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Article type : Special Issue-IEIC 6

# Special Issue IEIC6

# Observations of parasitoid behaviour in both no-choice and choice tests are consistent with proposed ecological host range

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**Running head**: Eadya *oviposition behaviour in tests*

**Keywords**: oviposition, Chrysomelidae, Braconidae, endemic beetle, host range testing, host acceptance, non-target impacts, *Eadya daenerys*, *Paropsis charybdis*, *Eucalyptus nitens*, Hymenoptera, Coleoptera, biological control agent

**Accepted:** 22 April 2020

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/EEA.12956

**Abstract**

The solitary larval endoparasitoid *Eadya daenerys* Ridenbaugh (Hymenoptera: Braconidae) is a proposed biological control agent of *Paropsis charybdis* Stål (Coleoptera: Chrysomelidae, Chrysomelinae), a pest of eucalypts in New Zealand. *Eadya daenerys* oviposition behaviour was examined in two assay types during host range testing, with the aim of improving ecological host range prediction. No-choice sequential and two-choice behavioural observations were undertaken against nine closely related species of New Zealand non-target beetle larvae, including a native beetle, introduced weed biocontrol agents, and invasive paropsine beetles. No behavioural measure was significantly different between no-choice and two-choice tests. In sequential no-choice assays the order of first presentation (target–non-target) had no significant effect on the median number of attacks or the attack rate while on the plant. Beetle species was the most important factor. Parasitoids expressed significantly lower on-plant attack rates against non-targets compared to target *P. charybdis* larvae. The median number of attacks was always higher towards target larvae than towards non-target larvae, except for the phylogenetically closest related non-target *Trachymela sloanei* (Blackburn) (Coleoptera: Chrysomelidae, Chrysomelinae). Most non-target larvae were disregarded upon contact, which suggests that the infrequent attack behaviour observed by two individual *E. daenerys* against *Allocharis* nr. *tarsalis* larvae in two-choice tests and the frass of *Chrysolina abchasica* (Weise) was probably abnormal host selection behaviour. Results indicate that *E. daenerys* is unlikely to attack non-target species apart from *Eucalyptus*-feeding invasive paropsines (Chrysomelinae). Non-lethal negative impacts upon less preferred non-target larvae are possible if *E. daenerys* does attack them in the field; however, this is likely to be rare.

**Abbreviated abstract**

In host range tests the parasitoid *Eadya daenerys* (Hymenoptera: Braconidae) attacked equally the beetle larvae of *Trachymela sloanei* and the target *Paropsis charybdis* (both leaf-feeding paropsines of *Eucalyptus* trees; Coleoptera: Chrysomelidae). In these assays *Eadya daenerys* spent more time searching, stinging larvae and frass. All other non-target beetle species feed on unrelated plant species in New Zealand, and *Eadya daenerys* spent more time resting and disregarding these larvae, and they were rarely attacked. [75 words]

**Graphic for Table of Contents**

Parasitoid Behaviour Graphical Abstract.tif

## INTRODUCTION

Accurate prediction of a parasitoid's host range prior to its introduction into a new country as a biological control agent is the most important aspect to ensuring only host specific natural enemies are introduced (Barratt et al., 2010). Whereas phylogeny is an invaluable starting point to predict parasitoid host range (Hoddle, 2004), other criteria such as ecological similarities of the target to non-target organisms in the new country are also very important (Kuhlmann et al., 2006; van Lenteren et al., 2006; Paynter et al., 2010). Although the safety of biological control can generally be improved by only introducing parasitoids that are host specific to the genus or species level (Van Driesche & Hoddle, 2016), how this specificity is assured from laboratory testing remains a challenging area of research.

Assay testing designs can influence the outcome of host specificity tests with parasitoids (Barton Browne & Withers, 2002; Van Driesche & Murray, 2004). A methodological consensus exists to guide the compilation of a host testing list and how to approach host testing from a risk assessment point of view (Kuhlmann et al., 2006; van Lenteren et al., 2006). However, there still remain variable predictions of ecological host range from comparing results produced by choice vs. no-choice test methods (Mansfield & Mills, 2004; Murray et al., 2010). There is continuing interest in characterising chemically-mediated responses from parasitoids to target and non-target cues during host range assessment to help resolve these current uncertainties (Avila et al., 2016). However, not all parasitoids respond to chemical cues in the laboratory. The parasitoid of Australian origin *Eadya daenerys* Ridenbaugh (Hymenoptera: Braconidae) (Ridenbaugh et al., 2018) was one such species that appeared to respond to both visual and chemical cues (T. Withers, pers. obs.). Behavioural observations of host finding and acceptance when target and non-target species are presented in more realistic arenas are, in this situation, a useful adjunct to physiological host range tests. By observing the interactions, we can learn to what extent the non-target plants are searched, whether or not the non-target species were contacted, and if oviposition occurred.

To assess its potential as a biocontrol agent for release in New Zealand (NZ), realistic arena assays were undertaken on *E. daenerys* with the aim to predict its ecological host range. The target pest is the invasive *Eucalyptus* tortoise beetle of the subtribe (or clade) Paropsina, *Paropsis charybdis* Stål (Coleoptera: Chrysomelidae, Chrysomelinae), that has been established in NZ since 1916 (Styles, 1970). Species in the Paropsina are primarily larval and adult herbivores of *Eucalyptus* and *Acacia* from Australia and Papua New Guinea (Reid, 2006). *Paropsis charybdis* specialises on the flush adult foliage of *Eucalyptus*, subgenus

*Symphyomyrtus* species, which have economic importance as exotic forestry species in NZ (Withers & Peters, 2017). *Paropsis charybdis* has two or three overlapping generations per year, as adults are long-lived. In NZ, larvae are present in November and December and again in February to April (Bain & Kay, 1989; Murphy & Kay, 2000; Jones & Withers, 2003). Field collections and sentinel-larval trials in the field in the country of origin revealed *E. daenerys* parasitizing larvae of four species within the closely related genera *Paropsis* and *Paropsisterna* (Peixoto et al., 2018). These two genera are in tribe Chrysomelini and subtribe or clade Paropsina (Reid, 2006). New Zealand has no native Paropsina species, only invasive pest species (Withers et al., 2017); however, there are a number of other native beetles and beneficial weed biological control agents in NZ in the same sub-family as the Paropsina, the Chrysomelinae (Withers et al., 2015).

To assess the non-target species that may be at risk from an introduced parasitoid we examined the Chrysomelidae sub-family relationships from Reid (1995) and Leschen & Reid (2004). This resulted in close consideration of all NZ species of Galerucinae, the sister sub-family to the Chrysomelinae, as well as NZ native and beneficial introduced Chrysomelidae (Withers et al., 2015, 2020). Two methods of assessing non-target risk were compared to ensure we had included the most appropriate species on the host testing list (Withers et al., 2018). The most important requirements were (1) non-targets sharing important biological traits with the target pest *P. charybdis*, and (2) including a range of species from closely to less-closely related to the target, based on a proposed phylogeny that examined co-evolution with host plant families derived from Australian chrysomelines (Jurado-Rivera et al., 2009). The required biological traits for non-targets to be a high priority for testing were (1) possessing soft-bodied, external leaf-feeding larvae; (2) having a generation of larvae active in late spring (November-December in the southern hemisphere); and (3) possessing a relatively large body (>6 mm long as an adult). This resulted in a host test list for which the top 10 included: a close relative pest of *Eucalyptus* sharing the same niche (tribe Paropsina), two large weed biocontrol agent Chrysomelinae beetles from Europe, a large Chrysomelinae pest of *Acacia*, the larger of NZ endemic Chrysomelinae sub-alpine beetles, two Galerucinae weed biocontrol agents, and two phylogenetically more distant weed biocontrol agents (in the chrysomelid subfamilies Criocerinae, and Cassidinae) (Table 1). In terms of testing galerucines, we chose to test exotic rather than endemic species, one from each of the two tribes Galerucini and Alticini. This was a practical choice as galerucines are characterised by soil-dwelling root feeders whereas alticine 'flea beetles' are usually miniscule but biologically diverse (Withers et al., 2020). The two weed biocontrol agents were larger than any NZ

species, spring-time active, leaf feeders, and easily obtainable. In total, nine species of non-target beetle larvae were presented to the parasitoid *E. daenerys* on their respective host plant to enable behavioural observations of their interactions, whereas the invasive pest *P. charybdis* was always the control target species.

*Eadya daenerys* is a braconid wasp from Australia with a bright orange head and black thorax and abdomen (Ridenbaugh et al., 2018). *Eadya* species approach host larvae while antennating and quickly insert their ovipositor into any-sized host larva. Oviposition takes approximately 1 s while a single egg is delivered directly into the haemocoel (Rice, 2005b). Paropsina host larvae have a range of defensive behaviours; however, many are slow to respond when densely aggregated on a host leaf. *Paropsis charybdis* larvae react aggressively to parasitoid attack by everting dorsal glands from between their eighth and ninth dorsal segments (Clark, 1930) and thrashing from side to side. This results in larger third and fourth instars often effectively dislodging *E. daenerys*. This defence behaviour impacts on attack rates as parasitoids contacted by dorsal gland secretions will stop searching to groom themselves (Rice, 2005a). To minimise disruption from defence behaviour, second-instar *P. charybdis* were consistently utilised. Following successful *E. daenerys* egg insertion, combined egg and larval development occurs in approximately 25 days at 18 °C within the host (Rice & Allen, 2009). A single fully grown *E. daenerys* larva emerges from the host's prepupa within the soil, spins a brown silk cocoon, and then undergoes an obligate pupal diapause until emergence the following summer (Rice, 2005b). *Eadya daenerys* is univoltine in Tasmania, Australia, with adults present from November to January and phenological knowledge suggests that *E. daenerys* could be effective against the spring larval generation of *P. charybdis* in NZ (AR Pugh, unpubl.; Withers & Peters, 2017). The behavioural observations during choice and no-choice tests were undertaken within Scion's insect containment facility in Rotorua, NZ. Physiological host development assays were also conducted (Withers et al., 2020), with both research studies completed prior to NZ government approval for *E. daenerys* field release being granted in 2019.

## MATERIAL AND METHODS

### Parasitoid culture

Due to difficulties in establishing a laboratory colony of *E. daenerys* in NZ, field-collected adults of unknown age were netted on the wing in *Eucalyptus nitens* (Deane et Maiden) Maiden (Myrtaceae) plantations in Tasmania, Australia (Moina forest 41°32'27"S, 146°04'38"E, Runnymede Forest 42°38'08.9"S, 147°33'57.9"E, Ellendale Forest

42°38'07.24"S, 146°45'04.24"E) between 28 November and 12 December 2014-2017. Adult female wasps were shipped within 7 days of collection in chilled boxes to the containment facility in Rotorua, NZ. Parasitoids were maintained in a temperature-controlled room (14-18 ℃), with a constant supply of honey for a maximum of 35 days before being used in experiments. In 2014 and 2015 *E. daenerys* were kept individually in glass vials at 18 ℃ but this reduced longevity to 14-18 days. In 2016 and 2017 they were maintained in individual ventilated 500-ml plastic containers lined with a paper towel, moistened with a spray of water droplets every 48 h, and kept at 14 ℃ and L14:D10. These conditions increased parasitoid longevity to 35 days.

**Beetle cultures**

The target (*P. charybdis*) colony was established each November from adults collected from various *E. nitens* plantations in the central North Island of NZ. All life stages were maintained in the laboratory at 18 ℃ and L14:D10 in large ventilated Perspex cages (1.0 × 0.8 × 0.6 m). Bases of cages were lined with paper towel and dead leaves. Twice per week adult beetles were provided with freshly cut branches of *E. nitens* foliage bearing adult leaves and new flush foliage, with their cut bases sitting in tap water. These foliage branches were stored for up to 1 week prior at 4 ℃ with bases sitting in water, covered by a large plastic bag to reduce transpiration. Egg laying commenced in late November and all egg batches were removed daily into individual Petri dishes. As eggs hatched, groups of 10-12 larvae were transferred onto cut flush foliage tips of *E. nitens* in 50-ml ventilated plastic containers for 4-5 days before being used in experiments as second instars.

Non-target beetle colonies (Table 1) were reared in the laboratory under the same conditions as *P. charybdis* to obtain larvae of the stage suitable for experiments (second-third instar and of similar body length to second instar *P. charybdis*), each species with a method adjusted slightly to optimise rearing success:

*Trachymela sloanei* (Blackburn) is a defoliating pest of *Eucalyptus* trees that invaded NZ in 1976. Both the larval and adult stages of the beetle feed on a number of *Eucalyptus* spp. (Selman, 1985). The eggs of *T. sloanei* are small and red-brown, and laid in a cluster stuck together in bark crevices. Hatching larvae are initially gregarious and feed nocturnally on young leaves of their host plants. *Trachymela sloanei* adults were collected in December of 2016 and 2017 from beneath the bark of large amenity eucalypts, in Napier city, Hawkes Bay, NZ, and maintained on a foliage mix of *Eucalyptus leucoxylon* subsp. *leucoxylon* F.Muell. and *E. nitens* in the form of freshly cut branches. Larvae hatching from egg batches laid

within corrugated cardboard or old leaves were transferred to unventilated 250-ml plastic containers along with flush foliage and tissue paper to mature.

*Gonioctena olivacea* (Förster) was introduced from the UK in 2007 as a biocontrol agent of the European legume scotch broom, *Cytisus scoparius* (L.) Link. Beetles are univoltine, adults appearing in spring to early summer and laying eggs on the leaf surface of host plants. Larvae feed on leaves and attain full growth through three instars. Adults were collected in spring (November 2015) from the Army Training area, Waiouru, NZ, and maintained in 4-l ventilated plastic containers on potted plants with the base of the container covered in damp filter paper. Experimental larvae were reared in ventilated 50-ml plastic vials with several sprigs of fresh *C. scoparius*, replaced every 2 days.

*Dicranosterna semipunctata* (Chapuis) is an invasive pest of the exotic tree species *Acacia melanoxylon* R. Br. and has been in NZ since 1996 (Murray & Withers, 2011). *Dicranosterna semipunctata* is probably univoltine in NZ, with oviposition beginning in spring (October to December) with the onset of new *A. melanoxylon* phyllode growth. Eggs are laid singly on the foliage, and larvae proceed through four instars, feeding solitarily. Larvae of *D. semipunctata* originated from unhatched eggs or L1 larvae collected in December 2015 and 2017 from the foliage of trees growing on Scion grounds, in Rotorua, NZ. They were maintained on *A. melanoxylon* phyllodes cut fresh daily and maintained with stems inserted into a water reservoir in a lower test chamber (535 ml) and held in place by a folded paper wick.

*Allocharis* nr. *tarsalis* Broun (identified by R. Leschen, Manaaki Whenua Landcare Research) is an uncommon endemic Chrysomelinae found feeding externally on leaves of *Veronica albicans* (Pétrie) Cockayne, in Kahurangi National Park, Northwest Nelson, NZ. A colony was started from 250 larvae collected in January 2017 from a number of sites between 1 200–1 400 m a.s.l. from near Mt Arthur (41°19’80”S, 172°69’51”E), maintained in 300-ml ventilated plastic containers on small sections cut from potted, organically grown, *V. albicans* with the base of the container covered in dampened *Sphagnum* spp. moss. The beetle is thought to be univoltine with overwintered adults emerging from hibernation in leaf litter in early summer. Eggs have never been seen but may be laid in leaf litter beneath the plant, the black larvae feed diurnally on foliage during December and January, scraping oval feeding holes, before pupating in the leaf litter and probably entering extended diapause (C. Wardhaugh, pers. obs.).

*Chrysolina abchasica* (Weise) is a weed biocontrol agent introduced from Georgia (Europe) in 2015 (Bieńkowski, 2011), against tutsan, *Hypericum androsaemum* L., an

evergreen or semi-evergreen shrub that grows to about 1.5 m tall (Groenteman, 2013). Adults were obtained from Manaaki Whenua Landcare Research and were maintained in 4-l ventilated plastic containers on numerous freshly cut sections of potted *H. androsaemum* with the base of the container covered in dampened vermiculite. Larvae were transferred to containers separate from adults to mature until at the stage suitable for experiments.

*Lochmaea suturalis* (Förster) was introduced in 1996 from Oakworth, northern UK, for the biological control of heather, *Calluna vulgaris* (L.) Hull. The colony originated from adults collected in spring (November 2015) from the Army Training area, Waiouru, NZ, and maintained in 4-l ventilated plastic containers on numerous freshly cut sections of heather with the bases sitting in damp florists foam. Larvae were transferred to containers separate from adults to mature until at the stage suitable for experiments and maintained on small cuttings replaced every 48-72 h.

*Agasicles hygrophila* Selman & Vogt is an Alticini flea beetle (Ge et al., 2012) introduced in 1984 from Argentina as a weed biocontrol agent against the semi-aquatic rooted weed *Alternanthera philoxeroides* (Mart.) Griseb, that grows in northern areas of NZ. Both adults and juveniles feed on the leaves of *A. philoxeroides*. Although the weed is aquatic, it can also spread to damp soil beyond the edges of infested water bodies. Three instars feed for a short period of just 2 weeks before boring a hole in the stem to pupate. Adults supplied from Manaaki Whenua Landcare Research were maintained in 1-l unventilated plastic containers on numerous freshly cut sections of *A. philoxeroides*. The plants originated from rooted cuttings which were maintained in a water trough in a glass house on Scion grounds, Rotorua, NZ (with permission from Bay of Plenty Regional Council). These plants were supplemented once every 2 months with phosphorous. Larvae were transferred to 250-ml plastic containers separate from adults and maintained on small cut sections, replaced every 48-72 h, to mature.

*Neolema ogloblini* (Monrós) was introduced into NZ from Brazil in 2011 as a weed biocontrol agent for *Tradescantia fluminensis* Velloso (Fowler et al., 2013), commonly known as tradescantia. Adult beetles lay eggs in small clusters on the underside of leaves. The larvae accumulate a faecal shield on their dorsal surface. Larvae of the first few instars sometimes feed gregariously on the leaf surface, but separate when they are older, feeding individually on the leaf. Multiple pairs of adults provided from the Manaaki Whenua Landcare Research colony were held in a large mesh sleeve cage enclosing potted plants of *T. fluminensis.* Larvae were removed from the infested plant upon the leaf on which they were feeding and transferred to 250-ml plastic containers separate from adults and maintained on small cuttings, replaced every 48-72 h, to mature.

*Cassida rubiginosa* Müller was introduced into NZ from Europe in 2008 as a weed biocontrol agent against thistle pasture weeds, mainly *Cirsium arvense* (L.) Scop. The beetle is univoltine with overwintered adults emerging from hibernation in early spring. Females lay oothecae, containing about 10 eggs, on the undersides of thistle leaves during spring. Larvae are leaf feeders, and pass through five instars, pupating attached to the leaf (Koji et al., 2012). Larvae accumulate a faecal shield on their dorsal surface. Adults originating from an infested site in Masterton, Wairarapa, were maintained in 4-l ventilated plastic containers on potted *C. arvense.* Egg batches were removed upon sections of cut leaf and maintained on damp filter paper until hatching. First instars were carefully transferred by camel hair brush to cut leaves held in 300-ml plastic containers, with a fresh leaf provided every 48 h, to mature.

**Behavioural observations in no-choice sequential tests**

Experimental arenas were large glass Petri dishes (140 mm diameter, 22 mm high). Experiments were conducted under both fluorescent and natural lighting within Scion's PC2 insect containment laboratory in Rotorua, NZ, at 20 ℃ and 75% RH. Eight second-instar beetle larvae were pre-settled 1-2 h prior to the assays onto a 5-8 cm sprig of their host foliage, in clean Petri dishes. In most cases (apart from the nocturnally active *T. sloanei*) an hour was sufficient for larvae to settle into rest or begin to feed. No attempt was made to remove frass associated with this feeding activity. For each replicate a reproductively active (not used prior in the same assay type) but not naive female *E. daenerys* was selected. Pre-screening involved holding a female in a cage within the 20 ℃ laboratory and introducing a *P. charybdis* (target) larva near the female in the same cage on a fine paint brush. If the female began to antennate or probe towards the larva it was considered to be in a suitable state for testing. The parasitoid was then caught and introduced into one of the glass arenas, alternating first contact with either the target host *Eucalyptus* leaves (on which eight *P. charybdis* larvae were already settled) for 10 min (T). After 10 min the parasitoid was caught in a vial and immediately moved by tapping the up-ended vial onto the non-target host (NT) foliage sprig (upon which eight non-target larvae of 2-4 mm length were already settled) in the NT arena, or vice versa, and observed for another 10-min period. Behavioural observations began the instant the parasitoid contacted foliage. Observations were recorded to the nearest second by coding the behaviour and location of the observed behaviour, as below.

**Behavioural observations in two-choice tests**

Experimental arenas were large glass Petri dishes (140 mm diameter, 44 mm high, i.e., double

the height of the no-choice arenas) with eight beetle larvae (second instar or 2-4 mm long) previously settled on a 5-8 cm foliage tip or sprig of their respective host plant (Table 1). The two sprigs were placed 4-5 cm apart. The female parasitoid was tapped from the vial into the dish midway between both sprigs of foliage and recording began when she first contacted either of the host plant sprigs. The same behaviours as listed below were recorded and analysed on the basis of which larval species the behaviour was directed towards, and/or upon which host plant the time foraging, attacking, or resting was spent. The two-choice assays ran for 25 min. We conducted a minimum of 12 replicates on each species with the exception of *T. sloanei*, for which insufficient larvae were obtained, and for *A.* nr. *tarsalis* with sufficient larvae for only eight choice assays. The only assays discarded were those in which a female never contacted or attacked a single *P. charybdis.*

At the conclusion of either type of behavioural assay, the eight target larvae were reared in a plastic container (174 × 118 × 28 mm) with small ventilation holes punched in the lid. The base of the container was lined with paper towel, and fresh foliage was supplied every 48-72 h. *Paropsis charybdis* larvae cease feeding after approximately 12 days at 20 ± 0.5 ℃. At this point, they were individually placed into a small (20 × 20 mm) Trace and Toile fabric pouch (McCall's, China), with the ends sealed with two staples to ensure no gap larger than 2 mm from which a pre-pupa could escape. This method had been developed to encourage emergent *E. daenerys* larvae to spin complete cocoons (Withers et al., 2020). Approximately 10 days later, pouches were opened and the contents recorded (an unparasitized beetle, a dead *P. charybdis* prepupa, or an *E. daenerys* larva or cocoon).

In the case of non-target larvae, those groups of larvae on which *E. daenerys* attacks had been observed were reared. Each week they were transferred to clean containers and any premature mortality, successful pupation to a beetle, or emergence of an *E. daenerys* larva was recorded. Transformation into a beetle pupa was indicative of not having been parasitized by *E. daenerys*. Any larvae or prepupae that died prior to pupation were frozen individually and then dissected under a microscope at increasing magnification (8×, 25×, and then 50×) to inspect for incomplete parasitism. Any larval parasitoid remains were noted, described in detail, and stored for future reference.

**Recorded behaviours**

Time of each behaviour was recorded to the nearest second, as well as the time *E. daenerys* moved onto or off foliage, whether by flying or walking. From this the total time each parasitoid spent on each plant species foliage was obtained. Once a larva was encountered by

the wasp the following behaviours were recorded:

- 'attack insertion' = a probe that involved the wasp inserting its ovipositor into a larva (target or non-target noted) for at least 1 s, previously reported as sufficient time for an egg to be laid, though we had no way of tracking if this occurred in each instance (Rice, 2005a).
- 'attack-fail' = unsuccessful ovipositor probing attempt resulting in either no ovipositor insertion into a larva (target or non-target noted) or for a shorter duration than the 1 s needed for egg deposition. This tended to occur most when larvae were moving rapidly.
- 'attack object' = ovipositor probing at beetle frass, an exuvia, or a piece of host plant such as a cut stem end.
- 'disregard' = encountering a larva but either ignoring its presence or actively rejecting it and moving away.

To summarise the propensity of *E. daenerys* to attack larvae in relation to time in contact with a beetle's host plant sprig we defined the parameter 'attack rate on plant' as the no. attack insertions / total time (min) on that host's plant.

No female parasitoid was tested more than once against any one non-target species in the same assay type but may have repeatedly been used on a different day against another non-target, and to oviposit into *P. charybdis* larvae for colony rearing (Withers et al., 2020).

**Statistical analysis**

To determine whether the number of attack insertions or other scored behaviours on the plant differed between beetle species, the Wilcoxon signed rank test was applied. This non-parametric test was used in favour of the t-test because sample distributions were not normally distributed. The null hypothesis was that the median difference between pairs of behavioural observations was zero (i.e., equal medians). A 95% confidence interval was applied. The Wilcoxon statistic W, in the comparison of 'T' vs. 'NT', is the numerator in the estimated probability of the number of pairs of 'T' being lower than 'NT'. The denominator is simply the product of the two sample sizes. Because measures of central tendency – such as the median, for which rates of attacks were identically zero for all non-targets, or mean (more subject to outliers further from zero, but still low for all non-targets) – fail (by definition of measures of central tendency) to identify behavioural extremes, we used box and whisker plots to visually express the range of behaviours exhibited by all parasitoids. This approach allowed us to identify behavioural extremes in activity in addition to behavioural 'norms'. Cases where boxes show a high degree of separation (i.e., no overlap), also demonstrate significant differences in sample medians. All analyses and data visualisation were performed

in R (R Core Team, 2019).

## RESULTS

### *Oviposition behaviour*

No-choice. The order of presentation (T–NT vs. NT-T) of no-choice sequential tests had no effect on the overall number of attack insertions (Wilcoxon test: W = 7699, P = 0.33), or the attack rate on plant (W = 7463, P = 0.61). Overall, the mean (± SE) number of attack insertions that occurred in 10 min towards target *P. charybdis* larvae was 6.0 ± 0.8, and the mean attack rate per min on that plant was 1.9 ± 0.3 with the order T-NT. With the order NT-T, the mean number of larval ovipositor insertions on *P. charybdis* was 5.4 ± 0.8 and the mean attack rate on plant was 2.4 ± 0.2. In contrast, the overall mean number of larval attack insertions towards all non-target larvae in 10 min was considerably lower at 0.7 ± 0.3 and a mean attack rate on plant of 0.4 ± 0.2 for the order T-NT. For the order NT-T, the corresponding mean values for non-targets were 0.2 ± 0.1 and 0.3 ± 0.2 for attack insertions and attack rate on plant, respectively.

The only non-target species that did not differ from *P. charybdis* in the median number of attack insertions received in no-choice tests was *T. sloanei* (median *T. sloanei* vs. *P. charybdis* = 1.5 vs. 2.5; Wilcoxon test: W = 42.5, P = 0.28); the box-and-whisker plots show a clear overlap of boxes only for *P. charybdis* compared with *T. sloanei* (Figure 1). All other paired non-target species received an identical median of zero attack-insertion counts, compared to the sometimes very high oviposition activity directed towards *P. charybdis* larvae, i.e., >30 attack-insertions in 10 min in one no-choice test (Figure 1). The same trend was reflected in the parameter of attack rates on plant, with no significant difference in median attack rates while *E. daenerys* was on *E. nitens* leaves, whether that was towards *T. sloanei* or *P. charybdis* larvae (Figure 2). Two-thirds of *E. daenerys* (62.5%) undertook at least one attack insertion on *T. sloanei* larvae. Whereas in all other non-target species in the no-choice assays, only a mean of 8% (range 0-25.0%) of female *E. daenerys* undertook an attack insertion on a non-target larva. Despite the close contact with all other non-target larvae when *E. daenerys* contacted their host plant sprig, median attack rates were all much less than towards *P. charybdis* larvae (Figure 2).

Two-choice. Unfortunately, with no *T. sloanei* larvae available for two-choice tests, direct comparison of *E. daenerys* behaviour towards both *Eucalyptus*-feeding species is not available. The median number of attacks on *P. charybdis* larvae was invariably higher than

against any non-target species (Figure 1), although there was no significant difference in the two-choice tests between *A.* nr. *tarsalis* and *P. charybdis*.

The attack rate of *E. daenerys* against *P. charybdis* larvae when on *E. nitens* foliage was higher than the attack rate on plant against most non-target species on their host plants (mean no. ovipositor insertions per min was 2-5 vs. 0-0.8; Figure 2). The observations showed that most of the time when *E. daenerys* was in contact with non-target plants bearing non-target species, the parasitoid predominantly sat, rested, or groomed. The only exception was with *E. daenerys* behaviour towards *A.* nr. *tarsalis* while on *V. albicans* foliage in two-choice tests (Figure 2). In this case, just two female parasitoids were responsible for a high attack rate on plant towards *A.* nr. *tarsalis* and in both cases these two individuals undertook more attack insertions against *P. charybdis* larvae than they did against *A.* nr. *tarsalis* larvae.

In two-choice assays (recall these did not include *T. sloanei*) only 15% of *E. daenerys* females on average undertook an attack-insertion against a non-target larva, compared to 100% undertaking attack insertions on *P. charybdis*, and this was despite all but two individual females making contact with non-target plants during the assays.

*Other parasitoid behaviours*

No-choice. We also recorded other relevant host-finding and acceptance or rejection behaviours undertaken by *E. daenerys* towards larvae in the two test types. Attack-fail counts (attempts to oviposit that did not result in successful insertion of the ovipositor) followed an identical pattern to attack insertion counts. This behaviour occurred significantly more frequently towards *P. charybdis* and *T. sloanei* larvae than against all other non-target species. The median number of attack-fails against all other non-target species was zero (Figure 3).

In stark contrast to successful and unsuccessful attack attempts on larvae, a different trend was apparent with *E. daenerys* attacking objects. This was ovipositor probing into either frass pellets, shed larval exuviae, or cut ends of plant sprigs. Object attacks occurred equally frequently within the assays in the presence of *T. sloanei*, *C. abchasica*, *G. olivacea*, and *P. charybdis*, but in other assays they occurred significantly more frequently in the presence of *P. charybdis* (Figure 4).

The only behaviour *E. daenerys* exhibited that occurred more often in tests with non-target species compared with *P. charybdis* was the 'disregard' of larvae directly encountered (Figure 5). Disregarding occurred significantly more often against *C. rubiginosa* larvae than in the pairwise comparison against *P. charybdis*. In no-choice tests, although the difference was not statistically significant, half the female *E. daenerys* either walked over or actively

avoided/disregarded *D. semipunctata* larvae that they encountered on *Acacia* foliage.

Two-choice. In two-choice tests, attack-fail counts were significantly higher in all assays towards *P. charybdis* than the non-target species, apart from failed attempts to oviposit in *A.* nr. *tarsalis* larvae (Figure 3). *Eadya daenerys* attacked objects consistently and significantly more times against *P. charybdis* objects, than against any non-target objects (Figure 4). Disregarding of larvae encountered during two-choice tests occurred significantly more often towards non-targets than towards *P. charybdis* in over half of the assays. For example, more than half of females tested disregarded all *D. semipunctata* larvae in choice tests, whereas less than a quarter of the same females ever disregarded a *P. charybdis* larva it encountered in the same test. The median number of times *P. charybdis* larvae were disregarded or ignored by *E. daenerys* in choice assays was between zero and once in 25 min (Figure 5).

**Rearing of attacked larvae**

Rearing of target and non-target replicates when attack-insertions were observed, compared to control larvae, was similar to the results of physiological host range assays reported in Withers et al. (2020). Many *P. charybdis* larvae died following the behavioural assays, potentially due to multi-parasitism (being over-stung) as the number of ovipositor insertions at times exceeded 30 stings into just eight larvae (Figure 1). Observed ovipositor insertions by *E. daenerys* against *C. abchasica* resulted in three larvae being internally parasitized, from which no *E. daenerys* emerged, whereas another five attacked larvae showed no evidence of parasitism. One of the *E. daenerys* larvae found in a *C. abchasica* larva was large, whereas the remainder were small. A similar outcome occurred with *A.* nr. *tarsalis* larvae; observed ovipositor insertions in behavioural assays resulted in some of the *A.* nr. *tarsalis* larvae failing to pupate. These were eventually killed for dissection after double the period of time needed for parasitoid development had passed. Four of the dissected larvae contained very small or encapsulated *E. daenerys* larvae, and one contained a moderate-sized *E. daenerys*. Internal parasitism of *A.* nr. *tarsalis* was not consistent, as another three attacked larvae were reared and showed no evidence of parasitism (one died but was empty upon dissection, two became adult beetles). No other species of non-target larvae were found to have been parasitized, not even those observed to have been attacked in assays and examined by dissection (e.g., *N. ogloblini*) (Table S1).

**DISCUSSION**

**Parasitoid behaviour**

We believe there is value in closely observing the interactions between potential biocontrol agents and the novel non-target organisms that will be present in the new environment. In this research we collected information to add to what we already knew about the parasitoid's physiological host range. Our observations suggest cues from *Eucalyptus* must play a key role in host location behaviour by *E. daenerys.* In the choice tests all but two *E. daenerys* contacted the non-target plant sprig within the arena, on which the non-target larvae were settled. However, *E. daenerys* females exhibited significantly different behaviour on non-target host plants than on *E. nitens*, the plant of its host. On a non-target sprig, if they stayed longer than a few seconds, *E. daenerys* were more likely to rest and groom. Significantly more disregards of larvae were recorded on non-target plant sprigs. In many cases the parasitoid walked right over the larva without any apparent recognition that the object was actually a potential host. Occasionally non-target larvae appeared to be recognised and the parasitoid immediately changed direction to avoid contact. Significantly fewer objects such as frass and exuviae were mistakenly oviposited into on non-target plants compared to on *E. nitens*. On *Eucalyptus,* presumably because the attractive 'objects' such as *P. charybdis* frass or a *P. charybdis* exuvia did not undertake defensive behaviour (e.g., thrashing), many *E. daenerys* did not move on after one attack but repeatedly attacked these objects, often in excess of 20× within an assay. The only observed exception to the trend was when two *E. daenerys* individuals exhibited object attacks on *H. androsaemum* in the presence of the non-target *C. abchasica*. *Chrysolina abchasica* larvae produce round black frass pellets similar in size to a first-instar paropsine larva, and this may have created a visually stimulating cue that induced those *E. daenerys* females to attack. Some object attacks were also observed into the darkly coloured cut end of a *H. androsaemum* sprig.

Overall, significantly less searching behaviour was observed on non-target plants compared to on *E. nitens* and this is reflected in attack behaviour against larvae. In the no-choice assays *E. daenerys* directed the same number of ovipositor insertions towards the two *Eucalyptus*-feeding species *P. charybdis* and *T. sloanei.* Whereas *D. semipunctata* larvae induced attacks from only one third of *E. daenerys* females. This non-target beetle *D. semipunctata* feeds on *A. melanoxylon*, a tree often found growing within similar habitats to *Eucalyptus* in their native ecosystems in Australia. In NZ, both tree species are often present within small forestry plantations and urban landscapes, creating geographical overlap in modified ecosystems. These trees are unrelated, must produce vastly different physical and chemical cues, and the lack of larval attacks, attack-fails, and the high number of disregards

suggest *E. daenerys* have no innate behavioural attraction to *Acacia*-feeding paropsines.

The proportion of *E. daenerys* attacking the other Chrysomelinae (*A.* nr. *tarsalis* or *C. abchasica)* was closer to one in five parasitoids. Apart from *T. sloanei*, all other non-target species were significantly less likely to receive an attack insertion, to attempt any attacks, or for their frass or exuviae to be probed (attack object), compared to *P. charybdis*. This was consistent across both no-choice and two-choice assay types and suggests that *Eucalyptus* plant and host cues are a pre-requisite to stimulate intensive host-searching behaviour in *E. daenerys.*

We need to continue the dialogue to improve understanding on biological control biosafety in our risk-averse regulatory environment, especially public perceptions on whether parasitoids will change their behaviour over time and in a new environment (Barratt et al., 2018). Descriptive data such as this are useful to help those less familiar with biological control to visualise how the host plant on which a parasitoid alights has a significant impact on subsequent behaviour. In this case reproductively active *E. daenerys* females were most likely to rest and groom on a non-target plant, whereas on a *Eucalyptus* plant they will initiate searching behaviour. This understanding feeds directly into and supports our risk assessments on the safety of host-specific biocontrol agents (Barratt et al., 2010).

**Testing methods**

The debate also continues on the relative usefulness of no-choice or choice tests for assessing the ecological host range of a parasitoid being proposed as a biological control agent (Van Driesche & Murray, 2004). No-choice tests provide the greatest assurance that the assays have revealed the limits of physiological host range (Kuhlmann et al., 2006; van Lenteren et al., 2006) but if run for too long relative host preferences can be obscured. This was the case in Mansfield & Mills (2004) where no-choice tests of the egg parasitoid *Trichogramma platneri* Nagarkatti were poor at separating hosts that were similar in host preference. Multiple-choice tests can produce more realistic results on host preference, especially for more host specific agents. From previous research, *E. daenerys* was expected to prefer *Eucalyptus*-feeding Paropsina species, as those species were completely physiological hosts (Withers et al., 2020). In this research both assay types supported this hypothesis. The only differences potentially observed were an increased propensity for some individual *E. daenerys* to repeatedly attack non-target larvae of *A.* nr. *tarsalis* in two-choice assays, more so than in no-choice assays. We agree with the recommendations of van Lenteren et al. (2006) that best practice in host specificity testing is still to first conduct no-choice tests before running choice tests to

ascertain relative host preferences. In this way the likely ecological host range of the biocontrol agent can be better predicted (Barratt et al., 2010).

**Behavioural attraction to non-targets**

*Eadya daenerys* used in our tests were of unknown mated status and unknown age which may have influenced parasitoid behaviour. A wide variability in activity was observed amongst *E. daenerys* in all tests but it was only the occasional individual undertaking attack-insertions on non-targets, whereas the majority of parasitoids did not. It is possible the less discriminating individuals were either in a state of oviposition host deprivation (Barton Browne & Withers, 2002) or nearing the end of their lives. For instance, three female parasitoids that undertook an attack-insertion on *N. ogloblini* almost all did so after more than 2 weeks of being held in captivity in 2016, but with the level of replication undertaken there was no consistent relationship between time held in captivity, and likelihood of non-target attack-insertions.

**Risk assessment conclusion**

Although phylogeny is the best initial starting point for predicting parasitoid potential host range (Haye et al., 2005), assessments of the risk posed to non-target species also need to include an examination of parasitoid behaviour towards more distantly related non-targets that share ecological similarities. None of the beneficial or native non-target species tested against *E. daenerys* share the same niche, even though they have exposed leaf-feeding larvae and belong to the same family or sub-family. Only the pest non-target species *T. sloanei* feeds on *Eucalyptus* and no other tested species have host plants in the Myrtaceae family. Four species from the same sub-family as the target (Chrysomelinae: *D. semipunctata*, *C. abchasica*, *G. olivacea*, and *A.* nr. *tarsalis*; Table S1) revealed some internal parasitism by *E. daenerys* in physiological assays (Withers et al., 2020) even though those species did not support complete development of the parasitoid. The results of rearing non-target larvae attacked in these assays supported this evidence, i.e., incomplete internal parasitism in *C. abchasica* and *A.* nr. *tarsalis* larvae (Table S1). We had previously concluded (Withers et al., 2020) that only *Eucalyptus*-feeding Chrysomelidae pests in the subtribe Paropsina with a final larval body size of at least 35 mg could be physiological hosts supporting complete development of *E. daenerys*, i.e., *Paropsisterna* and *Trachymela* species (Withers et al., 2020). This prediction still holds and is further strengthened by the attack rates and attack fails recorded here towards *T. sloanei* but will need to be tested in post-release impact studies once *E. daenerys* is confirmed to have established in the field (releases are planned for 2021). From the

observations now undertaken, we conclude it would be a very rare event that non-target species that do not feed on *Eucalyptus* will be attacked by *E. daenerys.*

**Acknowledgements**

Many thanks to other Scion staff including Justin Nairn, Matt Scott, Pam Taylor, Carl Wardhaugh, and University of Tasmania, Institute of Agriculture staff members Vin Patel and Steve Quarrell. Dean Satchell, NZ Farm Forestry Association, has also been an invaluable contributor to this research. Students have also been highly valued for their assistance with this project, in particular Stephanie Kirk, Mike Davy, Sean Gatenby, Katherine Moors, Elise Peters, and Amy Yasutake-Watson, in NZ, and Ray Ali, Hui Law, Meng Lim, Allanna Russell, and Rebekah Smart, in Australia. Thanks to landowners, including Forestry Tasmania, iFarm, PF Olsen, Oji Fibre Solutions (NZ) Ltd, the NZ Defence Force, the NZ Department of Conservation (DOC), and The Westervelt Company NZ Ltd, for access to field sites for collecting. Thanks to Manaaki Whenua scientists Ronny Groenteman, Hugh Gourlay, Chris Winks, Paul Peterson, and Richard Leschen, and AgResearch scientist Mike Cripps for their invaluable assistance with non-target beetles. *Allocharis* nr. *tarsalis* were collected under NZ Department of Conservation research authority 54216RES. Funding was from the NZ Ministry of Primary Industries Sustainable Farming Fund contracts 12-039 and 407964, the NZ Ministry of Business Innovation and Employment Strategic Science Investment Funding contract to Scion. The first author's presentation to the 6th International Entomophagous Insects Conference in Perugia, Italy, and the preparation of this publication was supported by the Better Border Biosecurity (B3, www.b3nz.org) research collaboration and a travel award from the NZ Plant Protection Society. Industry funding co-partners Southwood Exports Limited and Oji Fibre Solutions NZ Ltd (previously Carter Holt Harvey fibre solutions) are gratefully acknowledged, as are the Speciality Wood Products Partnership, and the NZ Farm Forestry Association.

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**Figure captions**

**Figure 1** Box and whisker distributions of the total number of successful attack-insertions counted for all *Eadya daenerys* in no-choice sequential and two-choice tests, lasting 10 and 25 min, respectively. Boxes represent the middle 50% of attack-insertions encompassed by the interquartile range (from the 25th to the 75th percentile), with the whiskers extending to the minimum and maximum values. The vertical line in each box represents the median, and the circle the mean. Where no box is visible all values are either at, or close to, zero. *Paropsis charybdis* target larvae are shown in grey, next to the non-target species they were paired against: *Trachymela sloanei*, *Gonioctena olivacea*, *Dicranosterna semipunctata*, *Allocharis* nr. *tarsalis*, *Chrysolina abchasica*, *Lochmaea suturalis*, *Agasicles hygrophila*, *Neolema ogloblini*, or *Cassida rubiginosa*. N represents the number of replicates of each species (with eight beetle larvae per replicate), and W the Wilcoxon test statistic. Asterisks indicate the level of significance: ***P<0.001, *0.01<P<0.05; ns, P>0.05.

**Figure 2** Box and whisker distributions of *Eadya daenerys* 'attack rate on plant' observed against a species of beetle larva in no-choice sequential and two-choice tests, lasting 10 and 25 min, respectively. The *Paropsis charybdis* target larvae are shown in grey, next to the non-target species they were paired against. See Figure 1 for explanation of the box-and-whisker

parts and the genus names. N represents the number of replicates of each species (with eight beetle larvae per replicate), and W the Wilcoxon test statistic. Asterisks indicate the level of significance: ***P<0.001, **0.001<P<0.01; ns, P>0.05.

**Figure 3** Box and whisker distributions of the total number of attempted attacks that failed (attack fail) counted for all *Eadya daenerys* in no-choice sequential and two-choice tests, lasting 10 and 25 min, respectively. The *Paropsis charybdis* target larvae are shown in grey, next to the non-target species they were paired against. See Figure 1 for explanation of the box-and-whisker parts and the genus names. N represents the number of replicates of each species (with eight beetle larvae per replicate), and W the Wilcoxon test statistic. Asterisks indicate the level of significance: ***P<0.001, **0.001<P<0.01, *0.01<P<0.05; ns, P>0.05.

**Figure 4** Box and whisker distributions of the total number of attack objects counted for all *Eadya daenerys* in no-choice sequential and two-choice tests, lasting 10 and 25 min, respectively. The *Paropsis charybdis* target larvae are shown in grey, next to the non-target species they were paired against. See Figure 1 for explanation of the box-and-whisker parts and the genus names. N represents the number of replicates of each species (with eight beetle larvae per replicate), and W the Wilcoxon test statistic. Asterisks indicate the level of significance: ***P<0.001, **0.001<P<0.01, *0.01<P<0.05; ns, P>0.05.

**Figure 5** Box and whisker distributions of the total number of times *Eadya daenerys* disregarded (rejected and actively moved away from, or walked over) a larva in no-choice sequential or two-choice test, lasting 10 and 25 min, respectively. The *Paropsis charybdis* target larvae are shown in grey, next to the non-target species they were paired against. See Figure 1 for explanation of the box-and-whisker parts and the genus names. N represents the number of replicates of each species (with eight beetle larvae per replicate), and W the Wilcoxon test statistic. Asterisks indicate the level of significance: ***P<0.001, **0.001<P<0.01, *0.01<P<0.05; ns, P>0.05.

**Supporting information**

Additional Supporting Information may be found in the online version of this article.

**Table S1** Summary of outcomes from rearing larvae observed to have been attacked by *Eadya daenerys* from no-choice (NC) and two-choice (2) tests

**Table 1** Summary of target and non-target beetle hosts tested against the proposed *Paropsis charybdis* biocontrol agent *Eadya daenerys*, from phylogenetically most closely to least closely related to the target host, based on a proposed phylogeny of Chrysomelinae in relation to their co-evolution to host plant lineages (Jurado-Rivera et al., 2009). BCA = biological control agent

| Sub-family | Chrysomelinae | | | | | Galerucinae | Criocerinae | Cassidinae |
|---|---|---|---|---|---|---|---|---|
| Clade | Paropsina | Gonioctenina | Dicranosternina | Phyllocharina | Chrysolinina | | | |
| Species tested | 2 | 1 | 1 | 1 | 1 | 2 | 1 | 1 |
| Genera | *Paropsis*, *Trachymela* | *Gonioctena* | *Dicranosterna* | *Allocharis* | *Chrysolina* | *Lochmaea*, *Agasicles* | *Neolema* | *Cassida* |
| Status in NZ | Exotic pests | Weed BCA | Exotic pest | Native | Weed BCA | Weed BCA's | Weed BCA | Weed BCA |
| Host plant | *Eucalyptus* spp. (Myrtaceae) | *Cytisus scoparius* (Fabaceae) | *Acacia melanoxylon* (Fabaceae) | *Veronica albicans* (Plantaginaceae) | *Hypericum* spp. (Hypericaceae) | *Calluna vulgaris* (Ericaeae), *Alternanthera* (Amaranthaceae) | *Tradescantia fluminensis* (Asteraceae) | *Cirsium* spp. (Asteraceae) |