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Improving conservation and translocation success of an endangered orchid, *Caladenia xanthochila* (Orchidaceae), through understanding pollination

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Abstract
Critical for conserving endangered orchids is identifying their pollinators and their distribution. *Caladenia xanthochila* is an endangered orchid that has floral traits characteristic of pollination by food foraging insects. We identified the pollinator(s), mechanisms of attraction and the presence of pollinators at natural, existing and potential translocation sites. Furthermore, we quantified pollination success at translocation sites and investigated the effect of rainfall on pollination success over 19 years at a natural site. We clarify if sharing of pollinators occurs with closely related species by comparing the CO1 barcoding region of the pollinators' DNA. *Caladenia xanthochila* was pollinated by a single species of thynnine wasp, *Phymatothynnus* aff. *nitidus*. *Caladenia xanthochila* produced 27.0 µg ± 7.1 sucrose on the labellum, while pollinators vigorously copulated with glandular clubs on the sepal tips, suggestive of a mixed pollination system. Pollination success of *C. xanthochila* was 7.6 ± 1.5% SE at the natural site and 16.1 ± 3.6% SE across the translocation sites. Furthermore, hand pollinations demonstrated that pollination was pollen limited. Pollination success was significantly related to average rainfall during the growth phase of the orchid ($P < 0.001$). Potential translocation sites for *C. xanthochila* were limited, with four of six surveyed lacking the pollinator. We found evidence for cryptic species of *Phymatothynnus*, with *C. xanthochila* pollinators being unique amongst the orchids studied. We recommend hand pollinations at translocated and remnant wild populations to boost initial recruitment. The evidence for cryptic species of pollinators further highlights the need for accurate identification of pollinators.

Keywords Orchidaceae · *Caladenia* · Pollination · Nectar · Cryptic species · Translocation · Conservation

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Introduction

The Orchidaceae has an estimated 30,543 species (POWO 2021) with a breathtaking diversity of floral forms (Dressler 1993) and specialised pollination strategies (Ackerman et al. 2023; Johnson and Schiestl 2016). Specialisation arises through floral cues that attract particular pollinators, whether food (Nilsson 1983), chemical fragrance (Dressler 1968; Schiestl et al. 1999, 2003) or visual (Kullenberg 1961; Paulus and Gack 1990) but also the structure of the flower, where pollinators have to be a particular size to remove and deposit pollinia (Anderson and Johnson 2008; Li et al. 2008; Phillips et al. 2020a). Fascinatingly, orchids rely on a single or a few pollinator species to set seed (Tremblay 1992; Schiestl and Schluter 2009; Phillips et al. 2020b). Specialisation on one or a few pollinators can facilitate speciation (Schiestl and Schluter 2009; Xu et al. 2011; Whitehead and Peakall 2014), species co-existence (Whitehead and Peakall 2014) and remarkable floral traits and morphologies adapted to particular pollinators (Johnson and Schiestl 2016). The specialisation of orchids to their pollinators can lead to vulnerability and local co-extinction when pollinators are lost (Pauw and Hawkins 2011) and restriction of an orchid's range or potential translocation sites based on the availability of pollinators (Phillips et al. 2015; Reiter et al. 2017).

At present, over 45% of all orchid species assessed under IUCN criteria are at risk of extinction (IUCN 2023; Wraith and Pickering 2018; Liu et al. 2020; Wagensommer et al. 2020). Threats facing orchid species include habitat destruction (Brummitt et al. 2015), illegal collecting (Hinsley et al. 2018), and introduced animals and plants either overgrazing or outcompeting species (Wraith and Pickering 2019). In some cases, the degradation or removal of habitat combined with reliance on a single or few species of pollinators has led to the loss of the pollinator (Pauw and Hawkins 2011; Phillips et al. 2015; Reiter et al. 2017), ultimately leaving populations unable to sustain themselves into the future (Phillips et al. 2015) without human intervention. The threatened nature of orchids has led to increasing conservation translocation efforts to sustain existing populations and create new populations to reduce the threat to the species (Reiter et al. 2016; Silcock et al. 2019). To conserve and translocate threatened species of orchids it is essential to understand: (1) the identity of the pollinator(s), (2) the effectiveness of the pollination mechanism and (3) the presence of the pollinator(s)' both at existing and potential translocation sites (Reiter et al. 2017; Phillips et al. 2020c).

Fifty-four percent of all orchid species are estimated to provide some type of reward to potential pollinators (Ackerman et al. 2023). The most common form of vector-dependent pollination involves nectar rewarding plants, where nectar is provided in a range, from trace amounts (i.e., 16.6 µg of sucrose in *C. colorata,* pollinated by wasps (Reiter et al. 2018)) to larger quantities (i.e., 40–300 µL, on average165 µL in *Angraceum sesquipedale* (Vandea: Angraecinae), pollinated by Hawkmoths (Wasserthal 1997; Arditti et al. 2012)). The other forty-six percent of orchid species are deceptive, either food deceptive where no nectar, pollen or reward is given to the pollinator, brood site mimics where insects mistake the orchid for a place to lay offspring, or sexually deceptive (Ackerman et al. 2023). Pollination by sexual deception generally involves mimicry by the orchid via chemical cues of female insects (Bohman et al. 2016; Peakall et al. 2010) and can be enhanced by visual or tactile cues (De Jager and Peakall 2016). Pollination by sexual deception has primarily been reported from the Orchidaceae but with two exceptions, one from each of the Asteraceae (Ellis and Johnson 2010) and Iridaceae (Vereecken et al. 2012). Pollination via sexual deception is common in Australian orchids, with this mechanism recorded in eleven orchid genera (Ackerman et al. 2023) including *Caladenia* (Stoutamire 1983).

Caladenia (Orchidaceae) consists of 284 species (Backhouse et al. 2019) found in a diversity of habitats (Hopper and Brown 2004; Jones 2006), with the centres of diversity in south-western and south-eastern Australia (Phillips et al. 2009). *Caladenia* is the most threatened genus of plant in Australia with 71 species nationally at risk of extinction (Critically Endangered, Endangered or Vulnerable) and three species that have become extinct since European settlement (Australian Government 2022; VicFlora 2022). Pollination mechanisms in *Caladenia* are diverse, with many species pollinated by sexual deception (i.e., Stoutamire 1983; Peakall and Beattie 1996; Phillips et al. 2009, 2017), food deception (Philips et al. 2020b; Phillips and Batley 2020), nectar reward (Faast et al. 2009; Reiter et al. 2018, 2019a, 2020) and provision of roosting sites (Reiter et al. 2019b). *Caladenia* are typically pollinated by Hymenoptera, predominately by thynnine wasps TiphiIdae subfamily Thynninae (Stoutamire 1983; Phillips et al. 2009, 2017). However, pollination can be facilitated by bees, for example the food rewarding *C. versicolor* (Reiter et al. 2019a) or food foraging bees in *C. hildae* (Phillips et al. 2020a). The majority of *Caladenia* pollinated by sexual deception of male wasps are dark green or red with an insectiform labellum or prominent calli (Peakall and Beattie 1996; Bower 2015; Bohman et al. 2017). *Caladenia* that are pollinated by food foraging insects generally have pink, white or yellow colourful flowers (Reiter et al. 2018; 2019a; 2019b), although there are exceptions, for example *C. abbreviata* is light yellow with a pink and white coloured labellum and is pollinated by sexual deception (Phillips and Peakall 2018). With such highly specialised (often only one or a few pollinators) and diverse pollination mechanisms, understanding the pollinator identity and distribution of threatened *Caladenia* species is critical

for effective conservation and translocation programs (Reiter et al. 2017; Phillips et al. 2020a, b).

Within *Caladenia* subgenus *Calonema* there is one morphological group predominately consisting of threatened species known as the 'reticulata group', which occurs in a variety of colours (white e.g., *C. pumila*; red e.g., *C. cruciformis*; red and white *C. calcicola*; and bright yellow *C. xanthochila*), all with clubs (condensed region of glands) at the tips of either or both sepals and petals (but not always all individuals of the species i.e., *C. flavovirens,* Kosky 2022). Species in the 'reticulata group' can have prominent dark calli (glandular lumps on the labellum) as in the case of *C. calcicola* or pale calli as with *C. pumila* and *C. xanthochila* (Fig. 1). A study by Swarts et al. (2014) based on morphological identification of pollinators (and limited microsatellite data of orchids) suggested that 10 species in the 'reticulata' group, despite the morphological differences between recognised plant species VicFlora (2023) were one morphospecies, as they shared a single pollinator, *Phymatothynnus* aff. *nitidus*. There is, however, evidence for cryptic species of thynnine wasp pollinators of orchids (Griffiths et al. 2011) with the mtDNA CO1 sequence locus being extremely effective at separating closely related species of thynnine wasp (Griffiths et al. 2011; Menz et al. 2015; Phillips et al. 2015). Furthermore, in orchids pollinated by sexual deception, pollinator specificity and isolation has led to strong reproductive barriers and speciation (Xu et al. 2011; Whitehead and Peakall 2014). The study by Swarts et al. (2014) has implications for the taxonomic status, conservation funding, conservation translocations and *ex situ* breeding programs of what are morphologically distinct nationally threatened species in general, and for *C. xanthochila* in particular. Thus, it is of conservation importance that the identity of the pollinators of *C. xanthochila* and other 'reticulata' group species that are currently accepted as taxonomically distinct orchid species (VicFlora 2023) are re-evaluated using molecular methods to test for pollinator crypsis within what have been morphologically identified as *P.* aff. *nitidus*.

To improve effective conservation of *C. xanthochila* we aim to: (1) identify the pollinator(s) at a wild site and existing and potential translocated sites in Victoria, Australia, using molecular techniques, (2) identify the pollination mechanism, (3) test for sharing of pollinators at wild sites with other members of the 'reticulata' complex, and (4) evaluate pollination success at natural and existing translocation sites, and the influence rainfall may have on pollination success.

Methods

Study species

Caladenia xanthochila is commonly referred to as the yellow-lipped spider-orchid and was previously widespread across the states of Victoria and South Australia, Australia, but has been reduced to one natural site in South Australia and two natural sites in Victoria (Fig. 2). *Caladenia xanthochila* is listed as Endangered under IUCN criteria under the *Flora and Fauna Guarantee Act 1988* in Victoria and Endangered nationally in Australia under the *Environment Protection Biodiversity and Conservation Act 1999*. *Caladenia xanthochila* is a summer-autumn dormant terrestrial orchid that flowers in September. Typically, *C. xanthochila* has one or rarely two flowers, on a scape to 32 cm tall. Flowers are of a bright yellow colour with perianth segments to 5 cm, the dorsal and lateral sepals have small brown terminal clubs (which is an area with tightly compacted glandular osmophores). Plants have a single

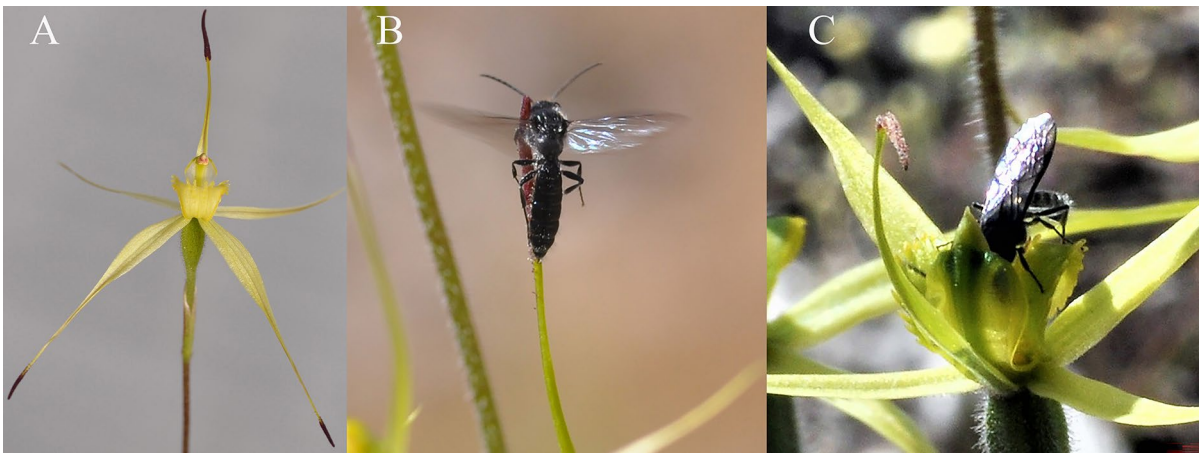

Fig. 1 **a** *Caladenia xanthochila* flower, **b** *Phymatothynnus* aff. *nitidus* copulating with the clubs of *C. xanthochila* and **c** *Phymatothynnus* aff. *nitidus* in the process of removing pollinia

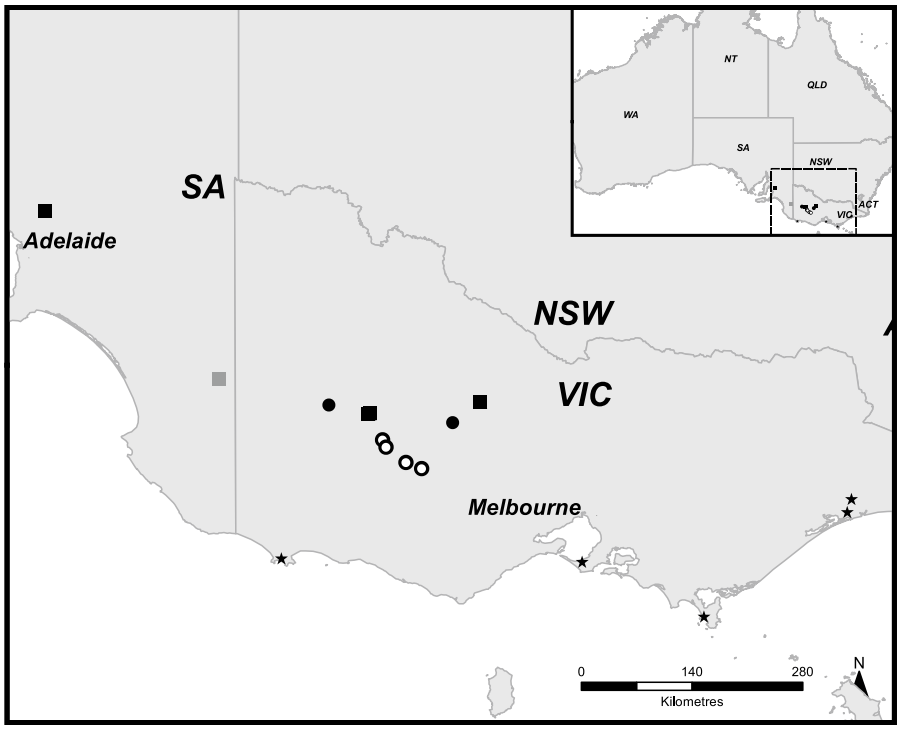

Fig. 2 Map of extant wild sites of *Caladenia xanthochila* (black square), translocation sites are within the footprint of the black squares in Victoria, extinct sites (grey square), potential translocation sites with pollinator present (black circle), potential translocation sites pollinator absent (white circle) and sites baited for pollinators of closely related species (black star)

hairy leaf to 17 cm long (VicFlora 2022). In Victoria *C. xanthochila* now only occurs at the following wild sites: Murtoa Golf Course, estimated 600 individuals, Barrabool FFR estimated 2 individuals (though not seen flowering in 10 years and possibly extinct) and Glenalbyn, estimated 2 individuals.

Study sites

Observations of pollinator presence and behaviour for *C. xanthochila* were undertaken in the vicinity of the two natural populations of *C. xanthochila* near Murtoa (Barrabool Flora and Fauna Reserve (FFR), Golf Club and private property and Glenalbyn (Kooyoora State Park and surrounds) and the conservation translocation sites ($N=6$) near the Murtoa wild site that were 200 m apart from each other (Fig. 2). Further pollinator surveys were undertaken at potential translocation sites in West Wail FFR (*C. xanthochila* previously recorded but no longer found), Dalyenong FFR, Ararat Regional Park, Illawara FFR, Mt Langi Ghiran State Park and Lake Lonsdale FFR (Fig. 2; Supplementary Material 1).

Translocation sites ($N=6$) were selected based on similar vegetation *Eucalyptus leucoxylon* (Myrtaceae) dominated open woodland with sandy loam soils, that were permanently protected and greater than 100 Ha, with minimal threats from introduced herbivores and weeds. Further sites chosen for translocation were those that had the pollinator presence confirmed either in this study or previous studies (Bower 2008; Wright 2009).

Propagation

Due to the endangered status of this species, rather than picking flowers from the wild for our pollinator experiments, we propagated the species for both pollination studies and conservation translocation using the following methods. *Caladenia xanthochila* seed from across the two Victorian sites was collected from > 30 hand-pollinated plants. Seed was cleaned and dried to15% RH and stored at − 20 °C until use. Seed was symbiotically sown with the mycorrhizal fungus *SerendipIta australiana* using the methods of Reiter et al. (2016), as previous research has shown *C. xanthochila* to associate exclusively with *S. australiana* (Reiter et al. 2020). Seed was surface sterilised in 0.05% NaOCl (Domestos) and drained over a Buchner funnel in a laminar flow. Seed was rinsed with sterile deionised water and then floated in a beaker of sterile water in the laminar flow. Seed was then pipetted onto sterile 3 µm pore filter paper (47 mm in diameter) over the Buchner funnel and the paper and seed plated onto Oatmeal Agar (OMA) (Clements and Ellyard 1979) without sucrose. Two 1-cm cubes of *Serendipita australiana* cultured on Fungal Isolation Media (FIM) (Warcup 1950; Clements and Ellyard 1979) were placed either side of the seed and paper. Plates were sealed with Parafilm® and kept in the dark for 16 h at 20 °C and for 8 h at 16 °C until protocorms formed. Once protocorms had formed, the dishes were transferred to a light regime with 16 h light 8 h dark, with the same temperature cycles. Once seedlings had reached > 1 cm in

green leaf they were transferred to flasks as per Reiter et al. (2016), containing a layer of OMA as a base covered by vermiculite as a surface. Plants were grown in flasks for 8 to 12 weeks and then transferred to the nursery on RBGV Biogro® Orchid terrestrial *Caladenia* mix.

Pollinator observations

Flowering potted plants of 2 to 5 years in age of *C. xanthochila*, containing 10 to 20 flowers were used as bait flowers to determine the presence of pollinators at the wild, existing translocation sites and potential translocation sites. Pollinator baiting involves presenting flowers in potential pollinator habitat to try and elicit a response from the pollinators. Baiting was undertaken using an alteration of the methods of Stoutamire (1974) and Peakall (1990), as described in Reiter et al. (2017). Bait flowers were presented by placing the pot on the ground for 6 min and observing any insects approaching the flowers. Bait plants were moved to a new position in the landscape every 6 min, with each new position being at least 100 m from the last, if pollinators responded a minimum of five trials were conducted per site. For sites where pollinators did not initially respond, trials were continued for 30 trials and repeated in a second year (to confirm absence). For both sexually deceived and food-seeking pollinators, the change of the position of the plants in the landscape elicits a new response (Peakall 1990; Reiter et al. 2018).

Surveys using the baiting method were undertaken during the flowering period of *C. xanthochila* in September to early October in 2016 (2 days), 2017 (5 days), 2019 (5 days) and 2020 (2 days). Thynnine wasps have previously been shown to only respond to bait flowers on warm days > 18 °C, with little to light winds and no rain (Stoutamire 1983). Observations of pollinators were made between 10 am and 4 pm on days with temperatures greater than 18 °C, light winds and no rain. For each individual insect approaching the flower, their behaviour was recorded as follows: whether they landed, the location of landing (sepal, petal, labellum, club), where they copulated with the flower, if any feeding behaviour was observed, pollinia removal, pollinia deposition or contact with the column.

Surveys for the pollinator were also conducted at five candidate conservation translocation sites, selected based on matching vegetation to the Victorian wild sites over 10 days in 2017, 2019 and 2020. Pollinator baiting was conducted over an area greater than 20 Ha (Fig. 2, Supplementary Material 1). Pollinators attracted to bait flowers at the conservation translocation sites were collected for DNA barcoding to test if it is the same species as at the wild site.

Given that Swarts et al. (2014) reported morphological evidence that endangered species in the 'reticulata' complex share the same thynnine pollinator we baited at the naturally occurring sites in 2014–2017 (using the above baiting methods) of three other Victorian members of the 'reticulata' complex assessed in Swarts et al. 2014; *C. calcicola* (Bats Ridge) (baited over 2 days), *C. robinsonii* (Cranbourne and Rosebud) (baited for over 2 days), *C. australis* (Baited for over 2 days), and two additional members not assed in that study *C. fitzgeraldii* (baited for over 2 days) and *C. peisleyi* (baited for over 2 days). Each species was baited for at the naturally occurring sites of the plant (See Supplementary Material 2 for details). Furthermore, for *C. xanthochila,* we baited at a naturally occurring site of *C. calcicola* and *C. robinsonii*, to check for pollinator attraction at these sites and if present compare the pollinator species attracted.

Representatives of insects at each site were collected for later morphological and molecular identification. Thynnine wasps were identified by Graham Brown at the Museum and Art Gallery of the Northern Territory using a series of unpublished keys to the Australian thynnine wasp fauna. Bees were identified by Dr Michael Batley at the Australian Museum.

DNA barcoding of pollinators in the 'reticulata' complex

We used DNA barcoding to resolve the number of wasp species involved in pollination of *C. xanthochila* from those collected through baiting ($N = 23$) at remaining wild sites in Victoria, translocation sites, and in addition Dalyenong FFR and West Wail FFR. Furthermore, given the morphological evidence that endangered species in the 'reticulata' complex share the same pollinator (Swarts et al. 2014), we used the methods of Griffiths et al. (2011) to test if these species do share pollinators. We compared pollinators attracted to *C. xanthochila* above, to wasp pollinators collected through baiting at their naturally occurring sites in 2014–2017 (using the above baiting methods) at the wild sites of three other Victorian members of the *Caladenia reticulata* complex assessed in Swarts et al. (2014); *C. calcicola* (Bats Ridge) ($N = 2$), *C. robinsonii* ($N = 7$), *C. australis* ($N = 4$), *C. fitzgeraldii* ($N = 7$), *C. peisleyi* ($N = 4$).

Previous work in thynnine wasps has shown that the mtDNA CO1 sequence locus is highly effective at distinguishing closely related species (Griffiths et al. 2011; Menz et al. 2015; Phillips et al. 2015). Sequencing was undertaken following the methodology of Griffiths et al. (2011). A multiple sequence alignment was performed in Geneious Prime ™ software Version 2023.0 (Kearse et al. 2012). A phylogenetic analysis was undertaken using PHYML (Guindon and Gascuel 2003) via a plug-in in Geneious Prime with 1000 bootstrap replicates using the GTR + G model of nucleotide substitution. Geneious was used to quantify the amount of genetic variation within the putative pollinator species based on percentage variation in the number of base pairs.

Do volatile chemical cues attract pollinators?

We used the methods of Phillips et al. (2013) to establish which part of the flower contains the sexual attractant for the pollinator. Sequential choice trials were conducted over 2 days in 2019, near the Barrabool FFR and Murtoa wild site. We conducted sequential choice trials where six flowers were dissected into the column, the labellum, the clubs, and the remaining floral display. Each piece of dissected tissue was pinned to the top of a wooden skewer using a black-headed pin and plasticine. A control containing a stick with a piece of plasticine and a pin only was used. Each trial involved presenting the column, labellum floral display and control in a line for 2 min before introducing the clubs for an additional 2 min, and then reversing the order, alternating the order of introduction between flower replicates. For each dissected flower, this trial was repeated at five sites more than 100 m apart. Floral visitors were scored as to whether they approached without landing, if they landed, and if they attempted copulation.

Nectar derivatisation and GC–MS analysis

We used the sampling methodology of Reiter et al. (2018) where flowers of *C. xanthochila* were sampled from the RBGV living *ex situ* collection (grown for translocation above) and placed into a glasshouse at 20 °C for 3 h prior to sampling. In short: For each of nine flowers, three drops of an aqueous solution of ribitol (5 mL, internal standard, 0.20 mg/mL) was added with a glass syringe onto three separate parts of the labellum, where the calli were present. The aqueous solution was subsequently collected with microcapillary tubes (5 mL) and immediately transferred to GC vials (2 mL) with inserts (50 µL). The three aliquots from each flower were combined in the same vial. Solutions taken from the flower were stored in a − 20 °C freezer until analysis. As we followed the same methodology as Reiter et al. (2018), we were able to compare our results with those from other nectar-secreting *Caladenia*, *C. colorata* (Reiter et al. 2018), *C. versicolor* (Reiter et al. 2019a), *C. concolor* and *C. arenaria* (Reiter et al. 2019b), *C. drummondii* (Phillips et al. 2021), and the nectarless *C. tentaculata* (Reiter et al. 2018). For further description of the GC methodology see Reiter et al. (2018). Tentative identification of sucrose was based on the comparison of retention index and mass spectrum with data from the mass spectral library (NIST-11) and confirmed by co-injection with a synthetic standard. Quantification was achieved. Quantification of glucose, fructose and sucrose was achieved by comparison of peak areas of total ion chromatograms (TIC) of nectar samples with the known amount of the internal standard ribitol. The response factor was calculated and included in the calculation of the amounts of analytes (Reiter et al. 2018).

Quantifying reproductive success

The wild population of *C. xanthochila* at Murtoa was demographically monitored over a period of 23 years, from 1999 to 2021. Fruit set was recorded for 19 years, in 2000–2014, 2017–2019 and 2021. Six translocation sites were demographically monitored between 2018 and 2022. Two of these sites were historic translocations of 240 plants (120 per population) from 2007 (Wright et al. 2009), where the pollinator presence was confirmed prior to translocation. Three sites were from a 2015 translocation of 365 plants (consisting of 3 subpopulations separated by 200 m of 230 plants, 87 plants and 48 plants, respectively) and one was a 2021 translocation consisting of 156 plants. Each plant was numbered and marked 10 cm away with a numbered disc and stainless-steel metal pin. Each year, plants were monitored for leaf emergence, flowering and fruit set. The proportion of flowering plants that set fruit each year was calculated and averaged across years to assess the natural pollination rate for this species.

We used a generalised linear model (GLM) with a negative binomial error distribution to investigate if the number of flowering *C. xanthochila* was related to average rainfall in the same year from emergence to flowering. Data from 2002, 2014 and 2017 were excluded due to the low flowering success in those years (< 4 flowers), leaving 16 years of flowering data at the natural site. A negative binomial model was used to account for overdispersion in the data. Translocation sites were not used in the analysis, due to the small number of years monitored combined with watering in the first year of translocation and nursery cultivated plants likely effecting the flowering rate.

A GLM with a binomial error distribution was used to investigate the relationship between pollination success and average rainfall. The distribution of residuals was checked visually to determine if models met assumptions. Significance of predictor variables was determined using likelihood ratio tests (X^2) comparing models with and without the variable of interest.

To calculate pollen transfer efficiency, pollinia removal and deposition was recorded across one wild site and five translocation sites in 2019 (this consisted of the above mentioned two 2007 translocations, and three 2015 translocations), each site was more than 200 m apart. Pollen transfer efficiency (range 0—1) was calculated as the number of stigmas with pollen present, divided by the number of paired pollinia removed. For example, if 10 pollinia were removed and 10 stigmas pollinated, the pollen transfer efficiency would be equal to 1 (100%).

Is fruit set in Caladenia xanthochila pollen limited?

We tested the ability of *C. xanthochila* to self-pollinate and if 100% pollination could be achieved when not resource limited. In 2019, fifteen *C. xanthochila* flowers were hand-pollinated under shade house conditions and 15 were left un-pollinated. In 2021, thirty *C. xanthochila* flowers were hand-pollinated at the wild site in Murtoa. Success of capsule formation was recorded.

Limits to reproductive success

For each of 35 petri dishes (each containing approximately 100 seeds) seed was scored as viable or not. Seed was scored as viable if it reached stage 5 which is where a green leaf is present (Warcup 1981) within six months. Viability of seed was assessed under a light microscope with a 2 × 2 cm grid with 2.5 mm squares dividing the grid, placed over each petri dish.

Results

Pollinator observations of *Caladenia xanthochila*

Pollinator baiting was undertaken in the natural range of *C. xanthochila* at the Murtoa Golf Course ($N = 3$ thynnines responded), Barrabool FFR ($N = 189$ thynnines responded), Glenalbyn (Kooyoora State Park)($N = 6$ thynnines responded), West Wail FFR ($N = 6$ thynnines responded) and outside of its known range in Dalyenong NCR ($N = 12$ thynnines responded), Illawarra SP ($N = 0$ thynnines responded), Lake Lonsdale ($N = 0$ thynnines responded) NCR, Mt Langi Ghiran ($N = 0$ thynnines responded) and Ararat RP ($N = 0$ thynnines responded) (Fig. 2). Thynnine wasps were attracted to the bait flowers in all reserves except Lake Lonsdale, Mt Langi Ghiran, Illawarra and Ararat. Minor responders (wasps that were attracted but did not land on the orchid) were detected at only Ararat RP and Mt Langi Ghiran SP. No potential pollinators were attracted to *C. xanthochila* at either Bats Ridge or Cranbourne Botanic Gardens (sites with populations of *C. calcicola* and *C. robinsonii*, respectively).

We observed the behaviour of the pollinators at the wild and nearby translocated sites near Murtoa in 2019, separately from the above surveys. Observations from these nearby sites could be combined as they were in the same patch of bush < 2 km apart and the pollinator was identified from CO1 barcoding (below) as the same species at all sites. Observations of pollinator behaviour assist with distinguishing between pollination systems based on food foraging or sexual deceit. Eighty thynnines were observed visiting flowers, of which three (3.8%) did not land. Of those that landed ($N = 77$ wasps), individuals would often move between sepals (33 landings), clubs (91 landings) and labellum (60 landings), with a further two wasps landing on the petals and 33 on the sepals. Pollinia were observed being removed on three occasions and deposited on one occasion (Fig. 1). Pollinia removal and deposition was not associated with copulation or mating behaviour by the pollinator. Pollinia were attached to the upper thorax of the wasp. Copulation and grappling by the pollinator with the flower only occurred on the clubs, which was observed for 47 out of the 91 landings on the clubs (51.6%) (Fig. 2). One individual bee *Leioproctus punctatus* landed on the labellum at Lake Lonsdale, appeared to forage on the labellum (mouth parts extended) but did not remove pollen.

Which part of the flower produces sexual attractant in *C. xanthochila*?

In our dissection experiment, no pollinators landed or approached the labellum, floral remains or control, regardless of the order in which they were presented. A total of 133 thynnid wasps responded to the clubs. Of these an average of 15.5 wasps ± 2.1 SE per replicate flower approached the clubs but did not land, whereas 6.0 wasps ± 1.8 SE per replicate flower landed on the clubs and grappled with the clubs.

Molecular identity of pollinators in the 'reticulata' complex

There was little genetic variation in the *P.* aff. *nitidus* (Murtoa, Barrabool FFR, Glenalbyn SP (Kooyoora), West Wail FFR and Dalyenong FFR) wasps attracted to *C. xanthochila*, with the difference in the CO1 barcoding region ranging from 0 to 1.42% ($N = 23$) (Supplementary Material 3). Based on genetic divergence, *C. xanthochila* does not share a pollinator with the following species from the 'reticulata' complex tested in this study: *C. calcicola*, *C. robinsonii*, *C. australis*, *C. fitzgeraldii* or *C. peisleyi*. Wasps attracted to these species showed greater than 8.00% difference in the CO1 region to the thynnine pollinator of *C. xanthochila*. There was 7–20% variation between thynnines attracted to species in the *C. reticulata* complex (Supplementary Material 3), apart from *C. fitzgeraldii* and *C. peisleyi*, which attracted wasps with less than 1% variation from each other.

Nectar derivatisation and GC–MS analysis

The surface of the labellum of *C. xanthochila* contained sucrose, with a maximum amount of 65 µg of sucrose

detected (mean = 27.0 ± 7.1 µg SE, $N = 9$). No glucose or fructose were detected.

Pollination success

Mean pollination success at the wild *C. xanthochila* population was 7.6 ± 1.5% (SE) ($N = 2024$, flowers over 16 years, range of flowers 12–241). The pollination rate of *C. xanthochila* at the Murtoa wild site varied between years with several years during the millennial drought (Watkins and Trewin 2007) having no pollination and the highest pollination rate recorded in 2009 of 17.2% ($N = 122$ Flowers).

Mean pollination success across the six translocated populations between 2018 and 2022 was 16.1 ± 3.6% (SE) ($N = 658$ flowers over 5 years, with a range of 16 to 128 flowers). The 2007 and 2015 translocation sites had pollination in all years except the 2007 translocation site 2 in 2019. The highest pollination rate was 33% ($N = 21$ flowers) in 2019 at the 2007 translocation site 1. Data for sites that had less than 10 flowering plants in a given year were not included in this analysis.

Pollination success of *C. xanthochila* at the wild population was positively related to average rainfall in the growing season of the plant ($X^2 = 36.60$, $P < 0.001$, Table 1, Fig. 3). There was no significant relationship between number of flowers and average rainfall during the growing season (Table 1, Fig. 3). However, there was a pattern whereby flowering only seemed to exceed 50 flowers in years with greater than 35 mm and less than 60 mm average rainfall during the growing season (Fig. 3).

In 2019, across the five translocation sites and one wild site, the percentage of flowers on which pollen was removed was 0.42 ± 0.07 (SE) and the percentage of flowers on which pollen was deposited was 0.16 ± 0.05 ($N = 201$). The pollen transfer efficiency was 0.49 ± 0.18.

Is *Caladenia xanthochila* pollen limited?

All 15 plants (100%) of *C. xanthochila* that were hand-pollinated set seed under shade house conditions, while those that were not hand-pollinated did not set seed. Of the plants

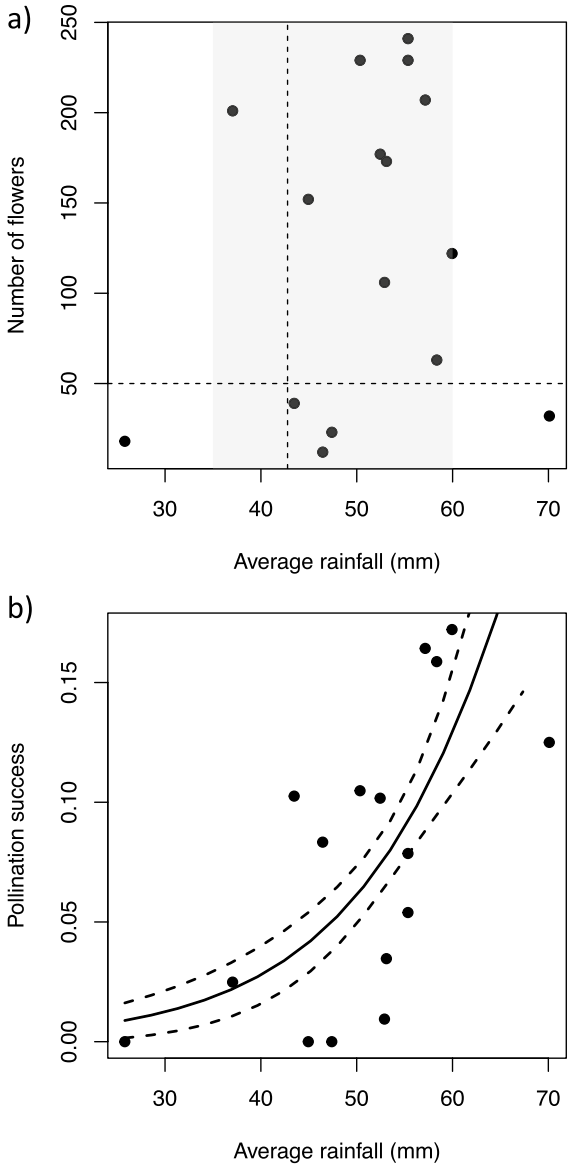

Fig. 3 Results of generalised linear models comparing the relationship between **a** number of flowers and rainfall and **b** pollination success and rainfall. The shaded area in **a** represents the range between 35 and 60 mm rainfall during the growing season, the horizontal dashed line is the threshold of 50 flowers and the vertical dashed line represents the average rainfall during the growing season (42.8 mm). The dashed lines in **b** represent the 95% confidence interval around the model prediction (solid line)

Table 1 Results of generalised linear models investigating the influence of rainfall on flowering and pollination success from 16 years of data for *Caladenia xanthochila*

Response variable	Explanatory variable	Est	s.e	df	X^2	P
Number of flowers	Intercept	3.773	1.050			
	Average rainfall	0.021	0.020	1	0.59	0.439
Pollination success	Intercept	− 6.838	0.798			
	Average rainfall	0.082	0.145	1	36.60	**< 0.001**

Significance of the explanatory variables is based on likelihood-ratio tests (X^2) comparing models with and without the variable of interest. Significant variables ($P < 0.05$) are in bold along with estimates (Est.) and standard errors (s.e) for significant predictors

hand-pollinated in the field, 22 of 30 (73.3%) set seed in 2019.

Limits to reproductive success

The mean viability of seed of *C. xanthochila* germinated to stage 5 was 14.64 ± 1.78% SE (*N* = 35 plates). The variability in seed viability between plates was from 0 to 45.09%.

Discussion

Highlights

We confirmed pollination of *C. xanthochila* by chemical attraction of an undescribed thynnine wasp, *Phymatothynnus* aff. *nitidus*. Pollinators attempted to copulate with the clubs of the orchid, however no copulatory behaviour of the pollinator was observed on the labellum of the orchid, which was coated with a small amount of sucrose. DNA barcoding of pollinators attracted to orchids in the *Caladenia* 'reticulata' complex revealed cryptic species of wasps. Furthermore, *C. xanthochila* does not share a pollinator in its natural range with five closely related species in the 'reticulata' complex. *Caladenia xanthochila* is pollen limited with large variation in pollination of wild plants among years.

How is the pollinator of *Caladenia xanthochila* attracted?

Caladenia xanthochila attracted one species of thynnine wasp, *Phymatothynnus* aff. *nitidus*. Through dissection experiments, we were able to demonstrate the primary chemical attraction was to the clubs of the orchid. Interestingly, 32% of the insect responses were to the labellum of the orchid, with 67% of pollinator activity on the clubs or sepals, a behaviour which could reduce the opportunity of pollination. Outside of the genus *Caladenia*, it is unusual for an orchid to produce a sexual response to somewhere else than the labellum, which typically has a structure that via colour or shape assists the pollinator to copulate with the orchid and ensure accurate transfer of pollen (Kullenberg 1961; Dafni and Bernhart 1990). Yet, there are exceptions in *Trigonidium obtusum* (Orchidaceae: Maxillariinae) (Singer 2002) where the sexual attractant alone does not directly result in pollination (i.e., the pollinator mating with the orchid does not remove or deposit pollinia) as is the situation with *C. xanthochila*.

With the bright yellow colouration of the flower, *C. xanthochila* is at odds with traditionally sexually deceptive *Caladenia,* which generally have dull green and or maroon-coloured flowers. There is a precedence for brightly coloured flowers that are sexually deceptive in *C. gardneri* (Phillips et al. 2017), which is pink and *C. hastata* (Reiter et al. 2017), which is white, which are both in the subgenus *Calonema*. In the subgenus *Phlebochilus*, Phillips and Peakall (2018) observed sexual deception in *C. abbreviata,* which has a brightly coloured lemon and white flower. The bright colour of *C. xanthochila* fits closely with other systems that attract food foraging insects, of which we know of several that provide nectar rewards in *Caladenia* such as *C. arenaria* (yellow) (Reiter et al. 2019a, b) and *C. colorata* (many colours yellow, pink and white, Reiter et al. 2018).

Interestingly, for a species that uses attraction by volatile chemical cues to induce copulation of the pollinator with the orchid, sucrose (27.0 ± 7.1 µg SE) was present on the labellum, and no copulatory behaviour was observed by the pollinator on the labellum. The amount of sucrose detected on the labellum of *C. xanthochila* is in the range of 0–65µg, while average amounts of sucrose detected on other *Caladenia* pollinated by food foraging thynnids or bees has been reported as 47.08 ± 24.57 µg, using the same method. Sexually deceptive species where copulation is associated with the labellum (e.g., *C. tentaculata)* have been found to have no sugar present on the labellum (Reiter et al. 2018). *Caladenia xanthochila* also attracted one individual female of *Leioproctus (Leioproctus) punctatus* which attempted to forage on the labellum. Given the bright yellow colour of the flower and the small amounts of sucrose present on the labellum it is possible that this species is transitioning towards a mixed pollination system, with the colour and nectar reward the potential attractant for the bee. Using large numbers of flowers to bait has been successful in attracting pollinators of other food foraging bee pollinated *Caladenia*, such as *C. versicolor* (Reiter et al. 2019a). Given that we only attracted one bee using this method with *C. xanthochila*, the transition towards a mixed system seems unlikely, however, it is predicted that species that are undergoing this transition pass through a phase where the traits of the flower are sub optimal for pollination by both pollinators (Muchhala 2007; Phillips et al. 2020b).

Further observations are required to detail the behaviour of the pollinator at the flower and if the small amount of reward provided by the flower leads to feeding behaviour of the wasps facilitating pollination. There is one documented case of thynnines attracted to clubs via chemical cues and then switching to feeding behaviour in *C. abbreviata* (Phillips and Peakall 2018), which is also brightly coloured. Interestingly, outside of the genus *Caladenia*, Kullenberg (1961) working with species of *Ophrys* in experimental addition of sucrose to the labellum was able to change the sexual behaviour of the pollinators to feeding behaviour. Further observational and nectar experiments are needed in *Caladenia* to determine the role sucrose

plays in pollination of species that use sexual cues for attraction of their pollinators.

Does *Caladenia xanthochila* share a pollinator with other closely related species in the 'reticulata group'?

The *Caladenia* 'reticulata' group comprises approximately 37 species (Jones 2021) of similarly shaped spider orchids, that come in a variety of colours with subtle differences to the floral morphology, timing of flowering and types of glandular osmophores on the sepals and petals. Swarts et al. (2014) reported that 10 morphospecies in the *C.* 'reticulata' complex shared the same pollinator, *Phymatothynnus* aff. *nitidus*, based on morphological identification of the wasps. Molecular studies of thynnine wasps have shown that across the geographic range of a thynnine species the genetic variation is typically < 3% (Menz et al. 2015; Phillips et al. 2015; Reiter et al. 2017). Our sequencing of pollinators from six species in the 'reticulata' complex, including four of the same species as those examined in Swartz et al. (2014), determined that five of the six species, including *C. xanthochila* did not share pollinators in their natural geographic range. Interestingly, we found evidence for pollinator sharing between *C. peisleyi* and *C. fitzgeraldii* with variation in the CO1 regions of less than 3%, indicating that where these species co-occur there is the potential to form hybrids. Some sexually deceptive orchid species have been shown to attract another potential pollinator outside their known geographic range, as found in *Chiloglottis* (Peakall et al. 2010; Table S2). While we did not bait with each of the related species outside of their natural geographic range, baiting with *C. xanthochila* at the *C. calcicola* and *C. robinsonii* sites did not attract pollinators to *C. xanthochila* though they did attract the pollinators of *C. calcicola* and *C. robinsonii*. Given the implications of our findings to other species of threatened *Caladenia* in the 'reticulata' complex, we refute the conclusions that species in the reticulata are one morphospecies (Swarts et al. 2014) and recommend a re-evaluation of pollinators including molecular identification within the complex. In addition, we recommend that all pollinator studies involving thynnids undertake molecular identification of pollinators, due to the occurrence of cryptic species (Griffiths et al. 2011).

Conservation of *C. xanthochila*

Caladenia xanthochila was found to have one pollinator species. Specialised pollination strategies are common in the Orchidaceae with the median number of known pollinator species across the family being one (Ackerman et al. 2023). Understanding the identity of pollinators is therefore essential for ensuring that pollinators are present at wild sites and at potential conservation translocation sites for threatened plant species (Reiter et al. 2016; Phillips et al. 2020c). We found that not all potential translocation sites of *C. xanthochila* with similar and suitable vegetation contained the pollinator, emphasising that pollinator surveys prior to translocation of this species are essential (Reiter et al. 2016, 2017).

There was a significant effect of rainfall during the growing season of *C. xanthochila* on pollination success. Consequently, reduced rain during June to September adversely affects the pollinator as well as the orchid. Our long-term monitoring of the wild site of *C. xanthochila* included the millennial drought, which lasted from the mid-1990s to 2009, in which both flowering and pollination of *C. xanthochila* were low. In particular, the flowering and pollination was affected by reduced rainfall in 2002, 2006 and post the millennial drought in 2014, where rainfall was reduced by 62.99% (average of 26.95 mm during the growing season), compared to the long-term average (42.78 mm during the growing season). The Wimmera region (which contains our long-term monitoring site) is predicted under high emission climate change scenarios to have up to a 26% reduction in rainfall with a 32% reduction in Spring (Clarke et al. 2019). We predict that with increasing weather extremes and therefore drought, there will be long-term effects on recruitment in *C. xanthochila* populations (due to reduced flowering and seed set). However, even under the worst-case scenario of high emissions (RCP 8.5), average rainfall alone is unlikely to be reduced enough to significantly affect flowering and pollination. Thus, translocations to enhance the numbers and populations of *C. xanthochila* in the Wimmera would be best placed in areas that are buffered from drought.

Pollen limitation is common in orchids that rely on a pollinator to transport pollen between or within plants, with a mean pollination rate of 20.7% for reward less species and 37.1% for rewarding species (Tremblay et al. 2005). Sexually deceptive *Caladenia* in Australia have a low pollination rate, with estimates of average reproductive success of 14 ± 3% (Phillips et al. 2009). The pollination rate in *C. xanthochila* of 7.6 ± 1.5% at the natural site falls below the average pollination rate for both rewardless and rewarding species, while the pollination rate across the six translocation sites of 16.1% ± 3.55 (SE) aligns with what would be expected of sexually deceptive *Caladenia.* The mean seed viability of *C. xanthochila* was 14.6%, which is low compared to other species of *Caladenia* (i.e., *C. arenicola* Batty et al. (2001); 9% seed viability in small populations of *C. rigida* compared to 36% in large populations, Faast et al. (2011)), further reducing the effective seed set. As we only scored pollination in the long-term data as fruit set it is possible that we were missing cases of pollinia deposition and due to resource limitation (field pollinations in our study led to 73% fruit set) did not observe fruit set. *Caladenia xanthochila*

is pollen limited in the wild, with hand pollinations in the shade house conditions leading to 100% fruit set. Given that an increase of up to 90% fruit set can be achieved with hand pollination, we recommend that hand pollination is undertaken periodically in seasonally wet years and several years after translocations to increase the likelihood of recruitment (in small populations) and successful establishment of multiple generations in the early stages of translocation.

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Author contributions NR wrote the first draft of the paper, conducted field work, pollinator baiting for *C. xanthochila* and *C. robinsonii*, propagation, nectar collection, translocations and monitoring. BB conducted the nectar analysis. GB provided morphological identification of thynnine pollinators. GP conducted long-term monitoring of *C. xanthochila* sites and field hand pollinations. MM conducted statistical analysis. MW conducted pollinator baiting of *C. xanthochila and C. calcicola*. NR, BB, GP, MM and MW edited the manuscript.

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Data Availability Data will be made available upon reasonable request to the first author.

Declarations

Conflict of interest The authors declare no conflict of interest.

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