Department of Biodiversity, Conservation and Attractions

GOVERNMENT OF WESTERN AUSTRALIA

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Biodiversity and Conservation Science

Pollination ecology of *Aluta quadrata* – Western Range Annual Research Report 1

for Rio Tinto Iron Ore
September 2022 to September 2023

Photos Carole Elliott

November 2023

Kings Park Science, Biodiversity and Conservation Science
Department of Biodiversity, Conservation and Attractions

Rio Tinto Iron Ore (RTIO) have commissioned Kings Park Science, DBCA, to undertake a 4-year research program to assess the pollination ecology and reproductive biology of the threatened plant species *Aluta quadrata* across the Western Range, Pilbara. This report outlines the progress of year 1 (2022-2023).

The conceptual design of the research program addresses the spatial and temporal variability in key parameters, to enable an assessment of potential disturbance from mining activities through a BACI (Before-After-Control-Impact) design.

The research program has six core themes: phenology (flowering), reproduction (fruit and seed set), pollination (pollinators), breeding system (self-compatibility), mating system (outcrossing rates) and population demography.

The research commenced in September 2022 with appointment of staff and implementation of the experimental design. The design had an initial selection of 11 accessible field sites covering the range of habitats *A. quadrata* occurs in across the Western Range distribution. Sites occur at varying distances from the primary disturbance location.

Field surveys identified that 2022 was a good flowering year, with most plants in heavy flower.

In September 2022, 218 plants from the 11 sites were bagged to collect fruit. Fruit matured, dropped into the bags and were collected in November 2022, and subsequently processed in the labs at Kings Park.

Key highlights

- Research commenced, key staff recruited, and research program implemented.
- First year of baseline information on pollination, reproduction, population demography and mating system completed.
- Fruit set was abundant in 2022 and provided seeds for study objectives.
- Flowering in 2023 was poor, only 25 of 294 monitored plants flowered (only at 5 sites).
- A diversity of floral visitors (20 spp.) was observed, dominated by flies.
- Plant reproduction and condition was highly variable among sites and plants.
- Fruit and seed production across years was highly variable.
- Seedling mortality was very high in 2022 (5 of 85 seedlings survived) and no recruitment observed in 2023.
- Protocols for seed germination, DNA extraction and mating system analysis are being optimised.
- Engagement and knowledge sharing with Yinhawangka Traditional Owners.

For plant reproduction assessment, approximately 45,000 fruit were screened for seed viability by x-ray. The mean percentage of fruit with viable seed (34%) in 2022 was higher than that from a collection made in 2018 (3%). The percentage of fruit with viable seed varied among sites (range = 12%-42%), with those at the eastern end of the distribution generally showing lower seed fill. Of the fruit without viable seed, 13% contained aborted seed, 30% were parthenocarpic, 19% were aborted fruit, and 4% were predated.

For mating system analysis, 4,725 fruit containing viable seed from 11 sites were pre-treated with wet/dry cycling for eight weeks, then placed on agar plates and incubated at 25°C for seed germination. DNA will be extracted from whole seedlings once they are large enough (another 5-8 weeks). DNA extraction protocols for seedlings and maternal leaf material have been assessed and optimised. Microsatellite markers for mating system parameter estimation are being screened and optimised, and comprehensive genotyping of maternal plants and offspring is commencing.

Population demography and plant health was assessed at multiple times during 2023, on 294 permanently tagged adult plants and 21 juveniles across all 11 sites. All adult and juvenile plants were still alive after the first year of monitoring. Parameters of plant health were largely consistent across all sites, with great variation

observed among individual plants. This included reproductive effort, leaf function and biomass estimates. We observed the recruitment of eighty-five seedlings in 2022, although all but five perished by September 2023. No seedling recruitment was detected in 2023. Soil collections were made for assessment of soil-stored seedbank. Processing of soils has commenced to extract any fruit.

In contrast to 2022, it was a very poor flowering year in 2023. Flowering was largely confined to only three sites (1-13 plants per site). As a consequence, planned research on pollinator communities was negatively impacted. Despite this, observations of the insect community were made on flowering plants at KPS14 and we identified at least 20 morpho-species visiting flowers. These were primarily fly species, and their behaviour was documented to try to identify effective pollinators.

An assessment of breeding system has progressed on flowering plants in the glasshouse at Kings Park. Floral morphology and phenology have been characterised and includes the observation of a unique pollen dehiscence mechanism that makes hand-pollination challenging, particularly in the field. Despite this, hand-pollination methods have been refined, optimised and implemented, at least for glasshouse conditions, and results are pending fruit development.

Implementation of the research program has included positive Traditional Owner engagement, particularly at sites of heritage significance (KPS14 and KPS6). Program extension has included supporting two Honours students (Murdoch University, University of Western Australia) with projects on molecular analysis of mating system and pollination biology.

Ultimately, research to date has highlighted the spatial and temporal variability in the pollination ecology and reproductive biology of *A. quadrata*, which is likely driven by climatic and landscape variability. Understanding the nature of this natural variability in establishing baseline responses is critical in the assessment of potential impacts.

Photo Carole Elliott

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PROJECT OVERVIEW

Rio Tinto Iron Ore (RTIO) have commissioned Kings Park Science, Department of Biodiversity, Conservation and Attractions (DBCA) to undertake a research project investigating the pollination ecology and reproductive biology of *Aluta quadrata* Rye & Trudgen (Myrtaceae) on Yinhawangka Country in the Western Range (Pilbara, Western Australia).

Aluta quadrata is a threatened plant species, classified as endangered under the *Biodiversity Conservation Act 2016*. The species is endemic to the Pilbara and grows on rocky ranges in habitat that includes steep rocky slopes, steep gorges, and gullies, with a preference for southern facing slopes of rugged topography in skeletal soils, including Brockman Iron Formation substrates (Rye and Trudgen 2000). It has a narrow distribution with three known populations (12-22km apart). Genetic studies have shown relatively high levels of population differentiation, leading to the species comprising three conservation or management units (Byrne et al. 2016; Coates et al. 2012). This project focuses on the population at the Western Range.

Defining the pollination ecology and reproductive biology of the species will enable a greater understanding of the critical factors that influence seed set and plant fitness. Here we focus on six research themes that characterise the variation in phenology, reproduction, pollinators, breeding system, mating system and population demography across multiple sites surrounding the proposed impact area. Addressing these issues will provide a basis for the conservation and management of vital population processes, and lay the foundations for understanding potential impacts of disturbance on these aspects of pollination ecology and reproductive biology.

Research will be carried out prior to and during the mining process, including the construction of a land bridge, which will be positioned within the extent of the species' distribution on Western Range. The research program will therefore enable an assessment of possible impacts on these biological parameters for this threatened species.

Project Objective:

- To establish a baseline of understanding of the spatial and temporal variability in pollination ecology, reproductive biology and mating system of *A. quadrata*, which will enable an assessment of impact on these parameters from mine expansion works in the Western Range area.

CONCEPTUAL DESIGN PROPOSED

The Before-After-Control-Impact (BACI) approach is a statistically powerful experimental design for the assessment of environmental impact from natural variability (Underwood 1994). The BACI experimental design requires replicate sites at the impact location with replicate control sites away from the impact location, which are then assessed before, during and after impact. In this way, temporal and spatial variation in parameters are controlled for, to enable the assessment of impact. Variations of the fundamental BACI design can include asymmetrical designs to increase power to control for natural variation at temporal and spatial scales in the parameters assessed (Underwood 1994).

We proposed to implement a BACI design for the assessment of multiple parameters of interest to establish a basis for understanding impact of mining activities on *A*. *quadrata*. This conceptual design consisted of 10 sites (originally proposed eight), with four replicates at or near the impact and six control sites away from the area of impact that will be measured before and after impact (Figure 1). Exact implementation of this design was then contingent on accessibility to sites and distribution of plants across the landform.

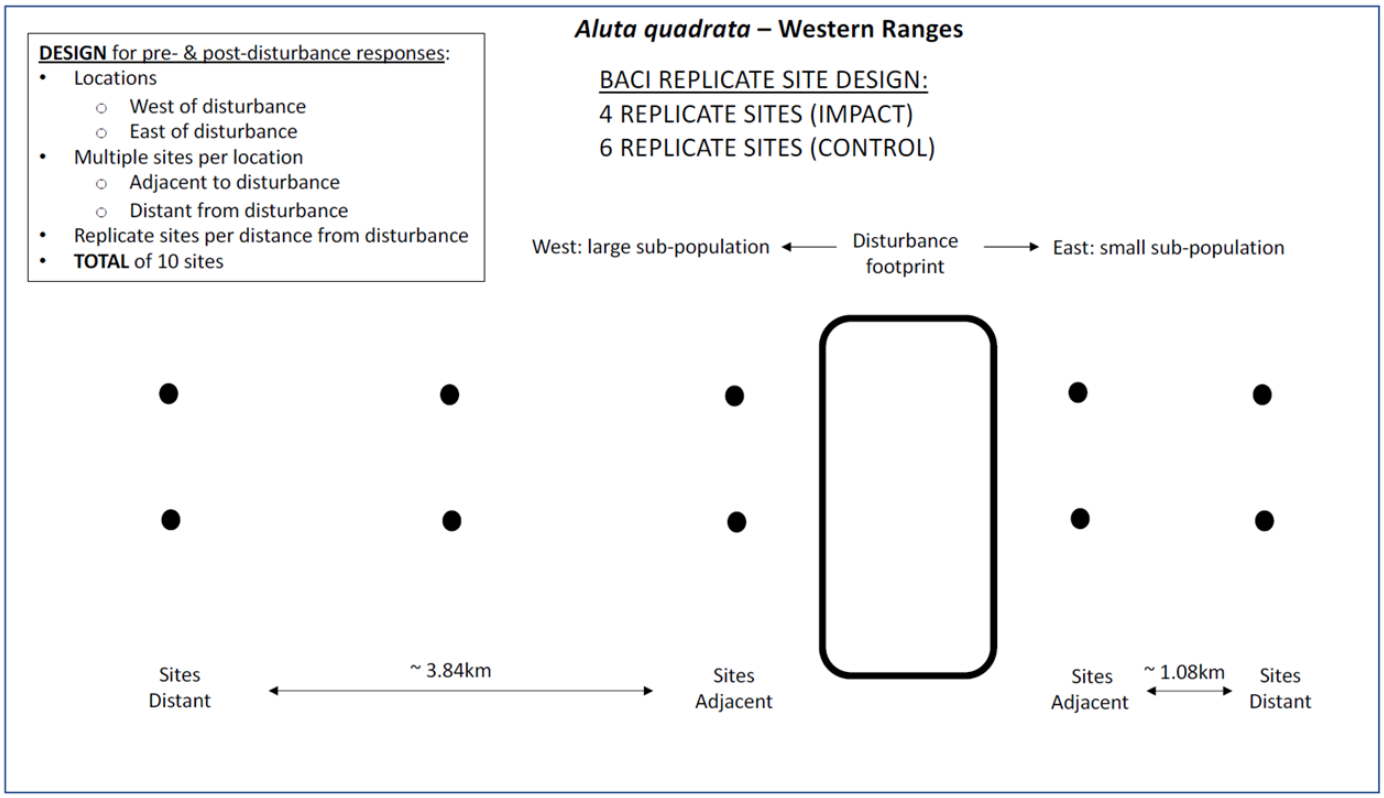

Figure 1. Conceptual BACI design for the assessment of impact on pollination biology parameters in *Aluta quadrata* at Western Range.

EXPERIMENTAL DESIGN IMPLEMENTED

The conceptual design described above was implemented at Western Range through extensive site surveys in 2022, with due consideration of logistic and safety issues. We have identified 11 field sites that enable the experimental design and provide some contingency as the program proceeds. These include seven sites west and four sites east of the proposed disturbance (Figure 2). These sites capture the range of habitat that the plants grow in and were classified into four broad habitat categories: 1) crest of a hill (crest of range); 2) on or at the base of a cliff (cliff area); 3) adjoining rock slope of creek or gully (creek rock slope, scree); and 4) creek bed (creek bed). In many sites, sampled plants occurred across multiple habitat types (see Population Demography theme, Table 5 for details). Incorporation of this classification into future analysis will enable characterisation of plant responses in relation to their habitats.

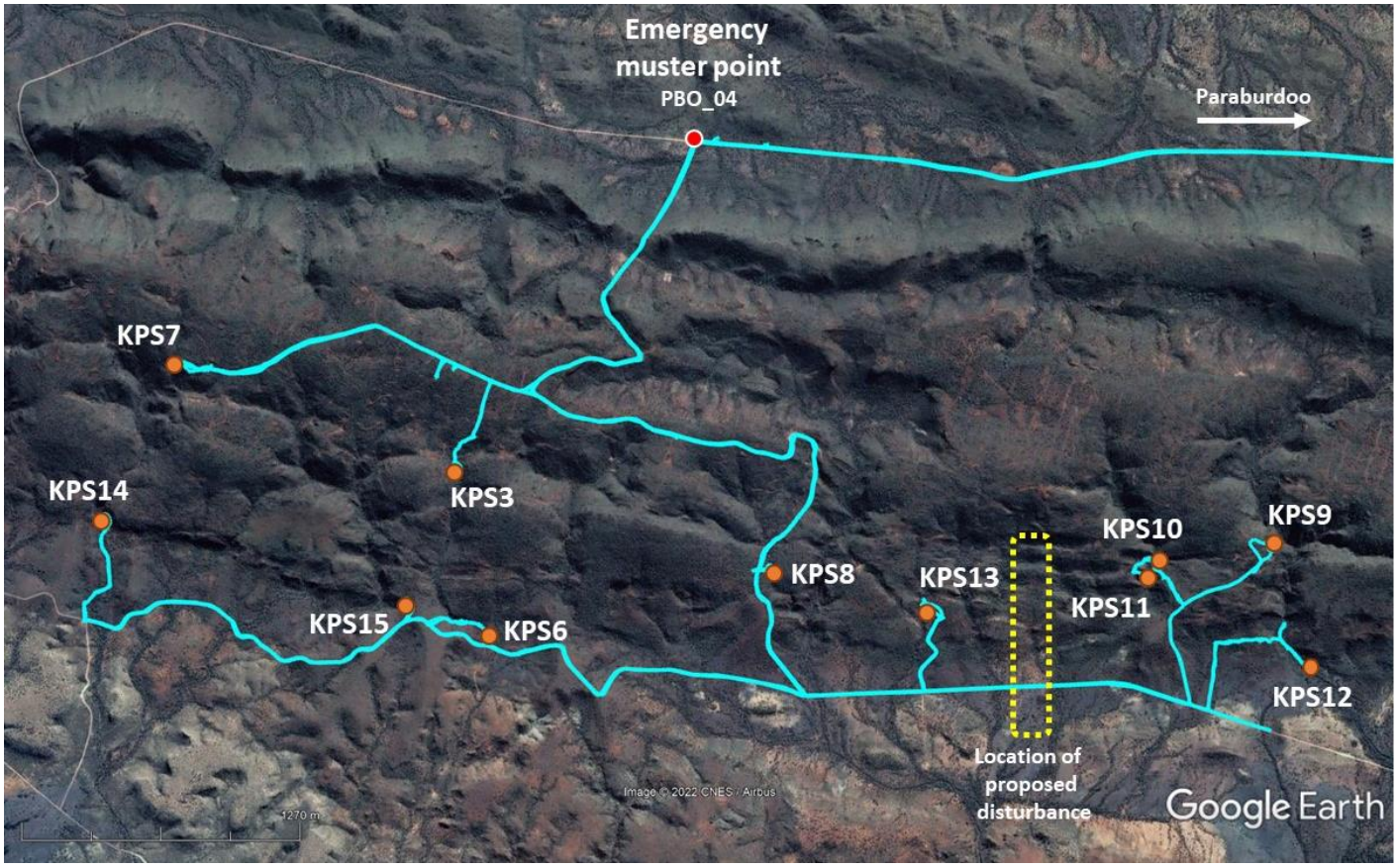

Figure 2. Potential study sites selected for *Aluta quadrata* at the Western Ranges as best fit for a BACI design (Figure 1) to assess the impact of disturbance on pollination biology parameters.

This research program shares the sampling/monitoring space with at least two other projects: an Ecophysiology Project (Lewandrowski et al. 2021-2024) and a Long-term Population Monitoring Project (Rio Tinto). Therefore, there may be a need to counteract any sampling pressure to the primary study sites by using some sites as secondary sites (e.g. Breeding System studies not dependent on site location). However, we monitored all 11 sites in this first year to allow flexibility should site choice become necessary, and/or the distances from disturbances these sites will experience changes.

This experimental design was implemented across the following six research themes 1) Phenology, 2) Reproduction, 3) Pollinators, 4) Breeding system, 5) Mating system and 6) Population demography. Each section outlines the research aims, method of data collection and analysis, progress and results, and the preliminary interpretation of the research.

1. PHENOLOGY

Aims

- Quantify the phenology of *A*. *quadrata,* and assess how the development of flowers, fruit and seed varies in response to seasonal changes in climate (e.g. rainfall, ambient temperature), plant size, location and habitat.
- Interpret flowering and seed production data to inform on the optimal timing of seed collection.

Understanding the drivers and possible impacts to plant phenology (e.g. timing of) requires the characterisation of how plants shift from a vegetative to reproductive state in response to environmental conditions (Rathcke and Lacey 1985), and would assist in the strategic planning of the timing and anticipated quantity of seed that may be collected in a given year (Morellato et al. 2016; Ritchie et al. 2017).

Method

The development phases of 294 adult plants from 11 sites were monitored during 2023 (Figure 2). For each tagged plant (16-32 plants per site), the vegetative or reproductive status of that plant was recorded during each monitoring period (Figure 3). Furthermore, floral parts were sampled to track floral development and document phenology of reproduction (5 plants per site; 3-5 reproductive units per plant).

Progress

The majority (80.5%) of plants failed to flower at all (e.g. remained vegetative). Some plants (11%) initiated reproduction (buds present in June) but these buds failed to develop into flowers by August/September. The remaining 8.5% of plants had buds that developed into flowers (and some fruit) by September (see Population Demography theme, Table 5 for details). Poor flowering for 2023 meant we could not collect a suitable number of floral samples during the reproductive phase to document plant phenology in detail. For the 2024 season, the tracking of floral and seed development outlined above will be implemented, should flowering occur.

Preliminary interpretation

- The spatial variability of whether a plant flowered in 2023 highlights the stochasticity in the system.
- *Aluta quadrata* plants do not/cannot flower every year – trigger is unknown but is likely related to climate, particularly rainfall, and the spatial location of the plant.

Figure 3. Vegetative (A) and reproductive (B and C) phases of *Aluta quadrata* (2023). Photos: Carole Elliott.

2. REPRODUCTION

Aims

- Generate baseline data on the variability of flower, fruit and seed production of mature plants of *A. quadrata* and investigate if this changes across sites and years.
- Determine if *A. quadrata* is pollen limited and investigate if this changes across sites and years.

Understanding how flower, fruit and seed production rates are influenced by the size and condition of plants, availability of resources, the breeding system, the availability or density of mates, plant-pollinator or plant-predator interactions provides information on spatial and temporal stochasticity, for which may vary between years depending on environmental conditions (e.g. Yates et al. 2008; Aguilar et al. 2006).

Method

Fruiting (2022)

Project commencement in September 2022 was after peak flowering, so we were unable to collect data on flowering and hence conversion to fruit for the 2022 season. However, almost all plants were observed to have many fruit, and an abundant amount of fruit was collected to investigate the quality of the fruit (e.g. amount of fruit with viable seed) produced in 2022. These fruit will also be used in the Mating System study.

Fruit were collected by placing organza bags on fruiting branches in September, which were then collected in November after the fruit had ripened and naturally dropped off plants into the bags. Fruit were kept dry for transport and stored in Kings Park's controlled environment drying room that is maintained at 15-20°C and 15-20% relative humidity until needed. Fruit were cleaned and a random sample from each plant assessed for quality (n = 30 fruit from each of 214 plants; Table 1) and morphological assessment (n = 5 fruit/plant for 10 plants/site). Fruit were placed on a gridded Perspex tray for imaging in a Faxitron Multifocus Digital X-ray Cabinet, to classify fruit quality. Five classes of fruit quality were defined as: aborted fruit (AF); parthenocarpic fruit (PC); fruit with aborted seed (AS); fruit with viable seed (S; as inferred from x-ray image); or predated fruit (PR; Table 1; Figure 4).

Morphological characteristics of fruit (weight, diameter, height) with viable seed (S) were measured to determine if the size of fruit produced (i.e. maternal resourcing) varied among the sites or plants monitored. Fruit morphology was measured using a Nikon SMZ18 microscope and imaged with a DS-Fi3 camera and NIS-Elements L software. This fruit quality classification can provide information on the outcome of pollination, fertilisation and predation for *A. quadrata* across the 11 sites for 2022.

Flowering (2023)

For 2023, field assessment of plant reproduction was conducted on 16-32 tagged plants per site (Figure 2; see Population Demography theme, Table 5 for details). Plants were randomly selected, and their size and health were measured (see Plant Demography research). Each plant was assessed for the conversion ratio of flowers to fruit and the production of viable seed, by tagging three random branches and counting reproductive units (e.g. bud, flower, fruit) for every monitoring period. At each site, 10-15 flowers per plant (if available on 5-10 untagged plants per site) will be collected for assessment of pollen load levels to provide information on the success rates of pollen being deposited on stigmas. Furthermore, a subset of flowers at each site will be augmented with additional pollen (i.e. hand-pollinated) to examine if the species is pollen limited (same

untagged plants if available). Depending on the sites chosen, plant density may need to be measured to account for variation in reproduction that occur due to influences of mate or resource availability, and also inform any differences observed in pollinator visitation due to floral density.

Table 1. Five classes of fruit quality (aborted (AF); parthenocarpic (PC); aborted seed (AS); seed (S); predated (PR)) employed here to provide information on the pollination, fertilisation and predation outcomes of reproductive effort among sites of *Aluta quadrata*.

CODE	Name	Classification	Description
AF	Aborted Fruit	Flowers that failed to develop into fruit	• Morphology: small, pale and disc on top of hypanthium is concave. • X-ray: centre of fruit is a very small, hollow sphere (no tissue) • Pollination or fertilisation absent or incomplete
PC	Parthenocarpic	Fruit develops without fertilisation of ovule	• Morphology: rust brown hypanthium, ruby red disc on top of hypanthium that is convex and reticulate-pitted (Rye and Trudgen 2000). • X-ray: unclear outline of moderately dense tissue that has irregular edges and wavy lines through it; tightly packed within the fruit • Unknown if pollination required to stimulate response
AS	Aborted Seed	Fruit develops but the seed fails to develop	• Morphology: rust brown hypanthium, ruby red disc on top of hypanthium that is convex and reticulate-pitted. • X-ray: clear outline of a distinct kidney-shape that is hollow (no tissue) roughly the size of a seed; with the other ovules squashed up to one side of the fruit wall (check for hole(s) in fruit wall) • Pollination and fertilisation occurred but seed development discontinued
S	Seed	Fruit and seed develop	• Morphology: rust brown hypanthium, ruby red disc on top of hypanthium that is convex and reticulate-pitted. • X-ray: clear outline of a distinct kidney-shape with tissue of a consistent and quite dense texture that fits tightly within the fruit; with the other ovules squashed up to one side of the fruit wall. Inferred to be viable seed. • Successful pollination, fertilisation and seed development
PR	Predated	Fruit but no seed – evidence of predation (insect larvae)	• Morphology: rust brown hypanthium etc. (as above), but tiny hole(s) might be observed drilled into fruit wall. • X-ray: clear outline of a distinct kidney-shape that is hollow, with hole(s) present in fruit wall; or insect larvae is present (Figure 4a inset) with a clear outline of a distinct kidney-shape that contains a smaller kidney-shape that is moderately dense with two tones of intensity (denser inner layer) and may appear segmented • Successful pollination, fertilisation and seed development but seed consumed by a predator (insect larvae unknown) before dispersal.

Data analysis – Fruiting (2022)

Initially, we grouped the five classes of fruit quality into those that contained seed (S) and those that did not (AF; PC; AS; PR). We fitted a generalised linear regression model, using a binomial function, to these two groups to analyse for any differences between fruit with seed versus those with no seed. We then tested for differences among the 11 sites by modelling independent linear regression models for each fruit quality class separately (e.g. identified whether there was a difference between sites in the proportion of aborted fruit), using the Tukey's pair-wise Honest Significant Difference test to determine any differences.

We assessed the morphological characteristics (weight, diameter, height) of fruit with seed to determine if there were any differences among sites. We used an ANOVA to assess each morphological variable separately (e.g. determine if fruit height differed among sites). Examination of statistical assumptions found that our data had unequal variance so we used Welch's ANOVA, which does not assume equal variance between groups. We then used Tukey's post-hoc pairwise analysis (t.test) to evaluate which pairs of sites had statistically different fruit morphology.

All parameters in this report were analysed in the statistical environment, R (R Core Team 2023), by fitting linear and generalised linear regression models, and performing post-hoc tests using the 'stats'package. We used the package 'ggplot2' to create some of the graphs, as well as the 'graphics' package (Wickham 2016).

Results and Progress

Fruiting (2022)

For the 2022 fruit collection, 34.7 ± 1.3% of sampled fruit contained viable seed (S: range of 0-80% per plant). Of the fruit that did not contain seed, 30.1 ± 1.4% were parthenocarpic (PC), followed by those with an aborted state at either the fruit (AF) or seed (AS) development stages, and predation rates were ~3.7 ± 0.6% (PR: range of 0-87% per plant; Figure 4).

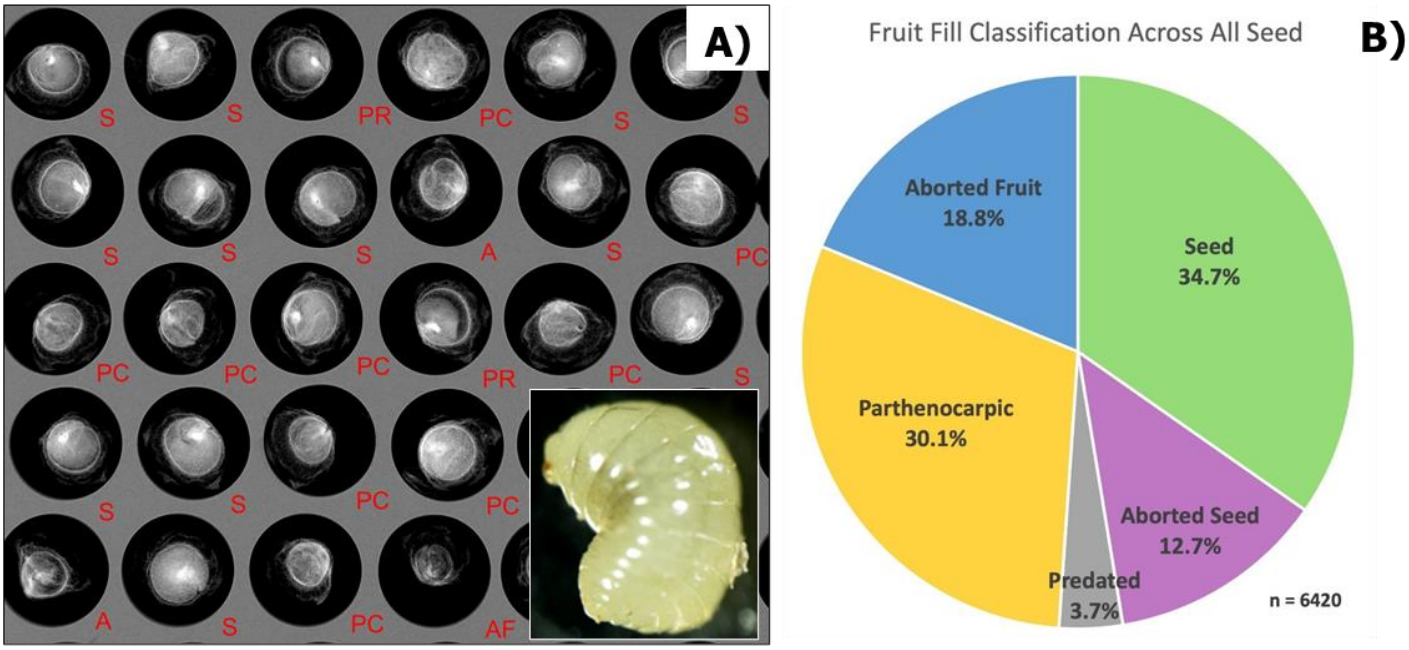

Figure 4. *Aluta quadrata* fruit quality for the 2022 collection (*n* = 6,420 fruit classified). A) Example of an x-ray image used to classify fruit, and the inset shows the larvae that predate on seed (plant KPS7_14; *n* = 30 fruit); and B) Overall percentage of each fruit quality class for the collection (aborted fruit (AF); parthenocarpic (PC); aborted seed (A); seed (S); predated (PR)).

Within each site, there was large variation in the fruit quality among the plants sampled (Figure 5). Comparing the proportion of fruit that contained viable seed against fruit without viable seed showed statistically significant differences among the sites. For example, KPS10 had the lowest amount of seed production (0.12 ± 0.02 SE of sampled fruit had seed; p <0.05) when compared to the other sites (0.21-0.47 of fruit had seed; Figure 5).

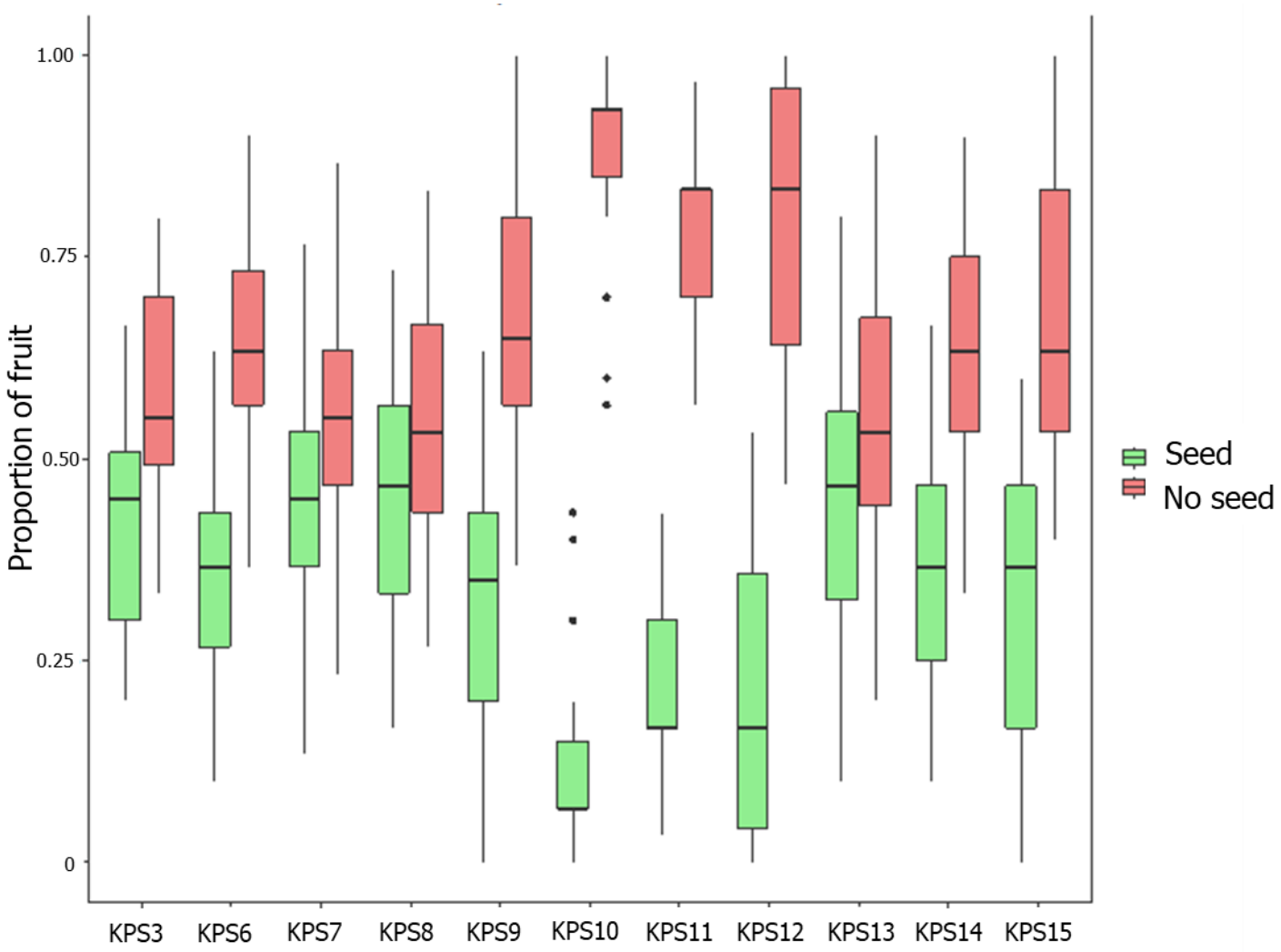

Figure 5. Fruit quality of 2022 collection of *Aluta quadrata*, comparing of the proportion of fruit with viable seed (S) against fruit without viable seed (AF; PC; AS; PR) for each of the 11 sites sampled (n = 30 fruit/plant for each site; KPS3-KPS15). Median represented by straight line through box (site mean not presented).

Further examination of the proportion of fruit with no viable seed showed that sites differed depending on the classification type of fruit with no viable seed. For example, our pairwise analysis indicated that KPS10 and KPS12 differ significantly from the rest of the sites (p <0.05), with higher mean proportion of aborted fruit (0.66 ± 0.07 and 0.42 ± 0.10, respectively; $F_{10,203}$ = 3.97; p <0.05) than the other sites (Figure 6A). In addition, KPS6, KPS9 and KPS10, had a significantly lower proportion of parthenocarpic fruit, than the other sites ($F_{10,203}$ = 3.86; p <0.05; Figure 6B). The proportion of fruit with aborted seed was significantly higher for KPS6 and KPS15 (0.20 ± 0.03 and 0.22 ± 0.05, respectively; $F_{10,203}$ = 14.7; p <0.05), with KPS10 having significantly lower aborted seed (0.05 ± 0.01). Proportion of predated fruit was similar among all sites ($F_{10,203}$ = 1.88; p = 0.0503; Figure 6C and 6D).

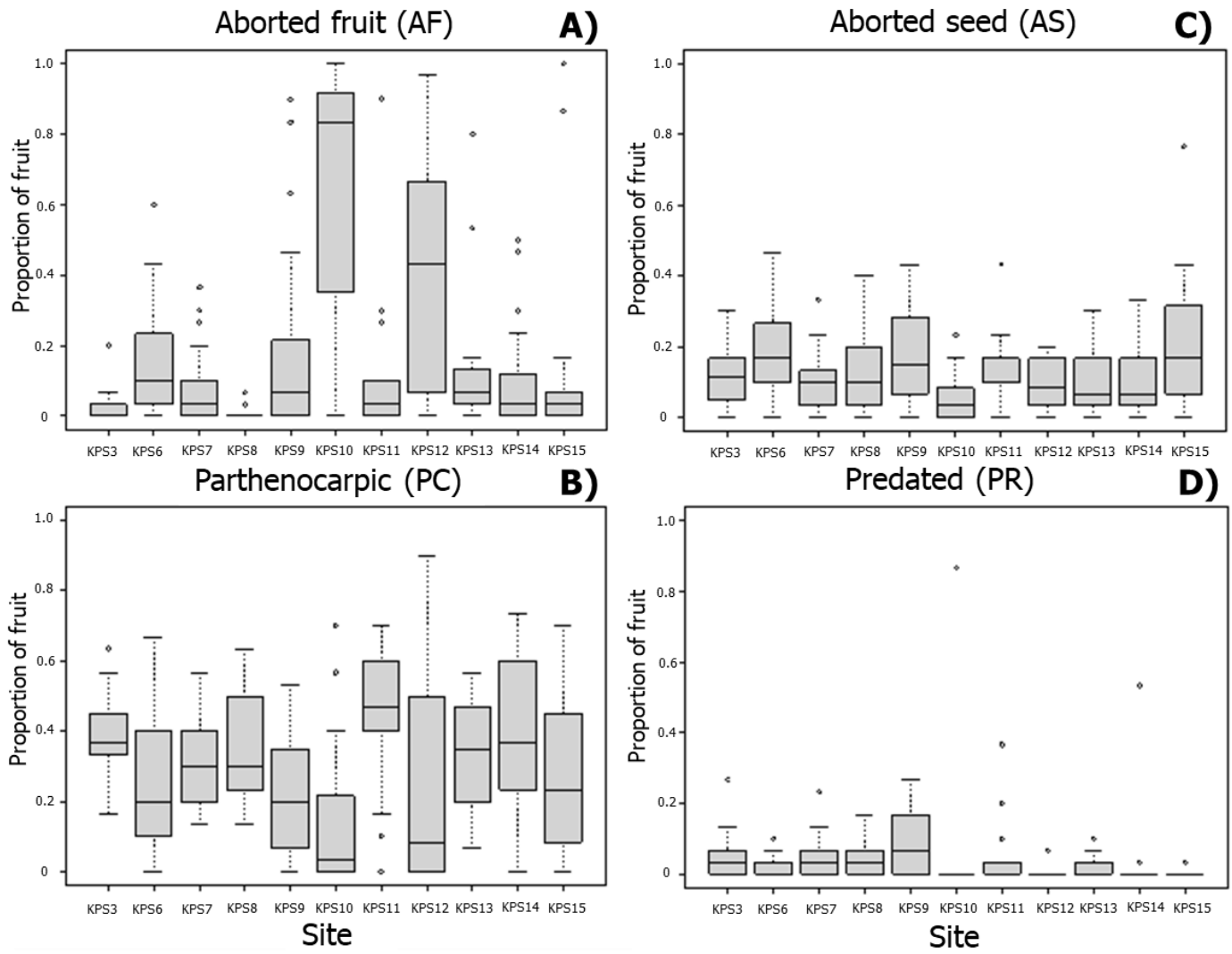

Figure 6. Fruit quality of 2022 collection of *Aluta quadrata*, comparing of the proportion of A) aborted fruit (AF); B) parthenocarpic fruit (PC); C) fruit with aborted seed (AS); and D) predated fruit (PR) for each site. Median represented by straight line through box (site mean not presented).

The morphological characteristics of fruit with viable seed (S) also showed large amounts of variation, indicating that fruit development differed among individual plants within a site (Figure 7). Analysis of these characteristics showed that fruit weight, diameter and height differed significantly among sites (F_{109} = 13.3-18.9; p <0.05; Figure 7D-F), with some sites having lighter and smaller fruit (e.g. KPS10, KPS12) on average than other sites (e.g. KPS3, KPS6).

Flowering (2023)

Unlike the flowering period of 2022 in which all plants were observed in reproductive state, 2023 was a very poor flowering year. There was extreme variation in the distribution of this poor flowering across sites and among individual plants (Table 2). Therefore, we could not tag three random branches and count reproductive units (e.g. bud, flower, fruit), as initially planned. Instead, for those plants that had initiated reproduction, we counted the number of reproductive units on the whole plant, for each monitoring time point. If the number of reproductive units on a plant was large (>300 reproductive units), an estimate was taken by counting the

number of reproductive units on a representative sized branch (e.g. branch units were ~20cm long) and then counting the number of these sized branches on that plant.

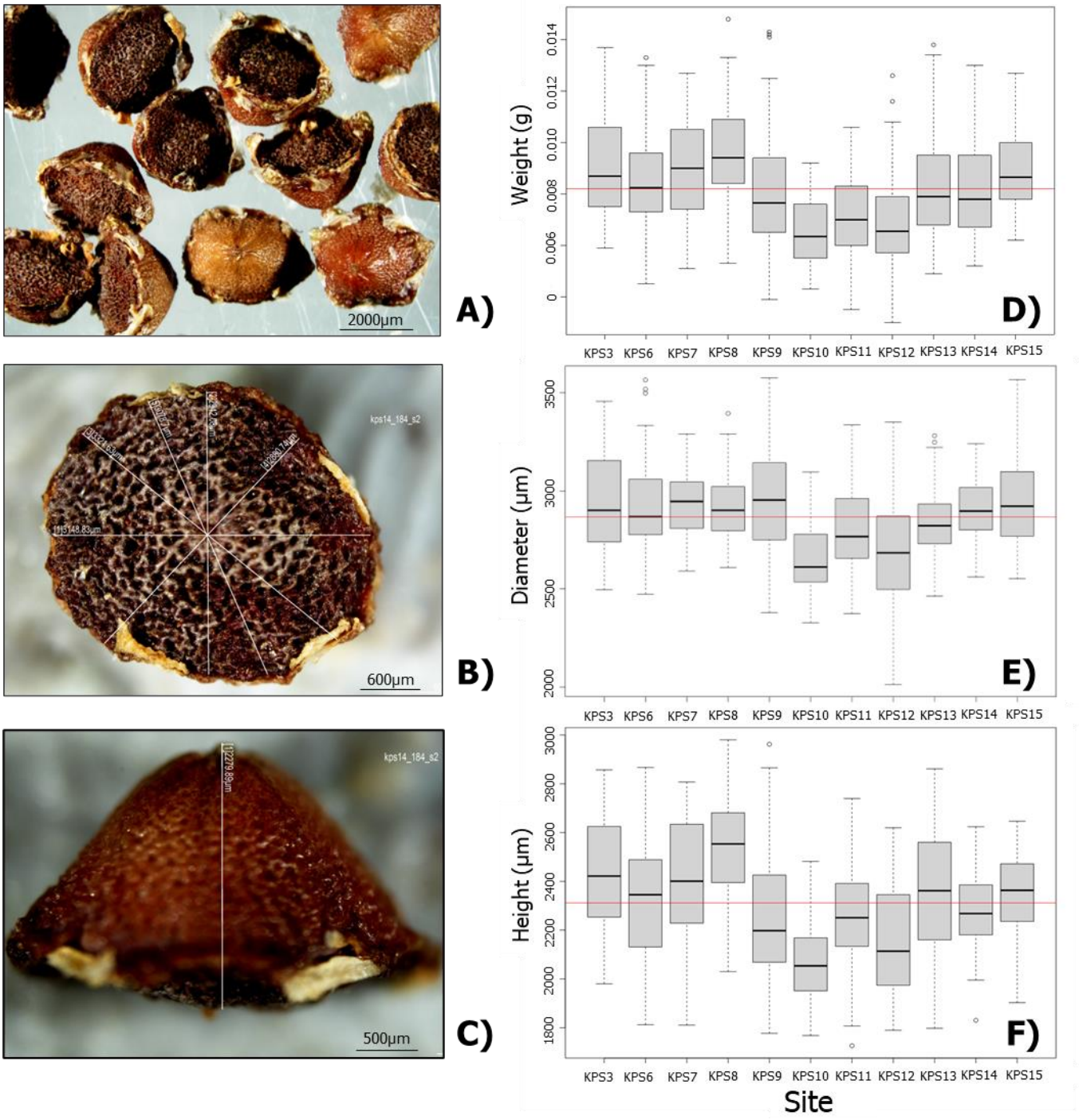

Figure 7. Morphological characteristics of fruit with viable seed (S; as determined by x-ray) of 2022 collection of *Aluta quadrata* (*n* = 550 fruit). A) Fruit cleaned and ready for measurements; B) View of convex and reticulate-pitted disc with the five diameter measurements (between the fruit bracts) that were averaged for each fruit; C) Side view of fruit showing the height measurement (base of fruit to the base of style) recorded; D) Fruit weight among the sites; E) Fruit diameter among the sites; and F) Fruit height among the sites. Median represented by straight line through box, with the overall sample mean being the red line through the plots. Photos: Emilie Perez-Wright and Nicole Maher.

As such, the reproductive plants were of two groups: those that were observed to initiate reproduction (buds present in June) but failed to develop into flowers by August/September 2023 and those few that produced flowers for 2023 (Table 2). Overall, most sites had one or more plants observed to be flowering, however, only three sites (KPS3, KPS7, KPS14) contained most of these reproductive plants (70%) and only one site (KPS14) had plants that were observed to be beginning to produce fruit from these flowers. In addition, the reproductive output estimated on a single plant was as low as one bud and as high as ~38K flowers, indicating that plant location (e.g. broad habitat or macrohabitat) may strongly influence reproductive effort under certain conditions.

Where possible (KPS14 only), we collected 10-30 flowers from one or two plants (June and July) and placed them in 70% ethanol. They are in storage awaiting pollen load assessment using fluorescence microscopy.

Table 2. The number of tagged plants that were vegetative (Group1) or reproductive at each site for 2023, including the range of reproductive units (i.e. bud, flower, fruit) estimated for plants that initiated reproduction by budding but failed to flower (Group2) and those that produced flowers (Group3).

	Group 1	Group 2		Group 3	
Site name	Number of vegetative adults	Number of plants that initiated reproduction (buds)	Number of buds per plant (i.e. initiated only)	Number of plants that produced flowers	Number of flowers per plant
KPS3	17	12	8 – 1,900	0	0
KPS6	28	0	0	1	12
KPS7	20	9	1 – 280	0	0
KPS8	22	1	1	7	10 – 85
KPS9	22	1	2	3	3 – 82
KPS10	27	1	23	0	0
KPS11	20	0	0	1	54
KPS12	22	1	5	0	0
KPS13	31	1	2	0	0
KPS14	12	6	5 – 290	13	5 – 38,448
KPS15	16	0	0	0	0
Total	237	32	…	25	…

Preliminary interpretation

- For *Aluta quadrata* (Western Range; 2022 collection), the mean proportion of fruit with viable seed was ~34.7 ± 1.3% and seed predation was ~3.7 ± 0.6% (range of 0-87% per plant).
- There is substantial year to year variation in seed production (Figure 8). Seed set in 2022 was much higher than that of a previous (2018) collection from the Western Range (Merritt and Erickson 2020), which reported a proportion of fruit with viable seed of ~3% (per plant).
- Seed set varied substantially among plants, from 0-80% in 2022 and 0-42% in 2018
- In 2023, flowering was very poor (only ~8.5% of plants flowered).
- Measures of fruit quality (aborted fruit (AF); parthenocarpic fruit (PC); fruit with aborted seed (AS); fruit with seed (S); and predated fruit (PR)) and fruit morphology (S: weight; height; and diameter)

varied substantially among plants and significantly among some sites. This indicates *A*. *quadrata* can experience highly variable outcomes of pollination, fertilisation, predation and maternal resourcing in response to certain environmental cues or triggers.

- The highly variable (spatial and temporal) reproductive capacity of *A*. *quadrata* is likely to be influenced by differences in habitat and climate (Lewandrowski et al. 2022). Environmental parameters, including climate of Western Range, will need to be examined to address the causative links. In addition, location of sites (broad habitat type) and individual plants (macrohabitat), and characteristics of individual plants (e.g. plant size), need to be examined to assess their relationship with patterns of variation in reproductive capacity from production, quality or morphology perspectives.
- For the 2024 season, the more detailed pollen load assessments and hand-pollinations outlined in the project proposal will be implemented, should flowering occur.

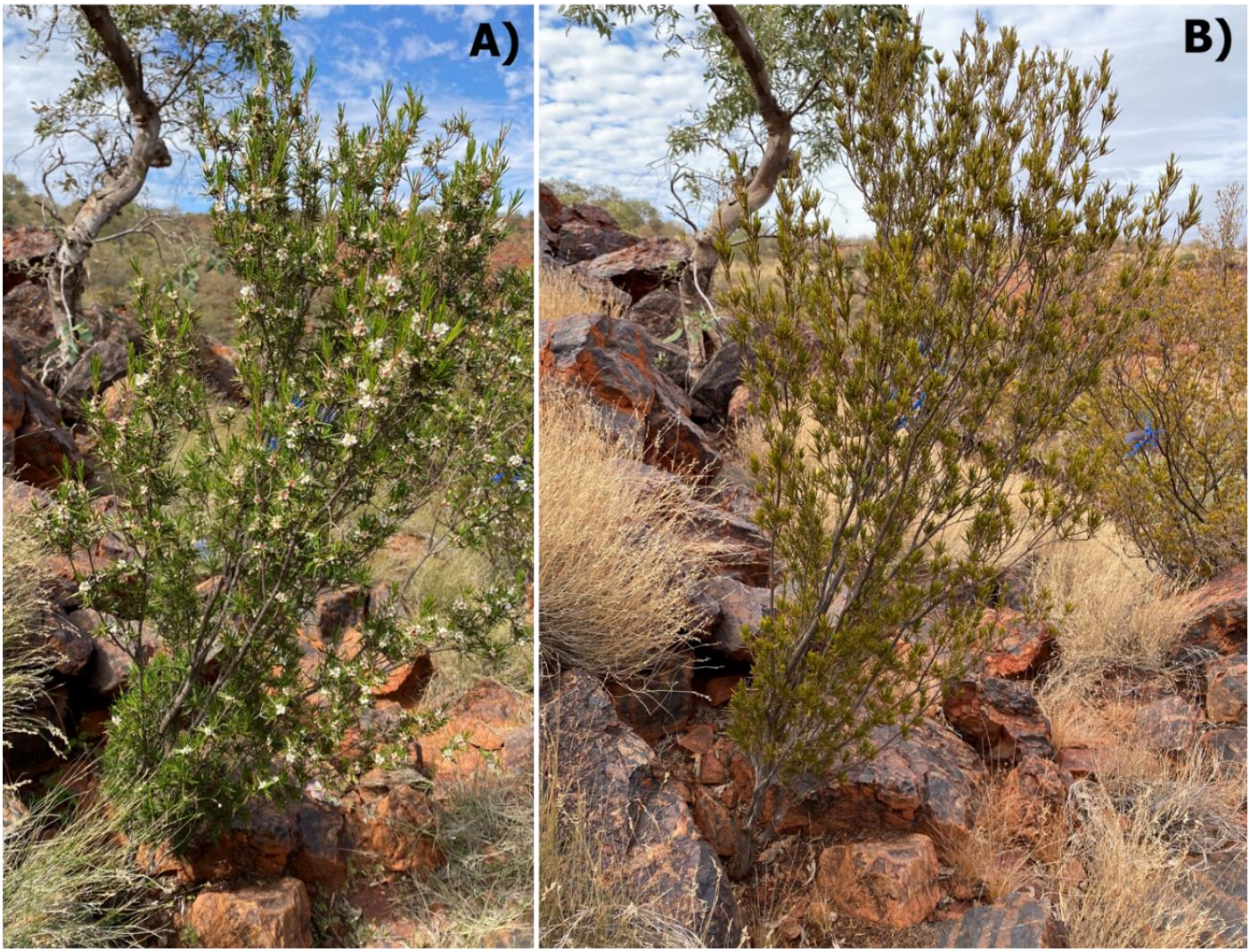

Figure 8. Example of year to year variation in *Aluta quadrata* reproduction. A) flowering plant at site KPS3 in August 2022; and B) the same plant 10 months later with no flowers (June 2023). Photos: Siegy Krauss.

3. POLLINATORS

Aims

- Identify the pollinator community of *A*. *quadrata.*
- Compare composition and visitation of pollinators to *A*. *quadrata* among different locations and years.

It is critical to identify pollinator diversity, quantify their activity and interaction with *A*. *quadrata* flowers, to understand how they vary between years depending on environmental conditions and what affects the dynamics of the pollinator community that the plant species may depend on.

Method

Identification of the pollinator community was to be done through a targeted collection of insect visitors to *A*. *quadrata* flowers. This involved sweep netting 10-15 plants per site during periods of highest insect activity, mid-morning, 2-3 times per season, and a generalised collection of insects at a site (2-5 vein or pan traps and/or 1 Malaise trap open during field trip) during the flowering period. Specimens of the insects that were observed interacting with flowers were collected into 70% ethanol to confirm their identity, but was done at the end of the observation time period to avoid disturbing insect behaviour. The insects were taxonomically identified and scored for the abundance of *A*. *quadrata* pollen on their bodies (measures of pollen presence dependent on the sampling method used).

Visitation rates and behaviour of floral visitors will be recorded by observing insects on plants either by surveys or cameras. Observers (2-3) will record the morpho-species and visitation behaviour for several periods, at 4-8 sites during peak flowering. Cameras will be deployed with 2-5 cameras at 4-8 sites, and footage collected 2-3 times per season using time-lapse modes (one image every 0.5 s to maximise battery life). In addition to sites within the *A*. *quadrata* population, sites in the surrounding or intermediate vegetation (i.e. no *A*. *quadrata*) will be sampled for insects (likely to be 2-5 vein or pan traps and/or 1 Malaise trap open during field trip) to assess their presence in alternate habitats that may facilitate insect movement between patches of *A*. *quadrata*.

Results and Progress

The lack of flowering in 2023 prevented the development and implementation of the planned survey methods across the 11 monitoring sites. However, there were 4-6 plants flowering at one site (KPS14) that provided an opportunity to conduct a study into the composition and behaviour of the pollinator community on *A*. *quadrata* under conditions of low flowering intensity (e.g. very few plants). Visitation rates, behaviour of floral visitors and their identity were recorded by three people observing insects for 1-2 hours on three different days (total observation time = 11 hours, plus opportunistic video) on four *A*. *quadrata* plants. Five insect specimens were collected (i.e. sweep net) for identification at the end of the observation time period to avoid disturbing insect behaviour, and several more were identified through photographs and video.

We found 20 insect morpho-species present on four *A*. *quadrata* plants at KPS14 and recorded observations of insect visitation to flowers for 19 morpho-species from five groups of insects (Table 3). Fly species were the dominant visitor to flowers, with four other groups of visitors observed (examples in Figure 9). A partial analysis of the dataset (not all data transcribed at time of writing) has shown insects feed differently to one another. An individual fly would visit 1-35 flowers in a continuous feeding bout, spending 1-10 seconds/flower

for large fly species and >2 minutes/flower for tiny fly species. In comparison, butterflies would visit 1-12 flowers in a continuous feeding bout, spending 30-90 seconds/flower. In general, individual insects would occasionally visit the same flower twice as it moved among flowers, but the dominant feeding patterns observed were movements to new flowers on the same plant.

Preliminary interpretation

- The floral visitors to *A*. *quadrata* at one site were diverse and dominated by Diptera species.
- Very poor flowering in 2023 meant that the full protocol could not be implemented as planned. For the 2024 season, these more comprehensive pollinator community surveys and observations of behaviour outlined in the project proposal will be implemented, should flowering occur.

Table 3. Morpho-species insects observed on *Aluta quadrata* (KPS14 only), June 2023.

Group	Field morpho-species ID	Family/Genus/Species	Visited flowers
Fly	Ant fly	Diptera sp.	Y
	Blue metallic fly with white spot	Diptera sp.	Y
	Bronze metallic fly	Diptera sp.	Y
	Bush fly	Diptera sp.	Y
	Bush fly (with blue on it)	Diptera sp.	Y
	Fly	Diptera sp.	Y
	Grey and white striped with yellow belly	Diptera sp.	Y
	Hoverfly	Diptera sp.	Y
	Hump-backed fly	Diptera sp.	Y
	March fly (black brown)	Diptera sp.	Y
	March looking fly (stripey)	Diptera sp.	Y
	Metallic green fly	Diptera sp.	Y
	Wingback fly	Diptera sp.	Y
	Yellow-backed fly	Diptera sp.	Y
Butterfly	Skipper	*Nacaduba biocellata*	Y
	Monarch	*Hypolimnas misippus*	N
Wasp	Orange & black wasp	*Abispa ephippium*	Y
	Wasp (small)	Hymenoptera sp.	Y
Hornet	Spider wasp	Pompilidae sp.	Y
Ant	Golden ant	*Polyrhachis* (*pilbara*?)	Y

Figure 9. Examples of floral visitors observed on *Aluta quadrata*, June 2023: A) Butterfly (*Nacaduba biocellata*; "Skipper"); B) Unidentified fly ("Hump-backed"); C) Unidentified fly ("Fly"); and D) Wasp (*Abispa ephippium*; "Orange and black wasp"). Photos: Carole Elliott.

4. BREEDING SYSTEM

Aims

- Identify the breeding system of *A*. *quadrata*.
- Determine if there are fitness differences associated with self- and cross-pollination.

Both self-compatible and self-incompatible plants can exhibit negative reproductive responses to disturbance (Aguilar et al. 2006) and knowledge of the breeding system is critical to understanding the potential impacts to plant fitness and population viability.

Method

Identification of the breeding system involved characterisation of floral features and hand-pollination experiments. Features of the pollen and stigma were characterised to identify the most likely breeding system of *A*. *quadrata* (de Nettancourt 1997). Hand-pollinations were carried out to determine if the species is capable of self-pollination or reliant on out-crossed pollen for seed set. Comparing naturally pollinated seed to these hand-pollinated seed, in the future, will determine the potential fitness effects (if any), coupled with the baseline patterns of seedling fitness, that may provide information on changes in seedling performance through time or after a disturbance event (Aguilar et al. 2019).

Buds and flowers were collected from plants in September 2022 (*n* = ~50 units) to examine floral morphology under a microscope to identify the most suitable hand-pollination technique that should be tested based on floral architecture.

The stage when stigmas are receptive for pollination will be determined via preliminary hand-pollination trials. This preparation work determines which stage of floral development should be targeted and what visual cues can be seen (e.g. surface changes of the stigma from smooth to rough) to enable effective hand-pollination. The hand-pollination experiment will consist of three treatments (control, self-pollination, out-crossed pollination) with replication, on multiple plants at 1-3 sites. The fruit generated from hand-pollinations will be collected and quality assessed (as described previously). This seed will be germinated to quantify any fitness effects among seedlings of different treatments (e.g. biomass, growth rates, fecundity).

The lack of flowering in 2023 prevented the development and implementation of the planned hand-pollination experiments in the field at Western Range. However, nursery grown plants from a previous project (Merritt and Erickson 2020) reached reproductive maturity in 2023 (e.g. they were 5 years old; also see Wright et al. 2019) and were able to be used to investigate floral morphology, floral phenology and develop the techniques required for hand-pollinating flowers (Figure 10).

Under glasshouse conditions, floral phenology was tracked by monitoring floral development from bud to open flower regularly (*n* = 18 plants; 145 buds). Hand-pollinations were carried out in the glasshouse on available flowers (Mar-Jun 2023) and this was to test and determine an appropriate technique. Hand-pollinations were carried out on tagged flowers (Figure 10B and 10C) with outcrossed pollen (*n* = 12 plants; 26 flowers) that were left to mature to see if fruit/seed developed and determine whether this technique was effective. Furthermore, to test the effectiveness of pollen transfer in the hand-pollination technique, we hand-

pollinated a subset of flowers (*n* = 30 outcrossed flowers; *n* = 4 selfed flowers), and 48 hours later harvested them and placed them in 70% ethanol to assess pollen loads and/or germination of pollen on the stigma.

Figure 10: Nursery grown *Aluta quadrata* plants were reproductively mature in 2023 (~5 years old) and were used to investigate floral morphology (A), phenology (B and C) and hand-pollination techniques (C and D). Photos: Carole Elliott.

Progress

Floral morphology

Preliminary observations of dissected buds and flowers (Figure 11) indicated a unique pollen dehiscence or display mechanism that may provide a significant challenge to hand-pollinations, particularly in the field. It appears that the anthers (<0.5 mm) retain the pollen within a sac structure that has two small pores. It is through these pores that the pollen is squeezed out as a "toothpaste-like" material and it is not clear what triggers this process to occur (e.g. age of bud/flower or changes in humidity/temperature). Figure 11 illustrates these structures and mechanisms from material collected from the field and processed in the laboratory. It shows the position and stage of anthers inside buds and flowers that are 2-3mm in size on the left-hand side (Figure 11A-C) and a time series over 20 minutes of how the pollen is expelled from the anthers on the right-hand side (Figure 11D-F).

Observations of flowers in the glasshouse in 2023 found that pollen could also be seen as threads in the flower in the absence of insects (Figure 11B), but these threads easily disappeared when they touched another surface. We theorise that pollen threads are held together by surface tension of a fluid. These observations flag a potential challenge in determining the timing, collection technique and application technique of pollen for hand-pollinations of these very small flowers with this unique system.

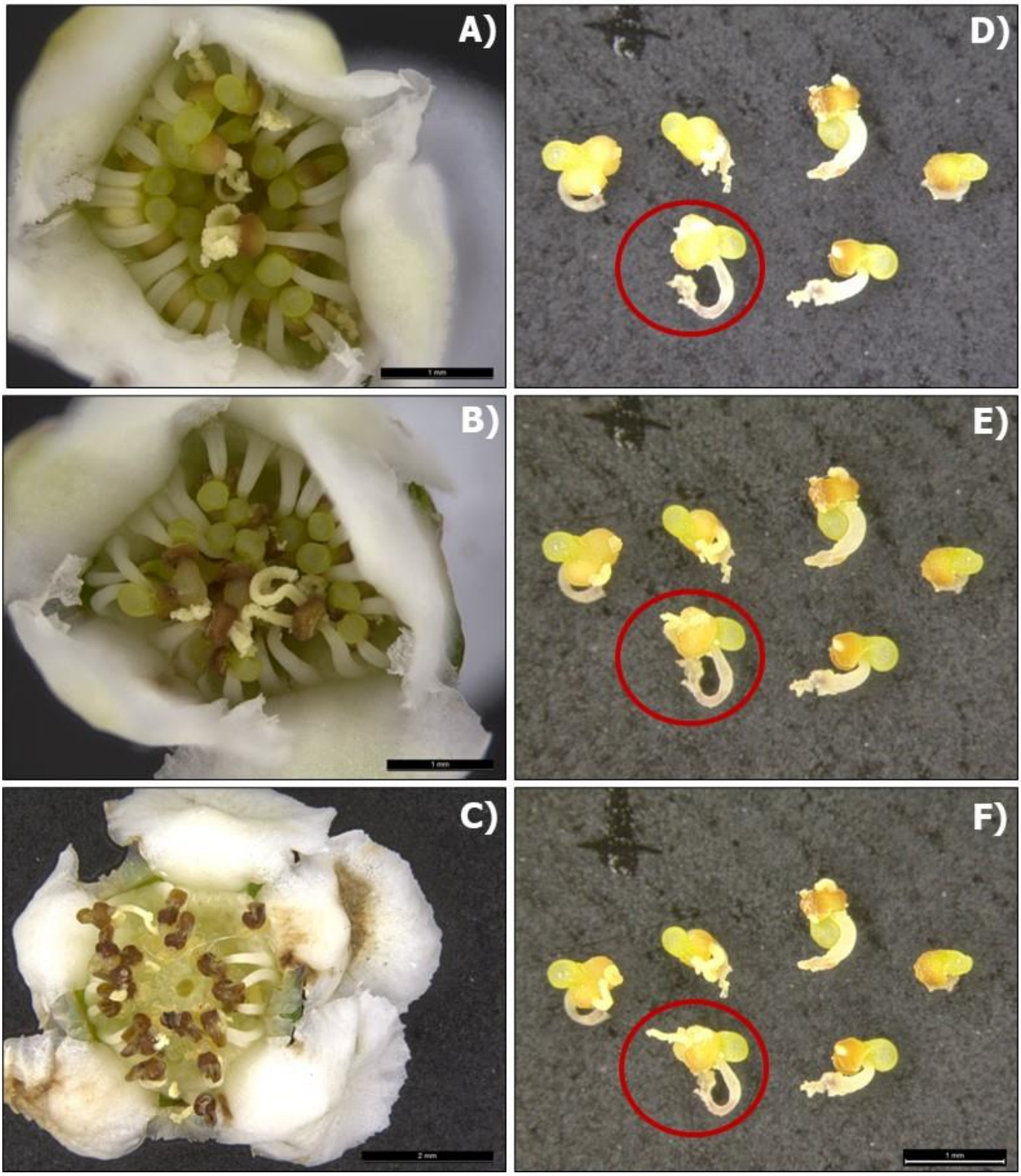

Figure 11: Floral structure and unique pollen dehiscence from *Aluta quadrata*. A) Status of anthers (i.e. all green and full) and pollen in an enclosed bud; B) Status of anthers and pollen in a bud just opening; C) Status of anthers (i.e. brown and most are empty) and pollen (i.e. limited) in a young flower (i.e. green hypanthium); D-F) Time series images (over ~20 minutes) of pollen being squeezed through two pores and being expelled like "toothpaste" from the two anther sacs, on six anthers excised from the flower bud under a microscope. Photos: Carole Elliott.

Floral phenology

Preliminary tracking of floral development in the glasshouse found it can take a minimum of 5 days for buds to transition to an open flower (~5.5% of buds) and many become open flowers after 12-21 days (~54% of buds), while the remainder were still at the bud stage after 21 days (~40.5% of buds; glasshouse cooler than ambient average of 30°C in March). Nectar was present on the floral disc surface and observed to accumulate in the absence of insects (~10µL in 2 hours). Pollen was observed to quickly dehisce as threads from anther sacs when conditions and maturity were reached (~within 1-2 hours) and anthers matured on a flower in stages but over a short window (~1-3 days). Further floral tracking is required to determine the maximum length of time plants delay buds developing into flowers; the stage of stigma receptivity; pollen viability and pollen longevity, and if these change at different temperatures.

Hand-pollinations

Preliminary hand-pollinations found that the most feasible technique (under glasshouse conditions) involved finding flowers with pollen threads, removing this anther with tweezers and bushing its threads onto the stigma of a flower that was fully open (stigma receptivity unknown). Alternate techniques included picking and using an entire flower to pollinate another flower, which prevented the accurate placement of pollen on recipient stigma, and collecting pollen threads onto tweezers/toothpick for transfer, but this method appeared to damage the stigma during placement and the pollen threads became invisible when placed on these tools. The flowers on nursery plants were large and fairly easy to manipulate, which may not be the situation for wild plants as they can have much smaller flowers. The glasshouse environment was constant, moderate and closed system, and this level of control is likely to greatly assist hand-pollination techniques more so than in the field, where temperature, air movement and insect activity are more unpredictable.

As fruit has not yet matured on the glasshouse plants, assessment of the success of hand-pollinations are yet to be completed. The assessment of pollen loads and/or pollen germination 48 hours after hand-pollinations have been collected, stored and are awaiting processing.

<u>Preliminary interpretation</u>

- *Aluta quadrata* demonstrated a capacity to delay development of buds – triggers are unknown.
- Flowers can produce a substantial amount of nectar under glasshouse conditions.
- Hand pollination techniques have been refined and optimised, at least for glasshouse conditions.
- For the 2024 season, the more comprehensive floral development and hand-pollination experiments outlined in the project proposal will be implemented, in the field and also in the glasshouse.

5. MATING SYSTEM

Aims

- Characterise the mating system of *A*. *quadrata* through genetic analysis.
- Assess the variability in mating system parameters within and among sites and years.

Understanding mating system parameters, such as outcrossing and inbreeding rates, provides information on gene flow dynamics, comparative plant fitness, and potentially the capacity of a population to respond to environmental changes.

Method

Sample collection and processing

Leaf and naturally pollinated fruit material were collected from 16-32 maternal plants at each of the 11 sites (Figure 2; see Table 5 for details) in 2022. Harvested leaf material was kept cool (esky or fridge) during transport to Kings Park laboratories (Perth). For each sample, a portion of leaf was freeze-dried (-56°C) for three days (and stored at room temperature), and another portion was stored directly at -80°C until needed.

Fruit from each maternal plant was collected by placing organza bags on branches in September (i.e. after peak flowering had occurred) and these bags were collected in November (i.e. when fruit had ripened and naturally fallen off the branches). Fruit was kept dry for transport and stored in Kings Park's controlled environment drying room at 15-20°C and 15-20% relative humidity until needed. Fruit was cleaned and imaged (*n* = ~45,000 fruit screened), using a Faxitron Multifocus Digital X-ray Cabinet, to identify the fruit with viable seeds (see Reproduction research theme; Table 1 for definition).

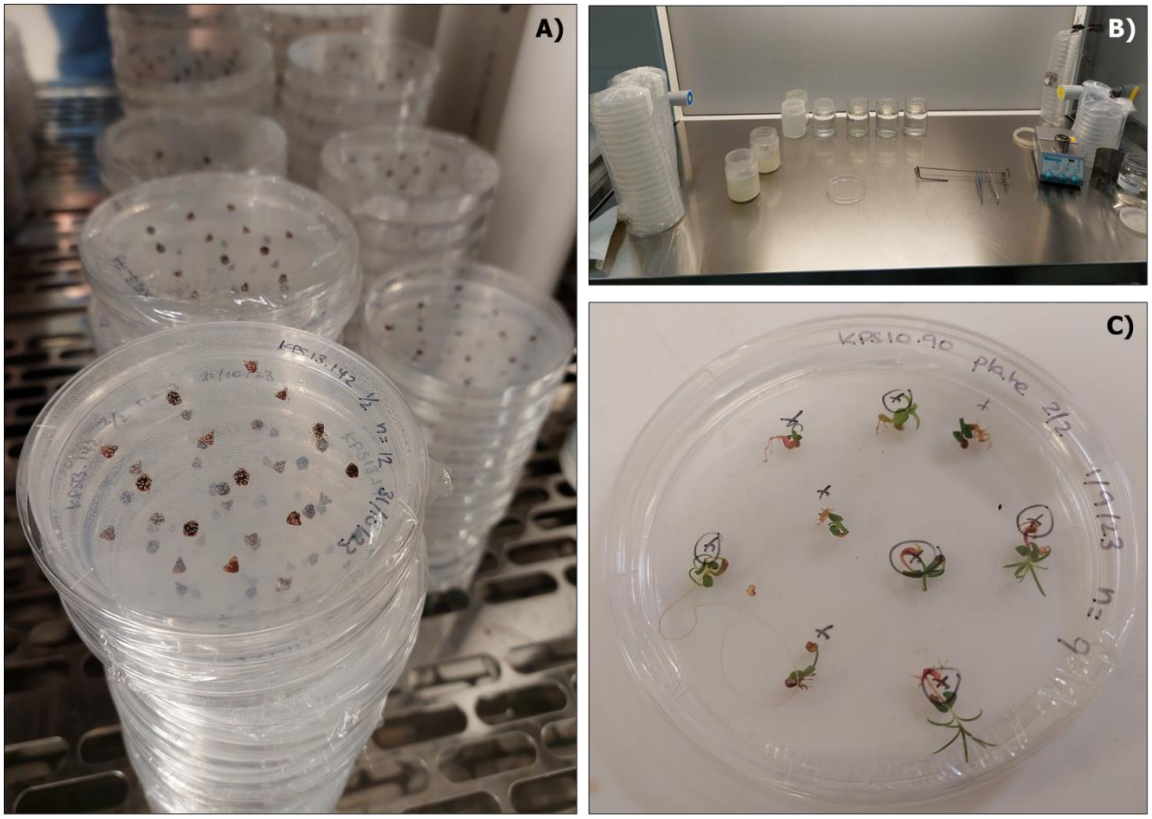

Figure 12: Germination of *Aluta quadrata*. A) Pre-treated fruit germinating on agar plates in incubator; B) Transfer of pre-treated fruit to germination plates; and C) Eight-week-old seedlings. Photos: Carole Elliott.

Samples were x-rayed until 50 fruit with viable seeds (Figure 4) were identified for each plant to ensure enough seedlings for mating system analysis (ideally *n* = 20). Nineteen plants were excluded due to having <20 viable fruit (8.7%). For samples with a small amount of fruit (ten plants with <25 viable fruit), seeds were manually excised from fruit under a microscope using a scalpel blade, placed on germination plates (*n* = 295 seed plated) and incubated at 25°C (see Merritt and Erickson 2020). For samples with an adequate number of viable fruit, they were germinated via a recommended pre-treatment of wet/dry cycling described in Merritt and Erickson (2020), which takes eight weeks to complete (189 plants with >25 viable fruit/plant). Following this, fruit were placed on germination plates (*n* = ~ 4,725 fruit plated) that contained 0.7% micropropagation agar (PhytoTech Labs), 1µM karrikinolide (Synthesised by G. Flematti, UWA School of Chemistry) and 1% plant preservative mixture (Plant Cell Technologies) and were sealed with gladwrap before placing in an incubator at 25°C (Figure 12). Preliminary trials determined the target harvest size of seedlings for a suitable quantity of DNA to be extracted.

Testing and optimisation of molecular markers

Leaf samples from eight maternal plants and eight seedlings (whole seedling, seed coat removed) were used to identify a suitable DNA extraction process that tested 1) freeze-dried, stored at -80°C, or fresh material; 2) grinding with ceramic or metal beads in a FastPrep machine; 3) speed of grinding in a FastPrep machine; and 4) grinding with or without extraction buffer.

Microsatellite markers (*n* = 35 primers) previously developed for *A*. *quadrata* (Byrne et al. 2016), were obtained (AlphaDNA, Canada) and optimised on leaf and seedling DNA. Optimisation requires testing a series of amplification temperatures, PCR conditions (e.g. cycling type, ingredients), sequencing conditions and families (i.e. seedlings from known maternal plant) to ensure unambiguous scoring of alleles for each marker (or locus) that prevents inaccurate or biased estimates of genetic parameters. Eight adult leaf samples (above) are being used to optimise PCR conditions and two families (i.e. maternal plant and their seven seedlings) are being used to confirm Mendelian inheritance, check for null alleles and confirm seedling amplification quality.

Data Analysis

Mating system parameters that will be assessed include outcrossing rate, bi-parental inbreeding, and correlated paternity, which will be compared among sites to assess spatial and temporal variation in mating system dynamics.

Progress

Sample processing

All fruit collected in 2022 have been cleaned and processed, with subsamples (*n* = 4,725) taken for assessment. Pre-treatment of fruit with the recommended germination technique is 75% complete. Initial germination has been observed on some plates (<25% of samples) and this process is currently at four weeks.

Preliminary testing determined that seedlings with cotyledons and 2-4 leaves provided >1,000ng of DNA, which required post-germination growth of 5-8 weeks on agar plates (fruit to target seedling size was 13-16 weeks minimum; Figure 13). Therefore, none of the seedlings above are currently at the appropriate harvest size for DNA extraction.

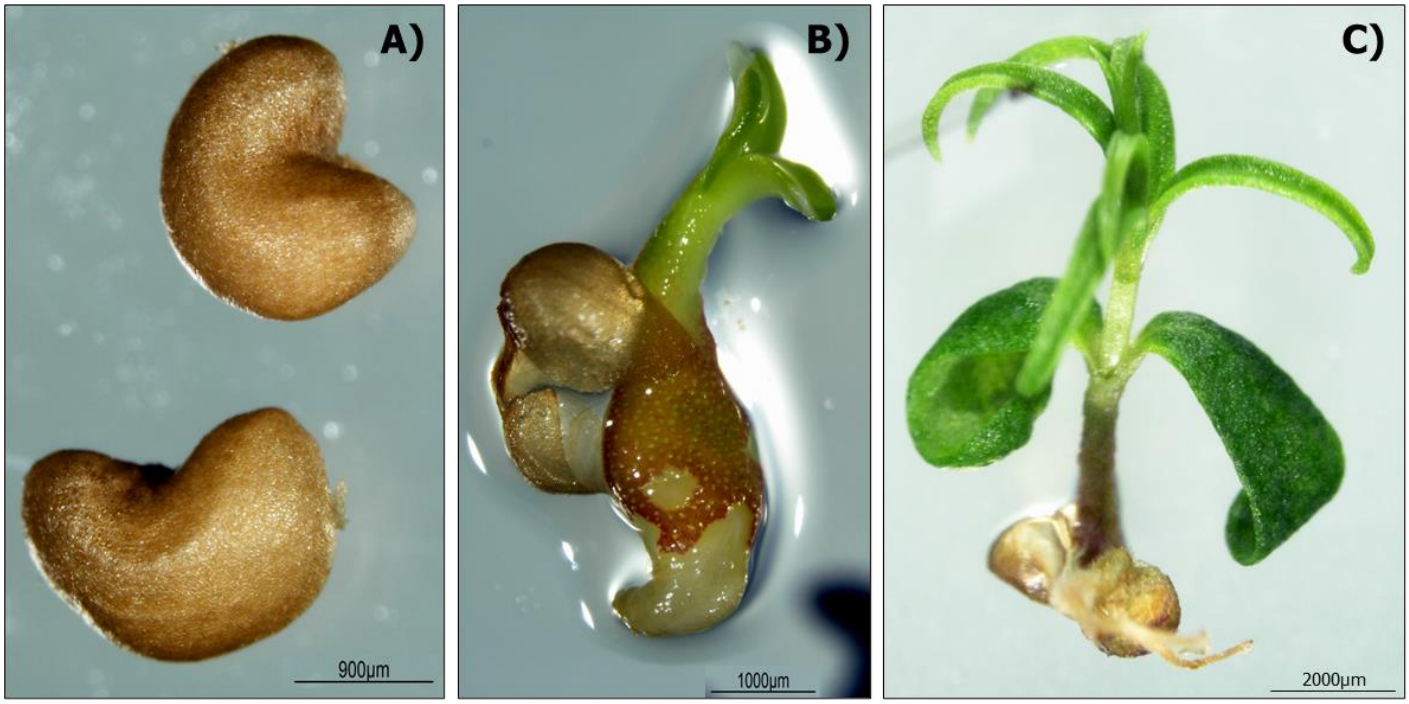

Figure 13: Germination of *Aluta quadrata* on agar plates. A) Seeds excised from fruit; B) Two week old seedling with radicle, attached seed coat and developing cotyledons; and C) Six week old seedling with roots, attached seed coat, cotyledons and three pairs of leaves (i.e. DNA extraction target size). Photos: Nicole Maher.

Testing and optimisation of molecular markers

Freeze-dried, stored at -80°C and fresh material had suitable quantity and quality (260/280 ratio >1.70; 260/230 ratio >1.80) of extracted DNA for adult leaves (200-600 ng/µl; 220 ng/µl; and 400-1000 ng/µl respectively) and seedlings (150-190 ng/µl; n/a; and 40-200 ng/µl respectively). Appropriate quality and quantity of DNA was obtained for all types of material with a modified Carlson extraction protocol (Carlson et al. 1991), where there were two wash steps (standard 5M NaCl and 70% ethanol) added to the end of the protocol, with the pellet being resuspended in a Tris-EDTA buffer. This extraction performed best when the extraction buffer was added after the material was ground with two metal beads at speeds of 4 m/sec for seedlings and 6 m/sec maternal plant leaves. As such, DNA was extracted from 218 maternal plants (freeze-dried material) collected from the field in 2022 with the modified Carlson extraction protocol (30-500 ng/µl) and are ready for microsatellite amplification.

Optimisation of amplification temperatures, PCR conditions (e.g. cycling type, ingredients), sequencing conditions and families (i.e. seedlings from known maternal plant) currently has undergone 30 rounds of testing and ten of the 35 primers have been optimised and are ready for use. The remainder are at various stages of testing. Preliminary assessments show low levels of polymorphism and we will therefore, need to continue to optimise as many primers as possible to analyse the mating system dataset.

Summary

- Optimised germination protocols for growing seedlings at scale.
- Target seedling size for DNA extraction takes 13-15 weeks (pre-treatment, germination, growth).
- Optimised DNA extraction protocols for leaf and seedling tissues, with 218 maternal plant samples ready for microsatellite assessment.
- Optimised PCR conditions and confirmed Mendelian inheritance for ~33% of the microsatellite primers.

6. POPULATION DEMOGRAPHY

Aims

- Develop baseline data on the recruitment, growth and survival of the population.
- Assess the soil seedbank and examine the persistence of seed in soil.
- Determine if population demographic responses change across different locations in the landscape (see proposed experimental locations).

Understanding demographic processes and their variability in relation to environmental conditions, seasonal patterns and life stages (i.e. seed, seedling, juvenile and adult plant) is key baseline information for determining population dynamics and detecting changes in the population over time.

Method

Plant vitals

Field assessment of population demography was conducted at all 11 sites on 294 permanently tagged plants (Figure 2). The size (e.g. dimensions), health (e.g. condition and growth monitored regularly (Table 6)) and macrohabitat (e.g. substrate) of plants (adult, juvenile, seedling) was measured using several techniques (Table 4). Adult plants (i.e. reproductively mature) were randomly selected to include variation in the plant sizes availably at each site. Plants that were shorter than ~50cm and had no evidence of reproduction in 2022 were tagged and classed as juveniles, with the same measurements taken as the adults. Seedlings were searched for in each site and their growth and survival was regularly monitored (Table 6).

Soil seedbank

Understanding soil seedbank dynamics involved sampling two types of soil, that underneath and away from *A*. *quadrata* plants (10 samples of each type/site; approved size 5cm x 5cm x 2cm), bi-annually before (July 2023) and after (November 2022) fruit had dropped from plants. This will provide information on the availability of seed at each site (i.e. recruitment capacity). Samples were transported to Kings Park for processing. We tested a range of sieve aperture sizes (500µm-2.36mm) to determine what was efficient and suitable for these sized fruit. Sieved samples were then examined under a microscope and all whole fruit (>75% intact) removed for x-ray assessment of seed fill and potentially germination testing.

Data analysis

We assessed plant size and plant health parameters to determine if there were any differences among sites. We used the non-parametric Kruskal-Wallis H test followed by the pair-wise Wilcox test because checks of the plant size found that they were not normally distributed and had unequal variance.

For plant health (e.g. chlorophyll fluorescence (Fv/Fm)), we used linear regression models to determine differences through time, followed by analysis of difference among sites through time.

Table 4. Description of the variables used to measure plant size, assess plant health (condition, growth) and classify the macrohabitat of tagged plants *Aluta quadrata*.

Variable	Measurement	Description
Plant size	Height (cm)	• Measure from the tip of the tallest stem to the ground in a vertical line (as gravity)
	Width (cm)	• Measure in two directions on the same plane and have the correct angles. o Longest (1): find the longest width (axis) of the plant and measure tip to tip o Perpendicular (2): find the longest width anywhere on the previous longest axis and measure tip to tip, maintaining a 90 degree angle to the longest axis
	Volume (cm^3)	• Calculate the volume of a plant as a cylinder: o Volume = $\pi r^2 h$ o Where h= height, and r^2 = [(average of two widths) / 2] ^{2}
	Number of stems	• At ground level: count how many stems emerge from the ground • Main stems: count how many main stems branch from the ground level stem(s)
	Stem size (cm)	• Measure the circumference of the ground level stem(s) (except for seedlings)
	Biomass estimate	• Count the number of stem tips (growing point of emerging leaves) on a plant o Small plant: stem tips counted on whole plant o Large plant: subsample the plant by selecting a representative unit of measure (e.g. branch length or stem density) and count each growing tip within this unit. Repeat this to obtain five replicate units for the plant. Then count the total number of these units on the plant. Calculate the average tip count for the five units and multiple this against the total number of units to estimate the total number of tips for a plant (e.g. vegetation biomass) o Seedling: the number of alive and dead leaves were counted until seedlings had more than three tips present
Plant health: condition	Leaf colour	• Visually assess and classify leaf colour of the majority of the leaves on the plant o Used four broad colour classes in descending order of plant condition: dark green (e.g. very healthy), light green (e.g. moderate health), yellow (e.g. poor health), or brown (i.e. dead)
	Chlorophyll fluorescence (Fv/Fm)	• Measure evaluates photosynthetic efficiency of leaves and plant stress using a fluorometer (Hansatech Pocket PEA). o Fv/Fm is between 0 and 0.85, with readings closer to 0.85 indicating higher chlorophyll performance (i.e. better function; Schönbeck et al. 2023) o Attach two clips on the plant and dark adapt the leaves (i.e. cover with clip and foil bag) for 15-20 mins before measuring with fluorometer
	Percentage of dead plant material	• Visually assess the percentage of leaf biomass material that is dead (i.e. brown)
Plant health: growth	Tip growth (cm)	• Measure the length of two soft stem tips (which indicates new growth) on a plant o New growth has a light-coloured stem (white/pale yellow or light green) where the new leaves emerge, that hardens over the growing period to a red/rust colour, and the following season turns light grey and is semi-hard o Measure from where the stem colour is white or light green to the apical meristem where the leaves emerge (i.e. NOT to the end of a leaf)
Plant macrohabitat	Substrate	• Visually assess the substrate habitat that the plant grows in o Classified the into five broad groups, where a plant grew in: 1) a crack within a rock/cliff substrate; 2) on a rock slope (similar amount of soil and loose rock); 3) a pocket of soil on mainly rock/cliff substrate; 4) soil only (minimal loose rock); 5) sand in creek bed (Figure 16).

Results and Progress

Plant vitals

The size of tagged adult plants varied substantially but this variation was mostly representative within all the sites (e.g. small and large plants were tagged at each site). The estimated volume of plants ranged from 0.01 – 40.7 m^3 and the estimated biomass ranged from 108 – 62,000 stem tips, among sites. However, there was a significant difference in the average size of tagged adult plants among sites (volume: Kruskal-Wallis = 33.397, *df* = 10, *p* <0.001; biomass: Kruskal-Wallis = 32.084, *df* = 10, *p* <0.001). Further analysis showed that KPS11 was driving this outcome, as it had significantly smaller-sized tagged plants on average compared to the other sites, which were not significantly different from each other (volume and biomass: *p* <0.05; Figure 14).

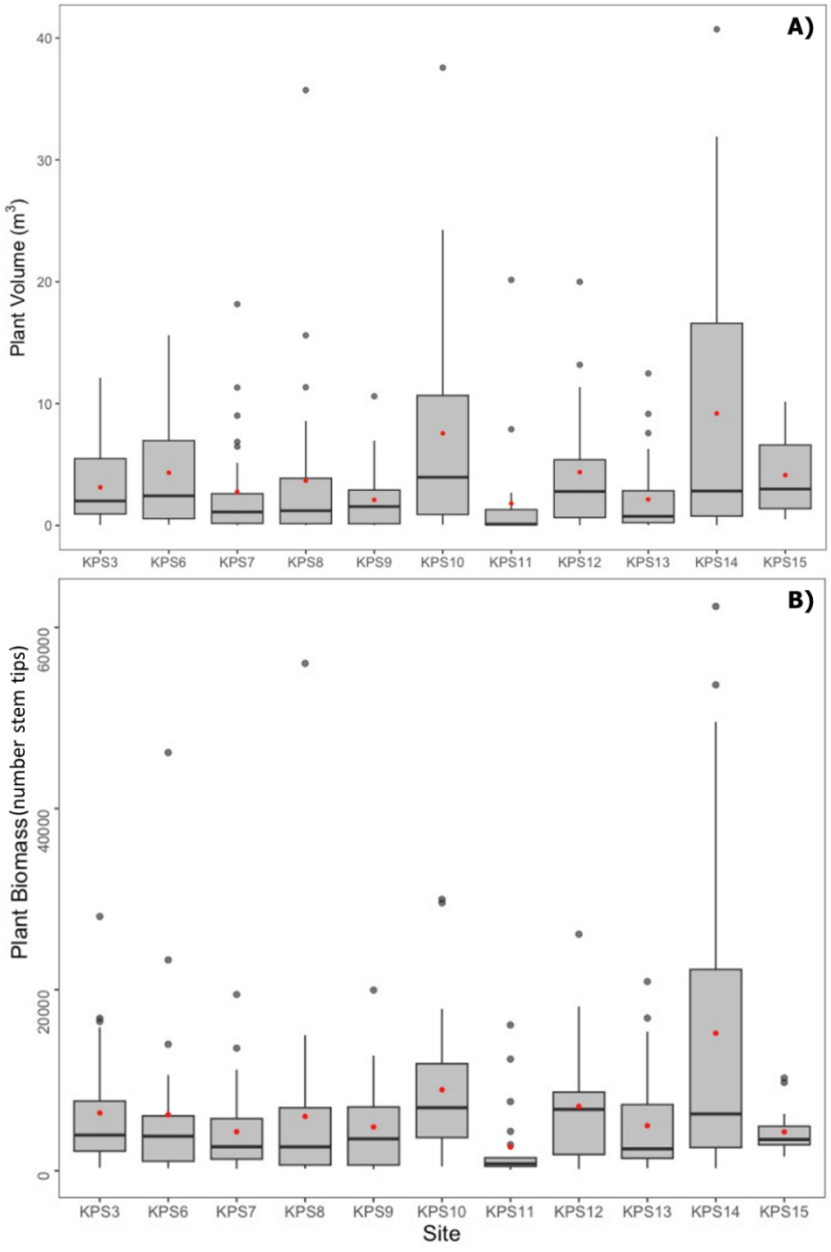

Figure 14. Estimated size of tagged *Aluta quadrata* plants at each of the 11 sites monitored. A) Volume of tagged plants (m^3); and B) Biomass of tagged plants (number of stem tips). Black lines through boxes indicate median values, red dots indicate mean values (*n* = 293 plants).

Regular monitoring of plant health (condition) showed most plants had light green leaf colour (60-73% of plants), followed by plants with dark green leaf colour (22-38% of plants), which was consistent during June, July and September (2023). In addition, the average percentage of a plant that was brown (i.e. dead leaves)

was low (range 0-5%, except two plants with 10% and 30% brown) over this period. Interestingly, overall averages for chlorophyll fluorescence were significantly different among the three time periods ($F_{2,1685}$ = 60.8; p <0.05), with July having the lowest average leaf function measures (Fv/Fm = 0.47) compared to June (Fv/Fm = 0.54) and September (Fv/Fm = 0.57). This has shown a measured change in average plant leaf function through time, with improvement in function from July to September. This indicates that plants on average might be responding to changes in the environment. For example, ecophysiological studies by Lewandrowski et al. (2023) indicate the timeframe between winter and spring to be an important threshold-period where plant health decreases significantly and plant-available water diminishes. Interestingly, this was not a continual decline in 2023 during this threshold-period, as early spring (e.g. September) had similar average leaf function to early winter (e.g. June; Figure 15A) indicating other environmental factors may have elicited this change in leaf function. However, these average estimates are an indication of reduced photosynthetic efficiency (i.e. an Fv/Fm < 0.75 indicates plant stress; Schönbeck et al. 2023) despite the large variation observed at site and individual plant levels (Fv/Fm ranged 0.12 – 0.88), and requires further study (Figure 15).

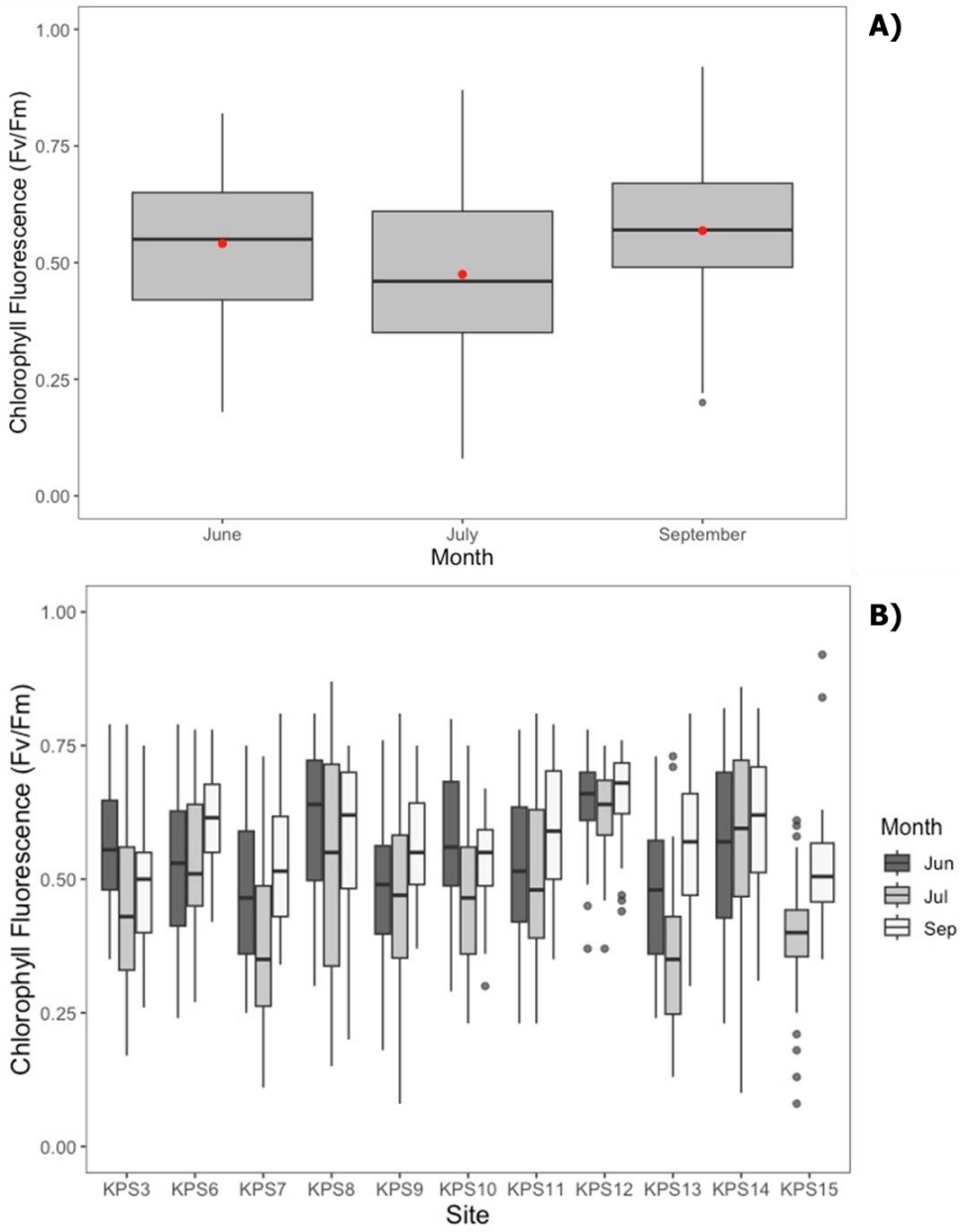

Figure 15. Chlorophyll fluorescence (Fv/Fm) of plants. A) Overall values across all plants for each monitoring period (June, July, September 2023); and B) Summarised for each site monitored, for each monitoring period. Black lines through boxes indicate median values, red dots indicate mean values (n = 293 plants).

The dominant macrohabitat of each individual tagged plant was cracks within a rock/cliff substrate (42.7% of plants; Figure 16) followed by plants in a pocket of soil on mainly rock/cliff substrate (23.6% of plants). Plants located on the creek bed (18.8% of plants) or on rock slopes (10.9% of plants) made up one third of the macrohabitat of tagged plants, with a small percentage of tagged plants being located in soil only (3.4% of plants; 0.6% unclassified at time of report).

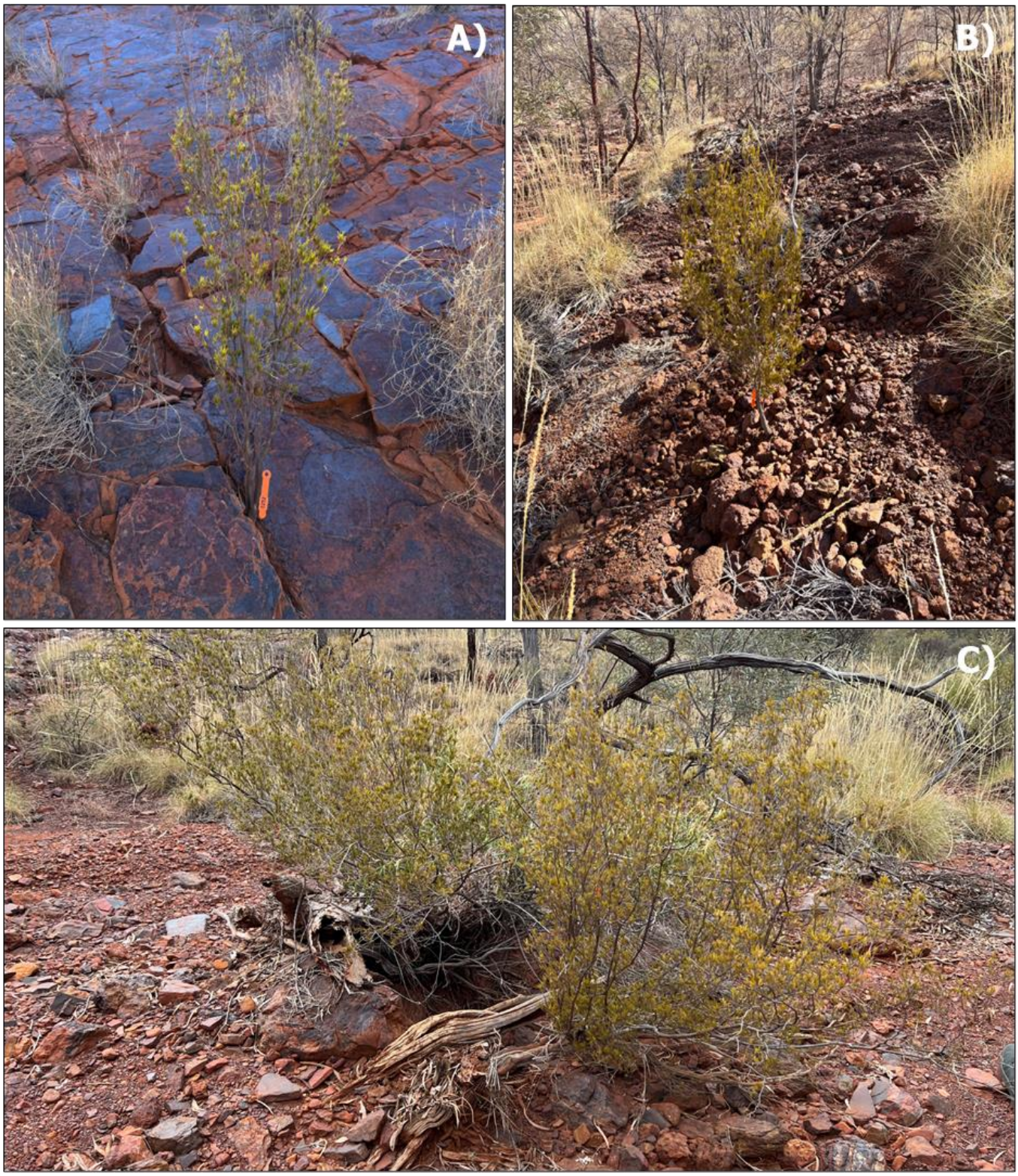

Figure 16. Examples of *Aluta quadrata* macrohabitats assessed as the substrate each plant was growing in. Plant located A) in a crack on rock/cliff substrate (KPS10); B) on a scree rock slope (KPS12); and C) on a creek bed (KPS6). Photos: Parry Aw.

There were 21 juvenile plants available for assessment at eight of the 11 monitoring sites (Table 5). For the 2022 recruitment season, we observed 85 seedlings emerge at nine of the 11 monitoring sites during July-September. However, only five seedlings (KPS11) survived their first year (Table 5). These seedlings were approximately 5cm tall with 12-25 leaves when measured in September 2023 (Figure 17). No recruitment was observed for the 2023 season.

Figure 17. Seedling recruitment and juvenile plants monitored at sites. A) Seedling condition in August 2022 (KPS3); B) Seedling condition in November 2022 (KPS11); C) Condition of surviving seedlings in September 2023 (KPS11); and D) Condition of juvenile plant (KPS13). Photos: Carole Elliott.

Table 5. Site characteristics (see Experimental Design for habitat definitions) and demographic data.

Site name	Average elevation (m)	Broad Habitat Type (site)	Number of plants fruit collected from	Number of tagged adults	Number of juveniles	Number of seedlings	Percentage of seedlings alive
KPS3	398.8	creek bed/creek rock slope	23	29	6	5	0%
KPS6	368.3	creek bed/creek rock slope	21	29	1	7	0%
KPS7	470.3	cliff area	22	29	2	5	0%
KPS8	407.4	creek bed/crest of range	20	30	1	4	0%
KPS9	448.0	crest of range	20	26	2	0	-
KPS10	403.3	cliff area	22	28	6	10	0%
KPS11	408.1	cliff area/crest of range	13	21	2	48	10.4%
KPS12	338.8	creek bed	17	23	0	0	-
KPS13	390.9	creek bed/crest of range	22	32	1	2	0%
KPS14	360.9	creek bed/creek rock slope	22	31	0	2	0%
KPS15	397.0	crest of range	16	16	0	2	0%
		Total	218	294	21	85	

Soil seedbank

We identified that samples needed to be sieved at 1.18mm (to remove fine silt/sand) and 4mm (to remove coarse stem and stone material) for optimal processing while retaining all fruit in the sample (Figure 18). Preliminary sieving has been completed for the 2022 soil collection.

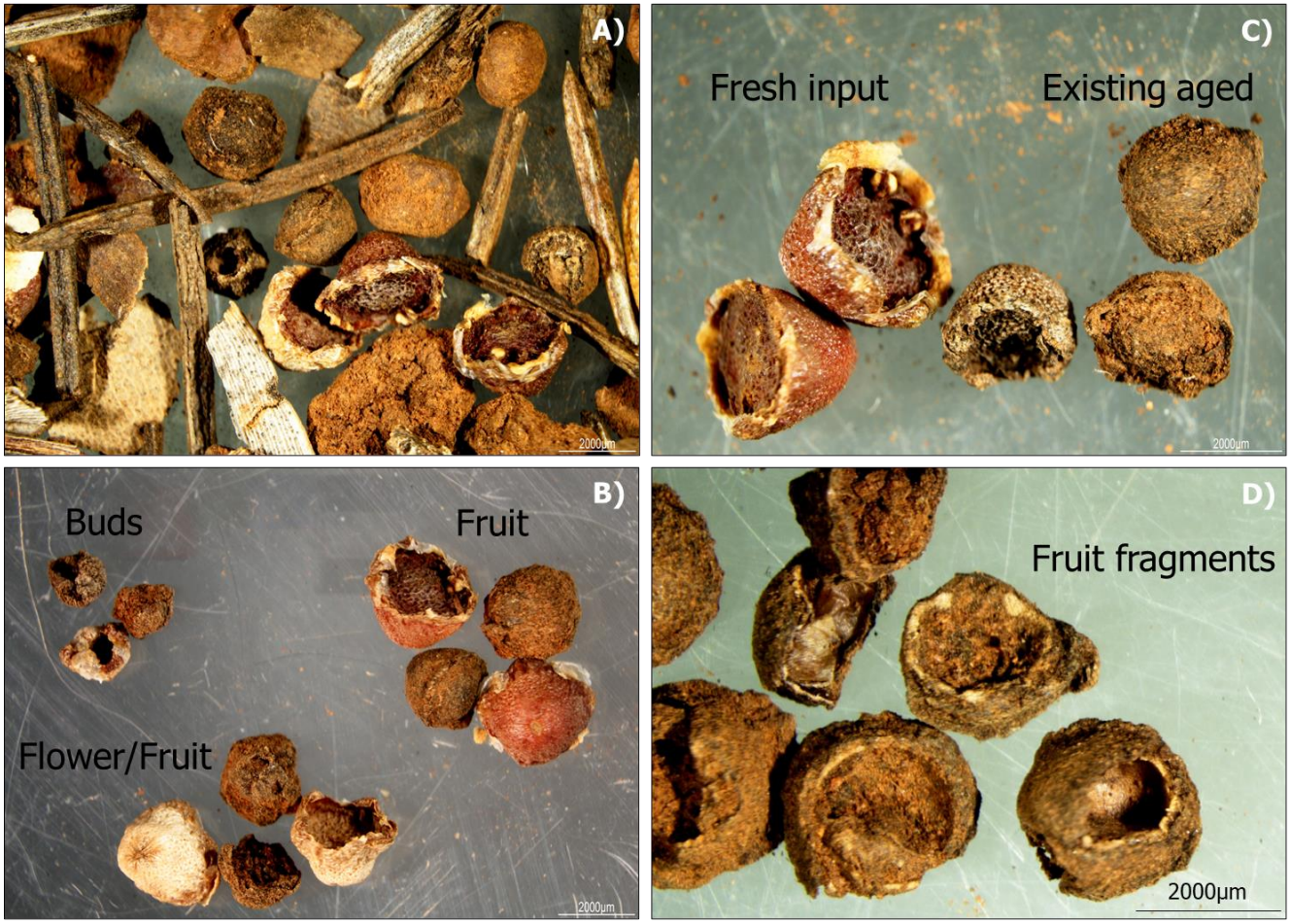

Figure 18. Soil seedbank samples from *Aluta quadrata* sites on the Western Range. A) Sieved soil seedbank sample showing remaining material for sorting; B) Sorted material showing buds, flowers, fruit that are fresh and aged; C) Fruit identified from sieved sample that are a fresh input from 2022 fruiting period (left) or are an existing aged fruit from previous fruiting periods (right); and D) Fragments of fruit present in the soil seedbank samples. Photos: Emilie Perez-Wright.

<u>Preliminary interpretation</u>

- Generally, the size of plants chosen and tagged for monitoring was representative among sites, except for KPS11 (which had significantly smaller plants).
- All adult and juvenile plants were still alive after the first year of monitoring.
- Recruitment was recorded in 2022, but not 2023, and seedling survival for 2022 was low (5.9%).
- Plant condition varied substantially at site and individual plant levels, but an overall change in condition was detected (at a statistical level) among the monitoring periods (Fv/Fm).
- Plants tagged for monitoring were located in varied macrohabitats that may influence plant responses.

CHALLENGES

The project's key performance indicators for commencement of the project have been completed as planned (Appendix 2 – adjusted for the different start date to the project). However, there have been a few important issues that have presented challenges to the operational and research capacity of the project:

- The Spring (rather than Autum) start to the project meant that we missed the beginning of the 2022 flowering-fruiting period. While we were able to capitalise on this fruiting period (i.e. samples collected for the Mating System study), we missed the opportunity to collect important reproductive data for the Phenology, Pollinator Communities, Reproduction or Breeding System studies in 2022.
- Following on from this, 2023 was a very poor year for flowering, which made it impossible to address some objectives and limited capacity to address others (Table 6).
- Issues with site access – administrative, weather, track conditions, availability of personnel to facilitate access. These challenges will need to be resolved on an ad-hoc basis should or when they occur.

The climatic conditions of the Western Range experienced so far, and the vastly different reproductive responses observed between years have indicated that the flowering-fruiting period of *A*. *quadrata* is sensitive to environmental conditions. This means that there is a potential risk to the collection of data that is dependent on the production of flowers and fruit within the planned experimental design and timeframe.

The timing of "impact", where "impact" is defined as the commencement of on-ground works for the land bridge and waste-rock dump, is unknown and we will continue to work with RTIO and local stakeholders to best align development of land bridge and waste-rock dump with time-sensitive mating system components of the study.

Figure 19. Monitoring, surveys and sampling of *Aluta quadrata* at the Western Range was a collaborative team effort (DBCA, Rio Tinto, Yinhawangka Aboriginal Corporation staff, and Murdoch University and University of Western Australia students). Photos: Carole Elliott and Parry Aw.

Table 6. Completed fieldwork schedule (Sept 2022 – Sept 2023) that shows the monitoring, surveys and sample collection activities completed on each field trip under each of the projects' six research themes.

			Research Themes					
Date	**Duration & Personnel**	**Site setup**	**Phenology**	**Reproduction**	**Pollinators**	**Breeding System**	**Mating System**	**Population Demography**
19-23 Sept 2022	5 days 4 people	Tag plants					Fruit bagging	Survey seedlings Map plants
22-29 Nov 2022	8 days 4 people						Fruit collection	Soil collection Monitor seedlings
19-26 Jun 2023	8 days 4 people	Tag plants	Monitor (status)	Monitor (flower)	Observation (floral visitors)	(glasshouse)		Survey seedlings Map plants Monitor (condition)
23 Jul – 3 Aug 2023	12 days 6 people	Habitat classified	Monitor (status)	Monitor (flower)				Monitor (growth, condition) Macro-habitat classified Soil collection
14-20 Sept 2023	7 days 6 people		Monitor (status)	Monitor (flower)			Fruit bagging	Monitor (condition)

Legend

Optimal
Good
Moderate
Poor
Not done

Note: Colour coding broadly categorises the research capacity achieved against the initial plans proposed for research program. Constraints were mainly due to the landscape scale lack of plant reproduction for the 2023 flowering-fruiting period and limited access to tagged plants at two sites.

LICENCES AND PERMITS

All permits and licences required to undertake the research trials (i.e. sourcing seed, plant and soil material) have been secured by research staff to undertake this work (DBCA licenses FT61000177-4; TFL 2223-0046 and FO25000484).

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Appendix 1: Research Program Schedule Progress [summer (S), autumn (A), winter (W), spring (S)].

THEME	Objective	Later start than plan		Year1				Year2				Year3				Year4				
		A	W	S	S	A	W	S	S	A	W	S	S	A	W	S	S	A	W	S
Commence project																				
Site choice																				
Phenology	ID stages																			
Reproduction	Fecundity																			
	Pollen limitation																			
Pollinators	Visitation rates																			
	Community ID																			
Breeding System	Hand-pollination																			
	Seedling fitness																			
Mating System	Collection																			
	Genotyping																			
Demography	Recruitment																			
	Plant health																			
	Soil seedbank																			
	Seed persistence																			
Quarterly meetings																				
Annual Reporting																				
Project Review																				

Appendix 2: Key Performance Indicators

KPI	THEME	Objective	Description	Completion Date	Reporting Period
		COMMENCE PROJECT	19TH SEPTEMBER 2022 - 18TH SEPTEMBER 2026		
1			Finalised experimental design (site selection)	Sept-Jun Year 1	Year 1 (Sept 2022-2023)
2	Phenology	ID stages	Studies of floral phenology development commenced	Jun-Nov Year 1	Year 1 (Sept 2022-2023)
3			Floral phenology development studies completed	Sept Year 4	Sept Year 1-4
4	Reproduction	Fecundity	Studies of reproductive biology commenced	Jun Year 1	Year 1 (Sept 2022-2023)
5			Reproductive biology studies completed	Sept Year 4	Sept Year 1-4
6		Pollen limitation	Studies of pollen limitation commenced	May Year 1	Year 1 (Sept 2022-2023)
7			Pollen limitation studies completed	Sept Year 4	Sept Year 1-4
8	Pollinators	Visitation rates	Studies of floral visitor behaviour commenced	May Year 1	Year 1 (Sept 2022-2023)
9			Floral visitor studies completed	Sept Year 4	Sept Year 1-4
10		Community ID	Studies of insect community composition commenced	May Year 1	Year 1 (Sept 2022-2023)
11			Insect community composition identified	Sept Year 4	Sept Year 1-4
12	Breeding System	Hand-pollination	Studies of characterising the breeding system commenced	Mar-Jun Year 1	Year 1 (Sept 2022-2023)
13			Breeding system characterised	Sept Year 3	Sept Year 1-3
14		Seedling fitness	Studies of seedling fitness commenced	May Year 3	Nov Year 4
15			Seedling fitness studies completed	May Year 4	Nov Year 4
16	Mating System	Collection	Material collection commenced	Nov Year 1	Year 1 (Sept 2022-2023)
17			Material processing completed	Jun Year 4	Sept Year 1-4
18		Genotyping	Studies of mating system patterns commenced	Jun Year 1	Year 1 (Sept 2022-2023)
19			Mating system pattern studies completed	Sept Year 4	Sept Year 1-4

20	Demography	Recruitment	Studies of recruitment commenced	Sept Year 1	Year 1 (Sept 2022-2023)
21			Recruitment studies completed	Sept Year 4	Sept Year 1-4
22		Soil seedbank	Studies of soil seedbank commenced	Nov Year 1	Year 1 (Sept 2022-2023)
23			Soil seedbank studies completed	Sept Year 3	Sept Year 3
24		Seed persistence*	Studies of seed persistence commenced	Nov Year 1	Sept Year 2
25			Short-term seed persistence studies completed	Sept Year 4	Sept Year 4

* Additional contract agreement not in place to commence this research component – see Project Proposal for details.