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Results of the First Year of a Sustainable Farming Fund Study to Locate a Spring-active Parasitoid in Tasmania for Potential Biological Control of *Paropsis charybdis*

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EXECUTIVE SUMMARY

Despite considerable efforts, the eucalyptus tortoise beetle *Paropsis charybdis* continues to defoliate *Eucalyptus nitens* plantations throughout New Zealand. This pest prevents expansion of this forest resource and requires constant management through aerial insecticide application.

In 2012/13 the Future Forests Research Diversified Species Theme partially-funded a Sustainable Farming Fund (SFF) research project named Contract 12-039 “Scoping Biological Control for Eucalyptus Tortoise Beetle Larvae”. This report summarises the 2012 Fieldwork conducted in Tasmania by the project team.

A parasitoid wasp of the spring-time larval stage of the eucalyptus leaf beetle *Paropsisterna agricola* in Tasmania is being studied as a potential biological control agent for New Zealand. The potential agent *Eadya paropsidis* was caught as adults on the wing from *E. nitens* plantations in northern Tasmania in December 2012 and brought into the laboratory in Hobart for behavioural testing.

Both sequential no-choice and two-choice testing methods examined the response of individual field-caught wasp females towards *P. agricola* or *P. charybdis* larvae, or both (a two choice method). Females behaved significantly more positively in attacking *P. agricola* larvae than in attacking *P. charybdis* larvae, but both species were attacked, and wasps reared out from them. This preference for attacking *P. agricola* may just be a result of the prior field experience they had unavoidably had.

Trials where laboratory-reared *P. charybdis* larvae were seeded out in groups into the wild and left there for three days to be attacked by any natural enemies were undertaken on *E. nitens* trees in five separate locations in Tasmania. In addition, field collections were made of wild *P. charybdis* larvae. Reared from these collections were natural enemies including both Tachinid flies and *E. paropsidis*. Some of the *E. paropsidis* reared have been sent overseas for identification, while the remaining individuals have been placed into over-wintering conditions in the laboratory. The results suggest this spring-active parasitoid *E. paropsidis* has good potential as a biological control agent for *P. charybdis* in New Zealand. This potential agent will be further evaluated in year 2 of the SFF study.

INTRODUCTION

Paropsine beetles (of which eucalyptus tortoise beetle is one species) are extremely diverse and abundant in their native Australian range, but they rarely cause substantial damage in natural and undisturbed forest. They have emerged as significant eucalypt defoliators only since the expansion of managed plantation forestry, particularly when host trees are planted outside their native range. In New Zealand since 1916, *Paropsis charybdis* has effectively prevented the commercial establishment of the highly favoured pulp species *Eucalyptus nitens* for a long time, until the introduction of the egg parasitoid *Enoggera nassaui*[1].

Paropsis charybdis completes two generations per year in New Zealand. The first generation of eggs are laid in spring from October onwards, and those laid early often escape any natural enemies [5]. After appearing in November, *E. nassaui* can control the latter portion of first generation eggs, and the second generation of eggs laid in summer time are controlled by *E. nassaui* as well as by a self-introduced primary egg parasitoid (*Neopolycystus insectifurax* Girault) which was first found here in 2000 [3].

Biological control of eucalyptus leaf beetle has been disrupted by the arrival of the hyperparasitoid that attacks *E. nassaui*, *Baeoanusia albifunicle*[4]. This difference in control between beetle generations was attributed to a mismatch between the climate requirements of *E. nassaui*, which was obtained from a frost-free area of Western Australia, and the conditions experienced in the central North Island of New Zealand [6]. To improve performance against the first *P. charybdis* generation, another biotype of *E. nassaui* was imported from a cooler climate (Tasmania) and established in the central North Island in 2000 [6, 7]. There is not yet any evidence that this Tasmanian biotype has been able to exert any better control of the first pest generation in New Zealand [10].

With the market projections for sustainably grown *E. nitens* continuing to increase, we undertook a fresh look at biological control prospects available to us for targeting the first generation of *P. charybdis*. The braconid wasp *Eadya paropsidis*, was the obvious first choice for consideration. It produces one generation per year and is responsible for high percentages of first generation parasitism of *Paropsisterna agricola* (Chapuis) in *E. nitens* plantations in Tasmania [8, 9]. Our first priority was to establish whether *E. paropsidis* would be effective against *P. charybdis* and be physiologically compatible.

A preliminary study undertaken in 2011 confirmed that *P. charybdis* was indeed a highly suitable physiological host for *E. paropsidis*[11]. Additional research is now being undertaken under a SFF research project – namely Contract 12-039 “Scoping Biological Control for Eucalyptus Tortoise Beetle Larvae”. This report summarises the outcome of the first year of research undertaken by the project team, and also under sub-contract to entomologists at the University of Tasmania (TIA) in 2012-13.

METHODS

Insects

Eadya paropsidis were caught as adults of unknown age on the wing from *E. nitens* plantations at Moina forest, Mersey District, northern Tasmania on 30 November and 6 December 2012. This forest had been sprayed approximately 24 months previously with alpha-cypermethrin to control leaf beetles. Adult female wasps were returned in chilled boxes to the laboratory within glass vials with mesh inserts in their lids, and provisioned with a male and a drop of liquid honey. They were maintained in an 18°C temperature-controlled cabinet for a maximum of seeven days before being used in experiments.

Control larvae of *Chrysophtharta agricola* (Chapuis) were obtained as eggs laid on juvenile foliage of *E. nitens* from Moina forest, and maintained in the laboratory on cut juvenile leaves of *E. nitens*.

The *Paropsis charybdis* colony was initiated from adults collected in 2011 from Hobart, Tasmania off *Eucalyptus ovata* and *Eucalyptus viminalis*, and maintained in a cage at the University of Tasmania with *E. viminalis* branches in a 20°C laboratory. Egg laying commenced in late November and as egg batches hatched, larvae were maintained on adult flush foliage of *E. nitens* before being used in experiments.

Behavioural Observations

- Experimental arenas were large glass petri dishes measuring 240 mm diameter x 45 mm high. Into each dish was placed a small cutting of *E. nitens*, either juvenile foliage (bearing *C. agricola*) or adult flush foliage (bearing *P. charybdis*). A drop of honey was smeared onto the flat glass lid of the dish. Experiments were conducted at bench height under both fluorescent and natural lighting within a laboratory at ambient (20-23ºC) temperature in Hobart, Tasmania.
- The methods chosen for testing the hypothesis of the behavioural preference of *E. paropsidis* consisted of one female parasitoid observed at a time with either eight target or eight non-target host larvae in experimental arenas, using a cross-over study of A-C or C-A sequence. Sixteen replicates were conducted.
- Parasitoids were introduced onto either the target host (eight larval *C. agricola*) for 10 minutes (A), then almost immediately moved on to the test host (*P. charybdis*) in the C arena and observed for 10 minutes, or vice versa (hence cross-over type study). Behavioural observations began once the parasitoid first encountered (characterised by active antennation) a larva in the first arena, and began immediately upon entering the second arena. To eliminate parasitoids that were not in a physiological state suitable for testing, those that did not show interest in any hosts within 30 minutes were excluded and trialled again the next day.
- Behavioural observations consisted of total frequencies of the number of times parasitoids attempted to attack larvae ‘attacks’ and proportion of the time spent actively searching on the foliage, versus being off the leaf. Also any attacks on frass (faecal pellets) were recorded. Larval probing behaviour consisted of the parasitoid stabbing forwards with its ovipositor. As it was not always possible to tell if the ovipositor had been successfully inserted for long enough (approximately one second [8]) for an egg to pass into the larva, resulting in a successful attack, probing and attacking were counted together. Each female parasitoid was tested only once for behavioural observations using the cross-over A-C or C-A sequence A (agricola) or C (charybdis), but those that were still alive were used on subsequent days in the two-choice assays (described below).
- Two-choice assays were carried out in the same arenas (all glassware had been cleaned and oven baked overnight at 90°C between replicates and treated to remove any chemical contaminants) using the same female parasitoids. One leaf of each foliage type bearing approximately eight second instar larvae as above was put into the dish at the same time, approximately 10 cm apart. The orientation of the leaf in relation to the laboratory was changed between each observation. Assays were run for a maximum period of 25 minutes. Females

were tested in a random order, then alternated between which leaf the female was encouraged to first alight upon. First leaf contact could never be totally controlled. Therefore first contact was assessed as a grouping variable in analyses (see below).

- Identical behavioural observations were recorded as described above, but in all cases the timing and type of leaf landed upon or larva contacted were recorded as either A (agricola) or C (charybdis).
- After the completion of the observations, target and non-target larvae were transferred to 200-ml plastic containers into which holes for air movement were made. They were then reared in a 20 deg C, 16:8 L:D Contherm incubator for up to three weeks after completion of experiments. Fresh foliage was supplied as required, and all larvae were monitored twice weekly for premature mortality, successful pupation, or emergence of an *E. paropsidis* parasitoid larva.

Data Analysis

We applied linear mixed-effects models using restricted maximum likelihood estimation (R-package nlme) to analyse the time the wasps spent on each leaf type and the rate of larval attacks (number of larval attacks standardised by time on the leaf). The fixed term of the model comprised 'host identity' and, in the case of the crossover study, we also incorporated the host 'exposure sequence' and the interaction between these two explanatory variables.

Sentinel Larval Studies

Sentinel larval trials involve placing laboratory-reared host larvae in the field, in the natural environment where the parasitoid is found, to assess levels of parasitism. These were conducted at the following sites: Ellendale, Moina, Runnymede, Pangarinda, and The Lea, Tasmania.

On each tree alongside a track, a branch of approximately 1 cm diameter was selected and tied down firmly to a stake in the ground, to prevent wind-thrash. The stake and the branch closer to the main stem were smothered in Tanglefoot™. Then branch foliage was clipped back to approximately 0.33 m^2 of foliage. All insects and spiders that were located on that foliage were carefully removed, in three separate inspections. When confident that the foliage was insect- and natural enemy-free, laboratory-reared larvae of either *P. charybdis,* (and at three sites also *P. agricola)* were released onto each branch. We attempted to minimise disturbance to the larvae during transfer, by either gently using a brush, or stapling and tying the foliage on which they had been feeding, onto the cleared branch so the larvae could transfer new foliage in their own time. Larvae were left for 72 hours.

Exactly 72 hours later we returned and carefully removed all larvae from each branch, putting them into plastic aerated containers, one for each replicate, and returning them to the laboratory in chilly-bins. Each replicate was split up so that no more than 20 larvae were put into each container, and larvae were fed on *E. nitens* foliage as required and reared to pupation within a Contherm chamber set at 20ºC and 16:8 L:D cycle.

Field Collections of Larvae

In field sites where larvae of *P. charybdis* (and a closely related *P. tasmanica*) were located, groups of larvae were brought back to the laboratory and reared to pupation. A sub-sample of the *E. paropsidis* reared from these field collections will be sent overseas for molecular and taxonomic identification. All remaining *E. paropsidis* (n=24) and Tachinidae (n=60) larvae that successfully pupated in the laboratory were transferred to a range of laboratory artificial over-wintering conditions, the results of which will be reported on in the next internal report.

Collections were made from natural populations at the following sites: The Lea, Runnymede, and Pangarinda, Tasmania.

RESULTS

Behavioural Observations

The time spent by *E. paropsidis* on leaves (residence time) presenting *P. agricola* was between three and eight times longer than on leaves with *P. charybdis*, depending on the laboratory method of host exposure given (Figures 1 and 4). In the crossover no-choice study, the rate of larval attacks differed significantly between the two Paropsine larval host species (P<0.001). *P. agricola* was attacked 2.7 times more often than *P. charybdis* (Figure 2). Given simultaneous two-host choice exposure, this significant difference in larval attack rate per residence time disappeared, although a slight preference for attacking *P. agricola* more quickly than *P. charybdis* remained (P<0.001, Fig. 5). Interestingly, frass derived from *P. charybdis* completely failed to attract the parasitoid wasp to oviposit, whereas *P. agricola* frass stimulated on average one mis-directed attack per two-minute period spent on the juvenile leaf type, irrespective of the laboratory method of host exposure given (no-choice crossover study: P < 0.001; two-choice study: P < 0.001; Figures 3 and 6).

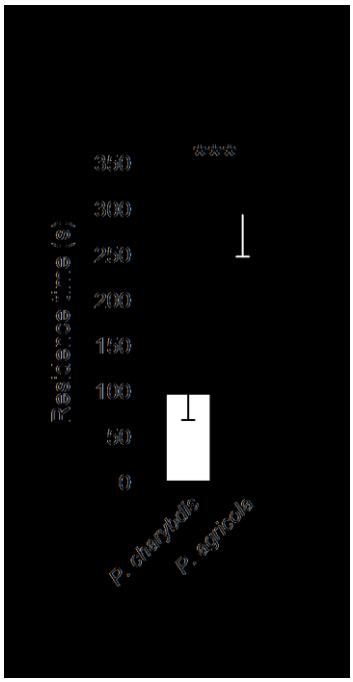

Figure 1. Average Total Residence time on the leaf type bearing the paropsine larvae either *P. charybdis* or *P. agricola* in cross-over no-choice study

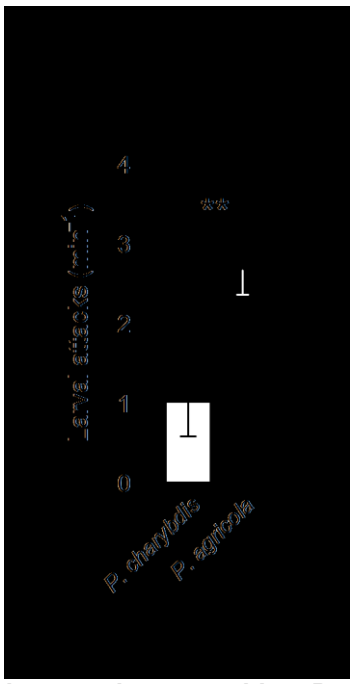

Figure 2. Mean number of larval attacks per minute on either *P. charybdis* or *P. agricola* in cross-over no-choice study

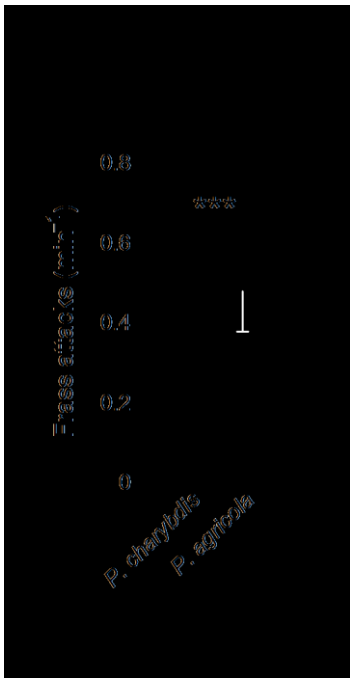

Figure 3. Mean number of frass pellets attacked per minute on either *P. charybdis* or *P. agricola*-bearing leaves in cross-over no-choice study. One sample t-test result for frass attack rate P = 0.0002

Two-choice Host Experiment

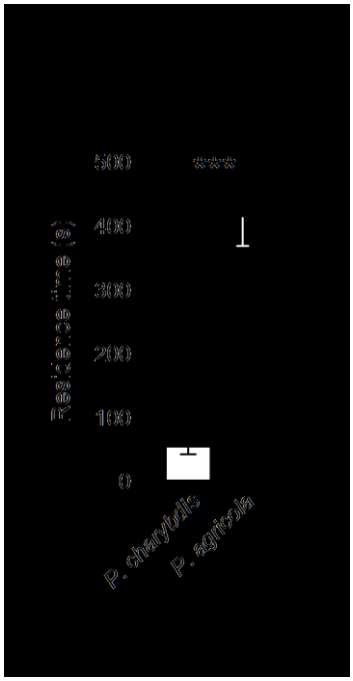

Figure 4. Average Total Residence time on the leaf type bearing the paropsine larvae either *P. charybdis* or *P. agricola* in two-choice study

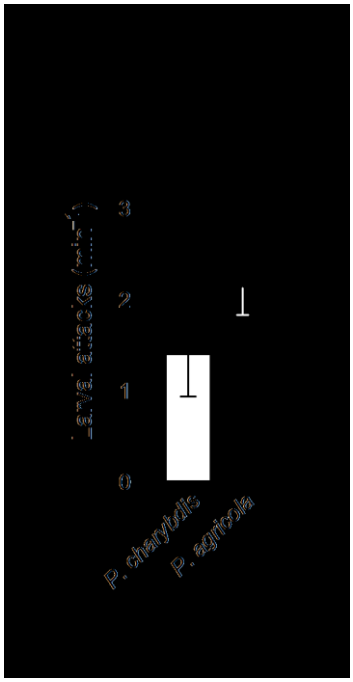

Figure 5. Mean number of larval attacks per minute on either *P. charybdis* or *P. agricola* in two-choice study

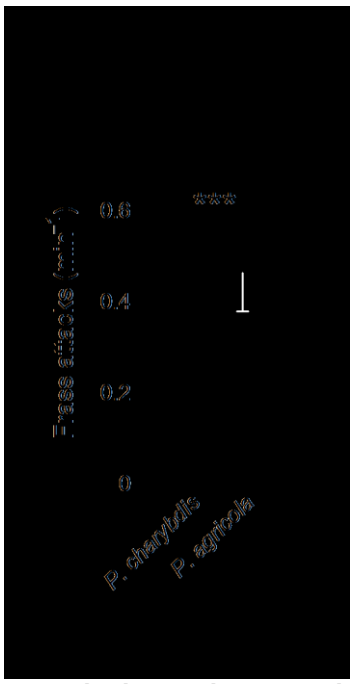

Figure 6.Mean number of frass pellets attacked per minute on either *P. charybdis* or *P. agricola*-bearing leaves in two-choice study. One sample t-test result for frass attack rate (two-choice study): P = <0.0001

All larvae that had been suspected of being attacked by *E. paropsidis* in the no-choice or two-choice assays were separately reared to pupation in the laboratory. Both paropsine species were hosts for *E. paropsidis*, but a lower number of both species of beetle larvae were parasitised by *E. paropsidis* in the two-choice tests than remained unparasitised. The opposite was the case for *P. charybdis* larvae in the no-choice tests (Table 1).

Table 1: Total survival of beetle larvae or *E. paropsidis* larvae to pupation according to experiment.

	n beetle larvae reared from no-choice	n beetle larvae reared from two-choice	N *E. paropsidis* reared from no-choice	n *E. paropsidis* reared from two-choice
P. charybdis	18	27	6	11
P. agricola	29	74	36	48

Sentinel Larval Studies

After being out in the field for 72 hours in the following field locations (Table 2), we returned the *P. charybdis* larvae (and *P. agricola* larvae also from three of the sites) to the laboratory for rearing. From these sentinel larval trials it was confirmed that *E. paropsidis* attacks the spring generation of *P. charybdis* in the wild in Tasmania. Furthermore the infestation rate was over 3% at four of the five sites where these sentinel larval trials were conducted. At three of the sites a greater percentage of larvae were infested with *E. paropsidis* than Tachinids. Only at the Moina site did Tachinid flies infest a far greater proportion of *P. charybdis* larvae (almost one third) than did *E. paropsidis* (Table 2).

Table 2: Percentage of recollected larvae that were confirmed to be parasitised by rearing out either *E. paropsidis* or Tachinidae following 72 hours in the field in sentinel larval trials, December 2013. The sample size recorded is the total number of larvae seeded into the field.

	% beetle larvae infested with *E. paropsidis*	% beetle larvae infested with Tachinidae	Total larvae
Ellendale:			
P. charybdis	5.0	4.1	188
P. agricola	6.0	9.6	194
Moina:			
P. charybdis	3.1	29.0	287
P. agricola	12.9	20.9	219
Runnymede:			
P. charybdis	6.0	0	150
Pangarinda:			
P. charybdis	6.25	0	150
The Lea:			
P. charybdis	0	0	150

Field Collections of Larvae

From locating wild field populations of *P. charybdis* and returning these larvae to the laboratory for identification and rearing, we are now able to confirm that *E. paropsidis* attacks the spring generation of *P. charybdis* in the wild in Tasmania. We were able to confirm this in three of the four wild populations located (Table 3). At the site where sentinel larvae were NOT attacked (The Lea, in Table 2), we later located a wild population of *P. charybdis* on a different tree, and these HAD been attacked by *E. paropsidis* (Table 3), specimens of which have been sent overseas for identification. This means *E. paropsidis* was found to be active in every site except for one (Kingston turn-off) in which we located wild populations or undertook sentinel larval trials with *P. charybdis*in December 2012.

Table 3: Percentage of wild-caught and collected *P. charybdis*larvae that were confirmed to be parasitised by rearing out either *E. paropsidis* or Tachinidae

	Number ofbeetle larvae infested with *E. paropsidis*of those collected
The Lea:	
P. charybdis	2 (from 7)
Runnymeade:	
P. charybdis	3 (from 10)
Pangarinda:	
P. charybdis	1 (from 4)
Kingston turn-off:	
P. charybdis	0 (from 2)

CONCLUSION

The first year of the SFF project “Scoping Biological Control for Eucalyptus Tortoise Beetle Larvae” has been a great success. Vin Patel (TIA) succeeded in rearing a large and healthy laboratory colony of *P. charybdis*, our target for biological control in New Zealand. With these larvae we were able to carry out a number of laboratory experiments and sentinel larval trials in the field. The laboratory experiments have revealed that field-caught female *E. paropsidis* (the spring-active natural enemy we are most interested in) readily attacks *P. charybdis* in the laboratory. Despite females showing a greater propensity to search juvenile leaves infested with the field host *P. agricola*, and to attack frass pellets of *P. agricola*, in a two-choice situation once the data were corrected for leaf residence time, attack rates were not significantly different between *P. agricola* and *P. charybdis*.

Unlike *P. Agricola* larvae, which are gregarious on juvenile leaves, *P. charybdis* feed independently and disperse all over their branches of adult flush foliage, making host location arguably more difficult for *E. paropsidis,* as they can locate only one at a time. The common field host *P. Agricola,* being more clustered together, are slower to thrash out at the parasitoid and the parasitoid is more efficiently able to locate and attack clusters of these species of larvae, and can attack a number of them in quick succession before they disperse in reaction to the attack. Considering that all females caught from Moina had undoubtedly had field experience of *P. agricola* prior to the laboratory trials, and may also have been reared from that host in the field, this is a promising result. Furthermore attacked *P. charybdis* larvae reared right through showed similar infestation levels to *E. paropsidis* to the *P. agricola* larvae. This backs up the preliminary findings of Withers [11]. If this parasitoid wasp were introduced into New Zealand it is likely that if the rearing host was *P. charybdis* and field experience was limited to infestations of *P. charybdis*, that female *E. paropsidis* search and attack behaviour would not be a limiting factor to biological control success.

The careful field searches conducted by Dean Satchell in December 2012 resulted in additional information. He observed that wild populations of *P. charybdis* in Tasmania are being readily attacked by *E. paropsidis.* This is very encouraging to the potential biological control project. Adding weight to this were the results of the sentinel larval trials, in which *E. paropsidis* infested a higher proportion of most larvae that had only been exposed to them in the field for 72 hours than did Tachinid flies in three out of four sites (Tachinidae are another common natural enemy of paropsine larvae in Tasmania [9]). In fact *E. paropsidis* was found to be active in every site except for one (Kingston turn-off) in which we located wild populations or undertook sentinel larval trials with *P. charybdis* in December 2012.

The second year of planned research under this SFF-funded project will answer many more of the important questions that arise from this study, including confirming parasitoid identity, and establishing laboratory rearing methods.

ACKNOWLEDGEMENTS

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APPENDICES

Image 1: Female *Eadya paropsidis* feeding on honey during a no-choice laboratory trial

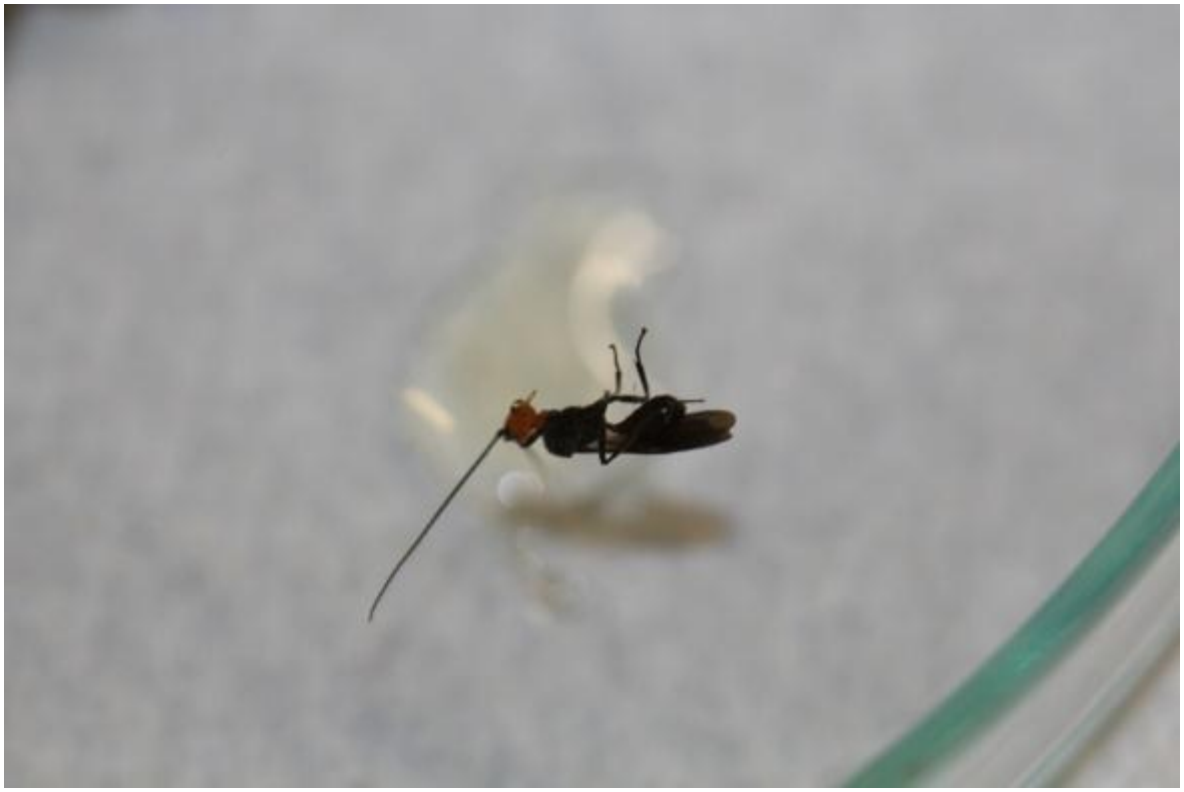

Image 2: Vin Patel sets up a secured branch for attaching sentinel larvae at the Moina field site.