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Pollination by multiple species of nectar foraging Hymenoptera in *Prasophyllum innubum*, a critically endangered orchid of the Australian Alps

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ABSTRACT

Context. Australia has numerous threatened species of terrestrial orchid, with a particularly high incidence of rarity in the genus *Prasophyllum* R.Br. Although there has been research on mycorrhizal associations and propagation, little is known about the reproductive ecology of threatened *Prasophyllum*. Understanding which animals are responsible for pollination and the impact of herbivores on reproduction may inform conservation actions. **Aims.** For the nationally Critically Endangered *Prasophyllum innubum*, we aimed to determine the pollinator species, test for self-pollination, quantify levels of reproductive success and herbivory, and identify herbivores. **Methods.** Pollinator observations were undertaken at wild populations of *P. innubum*, whereas an experiment testing for self-pollination was undertaken in shadehouse conditions. We quantified reproductive success and herbivory at two populations and attempted to identify herbivores using game cameras. **Key results.** Pollination occurred via three species of bee and a sphecid wasp, all of which attempted feeding on floral nectar. Fruit set averaged 72–84% at wild sites, whereas only 6% of flowers set fruit via self-pollination when insects were excluded. Just 4% of inflorescences were completely consumed by herbivores, and no herbivory was captured on camera. **Conclusions.** *P. innubum* has a generalist rewarding pollination system that confers high levels of reproductive success, with herbivory having little impact on reproduction. **Implications.** Pollinator availability is unlikely to restrict conservation translocation site selection of *P. innubum* because of a generalist pollination system. If herbivores are a threat for this species, it is likely to be through alteration of habitat rather than direct grazing.

Keywords: Australia, conservation, Halictidae, Hymenoptera, *Lasioglossum*, orchid, pollination, *Prasophyllum*, reproduction, Specidae.

Introduction

Globally, orchids are one of the most species-rich plant families and feature prominently on lists of threatened species in many countries (Phillips *et al.* 2020*a*). A potential limitation for effective orchid conservation is lack of knowledge of factors affecting reproductive success and pollinator availability. In particular, the orchid family is characterised by a very high incidence of species reliant on just one or few pollinator species (Ackerman *et al.* 2023; although see Catling 1983; Patt *et al.* 1989; Huber *et al.* 2005; Antonelli *et al.* 2009, for examples of species with generalist pollination strategies), meaning that habitat management will often need to account for the ecological requirements of particular pollinator species (Phillips *et al.* 2020*b*). Further, reliance on one or a few pollinator species means that knowledge of pollinator availability must be incorporated into site selection when aiming to establish new populations of a threatened orchid (Reiter *et al.* 2016, 2017). Alternatively, if pollination occurs via a range of animal species, this may favour higher levels of reproductive success and enable flexibility when managing sites for pollinators or selecting sites for conservation translocation.

The Australian orchid flora contains numerous species listed as threatened under the federal *Environment Protection and Biodiversity Conservation Act 1999* (Department of Climate Change, Energy, Environment and Water 2024). Many of these species are now restricted to a very small number of populations (e.g. Brown *et al.* 1998; Backhouse 2018), which has led to intensive conservation programs aimed at recovery of species in the wild (e.g. Reiter *et al.* 2021). *Prasophyllum* (leek orchids) is one of Australia's most threatened orchid genera, with 40 of 130 species being currently listed at the federal level as threatened with extinction (Department of Climate Change, Energy, Environment and Water 2024). The highest diversity of *Prasophyllum* is in temperate areas of south-eastern Australia, with a lesser diversity in south-western Australia (Jones 2021). Representatives of *Prasophyllum* occur in an exceptionally wide range of habitats, including coastal dunes, woodlands and forests, swamps, alpine herb fields, and granite outcrops (Jones 2021). However, many of the most threatened species are endemic to grasslands and associated damplands in south-eastern Australia (Jones 2021; Backhouse 2023). Although the natural geographic range of these species is often difficult to ascertain because of the extent of the clearing and degradation of these habitats (Williams and Morgan 2015), many *Prasophyllum* are restricted to a small number of populations containing just a few plants and are at imminent risk of extinction (Australian Government 2023; Backhouse 2023). This predicament has inspired research to identify the mycorrhizal partners of *Prasophyllum* (various fungi in the family Ceratobasidiaceae; Freestone *et al.* 2021, 2022), and develop propagation techniques tailored to this genus (e.g. Freestone *et al.* 2023). However, at this stage, there has been little research on the reproductive ecology of threatened *Prasophyllum*.

Species of *Prasophyllum* produce clustered spikes of small non-resupinate white, green, brown or reddish flowers, which in at least some species have been observed to produce a small quantity of nectar (Bates 1984*b*; Bernhardt and Burns-Balogh 1986; Peakall 1989). Although few studies have systematically quantified floral visitors to *Prasophyllum*, the trend from these studies and anecdotal observations are that *Prasophyllum* flowers can attract a wide range of insect groups (e.g. wasps, ants, flies, beetles and bees), but removal and deposition of pollen is typically primarily restricted to bees and wasps (Jones 1972; Bates 1984*b*; Bernhardt and Burns-Balogh 1986; Peakall 1989; Kuiter 2018). Among those *Prasophyllum* species where pollen vectors have been studied in greater detail, there is evidence that the relative contribution of different groups of pollinators varies among species. For example, Peakall (1989) found that *Prasophyllum fimbria* Rchb.f. was primarily pollinated by three families of bees and scoliid wasps, whereas Bates (1984*b*) observed at a site in South Australia that pollination of *Prasophyllum elatum* R.Br. occurred solely via a single species of thynnine wasp. Among smaller-flowered *Prasophyllum* species, there is also evidence that some species can produce seed when pollinators are experimentally excluded (Bates 1984*a*). In *Prasophyllum*, high levels of fruit set are often observed in the wild (e.g. Bernhardt and Burns-Balogh 1986; Elliott and Ladd 2002); however, observations of shade-house plants indicate that in many cases this does not arise through self-pollination (N. Reiter, pers. obs.). Given the evidence for interspecific variation in pollen vectors in *Prasophyllum*, we are not able to assume the pollination strategy for a given threatened species, or that suitable pollinators will necessarily be available at a candidate site for conservation translocation.

An important aspect of the ecology of terrestrial orchids, which is often overlooked in conservation translocations and the management of threatened orchids, is the potential for grazing by herbivores to limit reproductive success. Thus far, few studies have focused on the consumption of flowers and seed capsules of orchids (Petit and Dickson 2005; Faast and Facelli 2009; Karremans *et al.* 2023; Phillips *et al.* 2024), although these events are often reported in pollination studies (e.g. Ackerman and Montalvo 1990; Winkler *et al.* 2005; Sletvold *et al.* 2010; Nunes *et al.* 2016). In terms of florivory, some of the clearest evidence for an adverse effect comes from studies on *Caladenia* R.Br. in southern Australia (Petit and Dickson 2005; Faast and Facelli 2009). Here, Faast and Facelli (2009) showed that up to 94% of *Caladenia* flowers could be consumed in a given year, although the number is highly variable among years. For Australian orchids that occur in grasslands and associated habitats, herbivory by vertebrates is likely to be a conservation issue, given the potential for grazing by introduced herbivores such as horses, deer, rabbits and hares. Alternatively, in some regions, native herbivores may be reduced in abundance through conversion of native habitats to agriculture, leading to reduced grazing pressure on orchids. At present, there is no data on grazing of *Prasophyllum*, but management of herbivores is a potentially important conservation consideration.

Here, we study the pollination ecology and reproductive success of *Prasophyllum innubum* D.L. Jones, a summer flowering orchid that is restricted to a small area of subalpine swamps and peatlands within grasslands in the Southern Tablelands of New South Wales, Australia. *Prasophyllum innubum* is listed Critically Endangered under the federal *Environmental Protection and Biodiversity Act 1999*, owing to its highly restricted distribution and being threatened by grazing and trampling by feral horses and cattle, as well as drainage disturbance and hydrological changes (Department of Agriculture, Water and the Environment 2014). *Prasophyllum innubum* is the focus of a conservation program at the Royal Botanic Gardens Victoria, with the aim of establishing additional insurance populations and identifying potential management issues at existing sites. Therefore, there is a need to identify pollinators and establish whether grazing by vertebrates is likely to affect reproductive output. Here, we investigate (1) what are the pollinators of *P. innubum*; (2) whether *P. innubum* produces a nectar reward; (3) what proportion of flowers of *P. innubum* set fruit in the wild;

(4) whether *P. innubum* is capable of seed production without pollinators; and (5) how frequent is herbivory, and what animals are responsible for any damage to orchids?

Materials and methods

Study species and sites

Prasophyllum innubum is a terrestrial herb with a highly restricted distribution in the tablelands of south-eastern New South Wales, although there are suggestions that similar plants from the Victorian part of the Australian Alps may also be this species (Backhouse 2023). At present, *P. innubum* is known from just two nearby areas of grassland, with the most detailed population estimate being 4132 flowering plants in 2021 (Phillips *et al.* 2021*a*). It occurs in montane grass-tussock plains and peatlands at the edges of streams, ~1150–1200 m above sea level, where it grows in sphagnum hummocks and adjacent peaty and grassy areas (Jones 2007).

Prasophyllum innubum is a perennial geophyte that produces a single 30–90 cm inflorescence per plant, with 6–20 small non-resupinate brownish-green, purple flowers with a white or pinkish labellum (Fig. 1). Flowering extends from January to March, occurring principally in February (Jones 2007, 2021). In the taxonomic description of the species, Jones (2007) predicted that *P. innubum* is self-pollinating, but this has not been tested experimentally.

Our main study site was a large, unfenced population of *P. innubum* at MOP (site and name details for MOP are withheld because of the threat status of the orchid; voucher specimen numbers: MEL 2523474A, MEL 2523475A, MEL 2523473A). At this site, *P. innubum* primarily occurs in wetter areas of the plain dominated by *Poa costiniana* Vickery (Poaceae) tussocks and the rushes *Baloskion australe* (R.Br.) B.G.Briggs & L.A.S.Johnson and *Empodisma minus* (Hook.f.) L.A.S.Johnson & D.F.Cutler (Restionaceae). Co-flowering herbs included *Isotoma fluviatilis* McComb (Campanulaceae), *Stylidium montanum* Raulings & Ladiges (Stylidiaceae), *Leptorhynchos squamatus* (Labill.) Less. (Asteraceae), *Hypochaeris radicata* L. (Asteraceae), *Veronica subtilis* B.G.Briggs & Ehrend. (Plantaginaceae), *Gonocarpus tetragynus* Labill. (Haloragaceae), *Asperula conferta* Hook.f. (Rubiaceae), *Ranunculus graniticola* Melville (Ranunculaceae), *Arthropodium milleflorum* (DC.) J.F.Macbr. (Asparagaceae), *Spiranthes australis* (R.Br.) Lindl. (Orchidaceae), *Microtis rara* R.Br. (Orchidaceae), and *Oreomyrrhis eriopoda* (DC.) Hook.f (Apiaceae). In some areas, *P. innubum* grew among low shrubs (*Epacris gunnii* Hook.f., *E. breviflora* Stapf; Ericaceae).

During the pollinator-observation period, within the population at MOP, *P. innubum* made up 18.2% of the flowering community by number of flowers, making it the second-most numerically dominant flowering species after *Leptorhynchos squamatus* (37.7% of flowers; Supplementary Table S1). Other dominant co-flowering species included *Spiranthes australis* (15.0%), *Stylidium montanum* (10.8%) and *Veronica*

Fig. 1. (*a*) *Lasioglossum* (*Chilalictus*) *tamburinei* and (*b*) *Exoneura* (*Exoneura*) sp. visiting *Prasophyllum innubum*. They can both be seen carrying pollinia of *P. innubum*. Photographs by Tobias Hayashi.

subtilis (8.3%). A further twelve other flowering species were recorded, which each comprised < 2% of the flowering community (Table S1).

The secondary study site was at a population of *P. innubum* in a privately owned fenced area on MAP (site name and details of MAP are withheld because of the threat status of the orchid), which extends onto nearby unfenced land. At this site, *P. innubum* occurred in wetter drainage areas dominated by *Poa* tussocks (principally *P. sieberiana* Spreng. and *P. costiniana*). Co-flowering herbs included *Isotoma fluviatilis*, *Stylidium montanum*, *Bulbine bulbosa* (R.Br.) Haw. (Asphodelaceae), *Euphrasia collina* R.Br. (Orobanchaceae), *Stellaria angustifolia* Hook. (Caryophyllaceae), *Gonocarpus tetragynus* Labill. (Haloragaceae), *Brachyscome scapigera* (Spreng.) DC. (Asteraceae), *Leptorhynchos squamatus*, *Podolepis robusta* (Maiden & Betche) J.H.Willis (Asteraceae), *Craspedia aurantia* J.Everett & Joy Thomps. (Asteraceae), *Haloragis heterophylla* Brongn. (Haloragaceae), *Ranunculus graniticola*, and *Epilobium* sp. (Onagraceae).

A co-flowering population of approximately 40 plants of *Prasophyllum viriosum* D.L.Jones & D.T.Rouse was present approximately 300 m from the MAP population of *P. innubum*. However, no other *Prasophyllum* species were in flower within populations of *P. innubum* during the observation period. The pollinia of *P. innubum* can be distinguished in the field from the other orchid genera flowering at the site (*Corunastylis*, *Eriochilus*, *Genoplesium*, *Microtis*, *Spiranthes*) by either size or structure (see Pridgeon *et al.* 2001).

Pollinator observations

Observations of floral visitors were undertaken across 7 days in a 600 × 200 m area at MOP, between 27 January and 9 February 2021, with a total of 67 person-hours of observation. This population was used for pollinator observations because it had a greater number of plants in flower and there were no nearby co-flowering individuals of other *Prasophyllum* species. Observations were undertaken on sunny or partly cloudy days between 8 am and 6 pm, with temperatures being >18°C. Small patches of *P. innubum* with between 2 and 22 flowering plants were observed for 15-min periods, with two stationary observers (T. H., B. A.) working simultaneously at different patches (giving 123 15-min periods in total). Floral visitors and their behaviour were recorded, including whether they attempted to feed on flowers, how many flowers they visited per inflorescence, and whether they were carrying, removing or depositing *Prasophyllum* pollinia. After each 15-min period, the observer moved to a different patch of orchids greater than 15 m away, rotating between four and six patches throughout the day, with 16 patches being used across the study. In addition, five longer observation periods of 45 min to 3 h were undertaken at patches of flowering *P. innubum* plants, in areas where insects carrying *Prasophyllum* pollen had been seen earlier in the study. Opportunistic observations of floral visitors outside of the trials were also recorded. Floral visitors from 15-min periods, longer observation periods, and opportunistic observations were combined to quantify the range of floral visitors to *P. innubum*.

A high natural rate of pollination in *P. innubum* (see Results) hindered our ability to observe pollen deposition and removal, because there were generally few unvisited flowers on a given inflorescence. As such, during a subset of the observation periods described above, picked inflorescences with freshly opened flowers were added to help resolve the pollinator status of the insect species we observed. Depending on availability, between one and four fresh inflorescences that had not had any pollen removed or deposited were collected from both sites. These were placed in vials of water near wild *P. innubum* flowers during observation periods or opportunistically placed next to foraging insects. These potential pollinators were then allowed to feed on the flowers, and after each foraging event flowers were checked to see whether pollen had been removed or deposited. Inflorescences were replaced once pollen removal and deposition had occurred on multiple flowers.

Observation periods for potential nocturnal visitors were undertaken by two people on 7 and 8 March at MAP. MAP was chosen over MOP for night-time observations, because it was a safer, more-secure fenced site, far from the road. Because of the nearby co-flowering *P. viriosum* at the MAP population, at this site insects were considered to carry *P. innubum* pollen only if removal was observed. Observations were undertaken between 8:30 pm and 11 pm, representing eight person-hours of observation. Patches of flowering *P. innubum* with between three and six flowers were observed with a red torch light for 15 min, and any insect visitation was recorded. Additionally, moths seen in the vicinity of the orchid population were followed with red-light torchlight to determine whether they were visiting *P. innubum* inflorescences.

A representative sample of the floral visitors observed was captured for identification by using insect nets and either placed in 95% ethanol (most insects) or frozen (bees, Lepidoptera). Bees were identified by Michael Batley (Australian Museum), moths by Peter Marriott, and flower spiders by Narelle Murphy. Bee specimens were deposited in the Australian Museum, whereas other specimens were retained in the Royal Botanic Gardens Victoria pollinator collection. Pollinators were identified to species level where possible; however, flies from the families Bombyliidae, Calliphoridae and Syrphidae, which only visited the flowers, were recorded to family level only.

Testing for the presence of nectar

Because nectar droplets were not visible on the *P. innubum* flowers, we tested for the presence of sugar by using a method that does not require sampling nectar droplets (see Reiter *et al.* 2018). Sugar was sampled from 10 inflorescences on 8 February 2021 from MAP (11:30 am, partly cloudy to cloudy at times, ~18°C). Inflorescences were not bagged in advance to prevent nectar feeding by insects, so our quantification of sugar represents a measurement of the standing crop of a subset of flowers. An aqueous solution of ribitol (0.20 mg/mL) was dispensed on three flowers on the same inflorescence using a glass syringe. For each of the three flowers there were separate extractions from the labellum, where insects attempt to feed, and the dorsal sepal as a control, both using 5 μL of the ribitol solution. After 2 min, the extracts were collected with 5 μL microcapillary tubes and immediately transferred to gas-chromatography vials (2 mL) with glass inserts, giving 15 μL per plant for both the labellum and dorsal sepal. Samples were refrigerated at 4°C in transit and stored at −20°C until analysis.

For each of the extracts, the solvent was evaporated to dryness with a stream of nitrogen. Methoxyamine–HCl (20 μL of 20 mg/mL solution in pyridine; Sigma-Aldrich, St Louis, MI, USA) was added and the sealed vials were heated for 2 h in a heating block at 37°C. At the same temperature, the extracts were treated with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA, 35 μL; Sigma-Aldrich) in the same sealed vials for 1 h before gas chromatography–mass spectrometry (GC–MS) analysis (Lisec *et al.* 2006). GC–MS analysis was performed on an HP5972A mass selective detector connected to an Agilent 5890 GC equipped with a HP-5 column [(5% phenyl polysilphenylene-siloxane), 30 m × 0.25 mm × 0.25 μm film thickness, Agilent Australia], by using helium as the carrier gas. An Agilent 7673 autoinjector was used and injections (1 μL) were performed in splitless mode. The oven temperature started at 40°C and increased to 300°C at a ramp rate of 5°C/min and was maintained for 15 min. GC–MS data were transformed to.cdf or.mzML files and processed (ADAP chromatogram builder, chromatogram deconvolution, multivariate curve resolution) and aligned (ADAP aligner) with MZ Mine 2 (ver. 2.53, https://mzio.io/#mzmine) (Pluskal *et al.* 2010). Tentative identification of trimethylsilylated monosaccharides and sucrose was based on the comparison of retention indices and mass spectra with data from a mass spectral library (NIST-11). All tentative identifications were confirmed by co-injections with synthetic standards. Quantifications were achieved by comparison of peak areas of total ion chromatograms (TICs) of nectar samples with the known amount of the internal standard ribitol. The response factors for the respective carbohydrates sampled and the internal standard were included in calculations of the amounts of the analysed substances (see Reiter *et al.* 2018).

Rates of fruit set and herbivory in wild plants

To quantify rates of fruit set and herbivory, 10 quadrats (2 × 2 m) were set up at both MOP and MAP over an area of ~0.011 km^2 and 0.043 km^2 respectively. Quadrants were placed greater than 10 m apart. Within each quadrant, each inflorescence was marked with a wooden skewer and metal tag placed ~10 cm north of the plant. The number of inflorescences per quadrat ranged between 5 and 23. These quadrats were revisited at the end of the flowering season and the number of turgid capsules per inflorescence was recorded. Any missing or damaged inflorescences and capsules were noted. The mean percentage fruit set for a population was calculated by averaging across inflorescences.

Testing for self-pollination

Testing for self-pollination of *P. innubum* in the field was challenging, owing to how quickly pollination commenced after flowers opened, and damage to plants that had been covered to exclude pollinators (presumably by grazing mammals). As such, we performed our experiment with the plants available in the shade house at Royal Botanic Gardens Victoria (Cranbourne site). Nine plants were placed in 'mosquito domes' to exclude pollinators. Of these, four were emasculated (to test whether apomixis could occur), and the remaining five were left in their natural condition to test for self-pollination.

Herbivore observations

To document any trampling or herbivory of *P. innubum* by vertebrates, Reconyx HF2 Covert Pro camera traps were placed in orchid populations at MOP (five cameras) and MAP (four cameras). The cameras were tied to star pickets and suspended 40–50 cm above the ground. The cameras were set to high-sensitivity, rapid-fire mode to take 10 photos and one 10-s video per trigger. The nine cameras were deployed during the flowering season (29–31 January 2021) and retrieved at the end of the flowering season (22 February 2021) for a total of 5066 observation hours, monitoring a total of 47 inflorescences. Camera-trap photos and videos were scored for the presence/absence of vertebrates, vertebrate movement (passing through the frame or feeding near orchids) and any discernible damage to orchids (inflorescences trampled, eaten or pulled out of the ground).

Ethics statement

Our research was undertaken under the permit SL102461 from the New South Wales Department of Primary Industry and Environment.

Results

Pollinator observations

In total, 169 insects were recorded visiting *P. innubum* flowers, from at least 21 different species (Table 1). Of the 145 insects that displayed feeding behaviour on flowers, there were 75 bees, 25 flies, 21 wasps, 17 moths, six butterflies and one beetle. In total, 50 visitors were observed carrying *Prasophyllum* pollinia, with bees and wasps being the only

Table 1. Summary of insect visitors to *Prasophyllum innubum* flowers.

Type	Species	Number visiting flowers	Number caught	Number feeding	Number carrying pollinia	Number removing pollinia	Number depositing pollinia
Bee							
	Lasioglossum (*Parasphecodes*) *altichum* (Halictidae)	29	15	29	19	4	2
	Lasioglossum (*Chilalictus*) *tamburinei* (Halictidae)	13	8	12	9	3	4
	Lasioglossum (*Chilalictus*) *clelandi* (Halictidae)	3	3	3	0	0	0
	Lasioglossum sp. (Halictidae)	2	0	2	1	0	0
	Exoneura (*Exoneura*) sp. (Apidae)	28	12	28	9	5	4
	Amegilla (*Notomegilla*) *chlorocyanea* (Apidae)	3	1	1	0	0	0
	Paracolletes crassipes (Colletidae)	1	1	1	1	0	0
	Megachile erythropyga (Megachilidae)	1	1	1	0	0	0
	Leioproctus (*Leioproctus*) sp. (Colletidae)	1	1	1	0	0	0
Wasp							
	Prionyx sp. (Sphecidae)	20	6	17	9	1	2
	Sphex fumipennis (Sphecidae)	1	1	1	1	1	0
	Delta philanthes (Vespidae)	1	1	1	1	0	0
	Ichneumon promissorius (Ichneumonidae)	2	0	1	0	0	0
Moth							
	Helicoverpa punctigera (Noctuidae)	16	10	15	0	0	0
	Hippotion scrofa (Sphingidae)	1	0	1	0	0	0
Butterfly							
	Zizina otis (Lycaenidae)	4	2	3	0	0	0
	Vanessa kershawi (Nymphalidae)	3	0	3	0	0	0
	Anisynta dominula (Hesperiidae)	1	1	1	0	0	0
Fly							
	Syrphidae	16	2	12	0	0	0
	Other flies, including Bombyliidae and Calliphoridae	16	5	13	0	0	0
Beetle	Unidentified beetle	1	1	1	0	0	0

groups observed carrying pollinia. No nocturnal visitors were observed.

The most common floral visitors were female bees from the genera *Lasioglossum* (five species, primarily *L.* (*Parasphecodes*) *altichum* and *L.* (*Chilalictus*) *tamburinei*; Halictidae) and *Exoneura* (*Exoneura*) sp. (Apidae), with occasional visits from female bees of other genera (Table 1). Bees of all species displayed similar behaviours; they typically fed from the labellum of *P. innubum* flowers, often grasping the top and side of the labellum with their legs and feeding at the base of the labellum while facing downwards. The bees removed and deposited pollinia while probing the base of the labellum, with pollinia found on the clypeus between the eyes and below the antennae (Fig. 1). Of those bees carrying pollinia that were collected for identification, between one and five pollinia were seen on each individual bee (mean = 2.45 ± 0.2 s.e. pollinia; $N = 33$ bees). Nine of the 28 *Exonuera* spp. recorded were observed carrying pollinia (32%), compared with 29 of 44 *Lasioglossum* (66%) (Table 1). Five species of bee were observed carrying pollinia, namely *Lasioglossum* (*Parasphecodes*) *altichum* ($N = 19$), *Lasioglossum* (*Chilalictus*) *tamburinei* ($N = 9$), *Lasioglossum* sp. ($N = 1$), *Paracolletes crassipes* ($N = 1$; Colletidae) and *Exoneura* (*Exoneura*) sp. ($N = 9$). Deposition of pollen was observed only by *L.* (*Parasphecodes*) *altichum* ($N = 2$), *L.* (*Chilalictus*) *tamburinei* ($N = 4$) and *Exoneura* (*Exoneura*) sp. ($N = 4$).

Large nectar-feeding wasps were also regularly observed visiting and feeding at the labellum of *P. innubum* flowers. *Prionyx* sp. (Specidae) were the most frequently observed, with 10 individuals being observed carrying pollen, and two individuals removing and depositing pollen. The five *Prionyx* specimens captured were all female. Like the bees, pollinia were carried on the clypeus between the eyes and below the antennae. No other wasp species were observed to effect pollination.

Both bees and wasps typically fed on multiple flowers in each feeding event, often moving from one *P. innubum* inflorescence to another. In the 50 bee feeding events where these data were recorded, bees visited a total of 1–25 flowers (mean = 5.18 ± 0.69 flowers) across 1–4 inflorescences (mean = 1.46 ± 0.13 inflorescences). Similarly, in 18 feeding events, wasps visited a total of 1–18 flowers (mean = 5.83 ± 0.90 flowers) across 1–3 inflorescences (mean = 1.56 ± 0.15 inflorescences). On average, bees visited 3.41 ± 0.27 ($N = 50$) flowers per inflorescence, and wasps visited 3.77 ± 0.45 flowers ($N = 18$) per inflorescence. During our observations, it is possible the bees and wasps continued to visit other *P. innubum* inflorescences after leaving the view of the observer. Bees were occasionally observed going to flowers of *Stylidium montanum* and *Veronica subtilis* after visiting *P. innubum* flowers.

Moths, butterflies and flies were also common floral visitors, but were not observed carrying or removing pollinia (Table 1). The elongate proboscis of Lepidoptera did not appear to contact the reproductive structures while they fed at the flower. Similarly, the size and feeding behaviour of flies did not result in contact with the orchid's reproductive structures.

The flower spider *Australomisidia rosea* (Thomisidae) was regularly observed on *P. innubum* inflorescences, and was occasionally found feeding on *Lasioglossum* bees, including several bees carrying orchid pollinia.

Testing for the presence of nectar

For the 10 plants sampled, on average 151.3 ± 84.1 μg of monosaccharides and 2.0 ± 0.6 μg of sucrose was detected in total across the three labella sampled, whereas for the control samples on the dorsal sepal, there was 2.6 ± 1.4 μg of monosaccharides and 0.5 ± 0.3 μg of sucrose.

Rates of fruit set and herbivory

The number of flowers per inflorescence ranged between 3 and 23, with a mean of 11.5 ± 3.9. Fruit set averaged 85.4 ± 2.3% at MOP (from 99 inflorescences and 1133 flowers) and 72.4 ± 2.8% at MAP (from 107 inflorescences and 1232 flowers). A total of three inflorescences at MOP and five inflorescences at MAP did not set any fruit (excluding those eaten or aborted). A total of five inflorescences (5.1%) appeared to be entirely consumed by herbivores and two aborted before flowering at MOP, whereas three (2.8%) appeared to have been entirely eaten at MAP. Insect damage to particular flowers and seed capsules was recorded in 6 of 20 quadrats, on between one and three plants per quadrat (average 1.67 ± 0.33), and one to six flowers per plant (average 1.79 ± 0.43).

Testing for self-pollination

Of the five plants tested for self-pollination in the absence of pollinators, only two of them produced any seed capsules (3 of 12 flowers, 1 of 25 flowers). On average, 5.8 ± 4.8% of flowers produced fruit, which equated to four capsules from 106 flowers (compared with 85.4% and 72.4% of flowers forming fruit at wild sites). None of the four emasculated plants produced any fruit, from a total of 80 flowers.

Herbivore observations

In total, 3066 photos and videos were captured by the nine cameras. Twenty individual vertebrates were detected, including feral horses (7 individuals recorded, 3 moving through and 4 feeding near orchids), eastern grey kangaroos (7 individuals recorded, 4 moving through and 3 feeding near orchids), Australian magpie (2 individuals), and fallow deer (1 individual moving through). In addition, two unidentified animals and one unidentified macropod were detected passing through the field of view. In one instance, a feral horse was recorded trampling an inflorescence, but the other 46 inflorescences in view of the cameras were not damaged despite prolonged feeding and movements in close proximity to orchids.

Discussion

Detailed field observations showed that *P. innubum* attracts a large range of nectar-feeding insects, including bees, wasps, butterflies and moths. However, pollination occurs exclusively via the foraging of bees and wasps seeking nectar from the base of the labellum. Pollen-deposition events were observed for three species of bee (*Lasioglossum* (*Parasphecodes*) *altichum*, *L.* (*Chilalictus*) *tamburinei* and *Exoneura* (*Exoneura*) sp.), and one species of sphecid wasp (*Prionyx* sp.). However, it should be noted that even though these were the most frequent floral visitors, some of the other bee and wasp species observed carrying pollinia may also make a minor contribution to pollination, which we were unable to observe. The pollen vectors observed here are similar to those observed by Peakall (1989), where pollination of *P. fimbria* occurred by a range of bee species and solitary wasps (in that instance, in the Scoliidae). Our observations suggest a wider range of pollen vectors than for *P. elatum*, where pollination was almost entirely by a single species of thynnine wasp (Bates 1984*b*). However, it should be noted that observations elsewhere in the geographic range of *P. elatum* (Kuiter 2018) have shown additional wasp species acting as pollen vectors beyond those observed in Bates (1984*b*). Our observations of the importance of bees and to a lesser extent wasps are also in line with observations of *P. odoratum* (Bernhardt and Burns-Balogh 1986; Kuiter 2018).

By being pollinated at a single site by multiple species of bee and a species of sphecid wasp, *P. innubum* appears to have a more generalised pollination system than has been recorded in most other Australian orchids where pollination has been studied in detail. Although Australia has many species of orchid pollinated by sexual deception of particular species of male insects (e.g. Peakall 1989; Peakall *et al.* 2010;

Phillips *et al.* 2014*a*, 2014*b*, 2017), detailed studies of orchids pollinated by nectar-seeking insects have typically also shown pollination by one or few insects species (Phillips and Batley 2020), even across multiple populations of the orchid (Reiter *et al.* 2018, 2019*a*, 2019*b*; Scaccabarozzi *et al.* 2018; Phillips *et al.* 2020*b*, 2021*b*). This specialisation arises through some combination of floral traits that are attractive only to a particular subset of the pollinator community (Scaccabarozzi *et al.* 2018; Reiter *et al.* 2019*a*, 2019*b*), and pollinators of a particular size and shape being required to contact the reproductive structures (Reiter *et al.* 2018; Phillips *et al.* 2020*b*). *Prasophyllum* have relatively open-faced flowers where pollen is deposited on the head of the insect, meaning that a wider range of species may be capable of removing pollen. In the case of *P. innubum*, having numerous nectar-producing flowers with a white labellum may contribute to attracting a wide range of generalist pollen vectors, particularly because they were one of the most abundant nectar-producing plants at the time of flowering. We detected approximately 50 μg of monosaccharides per flower, which would be ~500 μg for a plant with 10 flowers open. Although out of necessity our method is not directly comparable with those in most other studies of orchids (e.g. those measuring sugar in droplets of liquid), our results suggest that *P. innubum* is likely to be rewarding, but with a lower quantity of sugar available than for many other orchids that provide a nectar reward (e.g. Johnson 1996; Galetto *et al.* 1997; Davies *et al.* 2005; Van der Niet *et al.* 2015). Interestingly, the generalist nature of pollination in *P. innubum* conforms to the results of plant-pollinator network studies undertaken at high-elevation regions of the Australian Alps, where generalist pollination systems are the norm across various plant families (Inouye and Pyke 1988; Johanson *et al.* 2019).

An interesting result evident in the bee community visiting *P. innubum* is that all specimens were females, regardless of species. As *P. innubum* produces nectar rather than a pollen reward, the female bias cannot be attributed to female bees visiting the flower to assist with provisioning their brood. Records of *L. altichum*, the bee species most frequently observed carrying pollinia in our study, suggest that the flowering of *P. innubum* approximately coincides with the beginning of the peak flying time of the bee (Atlas of Living Australia 2023). This rules out the possibility that males have emerged earlier than females and died off prior to the flowering of the orchid. One possibility for the absence of males is that they may be patrolling for females near nest sites, which could be located away from the swampy areas that *P. innubum* typically inhabits.

Our observations of pollination by sphecid wasps in the genus *Prionyx* is unusual in the context of Australian orchids. Although Peakall (1987) referred to observing a sphecid wasp pollinate *Prasophyllum plumiforme*, ours is the first formal documentation of pollination by sphecids in Australian orchids. Internationally, pollination of orchids by sphecids appears to be generally rare (see database of Ackerman *et al.* 2023), with two instances of specialised deception systems using only sphecids (Nilsson *et al.* 1986; Steiner *et al.* 1994) and some incidental records as visitors to orchid species that attract a range of nectar-seeking insects (e.g. Zhou *et al.* 2016; Pedersen *et al.* 2018). The latter observations conform to the trend outside of the orchids, where sphecids consume nectar from plants that attract a range of typically Dipteran and Hymenopteran species (e.g. Williams and Adam 1998; Robertson and Klemash 2003; Griffin *et al.* 2009). In this context, pollination by sphecids is unlikely to occur for most genera of Australian orchids but may be more widespread in larger-flowered *Prasophyllum*.

Prasophyllum innubum experienced very high levels of fruit set in the field, with the mean flowers per inflorescence being 72.4% and 85.4% at the two study sites. Experiments in the shade house, where plants were kept isolated from any insects, confirmed that this high fruit set is primarily arising from pollen vectors rather than self-pollination. This high level of fruit set is similar to some other *Prasophyllum* species with tall inflorescences, namely *P. elatum*, *P. fimbria* and *P. odoratum* (Bernhardt and Burns-Balogh 1986; Elliott and Ladd 2002). Although high visitation rates of insects are likely to drive the high levels of fruit set, the numerous flowers per inflorescence raise the possibility that some pollination events arise from geitonogamy. In our study, bees and wasps visited 3.41 and 3.77 respectively flowers per inflorescence (of a mean of 11.48 flowers per inflorescence for the population, on the basis of fruit-set data), although typically only some of these visits led to pollen transfer. In the pollen staining of experiments of Peakall (1989), geitonomagous transfers accounted only for 22% of pollinations in *P. fimbria*. As such, despite the numerous flowers on an inflorescence and the provision of a nectar reward, xenogamous pollination may predominate in large *Prasophyllum*.

Rates of complete herbivory of *P. innubum* inflorescences were less than 6% at both study sites. The complete consumption of the inflorescence is strongly suggestive of browsing by a vertebrate herbivore rather than damage by insects. However, we did not capture any instances of grazing on the camera traps, despite detecting a range of vertebrate herbivores, including feral horses and fallow deer. As such, we were unable to resolve which animal species were responsible for the consumption of inflorescences. However, given the low rates of browsing, we suggest that the bigger impact of introduced or overabundant herbivores would arise through altering the habitat through grazing, soil disturbance and changed nutrient cycling, rather than limiting reproductive output of the orchid (Driscoll *et al.* 2019; Eldridge *et al.* 2019). Indeed, grazing is listed as a threat in the Conservation Advice documents for several threatened *Prasophyllum* species from the Australian Alps, including *P. innubum*.

From a conservation perspective, given the generalist pollination strategy of *P. innubum*, availability of pollinators is unlikely to limit availability of sites to establish additional populations. However, surveying for their presence at candidate sites would be prudent, and could be achieved through vane traps or sweep-netting, both of which are effective approaches for surveying *Lasioglossum* in south-eastern Australia (Hall 2018; Reiter *et al.* 2019*a*). The evidence for pollination by bees in several of the larger species of *Prasophyllum* raises the possibility that hybrids could occur between *P. innubum* and closely related species (although this has not been documented at our study site). Although there is a difference in habitat and flowering time between *P. innubum* and most other *Prasophyllum* species, ideally one would select sites to reduce the possibility of hybridisation between early flowering *P. innubum* and late-flowering individuals of other *Prasophyllum* (particularly the morphologically similar *P. suttonii*). From our data, grazing of inflorescences is not a major concern for the conservation of *P. innubum*. However, given the damage caused by feral herbivores in other parts of the Australian Alps, understanding their impact to *P. innubum* habitat and selecting sites for translocation that are not subject to damage would be a high priority.

Conclusions

Although most Australian orchids studied so far have one or few pollinator species, in *P. innubum* we observed pollination by three species of bee and two species of sphecid wasp. The proportion of flowers setting fruit was very high (72% and 84%), whereas the level of fruit set when pollinators were experimentally excluded was only 6%. As such, this apparently generalist pollination strategy confers a high level of reproductive success. Despite occurring in a grassland where feral vertebrate herbivores were regularly recorded, rates of herbivory were low. From a conservation perspective, managing herbivores to prevent habitat alteration is likely to be more of a concern than preventing grazing directly on the orchids. Being pollinated by multiple species of Hymenoptera means that availability of pollinators is unlikely to place a strong constraint on selection of sites for establishing insurance populations of *P. innubum*.

Supplementary material

Supplementary material is available online.

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

Conflicts of interest. Noushka Reiter is an Associate Editor of *Australian Journal of Botany*. To mitigate this potential conflict of interest they had no editor-level access to this manuscript during peer review. The authors declare no other conflicts of interest.

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