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Hand-pollination increases seed set in the critically endangered orchid *Thelymitra kangaloonica*.

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Abstract: *Thelymitra kangaloonica* is a listed Critically Endangered sun-orchid (family Orchidaceae) with a highly restricted distribution. In recent years both individual abundance and capsule set rates have been low in this species. The flowers of *Thelymitra kangaloonica* possess some traits associated with autogamy (self-fertilisation) and others associated with entomophily (insect-pollination), however the pollination mechanisms have not been explicitly tested.

This study compared the capsule set rate of flowers cross-pollinated by hand among individuals, against flowers which received no treatment. We found that capsule set rate in hand cross-pollinated flowers was significantly higher than in flowers receiving no treatment (23.1% versus 4.4%). This suggests that *Thelymitra kangaloonica* relies primarily on entomogamy to achieve seed set. Hand cross-pollination may assist management of the species by increasing the number of seeds in the soil seed bank and potentially assist in increasing the population size. In addition, it facilitates seed collection for seed banking and development of an ex-situ population for future population augmentation or translocation.

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Introduction

Thelymitra kangaloonica is a large-flowered sun-orchid (family Orchidaceae) occurring in seasonal sedge swamp habitat on grey silty clay-loam and is currently known from just two swamps near Kangaloon on the Central Tablelands of NSW (Jeanes 2011). Monitoring at the two sites in 2016, 2017 and 2018 recorded a very small flowering population annually (maximum of 18 at a site in a season - DPIE unpublished data) although larger flowering events have been reported historically (D. Wilson anecdotal report). The species is listed as Critically Endangered under both Commonwealth and State legislation because of its small population and very limited distribution.

Sun-orchids only open their flowers on warm, sunny days (Bates 1984) and are known to be pollinated through a range of mechanisms including cleistogamy, mechanical selfing, and insect pollination (Cropper and Calder 1990, Sydes and Calder 1993). Insect-pollinated *Thelymitra* species generally have larger flowers that open freely on sunny days and have compact pollinia that are firmly attached to a functioning viscidium (Cropper and Calder 1990, Sydes and Calder 1993, Edens-Meier *et al*. 2013). In contrast, self-pollinating species tend to have flowers that do not open, or only open tardily on very hot days, and have friable, mealy pollen that does not adhere to the viscidium (Sydes and Calder 1993 and Edens-Meier *et al*. 2013).

Thelymitra kangaloonica is one of seven described taxa within the *Thelymitra aristata* complex (Jeanes 2011). Within this complex, the primary pollination mechanism for three species is entomogamy while the remaining four, including *Thelymitra kangaloonica*, are thought to be autogamous or facultatively autogamous based on pollen structure and coherency, and rates of capsule development (Jeanes 2011).

Flowers of *Thelymitra kangaloonica* have a combination of autogamous and entomogamous traits. Jeanes (2011) noted that the friable, mealy pollen as well as the high capsule set suggest that the species is facultatively autogamous whereas the large, freely opening flower, strong spicy fragrance and the functional viscidium indicate that it is at least capable of entomophily.

Seed collection as part of a conservation project for the species to establish an ex-situ population was planned as part of the NSW Government's Saving our Species Program. However, monitoring of the 14 plants observed in 2016 and 2017 revealed a complete absence of capsule development (DPIE unpublished data). In 2018, in collaboration with the Australian Botanic Garden, Mount Annan, hand cross-pollination was implemented in an attempt to improve capsule development rates to permit collection of seed for propagation. This presented an opportunity to test the impact of hand-pollination on capsule development within an experimental context, and so gain insights into the species' pollination biology. These insights have implications for management actions that may assist the conservation of this species, as we discuss below.

Methods

Study sites

We studied the population of *Thelymitra kangaloonica* at the two sites where the species is currently known near Kangaloon, NSW within the protected WaterNSW Metropolitan Special Area. The sites form part of the Coastal Upland Swamp Endangered Ecological Community also listed under State and Commonwealth legislation.

Population monitoring

Surveys to locate individuals were undertaken at both sites during the flowering period between 29th October and 14th November in 2016, 2017 and 2018. The presence of the species was first detected in Swamp 2 during exploratory surveys in 2017, meaning no surveys were conducted in this location in 2016. Surveys were only conducted on warm, sunny days when flowers were more likely to be open, therefore maximising detection probability. Each swamp was visited on at least three days each year and any flowering individuals were pegged and marked permanently with a thin metal stake and numbered brass label. All known individuals were checked in subsequent years. Non-flowering individuals, present only as an unidentifiable single basal leaf, were not recorded. For each plant the number of flowers per spike was recorded. Flowering individuals were checked weekly post-flowering for at least two weeks to determine if capsules had developed.

Hand-pollination

Hand cross-pollination was undertaken at both swamps in the 2018 flowering season. This was carried out by removing the pollinia from a flower of one individual plant using a new tooth pick, then gently rubbing the pollinia onto the stigma of a flower from a different plant. Only flowers which were open or beginning to open were selected for hand cross-pollination. For each hand pollination event, the individual plant and flower (counted from top down) of the pollen receiver and the pollen donor were recorded. Organza bags were placed over the flowering spike once capsule development was observed or if flowers were no longer active. All cross-pollination events were between individual plants within the same swamp.

Data analysis

Fisher's Exact Tests (Social Science Statistics 2019) were carried out to determine whether observed rates of capsule development differed between hand cross-pollinated and non-hand cross-pollinated flowers. Individual tests were carried out on 2x2 contingency tables for each swamp, as well as on the pooled data across both swamps.

Results

Population monitoring

The population of flowering individuals remained small over the three years of monitoring although it increased slightly at both sites over time (Table 1). No individuals recorded flowering in one year were recorded flowering the subsequent year; however, three individuals flowering in 2017 had a basal leaf but no flowering stem in 2018. This is likely to be an underestimate of total non-flowering individuals, as the leaf is quite cryptic in the swamp vegetation.

Table 1. Number of flowering *Thelymitra kangaloonica* individual plants found at each swamp across the three years of study. Note Swamp 2 was not formally surveyed in 2016.

	No. flowering individuals	
	Swamp 1	**Swamp 2**
2016	1	N/A
2017	5	8
2018	6	18

Pollination monitoring

The 24 flowering plants located in 2018 had a total of 162 flowers, with between 3 and 13 flowers per spike. Some individuals had finished flowering at the time of survey (all flowers on the spike were wilting and turning brown); 16 of the 24 flowering individuals had at least one flower available for cross-pollination. Intact pollinia were present in all individual flowers utilised for hand cross-pollination. The pollinia were observed to be cohesive and well-attached to a sticky viscidium, with which they could be easily removed. A total of 26 flowers across the 16 individuals were hand cross-pollinated (one to three flowers per individual plant). The remaining 136 flowers received no treatment.

A total of 11 flowers across eight plants developed capsules. A greater percentage of hand cross-pollinated flowers developed capsules (22.2% for Swamp 1 and 23.5% for Swamp 2) than untreated flowers (3.4% for Swamp 1 and 4.7% for Swamp 2; Table 2). This was statistically significant for Swamp 2 (Fisher's Exact Test, $P = 0.0346$) but not Swamp 1 ($P = 0.1703$). The comparison was statistically significant for the pooled data ($P = 0.0101$).

Table 2. Comparison of capsule development of hand cross-pollinated versus untreated flowers across the two swamps.

	No. flowers	No. flowers capsule developed	% capsule development
Swamp 1			
Hand-pollinated	9	2	22.2
Untreated	29	1	3.4
Total	38	3	7.89
Swamp 2			
Hand-pollinated	17	4	23.5
Untreated	107	5	4.7
Total	124	9	7.26

Discussion

Seed capsule development was much higher when hand cross-pollination was undertaken compared to when no treatment was applied. In combination with the functional viscidium, cohesive pollen, strong fragrance, and large, freely opening flowers (Jeanes 2011), this suggests that *Thelymitra kangaloonica* is more strongly dependent upon insect pollination than thought by Jeanes (2011).

Jeanes (2011) based his conclusions on the pollination mechanism of *Thelymitra kangaloonica* on analysis of herbarium specimens and discussions with orchid enthusiasts who had observed the species. The "friable and mealy" pollen observed was one of the reasons he suggested that the species was autogamous. In this study we observed that the viscidium was sticky, the pollinia were well-attached and the pollen held together well during transfer between flowers. It is possible that the pollen Jeanes (2011) observed had become "mealy" with age as when we investigated older flowers in the field the pollinia tended to disintegrate when we attempted to remove them with the tooth pick and the pollen within was friable and mealy.

A high level of seed capsule set in the *Thelymitra* genus, as reported by Jeanes (2011), is typically associated with autogamy. Again, Jeanes based his observations on capsule set in *Thelymitra kangaloonica* on preserved herbarium specimens which had high capsule set but were lacking in information on species abundance at the time of collection (Jeanes pers. comm.). In contrast, we observed low seed capsule set rates during a period of low species abundance. This inconsistency in seed capsule set may be a result of variable insect pollination rates based on the size of the floral display in any one season. This is particularly likely if insect pollination is by food deception, rather than olfactory communication, and insect pollinated *Thelymitra* are thought to be primarily food deceptive rather than sexually deceptive (Jones 2006). In a year when many *Thelymitra kangaloonica* are flowering the floral display may be large enough to attract many pollinators to the area leading to high pollinator visitation rates, resulting in high seed capsule set by entomophily. When there are few *Thelymitra kangaloonica* flowering, the display may be insufficient to attract many pollinators leading to low seed capsule set by entomophily. This phenomenon has previously been observed in other orchid species such as *Listera ovata* (Brys *et al*. 2008) and *Myrmecophila christinae* (Parra-Tabla and Vargas 2007). The floral display present is not necessarily limited to flowers of that single species but may include synchronous flowering by other species, typically somewhat similar in appearance. We did not collect data on other flowering species as part of our surveys although the related *Thelymitra ixoides* and *Thelymitra pauciflora* were observed in low numbers in the broader vicinity but not within the swamp habitat of *Thelymitra kangaloonica.*

Insect-mediated and putative cross-pollination has only been confirmed in eight large-flowered *Thelymitra* species. These species are *Thelymitra media* and *Thelymitra aristata*

(Jones 1981), *Thelymitra megcalyptra* (Bernhardt and Burns-Balogh 1986), *Thelymitra antennifera* (Dafni and Calder 1987), *Thelymitra epipactoides* (Cropper and Calder 1990), *Thelymitra ixoides* (Sydes and Calder 1993), *Thelymitra macrophylla* and *Thelymitra crinita* (Edens-Meier and Bernhardt 2014). Generally, pollinators were female native bees of the families Apidae, Colletidae, and Halictidae; although pollen has also been observed on a solitary wasp (*Eurys* species) and hoverfly (*Syrphus damaster*) (Edens-Meier and Bernhardt 2014). We did not observe any likely insect pollinators incidentally while in the field however no targeted surveys were undertaken.

This research does not preclude autogamy occurring in the species. A low number of seed capsules developed in the absence of hand cross-pollination, however we cannot determine if these were pollinated via entomophily or autogamy. In this field study, recently opened or opening flowers were observed to possess cohesive pollinia well-attached to, and easily removed by, a sticky viscidium. This feature, combined with the low rate of capsule set in the absence of hand cross-pollination, suggest entomophily is the dominant mode of pollination and autogamy – if present – is likely to be facultative rather than obligate. A future study comparing the effectiveness of hand-pollination within individual flowers could be undertaken to determine if autogamy is possible but is a low priority given the status of the species.

Seed capsule set and development is not solely determined by pollination (Elliott and Ladd 2002). Production of seed and fruit is costly and resource availability has been demonstrated to limit production in some cases (e.g, Lloyd 1980 and Stephenson 1981). Given that seed capsule set increased with hand-pollination in a season it is likely that seed capsule set is primarily limited by pollination. However, it has been demonstrated that plants can be pollinator-limited within a season but resource-limited over their lifetime (e.g, Montalvo and Ackerman 1987). As no individuals flowering in 2016 or 2017 then flowered the subsequent year, it may indicate that resource availability is limiting reproduction over consecutive years, however further evidence would be required to confirm this. There has been little research into either pollinator or resource limitations in the *Thelymitra* genus. Not all individuals flower each year so multi-year studies, tracking individuals' reproductive status and outcomes as well as environmental conditions, may help explain some of the high variability observed in flowering events and seed capsule set.

As a critically endangered orchid, the small, flowering population size and very low levels of natural capsule set over the last three years is a concern. It is likely that seed production is the only means of population increase in this species as it does not appear capable of producing multiple replacement tubers each year. This is based on the lack of any clumping of individuals observed over the last 3 flowering seasons, as seen in some other species such as *Diuris arenaria* (K. Sommerville unpublished data). The success of hand cross-pollination in increasing capsule set and development offers a potential management action to benefit the species. Increased seed production through hand-pollination would lead to an increased soil seed bank and potentially assist in increasing the population size as well as allow for seed collection for seed banking and development of an ex-situ population for future population augmentation or translocation.

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