Final report

Integrated management and development of additional agents for Parkinsonia

Project code: B.WEE.0148

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Abstract

Parkinsonia aculeata (parkinsonia) is a neotropical shrub/tree species that was introduced in Australia as an ornamental species and for its potential value as a hedging and fodder plant. It has since spread to occupy over 8000km2 of the rangelands of northern Australia, and forms dense thickets in floodplains and grasslands, and along water courses and bore drains. It has negative impacts on the pastoral industry and rangeland production systems through limiting pasture growth, restricting stock access to water and impeding mustering. Mechanical and chemical control methods for parkinsonia exist and are already being effectively used by land managers wherever possible. But these management tactics require repeat application and are not always possible in all parkinsonia infestations (e.g. in difficult terrain or in sensitive riparian environments). At present, widespread prickle bushes like parkinsonia can have control costs between $2-$300/ha/y depending on the density of infestations.

Having a landscape-scale, self-perpetuating form of control like biological control in these systems may aid in the integrated management of parkinsonia. This was the basis for the research pipeline of previous projects funded by MLA to identify candidate biological control agents, test their safety through quarantine risk assessment and only mass rearing and release of agents that are sufficiently host specific to the target weed. This project focused on the continued mass release of two leaf-defoliating moths, while also developing molecular and pheromone tolls to assist with their monitoring and evaluation. Additionally native range and quarantine research was undertaken to determine if an additional stem-galling biocontrol agent could be developed for deployment against this weed of rangeland Australia.

Based on the monitoring and evaluation presented both leaf-defoliating agents have established self-sustaining populations across the range of *P. aculeata* in northern Australia and there is evidence these populations are moving across the landscape. At surveyed release sites 52% of plants had no evidence of leaf defoliation, 45% of plants had evidence of leaf drop which could not be directly attributed to larval feeding on inspection, 3% of plants were classified as defoliated where <50% leaves were present. Native range and quarantine research on the stem-galling biocontrol agent was challenging but research approaches to troubleshoot the colony initiation for risk assessment are detailed as future research considerations.

The key benefit to the pastoral industry is the presence of two additional leaf-defoliating moth biological control agents as a persistent landscape scale weed management tool in the integrated weed management toolbox for parkinsonia. This will enable land managers to prioritise where in the landscape they can deploy other management tactics**.**

Executive summary

Background

The project aimed to control the invasive *Parkinsonia aculeata*, a shrub/tree species causing significant ecological and economic damage in northern Australia, that can incur control costs between $2-$300/ha/y depending on the density of infestations, by releasing an approved leaf-defoliating biological control agent which had not yet received substantial release effort. And developing tools to assist with the monitoring and evaluation of the two closely related leaf-defoliating agents once they were established across northern Australia.

Objectives

1. This project aimed to build on previous research investments made into parkinsonia management by focusing rearing and release efforts on *Eueupithecia vollonoides*.
2. To simplify the identification process of the morphologically identical moth agents molecular tools were developed. These molecular diagnostic tools have become increasingly important in monitoring establishment and impacts of agents, and to determine where each moth species has established relative to one another.
3. Chemical components of the female sex pheromones of *E. vollonoides* and *Eueupithecia cisplatensis* were characterised to determine if a pheromone lure that could be deployed for monitoring and aggregating populations of the agents across northern Australia.
4. The final component of the project focused on investigating the applicability of an additional weed biocontrol agent with a different mode of action to existing approved agents for *P. aculeata.*

Methodology

Multiple methodological approaches were used in the project to meet the above objectives including native range survey and exploration, mass rearing and release, field monitoring and insect survey, molecular barcoding, population genetics, chemical ecology, behavioural ecology, stakeholder engagement surveys, quarantine risk assessments.

Results/key findings

Based on the surveys presented both *E. cisplatensis* and *E. vollonoides* have established self-sustaining populations across the range of *P. aculeata* in northern Australia and there is evidence these populations are moving across the landscape. Ten years after initial releases commenced population densities of both moth species are not yet at the levels determined by laboratory studies to start chronically defoliating and stressing trees. This is evidenced by defoliation observed at release sites where 52% of plants had no evidence of leaf defoliation, 45% of plants had evidence of leaf drop which could not be directly attributed to larval feeding on inspection, 3% of plants were classified as defoliated where <50% leaves were present. When landholders and managers across rangeland northern Australia were surveyed 84% percent of respondents agreed or strongly agreed that parkinsonia was a severe problem for the grazing industry.

Benefits to industry

The key benefit to the pastoral industry is the presence of two additional leaf-defoliating moth biological control agents as a persistent landscape scale weed management tool in the integrated

weed management toolbox for parkinsonia. This will enable land managers to prioritise where in the landscape they can deploy other management tactics**.**

Future research and recommendations

Based on the results of the project, further native range and quarantine risk assessment to develop an additional weed biocontrol tool for *P. aculeata* should be undertaken to build on the knowledge of *N. aculeata* gained during B.WEE.0148. Any future research on *N. aculeata* should be focused on further understanding the ecology of the galling insect so that it can be reared under Australian quarantine conditions and undergo host-specificity testing.

Because *P. aculeata* is such a widespread weed across the rangelands of northern Australia monitoring and evaluation of weed management would be enhanced if a cost-effective, scalable and accurate approaches are developed. This includes building on the development of pheromone-based tools for *E. vollonoides* and *E.cisplatensis* which can monitor the presence of these agents but may also be able to aggregate them into areas of high weed density.

Table of contents

1. Background

1.1 *Parkinsonia aculeata* and the pipeline of biocontrol research

Parkinsonia is a shrub/tree species that occupies over 8000km2 of the rangelands in northern Australia. It has negative impacts on the pastoral industry and rangeland production systems through limiting pasture growth, restricting stock access to water and impeding mustering. At present, widespread prickle bushes like parkinsonia can have control costs between $2-$300/ha/y depending on the density of infestations. Reducing some of these control costs and improving pasture productivity can therefore assist in enhancing the profitability of rangeland production systems.

Having a landscape-scale, self-perpetuating form of control like biological control in these systems may aid in the integrated management of parkinsonia. This was the basis for the research pipeline of previous projects funded by MLA (B.NBP.0366; B.NBP.0620; B.WEE.0134; B.WBC.0060) to identify candidate biological control agents, test their safety through quarantine risk assessment and only mass rearing and release of agents that are sufficiently host specific to the target weed (Figure 1). This project (B.WEE.0148) extended this pipeline of research by; 1) releasing additional leaf defoliating biological control agents (risk assessed during project B.NBP.0620, and commenced releases in projected B.WEE.0134 & B.WBC.0060) 2) developing new tools to ease the monitoring of their establishment and, 3) investigating an additional stem-galling fly that was identified as a potential agent in B.NBP.0366. This study aims to improve rangeland productivity and profitability by minimising the impacts of parkinsonia using biological control.

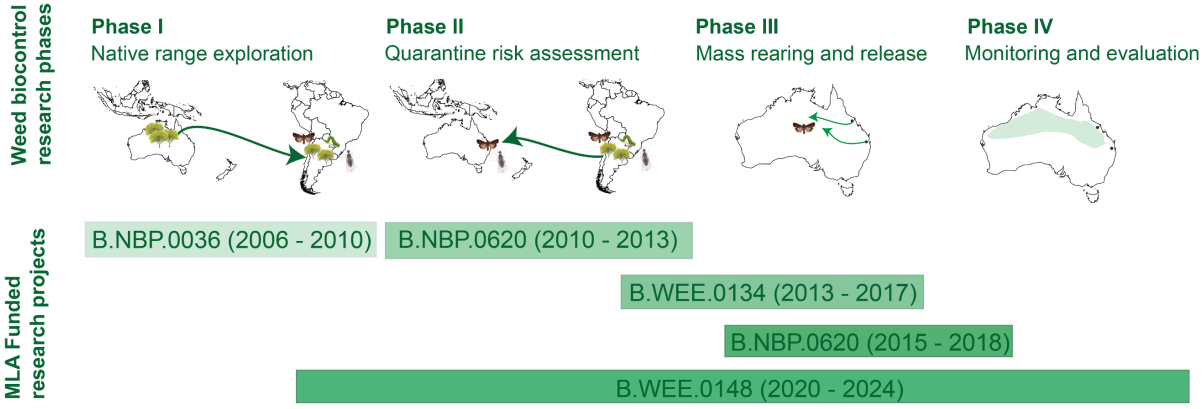


Figure 1: Pipeline of MLA funded parkinsonia research projects to identify, develop and implement biological tools for this weed of northern rangeland Australia. Green bars indicate each project and the section of the biocontrol research pipeline they focused on. The current project (B.WEE.0148) contained research components that delivered into each of the four biocontrol research phases.

1.2 *Eueupithecia*, a general introduction to the two leaf-defoliating moth species

Eueupithecia was historically considered a monotypic genus, with only *E. cisplatensis* described (Sihvonen et al. 2011). Specimens collected on *P. aculeata* from different localities of Argentina, were initially identified as *E. cisplatensis* by Axel Hausmann (Bavarian State Collection of Zoology). Difficulties maintaining cultures (during project B.NBP.0620) with moths collected from both the

northern and southern parts of the moth's distribution in Argentina suggested that a cryptic species may be present. Further taxonomic examination resulted in the description of a new species (*Eueupithecia vollonoides* Hausmann) based on reproductive anatomy and COI barcoding (Hausmann et al. 2016). Although there are striking differences in female and male genitalia, significant and consistent differential external features in colour or pattern of adults or larvae have not been found (Hausmann et al. 2016).

Eueupithecia cisplatensis and *E. vollonoides* are leaf-feeders and have a similar life-history. The female moth lays her eggs on the leaves of parkinsonia. Development of the moths at a temperature of 25-28^{o}C has the following timelines. Eggs hatch after 5-7 days and newly hatched larvae (caterpillars), less than 2mm long, begin feeding on the leaves (Figure 2A). The caterpillars (called loopers because of how they move) continue feeding for around 15 days and grow to approximately 2cm in length before pupating. Adult moths (Figure 2B) emerge from the cocoons after 5-7 days and mate. Female moths then lay their eggs and the cycle begins again.

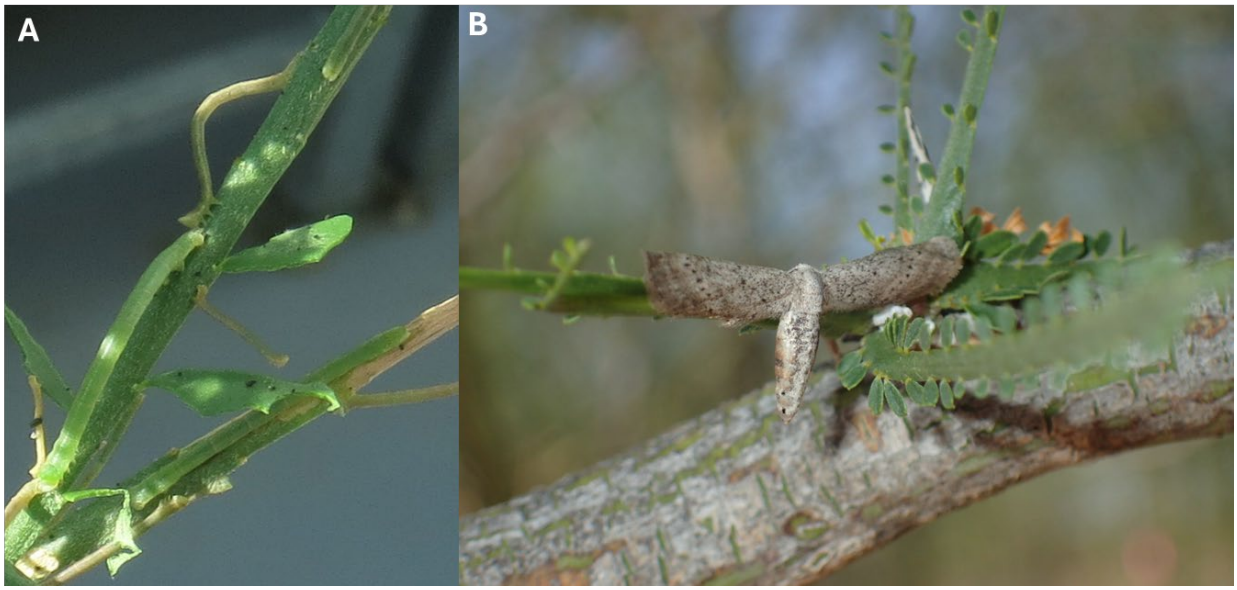


Figure 2: Photos of *Eueupithecia cisplatensis* looper caterpillars in culture in the laboratory (A) and an adult *Eueupithecia* sp. observed on *Parkinsonia aculeata* at field site in northern Queensland.

Despite similarities in their biology, the two species appear to have slightly different bioclimatic requirements in their native range (Hausmann *et al.* 2016; Mukherjee et al. 2021). Surveys in Argentina discovered a distinct distribution for each of species, with *E. cisplatensis* occurring in the coastal, slightly cooler and more humid southeast and *E. vollonoides* occurring in the inland, hotter and drier northwest of northern Argentina (Hausmann *et al.* 2016; Mukherjee et al. 2021). Physiological studies completed in project B.WBC.0060 on *E. cisplatensis* and *E. vollonoides* informed bioclimatic models published by Mukherjee et al. 2021 have guided release efforts across northern Australia of the two moth species across the consecutive MLA funded projects.

More than 1 million *E. cisplatensis* moths and 285,000 *E. vollonoides* moths were released during the previous project and achieved establishment at over 50% of release sites (B.NBP.0620). It was anticipated that these leaf defoliating agents will chronically stress the parkinsonia in areas where it has established, but it is not known how long it will take to get to this point, or if this level of stress will be seen across the landscape. What is known is that this agent is unlikely to take out mature plants entirely.

This project intended to build on previous research investments by focusing rearing and release efforts on *E. vollonoides* so that similar numbers of this species are released across bio-climatically optimal sites in Queensland, Northern Territory, and Western Australia.

1.3 *Neolasioptera aculeata* a stem galling fly prioritised for biocontrol risk assessment screening

Neolasioptera aculeata (Figure 3A) is a stem-galling fly native to the northern distribution *of P. aculeata* in Argentina, that was identified during previous native range surveys (MLA project B.NBP.0366) (Figure 1) as a potential biocontrol agent (Mc Kay et al. 2014). This species induces woody galls (ovoid growths) at stem and spine junctions of parkinsonia plants (Figure 3B-D), within which the larvae of the insect develop. Galls function as metabolic sinks, and therefore we anticipate that these galls on parkinsonia will reduce the plant's growth and reproduction and reduce the potential of plants to flower and set mature, viable seeds (Harris & Shorthouse 1996).

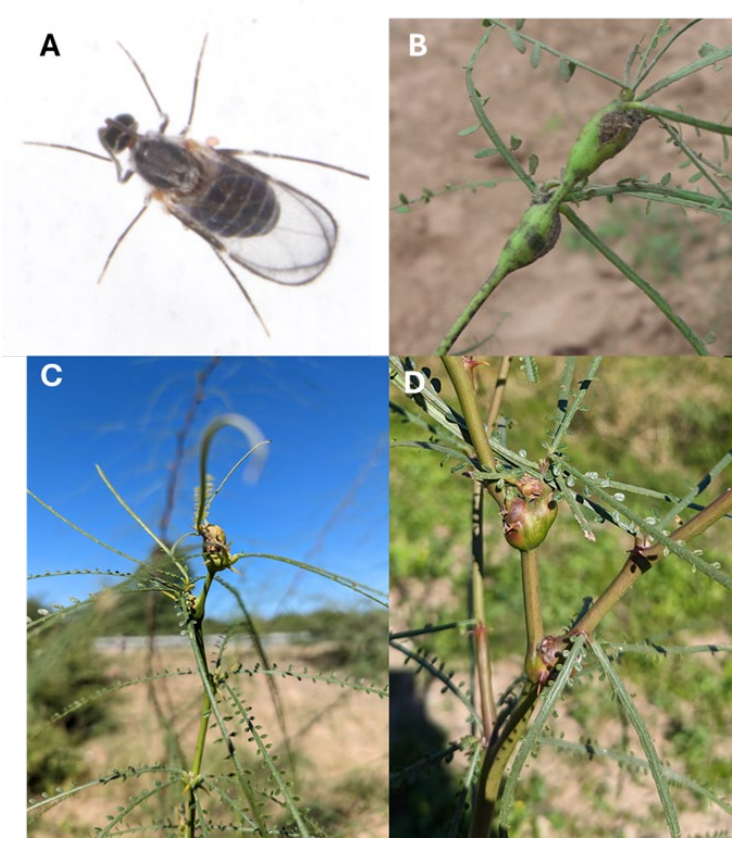


Figure 3: A) *Neolasioptera aculeata* adult B-D) photos of galls on *P. aculeata* in the native range of Argentina.

It was identified while developing the B.WEE.0148 project proposal that *N. aculeata* warranted further investigation as the species has the capacity to reduce the growth and reproduction of parkinsonia, using a different mode of action to the two leaf-defoliating moths but its ecology, life history and host-specificity was yet to be comprehensively evaluated. The species also needed to undergo a rigorous risk assessment to determine if it is sufficiently host specific to *P. aculetae* to be considered for release in Australia.

2. Objectives

Specifically, the project which commenced in January 2020 set out to achieve the following nine project objectives:

1. Up to 100,000 *E. vollonoides* will have been mass-reared and released across bio-climatically optimal sites (in Queensland, Northern Territory, and Western Australia) where this species has not yet been released
2. Have increased the number of release sites for *E. vollonoides*, from 19 (current) to ~50 across Northern Australia
3. Develop molecular diagnostic and pheromone tools to help monitor and track *E. vollonoides* establishment and *E. cisplatensis* persistence at up to 20 release sites across Australia
4. Three draft manuscripts as appropriate, based on outputs from either diagnostic, pheromone, host testing, rearing R&D
5. Report on the impact of *E. cisplatensis* and *E. vollonoides* on parkinsonia at least 19 existing sites based on presence of *E. cisplatensis* / *E. vollonoides*, initial spread, establishment of self-sustaining population, and an assessment of plant health where relevant
6. Worked with collaborators in Argentina to develop an appropriate laboratory rearing method for the stem-galling fly so that the potential agent can be reared successfully in quarantine for host testing in Australia
7. Pending success in developing a rearing method, and approval to import a stem-galling fly, complete host specificity screening of the stem-galling fly
8. Pending successful host specificity testing with the agent being specific to parkinsonia, lodge an application to the Department of Agriculture & Water Resources to release the stem-galling fly
9. Successfully trained two PhD students

3. Methodology

3.1 Mass-rearing and release of *Eueupithecia vollonoides*

Eueupithecia vollonoides colony was established at the Tropical Weeds Research Centre (TWRC), Charters Towers in February 2020 from eggs collected from the CSIRO Brisbane colony established in late 2019 from a reimportation of this species from Argentina. Rearing was done under optimal environmental conditions for the plant and *E. vollonoides*. Colonies were maintained as follows; eggs laid by female moths were maintained in the laboratory until neonates hatched; these were then transferred onto the leaves of parkinsonia plants growing in cages in an air-conditioned greenhouse (ca 25-28^{o}C; 50-60% RH). After completion of their development through larval and pupal stages, newly emerged adults were collected daily from colony cages and paired with adults emerging from different cages (to ensure an adequate mix of their genetic diversity and limit the likelihood of any negative inbreeding effects). These mating pairs were confined in plastic containers (17 x 11 x 5 cm) to ensure mating and oviposition. These containers were lined with moistened paper towels to maintain a high level of humidity to prevent desiccation of eggs laid.

By May 2020 the TWRC colony was large enough to initiate mass releases using the pupal release method established in the previous MLA project (B.WBC.0060). Releases of pupae enabled *E. vollonoides* to escape predation, and the adults emerging from puparia can find optimal microhabitats within parkinsonia foliage for egg deposition and larval development. Therefore, field

releases in this project focussed exclusively on release of pupae. When pupae were hand-collected from colony cages they were released in the field in a plastic container placed within a pyramid shelter (Delta trap) hung on parkinsonia foliage (Figure 4).

Figure 4: Pyramid shelter (Delta traps) hung in parkinsonia trees to provide shelter for *Eueupithecia vollonoides* pupae until they emerge as moths.

The aim of this project was to release *E. vollonoides* at up to 30 new release locations. Release sites were selected based on Parkinsonia abundance and climate suitability for *E. vollonoides* predicted by bioclimatic modelling (Mukherjee et al. 2021). Previous *E. cisplatensis* release sites or sites where *E. vollonoides* was released but failed to establish during the previous project were also considered.

Collaborators were identified and contacted in 2020 to participate in the mass release of *E. vollonoides*. They included Mt Isa City Council, Cloncurry Shire Council, Flinders Shire Council, Central Highlands Regional Council, Isaac Regional Council, McKinlay Shire Council, Richmond Shire Council, Winton Shire Council, Barcaldine Regional Council, DAF, Fitzroy Basin Association, Department of Primary Industries and Regional Development – Western Australia, Department of Environment and Natural Resources – Northern Territory, Ord River System (WA), Western and Central Queensland LGA's, Fitzroy Basin Association, and co-ordinated landholders within region/properties identified.

3.2 Development of molecular diagnostic tools

3.2.1 CO1, 28S and CAD barcoding

A total of 102 samples of *E. cisplatensis* (n = 45) and *E. vollonoides* (n = 57) from laboratory colonies maintained at CSIRO quarantine facilities, from Argentinian native range and from the introduced range in Australia were used in the study (Appendix 1, Table 7). Samples from Argentina were collected by beating *P. aculeata* foliage over a one square metre sheet, while individuals from Australia were trapped using delta sticky traps in Burketown, QLD, where *E. vollonoides* were released as part of the biological control program.

DNA was extracted from *E. cisplatensis* and *E. vollonoides* samples that had been stored at -20°C using the spin column DNA extraction protocol described by Ridley et al. (2016). Briefly, either 3 legs or 'head + thorax' were homogenized in 200µl of lysis buffer and 5µl of Proteinase K, then digested overnight at 55°C. Lysate, binding buffer (4M GuHCl) and ethanol were combined in equal parts and added to an EconoSpin mini spin column (Epoch Life Science, Texas, USA). The spin column was washed twice with 500µl of 70% ethanol, and then the DNA was eluted using 100µl of 10mM Tris. Eluted DNA was quantified using a Qubit fluorometer (Thermo Fisher Scientific, Massachusetts, USA) following the manufacturer's protocol.

The gene regions COI, 28S and CAD were PCR amplified using the primers listed in Appendix 1, Table 8. A 20 µl reaction containing 1× MyTaq HS buffer, 0.2 µm of forward primer, 0.2 µm of reverse primer, 1 unit my MyTaq HS DNA polymerase (Bioline, United Kingdom) and 3.0 µl of DNA template were amplified using the following PCR conditions. One cycle of 95°C for 2 min and then 40 cycles of the following: 95°C for 30 s, annealing for 45 s and 72°C for 1 min 15 s. A final cycle of 72°C for 10 min was used. Annealing temperatures were 51°C for COI and 48°C for 28S and CAD. All PCR products were cleaned by adding 1 unit of Exonuclease I and 1 unit of Antarctic Phosphatase (New England Biolabs, United States) and heating at 37°C for 30 minutes. PCR products were sequenced in both directions using Sanger sequencing by Macrogen (South Korea).

Sequence data were trimmed, aligned, and checked using CodonCode Aligner version 4.1.1 (CodonCode Corporation, Centerville, MA, USA) and priming sites were removed. Sequences were checked manually for problems typical of each gene region, including the presence of pseudogenes, including stop codons, large numbers of protein coding changes and GC bias. Nuclear DNA haplotypes were reconstructed using the PHASE algorithm as implemented in DNAsp 6.12.01 using 10,000 interactions with 1,000 burn-in, an output probability of 0.9, and both the hybrid and recombination models (Rozas 2017). Popart was used to create haplotype networks using the TCS network model (Leigh and Bryant 2015; Clement 2002).

3.2.2 Populations genetics

Larval *Eueupithecia* of both species were collected from sites in their native range of Argentina and Paraguay and across the introduced range in Australia (Table 1, Figure 5). Field specimens were sampled by beating *P. aculeata* foliage over a 1 m^2 sheet. Where possible, about 10 larvae were collected from each *P. aculeata* tree and from about 10 trees per site. Adult hybrids of *E. vollonoides* females mated with *E. cisplatensis* males from laboratory reciprocal cross-mating experiments (Murray et al., unpublished thesis chapter, 2024)) were also included in the analysis. The insects used to produce these hybrids were obtained from Australian laboratory cultures of *E. vollonoides* females and a combination of Australian field-collected and laboratory-cultured *E. cisplatensis* males. Hybrid individuals were reared to adulthood before their DNA was extracted.

DNA was extracted from the moth tissue using a modified silica plate extraction protocol (Ridley et al. 2016). Briefly, the protocol involved separating single larvae or the bodies of adult moths (with the genitalia and wings removed) individually into 1.5 mL microcentrifuge tubes. Three 4mm stainless steel balls and 300µL of lysis buffer and 3µl proteinase K (≥20 mg/mL) were added to each tube before maceration in a Bullet Blender Homogenizer (5 Storm (BBY5M), Next Advance, New York USA), set to maximum speed for 10 minutes. Samples were incubated overnight at 50°C on a dry block heater/shaker. RNA was digested by adding 2µl of RNAse A (10mg/mL) and incubating for ~30mins at 37°C. Samples were spin-column purified using EconoSpin spin column plates from Epoch Life Sciences (Missouri City, Texas, USA).

Table 1: The collection details for each sample included in the population genetics analysis of both species of *Eueupithecia*. Locations of these samples mapped in Figure 4.

Site	Species	Country	Province	Latitude	Longitude	Year	Source	n
Arg-D	*E. cisplatensis*	ARG	BA	-36.8147	-59.1306	2020	Field	9
Arg-E	*E. cisplatensis*	ARG	BA	-35.0149	-58.4476	2020	Field	6
Arg-G	*E. cisplatensis*	ARG	BA	-34.9455	-57.8476	2020	Field	7
Arg-F	*E. cisplatensis*	ARG	BA	-34.6715	-59.0337	2020	Field	10
Arg-C	*E. cisplatensis*	ARG	C	-29.7534	-58.0858	2019	Field	5
Arg-B	*E. cisplatensis*	ARG	C	-29.0186	-58.4961	2019	Field	10
Arg-H	*E. vollonoides*	ARG	C	-28.5735	-58.7219	2018	Field	4
Arg-A	*E. vollonoides*	ARG	C	-28.5718	-58.7217	2019	Field	5
Arg-I	*E. vollonoides*	ARG	C	-28.2741	-58.6457	2018	Field	1
Arg-K	*E. vollonoides*	ARG	F	-25.2911	-57.7282	2019	Field	4
Par-A	*E. vollonoides*	PAR	PHC	-25.0112	-57.558	2019	Field	9
Par-B	*E. vollonoides*	PAR	PHC	-24.8848	-57.6828	2019	Field	9
Arg-L	*E. vollonoides*	ARG	F	-24.7021	-60.5938	2019	Field	4
Arg-M	*E. vollonoides*	ARG	S	-23.4292	-64.1455	2019	Field	2
Arg-J	*E. vollonoides*	ARG	S	-23.2043	-64.0934	2019	Field	6
Hyb	*E. cisplatensis* ♂ *X* *E. vollonoides* ♀	AUS	NA	NA	NA	2022	Culture	15
AB	*E. cisplatensis*	AUS	CT	-19.9091	146.2542	2020	Field	2
Big_Bend	*E. cisplatensis*	AUS	CT	-19.8526	146.1336	2020	Field	2
BRFC	*E. cisplatensis*	AUS	CT	-19.8063	146.0692	2020	Field	9
MB	*E. cisplatensis*	AUS	CT	-19.998	146.435	2020	Field	1
R4	*E. vollonoides*	AUS	BT	-17.8178	139.4272	2021	Field	2
AD2	*E. vollonoides*	AUS	FW	-18.7353	139.2098	2021	Field	5
AD9	*E. vollonoides*	AUS	FW	-19.1563	140.1956	2021	Field	3
AD11	*E. vollonoides*	AUS	MI	-20.4213	139.087	2021	Field	3
AD13	*E. vollonoides*	AUS	MI	-20.573	138.6101	2021	Field	8
S1	*E. vollonoides*	AUS	MI	-19.6232	138.3052	2022	Field	3
S2	*E. vollonoides*	AUS	MI	-19.6227	138.3067	2022	Field	2
S5	*E. vollonoides*	AUS	MI	-20.389	137.7395	2022	Field	1
S7	*E. vollonoides*	AUS	MI	-20.9593	137.8968	2022	Field	2
S8	*E. vollonoides*	AUS	MI	-20.6241	137.6631	2022	Field	3
S9	*E. vollonoides*	AUS	MI	-20.6603	137.6803	2022	Field	3
S10	*E. vollonoides*	AUS	MI	-19.9501	138.107	2022	Field	3
S11	*E. vollonoides*	AUS	MI	-19.949	138.1079	2022	Field	5
S12	*E. vollonoides*	AUS	MI	-20.5754	139.5712	2022	Field	2

Native range field samples are grouped by site with n varying from 1-10 individuals per site, with those from Argentina (ARG) being randomly assigned a site letter in from A to M, and those from Paraguay (PAR) assigned either A or B. The experimental hybrids (Hyb) (n= 15) included for comparison were obtained from cultured insects in Australia (AUS), and therefore their location details are listed as NA. Provinces: BA= Buenos Aires, C= Corrientes, F= Formosa, PHC= Presidente Hayes / Central, S= Salta, CT= Charters Towers, BT= Burketown, FW= Four ways, MI= Mt. Isa.

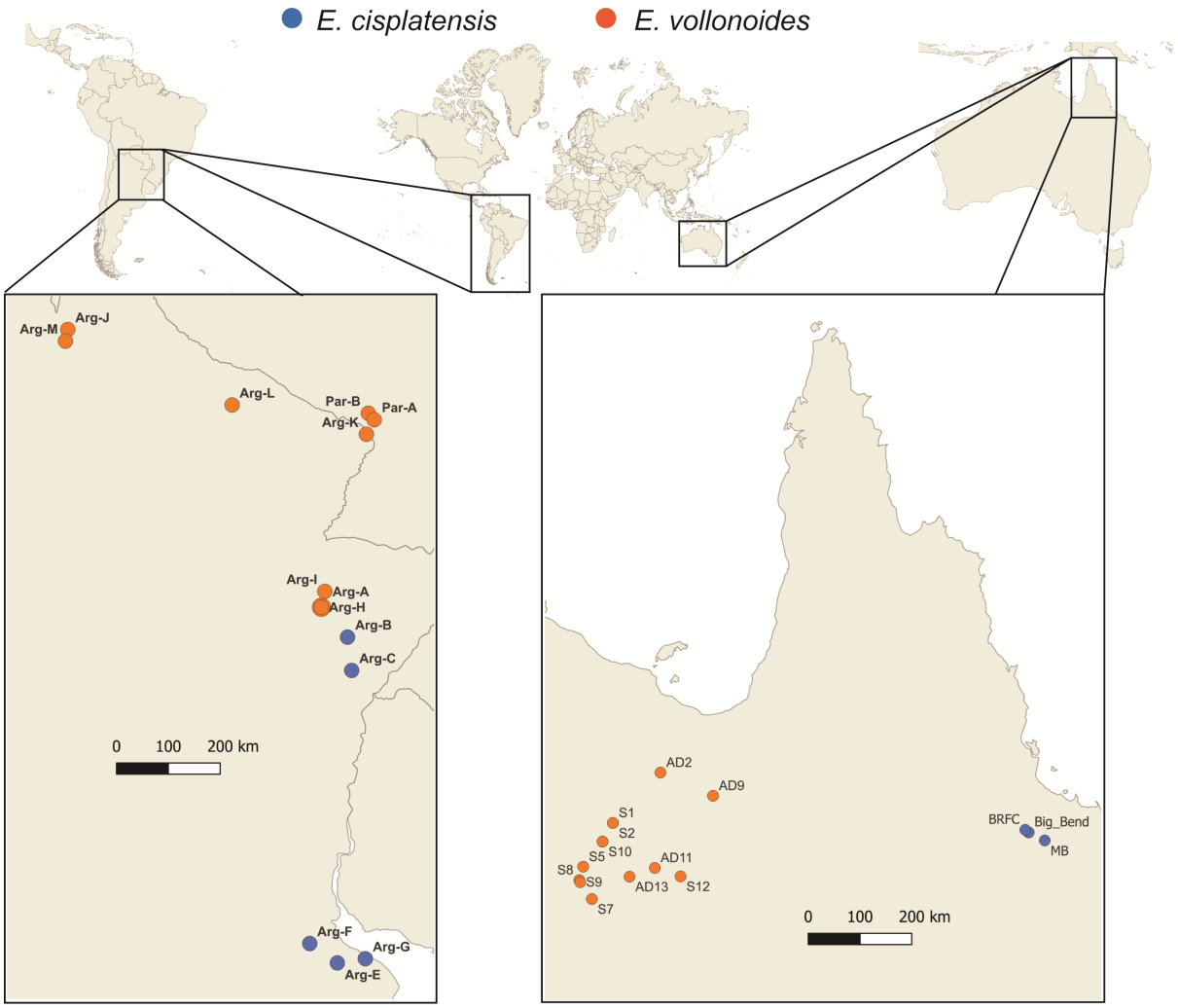


Figure 5: Locations of *Eueupithecia cisplatensis* and *Eueupithecia vollonoides* samples included in population genetic analysis (Table 1).

Preparation of GBS libraries followed the protocol described in the methods of Etebari et al. (2021) and Hereward et al., (2020), which is based on the methods of Elshire et al. (2011), Poland et al. (2012) and Peterson et al. (2012), with barcodes developed from the protocols of Caporaso et al. (2012). Briefly, DNA was quantified using a Qubit Fluorometer (Thermo Fisher Scientific, Australia) and normalised for priority samples with DNA concentrations of 2.5 ng or higher. A restriction enzyme digest using the enzymes PstI and MspI was performed for 100 ng (40 µL at 2.5 ng) DNA from each sample. Adapters were ligated onto the ends of cut fragments, and individuals that shared a 96-well plate were pooled. A dual-wash spin column method was used to purify each pooled plate. A BluePippin (Sage Science, Massachusetts USA) was used to select a 300-400 bp window from each purified library pool. The size-selected libraries were then amplified by PCR, and then the amplified libraries were quantified before equimolar pooling. The final pool was purified using AMpure paramagnetic beads (Beckman Coulter Life Sciences, Lane Cove New South Wales, Australia), vacuum dried, and posted to Novogene (Bejing, China) for sequencing using Illumina HiSeq X 150 bp paired-end sequencing (Etebari et al., 2021; Caporaso et al., 2012)

STACKS (version 2.59) was used to demultiplex and process the sequenced reads. STACKS was used to optimise the map and parameter assembly of loci using the de novo “80% rule” method described

by Paris et al. (2017) by varying the m parameter from 1 to 8 (with the n parameter = m+2). The combination that produced the greatest number of polymorphic loci after filtering was used (DeRaad, 2022; Knaus & Grünwald, 2017). The dataset was filtered further using SNPfiltR 1.0.1 and recovered 6,482 SNPs with 11.63% missing data after filtering by read depth, genotype quality and SNP completeness (DeRaad, 2022; Knaus & Grünwald, 2017). Analyses that were performed using R packages all used R 4.3.2 (R Core Team, 2024).

Pairwise population F_{ST} and its confidence intervals were calculated as described by Weir & Goudet (2017) using *betas* from hierfstat. Lower and upper confidence intervals were 5% and 95% respectively and were calculated with 1000 bootstrap replicates. The *randtest* function from ade4 1.7-22 (Dray & Dufour, 2007) was used to perform IBD analysis. Tests for IBD within and across species were performed using *mantel.randtest* (ade4) with 1000 permutations, using the matrix transformations described by Rousset (1997).

One random SNP from each locus was used for the population structure analyses to avoid linkage bias. Analysis of population structure was performed with STRUCTURE 2.3.4 (Falush et al., 2003, 2007; Hubisz et al., 2009; Pritchard et al., 2000). Population parameters (K) from 1 to 8 were tested, with 10 replicates each. The "admixed" and "alleles correlated" models were used with population alpha priors set to 1/K (Wang, 2017). Replicates of K were separated into major and minor clusters using CLUMPAK (Kopelman et al., 2015). Meaningful K values for all analyses were determined using the ΔK value (Evanno et al., 2005) for STRUCTURE results. A PCA was conducted to contrast ordination-based analysis of population structure with the individual assignment analyses from STRUCTURE and snapclust. The PCA was performed using the *dudi.pca* function from ade4 (Dray & Dufour, 2007).

3.3 Development of pheromone tools

Initial tests of excised dorsal glands were conducted using *E. cisplatensis* and *E. vollonoides* moths reared in the laboratory at the Australian Commonwealth Scientific and Industrial Research Organisation (CSIRO) Ecosciences Precinct in Brisbane. All subsequent tests used *E. vollonoides* individuals that were readily available in large numbers from the mass rearing facility at Charters Towers (Tropical Weeds Research Centre (TWRC), in Queensland). Only some of the subsequent tests were conducted on *E. cisplatensis,* and these were done using wild-caught individuals and their offspring, when they were available. Initial investigations into the pheromone glands of these moths identified a dorsally located small white gland, from which several compounds were identified (here throughout referred to as the "dorsal gland"). Subsequent research into the calling behaviour of *Eueupithecia vollonoides* revealed an alternative gland structure that was associated with the mating behaviour of these moths and is referred to throughout as the "terminal abdominal gland".

3.3.1 Female terminal abdominal pheromone glands and "calling" behaviour

Virgin female moths (one day post-emergence, n=12) were placed in 30mm diameter plastic Petri dishes on the day of their eclosion and they were immobilised by adhering their legs to a 10mm x 10mm piece of adhesive insect trap. Females were recorded undisturbed under red LED light using Panasonic HC-V380 video cameras for the entire scotophase and first half of the photophase (i.e. the limit of camera storage). Limiting the movement of the females was necessary to keep their abdomens within view of the video camera, so that calling might be observed. The video was then reviewed in 30 second increments and any behaviour suggestive of typical moth "calling" postures

was inspected closely and characterised. Call duration, number of calls per observational period and the duration between bouts of calling were also recorded. Following initial review of the video recordings, a bout of calling was defined as the duration between a female extruding her (presumed) pheromone gland from her abdominal tip, before retracting it again.

Virgin females (one to three days post-emergence, n=10) were used for pheromone gland dissections. Dissections were conducted between 0000h and 0400h, when females were most likely to engage in calling. Females were immobilised by grasping their head and thorax dorso-ventrally with feather-touch forceps. The entire abdomen was then removed from the thorax using a dissection probe to pull the thorax away from the first abdominal segment. The remainder of the female was immediately euthanized in 100% ethanol. Gentle pressure was applied to the length of the excised abdomen until the ovipositor and pheromone gland complex was extruded past the final abdominal segment. Using a micro dissection probe (a minutin pin inserted into a matchstick), the papillae anales (PA) were pierced and gently pulled away from the remainder of the abdominal tip. In most cases, this process separated the PA, vaginal chamber (VC), accessory glands (AG), and saccular pheromone gland (SPG) cleanly, as an entire piece from the rest of the genitalia. The dissection probe was then used to clear away any remaining irrelevant structures. The pheromone gland and ovipositor complex were then photographed and the PA, AG and SPG were measured, until each structure had been successfully dissected and measured in 10 females. Dissection probes were sterilised in 100% EtOH after each gland was removed.

3.3.2 Excised dorsal gland and synthetic lure field trapping tests

Initial reciprocal trapping tests were conducted at *a P. aculeata* infestation in Burketown, Qld, where *E. vollonoides* was known to be well established to ascertain if there is a pheromone attraction component in this species. These tests used crude extracts of individual glands from females of each species suspended in hexane and paraffin oil, independently from one another, 200µl of which was loaded into rubber septa as lures. These were tested alongside traps baited with live virgin females (of both *Eueupithecia* species) and control traps (that were either empty cages, or cages containing rubber septa loaded with only hexane). Traps were suspended from the top of steel placed amongst *P. aculeata* trees.

In May 2022 and May/June 2023 reciprocal pheromone trapping was conducted at three sites along a 45km stretch of the Burdekin River near Charters Towers. *Eueupithecia cisplatensis* is well established at this location and this was confirmed at each site by beat-sheet sampling from trees for *Eueupithecia* larvae prior to deploying traps and then performing COI analysis on those samples. White delta traps with sticky liners were baited with plastic mesh cages suspended from the inner apex of the trap. Cages contained either: (1) a live virgin female *E. cisplatensis,* (2) a live virgin female *E. vollonoides* (with these two being positive controls), (3) a lure consisting of 100ng of either Z3,Z6,Z9-octadecatriene, Z3,Z6,Z9-nonadecatriene, Z3,Z6,Z9-eicosatriene or Z3,Z6,Z9-heneicosanetriene prepared in hexane, (4) a lure containing hexane solvent alone or (5) the cage was left empty (negative control). The delta traps were placed at random in infestations of *P. aculeata* at a height of 1-2m above the ground and at least 5m from other traps. Females were used on consecutive nights and replaced weekly when trap catches were recorded. Each trap was rotated around the infested site weekly. Catches from 2022 and 2023 were pooled because the numbers of males caught in the live female treatments were low, and because results did not vary greatly across seasons or sites (Kruskal-Wallis tests: χ^2= 3.6, 2.88, df=1,4, p= 0.576, 0.577, respectively).

3.3.3 Chemical analyses and synthetic lure preparation of the dorsal gland

Dorsal glands were extruded by applying slight pressure to the female's abdomen and were excised by gently scraping the everted gland from the body using the edge of a small filter paper. Glands were then immersed in chromatographically pure n-hexane for 15 min. The n-hexane extracts were transferred to a clean conical glass vial and kept in a freezer at -10℃ if not used immediately. Samples from five females were pooled for each species, and then each were concentrated under a gentle stream of pure N2 before analysis.

The extracts were initially analysed by gas chromatography using a HP 6890 gas chromatograph fitted with a flame ionization detector (GC-FID) and silica capillary column (DB-5, 30 m × 0.25 mm × 0.25 µm). Samples were analysed by autosampler splitless injection and held initially for five minutes at 100°C before ramping the column temperature by 10°C every minute until 300°C was reached, at which point this temperature was maintained for 10 minutes.

The peaks observed in the GCFID analyses were identified by sending the samples for third party analysis by gas chromatograph mass spectrometry (GCMS) at the School of Agriculture and Food Science, the University of Queensland (Australia). The injector and detector temperatures were initially 250°C and the oven temperature was programmed to begin at 80°C for two minutes, before ramping by 4°C every minute until 180°C was reached. After this point, the oven ramped by 10°C per minute until 240°C was reached, at which point isothermal conditions were maintained for 12 minutes.

Following the putative identification of the compounds present in the gland extracts, some of the compounds were synthesised and prepared as 100ng doses in hexane for loading into red rubber septa by Dr Hongxia Liu (Shanxi Agricultural University, Shanxi Province, China). The septa were tightly sealed in opaque foil covered vacuum bags and shipped to Australia for testing but were stored at -20°C until use.

3.3.4 Wind tunnel tests of dorsal gland extracts

Wind tunnel assays were based on the protocols of Charlton & Cardé (1990), Royer & Mc Neil (1993), Allison & Cardé (2008) and Fang et al. (2018). The floor of the wind tunnel was covered with ~40 non-overlapping red paper dots (~6cm diameter) to provide visual reference points for optomotor regulation (Allison & Cardé, 2008; Kuenen & Baker, 1982). Wind speed was set to ~40cm/s. Three "Twilight Red Light Blue Blocking" LED torches (BlockBlueLight, Melbourne, Australia), which emit only red light in the wavelength of 625nm, were top mounted at even intervals on the outside of the wind tunnel ceiling. A dark piece of material was draped over one side of the wind tunnel to exclude artificial light that entered the laboratory from outside.

Odour treatments included: virgin *E. cisplatensis* females, virgin *E. vollonoides* females, or lures made of a 100ng dose of each synthetic compound (on its own) prepared in hexane (Z3,Z6,Z9-octadecatriene, Z3,Z6,Z9-nonadecatriene, Z3,Z6,Z9-eicosatriene or Z3,Z6,Z9-heneicosanetriene, see results for details). Odour treatments were placed in mesh cages and mounted on platforms about 30cm high and 10cm downwind from the fan. Platforms were made from bamboo skewers, masking tape and polystyrene pieces roughly 25cm by 15cm. Virgin females were kept individually in these cages for a minimum of 30 minutes prior to being tested. Test males were kept alone in a plastic film canister with aeration holes in the lid. Prior to each assay, the canisters were affixed horizontally with mounting putty to a platform 30cm high and 120cm downwind of the odour treatment. At the beginning of each assay the lid of the canister was removed, and the male's behaviour was

observed. The following behaviours were recorded: (1) taking flight, (2) upwind flight towards the odour source, (3) arrestment of in-flight upwind progress, (4) landing at the odour source, and (5) initiating courtship behaviour. Males that failed to take flight within three minutes or failed to undertake upwind flight towards the odour source within five minutes were classified as unresponsive. Other studies typically exclude males after shorter intervals of inactivity, but additional time was given in these trials because of the covert nature of female *Eueupithecia* calling behaviour, which is not visible to the naked eye, so confirmation of the female's calling during the wind tunnel trial was not possible.

Males were tested only once for each odour treatment and re-caged and put aside for at least 15mins before being exposed to a different treatment. Clean air was allowed to pass through the wind tunnel for three minutes between treatments. The order in which males were exposed to treatments was randomised for each individual across the scotophase.

3.3.5 Glasshouse tests of dorsal gland extracts

Trap catches in the field during the May 2022 field tests were low, so tests were repeated in the glasshouse to determine whether laboratory tests could substantiate the results obtained from the field. Known numbers of *E. cisplatensis* and *E. vollonoides* males were released so that trap catches could be interpreted relative to the density of males present in the glasshouse. Initially, delta traps were baited with live virgin *E. vollonoides* females, and control traps were the same, but without the moths. Also, other delta traps were deployed simultaneously, and they held rubber septa impregnated with 100ng of either Z3,Z6,Z9-octadecatriene, Z3,Z6,Z9-nonadecatriene, Z3,Z6,Z9-eicosatriene or Z3,Z6,Z9-heneicosanetriene, with each prepared in hexane. The compounds were those identified in the putative dorsal pheromone gland. These traps were hung at ~1.5-2m intervals along a ~9m transect inside a 51m2 glasshouse. Male moths were released each afternoon into the space as they became available, and the traps were checked for catch daily and simultaneously randomised again along the transect (other than weekends) for three weeks from August-September 2022. Subsequent trapping tested only control traps and *E. vollonoides* females, with each treatment trap containing multiple female *E. vollonoides*. Females were added as they became available, and dead ones removed, to increase the likelihood that at least one female was calling each evening. During this time, 90 virgin females were used, in total, as attractants, with between 1-31 being added each release day. A total of 187 virgin male adults was released into the glasshouse, with 3 to 46 male adult moths let go in each instance.

3.3.6 Chemical analyses and synthetic lure preparation of the terminal abdominal gland

Pooled samples of *E. vollonoides* and *E. cisplatensis* virgin females were extracted and prepared by dissecting out 5-10 pheromone glands during the usual time of peak calling and immersing them in the equivalent of 10µL of hexane for ~5 minutes per gland, before transferring the supernatant to a new vial. Samples were stored at -20°C until use.

The pooled samples of each moth species were concentrated with a gentle stream of analytical grade argon gas and the samples were analysed by means of GCMS and manual injections (Agilent 7890B GC coupled with an Agilent 5977A MS) at Plant and Food Research, Lincoln, New Zealand. A synthetic formulation of the compound (11Z,13Z)-hexadeca-11,13-dienal, identified by GCMS and believed to be involved in pheromonal activity of both species, was then synthesised by Dr Andrew Twidle (Plant and Food Research, New Zealand) and concentrated under argon gas. This was then stored in an opaque vial at between -20 and -80°C until use.

For biological testing, 1µl and 5µl doses of this compound were prepared in 100µl of distilled analytical grade hexane before being eluted into red rubber septa and then sealed in opaque glass vials at -20°C until testing.

3.3.7 Field tests of the terminal abdominal gland extracts

These tests were conducted in the same manner as those described above, using delta traps hung *in P. aculeata* trees containing cages that were either empty, contained a live virgin *E. vollonoides* female, a rubber septum lure with either 1µl or 5µl of (11Z,13Z)-hexadeca-11,13-dienal prepared in 100µl of distilled hexane, or with only 100µl distilled hexane. Trap catches were recorded weekly for three weeks.

3.3.8 Y-tube tests of the terminal abdominal gland extracts

Olfactometer tests were conducted under 625nm wavelength monochromatic red LED lights, during hours 6-12 of the scotophase, to cover the diel mating period of each species (Murray et al., 2014). A glass y-tube (5.5cm diameter, with each arm 24cm long, as was the introduction chamber stem), cleaned with acetone and baked at a minimum of 150°C overnight, was placed in a windowless room and the light source suspended ~32.5 cm above the tube, centrally, at the intersection of the odour arms and introduction chambers. An activated charcoal (1.5g) filtered air current was humidified and used to deliver the odour treatments into the y-tube at about 150-200mL/min. Treatments of 1µl and 5µl doses of (11Z,13Z)-hexadeca-11,13-dienal were prepared in 100µl of distilled hexane and eluted onto rubber septa. A rubber septum impregnated with 100µl of distilled hexane served as a control. Virgin males (1-4 days post-emergence (mean = 2.7 days)) were placed in the introduction chamber and observed for 10 minutes. Three males were individually observed before the y-tube was inverted, odour sources rotated, and observations resumed. Before this, the tube was flushed with clean air between tests, and the two treatment doses (1µl and 5µl) were alternated after six males were tested until a total of 49 males had been tested.

3.3.9 Headspace extraction lures

The chemical composition of crude gland extracts may differ substantially from the pheromone profile of the volatiles emitted during female calling, so a headspace-based lure was developed for *E. vollonoides*. The headspace was extracted continuously through the entire night to encompass the entire calling period of all females. Twenty-eight virgin females were sealed inside a clean glass jar using low volatility plastic oven bags that had been baked out at 50°C for a minimum of 24hrs prior to use. The sealed jars were secured with a combination of masking and Teflon tape. A trap for volatiles was made by loading 250mg of matrix porous polymer adsorbent (Porapak Q 80-100 mesh, Merck Life Sciences, Victoria, Australia) into a Pasteur pipette, which was plugged with glass wool and inserted through the lid of the jar. The exposed end of the pipette was attached to clean plastic hosing and a vacuum that was set to ~500mL/min. A Pasteur pipette packed with 300mg of activated charcoal and glass wool was also inserted into the jar to remove contaminants from the incoming air passively. The air was allowed to flow through this apparatus overnight. The following morning the Porapak trap was eluted using several mL of analytical grade hexane until at least 500µl of elute was obtained. The elute was evaporated onto the surface of a rubber septum using a gentle stream of analytical grade nitrogen before sealing it with an opaque jar and freezing at -20°C until use. This lure was tested in the field during the 2022 field trapping season in the same manner as described above.

3.4 Assessment of establishment, spread and impact of *E. cisplatensis* and *E. vollonoides*

3.4.1 Field surveys

Selected sites were monitored with Queensland sites being more heavily surveyed initially due to border closures and lockdowns between 2020 until March 2022. Larvae are very good at mimicking parkinsonia foliage or thorns, which means detecting their presence by searching plants is difficult and laborious. Therefore, the beat-sheet method was employed to determine the presence and density of larvae at sites. Up to ten *P. aculeata* plants close to the release area at a site were randomly selected. A standardized number of 10 beats per tree at each site was used to beat the healthy foliage to dislodge any insects present onto the beat-sheet placed beneath the foliage. The beat-sheet was then examined to record the numbers of larvae, and the presence of other insects (particularly, predatory insects). The presence of larvae after at least one wet season-dry season cycle was determined to be the minimum evidence acceptable to confirm establishment; this period ensured that the released insects had not only survived the release, but that the local site was able to sustain multiple generations of the insects. Once populations were recorded as having established, any spread from the original release sites was also monitored using the beat-sheet method.

To determine the impact of *E. cisplatensis* and *E. vollonoides* population measures and plant parameters were measured at each site. Plant population measures included density and spread of the *P. aculeata* infestation and number of each life stages present within a 20 x 20 m quadrat. Up to 10 plants within the 20 x 20 m quadrat were measured for height, canopy density and leaf defoliation.

3.4.2 Stakeholders survey

An online survey was developed as part of a Masters research project based at the University of Queensland. The survey was developed with input from Dr Michelle Rafter (CSIRO), Dr Barton Loechel (CSIRO), Dr Raghu Sathyamurthy (CSIRO) and Prof Gimme Walter (UQ). The purpose of the survey was to quantify and better understand the from the perspective of stakeholders the impact of parkinsonia on agriculture, and how management tools, such as biological control, were perceived to be working to mitigate the impacts of the weed.

The survey was deployed using the platform Survey Gizmo and was conducted with human ethics permission form UQ and CSIRO. Survey participants were anonymous and were able to skip questions if they did not want to answer so the number of responses for each question will differ and are indicated in the results section below.

3.5 Native range research of stem-galling fly *Neolasioptera aculeata*

Eight native range surveys for *N. aculeata* were conducted in between 2019 and 2024. Each time surveys were focused on the northern distribution of *P. aculeata* in Salta province (Figure 6) where *N. aculeata* are prevalent and an area in which our collaborators from Foundation for the Study of Invasive Species (FuEDEI) were able to secure the appropriate sampling permits from regional authorities (Appendix 2.1).

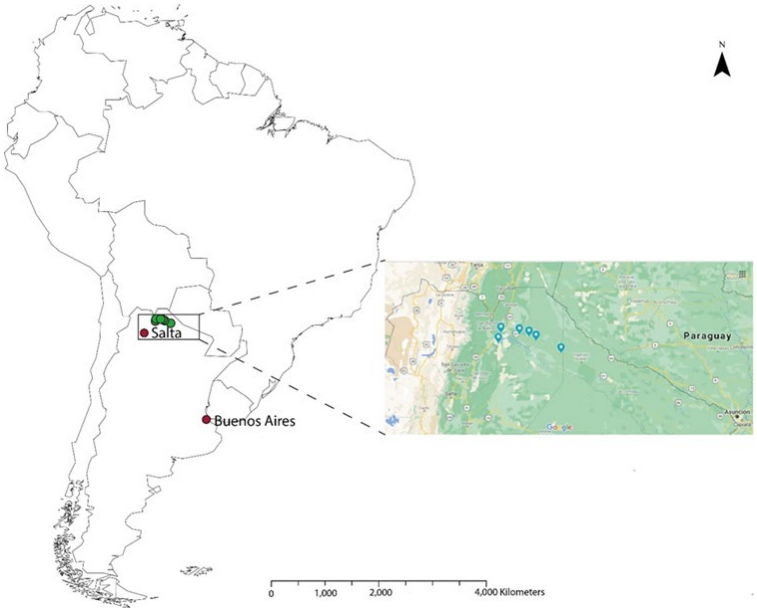


Figure 6: Map indicating sites sampled for *Neolasioptera aculeata* stem galls during 2019, 2021, 2022, 2023 and 2024. Red dots show the cities of Buenos Aires (where FuEDEI is located) and Salta (the major city closest to known *Neolasioptera aculeata* locations). Field sites in Salta Province are indicated by green dots on the inset google map (https://goo.gl/maps/EvHscUVS4uRhqE2m9).

Parkinsonia aculeata plants occurring mainly along the roadsides, rangelands and riparian regions were visually inspected. *Neolasioptera aculeata* galls were collected by cutting gall bearing *P. aculeata* stems/branches with secateurs. These stem fragments were kept in plastic containers and transported to the laboratory. This collection method evolved over the course of the project with the aim of increasing emergence rates of *Neolasioptera aculeata* adult flies from the collected galls. Field collected stems containing galls were placed directly into moist florist foam (Figure 7) to ensure that the collected galls stay green and do not dry up and harden. These florist foam bricks containing galls were placed in coolers for transport to the FuEDEI laboratory in Buenos Aires.

Figure 7: Field collected galls placed into moist florist foam for transport back to the laboratory cooler.

Back in the laboratory, field collected galls were put in plastic jars at room temperature for subsequent emergence of adult specimens (Figure 8). When *N. aculeata* adults began to emerge from the field collected galls an equivalent number of male and female adults (10-15 total) were confined within several rearing devices, different sized sleeves and cages as detailed in Table 2 and Figure 8. Throughout the project FuEDEI maintained healthy plant stock of *P. aculeata* by practicing good plant hygiene within plant growing areas to ensure material was free from mites and other pests. A seaweed solution was also applied to the plant every two weeks to ensure they were well fertilized and actively growing. It was these plants that were exposed to emerging *N. aculeata* adults to oviposit on an initiate gall development. Based on these experiments a rearing protocol was developed and provided to the Australian research team (Appendix 3).

Figure 8: *Neolasioptera aculeata* galls in jars to collect emerging adults for use in different rearing devices B) 500cm^3 polyester gauze bag, C) BugDorm-4S4590 Insect Rearing Cage, D) 1-litre polyester gauze bag.

Table 2: Detailed information on *Neolasioptera aculeata*: date of emergence of adult and assembly of rearing devices (February-March 2021).

Rearing attempt	Date	Number of adults (males/females)	Rearing method
1	3 February	4♂ / 6♀	500cm^3 opened-ended plastic bottle
2	6 February 8 February	1♂ / 8♀ 2♂ / 3♀	BugDorm-4S4590 Insect Rearing Cage
3	9 February	1♂ / 2♀	500cm^3 polyester gauze bag
4	11 February	2♂ / 2♀	500cm^3 polyester gauze bag
5	12 February	1♂ / 4♀	500cm^3 polyester gauze bag

Rearing attempt	Date	Number of adults (males/females)	Rearing method
	13 February	4♀	
6	15 February	2♂ / 8♀	500cm^3 polyester gauze bag
7	17 February	5♂ / 15♀	10-litre polyester gauze sleeve
8	18 February	2♂ / 5♀	500cm^3 polyester gauze bag
9	19 February	5♂ / 11♀	500cm^3 polyester gauze bag
10	22 February	60 adults	BugDorm-4F4590 Insect Rearing Cage
11	24 February	20 adults (at least 5♂)	5-litre polyester gauze sleeve
12	26 February	4♂ / 8♀	1-litre polyester gauze bag
13	1 March	3♂ / 4♀	500cm^3 polyester gauze bag

3.6 Quarantine research of stem-galling fly

3.6.1 *Neolasioptera aculeata* quarantine importations

Permits to import conditionally non-prohibited goods issued by the Department of Agriculture, Water and Environment for *Neolasioptera aculeata* (Parkinsonia gall fly) was issued on the 24th of June 2021 (Appendix 2.3). Testing permit also issued by the Department of Agriculture, Water and Environment for *Neolasioptera aculeata* (Parkinsonia gall fly) was issued on the 23rd of December 2021 (Appendix 2.4). Native range provincial collection and export permits issued by Salta Province, Argentina (Appendix 2.1 & 2.2).

Imports were undertaken by hand carriage from Argentina by either sub-contracted collaborators (FuEDEI) or project team members. Galls were unpacked in the unpacking room of the quarantine facility (Figure 9) and transferred into moist florist foam. Gall material was then transferred into the quarantine lab where it was kept in a sealed Perspex handling box which was placed within a fine mesh cage. The Department of Agriculture, Water and Environment permit conditions required double containment of the Argentinean *P. aculeata* plant material while waiting for adult flies to emerge from the galls (Figure 9). As adult flies emerged, they were transferred onto Australian *P. aculeata* plant material. Once the holding extensions as per the import permit conditions were exhausted, the Argentinean plant material was destroyed.

Figure 9: Galled *Parkinsonia aculeata* material from Argentina held under double containment in the quarantine lab.

3.6.2 *Neolasioptera aculeata* quarantine emergence and rearing

The double containment holding box of imported galled material was monitored twice daily for adult *N. aculeata* emergence. Any other insects that emerged from the imported material were also recorded, photographed and stored in ethanol and the Department was informed of their presence when applying for holding extensions as per the import permit conditions.

Once *N. aculeata* began emerging from imported galled material in the double containment holding box, they were carefully removed by capturing them in a specimen jar. They were then observed under a dissecting microscope to determine their sex. Newly emerged flies were then confined to pest free *P. aculetae* stems using several approaches including plastic holding containers (Figure 10A) or placing plants in small cages (Figure 10B) to encourage their interaction with the Australian *P. aculeata* plant material. After 7 days when the emerged *N. aculeata* had died the exposed *P. aculetae* plants were trimmed to remove excess foliage to minimise the likelihood of spider mite infestation, and fertilised once a week with Seasol while observed for gall development.

In January 2023 several female *N. aculeata* were removed from the holding containers a day after emergence and dissected to determine what eggs of this species look like. Plants exposed to *N. aculeate* were then inspected under a high magnification dissection microscope for evidence of oviposition and larval activity.

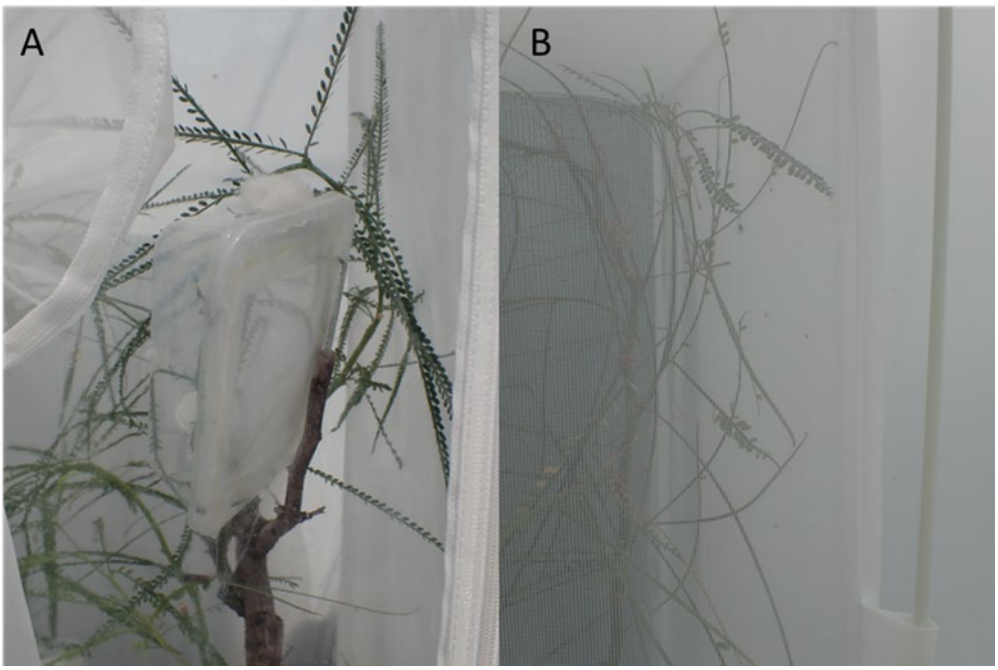


Figure 10: Confining newly emerged *Neolasioptera aculetae* to *Parkinsonia aculetae* by a small holding container secured to a single stem (A) or a small cage (B).

3.6.3 *Host test list refinement for Neolasioptera aculeata*

Since the host specificity testing was undertaken for *E. cisplatensis* and *E. vollonoides* the Fabaceae plant family has been re-circumscribed by the Legume Phylogeny Working Group. The Fabaceae family has traditionally been divided into three subfamilies: Caesalpinioideae, Mimosoideae, and Faboideae (syn. Papilionoideae). The recent revision now recognises six subfamilies: the Mimisoideae is now considered a distinct clade nested within Caesalpinioideae (and is currently referred to informally as the mimosoid clade), four new subfamilies were described (Cercidoideae, Detarioideae, Duparquetioideae, and Dialioideae), whereas the Faboideae subfamily remains relatively unchanged (Azani et al., 2017). Only five of the six subfamilies are present in Australia; no species in subfamily Duparquetioideae occur in Australia.

Consequently, the plant test list for inclusion in host-specificity testing experiments for *N. aculeata* was refined by prioritising Australian plant species for inclusion in line with the revisions to the Fabaceae family. The prioritised test species have been identified based on their phylogenetic relatedness to the target weed, according to the centrifugal phylogenetic method (Briese 2003; Gilbert et al. 2013; Wapshere, 1974). This method is underpinned by evidence that specialist herbivores are evolutionarily more likely to feed on non-target species closely related to the target weed, relative to species that are more distantly related.

The revised test list was sent to several Fabaceae plant taxonomic specialists for review to ensure that it is correct in terms of genera placement and plant species included. The revised test list was also posted on DAFF website and sent to biocontrol practitioners for public comment. This process is important to ensure that if an application for release is submitted the host specificity testing is comprehensive enough for an adequate risk assessment to be undertaken by the department.

4 Results

4.1 Mass-rearing and release of *Eueupithecia vollonoides*

Since 2019 releases of ~184,852 *E. vollonoides* were made at 65 sites across northern Australia (Figure 11A). This met the first and second objectives of this project to release up to 100,000 *E. vollonoides* at ~50 sites across bio-climatically optimal sites in Queensland, Northern Territory and Western Australia. Release intensity varied across sites, with between 300-7508 individual *E. vollonoides* released at each (mean = 2286) (Figure 11B). To achieve the releases of *E. vollonoides* over such an extensive area required Local Government Area (LGA) and producer involvement throughout the project (n = 71) (Table 3).

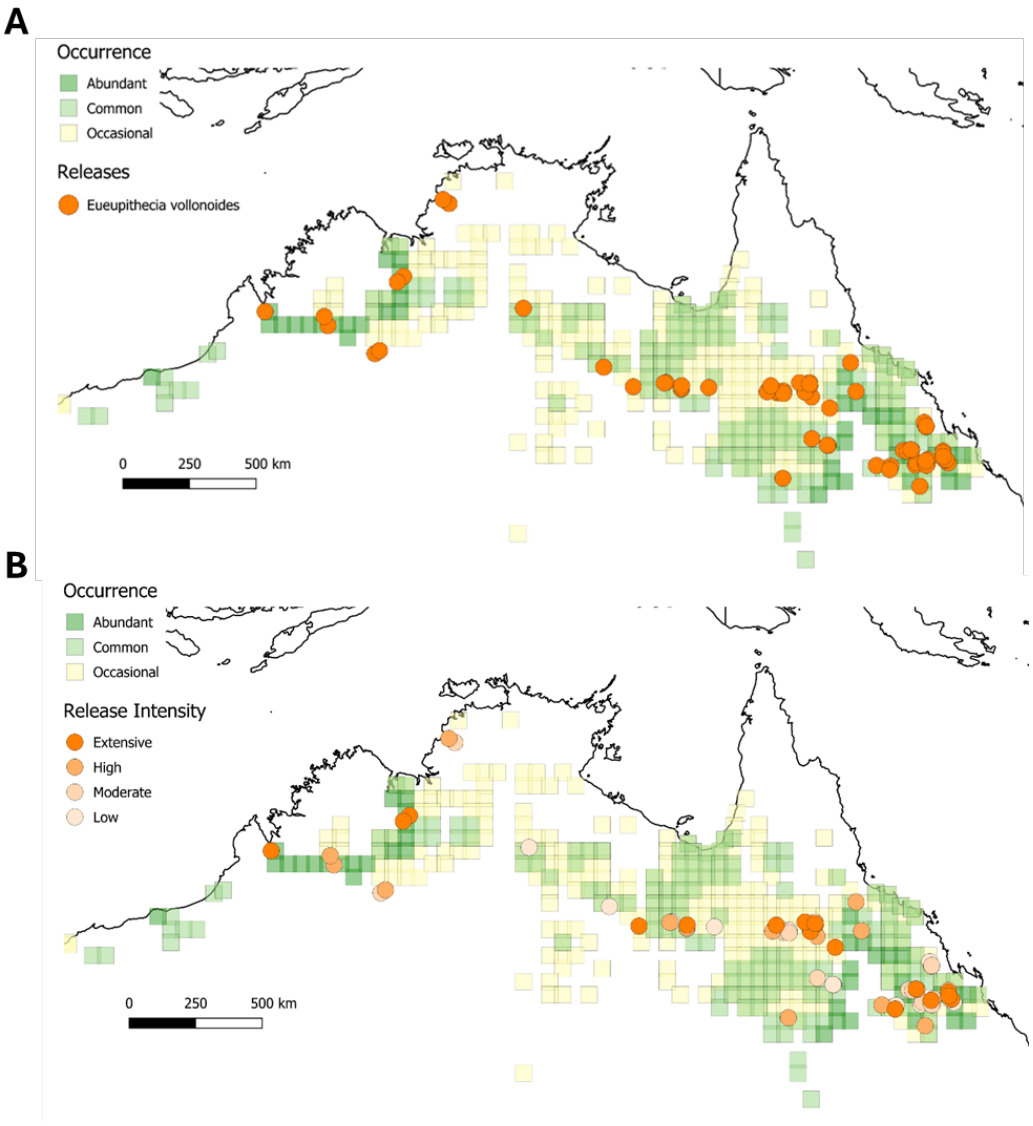


Figure 11: A) The release locations of *Eueupithecia vollonoides* (orange) between 2019-2023 plotted over a map of the occurrence incidence of *Parkinsonia aculeata* across northern Australia (green gradient). B) The same release locations of *Eueupithecia vollonoides* plotted with relative release intensity (orange gradient). Site coloration increases in saturation (light orange to dark orange) with release intensity relative to interquartile range from Low (300-1238) to Moderate (1239-2286), High (2287-3712) and Extensive (3713-7508).

Table 3: Local Government Areas, Natural Resource Managers and property owner locations engaged in the mass release of *Eueupithecia vollonoides* across Queensland (QLD), Northern Territory (NT) and Western Australia (WA). Property owner details not included for privacy reasons.

State	Location(s)	Organisation / Properties involved
NT	Darwin, Tennant Creet, Alice Springs, Adelaide River NT, Avon Downs, Austral Downs, Brunette Downs, Anthony Lagoon, Eva Downs, Newcastle Waters, Powell Creek Tandyidgee, Alroy and Dalmore Downs, Daly River	Department of Natural Resources, Environment, the Arts and Sport, Department of Environment, Parks and Water Security, Australian Agricultural Company Limited, Baldy Bay Ptd Ltd, Consolidated Pastoral Company (CPC), Malak Malak Lands Trust, NT Government
QLD	Barkly Downs, Boulia Burketown, Charters Towers, Cloncurry Four Ways, Normanton, Hughenden, McKinlay, Mount Isa, Prairie Rockhampton, Townsville, Winton, Julia Creek, Emerald, Springsure, Capella, Clermont, Richmond, Comet, Burleigh, Bluff, Goowarra, Mount Gardiner, Duaringa, Rolleston, Blackall, Dutton River, Muttaburra, Blackwater, Balcomba, Torrens Creek, Ayr	Barkly Downs Station, Boulia Shire Council, Burke Shire Council, Carpentaria Land Council Aboriginal Corporation QDAF, Tropical Weeds Research Centre, Lorraine Station, Carpentaria Shire, Wild River Ranger Team, Southern Gulf NRM, Mount Isa City Council, Mount Isa Waterboard Authority, Southern Gulf NRM, Flinders Shire Council, Fitzroy River & Coastal Catchments, Winton Shire Council, Fitzroy Basin Assoc, Headingly Station, Charters Towers Regional Council, Townsville City Council, Burdekin Shire Council, Isaac Regional Council
WA	Broome, Derby, Kimberley, West-Fitzroy Crossing, Karratha, Kununurra Kununurra, Kimberley, East-Ord River	PMMC (Pilbara Mesquite Management Committee) WA Dept of Agriculture & Food, NRM WA, Kimberley Nature Project, Kimberley Rangelands Biosecurity Association

4.2 Development of molecular diagnostic tools

4.2.1 CO1, 28S and CAD barcoding

Eueupithecia cisplatensis and *E. vollonoides* collectively had 5, 13 and 18 haplotypes respectively at a 658 base pair (bp) fragment the COI mtDNA gene, a 702-705bp fragment of the 28S rDNA gene and a 685bp fragment of the CAD nuDNA gene, (Figure 12). Haplotypes were obtained from 100, 95 and 90

individuals for COI, 28S and CAD respectively (Appendix 1, Table 9). Phased haplotypes for the 28S gene were identical whether the hybrid or recombination model was used. Phased haplotypes for the CAD gene differed for two individuals, ECAJu13n6 and EVAJ13n6 (Appendix 1, Table 9). The hybrid PHASE model produced the most appropriate result for the CAD gene, as it more appropriately resolved the position of haplotype CAD-hap14 as an intermediate haplotype of the two main haplotype groups.

The COI haplotype network shows two divergent haplotype groups, and each haplotype group is associated with one *Eueupithecia* species, in both the native range and laboratory culture samples (Figure 12a). The field collected *E. vollonoides* individuals from the Australian range shared COI haplotypes with the *E. vollonoides* haplotype group, those from the *E. vollonoides* laboratory culture and *E. vollonoides* from the native range (Figure 12a). The minimum pairwise identity (pairwise ID) is 4.0% between *E. cisplatensis* and *E. vollonoides* using all available COI sequences, those from this study and those of Hausmann et al. (2016). The haplotype associations of individuals are indicated in Appendix 1, Table 9.

The CAD gene region had two haplotype groups (Figure 12b) and each was mostly associated with a single species, but also the alternate species at a low frequency. A few individuals from the *E. cisplatensis* laboratory culture shared a CAD haplotype with *E. vollonoides* of laboratory origin, and the reverse was true, with a few individuals from the *E. vollonoides* culture sharing a CAD haplotype with *E. cisplatensis* of native and laboratory origin (Figure 11b). Of the haplotypes assigned to a species atypical for the haplotype group, 60% (3/5) were unique to that species (CAD-Hap13, CAD-Hap16 and CAD-Hap17 in Figure S1). Two haplotypes were intermediate between the two groups. All Australian field collected individuals shared CAD haplotypes with *E. vollonoides* (Figure 12b).

The 28S gene had three high frequency haplotypes that were differentially associated with the two *Eueupithecia* species, but the haplotypes were poorly differentiated overall (Figure 12c). Two of the three 28S haplotypes found at a high frequency were shared by the laboratory *E. cisplatensis*, native *E. cisplatensis* and the laboratory *E. vollonoides* (Figure 12c). The third 28S haplotype found at a high frequency was shared by both *E. cisplatensis* and *E. vollonoides* of laboratory and native origin, and all individuals which were trapped in the introduced range (Figure 12c). Overall, the 28S haplotype network was similar to the CAD haplotype network, but with no identifiable haplotype groups.

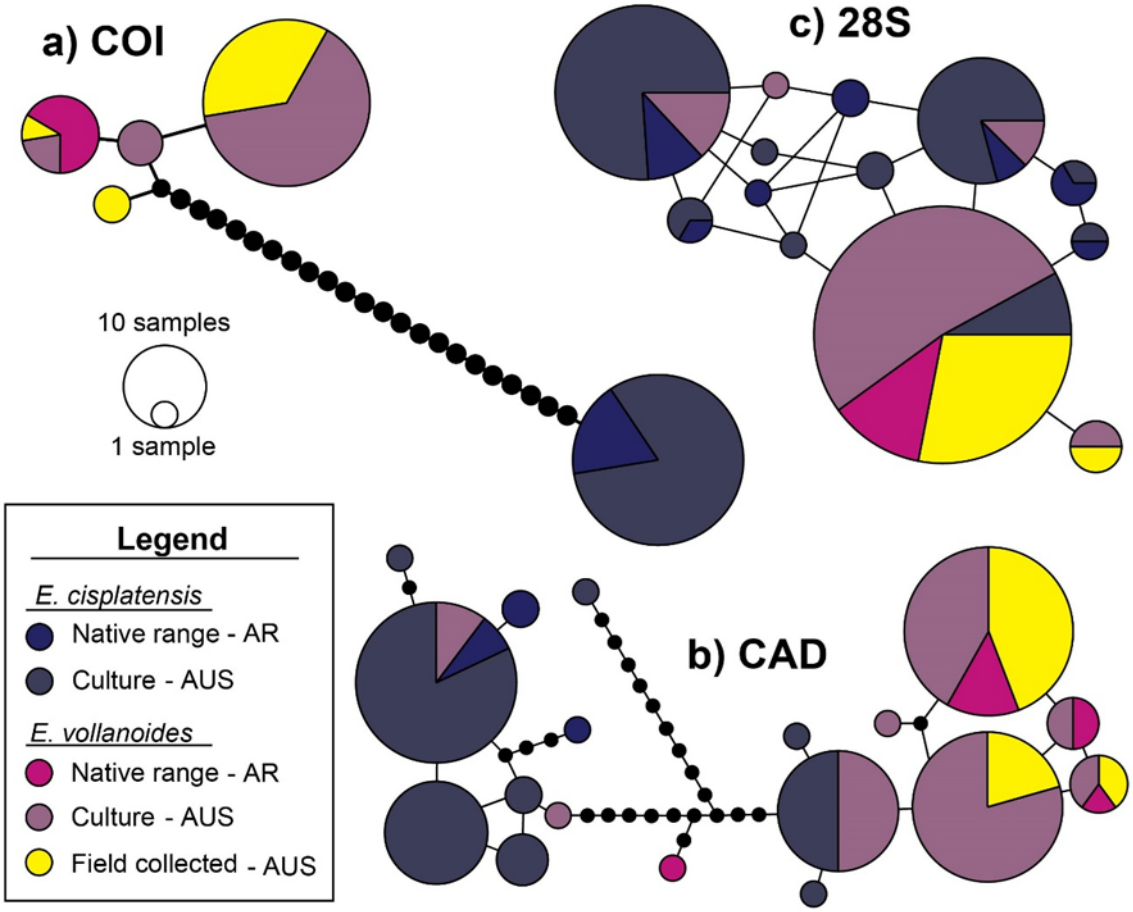


Figure 12: Haplotype networks of the (a) COI gene region, (b) CAD gene region, and (c) 28S gene regions for *Eueupithecia cisplatensis* and *Eueupithecia vollonoides* individuals. Each individual is coloured according to their origin (cultured in lab, collected in native range and field collected in the introduced range). The size of each circle represents the number of sequences that were found with each haplotype. In the legend, AR indicates Argentina, and AUS indicates Australia.

4.2.2 Population genetics – Native range

Population genetic statistical methods (Evanno's Delta K method (Evanno et al. 2005)) indicate that K = 2 best represented the genetic structure of the native range samples and, indeed, separated them into the same *E. cisplatensis* and *E. vollonoides* groupings as COI analyses. The known experimental hybrid samples are clearly recognisable as admixed clusters, though not with a 50:50 assignment ratio to the two parental species clusters. However, *E. cisplatensis* samples from sites Arg-B and Arg-C, which occur within the zone of sympatry, do show a similar but not identical pattern of population assignment than that observed in the hybrids (Figure 13). The sympatric zone is defined by a 100km radius around the 29° south latitude, as this is the area highlighted by Rafter et al., (2022a) as the potential area of sympatry, and surveys of the spread of *Eueupithecia* (in Australia), have indicated that these insects can establish a minimum of 100km away from release sites (see Section 4.4.1). The next best Delta K indicated by this method was K = 5 (Figure 13, bottom), and the Pritchard's best probability by K method (see supplementary Figure S5.1 (bottom)) indicated K = 3 which provided additional insight into the genetic population structure of Eueupithecia (Figure 13, middle).

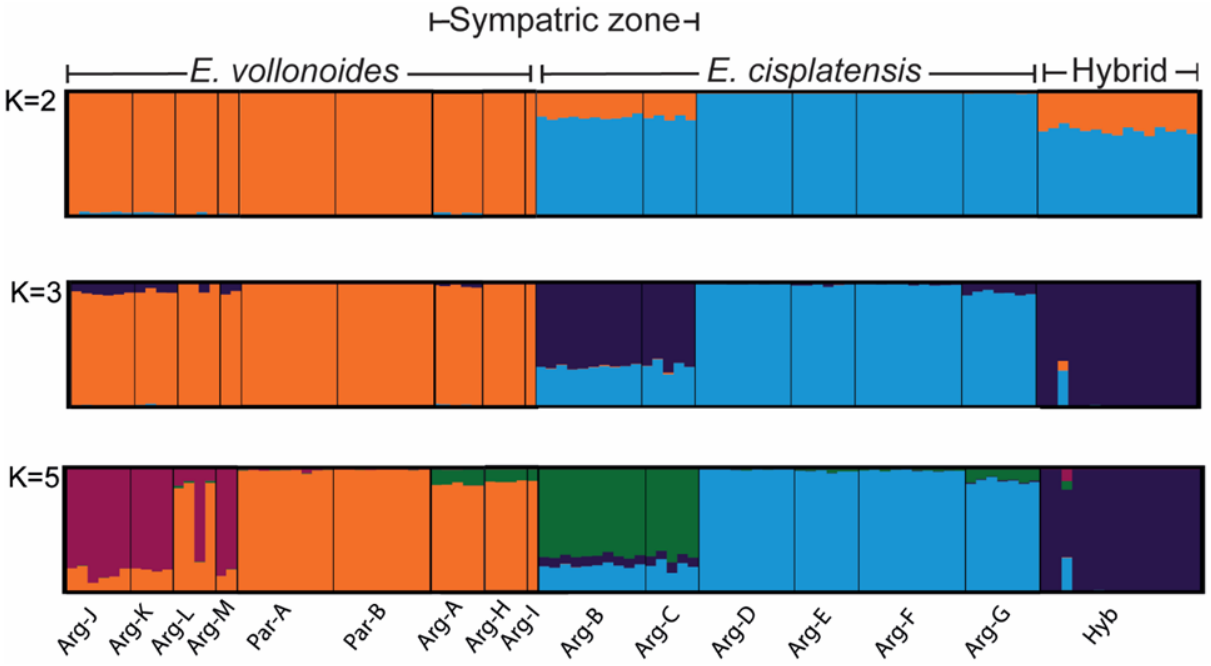


Figure 13: STRUCTURE plots generated using CLUMPAK indicating the assignment of *Eueupithecia* native range and hybrid individuals (from laboratory cross-mating experiments) to K populations. Sites Arg-A, Arg-H, Arg-I, Arg-B and Arg-C fall within the "sympatric zone". Notably, the most informative K values as determined by the Evanno method are K = 2 and K = 5, and the most informative as determined by the Pritchard method is K = 3. The "Hyb" population consists of those individuals produced from laboratory cross experiments where *E. cisplatensis* males mated with *E. vollonoides* females.

Principal component analysis separated the native range populations of *E. vollonoides* and *E. cisplatensis* moths into four main clusters, two within *E. vollonoides* and two within *E. cisplatensis* (Figure 14). The Australian laboratory hybrids formed their own relatively dense cluster (except for the individual "hybM1", and clustered most closely to the northern populations of *E. cisplatensis* (Arg-B and Arg-C) (Figure 14). The specimens associated with the major native range clusters reflect and support the known historical distribution of each species, as was determined by earlier morphological analyses and COI analyses (Hausmann et al., 2016; Rafter et al., 2022b & Section 4.2.1 above). The clusters within the *E. cisplatensis* samples formed two further clusters that were more disparate than those clusters observed within the *E. vollonoides* samples. That is, samples from the northern distribution of *E. cisplatensis* (sites Arg-B and Arg-C) clustered separately from the southern samples (Arg-D, Arg-E, Arg-F, Arg-G) (Figure 14). *Eueupithecia* sample sites are mapped relative to the distribution of its host plant *P. aculeata,* which is based on occurrence records from human observations and preserved specimens and is represented as grey circles in Figure 5.2. (GBIF.org, 2024).

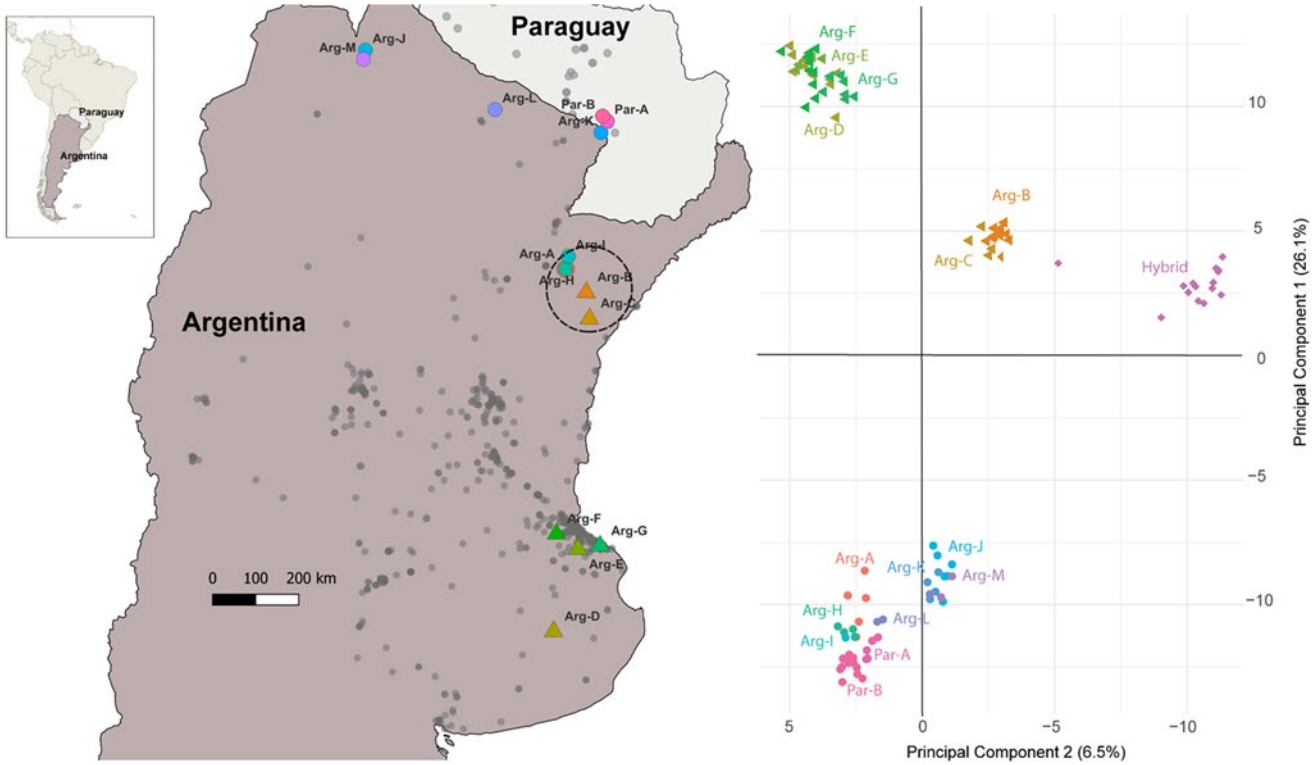


Figure 14: (LEFT) A map of sample sites of *Eueupithecia cisplatensis* (triangles) and *E. vollonoides* (circles) throughout the native range of Argentina (Arg-A to Arg-M) and Paraguay (Par-A and Par-B), with a map of South America inset. The sympatric zone of their distribution is indicated by the dashed circle encompassing those sites within a 100km radius of the 29° south latitude. Small grey circles represent the occurrence records of *Parkinsonia aculeata* from human observations and preserved specimens, as pooled by GBIF.org (2024). (RIGHT) A principal component analysis (PCA) depicting the genetic relationship of individuals across all the native range sites, including laboratory samples of offspring produced from *E. cisplatensis* males crossed with *E. vollonoides* females ("Hybrid", diamonds). Sites are coloured according to the proportional assignment of individuals sampled from them to the PCA clusters.

4.2.3 Population genetics – Introduced range

Eueupithecia cisplatensis (blue) samples cluster together and depict a clearly differentiated genetic group from the two *E. vollonoides* (orange) clusters (Figure 15). The two distinct clusters of *E. vollonoides*, are likely the result of temporal genetic structure present in the native range material used to mass rear and release this species, first in 2014 and then later from 2019 onwards (Figure 15).

Structure analysis of field samples collected in Australia, indicate, that much like the native range, *E. cisplatensis* and *E. vollonoides* exist as highly discrete gene pools and are likely to maintain their distinct species boundaries in Australia.

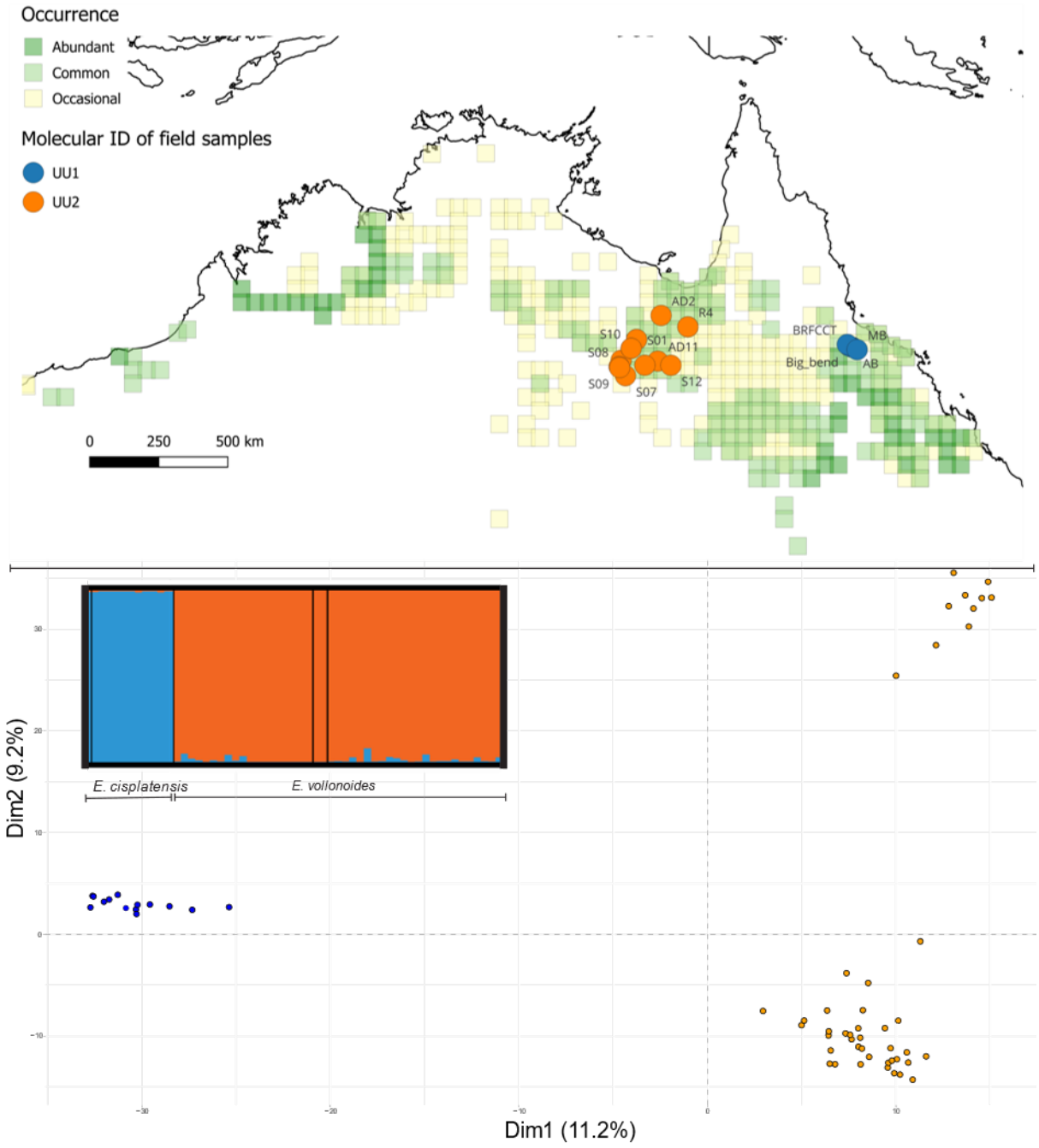


Figure 15: (TOP) A map illustrating the occurrence of *Parkinsonia aculeata* (green gradient) in northern Australia with *Eueupithecia cisplatensis* (UU1, blue) and *E. vollonoides* (UU2, orange) sample sites overlaid, from which genotyping-by-sequencing (GBS) analysis was conducted to explore the population genetics of established *Eueupithecia* populations in Australia. (BOTTOM) A PCA analysis of the GBS data collected from the field sites listed in the map. (INSET) a STRUCTURE plot of the population genetics of *Eueupithecia* in Australia.

4.3 Development of pheromone tools

4.3.1 Female pheromone glands and "calling" behaviour

Dissection revealed the putative pheromone glands are a lobed sac, 'milky' in appearance. They are off-white (almost yellow) in colour, becoming amber-brown towards the abdominal tip where the gland is extruded (Figure 16a-c). Externally, the visible portion of the glands extend from the papillae anales, (Figure 16d) and the internal portion is located behind the vaginal chamber, extending back into the abdomen, parallel to the accessory glands (Figure 16e). Generally, the putative pheromone gland terminates its length at a similar position to where the lateral accessory gland reservoirs (Buntin and Pedigo, 1983) transitions to the major length of the remaining accessory gland (Figure 16e-f). The pheromone glands are delicate, membranous and easily pierced. The entire glandular structure may pulsate rhythmically for several minutes following dissection. The gland measured on average 1707.5µm ± 97.6 µm (1SE, n=10) in length.

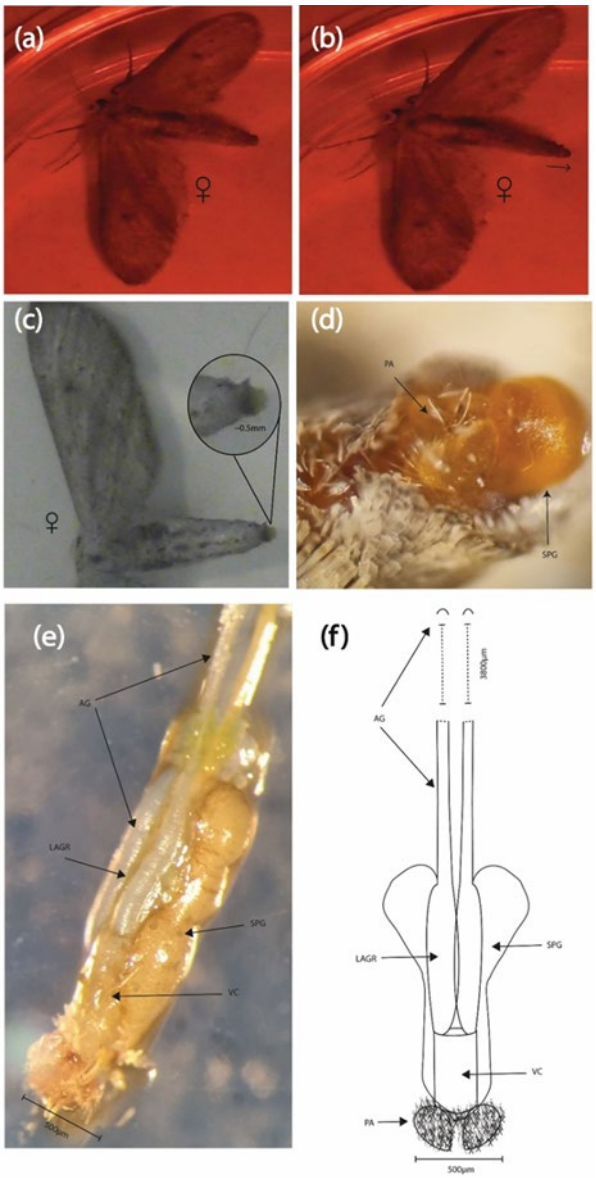


Figure 16: External and internal terminal pheromone gland anatomy of *Eueupithecia vollonoides* females. (a) Female before calling. (b) Pheromone gland and ovipositor complex is extruded about

500µm beyond the papillae anales during calling. (c) Calling female, with the pheromone gland and ovipositor complex shown in more detail (inset). (d) A close-up image of the extruded pheromone gland, photographed from the underside of a calling female, illustrating the saccular pheromone gland (SPG) parallel to the papillae anales (PA). (e) Ventro-medial view of the internal structure of the pheromone gland complex with the accessory glands (AG), lateral accessory gland reservoir (LAGR), saccular pheromone gland (SPG), vaginal chamber (VC) and papillae anales (PA) labelled. (f) Schematic of the dissected pheromone gland complex.

Analyses of the video recordings of 12 virgin females observed without males showed that 67% engaged in the periodic extrusion and retraction of their terminal abdominal segments and presumed pheromone gland complex beyond their papillae anales, an action often associated with calling (Figure 16a-b). The visible portion of this complex presents as a moderately translucent yellow to amber structure between 100 and 500µm long, depending on the extent of extrusion (Figure 16b-c). The small size of this gland complex means calling is not readily visible to the naked eye, especially under long wavelength red light, which is near the end of the light spectrum visible to humans. During calling, the extruded abdominal complex pulsates consistently with varying degrees of contraction, and the female slowly tilts her abdominal tip upwards, although this behaviour is slight, not always observable, and not readily differentiated from other non-calling postures.

The completion of a calling bout was identifiable when the female (n=8) began to rock back and forth, with shallow beating of her wings and her front legs extended. Following these behaviours, the female retracted her abdominal complex to be flush with the papillae anales. Often this complex was retracted into the abdominal tip after which the female became still.

Calling began between 0135h and 0407h with all calling females calling into the photophase. However, calling in one of the females continued as late as 1125h in the morning. Call duration varied from 2 to 430 minutes ($\bar{x}$=113 minutes, n=8) with the period of latency between calls ranging from 0.5-58.8 minutes ($\bar{x}$= 8.2 minutes, n=4). The behaviour of the females that did call was variable, with individuals engaging in 1-7 calls each ($\bar{x}$= 2.8, n=8). Most engaged in both short and long calls. Females that called only once (“single callers”) (n=4), called continuously for significantly longer ($\bar{x}$=291.5 mins) than those that called multiple times (“multiple callers”) (n=4) ($\bar{x}$=70.1 mins per call, $\bar{x}$=4.5 calls per multiple caller) (pairwise t test; p= 0.007717) (Figure 17 (left)). However, the overall time spent calling was not significantly different across the single and multiple calling females once the intervals between calls were removed from the sequences of the multiple callers (Figure 17 (right)).

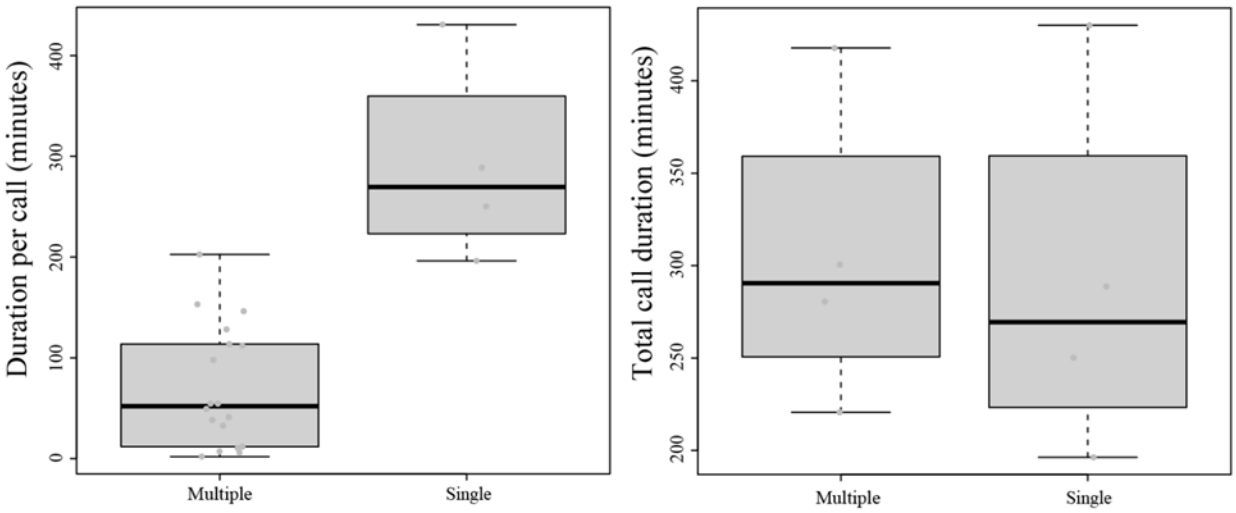


Figure 17: Mean duration of individual calls made by females that engaged in multiple calls ($\overline{x}$= 70.1 minutes ± 13.8, n= 4) or single ones ($\overline{x}$= 291.5 minutes ± 43.4, n= 4) (±1SE). (right) Total time spent calling across females that engaged in multiple calls ($\overline{x}$= 315.5 ± 33.9, n= 4) or single ones ($\overline{x}$= 291.5 minutes ± 43.3, n= 4) (with pauses removed from multiple callers).

4.3.2 Excised dorsal gland field trapping tests

Live virgin females of both species attracted male *E. vollonoides* moths at Burketown, although significantly more were caught by conspecific females (Figure 18). In comparison, none of the dorsal gland extracts succeeded in trapping any *Eueupithecia* (Figure 18).

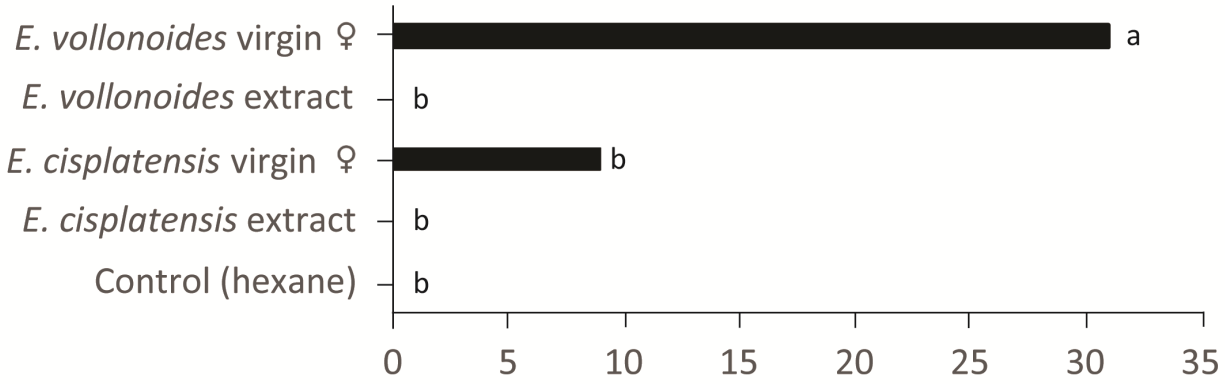


Figure 18: The *Eueupithecia vollonoides* males caught in pheromone traps during field assays conducted in 2018. Only those traps baited with live virgin *E. cisplatensis* and *E. vollonoides* females (n=12 of each species on each of the two trapping occasions) attracted *E. vollonoides* males, with significantly more being attracted by *E. vollonoides* females (ANOVA and post hoc Tukey's tests p = < 0.05, n= 24 per treatment).

No *E. cisplatensis* males were caught in traps with any of the dorsal gland synthetic lure preparations, nor by the control traps in subsequent field trapping tests in 2022 and 2023. Only those traps baited with live *E. cisplatensis* or *E. vollonoides* virgin females caught wild males (Kruskal-Wallis and Dunn's post hoc tests: χ^2= 54.88, df=7 p= <0.0001), (Figure 19). All treatments, except for those traps baited with *E. cisplatensis* females, caught non-target lepidopteran species. Although the

non-target moths were not identified, at least one hawk moth (Sphingidae) and several Arctiinae were captured.

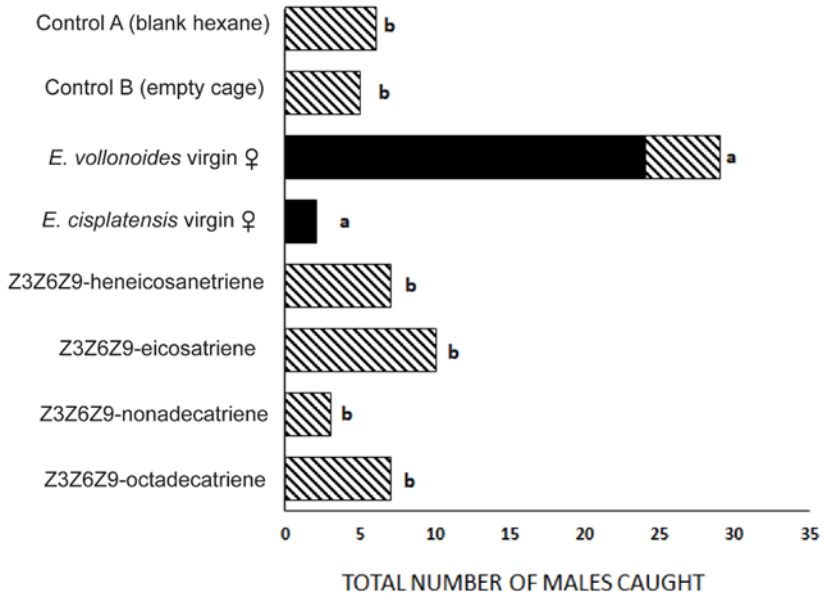


Figure 19: Number of *Eueupithecia cisplatensis* males (solid bar) and other lepidopterous males (hatched bar) caught during field trapping tests in 2022 and 2023. The traps were baited with either hexane-impregnated rubber septa (control A, n= 4), rubber septa impregnated with hexane and 100ng of one of four synthetic compounds (n= 4 for each), an empty cage (control B, n= 4), or cages baited with either *E. vollonoides* (n= 4) or *E. cisplatensis* (n=2) females. Superscript indicates the significant relationships across treatments for the number of *Eueupithecia cisplatensis* males caught. No significantly different trap catches were reported for other lepidoptera, across treatments.

4.3.3 Chemical analyses and synthetic lure preparation of the dorsal gland

GCMS analyses of pooled samples of the dorsal gland tentatively identified eight chemical compounds in crude extracts derived from *E. cisplatensis* (Figure 20, Table 4), three of which (Z3,Z6,Z9- heneicosanetriene, Z3,Z6-9,10-epo-eicosatriene and Z3,Z6-9,10-epo-octadecatriene) were also identified in *E. vollonoides*.

Synthetic formulations of four compounds identified that were in the extracts of *E. cisplatensis* (Z3,Z6,Z9-octadecatriene, Z3,Z6,Z9-nonadecatriene, Z3,Z6,Z9-eicosatriene and), were prepared in lures as per Section 6.2.2.2. Z3,Z6,Z9-heneicosanetriene was also identified in *E. vollonoides*.

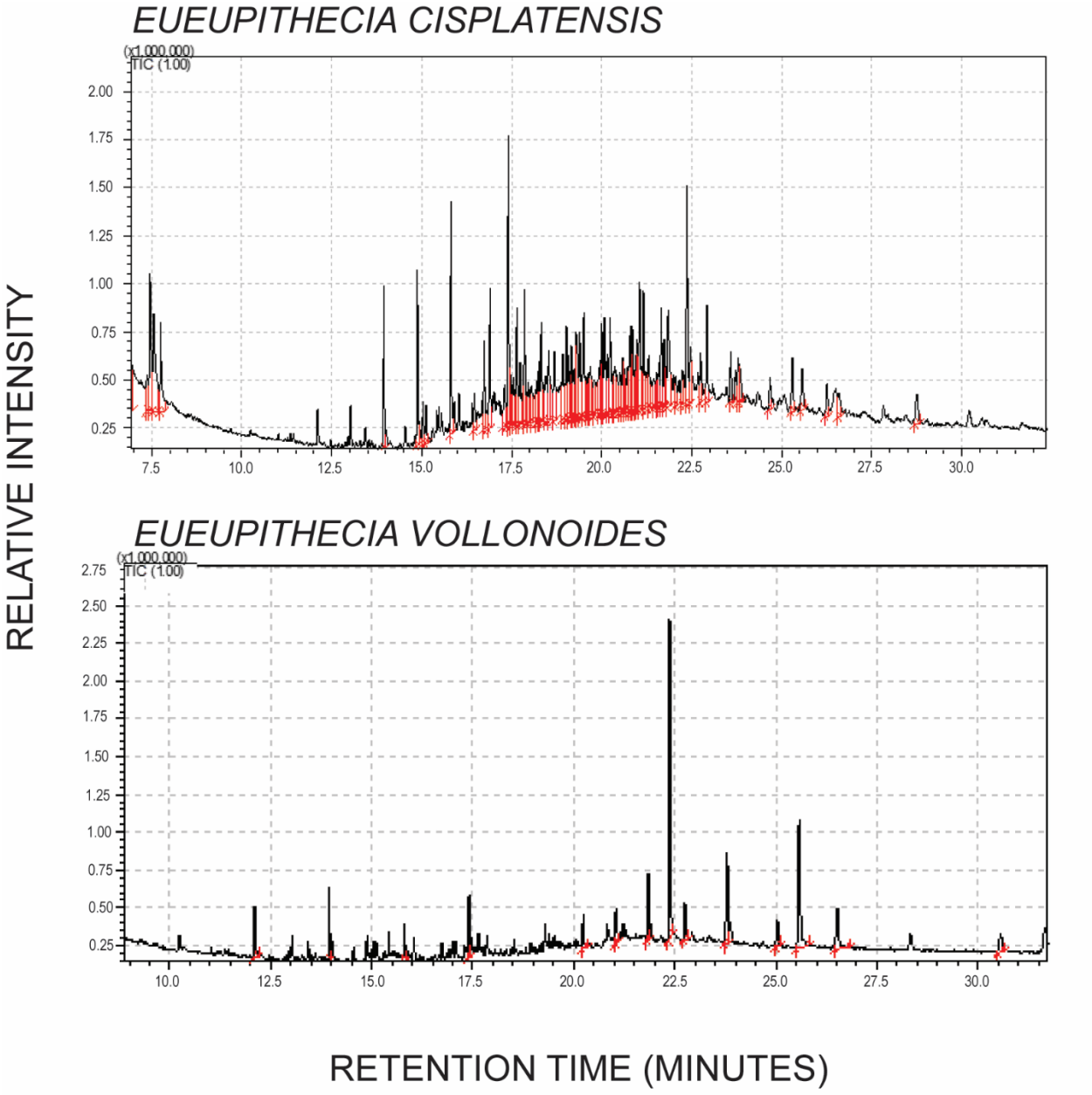


Figure 20: Chemical trace outputs from GCMS analyses of concentrated female pheromone gland extractions, in hexane, taken from *Eueupithecia cisplatensis* (top) and *Eueupithecia vollonoides* (bottom) (n = 5 glands per species). Relative intensity (m/z) refers to the amount of ion produced in relation to that of the most abundant ion (the base peak). Retention time (minutes) is a measure of the time taken for the solute to pass through the chromatography column and detected by the MS. This time differs by the chemical constitution of each compound within a sample. Together, relative intensity and retention time can help to infer the identification of the chemical compounds within a sample and their relative proportional abundance.

Table 4: Tentative identifications of the chemical components present in female dorsal pheromone glands of *Eueupithecia cisplatensis* and *Eueupithecia vollonoides*, by means of GCMS analyses. NA= the compound was not identified for that species.

RETENTION TIME	**TENTATIVE IDENTIFICATION**	
	Eueupithecia cisplatensis	***Eueupithecia vollonoides***
18.690	Z3Z6Z9- octadecatriene / Z3Z6Z9- eicosatriene	NA
20.383	Z3Z6Z9- nonadecatriene / Z3Z6Z9- eicosatriene	NA
21.844	Z3Z6Z9- nonadecatriene	NA
22.011	Z3Z6Z9- eicosatriene	NA
22.368	Z3Z6-9,10-epo- eicosatriene	Z3Z6-9,10- epo- eicosatriene
23.794	Z3Z6Z9- heneicosanetriene	Z3Z6Z9- heneicosanetriene
24.021	Z3Z6-9,10-epo- heneicosanetriene	NA
25.579	Z3Z6-9,10-ep- octadecatriene	Z3Z6-9,10-epo- octadecatriene

4.3.4 Wind tunnel tests of dorsal gland extracts

Neither *E. cisplatensis* nor *E. vollonoides* males undertook what could be observed as odour-mediated flight to any of the stimulus odours in the wind tunnel. Most (65%, n=76) remained still within their release container for the duration of their assay, but some took flight once their container lid had been removed (presumably disturbed by this action), and typically came immediately to rest on the observation platform or on some nearby surface of the wind tunnel for the remainder of the assay (34.2%).

4.3.5 Glasshouse tests of dorsal gland extracts

Only a single male *Eueupithecia* moth was caught during the initial glasshouse trapping trial between August and September 2022. It was caught in a trap baited with a live virgin *E. vollonoides* female. None of the single compound baited traps, nor the controls, caught any of the males released. From September 2022 to February 2023, when only *E. vollonoides* female baited traps and control traps were deployed, only a single male moth was caught, in the treatment trap. The trap catch data are graphed relative to the numbers of males released and females used as bait in Figure 21.

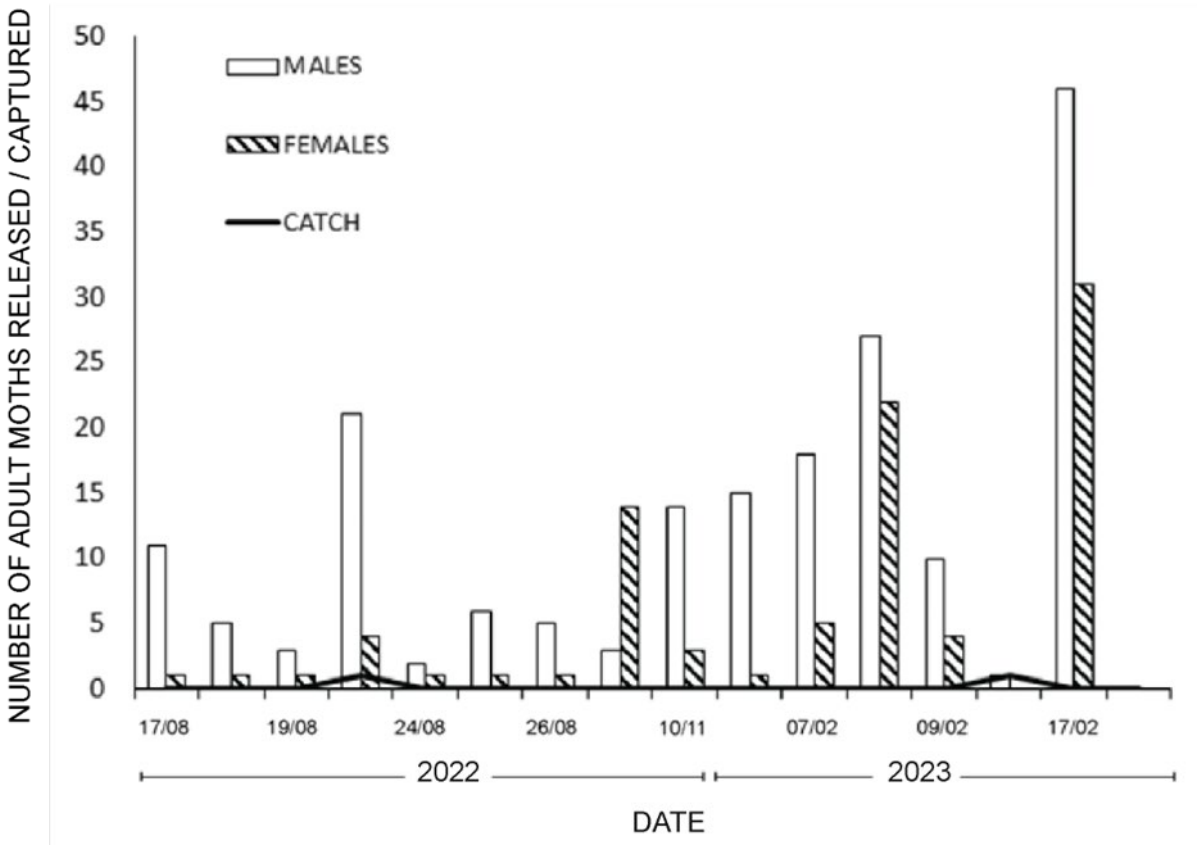


Figure 21: The number of E*ueupithecia vollonoides* males (solid bars) released into the glasshouse trapping tests and the number of females used as trap bait (hatched bars), from August 2022 to February 2023, with trap catches indicated by the black line. Total of 90 females were used as bait, and a total of 187 males were released into the glasshouse during this time.

4.3.6 Chemical analyses and lure preparation of the terminal abdominal gland

GCMS analysis of pooled crude extracts of these structures revealed a common compound present in the chemical profiles across *E. cisplatensis* and *E. vollonoides*, namely (11Z,13Z)-hexadeca-11,13-dienal. This identification was confirmed by isolating the relevant peak in the GCMS trace and comparing it to the profile of a synthetic library sample of this compound (Figure 22).

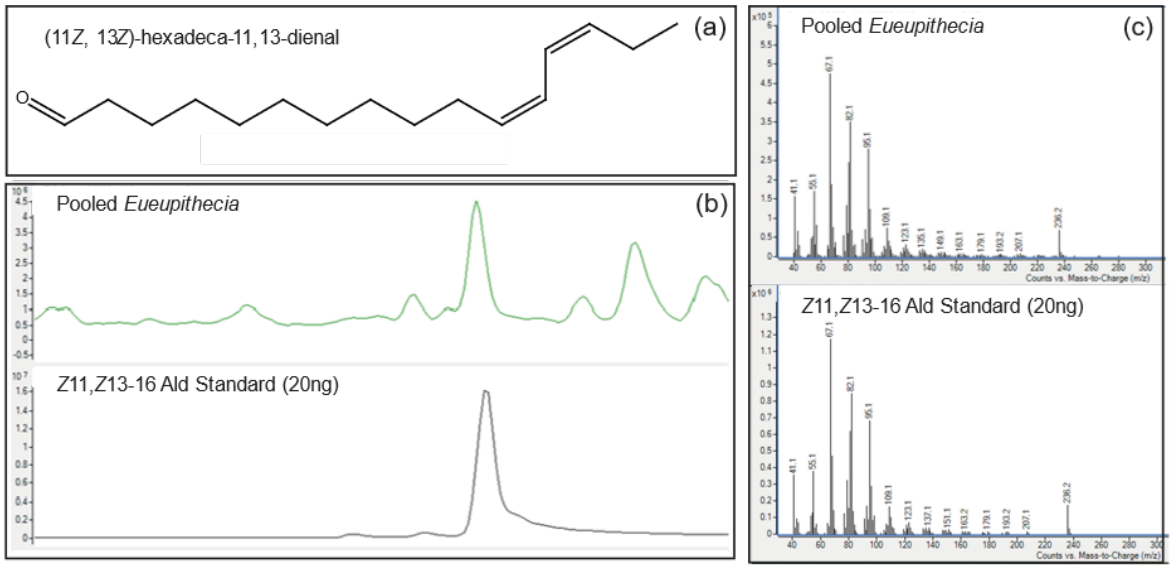


Figure 22: (a) Molecular structure of (11*Z*,13*Z*)-hexadeca-11,13-dienal. (b) Mass spectrum peak of interest in the trace from a pooled *Eueupithecia vollonoides* sample (top) compared to a 1µL injection of 20ng (11*Z*,13*Z*)-hexadeca-11,13-dienal (bottom). (c) A extracted ion chromatogram (EIC) for the peak of interest (top) compared to the EIC of synthetic (11*Z*,13*Z*)-hexadeca-11,13-dienal (bottom).

4.3.7 Field tests of terminal abdominal gland extracts

Only traps baited with live virgin female moths, in this case *E. vollonoides,* caught wild males, all of them *E. cisplatensis* (Figure 23). Trap catches range from one to five males per week of the trial. These traps caught no other species of Lepidoptera, whereas all other trap treatments in these field tests caught non-target moth species, although not in significant numbers across treatments (Kruskal-Wallis tests: χ^2= 2.36, df=4 p= 0.67) (Figure 23).

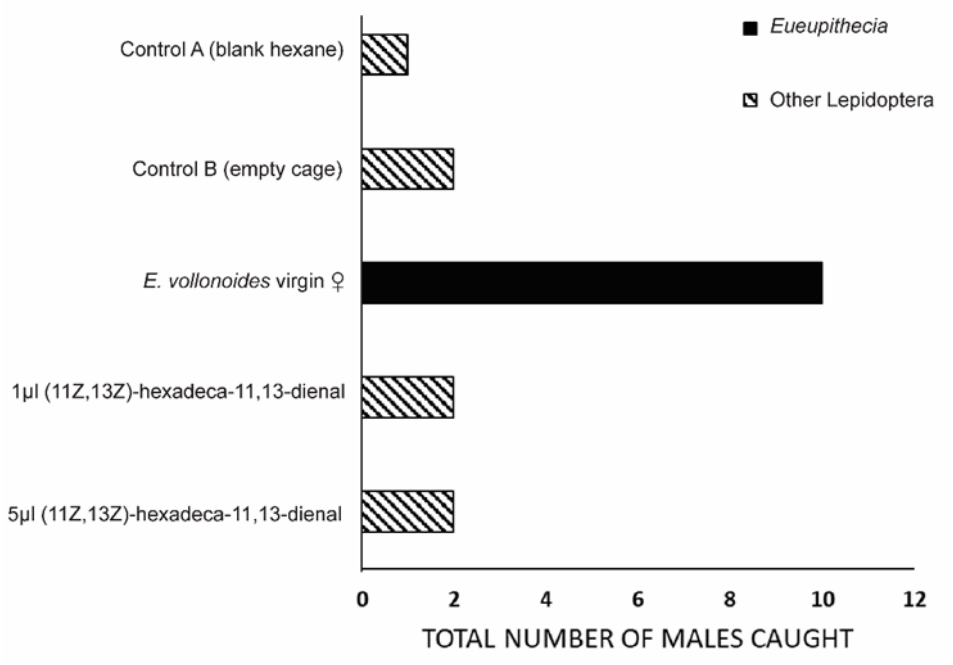


Figure 23: The number of *Eueupithecia cisplatensis* males (solid bars) and other lepidoptera (hatched bars) caught during field trapping assays in 2024. Delta traps were baited with either hexane-impregnated rubber septa (control A), rubber septa impregnated with 1µl or 5µl of (11Z,13Z)-hexadeca-11,13-dienal in 100ul of hexane, nothing at all (control B) or with *E. vollonoides* females (n= 3 per treatment).

4.3.8 Headspace extraction lures

Only the traps baited with live virgin *E. vollonoides* females caught *E. cisplatensis* males over the course of the four weeks in the field, although in low numbers (n= 4). Nothing was caught in the control trap, whereas both treatments (*E. vollonoides* headspace lure and live virgin females) caught other miscellaneous Lepidoptera (Figure 24). Neither outcome was significant (Kruskal-Wallis tests: $\chi 2 = 5.98$, df=4 p= 0.20).

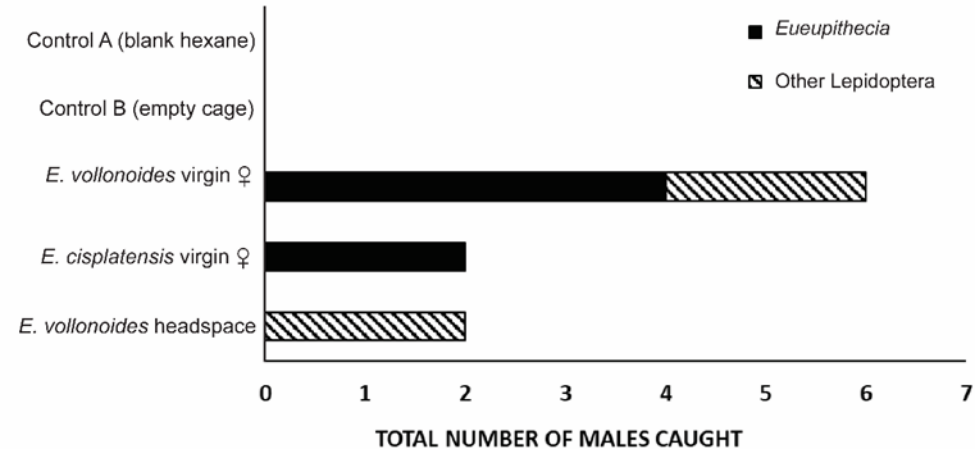


Figure 24: The number of *Eueupithecia cisplatensis* males (solid bars) and other lepidopterous males (hatched bars) caught during field trapping assays in 2022. Delta traps were baited with either hexane-impregnated rubber septa (control A, n= 6), rubber septa impregnated with the hexane eluted headspace extract of 28 female *Eueupithecia vollonoides* (n= 3), an empty cage (control B, n= 6) or cages baited with live *Eueupithecia vollonoides* (n= 21) or *Eueupithecia cisplatensis* (n= 12) females.

4.4 Assessment of establishment, spread and impact of *Eueupithecia*

4.4.1 Field results

Of these release sites established in this project, 14 (21.5%) were surveyed between 2020-2024 to determine establishment and this was confirmed at 10 (~72%) sites (Figure 25). *Eueupithecia vollonoides* populations established at release sites which had been subjected to higher intensity initial releases, with an average of 3,466 *E. vollonoides* released per site. By contrast, surveyed release sites that had no evidence of moth establishment were typically sites that received releases of lower intensity (mean = 1,973), although there was no significant relationship identified between the intensity of initial release and the likelihood of establishment across the 14 surveyed sites (ANOVA, p =0.3276).

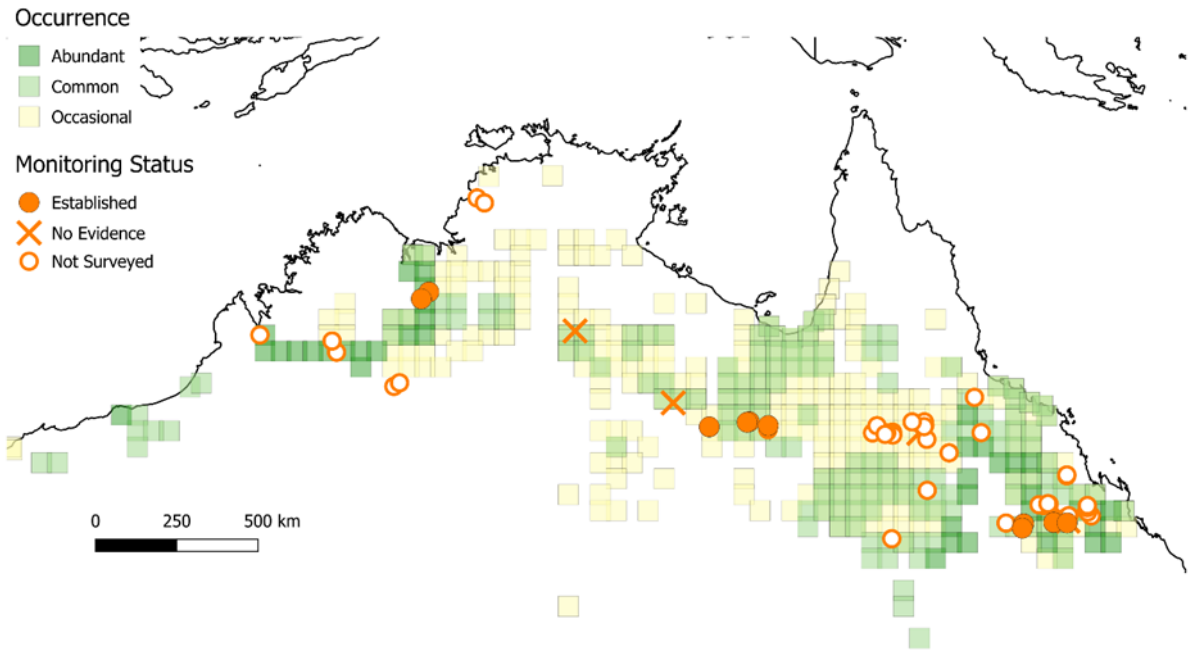


Figure 25: Monitoring survey details of all *Eueupithecia vollonoides* release sites from 2019-2023. Solid orange circles represent surveyed release sites where populations have established, orange crosses indicate release sites that were surveyed but no evidence of *E. vollonoides* was found, and hollow orange circles indicate release sites that are yet to be surveyed.

Surveys were also conducted ad hoc at *Parkinsonia aculeata* infestations throughout the plant's distribution alongside monitoring surveys of existing release sites (Figure 26).

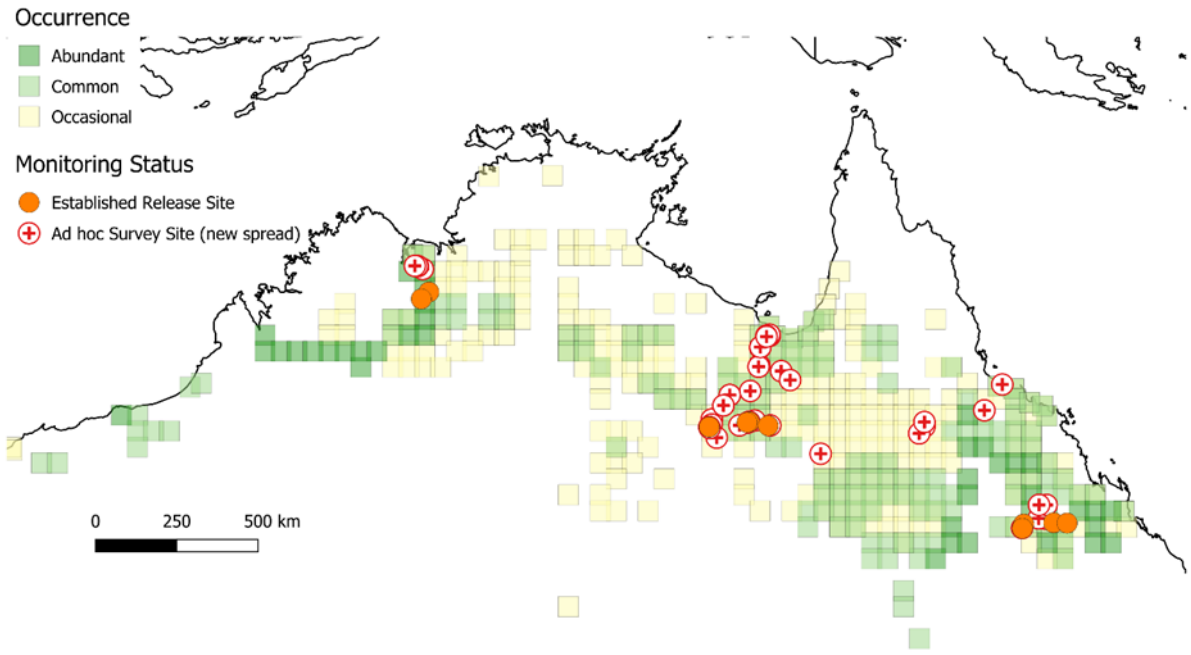


Figure 26: All sites surveyed between 2019 and 2024 where *Eueupithecia* spp. populations were established at either new spread sites (red crosses inside red circles) or at previous release sites (solid orange circles).

Established *Eueupithecia* spp. populations were identified at 44 sites where the insect had not previously been released. The likely release site which provided the source material for the

establishment of new spread sites was determined by identifying the closest release site (across all releases from 2013-2023) where establishment of the agent had been confirmed (Figure 27).

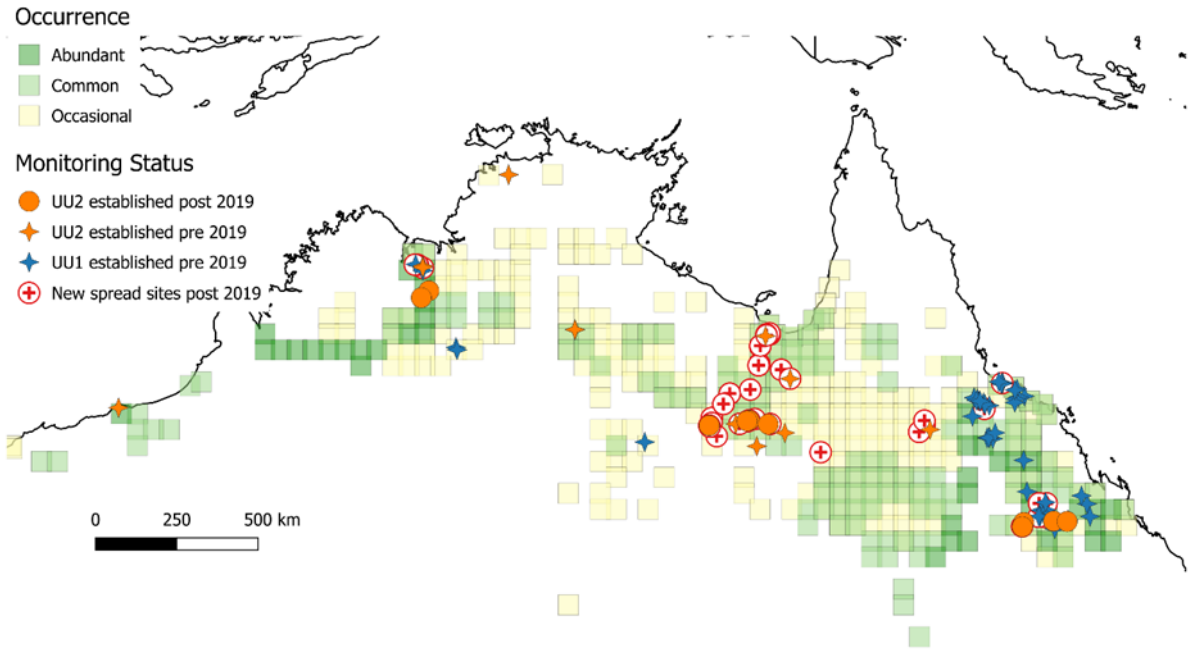


Figure 27: New spread sites (red cross in red circles) relative to the potential spread source of release populations of *E. vollonoides* (UU2) at sites that were established either before (orange star) or after (orange circle) 2019, or release populations of *Eueupithecia cisplatensis* (UU1) at sites established pre-2019 (blue stars).

New spread sites were established on average ~45km from the nearest surveyed release site where establishment had been confirmed (range 0.1- 136km). Because spread surveys were conducted ad hoc during the monitoring of release sites, the average spread distance of ~45km is likely to be a very conservative representation of spread potential, as spread surveys were conducted within the same immediate vicinity of release sites.

The dispersal ability of moths varies greatly across groups (particularly at the superfamily level) with some migratory species such as the Bogong moth (*Agrotis infusa*, Noctuidae) able to travels more than 1000km in a lifetime (Warrant et al., 2016). While there is not sufficient data to determine the dispersal ability of *Eueupithecia* moths, it is generally accepted that larger species with robust wings (like Noctuids) are more likely to be strong flyers and therefore capable of greater dispersal than smaller species with more delicate wings, like that of Geometridae, to which *Eueupithecia* belongs (Truxa and Fiedler, 2012). Surveys of *P. aculeata* infestations should be conducted at radial intervals of ~200km (for example) from existing releases sites to determine the full dispersal capacity of *E. cisplatensis* and *E. vollonoides.*

The spread potential of these insects in indicated in Figure 28. All sites where *Eueupithecia* has been recorded (both established release sites and new spread sites) are indicated by black points. Mean and maximum spread potential is illustrated by solid circles of 45km (mean spread observed) and dashed circles of 130km (maximum spread observed).

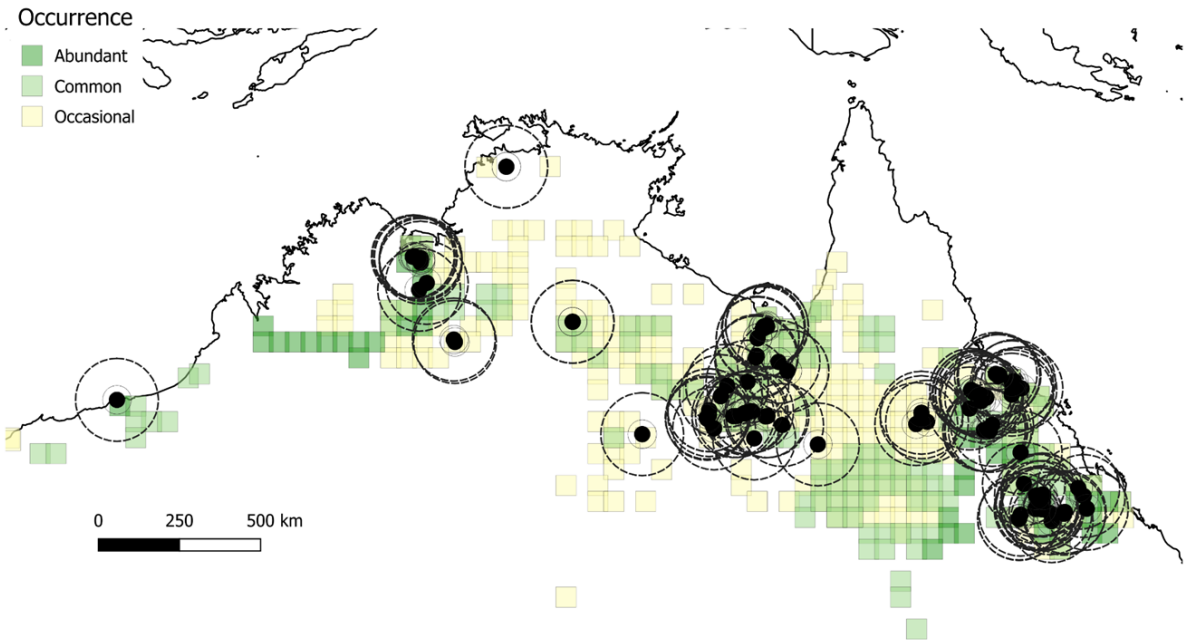


Figure 28: All established *Eueupithecia* species sites (release and new) from 2013 to 2024 (black dots) depicted with buffers to indicate the potential distance of insect spread from known sites. The buffers represent the mean (thin solid black circles) and maximum (dashed circle) observed dispersal of new *Eueupithecia* populations from release sites known to be established.

In total 127 sites were surveyed for both individual and population measures in response to the presence of the moth biocontrol agents (impact). These sites were for analysis divided into 'release sites', where known releases of either *E. cisplatensis* or *E. vollonoides* had occurred (n = 49), 'new monitoring sites' where either *E. cisplatensis* or *E. vollonoides* had spread to and established (n = 44) and 'no larvae sites' where no larvae were sampled from *P. aculeata* (mix of known release locations and new monitoring sites for spread) (n = 27).

The mean density of larvae at known release sites is 1.5 larvae per $2m^2$ of tree sampled (range 0-11 per tree). The mean density of larvae at 'new monitoring sites' sites is 2.6 larvae per $2m^2$ of tree sampled (range 0-14 per tree). During the study no larvae were detected at 27 of the evaluation sites.

The average size of *P. aculeata* infestations at surveyed release sites was 698.1 m^2, 494.6 m^2 at new monitoring sites and 901.3 m^2 at sites where no larvae were observed during beat sampling ('No larvae sites'). Demographic life stages of *P. aculeata* present was dominated by adult trees (> 100 cm tall) with 62.5% of release sites, 28.5% of new monitoring sites and 32.6% of no larvae sites having either seedlings or juveniles present (Figure 29A). The density of *P. aculeata* infestations at plots varied from moderate (5-10 plants) to dense (> 10 plants) at surveyed release, new monitoring and no larvae sites (Figure 29B).

At surveyed release sites 52% of plants had no evidence of leaf defoliation (75-100% leaves present) and 45% of plants had evidence of leaf drop (50-75% leaves present) which could not be directly attributed to larval feeding on inspection, 3% of plants were classified as defoliated where <50% leaves were present (Figure 29C). At spread sites 56% of plants had no evidence of leaf defoliation and 43% of plants had evidence of some leaf drop (50-75% leaves present), only 1 % of plants were classed as defoliated (<50% leave present) (Figure 29C). At sites where no larvae were detected 57%

of plants had no evidence of leaf defoliation (75-100% leaves present) and 39% of plants had evidence of leaf drop (50-75% leaves present), 5% of plants had <50% leaves present (Figure 29C).

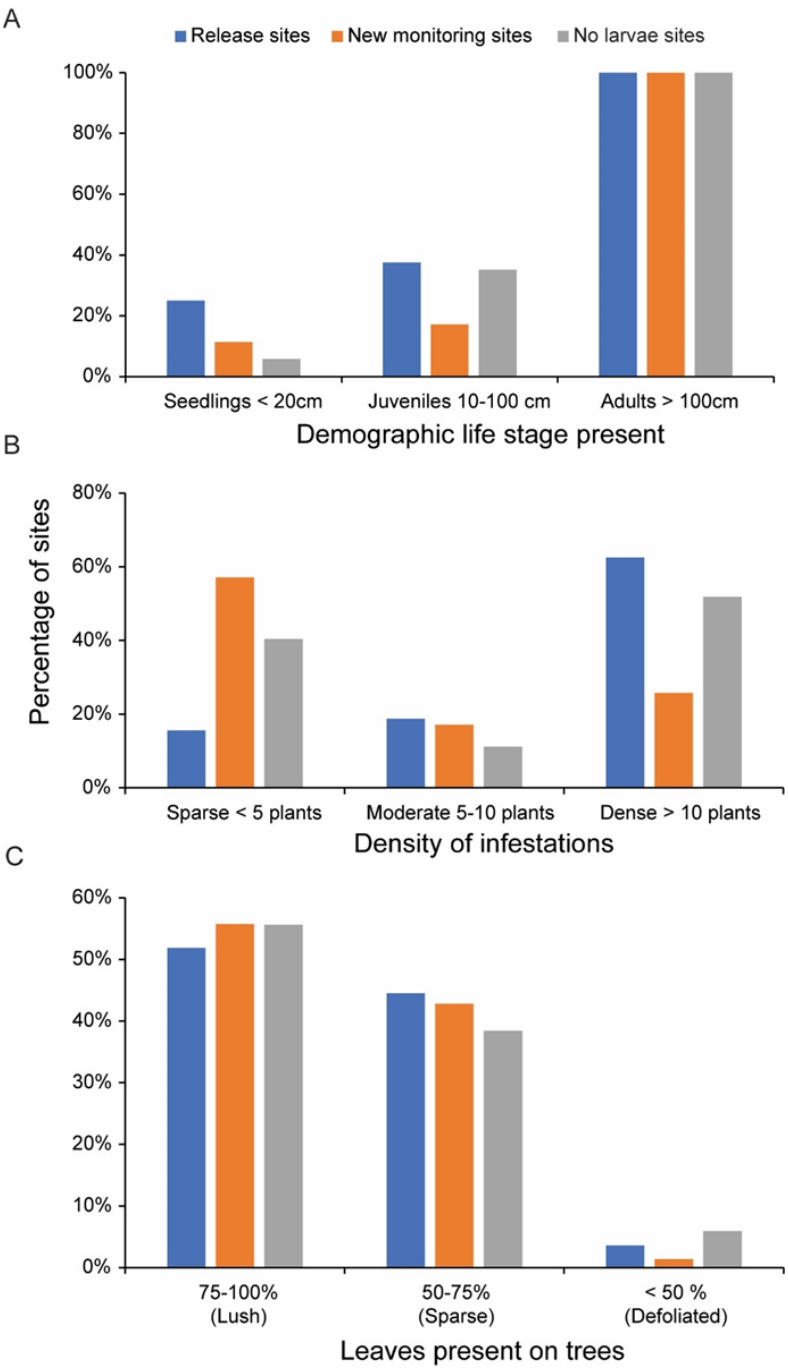


Figure 29: Percentage of sites where A) each demographic life stage was present, B) the density *Parkinsonia aculeata* and C) percentage of leaves observed on trees. Release sites (blue bars) n = 49, new monitoring sites (orange bars) n = 45, sies where no larvae were recorded (grey bars) n = 27.

Larval defoliation experiments of *P. aculeata* plants (~1m tall) in the glasshouse indicate that over 50% defoliation can occur when initial densities of 20 larvae/plant and near complete defoliation occurred at neonate densities of 50 larvae/plant (B.WEE.0134). Defoliation at these levels was enough to impact the rate of change of plant height but not basal stem diameter (B.WEE.0134).

When the larval densities required to negatively impact *P. aculeata* seedlings in the glasshouse are juxtaposed against present larval densities observed in the field (1.5 larvae per tree) the population is not sufficiently large enough or uniformly distributed across sites to be having the desired impact on the weed. In addition to this *P. aculeata* drops leaf material when environmentally stressed, in response to infection by plant pathogens (dieback) and when fed on by lepidopterans, such as *E. cisplatensis, E. vollonoides* and another native lepidopteran present on *P. aculeata*. Thus, when plants do exhibit 50-75% loss of leaf material it is difficult to attribute to *Eueupithecia* specifically.

4.4.2 Stakeholder survey

The survey attracted 64 respondents. Most of the respondents indicated that their farm or commercial enterprise is in Queensland but there were a few from the NT and WA. Thirty-nine percent of respondents identified as farm owners or managers, 39% as extension officer, agricultural specialist or natural resource land manager and the remainder as community interest group members (11%), agricultural consultants (7%) and researchers (4%). Of the respondents that own or manage a farming enterprise most preferred not to indicate the size or value of their holdings but of the responses received the average farm size was ~55, 000 HA.

Parkinsonia is clearly seen as a problem by most and in need of control. Eighty-one percent of respondents indicated they knew parkinsonia well while the remaining 19% were familiar with it (none of the respondents were unfamiliar). Eighty-four percent of respondents agreed or strongly agreed that parkinsonia was a severe problem for the grazing industry. Fourteen percent disagreed with this statement and the remaining 2% were neutral (Figure 30). The number of participants indicating that they disagreed with the following statement 'Do you agree that controlling parkinsonia is costly?' increased to 26.2% (disagree and strongly disagree combined). Neutral responses also rose to 14.3%, although over half the respondents (59.5%) either agreed or strongly agreed with the statement (Figure 30). Most respondents agreed that controlling parkinsonia is beneficial (71.5% agree or strongly agree) while 11.9% were neutral and 16.6% either disagreed or strongly disagreed with the statement (Figure 30).

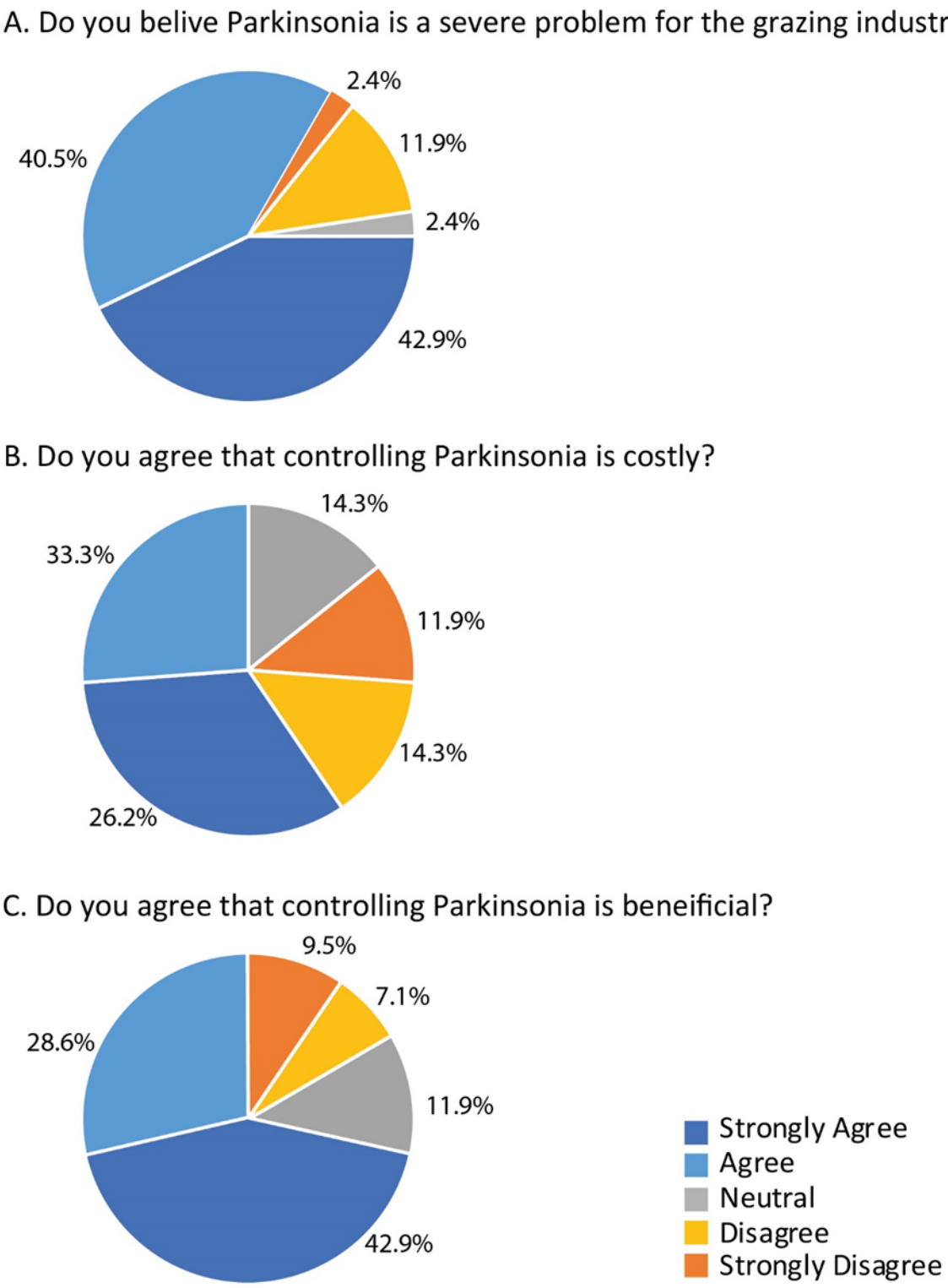


Figure 30: Responses of participants to questions on A) the severity, B) cost, and C) benefit of controlling parkinsonia (n = 42 for each question).

Survey participants were also asked about their experience with parkinsonia on their own properties. Just over 50% of respondents rated parkinsonia as a major or significant problem on their properties while 41.5% indicated it was a minor pest (Figure 31). Survey participants were asked to rank on a scale of 1-10 how much effort they have had to expend to control parkinsonia on their properties (Figure 31). For this question there was an even spread with peaks at **1.** I have had to do

nothing (17.1%), **5.** which would indicate moderate effort (17.1%), **7.** (14.6%) and **10.** (12.2%) which would indicate significant control effort (Figure 31). This spread in control effort does correlate to the responses to the previous question 'How much of a problem is parkinsonia on your property?'. These responses combined with the results from the previous figure, suggest that most respondents perceive parkinsonia to be a weed threat to their property.

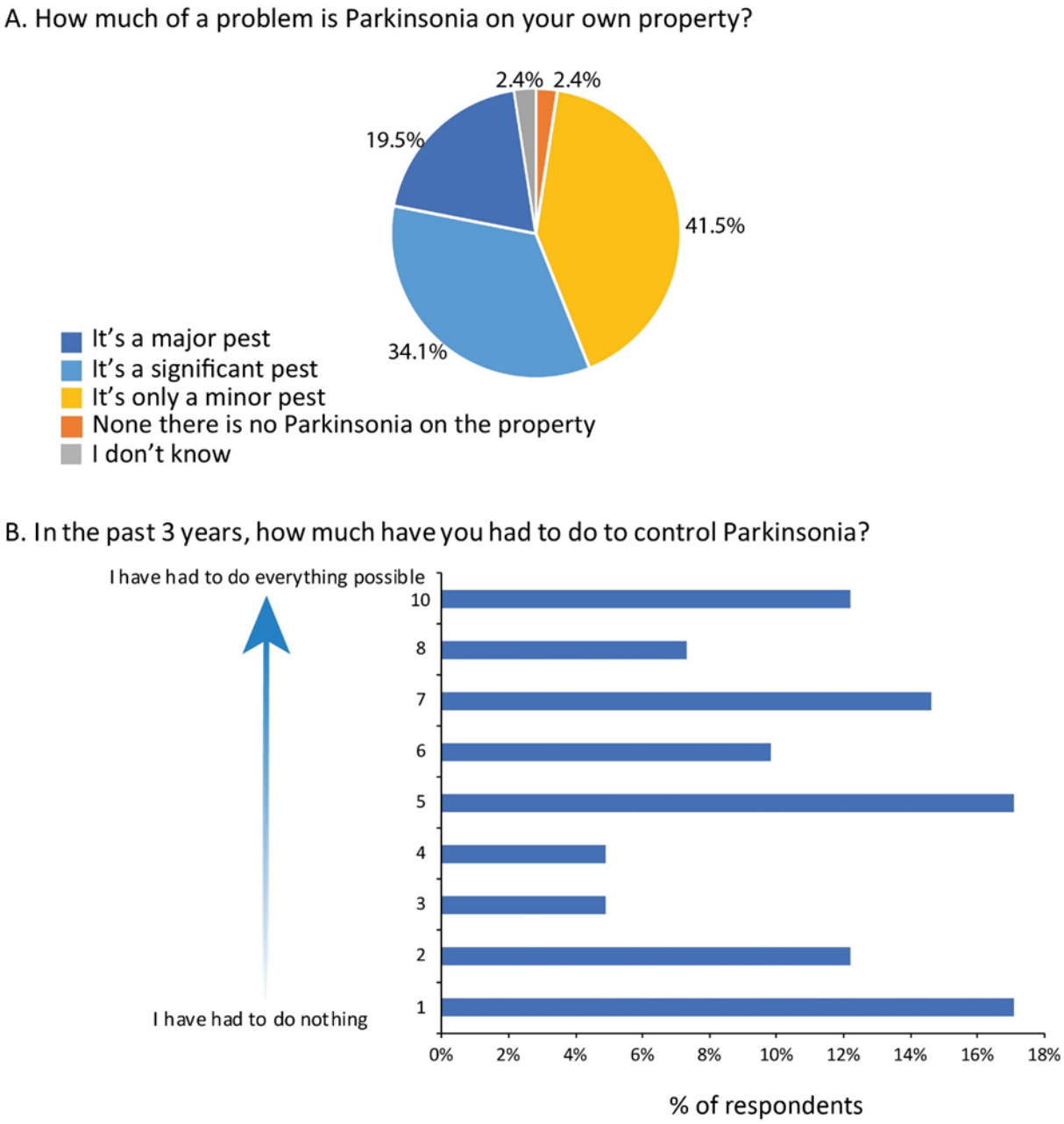


Figure 31: Responses of participants to questions about A) the degree of severity of parkinsonia on their property and B) the control effort they have gone to in the past 3 years (n = 41 for each question).

In terms of parkinsonia impact, survey participants were asked to rank (in order of importance to them) eight known negative impacts that stem from the weed. They were also invited to write about their own negative impacts if it was not captured in the question. The top four ranked negative impacts were 1. Loss of pasture productivity, 2. Increased management costs, 3. Impedes mustering, and 4. Associated environmental impacts from mechanical control (Figure 32). Negative impacts that

were written in by respondents included decrease in land value, loss of biodiversity and environmental

Negative impact	Overall Rank	Rank Distribution	Score	No. of Rankings
Loss of pasture, decreased pasture productivity	1		255	32
Increased management cost	2		234	32
Impedes mustering	3		198	32
Associated environmental impacts from mechanical control – erosion, loss of top-soil, sedimentation of water courses	4		161	28
Accessibility to water	5		154	27
Associated impacts from chemical control (e.g., chemical residues in the environment)	6		152	29
Increase vehicle maintenance	7		90	25
Animal/stock injury due to weed entanglement	8		79	23
Others	9		35	8

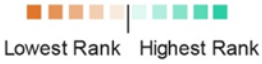


Figure 32: Documented negative impacts of parkinsonia ranked from highest to lowest based on respondent feedback (n= 42 responses).

In terms of current management, 87.5% of respondents indicated they controlled the plant using chemical methods, while 40% used mechanical control methods, 35% biological control and 17.5% fire. More than one control option could be selected and ~80% of respondents indicated that they use multiple management techniques for parkinsonia. Ten percent of respondents said they used other management methods, which ranged from camels or goats to no control at all. Survey participants were then asked if they agreed that using biological control is a cost-effective way of controlling parkinsonia? Forty percent of respondents were neutral to this statement while 28.6% and 20% of respondents agreed or strongly agreed respectively (Figure 33). A small proportion of respondents (2.9% and 8.6%) either disagreed or strongly disagreed with this statement (Figure 32). This pattern around biological control acceptance was further reflected in the follow-up question around if the survey participants would recommend biological control to other land holders. Forty percent of respondents indicated they were likely to recommend parkinsonia biological control (ranking ≥ 8), 31.5% were neutral (ranks 5-7), and 22.9% indicated they were unlikely to recommend parkinsonia biological control (ranks ≤3) (5.7 % declined to rank) (Figure 33).

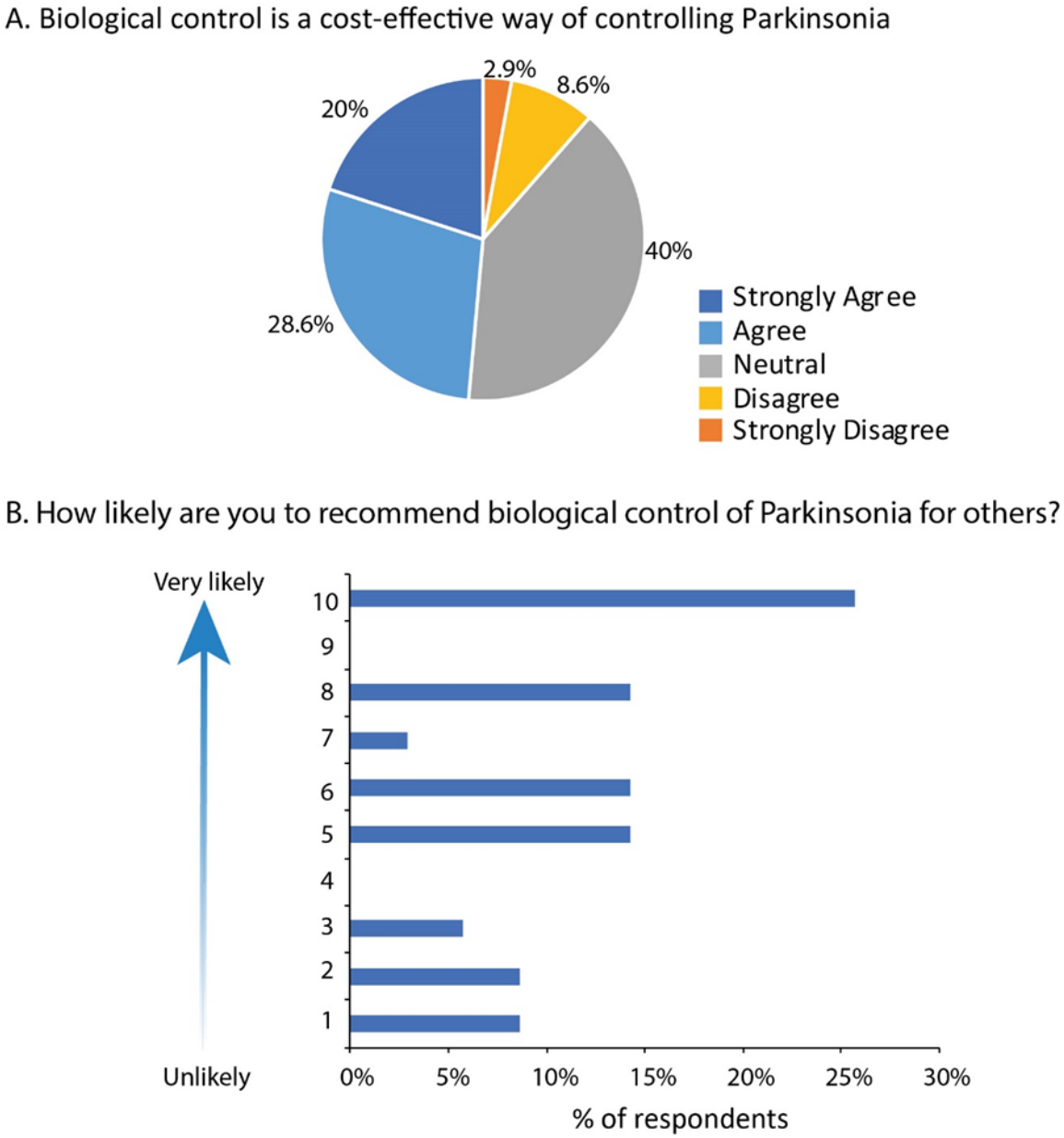


Figure 33: Survey participants perspectives on A) biological control of parkinsonia and B) if they would recommend parkinsonia biological control to others (n = 35 responses for both questions).

Main findings of the survey indicate that parkinsonia is viewed as a problematic plant that impacts negatively on rangelands. A range of management tools are currently used, with chemical control by far the most common among respondents. Much can be done to improve the uptake of biological control including providing information on the climatic suitability of biological control agents, the potential effectiveness of biological control programs and the process by which they can obtain agents.

4.5 Native range research of stem-galling fly *Neolasioptera aculeata*

Field work to study and collect galled *P. aculeata* material was conducted in Salta province beginning in November 2019 through till April 2024 (Table 5). The number of galls available across these surveys varied dependent on the time of year with surveys in March not yielding large numbers of galls (Table 5).

Table 5: Native range surveys for *Neolasioptera aculeata*

Date	Location	Number of trees and sites sampled	Number of galls collected
November 2019	Salta and Formosa provinces	125 plants at 5 sites	143
January 2021	Salta province	256 plants at 6 sites	388
September 2021	Salta province	200 plants at 3 sites	600
December 2021	Salta province	100 plants at 1 site	120
March 2022	Salta province	90 plants at 3 sites	58
June 2022	Salta province	210 plants at 5 sites	342
November 2022	Salta province	255 plants at 9 sites	184
March 2023	Salta province	22 plants at 4 sites	176
May 2023	Salta province	195 plants at 12 sites	296
April 2024	Salta province	257 plants at 3 sites	306

Most rearing attempts undertaken at FuEDEI proved unsuccessful. Incipient gall development was observed on exposed *P. aculeata* material in December 2021. Of the eight galls observed half had failed to progress (went dry) and the other half remained healthy in April 2022, indicating that the development duration of larvae in galls is protracted (~4 months). Subsequent incipient gall development was also observed in a BugDorm ® cage (MegaView Science Co., Ltd. Tawain) in April 2022 where 16 potted *P. aculeata* plants were exposed to 60 field collected galls. Three galls were observed on the shoot tips of three separate plants about 1 month after exposure (Figure 34).

Figure 34: The three incipient galls observed on exposed *P. aculeatae* plants at FuEDEI in April 2022.

4.6 Quarantine research of the stem-galling fly

4.6.1 *Neolasioptera aculeata* quarantine importations

Field collected *P. aculeata* stems infested with *N. aculeata* galls were imported on 5 sperate occasions into the Dutton Park Approved Arrangements (Quarantine) facility (Table 6). The first importation of 300 galls, hand carried from Argentina by Dr Willie Cabrera Walsh, Director of FuEDEI, arrived on the 4th of July 2022. (Figure 35A). This was an achievement at the time as couriers who would handle live insects were still not operating out of South America at this time and flights from Argentina to Australia which did not route through the USA had only just resumed (internationals cannot hand carry insects under quarantine through the USA).

Table 6: Timings, permits and import methods of field collected *Parkinsonia aculeata* stems infested with *Neolasioptera aculeata* galls.

Import No.	Date received	Import permit	Entry No.	Import method	No. Galls
1	4/07/2022	0005450451	NA22026220	Hand carry - Dr Willie Cabrera Walsh	300
2	21/11/2022	0005450451	QA22028307	Hand carry – Dr Fernando McKay	300
3	24/11/2022	0005450451	AEWEG6CCP	Test of World Courier system	150
4	4/04/2023	0007143194	BAB078583	Courier	150
5	20/05/2023	0007143194	NA23047004	Hand carry – Dr Michelle Rafter	300

Figure 35: Importation of the *Neolasioptera aculeata* galled *Parkinsonia aculeata* material from Argentina A) in July 2022 by Dr Willie Cabrera Walsh (FuEDEI), B) in November 2022 by Dr Fernando McKay, C) imported galled material held in florist foam, D) the double containment of imported galled material on florist foam that was held for up to 12 weeks under holding extensions to the import permit conditions granted by DAFF entomologists.

Under the import permit Argentinean plant material can be kept in a double containment area for a period of four weeks. After the initial four-week period we applied for extensions to hold on to the material as flies continued to emerge. The plant material was kept for a total of 12 weeks after each import. At this time point the department would not grant any further extensions and the imported material was destroyed.

4.6.2 *Neolasioptera aculetae* quarantine emergence and rearing

Across four importations of galled material from Argentina (imports 2 and 3 combined as they occurred within a few days of one another) significant emergence was observed in the July 2022 and November 2022 imports, 43.7 and 55.5% of imported galls respectively (Figure 36). This fell significantly with the imports in April and May 2023 to 7.3% and 6.3% respectively (Figure 36).

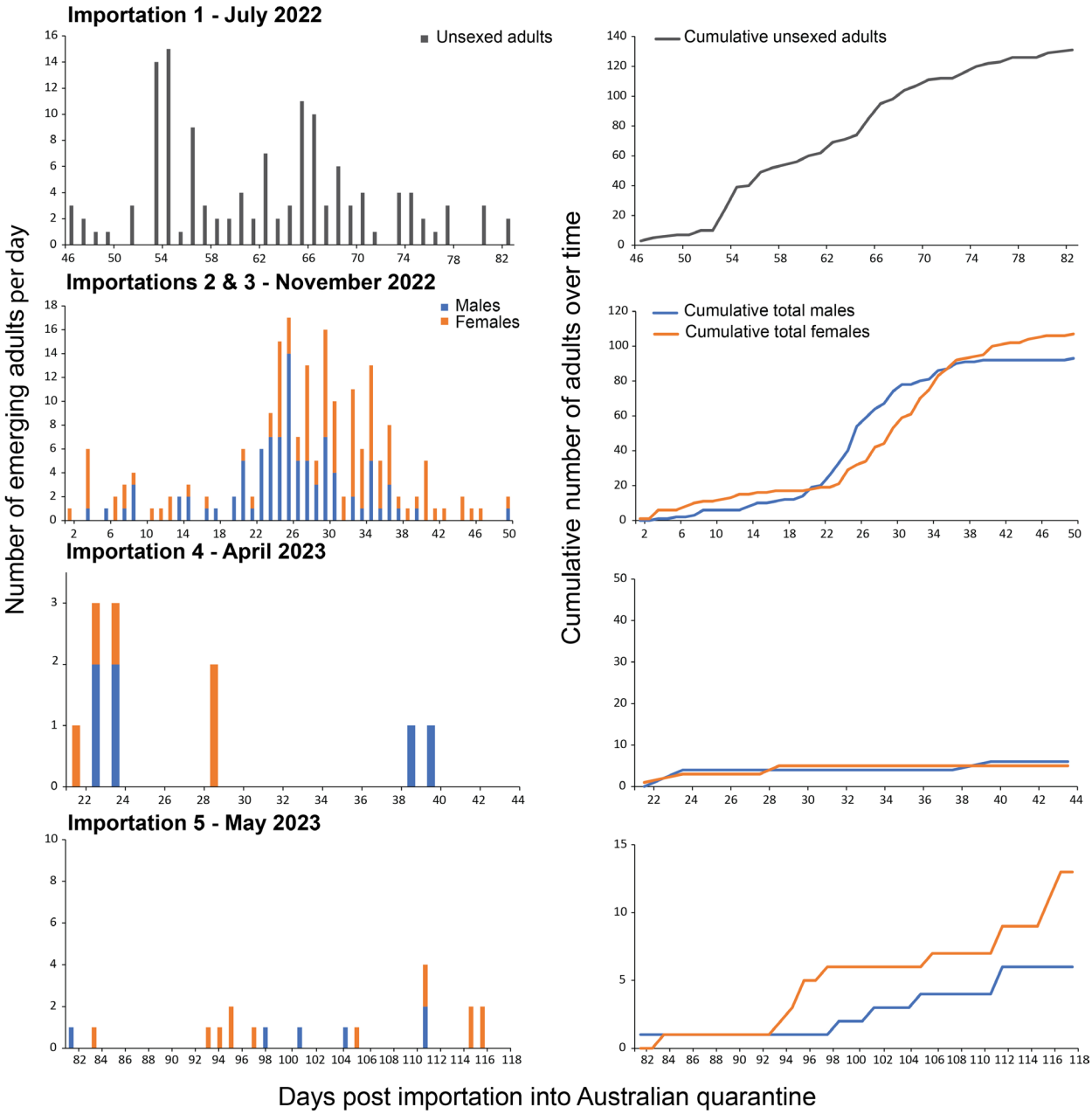


Figure 36: Emergence of *Neolasioptera aculeata* from imported galls in Australian quarantine in numbers (left hand bar graphs) and cumulative number of emerged adults over time (right hand line graphs) over four importations in 2022 and 2023. The two November 2022 hand carry and courier have been combined into a single entry. A few incipient galls were observed on Australian *P. aculeata* exposed material from the November 2022 importation (Figure 37), but these failed to progress. This may be due to insufficient humidity in the quarantine glasshouse, or the plants were not at the correct stage for gall development.

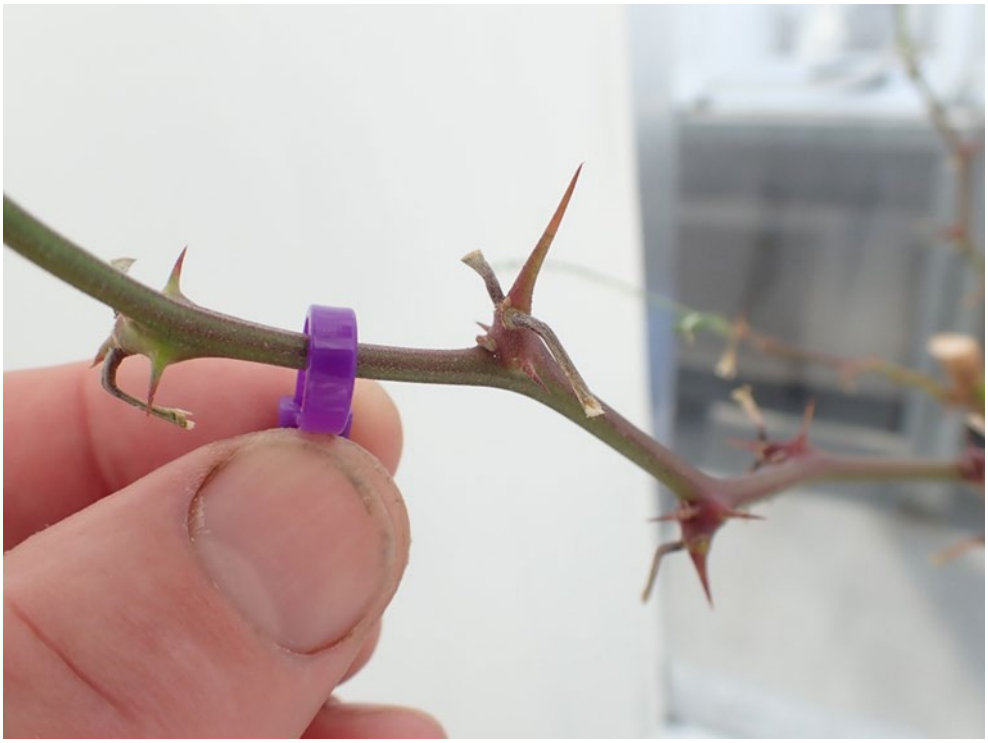


Figure 37: Initial gall development at three apical nodes on *Parkinsonia aculeata* material exposed to *Neolasioptera aculeata* adults from the November importation 2022.

Dissections of female *N. aculeata* in January 2023 found the eggs of this species are conspicuous bright orange ovoids (Figure 38A). This discovery enabled plants exposed to *N. aculeate* to be inspected under a high magnification dissection microscope for evidence of oviposition and larval activity. Several eggs were observed on plants, and most were laid on the axillary bud (Figure 38B-C). These eggs were observed to see if they progressed into evidence of gall activity. Small larvae were observed during these observations (Figure 38D), but some eggs desiccated without hatching indicating that they may not have been fertilised.

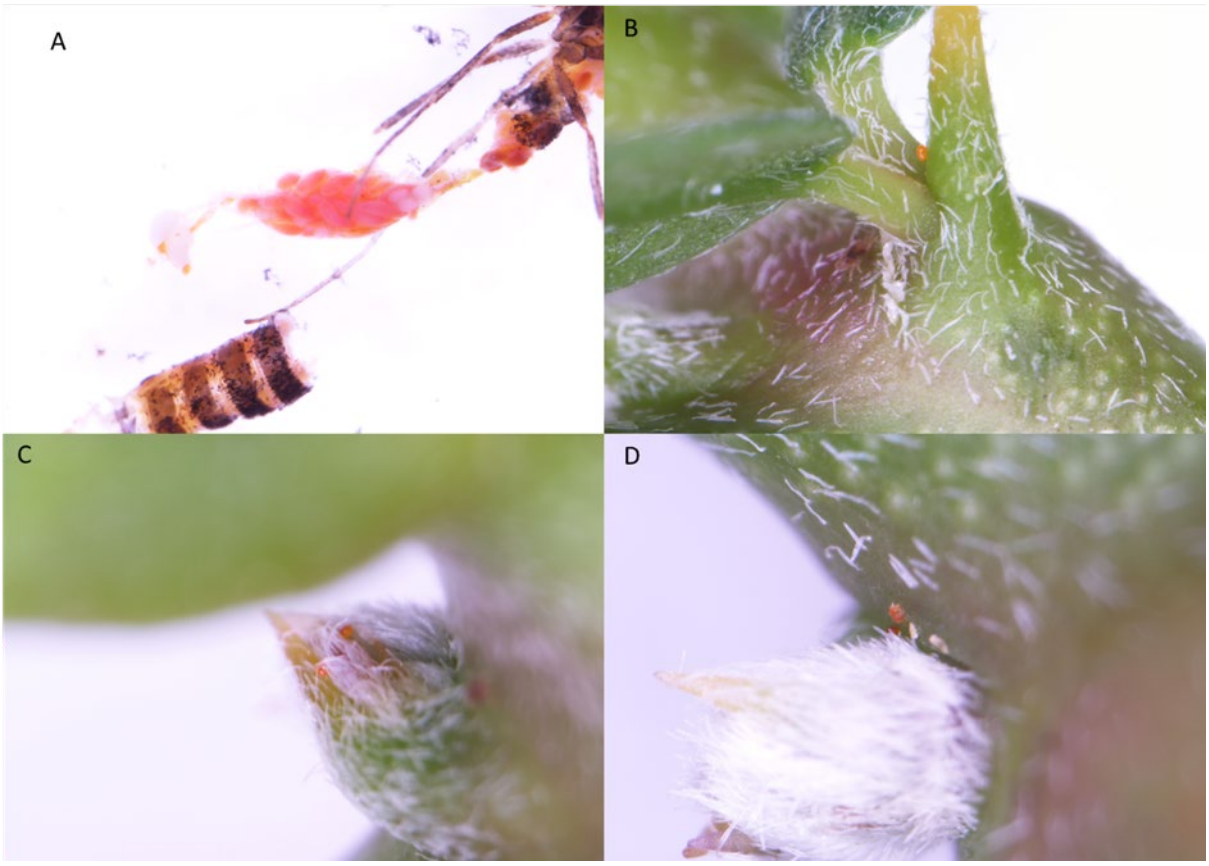


Figure 38: Microscope dissection of female *Neolasioptera aculetae* to reveal eggs (A), observation of a single egg laid on *Parkinsonia aculeata* (B), multiple eggs laid on *Parkinsonia aculeata* axillary bud (C), emergence of larvae from eggs (D).

Although the April and May 2023 imports contained the same number of galls as previous imports the flies emerged sporadically and asynchronously in terms of male and female availability. Researchers requested four extensions from DAFF to hold the May 2023 galled material for 16 weeks after importation. The first male fly emerged from the material 11 weeks after May 2023 import, and subsequent emergence was sporadic with male and female flies only emerging simultaneously once on the 112 days after import (Figure 36). After four extensions to hold imported plant materials DAFF did not permit the material to be held any longer and the material was autoclaved. In total 21 *N. aculeata* emerged from the May 2023 imported galls. Dissection of March 2023 galled material and comparison with the July 2022 imported material revealed that the galls from the March 2023 import were smaller on average (5.31 mm vs 8.45 mm) and contained on average half as many pupal chambers (2 vs 4) (Figure 39).

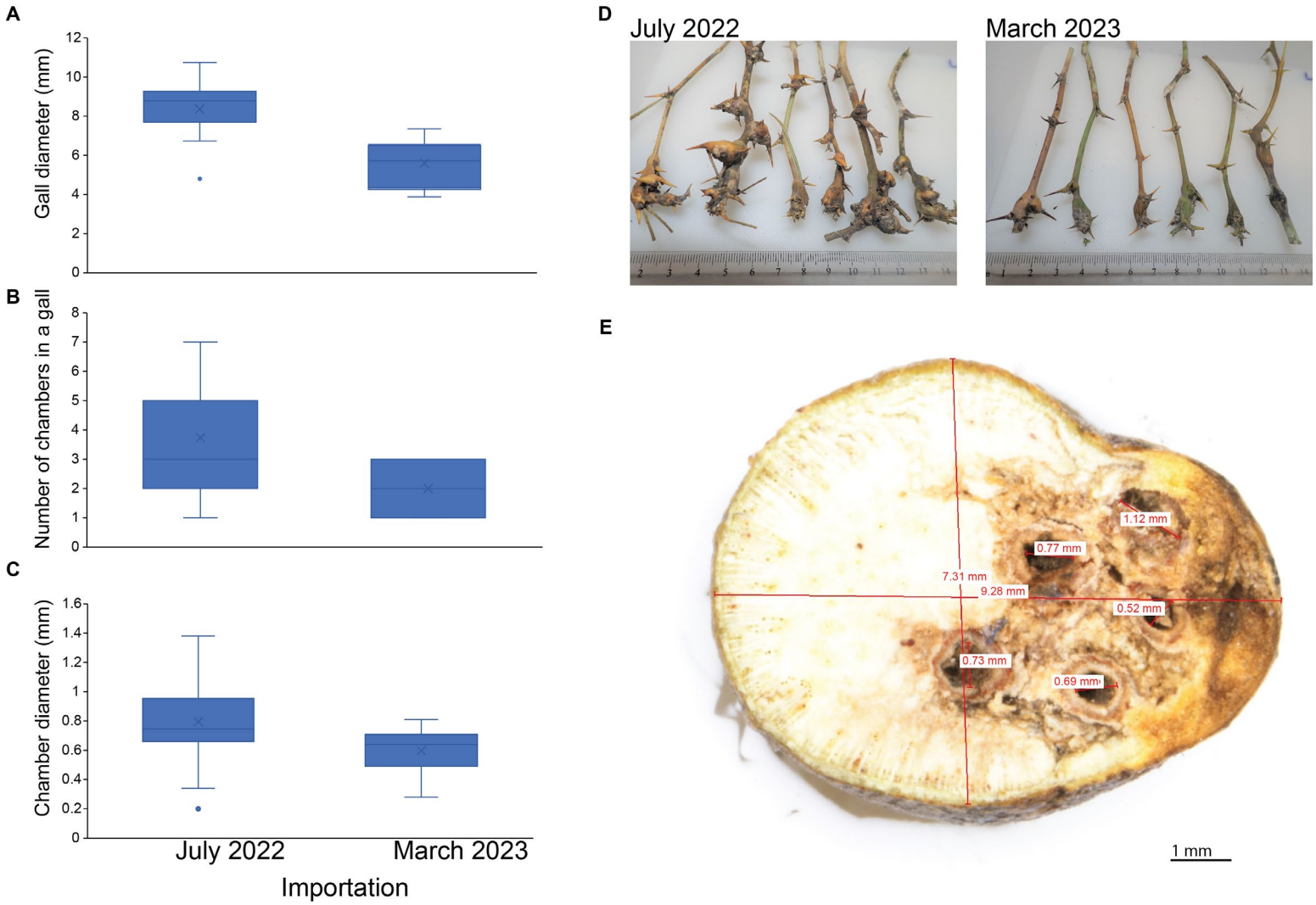


Figure 39: A) Mean cross sectional gall diameter of galls imported in July 2022 and March 2023, B) number of chambers counted within each dissected gall, C) mean diameter of chambers within a gall, D) photo illustrating how galls from the July 2022 and March 2023 shipments, F) cross sectional gall dissection and chamber measurement.

The lack of emergence may be caused by the following factors:

1. Galls were collected from a subset of known sites in the native range
2. Galls collected developmentally too early or from plants with smaller stem diameters. All the galls and stems were smaller than previous shipments.
3. This importation was couriered (not hand carried), and may have been irradiated during the transportation process which may impact development
4. Galls also had a higher fungal load than previous shipments which caused the stem material to decay faster. This could again be attributed to being couriered and the method of packing which include wrapping stems in cotton wool which provides an enlarged surface area for fungal growth. In addition, it was revealed from the collaborator post import that it was persistently raining when these galls were collected in the field.

The lack of *N. aculeata* emergence from imported galls has severely hampered our ability to initiate the planned host specificity testing. No further importations were undertaken after May 2023 due to collection permitting issues at the provincial level in Argentina, which were resolved in March 2024.

4.6.3 Host test list refinement for *Neolasioptera aculeata*

The revised proposed test list currently includes 28 plant species in total, 23 native species and 5 of commercial importance. Australian species in the mimosoid clade, which now sits as a sister clade to that of the target weed *P. aculeata* were prioritised for testing. Lower priority was then given to those genera that were moved to relatively more distantly related subfamilies. The revised test list is provided in Appendix 4. The priority test list included *Peltophorum pterocarpum, Delonix regia* (same clade as *P. aculeata*), *Erythrophleum chlorostachys, Acacia baileyana* and *Acacia holosericea* (close sister clades *to P. aculeata*).

5 Conclusion

5.1 Key findings

- Based on the surveys presented both *E. cisplatensis* and *E. vollonoides* have established self-sustaining populations across the range of *P. aculeata* in northern Australia and there is evidence these populations are moving across the landscape.
- Measured population densities of both species remain low at both nursery release and adventive monitoring sites and have not yet reached a sufficient density to achieve the desired level of leaf defoliation (as determined in B.WEE.0134).
- Stakeholder engagement revealed that parkinsonia is still viewed as a problematic plant that impacts negatively on rangelands. A range of management tools are currently used, with chemical control by far the most common among respondents.
- Known hybrid individuals (from the laboratory) appear only as their maternal (*E. vollonoides*) species in phylogenetic illustrations of their CO1, potentially masking hybrid individuals in collected samples.

- If significant levels of hybridisation were found in the native range of *Eueupithecia*, we would not be aware of this using CO1 barcoding alone, and further, we would not know that such hybrids are more genetically like *E. cisplatensis*.
- The lack of introgression observed in sympatry in the native range of Argentina and the introduced range in Australia, suggests that despite evidence of pheromone cross-attraction in the introduced range of Australia, and laboratory evidence for asymmetrical hybridisation; these moths operate as discrete species in nature.
- Mating behaviour in *Eueupithecia* species begins with pheromonal stimulation which mediates the localisation of potential mates.
- Reciprocal pheromone trapping tests found males are attracted to the pheromones of females of both species, and cross-mating tests in confinement revealed asymmetrical hybridisation is possible between *E. vollonoides* females and *E. cisplatensis* males, but not between *E. cisplatensis* females and *E. vollonoides* males.
- Preliminary analysis of crude pheromone gland extracts reveals the presence of a common compound (tentatively characterized as a type I moth sex pheromone, (11Z,13Z)-hexadeca-11,13-dienal) present in samples of pooled female gland extracts of each species.
- If (11Z,13Z)-hexadeca-11,13-dienal proves to be biologically active in the mate attraction of Eueupithecia, this compound could contribute a major chemical signature to the pheromone profile of each species and thus explain the field cross attraction of males to the pheromone of both species.
- Large numbers of adult *N. aculetae* can emerge from galls collected in the field and imported directly into Australian quarantine, but it is not reliable and incipient gall induction on Australian material failed to progress.
- We have learnt some key details around the ecology of *N. aculetae* such as, their oviposition sites are external and eggs are not inserted into the stem, but more needs to be learnt about this species ecology to rear it successfully under quarantine conditions.

5.2 Benefits to industry

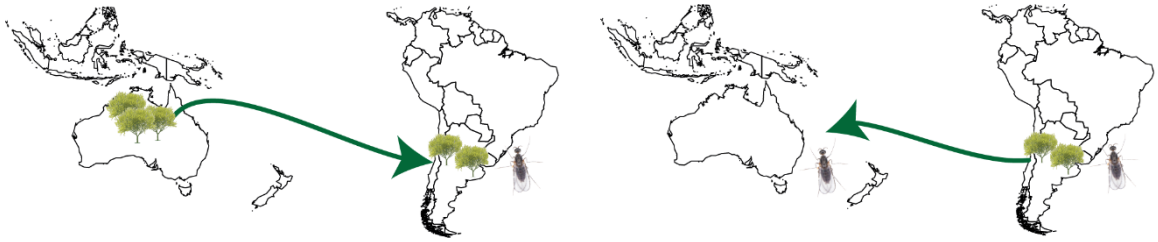
The key benefit to the pastoral industry is the presence of two additional leaf-defoliating moth biological control agents as a persistent landscape scale weed management tool in the integrated weed management toolbox for parkinsonia. This will enable land managers to prioritise where in the landscape they can deploy other management tactics (e.g. in areas where the agents have failed to establish for some reason or are easy to access by other control tactics), while biological control is a chronic stressor in areas where it has established. A related benefit is that the network of collaborators forged during the life of this project can be used to further the biological control and integrated management of other similarly widely distributed rangeland weeds.

6 Future research and recommendations

Based on the progress made in this project two future research avenues exist to develop biocontrol/weed management tools for *P. aculeata.* The first option focuses on Phases I and II of the biocontrol research pipeline and the second option is centred in Phase IV – monitoring and evaluation.

Phase I
Native range exploration

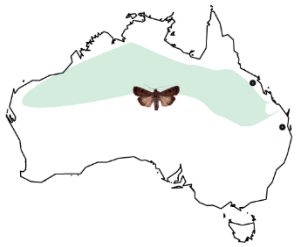
Phase II
Quarantine risk assessment



Further native range and quarantine risk assessment to develop an additional weed biocontrol tool for *P. aculeata* could be undertaken to build on the knowledge of *N. aculeata* gained during B.WEE.0148. Any future research on *N. aculeata* should be focused on the following aspects to ready the insect for importation into the restrictive Australian quarantine conditions for insect rearing:

1. Population genetics analysis of *P. aculeata* across the native South American and invasive Australian range. The last genetic analysis of P. aculeata was conducted using three chloroplast gene regions, and amplified fragment length polymorphism (AFLP) markers were used to reconstruct the intraspecific phylogeny (Hawkins et al. 2007) and only a single sample from Argentina. As the stem-galling fly is only located from a limited zone within the distribution of *P. aculeata* within Argentina specifically, the genetic match of the *P. aculeata* sustaining *N. aculeata* in the field needs to be investigated to both the plant material being used to culture it in Buenos Aires and to the invasive Australian population.
2. Investigations of if microbial or bacterial symbionts are involved in gall induction and *N. aculeate* development. Incipient gall development was observed during B.WEE.0148 but perhaps moving insects from the field into the laboratory setting a crucial symbiont was lost in the process (Hammer et al. 2021).

Phase IV
Monitoring and evaluation



The pipeline of MLA funded biocontrol research has resulted in self-sustaining populations of both *E. cisplatensis* and *E. vollonoides* becoming established across the invasive range of *P. aculeata* in Australia. Measured population densities of both species remain low at both release and adventive monitoring sites and have not yet reached a sufficient density to achieve the desired level of leaf defoliation.

During this project field trapping using live virgin *Eueupithecia* has demonstrated that there is a pheromone cue to mate attraction. Further work is needed to determine the active chemical

constituent and determine if a synthesised blend can be developed for deployment in the field. An assistive pheromone tool could be used not only to monitor moth presence at sites but could be deployed to lure and aggregate moths in *P. aculeata* infestations. It would be based on current spread, establishment and impact measures be desirable to increase population densities of the agent at sites where Parkinsonia impacts need to be mitigated

Because *P. aculeata* is such a widespread weed across the rangelands of northern Australia monitoring and evaluation of weed management would be enhanced if a cost-effective, scalable and accurate mapping tool could be developed. Remote sensing approaches have developed over the past 5 years and preliminary discussions with subject matter experts indicate that parkinsonia is likely to be amenable to remote sensing using high-resolution satellite imagery and gradient boosting (as detailed in Shendryk et al. 2020) combined with Machine Learning (ML). Though out the MLA funded pipeline of research CSIRO and the project partners have a considerable database of known *P. aculeata* infestation locations which could be used to train the ML algorithm (Shendryk et al. 2020).

As multiple biocontrol agents have been released for *P. aculeata* it would be worth investigating if the remote sensing ML approach could be applied to historical high-resolution satellite imagery to quantify temporally if *P. aculeata* populations are contracting, expanding or simply moving around the landscape and if there is a spatial/population response to biocontrol over time.

7 Publications and engagement

7.1 Peer reviewed publications and publications in preparation

Rafter, M. A., McKay, F., Parisi, M., Sosa, A., Heard, T. A., White, A., Fichera, G., Brookes, D., Nagalingam, K., Kaye, L., & Raghu, S. (2022). Biology, host specificity and DNA barcoding of cryptic *Eueupithecia* species (Lepidoptera: Geometridae), and implications for biological control of *Parkinsonia aculeata* (Fabaceae) in Australia. Austral Entomology, 61(1), 124–132. https://doi.org/10.1111/aen.12586

Murray, C-E., Rafter, M.A., & Walter G.H. (Accepted). Laboratory lighting and characterizing the "discreet" mating behavior of a small moth, *Eueupithecia vollonoides* (Lepidoptera, Geometridae, Sterrhinae).

Journal of Insect behaviour

Murray, C-E., White, A., Pukallis, K., Brookes, D., & Rafter, M.A. (in prep). Establishment, spread and impact of two leaf defoliating agents (*Eueupithecia cisplatensis* and *E. vollonoides*) on the rangeland weed *Parkinsonia aculeata.*

Target journal: BioControl

Murray, C-E., Pukallis, K., Walter, G.H. & Rafter, M.A. (in prep). Developing pheromone lures to aggregate and monitor *Eueupithecia* moths to improve biological control.

Target Journal: Biological Control

Murray, C-E., Rafter, M.A., Twidle, A.M., Brookes, D., White, A., Murree, K., & Walter, G.H. (in prep). Comparative mating behaviour of *Eueupithecia cisplatensis* and *E. vollonoides* - differentiating species for biological control.

Target Journal: Biological Control

Murray, C-E., Rafter, M.A., Brookes, D., White, A., Murree, K., & Walter, G.H. (in prep). The population genetics and species delimitation of *Eueupithecia cisplatensis* and *E. vollonoides*, and the implications for the biological control of *Parkinsonia aculeata* in Australia.

Target Journal: Evolutionary Applications

Murray, C-E., Rafter, M.A., Brookes, D., White, A., Murree, K., & Walter, G.H. (in prep). *Eueupithecia cisplatensis* and *E. vollonoides*, biological control agents of *Parkinsonia aculeata* - do they hybridise in the native range?

Target journal: Evolutionary Ecology

7.2 Conference presentations

Murray, C-E., Rafter, M.A., Brookes, D., Murree, K., White, A., McKay, F., & Walter G.H. (2024). Two cryptic species on one weed- how clarifying their species status using molecular and behavioural tests can inform the screening, mass-rearing, and monitoring protocols of biological control agents. In: 23rd Australasian Weeds Conference; 25 -29 Aug 2024; Brisbane.

Murray, C-E, Rafter, M. A., Brookes, D., & Walter, G. H. (2023). Two host-specific cryptic species on one weed- will disentangling their species status and ecology help improve biocontrol? In: XVI International Symposium on Biological Control of Weeds; 07 - 12 May 2023; Puerto Iguazú, Misiones, Argentina.

Rafter, M. A., Pukallus, K., Su, W., Walter, G.H., White, A. (2022) Parkinsonia biological control: Establishment, spread and impact of UU1 and UU2 across northern Australia. In: 22nd Australasian Weeds Conference 2022; 25 to end of 29 Sep 2022; Adelaide. Council of Australasian Weed Societies and the Weed Management Society of South Australia Inc.; 2023. 236.

Murray, C-E., Rafter, M.A., Walter, G. H. (2022) Pheromone Trapping for Monitoring the Establishment and Spread of *Eueupithecia cisplatensis* and *E. vollonoides*, Biological Control Agents for Parkinsonia aculeata. In: 22nd Australasian Weeds Conference 2022; 25 to end of 29 Sep 2022; Adelaide. Council of Australasian Weed Societies and the Weed Management Society of South Australia Inc.; 2023. 237.

7.3 Media

Radio Interview, ABC North West Queensland, Michelle Rafter on the Parkinsonia Biocontrol program. 28th July 2020.

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9 Appendices

Appendix 1: CO1, 28S and CAD barcoding resources

Table 7: The number of Eueupithecia cisplatensis and Eueupithecia vollonoides individuals that sequences were successfully obtained for is listed for each of the gene regions that were included in this study. AR indicates samples from Argentina, and AUS indicates samples from Australia.

Gene Region	*E. cisplatensis* Native range - AR	*E. cisplatensis* Culture - AUS	*E. vollonoides* Native range - AR	*E. vollonoides* Culture - AUS	Field Collected - AUS
CO1	8	36	6	32	18
28S	7	35	6	34	15
CAD	3	34	5	33	14

Table 8: Gene regions and primers used in mitochondrial and nuclear gene barcoding of *E. vollonoides* and *E. cisplatensis* samples.

Gene Region	**Primers**	**Primer sequence (5' > 3')**	**Annealing temp. (°C)**	**Fragment length (bp)**	**Source**
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	51	666	Folmer *et al.* 1994; Foottit *et al.* 2008
	HCO2198	TAAACTTCWGGRTGWCCAAAAAATCA			
28S	A335	TCGGARGGAACCAGCTACTA	48	646-649	Dowton & Austin, 1998, Whiting *et al.* 1997
	S3660	GAGAGTTMAASAGTACGTGAAAC			
CAD	CAD821F	AGCACGAAAATHGGNAGYTCNATGAARAG	48	690	Wild & Maddison, 2008
	CD1098R	GCTATGTTGTTNGGNAGYTGDCCNCCCAT			

Table 9: Details of the *E. vollonoides* and *E. cisplatensis* samples used in mitochondrial and nuclear gene barcoding. The haplotype code for the COI, CAD and 28S genes are also listed for individuals where sequence data was obtained. 28S and CAD have two haplotypes each because they are phased (i.e., the haplotypes for these individuals have been estimated from a single ambiguous sequence).

Name	**Species**	**Species code**	**Origin**	**Location**	**COI**	**28S-1**	**28S-2**	**CAD-1**	**CAD-2**
ECAA18n1	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap06	28S-Hap06	CAD-Hap08	CAD-Hap06
ECAA18n2	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap08	CAD-Hap08
ECAA18n3	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap10	28S-Hap06	CAD-Hap16	CAD-Hap04
ECAA18n4	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap11	28S-Hap04	CAD-Hap06	CAD-Hap03
ECAA18n5	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap08	CAD-Hap04
ECAA18n6	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap06	CAD-Hap06
ECAA18n7	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap08	CAD-Hap08	CAD-Hap04
ECAA18n8	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap17	CAD-Hap04
ECAA18n9	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap06	28S-Hap06	CAD-Hap08	CAD-Hap06

ECAA18n10	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap08	CAD-Hap08	CAD-Hap08
ECAA18n11	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap01	CAD-Hap01
ECAA18n12	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	-	-
ECAA18n13	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	-	-	-	-
ECAA18n14	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap08	CAD-Hap08	CAD-Hap08
ECAA18n15	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap08	CAD-Hap06
ECAA18n16	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap06	CAD-Hap06
ECAA18n17	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap06	CAD-Hap06
ECAA18n18	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap05	CAD-Hap05
ECAAu13n1	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap08	CAD-Hap03	CAD-Hap03
ECAAu13n2	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap08	CAD-Hap03	CAD-Hap03
ECAAu13n3	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap08	CAD-Hap03	CAD-Hap03

ECAAu13n4	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap09	28S-Hap06	CAD-Hap06	CAD-Hap03
ECAAu13n5	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap03	CAD-Hap03
ECAAu13n6	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap03	CAD-Hap03
ECAAu13n7	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap06	CAD-Hap03
ECAAu13n8	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	-	-	-	CAD-Hap03	CAD-Hap03
ECAJu13n1	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap03	CAD-Hap03
ECAJu13n2	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap02	CAD-Hap03	CAD-Hap03
ECAJu13n3	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap03	28S-Hap06	CAD-Hap03	CAD-Hap03
ECAJu13n4	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap03	CAD-Hap03
ECAJu13n5	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap05	CAD-Hap03	CAD-Hap03
ECAJu13n6	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap03	CAD-Hap14
ECAJu13n7	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap05	28S-Hap04	CAD-Hap03	CAD-Hap03

ECAJu13n8	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap03	CAD-Hap03
ECAJu13n9	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap08	CAD-Hap03	CAD-Hap03
ECAF18n2	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	COI-Hap01	COI-Hap01	COI-Hap01	COI-Hap01
ECAF18n3	*Eueupithecia cisplatensis*	UU1	lab colony	N/A	COI-Hap01	COI-Hap01	COI-Hap01	COI-Hap01	COI-Hap01
ECAnat1	*Eueupithecia cisplatensis*	UU1	native	Entre Rios, Corrientes	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap12	CAD-Hap03
ECAnat2	*Eueupithecia cisplatensis*	UU1	native	Entre Rios, Corrientes	COI-Hap01	28S-Hap08	28S-Hap07	-	-
ECAnat3	*Eueupithecia cisplatensis*	UU1	native	Entre Rios, Corrientes	COI-Hap01	28S-Hap08	28S-Hap02	-	-
ECAnat4	*Eueupithecia cisplatensis*	UU1	native	Entre Rios, Corrientes	COI-Hap01	28S-Hap08	28S-Hap04	CAD-Hap03	CAD-Hap03
ECAnat5	*Eueupithecia cisplatensis*	UU1	native	Entre Rios, Corrientes	COI-Hap01	-	-	CAD-Hap02	CAD-Hap02
ECAnat6	*Eueupithecia cisplatensis*	UU1	native	Entre Rios, Corrientes	COI-Hap01	28S-Hap13	28S-Hap02	-	-
ECAnat7	*Eueupithecia cisplatensis*	UU1	native	Entre Rios, Corrientes	COI-Hap01	28S-Hap08	28S-Hap07	-	-
ECAnat8	*Eueupithecia cisplatensis*	UU1	native	Entre Rios, Corrientes	COI-Hap01	28S-Hap09	28S-Hap03	-	-

EVAA14n1	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap10	CAD-Hap11
EVAA14n2	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap11	CAD-Hap11
EVAA14n3	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap10	CAD-Hap11
EVAA14n4	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap11
EVAA14n5	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap09
EVAA14n6	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	-	-	CAD-Hap11	CAD-Hap11
EVAA14n7	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap09
EVAA14n8	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap11
EVAA14n9	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap11
EVAA14n10	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap02	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap07
EVAA14n11	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap01	28S-Hap06	CAD-Hap07	CAD-Hap11
EVAA14n12	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap07

EVAD17n3	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-H03	28S-Hap08	28S-Hap04	CAD-Hap03	CAD-Hap03
EVAD17n5	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap01	-	-	CAD-Hap08	CAD-Hap08
EVAD17n6	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap01	28S-Hap06	28S-Hap06	CAD-Hap08	CAD-Hap08
EVAD17n7	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap01	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap08
EVAD17n8	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap01	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap08
EVAD17n9	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap01	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap08
EVAD17n10	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap12	CAD-Hap07	CAD-Hap03
EVAD17n11	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap01	28S-Hap08	28S-Hap08	CAD-Hap07	CAD-Hap03
EVAD17n12	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap01	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap07
EVAD17n13	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap08	28S-Hap04	CAD-Hap08	CAD-Hap11
EVAD17n14	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap08	28S-Hap04	CAD-Hap08	CAD-Hap08
EVAD17n15	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap07

EVAD17n16	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap08
EVAJ13n1	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap11	CAD-Hap11
EVAJ13n2	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap07
EVAJ13n3	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap11
EVAJ13n4	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap11
EVAJ13n5	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap07
EVAJ13n6	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap02	28S-Hap06	28S-Hap06	CAD-Hap18	CAD-Hap13
EVAJ13n7	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap11
EVAS13n8	*Eueupithecia vollonoides*	UU2	lab colony	N/A	COI-H02	28S-Hap01	28S-Hap06	CAD-Hap11	CAD-Hap11
EVAnat13	*Eueupithecia vollonoides*	UU2	native	San Roque (98597)	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap09	CAD-Hap11
EVAnat14	*Eueupithecia vollonoides*	UU2	native	San Roque (98597)	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap10	CAD-Hap11
EVAnat15	*Eueupithecia vollonoides*	UU2	native	San Roque (98597)	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap10	CAD-Hap11

EVAnat16	*Eueupithecia vollonoides*	UU2	native	San Roque (98597)	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap11	CAD-Hap11
EVAnat17	*Eueupithecia vollonoides*	UU2	native	Saladas (98549)	-	28S-Hap06	28S-Hap06	CAD-Hap15	CAD-Hap11
EVAnat18	*Eueupithecia vollonoides*	UU2	native	Saladas (98549)	COI-Hap03	28S-Hap06	28S-Hap06	-	-
EspBV1	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap11
EspBV2	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap11	CAD-Hap11
EspBV3	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap11
EspBV12	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap11	CAD-Hap11
EspBV16	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap03	28S-Hap06	28S-Hap06	CAD-Hap07	CAD-Hap09
EspBV17	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap03	28S-Hap01	28S-Hap06	CAD-Hap07	CAD-Hap11
EspBV19	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap03	28S-Hap06	28S-Hap06	-	-
EspBV21	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap03	28S-Hap06	28S-Hap06	-	-
EspBV22	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap04	28S-Hap06	28S-Hap06	CAD-Hap09	CAD-Hap11

EspBV23	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	-	-	-	CAD-Hap07	CAD-Hap11
EspBV24	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap03	28S-Hap06	28S-Hap06	-	-
EspBV25	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap04	28S-Hap06	28S-Hap06	CAD-Hap11	CAD-Hap11
EspBV26	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap04	28S-Hap06	28S-Hap06	CAD-Hap11	CAD-Hap11
EspBV27	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap03	-	-	CAD-Hap11	CAD-Hap11
EspBV28	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap03	28S-Hap06	28S-Hap06	-	-
EspBV29	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap02	28S-Hap01	28S-Hap06	CAD-Hap07	CAD-Hap11
EspBV30	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap02	28S-Hap06	28S-Hap06	-	-
EspBV31	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap02	-	-	CAD-Hap07	CAD-Hap11
EspBV33	*Eueupithecia vollonoides*	UU2	Field collected in Australia	Burketown, Queensland	COI-Hap02	-	-	CAD-Hap11	CAD-Hap11

Appendix 2: Permits

2.1 Salta province sampling permit for *Neolasioptera aculeata*

GOBIERNO DE LA PROVINCIA DE SALTA
Ministerio de Producción y Desarrollo Sustentable.
Secretaría de Ambiente y Desarrollo Sustentable.

RESOLUCION Nº 000114
SECRETARIA DE AMBIENTE Y DESARROLLO SUSTENTABLE
MINISTERIO DE PRODUCCION Y DESARROLLO SUSTENTABLE
PEDIENTE. Nº 00227-768/2021

VISTO el pedido presentado por el Dr. Fernando Mc Kay, con domicilio particular denunciado en calle Teodoro García 2459 – Buenos Aires, investigador de la Fundación para el estudio de Especies Invasivas (FuEDEI), solicitando autorización para realizar trabajos de investigación; en el marco del Proyecto: "**Evaluación de *Neolasioptera aculeatae* como agente de control biológico de *Parkinsonia aculeata* en Australia**", durante los años 2021 al 2023, y;

CONSIDERANDO:

Que la Ley Nº 7070 de Protección del Medio Ambiente tiene por objeto establecer las normas que deberán regir las relaciones entre los habitantes de la provincia y el medio ambiente en general, los ecosistemas, los recursos naturales, la biodiversidad, entre otros, a fin de asegurar y garantizar el desarrollo sustentable, la equidad intra e intergeneracional y la conservación de la naturaleza;

Que mediante Resolución Nº 091/05 de la Ex Secretaría de Medio Ambiente y Desarrollo Sustentable, se reglamentaron las actividades de investigación en todo el territorio de nuestra provincia;

Que, el Dr. Mc Kay, presentó solicitud para realizar trabajos de investigación, adjuntando un proyecto que cumple con los requisitos establecidos en la norma anteriormente mencionada;

Que el investigador presento protocolo de medidas para Covid, según lo solicitado por el Programa Biodiversidad, y en el marco de la pandemia por coronavirus;

Que el objetivo general del proyecto es realizar estudios de biología y experimentos de especificidad en Argentina y Australia para determinar el potencial de utilizar el mosquito agallícola (*Neolasioptera aculeatae*) como agente de control biológico en poblaciones de la brea de agua o cina cina (*Parkinsonia aculeata*);

Que la mosquita agallícola es un fitófago que funciona como controlador biológico natural de esta especie de fabaceae, ya que forma agallas en la planta deteniendo su crecimiento;

Que el área de estudio comprende Banquinas y caminos de: la localidad de Dragones, la localidad de Embarcación, y la localidad de Yuchán;

Que en lo que respecta a la metodología se realizarán observaciones y colecta de insectos de la especie *N. aculeatae* asociados a la brea de agua, en distintas zonas de la provincia;

1

"Gral. Martín Miguel de Güemes,
Héroe de la Nación Argentina"
Ley 7389

Secretaría de Ambiente y Desarrollo Sustentable

Ministerio de Producción y Desarrollo Sustentable
Gobierno de Salta

SALTA, 01 de Febrero de 2.022

CÉDULA DE NOTIFICACIÓN

Dr. FERNANDO MC KAY
Santiago del Estero Nº 2245 – Oficina 14 – 2º Piso
Ciudad de Salta

Oficina de Trámite: Programa Biodiversidad

REF.: Expte. Nº 227-768/2021-0 - Dr. FERNANDO MC KAY, Investigador de la Fundación para el Estudio de Especies Invasivas, solicita permiso de recolección de la mosquita agrícola, Neolasioptera aculeatae, a realizarse en sitios ubicados en la banquina de la Ruta 81 en la localidad de Dragones .- ----------

Por la presente Cédula NOTIFICO y hago saber a Ud. que la Secretaría de Ambiente y Desarrollo Sustentable ha emitido Resolución Nº 000044 de fecha 01 de Febrero de 2.022, cuya copia certificada por este Despacho, se adjunta a la presente en cuatro (4) fs. útiles.

QUEDA UD. DEBIDAMENTE NOTIFICADO.

MIRIAM R. ARIAS DE LALÚ
DESPACHO
SECRETARÍA DE AMBIENTE

---------- *Lo notificado es pasible de ser recurrido a través del Recurso de Aclaratoria en un plazo de tres (3) días, Recurso de Revocatoria o Reconsideración en un plazo de diez (10) días a partir de su notificación, según lo dispuesto por los arts. 176, 177 y ccdtes de la Ley de Procedimientos Administrativos (Nº 5348).*----------

------- *Art. 149 L.P.A. Salta"...Cuando la Cédula no fuere recibida personalmente por el destinatario, el oficial notificador deberá dejar constancia en la copia, el documento de identidad que le fuere exhibido y el vínculo de relación existente entre las personas que reciba y el destinatario. Cuando el empleado no encontrare a la persona a la cual va a notificar y ninguna de las otras personas de la casa quiera recibirla, la fijara e la puerta de la misma, dejando constancia en el ejemplar destinado a ser agregado al Expediente*----------

Observaciones: ----------

FIRMA	ACLARACIÓN	FECHA	HORA	L.D.	L.I.		BAJO PUERTA
						REFERENCIAS:	
		VÍNCULO		DOCUMENTO			

MARCAR EN CASO DE NO ENTREGA									
01	ENTREGADO	04	NO SE UBICA DOMICILIO	07	NO COINCIDE C.P.	10	RECHAZADA POR TITULAR	13	PERSONA NO HABILITADA
02	DESCONOCIDO	05	NO RESPONDE	08	FALLECIDO	11	DE VIAJE	14	CASOS ESPECIALES
03	SE MUDÓ	06	NO EXISTE NÚMERO	09	DOMICILIO INCOMPLETO	12	NO RECIBE	15	OTROS

FIRMA NOTIFICADOR	OBSERVACIONES

1º VISITA ENTREGA EN MANO CON FIRMA O BAJO PUERTA CON REFERENCIAS.-

mral.

2.2 Argentinean export permit for *Neolasioptera aculeata*

República Argentina - Poder Ejecutivo Nacional
Las Malvinas son argentinas

Nota

Número: NO-2022-119727699-APN-DNPV#SENASA

CIUDAD DE BUENOS AIRES
Lunes 7 de Noviembre de 2022

Referencia: Permiso de envió de ejemplares de Neolasioptera aculeatae.Destino: CSIRO, Brisbane, Australia. EX-2022-110874780-APN-DIEF#SENASA.

A: Dr. Guillermo Cabrera Walsh (FuEDEI),

Con Copia A: Sergio Daniel Chireno (DCRNN#SENASA), Laura Elizabeth Maly (DCRM#SENASA), Carlos Alberto Selas (DCRM#SENASA), Gabriel Eduardo Pappalardo (DCRM#SENASA), Pablo Cortese (DIEF#SENASA), Marcelo Sanchez (DIEF#SENASA), Juan Ignacio Puente (DIEF#SENASA), Pamela Adriana Pascal (DIEF#SENASA), Patricia Susana Gomez (DIEF#SENASA),

---

De mi mayor consideración:

Me dirijo a Usted a fin de informarle que esta Dirección Nacional de Protección Vegetal autoriza el envío de ejemplares vivos de *Neolasioptera aculeatae* Gagné (Diptera:*Cecidomyiidae*), enemigo natural de la especie *Parkinsonia aculeata*, con destino investigación en instalaciones de cuarentena del CSIRO Dutton Park del CSIRO (Commonwealth Scientific and Industrial Research Organisation*)*, Brisbane, Queensland, Australia, ubicado en 41 Boggo Rd, Dutton Park QLD 4102, Australia.

La presente autorización se extiende bajo las siguientes condiciones:

- Partida compuesta por aprox. 1.000 (mil) larvas de *N. aculeatae.* en agallas de *Parkinsonia aculeata.*
- Origen del Material: Colecta científica a campo en la localidad de Embarcación, del departamento de General Jose de San Martin, de la provincia de Salta.
- Condiciones de embalaje: Las agallas deberán ir envueltas en papel húmedo en 6 (seis) recipientes plásticos sellados con cinta adhesiva, dispuestas dentro de 2 (dos) bolsas de tela doble selladas. Las bolsas colocadas en una conservadora que a su vez, se ubicara en una caja de cartón, envuelta en papel.

2.3 Australian import permit

Australian Government
Department of Agriculture, Water and the Environment

Permit to import conditionally non-prohibited goods
This permit is issued under *Biosecurity Act 2015* Section 179 (1)

Permit: 0005450451

Valid for: multiple consignments between 24 June 2021 and 29 March 2023

This permit is issued to: CSIRO
41 Boggo Road
DUTTON PARK QLD 4102
Australia

Attention: Dr Raghu Sathyamurthy

This permit is issued for the import of Biological or Plant Products (Non-standard goods).

Exporter details:	Various exporters
Country of export:	Various countries

This permit includes the following good(s). Refer to the indicated page for details of the permit conditions:

1. Biological control agents - Plant		
Description:	Neolasioptera aculeatae (Parkinsonia gall fly)	
End use:	Host specificity testing	
Country of origin:	Argentina; Paraguay	
Permit Conditions:	Biological control agents (insects)	Page 3

NOTE: Where a good has more than one set of permit conditions please read each set to determine which set of permit conditions applies to a specific consignment.

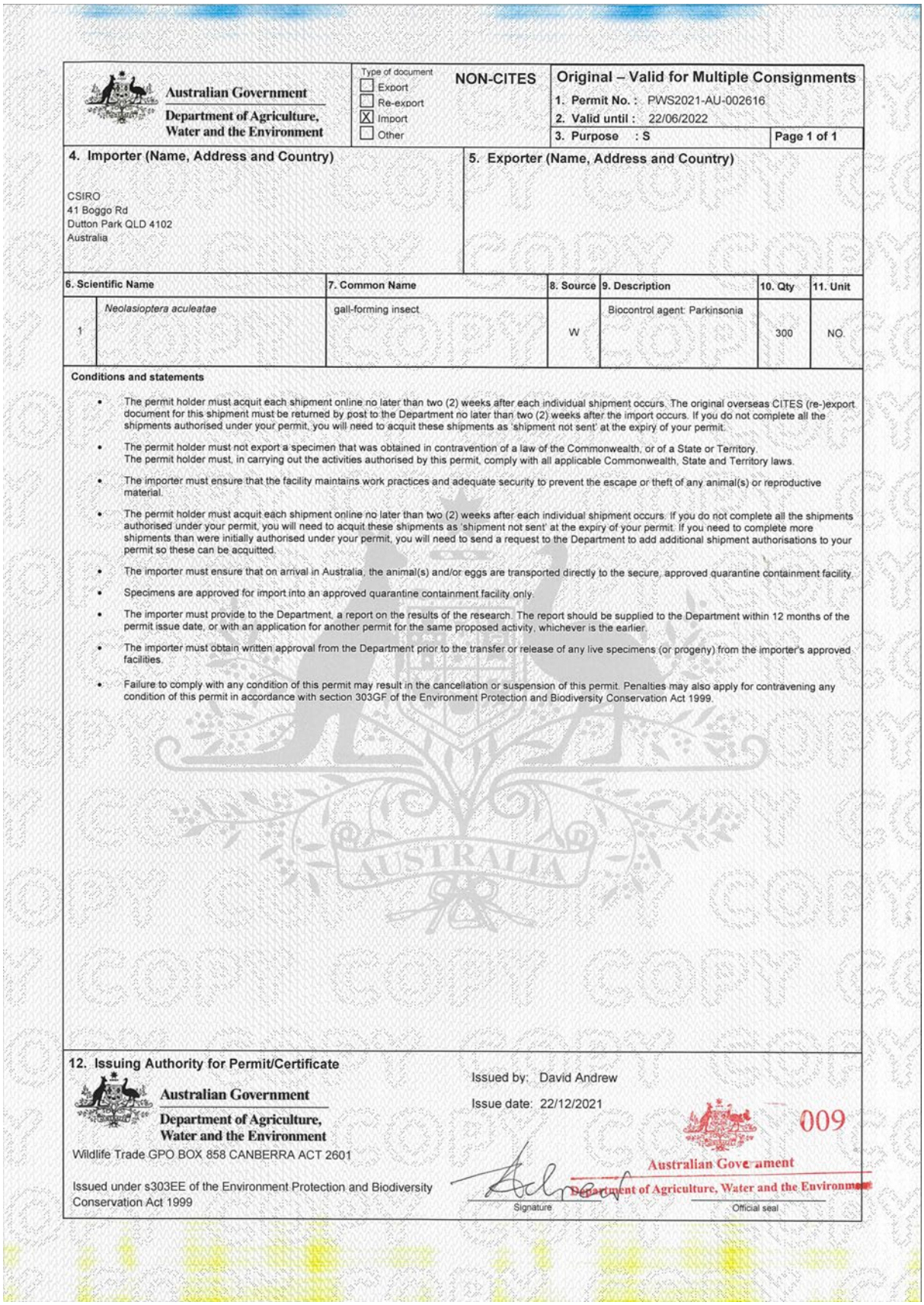
---------- **End of commodity list** ----------

This permit is granted subject to the requirement that fees determined under section 592(1) are paid.

Adam Zur
Delegate of the Director of Biosecurity Date: 24 June 2021

T +61 2 6272 3933 | 18 Marcus Clarke Street | GPO Box 858 | **agriculture.gov.au**
F +61 2 6272 5161 | Canberra City ACT 2601 | Canberra ACT 2601 | ABN 34 190 894 983

2.4 Australian quarantine testing permit



Australian Government
Department of Agriculture, Water and the Environment

Type of document: ☐ Export ☐ Re-export ☒ Import ☐ Other

NON-CITES

Original – Valid for Multiple Consignments

1. Permit No. : PWS2021-AU-002616
2. Valid until : 22/06/2022
3. Purpose : S

Page 1 of 1

4. Importer (Name, Address and Country)

CSIRO
41 Boggo Rd
Dutton Park QLD 4102
Australia

5. Exporter (Name, Address and Country)

	6. Scientific Name	7. Common Name	8. Source	9. Description	10. Qty	11. Unit
1	*Neolasioptera aculeatae*	gall-forming insect	W	Biocontrol agent: Parkinsonia	300	NO

Conditions and statements

- The permit holder must acquit each shipment online no later than two (2) weeks after each individual shipment occurs. The original overseas CITES (re-)export document for this shipment must be returned by post to the Department no later than two (2) weeks after the import occurs. If you do not complete all the shipments authorised under your permit, you will need to acquit these shipments as 'shipment not sent' at the expiry of your permit.
- The permit holder must not export a specimen that was obtained in contravention of a law of the Commonwealth, or of a State or Territory. The permit holder must, in carrying out the activities authorised by this permit, comply with all applicable Commonwealth, State and Territory laws.
- The importer must ensure that the facility maintains work practices and adequate security to prevent the escape or theft of any animal(s) or reproductive material.
- The permit holder must acquit each shipment online no later than two (2) weeks after each individual shipment occurs. If you do not complete all the shipments authorised under your permit, you will need to acquit these shipments as 'shipment not sent' at the expiry of your permit. If you need to complete more shipments than were initially authorised under your permit, you will need to send a request to the Department to add additional shipment authorisations to your permit so these can be acquitted.
- The importer must ensure that on arrival in Australia, the animal(s) and/or eggs are transported directly to the secure, approved quarantine containment facility.
- Specimens are approved for import into an approved quarantine containment facility only.
- The importer must provide to the Department, a report on the results of the research. The report should be supplied to the Department within 12 months of the permit issue date, or with an application for another permit for the same proposed activity, whichever is the earlier.
- The importer must obtain written approval from the Department prior to the transfer or release of any live specimens (or progeny) from the importer's approved facilities.
- Failure to comply with any condition of this permit may result in the cancellation or suspension of this permit. Penalties may also apply for contravening any condition of this permit in accordance with section 303GF of the Environment Protection and Biodiversity Conservation Act 1999.

12. Issuing Authority for Permit/Certificate

Australian Government
Department of Agriculture, Water and the Environment
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Appendix 3: *Neolasioptera aculeata* (Cecidomyiidae) Rearing Manual

1. Maintain healthy plant stock.

   - Keep plant stock free from mites and other pests.
   - Maintain good plant hygiene to ensure that your glasshouse plants remain pest free.
   - Feed plants with seaweed solution every two weeks.

2. Place field collected stems with galls directly into wet florist foam.

   - Place with gall into a large container with wet florist foam.
   - Galls stay greener for longer and as don't dry and harden as quickly, adults that emerge are healthier and more robust.
   - When receiving colony-reared galls from the native range, also place stems into wet florist foam until all adults have emerged.

3. Place field collected galls (in florist foam) into cages with healthy plants.

   - Place fresh plant stock (either seedlings or larger potted plants) into cages.
   - Attach florist foam bouquets to branches using wire.
   - For larger containers of galls, ensure that galls are in contact with lower leaves and branches in the cage.

4. Initiating a colony.

   - Check the field collected galls for emergence holes and the cage and plants for adults. Record numbers on rearing card.
   - Label branches where you see oviposition.
   - One month post female exposure, there is usually early signs of galling at the actively growing stems
   - After one-two months, fresh F1 adults will begin to emerge. Remove F1 adults from cage and set up onto fresh plants in new cages or oviposition cylinders. This will ensure that the colony is free from parasitoids.

5. Other notes

- Galls grow larger when the plant is actively growing at the oviposition site. It is important to provide plants that have young, active growth so that gall formation can occur.
- Setting up in clear rearing cylinders enables you to examine the oviposition behaviour of the female.
- Ensure that the females aren't aggregated in the top corner of the cylinder/cage/container. Gently move them onto the plant material if you see this occurring.
- Modified take-away containers are also great for covering galls for adult emergence. This is particularly useful if you want to determine which branches adults emerge from first, if you are working with seedlings, if glasshouse space is tight or there are simply not enough cages available for colony requirements.
- Fertilise plants every two weeks with seaweed solution (Seasol) every two weeks, to keep the plants (and galls) growing.
- Fertilised, healthy plants produce a lot more galls than unfertilised plants. The galls also tend to be larger, and the adults that emerge are also larger.

Appendix 4: Proposed plant host test list for assessing risk of biological control agents for *Parkinsonia aculeata* L. (Fabaceae: Caesalpinioideae)

Background

This document presents a proposed test list of non-target plant species for inclusion in host-specificity testing experiments for potential biological control agents on *Parkinsonia aculeata* L. (Fabaceae: Caesalpinioideae, the "target weed") (Table 1). It was developed based on the relatively recent re-circumscription of the Fabaceae family by the Legume Phylogeny Working Group (LPWG) and the most recent phylogenetic information available in the literature (Azani et al. 2017; Koenen et al. 2020), as supported by the Angiosperm Phylogeny Website (Stevens 2001 onwards).

The test species have been identified based on their phylogenetic relatedness to the target weed, according to the centrifugal phylogenetic method (Briese 2003; Gilbert et al. 2013; Wapshere, 1974). This method is underpinned by evidence that specialist herbivores are evolutionarily more likely to feed on non-target species closely related to the target weed, relative to species that are more distantly related. Within such a phylogenetic/evolutionary framework, an ecological emphasis is also placed on endemic species, species of economic importance and those that are likely to overlap biogeographically with the target weed, where possible.

The included test species differ marginally from previous lists developed for *P. aculeata* due to the recent major taxonomic revision of the legume family (Fabaceae, syn. Leguminosae) by the LPWG (Azani et al. 2017). The Fabaceae family has traditionally been divided into three subfamilies: Caesalpinioideae, Mimosoideae, and Faboideae (syn. Papilionoideae). The recent revision now recognises six subfamilies: the Mimisoideae is now considered a distinct clade nested within Caesalpinioideae (and is currently referred to informally as the mimosoid clade), four new subfamilies were described (Cercidoideae, Detarioideae, Duparquetioideae, and Dialioideae), whereas the Faboideae subfamily remains relatively unchanged (Azani et al., 2017). Only five of the six subfamilies are present in Australia; no species in subfamily Duparquetioideae occur in Australia. Relative to previous test lists, the new test list prioritises species in the mimosoid clade, which now sits as a sister clade to that of the target weed. Lower priority is given to those genera that were moved to relatively more distantly related subfamilies.

The test list includes one representative species from each of the native Australian genera in subfamily Caesalpinioideae except for *Paraserianthes* (Table 1, Fig. 1). The latter genus was excluded from the test list because it is restricted to the southern coastline of the Australian continent and does not overlap geographically with *P. aculeata*. There are no other *Parkinsonia* species native to Australia. Only one native Australian species, *Peltophorum pterocarpum* (DC.) Backer ex K.Heyne, shares the Peltophorum clade with P. aculeata (Table 1). However, there are numerous ornamental and streetscape species in this clade, and a representative species has been included in the test list (*Delonix regia* (Bojer ex Hook.) Raf.). Two *Acacia* species are included in the test list: one species with bipinnate leaves (*Acacia baileyana* F.Muell.), that has morphological similarity to the target weed but does not overlap with it georaphically, and one species with phyllodes (*A. holosericea* A.Cunn. ex G.Don.) that overlaps geographically with the target weed (Fig. 1, top right). Individual representative species from the remaining four subfamilies in family Fabaceae (Faboideae, Dialioideae, Detarioideae, and Cercidoideae), as well as the economically significant species, *Cajanus cajan* (L.) Millsp. (pigeon pea) and *Vicia faba* L. (broad bean), are also included in the test list.

Any suggestions for plant species substitutions or additions are welcomed, but we kindly ask that they be justified within the phylogenetic/evolutionary framework approach taken to develop the

host test list. We also kindly request that when proposing these additions/substitutions that you provide us with details as to where accessions of the plant species you would like added to this test list can be obtained. Feedback and comments on this proposed plant host test list can be addressed to Dr Michelle Rafter (michelle.rafter@csiro.au; 07 3833 5549).

Proposed plant host test list

Table 1: List of proposed plant species to be included in testing the proposed biological control agent for the target weed, *P. aculeata*.

Taxonomy				Test species	Relationship to target weed	Taxon status[1]	Geographic overlap with *P. aculeata*[2]	No. Australian species[3] (native/ naturalised)	Percentage coverage of the genus present in Australia
Family	**Subfamily**								
Family Fabaceae	Subfamily Caesalpinioideae	Peltophorum clade		*Parkinsonia aculeata* L.	Target weed				
				Peltophorum pterocarpum (DC.) Backer ex K.Heyne	Same clade	Native	Yes	1/0	100 %
				Delonix regia (Bojer ex Hook.) Raf.	Same clade	Ornamental	Yes	0/1	NA
		Dimorphanda Group B clade	Informal mimosoid clade	*Erythrophleum chlorostachys* (F.Muell.) Baill.	Sister clade	Native	Yes	1/0	100 %
				Acacia baileyana F.Muell.	Sister clade	Native	Limited	> 1000	0.2 %
				Acacia holosericea A.Cunn. ex G.Don	Sister clade	Native	Yes	> 1000	0.2 %
				Adenanthera abrosperma F. Muell.	Sister clade	Native	Yes	1/1	100 %
				Albizia lebbeck (L.) Benth.	Sister clade	Native	Yes	5/0	20 %
				Archidendron hendersonii (F.Muell.) I.C.Nielsen	Sister clade	Native	Yes	10/0	10 %
				Archidendropsis basaltica (F.Muell.) I.C.Nielsen	Sister clade	Native	Yes	3/0	33 %

Taxonomy								
Family	**Subfamily**	**Clade**	**Test species**	**Relationship to target weed**	**Taxon status[1]**	**Geographic overlap with *P. aculeata*[2]**	**No. Australian species[3] (native/ naturalised)**	**Percentage coverage of the genus present in Australia**
			Cathormion umbellatum subsp. *moniliforme* (DC.) Brummitt	Sister clade	Native	Yes	1/0	100 %
			Dichrostachys spicata (F.Muell.) Domin	Sister clade	Native	Yes	2/0	50 %
			Entada phaseoloides (L.) Merr.	Sister clade	Native	Limited	2/0	50 %
			Leucaena leucocephala (Lam.) de Wit	Sister clade	naturalised	Yes	0/1	NA
			Neptunia major (Benth.) Windler	Sister clade	native	Yes	5/1	20 %
			Pararchidendron pruinosum (Benth.) I.C.Nielsen	Sister clade	native	Yes	1/0	100 %
			Senegalia albizioides[4] (Pedley) Pedley	Sister clade	Native	Limited	3/2	33 %
			Vachellia bidwillii (Benth.) Kodela	Sister clade	Native	Yes	9/3	11 %
		Cassieae clade	*Chamaecrista nomame* (Siebold) H.Ohashi	Same subfamily	Native	Yes	12/1	8.3 %
			Senna artemisioides subsp. *artemisioides* (Gaudich. ex DC.) Randell	Same subfamily	Native	Yes	~ 50/7	2 %
			Cassia brewsteri (F.Muell.) Benth.	Same subfamily	Native	Yes	2/2	50 %

Taxonomy								
Family	**Subfamily**	**Clade**	**Test species**	**Relationship to target weed**	**Taxon status[1]**	**Geographic overlap with *P. aculeata*[2]**	**No. Australian species[3] (native/ naturalised)**	**Percentage coverage of the genus present in Australia**
		Caesalpinieae clade	*Caesalpinia bonduc* (L.) Roxb.	Same subfamily	Native	Yes	4/1	25 %
			Mezoneuron scortechinii F.Muell.	Same subfamily	Native	Limited	5/0	20 %
	Subfamily Faboideae		*Hovea acutifolia* A.Cunn. ex G.Don	Sister subfamily	Native	Limited	34/0	2.9 %
			Cajanus cajan (L.) Huth	Sister subfamily	Cultivated	NA	16/2	NA
			Vicia faba L.	Sister subfamily	Cultivated	NA	0/9	NA
	Subfamily Dialioideae		*Petalostylis labicheoides* R.Br.	Same family	Native	Yes	2/0	50%
	Subfamily Detarioideae		*Tamarindus indica* L.	Same family	Naturalised / cultivated	Yes	1/0	100 %
	Subfamily Cercidoideae		*Lysiphyllum hookeri* (F.Muell.) (syn. *Bauhinia hookeri* F.Muell.)	Same family	Native	Yes	6/4	16.7 %

[1]Taxon status derived from the Australian Plant Census (APC) (APC, 2022). [2]Geographic overlap with *P. aculeata* derived from distribution records downloaded from the Atlas of Living Australia (ALA) (ALA, 2022). [3]Number of Australian species consistent with the APC (2022). [4]*Senegalia albizioides* is a

rare endemic species restricted to the Cape York Peninsula. If experimental plants are unable to be sourced, this species may be substituted by one of the *Senegalia* species naturalised in Australia, as also recommended in Taylor and Dhileepan (2019).

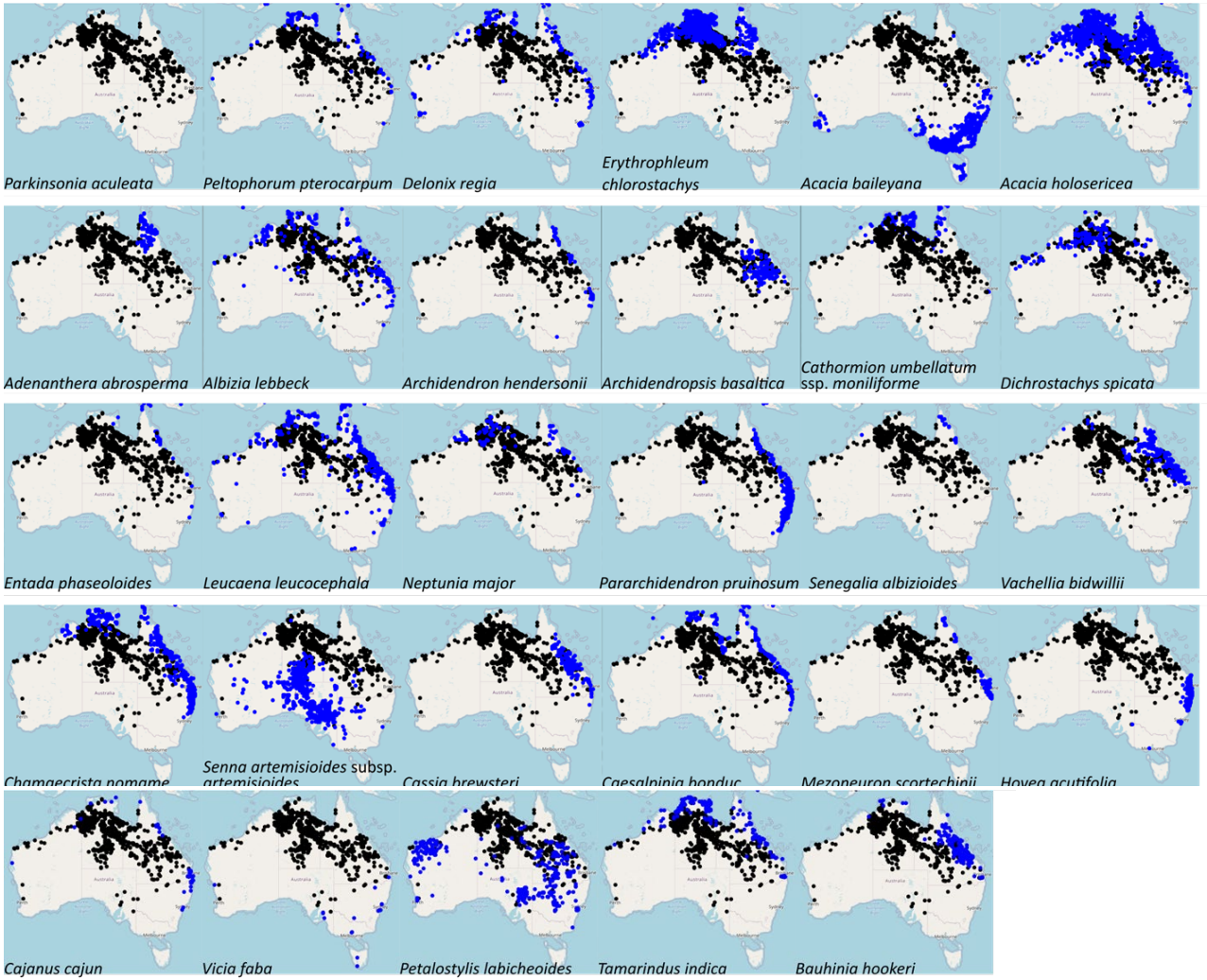

Figure 1. The spatial distribution of host test list species (blue) in relation to the target weed, *Parkinsonia aculeata* (black). Species records from the ALA (2022).

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