Environmental factors influencing fruit production and seed biology of the critically endangered *Persoonia pauciflora* (Proteaceae)

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Abstract The factors that influence seed production and seed dormancy in rare plant species are crucial to their conservation yet are often poorly understood. In this study, we examined the breeding system and seed biology of the critically endangered Australian endemic species *Persoonia pauciflora* through a series of experiments. Pollinator visitation surveys and manipulative hand-pollination treatments were conducted to investigate the breeding system and subsequent seed production. We used an experimental seed burial to examine the breakdown of the woody endocarp and changes to germination over time. Seed germination response under simulated local seasonal conditions was also examined. *Persoonia pauciflora* was found to be predominantly pollinated by native bees, and cross-pollinated flowers produced significantly more mature fruit ($18 \pm 3\%$) than self-pollination treatments ($2\text{--}3\%$). The average strength of *P. pauciflora* pyrenes buried in soil significantly decreased over two years, from 413.3 ± 14.6 N to 130.8 ± 11.8 N. Post-burial seed germination was cyclical, with highest final germination percentage occurring following late-summer exhumations ($75 \pm 2\%$ and $74 \pm 14\%$, respectively). When seeds were incubated under different local seasonal conditions, germination at local summer temperatures was more likely when compared with autumn/spring and winter temperature-

incubated seeds. Our study highlights the importance of ensuring appropriate biotic pollen vectors are present in the local landscape for maximising viable fruit production for this species. In addition, our data indicate that recruitment will most likely occur after the endocarp has suitably weakened, allowing physiological dormancy of the embryo to be relaxed and germination to commence following summer temperatures.

Keywords breeding system · endocarp · *Leioproctus* · *Persoonia pauciflora* · pollination · seed germination

Introduction

Numerous factors operating at a range of spatial scales can affect plant abundance or rarity (Schemske et al. 1994). The most important ecological information for conserving rare species is their reproductive traits, as these are associated with species persistence in the landscape. The regeneration niche, originally defined by Grubb (1977), highlights the crucial role of plant reproductive factors for population persistence, with viable seed production noted as a major component of this niche. Important considerations include both species and environmental factors that influence seed production, such as breeding system and pollen vectors. The potential for flow-on effects from seed production means that subsequent factors associated with successful seedling establishment, such as seed dormancy and germination, are also key traits that define the reproductive niche (Grubb 1977; Bykova et al. 2012; Jiménez-

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Alfaro et al. 2016). Seed bank dynamics are a particularly important inclusion in threatened species management, as dormant seeds and adult plants are affected by different suites of external environmental stresses (Pavlik et al. 1993; Donohue et al. 2010). If the requirements for seed germination are ascribed to specific triggers, such as the chemical stimulants in smoke, for example, then populations may become locally extinct in areas where those triggers, such as fires, are not common in the landscape. Indeed, changes in any component of a species' reproductive niche could cause range-shifts through population expansion or extinction, and rare species are likely to be sensitive to even small changes in the reproduction niche (Grubb 1977; Bykova et al. 2012).

The reproductive niche is an especially important consideration for Proteaceae, as species from this expansive plant family often exhibit a very low fruit-to-flower ratio, sometimes as low as 0.01% (Carolin 1961; Lamont and Barrett 1988). Although numerous contributing factors have been documented (Groom and Lamont 1998), key knowledge gaps remain. The genus *Persoonia* is endemic to Australia and is a good model in which to study key reproductive traits in Proteaceae, as numerous species have received considerable attention focussed on the mechanisms that affect seed production and germination (Bernhardt and Weston 1996; Weston 2003; Rymer et al. 2005; Chia et al. 2016b; Emery and Offord 2018). Several species are known to have breeding systems that preferentially select outcrossed pollen (Krauss 1994; Cadzow and Carthew 2000; Wallace et al. 2002; Rymer et al. 2005). For example, Krauss (1994) reported 20% of outcrossed flowers from *P. mollis* plants produced mature fruit, compared with only 1% of self-pollinated flowers. Fruit set was also very high in *P. rigida* when flowers were left open to outcrossing (67.4%; Trueman and Wallace 1999). This could not be matched by manual cross-pollination, suggesting that manual hand-pollination does not effectively mimic natural pollinator behaviour to achieve similar pollination success (Trueman and Wallace 1999). *Leioproctus* (sub-genus *Cladocerapis*) bees are widely reported to be frequent visitors to eastern Australian *Persoonia* species (Bernhardt and Weston 1996; Wallace et al. 2002; Rymer et al. 2005). When foraging on *Persoonia* flowers, *Leioproctus* bees contact the stigma as they collect pollen and nectar, making them effective pollinators of the genus (M. Batley pers. comm.).

Persoonia fruits contain a woody endocarp, which is noted as a mechanical dormancy mechanism in the genus, and germination only occurs if the endocarp is at least

partially removed (Bauer et al. 2004; Chia et al. 2016b). Within the endocarp is one or two seeds, comprising an outer testa that holds an embryo and cotyledons. Some *Persoonia* species have a physiologically dormant embryo that requires specific environmental cues (i.e. temperature and moisture) for germination (Bauer and Johnston 1999). Furthermore, if physiological dormancy is alleviated, the embryo must then have enough growth potential to break through the endocarp (Nikolaeva 1969). In an ecological context, the window of conditions required for relaxing physiological dormancy in the embryo should reflect the local climate, meaning that variation in germination may be apparent at fine scales (Jiménez-Alfaro et al. 2016). This intricate relationship between the endocarp and embryo suggests that *Persoonia* germination traits – and the seed biology more generally – should be examined in both laboratory and field environments (Baskin et al. 2006; Emery and Offord 2018).

Seed burial trialling is a common method to examine the effects of dormancy over time. This is because the initial age and viability of the seeds are known, allowing the effects of the environment on dormancy release and germination to be interrogated. For example, following a two-year soil burial, Norman and Koch (2008) found *P. longifolia* endocarps did not significantly decline in hardness, and still required chipping to stimulate germination. More recently, Chia et al. (2016b) reported the germination of exhumed *P. longifolia* seeds was cyclical, and highest following 30 months of burial. It was determined that the combination of endocarp weakening caused by significant wet/dry cycles, coupled with warm summer temperatures breaking physiological dormancy, allowed seeds to subsequently germinate (Chia et al. 2016b). Temperature, in particular, has a pivotal role in controlling physiological dormancy, as many species require a period of constant temperatures within a threshold, or the correct alternating temperature pattern for relaxing dormancy (Offord and Meagher 2001). Additionally, this temperature window varies according to the species and the geographic distribution of its populations. Thompson et al. (1977) illustrated this by screening 292 plants for the alternating temperature and light regimes in which germination was promoted. The authors noted that the response of species to *ex situ* conditions reflected the conditions experienced *in situ*. In this way, species could be grouped according to habitat (Thompson et al. 1977). In a comprehensive study on 43 species in Western Australia, Bell et al. (1995) demonstrated that the relationship between

germination and temperature was dependent on a species' life history, and suggested that understorey species were likely to have more specific germination requirements compared to overstorey species.

With nine species currently threatened, endangered or critically endangered, there are important conservation outcomes for understanding the link between the environment and seed traits in *Persoonia* (Rymer et al. 2005; Nield et al. 2015; Chia et al. 2016a, b; Emery et al. 2018). Of the 99 known *Persoonia* species, *P. pauciflora* P.H.Weston is the only critically endangered species currently listed under both the Australian *Environment Protection and Biodiversity Conservation Act 1999* and the New South Wales (NSW) *Biodiversity Conservation Act 2016*. *Persoonia pauciflora* has a highly restricted distribution within the Broad-leaved

Ironbark (*Eucalyptus fibrosa*) and Spotted Gum (*Corymbia maculata*) woodlands around North Rothbury in the lower Hunter Valley region of NSW (Fig. 1). Spatially, the species encompasses a linear range of 4.3 km, a total area of occupancy of 29 ha, and around two-thirds of the plants occur on a single private property (Office of Environment and Heritage 2012). *Persoonia pauciflora* is a small, spreading shrub that grows between 0.1–1.4 m high and 0.4–1.6 m wide, and occurs on the silty sandstone soils derived from the geological Farley formation (Weston 1999). Flowers comprise four yellow recurved tepals that range between 4.5–8.0 mm long, four stamens, and a single gynoecium containing one ovule that produces a fleshy, drupaceous fruit (Fig. 2; Weston 1999). Peak flowering is from December to May; however, as is typical of this genus,

Fig. 1 Map showing the approximate spatial distribution of *Persoonia pauciflora*. The species is confined to the town of North Rothbury and surrounds (b), situated in the Hunter bioregion of

NSW (a) approximately 50 km WNW of Newcastle. Plant records are not shown due to the species threatened status

Fig. 2 **a** – *Persoonia pauciflora* is an erect spreading shrub with lime-green foliage. **b** – The small, inconspicuous yellow flowers are characterized by four yellow recurved tepals, four stamens, and a single gynoeceum. **c** – Each pollinated ovule slowly matures into a green fleshy fruit. Photos: N. Emery

flowering can also occur randomly throughout the year. *Persoonia pauciflora* does not appear to have a lignotuber (present in some *Persoonia* species), relying solely on recruitment in the soil seedbank. The species is currently threatened by habitat fragmentation and loss due to urban development. Several *P. pauciflora* seedlings have been observed in the wild, but little is known of its reproductive niche.

In this study, we performed a series of experiments that each examined a component of the reproductive niche of *P. pauciflora*, specifically focused towards factors that might influence seed production and germination traits. The aims of this study were to: (1) investigate the visitation rates of native and exotic pollinators, (2) assess the level of self-compatibility and its impact on seed production, (3) determine the rate of endocarp breakdown and how seed germination changes following soil burial, and (4) examine the seasonal temperature regime in which seeds are most likely to germinate. Several experimental translocations of *P. pauciflora*, including population supplementation and assisted colonization, have recently occurred (Emery et al. 2018). The data from the present study can be used to help determine and monitor translocation outcomes. More broadly, this study provides a model for other rare flora for investigating the reproduction niche and its potential impact on persistence in the landscape.

Materials and methods

Study sites

Pollinator visitation was surveyed on the largest natural population of *P. pauciflora*, occurring on a private property in North Rothbury, NSW, located in the approximate centre of the species distribution (termed Site 1). Approximately 600 plants have been surveyed to occur on the property. We collected fruits from plants on the property in October 2015 and again in October 2016. Since fruits are only mature when dropped from the maternal plants, only recently dropped fruits found on the ground below plants were collected (Catelotti and Offord 2017). Fruits were also collected in October 2016 from another property that has the second-largest population surveyed (around 200 plants; termed Site 2). We buried pyrenes (i.e. endocarp and seed within; flesh cleaned and removed) on a conservation offset land in North Rothbury (termed Site 3). This site had several extant plants and was previously used for an experimental conservation planting. Hand-pollination treatments to investigate the breeding system were conducted at the Australian Botanic Garden Mount Annan (ABGMA) using a potted collection of 88 plants. These plants were propagated using plant material collected in 2009 from the same population that was surveyed for pollinator visitation in this study. The potting medium used to

grow the propagated plants comprised crushed quartz and coir fibre.

Pollinator visitation experiment

Thirty-four randomly selected *P. pauciflora* plants were tagged within a 60 m² area where the study species density was deemed to be highest. Tagged plants encompassed different ages, and above ground plant height ranged from 45 to 125 cm (average 79.1 ± 3.2) and the number of flowering branches per plant during surveys ranged from 2 to 323 (average 93.3 ± 12.2). Surveys were conducted over five days during November and December 2015. Tagged plants were randomly observed for pollinator visitation for 10 min each, between 08:00 h and 17:00 h each day. A pollinator was recorded if it was observed gathering pollen or nectar from a flower. To determine pollinator effort, the number of flowers visited per plant before the insect flew away from the plant was also recorded. Visitor species identities were recorded and classified as native or exotic species (i.e. European honeybees), and examples of any unidentified species in the field were caught and taken back to the laboratory for identification.

Data for pollinator abundance and pollinator effort showed a Poisson distribution and analyses were conducted as generalized linear mixed models using the *lme4* package (Bates et al. 2018) in R (v. 3.4.0). Survey days and plants were included as random effects and bee type (native bee or honeybee) as a fixed effect in both models.

Breeding system

To assess the degree of self-compatibility of *P. pauciflora*, we randomly tagged 30 plants from the potted collection at ABGMA for hand-pollination treatments in November 2015. These plants were grown in a low organic, high drainage soil mix containing crushed quartz and coir fibre. All tagged plants were reproductively mature and were 47 ± 10.2 cm tall on average, spaced 30–50 cm apart and watered daily. From the available flowering branches, we randomly selected four branches per plant and assigned each of the four branches to one of the following pollination treatments:

- (i) Closed/autogamy: the branch was bagged prior to flower opening and not manipulated further;
- (ii) Self: branches were bagged prior to flowering. As flowers opened, pollen from dehiscent anthers was collected and brushed against the stigma of the same flower;
- (iii) Geitonogamy: branches were bagged prior to flowering. As flowers opened, self-pollen was removed and stigmas were brushed with pollen collected from at least five flowers on different non-bagged branches of the same plant; and
- (iv) Crossed/xenogamy: flowers were treated the same as (iii) except that flowers were brushed with pollen collected from at least five flowers from a genetically different plant.

The number of flowers on each tagged branch was counted prior to any treatment. Any flowers that had already opened or senesced on tagged branches were not counted. Stigmas were receptive prior to any manual pollination treatment (i.e. sticky glistening stigmatic surface). Pollinators were excluded using large organza bags with a mesh size sufficient to exclude visitors while allowing air and moisture to pass through. Fruit set was recorded in February 2016, determined as the number of swollen ovaries on a tagged branch. The number of fruits maturing on tagged branches was recorded in August 2016, where fruit maturation was defined as the presence of a green, fleshy drupe with purple spots or striations visible on the pericarp. Each fruit was cut in half using secateurs and the presence of a viable seed was recorded when the white embryo was visible.

The effect of pollination treatment on fruit set and fruit maturation was analysed separately. Fruit set and maturation proportional data were log-transformed to satisfy assumptions of normal distribution prior to analysis in R (version 3.4.0). We used a two-factor linear model to determine if there were significant differences between the number of fruits on branches and recorded data for fruit set or maturation. We investigated pairwise comparisons between treatments using a post-hoc Tukey's HSD test where applicable.

Seed burial experiment

To examine the rate of endocarp breakdown in the soil, we buried 600 three-month old pyrenes at Site 3. At the burial site, six 0.5×2 m plots were established adjacent to one another. Each plot had four replicate sets of 25 pyrenes that were buried under 2–5 cm of soil. For each

replicate, pyrenes were mixed with local soil and sealed in organza mesh bags (90×120 mm), and then bags were buried in the soil using metal pegs to mark their locations. Pyrenes were buried at the end of summer, in February 2016, and exhumed at six-monthly intervals over 24 months, until February 2018. One bag per plot was exhumed at each interval to retrieve a total of 150 pyrenes. A further 150 fresh pyrenes (i.e. 0 months burial) were also tested in February 2016 as below.

Following exhumation, the six replicate pyrenes bags were bulked and cleaned of soil using sieves and brushes in the laboratory at ABGMA. Twenty-five pyrenes were randomly chosen for assessing endocarp strength via penetrometer force (N), using an Enpaix Digital Force Gauge (EFG1000 and manual test stand; SI Instruments, South Australia). A pyrene was placed longitudinally on a flat metal surface and cracked using a flat metal probe attached to the penetrometer. The peak force required to crack the endocarp was recorded for each pyrene.

Mean pyrene weight was assessed by weighing a further 25 exhumed pyrenes chosen at random. The permeability to water was measured by then soaking pyrenes in deionized water for 30 h. During this time course, the pyrenes were removed from the water at 0.5, 1, 2, 4, 8, 24 and 30 h time points, then lightly dried and weighed. The percentage increase in seed mass per time interval was calculated as the mean imbibed weight divided by the mean non-imbibed weight. Following imbibition, endocarp strength was tested on pyrenes using the EFG1000 as outlined above.

The remaining 100 pyrenes were used for germination testing. Pyrenes were soaked in deionized water overnight to soften the endocarps. A mounted hand vice was used to extract seeds from the endocarps, and seeds were transferred to a weak bleach solution (1–2%) for 15 min to help minimize microbial contamination during incubation. Bleach-treated seeds were then transferred into deionized water and soaked for a further 15 min. Seeds were sown on water agar plates (0.7% w/v) in replicates of 25 and incubated at a 10/25°C temperature regime with a 12 h/12 h light/dark photoperiod for ten weeks. Seeds with a visible radicle of > 2 mm were scored as germinated, and then removed.

General linear models were used to examine changes to pyrene strength and the proportion of germination over the two-year burial period. Final germination was adjusted following viability checks of non-germinated seeds. Seeds with a firm, white embryo were scored as viable following the ten-week germination period.

Variables with significant differences were then analysed using a Tukey HSD post-hoc test. The data were analysed in R (v. 3.4.0).

Simulated seasonal germination experiment

To examine the effect of temperature on germination probability, we collected approximately 1,800 fruits from approximately 40–50 plants at each of Site 1 and Site 2 in September 2016. The outer fleshy layers of the fruits were removed, and the remaining pyrenes were cleaned in the laboratory at ABGMA. Seeds were extracted from endocarps as described for the seed burial study. A factorial treatment combination of two collections sites and three incubation temperature regimes was applied. The factorial design was replicated on both water agar (0.7% w/v) and agar containing the germination stimulant gibberellic acid (GA₃ agar), which has been shown to increase germination success in some *Persoonia* species (Nancarrow 2001; Mullins et al. 2002).

For each treatment combination, a total of 96 seeds were sown into sterile plastic cell culture plates with 24 flat-bottom wells (Corning® Costar® TC-treated; 3.4 mL well capacity). Each well contained approximately 2 mL of agar, and one seed was sown per well. Well-plates were used over traditional petri plates due to their ability to isolate seeds and, therefore, any possible fungal contamination. GA₃ agar was prepared as per water agar, but with 250 ppm gibberellic acid powder dissolved into the molten agar immediately prior to pouring. Seeds were incubated for ten weeks at one of three different alternating temperature regimes that reflected seasonal averages in North Rothbury between 2014 and 2016. These were: (1) 5/18°C (winter), (2) 10/25°C (autumn and spring; but hereafter referred to as ‘autumn’), or (3) 18/29°C (summer; Cessnock Airport, Australian Weather Station; www.bom.gov.au). Plates were checked on a weekly basis and seeds with a protruding radicle of > 2 mm were recorded as germinated.

Germination on both agar treatments was recorded as a weekly count for each plate replicate and then analysed using a linear model to examine the interactive effects of incubation temperature and site. We used weekly germination checks as a repeating measure in the models. Post-hoc tests using Tukey’s HSD were run where necessary to identify significant differences. Time-to-event models were performed to analyse the germination rate (g_r) and time to 50% germination (T_{50}), relative to the maximum germination among

experiment treatment combinations. These regression models take into consideration that a seed may have germinated at any point within a survey interval (Ritz et al. 2013). Furthermore, the model assumes that non-germinated viable seeds at the end of the experiment may still germinate if the experiment was to continue (i.e. right-censored data). The data were analysed in R (version 3.4.0), with the time-to-event models analysed using the ‘drc’ R package (Ritz et al. 2016).

Results

Pollinator visitation

During the five days of surveys, 55 European Honeybees (*Apis mellifera*) and 270 native bees (including *Leioproctus incanescens*, *L. speculiferus*, *Amegilla* sp. and *Tetragonula carbonaria*) were recorded on *P. pauciflora* flowers. *Leioproctus* species accounted for around 95% of native bee observations. There was a significant difference in the total number of honeybees compared to native bees across the survey period ($P < 0.001$; Fig. 3). There were few instances of high bee abundance on plants over the five survey days ($< 15\%$ of observations). Although there was a 5:1 ratio of native bees to honeybees, they displayed similar foraging

efforts ($P = 0.06$; Fig. 3). Native bees foraged on an average of 6.53 ± 0.44 flowers per plant per ten minutes before moving to an adjacent plant, while honeybees foraged on an average of 6.45 ± 0.80 flowers. Foraging effort was similar among the survey days and plants.

Breeding system

Due to inclement weather and damage to tags, data for the breeding system assessments were obtained from 27 plants rather than the original 30. The number of flowers per branch ranged among pollination treatments from 21 ± 2 to 29 ± 2 , which did not represent a significant variation ($P = 0.113$). Self-compatibility was observed in the species, as fruit set occurred in autogamy ($9 \pm 2\%$), self ($8 \pm 2\%$) and geitonogamy ($11 \pm 5\%$) treatments (Fig. 4). By contrast, $23 \pm 5\%$ of xenogamously pollinated flowers had set fruit, which was significantly higher than all other treatments ($P = 0.003$; Fig. 4). A similar pattern was observed for the number of fruits maturing per branch, with xenogamously treated branches exhibiting significantly higher values ($18 \pm 3\%$) than the remaining three treatments, which ranged from $2 \pm 1\%$ to $3 \pm 1\%$ ($P < 0.001$; Fig. 4). At the time of recording fruit maturation, the number of fruits on tagged branches was significantly lower across treatments compared to when fruit set was recorded ($P = 0.001$).

Fig. 3 The mean ($\pm SE$) number native bees, honeybees and the mean number of flowers visited per *Persoonia pauciflora* plant (per 10-min observation). An asterisk (*) denotes a significant difference between native bees and honeybees from the generalized linear mixed model

Fig. 4 Average percentage of fruits on tagged branches of *Persoonia pauciflora* plants ($n = 27$) across pollination treatments. Different lower-case letters indicate significant a difference in percent fruit set and percent fruits matured between treatments

Seed burial

The average strength of dry *P. pauciflora* pyrenes buried in soil significantly decreased over time from 413.3 ± 14.6 N to 130.8 ± 11.8 N ($P < 0.001$; Fig. 5a). Of the 68% decrease in pyrene strength after a two-year burial, 42% of this decline occurred during the first six months. Pyrene strength following imbibition also significantly decreased from 304.7 ± 10.6 N to 84.5 ± 8.8 N after two years of soil burial ($P < 0.001$). Imbibing fresh pyrenes resulted in a 26.3% decrease in endocarp strength compared to the dry endocarp strength. Endocarp fracturing was visible after twelve months of burial; however, no *in situ* germination was observed. Fresh pyrenes had an average dry weight of 160 ± 4 mg, which declined to 110 ± 5 mg after 12 months in the soil (i.e. 68% of the average dry weight of fresh pyrenes), and then remained stable over the second 12 months in the soil (Fig. 5b). Fresh pyrenes had an average weight increase of 42% (from 160 ± 4 mg to 228 ± 7 mg) following 30 h of imbibition, whereas water uptake for pyrenes buried for 6–24 months was lower (8–28% weight increase; Fig. 5b). Water uptake by fresh pyrenes was greatest in the first 8 h and plateaued thereafter. Across all burial durations (6–24 months), water uptake was greatest between 8 and 24 h before plateauing at 30 h (Fig. 5b).

The *ex situ* germination percentage of fresh seeds was $13 \pm 3\%$. Germination of exhumed *P. pauciflora* seeds occurred from all burial intervals, with the seed batches displaying a seasonal, cyclic germination throughout the two-year burial period (Fig. 5c). Percent germination was similar between the two winter-exhumed seed batches ($26 \pm 4\%$ for 6 months, and $26 \pm 6\%$ for 18 months). No significant difference was detected between the germination of fresh seeds and seeds buried for 6 months ($P = 0.244$) or 18 months

Fig. 5 **a** – Penetrometer force ($N \pm SE$) required to crack *Persoonia pauciflora* pyrenes following soil burial over two years. Pyrenes were cracked when dry (open circles) and following 30 h water imbibition (grey circles). **b** – Average weight ($\pm SE$) of *P. pauciflora* pyrenes measured after imbibing water for 0, 0.5, 1, 2, 4, 6, 8, 24 and 30 h following soil burial for up to two years. **c** – Final germination percentage ($\pm SE$) of *P. pauciflora* seeds sown on water agar and incubated at $10/25^{\circ}\text{C}$ following soil burial over two years, and adjusted for viability

($P = 0.214$). Compared to fresh seeds, percent germination significantly increased following summer-exhumations at 12 and 24 months ($75 \pm 2\%$ and $74 \pm 14\%$, respectively; both $P < 0.001$). The initial viability of fresh seeds was 92%, which declined to 71% over two years of burial in the soil (Fig. 5c).

Simulated seasonal germination

The germination of *P. pauciflora* seeds on water agar was highly variable across seasonal temperature regimes and sites, with a significant interaction identified between temperature and site ($P < 0.001$). The highest germination percentage on water agar was exhibited by seeds collected from Site 1 and incubated under the autumn temperature regime ($51 \pm 4\%$; Fig. 6). By comparison, the lowest germination percentage on water agar occurred for seeds collected from Site 2 and incubated under the winter temperature regime ($4 \pm 2\%$; Fig. 6). Germination on water agar was significantly lower for both study sites when seeds were incubated at winter temperatures ($P < 0.001$). Germination was more consistent among the temperature regimes and site when seeds were sown on GA₃ agar, varying between $15 \pm 6\%$ and $25 \pm 5\%$ (Fig. 6). A significant interaction between site and temperature was concluded from the linear model ($P = 0.001$), with germination percentage increasing on GA₃ agar compared to water agar for seeds collected from Site 2, while the converse was true Site 1 seeds.

Seeds germinated on water agar at the winter temperature had a significantly slower g_r than those germinated at summer and autumn temperatures for both study sites ($P < 0.001$ and $P = 0.009$, respectively). Seeds from Site 2 had a significantly higher g_r on water agar under the summer temperature regime than the autumn temperature regime ($P = 0.042$). By comparison, for Site 1 seeds the g_r was statistically similar when incubated at summer and autumn temperature regimes ($P = 0.868$; Table 1). T_{50} was significantly slower when seeds from either site were incubated at the winter temperature regime on water agar, compared to the summer and autumn regimes. There was no significant difference in T_{50} detected between the summer and autumn temperature regimes for either site (Table 1). On GA₃ agar, g_r and T_{50} were each statistically similar across the three temperature regimes and two Sites (Table 1).

Discussion

Pollinator visitation

Our results showed that *Persoonia pauciflora* is predominantly visited by native bees. *Leioproctus incanescens* and *L. speculiferus* were the most common visitors recorded during surveys, with peak visitation in the morning. These native bees are solitary foragers and have anatomical features, including a flattened clypeus and spines on the basitarsi of females, that make them specialist pollen and nectar gatherers from *Persoonia* flowers (Rayment 1950; Maynard 1992; Wallace et al. 2002). *Leioproctus* do not solely visit *Persoonia*, having previously been recorded on plants such as *Leptospermum* sp., *Lomatia silaifolia* and *Claoxylon australe* (Maynard 1992). Both male and female *Leioproctus* are known to forage on *Persoonia* flowers (Maynard 1992), and this was found to be the case for *P. pauciflora* plants with both sexes recorded in our surveys. Studies have recorded *L. incanescens* and *L. speculiferus* as visitors of up to eight and twelve eastern Australian *Persoonia* species, respectively (Bernhardt and Weston 1996; Wallace et al. 2002). *Leioproctus* species have also been documented to visit rare and common sub-species of *P. mollis* (Rymer et al. 2005). Though Bernhardt and Weston (1996) noted that *Exoneura* sp. frequently visit *Persoonia*, we did not record these bees in our surveys. Further seasonal surveys would help determine whether other native bee species visit *P. pauciflora* flowers.

Honeybees were also commonly observed visiting *P. pauciflora* flowers. Pollinator observations of several endangered *Persoonia* species, including *P. bargoensis*, *P. mollis* subsp. *maxima* and *P. glaucescens*, found honeybees to be the dominant floral visitor (Field et al. 2005; Rymer et al. 2005). However, honeybees may be less efficient pollinators than *Leioproctus* bees, as occasionally honeybees do not pollinate flowers since they tend to mix the nectar and pollen together on their bodies, causing pollen grains to germinate prematurely (Paton 1997). Although significantly less abundant than native bees, honeybees foraging effort on *P. pauciflora* flowers was statistically similar to native bees. Several population factors are also known to influence pollinator presence and foraging behaviour, including plant density, abundance, floral display size and their interactions (Field et al. 2005). For example, within-plant foraging efforts of pollinators were significantly higher in a low-

Fig. 6 Observed proportion of germinated *Persoonia pauciflora* seeds relative to the total from two sites under three season temperature regimes and two agar mediums using time-to-event models. Weekly germination totals are represented by points and shaded areas represent 95% CIs

density *P. bargoensis* population when compared with a high-density population (Field et al. 2005). Such specificity in observing chiefly *Leioproctus* species in our study could be an indication of the extremely narrow geographic distribution of *P. pauciflora*, low plant abundance and density, a lack of diversity of bees in the local area, or only a subset of local bees are attracted to *P. pauciflora*. Furthermore, it is possible that *Leioproctus* species often co-occur in habitats where *Persoonia* species are present. Since *P. linearis* co-occurs with *P. pauciflora*, these habitats may then be more attractive to *Leioproctus* bees due to a higher abundance of flowers. However, it is also unlikely that *Persoonia* species rely solely on *Leioproctus* species, as other bees including *Tetragonula*, *Xylocopa*, *Exoneura* and *Apis*, have been recorded visiting flowers (Bernhardt and Weston 1996; Wallace et al. 2002; Rymer et al. 2005).

Breeding system

Our results indicate that *P. pauciflora* has a self-compatible breeding system; however, the species is a preferential out-crosser, as xenogamously pollinated flowers produced significantly more fruit than any other

pollination treatment. Self-compatibility is thought to be an evolutionary strategy of rare plants to ensure some level of reproductive success when pollinators are limited (Kunin and Gaston 1993). Typical of Proteaceae, *P. pauciflora* was found to have a very low fruit-to-flower ratio, and the cross-pollinated fruit set was comparatively lower than other previously studied *Persoonia* species (Carolin 1961; Trueman and Wallace 1999; Wallace et al. 2002; Rymer et al. 2005). Furthermore, the fact that significantly fewer fruits remained on branches compared to the number of recorded mature fruit could indicate a possible post-zygotic mechanism that terminates self-pollinated fruit. This mechanism was originally identified in *Persoonia* by Krauss (1994), who noted selfed fruit to be terminated on *P. mollis* plants between 4 and 30 weeks post-pollination. A low number of matured fruits could also be a factor of rarity and inbreeding. In common with our study, two other endangered species, *P. glaucescens* and *P. mollis* subsp. *maxima*, both produced mature fruits in only less than 20% of out-crossed flowers (Rymer et al. 2005).

Seed burial

Pyrene burial within the Broad-leaved Ironbark and Spotted Gum woodland distribution of *P. pauciflora* resulted in the gradual degradation of the endocarp and cyclical germination over the two years. The average strength of *P. pauciflora* endocarps declined by around 40% following six months of soil burial, and by almost

Table 1 Germination of *Persoonia pauciflora* seeds from two sites incubated under three seasonal temperature regimes

		Germination temperature	g_{final} [%]	T_{50} [days]	g_r [slope]
Water agar	Site 1	Summer (18/29°C)	38 ± 6 ^a	39.6 ± 3.0 ^a	-5.0 ± 0.9 ^a
		Autumn (10/25°C)	51 ± 4 ^b	34.5 ± 2.1 ^a	-4.8 ± 0.7 ^a
		Winter (5/18°C)	24 ± 5 ^c	48.7 ± 1.9 ^b	-10.9 ± 2.6 ^b
	Site 2	Summer (18/29°C)	33 ± 3 ^{ac}	42.7 ± 4.0 ^a	-4.8 ± 1.0 ^a
		Autumn (10/25°C)	9 ± 1 ^d	36.6 ± 2.7 ^a	-8.4 ± 2.7 ^b
		Winter (5/18°C)	4 ± 2 ^d	55.1 ± 2.5 ^b	-65.1 ± 166.7 ^c
GA ₃ agar	Site 1	Summer (18/29°C)	25 ± 5 ^a	30.7 ± 1.8 ^a	-6.3 ± 1.3 ^a
		Autumn (10/25°C)	18 ± 2 ^{ab}	31.7 ± 1.7 ^a	-7.8 ± 1.8 ^a
		Winter (5/18°C)	19 ± 3 ^b	35.3 ± 2.4 ^a	-6.5 ± 1.6 ^a
	Site 2	Summer (18/29°C)	15 ± 6 ^b	57.8 ± 33.7 ^a	-2.8 ± 1.2 ^a
		Autumn (10/25°C)	23 ± 4 ^{ab}	38.1 ± 3.3 ^a	-5.2 ± 1.2 ^a
		Winter (5/18°C)	23 ± 4 ^b	39.0 ± 4.7 ^a	-4.1 ± 1.0 ^a

Seeds were sown on either water agar or water agar infused with GA₃. Different lower-case letters indicate significant differences between site and germination temperature treatments. Each agar medium was analysed separately

Fig. 7 Daily maximum temperature (°C) and monthly rainfall (mm) over the two years of the soil burial experiment. Climate data was recorded from the Cessnock Australian Weather Station and downloaded from the Bureau of Meteorology website (www.bom.gov.au)

70% following two years of soil burial. Pyrenes also began imbibing within the first hour of soaking, confirming that the endocarp is water-permeable. Although natural fracture lines on buried endocarps were visible by 12 months, no *in situ* germination was observed throughout the burial time (N. Emery pers. obs.). In contrast to our results, in a study of *P. longifolia* there was no significant change in the strength of endocarps following two years soil burial (Norman and Koch 2008). This difference in endocarp degradation between these two species might be attributed to the fact that the fresh *P. longifolia* endocarps reported by Norman and Koch (2008) were around 150% stronger than fresh *P. pauciflora* endocarps (around 600 N and 400 N, respectively). The rate of *in situ* endocarp degradation and subsequent germination is also directly influenced by seasonal climate and local site factors (Chia et al. 2016a, b).

Maximum germination percentage occurred following late-summer exhumation at 12 and 24 months burial (February 2017 and 2018; 75% and 74%, respectively). For *P. longifolia* seeds, an increase in germination success to 40% has been reported following 8 months burial; but these seeds required endocarp chipping, GA₃ and smoke-water treatments to promote germination (Mullins et al. 2002). Similarly, chipped and GA₃ treated *P. longifolia* seeds germinated to 37% following 21 months burial in the soil in another trial (Norman and Koch 2008). When *P. longifolia* endocarps were buried

for three years under field and nursery conditions, germination reached 64% and 42%, respectively (Chia et al. 2016b). These results suggested the endocarp was the main barrier to germination. *Persoonia virgata* and *P. sericea* also required at least half of the endocarp to be removed for seeds to germinate, with chipping being insufficient for germination to occur (Ketelhohn et al. 1996; Bauer et al. 2004). Here, in the absence of the endocarp, *P. pauciflora* germination significantly varied throughout the soil burial trial, indicating that dormancy was not solely caused by the endocarp. That germination percentage was significantly higher following retrieval from the soil in late summer (12 and 24 months) compared to winter retrieval (6 and 18 months) suggests that the *P. pauciflora* embryos exhibited cyclical physiological dormancy. Summer conditions during the seed burial experiment were hotter and drier when compared to the local long-term average (29.6°C and 256.6 mm rainfall over summer). Around 180 mm of rain occurred over both summer seasons during the seed burial experiment and coupled with hot summer maximum temperatures (32.5 ± 0.4°C daily average during experiment) may have provided suitable environmental conditions to relax physiological dormancy (Fig. 7). However, it is possible that an initial period of cool winter temperatures was also required to relax physiological dormancy (i.e. cold stratification). Future burial trials should aim to exhumate pyrenes monthly to ascertain whether both cold and warm temperatures relax

dormancy. Cyclical germination has also been reported in buried *P. longifolia* seeds, with significantly higher germination occurring following autumn retrieval compared with late winter or early spring retrieval (Chia et al. 2016b).

Coupled with the cyclical germination and decline in endocarp strength, viability of fresh *P. pauciflora* seeds was 92% and steadily declined to 71% over two years of soil burial. This confirms that *P. pauciflora* has the potential to form an accumulating soil seed bank. The observed decline in seed viability over time resulted in an increased prevalence of microbial contamination of incubated seeds. Mixed results of long-term persistence of seeds in the soil seed bank have been reported in several *Persoonia* species. In two separate studies on *P. longifolia*, one found no significant loss of viability following two years soil burial, and the other a decline in viability from 93% to 68% after three years soil burial (Norman and Koch 2008; Chia et al. 2016b). *Persoonia pinifolia* seeds were estimated to exhibit an average decline in viability of 50% after one year in the soil (Auld et al. 2007). The potential for seeds to survive this timeframe is partially determined by the local ambient conditions. For example, *P. longifolia* seeds have been noted to rapidly lose viability when stored at 4°C (Mullins et al. 2002). A significant decline in viability of *P. mollis* subsp. *nectens* buried seeds was also recorded following a burn (McKenna 2007).

Seasonal germination

Germinating *P. pauciflora* seeds under different seasonal temperature regimes – simulating those occurring in its natural locality – suggested that germination is most likely to occur during summer, following the relaxing of physiological dormancy with warmer temperatures. However, seeds from different local sites showed variation in the width of their temperature window, with seeds from Site 1 having a greater potential to also readily germinate when conditions are cooler in autumn and spring. The capacity to germinate extracted *Persoonia* seeds has been met with mixed success (McIntyre 1969; Bauer and Johnston 1999; Nancarrow 2001; Mullins et al. 2002; Bauer et al. 2004). As many studies have approached germinating *Persoonia* with a horticultural impetus, few have described the temperature conditions required for germination in *Persoonia*. In a seasonal field trial, *P. longifolia* germination was

reported to occur during the transition from winter to spring (August to September) in south-west Western Australia (Chia et al. 2015). When *P. longifolia* seeds were incubated under *ex situ* winter temperatures, germination was reported to be highest at 20°C, or under a 10/20°C cycle (Chia et al. 2016b). Micro-site variation may explain the wider temperature window for the germination of *P. pauciflora* seeds from Site 1 compared to seeds from Site 2.

The physiological dormancy mechanism imposed by the embryo may also be species-specific or vary among years and population cohorts depending on conditions during fruit maturation on the maternal plant. The use of GA₃ has been demonstrated to alleviate physiological dormancy and increase germination success in several *Persoonia* (Ketelhohn et al. 1996; Nancarrow 2001; Mullins et al. 2002; Bauer et al. 2004). In our study, GA₃ elicited similar germination responses among sites and temperature regimes. When compared with germination on water agar, overall germination was either reduced or increased on GA₃ agar. This complex germination response might be explained by a combination of the increased microbial contamination of seeds observed on the GA₃ agar, and a varying degree of receptivity of *Persoonia* seeds to GA₃. For example, in a comparative germination study, *P. levis* germination was positively affected by GA₃, whereas *P. myrtilloides* seeds had no response (Nancarrow 2001). Microbial contamination has been reported to negatively affect the germination of numerous *Persoonia* species since initial trials by McIntyre (1969), and is one of the main limitations of *ex situ* germination experiments. Despite our best efforts to surface sterilize seeds, contamination in our experiment was most prevalent on GA₃ agar and often originated from within the seed, indicating endogenous microbes were still present. Microbial growth could be promoted by GA₃ as similar chemical derivatives have a microbial origin (Brian et al. 1954).

Conclusions

The set of experiments presented here encompass several components of the reproductive niche of *Persoonia pauciflora*. Biotic and abiotic factors can influence the reproductive niche at multiple developmental stages – from flower development and seed production to seed dormancy and germination. Indeed, reproductive traits are often more sensitive to changes in external

conditions than other vegetative traits (Snider and Oosterhuis 2011). These complex mechanisms make studies on the reproductive niche of vital importance, particularly in rare species, as these traits will ultimately define population persistence and overall species distributions (Bykova et al. 2012). Other external environmental factors not examined in this study, such as soil microsite factors that vary over time, could also ultimately determine seedling establishment.

For this critically endangered species, a partially self-compatible breeding system means that viable fruits should be seasonally produced, as some fruits may still reach full maturity in the absence of pollinators. We have demonstrated that a cross-pollination mechanism produced significantly greater numbers of fruit, which is important as plants with a self-compatible breeding system that are incapable of out-crossing without a biotic vector to transfer pollen are at the greatest risk of inbreeding depression. Local bee species, in particular *Leioproctus* spp. that were observed as floral visitors in a natural population, may then form an important interaction with *P. pauciflora* to increase the likelihood of cross-pollination and, therefore, a larger and more genetically diverse fruit cohort. Following fruit drop, the environmental conditions are then critical for breaking dormancy and controlling germination. The woody endocarp is a mechanical barrier that restricts germination and must suitably weaken over time to permit embryo growth. Coupled with the slow decline in seed viability, there is potential for an accumulating seed bank to form while endocarps weaken in the soil. A second set of environmental parameters are then required to relax physiological dormancy within the embryo, and summer temperature conditions were found to be important for regulating this trait. Similarly, germination was more likely to occur when seeds were exposed to warmer local temperatures. Overall, the insights from this study into the ecological interactions and mechanisms with seed production, dormancy and germination that define the reproductive niche will play a vital role in the development of future propagation, restoration and management policies for *P. pauciflora* and other related species.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest with the experiments in this study. Fruit collections were made according to NSW Scientific licence conditions (SL100569).

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