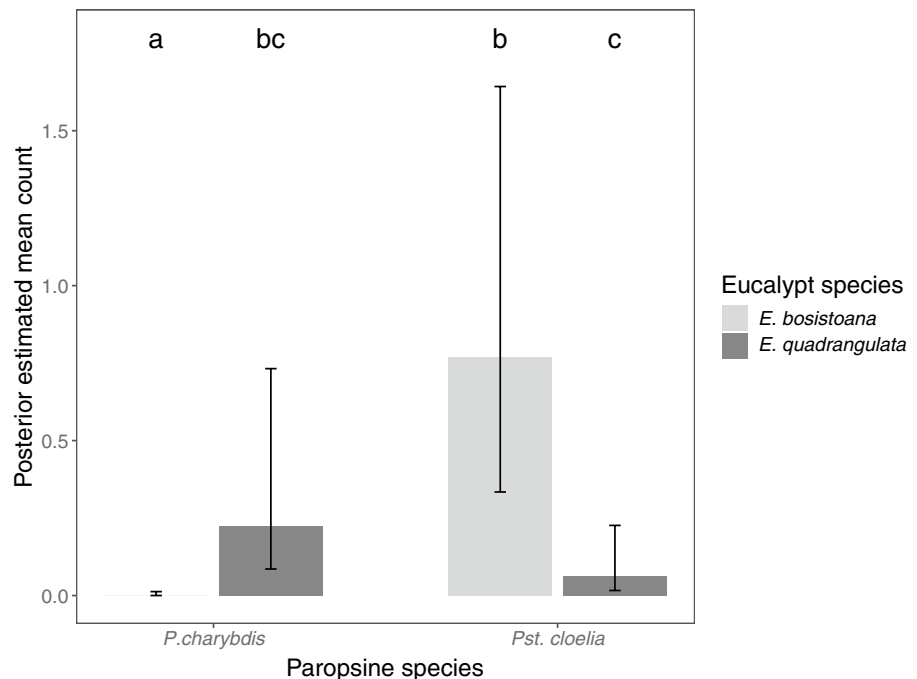
considerably more likely for *Pst. cloelia* than for *P. charybdis* (OR: 4.44 (1.81–11.51)) (Table S4). Both no-choice and choice tests show similar trends for both beetles and parasitoids, with the only difference of *P. charybdis* egg fate being considerably more likely ‘larvae hatched’ in choice tests independent of parasitoid species (OR: 0.20 (0.07–0.73) and 0.04 (0.01–0.20) for *E. nassau* and *N. insectifurax*, respectively) (Table S5).

Field trial

Egg batch abundance

With posterior estimated mean counts of 0.22 and 0.00, *P. charybdis* egg batches were considerably less abundant on *E. bosistoana* than on *E. quadrangulata* ($P>0.99$), respectively. Conversely, *Pst. cloelia* egg batches were more abundant on *E. bosistoana* than on *E. quadrangulata* ($P>0.99$) with posterior estimated mean counts of 0.77 and 0.06, respectively. On *E. bosistoana*, *P. charybdis* was less abundant than *Pst. cloelia* with a posterior probability of $P>0.99$, whereas there was no evidence for differences in egg batch abundance of both paropsines on *E. quadrangulata* ($P=0.06$) (Fig. 2).

Fig. 2 Posterior estimated mean count (i.e., expected number of egg batches per branch) and the respective posterior 95% CIs shown as error bars per beetle species and eucalyptus tree species (*E. bosistoana* and *E. quadrangulata*) in the Marlborough field trial, New Zealand. Groups that share the same letters have the posterior 95% CI for the ratio of counts containing 1.0, indicating insufficient statistical evidence for the difference between them



Parasitism

For field-collected eggs, the probabilities of egg fates varied with both beetle and eucalyptus species. On average over the season and both beetle generations, the predicted most likely fate of *P. charybdis* eggs on *E. bosistoana* and *E. quadrangulata* was ‘dead’ ($P_{\max}=0.64$ and $P_{\max}=0.96$, respectively). The least likely fate was ‘*N. insectifurax*’ ($P_{\min}>0.99$) and ‘larvae hatched’ ($P>0.99$) for *E. bosistoana* and *E. quadrangulata*, respectively (Fig. 3, Table S7). Fate of *P. charybdis* eggs was considerably more likely ‘larvae hatched’ (OR: 0.04 (0.01–0.24)) and less likely ‘*N. insectifurax*’ (OR: 8706.47 (35.66–3,979,711.16)) on *E. bosistoana* than on *E. quadrangulata* (Table S8).

The predicted most likely fate for *Pst. cloelia* eggs on *E. bosistoana* was ‘larvae hatched’ ($P_{\max}=0.73$) and on *E. quadrangulata* eggs had the most likely fate of ‘dead’ ($P_{\max}=0.91$) (Fig. 3, Table S7). On both *E. bosistoana* and *E. quadrangulata*, the least likely fate of *Pst. cloelia* eggs was ‘*N. insectifurax*’ ($P_{\min}>0.99$ and $P_{\min}=0.78$, respectively). However, the probability of egg fates ‘dead’ and ‘larvae hatched’ were not considerably different (OR: 3.81 (0.65–17.81) and 0.27 (0.07–1.55), respectively) between the two eucalyptus species (Fig. 3, Table S8). Fates of *Pst. cloelia* eggs generally did not vary between eucalyptus

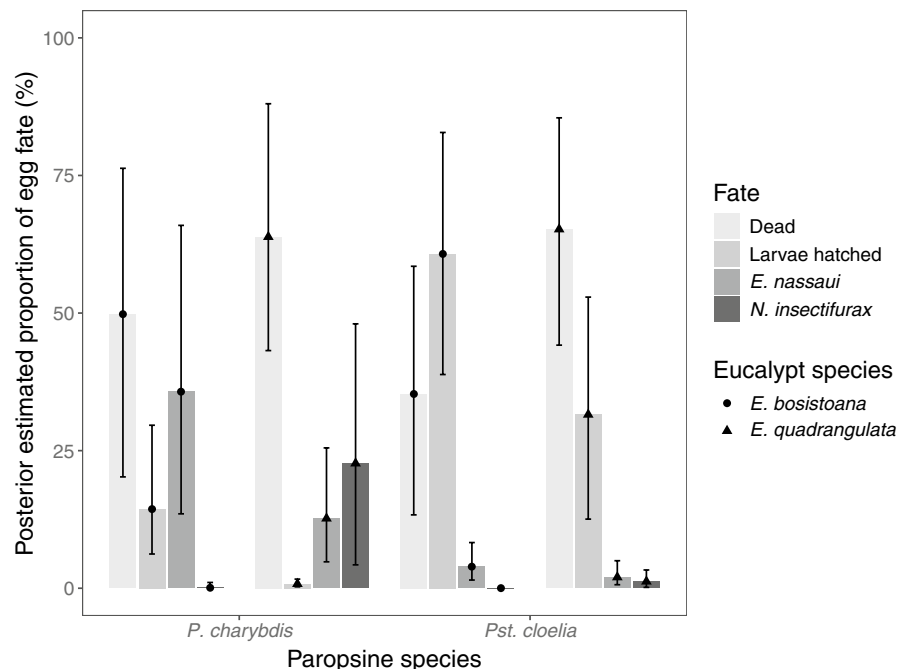
hosts, but eggs were considerably more likely to be parasitised by ‘*N. insectifurax*’ on *E. quadrangulata* than on *E. bosistoana* (OR: 49.34 (11.12–162.51)) (Table S8).

On both *E. bosistoana* and *E. quadrangulata*, the fate of ‘larvae hatched’ was considerably less likely for *P. charybdis* than for *Pst. cloelia* eggs (OR: 10.35 (5.17–23.35) and 63.00 (35.04–107.66) on *E. bosistoana* and *E. quadrangulata*, respectively) and the fate of being parasitised by *E. nassau* was considerably higher for *P. charybdis* than for *Pst. cloelia* eggs (OR: 0.07 (0.03–0.15) and OR: 0.13 (0.08–0.23) on *E. bosistoana* and *E. quadrangulata*, respectively). However, the fate ‘dead’ was evenly likely for the two paropsines on both eucalypt host species (OR: 0.52 (0.26–1.12) and OR: 1.04 (0.33–2.92) on *E. bosistoana* and *E. quadrangulata*, respectively). Parasitism by *N. insectifurax* was considerably more likely for *P. charybdis* than for *Pst. cloelia* eggs on *E. quadrangulata* (OR: 0.04 (0.02–0.06)), but evenly likely for both paropsines on *E. bosistoana* (OR: 6.79 (0.02–2954.52)) (Table S9).

Parasitoid offspring size

Beetle host species had a considerable effect on the body size as indicated by wing length of the emerging

Fig. 3 Posterior mean estimated proportions and the respective 95% CIs shown as error bars of the egg fates of *P. charybdis* and *Pst. cloelia* eggs collected from the two eucalyptus host trees *E. bosistoana* and *E. quadrangulata* in the Marlborough field trial, New Zealand



parasitoid offspring, whereas eucalypt host species upon which the egg had been laid did not (Fig. 4). *N. insectifurax* wing length was consistently slightly longer than *E. nassaui*. Combining parasitoid and eucalyptus species, the predicted wing length for parasitoid offspring emerging from *P. charybdis* was on average 36% larger than for those emerging from *Pst. cloelia* (95% CI: 21.3–55.7%).

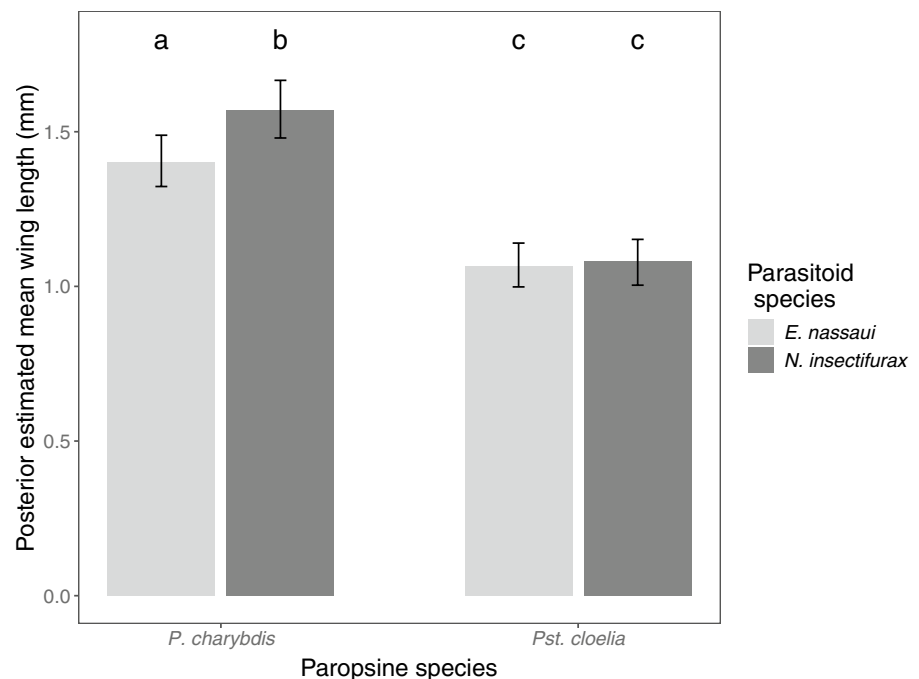
Discussion

We have shown that, under both laboratory and field conditions, *P. charybdis* and *Pst. cloelia* are suitable hosts for the two paropsine egg parasitoids *E. nassaui* and *N. insectifurax*. These findings are consistent with previous studies from Australia (Mo and Farrow 1993; Murphy 2006). However, we found that both parasitoid species clearly preferred *P. charybdis* over *Pst. cloelia* eggs, with lower egg parasitism and a higher proportion of beetle larvae hatching from *Pst. cloelia* compared to *P. charybdis*. There was no indication that the presence of the more preferred *P. charybdis* increased parasitism of *Pst. cloelia*, as *Pst. cloelia* egg fates showed no clear difference between choice and no-choice laboratory trials or between eggs collected from the two eucalyptus species in

the field trial, on which relative abundance of the paropsines varied.

There are several possible explanations for the parasitoids' preferences for *P. charybdis* eggs, including natal host, host size, chorion composition and structure, and egg arrangement within the batches. The natal host alone may not influence parasitoid host preferences, as shown in the idiobiont pupal parasitoid *Pachycrepoideus vindemiae* Rondani (Hymenoptera: Pteromalidae), that only displayed a preference for its natal host after gaining experience attacking it (Morris and Fellowes 2002). All *E. nassaui* and *N. insectifurax* individuals used in the laboratory trials emerged from *P. charybdis* eggs, so we cannot rule out that they retained host preferences for their natal host. The female *E. nassaui* and *N. insectifurax* used in laboratory assays had not been permitted to oviposit within *P. charybdis* eggs. However, females identified for choice tests had interrupted encounters with them during pre-screening. We have not experimentally verified the impact of this experience on oviposition preferences, but this method was effectively used in Murray (2010), and significant differences in host preferences for beetle species eggs and physiological suitability was revealed in that study. To examine the role of natal host on host preferences of both these parasitoids, parasitoid colonies would

Fig. 4 Posterior estimated mean wing length (in mm) of *E. nassaui* and *N. insectifurax* offspring emerging from *P. charybdis* or *Pst. cloelia* eggs collected from *E. bosistoana* and *E. quadrangulata* host trees in the Marlborough field trial, New Zealand. For groups with different letters, the posterior 95% CI of the ratio of posterior estimated mean lengths did not include 1.0, i.e., there was sufficient statistical evidence for a difference



need to be developed from both paropsine hosts and their preferences compared, a task beyond the scope of this study, but one we plan to investigate. It will be important to establish whether the smaller parasitoids that did emerge from *Pst. cloelia* are reproductively viable, as well as whether they retain behavioural preference for *P. charybdis* eggs.

Both *E. nassaui* and *N. insectifurax* are believed to be idiobiont solitary parasitoids that prevent further development of the host embryo, hence initial host size is of particular importance for parasitoid development (Murray 2010). *Pst. cloelia* eggs are smaller in size (~2.0 mm) compared to *P. charybdis* eggs (~2.6 mm) (De Little 1979). Although both *E. nassaui* and *N. insectifurax* can parasitise and fully develop in *Pst. cloelia* eggs, progeny of both parasitoids emerging from *Pst. cloelia* eggs were considerably smaller than those emerging from *P. charybdis* eggs (Fig. 4). Previous laboratory studies also observed that different-sized paropsine host eggs supported development of both species, and that offspring size varied with paropsine host (Murray 2010; Tribe 2000). Although it is not the only determinant of parasitoid body size, larger host size commonly leads to increased offspring size (Häckermann et al. 2007; Liu et al. 2011) as host size is generally correlated with resource availability (Liu et al. 2011; Nicol and Mackauer 1999). In turn, larger parasitoids can be related to increased fitness and reproductive parameters that may vary with sex. For example, increased body size in females may increase longevity, lifetime fecundity, and egg load, as well as dispersal and host-finding ability (Bezemer and Mills 2003; Saeki and Crowley 2013), in males it may increase longevity and mating success (Sagarra et al. 2001). Host size may not affect both sexes equally. For example, for the typically idiobiont egg parasitoid *Anastatus disparis* Ruschka (Hymenoptera: Eupelmidae), host egg size only significantly affected the size of female but not male offspring (Qi et al. 2024). Accordingly, choosing larger host eggs may provide a fitness advantage for *E. nassaui* and *N. insectifurax*. However, the extent and kind of effect remains a subject for future research.

Chorion structure is recognised as a physical defence against parasitism and as an important trait for host recognition and acceptance (Mansfield and Mills 2004). In laboratory trials investigating the host acceptance of *E. nassaui*, Murphy (2006) found that

this parasitoid preferred *Paropsis* species (including *P. charybdis*) over the congener *Paropsisterna* species (including *Pst. cloelia*) in Tasmania, Australia, which was attributed to the generally smooth chorion structure of *Paropsis* species eggs compared to the spine-like chorion structure of many *Paropsisterna* species eggs. Moreover, *P. charybdis* eggs are laid in a single, easily accessible layer, whereas *Pst. cloelia* often oviposits batches in untidy heaps with eggs mounted on top of each other. This may provide additional protection against parasitism for *Pst. cloelia* as the eggs located beneath others may be physically difficult for wasps to successfully antennate and oviposit in (Murphy 2006).

We assume that egg age had no effect on the outcome of the laboratory tests as egg batches presented to parasitoids were always less than 24 h old and both parasitoids are known to prefer freshly laid host eggs (Murray 2010). However, this cannot be excluded for results of the field trial since egg batch age could not be determined at time of collection.

The laboratory assays involving no-choice and choice tests were methodologically consistent with Murray (2010) as the duration of egg exposure is important to ensure assays are long enough for each parasitoid to encounter all potential host eggs, but short enough to avoid superparasitism. Murray (2010) had detailed the appropriate methods to take account of the different behaviour of the two species. In the Murray (2010) assays false positive parasitism was observed. For instance, laboratory trials predicted that the *Acacia*-feeding paropsine *Dicranosterna semipunctata* (Chapuis) was within the physiological host range of both egg parasitoids. Two choice tests revealed a mean of 83–89% *P. charybdis* egg parasitism compared to 40% and 23% *D. semipunctata* egg parasitism by *E. nassaui* or *N. insectifurax*, respectively. Despite being physiological hosts, no *D. semipunctata* parasitism has ever been recorded from field collections. Conversely, our field trial revealed similar probabilities of *Pst. cloelia* parasitism do occur in the field compared to laboratory trials. Numerous factors could explain this discrepancy in the usefulness of laboratory trials between these two studies. Host plant cues is the most obvious difference between the trials and is known to strongly affect parasitoid host searching behaviour (Avila et al. 2016). Chemical and visual cues of eucalyptus foliage may be an important habitat cue that retains both parasitoid species on

Eucalyptus hosts and not on *Acacia*, thus ecologically separating them from *D. semipunctata* eggs.

Importantly, in addition to successful parasitism, a proportion of eggs of both paropsines died during rearing in the laboratory in the presence of parasitoids. Results from laboratory choice tests suggest that this mortality was associated more with *E. nassau* than *N. insectifurax*. However, this trend was not observed in no-choice tests. Mortality of both *P. charybdis* and *Pst. cloelia* eggs collected in the field from *E. quadrangulata* (63.8% and 65.2%, respectively) was higher than mortality observed in any beetle-parasitoid combination during the laboratory trials. Although the cause of egg mortality is unknown, dead eggs from both laboratory trials and field collections were often marked with oviposition scars, sometimes multiple scars. Mortality of eggs with oviposition scars could have been caused by unsuccessful parasitism, probing without oviposition, or repeated oviposition into the same host (superparasitism or multiparasitism). It is also possible that, after a parasitoid successfully oviposited in a paropsine egg, both host and parasitoid died from unknown causes without developing further. Moreover, both *E. nassau* and *N. insectifurax* are known to probe host eggs without oviposition (T. Withers, pers. comm.), possibly to gain additional chemical or physical information on host quality and developmental state (Murray 2010). Another paropsine parasitoid, *Signiphora* sp. Ashmead (Hymenoptera: Signiphoridae), would probe *Trachymela tincticollis* (Blackburn) (Coleoptera: Chrysomelidae) eggs to assess developmental state and only oviposit when yolk was absent (Tribe 2000). Keinan et al. (2012) suggests that repeated probing may lower host defences and increase parasitoid offspring survival. However, probing may also kill eggs irrespective of whether a parasitoid oviposits (Tribe 2000). *E. nassau* tends to parasitise batches more quickly and move on with host searching immediately after ovipositing compared to *N. insectifurax*, which tends to guard egg batches after ovipositing. Hence *E. nassau* may be more inclined to encounter and probe less suitable hosts nearby (Murray 2010). *D. semipunctata* eggs were readily accepted and probed by *E. nassau* in laboratory trials, but the wasps seemed to probe them more often than *P. charybdis* eggs (although this has not been quantified) (Murray 2010). Nevertheless, only 40% of *E. nassau* offspring emerged from the probed *D. semipunctata*

eggs, adding weight to the hypothesis that excessive probing causes egg death (Murray 2010). Both parasitoids were shown to perform multiparasitism and avoided superparasitism in Murray (2010) laboratory trials. *N. insectifurax* seemed to purposely select for eggs already parasitised by *E. nassau* and was observed puncturing eggs of *E. nassau* with their ovipositor (Murray 2010). *E. nassau*, on the other hand, appeared to only be able to select hosts to avoid (self-) superparasitism and treated all other hosts the same (Murray 2010). Superparasitism and multiparasitism can delay or prevent offspring development (Potting et al. 1997). Another possible explanation for the high rate of dead field collected eggs is hyperparasitism by *B. albifunicle* or *Tetrastichus* sp., the latter being a new *E. nassau* hyperparasitoid in New Zealand found in field-collected eggs. In laboratory trials, Tribe (2000) observed the hyperparasitoid *Neb-latticida* sp. Girault (Hymenoptera: Encyrtidae) probing eggs to locate host parasitoid larvae within the eggs, which increased egg mortality of unparasitised *T. tincticollis* eggs by 23%. Murray (2010) found in laboratory trials that *B. albifunicle* sometimes lays multiple eggs in one paropsine egg. Future research could reveal the reason for the arrested development of these field-collected eggs with oviposition scars by inspecting dissected eggs under a microscope at 100–200× magnification to visualise any parasitism structures (Murray 2010) or employing molecular methods to test for the presence of parasitoid DNA within the eggs (Jones et al. 2005).

The indirect effects of parasitoids causing egg death and unsuccessful parasitism are often overlooked as biocontrol services (Abram et al. 2016). For example, indigenous North American (Abram et al. 2014) and European egg parasitoids (Haye et al. 2015) readily oviposited in the brown marmorated stink bug, *Halymorpha halys* Stål (Hemiptera: Pentatomidae), in laboratory experiments, but parasitoid development was rarely completed and a substantial proportion of eggs aborted and/or parasitoids died early in development (Abram et al. 2014; Haye et al. 2015), leading to a potential underestimation of attack rates of these species in the field. Here, results showed that the combined direct and indirect effect of *E. nassau* through parasitised and dead eggs reduced the estimated proportion of hatched *Pst. cloelia* larvae in comparison to assays with *N. insectifurax* by 41% and 33% in no-choice and choice tests,

respectively. However, as this effect was not observed when paired with *N. insectifurax* or in field-collected eggs, it is unlikely that the total impact of parasitoids on *Pst. cloelia* has been underestimated.

Predicted parasitism rates of field-collected *P. charybdis* eggs in this field study in Marlborough were considerably lower (as a combination of both parasitoids $\leq 36\%$) than those in previous field studies collected from *E. nitens* plantations in the North Island, which generally increase over time and reach up to 100% parasitism rates towards the end of the season (March–April) (Jones and Withers 2003; Mansfield et al. 2011; T. Withers, pers. comm.). However, a direct comparison with the previous studies is not possible as in the present study we used an analysis approach to detect general parasitism trends during the entire season to address the high variability in the data and to allow comparability with the laboratory trials, which does not reveal the effect of time. The low abundance of the more preferred *P. charybdis* eggs on both eucalyptus hosts at the Marlborough site compared to *E. nitens* plantations in the North Island (Jones and Withers 2003) could have led to lower abundance of both parasitoid species, resulting in the lower parasitism rates reported herein. Additionally, the more complex, multi-species system, created by the arrival of *Pst. cloelia* may have reduced parasitoid efficiency on *P. charybdis*. Quantifying *P. charybdis* parasitism in other North and South Island sites with and without *Pst. cloelia* present could verify this.

Overall, the results presented here show that the direct and indirect impacts of the two egg parasitoids *E. nassau*i and *N. insectifurax* on *P. charybdis* in laboratory and field trials remain high, ranging from 76 to 99%, indicating a successful biological control impact. Unfortunately, potential impacts are considerably lower (21–68%) on *Pst. cloelia*. We therefore conclude that the existing egg parasitoids are not expected to suppress populations of this emerging pest in New Zealand unless parasitoids that emerge from *Pst. cloelia* over time develop a preference for that species. Future research could investigate whether other potential specialist biological control agents exist in Australia that could control *Pst. cloelia* in New Zealand. However, this would require considerable investment and time. An achievable short-term benefit would be the identification of generalist predators already present in New Zealand that feed on paropsines and identify potential augmentative/conservation biological control practices

that can support all paropsine species pest management in this country.

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Author contributions CW designed and carried out the field trial and wrote the first draft of the manuscript. All authors commented on previous versions of the manuscript. TMW and SMP helped designing the field trial as mentors. TMW designed and performed the laboratory trials with MAD. EM carried out all statistical analyses and generated graphs of the results. All authors read and approved the final manuscript.

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Declarations

Competing interests The authors declare that they have been recipients of research funding from the Specialty Wood Products (SWP) Partnership that included contributions from a consortium of forestry, timber, and power companies supporting sustainably managed eucalypt plantations in New Zealand, including Vineyard Timbers Ltd, Southwood Exports Ltd, MBIE, Forest Growers' Levy Trust, Juken Ltd, Proseed Ltd, Ernslaw One, New Zealand Farm Forestry Association, Timberlands Ltd, Lake Taupo and Lake Rotoaira Forest Trust, Te Tumu Paeroa, Blakely Pacific Ltd, City Forests Ltd, Marlborough Lines. TW and CW are recipients of research funding from an MPI (New Zealand Ministry for Primary Industries) Sustainable Food and Fibre Futures grant which includes funding from Southwood Exports Limited, Oji Fibre Solutions (New Zealand) Limited, and MPI. The authors do not consider this funding alters their interpretation of the results, and the views reported are to inform for public good.

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