Hort Innovation

Final Report

Integrated pest and disease management – Phase II (Child of AP14014)

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Integrated pest and disease management – Phase II (Child of AP14014) AP15001

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Content

Summary

Apple and pear growers in Australia contend with a range of insect pests that affect productivity, fruit quality, and economic viability. The key pest in all Australian growing regions except Western Australia is the codling moth. The Australian industry has for many years been investing in research to reduce reliance on synthetic pesticides by adopting biorational (ecologically benign, highly selective or behavior modifying) techniques integrated with biological control and agronomic practices to provide economically and environmentally sustainable pest, disease and crop management. One of the results from a 5-year national Orchard Productivity R&D program IPDM sub project was approval to import and release a biocontrol agent (*Mastrus ridens*) against codling moth to supplement existing biorational techniques such as pheromone-mediated mating disruption. Phase II of the IPDM sub-project released Mastrus into sites in Qld, NSW, SA, Tas, and Victoria.

Mastrus were released at two orchards in the Stanthorpe area of Queensland in late April 2016. Approximate numbers of *Mastrus* released were 20,480 at the first site and 17,280 at the second site. Releases were made during April 2017 in five NSW orchards (2 in Batlow; 2 in Orange, and 1 near Young), one orchard in Tasmania (Grove) in May 2017, two orchards in South Australia and one in NW Victoria in May 2018, one orchard at Ardmona Victoria in spring 2018, and another in Batlow NSW in autumn 2019. A total of approximately 50,000 Mastrus were released at each site. Adult parasitoids were evident at all sites for a week after release. In the season following release, codling moth populations crashed at all release sites bar one (2019 Batlow site which has not yet completed a follow up season), resulting in much reduced levels of damage.

To ensure growers and their advisors have good information to help them support Mastrus populations within orchards to their advantage, the research team investigated the direct and indirect effects of common pesticides on the parasitoid. Some pesticides were highly toxic to the adult wasps, whilst others had no direct toxic effect on adults but influenced fertility and subsequent performance of the next generation of wasps. Others were moderately toxic to adult Mastrus at field rates but did not have any effects on fertility or performance of subsequent generations.

Mastrus are reared by exposing laboratory-reared cocooned (hibernating) codling moth larvae to adult Mastrus in special cages that allow the adult wasps to search for and parasitize the codling moth larvae. The parasitized larvae can then be stored for a couple of months before release in large numbers in orchards. The quality and number of the codling moth larvae exposed to Mastrus is important because poor quality larvae and/or overcrowding of the Mastrus may generate poor quality parasitoids. Guidelines for production of the parasitoid and its host codling moth have been developed so that commercial producers of biocontrol agents can assess feasibility of incorporating Mastrus into their suite of biocontrol agents for sale.

Further work is required to develop a cost-effective method of assessing establishment of the parasitoid, and there is an ongoing need to test new pesticides for their direct and indirect effects on survival of Mastrus.

Keywords

Codling moth; biocontrol; integrated pest management; Mastrus ridens; pesticides

Introduction

Orchardists require economically viable production techniques that allow them to meet market requirements so that they can stay in business. The PIPS (Productivity Irrigation Pests and Soils) program conducted from 2009-2014 was established to develop new tools and knowledge for use by fruit growers. The Integrated Pest and Disease Management (IPDM) subproject team introduced a biocontrol agent (*Mastrus ridens*) against codling moth to supplement existing biorational pest management approaches such as pheromone mediated mating disruption; investigated the biotypes of woolly apple aphid present in Australia in preparation for introduction of a predator to supplement control exerted by the parasitoid wasp *Aphelinus mali*; developed an improved scab management program; and reviewed the interactions between various control measures, pests and diseases, and agronomic practices in the orchard. A second phase, PIPS2 was contracted via tender to run from 2015-2020 with an IPDM component tasked to complete field releases of *Mastrus ridens* in pome fruit orchards in eastern Australian states and to evaluate its effectiveness as a biocontrol agent against codling moth. One of the desired outcomes identified in the Apple and Pear Industry strategic plan was to improve industry profitability and global competitiveness. The plan included the strategy of continuing to build the body of knowledge around pest and disease management and prevention, considering both biosecurity risk mitigation and cost reduction. Although pesticides will remain important tools for intervention their use, and the range of available activity groups, will be limited due to restrictions imposed by reaction to consumers, market demands, development of resistance in target pests, and costs. Advances in biological control, enhanced resistance traits in crops and biorational (ecologically benign, highly selective or behavior-modifying) techniques integrated with agronomic practices will provide opportunities for more stable pest, disease and crop management. During the tender process the scope of the project was reduced to completion and evaluation of the Mastrus releases and the timeframe was reduced from five down to four financial years to meet budgetary constraints. The significance of the project for industry is that if Mastrus establishes and performs as well as it has in other countries then Australian pome fruit growers will have access to biorational tools targeting all stages of their most damaging pest, the codling moth.

Methodology

A large component of the project involved rearing the parasitoid (*Mastrus ridens*) and the host (codling moth) in separate cultures. The cultures were separated spatially and temporally so that the parasitoid could not inadvertently contaminate the stock culture of codling moth. Other aspects of the project involved pesticide testing, field releases of parasitoids, monitoring for establishment of the released parasitoids and detection of hyper-parasites, assessment of parasitoid efficacy against the pest, and communication and awareness activities.

Codling moth culture:

A laboratory culture of codling moth was maintained in the Invertebrate & Weed Sciences laboratory at the Tatura Centre of Agriculture Victoria. Codling moth larvae were reared individually in small test tubes containing an artificial diet based on the modifications made by Ashby et al. (1985) to Brinton's diet (Brinton et al. 1969) and used by Sandanayaka et al. (2018). Larvae destined for the stock culture were maintained at 24 ± 1°C, 16L:8D photoperiod, and allowed to cocoon in the tubes. Pupae were removed and placed into emergence cages fitted with an oviposition tube containing honey water to facilitate collection of adults, mating and oviposition. Egg sheets collected from oviposition tubes were placed into plastic containers until eggs matured to the black head stage, indicating imminent hatching, and then used to seed fresh culture tubes.

Larvae destined to be hosts for Mastrus ridens were separated from the stock culture as first instars in freshly seeded culture tubes and maintained in a diapause room at 17± 1°C, 50±10% RH, and 6L:18D photoperiod until larvae had reached 5th instar and were seeking cocooning sites in the cotton wool plugs of the culture tubes. These mature larvae were manually removed from the tubes and presented with rolls of corrugated cardboard in which to establish cocoons. Each roll received 50 larvae and was placed in a lidded petri dish before being stored at 4 ± 1°C in constant darkness until needed for exposure to Mastrus.

Mastrus culture:

The Mastrus culture was maintained at 21± 1°C and 16L:8D photoperiod at the Agribio Centre of Agriculture Victoria, using similar methods to those of Sandanayaka et al (2011) including accumulation of parasitized rolls in cool storage (Sandanayaka et al., 2015).

The effect of cold storage of parasitized larvae on yield of Mastrus was investigated by storing rolls containing parasitized codling moth larvae for 12, 37, 80, 112, 131, 150, 171, 185, 203, and 220 days at 4± 1°C before incubating them at 22± 2°C, 55± 5% RH and 16L:8D photoperiod for 20 days. Emerging adult Mastrus were captured daily, counted and sexed to allow yield and sex ratios to be calculated.

Influence of host larval size on yield and sex ratio of Mastrus was investigated by allowing Mastrus to parasitize either large or medium sized 5th instar diapausing codling moth larvae, then incubating the parasitized larvae at 22± 2°C, 55± 5% RH and 16L:8D photoperiod. Emerging Mastrus were then collected, counted and sexed.

Field releases:

Releases of Mastrus were made in autumn into orchards that were either organic, transitioning to organic, or orchards reported as neglected and infested with codling moth. All sites had active codling moth damage visible at the time of release and high levels of diapausing larvae were expected to have already cocooned on the trees. Parasitized codling moth diapausing larvae in corrugated cardboard rolls were placed in chilled Styrofoam boxes or Eskies for transport to the field sites either by car (NSW, SA, and Victoria) or air plus car (Qld and Tasmania). Transport by air had to be made via specialized pet cargo to satisfy airline concerns about transporting live wasps (despite detailed explanations of the type and wasp and them posing no risk to humans!). Modified fruit fly traps were used to contain the rolls when placed on trees. Four rolls containing the parasitized larvae were placed in each modified fruit fly trap and the traps were then distributed across the release site, a single block of apples in an orchard. The modified fruit fly traps allowed the wasps to safely emerge over a few days and disperse onto the lower trunks of the trees, where codling moth larvae spin their cocoons under the bark. An estimated 480 Mastrus were released from each modified trap and ten traps were used in each release orchard.

Monitoring establishment:

Establishment was initially monitored using sentinel corrugated cardboard rolls containing non-parasitized diapausing codling moth larvae from the culture at Tatura. This technique had been used at the original release

site during the pilot PIPS project and had confirmed movement of Mastrus more than 40m from release trees.

Later monitoring attempts included use of corrugated cardboard trunk bands (Charles et al., 2019) deployed in late summer-autumn to capture wild codling moth larvae seeking cocooning sites, which would then attract Mastrus, other parasitoids, or hyper-parasites if present. Other techniques investigated included use of various trap designs baited with codling moth larvae and/or larval aggregation pheromone (Jumean et al 2005a, 2005b).

Pesticide bioassays:

Bioassays to assess the impact of various pesticides on Mastrus were conducted using commercial formulations of pesticides registered for use on apples and pears. The registered field rates of each pesticide were used as guides to establish stock solutions that were used in serial dilutions representing various multiples or fractions of the field rate. Test arenas consisting of plastic petri dishes were sprayed with uniform small droplets in a Potter Spray Tower delivering 1mL of test solution at 4 psi. After the sprayed arenas had dried, 10 male and 10 female Mastrus were placed into each arena and exposed to the pesticide residue for 48 hrs under constant light at 20 ^{o}C to observe mortality. Petri dishes sprayed with water were used as untreated controls. Pesticides were then rated according to the IOBC accepted toxicity level criteria for laboratory testing (Hassan et al. 1985). Survivors from the treatments were then given access to rolls containing diapausing codling moth larvae to parasitize. The next 2 generations of Mastrus were then observed for effects on sex ratio and fecundity.

Extension:

Industry awareness and extension activities conducted included articles in industry journals, training events, participation as a presenter in Future Orchards Farm Walks, speed updating sessions at APAL annual conferences, and presentations incorporated in to the IPDM training workshops conducted in Qld, SA, NSW, Tas, and Victoria for Hort Innovation project AP16007: An integrated pest, disease and weed management program for the Australian apple and pear industry.

Outputs

A Monitoring and Evaluation Plan was completed. Progress towards outputs identified in the plan in are summarized below. More detail is presented in the sections related to outcomes, monitoring and evaluation, and appendices.

Output 1: *M. ridens* established in orchards in Qld, NSW, Vic, Tas and SA.

Mastrus ridens was released into 16 sites over the life of the project (Table 1). In all cases there was a dramatic reduction in damage caused by codling moth in the season following release of the parasitoid. Sentinel bands consisting of 25mm wide x 1200 mm long corrugated cardboard bands containing diapausing codling moth larvae from the laboratory colony were deployed in 2016 at six sites in the original (2014) release orchard. The bands were placed low on trees near where codling moth larvae would be expected to spin cocoons. The bands were changed after 7-10 days to allow Mastrus time to find the larvae but not give the codling moth larvae enough time to break diapause and emerge as adult moths. One female and one male Mastrus emerged from a total of 24 bands, indicating that Mastrus had survived two seasons in the orchard. As most bands had earwigs present and ants were also present in bands from several locations, it is likely that these insects preyed on the codling moth larvae, as evidenced by few signs of larvae or spent pupal cases present. Further banding was postponed due to a restriction in capacity of the laboratory culture. This was due to the restricted import of one of the key components of the codling moth artificial diet, cholesterol: the suppliers notified the project team that they could no longer supply it without a permit from AQIS. The permit was issued several months later but the problem of ants and earwigs attacking the larvae in sentinel bands had not been resolved, despite testing several methods of restricting ant and earwig access without impacting access by Mastrus. In autumn 2019, before codling moth larvae were leaving infested fruits, fruit damage levels were assessed, and empty bands were deployed to provide cocoon sites for codling moth larvae (that would hopefully become parasitized) in all orchards that had received Mastrus. The bands were retrieved in winter 2019 and examined for presence of Mastrus or signs that Mastrus had been present. In most cases there were very few codling moth larvae recovered, probably because this reflected the low populations in the orchards after the initial populations crashed when Mastrus was released. Almost all bands contained earwigs, spiders and sometimes ants.

Table 1: Mastrus release sites and approximate numbers of Mastrus released.

Location	State	Year	Number of Mastrus
Merrigum	Vic	2014	50,000
St Germains	Vic	2016	10,000
Applethorpe x2	Qld	2016	38,000
Orange x2	NSW	2017	50,000
Batlow x2	NSW	2017	50,000
Batlow x1	NSW	2019	5,000
Young	NSW	2017	9,000
Grove x2	Tas	2017	32,000
Ashton	SA	2018	16,000
Loebethal	SA	2018	16,000
Murrayville	Vic	2018	1,500
Ardmona	Vic	2018	6,000

Output 2: Identification of hyper-parasites that could inhibit the success of codling moth biocontrol by *M. ridens*.

No hyper-parasites were recovered from any of the 310 sentinel bands deployed. This is not surprising given the low numbers of Mastrus recovered. A pupal parasitoid from the Ichneumonid wasp genus *Gotra*, which contains six described species in Australia including *Gotra pomonellae*, was detected at one site in Victoria. This pupal parasitoid would not parasitize Mastrus because codling moth larvae parasitized by Mastrus do not proceed to the pupal stage. *Gotra* could be a useful addition to the biocontrol arsenal because it would attack codling moth larvae that had avoided Mastrus and managed to pupate.

Output 3: Identification of pesticides hat impact survival of *M. ridens*.

A review of the literature failed to identify a standard bioassay for testing toxicity of pesticides to parasitoid wasps. A hybrid bioassay was developed for contact toxicity, accounting for the nature of the pesticides to be tested, the equipment available, and the size and behavior of the wasps. Preliminary testing of the protocol for the bioassay indicated ease of use, safety to the operator, and very low mortality in the check (untreated control) treatment if food or water was provided. The protocol used serial dilutions in water of commercial registered products formulated for use on pome fruit, applied to both the inside base and lid of petri dishes using a Potter Spray Tower, allowing the spray to dry and then exposing adult Mastrus by enclosing them in the petri dishes for 48 hrs with access to food and water. Hassan et al. (1985) claim that there is general agreement that laboratory bioassays should be used for initial screening of pesticides, and that there is no justification for field testing of pesticides that were harmless in the laboratory. They recommended that pesticides that were slightly harmful, moderately harmful, or harmful in laboratory testing should probably be further tested in the field. Field testing requires established populations of the target organism and, because we did not have high populations established in the field, we investigated sub-lethal effects by assessing various traits in the subsequent generations of Mastrus bred from survivors of the direct toxicity testing. High priority pesticides were identified based on their use pattern, target pest range, and potential life span in the market. Each batch of testing required approximately 1000 high quality wasps to be reared while the main culture was being bulked up in preparation for field releases. Results are presented in Table 2 below.

Table 2: Summary of direct toxicity and sub-lethal effects (fertility and fertility of offspring) resulting from exposure of *Mastrus ridens* adults to dried residue of pesticides registered for use on pome fruit. Key to ratings (with IOBC accepted scale (Hassan et al. 1985) enclosed in brackets): L= Low (harmless); M = Moderate (slight-moderately harmful); H= High (high-very high); NS=no survivors to test

Pesticide		**Direct Toxicity**	**Fertility**	**Offspring Fertility**
Type	**Trade name**			
Fungicide	Chorus	L	M	L
	Ziram	L	M	L
	Dithane	L	M	L
	Rubigan	L	M	L
Miticide	Sorcerer	L	M	L
Insecticide	Cormoran	M	L	L
	Altacor	L	M	M
	Avatar	H	NS	
	Samurai	H	NS	
	Insegar	L	Not tested	
	Success	H	NS	

Output 4: Guidelines for commercial suppliers of biocontrol agents.

Development of guidelines for suppliers of biocontrol agents is at a very early stage. Two suppliers have expressed interest. One has visited the rearing facilities for both codling moth and Mastrus and was preparing a business case. The rearing methods for both host and parasitoid are documented but may require further development to be economically viable. Investment in automation of several stages in codling moth production would improve capacity to produce enough Mastrus in the large numbers required for greater distribution across fruit growing regions. Further work is also required to develop safeguards that prevent in-breeding, and the resultant genetic bottlenecks, of the parasitoid. This also applies to calculations of numbers and genetic diversity of Mastrus to release at given sites.

Output 5: Guidelines for growers on how to manage codling moth in apple orchards.

This output might better have been reworded to "Guidelines for growers on how to utilize Mastrus to assist management of codling moth in apple orchards". Despite the dramatic reduction of codling moth populations and damage at release sites, the development of guidelines for growers should be built on evidence of establishment supported by robust data on parasitoid populations and parasitism within orchards. This data is still pending as a result of low recovery of Mastrus in sentinel bands. Despite trialing several methods to improve monitoring, it has not been possible to test various release strategies. The design and adoption of effective Mastrus monitoring methods should be a priority for future work. In the meantime, the data on chemical toxicity, produced for output 3 above, has been disseminated in industry journals, workshops, APAL conferences, and farm walks so that growers and their advisors can begin planning spray programs that would allow survival of Mastrus.

The chapter on management of codling moth, and other information packages, being developed in Project AP16007 (An Integrated Pest Disease and Weed Management Program for the Australian Apple and Pear Industry) will include information on how to integrate biological control agents, including Mastrus, into industry IPDM programs.

Activities supporting delivery of outputs

This project, AP15001, was a subproject within the larger PIPS 2 project, which included an independent Program Coordinator responsible for the mid-term review and the overall M&E Plan. Reports were submitted to the Independent Program Coordinator, and discussed with the other participants in the program and representatives from APAL and Hort Innovation at meetings on:

- 01 December 2015
- May 2017
- December 2017
- June 2018
- December 2018
- June 2019

Industry communication products presented for the broader industry include:

- APAL Research Speed Updating, Launceston May 2017
- Future Orchards Northern Loop Autumn 2017- Stanthorpe, Orange, Batlow, Shepparton
- Pome Zone 2016
- Williams D. (2016). Advances in codling moth control in orchards. *Australian Fruitgrower* 10(4): 31-33
- Williams D (2018). A new biocontrol agent and mass-trapping of codling moth. *Tree Fruit*
- Williams D (2018). Integrated Pest Management. APAL Research Speed Updating, Brisbane June 2018
- Williams D (2018). IPDM: Bringing it all together. Horticulture Conference Shepparton August 2018
- Williams D (2018). Killer wasps and better pheromones target codling moth. *Australian Fruitgrower* 12(3): 26-27
- Williams D (2018). A new biocontrol agent and mass-trapping of codling moth. *Orchard plant Protection Guide for Deciduous Fruits in NSW 2018-19:* 11-13
- Williams D (2018) New methods to target codling moth. *Australian Tree Crop Oct/Nov 2018:* 12-13

Training events conducted for growers and industry support organisations include

- ADAMA Field and Technical staff training day
- Bayer CropScience Field and Technical staff training day
- Seminar at AgriBio 13 May 2016
- Regional Innovation Forum 18 May 2016
- Adama Cormoran Launch
- Mastrus featured in IPDM workshops delivered as part of Hort Innovation project AP16007: An Integrated Pest, Disease, and Weed Management Program for the Australian Apple and Pear Industry.
    - Applethorpe Qld
    - Orange & Batlow NSW
    - Tatura & Bundoora Vic
    - Tasmania
    - South Australia.

Outcomes

The end-of-project outcomes identified in the M&E Plan were:

- Improved awareness by industry of the effectiveness of *M. ridens* as a biocontrol agent for codling moth in Australian pome fruit orchards
- Improved knowledge of hyper-parasites and impact of commonly used pesticides that could inhibit the success of codling moth biocontrol by *M. ridens*.
- Improved awareness by industry of how to manage codling moth using an integrated approach.

Measures to assess these outcomes were:

- Percentage of fruit growers/ advisors aware of *M. ridens* as a biocontrol option
- Percentage of pome fruit growers/ advisors aware of pesticides harmful to *M. ridens*
- Number of biocontrol agent producers aware of how to produce codling moth and *M. ridens*

Outcome 1: Improved awareness by industry of the effectiveness of *M. ridens* as a biocontrol agent for codling moth in Australian pome fruit orchards

The project has confirmed the potential of the parasitoid to enhance the management of codling moth in Australia, despite the lack of hard data to support evidence for establishment of *Mastrus ridens*. Mastrus was deliberately released into orchards with high populations of codling moth to give it a chance of establishing. Most of the selected sites were either under organic production, transitioning to organic production, or in one case "neglected" in preparation for another use. In all cases the populations of codling moth crashed dramatically after release of Mastrus. The release sites were relatively small, which means that codling moth in near-by blocks could migrate back in to the release sites but conversely Mastrus could move into the rest of the orchard in search of prey. The pattern of population crash and slow return has been observed in other countries where Mastrus has been released.

This information has been provided to industry in written form (Australian Fruitgrower; Tree Fruit; Australian Tree Crop; Orchard Plant Protection Guide for Deciduous Fruits in NSW), oral presentations (APAL speed research updating; Future Orchards field days; Industry conferences and seminars; Training sessions for agronomists and chemical company staff), and in focus groups/ local workshops conducted as part of project AP16007.

Outcome 2: Improved knowledge of hyper-parasites and impact of commonly used pesticides that could inhibit the success of codling moth biocontrol by *M. ridens*.

No hyper-parasites were detected but earwigs, ants and spiders were present in sentinel bands used to monitor establishment of Mastrus. All Australian pome fruit growers have access to the Australian Fruitgrower, and many growers and service providers access Australian Tree Crop. The pesticide impacts study has been reported in both journals and it has also been reported at the last two APAL conferences and at the AP16007 workshops and Future Orchards field walks.

Outcome 3: Improved awareness by industry of how to manage codling moth using an integrated approach

The industry now has tools that target each life stage of codling moth. These tools have been used in some cases as stand-alone treatments and been found wanting. In a true IPM approach these tools should be used to complement each other. For example, pheromone mediated mating disruption delays mating and therefore reduces the number of eggs laid by female moths. However, if the population of moths emerging in spring is too large the reduction in egg laying will not be enough to avoid economic damage if no other controls are used. Egg parasitoids (e.g. *Trichogramma*) are available commercially and ovicidal sprays are also available. Timing of both is critical. Larvae that escape the egg parasitoids or the ovicidal sprays can be treated with "soft" chemicals that are relatively safe on non-target organisms but again, with codling moth, the timing is critical because there is a short window of opportunity before the larva penetrates the fruit and becomes inaccessible to pesticides or biocontrol agents. The next susceptible stage is when the mature larvae leave the infested fruit and seek cocoon sites in which to pupate and transform to adult moths. This cocooning stage is the target of Mastrus. Codling moth in Australia can complete between one and three generations per season depending on latitude and temperature. In areas where two to three generations occur Mastrus can help reduce populations between generations during the fruit growing season when the codling moth larvae are susceptible for short time periods and reduce the size of the overwintering population when the larvae have entered diapause (hibernation). In areas with only one

generation per season Mastrus will have access to various levels of diapausing larvae most of the year until it decimates the population. As with any introduced biological control agent, it is highly specific to its host and its own population size will fluctuate according to available host resources. If hosts are difficult to find, then the parasitoid will disperse seeking new populations of codling moth to parasitize. The ability of Mastrus to crash overwintering codling moth populations provides a better probability that mating disruption can reduce egg laying to low levels and that in turn provides incentives for growers to use "softer" options during the fruit growing season. The discovery of the pupal parasitoid Gotra in a Victorian orchard also provides an opportunity to complement the activity of Mastrus because Gotra targets pupae formed by codling moth larvae that escaped Mastrus.

This information has been delivered in workshops, seminars, industry conferences, and particularly emphasized in IPDM workshops and case study meetings conducted in Qld, NSW, Victoria, Tasmania and South Australia as part of AP16007. Growers and consultants attended these activities. IPDM workshops and case study meetings in Western Australia did not involve discussion on codling moth because it is not currently present in that state.

Measures

The percentage of growers/advisors aware of *M. ridens* and aware of pesticides harmful to *M. ridens* would be similar because both topics were raised at the same time during oral and written presentations. Large meetings such as conferences do not lend themselves to the use of evaluation sheets for individual presentations. Industry surveys are also notoriously difficult to conduct and usually result in poor response rates. Grower and advisor awareness have been enhanced by inviting advisors to bring their clients to training sessions and workshops, and the involvement of companies like Bayer and Adama requesting training for their staff and resellers. At least 100 growers have been involved in direct contact with the project team during workshops and training sessions delivered either specifically for this project or for AP16007 (which is proving to be a valuable platform for generating grower and advisor interest).

The mid-term review conducted by the independent coordinator concluded that the project had demonstrated effectiveness of Mastrus; that improved management of codling moth will not only reduce costs and environmental impact but should also facilitate access to key export markets; and that communication had been effective.

Monitoring and evaluation

Effectiveness

The project has met the short-term outcomes identified in the M&E Plan. These have been discussed in detail in the outcomes section of this report. This means that growers who read industry journals, attend workshops and field days, or use consultants who attend such activities are aware of the effectiveness of *M. ridens* as a biocontrol agent for codling moth, the impact of commonly used pesticides that could inhibit the success of codling moth biocontrol by *M. ridens*, and they also have awareness of how to manage codling moth using an integrated approach. To achieve this the project team involved local advisors, chemical resellers, consultants, and chemical company staff, in addition to fruitgrowers, in the target audience for awareness and training activities. The team also took advantage of work they were also conducting in project AP16007 involving much the same audience to achieve efficiencies in travel and time.

It is too early yet to measure adoption because the question of establishment of the parasitoid needs more than the circumstantial evidence of initial effect on codling moth populations. The initial intent for measuring establishment was to use sentinel bands containing diapausing codling moth larvae from the laboratory culture. This was not effective for several reasons. Firstly, there were large numbers of earwigs, ants, and spiders in the organic orchards in which Mastrus was released and these generalist predators appeared to have consumed the sentinel codling moth larvae before, or perhaps during, parasitism by Mastrus. Secondly, it appears that the laboratory reared codling moth larvae may not be emitting aggregation pheromone. Mastrus uses the codling moth aggregation pheromone to find larval cocoons in their hibernacula. A small-scale laboratory study to confirm this failed to detect any pheromone components in the headspace of vials containing diapausing lab-reared codling moth larvae. Further work is required to determine if this was a result of the storage time of the diapausing larvae, lack of appropriate precursors in the artificial diet used to feed the larvae, or adaptation to the rearing conditions in the laboratory. Thirdly, birds such as choughs foraging in the orchards often destroyed the sentinel bands while attempting to access the larvae. Finally, in most cases the initial releases of Mastrus resulted in crashing of the codling moth population at the release sites, resulting in very few larvae being available at the sites to support the Mastrus population. In situations where a freshly emerged female Mastrus does not encounter a host nearby she begins to forage further from her emergence site and can disperse significant distances in search of prey. This means that establishment may have to be monitored at considerable distances away from the original release sites. The project team is keen to investigate the use of synthetic aggregation pheromone as a lure to monitor presence of Mastrus.

The issues affecting measurement of establishment may also have impacted our ability to detect hyper-parasites since, by definition, we needed Mastrus larvae to be parasitizing codling moth larvae so that we could detect hyper-parasites attacking Mastrus.

Relevance

Industry levy payers, as represented by growers attending communication activities, are very interested in using Mastrus integrated into their pest management programs. Pest management is still high on the agenda for the Apple and Pear Strategic Investment plans. Feedback from the mid-term review included the comment by a BCA supplier that "….if we were to reduce pesticide costs by 50% it doesn't mean that much but if can help growers produce a better product, preserve the chemicals they can use and open export markets then that is a good result".

Process appropriateness

Regular updates have been provided through written media such as industry journals, oral presentations at industry conferences and other events such as orchard walks, field days, and IPDM workshops. Over 100 growers and their advisors have been engaged in face to face meetings in either group or individual interactions. Further evaluation of this question will be conducted by the independent coordinator of the PIPS 2 program when that overall program is evaluated.

Efficiency

One of the strengths identified in the mid-term review was the ability to adaptively manage research activity to mitigate risks. An example highlighted by the review was our response to the difficulty of measuring establishment using the accepted practices from other countries. When we experienced issues with the sentinel banding techniques we explored a range of other options including design of traps to protect bands, and the use of

aggregation pheromone instead of larvae to attract Mastrus.

Another example of efficiency is combining travel for more than one project, wherever possible, to reduce time and costs associated with field work. Travel related to case studies associated with AP16007 were combined with visits to Mastrus release sites in the same areas in Qld, NSW, Tas, and SA. Similarly, the workshops in the eastern states for AP16007 were used as vehicles to generate greater awareness of the Mastrus work.

Recommendations

Further work is required to develop better tools for detecting and monitoring presence of Mastrus, not just for demonstrating establishment but for on-going management of the parasitoid and codling moth populations. This should be a high priority for future funding.

The laboratory culture of Mastrus has been through more than 100 generations since its introduction originally to California then Argentina and New Zealand before a small number of wasps were imported into Australia. Genetic diversity is therefore narrow and there is a need to import fresh genetics to avoid bottlenecks due to in-breeding. Codling moth is now designated as a target for biological control and *Mastrus ridens* is approved for importation and release in Australia. Testing genetic diversity of the laboratory culture and sourcing new populations of *Mastrus ridens* to provide improved genetic diversity should be a priority.

There is also a need to investigate the impact, on genetic diversity, of the number of Mastrus released and the spatial distribution of release sites. This is a critical requirement for development of guidelines for suppliers of biocontrol agents.

On-going support of pesticide testing is required to keep ahead of, or at least abreast of, new developments and use patterns for pesticides. This is particularly important for fungicides because the pesticide testing in this current project indicated that fungicides appear to have sub-lethal effects impacting on fertility or fecundity of Mastrus. Investigation of the mechanisms driving sub-lethal effects would also be warranted.

Parasitoid wasps generally require a source of energy soon after emergence as adults. Laboratory cultures provide this in the form of honey water. Australian orchards generally do not have many nectar sources in the inter-row sward or nearby. Further work is warranted to investigate ways of improving availability of suitable nectar sources for not just Mastrus but also for generalist parasitoids and predators that could help reduce the troughs and peaks observed in pest populations in disturbed ecosystems.

Refereed scientific publications

None produced but some are being considered for production in the next 12 months

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Appendices

Appendix 1: Field release of *Mastrus ridens*

Appendix 2: Monitoring the establishment of *Mastrus ridens* at release sites

Appendix 3: Toxicity of pesticides to *Mastrus ridens*

Appendix 4: Understanding the effect of cold storage of parasitized codling moth larvae on yield of the parasitoid *Mastrus ridens*

Appendix 5: Influence of larval host size on yield and sex ratio of the codling moth parasitoid *Mastrus ridens*

Appendix 1: Field release of Mastrus

Release of Mastrus in South Australia

Two sites in the Adelaide Hills were identified as suitable for release of Mastrus. One site is in Loebethal and the other is in Ashton. Mark Staniford (an agronomist at E.E. Muir and Sons in Lenswood) assisted with identification of the blocks, grower liaison, and release of the Mastrus. Both sites had a history of codling moth infestation and the trees were 40-60 years old Granny Smith whose scaly bark would provide sites for codling moth larvae to establish hibernacula. Four corrugated cardboard bands, each containing about 50 parasitized codling moth larvae, were placed in modified fruit fly traps (Lynfield traps with a large opening, covered with plastic 15mm x 10mm mesh that allows Mastrus to exit but retains the bands, in the base) to protect the bands from predation by insectivorous birds. One trap was attached to a lower limb, close to the trunk, on each of nine trees in each release site. GPS coordinates were recorded for each trap location to allow follow up inspections. Release took place 16 May 2018 in good weather conditions. The Mastrus colony generally produces an average of about 6 Mastrus adults per codling moth larva. We therefore calculated that about 10,000 wasps would emerge at each of the two release sites. An additional two bands from the shipment were retained and returned to the DEDJTR AgriBio laboratory to assess emergence rate in case storage and transport had affected survival of the wasps.

Figure 1: Mastrus emerged from corrugated cardboard band

Figure 2: Mark Staniford (E.E. Muir & Sons, Lenswood) releasing Mastrus

Release of Mastrus in Queensland

Two sites near Stanthorpe in the Queensland Granite Belt were selected after consultation with local researchers, consultants and growers. Farm Use Agreements were developed for each property and signed by the orchardists. Parasitized codling moth diapausing larvae in corrugated cardboard bands were placed in chilled Eskies and consigned to Brisbane by air mid-afternoon on 26th April 2016 via specialist pet cargo, alongside modified fruit fly traps used to contain the bands when placed on trees. The cargo was collected in Brisbane and driven to Stanthorpe on the 27th April. *Mastrus* were released into the orchards on the 27th and 28th April 2016

Four bands containing the parasitized larvae were placed in each modified fruit fly trap, and the traps were then distributed across the release site, a single block of apples in an orchard. The coordinates of each release site were recorded by GPS to make it easy to find on follow up visits. Approximately 20,480 *Mastrus* were released at site 1, using 32 trees. At site 2 approximately 17,280 *Mastrus* were released, using 27 trees. *Mastrus* adults were observed emerging during the release process.

Release of Mastrus in NSW, Tas, and Victoria

Releases were also conducted in two orchards in Orange NSW, an organic orchard south of Young NSW, three orchards in Batlow NSW, two blocks in an organic orchard at Grove Tasmania, a small block of apples in Murrayville NW Victoria, a block of organic apples at St Germains in Northern Victoria and a block of pears in Ardmona Victoria. The release methodology was the same as for the releases in South Australia. Reference specimens from each release were provided to the relevant authorities in each state for lodging in their reference collections.

Appendix 2: Monitoring the establishment of Mastrus ridens at release sites

Corrugated cardboard bands have been used to recover Mastrus from orchards in USA (Caprile & Mills, 2004), Argentina (Tortosa *et al*, 2014) and NZ (Davis *et al*, 2018;) but attempts in Australia have been hampered by the activity of insectivorous birds such as white winged choughs, earwigs, and ants. The birds destroy the bands while searching for larvae. The earwigs and ants consume the larvae in the bands, destroying evidence of Mastrus in the process.

There are very few efficient traditional sampling protocols for natural enemies, despite the importance to IPM of estimating their abundance. Natural enemies are known to respond to plant volatiles that may be useful as lures in traps designed for monitoring natural enemies. Traps baited with methyl salicylate plus phenylacetaldehyde performed well in traps for monitoring hymenoptera (Jones *et al,* 2016) but they did not test Mastrus. Jumean *et al* (2005a) identified a larval aggregation pheromone of codling moth. Jumean *et al* (2005b) demonstrated that Mastrus uses the codling moth larval aggregation pheromone to detect presence of cocooned larvae. Jumean *et al* (2006) used the pheromone to treat corrugated cardboard bands to enhance trapping of codling moth larvae searching for cocooning sites. The amount of pheromone applied was measured as larval hour equivalents (LHE) which are defined as the amount of pheromone emitted by a larva during an hour. Bands treated with 10^5 or 10^6 LHE per 30cm length of band captured significantly more larvae than bands treated with 10^4 LHE or untreated bands. Their experiment used polyurethane as a pheromone-dispensing matrix but most of the pheromone components were released too rapidly to be practical in the field. The eight major components of the aggregation pheromone had different release rates with initial rates measured from 30cm bands ranging from 0.005 mg/day for geranylacetone to 1.2 mg/day for (E)-2-nonenal (Jumean *et al*, 2006). Further work is required to formulate a better dispensing system.

We tested the behavior of the laboratory reared Mastrus towards the aggregation pheromone in both the wind tunnel and y-tube olfactometers at Agribio. We also tested if the laboratory reared codling moth larvae were producing aggregation pheromone, by collecting the headspace volatiles from a chamber containing cocooned diapausing codling moth larvae and then running them through a GC-MS.

Results

Sentinel bands established at release sites in Tasmania and NSW

The use of sentinel bands has been problematic, with predation by earwigs and spiders destroying codling moth larvae in the bands. Experience in California where Mastrus has been released is that in the first, and frequently second and third season after release, very few codling moth larvae are detected in bands and those larvae in the bands had low rates of parasitism. The low levels of codling moth larvae indicated that Mastrus was being effective, but also suggested that very high numbers of bands would be required to detect the pest and the parasitoid. A similar problem arose in the Tasmanian release site on 4 May 2018 when eighty trees were inspected, including scraping the bark on all trees to look for codling moth hibernacula, and only 14 codling moth larvae and no Mastrus were found. Bands left on the trees after release were also inspected but all were empty. According to the orchardist codling moth damage in the 2017-18 season was less than 1.0%, the lowest since the orchard transitioned to organic. Similar results were obtained in NSW at Batlow and Orange where virtually no damage from codling moth occurred in release blocks despite other blocks nearby having damage.

Bands collected from field and contents reared to determine presence of Mastrus and any hyper-parasites or other natural enemies.

All bands left in the orchards the season after release of Mastrus were empty when inspected. The advantage of corrugated cardboard bands is that it is easy to see if there are hibernacula in the corrugations without having to open them. There is no need to collect empty bands. Bands placed in release orchards in the autumn of 2019 were recovered in July 2019. One band collected from Batlow had a single codling moth larva present and all bands had spiders and earwigs present but no signs of Mastrus. The organic orchard near Young had an average of 50.8 codling moth larvae per band, 9 cadavers that may have been the result of Mastrus attack, and 70% of the bands contained earwigs and spiders. Despite the high numbers of codling moth larvae in the bands the percentage fruit damage when the bands were established averaged 30% and the grower indicated that was a

good result. Bands recovered from one release site in Queensland had an average of 1.1 codling moth larvae/ band, with a total of 3 cadavers in the 10 bands, no earwigs and only two bands had spiders present. Bands from the other Queensland release site had an average of 2.7 codling moth larvae/ band, no signs of Mastrus or earwigs but spiders were present in 60% of the bands. Bands recovered from Ardmona were completely empty. Bands from South Australia and Tasmania had not arrived in the mail at time of preparation of this report.

The use of sentinel bands containing diapausing codling moth larvae from the laboratory culture was not effective for several reasons. Firstly, there were large numbers of earwigs, ants, and spiders in the organic orchards in which Mastrus was released and these generalist predators appeared to have consumed the sentinel codling moth larvae before, or perhaps during, parasitism by Mastrus. Secondly, it appears that the laboratory reared codling moth larvae may not be emitting aggregation pheromone. Mastrus uses the codling moth aggregation pheromone to find larval cocoons in their hibernacula. A small-scale laboratory study to confirm this failed to detect any pheromone components in the headspace of vials containing diapausing lab-reared codling moth larvae. We also failed to elicit definitive responses to aggregation pheromone by lab-reared Mastrus. Further work is required to determine if this was a result of the storage time of the diapausing larvae, lack of appropriate precursors in the artificial diet used to feed the larvae, or adaptation to the rearing conditions in the laboratory. Thirdly, birds such as choughs foraging in the orchards often destroyed the sentinel bands while attempting to access the larvae. Finally, in most cases the initial releases of Mastrus resulted in crashing of the codling moth population at the release sites, resulting in very few larvae being available at the sites to support the Mastrus population. In situations where a freshly emerged female Mastrus does not encounter a host nearby she begins to forage further from her emergence site and can disperse significant distances in search of prey. Hougardy & Mills (2006) reported that Mastrus moved into areas with higher codling moth populations after reducing populations in the initial release sites. This means that establishment may have to be monitored at considerable distances away from the original release sites. The project team is keen to further investigate the use of synthetic aggregation pheromone as a lure to monitor presence of Mastrus.

The issues affecting measurement of establishment may also have impacted our ability to detect hyper-parasites since, by definition, we needed Mastrus larvae to be parasitizing codling moth larvae so that we could detect hyper-parasites attacking Mastrus since there is little value in looking for hyper-parasites where the host does not exist.

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Appendix 3: Toxicity of pesticides to *Mastrus ridens*

Introduction

Integrated pest Management (IPM) in Australia arguably began when the use of biological control agents for pest and weed control was integrated with other management techniques in the early 1900s (Wilson 1960). The pome fruit industry developed national guidelines for integrated fruit production that supported the integration of pesticide resistant strains of predators and parasitoids with selective use of pesticides (Williams, 2000a, b). Low-volume (concentrated) spray application can affect predators and parasitoids (Cole and Laukart, 2000) and it is important to understand and account for these impacts (Williams and Il'ichev, 2003). This is particularly important when the predators and parasitoids are introduced biocontrol agents in the early stages of establishment (Hassan and Oomen, 1985).

Despite development and publication of standard methods for testing side-effects of pesticides on biocontrol agents (Hassan *et al* 1985) there appears to be no universally accepted method available. Rathman *et al* (1990) used pesticide treated clear plastic cups to bioassay resistance in *Diglyphus begini* (Ashmead), a hymenopteran parasitoid of *Liriomyza* spp. leafminers. Scott *et al* (1991) used a parasitoid selectivity ratio (PSR), defined as [EC_{50} parasitoid]/ [EC_{50} host] where EC_{50} is the effective topically applied concentration of technical grade pesticide that caused 50% emergence from pupae, to investigate comparative toxicity to immature hosts and their parasitoids. Shean and Cranshaw (1991) used treated-vial bioassays to evaluate mortality of adult hymenopteran parasitoids *Aphelinus semiflavus* Howard and *Diaretiella rapae* (McIntosh) after exposure to pesticide residues. They also evaluated mortality of immature parasitoids after mummified hosts on excised leaf discs were dipped in aqueous solutions of pesticides, allowed to dry, and then placed in petri dishes until emergence of parasitoids from the mummies. Rathman *et al* (1992), using the technique of Rathman *et al* (1990), found that size differences between the sexes contributed to male parasitoids (*D. begini*) being more susceptible than females. Brunner *et al* (2001) used a Potter spray tower (Burkhardt, Rickmansworth, UK) to apply a fine spray of pesticide solution to anesthetized parasitoids on a filter paper disc that was then transferred to a petri dish. Prabhaker *et al* (2007) used a petri dish bioassay in which leaf discs dipped in pesticide solution were allowed to dry and then placed onto agar beds in petri dishes before adult parasitoids were added. Miller *et al* (2010) advocated the treated-vial bioassay for monitoring insecticide resistance in pest species.

The biological control agent *Mastrus ridens* (Hymenoptera: Ichneumonidae) was approved for importation and release in Australia against codling moth *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) in 2013 to supplement pheromone-mediated mating disruption. The parasitoid is being released into orchards in the eastern states (Qld, NSW, SA, TAS, and Southern Victoria). *M. ridens* searches for fifth instar codling moth larvae in cocooning sites under bark on the butts of trees. It oviposits into the cocoon and the *M. ridens* larvae develop fully and pupate inside the codling moth cocoon. The cryptic nature of the parasitism means that the adult wasp is the most likely stage to be affected by pesticides applied in the orchard. Establishment of the parasitoid may be negatively impacted by grower application of pesticides toxic to *Mastrus.*

This paper reports on the results of bioassays conducted to test susceptibility of the laboratory strain of *Mastrus ridens*.

Materials and methods

Laboratory reared adult *Mastrus ridens* were obtained from a colony maintained at the DEDJTR AgriBio Centre. The parasitoids were reared on diapausing codling moth larvae supplied from a stock colony of codling moth maintained at the DEDJTR Tatura Centre. The *M. ridens* adults were visually assessed for quality (based on size and activity), sexed, and tubes containing 10 males and 10 females were placed in a coolroom while the test arenas were prepared.

Test arenas consisted of 87 mm diameter plastic petri dishes with the internal surfaces of both the base and the lid sprayed with uniform small droplets in a Potter Spray Tower (Burkhardt, Rickmansworth, UK) delivering 1 mL of test solution at 4 psi. After spraying, the arenas were placed in a fume cupboard and allowed to air dry. Small containers for water and honey agar were placed in the bottom of each arena after the arenas had dried.

The cooled tubes of insects were transported from the coolroom to the laboratory in an insulated foam box. Each arena received the contents of one tube of insects (10 males, 10 females) and was placed on a bench under constant light and temperature (20^{o}C) for 48 hrs to observe mortality.

A preliminary bioassay was conducted using a commercial formulation of the newly registered pesticide

Cormoran® (80g/L acetamiprid plus 100g/L novaluron) at field rate (70mL product/100L water) with test insects having access to either no food or water, food but not water, or food plus water. An untreated control consisted of water-sprayed arenas with insects having access to the same combinations of water and food as the insecticide treated insects.

For all bioassays the registered formulation was used as the source of pesticide. The registered field rate for control of codling moth was used as a guide and stock solutions based on a multiple of that rate were made by dissolving or suspending the appropriate amount of product in water. Serial dilutions were then made from the stock solutions. All flasks containing the serial dilutions were agitated by gentle shaking to ensure the products remained in suspension throughout the test period. Treatments were the different concentrations of product in the solution, there were 5 replications for each treatment and water was sprayed as an untreated control.

Mortality was assessed as either obviously dead or moribund individuals that were non-responsive.

Treatments that resulted in low or moderate direct toxicity were subjected to studies of the sublethal effects of exposure. New batches of test solutions were used to expose Mastrus for 24 hrs, using the same protocols as for the initial bioassays. After 24 hrs exposure all remaining live females in each treatment were removed, pooled according to treatment, and placed into oviposition cages labelled by treatment. Each oviposition cage contained 5 corrugated cardboard rolls with 50 diapausing codling moth larvae per roll (250 larvae/cage), water and food (10% sugar and honey agar). These F_0 female Mastrus were given an oviposition period of 6 days, after which the parasitized rolls were removed and placed into emergence cages. Yield of F_1 adult Mastrus was recorded for each treatment and converted to yield/ F_0 female. Sub-samples of male and female F_1 Mastrus in a ratio of approximately 2 females/male were then transferred to fresh oviposition cages to produce the F_2 generation

In late November 2018 we had the opportunity to conduct a preliminary bioassay on two additional insecticides, Insegar and Success, and a potential new product Sero-X. At the time we had limited resources and were not able to investigate sub-lethal effects but did conduct a direct toxicity bioassay using the field rates for each product.

Results

Preliminary Bioassay.

There were no significant differences in mortality between water-treated control and Cormoran®-treated arenas in the presence of food or food plus water, but mortality after 24hrs exposure in the absence of food plus water was significantly higher in Cormoran®-treated arenas for males and weakly for females. Differences disappeared at 48 hrs (Table 1). Control mortality above 20% is considered unacceptable and, consequently, all further bioassays were conducted with food plus water available to the insects in the arenas.

Table 1: Mortality of adult *Mastrus ridens* in arenas that had been sprayed with either water (control) or Cormoran® at registered rate (treated), when no water or food was provided to the insects over the exposure period.

	Female				Male			
	No. dead at 24 hrs		No. dead at 48 hrs		No. dead at 24 hrs		No. dead at 48 hrs	
Replicate	Control	Treated	Control	Treated	Control	Treated	Control	Treated
1	1	2	5	6	3	5	8	8
2	1	1	4	4	2	2	7	7
3	1	2	4	7	0	3	7	6
4	2	2	7	6	3	6	8	8
5	0	2	0	5	0	6	4	8
Total	5	9	20	28	8	22	34	37
% mortality	10	18	40	56	16	44	68	74
p-value	0.05		0.106		0.022		0.265	

Dose-response bioassays

Results for Cormoran® (Table 2) suggest that this product is harmless at rates up to 2xField rate for males and 4xField rate for females, based on the IOBC accepted toxicity level criteria for laboratory testing (Hassan *et al* 1985).

Results for Avatar® (Table 3) suggest that this product is harmful at field rate and higher, and that rates at or above 25% of the field rate are harmful at greater than 24 hrs exposure time.

Results for Altacor® (Table 4) demonstrate that this product is harmless at application rates up to 4xField rate

Table 2: Mortality of adult *Mastrus ridens* in arenas that had been sprayed with aqueous solutions of Cormoran® at registered field rate (FR) and multiples of that rate ranging from 0 (control) – 16x registered rate, when the insects had access to food and water in the arenas over the exposure period. Asterix * indicates IOBC accepted toxicity level based on Hassan *et al* (1985) criteria for laboratory testing. *=harmless; **= slightly harmful; ***= moderately harmful; ****= harmful

Rate (xFR)	% mortality			
	Female		Male	
	24 hrs	48 hrs	24 hrs	48 hrs
Control (0)	0*	0*	0*	0*
1x	0*	0*	4*	6*
2x	10*	14*	26*	38*
4x	38*	46*	76**	76**
8	64**	82***	86***	86***
16x	100****	100****	96***	100***

Table 3: Mortality of adult *Mastrus ridens* in arenas that had been sprayed with aqueous solutions of Avatar® at registered field rate (FR) and multiples of that rate ranging from 0 (control) – 16x registered rate, when the insects had access to food and water in the arenas over the exposure period. Asterix * indicates IOBC accepted toxicity level based on Hassan *et al* (1985) criteria for laboratory testing. *=harmless; **= slightly harmful; ***= moderately harmful; ****= harmful

Rate (xFR)	% mortality			
	Female		Male	
	24 hrs	48 hrs	24 hrs	48 hrs
Control (0)	2*	2*	4*	4*
0.25x	70**	100****	50**	100****
0.5x	80***	100****	80***	100****
1x	100****	100****	100****	100****
2x	100****	100****	100****	100****
4x	100****	100****	100****	100****

Table 4: Mortality of adult *Mastrus ridens* in arenas that had been sprayed with aqueous solutions of Altacor® at registered field rate (FR) and multiples of that rate ranging from 0 (control) – 16x registered rate, when the insects had access to food and water in the arenas over the exposure period. Asterix * indicates IOBC accepted toxicity level based on Hassan *et al* (1985) criteria for laboratory testing. *=harmless; **= slightly harmful; ***= moderately harmful; ****= harmful

Rate (xFR)	% mortality			
	Female		Male	
	24 hrs	48 hrs	24 hrs	48 hrs
Control (0)	0*	0*	0*	0*
0.03125x	0*	0*	6*	6*
0.0625x	0*	0*	0*	0*
0.125x	0*	0*	0*	2*
0.25x	0*	0*	0*	0*
0.5x	0*	0*	0*	2*
1x	0*	0*	0*	0*
2x	0*	0*	0*	0*
4x	2*	2*	0*	0*

Sub-lethal impacts of pesticides

Cormoran at 0.25x field rate did not appear to affect yield of the F_1 or F_2 compared to the untreated control but the results of exposure to higher rates suggest there was a slight negative effect. Altacor at 0.25 - 2X field rate had a negative impact on yield of both male and female F_1 but the yield of F_2 females was not affected although the yield of males was. The fungicides Ziram and Dithane had no impact on yield of F_1 females but reduced yield of F_1 males but the effects on yield of F_2 males and females were minor. The fungicides Chorus and Rubigan and the miticide Sorcerer each had negative impact on yield of male and female F_1 but not on yield of F_2 (Table 5)

Table 5: Mortality of F0 adult Mastrus ridens exposed to pesticides, and sublethal effects on yield of adults in subsequent generations (F1, F2). Mortality was corrected using the Henderson-Tilton formula to correct for control mortality.

Treatment	corrected % mortality		F_1/F_0 female			F_2/F_1 female		
	F	M	F	M	Total	F	M	Total
Control 1			1.91	4.38	6.3	1.07	5.34	6.41
Cormoran - 0.25 x Field rate	21.28	37.93	2.14	4.27	6.41	1.11	4.33	5.44
Cormoran - Field rate	27.66	72.41	1.44	4.24	5.68	0.98	4.1	5.08
Cormoran - 2 x Field rate	27.66	62.07	1.47	2.24	3.71	0.66	3.88	4.54
Altacor - 0.25 x Field rate	0.00	44.83	1.38	2.11	3.49	1.47	4.33	5.8
Altacor - Field rate	0	13.79	1.71	3	4.71	1.31	3.71	5.02
Altacor - 2 x Field rate	2.13	51.72	0.98	1.52	2.5	1	3.67	4.67
Control 2			1.96	8.04	10	3	5.7	8.7
Chorus -Field rate)	2	0	1.71	3.59	5.31	2.43	5.23	7.65
Ziram – Field rate	2	0	1.98	4.65	6.63	3.03	5.61	8.64
Dithane – Field rate	2	0	2.22	4.29	6.51	2.78	4.91	7.69
Rubigan – Field rate	0	0	1.44	4.68	6.12	2.9	5.04	7.94
Sorcerer – Field rate	0	0	1.02	4.26	5.28	3.27	5.43	8.71

Limited field rate bioassays

Sero-X and Insegar applied at field rates had no direct toxicity on adult Mastrus after 48 hrs exposure to dried residues. Success at field rate had a negligible effect on Mastrus adults after 24hrs exposure but was highly toxic by 36 hrs exposure (Table 6)

Table 6: Mortality of Adult Mastrus ridens exposed to dried residues of pesticides. Mortality was corrected using the Henderson-Tilton formula to correct for control mortality.

Treatment	Corrected % Mortality					
	24 hr		36 hr		48 hr	
	female	male	female	male	female	male
Sero X 1.0ml/L	0.00	0.00	0.00	0.00	0.00	0.00
Insegar Field rate	0.00	0.00	2.00	0.00	2.00	0.00
Success Field rate	3.33	0.00	83.33	100.00	83.33	100.00

Discussion

The preliminary bioassay was designed to indicate any problems with control mortality. The arenas supplied with food alone, water alone, or both food and water had negligible mortality in the control treatment even after 48hrs, whereas significant control mortality occurred at 36 hrs and beyond in arenas without food and water. Those results gave confidence that supplying food and water in the arenas would improve the accuracy of the bioassays. The techniques used by Brunner *et al* (2001) involved directly spraying the insects, whereas our target insects would be more likely to be exposed to pesticides by foraging over tree parts containing pesticide residues. Prabhaker *et al* (2007) used leaf discs dipped in pesticide solution as a substrate for contact with pesticide residue. The dipping technique is difficult to control and to standardize the amount of residue. The Potter Spray Tower applies a consistent volume of spray in uniform drop size distributed evenly across the test arena. The petri dish arenas gave plenty of room for the insects to move around and the clear lids made observation easy. The depth of the dish bottoms also allowed small containers for food and water to be placed in the arenas without impacting on lids.

Hassan *et al* (1985) claim that there is general agreement that laboratory bioassays should be used for initial screening of pesticides, and that there is no justification for field testing of pesticides that were harmless in the laboratory. They recommended that pesticides that were slightly harmful, moderately harmful, or harmful in laboratory testing should probably be further tested in the field. According to their criteria neither Cormoran® (80g/L acetamiprid plus 100g/L novaluron) nor Altacor® (360g/kg chlorantraniliprole) were harmful in the laboratory testing at field rate or twice the field rate, and therefore there is no need to test them in the field. Avatar® (300g/kg indoxacarb) on the other hand was highly toxic and may warrant further investigation in the field to determine the duration of the effect and thus provide an insight into potential safe windows of opportunity to apply it in orchards without disrupting the parasitoid. The results from testing Insegar and Success suggest there would be value in testing dose response for those products, as well as sub-lethal effects of doses that do not exhibit direct toxicity. Similarly, further dose response work with Avatar to determine a no-effect level may provide information that, couple with residue degradation data, could inform decision making to improve timing of spray applications to minimize impacts on Mastrus. Further work would also be warranted to understand the mechanisms behind the sub-lethal effects of the pesticides that had shown little effect in direct toxicity testing.

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Appendix 4: Understanding the effect of cold storage of parasitized codling moth larvae on yield of the parasitoid *Mastrus ridens*

Introduction

Mastrus ridens (Horstmann) (Hymenoptera: Ichneumonidae) is a parasitoid wasp that attacks cocooned codling moth (CM) larvae (*Cydia pomonella* (L.) (Lepidoptera: Tortricidae) (Mills 2005; Devotto et al. 2010; Sandanayaka et al. 2011). Eggs laid on the CM larva within its cocoon after the codling moth larva has been paralysed hatch as parasitoid larvae that consume their host before spinning their own cocoon (Sananayaka et al 2011; Charles et al 2013). The mean duration of the immature parasitoids (egg to mature larvae) is 15.5 days at 21°C (Charles et al 2013). Adult females and males live for 18±3.6 and 17.2±5.2 days respectively when reared at 25°C (Devotto et al, 2010).

Storing parasitoid rolls in a cool room (4°C) can allow greater flexibility in production and synchronize rearing in the laboratory with the timing of field release (van Lenteren & Tommasini 2003, Colinet & Boivin 2011). However, prolonged storage at low temperatures may also result in deformation (Tezze & Botto 2004), increased mortality due to poor immature survival, reduced adult longevity, and poor reproductive performance (Rundle et al 2004).

The work reported herein was part of a larger project in which *M. ridens* was released in the pome fruit growing areas in NSW, VIC, TAS, SA and QLD. Adult Mastrus were released to coincide with the seasonal appearance of cocooning CM larvae. This required rearing more than 40,000 healthy *M ridens* for release and colony maintenance. To achieve that number of adult wasps in the limited rearing space available batches of parasitized codling moth larvae in corrugated cardboard rolls were stored in a cool room at 4ºC until enough numbers had accumulated to perform a field release.

The impact of cold storage at 4°C on yield of Mastrus adults from parasitized CM larvae was studied to assist with optimisation of culture methods and field releases.

Materials and methods

Mastrus ridens were sourced from the laboratory colony maintained in the Agriculture Victoria Research facility at AgriBio, Bundoora. The wasps were reared on diapausing codling moth larvae supplied from the laboratory colony maintained at Agriculture Victoria, Tatura. Approximately 40 female and 20 male 1-4-day old Mastrus adults were released in each insect cage (Bug dorm: 47.5 x 47.5 x 47.5) containing honey-agar diet and water at 22°C±2°C and 16 h photoperiod. Each cage had corrugated cardboard rolls, each containing 50 good quality (large) diapausing CM larvae, suspended in it at a ratio of 1 roll per 10 female Mastrus. The rolls remained in the cage for 15 days to give maximum opportunity for female Mastrus to parasitize the CM larvae.

At the end of the 15 days exposure to Mastrus the rolls were labelled with the date and then stored in bags in the cool room at 4° C and total darkness until required. Parasitized rolls from the cool room were randomly selected after 12, 37, 80, 112, 131, 150, 171, 185, 203, and 220 days storage. These storage times were allocated as treatments and a single roll from each treatment was used for each replicate. Storage times of 37 and 80 days were replicated 3 times, but the others were only replicated twice because of the need to supplement the stock culture. Emergence cages consisting of a 5.5 L vented plastic container (20 x 19 x 19 cm) containing honey-agar diet and water for emerging adults and one parasitized roll in each cage were kept in the climate chamber at 22±2° C, 55±5% RH and a 16-hr photoperiod to allow adult Mastrus to emerge over a 3-week period or until no further Mastrus emerged. Mastrus emerging in the cages were removed daily Monday-Friday, counted, and sexed to allow sex ratios and yield per roll to be calculated.

The cumulative emergence was computed and analysed statistically with the 2-sample Kolmogorov-Smirnov test using Genstat 19.1 software to assess similarity between the underlying distributions of emergence resulting from paired treatments. This is done by

comparing their empirical cumulative distribution functions. The test statistic MD is defined as MD = max(abs{$S_1(X) – S_2(X)$}) and forms the basis for significance tests. MD is the maximum absolute difference between the two cumulative distribution functions S_1 and S_2 where $S_k(X)$ = (number of scores in sample $k \leq X$) / (size of sample k) for k= 1,2. The chi-square approximation (with 2 degrees of freedom) to the test statistic MD is

$$Chi = 4 \times MD^2 \times ((n_1 \times n_2 / (n_1 + n_2))$$

Where n1, n2 are the sizes of the samples (Siegel 1956). The p values are derived from this chi-square approximation to the test statistic MD.

Results and Discussion

The yields of Mastrus from the parasitized rolls stored in the cool room for 37 and 12 days (Treatment 9 and 10 respectively) were very similar, however after 11 days Mastrus emergence from treatment 9 had ceased but in treatment 10 emergence continued for almost 19 days. For treatment 8 (80 days cold storage), the yield was lower than treatments 9 and 10.

Storage times longer than 80 days (i.e. treatments 1 to 7) resulted in low yields of Mastrus ranging between 2 to 16.5 Mastrus per roll, with the highest yield from treatment 7 (112 days storage) (Fig 1). Emergence started 4-5 days post-cold storage in treatments involving 12 to 112 days of cold storage. There was no emergence in the first 12 days post-cold storage from the rolls stored longer than 112 days (Figs 1 to 3).

The results of the 2-sample Kolmogorov-Smirnov test for female yield from treatment 10 (12 days cold storage) was significantly different ($p=0.041$) to that for treatment 5 but not the other treatments (Table 1) whereas male yield from treatment 10 was significantly different ($p<0.05$) to those from treatments 1-5 but not treatments 6-9 (Table 2). The total (male + female) yield from treatment 10 was significantly different ($p<0.05$) to that in treatments 1-7 but not treatments 8 and 9 (Table 3).

More males than females emerged in all treatments. Comparison of male and female emergence suggests that female pupae may be more sensitive than male pupae to cold storage, given that males subjected to treatment 7 (112 days cold storage) emerged after 5 days of return to warm conditions whereas no females from the same treatment emerged until 14 days in warm conditions (Figs 2 and 3). Sandanayaka et al (2015) suggested that 7 to 10 days old Mastrus ridens larvae can be stored up to 12 weeks (84 days) at 4° C without any negative effect on adult emergence and quality. They reported that although eggs survived and developed normally at 22 ° C, eggs stored at 4° C for 4 weeks had very low survival. They also reported that 1st instar larvae were not very cold tolerant, with very high mortality after 4 weeks cold storage. Pre-pupae and pupae also suffered high mortality in extended cold storage. The delayed emergence, and low overall yield, resulting from treatment 7 of our study suggests that pre-pupae, pupae and eggs were probably killed by the cold exposure and the delayed emergence of females resulted from development of surviving larvae.

No obvious behavioural or physical differences were observed among males from any treatment. Some females that emerged from cold-storage for longer than 150 days had curved ovipositors and deformed wings.

In our study, since the parasitized rolls had been exposed to Mastrus for 15 days, it is likely that they contained the full range of immature stages (eggs, larvae and pupae) when they were placed in cold storage. Low temperatures reduce the metabolic rate of insects and prolonged cold storage may generate a temporary quiescent state (Leopold 1998) but, unless the insects enter diapause, long term cold storage may eventually cause death. Little is known about the lower developmental threshold of *Mastrus ridens*, or whether it enters diapause, but anecdotally it appears to be active at temperatures that inhibit codling moth development. Further study of cold temperature behaviour would be useful to provide guidance on timing of field releases.

Conclusions

Cold storage of parasitized CM rolls for more than 80 days is detrimental to Mastrus emergence and quality. Sandanayaka et al (2015) and our current studies suggest that later instar larvae are less sensitive to prolonged cold storage. It may be more productive to expose CM larvae to Mastrus females for a shorter period and then incubate the rolls at 22°C±2°C to allow further development of a more uniform population of the less cold sensitive larval stage before storing the rolls in the cool room. Mastrus females lay most of their eggs, and higher quality eggs, within a week of their emergence. Further study is required to confirm the benefits, such as condensed emergence and greater fitness during field releases, of such an approach.

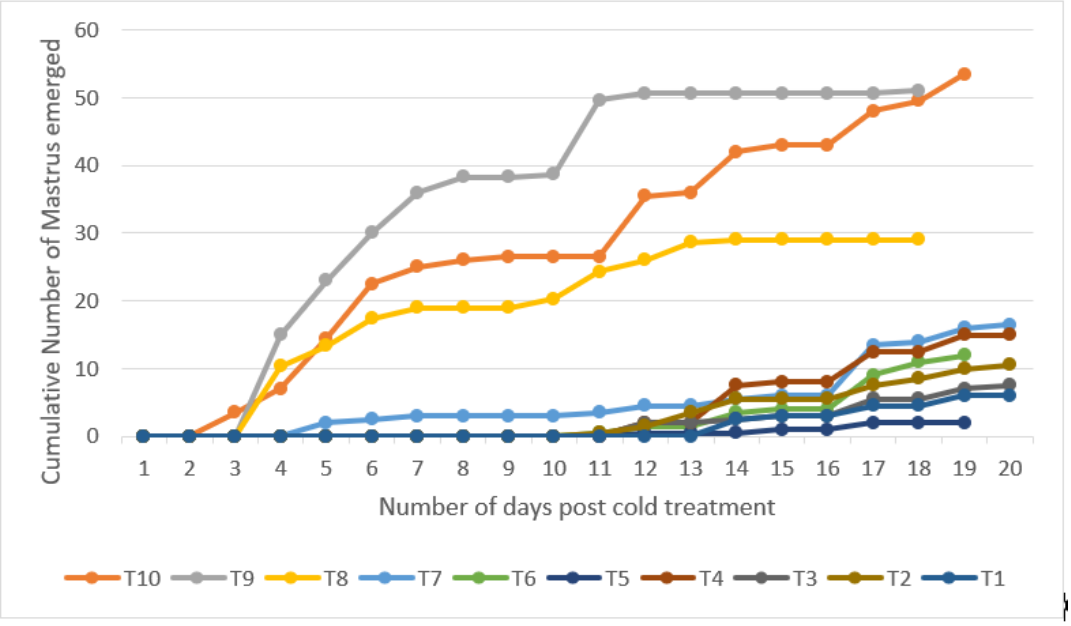

Fig 1. Cumulative emergence of Mastrus (total of both sexes) after being subjected to different periods of cold treatment of parasitized CM rolls. Number of days in the cold room were: T1= 220; T2= 203; T3= 185; T4= 171; T5= 150; T6= 131; T7= 112; T8= 80; T9 = 37; and T10= 12.

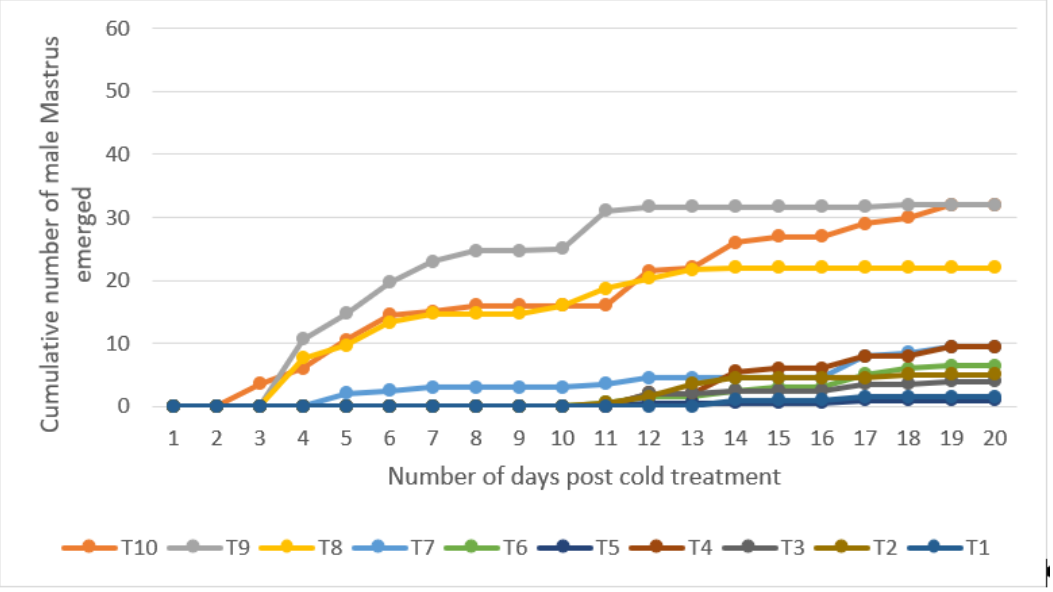

Fig 2. Cumulative emergence of male Mastrus emerged after being subjected to different periods of cold treatment of parasitized CM rolls. Number of days in the cold room were: T1= 220; T2= 203; T3= 185; T4= 171; T5= 150; T6= 131; T7= 112; T8= 80; T9 = 37; and T10= 12.

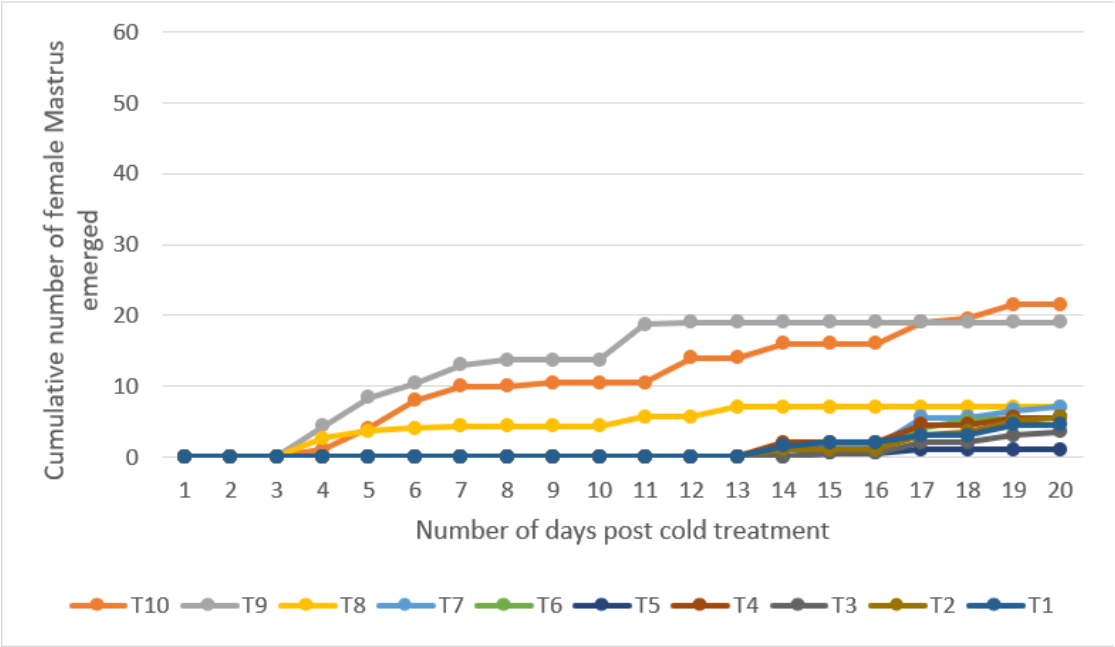

Fig 3. Cumulative number of female Mastrus emerged after being subjected to different periods of cold treatment of parasitized CM rolls. Number of days in the cold room were: T1= 220; T2= 203; T3= 185; T4= 171; T5= 150; T6= 131; T7= 112; T8= 80; T9 = 37; and T10= 12.

Table 1: p-values derived from 2-sample Kolmogorov-Smirnov tests: Female Mastrus emergence									
	Treatment (days cold storage)								
T1 (220)	T2 (203)	T3 (185)	T4 (171)	T5 (150)	T6 (131)	T7 (112)	T8 (80)	T9 (37)	T10 (12)
T1	0.951	0.951	0.819	0.638	0.951	0.951	0.714	0.232	0.086
T2		0.951	0.819	0.638	0.951	0.951	0.877	0.374	0.165
T3			0.819	0.819	0.951	0.951	0.714	0.232	0.086
T4				0.638	0.951	0.819	0.529	0.339	0.086
T5					0.638	0.638	0.356	0.122	0.041*
T6						0.951	0.714	0.374	0.165
T7							0.773	0.374	0.165
T8								0.411	0.193
T9									0.591

Table 2: p-values derived from 2-sample Kolmogorov-Smirnov tests: Male Mastrus emergence									
	Treatment (days cold storage)								
T1 (220)	T2 (203)	T3 (185)	T4 (171)	T5 (150)	T6 (131)	T7 (112)	T8 (80)	T9 (37)	T10 (12)
T1	0.638	0.819	0.449	0.951	0.287	0.165	0.024*	0.048*	0.002*
T2		0.951	0.638	0.638	0.819	0.638	0.052*	0.218	0.041*
T3			0.638	0.819	0.638	0.449	0.052*	0.182	0.017*
T4				0.449	0.638	0.638	0.306	0.306	0.041*
T5					0.287	0.165	0.024*	0.048*	0.002*
T6						0.951	0.052*	0.218	0.086
T7							0.106	0.356	0.086
T8								0.607	0.449
T9								0.275	0.449

Table 3: p-values derived from 2-sample Kolmogorov-Smirnov tests: Total Mastrus emergence									
	Treatment (days cold storage)								
T1 (220)	T2 (203)	T3 (185)	T4 (171)	T5 (150)	T6 (131)	T7 (112)	T8 (80)	T9 (37)	T10 (12)
T1	0.449	0.819	0.638	0.638	0.638	0.086	0.113	0.182	0.007*
T2		0.638	0.638	0.287	0.951	0.638	0.232	0.122	0.017*
T3			0.819	0.638	0.819	0.287	0.193	0.218	0.017*
T4				0.449	0.819	0.165	0.306	0.232	0.017*
T5					0.449	0.041*	0.024	0.098	0.002*
T6						0.449	0.193	0.218	0.041*
T7							0.193	0.218	0.041*
T8								0.411	0.290
T9									0.160

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Appendix 5: Influence of larval host size on yield and sex ratio of the codling moth parasitoid *Mastrus ridens*

Introduction

Codling Moth (*Cydia pomonella* (L.) (Lepidoptera: Tortricidae) is one of the most devastating pests of apple worldwide. It is a key pest for the Australian apple industry because it directly attacks the fruit and chemical control often leads to secondary pest outbreaks by killing predators and parasitoids that help to maintain secondary pest populations at low levels. *Mastrus ridens* (Horstmann) (Hymenoptera: Ichneumonidae) was identified as a potential biocontrol for Codling Moth (CM), imported into quarantine in Australia, approved for field release after satisfying regulatory requirements, and released in field sites in May 2014 as part of the Hort Innovation program AP09031 (Williams et al, 2014). It is a specialised ecto-parasitoid that attacks 5th instar larvae in their cocoons.

The next phase of work aimed to release and establish populations of Mastrus in apple growing districts in eastern Australia to control CM. To produce enough Mastrus for such releases required development of an effective rearing method which in turn required an understanding of the impact of host quality on yield and quality of Mastrus produced in the rearing facility. Host body size had been reported as having an impact on Mastrus oviposition whereby Mastrus females selectively lay more eggs on larger hosts and fewer eggs on smaller hosts, probably to allocate equal resources for offspring (Sandanayaka et al, 2011). Our colony had experienced declining yield of Mastrus and, as part of an investigation into the cause of the decline, a small-scale experiment was conducted to assess if the size of the host larvae in the codling moth culture was a contributing factor.

Materials and Methods

A colony of *Mastrus ridens* maintained at 22±2° C, 55±5% RH and a 16-hr photoperiod (provided by fluorescent lighting) was reared on diapausing CM larvae that had cocooned in rolls of corrugated cardboard. Each roll contained fifty fully developed 5th instar larvae from a laboratory culture maintained at 25 ±2° C, 55±5% RH and 8L/16D photoperiod before being allowed to spin cocoons in the cardboard rolls. For this experiment the diapausing CM larvae had been classified into either medium or large size (Figure 1) before being transferred to cardboard rolls labelled with larval size and date transferred to the roll. The rolls were then stored in a cool room at 4° C and total darkness until required.

Approximately 40 female and 20 male 1-4-day old Mastrus adults were released into each insect cage (Bug dorm (47.5 x 47.5 x 47.5 cm) containing honey-agar diet and water at 22±2° C and 16 h photoperiod. Corrugated cardboard rolls containing sized CM larvae were placed in each cage for 15 days, at a ratio of 4 rolls per 40 Mastrus females, to give maximum opportunity for the Mastrus to parasitise the CM larvae. At the end of the 15-day exposure period the rolls were removed from the cages and placed into labelled 5.5L vented plastic containers (20 x 19 x 19 cm), with honey-agar diet and water for emerging adults and kept in the climate chamber at 22±2° C, 55±5% RH and a 16-hr photoperiod for parasitoids to complete their development. A total of 20 parasitized rolls containing medium sized CM larvae and 22 parasitised rolls containing large sized CM larvae were used. A maximum of 3 parasitised rolls were placed in each container to allow easier collection of emerging adult Mastrus.

All Mastrus that emerged from parasitised rolls were counted and sexed to allow calculation of Mastrus yield per CM larvae and male-female ratios.

Data were analysed using 2-tailed T-Test and Mann-Whitney U-Test.

Figure 1: Larval size comparisons: Larvae on the left of the image were considered "large" and those on the right of the image were considered "medium" size

Results and Discussion

The yield of Mastrus adults per medium sized CM larva averaged 1.73/larva with a sex ratio of 1.43 Male: 1 Female. The yield from large CM larvae averaged 2.06/larva with a sex ratio of 1.52 Male: 1 Female. There were no significant effects ($p<0.05$) of host larval size on total yield of Mastrus, yield of Mastrus males, yield of Mastrus females, or sex ratio (Table 1). T-tests assume normal distribution and equality of variance but since this is difficult to determine with small sample sizes, we conducted a Mann-Whitney U-Test to confirm the lack of significance identified in the T-Test.

Brood size tends to be very variable in parasitic Hymenoptera but the cause of this is not very clear. Many parasitoids lay more eggs in larger hosts (Hardy et al 1991, Vet et al 1993) and Sandanayaka et al (2011) demonstrated this with Mastrus in both choice and non-choice tests. Our current study suggests a trend towards similar results where larger sized host larvae yielded more Mastrus compared to the yield from medium sized host larvae. Possible explanations for the differences between our findings and those of Sandanayaka et al (2011) are (i) Our sample size may have been too small to detect differences, (ii) the effects observed in our Mastrus colony may have been due to loss of weight in the host larvae during storage before exposure to the parasitoid, or (iii) impact of storage conditions on parasitised larvae. The latter is examined in another experiment.

Most parasitoids that can detect if the host has already been parasitised will either reject those hosts or super-parasitise the host by laying their own eggs on the parasitised host, often after destroying eggs laid by the earlier parasitoid (van Alpen and Visser 1990). A single female Mastrus might parasitise a maximum of 4 CM larvae/day, could lay 6-20 eggs within 24 h at the beginning of the reproductive period, and super-parasitism is common when competition for hosts is high (Sandanayaka et al 2011). Female parasitoids are also known to feed on sub-standard host larvae. Although Hougardy & Mills (2007) declared Mastrus to not be a host-feeding species, Sandanayaka et al (2011) reported host feeding by Mastrus in the laboratory under competitive conditions in restricted arenas. Super-parasitism and host-feeding can affect the yield of Mastrus/host larva.

Table 1: Yield of *Mastrus ridens* per host larva from medium and large sized CM larvae in a no-choice test, where Mastrus could oviposit for 15 days.

	Total Mastrus/CM		Mastrus males/CM		Mastrus Fem/CM		sex ratio (M:F)	
	medium	large	medium	large	medium	large	medium	large
	2.15	2.14	1.09	1.24	1.06	0.90	1.03	1.38
	0.72	1.63	0.49	0.86	0.23	0.77	2.13	1.13
	1.69	2.19	1.07	1.38	0.62	0.81	1.72	1.71
	2.09	2.33	1.15	1.35	0.94	0.97	1.22	1.39
	2	2	1.11	1.33	0.89	0.67	1.25	1.97
	1.48		0.74		0.74		1.00	
	2.01		1.26		0.75		1.68	
mean	1.73	2.06	0.99	1.23	0.75	0.82	1.43	1.52
Variance	0.257	0.071	0.074	0.045	0.073	0.014	0.177	0.108
t-value (p)	-1.29 (0.225)		-1.67 (0.126)		-0.59 (0.568)		-0.37 (0.720)	
U-value	9.5		6		14		14	

Conclusion

The literature suggests that supplying good quality hosts (larger CM larvae) might improve yields of Mastrus, and there is a trend in our data that supports that suggestion. Our results yielded an average of 2.06 Mastrus/CM larva from the large sized larvae. This is at the lower end of the range (2-5 Mastrus/ CM larva) obtained by Sandanayaka et al (2011) and suggests that some other factor in addition to size may have contributed to lower yields.
In addition to improving the size of CM larvae presented to Mastrus, methods to ensure reduced competition for host resources and the effects of host storage prior to exposure to Mastrus should be explored.

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