#### Final report to the AOF (Project 303.15).

Floral Presentation, Pollination and Reproductive Success in *Corunastylis* species Zong-Xin Ren<sup>A</sup>, Wendy Grimm<sup>B</sup>, Brian Towle<sup>C</sup>, Qi Qiao<sup>D</sup>, Daniel J. Bickel<sup>E</sup>, Soraya K. M. Outim<sup>F</sup>, Peter Bernhardt<sup>G, H</sup>

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Abstract. We observed and recorded floral presentation and the reproductive ecology of seven Corunastylis species native to New South Wales or Victoria in 2016. Auricle lobes of the column wings of Corunastylis filiformis, C. fimbriata and C. ruppii (NSW) secreted a fluid consumed by their pollinators. Scent analyses of these species identified six compounds. In New South Wales, these three species and C. rufa were pollinated exclusively by Conioscinella species (Chloropidae). The dominant carriers of pollinaria were females of the same morphotype. A fly could carry a maximum of two, whole pollinaria on its thorax. Microscopy showed that rates of

pollinarium removal were higher than rates of pollen deposition on receptive stigmas in three species in NSW. Bagging experiments showed low rates of self-pollination and no fruit set. In the absence of bagging, the rates of fruit set in inflorescences in four species (NSW and Victoria) exposed to chloropids varied from 35-91%. The highest conversion rate of flowers into fruit was in the Victorian, C. ciliata, which also produced the lowest number of flowers/scape. Seed set varied in three species in NSW with >42-70% of seeds containing fully developed embryos

#### Introduction

The estimated 50 species placed currently in the genus *Corunastylis* (sensu Jones and Clements 2002, 2018) often show floral characters atypical of related, terrestrial orchids native to Australia. The majority of these "midge orchids" bloom in the dead heat of summer through autumn and the act of flowering may be triggered by summer rain (Canackle et al. 2020). These species produce some of the smallest and "hairiest" flowers of native, terrestrial, orchids. However, some Corunastylis species show another feature atypical to most native wildflowers. Several species continue to produce and maintain extensive populations during or following human degradation of native vegetation. This includes, but is not limited to, populations colonizing margins along trails maintained in national parks, mown roadside verges or in paddocks planted with non-native grasses (Bernhardt et al. 2017). How do these species maintain sexual reproduction while exposed to summer heat and non-native competitors?

Research on pollination of *Corunastylis* began with Garnet (1940) when these orchids were still treated as a subgroup in the genus Prasophyllum. Garnet potted four Victorian species and left them on his open windowsill to bloom. They were visited readily by tiny flies that fed on secretions at the base of the labellum's callus. As usual, the pollinarium was deposited on the back of the insect's thorax, as it exited the flower. As their attachment stalks (stipes) dried they went through the same bending motions as as Darwin (1862) first described in terrestrial orchids native to England. Garnet's pollinators were identified as Caviceps flavipes and Oscinosoma subpilosa in the family Chloropidae (eye flies). Fruit and seed set rates were not addressed.

There was little additional research on pollination in midge orchids until recently. We are indebted to Kuiter (2016, 2018) for his ongoing photography and observations of chloropids taken while these insects visited flowers and transported pollinaria in Victorian populations. He often worked in sites dominated by naturalized vegetation. Bower *et al.* (2015) studied a rare species, *C.* (*Genoplesium*) *littoralis* (<u>D.L.Jones</u>)

D.L.Jones & M.A.Clem. Once again, most of the pollinators were chloropids but they were placed in the genera *Conioscinella* and *Cadrema*. As usual, the vast majority of pollinators drank fluids at the base of the labellum callus but most were identified as females. Ten flowering stalks had 71% of their pollinaria removed by insects and 44% of those flowers set fruit.

Our consortium of scientists from Australia, America and China made observations on floral and fruit biology and collected pollen bearing insects of *Corunastylis* species from 2015-2019 working in multiple sites in New South Wales and Victoria and in laboratories in Kunming,

Yunnan, and Saint Louis, Missouri. We present our results on the reproductive biology of the following species: C. archeri (R.S.Rogers)D.L.Jones & M.A.Clem (Vic.), C. ciliata (Ewart & B. Rees) D.L. Jones & M.A. Clem. (Vic.), C. filiformis (Fitzg.) D.L.Jones & M.A.Clem (NSW), C. fimbriata (NSW), C. morrisii (Nicholls) D.L.Jones & M.A.Clem. (NSW), C. rufa (R.Br.) D.L.Jones & M.A.Clem. (NSW), C. ruppii (R.S.Rogers) D.L.Jones & M.A.Clem (NSW) and the possible hybrid of C. archeri x ciliata (Vic.). Due to constraints of time, a limited budget budget, and the disproportionate sizes of populations it was not possible to record the same data or perform the same analyses on each species.

#### **Materials and Methods**

*Study sites*. Our study sites in NSW and Victoria for 2016 are described in full in Ren *et al*. (2020). The

reader is reminded that our sites in Arcadia (Bloodwood Road), Kulnura, Royal National Park, Ku-ring-gai Chase National Park, Crib Point, and the Langwarrin Flora and Fauna Reserve showed a history of human disturbance. These included varied attempts to clear vegetation for different purposes including the preparation of land for cultivation (abandoned; Fig. 1), maintaining bush tracks, and mowing roadside verges.



Figure 1. Arcadia (Bloodwood Road) site for the largest population of

*C. fimbriata*. Bushland cleared originally for an orchard that was never planted.

*Recording floral* characteristics. The reader is also directed to Ren et al (2020). Over the 2016 season we recorded comparative flowering periods and the number and development of flowers on inflorescences. We also made observations of labellum movements as some species are recorded as having "trembling" labella (Jones 2006). We looked for the origin of floral secretions within these sites while wearing 3.5 X Opti-visors. Specimens preserved in 70% ethanol were examined under light and epifluorescence microscopy. Scent collection protocols and analyses of C. filiformis, C. *fimbriata* and *C. ruppii* followed Edens-Meier *et al.* (2014, and see Fig. 2).



Figure 2. Scent removal of an inflorescence and adjacent Control bag. (Lower left) Inflorescence isolated from pollinators in a green organza bag.

Observations, collections of visiting insects and their

### pollinaria

We made close observations of insect behaviour on flowers of the four *Corunastylis* species in NSW. Kuiter (2016, 2018) has observed and collected the pollinators associated with Victorian species in the Crib Point and Langwarrin sites. Insects visiting flowers or clinging to flowering stems were aspirated and preserved by freezing. As visitors to C. ruppii were few we also aspirated specimens from adjacent and overlapping grass blades and woody twigs. After recording the number and position of pollinaria on these samples, under a dissecting microscope, we preserved them for identification by storing in 70% ethanol. Preservation in an ethanol solution means they can be used for molecular systematics in the future. However, there is no current taxonomic treatment for flower-visiting, Australian chloropids. Drs. Bickel and Outim recorded the standard morphological characters of Diptera and segregated specimens into 6 morphotypes. Each morphotype was given a number instead of a name. We also recorded

pollinia deterioration (loss of pollen units in pollen sacs) on insect bodies as fragmentation of pollinia often indicates previous contact(s) with receptive stigmas (Tao *et al.* 2018b).

Rates of mechanical self-pollination vs. insect-mediated pollination Analyses and collections were restricted to the largest populations in NSW of *C. filiformis, C. fimbriata* and *C. ruppii* (above). We selected, at random, inflorescences in bud and divided them into open (not bagged) and bagged controls (Fig. 3).



Figure 3. Bagged inflorescence of *C. ruppii* with supportive bamboo skewer and jeweler's tag label.

To determine whether mechanical self-pollination (autogamy) can occur in the absence of visiting insects we labeled each inflorescence. Each inflorescence designated as bagged was then covered with a plain weave, synthetic fiber, organza bag equipped with drawstrings. To stabilize the slender stalks, a bamboo skewer was inserted into the earth next to each inflorescence before including it within the same bag as the inflorescence. When drawstrings were pulled insects could not enter from the bottom. Open and bagged inflorescences in this series were excised after the majority of floral organs on all flowers withered or their labella abscised. We could not wait for all flowers to wither naturally as the callose composing pollen tubes breaks down as the ovaries swell and styles dehydrate and abscise (Lipow et al. 2002).

Fixation, storage and preparation for viewing pollen tubes in pistils under epifluorescence followed the methods of Lipow *et al.* (2002) and Edens-Meier *et al.* (2010). Under a Zeiss Axioskop 40 or a Zeiss Axio Imager M2. Completion of this work was severely delayed by the Biology Building (Macelwane Hall, Saint Louis U.) fire in May 2017 which destroyed the original Zeiss-Axioskop-40.

# Proportions of pollinarium removal vs. pollinia fragment deposition on stigmas

The protocols also required that we examine flowers on whole inflorescences of these three species (collected in 2016) prior to fixation. The fresh stalk was placed under a dissecting microscope and we recorded whether pollinia fragments on the stigma were visible (Fig. 4) and whether the whole pollinarium had been removed prior to collection based on whether its anther was empty and the viscidium was missing.



Figure 4. Column of *C ruppii* showing empty anther sacs and pollinia fragments on sigma.

## Fruit and seed set proportions (2016)

To compare the conversion rate of gynoecia into fruit among three species exposed to pollinators we selected and tagged flowering stems at random of *C. filiformis*, *C. fimbriata* and *C. ruppii* at three sites in New South Wales (see above). We counted the number of flowers and buds excluding the terminal bud that never opens (Ren *et al.* 2020). As these three-species showed rapid fruit set we were able to count capsules on surviving scapes < 3 weeks after all individuals in the population stopped flowering. We excluded all tagged inflorescences that showed signs of animal damage.

Calculation of fruit set at the Crib Point population of *C. ciliata* (see above) differed as all specimens were already in fruit. As this species produces very few flowers (above) it was not necessary to apply the same protocol used on the three species in New South Wales. By viewing the fruiting scape with an Opti-visor, it was easy to determine the original number of flowers on a scape by counting fruits and then adding the number of pedicel abscission scars and/or any remaining withered flowers with shriveled ovaries (see Ren *et al.* 2020).

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#### Seed set

To minimize disturbance to the local seed bank we only tested 13-21 capsules (one capsule per scape) for *C*. *filiformis*, *C. fimbriata* and *C. ruppii*. All seeds in each capsule were extracted, placed on an individual petri dish and examined under a dissecting microscope. Each seed was assigned to one of two categories following Ren *et al.* (2014): seed with a developed embryo, or aborted seed (embryo undeveloped-collapsed or empty seed coat; see below).

#### Results

#### Floral Presentation

Flowering in individual populations of *C. filiformis, C. fimbriata* and *C. ruppii* was asynchronous with new scapes emerging in bud as older flowering stems began setting fruit. Populations could remain in

bloom up to a month. Opening of buds on a scape usually occurred from the bottom up (acropetal, sub-acropetal).

Observations and analyses were published in Ren *et al.* (2020) and are summarized, as follows. The three studied in NSW produced more than twice as many flowers/scape, as *C. archeri*, *C. ciliata*, their prospective hybrid and *C. morrisii* in Victoria. The three species in NSW did not secrete fluids at the bases of their labella. Instead, transparent droplets were found at the apices of the auricle lobes that make up half the column wings in this genus (Fig. 5).



Figure 5. Note the droplet on the exposed auricle lobe of *C. fimbriata* and the unidentified *Conioscinella* sp. bearing pollinaria with erect stipes.

These auricles contained enlarged cells filled with raphides and these cells were especially congested at auricle apices (Fig. 6). The number of raphides in auricles varied between species but also varied among flowers on the same plant. Small flies that visited were observed to perch on flowers and scapes regurgitating transparent droplets (Ren *et al.* 2020; and see below).



Figure 6. Two lobes of the column wing of *C. filiformis* showing the papillose margins of the staminodium (above) and swollen cells containing black raphides in the auricle lobe much congested at the lobe apex.

*Corunastylis ruppii* produced less observable fluid than *C. filiformis* and *C. fimbriata* and also lacked a trembling labellum. The labellum of *C. ruppii* moved freely when probed with insect pins. A total of six scent molecules were detected in these flowers with *C. ruppii* producing five different molecules. *Corunastylis fimbriata* was the only species to emit Nepetalactone, a scent identified in commercial catnip.

#### Insect Vectors of Pollinaria

Chloropid flies were most likely to visit open flowers of *C. filiformis, C. fimbriata, C. rufa* and *C. ruppi* in NSW from early morning, after dew evaporation, through mid-day. However, the majority of the chloropids we observed and collected, during morning hours of observation, appeared to remain on the same inflorescence. We rarely saw a chloropid move from one flower to a second inflorescence. The majority appeared to have landed first on the erect-sub erect and non-resupinate labellum before crawling down towards the column. We caught a total of 323 flies on these four species and on adjacent vegetation in *C. ruppii* (see above). All belonged to the genus *Conioscinella* (Chloropidae). Of the 323 specimens segregated into 6 morphotypes 96% belonged to morphotype 2 regardless of *Corunastylis* species or site in 2016.

We were unable to see the transfer of pollinaria to the body of the chloropid and the transfer of pollen to the receptive stigma. However, all pollinaria were deposited on the dorsum of the thorax. When we removed whole pollinaria from fresh flowers by touching the rostellum with a probe or pin the stipes were held erect to sub-erect upon release. Erect stipes observed on chloropids, following aspiration, bent forward over time until the pollinia touched, or almost touched, the bristles on the head (Fig. 7).



Figure 7. Unidentified female *Conioscinella* sp. (collected on *C. filiformis*) with bent stipe and the pollinium contacts bristles on the head

Out of a total of 277 specimens of *Conioscinella* morphotypes collected on the four species in New South Wales (above) a total of 80 flies carried the pollinaria of a *Corunastylis* species. The majority of pollinaria-carrying flies carried only one pollinarium/thorax (n = 66). The remaining 14 carried

two (Fig. 8).



on C. fimbriata, with each carrying two pollinaria.

A total of 31 pollinaria showed erosion/fragmentation of their pollinia suggesting previous contact with receptive stigmas. The number of flies carrying pollinaria of the *Corunastylis* species they visited varied from species to species. Out of 52 chloropids collected on flowers of *C*. *ruppii*, and on adjacent vegetation, only four carried pollinaria of the host plant. This may be contrasted with the 163 chloropids collected on *C. fimbriata* with 65 carrying pollinaria. Out of the four species studied in NSW, chloropids visiting *C. fimbriata* carried the greatest number of pollinaria (81%) as well as the highest percentage of individuals carrying two pollinaria/thorax and the highest percentage of fragmenting pollinia.

Coniosciella specimens were segregated into 6 morphotypes with four morphotypes aspirated on C. filiformis and C. fimbriata. However, out of 323 specimens, 96% belonged to the same morphotype (M2) regardless of Corunastylis species or collection site. Males (n = 65) were vastly outnumbered by females (n =258) on all orchid species except for *C. ruppii* (males = 60%). As C. ruppii received so few floral visits we aspirated insects on adjacent vegetation so this may be where the "boys" preferred to congregate. Only two males carried one pollinarium apiece and both were

aspirated on flowers of *C. filiformis* at the Kulnura site. *Floral Predation and Food Webs.* 

Whole flowers of *C. fimbriata* were also consumed by grasshoppers including a female of Exarna includens Walker, collected en copula, at the Arcadia (Bloodwood Road) site (Fig.8). Populations of Corunastylis species, especially C. fimbriata, at Arcadia appeared to be centers of food webs while they remained in bloom. We observed small spiders catching chloropids on the inflorescences and on overlapping stems of other plants. At the Bloodwood site, populations of basal rosettes of the carnivorous Drosera spathulata Labill. and D. pygmaea DC grew under and adjacent to C. fimbriata. We observed the remains of chloropids on their leaves.



Figure 8. Female of *Exarna includens* consumes flowers of *C. fimbriata.* 

Rates of self-pollination in bagged flowers versus pollen deposition and pollen tube growth in open flowers

The presence of pollinia fragments on stigmas, and pollen tubes penetrating styles in bagged flowers of C. *filiformis, C. fimbriata* and *C. ruppii* was always lower than in inflorescences exposed to insect visits. Inflorescences exposed to insects always contained more pollen tubes in their styles (Fig. 9). While the rate of mechanical self-pollination was highest in *C. ruppii* it accounted for only 4% of flowers distributed among 14 inflorescences.



Figure 9. Dissected and squashed stigma (anther removed) of *C. fimbriata* under epifluorescence showing deposition of pollinia fragments and dense pollen tube penetration (white) of the style.

Fruit and Seed Set

None of the bagged inflorescences of *C. filiformis* (n = 6), *C. fimbriata* (n = 17), and *C. ruppii* (n = 14) showed signs of fruit set. Fruit set in inflorescences exposed to insects varied significantly between the Victorian *C. ciliata* (Mean = 6.2 flowers/scape; 5.7 fruits/scape) versus C. filiformis (Mean = 14.6 flowers/scape; 6.0 fruits), *C. fimbriata* (Mean = 15.7 flowers/scape; 6.4 fruits) and *C. ruppii* (Mean = 15.1 flowers; 5.1 fruits/scape). Differences in the conversion of flowers into fruit was not significant among the three species from NSW.

The proportion of seeds containing embryos (Fig. 10) differed among *C. filiformis, C. fimbriata* and *C. ruppii* in New South Wales. In 2016, *C. filiformis* (Kulnura site) showed the highest percentage of seeds with developed embryos (70.3%). This was significantly higher than those observed in *C. ruppii*(42.7%) but not significantly different from *C.fimbriata* (50.3%) based on the Kruskal-Wallis Test.



Figure 10. Sample of seeds of *C. ruppii.* Seeds with fully developed embryos are swollen with ribbed-articulated coats while undeveloped seeds are white or resemble brown scales or chaff.

# Discussion

Floral Presentation

Good things come in small packages. While the genus Corunastylis may contain species offering the smallest flowers of any of Australia's southern, temperate and ground-dwelling orchids a closer examination of floral organs and their tissues indicates considerable novelty within this lineage. What is the function of auricle secretion versus labellum callus-based secretions? The same may be asked for variation in scent production among three species. All three attracted the same pollinator morphotype, placed their pollinaria on the same place on the same insect and stipes bent down in the same direction. More histological and biochemical analyses (scents and reward secretions) should be considered along the same lines as ongoing pollination by small flies in studies of Neotropical *Pleurothallis* species and their allies (Bogarin et al. 2018; Borba & Semir 2001).

Ultimately, this will help answer important questions. For example, do flowers of *Corunastylis* species mimic the bodies of wounded insects bleeding hemolymph that is consumed by small, opportunistic, flies (Bower *et al.* 2015) or does the presentation and reward cater to chloropids that consume the eye secretions of vertebrates (Ren *et al.* 2020)?

# *Chloropids as Pollinators of* Corunastylis *species and their ecological implications*

Based on the preceding literature we anticipated pollination by chloropids of four *Corunastylis* species in New South Wales. Based on previous work by Bower *et al.* (2015) we may have predicted that most of the pollinators would be female. What was not predictable was that the vast majority of pollinators would be so similar in morphology that they are probably the same species. The same insects appear to pollinate the same *Corunastylis* species in NSW although orchid populations are isolated by many kilometres. The same insects appear to pollinate different *Corunastylis* species when they are sympatric and co-blooming.

This may help to explain why reproductive success continues in Corunastylis species that have colonised disturbed habitats. The primary pollinators may benefit from the same man-made disturbances as their orchids. This also helps explain records of six hybrids amongst eight Corunastylis species in Australia (Backhouse et al. 2019). As different Corunastylis species may share the same pollinator and the pollinaria are deposited on the same place and bend towards the same angle the usual pre-fertilization barriers to hybridization (Grant

1994) appear weak in this lineage. Rejection of hybrid crosses could occur after fertilization, if it occurs at all.

As they exploit the same pollinator resource and are sympatric and co-blooming the four midge orchid species in NSW may be competing with each other for the same pollinator resource. This may be reflected in the evolution of divergent and exaggerated, floral advertisements (scent, pigmentation patterns, pubescence, labellum flexibility, etc.). Corunastylis ruppii appears to offers smaller volumes of auricle secretions, but a more complicated scent. Is it exploiting naïve insects that would be better fed by species like *C. fimbriata* and *C. filiformis*?

... The *C. fimbriata* population at Arcadia (Bloodwood) appears to be at the center of an interesting food web. Flowering at a time of year, in which, most low growing, herbaceous species are vegetative or dormant their flowers become food for grasshoppers but their pollinators become food for other arthropods and carnivorous plants. One wonders if these chloropids play any other roles as pollinators of late blooming wildflowers with tiny, massed flowers like some Actinotus species? We examined the stigmas of *C. fimbriata* during the epifluorescence squash series and occasionally found eudicot grains of unidentified flowering plants on stigmatic surfaces but could not trace their origins to resident species with certainty. They were definitely not the triangular grains associated with resident Myrtaceae or spiny Asteraceae (unpublished). Fruit and Seed Set

The conversion rate of flowers into fruit in *C. ciliata* (Vic) was dramatically higher than those three species

in NSW although these latter species produced at least twice as many flowers/inflorescence in the same year. Fruit set in *C. ciliata* was also far higher than the >42% conversion ratio of flowers into fruit recorded previously in *C. littoralis* D.L. Jones, a species producing as many as 30 flowers/inflorescence (Bower et al. 2015). We are not suggesting that fruit set rates decline in Corunastylis species as distributions move from south to north or that the number of flowers on an inflorescence increases in the same direction. This was a coincidence as we did not have enough specimens of *C. rufa* in NSW to count and record fruit set. This species produces1-25 flowers/scape (seePlantNet;

https://plantnet.rbgsyd.nsw.gov.au/cgi-bin/NSWfl.pl? page=nswfl&lvl=sp&name=Genoplesium~rufum).

We confirm here that the conversion rate of flowers

into fruit is usually higher in reward-producing orchid species than in cross-pollinated food mimics (Tremblay *et al.* 2005; Edens-Meier & Bernhardt 2014). Furthermore, the conversion ratio of flowers into fruit is higher in rewarding orchids producing fewer flowers/scape compared to closely related species offering many flowers/scape, as hypothesized by Jersáková and Kindlmann (2004).

Our observations have suggested that chloropids visit a flower on a stem of *Corunastylis* and linger for hours unlike other orchid pollinators. Considering the minutes it takes for stipes to bend downwards on fly bodies doesn't this imply that a lot of flowers on the same stem will ultimately be self-pollinated by a fly visiting more than one flower on the same plant (geitonogamous pollination)? Shouldn't this result in a decline in maternal fitness visible as empty seeds? It may be a recurrent problem in *C. ruppii* with only 42% well-developed seeds in capsules as it received so few visitors carrying pollinaria. It may be less of a problem in C. fimbriata (50%) and C. filiformis (70%) if we compare their frequencies of seed set under insect-pollination to hand-mediated, cross-pollinated orchids belonging to unrelated species. Habenaria *limprichtii* Schltr. offered nectar and the ratio of developed seeds inside hand-pollinated, outcrossed fruits was 62% (Tao et al. 2018b). Full embryonic development in nectar-secreting Spiranthes sinensis (Pres.) Ames was 72% for hand pollinated, outcrossed fruits (Tao et al. 2018a). Calanthe yaoshanensis Z.X. Ren & H. Wang (Ren et al. 2014), a food mimic, had embryonic development at 70% for hand-pollinated, insect-mediated outcrossed fruits (Ren et al. 2014).

#### Conclusions

Continued field and lab work on the reproductive biology of *Corunastylis* species has considerable merit as they are successful orchids benefiting from disturbances made by people. Future investigations of these small flowers could provide more information on how attractants and rewards may evolve under competition for the same common and widespread pollinator. This includes modifying atypical organs to secrete rewards and produce atypical scents. As four species in New South Wales share the same pollinator, deposit their pollinaria on the same part of the insect's body, and grow and bloom together in the same sites the more common isolation mechanisms preventing hybridisation, may not apply here. Unlike many "weedy" species, mechanical self-pollination appears unimportant in some Corunastylis species but a high conversion rate of flowers into fruit may depend, at least in part, on the sheer number of flowers on a stem (less means more). Currently, the frequency of developed embryos in two,

insect-pollinated *Corunastylis* species parallels frequencies in other, unrelated, orchids when they are carefully hand-pollinated. As these small orchids may make useful model systems to better study reproductive success and the evolution of novel traits we recommend a greater effort from native orchid horticulturists to adapt them for future research in bush and glass houses.

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### References

Backhouse GN, Bates RJ, Brown AP, Copeland LM (2019) 'Checklist of the orchids of Australia including its island territories.' (Melbourne, Victoria, Australia) Bernhardt P, Edens-Meier R, Grimm W, Ren ZX, Towle
B (2017) Global collaborative research on the
pollination biology of rare and threatened orchid
species (Orchidaceae). *Annals of the Missouri Botanical Garden* 102, 364-376.

Bogarín, D., Fernández, M., Borkent, A., Heemskerk, A., Pupulin, F., Ramírez, S., Smets, E., Gravendeel, B. (2018). Pollination of *Trichosalpinx* (Orchidaceae: Pleurothallidinae) by biting midges (Diptera: Ceratopogonidae). *Botanical Journal of the Linnaean Society* **186**, 510–543.

Borba, E.L. and Semir, J. (2001). Pollinator specificity and convergence in fl Dr. y-pollinated *Pleurothallis* (Orchidaceae) species: A multiple population approach. *Annals of Botany* **88**, 75–88. Bower CC, Towle B, Bickel D (2015) Reproductive success and pollination of the Tuncurry Midge Orchid (*Genoplesium littorale*) (Orchidaceae) by Chloropid flies. *Telopea* 18, 43-55.

Canackle L, Armstrong R, Briggs J, McCreery D (2020)
'Late summer and autumn rains spark new hope for three endangered midge orchids in South-east NSW.' *Australasian Plant Conservation* 29, 15-20.

Darwin C (1862) 'On the various contrivances by which British and foreign orchids are fertilised by insects, and on the good effects of intercrossing.' (John Murray: London, England) Edens-Meier R, Bernhardt P (2014) The Sun orchids (*Thelymitra*) then and now: Large flowers versus small flowers and their evolutionary implications.
In 'Darwin's Orchids: Then & Now'. (Eds R
Edens-Meier, P Bernhardt) pp 173-198. (University of Chicago Press: Chicago, USA).

Edens-Meier, R.M., Raguso, R., Westhus, E. and Bernhardt, P. 2014. Floral fraudulence: Do blue *Thelymitra* species (Orchidaceae) mimic *Orthrosanthus laxus* (Iridaceae)? *Telopea* 17, 15 – 28.

Edens-Meier R, Vance N, Luo YB, Li P, Bernhardt P
(2010) Pollen pistil interactions in North American and Chinese *Cypripedium* L. (Orchidaceae). *International Journal of Plant Sciences* 171, 370-381. Garnet JR (1940) Observations on the pollination of orchids. *Victorian Naturalist* **56**, 191-197.

Jersáková J, Kindlmann P (2004) Reproductive success and sex variation in nectarless and rewarding orchids. *International Journal of Plant Sciences* **165**, 779-785.

Grant V (1994) Modes and origins of mechanical and ethological isolation in angiosperms. *Proceedings of the National Academy of Sciences* **91**, 3-10.

Jones, D.L. 2006. A complete guide to native orchids of Australia including the island territories. New Holland Publishers (Australia) Pty, Ltd. Sydney, New South Wales. Jones DL, Clements MA (2018) Newly recognised and reinstated species of Nomenclatural notes arising from studies into the tribe Diurideae (Orchidaceae). *The Orchadian* **13**, 437-468.

Kuiter R (2016) 'Orchid pollinators of Victoria', 4th End. (Aquatic Photographics: Seaford, Victoria, Australia).

Kuiter R (2018) 'Pollination of Victoria's leek orchids & their allies.' (Aquatic Photographics: Seaford, Victoria, Australia).

Lipow SR, Bernhardt P, Vance N (2002) Comparative rates of pollination and fruit set in widely separated populations of a rare orchid (*Cypripedium*  *fasciculatum*). *International Journal of Plant Science* **163**, 775-782.

Ren ZX, Grimm W, Towle B, Qiao Q, Bernhardt P
(2020) Comparative floral traits in *Corunastylis*(Diurideae; Orchidaceae) with novel applications: do some species bleed or blink? *Muelleria* 39, 27-38.

Ren ZX, Wang B, Bernhardt B, Camilo G, Li DZ (2014) Which food-mimic floral traits and environmental factors influence fecundity in a rare orchid, *Calanthe yaoshanensis? Botanical Journal of the Linnean Society* **176**, 421-433.

Tao ZB, Ren ZX, Bernhardt P, Liang H, Li HD, Zhao YH, Wang H, Li, DZ (2018a) Does reproductive isolation reflect the segregation of color forms in *Spiranthes*  *sinensis* (Pers.) Ames complex (Orchidaceae) in the Chinese Himalayas? *Ecology and Evolution* **8**, 5455-5469.

Tao ZB, Ren ZX, Bernhardt P, Wang WJ, Liang H, Li
HD, Wang H (2018b) Nocturnal hawkmoth and
noctuid pollination of *Habenaria limprichtii*(Orchidaceae) in sub-alpine meadows of the Yulong
Snow Mountain (Yunnan, China). *Botanical Journal of the Linnean Society* 187, 483-498.

Tremblay RL, Ackerman JD, Zimmerman JK, Calvo RC (2005) Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. *Biological Journal of the Linnean Society* **84**, 1–54.