THE REPRODUCTIVE ECOLOGY OF TWO TERRESTRIAL ORCHIDS, *CALADENIA RIGIDA* AND *CALADENIA TENTACULATA*

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DECLARATION

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Published works contained within this thesis:
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Phillips RD, **Faast R,** Bower CC, Brown GR, Peakall R (2009) Implications of pollination by food and sexual deception for pollinator specificity, fruit set, population genetics and conservation of *Caladenia* (Orchidaceae). *Australian Journal of Botany* **57**:287-306.

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Cover photos: *Caladenia rigida* (left) and *Caladenia tentaculata* (right). Photos by author.

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THESIS SUMMARY

The reproductive outcome of plants is often determined by a multitude of interacting factors operating at both the plant level and the population level. For many plants, fruit production and the subsequent release of seeds are paramount for the persistence of the species. Understanding the processes that influence variation within and among populations is therefore crucial for the successful long-term management of threatened plants. While abiotic factors such as resource availability and environmental conditions can influence seed production directly through their effects on plant growth, biological interactions such as those between plants and pollinators or herbivores can be equally important. The relative intensity and direction of such interactions are often determined by the nature of the plants themselves, or by characteristics of the plant population or the habitat in which it occurs.

This thesis examines the processes that influence spatio-temporal variation in the reproductive success of two terrestrial orchids, *Caladenia rigida* and *Caladenia tentaculata*. The study was carried out over three years (2005 – 2007), in several populations located in the Mount Lofty region of South Australia. A detailed investigation of the pollination strategy employed by *C. rigida* revealed that this species is a generalist, being pollinated by a suite of food-seeking insects, possibly attracted by the presence of small amounts of nectar. Successful pollination and seed release for *C. rigida* was highly variable across space and time. Furthermore, both measures were consistently higher than for the sexually deceptive species, *C. tentaculata*, leading to the suggestion that the highly specialised pollination syndrome of the latter species may place it at a reproductive disadvantage. Pollination success of *C. rigida* was influenced by the height of flowers, but not by the local density of conspecifics. Small populations of *C. rigida* did not produce capsules when environmental conditions were stressful, suggesting that resource availability may indirectly restrict reproductive success by limiting the availability of pollinators. Poor seed quality in some populations may also be attributed to reduced population size.

Both orchid species were subject to intense levels of vertebrate florivory and capsule predation, leading to significant reductions in seed output. A herbivore exclusion experiment was carried out to help elucidate the size and type of herbivores, and video-

surveillance identified birds as a predominant florivore in some populations. The intensity of florivory varied within and among populations, as well as among years, in response to several factors including flower height, the local density of conspecifics, concealment amongst neighbourhood vegetation and proximity to the habitat edge. Spatio-temporal variation in seed release was thus the net outcome of processes acting on both mutualistic and antagonistic interactions.

This work provides valuable baseline data of factors that influence the reproductive ecology and, hence, population dynamics of *Caladenia* species. Implications for the conservation and management of threatened populations are discussed, with respect to both short-term and long-term goals. The thesis is presented as a series of five manuscripts. Two of these have been published, and the remaining three have been prepared for submission as publications.

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CHAPTER 1

INTRODUCTION

1.1 General introduction

One of the key goals of ecology is to understand how interactions among organisms affect population dynamics and community structure. The abundance and distribution of plant species is contingent on their reproductive success, and for most plants, factors affecting seed production are critical elements influencing population dynamics.

Seed production can be constrained by the availability of pollen or resources, or both (Haig and Westoby 1988b; Zimmerman and Aide 1989; Ashman *et al.* 2004) and these, in turn, are controlled by intrinsic factors such as plant traits, as well as extrinsic abiotic and biotic influences (Lee and Bazzaz 1982; Lawrence 1993; Ghazoul 2005; Knight *et al.* 2005). Plant-animal interactions such as herbivory, pollination, florivory and seed or fruit predation play a pivotal role in determining the number of seeds that contribute to subsequent generations. Spatial and temporal variability in the relative intensity of these interactions can lead to substantial variation in seed production among individual plants, among populations and among seasons or years (Pettersson 1991; Jennersten and Nilsson 1993; Herrera 2000; Petit and Dickson 2005; Kolb *et al.* 2007; Shimono and Washitani 2007; Ågren *et al.* 2008; Toräng *et al.* 2008). A holistic understanding of plant population dynamics therefore relies on an integrated approach that considers the outcomes of multispecies interactions (Strauss and Irwin 2004).

Amongst plant families, the Orchidaceae is arguably one of the most charismatic, having captivated the attention of conservationists, growers and enthusiasts worldwide. Comprising an estimated 25,000 species, orchids are among the most widely distributed and diverse families of plants (Cribb *et al.* 2003). Orchids have adapted to extreme habitat conditions around the planet and are considered to be one of the most highly evolved plant families. Such diversification, particularly with respect to pollination strategies, has provided researchers with valuable opportunities to investigate elements of plant reproductive success. Orchids may be particularly susceptible to modifications of plant-pollinator interactions, because fecundity is usually pollen limited rather than resource limited (Tremblay *et al.* 2005). Furthermore, the great variation of traits among species makes them ideal candidates for comparative studies.

Understanding how plants respond to changes in biotic interactions forms an essential component of the conservation and management of individual plant species and ecological communities, particularly in light of human-induced modifications of landscapes. Development, agriculture, grazing and hydrological changes inevitably lead to habitat destruction and consequent fragmentation of once-continuous habitat. Populations within remnants are likely to experience drastic alterations in their size and density, as well as in the characteristics of the surrounding habitat, potentially leading to modification or disruption of important biological interactions (Aizen and Feinsinger 1994; Kearns and Inouye 1997; Aguilar *et al.* 2006; Pauw 2007).

In the Mount Lofty region of South Australia (an area of approximately 7,800 km^2), less than 13% of the original native vegetation remains and the majority of remnants are highly fragmented and degraded (SADEH 2009). According to the Census of South Australian Vascular Plants (Barker *et al.* 2005), a large number of South Australia's 234 orchid species are listed as vulnerable (30), rare (26), or endangered (47). Present management strategies for threatened orchids include hand pollination, weed eradication and protection from grazing (Quarmby 2006), all of which are likely to be crucial for the short-term survival and recovery of these species. However, detailed knowledge of the various factors that influence the reproductive success of orchids, and how responses to such factors vary both spatially and temporally, will provide valuable baseline information for the design of successful long-term management regimes.

This thesis presents a detailed investigation of three of the key interactions influencing plant reproductive ecology, namely those with pollinators, herbivores and seed predators. Each of these interactions can respond to characteristics at the level of the individual plant, the plant population, or the habitat in which they grow. At the plant level, pollination syndrome and the degree of pollination specialisation can dramatically affect the quantity and/or quality of pollen that a plant receives (Knight *et al.* 2005; Tremblay *et al.* 2005; Kindlmann and Jersakova 2006). Traits such as floral display, flower height or flower size are associated with attracting pollinators, but can also increase a plant's likelihood of being detected by herbivores (Brody and Mitchell 1997; Ehrlén 1997; Gómez 2003; Toräng *et al.* 2008). Similarly, population attributes such as the number of individuals or their spatial arrangement, can have an effect on pollination success as well as on the intensity of predation (Sih and Baltus 1987; Kunin 1997; Ågren *et al.* 2008; Sletvold and Grindeland 2008). Other extrinsic features, such as the composition or structure of the surrounding

vegetation or proximity to habitat edges, have also been shown to influence both mutualistic and antagonistic interactions (Jules and Rathcke 1999; Petit and Dickson 2005; Miller *et al.* 2006; Juillet *et al.* 2007).

The factors described above affect the number of seeds that are released by the plant by acting directly on seed production or seed predation. However, the ability of those seeds to germinate and recruit is crucial for the maintenance and growth of populations. The quality of the seeds produced can also be influenced by factors that are intrinsic to the plant, such as size or maternal genotype (Andersson 1993; Griffin and Barrett 2002), as well as ecological factors, such as resource availability, the size or density of the population, or the composition of neighbourhood vegetation (Menges 1991; Oostermeijer *et al.* 1998; Wallace 2003). Ultimately, the long-term persistence of plant populations will depend on whether population growth is limited by the availability of viable seeds, or the availability of microsites that are suitable for the establishment of seedlings (Eriksson and Ehrlén 1992; Ackerman *et al.* 1996; Moore and Elmendorf 2006).

The model species chosen for this research are terrestrial orchids of the genus *Caladenia*. This genus provides a unique opportunity for comparative studies because it comprises species that employ food-advertising (generalist) and sexually-deceptive (specialist) pollination strategies. These contrasting pollination strategies are likely to attract different types and numbers of pollinators, and could therefore have quite different implications for the final reproductive success of these orchid species. The two focal species are *Caladenia rigida,* an endangered orchid that is endemic to the Mount Lofty region of South Australia, and *Caladenia tentaculata*, a widespread species, common throughout the south-eastern part of Australia. Prior to this study, *C. rigida* was thought to employ a pollination syndrome involving both food deception and sexual deception (Bates 1984a; Bickerton 1997); however, no detailed investigations of the pollinators involved had been undertaken. The pollination strategy of *C. tentaculata* is well-described, with the orchid attracting a single-species of thynnine wasp through sexual deception (Bates 1996; Peakall and Beattie 1996). A third species, *Caladenia carnea*, was included for comparative studies of pollination strategies. This food-deceptive orchid is also widespread and common throughout south-eastern Australia.

1.2 Research aims and thesis outline

The goals of this research are to identify biological interactions that play an important role in determining the reproductive success of two terrestrial orchid species (*C. rigida* and *C. tentaculata*), and to characterise factors that influence the strength and direction of these interactions.

The specific aims are to:

i) Characterise the pollination syndrome employed by *C. rigida.*

ii) Evaluate the intensity and importance of antagonistic interactions affecting the reproductive success of *C. rigida* and *C. tentaculata* and identify potential herbivores.

iii) Compare spatio-temporal variation in pollination success and seed release for *C. rigida* and *C. tentaculata*.

iv) Assess the influence of a variety of intrinsic and extrinsic factors on both pollination and antagonistic interactions.

v) Determine whether seed production and subsequent seed release is an accurate prediction of reproductive output and the recruitment potential of seeds.

CHAPTER 2 provides an overview of the pollination biology of orchids in general and *Caladenia* more specifically. All original data are presented in CHAPTERS 3 to 7 and APPENDICES A to C.

A comprehensive examination of the pollination strategy of *C. rigida* is presented in CHAPTER 3. Pollinators are identified and the production of scent and nectar is investigated. The importance of antagonistic interactions is examined in CHAPTER 4, where variation in grazing intensity is assessed among populations and among years. The impact of grazing on final reproductive output is evaluated using herbivore exclusion cages. With the aid of video-surveillance, one of the predominant florivores of *C. rigida* is identified. The effectiveness of various cages as protection against grazing without impeding pollination is also evaluated. A copy of video footage is provided on a CD inside the back cover of the thesis.

In CHAPTER 5, spatial and temporal variation in the pollination success and seed release of *C. rigida* and *C. tentaculata* are compared and discussed with regard to their contrasting pollination strategies. The response of pollination success to reductions in population size

is also investigated. To minimise the effects of differences in habitat and environmental conditions, the reproductive success of *C. tentaculata* is compared with the food-advertising species, *C. carnea*, co-flowering at the same site.

Factors that can influence both pollination success and the risk of florivory and capsule predation in *C. rigida* are explored in CHAPTER 6. The focus here is on features of the plant, population and surrounding habitat that have the potential to affect the apparency of flowers; specifically flower height, density of conspecific flowers and concealment amongst neighbouring vegetation. In addition, a vegetation removal experiment was used to evaluate the impact of neighbourhood plants on biological interactions as well as on plant emergence and flowering.

Population maintenance and growth relies on the production of viable seeds and subsequent recruitment of seedlings. This issue is addressed in CHAPTER 7, where seed quality is examined among *C. rigida* populations in two regions of the species' distribution. These data, together with calculations based on recruitment from other *Caladenia* species, are then used to estimate whether seed production is sufficient to maintain the smallest populations of *C. rigida.*

APPENDICES A, B and C present additional work that supplements or supports the research presented in previous chapters, but was not included in the manuscripts submitted for publication due to space constraints. Potential pollinators captured bearing *C. carnea* pollinia are described in APPENDIX A. The abundance and nature of heterospecific flowers has implications for pollination success and seed quality. This is addressed in APPENDIX B by assessing the floristic community in populations of *C. rigida* and *C. tentaculata* and overlaying this with the phenology of flowering for both species of orchid. In APPENDIX C, the influence of apparency on the risk of florivory is examined for *C. tentaculata* and compared with the findings for *C. rigida*.

CHAPTER 8 consolidates the main outcomes from CHAPTERS 3 to 7 and discusses the overall significance of the research, with particular reference to implications for the management of threatened plants and future research directions.

Notes on Chapter Style

Each of the data chapters (3 to 7) has been written in a style suitable for publication in a scientific journal. Accordingly, the text reflects multiple authors. Each chapter can therefore be regarded as a stand-alone body of work, but collectively the chapters supplement each other and are presented in a sequence that follows the most logical progression. The data and text in each chapter are as they appear in the published or submitted manuscripts; however, some tables and figures have been enlarged or re-formatted to provide consistency among the chapters comprising this thesis. Literature cited throughout all of the chapters appears at the end of the thesis, rather than at the end of each of the results chapters. Where applicable, additional data or information that are made available by the publishing houses as online supplementary material, are included at the end of the relevant chapter.

A statement of authorship, detailing the contributions of all co-authors and the publication status of the manuscript, is provided at the beginning of each results chapter. At the time of thesis submission, two papers (CHAPTERS 3 and 4) are published, and reprints of these are included in APPENDIX D, along with permission from the publisher to reproduce these publications as thesis chapters. The remaining manuscripts (CHAPTERS 5, 6 and 7) have been prepared to submit for publication. During the course of my PhD studies, I also contributed to two other publications that are related to my research but do not address the specific aims of this thesis. Reprints of these manuscripts are provided in APPENDIX E.

BACKGROUND TO *CALADENIA*

2.1 Introduction to Australian terrestrial orchids and the genus *Caladenia*

Australia provides habitats for approximately 1300 species of orchids, the majority being terrestrial and confined to the more temperate southern regions of the continent (Jones 2006). Unfortunately the habitats of many orchids, such as open woodland or swamps, have been preferred for agricultural development, drastically reducing the distribution of orchid species. While some species that were once widespread and common are now rare, others had restricted and sparse distributions prior to human intervention and others still remain widespread and common (Jones 2006). The decline of orchid populations in Australia has been largely attributed to habitat destruction and the subsequent effects of habitat fragmentation (Todd 2000; Coates *et al.* 2003).

The genus *Caladenia* R.Br. contains 376 species and subspecies, of which 366 occur in Australia, making it the largest genus in the continent. *Caladenia* occupy a diverse range of habitats from tropical to sub-alpine zones, and although their distribution extends to islands of New Zealand, Indonesia and New Caledonia, greatest abundance and diversity occurs in the south-eastern and south-western parts of Australia (Jones 2001; Phillips *et al.* 2009a). The taxonomic status of the genus remains controversial (Hopper and Brown 2004; Jones and Clements 2005); however, for the purposes of this thesis the nomenclature adopted by the State Herbarium of South Australia is followed. This considers *Caladenia* sensu lato as a single genus (as per Hopper and Brown 2004), comprising six subgenera (*Caladenia*, *Stegostyla, Elevatae, Phlebochilus, Drakonorchis* and *Calonema*).

Amongst nationally threatened flora, *Caladenia* are represented at a disproportionately high level. One third of orchids listed under the Environment Protection and Biodiversity Conservation Act (1999) belong to *Caladenia*, and the genus contributes to almost 5% of the total number of listed plants (Dixon and Hopper 2009). In South Australia, sixty-four species of *Caladenia* have been recorded (Barker *et al*. 2005). Twenty-five species occur in the southern Mount Lofty region, and of these, 12 are listed as vulnerable, rare or endangered. The 2007 – 2012 Recovery Plan for Twelve Threatened Orchids in the Lofty Block Region of South Australia encompasses eight *Caladenia* species, all of which are nationally threatened (Quarmby 2006). The major threats contributing to the conservation

status of these species include vegetation clearance, habitat fragmentation, weed invasion, herbivory, pollinator declines and changes to fire regimes (Quarmby 2006).

2.2 Orchid floral biology

Orchid flowers are always zygomorphic and exhibit a vast range of morphological variation, but all are characterised by three basic features that distinguish them from other families of flowering plants:

1. The labellum, a modified petal that often forms a landing platform for pollinators and acts to guide insects to nectaries, or in some cases mimics the female form of certain insect species.
2. The column, formed by the fusion of stamens and styles, presenting the anthers at its apex with the stigma just beneath them.
3. Pollinia, tightly packed masses of pollen grains, which can be sectile and friable, but in most cases are sessile and transferred as a single unit to the pollinator.

Fig. 2. 1 Floral structure of *Caladenia* sp. (eg, subgenus *Calonema*). Diagram adapted from Backhouse and Jeanes (1995).

Genus Caladenia

Caladenia are terrestrial orchids. They are characterised by the production of a single, usually hairy, basal leaf and a flower consisting of a labellum that is morphologically distinct to the remaining petals and sepals (Fig. 2. 1). The labellum contains rows of conspicuous gland-like hairs or calli of varying colour, from which the genus derives its name (Greek: *Kalos*, beautiful; *adenos*, gland). Most species provide no obvious nectar and only one or a few flowers are produced per plant. Cross-pollination is facilitated by two triangular flaps that cover the compact pollinia. These are held shut when an insect pushes past them into the flower and are lifted when its thorax catches them upon backing out (Erickson 1965). A sticky secretion is smeared onto the insect's back as it passes by the rostellum, firmly adhering to and dislodging the now exposed pollinia (Bower 2001). Upon the next visit to an orchid flower, the pollinia are transferred to the sticky surface of the stigma to effect pollination (Fig. 2. 2a). *Caladenia* have four, bilobed, coherent (sessile) pollinia such that one pollination event can lead to the production of seeds that are all fathered by one individual.

Flowers usually close within one or two days of receiving pollinia, and the ovary gradually swells over the next 6 – 8 weeks to produce a capsule. Once mature, the capsule turns from green to yellow (Fig. 2. 2b) and then brown upon dehiscence (Fig. 2. 2c). Almost all orchids produce thousands to millions of tiny seeds (each weighing 0.3 - 24 μg), suggestive of long-distance passive dispersal (Arditti and Ghani 2000). The seeds lack an endosperm and germination is dependent upon a symbiotic relationship with soil-borne mycorrhizal fungi (Batty *et al.* 2001a; Zettler *et al.* 2003). These endophytes occupy a swollen region, known as the collar, located at the base of the leaf, just beneath the soil surface. Most Australian terrestrial orchids become dormant to enable them to survive hot, dry summers. They are deciduous and their roots form tuberoids or storage organs. Within *Caladenia*, most species only reproduce from seed and occur as solitary plants or in sparse groups, producing a single replacement tuber annually; however, some species also reproduce vegetatively via clonal propagation. *Caladenia* shoots emerge within the remains of the stem produced in the previous year, a characteristic that greatly facilitates the long-term monitoring of individual plants.

Fig. 2. 2 Stages of pollination in *Caladenia*. (*a*) Pollinia (arrow) deposited on stigma of *C. tentaculata.* (*b*) Maturing *C. rigida* capsule. (*c*) Dehiscent *C. rigida* capsule.

2.3 Pollination strategies

While the majority of the world's orchids provide a nectar reward, Australian terrestrial orchids are characterised by a high proportion of non-reward species that use some form of deceit to achieve pollination (Dafni and Bernhardt 1990; Adams and Lawson 1993). Several mechanisms for the evolution of deception have been put forward and it remains a highly debated topic (Dafni and Bernhardt 1990; Nilsson 1992; Tremblay *et al.* 2005). The resource limitation hypothesis suggests that plants in harsh environments have forgone the production of costly nectar to maximise the long-term production of offspring. However, as most orchids are pollination limited, deception is more likely to have evolved as a response to competition for limited pollinators, for example when population density is low or when competition with other co-blooming species is high.

Caladenia is a particularly interesting genus in that it contains species that employ food-deceptive and sexually deceptive pollination syndromes. Only one other genus, *Disa*, contains species representing both of these syndromes; however, in this case food-advertising species tend to me more specialised than those within *Caladenia* (Phillips *et al.* 2009b; Schiestl and Schlüter 2009).

Food deception

The majority of deceptive species lure insects by falsely advertising the presence of food, using fragrance and/or visual cues such as bright colours and fake structures that resemble nectaries or pollen. Mimicry systems can be broadly grouped into two types based on how well the mimics imitate their models (Nilsson 1992; Schiestl *et al.* 1999). Close resemblance of the deceptive species to a model species or guild, is known as Batesian or

guild mimicry, the latter being exemplified by the rewardless *Duiris maculata* which mimics legume flowers (Indsto *et al.* 2006). Non-model or generalised mimicry refers to species that do not mimic any other species, but employ a general floral signal such as colour or scent to falsely advertise the presence of a reward (Dafni and Bernhardt 1990).

Early observations suggested that the pollinators of food-advertising *Caladenia* may obtain nectar rewards (Erickson 1951; Stoutamire 1983), but a lack of visible nectar has led to the general acceptance that most of these orchids are food-deceptive (Dafni and Bernhardt 1990; van der Cingel 2001; Bates 2006). Two reports mention the production of small amounts of nectar (*C. nana*, Hopper and Brown (2001); *C. paludosa*, Dixon and Tremblay (2009)), but provide no empirical evidence. Amongst *Caladenia*, food-deception is represented in all but one subgenus. Species of *Caladenia*, *Stegostyla* and *Elevatae* are small-flowered and sometimes scented, and are considered to be pollinated mainly by native bees (Dafni and Bernhardt 1990; van der Cingel 2001; Bates 2006). However, food-deception has also been reported in some of the more brightly coloured spider orchids within *Phlebochilus* and *Calonema,* which are characterised by long, filamentous sepals and petals (Stoutamire 1983; Phillips *et al.* 2009b). There is no evidence for mimicry of any model species, indicating that non-model deception is the most likely means of attracting pollinators. Pollen vectors have only been positively identified for a handful of food-deceptive *Caladenia* (reviewed in Phillips *et al.* 2009b). In most cases, suggested pollinators are based on opportunistic observations that fail to demonstrate the transport of pollinia.

Sexual deception

Orchids of several Australian genera (*Caladenia, Drakaea, Chiloglottis, Calochilus* and *Spiculaea*) exploit the distinctive reproductive behaviour of thynnine wasps (Tiphidae) (Kimsley 2002). Male wasps are attracted to orchid flowers by kairomones that simulate the sexual pheromones produced by the female insects. Males are often observed flying upwind in a zigzag motion towards the flowers and then circling them (Stoutamire 1983). While chemical attractants operate at long range, visual stimulation is provided at close proximity by the insectiform appearance of the calli. Orchids employing sexual deceit have high pollinator specificity, with only one or a few insect taxa effecting pollination (Bower 1992; Phillips *et al.* 2009b). In many cases, the unique behaviour of sexually attracted insects promotes outcrossing and long-distance pollen flow in the orchids they pollinate (Peakall and Beattie 1996).

Sexually deceptive *Caladenia* belong to the subgenera *Phlebochilus, Drakonorchis* and *Calonema* and are typically dull shades of green, brown or maroon with long, filamentous perianth segments. Kairomones are produced in expanded clubs at the tips of the lateral sepals (Stoutamire 1983) but may also be produced by the labellum calli as demonstrated in other species (Ascensao *et al.* 2005). A detailed description of thynnine pollination of *C. tentaculata* is provided by Peakall and Beattie (1996). The male wasp lands on the labellum, grasps the female decoy and attempts to copulate. In doing so, he is thrown against the column by the mobile labellum such that his thorax is brought into close proximity of the stigma allowing for pollen transfer. Pollinia removal occurs as the insect retreats.

The specific attraction of wasps to sexually deceptive flowers has been exploited in a technique known as pollinator baiting, providing a valuable tool for identifying pollinators (Stoutamire 1983; Peakall 1990; Bower 1996). Artificially presented flowers placed in a suitable habitat elicit a rapid response by male wasps, peaking within one minute and then rapidly declining over the next 10 - 15 minutes. This behaviour has been attributed to strong competition for newly emergent female wasps (Peakall 1990). Baiting is most successful when flowers are presented at least 20 m from the orchid patch, as male wasps learn to avoid areas of unrewarding orchids (Wong *et al.* 2004).

Intermediate or dual pollination strategy

Several authors refer to the existence of orchid species that utilise more than one strategy to attract pollinators (Dafni and Bernhardt 1990; Bates 2006; Jersakova *et al.* 2006); however, very few examples of dual pollination syndromes have been described in detail. Two species of *Epipactis* (*E. consimilis* and *E. thunbergii* from Israel and Japan, respectively) are pollinated by syrphid flies that collect nectar as well as lay their eggs on the labellum, the hypochile of which apparently resembles aphids (the food source of their larvae). Stoutamire's (1983) observations that *Caladenia patersonni* (now *C. longicauda*) is pollinated by a suite of insects including native bees and thynnine wasps, are often misquoted as evidence for an intermediate state utilising both food and sexual deception. In fact, Stoutamire (in Bates 1990) states that "they attract an assortment of bees, wasps and flies without sexual attraction" and that the wasps are likely to be seeking food in the orchid (Stoutamire 1983).

Caladenia rigida is also thought to utilise a dual pollination strategy. The species has been shown to be pollinated by native bees (Bates 1984a; Bickerton 1997), and the capture of a male thynnine wasp displaying mate-seeking behaviour above a flower led to speculations that this species is also sexually deceptive (Bickerton 1997). However, baiting experiments have so far been unsuccessful in attracting wasp pollinators to the flowers of *C. rigida* (Bates 1996).

2.4 Description of study species

Caladenia rigida

Caladenia rigida R.S.Rogers (subgenus *Calonema*) is commonly known as the rigid white spider orchid. It produces a single flower (sometimes two, and rarely, three), borne at the end of a slender, green or reddish hairy stem, 10 – 30 cm long. Flowers measure 4 - 6 cm across and have filamentous segments that are crystalline white, with a longitudinal red stripe along the underside (Fig. 2. 3a). The dorsal sepal is held erect and incurved over the column while petals and lateral sepals are stiffly spread out, sometimes recurved. Sepals terminate in red to brown (sometimes yellow) cylindrical clubs. The labellum is ovate with white-tipped, reddish teeth along the margin, and four rows of basally clubbed calli, which are red with white tips. Morphological variants with no red colouring are sometime encountered. The column is about 10 mm long with two sessile yellow glands at the base. The leaf of *C. rigida* is lanceolate and varies in length from 3 – 20 cm.

Caladenia rigida is endemic to the Mount Loft Ranges of South Australia. It occurs in three disjunct areas and currently comprises 24 known sub-populations (Quarmby 2006). Historical records indicate that the species' extent of occurrence has declined from over 1150 km^2 to less than 460 km^2, with at least 18 subpopulations becoming extinct over the last century (Quarmby 2006). This loss has been primarily attributed to habitat destruction and fragmentation. Consequently, the species is listed as Endangered in South Australia (National Parks and Wildlife Act 1972) and Endangered in Australia (Environment Protection and Biodiversity Conservation Act 1999).

Fig. 2. 3 (*a*) *Caladenia rigida.* (*b*) *Caladenia tentaculata*

Caladenia tentaculata

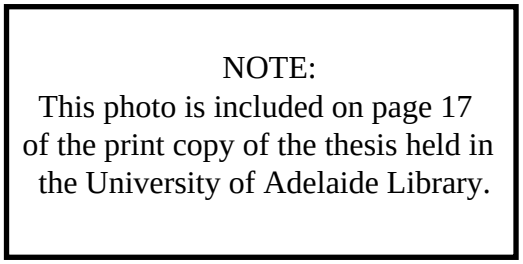
Caladenia tentaculata Schldl. (subgenus *Calonema*), or the king spider orchid, is regarded as widespread and common in higher rainfall parts of South Australia, as well as in Victoria and parts of New South Wales (Bates 2006; Jones 2006). Typically, a single flower is produced, however robust individuals sometimes produce two or three. Flowers are large, 6 - 10 cm across, on hairy stems 15 – 60 cm tall. The filamentous floral segments are green with a red central stripe and sepals terminate in thickish yellow to brown slender clubs (Fig. 2. 3b). The labellum is white with a maroon apex, long green marginal teeth and four rows of densely crowded, maroon calli up to 1.7 mm long. The dorsal sepal is incurved, lateral sepals are deflexed at the base often with upswept tips, and petals are swept downwards. The labellum is very loosely hinged and highly mobile. The species has a large, hairy leaf, 8 - 15 cm x 1 - 2 cm.

Fig. 2. 4 *Caladenia carnea*

Caladenia carnea

Caladenia carnea R.Br. (subgenus *Caladenia*) is commonly referred to as the pink fairy or pink finger orchid. The species is common in many parts of South Australia and its distribution lies throughout the eastern states of Australia (Bates 2006; Jones 2006). One to four flowers are borne at the end of a 10 - 25 cm-tall stem. Flowers range from white to pink and measure 2 - 3 cm across (Fig. 2. 4). The labellum and column are striped with red transverse bars. Floral segments are ovate lanceolate; the dorsal sepal erect with lateral sepals and petals spread out. The labellum is tri-lobed; side lobes are erect and entire and the midlobe is recurved with short marginal teeth and 2 - 4 rows of yellow, clubbed calli. The leaf is sparsely hairy, thin and linear, up to 10 cm x 0.4 cm.

CHAPTER 3



Native bee on Caladenia rigida (Photo by Jason Tyndall)

Chapter 3: Preamble

CHAPTER 3 investigates the pollination strategy of *Caladenia rigida*. Potential and confirmed pollinators were captured through direct observation and trapping. The nectar and scent status of flowers is also examined.

In addition, pollinators carrying pollinia from *C. carnea* were identified, and these findings are presented in APPENDIX A.

This chapter has been published in the *Australian Journal of Botany* (2009), with myself as principle author and Lachlan Farrington, José M. Facelli and Andrew D. Austin as co-authors. Permission to reproduce this manuscript herein has been granted by the publisher (APPENDIX D).

Contributions and signatures of authors:

Renate Faast

Sought and won funding for the capture of insects, collected and analysed nectar samples, stained for osmophores, interpreted all data and prepared manuscript as principle and corresponding author.

Signed.... ..... Date. 29th June 2010

Lachlan Farrington

Developed and implemented techniques for the molecular identification of pollinia and assisted with Malaise trapping and baiting for pollinators.

Signed.. ..... Date. 30:11:2009

José M. Facelli

Sought and won funding, supervised development of research and evaluated manuscript.

Signed..... ..... Date. 15/06/2010

Andrew D. Austin

Sought and won funding, advised on aspects of research and evaluated manuscript.

Signed..... Date. 30th June 2010

BEES AND WHITE SPIDERS: UNRAVELLING THE POLLINATION SYNDROME OF *CALADENIA RIGIDA* (ORCHIDACEAE)

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Abstract

Orchids of the genus *Caladenia* have been shown to utilise two quite different pollination strategies; species-specific sexual deception of thynnine wasps and a more generalist strategy, attracting a larger spectrum of foraging insects. While baiting techniques have enabled the identification of numerous pollinators of sexually deceptive *Caladenia*, insects that pollinate food-advertising species have received little attention. The current study employed a multidisciplinary approach to better evaluate the pollination syndrome of the white spider orchid *Caladenia rigida* R.S.Rogers, a species previously reported to utilise both food and sexual deception. This included the observation and capture of potential pollinators of *C. rigida* through direct observation, pantraps, Malaise traps and pollinator baiting experiments, using molecular techniques to identify orchid pollinia isolated from carrier insects. We describe a suite of generalist insects visiting and bearing pollinia from *C. rigida*. In addition, samples collected from the labellum and column of *C. rigida* contained sugars at levels comparable to those of a known nectar-producing orchid, *Microtis parviflora* R.Br. Potential osmophores in the clubs and calli stained positively with neutral red and whilst this character is often associated with sexual deception, we found no evidence for this secondary pollination syndrome in *C. rigida*. This is the first study to provide a detailed description of the pollinators and pollination syndrome of a non-sexually deceptive species within the genus *Caladenia* and the first report to provide evidence of nectar production by a species within this genus.

3.1 Introduction

The use of two distinct pollination strategies has earned *Caladenia* a reputation as being unique amongst Australia's orchid genera. While the majority of its species utilise sexual deception to lure pollinators, some are reported to attract visitors through the 'promise' of food (Stoutamire 1983; Bates 1984b). The lure of specific kairomones has greatly aided the capture of sexually deceived pollinators particularly with the use of the "baiting" technique first described by Stoutamire (1979). However, the attraction of insects to food-advertising orchids is far less targeted and capture of these visitors is often more serendipitous, perhaps explaining the paucity of information detailing the pollination of these flowers. Several authors have implied that some species employ a dual or intermediate pollination syndrome (Dafni and Bernhardt 1990; Bates 1996; van der Cingel 2001); however, there is little empirical evidence to support this.

Whilst the implications of pollination syndrome on orchid reproductive biology are treated in detail elsewhere ((Phillips *et al.* 2009b), it is worth considering exactly why an understanding of this life history variable is important. Primarily, differences in pollinator behaviour in response to floral density, as well as the abundance and diversity of co-flowering species, may ultimately influence fruit set and gene flow patterns of orchids. Consequently, a sound knowledge of pollination syndrome and pollen vectors provides a fundamental basis for conservation initiatives considering the habitat requirements not only of orchids but also of the organisms with which they interact.

The endangered *Caladenia rigida* is one example cited as using both sexual and food deception. Bates (1984a) reported pollination by native bees, *Exoneura* spp. and suggested mimicry of co-blooming *Burchardia* spp. The inference that the orchid uses a dual pollination syndrome is drawn from observations by Bickerton (1997) who noticed two native bees visiting *C. rigida* and a species of *Phymatothynnus* (Tiphiidae) exhibiting mating behaviour above a bagged flower. The presence of clubbed sepals (presumed to be osmophores) was taken as further evidence for sexual deception. *Caladenia* species that are sexually deceptive are usually dull coloured in hues of green and maroon, whereas food-advertising species are often brightly coloured and scented (Stoutamire 1983; Bates and Weber 1990). Colour-wise, *C. rigida* falls into the latter category, having a glistening white flower with red labellum calli and fringe, but the flowers produce no discernable scent. The sepals bear red to brown glandular clubs and, before the present study, the species was thought to offer no reward (Bates 1984a).

In this study we further characterise the pollination syndrome of *C. rigida* by employing several methods to capture and identify pollinators. Other authors have identified pollinators of *Diuris* species by netting insects bearing pollinia whilst they were foraging on nearby shrubs (Beardsell *et al.* 1986; Dafni and Calder 1987; Indsto *et al.* 2006). However, being an early flowering species, *C. rigida* has few co-blooming plants making this option less likely to succeed. Given our past experience, the rarity of observing a natural pollination event led us to employ insect trapping techniques in conjunction with DNA sequencing to identify pollinia carried by putative pollinating agents. The use of molecular markers to determine the origin of pollinia recovered from insects has been amply demonstrated as a tool for identifying orchid-pollinator relationships (Widmer *et al.* 2000; Indsto *et al.* 2006). Farrington *et al.* (2009) have developed a chloroplast DNA marker system capable of inferring species of origin from the pollinia of several species of *Caladenia*, including *C. rigida* and this has provided a valuable technique for the current study. We also investigated the possibility of nectar and scent production in *C. rigida*, providing a more detailed analysis of the floral biology and hence, pollination syndrome of this species.

3.2 Materials and methods

Caladenia rigida (rigid white spider orchid) is listed as endangered at both the State and Federal levels (Quarmby 2006). It is endemic to the Mount Lofty region, South Australia and although once widespread, it has undergone a substantial decline in its former range. The species occurs on brown to yellow podsolic soils with mottled clays and course quartz-gravel or sandstone pebbles (Bates 1984a), and is restricted to ridge tops and associated slopes. A single leaf emerges in late autumn and buds usually begin to open at the end of August (early spring), when few other species are flowering. Plants usually bear a single flower, growing 10 to 30 cm tall, although some individuals produce two or very rarely, three flowers. Typically, flowers are white with red calli and labellum fringes and earn their name from stiffly held lateral sepals and petals. Sepals (and sometimes petals) terminate in red to brown clubs, although rare morphs in which clubs are coloured yellow or are totally absent can also be found. Plants within populations often have a patchy distribution occurring in loose clusters interspersed with isolated individuals.

Site locations and description

Pollinator studies were carried out during the beginning of the *C. rigida* flowering season from 31 August to 18 September, 2006 and 27 August to 21 September, 2007. We chose this time for observations because monitoring of tagged plants had previously shown that at least 75% of pollinations occur within the first three weeks of flowering. We examined three populations of *C. rigida* located in the Mount Lofty Ranges, South Australia. One population was within Mount Crawford Native Forest Reserve (Mt. Crawford), one near Millbrook Reservoir (Millbrook) and another adjacent to South Para Reservoir (South Para). The conservation status of this species prevents publication of the exact locations of the populations.

The first two populations occur in *Eucalyptus obliqua* L'Her woodland with an understorey dominated by *Acacia pycnantha* Benth., *Pultenaea daphnoides* J.C.Wendl., *Leptospermum myrsinoides* Schltdl., *Hibbertia exutiacies* N.A.Wakef., *Platylobium obtusangulum* Hook and *Lepidosperma* spp. The South Para site is open woodland consisting of patches of *Allocasuarina verticillata* (Lam.) L.A.S.Johnson and *Eucalyptus fasciculosa* F.Muelll. with an understorey dominated by *Acacia paradoxa* D.C., *Hibbertia* spp., *Gonocarpus elatus* (A.Cunn. ex Fenzl) Orchard and *Lepidosperma* spp. The furthest distance between these sites was 13.8 km. Soils at the sites range from fine sandy loam to fine sandy clay with a pH of 6.0 - 6.5 (DEHSA database). According to the Bureau of Meteorology, South Australia, the average annual rainfall ranges from 684 mm (1968 - 2007) at the South Para weather station (2.9 km from the South Para site) to 860 mm (1914 - 2007) recorded at the Millbrook weather station (2.8 km and 6.6 km from the Millbrook and Mt Crawford sites, respectively). Pollination success of *C. rigida* is highly variable among populations and years (R. Faast, J. M. Facelli, A. D. Austin, unpubl. data; CHAPTER 5) and ranged at these study sites from 13 to 23% (n = 22 - 65) in 2006 and from 26 to 81% (n = 51 - 96) in 2007.

Direct observation of floral visitors

Observations of insects visiting *C. rigida* were made for a total of 24 h over seven days (two days at Mt Crawford, three days at Millbrook and two days at South Para). We recorded the behaviour of all insects entering the vicinity of the flowers, as well as the presence, absence and position of orchid pollinia, the time of day and weather conditions (details obtained from Bureau of Meteorology, South Australia). Where possible the insects were captured with a net and killed in 80% ethanol for later identification. Some

insects were observed and/or captured incidentally during the study period while monitoring plants for other studies. Plant vouchers are lodged with the State Herbarium of South Australia and insect specimens are lodged with the South Australian Museum.

Insect trapping

In 2007, pantraps (yellow or blue plastic dishes filled with water containing a small amount of detergent) were placed among patches of at least 10 orchid flowers on eight days during the study period (two days at Mt. Crawford, three days at Millbrook and three days at South Para). At each site, 20 - 40 traps (five of each colour at 2 - 4 patches) were set up in the morning and left in place until the afternoon (~ 1000 - 1600 hours). Contents of pantraps were sorted to separate out insects bearing orchid pollinia and then were stored in 80% ethanol. In 2006 (21 August to 23 October), we set up one large and one small Malaise trap at Millbrook and at South Para. In case insects laden with pollinia have difficulty flying upwards into large traps, we constructed smaller versions. All linear dimensions were scaled down to one half of the standard design (Townes 1972). The following year, one large and two small Malaise traps were erected at the Millbrook site from the 29 August to 21 September. These were placed in the vicinity of, or directly over, large patches of *C. rigida* (at least 20 flowers) at right angles to a natural flyway, and collecting bottles were emptied once per week.

Pollinator baiting experiments

To attempt capturing sexually-attracted male thynnine wasps, we carried out baiting experiments as described by Peakall (1990) and Bower (1996). These took place over four days at three sites in 2006 and one day at Millbrook in 2007, totalling approximately 5.5 h. Briefly, three *C. rigida* flowers were artificially presented along insect flyways for 5 - 10 min at a distance of at least 20 m from the orchid population. Bait flowers were relocated 10 - 15 times (10 - 20 m apart) at the same site. All experiments were restricted to sunny days above 20^{o}C between 1100 and 1500 hours, to ensure optimal flight activity of male thynnine wasps (Stoutamire 1983).

Neutral red staining

Although *C. rigida* does not produce a perceptible scent, we examined the possibility of odour secretion by staining flowers with neutral red to reveal the presence of putative osmophores. Whole flowers were soaked in a solution of 0.1% neutral red for 20 - 30 min and then rinsed for 18 h in water (Dafni 1992). The calli and clubs of *C. rigida* are

normally coloured red, so we selected three rare morphs where one or both of these structures were white or pale yellow: white calli, red sepal clubs and no clubs on the petals (WR); white calli and yellow clubs on sepals and petals (WY); red calli, yellow sepal clubs and no clubs on petals (RY).

Nectar chromatography

As *C. rigida* does not produce obvious amounts of nectar, the labellum calli or the base of the column was rinsed with 3 μL of Milli Q water and the aliquot stored at -20^{o}C until use. We sampled a total of 15 flowers from three populations. Samples were also collected from the sexually deceptive *Caladenia tentaculata*, as well as the orchids *Microtis parviflora* R.Br. (shown to produce nectar by Peakall and Beattie (1989)) and *Dipodium roseum* D.L.Jones & M.A.Clem. (belonging to a genus containing extra floral nectaries (Bates 1985)). To prevent removal of nectar by insects prior to collection, mesh bags were placed over 11 of the *C. rigida* buds and *C. tentaculata* buds a few days prior to anthesis. We included samples from *E. obliqua* and *Drosera whittakeri* Planch. as controls. *Eucalyptus* species are known to produce nectar (Davis 1997) and although the specific status of *D. whittakeri* is unknown, other species within this genus have been shown to lack nectaries (Murza and Davis 2003). Thin layer chromatography was performed by spotting nectar samples and sugar standards (1% sucrose, 1% glucose and 1% fructose) onto Whatmann's No. 1 chromatography paper and separating sugars overnight in a solution of n-butanol: acetic acid: water (12: 3: 5) as described by Turner and Conran (2004). Dried chromatographs were developed in an indicator solution of aniline (1%), diphenylamine (1%) and phosphoric acid (4%) and baked at 100^{o}C for 5 min. Relative intensity of sugars based on subjective assessments of the size and colour of sugar spots were coded according to the classification system outlined by Percival (1961) where: S, G and F represent sucrose, glucose or fructose, respectively; uppercase indicates an abundance of the sugar, lower case indicates a trace of the sugar, and bold letters denote a preponderance of the sugar.

3.3 Results

Direct observation of floral visitors

A total of 25 insects (seven native bees at Mt Crawford and Millbrook, 14 syrphid flies at all three sites, and three honeybees (*Apis mellifera* L.) and one calliphorid fly at South Para) were observed either showing interest in or alighting on *C. rigida* flowers (Table

3.1). Most observations of floral visitation (particularly of native bees) were made between 1030 and 1500 hours on warm (20 - 25^{o}C), sunny days with a moderate to strong northerly wind (15 - 40 km h^{-1}). Syrphid flies and honeybees were also observed in cooler and calmer conditions.

All of the native bees landed either directly on the labellum or on a petal, and then crawled onto the labellum before progressing towards the base of the column. The native bees were identified as *Lasioglossum* (*Chilalictus*) *clelandi* Cockerell (Halictinae, 4 specimens), *Exoneura* sp. (nr *bicolor*) (Apidae, 2 specimens) and *Homalictus (Homalictus) punctatus* Smith (Halictinae, 1 specimen) and all were female. One *L. clelandi* carried a pollinium attached to its thorax (Fig. 3.1*a*) and although becoming momentarily stuck, struggling for several seconds to exit the labellum, no pollinia deposition was witnessed. DNA extracted from the pollinia corresponded to the predominant *C. rigida* chloroplast DNA haplotype (Farrington *et al.* 2009). Pollinia were found on the stigma of a flower following the visit by *H. punctatus*; however, since the prior status of the flower is unknown we cannot be certain that this insect effected pollination. One of the *Exoneura* sp. (nr *bicolor*) was seen exiting one *C. rigida* flower and then entering another.

Four syrphid flies approached and inspected *C. rigida* flowers but avoided landing. Two of these had pollinia attached to their heads. Nine syrphids alighted (eight on the labellum, one on a petal). One appeared to be inspecting the top of the column around the pollinia and three were observed to probe the calli with their proboscis, progressing in a side-to-side fashion towards the base of the column. One of these flies became temporarily stuck but did not remove pollinia upon breaking free. Another carried a pollinium attached dorsally to its thorax (Fig. 3.1*f*) but was not captured, and we found one fly lodged upside down inside a flower. All of the syrphid flies were *Simosyrphus grandicornis* Macquart, a widespread species, indigenous to Australia.

Two honeybees landed on the labellum of *C. rigida* flowers and crawled towards the base of the column. One of these had fragments of pollinia from *C. rigida* (as shown by DNA sequencing) on its head (Fig. 3.1*d*) and we found pollinia on the stigma after capturing the insect; however, the pollination status of the flower prior to the visit is unknown. Another honeybee approached a *C. rigida* flower but avoided landing. A calliphorid fly alighted on a *C. rigida* flower, probing the calli with its proboscis as it worked its way towards the base of the column. Several other flies belonging to the families Muscidae, Calliphoridae

and Tabinidae were found lodged dead inside *C. rigida* flowers; however, there was no evidence of pollinia deposition or removal (Table 3.1).

Table 3. 1 Number of insects observed on *Caladenia rigida* or caught in pantraps.
Letters in parentheses indicate site (MC, Mt Crawford; MB, Millbrook; SP, South Para); the number of insects in yellow (Y) and blue (B) pantraps are indicated

Insect taxon	No of insects observed on the flower (with pollinia)	No. of insects observed on the flower (no pollinia)	No. of insects inspecting the flower (no landing)	No. of insects captured in pantraps (with pollinia)
Hymenoptera				
Lasioglossum clelandi	1 (MB)	2 (MC), 1 (MB)		3 (MC) - 1Y,2B
Exoneura sp. (nr *bicolor*)		1 (MC), 1 (MB)		
Homalictus punctatus		1 (MC)		
Lipotriches flavoviridus				1 (SP) - 1Y
Apis mellifera	1 (SP)	1 (SP)	1 (SP)	
Diptera				
Simosyrphus grandicornis	1 (SP)	2 (MC), 6 (MB), 1 (SP)[B]	4 (MB)[A]	1 (MC) - 1Y
Conopidae				1 (SP) - 1Y
Calliphoridae		2 (MB)[B], 1 (SP)		
Muscidae		1 (MC)[B], 3 (MB)[B], 3 (SP)[B]		
Tabanoidea		1 (MC)[B]		

[A]Two syrphid flies carried pollinia; [B] Insects found trapped inside the flower.

Insect trapping and identification of pollinia

Identification of potential pollinators of *C. rigida* relied upon capturing insects bearing orchid pollinia. Four native bees (all females) carrying pollinia were caught in pantraps (two in yellow and two in blue traps). Three of these were *L. clelandi* with pollinia attached dorsally to their thorax (Fig. 3.1*b* & 3.1*c*) and DNA sequencing matched these pollinia to the predominant *C. rigida* chloroplast DNA haplotype (see Farrington *et al.* 2009). The fourth bee, *Lipotriches (Austronomia) flavoviridus* Cockerell, had pollinia attached to the dorsal surface of its head. These pollinia were morphologically distinct to those of *C. rigida* and DNA sequencing confirmed their origin from a co-flowering orchid genus, *Diuris*. A loose *C. rigida* pollinium, presumably dislodged from an insect, was found in one yellow trap containing five native bees (three *L. clelandi* and two unidentified *Lasioglossum* spp., all female) and a single species of chalcidoid wasp. Microscopic examination confirmed that none of the remaining native bees caught in pantraps carried

remnant pollinia (42 specimens from Mount Crawford, 13 from Millbrook, seven from South Para). The majority of native bees (49 out of 62 specimens) were captured in yellow pantraps. We also trapped one syrphid fly, *S. grandicornis,* and a dipteran, Conopidae sp. (Fig. 3.1*e*), both with *C. rigida* pollinia attached to the head. We found no fragments of pollinia on the remaining syrphids, all of which were captured in yellow pantraps (one at Millbrook and four at South Para).

The contents of Malaise traps were inspected for insects bearing orchid pollinia; however, over the entire trapping period only one specimen, Cecidomyiidae, was found with pollinia from a small, unidentified species of orchid (probably a co-flowering *Pterostylis* sp.). Very few insects were captured in the small Malaise traps.

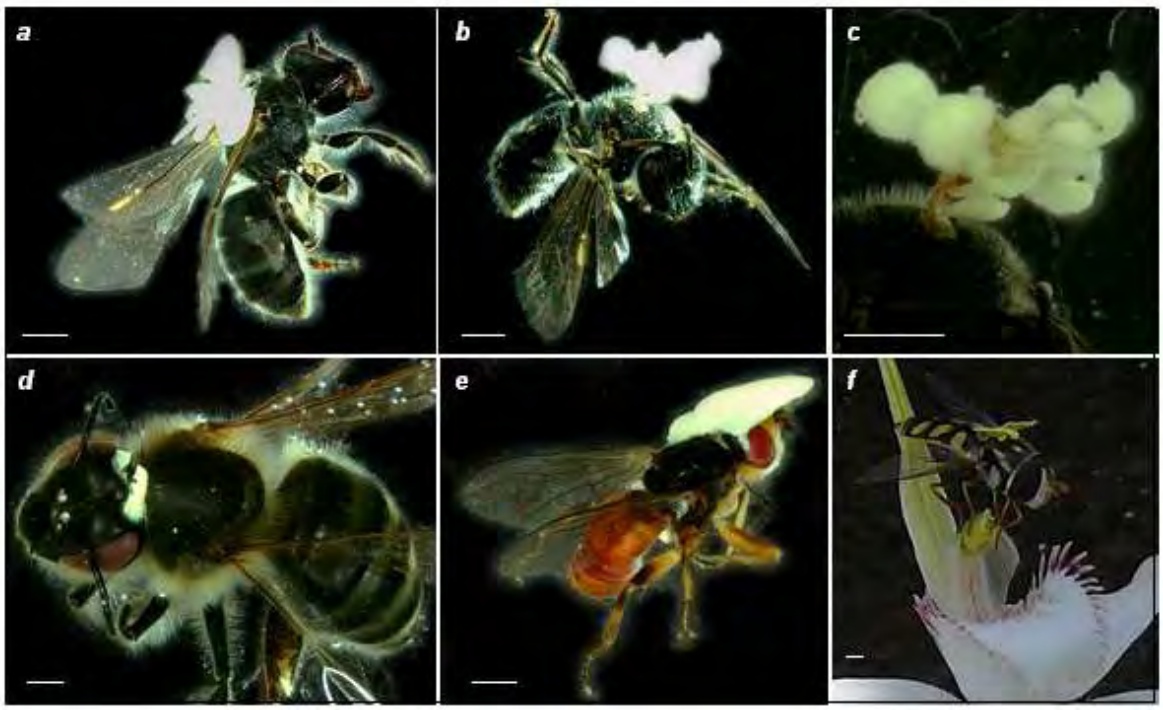

Fig. 3. 1 Potential pollinators of *Caladenia rigida*. Insects captured carrying *C. rigida* pollinia, (*a*, *d*) by direct observation or (*b*, *e*) in pantraps or (*f*) observed on *C. rigida* flower but not captured. (*a*, *b*) *Lasioglossum clelandi*. (*c*) Close-up showing multiple sets of pollinia from (*b*). (*d*) *Apis mellifera* with fragments of pollinium. (*e*) Conopidae sp. (*f*) *Syrphus* sp. Scale bar = 1 mm.

Pollinator baiting experiments

Sexually deceptive orchids usually attract wasps within minutes of presentation (Bower 1996; Peakall and Beattie 1996); however, we were unable to attract any insects to *C. rigida* bait flowers. In 2007, the trials were carried out on a day with high insect activity, including thynnine wasps (R. Leijs, pers. comm.) but we saw no response to bait flowers placed along the paths of patrolling males.

Neutral red staining

The calli tips and sepal clubs of *C. rigida* were found to absorb neutral red (Fig. 3.2), suggesting likely scent production in these areas (Stern *et al.* 1986). Clubs on the petals of the yellow-clubbed form (WY) also stained red (Fig. 3.2*d*). In morphs lacking clubs on their petals (WR & RY), there was no staining of the petal tips (Fig. 3.2*f*). Pollen grains commonly absorb neutral red due to their porous nature and the production of volatile oils (Dafni 1992), thus the deep red staining of pollinia seen in all three *C. rigida* flowers was not surprising.

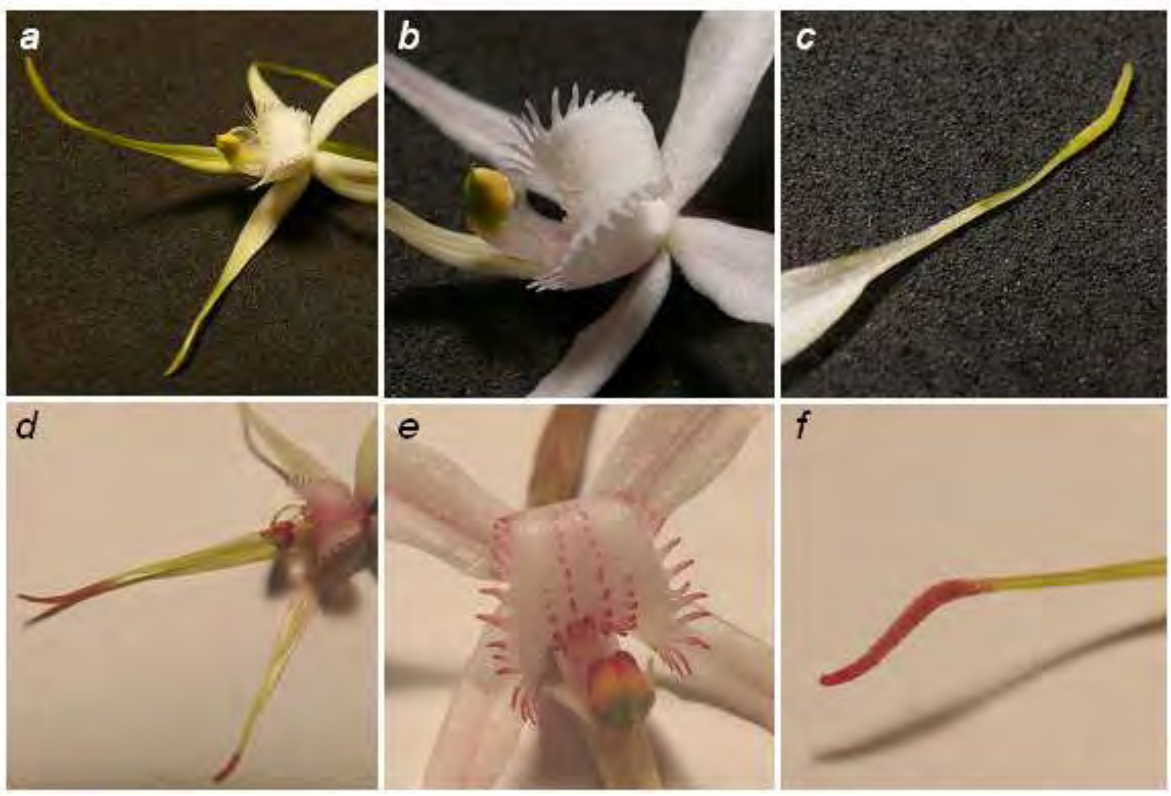

Fig. 3. 2 Neutral-red staining of pale morphs of *Caladenia rigida*. (*a*, *d*) WY, (*b*, *e*) WR and (*c*, *f*) RY (see Materials and methods for abbreviations). (*a–c*) Unstained and (*d–f*) stained flowers. (*d*, *e*) Calli and (*d*, *f*) clubs stain positively.

Nectar chromatography

As described above, insect visitors to *C. rigida* were sometimes observed probing the labellar calli and base of the column with their proboscis, as if searching for nectar. To determine whether *C. rigida* offers any reward to visiting insects, 18 samples collected from the calli and/or column base, were subjected to thin layer chromatography (Table 3.2, Fig. 3.3). Sugar was detected in 14 samples, with sucrose predominant in 12 of these (S or s). Six flowers produced glucose (Sg, **S**fG, G or g) and one sample contained fructose (**S**fG). Sugars were present in samples from both the labellar calli and the base of the column (Fig. 3.3) at levels comparable to those of *M. parviflora* (G, fG) and *D. roseum* (SFG, Sfg). Three *C. rigida* flowers had no detectable nectar. None of the four samples collected from *C. tentaculata* or from *D. whittakeri* contained sugars whereas nectar from *E. obliqua* was sucrose rich (**S**FG).

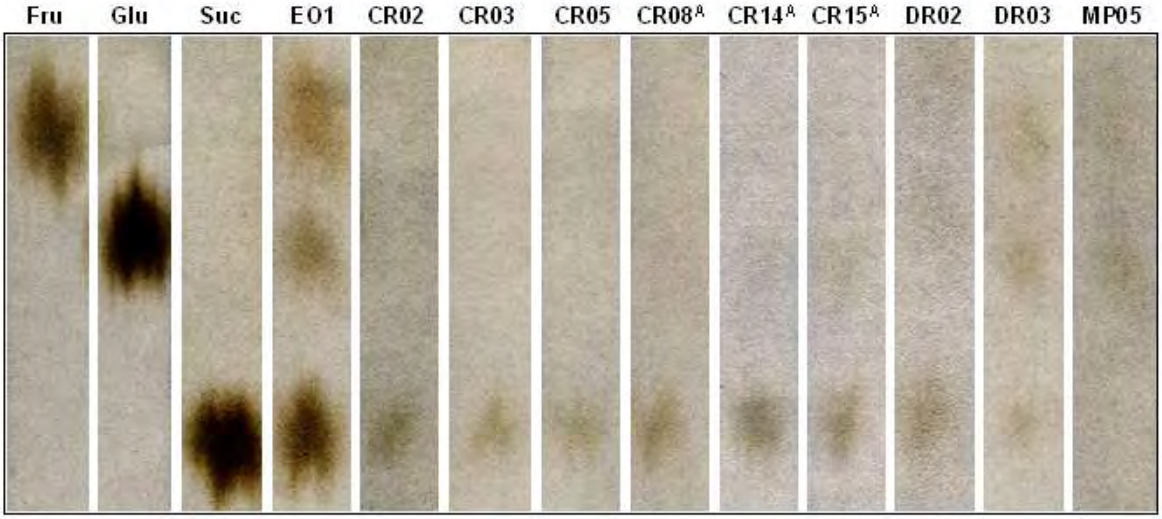

Fig. 3. 3 Thin-layer chromatography of nectar samples. Fruc, 1% Fructose; Gluc, 1% Glucose; Suc, 1% Sucrose; EO, *E. obliqua*; CR, *C. rigida*; DR, *D. roseum*; and MP, *M. parviflora*. [A]samples collected from the labellar calli; all other samples were collected from the base of the column.

Table 3. 2 Thin layer chromatography of nectar samples collected from *Caladenia rigida.* MC, Mt Crawford; MB, Millbrook; SP, South Para; O, Other. Samples with the same ID were collected from the same flower. Nectar codes according to Percival (1961): S, sucrose; F, fructose; G, glucose; uppercase, easily detectable levels; lower case, trace amounts; bold, a preponderance of the sugar; -, no detectable sugars.

Species	Site	Flower ID	Nectar code
Caladenia rigida	MB	CR01[C]	s
		CR02[C]	S
		CR03[C]	S
		CR04[C]	s
		CR05[C]	S
	MC	CR06	-
		CR06[A]	-
		CR07	G
		CR07[A]	g
	SP	CR08[C]	s
		CR08[AC]	S
		CR09[C]	s
		CR10[C]	Sg
		CR11[C]	**S**fG
		CR12[C]	-
		CR13[C]	-
		CR14[A]	Sg
		CR15[A]	Sg
C. tentaculata	SP	CT01[BC]	-
		CT02[BC]	-
		CT03[BC]	-
		CT04[BC]	-
Microtis parviflora	MB	MP01	-
		MP02	-
		MP03	G
		MP04	g
		MP05	fG
Dipodium roseum	O	DR01	-
		DR02	Sfg
		DR03	SFG
Eucalyptus obliqua	O	EO1	**S**FG
		EO2	SFG
Drosera whittakeri	SP	DW01	-
		DW02	-
		DW03	-

[A] Samples were collected from the labellar calli; all other samples were collected from the base of the column. [B]For *C. tentaculata,* both the calli and the base of the column were sampled and pooled.
[C]Flowers bagged as buds.

3.4 Discussion

The evidence provided here demonstrates that *C. rigida* utilises a generalist food-advertising pollination syndrome, which attracts a diverse range of hymenopterans and dipterans. Pollination by sexual deceit does not appear to play an important role in the reproductive success of this species. Furthermore, it is clear that *C. rigida* offers at least some reward in the form of sucrose-rich sugars and to our knowledge this is the first documented evidence of nectar production in a member of the orchid genus *Caladenia*.

Pollinators

Caladenia rigida flowers were visited by at least nine species of insects from six families. Although we did not directly observe transfer of pollinia between *C. rigida* flowers, we captured insects bearing pollinia from this orchid species. According to the criteria proposed by Adams and Lawson (1993), only insects directly observed to remove pollinia from the anther of one plant and deposit it onto the stigma of a conspecific flower can be classed as "confirmed" pollinators. Observation of only one of these events leads to a classification of "probable" pollinator. Given the rarity of witnessing the complete pollination process, Weston *et al*. (2005) argue that the above methodology does not allow for scientific inference. We also infer that insects, having visited and correctly positioned themselves for the uptake of pollinia from one flower, are capable of repeating this process for subsequent deposition. The capture of insects bearing several sets of pollinia (see Fig. 3.1*c*) is proof that multiple floral visits do occur and provides further support for their classification as pollinators of this orchid.

The most frequent visitors and pollinators of *C. rigida* were native bees and syrphid flies. The only native bees transporting pollinia were *L. clelandi*; however, the size and behaviour of the two other species seen entering flowers (*Exoneura* sp. (nr *bicolor*) and *H. punctatus)* was very similar to that of *L. clelandi*, rendering them possible candidates as pollinators. Although we made no attempts to assess the abundance and diversity of these insect species, the larger number of *L. clelandi* observed or trapped may reflect their higher local abundance rather than a specific attraction to *C. rigida*. Emergence patterns and foraging behaviour of native bees could explain why all of the bees captured (including those in pantraps with no pollinia) were females. Fertile female halictine bees emerge early in spring and provision their nests with nectar and pollen (Rayment 1935; O'Toole and Raw 1991). In many cases, males are produced only in the last brood in late summer

and early autumn and would account for the lack of male bees captured in our study. While some studies report orchid pollination by native bees in warm but calm weather (Beardsell *et al.* 1986; Bernhardt and Burns-Baloghm 1986b), the majority of our observations and trappings of bees were made on days with a moderate to strong northerly wind. Bates (1982; 1984a) reported similar conditions whilst observing bee pollination of *C. rigida* and *C. congesta*. The lack of a strong northerly wind on the days we visited South Para could explain the relatively few observations of native bees at this site.

Syrphid flies have been reported to hinder orchid reproduction either by becoming trapped inside flowers (Bates 1984a) or by effecting self-pollination (Dickson and Petit 2006), which can result in a reduction of seed viability (Tremblay *et al.* 2005). We saw no self-pollination events and observed only one imprisonment, but we did find evidence of pollinia removal by several of these flies and infer that they are also capable of transferring pollinia since they were found outside of flowers. Myophilous pollination syndromes have been described for numerous orchid subfamilies (Christensen 1994) and at least two species of *Epipactis* in the northern hemisphere are predominately pollinated by syrphid flies (Ivri and Dafni 1977; Sugiura 1996). Within Australia, hover flies have been reported as the primary pollinators of the nectar-producing orchid *Prasophyllum odoratum* (Bernhardt and Burns-Baloghm 1986b), and secondary pollinators of a food mimicking species, *Thelymitra antennifera* (Dafni and Calder 1987)). Given the widespread distribution of this indigenous species, their importance as significant pollinating agents should not be underestimated. Syrphidae are active over a wider range of weather conditions (R. Faast, pers. obs.; see also Bernhardt and Burns-Balogh 1986b), thus providing opportunities for pollination when native bees are idle. The other dipteran, Conopidae, found bearing a pollinium may also be an effective pollinator of *C. rigida*; however, the lack of direct observations and the capture of only one specimen suggest that this is a less frequent event. Several other flies, belonging to the Muscidae and Calliphoridae were found lodged inside flowers with no evidence of pollinia removal or deposition, making them unlikely pollinators. It is possible that the broader body shape of these insects, compared to that of the above pollinators, prevents them from making contact with the orchid's reproductive structures and results in a tighter fit when pushing down towards the base of the labellum, ultimately leading to their entrapment.

The type of insect responsible for effecting pollination may have important consequences for the reproductive success of *C. rigida*. The positioning of *C. rigida* pollinia on insects

differed between species, with deposition on native bees always on the dorsal surface of the thorax, whilst dipterans carried pollinia on their head or thorax. Differential but consistent placement of pollinia can prevent interspecific hybridisation between co-blooming species (Bernhardt and Burns-Baloghm 1986a). In the case of *C. rigida*, less specific deposition of pollinia on dipterans and the introduced honeybee could therefore lead to higher incidences of interbreeding, a proposal requiring further exploration. The observation and capture of *A. mellifera* bearing *C. rigida* pollinia remnants was surprising given their much larger body size and further studies are required to determine whether they make a significant contribution to this orchid's pollination. There are few reports of terrestrial orchid pollination by honeybees in Australia and some authors have expressed concerns about displacement of native bees by this introduced species and the associated consequences for orchid reproduction (Stoutamire 1983; Adams and Lawson 1993). Furthermore, differences in foraging ranges and dispersal distances between native bees and honeybees can affect gene flow patterns within and between plant populations (Rymer *et al.* 2005). More comprehensive studies investigating both the abundance and diversity of insects across a number of sites, and the pollination efficiency of different taxa, are required to determine their relative importance for orchid reproductive success.

Pantraps proved to be a novel and effective technique for capturing *C. rigida* pollinators, providing a simple and more time-efficient alternative to direct observation. Trials with other coloured dishes and coordinating trapping with favourable weather conditions should help to optimise the method further. This technique relies on the accurate identification of pollinia recovered from insects and we have successfully achieved this with molecular methods. Furthermore, the nature of insect collection requires that the ensuing DNA technique is robust enough to accommodate the rigours of less than ideal storage conditions, and chloroplast markers as opposed to AFLP's, are particularly suitable in this application (Farrington *et al.* 2009).

Nectar and scent production

We detected sugars in samples collected from the labellum and column base of *C. rigida*. The majority of flowers produced sucrose-rich nectar; however, the amount and ratios of sugars varied. Many factors can account for such intraspecific variation including climatic conditions, as well as temporal, morphological and phenological variation (Dafni 1992). Although our chromatography results are not quantitative, levels of sugars in some samples appeared, on the basis of visual inspection, to be similar to those detected in *M. parviflora*,

a species shown to produce hexose-rich nectar (Peakall and Beattie 1989). The bee pollinated *D. roseum* produces nectar through extra-floral nectaries located at the base of floral bracts; however, the flower itself is reported to be nectarless (Bates 1985). We included this species as a negative control for our nectar chromatography but detected sucrose, glucose and fructose at the base of the column, again at levels comparable to those of *M. parviflora*, which might suggest that this flower also provides some reward. As expected, *C. tentaculata* produced no detectable nectar, confirming its status as a truly deceptive species. Detailed examination of *C. rigida* is required to elucidate the source of sugars and their concentrations.

The detection of sugars was surprising given the general acceptance that *Caladenia* are deceptive and nectarless (Dafni and Bernhardt 1990; van der Cingel 2001; Bates 2006). The only published reference of a *Caladenia* species producing nectar (*C. nana* in Hopper and Brown 2001) provides no details. Classification of nectar status based solely on morphological criteria such as the presence of nectar or nectaries may fail to detect small amounts of concentrated or crystallised sugars. Desiccation of exposed nectar can lead to the formation of crystals that remain available to insects able to reconstitute them with saliva (Kevan and Baker 1983). Recent research examining *Maxillaria* spp. (a genus previously assumed to be rewardless), revealed nectar secretion at the ventral surface of the column (Stpiczynska *et al.* 2003) or on the labellar surface via modified stomata (Davies *et al.* 2005), in both cases leading to its accumulation at the base of the column. Our results lend support to an earlier suggestion by Stoutamire (1983) that the labellar calli of food-advertising *Caladenia* produce surface secretions. A recent report by Indsto *et al.* (2007) detected nectar in *Diuris alba*, which also belongs to a genus generally regarded as lacking rewards. The use of techniques, such as chromatography, capable of detecting small amounts of sugars may reveal nectar secretion by many more species currently considered as food deceptive.

Few Australian taxa have been reported to produce food rewards. Species within four temperate genera, *Acianthus, Microtis, Prasophyllum* and *Spiranthes* have functional nectaries and in all but one case nectar accumulates at the base of the labellum (Dafni and Bernhardt 1990). At present we do not have direct evidence to show that the amount of nectar produced by *C. rigida* attracts pollinators or sustains their interest. However, several insects in this study seemed to respond in a manner consistent with the distribution of sugars documented. Furthermore, surveys of the abundance of co-flowering species at

the peak flowering time of *C. rigida* have revealed few alternative sources of nectar suggesting little competition for pollinators (R. Faast, J. M. Facelli, A. D. Austin, unpubl. data; APPENDIX B). To what extent the presence of a reward influences the pollination biology of *C. rigida* and other species within this genus, deserves further investigation. Given this uncertainty, we suggest the use of the term "food-advertising" to describe pollination strategies that advertise the presence of a reward but may or may not produce one. This term therefore encompasses both food rewarding and food deceptive strategies.

Although orchids are generally pollination limited rather than resource limited (Tremblay *et al.* 2005), the production of concentrated sugars as opposed to obvious amounts of liquid may be a strategy for *C. rigida* to conserve water and/or carbohydrate resources, while maximising its pollination success. The energetic costs associated with nectar production have been proposed as one of the drivers for the evolution of deception and costs for seed production have been demonstrated (Pyke 1991; Ordano and Ornelas 2005). Further, following on from a proposal by Irwin *et al.* (2004) that production of dilute nectar deters nectar robbers, it is conceivable that small amounts of sugars, possibly requiring reconstitution, could provide *C. rigida* with a similar advantage.

Caladenia rigida produces no discernible scent, yet we identified two regions staining positively for neutral red. However, being a presumptive stain only, which also stains nectaries (Dafni 1992), interpretation of these results should remain tentative. Trichomes located at the apex of sepals or on the labellum have been associated with scent emission in food advertising and sexually deceptive species (Stoutamire 1983; Davies and Turner 2004; Ascensao *et al.* 2005). Stoutamire (1983) links the presence of clubs with sexual deception, based on the absence of these structures in food-advertising species such as *C. patersonii* (now *C. longicauda*). Whether the clubs in *C. rigida* do indeed release wasp-luring kairomones, or whether they emit odours to attract food-seeking insects, requires further examination with more sensitive techniques such as gas chromatography of flower volatiles.

Implications of generalist and rewarding pollination syndromes

Generalist plants utilising a broad range of pollinating vectors are expected to be less sensitive to changes in pollinator abundance or behaviour than those relying on a single pollinator (Bond 1994). This implies that *C. rigida* should be better buffered against pollination failure than sexually deceptive species and suggests that other factors such as

herbivory, genetic structure or resource availability account for its current conservation status. These are the subjects of ongoing investigations.

The production of nectar by *C. rigida* challenges previous notions that the species is purely food deceptive (Bates 1984a; Bickerton 1997). The presence or absence of floral rewards has several implications for pollination ecology, particularly with respect to pollinator behaviour. Whereas pollinator learning can lead to a reduction in reproductive success of deceptive species at high density (Peakall 1990; Ferdy *et al.* 1999; Wong and Schiestl 2002), rewarding flowers can benefit from repeat visits and greater attraction of pollinators (Sih and Baltus 1987; Makino and Sakai 2007). This may explain the higher fruit set found for nectariferous orchids compared to nectarless species (Neiland and Wilcock 1998; Tremblay *et al.* 2005). Deceived pollinators are also more likely to leave the patch thus promoting outcrossing whilst nectar production can encourage self or biparental pollination (Dressler 1990; Nilsson 1992; Jersáková and Johnson 2006), although other studies have found no evidence for increased geitonogamy or inbreeding depression in nectariferous orchids (Smithson and Gigord 2001; Smithson 2006). Outcrossing is also influenced by differences in the types of pollinators utilised by generalists versus sexually deceptive orchids. Mark and recapture trials demonstrated considerably shorter flight distances for native bees feeding on the nectar of *Prasophyllum fimbria* (Peakall 1989), compared to sexually deceived wasp pollinators of *C. tentaculata* or *Drakea glyptodon* (Peakall 1990; Peakall and Beattie 1996). Furthermore, these differences were reflected in the pollen flow distances for the two pollination strategies and therefore have implications for the fine-scale genetic structure of orchid populations.

Evidence for a dual pollination syndrome?

Our results do not support the existence of a dual pollination syndrome in *C. rigida*. Considering the reasonably high rates of pollination in 2007, we expected to attract at least some wasps if sexual deception plays an important role in this orchid's pollination strategy. Evidence for such a dual strategy is based on a single observation of a thynnine wasp displaying mate-searching behaviour above a bagged flower (Bickerton 1997). The orchid may attract a rare wasp that is in low abundance or no longer present at the sites we studied. The wasp captured by Bickerton (1997) was also located at the Millbrook site and extensive baiting experiments carried out at around the same time failed to attract wasps to *C. rigida* (Bates 1996). In our study, several species of thynnine wasps were captured in Malaise traps; however, none carried orchid pollinia (data not shown). It is possible that

thynnine wasps seek nectar from *C. rigida*, as described for some rewarding species (Bates 1984c), but in this case mate-searching behaviour would not be expected. Regardless, our data lead us to conclude that *C. rigida* is primarily pollinated by a suite of generalist species seeking food rewards, and that pollination by sexual deceit does not make a significant contribution to the reproductive success of this species.

Conservation implications

Conservation measures for orchids are usually targeted at the threatened species in question; however, long-term management plans must also consider the habitat requirements of their mutualists. The identification of pollinators and an understanding of pollination syndrome form important prerequisites for the implementation of such plans, yet very few food-advertising orchids have been successfully studied. Clearly, *C. rigida* relies upon a habitat supporting an active community of native bees and syrphid flies; however, other studies suggest that this early-flowering orchid may also benefit from a lack of co-blooming nectariferous plants (R. Faast, unpublished data), an important consideration when planning revegetation projects.

The procedures described by us are readily transferable to other species; in particular pantraps offer a simple and cost-effective method for capturing potential pollinators, provided that techniques are available for the subsequent identification of pollinia. Two species of native bees have also been identified as pollinators of *Caladenia carnea* using pantraps placed among flowers of this orchid species (Farrington *et al.* 2009). However, the use of direct observation or pantraps may be less effective for rare or sparsely distributed flowers, or when pollination rates are low. When considering the habitat requirements of pollinators, the above techniques could be extended to include isolation of pollen from captured insects to determine which co-blooming species are also visited.

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CHAPTER 4

The remains of *Caladenia rigida* following florivory
(Photo by author)

Chapter 4: Preamble

CHAPTER 4 examines the impact of antagonistic interactions (herbivory, florivory and capsule predation), on *Caladenia rigida* and *Caladenia tentaculata.* Video surveillance and an exclusion experiment were carried out to help elucidate the types of herbivores responsible. Caging also provided a means of assessing the potential cost of herbivory on the final reproductive output of these orchids.

This chapter has been published in the *Australian Journal of Botany* in 2009, with myself as principle author and José M. Facelli as co-author. Supplementary material made available as an Accessory Publication on the publisher's website, is provided at the end of the chapter. Permission to reproduce this manuscript herein has been granted by the publisher (APPENDIX D).

Video footage showing all animals recorded during the surveillance period is provided on a CD inside the back cover of the thesis.

Contributions and signatures of authors:

Renate Faast

Sought and won funding for video-surveillance and herbivore exclusion experiment, collected and analysed all data and prepared manuscript as principle and corresponding author.

Signed..... ..... Date..29th June 2010

José M. Facelli

Sought and won funding, supervised development of research and evaluated manuscript.

Signed..... ..... Date..15/06/2010.....

GRAZING ORCHIDS: IMPACT OF FLORIVORY ON TWO SPECIES OF *CALADENIA* (ORCHIDACEAE)

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Abstract

Herbivory is considered a major threat in many of Australia's orchid species recovery plans. Kangaroos and rabbits are the most commonly implicated herbivores; however, no studies have attempted to confirm their role. Regular monitoring of several populations of *Caladenia rigida* R.S.Rogers and *C. tentaculata* Schldl. over three years in the Mount Lofty Ranges, South Australia, revealed that up to 94% of flowers and 36% of seed capsules were browsed, whereas leaf herbivory was less prevalent. Furthermore, patterns of herbivory varied markedly among sites and across years. In two seasons, predation of *C. rigida* flowers inside a kangaroo and rabbit-proof exclosure was equal to or higher than outside the exclosure. Florivory within populations was influenced by proximity to the habitat edge, but the direction of this response differed among sites. Various types of mesh cages were erected around plants to elucidate the size and type of herbivores. Plants protected from florivores were almost three-times more likely to release seed than were exposed plants; however, some cage types reduced pollination. Video-surveillance confirmed the role of the white-winged chough, *Corcorax melanorhamphos,* as a florivore. This is the first study to unequivocally identify a herbivore, quantify the intensity and extent of floral herbivory across a range of populations, and assess the potential cost of florivory to the direct reproductive output of orchids.

4.1 Introduction

Herbivory is an important biotic interaction that can have negative consequences for a plant's reproductive success and fitness. Experimental manipulations of several terrestrial orchid species have demonstrated that defoliation can lead to decreased seed production, reduced leaf growth and flowering in subsequent years, as well as increased dormancy (Vallius 2001; Shefferson *et al.* 2005; Pellegrino and Musacchio 2006; Shefferson *et al.* 2006). The impacts of floral herbivory (florivory) however have been less studied, particularly amongst the Orchidaceae. Studies of herbaceous and shrub species show that partial florivory by invertebrates may reduce the attractiveness of flowers, indirectly affecting reproductive success by lowering pollinator visitation rates (Krupnick *et al.* 1999; Sanchez-Lafuente 2007). In contrast, vertebrate florivores often consume the entire inflorescence, directly affecting the plant's reproductive output by the consumption of sexual structures (Cooper and Wookey 2003; Tobler *et al.* 2006). Complete flower removal reduces the number of flowers available for pollination, and under intense grazing pressure the subsequent decrease in total seed production can lead to reductions in population growth rate (Garcia and Ehrlen 2002; Gregg 2004) and depletion of the soil seed bank (Kuijper *et al.* 2006). Many herbaceous plants can tolerate florivory by initiating more flowers (Inouye 1982; Wise *et al.* 2008); however, most terrestrial orchids produce a single, non-regenerating inflorescence making these plants particularly vulnerable to the consequences of florivory.

While florivory can have obvious implications for individual plants, few studies have assessed spatial patterns of florivory at the population or landscape scale. Cooper and Wookey (2003) found large geographical variation in the intensity of floral herbivory both within and among populations, largely due to differences in herbivore densities. Habitat fragmentation can affect herbivore activity because it increases the proportion of edges around patches of remnant vegetation. Although the impacts of edge effects on biotic interactions such as pollination or herbivory are not well documented, Jules and Rathcke (1999) attributed reductions in the recruitment of a perennial herb to higher pollination limitation and seed predation within 100 m of forest edges.

Along with habitat loss and fragmentation, grazing by invertebrates and vertebrates is listed as one of the major threats facing many Australian terrestrial orchids (Todd 2000; Duncan *et al.* 2005; Quarmby 2006). In South Australia alone, herbivory poses a moderate

to high threat to 11 of the species encompassed by the 'Recovery Plan for Twelve Threatened Orchids in the Lofty Block Region' (Quarmby 2006); seven of these species belong to the genus *Caladenia*. Macropods (eg. kangaroos) and the introduced rabbit (*Oryctolagus cuniculus*) are thought to be the predominant herbivores, but other species cited include the hare (*Lepus capensis*), possums, deer, snails, caterpillars and other invertebrates. The evidence provided for the identity of potential herbivores, however, is largely anecdotal, usually based on the presence of animals in the vicinity of orchid populations, or the abundance of scats, diggings or tracks. The only study that reports direct observation of orchid grazing by kangaroos (Petit and Dickson 2005), also found that culling programs that reduced kangaroo numbers had little effect on the level of orchid grazing. Prompted by observations that in some years, *Caladenia behrii* Schldl. flowers within a kangaroo- and deer-proof exclosure were more likely to be browsed than those outside, Petit and Dickson (2005) added birds (white-winged chough, *Corcorax melanorhamphos*) and the sleepy lizard (*Trachydosaurus rugosus*) to the list of potential vertebrate herbivores, although neither of these animals were observed eating orchids. Research carried out in one population of *Caladenia rigida* R.S.Rogers found that almost 35% of flowers were browsed (Bickerton 1997) with kangaroos (*Macropus* spp.) and rabbits being listed as the chief offenders (Bates 1984a; Quarmby 2006). Cages are routinely erected to protect orchids from grazing (J. Quarmby, pers. comm.); however, their effectiveness against herbivores and their potential to impede pollinators has not been investigated.

Specifically, this study aimed to quantify herbivory in two species of terrestrial orchids, *C. rigida* and *Caladenia tentaculata* Schldl., comparing spatial and temporal dynamics of florivory at both the local and landscape scale. Within populations, we examined the intensity of herbivory with respect to distance from the nearest edge. In addition, we attempted to identify orchid florivores using video-surveillance and also compared the exclusion effectiveness of several styles of wire-mesh cages. A final objective was to directly compare seed release in the presence and absence of grazing through herbivore exclusion.

4.2 Methods

Study species

The rigid white spider orchid*, C. rigid*a, is a perennial orchid that is endemic to the Mount Lofty Ranges, South Australia where it is restricted to areas along ridge tops. Although once wide-spread, recent declines in its range have led to it being listed as endangered at both the national and state levels (Quarmby 2006). While land clearance and habitat fragmentation are assumed to be the main causes of its decline, herbivory and lack of recruitment are also regarded as major threats (Quarmby 2006). Its current distribution includes several large populations located in the northern area of its range, and a few small populations in the southern area. *Caladenia tentaculata* (king spider orchid) is a widespread and common species occurring throughout south-eastern Australia, in a range of forest and woodland habitats (Bates 2006).

Caladenia orchids replace their single tuber every year and can either remain dormant (below ground), emerge as a single leaf, or produce a leaf and a flower stem. *Caladenia rigida* has a narrow leaf, 3 - 20 cm long and mature plants usually produce a single white flower, although sometimes two or rarely, three flowers are produced. The flowers measure 4 - 6 cm across and are born at the end of a 10 - 30-cm-tall stem. Flowering usually begins early in spring (towards the end of August) and flowers remain open for up to four weeks, unless pollinated. The species is pollinated by a suite of generalist insects including native bees and syrphid flies (Faast *et al.* 2009). *Caladenia tentaculata* produces a single leaf, 8 - 15 cm long and green and maroon flowers (usually one, but up to three) which are 6 - 10 cm across, ranging in height from 15 - 60 cm. Buds of this species open mid-spring (around the beginning of October) and remain open for four to six weeks. This species is sexually deceptive, being pollinated by the male of a thynnine wasp, *Thynnoides pugionatus* (Bates 1996). Both orchid species tend to be patchily distributed, often occurring in loose clusters interspersed with isolated individuals.

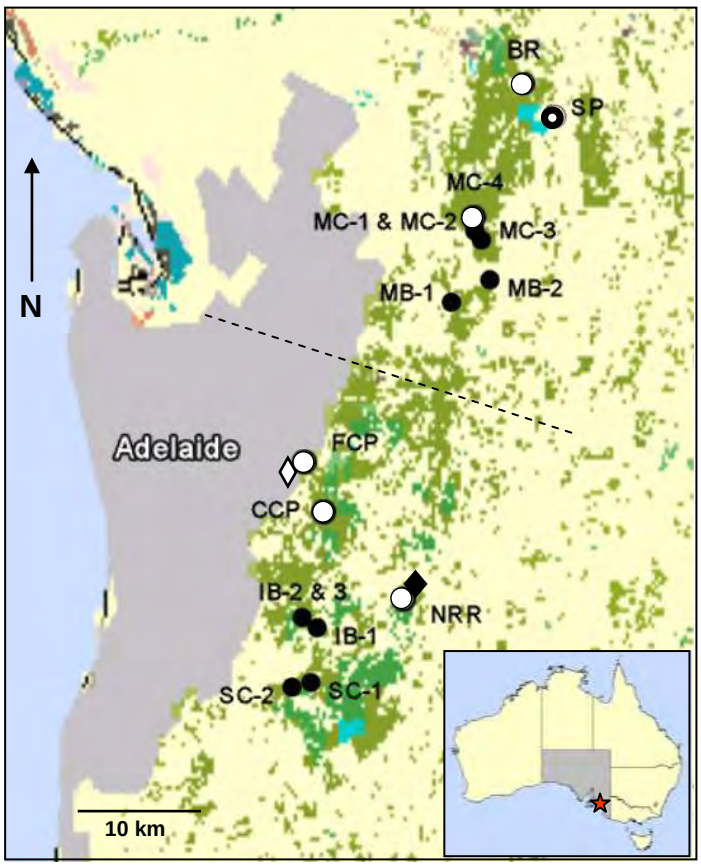

Fig. 4. 1 Map showing location of study sites. (●) *C. rigida* sites, (○) *C. tentaculata* sites; (⊙) site with both species. Light grey shading shows the built-up area of the city of Adelaide, dark green represents areas of extant native vegetation. The dashed line delineates the arbitrary separation between northern and southern sites. (◊) Kensington Upper weather station: average annual rainfall, 622 mm (1897 – 2007); (♦) Bridgewater weather station, average annual rainfall, 1041 mm (1861 – 2007, Bureau of Meteorology, South Australia). Map source: Australian National Resources Atlas.

Site locations

During 2005, 2006 and 2007, we monitored 11 *C. rigida* and six *C. tentaculata* populations (Table 4.1, Fig. 4.1). In 2005 we selected five populations of *C. rigida* located in the northern region of the Mount Lofty Ranges. Two populations were in the Mount Crawford Native Forest Reserve, one (MC-1) being inside a 0.5 Ha, 1.8 m high kangaroo-, deer- and rabbit-proof exclosure, erected in 2001. The second population (MC-2) was located outside the exclosure approximately 50 m to the north. Another two populations were in forested land surrounding Millbrook Reservoir (MB-1 and MB-2) and a fifth population was adjacent to South Para Reservoir (SP). We also selected three populations of *C. tentaculata* in the northern Mt Lofty Ranges at South Para Reservoir (SP), Barossa Reservoir (BR) and Mt Crawford NFR (MC-4). The northern sites are within large areas (several hundred hectares) of native vegetation (Table 4.1) with the exception of the South Para site, which is located in a small fragment of native woodland surrounded by pine

plantations and farmland. Two sites containing *C. tentaculata* were located in more urbanised areas; one in a small isolated reserve (Nation Ridge Road Bushland Reserve, NRR) situated in a residential part of the Mount Lofty Ranges and the other in Ferguson Conservation Park (FCP) in the foothills of suburban Adelaide. In 2006, we selected another six populations of *C. rigida*. One of these (MC-3) was again located within Mt Crawford NFR at a site that had undergone an autumn fuel reduction burn in 2003. The remaining populations were in the southern Mt Lofty Ranges in Scott Creek Conservation Park (SC-1 and SC-2) and on privately owned land at Ironbank (IB-1, IB-1 and IB-3). All of these populations were within large areas of native vegetation (Table 4.1). Annual rainfall in 2006 was well below average across sites (336 - 669 mm, Bureau of Meteorology, South Australia; see Fig. 4.1 for average annual rainfall), coinciding with low rates of emergence and flowering for both *C. rigida* and *C. tentaculata* (data not shown). In 2007, we monitored an additional population of *C. tentaculata,* located in Cleland Conservation Park (CCP). We designated sites as "northern" or "southern" based on their geographical location with respect to the city of Adelaide and spatial separation from each other (Fig. 4.1, Table 4.1). Due to the conservation status of *C. rigida* the exact locations of populations cannot be disclosed.

Site descriptions

Sites MC, MB, IB and SC are characterised by woodlands of *Eucalyptus obliqua* L'Her, *E. leucoxylon* F.Muell. and *E. fasciculosa* F.Muell. with an understorey dominated by *Acacia pycnantha* Benth., *Pultenaea daphnoides* J.C.Wendl., *Leptospermum myrsinoides* Schltdl., *Hibbertia* spp. and *Lepidosperma* spp. The SP site is an open woodland comprised of *E. fasciculosa* and *Allocasuarina verticillata* (Lam.) L.A.S.Johnson with a more sparse shrub layer including *Acacia paradoxa* DC., *Hibbertia* spp. and *Lepidosperma* spp. *Caladenia tentaculata* is found in similar habitats to *C. rigida* (MC-4, SP, NRR) as well as in woodlands consisting of *E. fasciculosa* and *Callitris preissii* Miq. over an understorey dominated by *Calytrix tetragona* Labill., *A. paradoxa* D.C. and *Hibbertia sericea* (R.Br. ex D.C.)Benth. (BR and FCP). At CCP, a canopy of *Eucalyptus baxteri* (Benth.)Maiden & Blakely ex J.M.Black and *E. obliqua* dominates an understorey of *A. pycnantha*, *Arthropodium fimbriatus* R.Br. and *Hibbertia* spp.

Table 4. 1. Location of study sites containing populations of *C. rigida* and *C. tentaculata.* The number of flowering plants at each site is shown as the range observed over the survey period

Site	Location	No. flowering plants (2005 – 2007)	Area of native vegetation (Ha)
C. rigida			
MC-1 [A]	Mt. Crawford NFR (within exclosure)	~ 80 - 200	>450
MC-2 [A]	Mt. Crawford NFR (outside exclosure)	~100 - 200	>450
MB-1 [A]	Millbrook Reservoir	~ 80 - 200	480
MB-2 [A]	Millbrook Reservoir	~ 120 - >500	150
SP [A]	South Para Reservoir	~ 400 - >1500	4.5
MC-3 [A]	Mt. Crawford NFR	~100 - 200	>440
IB-1 [B]	Ironbank – private	~ 50 - 100	~350
IB-2 [B]	Ironbank – private	15 - 23	~350
IB-3 [B]	Ironbank – private	20 - 60	~350
SC-1 [B]	Scott Creek Conservation Park	0 - 19	~200
SC-2 [B]	Scott Creek Conservation Park	0 - 7	~200
C. tentaculata			
SP [A]	South Para Reservoir	~80 - 300	4.5
BR [A]	Barossa Reservoir	~50 - 200	> 230
MC-4 [A]	Mount Crawford NFR	~80 - 300	>450
NRR [B]	Nation Ridge Road Reserve	~120 - 200	0.5
FCP [B]	Ferguson Conservation Park	1 - 50	7
CCP [B]	Cleland Conservation Park	80 - 100	990

[A] Northern sites, [B] Southern sites

Intensity of florivory

At each site, we labelled up to 120 flowering plants with a concealed tag. We monitored plants every one to two weeks throughout the flowering season recording floral, stem and leaf herbivory and capsule predation. As a result of this monitoring regime it was not always possible to determine at exactly what stage the flower was removed (eg. buds may have opened in the intervening time period), therefore our measure of florivory included removal of buds or open flowers. Capsule predation may have been underestimated if capsule formation and subsequent grazing occurred between monitoring time points. We determined the phenology of flowering and florivory by calculating the number of flowers (or browsed flowers) at each visit as a proportion of the total number of flowering plants monitored. Plant vouchers are lodged with the State Herbarium of South Australia.

We assessed floral and leaf herbivory for all plants and categorised these as: no damage; less than half browsed; or at least half browsed. For plants affected by florivory, we

categorised the degree of stem herbivory by estimating how much of the stem had been removed (the base of the ovary was still evident in plants with intact stems). The majority of browsed flowers had the entire perianth removed with only a few incurring partial damage to tepals (1.6% and 2.8% of *C. rigida* and C. *tentaculata* flowers, respectively) apparently caused by invertebrates. We omitted these latter plants from all subsequent analyses of florivory and stem herbivory. In 2007, experimental manipulations for an unrelated study at MB-1 involved caging some of the orchid plants and we left these out of the analyses, along with plants included in the herbivore exclusion experiment at SP.

Distance to edge

To examine spatial patterns of herbivory within populations, we estimated the distance of each flowering plant from the nearest edge (dirt road or fire track) within categories of 0 - 5 m, 5 - 25 m and > 25 m. We did not assess edge effects in 2006, as rates of emergence were very low for both orchid species. In 2007, we only analysed sites MC-1, MC-2 and MB-2, due to small sample sizes or experimental manipulations being carried out at the other sites. For *C. tentaculata*, plants rarely occurred in more than two distance categories at each site, so we excluded this species from analyses of edge effects.

Video surveillance

In an attempt to positively identify herbivores of *C. rigida* and *C. tentaculata* we set up video surveillance equipment in the vicinity of selected orchid patches, in 2007. This consisted of a VHS video event recorder (used in security systems) connected to a domestic passive infrared sensor, a low lux black and white video camera (Ikegami Electronics, U.S.A.) and an infrared light made up of 36 light emitting diodes, all powered by a 12 Volt deep-cycle battery. A wide-aperture lens on the camera allowed for filming in low light conditions. We programmed the video recorder to record for 30 seconds after being triggered by the sensor. We placed the camera and light at a distance approximately 1 - 2 m from the orchid patch, and located the remaining equipment at least 5 m away to minimise noise disturbance. We concealed all equipment with vegetation and camouflage-coloured material, and serviced batteries and videotapes every two to three days.

We carried out video surveillance for a total of 28 and 34 days in populations of *C. rigida* and *C. tentaculata*, respectively. For *C. rigida* the camera was in place from 3 - 7 September inside the MC-1 exclosure and then from 13 September at the SP site. After 14 days at SP, the target flowers remained intact despite extensive florivory of surrounding

plants, prompting us to move the camera to another patch of *C. rigida* (previously protected with cages) and leaving it in place for a further eight days. We relocated the camera to a patch of *C. tentaculata* flowers at SP on 11 October and moved it to non-browsed patches of flowers as necessary (on one occasion flowers were browsed without triggering the camera). By 25 October few intact flowers remained outside of cages, so we placed three vials (concealed) of cut flowers (five flowers in total) in front of the camera, replacing them as necessary until 13 November when filming ceased.

Herbivore exclusion experiment

In 2007, we set up a herbivore exclusion experiment at the SP site, which contains populations of both *C. rigida* and *C. tentaculata* shown to suffer from high rates of florivory in the previous two years. Kangaroos are abundant within this area and emus (*Dromaius novaehollandiae*) and hares have also been sighted (R. Faast, pers. obs.). Other potential herbivores that may occur at the site include deer, rabbits (although no evidence of their presence has been observed), possums, white-winged choughs, sleepy lizards and invertebrates. We placed four types of cages and an uncaged control around randomly chosen orchid plants to selectively exclude different herbivores. We located and caged previously tagged plants in July (*C. rigida*) and August (*C. tentaculata*) when plants bearing buds were easily distinguished but prior to florivore damage.

To minimise the possible effect of surrounding vegetation on orchid florivory, we assessed the amount of vegetation below 50 cm within a 30 cm radius of each orchid plant by assigning a vegetation score (VS), previously shown to be positively correlated with vegetation biomass (data not shown). The maximum VS of 25 represents vegetation present at every 10 cm interval between ground level and 50 cm in height, at each of five points (centre plus 20 cm from orchid plant at the four compass points). In the herbivore exclusion experiment, we included only plants with a VS of less than six, that were therefore relatively exposed.

In total, we erected 60 cages (15 of each type) around each of the two species of orchids, along with 15 uncaged controls. Type 1 cages were 1.2 m tall, 0.9 m wide, open cylinders made of weld mesh with 100 mm squares, designed to exclude large vertebrates such as kangaroos and deer. Holes in the bottom row of these cages were enlarged to 20 cm wide by 10 cm high, to allow easier access to small animals such as rabbits and birds. Type 2 cages were 0.3 m tall, 0.5 m wide, open cylinders made of chicken wire with a 40 mm

hexagonal mesh size, designed to exclude rabbits and possibly hares, but allow access to kangaroos, deer and birds. We demonstrated the effectiveness of these cages with domestic rabbits prior to use. Type 3 cages were made of the same mesh as Type 2 cages, but were 0.6 m tall and 0.5 m wide with the top enclosed to exclude vertebrate herbivores, but allow access to invertebrate florivores and pollinators. Type 4 cages consisted of green plastic-coated bird mesh with 12.5 mm squares made into a 0.9 m x 0.5 m cylinder with an enclosed top, for the exclusion of all vertebrate herbivores. This latter type of cage is routinely used as a management tool to protect threatened orchids from herbivory (J. Quarmby, pers. comm.); however, their potential to hinder the movement of pollinators has never been assessed. Detailed descriptions for the construction of Type 3 and Type 4 cages are provided in Fig. 4S.1. To account for the possibility that florivory may not be even across the site, we treated patches of orchids that were more than 20 m apart, separately. Within patches, we randomly assigned a cage type or control to selected tagged plants, ensuring an equal number of each type. For *C. rigida*, cages were distributed among four patches, each with two to six replicates of every cage type. For *C. tentaculata*, there were two patches with four and 11 replicates of each cage type.

We collected data for all conspecifics encompassed within each cage. We did not include plants within 10 cm of the inside of Type 1 cages, as these may still have been within reach of the herbivores they were designed to exclude. In the case of uncaged control plants, we recorded the status of all conspecifics within 25 cm of the tagged plant. We monitored an additional 85 *C. rigida* and 45 *C. tentaculata* flowers (uncaged) as part of an ongoing study at the SP site. We recorded pollination success (capsule production), seed release (dehiscing capsule) and florivory once a week throughout the flowering season. Although capsule production is not a direct measure of pollination success, it was not practical to assess pollinia deposition for caged flowers. Furthermore, orchids are generally not resource limited (Tremblay *et al.* 2005) and we have found that 97% of *C. rigida* flowers with pollinia deposited go on to produce a capsule (Faast R., Facelli J.M. and Austin A.D. unpubl. data; CHAPTER 5). Capsule predation was only recorded for tagged plants.

Data analysis

We analysed intensity of florivory data (binary response: browsed or not browsed) using logistic regression to test the effect of site and year and their interaction. Only those sites with three years of data were included in tests of florivory among years. Mean rates of florivory were compared between northern and southern populations with Mann Whitney

U tests. We used nominal logistic regression to analyse patterns of stem herbivory across sites separately for each year, excluding sites with less than ten browsed plants. The influence of distance-to-edge and site on the risk of florivory was also examined using logistic models. We employed likelihood ratio tests to assess the significance of factors. Non-significant interaction terms were excluded and the analyses repeated to retain the most parsimonious models (Underwood 1997).

For the herbivore exclusion experiment, we used logistic regression to assess the binary responses of florivory, pollination and seed release as a function of cage type and patch, and their interaction. These analyses were based on the total number of flowering plants within each cage type, rather than individual cages. While this may not be ideal, the patchy distribution of orchids led to large variations in the number of plants inside each cage, preventing meaningful analysis of cage replicates. To determine differences between cage types we performed post-hoc pairwise comparisons (Fisher's exact tests) using sequential Bonferroni corrections (adjusted to $\alpha = 0.05$). Our measure of seed release refers to the final number of dehiscing capsules relative to the total number of flowers present at the commencement of the study (grazed and ungrazed). As only one capsule (on a control plant) was grazed, seed release closely represents the total number of capsules produced. In order to determine whether pollinators are affected by cage type, we assessed capsule formation as a function of the number of ungrazed flowers and refer to this as pollination of available flowers. We used the statistical package JMP 4.0 (SAS Institute, Cary Indiana) for all tests.

4.3 Results

Intensity of florivory

Levels of florivory experienced by *C. rigida* and *C. tentaculata* were highly variable among populations and among years (Fig. 4.2). For *C. rigida*, florivory ranged from zero to 94%; however, a significant site by year interaction ($\chi^2 = 79.6$, d.f. = 8, $P < 0.0001$) indicates that the difference among sites is dependant upon the year. Single factor analyses revealed between-site differences in each year (2005: $\chi^2 = 78.5$, d.f. = 4, $P < 0.0001$; 2006: $\chi^2 = 140.5$, d.f. = 8, $P < 0.0001$; 2007: $\chi^2 = 241.8$, d.f. = 10, $P < 0.0001$). In 2005, there were fewer browsed flowers inside the exclosure (MC-1) than outside (MC-2), but this pattern was reversed in 2007 (Fig. 4.2*A*). Examination of the phenology of florivory revealed that 70 - 90% of flowers were browsed within the first two weeks of the peak

flowering period (data for 2007 is provided in Fig. 4S.2*A*). On average, more plants were attacked by florivores in the northern populations (2006: 45.4 ± 8.6% (mean ± s.e.); 2007: 66.5 ± 8.9%) than in the south (2006: 5.2 ± 5.2%; 2007: 14.6 ± 4.3%) (Mann Whitney U tests: 2006, $P = 0.024$; 2007, $P = 0.0043$). Predation of *C. rigida* capsules also differed among sites in 2005 ($\chi^2 = 11.56$, d.f. = 4, $P = 0.0209$, Fig. 4.3); however, the low number of fruits produced at some sites in 2006 and 2007 prevented meaningful statistical analysis for these years.

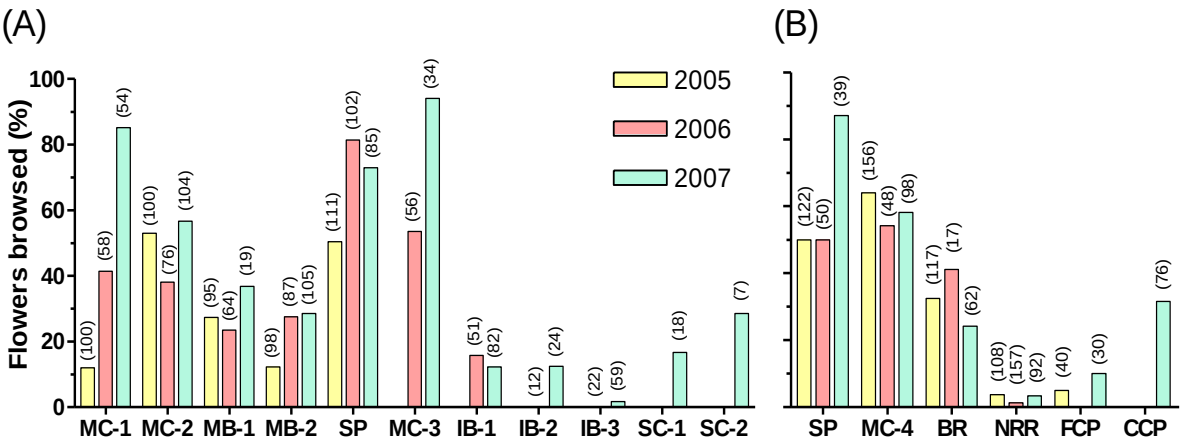

Fig. 4. 2 Florivory of *C. rigida* (*A*) and *C. tentaculata* (*B*). Percentage of flowers browsed at each site, for each of three years. Numbers in parentheses denote the total number of flowering plants monitored at each site. Only one *C. tentaculata* plant flowered at FCP in 2006 so this site was excluded from analyses.

Between 1% and 87% of *C. tentaculata* flowers were browsed (Fig. 4.2*B*), and differences among sites varied across years (significant site by year interaction: $\chi^2 = 29.8$, d.f. = 6, $P < 0.0001$). Separate single-factor analyses revealed differences among sites in all three years (2005: $\chi^2 = 126.8$, d.f. = 4, $P < 0.0001$; 2006: $\chi^2 = 97.8$, d.f. = 3, $P < 0.0001$; 2007: $\chi^2 = 123.5$, d.f. = 5, $P < 0.0001$). Most florivory (65 - 97%) occurred over a two week period during the peak flowering time (Fig. 4S.2*B*). Rates of *C. tentaculata* pollination and hence capsule production were very low in the northern sites (1.7 ± 0.87%) for all three years of this study, so we did not analyse the extent of capsule predation for this species.

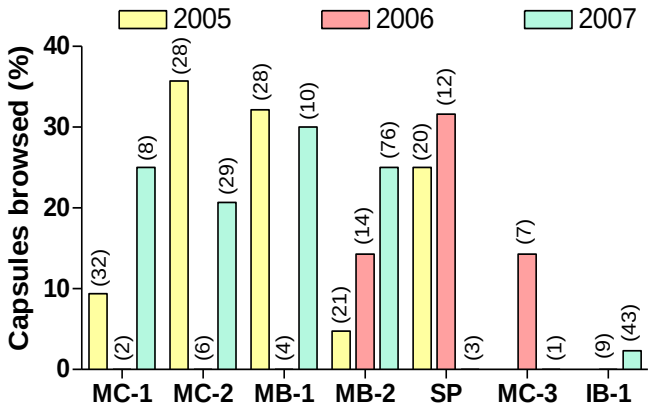

Fig. 4. 3 Capsule predation of *C. rigida*. Percentage of capsules browsed at each site, for each of three years. No capsules were predated at sites IB-2, IB-3, SC-1 & SC-2. Details as for figure 4.2.

Leaf and stem herbivory

The degree of leaf herbivory for *C. rigida* was low at all sites with only 0 - 8% of plants having more than half of their leaf browsed and 70 - 91% left with an intact leaf at the time of withering. For *C. tentaculata*, 0 - 17% of plants had more than half of their leaf browsed whereas 64 - 97% still had their leaf intact at the end of the flowering period. The pattern of stem herbivory varied among sites for both species of orchid (Fig. 4.4) and for *C. rigida* these differences were significant for each year of monitoring (2005: $\chi^2 = 45.65$, d.f. = 8, $P < 0.0001$; 2006: $\chi^2 = 47.36$, d.f. = 10, $P < 0.0001$; 2007: $\chi^2 = 53.93$, d.f. = 10, $P < 0.0001$). In 2005 and 2006, most of the browsed plants at sites MC-1, MC-2 and SP had little or no stem herbivory, whereas at the two MB sites, the proportion of plants with part of the stem removed was considerably higher (Fig. 4.4*A*). In 2007, patterns of stem herbivory inside and outside the exclosure were different ($\chi^2 = 20.51$, d.f. = 2, $P < 0.0001$) prompting further examination of the data. When we included only browsed flowers that were known to be open at the time of florivory (as opposed to inclusion of buds) there was only a marginal difference between inside (MC-1) and outside (MC-2) the exclosure ($\chi^2 = 3.34$, d.f. = 1, $P = 0.068$, Fig. 4.4). For *C. tentaculata*, the patterns of stem herbivory also varied among sites in 2005 and 2007 (Fig. 4.4*B*, $\chi^2 = 48.66$, d.f. = 4, $P < 0.0001$ and $\chi^2 = 26.71$, d.f. = 6, $P < 0.0001$, respectively).

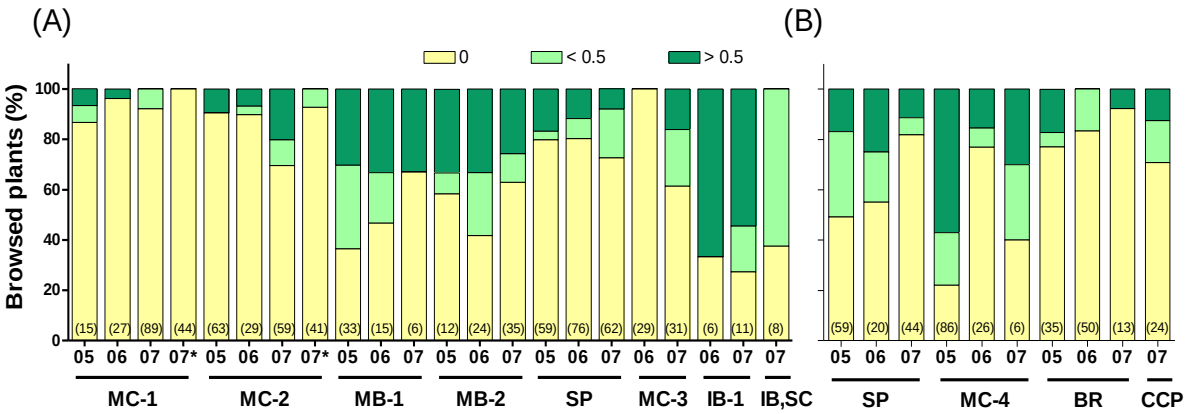

Fig. 4. 4 Degree of *C. rigida* (*A*) and *C. tentaculata* (*B*) stem herbivory. At each site, the percentage of browsed plants with intact stems (0) or with less than half (<0.5) or at least half (>0.5) of the stem removed is indicated for each year. Site IB,SC includes IB-2, IB-3, SC-1 & SC-2. *Stem herbivory of open flowers only (see text). Numbers in parentheses denote the total number of browsed plants at each site.

Distance to edge

We found that although the distance of a *C. rigida* flower from the nearest edge affected its risk of being browsed (Table 4.2), the direction of this effect differed among sites in both 2005 and 2007 (distance/site interaction, $\chi^2 = 12.9$, d.f. =7, $P = 0.075$ and $\chi^2 = 13.6$, d.f. = 4, $P = 0.0085$, respectively). Analysis of each site separately showed significant edge effects in 2005 at MB-1 and SP with florivory increasing away from the edge (Table 4.2). A similar (but non-significant) trend occurred at sites MC-1 and MC-2. In 2007, florivory increased away from the edge at MC-2 but at MB-2, plants within 5 m of the edge were at highest risk of being browsed.

Table 4. 2. **Percentage of *C. rigida* flowers browsed at each site within three distance classes from the habitat edge.**

Site	0 – 5 m	5 – 25 m	>25 m	χ^2
2005				
MC-1	4.8 (21)	10 (10)	18.1 (72)	2.95_2
MC-2	38 (21)	57.9 (38)	64.7 (51)	4.3_2
MB-1	0 (9)	31.7 (82)	69.2 (13)	14.36_2^{**}
MB-2	11.1 (9)	14.5 (62)	10.7 (28)	0.29_2
SP	(0)	11.1 (9)	56.1 (107)	7.47_1^{*}
2007				
MC-1	82.8 (29)	91.7 (12)	88.1 (67)	0.75_2
MC-2	36.8 (19)	53.2 (47)	75.0 (44)	9.4_2^{**}
MB-2	75 (12)	35.6 (87)	36 (25)	6.92_2^{*}

The number of flowers in each distance class is indicated in parentheses. Subscripts denote degrees of freedom. $^{*}P < 0.05$, $^{**}P < 0.01$.

Video surveillance

We obtained video footage of a white-winged chough removing and eating two consecutive *C. rigida* flowers within the exclosure at MC-1 at 0842 hours, 4 September (Fig. 4.5). The recording clearly shows the bird plucking off a flower and consuming it, then pulling another one off at the base of the ovary and swallowing it. In both instances, the flower stem was left intact. A few days later, we observed a white-winged chough foraging amongst the litter at MC-2, and eating a *C. rigida* flower. Despite high levels of florivory at SP, we did not obtain footage of any animals consuming *C. rigida* flowers at this site. The common brushtail possum (*Trichosurus vulpecula*) was recorded moving slowly through the orchid patch on two occasions but did not remove any flowers. We were unable to get any direct evidence of *C. tentaculata* florivory using video surveillance. We recorded a grey currawong (*Strepera versicolor*) picking at a cut *C. tentaculata* flower but releasing it, and a western grey kangaroo (*Macropus fuliginosus*) grazing amongst the orchid patch but not eating flowers.

Fig. 4. 5 Excerpts of video footage showing a white-winged chough picking and eating a *C. rigida* flower at site MC-1. Arrows show intact stems of *C. rigida* remaining after florivory.

Herbivore exclusion experiment

Cages had a significant effect on the probability of florivory for both *C. rigida* and *C. tentaculata* (Fig. 4.6); however, the effectiveness of each cage type was not the same for the two species. Although *C. rigida* florivory differed among patches ($\chi^2 = 17.47$, d.f. = 3, $P = 0.0006$), the effects of cages ($\chi^2 = 144.0$, d.f. = 4, $P < 0.0001$) were the same in all patches (non-significant interaction between cage and patch). The rate of *C. rigida* florivory ranged from 2.7 - 19.6% among the four patches. Cages clearly provided substantial protection against grazing by vertebrate herbivores. Cages designed to exclude all vertebrates (Types 3 & 4) as well as those excluding rabbits and hares (Type 2) were the most effective at reducing herbivory (less than 4.2% browsed, Fig. 4.6). Although flowers within cages that excluded only large herbivores (Type 1) were at greater risk of

being browsed than those inside other cage types, rates of florivory were considerably lower (16%) than those of uncaged control plants (59%).

For *C. tentaculata* rates of florivory also differed between patches ($\chi^2 = 71.9$, d.f. = 1, $P < 0.0001$), with only 9.1% of flowers browsed in patch 1 compared to 69.5% in patch 2. However, the effects of cage type ($\chi^2 = 81.81$, d.f. = 4, $P < 0.0001$) were consistent across the two patches (non-significant interaction between cage type and patch). Only 6% of flowers were browsed inside cages with the smallest mesh size (Type 4), designed to exclude all vertebrates, whilst over 77% of control plants suffered from florivory (Fig. 4.6). Type 3 cages (also designed to exclude all vertebrates, but with larger mesh size) offered moderate protection, whereas flowers within Type 1 (excluding large vertebrates) and Type 2 cages (excluding rabbits and hares) were just as likely to be grazed as their uncaged counterparts.

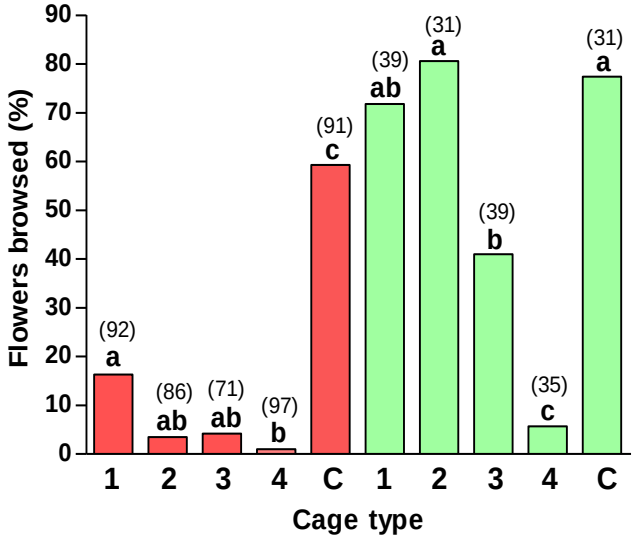

Fig. 4. 6 Percentage of flowers of *C. rigida* (red bars) and *C. tentaculata* (green bars) browsed within four cage types, 1 to 4, or as uncaged controls, C, as described in the methods. Bars with the same letters within each species are not significantly different. Numbers in parentheses denote the total number of flowers within each cage type.

For *C. rigida*, cage type and patch each influenced the pollination of available flowers (cage type: $\chi^2 = 15.68$, d.f. = 4, $P = 0.0035$; patch: $\chi^2 = 14.1$, d.f. = 3, $P = 0.0028$), whereas the cage type by patch interaction was not significant. Flowers inside cages with the smallest mesh size (Type 4) were less likely to be pollinated (10%) than uncaged control flowers (32%) (Fig. 4.7). When browsed flowers were taken into account, there was only a marginal effect of cage type on the number of plants that released seed ($\chi^2 = 9.48$, d.f. = 4, $P = 0.0502$). Flowers within Type 2 cages had the highest rate of seed release (Fig. 4.7); however, none of the differences between cage types were statistically significant.

Pollination rates of *C. tentaculata* at SP were very low in 2007 (< 1%) preventing evaluation of the impact of cages on the pollination success of this species.

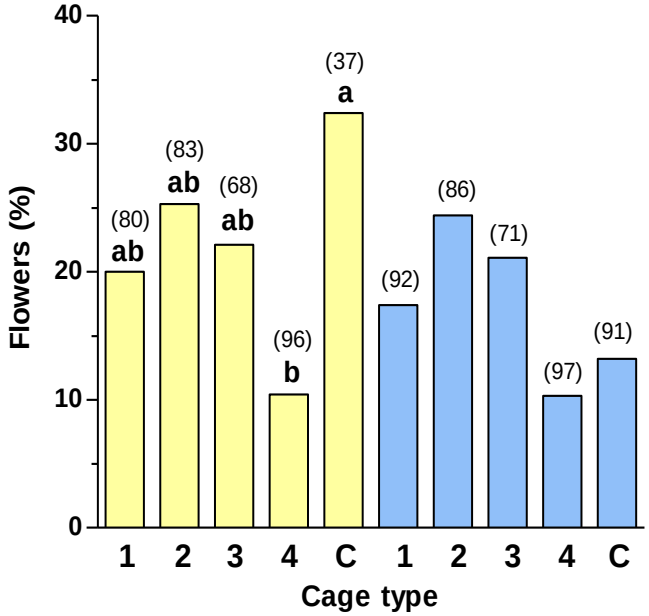

Fig. 4. 7 Percentage of *C. rigida* flowers producing capsules within four cage types, 1 to 4, or as uncaged controls, C. Yellow bars: pollination of available flowers (excludes grazed flowers); blue bars: seed release relative to the total number of flowers monitored (includes grazed flowers). Details as for figure 4.6. Pairwise comparisons for seed release were not statistically significant.

Impact of florivory on reproductive output

To compare total seed output for *C. rigida* in the presence and absence of grazing, we pooled the data for cage types 1, 2 and 3 (excluding Type 4 cages, as these had been shown to reduce pollination success) to provide the proportion of protected plants that released seed. To obtain data for unprotected plants we included all uncaged *C. rigida* flowers monitored at the SP site (ie. counting those not part of the herbivore exclusion experiment), thus reducing unevenness in sample sizes between treatments and increasing the statistical power of our analysis. Seed release of caged flowers (20.8%, n = 197) was significantly higher than that of uncaged plants (7.4%, n = 108) (Fisher's exact test, $P = 0.0018$). The reduction in reproductive success was mostly attributable to floral herbivory as only one out of ten capsules produced by uncaged plants was browsed, and none of the capsules within cages suffered predation. Of all capsules produced by tagged plants in 2007 (n = 26), one aborted without dehiscing. We found no evidence of damage to capsules by invertebrates at this site.

4.4 Discussion

Patterns of florivory and herbivore identification

We have shown that florivory has a complex pattern of spatial and temporal occurrence for both *C. rigida* and *C. tentaculata*. Over 80% of flowers were grazed at some sites and up to 36% of capsules suffered from predation indicating that herbivory can severely reduce the reproductive success of these orchids both prior to (by reducing the number of available flowers) and after pollination. Herbivory of leaves was relatively low at all sites for both orchid species and seems unlikely to have a major impact on the reproductive success of these orchid populations as a whole, at least when compared with the probable effect of florivores on the reproductive organs. It is possible however, that herbivory of younger leaves occurred earlier in the season leading to subsequent misclassification of plants as non-emergent. For example, a survey at MC-1 in June 2007 revealed that 6% (n = 131) of emergent leaves were no longer apparent when monitoring began in August (data not shown). That herbivores prefer orchid flowers is perhaps a reflection of their nutrient content. While this has not been determined specifically for orchids, analyses of several plant species indicate that flowers contain higher levels of sugars than their foliage, and may therefore provide herbivores with a readily metabolisable energy source (Held and Potter 2004). A recent study detected nectar on the surface of *C. rigida* but not *C. tentaculata* flowers; however, the sugar content of the entire flower was not analysed (Faast *et al.* 2009).

White-winged choughs were recorded eating *C. rigida* flowers thus providing the first evidence of orchid florivory by an avian species. These large, ground-foraging birds occur throughout south-eastern Australia and are highly social, living in groups of up to 20 (Rowley 1965). Predominantly insectivorous, they are also opportunistic foragers feeding on seeds, berries and (rarely) flowers (Barker and Vestjens 1990). White-winged choughs have also been reported to dig up and eat the tubers of the orchids *Pterostylis despectans* (Nicholls)M.A.Clem. & D.L.Jones (Duncan *et al.* 2005) and *Caladenia rosella* G.W.Carr (Todd 2000). In the case of *C. rigida*, video footage as well as direct observation of the birds' behaviour, indicated that the removal of orchid flowers was quite deliberate and selective and we did not see them consume any other flowers. It is therefore conceivable that large groups of birds could inflict serious damage to a population of orchids in a relatively short amount of time.

Some of the geographical variation in orchid florivory may be explained by the distribution or home ranges of white-winged choughs. Although we do not have accurate records of the species' fine-scale distribution, we noticed large groups of the birds upon every visit to the MC-1, MC-2 and SP sites in 2007. We occasionally observed groups in the vicinity of MB-1 and MC-3, but did not see them at any of the remaining sites. It is possible that the large temporal changes in the effectiveness of the MC-1 exclosure are due to yearly fluctuations in the local occurrence of florivores (eg. choughs) or patchiness in their foraging patterns. Higher grazing pressure inside the exclosure may be coincidental or could reflect a behavioural response by the birds, perhaps preferring the habitat associated with vegetation protected from other grazing herbivores. A similar study of *C. behrii* also found that the protection offered by a kangaroo- and deer-proof fence was highly variable among years (Petit and Dickson 2005).

Levels of *C. rigida* florivory were generally lower at sites where white-winged choughs were not observed, but a considerable proportion of flowers were still browsed, particularly at the northern sites. Although we cannot rule out choughs frequenting these areas, it is likely that other herbivores also feed on orchids. This is supported by the different patterns of stem herbivory recorded at these sites. In the exclosure in 2007, all browsed plants had little or no stem herbivory, whereas over 20% of plants outside had more than half of their stem removed. When we only included plants known to be in flower at the time of grazing, this was reduced to zero, suggesting that plants with stem damage were browsed at the bud stage, by herbivores unable to access the exclosure. For example, Petit and Dickson (2005) mention that kangaroos prefer the buds of *C. behrii*, and that rabbits tend to first graze the stems of plants. Additionally, video evidence clearly shows a chough picking off orchid flowers and leaving the stems intact. Similarly, different species of herbivores could account for the higher degree of stem damage seen at the MB and southern sites.

Geographical variation in florivory was most striking when comparing the northern and southern areas. The latter sites are located within an urbanised region of the Mt Lofty Ranges, which can influence the distribution, density or behaviour of vertebrate herbivores (Garden *et al.* 2006). In addition, the smaller population size of *C. rigida* in the southern sites (Table 4.1) may make orchids more difficult to find. Groom (2001) for example, demonstrated that the herbaceous plant, *Clarkia concinna concinna*, was more likely to escape predation by deer in smaller, isolated patches. Although we have not directly

identified the herbivores of *C. tentaculata*, spatial and temporal heterogeneity in florivory and stem damage is also likely to be the result of different herbivore species. Two of the southern *C. tentaculata* populations (NNR & FCP) occur in small fragments surrounded by urban environments, which sustain few vertebrate herbivores other than birds and possums (R. Faast, pers. obs.), and probably accounts for the negligible levels of florivory at these sites.

Interestingly, we did obtain video footage for two of the species listed as potential herbivores (western grey kangaroo and common brushtail possum) but neither of these animals showed any interest in the orchid flowers. Indeed, the kangaroo spent a considerable time grazing amongst the orchid patch, at one point within a few centimetres of an intact *C. tentaculata* flower. Aside from NNR and FCP, kangaroos are common at all sites (R. Faast, pers. obs.); we saw hares at MB-1 and SP, and along with all of the other potential herbivores listed earlier, they are likely to occur at most sites. In addition, we observed emus or their scats at MB-2 and SP and, given that they are known florivores (Quinn 1996), it is plausible that orchid flowers also form part of their diet. Our recording of a grey currawong does not provide direct proof of orchid herbivory; however, the interest shown in the flower makes these birds potential candidates as florivores. Being a cut flower, the lack of resistance may have discouraged the animal. Although widespread and common throughout southern Australia, currawongs usually forage alone or in pairs (Schodde and Tidemann 1986), so are unlikely to inflict the same extent of damage as large groups of white-winged choughs. The use of video surveillance to identify herbivores was only partially successful. Flowers in front of the camera were sometimes left intact whereas surrounding flowers were heavily browsed. It is unlikely that the camera set-up itself deterred herbivores as grazing occurred in close proximity. Furthermore, the set-up is routinely used to capture images of nocturnal mammals with no apparent disturbance to their behaviour (P. Moyle, pers. comm.). The lack of activity in front of the camera may simply be coincidental, and once surrounding flowers were removed the remaining patch was probably less conspicuous to herbivores.

Edge effects

Spatial variation in florivory at the population level was demonstrated by the differential grazing intensity among orchid patches and with respect to distance from the nearest fire track or road. At most sites, this edge effect resulted in a decreased likelihood of orchid herbivory closer to the edge but at MB-2 we found the opposite situation in 2007, and no

edge effect in 2005. Again, this difference could be attributed to the identity of the predominant herbivores at each site and their behavioural response to edges. Sites at which proximity to the edge appeared to benefit orchids, coincided with those frequented by white-winged choughs, whereas these birds were never recorded at MB-2. Choughs may prefer to forage away from the edge, avoiding predators, or in response to differential prey distribution (eg. litter accumulation and hence invertebrate density may be higher within the forest interior). In contrast, herbivores preferring more open habitats and moving along tracks are more likely to encounter orchids near the edge. Most studies of edge effects have found an increased risk of vertebrate herbivory at forest edges (Jules and Rathcke 1999; Wahungu *et al.* 1999; Bach and Kelly 2004); however, Cadenasso and Pickett (2000) demonstrated that although this held true for meadow voles (*Microtus pennsylvanicus*) in the United States, seedling herbivory by white-tailed deer (*Odocoileus virginianus*) was higher in the forest interior. Such edge effects are usually attributed to differences in herbivore density or habitat preferences, but changes in the microclimate associated with edges can have indirect influences. For example, the composition and structure of vegetation has been shown to differ between interior and edge habitats (Watkins *et al.* 2003) resulting in changes in the abundance and activity of herbivores (Kollmann and Buschor 2002). Furthermore, changes in the density and hence apparency of the target plants themselves may affect their risk of herbivory (R. Faast, unpubl. data). All these factors need to be investigated in detail in order to gain a better understanding of the influence of habitat edges on orchid florivory.

Herbivore exclusion experiment

The level of protection provided by the herbivore exclusion cages differed considerably between the two species of orchids. All cage types were highly effective against herbivores of *C. rigida*, but only fully enclosed fine-meshed cages protected *C. tentaculata*. Difference in the identity of herbivores is one explanation for these results; however, the apparency or accessibility of the orchid flowers may also influence their risk of florivory. *Caladenia tentaculata* flowers were considerably taller (31.9 ± 6.8 cm) than those of *C. rigida* (20.3 ± 6.1 cm) and often protruded above the top of Type 2 cages, putting them within easier reach of herbivores. Inside closed-top Type 3 cages all of the *C. tentaculata* flowers affected by florivory had grown within 10 cm of the mesh, indicating that herbivores were able to gain access through the 40 mm holes. In contrast, *C. rigida* flowers did not grow tall enough to be within reach.

We did not obtain direct evidence of florivory by white-winged choughs at the SP site but we regularly saw large groups of birds foraging amongst the *C. rigida* population. Patterns of *C. rigida* stem herbivory are consistent with that observed for choughs and if they are the predominant herbivore at the site, our results suggest that the birds were deterred by all cage types. In contrast, florivores gained access to all but the best protected *C. tentaculata* flowers. Cages designed to exclude large herbivores offered no protection, making kangaroos and deer unlikely candidates, whereas rabbits and hares should have been excluded from the open top (Type 2) cages. It is possible that more than one species of herbivore is responsible for the damage within different cage types or that possums and birds could gain access to three of the cage types, but the low incidence of *C. rigida* florivory within all cages remains puzzling. Perhaps the large population of uncaged *C. rigida* flowers provided ample food supply, whereas later in the season, the less abundant *C. tentaculata* flowers (Table 4.1) became more sought after.

From a management perspective, an important outcome of the herbivore exclusion experiment is the potential of some cages to reduce the reproductive success of *C. rigida*. We demonstrated that flowers inside fine-meshed cages had one-third the probability of being pollinated and setting seed than uncaged plants that had escaped florivory, suggesting that the pollinators of this species are deterred or impeded by this type of cage. However, protection against grazing meant that there were more flowers available for pollination so that the proportion of plants actually releasing seed was similar for caged and uncaged plants. Cages with a larger mesh size (eg. 40 mm) were a more effective management tool as these excluded herbivores without significantly reducing pollination. Unfortunately we were unable to assess the impact of cages on the pollination success of *C. tentaculata*. This species is sexually deceptive and pollinated by a thynnine wasp, which may behave quite differently to the generalist pollinators of *C. rigida*. While fine-meshed cages could also impede the passage of these larger insects, a strong attraction to the kairomones emitted by these orchids may be enough to overcome any hindrance. Further studies are therefore required to assess the impact of cages on the pollination success of sexually deceptive orchids.

Implications of florivory

One of the aims of this study was to determine the potential reproductive output of a population of orchids in the absence of grazing. We found that caged *C. rigida* plants at the SP site produced approximately three-times more capsules than unprotected plants,

demonstrating that florivory substantially reduces the reproductive potential of this orchid population. The large spatial and temporal variations in the severity of florivory both within and among populations have important implications for plant populations both locally and at the landscape scale. Cooper and Wookey (2003) suggested that a patchy spatial distribution of grazing by reindeer allows some plants to escape florivory, providing valuable opportunities for reproduction. Plants that occur as dispersed clusters or amongst vegetation may also escape predation (Gregg 2004). Similarly, yearly fluctuations in the intensity of herbivory could allow populations to recruit and "recover" in some years. However, some of the sites in this study suffered from considerable grazing pressure in all three years, raising the question as to whether such populations can be maintained in the long-term. Given the limited seed dispersal of orchids (Peakall and Beattie 1996; Jacquemyn *et al.* 2007a), recruitment from nearby less-grazed sites is unlikely, particularly in light of habitat fragmentation, which has resulted in sites such as SP being isolated by more than 3 km to the nearest *C. rigida* population. Historically, we do not know whether orchid populations have always suffered from high rates of predation, or whether the present landscape has changed patterns of herbivore activity.

The effects of florivory on the reproductive success of orchids are multiple and possibly complex. At the population level, factors such as the degree of pollen limitation, seedling recruitment, population size and density can all influence orchid reproductive success (Tremblay *et al.* 2005) and hence their ability to tolerate grazing. Floral herbivores directly affect plant reproductive success by reducing the number of flowers available for pollination and consuming flowers that may already have pollinia deposited. Consumption of one flower not only results in the loss of both female and male reproductive function, but also has the potential to deprive another flower from receiving pollinia. Given that the reproductive success of most orchids is already constrained by pollen limitation (Tremblay *et al.* 2005), high levels of herbivory are likely to limit seed production even further. Florivory can also indirectly influence the pollination success of those flowers that remain. For example, pollination of nectar producing flowers has been shown to be density dependent (Sih and Baltus 1987; Feldman 2006), so a decrease in the number of conspecific flowers could exacerbate the direct impacts of florivory. Our results for *C. rigida* show that flowers escaping florivory and achieving pollination are still at considerable risk of capsule predation, further reducing total seed output. Species with a long-lived seed bank may be better adapted to deal with temporal variation in florivory by

delaying germination over several years; however, data from another *Caladenia* species, *C. arenicola*, suggests that seeds of this genus do not persist for more than one year (Batty *et al.* 2001a). Loss of reproductive potential will therefore affect an entire year's recruitment. The consequences of reduced seed production will depend upon the availability of suitable recruitment sites, and at present there is little information available regarding the degree of seed limitation versus microsite limitation for *Caladenia*. The longer-term effects of flower loss on plant traits and dormancy in subsequent years have not been determined for orchids; however, studies of other species have demonstrated changes in resource allocation in response to florivory (McCall and Irwin 2006; Tobler *et al.* 2006).

Conclusions and management recommendations

Our findings provide direct evidence of intense grazing pressure in several populations of *C. rigida* and *C. tentaculata,* and we have shown that at least for *C. rigida*, total seed output is significantly reduced as a result. We have identified white-winged choughs as the dominant florivore of *C. rigida* and possibly *C. tentaculata* at some sites; however, geographical variations and patterns of stem herbivory suggest that other species of vertebrate herbivores also consume orchid flowers. Within populations, spatial differences associated with edges or patches may help some orchids escape grazing. Although it seems likely that high levels of grazing pressure would substantially impact orchid demography, the long-term consequence of floral herbivory on the population dynamics of orchids requires further investigation.

Herbivore exclusion can be an effective management tool with respect to increasing reproductive success; however, important considerations include selection of mesh size to optimise pollination success, and timing the erection of cages prior to anthesis (as most florivory occurs early in the flowering season). In the case of food-advertising orchids such as *C. rigida*, Type 3 cages offer the best protection against grazing without impeding pollinator activity. For taller species of orchids, we recommend the use of taller and wider cages to ensure that flowers are kept at least 10 cm from the mesh. Further video surveillance is required to determine the extent of stem damage incurred by different herbivores; however, once identified, assessment of stem herbivory may aid management decisions by providing a quick indicator of the type of herbivore active at a particular site. At sites where white-winged choughs are the primary florivore, edge effects suggest that caging efforts can be concentrated away from the habitat edge.

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SUPPLEMENTARY MATERIAL

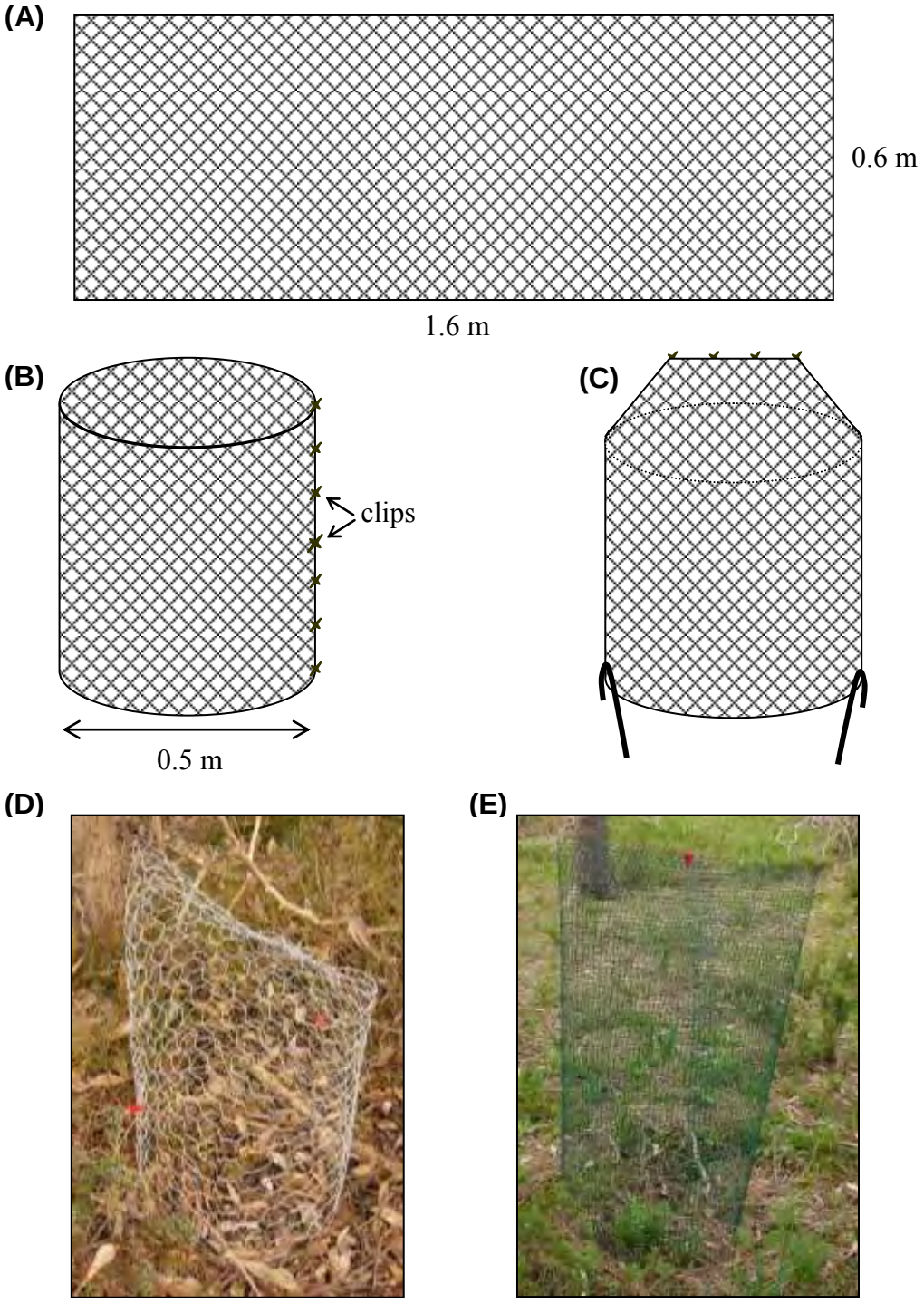

Fig. 4S. 1 Design and construction of Type 3 cages, which exclude kangaroos, deer, rabbits, hares, possums and large birds (provided flowers remain at least 10 cm from mesh). (*A*) Galvanised chicken wire (40 mm x 40 mm hexagonal mesh), 0.6 m wide was cut to 1.6 m lengths. (*B*) This was shaped into a cylinder and fastened by bending over the cut edges, or using fencing clips. (*C*) The top was fastened shut using wire or fencing clips and the cage was secured into the ground using three or four tent pegs or (more economical) 20 cm stainless steel irrigation stakes (these are designed for ½" poly-tubing and are available from irrigation suppliers). Type 4 cages were constructed in a similar way using 0.9 m wide green plastic-coated bird mesh with 12.5 mm squares. (*D*) Type 3 cage *in situ* (*E*) Type 4 cage.

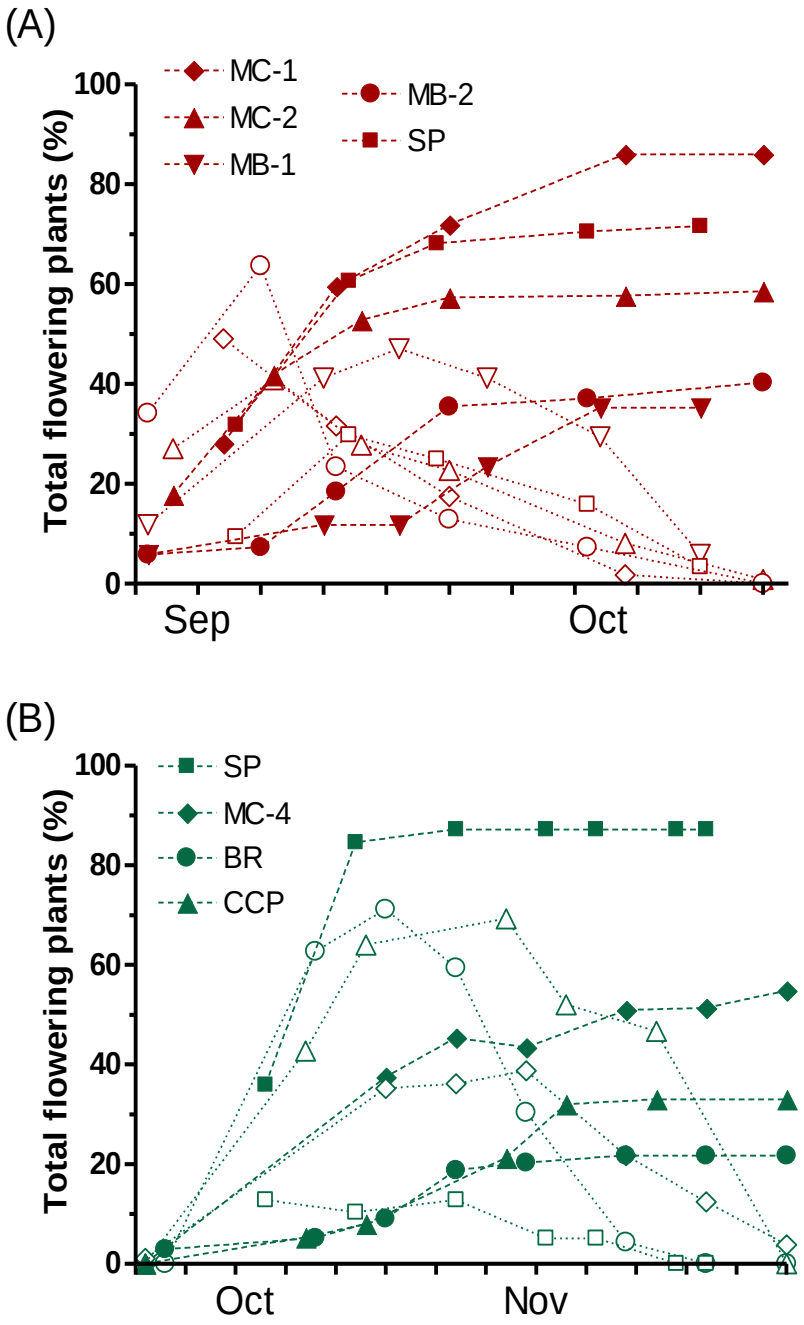

Fig. 4S. 2 Phenology of florivory for (*A*) *C. rigida* and (*B*) *C. tentaculata* in 2007, at sites with high levels of florivory. Closed symbols show percentage of flowers browsed over time, and open symbols show the percentage of open flowers. Similar trends were observed in 2005 and 2006 (data not shown).

CHAPTER 5

Caladenia rigida (Photo by author)

Chapter 5: Preamble

CHAPTER 5 investigates spatial and temporal variation in the pollination success of *Caladenia rigida* and *Caladenia tentaculata*. Differences in the magnitude and patterns of pollination success between the two species are discussed in terms of their contrasting pollination strategies. To alleviate the possible influences of both habitat and climatic variation, pollination success of *C. tentaculata* is compared with that of a third species, *Caladenia carnea*, co-flowering at one site. The impact of grazing on final seed release is also examined.

This chapter has been prepared as a submission for publication. Supplementary material, intended for electronic viewing via the publisher's website, is included at the end of the chapter.

Contributions and signatures of authors:

Renate Faast

Designed experiments, collected and analysed all data and prepared manuscript as principle and corresponding author.

Signed.... ..... Date.29th June 2010..

José M. Facelli

Sought and won funding, supervised development of research and evaluated manuscript.

Signed..... ..... Date.....15/06/2010.....

Andrew D. Austin

Sought and won funding, advised on aspects of research and evaluated manuscript.

Signed.... ..... Date.30th June 2010

SPATIO-TEMPORAL VARIATION IN POLLINATION AND SUCCESSFUL SEED RELEASE IN TWO TERRESTRIAL ORCHIDS WITH CONTRASTING POLLINATION STRATEGIES.

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Abstract

Pollination strategy is predicted to play an important role in determining the magnitude of pollen limitation in animal-pollinated plants. The orchid genus *Caladenia* provides an ideal opportunity to investigate the consequences of different pollination strategies, as it includes species that are highly specialised and others that utilise a more generalist strategy. We compared spatio-temporal variation in pollination and the subsequent release of seeds for a generalist species, *Caladenia rigida*, with that of a congeneric pollination specialist, *Caladenia tentaculata*. The study was carried out over three consecutive years across multiple populations within the Mount Lofty Ranges in South Australia. At one site we also evaluated the pollination success of *C. tentaculata* and a co-flowering generalist, *Caladenia carnea*, allowing us to compare pollination strategy in the absence of both climatic and habitat variation. Both pollination and successful seed release were highly variable among populations and among years for *C. rigida*. During a drought year small populations of *C. rigida* failed to set fruit, suggesting that Allee effects may only manifest themselves under conditions of environmental stress. Pollination success of the specialist was consistently lower than that of the two generalist species. In all three species, seed output was severely reduced through interactions with antagonists (florivores and capsule predators). To our knowledge, this is the first study to directly compare a generalist pollination strategy with that of a highly specialised sexually deceptive strategy, across similar spatial and temporal scales. Our results indicate that the degree of specialisation may have important implications for fruit production in *Caladenia*. Temporal variation in the expression of Allee effects suggests that factors other than population size are important for determining the long-term viability of fragmented populations.

5.1 Introduction

Seed production in plant populations is often limited by the amount or quality of pollen received by flowers (Burd 1994; Ashman *et al.* 2004), and variation in plant-pollinator associations has been shown to drive patterns of pollen limitation in a range of species (Oostermeijer *et al.* 1998; Kindlmann and Jersakova 2006; Ågren *et al.* 2008). Such patterns are usually caused by changes in pollinator communities across time and space (Herrera 1988; Petanidou *et al.* 2008); however, pollen limitation can also be governed by plant traits, as well as factors that are intrinsic to plant populations themselves such as population size or density, eg. Allee effects (Ghazoul 2005; Knight *et al.* 2005).

It has been argued that variation in the magnitude of pollen limitation depends on the species' pollination strategy (Knight *et al.* 2005). Species with a broad spectrum of pollinators are expected to be better adapted to fluctuations in pollinator assemblages than those that are pollinated exclusively by one or a few pollinators. Alternatively, specialisation may have evolved to take advantage of the most abundant or most efficient pollinator (Stebbins 1970). Specialist species should therefore benefit when the abundance of their pollinators is reliable, but will be sensitive to temporal fluctuations in pollinator services (Waser *et al.* 1996). Empirical support for these predictions has been difficult to obtain. A recent study showed that seed set failure in pollination specialists was linked to the loss of their pollinator in small conservation areas, while a co-occurring generalist remained unaffected (Pauw 2007). Others have found no association between pollinator specificity and vulnerability to habitat fragmentation (Aizen *et al.* 2002; Donaldson *et al.* 2002). Studies analysing the influence of pollination strategy on plant reproductive success are usually based on meta-analyses of data pooled across broad geographic (eg. across biomes) and/or temporal scales (different flowering seasons). Direct comparisons between congeneric species subject to similar ecological and environmental conditions, could therefore provide a valuable approach to test the predictions of these studies, while avoiding the potentially confounding effects of phylogenetic constraints and regional differences.

The persistence of plant populations relies not only on the production of fruit but also on the maturation and survival of seeds that contribute to the next generation, yet research studies and monitoring programs rarely take the latter into consideration. For example, interactions such as herbivory or seed predation also vary across space and time (Ågren *et*

al. 2008; Faast and Facelli 2009), and may ultimately affect patterns of seed output. If variation among populations or through time is high, a single-population or single-year study will provide very different outcomes depending on the time and site chosen. Furthermore, plant traits that are associated with pollination strategy may also influence interactions with herbivores (Ehrlén 1997; Gómez 2003). This emphasises the importance of assessing the nature and degree of spatio-temporal variability of both mutualistic and antagonistic interactions, when considering the long-term fitness of plant populations.

The Orchidaceae comprise a high proportion of pollination specialists (Tremblay 1992; Schiestl and Schlüter 2009) and over one third of orchids employ a pollination strategy based on deceit (Nilsson 1992). Most comparative studies of pollination success have focused on the advantages of rewarding over deceptive strategies (Johnson and Bond 1997; Neiland and Wilcock 1998; Tremblay *et al.* 2005; Kindlmann and Jersakova 2006; Jacquemyn *et al.* 2009), and are often based on species from several genera with varying degrees of pollination specialisation. However, to our knowledge, no studies have been carried out comparing the reproductive success of orchids that rely on a single pollinating vector versus those that are pollinated by several species.

The genus *Caladenia* provides a unique opportunity for comparative studies of species with different pollination syndromes, as it includes both generalist and specialist species. The latter are usually highly specific, each species being pollinated through the sexual deception of a single species of thynnine wasp (Phillips *et al.* 2009b). Based on a meta-analysis of data encompassing 20 species across the Australian continent, Phillips *et al.* (2009b) showed that fruit set in generalist (food-advertising) *Caladenia* was more than two-fold higher than sexually deceptive congeners (36% versus 14%). However, to date there is little information on how fruit set and seed release for species with contrasting pollination strategies varies in space and over time, and with population size.

In the present multi-year study we investigated the degree of spatial and temporal variation in the pollination success and seed release of two *Caladenia* species across several sites. These species utilise quite different pollination strategies. *Caladenia rigida* is a generalist food-advertising orchid that is pollinated by several taxonomically diverse insects, while the sexually deceptive *Caladenia tentaculata* relies on a single species of thynnine wasp. At one of the sites, these species co-occurred, allowing us to compare pollination strategies while eliminating the potential confounding influences of habitat characteristics. At

another site, we compared the pollination success of *C. tentaculata* with that of a generalist, *Caladenia carnea*, which flowered at the same time, allowing us to assess contrasting pollination strategies in the absence of both habitat and climatic variation. None of the sites had all three species present. We have previously demonstrated substantial spatio-temporal variation in florivory and capsule predation for *C. rigida* and *C. tentaculata* (Faast and Facelli 2009) and investigate this further by evaluating the impacts of grazing on the successful release of seeds. Our findings will help to refine our understanding of the effectiveness of both pollination strategies across multiple populations and years.

Specifically, the questions we addressed were: (i) Does pollination success in *C. rigida* and *C. tentaculata* vary across space and time, and what factors could explain this variation? (ii) Are interactions with antagonists important in determining the proportion of the population that actually releases seed? (iii) Does the magnitude and pattern of pollen limitation differ between the generalist and the specialist species?

5.2 Methods

Study species

Caladenia is a genus of terrestrial, deciduous orchids that produce a single replacement tuber each year. Plants may remain dormant within a season or emerge in autumn as a single basal leaf with or without a flower (Dixon and Tremblay 2009). *Caladenia* species are self-compatible and most reproduce only from seed.

Caladenia rigida (subgenus *Calonema*) is endemic to the Mount Lofty Ranges, South Australia (Fig. 5.1). Habitat loss and fragmentation over the last century have contributed to reductions in its range and abundance, and the species is listed as endangered based on IUCN criteria (Quarmby 2006). Flowers begin to open in late August (early Spring) and plants usually produce a single, predominantly white flower. Unless pollinated, flowers remain open for up to four weeks. *Caladenia rigida* is a generalist food-advertising orchid, attracting a range of confirmed and putative pollinators including native bees (genera *Lasioglossum, Exoneura*, *Homalictus*), syrphid flies (*Simosyrphus*), honey bees (*Apis mellifera*) and conopid flies (Conopidae) (Faast *et al.* 2009). Small amounts of sugars have been detected on the labellum and at the base of the column (Faast *et al.* 2009), but

whether they attract or sustain pollinators has not been established. Either way, the pollination service is provided by a broad spectrum of food-seeking insects.

Caladenia tentaculata (subgenus *Calonema*) occurs throughout the Mount Lofty Ranges and is widespread and common in south-eastern Australia (Bates 2006; Jones 2006). It typically produces one green and maroon flower, which usually opens in early October (Spring) and can remain open for up to five weeks. The species is pollinated by the male of a sexually deceived thynnine wasp, *Thynnoides pugionatus* (Bates 1996).

Caladenia carnea (subgenus *Caladenia*) is also common in the Mount Lofty Ranges and is widespread throughout eastern Australia (Bates 2006; Jones 2006). This species normally produces one to three pink flowers which, at the study site, open in late-September and stay open for up to four weeks. *Caladenia carnea* utilises a generalist food-advertising pollination syndrome, attracting several species of native bees, and does not produce any obvious nectar (Bates 1984b; Adams *et al.* 1992; Farrington *et al.* 2009).

Study sites

During the flowering season of 2005, we monitored pollination success and capsule dehiscence for *C. rigida* and *C. tentaculata* at five different sites for each species. In 2006 and 2007, six more *C. rigida* populations and one *C. tentaculata* population were located and incorporated into the study. Populations varied in size and occurred within two distinct regions in the Mount Lofty Ranges, South Australia (Table 5.1, Fig. 5.1). Two of the *C. rigida* populations (MC-1 and MC-2) were adjacent to each other, the first being enclosed by a kangaroo- and rabbit-proof fence. Neither of the SC-1 and SC-2 populations produced any flowering individuals in 2006, so these sites are only included in analyses for 2007. At one of the sites, SP, *C. rigida* and *C. tentaculata* co-occurred. *Caladenia carnea* only grew at the CCP site (co-occurring with *C. tentaculata*).

Vegetation at the study sites is comprised of *Eucalyptus* woodlands with an understorey dominated by *Acacia, Leptospermum*, *Hibbertia* and *Lepidosperma* species (for details, see Faast and Facelli (2009)). Rainfall data were obtained from the Australian Government Bureau of Meteorology for seven weather stations located within 1.3 to 6.5 km of each of our study sites (Fig. 5.1). We calculated annual rainfall and monthly averages for September and October (the peak flowering time of *C. rigida* and *C. tentaculata*) for 2005, 2006 and 2007 as well as long-term annual rainfall (based on data collected over 40 to 147

years) (Table 5.2). We averaged data separately for northern and southern sites (three stations in each) as these regions experience different weather patterns. Site FCP is located on the Adelaide plains and is considerably drier than the Mount Lofty Ranges, so rainfall from the closest weather station is shown separately.

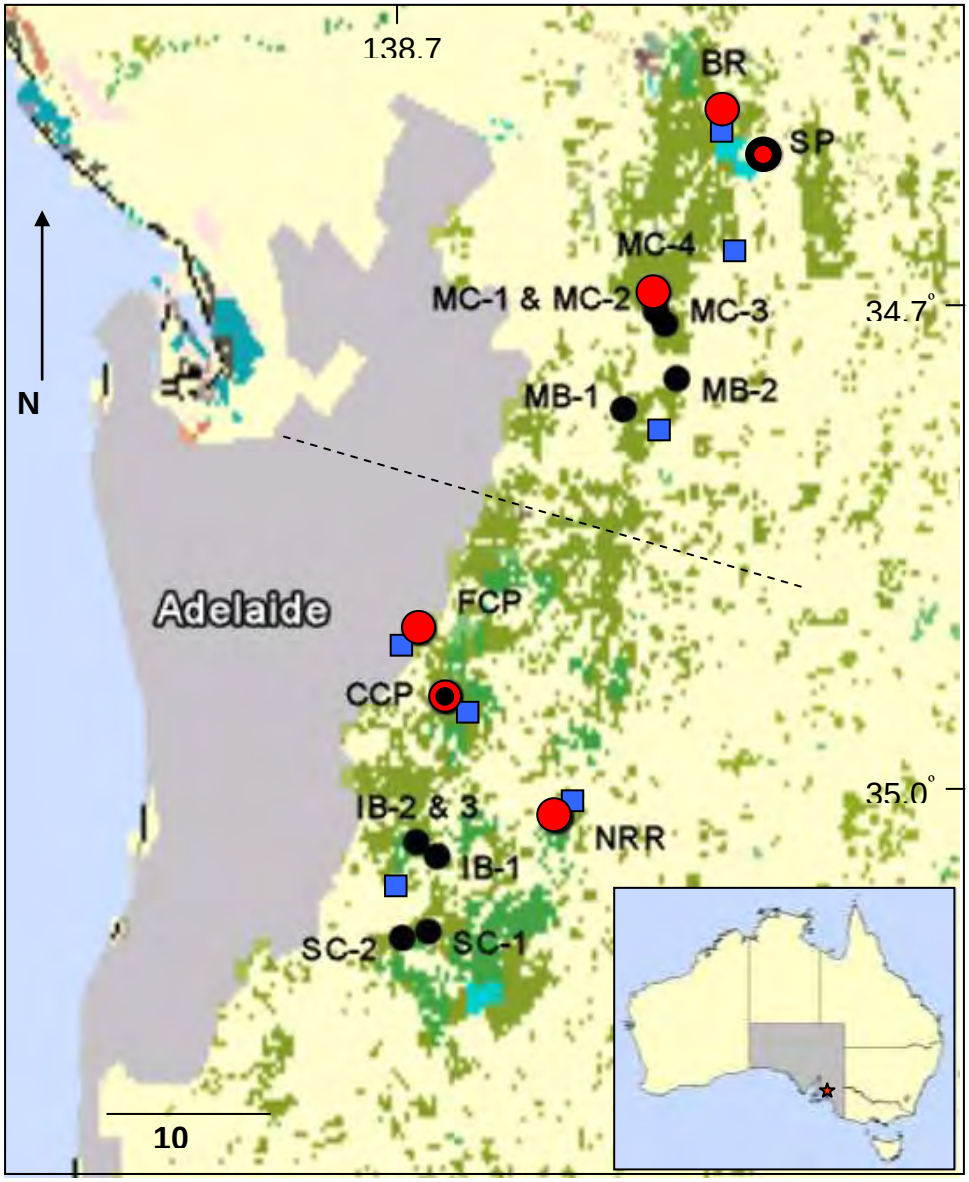

Fig. 5. 1 Map showing location of study sites and weather stations in the Mount Lofty Ranges, South Australia. (●) *Caladenia rigida*; (●) *C. tentaculata*; (◉) *C. rigida* and *C. tentaculata*; (◉) *C. tentaculata* and *C. carnea*; (■) Weather stations (Bureau of Meteorology, South Australia). Grey shading shows the urban area of the city of Adelaide, green represents areas of extant native vegetation. An arbitrary separation between northern and southern populations (based on their spatial separation and their direction from the city of Adelaide) is delineated with a dashed line. Map source: Australian National Resources Atlas.

Table 5. 1 Location and description of study sites containing populations of *Caladenia rigida*, *C. tentaculata* or *C. carnea.*

Species	Site	Location	No. flowering plants [D]			Area of native vegetation (Ha)
			2005	2006	2007	
C. rigida	MC-1[AC]	Mt. Crawford NFR	150	80	200	>450
	MC-2 [A]	Mt. Crawford NFR	150	100	200	>450
	MC-3 [A]	Mt. Crawford NFR		100	100	>450
	MB-1 [A]	Millbrook Reservoir	150	80	200	480
	MB-2 [A]	Millbrook Reservoir	250	120	>500	150
	SP [A]	South Para Reservoir	800	400	>1500	4.5
	IB-1 [B]	Ironbank – private land		50	100	~350
	IB-2 [B]	Ironbank – private land		15	23	~350
	IB-3 [B]	Ironbank – private land		20	60	~350
	SC-1 [B]	Scott Creek CP		0	19	~200
	SC-2 [B]	Scott Creek CP		0	7	~200
C. tentaculata	SP [A]	South Para Reservoir	120	80	300	4.5
	BR [A]	Barossa Reservoir	150	50	200	>230
	MC-4 [A]	Mt. Crawford NFR	120	80	300	>450
	NRR [B]	Nation Ridge Rd Reserve	120	180	200	0.5
	FCP [B]	Ferguson CP	40	1	50	7
	CCP [B]	Cleland CP		80	100	990
C. carnea	CCP [B]	Cleland CP	300	200	500	990

[A] Northern sites, [B] Southern sites, [C] Plants within herbivore exclosure. [D] All flowering plants were counted at sites with less than 60 flowers, whereas in larger populations the number of flowering plants is based on estimations. Area of native vegetation was estimated from available mapping. NFR, Native Forest Reserve; CP, Conservation Park.

Table 5. 2 Rainfall (mm) averaged from weather stations in northern and southern regions (mean ± s.e.m.) and at the FCP site.

Region / Site	Period	2005	2006	2007	Long-term
Northern	September	73.5 ± 4.7	45.8 ± 7.1	64.3 ± 8.6	87.1 ± 5.3
	October	159.7 ± 4.0	0.2 ± 0.0	29.7 ± 1.4	66.3 ± 3.4
	Annual	889.2 ± 36.8	485.1 ± 12.0	706.0 ± 3.1	760.9 ± 51.7
Southern	September	97.7 ± 1.0	60.6 ± 5.0	56.9 ± 7.5	108.0 ± 3.7
	October	181.7 ± 5.1	2.0 ± 0.8	42.4 ± 1.6	80.0 ± 3.6
	Annual	1075.1 ± 35.7	643.1 ± 13.7	913.8 ± 44.5	985.3 ± 33.6
FCP	September	60.2	35.8	29.8	54.6
	October	100.4	1.2	34.4	45.8
	Annual	670.4	336.2	569.2	622.6

Pollination and seed release

At each site we randomly selected and tagged up to 120 flowering plants and monitored these every 1 - 2 weeks from the beginning of the flowering season through to the dehiscence of mature capsules. For all three species, we recorded capsule formation, florivory, capsule abortion, capsule predation and dehiscence. To account for the high rates of florivory at some sites, we calculated pollination success as the number of capsules produced relative to the number of flowers available for pollination, excluding grazed plants. To evaluate the proportion of the population that was able to contribute to subsequent generations, we calculated the number of dehiscing capsules relative to the total number of tagged flowers (including grazed flowers and capsules). While the first aspect reflects only the interaction between pollinators and plants, the latter also takes into account interactions with antagonists as well as the effect of resource availability.

Severe drought conditions during 2006 (Table 5.2) reduced the emergence and flowering rates of all three orchid species at most sites. In this year there was only one flowering *C. tentaculata* plant at FCP, so we excluded this from analyses. In 2007, high rates of florivory forced us to exclude site MC-3 from analyses of *C. rigida* pollination success and we omitted site MB-1 when analysing the proportion of dehiscent capsules due to an experiment involving herbivore exclusion.

In 2007, we recorded pollinia deposition in 70 *C. rigida* flowers across all sites; 97% of these subsequently produced a capsule. In *C. tentaculata*, 12 out of 14 flowers (86%) that were observed to receive pollinia went on to set fruit. These results confirm that capsule formation is a reliable estimate of the female component of pollination success for both orchid species. Given the low pollination rates observed for *C. tentaculata,* we also determined male reproductive success (pollinia removal) for this species in 2007 at all sites except SP. Results for pollinia removal and visitation rates for *C. tentaculata* are available as Supplementary Material (S5.1). We were unable to assess pollinia removal for *C. rigida* without physically manipulating and potentially damaging the flowers. To establish whether potential pollinators of *C. tentaculata* were present at each site we used wasp baiting methods as described by Peakall (1990) and Bower (1996). Detailed methods and results are provided as Supplementary Material (S5.2).

Supplemental pollination

To determine whether capsule production was limited by insufficient deposition of pollinia, we hand-pollinated an additional 30 *C. tentaculata* flowers at the SP site in 2005, and 20 *C. rigida* flowers (ten each at MB-1 and SP) in 2006. We transferred a single pollinium to the stigma of recipient flowers located at least 5 m away and recorded capsule formation and dehiscent capsules as above.

Data analysis

To test whether the number of capsules produced and the number of dehiscent capsules differed between natural and hand-pollinated flowers, we employed Fisher's exact tests. At sites with three years of data, we used multiway contingency tests (Wald's statistic) to analyse patterns of pollination success and dehiscent capsules (binary responses) while testing for interactions between sites and years. To detect differences among sites (for each year) or among years (for each site) we used separate chi-squared tests of independence, or Fisher-Freeman-Halton (FFH) exact tests when assumptions of the chi-square statistic were not met (Freeman and Halton 1951). Significance levels of post-hoc comparisons (Fisher's exact tests) were adjusted using the sequential Bonferroni method ($P = 0.05$). To compare mean pollination success or dehiscent capsules between *C. rigida* and *C. tentaculata* we used unpaired *t*-tests (two-tailed). We tested for different variances using Levine's test for equal variance, and employed Welch-corrected *t*-tests when data were heteroscedastic.

We quantified temporal variability (S_T) using a measure described by Lehman and Tilman (2000) for calculating the temporal stability of ecological communities. Unlike the coefficient of variation, which measures sampling error, variability (S) represents actual changes through time or across space. For each population with three years of data, we calculated $S_T = \mu / \delta$, where μ is the mean pollination success and δ is the standard deviation that results from temporal variation in pollination success. Populations with a higher average annual variation, relative to the mean, will have a smaller S value. Similarly, spatial variability in pollination success (S_S) was calculated for each year using the standard deviation based on variation among populations.

We used Fisher's exact tests to directly compare rates of pollination or dehiscent capsules between *C. tentaculata* and *C. rigida* at the SP site and *C. tentaculata* and *C. carnea* at the

CCP site. Statistical analyses were carried out in JMP 4.0 (SAS Institute) and SPSS 15.0, except for Fisher-Freeman-Halton exact tests, which were done in StatXact 8 (Cytel).

5.3 Results

Supplemental pollination

Capsule production was increased by supplemental pollination for both *C. rigida* and *C. tentaculata* (Table 5.3). The proportion of dehiscent capsules was lower than pollination success for both species as a result of capsule abortion (*C. rigida*: 7%; *C. tentaculata*: 6%), capsule predation (*C. rigida*: 33%) or snapped stems (*C. tentaculata*: 12%). Despite this, more hand-pollinated flowers released seed than naturally pollinated flowers for both species (Table 5.3).

Table 5. 3 Percentage of *Caladenia rigida* and *C. tentaculata* plants pollinated and releasing seed following natural and hand pollinations.

Species	Site	Pollination method	
		Natural	Hand
Pollination			
C. rigida	MB-1	8.2 (49)	87.5 (8)***
	SP	22.7 (22)	100 (8)**
C. tentaculata	SP	1.7 (60)	56.7 (30)***
Seed release			
C. rigida	MB-1	5.4 (74)	50 (10)**
	SP	1.8 (113)	40 (10)**
C. tentaculata	SP	0.83 (121)	46.7 (30)***

Number of flowers is shown in parentheses. Significant differences between natural and hand pollinations were assessed with Fisher's exact tests. $^{**}P < 0.01$; $^{***}P < 0.0001$.

Spatio-temporal variation in pollination success

C. rigida

Pollination of *C. rigida* was highly variable among populations and among years, ranging from zero to 81% of available flowers (Fig. 5.2A). For sites with three years of data, we detected a significant site by year interaction (Table 5.4), indicating that the magnitude of among-year variation differed among sites. Single factor analyses for each year revealed that pollination success was population dependent in 2006 and 2007 (Table 5.4); however,

pairwise comparisons only detected differences between individual sites in 2007 (Fig. 5.2A). Mean pollination success of *C. rigida* ranged from 12% in 2006 to 55% in 2007 (Table 5.5).

In 2006, there were no pollinated *C. rigida* flowers in the two smallest populations (IB-2 and IB-3, < 25 flowering plants), whereas in the neighbouring IB-1 population (~ 50 flowering plants) the pollination rate was 21% and comparable to that of larger populations (21.5 and 22.7% at MB-2 and SP, respectively). To separate possible confounding effects of region and population size, we analysed the southern sites separately and found significant differences among these sites (FFH test, $P = 0.014$) with pairwise comparisons revealing that pollination success in the largest population, IB-1, was higher than in IB-3 ($P = 0.023$), and marginally higher than in IB-2 ($P = 0.098$). In contrast, during 2007, the three smallest populations (IB-2, SC-1, and SC-2), all comprising less than 25 flowers, experienced rates of pollination that were well within the range observed for larger populations (Fig. 5.2A). At the southern sites alone, among-population variation was significant in 2007 (FFH test, $P = 0.004$), and post-hoc tests revealed a higher proportion of flowers pollinated at site IB-1 than at IB-3 ($P = 0.001$), but no differences between the remaining sites. The overall rate of capsule abortion was low in all years, ranging from 0.6% in 2005 to 2.5% in 2007.

C. tentaculata

For the specialist, *C. tentaculata*, the proportion of available flowers producing a capsule ranged from zero to 16% (Fig. 5.2A). Pollination rates were too low to test for site by year interactions and FFH exact tests did not detect differences among sites in any year, or among years for any site (Table 5.4). Similarly, pollinia deposition, pollinia removal and total visitation did not differ among populations in 2007 (Supplementary Material, S5.1). Capsule abortion for this species ranged from 0% in both 2005 and 2006 to 6.7% in 2007. The number of wasp responses to bait flowers varied among sites with most activity observed at CCP (Supplementary Material, S5.2).

C. carnea

Pollination success of the generalist, *C. carnea,* was not different among years (Fig. 5.2A). No capsules were aborted in 2005 or 2006, while 2% aborted in 2007.

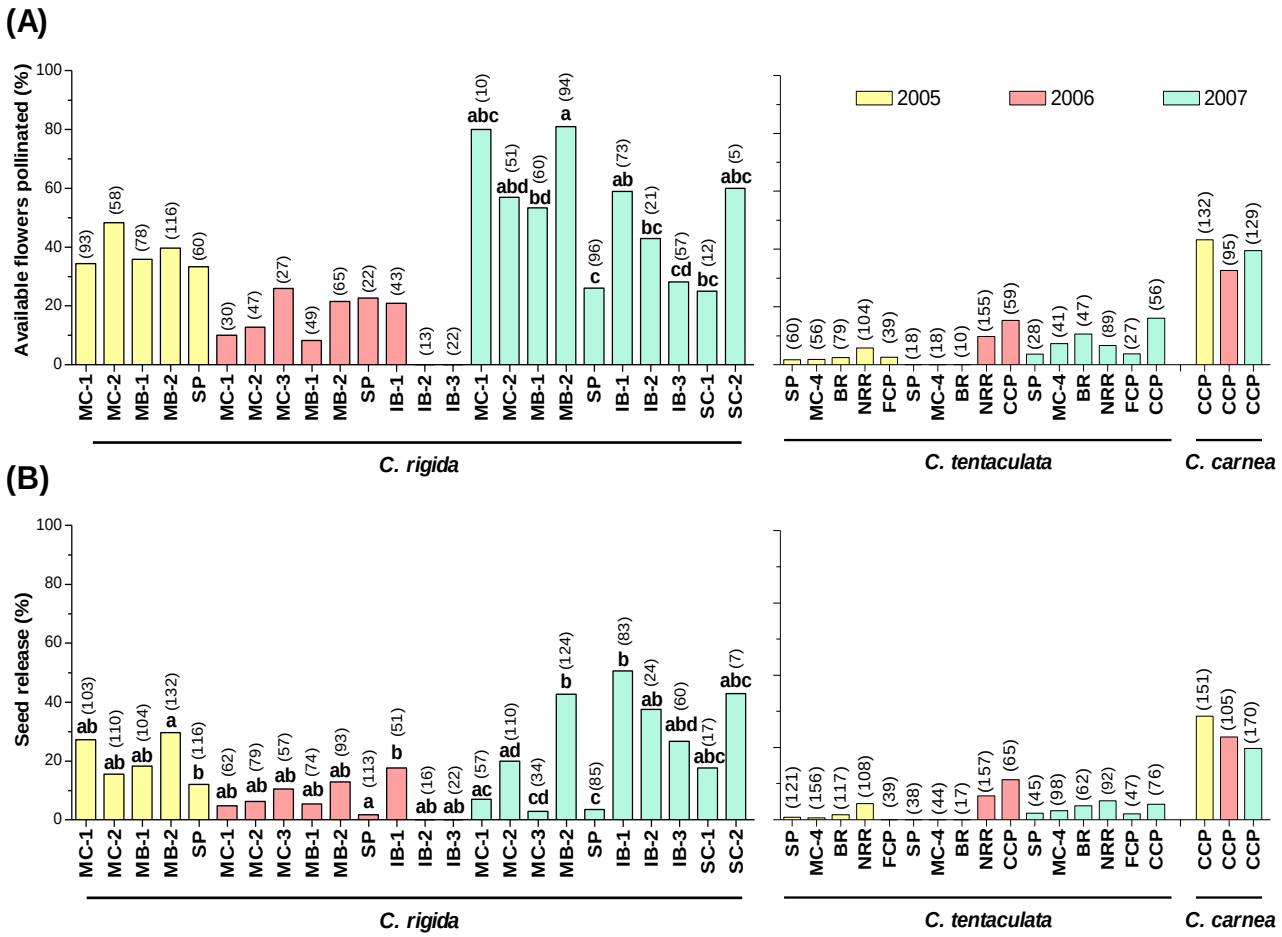

Fig. 5. 2 Percentage of available flowers pollinated (A) and percentage of flowers releasing seed (B) at each site for *Caladenia rigida*, *C. tentaculata* and *C. carnea* in 2005, 2006 and 2007. Numbers in parentheses indicate the number of monitored flowers. Sites with the same letter, within a particular year, are not significantly different ($P > 0.05$).

Table 5. 4 Comparison of *Caladenia rigida* and *C. tentaculata* pollination and successful seed release among sites and years.

	C. rigida				*C. tentaculata*	
	Site x Year	Year	Site	*n*	Site	*n*
Pollination success	33.3_8 *** [A]	61.0_2 *** [A]	17.9_4 ** [A]	929		
2005			3.9_4 [B]	405	2.4_4 [C]	338
2006			15.7_8 * [C]	318	5.5_4 [C]	260
2007			79.4_9 *** [B]	481	5.3_5 [C]	288
Seed release	18.4_6 ** [A]	27.3_2 ** [A]	47.3_3 *** [A]	1184		
2005			16.4_4 ** [B]	565	7.4_4 † [C]	541
2006			20.1_8 ** [C]	567	11.8_4 ** [C]	321
2007			92.0_9 *** [B]	601	2.4_5 [C]	420

Wald's statistic (χ^2) is shown for each factor, with degrees of freedom as subscripts. [A]Sites with three years of data were analysed using multiway contingency tests. Differences among sites in a single year were analysed with [B]Chi-square or [C]Fisher-Freeman-Halton exact tests. Site by Year interactions were not assessed for *C. tentaculata* due to low pollination rates. *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$; †$P = 0.06$.

Spatio-temporal variation in the release of seed

C. rigida

The proportion of *C. rigida* plants that released seed was highly variable among sites and years (Fig. 5.2B), with a significant site by year interaction for those sites with three years of data (Table 5.4). Analysis of separate years revealed differences among populations in each year of the study (Table 5.4). In all three years, the mean percentage of dehiscent capsules was approximately half of the percentage of capsules initiated (Table 5.5), indicating that final reproductive output is determined by other factors in addition to interactions with pollinators.

C. tentaculata

For *C. tentaculata*, the proportion of dehiscent capsules was population dependent only in 2006 (significant site effect, Table 5.4, Fig. 5.2B); however, Bonferroni-corrected pairwise comparisons did not detect differences between individual sites. Low numbers of dehiscent capsules prevented meaningful tests for site by year interactions. Over the study period, between 50 – 70% of initiated fruit progressed through to capsule dehiscence (Table 5.5).

C. carnea

In *C. carnea*, 63 – 91% of capsules went on to dehisce, across the three study years (Fig. 5.2).

Comparison of species

Mean pollination success of *C. rigida* was higher than that of *C. tentaculata* in 2005 and 2007 (Table 5.5). In general, temporal variability was lower (ie. higher *S* values) for *C. rigida* than for *C. tentaculata* (Table 5.6). Spatial variability was also lower for *C. rigida* in all three years (Table 5.6). Comparisons of *C. rigida* and *C. tentaculata* growing at the same site (SP) revealed higher pollination success for *C. rigida* in each year (Fisher's exact tests, 2005: $P < 0.001$; 2006: $P = 0.05$: 2007: $P = 0.008$) and considerably lower temporal variability (Table 5.6). Pollination of *C. carnea* was at least 2-times higher than that of *C. tentaculata* at the same site, for both years of the study (Fisher's exact tests, 2006: $P = 0.023$; 2007: $P = 0.004$). Although *C. carnea* flowers began to open about one week earlier than *C. tentaculata* at the CCP site, phenological comparisons showed that the timing of pollination events coincided with each other (Supplementary Material, S5.3).

Taking into account the effects of grazing, more C. *rigida* capsules released seed compared to *C. tentaculata*, in 2005 and 2007 (Table 5.5). Where the two species occurred at the same site (SP), *C. rigida* had a higher proportion of dehiscent capsules than *C. tentaculata* in 2005 (Fisher's exact test, $P < 0.001$). In 2006 and 2007, the percentage of *C. carnea* flowers releasing seed was two- to four-times higher than *C. tentaculata* (Fisher's exact tests, 2006: $P = 0.038$; 2007: $P < 0.001$).

Table 5. 5 Comparison of mean values (averaged across all sites) of pollination and successful seed release for *Caladenia rigida* and *C. tentaculata.*

Parameter		Mean ± s.e.m. (%)		t-test $_{df}$
		C. rigida	*C. tentaculata*	
Pollination success				
	2005	39.4 ± 3.0 (5)	2.9 ± 0.1 (5)	11.9_8 ***
	2006	12.2 ± 3.5 (9)	6.2 ± 7.1 (6)	1.3_{13} [A]
	2007	55.5 ± 7.9 (10)	8.0 ± 1.9 (6)	5.8_{10} [A] ***
Seed release				
	2005	20.7 ± 3.4 (5)	1.7 ± 1.0 (5)	5.3_5 [A] ***
	2006	6.6 ± 2.0 (9)	4.4 ± 2.9 (6)	0.6_{12}
	2007	25.8 ± 5.8 (10)	4.0 ± 0.7 (6)	3.7_9 [A] **

[A]Welch-corrected t-test. **$P < 0.01$; ***$P < 0.0001$. All analyses are based on arcsine-transformed data. Number of sites is indicated in parentheses.

Table 5. 6 Temporal (S_T) and spatial (S_S) variability ($S = \mu/\delta$) of pollination success of *Caladenia rigida* and *C. tentaculata*, relative to the average number of capsules produced.

		C. rigida	*C. tentaculata*
Temporal Variability (S_T)			
		1.2(MC-1)	0.8 (MC-4)
		1.7 (MC-2)	0.8 (BR)
		1.4 (MB-1)	3.6 (NNR)
		1.6 (MB-2)	
		5.0 (SP)	1.0 (SP)
Spatial Variability (S_S)			
	2005	6.3	1.7
	2006	2.3	0.9
	2007	2.6	1.9

Sites for which S_T was calculated are indicated in parentheses.

5.4 Discussion

Both female pollination success and the successful release of seeds varied considerably among populations and among years for the generalist, *C. rigida*. Pollination success of this species was largely determined by climatic factors rather than site characteristics, and this became particularly important when population sizes were small. Significantly, pollination and subsequent seed release by the specialist, *C. tentaculata*, was consistently lower than that of both generalists, *C. rigida* and *C. carnea*, suggesting that specialisation may place this species at a reproductive disadvantage. In all three species, interactions with both pollinators and herbivores were the primary factors limiting the successful release of seeds.

Spatio-temporal variation in pollen limitation

Supplemental pollination demonstrated that both *C. rigida* and *C. tentaculata* were strongly pollen limited. *Caladenia tentaculata* produced fewer capsules, perhaps because hand-pollination of this species was carried out later in the season when the age of some flowers may no longer have been optimal for receiving or donating pollinia (Light and MacConaill 1998). Differences in the number of capsules produced by natural *v.* hand pollinations are unlikely to stem from differences in the quality of the pollen transferred (Aizen and Harder 2007), as supplemental pollination with self- and cross-pollen has been shown to enhance capsule formation equally in *C. rigida* (Bickerton 1997). Since rates of capsule abortion were low for both natural and hand-pollinations, we conclude that fruit production in populations of *C. rigida* and *C. tentaculata* is primarily constrained by the availability of pollen rather than resources. Pollen limitation prevails amongst the Orchidaceae (Tremblay *et al.* 2005). However, our data suggest that in favourable years, some populations of *C. rigida* may be less limited by pollen availability. In 2007, rates of natural pollination in two populations approached those observed for hand-pollinated plants in the previous (drought) year. This is consistent with environmental conditions having an indirect effect on reproductive success by limiting the availability of pollinators. Studies of supplemental pollination in favourable years should help to resolve this.

Capsule production by *C. rigida* varied substantially among populations in 2006 and 2007, and also among years. However, variation among sites was not constant among years, indicating that site characteristics are not, *per se*, the main determinants of capsule production (ie. none of the populations performed consistently well or consistently poorly).

Annual rainfall in 2006 was well below long-term averages (Table 5.2), and is the most likely explanation for the three- to four-times lower pollination success observed for *C. rigida* in this year compared to 2005 and 2007. A reduction in fruit set during dry years is usually attributed to resource limitation (Jennersten and Nilsson 1993; Severns 2003; Maad and Alexandersson 2004). However, we recorded an increase in capsule production following hand-pollination in the drought year, and found similar rates of capsule abortion across all years, indicating that low fruit set in 2006 was the result of pollen limitation rather than resource constraints.

Spatial and temporal variability in fruit production has been documented for a number of orchid species (Alexandersson and Ågren 1996; Ehlers *et al.* 2002; Kindlmann and Jersakova 2006; Tremblay and Ackerman 2007) and is likely to stem from differences in pollinator assemblages in response to resource availability and habitat characteristics (Herrera 1988; Knight *et al.* 2005). Annual and regional fluctuations in climatic conditions influence not only the dynamics of insect populations (eg. population size, timing of emergence) (Herrera 1988; Roubik 2001), but also the activity of various insect species (Light and MacConaill 2002). For example, the native bee pollinators of *C. rigida* are particularly active on warm, windy days, while syrphid flies and honeybees also effect pollination in milder weather (Faast *et al.* 2009).

Several studies have identified a threshold number of flowering individuals, below which reproductive success is limited (Lamont *et al.* (1993), 6 individuals; Jacquemyn *et al.*, (2007b), 50; Spigler and Chang (2008), 15). In all of these cases, the species investigated bear inflorescences with multiple flowers (10 - 100s). Given that *C. rigida* usually produces a single flower, this species should be even more susceptible to the effects of population size. Indeed, in one year of our study, populations of *C. rigida* with less than 25 flowering individuals produced no capsules, whereas pollination in a neighbouring population of about 50 flowers was comparable to that of larger populations. While it appears that a threshold number of conspecific flowers is required to ensure capsule production, we saw no evidence for this in 2007, when weather conditions were closer to the long-term average. This suggests that small populations are prone to reproductive failure when pollinator services are low, but are more resilient during favourable years. This is supported by a recent study of two woodland orchids, demonstrating lower and more variable fruit production in small populations, particularly in response to extreme weather conditions (Jacquemyn *et al.* 2009).

A positive relationship between any component of individual fitness and numbers of conspecifics is defined as an Allee effect (Stephens *et al.* 1999). In our study, pollination of *C. rigida* may be subject to Allee effects, but the strength of these effects appears to be modified by environmental conditions. Long-term persistence may therefore depend on the frequency of years that favour pollination, as well as the potential of immigration from nearby populations. Accordingly, differences in flower abundance only partly explain among-population variability in pollen limitation. Reduced reproductive success in small populations is usually attributed to changes in the frequency or quality of pollination as a result of decreased pollinator attraction or changes in pollinator foraging behaviour (Waites and Ågren 2004; Jacquemyn *et al.* 2007b). As mentioned earlier, pollen quality is unlikely to be the cause of reproductive failure in *C. rigida*, as capsule formation is not affected by self-pollination (Bickerton 1997).

In contrast to *C. rigida*, capsule production by *C. tentaculata* was low in all populations throughout the study period. The percentage of pollinated *C. tentaculata* flowers averaged across all sites and years was 5.5 ± 2.3% (s.e.m.); considerably lower and less variable than that reported by Peakall and Beattie (1996). In their study, pollination ranged from 12% to 82% in successive years and averaged 36.4 ± 11.9% across three populations and two years. Differences between the two studies most likely reflect both temporal and spatial variations in pollination success; the populations monitored by Peakall and Beattie (1996) are located in eastern Australia and experience markedly different environmental and climatic conditions. Such high variability between studies of the same species emphasises the value of making comparisons within the same geographical region and within the same flowering seasons. Clearly, longer-term studies are required to determine whether populations of *C. tentaculata* in the Mount Lofty Ranges are also subject to large fluctuations in pollination success.

Capsule production by *C. tentaculata* in our study was also lower than that of sexually deceptive specialists from other genera (35.9 ± 6.2%), as well as other *Caladenia* species (14.0 ± 3%) (Phillips *et al.* 2009b). Since fertilisation of most of the orchids included in the above analyses relies on a single species of wasp, differences in pollination success will be tightly linked to variation in pollinator abundance. Knowledge of the population dynamics of thynnine wasps is lacking but essential for understanding spatio-temporal variation in pollination success, both within and among species and genera. According to our baiting experiments, the activity of thynnine wasps varied substantially among

populations and we failed to attract any wasps at the SP and MC-4 sites. Although the site with the highest activity (CCP) also had the highest rates of capsule production, there was no clear association at the remaining sites. Differences in pollinator behaviour could account for the low rate of capsule production compared to other sexually deceptive species. Peakall and Beattie (1996) found that only 7.5% of floral visits to *C. tentaculata* resulted in behaviour required for successful pollination, whereas for *Chiloglottis reflexa*, 80% of wasp visits resulted in attempted copulation (Handel and Peakall 1993). This low conversion from attraction to pollination may also explain the lack of correlation between wasp activity and capsule production observed in the present study.

The smallest population of *C. tentaculata* ranged from one (in 2006) to 50 (in 2007) flowering individuals and only produced two capsules throughout the entire study period. However, low rates of pollination at the remaining sites prevent us from drawing any conclusions about the impact of population size. Although sparsely spaced flowers of *C. tentaculata* are not disadvantaged with respect to pollinator visitation (Peakall and Beattie 1996), specialisation may make this species particularly vulnerable to pollinator loss when population sizes are small (Bond 1994). In small habitat fragments, reduced abundance of pollinators may be more important than the size of the orchid population itself. For example, we attracted only one wasp at FCP and none at SP, the two smallest and most isolated habitat fragments. Being parasitoids of beetle larvae (Austin *et al.* 2004), thynnine wasps are potentially highly vulnerable to the impacts of habitat fragmentation (Tscharntke *et al.* 2002).

Spatio-temporal variation in the release of seed

We have demonstrated that for all three study species, interactions with florivores played a major role in determining whether a plant released seed. Despite relatively high levels of pollination success, the proportion of *C. rigida* plants with dehiscent capsules was severely reduced in several populations. Loss of seeds following successful pollination is often attributed to abortion of fruits as a consequence of resource constraints or genetic makeup (Kärkkäinen *et al.* 1999; Severns 2003; Ågren *et al.* 2008). *Caladenia rigida* capsules rarely aborted and the loss of mature seeds was attributed firstly, to a reduction in the number of flowers available to pollinators as a result of florivory, and secondly, to predispersal predation of capsules. A concurrent study demonstrated that populations of *C. rigida* and *C. tentaculata* are subject to substantial rates of vertebrate herbivory, and that the intensity of this interaction varies at both the spatial and temporal scales (Faast and

Facelli 2009). At some sites (eg. MC-1, MC-3 and SP) over 80% of flowers were browsed and up to 35% of capsules were eaten. The magnitude of losses was less severe in the southern populations, reflecting the lower rates of herbivory observed in this region (Faast and Facelli 2009). Differences between pollination success and the release of seeds in *C. carnea* can also be attributed to florivory and capsule predation (R. Faast, unpublished data).

Patterns of capsule dehiscence can be interpreted differently at different sites. Despite large variation in the level of pollination success at site MC-1 between 2006 and 2007, the proportion of plants releasing seed was similarly low in both years. Therefore, increasing pollination in this population will not lead to greater seed output unless grazing pressure is simultaneously reduced. In contrast, supplemental pollination at other sites substantially increased the proportion of plants with dehiscent capsules in both *C. rigida* and *C. tentaculata*. This suggests that enhanced pollinator services can compensate for the impact of herbivory, but that this benefit may be site-specific. Future research should include hand-pollination over multiple years, to determine whether this increase in seed release also occurs when natural levels of pollination and/or intensity of florivory are higher.

Comparison of species

We have documented a stark contrast in capsule production and successful seed release between congeneric species with different pollination strategies. Mean rates of *C. rigida* pollination were 2 to 13-fold higher, and *C. carnea* pollination was at least 2-fold higher than that of *C. tentaculata*. This difference could reflect more efficient pollen transfer by food foraging insects, compared to sexually deceived wasps (Peakall and Beattie 1996; Phillips *et al.* 2009b). However, pollinator behaviour cannot fully explain the observed differences as Peakall and Beattie (1996) have shown that sexual deception in *C. tentaculata* is highly successful in some years. *Caladenia tentaculata* also showed greater annual and spatial variability in pollination success, relative to average capsule production, compared to *C. rigida*. The magnitude of losses between capsule initiation and capsule dehiscence was similar for *C. rigida* and *C. tentaculata*, indicating that pollination strategy does not have a significant influence on antagonistic interactions.

Evaluation of the performance of a generalist and a specialist at the same site has allowed us to eliminate habitat characteristics as a possible source of variation. Comparison of

C. rigida with *C. tentaculata,* however, does not completely remove the influence of seasonal climatic variation, as the flowering phenologies of the two species do not overlap. We have overcome this caveat by comparing co-flowering species, *C. tentaculata* and *C. carnea,* at the same site and have found that in the absence of confounding environmental effects (both habitat and climatic variation) the pollination specialist species was subject to significantly higher levels of pollen limitation. Ideally, future research should compare the pollination success of *C. carnea* and *C. tentaculata* (and other co-flowering congeners), across several populations and in different geographic regions, to test whether our tentative conclusions hold true over a greater range of species and environmental conditions.

Our results are consistent with findings that plants depending on a single pollinator are more prone to pollen limitation than those utilising several pollinating taxa (Bond 1994; Knight *et al.* 2005; Phillips *et al.* 2009b). Pollinator redundancy is likely to play a key role in maintaining levels of reproductive success in generalist plant species across a broad range of ecological and environmental conditions. In contrast, spatio-temporal variation in pollinator abundance, coupled with a low rate at which attraction is converted to successful pollination, may expose *C. tentaculata* to a high risk of reproductive failure. As a result of anthropogenic modifications, the habitat and environmental conditions experienced by the species in this study are likely to be quite different to those that have shaped evolutionary pathways. As such, the current study has focused on the demographic consequences of pollination strategy under present conditions. Species that undergo great population fluctuations may be more vulnerable to stochastic extinction if they experience a number of consecutive years of low recruitment (Bond 1994). Storage mechanisms, such as longevity or persistent seed banks, may enable some species to withstand extended periods of unfavourable conditions (Henle *et al.* 2004). Although anecdotal observations imply that some *Caladenia* species can live for up to 20 years *ex situ*, there is no accurate data available for longevity in the field. The persistence of orchid seed banks can vary substantially among species (Whigham *et al.* 2006), but the only study of *Caladenia* has shown that the seeds of *C. arenicola* survive for less than one year (Batty *et al.* 2001a). An increase in the interval between favourable years as a result of changing climatic conditions could therefore be detrimental to the long-term survival of orchid species, particularly those relying on one or a few species of pollinators. Generalist species may be more resilient to unfavourable and unpredictable climatic conditions in the short term;

however, further studies exploring the effectiveness of different pollinators are required in order to understand the long-term consequences of changes in pollinator assemblages.

Implications for conservation

This study has demonstrated that the critical effects of population size may only become evident in extremely unfavourable years. Small populations are known to be more susceptible to stochastic variation (Stephens *et al.* 1999). Our interpretation is that here we have an indirect effect of environmental conditions through reduced pollinator services (since hand-pollination did increase capsule production under unfavourable conditions). The importance of reduced fecundity in small populations may become particularly relevant if predictions of increased drought frequency hold true (Mpelasoka *et al.* 2008). Although for *C. rigida*, relatively good rates of pollination appear to alleviate the problems of low abundance in suitable years, the genetic consequences of reduced population size have not been addressed here. For instance, inbreeding depression has been shown to cause reductions in orchid seed quality (Ferdy *et al.* 2001; Wallace 2003). Assessment of the long-term viability of small populations of *C. rigida* will therefore require more detailed investigations of post-pollination stages such as seed viability and germination.

The substantial variation in space and time of the processes observed here highlights the importance of surveying multiple populations over several years to obtain an accurate assessment of the reproductive ecology of plant populations. In the present case, a single year study of *C. rigida* would have provided extremely contrasting interpretations depending on the year chosen, potentially justifying quite divergent management decisions. Furthermore, such variability is likely to affect the accuracy of meta-analyses based on data collected in different flowering seasons and from different regions. Our results also emphasise the importance of monitoring successful seed release in conjunction with fruit production, to account for complex interactions with both mutualists and antagonists. Finally, differences in the response to pollinator fluctuations suggest that pollination specialists and generalists could benefit from different management regimes. For instance, conservation and management of the habitat requirements of thynnine wasps is likely to be critical for the long-term persistence of sexually deceptive orchids.

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SUPPLEMENTARY MATERIAL

S 5.1 Male reproductive success and visitation rates for C. tentaculata

In addition to pollinia removal and deposition, we have included a measure of total visitation for *C. tentaculata*. Visitation represents all flowers that had pollinia deposited or removed, but assumes that flowers in which both events were recorded at the same time point were visited only once. This is likely to underestimate true visitation rates as not all visits necessarily result in the removal or deposition of pollinia.

Chi-square analysis revealed no difference among sites for pollinia deposition, pollinia removal or total visitation in *C. tentaculata* (Fig. S 5.1). Overall rates of pollinia deposition and removal did not differ; however, total visitation was significantly higher than pollinia deposition (visitation: 19.3 ± 0.03% (mean ± s.e.m.); pollinia deposition: 9.6 ± 0.02%; $t_8 = 2.5$, one-tailed $P = 0.02$).

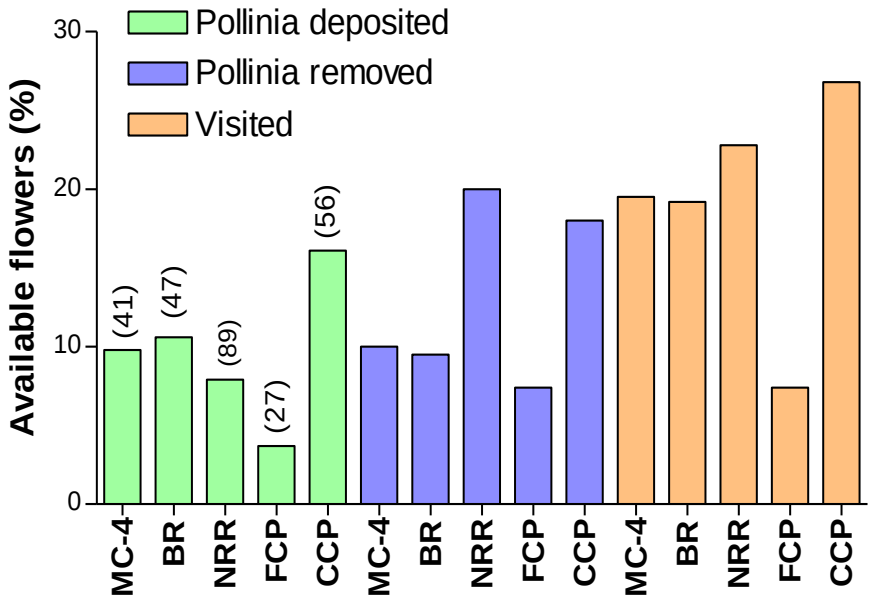

Fig. S 5. 1 The percentage of available *Caladenia tentaculata* flowers in 2007, with pollinia deposited, pollinia removed or visited. The number of flowers available for pollination at each site is shown in parentheses.

S 5.2 Pollinator baiting trials with Caladenia tentaculata

The strong attraction of thynnine wasps to the kairomones emitted by sexually deceptive orchid flowers can be exploited as a means of capturing pollinators. We used this "baiting" technique (Peakall 1990; Bower 1996) during the flowering season of 2007 to determine whether the pollinators of *C. tentaculata* were present at our sites. Three bait flowers were artificially presented adjacent to, but away from, the orchid patch and relocated every five minutes to a distance at least 20 m away. We conducted all trials between 1100 and 1500 h on warm, sunny days considered to be ideal for wasp activity. At some sites, multiple trials (10 x 5 min exposures) were conducted on different days. Trials at CCP and FCP were carried out on the same day. Since encounters between wasps and bait flowers are usually brief and do not always involve contact, we define a positive response as one in which a wasp was actively attracted to a bait flower, but did not necessarily land.

The number of responses by thynnine wasps at bait flowers was highly variable among sites (Table S 5.1). Most activity was observed at CCP, where on both days, wasps were attracted within seconds of presenting the flowers, several carrying pollinia. Of the 29 wasps responding at this site, 11 made contact with the flower; however no transfer of pollinia was observed. Two specimens were captured and identified as *Thynnoides pugionatus* (G. Brown, personal communication). Responses at the remaining sites were much lower, with no wasps observed at the SP and MC4 sites.

Table S 5. 1 *Caladenia tentaculata* pollinator baiting experiments carried out in 2007.

Site	Trial No.	No. wasp responses
SP	1	0
	2	0
	3 [b]	0
BR	1 [b]	3
MC4	1 [b]	0
	2	0
NRR	1	4
FCP	1	1
	2 [a]	0
CCP	1	19
	2 [a]	10

Each trial consisted of 10 x 5 minute exposures.
Trial numbers with the same letter superscript were conducted on the same day.

Phenology of C. tentaculata and C. carnea flowering and pollination

To confirm that *C. tentaculata* and *C. carnea* flowered at the same time at the CCP site, we calculated the percentage of open flowers and pollinated flowers at each monitoring time point over the flowering season. Although *C. carnea* flowers opened a few days earlier than *C. tentaculata*, most pollination events occurred over the same time period (Fig. S 5.2).

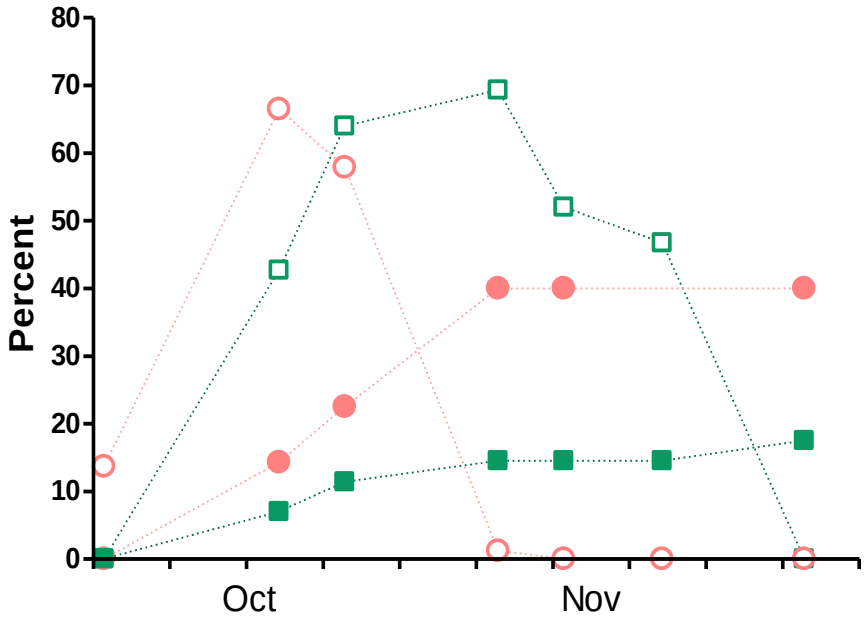

Fig. S 5. 2 Phenology of flowering (open symbols) and pollination (solid symbols) for *Caladenia tentaculata* (squares) and *C. carnea* (circles) at the CCP site in 2007. Note that the percentage of pollinated flowers is cumulative while the percentage of open flowers is non-cumulative.

CHAPTER 6

MB2 site at Millbrook Reservoir (Photo by author)

Chapter 6: Preamble

CHAPTER 6 examines the extent to which features that have the potential to affect a flower's apparency can influence both mutualistic and antagonistic interactions. The influence of flower height, density of conspecific flowers and concealment amongst neighbouring vegetation was assessed with respect to the pollination success, florivory and capsule predation of *Caladenia rigida*. Vegetation removal experiments were used to evaluate the impact of neighbourhood plants on biological interactions as well as on plant emergence and flowering.

This chapter has been prepared as a submission for publication in *Oecologia*. Factors affecting the apparency of *Caladenia tentaculata* flowers, with respect to their risk of herbivory, were also investigated and these findings are presented in APPENDIX C.

Contributions and signatures of authors:

Renate Faast

Designed experiments, collected and analysed all data and prepared manuscript as principle and corresponding author.

Signed..... ..... Date.29th June 2010.

José M. Facelli

Sought and won funding, supervised development of research and evaluated manuscript.

Signed..... ..... Date.....15/06/2010.....

Andrew D. Austin

Sought and won funding, advised on aspects of research and evaluated manuscript.

Signed..... ..... Date.30th June 2010.

TO HIDE OR NOT TO HIDE: THE INFLUENCE OF APPARENCY ON THE POLLINATION AND HERBIVORY OF AN ENDANGERED TERRESTRIAL ORCHID.

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Abstract

Apparency influences the rate at which a plant is discovered by both its pollinators and its herbivores. The degree of apparency for any individual plant can be determined intrinsically by its genotype, as well as extrinsically by characteristics of the local environment and the plant community. This study simultaneously examines the influence of apparency on the pollination success and vertebrate herbivory of an endangered terrestrial orchid, *Caladenia rigida* R.S. Rogers. We assessed the effect of floral height, the local density of conspecifics, and concealment amongst neighbouring vegetation, on pollination and successful seed release among five orchid populations, over three years. Seed release was the net outcome of conflicting interactions with pollinators, florivores and fruit predators and varied both spatially and temporally. However, we found no strong evidence for a compromise between pollination success and predation risk for any of the factors examined. Floral height had a positive effect on pollination success and the subsequent release of seed in one year, while both local density and concealment affected the risk of floral browsing in all three years of the study. Experimental removal of neighbouring vegetation did not affect the final proportion of plants that released seed, but increased the rate of discovery by florivores. Our data suggest that pollinators and herbivores respond to different visual and/or olfactory cues when locating flowers of *C. rigida*. Based on different predation rates for flowers and seed capsules, we propose that under intense grazing pressure, pollination soon after anthesis can provide plants with a reproductive advantage.

6.1 Introduction

The ability to attract pollinators is essential for the reproductive success of plants relying on animals for ovule fertilization. As such, the ease with which plants are located, or their apparency, can be an important determinant of pollination success. Many plants have evolved traits that optimise their attractiveness, such as colour, size or stature, or the number of flowers produced (Jersáková and Kindlmann 1998; Tremblay 2005; Gomez *et al.* 2006; Brys *et al.* 2008). These intrinsic traits are often genetically controlled and can be subject to pollinator-mediated selection. However, characteristics of the biotic community can also play a role in determining whether, or how often, a particular plant is visited. For instance, the abundance and density of conspecific plants, the composition of neighbouring vegetation, as well as the diversity and behaviour of the pollinating vectors, can all influence visitation, pollination and fruit set (Sih and Baltus 1987; Herrera 1995; Juillet *et al.* 2007; Brys *et al.* 2008).

The contribution of a plant to subsequent generations is determined by interactions with both mutualists (eg. pollinators or seed dispersers) and antagonists (eg. herbivores, florivores or seed predators), yet these associations are rarely studied in combination. Some traits or habitat characteristics that enhance a plant's attractiveness or apparency to mutualists may simultaneously increase the risk of discovery by enemies (Feeny 1976). Floral display and flower height have been shown to present a trade-off between fruit production and grazing or seed predation for several species (Gómez 2003; Ågren *et al.* 2006; Kolb *et al.* 2007), and selection on plant traits therefore represents a delicate compromise between these opposing interactions.

Population size and local plant density can also have important consequences for both pollination and herbivory. Large or dense displays of flowers may present a stronger visual signal attracting pollinators and herbivores from afar. In addition, aggregated plants can benefit from having more pollen donors nearby, and the pollinators themselves may prefer patches with shorter inter-plant distances (Sih and Baltus 1987; Cheptou and Avendaño 2006; Le Cadre *et al.* 2008). Similarly, optimal foraging theory suggests that the probability of predation should increase with prey density (MacArthur and Pianka 1966). While several studies support this hypothesis (Jennersten and Nilsson 1993; Ehrlén 1996; Sletvold and Grindeland 2008), the intensity and direction of density-dependency varies among plant-herbivore interactions and is influenced by traits such as the search and detection method employed, motility, and diet breadth of the herbivore (Kunin 1999;

Masumoto *et al.* 2000; Shea *et al.* 2000). Theory predicts that while large aggregations may be more conspicuous to predators that locate their prey visually, the detection risk for each individual may be disproportionately lower compared to solitary individuals (Vine 1973). In addition, visual or physical interference by neighbouring vegetation can offer significant protection against grazing by vertebrate herbivores (Callaway *et al.* 2000; Facelli and Temby 2002; Miller *et al.* 2006). Plants that are concealed may evade detection by predators, but few studies have examined whether concealment renders plants more difficult to find by their mutualists.

Orchids may be particularly vulnerable to factors that limit pollinator interactions because fecundity is usually restricted by the availability of pollen rather than resources (Tremblay *et al.* 2005). Numerous studies have investigated relationships between orchid pollination success and plant attributes, population size or density, and habitat characteristics (reviewed in Tremblay *et al.* 2005). For example, correlations between flower height and pollen transport have been attributed to visual attractiveness (Kropf and Renner 2005), pollinator preferences (Handel and Peakall 1993), or both (Dickson and Petit 2006). The local density of conspecific flowers has been shown to have positive (Tremblay *et al.* 2007; Brys *et al.* 2008), neutral (Meléndez-Ackerman and Ackerman 2001), or negative (Internicola *et al.* 2006) effects on pollination or seed production in orchids. To our knowledge, however, no studies have investigated the impact of plant traits or density on successful seed release in orchids; that is, the outcome of interactions with both pollinators and herbivores.

The influence of microhabitat on the pollination success of orchids also varies among species. While some species produce fewer capsules when growing amongst vegetation compared to in the open (Inoue 1985; Petit and Dickson 2005), some benefit (O'Connell and Johnston 1998; Juillet *et al.* 2007), and others are unaffected by plant cover (Inoue 1985; Handel and Peakall 1993). Microhabitat has also been shown to affect the risk of orchid predation (Inoue 1985; Gregg 2004; Petit and Dickson 2005). This is important not just conceptually, but also because management recommendations for threatened terrestrial orchid species often include strategies aimed at removing competing vegetation (Todd 2000; Coates *et al.* 2003; Kull *et al.* 2006). This is largely based on observations that disturbances such as fire, coppicing or mowing can promote emergence and flowering in some species (Wotavova *et al.* 2004; Coates *et al.* 2006; Jacquemyn *et al.* 2008; Coates and Duncan 2009). However, most comparisons of the impacts of such disturbances are

made across widely separated populations and are subject to site-specific influences. Furthermore, subsequent effects on fruit set are only rarely considered (Wake 2007; Jacquemyn *et al.* 2008). While large-scale removal of vegetation may stimulate flowering of the focal species, a concomitant reduction in food and nesting resources for potential pollinators may negate the benefits of increased flowering, particularly if the focal plants offer little or no reward themselves. In addition, the absence of cover vegetation may leave both pollinators and focal plants more vulnerable to predation.

Caladenia rigida R.S.Rogers is an endangered terrestrial orchid, endemic to the Mount Lofty Ranges in South Australia. The single-flowered species is pollen limited and pollination success varies both temporally and spatially (CHAPTER 5). Intense vertebrate florivory and capsule predation significantly reduce the proportion of flowers that release seed relative to rates of pollination success, particularly within the northern region of the species' distribution (Faast and Facelli 2009, CHAPTER 5). Conservation management of this species therefore requires careful consideration of factors that influence interactions with both mutualists and antagonists. Fire is reported to promote the flowering of *C. rigida*, and the use of fire or slashing has been recommended as a management tool (Bates 1995). However, the influence of vegetation removal on reproductive output is unknown.

In the present study, we assessed whether factors that may increase the apparency and hence pollination success of *C. rigida,* also increase the orchid's risk of predation. As flowers of *C. rigida* are considerably more conspicuous than its capsules (at least from a human perspective), we also examined separately the relative risk of predation of these two life-states. Given the spatial and temporal variability of reproductive success for this species (CHAPTER 5), the study was carried out across five populations over three years. In addition we set up vegetation removal experiments at two spatial scales; one involving removal of vegetation in the immediate vicinity of selected orchids (30 cm radius), and the other consisting of slashing all vegetation within the surrounding area (~900 m^2). We expect that these treatments could affect orchid reproductive success differently. Localised removal of vegetation is likely to affect the microclimate around the target plant (eg. light availability, soil moisture) as well as floral apparency, but it is unlikely to influence the abundance of insects amongst the orchid population. In contrast, removal of vegetation over a larger area might have additional implications by altering the overall biotic and abiotic environment.

We specifically addressed the following questions: 1. Do characteristics that can affect the apparency of flowers (ie. flower height, local density of conspecifics, and concealment amongst neighbouring vegetation) influence pollination success, browsing and hence final seed release of the orchid? 2. Are flowers and capsules equally at risk of predation? 3. Does the removal of neighbourhood vegetation affect biotic interactions with the orchid?

6.2 Methods

Species description

Caladenia rigida, the rigid white spider orchid, is a tuberous, perennial orchid that produces one elongate leaf in late autumn and typically bears a single, predominantly white flower measuring 4 - 6 cm across, at the end of a 10 - 30 cm stem. The orchid is non-clonal, producing a replacement tuber annually, and within a season can exist in one of three states: dormant tuber, vegetative (leaf only) or reproductive (leaf and flower). Buds begin opening early in spring, and unless pollinated, remain open for up to four weeks. *Caladenia rigida* is a food-advertising orchid pollinated by a diverse suite of generalist insects, including native bees and syrphid flies (Faast *et al.* 2009). A recent study detected concentrated sugars on the labellum and at the base of the column (Faast *et al.* 2009), but their role in attracting and sustaining the interest of pollinators has not been determined. Once pollinated, flowers close within 1 - 2 days to form a green capsule that takes 6 - 8 weeks to ripen and dehisce. Within populations, plants are often patchily distributed as loose clusters interspersed with isolated individuals.

Caladenia rigida is subject to high rates of florivory (up to 94%) and a recent study identified *Corcorax melanorhamphos* (the white-winged chough) as a predominant florivore in several populations (Faast and Facelli 2009). The birds consume the entire perianth and are quite deliberate and selective in their predation of *C. rigida*. Floral damage by invertebrates is low but florivory of *C. rigida* at sites not frequented by white-winged choughs suggests that other vertebrates also consume orchid flowers. Some populations also suffer from high rates of capsule predation (up to 35%), and although the herbivore(s) responsible have not been identified, the success of exclusion guards indicate that vertebrates rather than invertebrates are implicated (Faast and Facelli 2009).

Study sites

The study was carried out from 2005 to 2007, in five populations of *C. rigida* located in the northern region of the species' distribution in the Mount Lofty Ranges, South Australia. Each population contained more than 150 reproductive individuals (in 2005). Two populations were adjacent to one another in Mount Crawford Native Forest Reserve (MC1 and MC2), the first being within a kangaroo- and deer-proof exclosure. Two were located close to Millbrook Reservoir (MB1 and MB2) and one was near South Para Reservoir (SP). Detailed site descriptions are provided in Faast and Facelli (2009).

The MC and MB sites are *Eucalyptus* woodlands (*E. obliqua*, *E. leucoxylon* and *E. fasciculosa*) with an understorey dominated by *Acacia pycnantha*, *Pultenaea daphnoides*, *Leptospermum myrsinoides*, *Hibbertia* spp., *Platylobium obtusangulum* and *Lepidosperma* spp. At the SP site, *C. rigida* occurs in open woodland comprised of *E. fasciculosa* and *Allocasuarina verticillata* with a sparse shrub layer including *Acacia paradoxa*, *Hibbertia* spp., *Gonocarpus elatus* and *Lepidosperma* spp. Average annual rainfall in the region is 761 mm (Australian Government Bureau of Meteorology) in a Mediterranean-type climate with cool, wet winters and hot, dry summers.

Monitoring plant status and local environs

At each site, at least 100 flowering plants were randomly selected and labelled with a concealed metal tag. We monitored plants every one to two weeks throughout the reproductive season, recording flower height (vertical distance from the ground to the base of the ovary), pollination success (pollinia deposition or capsule formation), florivory (removal of entire perianth), capsule predation and capsule dehiscence (ie. successful seed release). In a concurrent study we demonstrated that 97% of pollinia depositions in *C. rigida* lead to the formation of a capsule (CHAPTER 5), justifying the use of either of these measures when recording pollination success. Flower height was not obtained for all flowers because many were browsed before measurements could be taken. Most florivory occurred at the beginning of the flowering season (Faast and Facelli 2009), thus reducing the number of flowers available for pollination. We therefore strived for a more accurate assessment of factors that may influence pollination, by excluding grazed flowers (but not grazed capsules) from our analyses, and we refer to this as the pollination of available flowers. Successful seed release was calculated as the proportion of dehiscent capsules relative to the total number of tagged flowers, including grazed flowers and capsules.

We obtained an estimate of floral concealment by categorising each flower as exposed (no vegetation within 10 cm of the flower), concealed (some vegetation within 10 cm) or well-concealed (flower coming into contact with and hidden amongst vegetation). Orchid plants showed no association with any particular neighbourhood species and grew amongst any of the low heath species representing the understorey, including *Hibbertia* spp., *P. obtusangulum*, *Lepidosperma* spp. or rarely, *Xanthorrhoea semiplana*. Despite intense browsing of orchids at some sites, the surrounding vegetation was not noticeably grazed and the degree of floral concealment remained constant throughout the flowering season (R. Faast, pers. obs.). We therefore analysed the influence of concealment on pollination, florivory, capsule predation and capsule dehiscence of all tagged flowers, based on assessment at the beginning of the monitoring period.

At each monitoring time we quantified the number of open conspecific flowers within a 35 cm radius of the focal plant, and we refer to this as the local density of conspecifics. A density of one represents a solitary focal plant. Local density is a dynamic measure, varying throughout the monitoring period as nearby flowers are pollinated or browsed. When attempting to tease apart the influence of this factor, it is important to consider the local environment as perceived at the time of pollination or florivory. As such we chose a "snapshot" approach by selecting the monitoring time with the highest rate of pollination or florivory and analysing the effect of density recorded at that time. In contrast, when determining the impact of local density on successful seed release (final reproductive output) we used the local density recorded at the peak of the flowering season. We did not assess the influence of density on capsule predation because the number of capsules consumed at any one time point was too low to permit meaningful statistical analyses.

In 2006, severe drought conditions (average rainfall of 485 mm for the northern sites) coincided with low rates of flowering, pollination success and hence, seed release (CHAPTER 5), and so we have only assessed impacts on florivory in that year. In 2007, we included only the control plants from the slashing experiment at the MC1 site (see below). However, this site was excluded when testing the influence of flower height and concealment on the pollination of available flowers, as there were only seven non-browsed plants at the end of the monitoring period. Plants at the MB1 site were caged in 2007 to exclude herbivores, so data from this site were omitted from analyses of florivory and seed release.

Vegetation removal experiments

Two vegetation removal experiments, differing in spatial scale, were carried out in the autumn of 2007 when orchid leaves were just beginning to emerge, but well before the emergence of floral buds. Since the amount of surrounding vegetation varied among orchid plants, all tagged plants were assigned a "vegetation score" (VS) prior to treatment. This involved assessing the presence of vegetation at 10 cm increments from ground level up to 50 cm in height, at each of five points (centre plus 20 cm from the orchid plant at each major compass point). The maximum VS of 25 therefore represents a plant that is completely surrounded by vegetation within a radius of 30 cm. In both experiments, vegetation within 30 cm of each target plant was pruned to 5 cm above ground level using hand-secateurs, and collected, dried and weighed. The dry weight of vegetation collected in the vicinity of orchid plants was highly correlated with the VS assigned to them (Spearman Rho, $r_s = 0.65$, $n = 81$, $P < 0.0001$), validating the use of VS as an indictor of vegetation biomass. To determine whether removal of surrounding vegetation affects emergence and flowering in subsequent years, we recorded emergence status as non-emergent (NE) or emergent (E), and flowering status of emergent plants as leaf (L) or flower (F), during the flowering seasons of 2007 and 2008.

Slashing experiment

To examine the impact of standing vegetation on the behaviour of pollinators and herbivores, we carried out a broad scale slashing experiment at MC1. An area of 30 m x 30 m, encompassing approximately half of the tagged orchid plants was assigned to a slashing treatment while the remaining tagged plants remained as non-slashed controls. Following the removal of vegetation around target plants, intervening understorey vegetation was slashed to 10 cm above ground level with a brush-cutter, then raked and removed from the site. Pollination success, florivory and capsule dehiscence of tagged plants were monitored throughout the following flowering season. Due to high rates of florivory at this site we analysed the impact of slashing on the pollination of available flowers (excluding grazed flowers).

Localised vegetation removal

At the MB1 site, we selected 72 tagged plants that were at least 60 cm apart to prevent overlap between treatments. To ensure that the amount of vegetation removed was relatively consistent between treatment and control plants, pairs of orchids were matched up as closely as possible, based on their VS. Within each pair, one plant was randomly

assigned to the vegetation removal treatment and the other was left as a control. We placed protective guards around all plants to allow us to assess pollination success in the absence of florivory. Cylindrical guards (0.6 m tall x 0.5 m wide) were made of 40 mm hexagonal wire mesh with an enclosed top and have been demonstrated to effectively exclude florivores of *C. rigida* without impeding pollinators (Faast and Facelli 2009). Of the tagged plants, 52 produced flowers (26 in each treatment) and these were monitored throughout the subsequent flowering season for successful pollination and seed release. In addition, we monitored all other *C. rigida* flowers within each guard. In a concurrent study, we demonstrated that both seed number and the proportion of viable seeds are highly correlated with capsule width (CHAPTER 7). We therefore assessed the effect of vegetation removal on seed output by measuring the width of capsules (using vernier callipers) just prior to dehiscence.

Data analysis

We examined the effect of concealment and population on flower height using two-way ANOVA followed by Tukey-Kramer HSD post-hoc comparisons to detected differences in flower height among concealment categories. To account for the relationship between flower height and concealment, we first used logit models that included three- and two-way interactions between the explanatory variables (concealment, flower height and population) testing for their effect on each of the binary dependent variables (pollination, florivory, capsule predation and capsule dehiscence). None of these interactions were significant ($P > 0.25$), so we analysed the relationship between the dependent variables and concealment or flower height separately, in each case including their interaction with population. This provided us with a greater sample size and hence statistical power when assessing the influence of concealment (as we did not have height data for all flowers). Logit models were also employed to evaluate the effect of local density (and its interaction with population) on each of the dependent variables. We employed likelihood ratio tests (G^2) to assess the contribution of individual explanatory variables within each model. Non-significant interaction terms were removed to retain the most parsimonious model (Underwood 1997). The direction of significant effects was determined using values of the coefficient estimate (B) and the corresponding odds ratio. Where interactions between population and flower height or density were significant ($P < 0.05$), we carried out single-factor analyses for each population using logistic regression. We used logit models to determine the effect of flower status (open flower versus pollinated capsule) on the risk of

predation, followed by Fisher's exact tests at individual sites when flower status x population interactions were significant.

Spearman's rho correlation coefficient was used to measure the strength of the relationship between VS and dry weight of collected vegetation. For the vegetation removal experiment, we employed contingency analyses (Fisher's exact tests) to test for differences in pollination, florivory and capsule dehiscence between vegetation removal and control treatments. We expect that the impact of these treatments depends on the amount of vegetation removed, so we also analysed orchids with a pre-slashing VS >10, separately. Fisher's exact tests were employed to assess the influence of vegetation removal on subsequent emergence status (NE, E) or flowering status (L, F) in 2007 and 2008. We used the statistical package JMP 4.0 (SAS Institute) for ANOVA, correlation and contingency analyses, and SPSS 15.0 for logit models.

6.3 Results

6.3.1 Influence of apparency on biotic interactions

Flower height

Floral height varied among populations and among categories of concealment in 2005 (ANOVA, population: $F_{4,318} = 11.7$, $P < 0.0001$; concealment: $F_{2,318} = 7.9$, $P = 0.0005$), whereas in 2006 variation among concealment categories was only marginally significant (population: $F_{4,296} = 2.8$, $P = 0.026$; concealment: $F_{2,296} = 2.5$, $P = 0.08$). In 2007 there was no relationship between flower height and concealment. Post-hoc comparisons revealed that well-concealed flowers (height = 23.9 ± 0.6 cm (mean ± s.e.m.)) were taller than concealed (21.8 ± 0.4 cm) and exposed (20.8 ± 0.5 cm) flowers in 2005.

Pollination success averaged 15% in 2006 to 59% in 2007 (Table 6.1) and was positively related to flower height in 2005 but not in 2007 (Table 6.2). The overall percentage of browsed flowers also varied among years (Table 6.1) and was not influenced by flower height in 2005 or 2006 (Table 6.2). However, in 2007 the effect of height on florivory varied among populations (significant height x population interaction, Table 6.2) and single factor analysis detected a negative effect of height in the MB2 population. A similar result was obtained when the analysis was repeated on exposed flowers only (data not shown), confirming that concealment was not confounding the effect of flower height on florivory.

Among years, an average of 15 - 25% of capsules were eaten (Table 6.1) but the height of seed capsules did not influence their probability of being browsed (Table 6.2). Capsule abortion was low in all three years (Table 6.1) and the proportion of plants releasing seeds is therefore a function of capsule formation and capsule predation. The probability of seed release increased with flower height in 2005 and 2007 (Table 6.2).

Table 6. 1 Pollination success, florivory, capsule abortion, capsule predation, capsule dehiscence and flower height of *Caladenia rigida*, averaged across populations (mean ± s.e.m.) for each year.

Population	2005	2006	2007
Pollination (avail. flwrs) (%)	38.3 ± 2.7	15.0 ± 3.0	59.4 ± 10.1
Browsed flowers (%)	20.5 ± 3.4	42.4 ±10.3	60.9 ± 12.2
Aborted capsules (%)	0.6 ± 0.6	3.3 ± 3.3	5.4 ± 2.9
Browsed capsules (%)	21.4 ± 6.1	15.3 ± 9.1	25.2 ± 1.9
Dehiscent capsules (%)	20.5 ± 3.4	6.2 ± 1.8	18.3 ± 8.9
Flower height (cm)	21.7 ± 1.0	22.3 ± 0.6	23.2 ± 0.8

Table 6. 2 Effect of flower height, population and their interaction on the pollination of available flowers, florivory, capsule predation and capsule dehiscence of *Caladenia rigida.* Likelihood ratios (G^2) are shown for each factor, with degrees of freedom as subscripts. Non-significant interactions were removed from the model and the analysis repeated.

	Year	Population x Flower ht	Population	Flower height	Individual sites [a]	N
Pollination avail.	2005	1.7_4	4.0_4	**11.0_1***** [b]		322
	2007	3.3_3	**58.8_3*****	1.7_1		344
Florivory	2005	$<0.01_4$	9.0_4 [†]	0.01_1		224
	2006	6.1_4	**48.1_4*****	0.9_1		234
	2007	**8.0_3***	**29.3_3*****	3.6_1 [†]	MB2** [c]	150
Capsule predation	2005	2.0_4	3.3_4	0.1_1		101
	2007	0.9_3	2.8_3	0.5_1		130
Capsule dehiscence	2005	2.7_4	1.7_4	**10.4_1**** [b]		326
	2007	5.6_3	**29.2_3*****	**8.5_1**** [b]		282

[a] Significant population by flower height interactions were followed by single-factor analyses at each site. [b] Positive relationship; [c] Negative relationship. *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$; [†]$P < 0.1$

Concealment in neighbouring vegetation

The concealment of flowers did not influence their probability of being pollinated but had a significant effect on florivory in all three years. While the intensity of florivory varied among populations, there were no population x concealment interactions, and the risk of being browsed was consistently higher for exposed flowers (Fig. 6.1, Table 6.3). In 2005, exposed capsules were also at greater risk of being consumed (Table 6.3) but we did not detect an effect in 2007, probably due to the scarcity of well-concealed capsules (seven). Well-concealed flowers were more likely to release seeds in 2005, but there was no relationship in 2007 (Table 6.3).

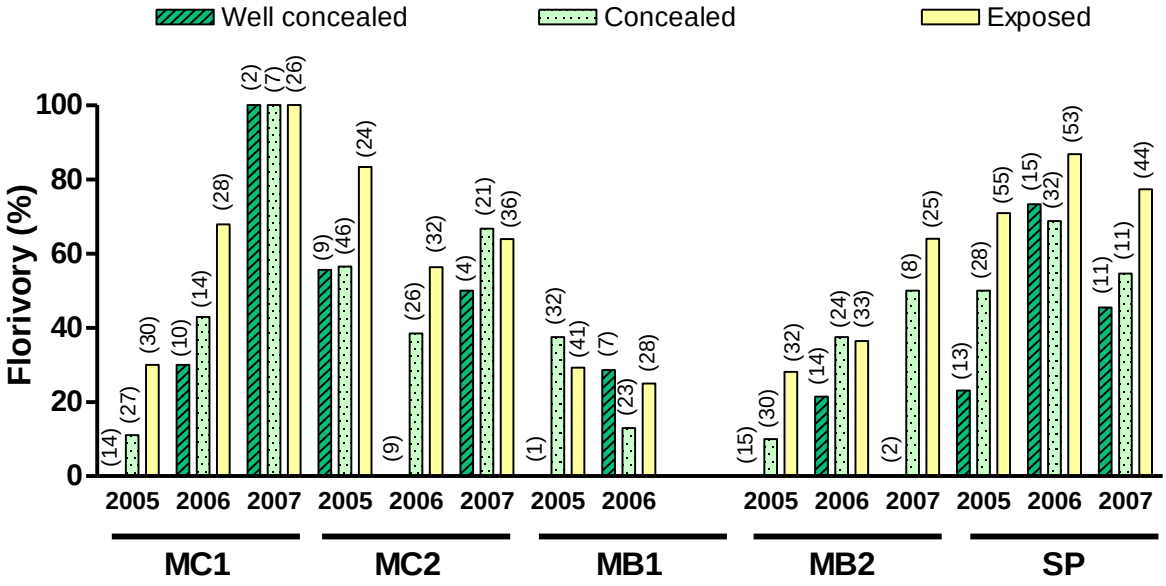

Fig. 6. 1 Percentage of flowers browsed within each category of concealment, across the three study years at each site. A caging experiment at MB1 in 2007 precluded the use if this population for analyses of florivory. Numbers in parentheses indicate the number of monitored flowers.

Table 6. 3 Effect of concealment, population and their interaction on the pollination of available flowers, florivory, capsule predation and capsule dehiscence of *Caladenia rigida.* Likelihood ratios (G^2) are shown for each factor, with degrees of freedom as subscripts. Non-significant interactions were removed from the model and the analysis repeated.

	Year	Population x Concealment	Population	Concealment	N
Pollination avail.	2005	6.7_8	**10.0_4***	0.2_2	369
	2007	10.7_6	**32.0_3*****	1.7_2	288
Florivory	2005	11.8_8	**76.2_4*****	**24.6_2***** [c]	397
	2006	10.9_8	**67.1_4*****	**13.9_2***** [c]	348
	2007	3.4_6	**28.2_3*****	**6.1_2*** [c]	197
Capsule predation	2005	7.2_8	**11.3_4***	**6.9_2*** [c]	127
	2007	0.1_4	3.5_3	1.7_2	124
Capsule dehiscence	2005	4.1_8	7.5_4	**5.8_2*** [b]	530
	2007	4.5_6	**51.5_3*****	0.55_2	321

[b] Positive relationship; [c] Negative relationship. *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$.

Local density of conspecifics

The range and mean number of flowers within a 35 cm radius of target plants varied among sites in all three years (Table 6.4); however, the local density of *C. rigida* flowers did not influence their probability of being pollinated (Table 6.5). The risk of florivory was dependent on the local density of conspecifics, and the strength and direction of this effect varied among populations, as indicated by a significant density by population interaction in 2005 and 2007 (Table 6.5). Testing the main effect of density at each site separately detected a positive relationship with florivory at two sites in 2005, whereas in 2007 the relationship was positive at two sites, but negative at another (Table 6.5). In 2006, the effect of density was consistent among sites, and the probability of florivory increased with the number of nearby conspecific flowers. Seed release was strongly influenced by the local density of conspecifics in 2007; however, this effect varied among populations (significant density x population interaction, Table 6.5) and analysis of individual populations revealed a negative relationship with capsule dehiscence at two sites. In 2005 there was no relationship between capsule dehiscence and local density.

Table 6. 4 Number of flowering plants and the range of local density (number of conspecifics within 35 cm of target plant) recorded within populations of *Caladenia rigida* in each of the study years.

Population	No. flowering individuals			Local density of conspecifics		
	2005	2006	2007	2005	2006	2007
MC1	150	80	200	1 – 10 (2.5)	1 – 5 (2.2)	1 – 10 (3.4)
MC2	150	100	200	1 – 4 (1.8)	1 – 4 (1.8)	1 – 5 (2.1)
MB1	150	80	200	1 – 10 (2.7)	1 – 3 (1.7)	1 – 11 (3.5)
MB2	250	120	500	1 – 4 (1.5)	1 – 4 (1.7)	1 – 9 (2.3)
SP	800	400	1500	1 – 15 (4.1)	1 – 13 (3.6)	1 – 26 (6.5)

Mean values for local density are shown in parentheses.

Table 6. 5 Effect of local density, population and their interaction on pollination, florivory and capsule dehiscence of *Caladenia rigida.* Likelihood ratios (G^2) are shown for each factor, with degrees of freedom as subscripts. Non-significant interactions were removed from the model and the analysis repeated

	Year	Population x Density	Population	Density	Individual sites [a]	N
Pollination	2005	6.0_4	2.5_4	0.34_1		421
	2007	4.9_4	$\mathbf{18.1_4}$**	0.5_1		318
Florivory	2005	$\mathbf{12.7_4}$ **	$\mathbf{36.4_4}$***	$\mathbf{5.3_1}$*	MB1*** [b], SP ** [b]	288
	2006	6.8_4	4.2_4	$\mathbf{5.0_1}$* [b]		239
	2007	$\mathbf{9.9_4}$*	$\mathbf{12.6_4}$**	$\mathbf{4.2_1}$*	MC1† [b], MC2† [b], SP† [c]	245
Capsule dehiscence	2005	7.3_4	$8.5_4^{\dagger}$	0.03_1		530
	2007	$\mathbf{8.0_3}$*	$\mathbf{26.4_3}$***	$\mathbf{10.0_1}$**	MC1† [c], MC2** [c]	354

[a] Significant population by density interactions were followed by single-factor analyses at each site.
[b] Positive relationship; [c] Negative relationship. *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$; $^{\dagger}P < 0.1$

Flower status

Flower status (open versus pollinated capsule) affected the risk of predation in 2007 ($G^2_1 = 40.0$, $P < 0.001$). Although the strength of this effect varied among populations (flower status x population interaction, ($G^2_3 = 8.8$, $P = 0.032$), capsules were less likely to be browsed in all of the four populations analysed in 2007 (Fig. 6.2). There was no relationship between flower status and predation in 2005, when levels of florivory were generally lower (see Table 6.1).

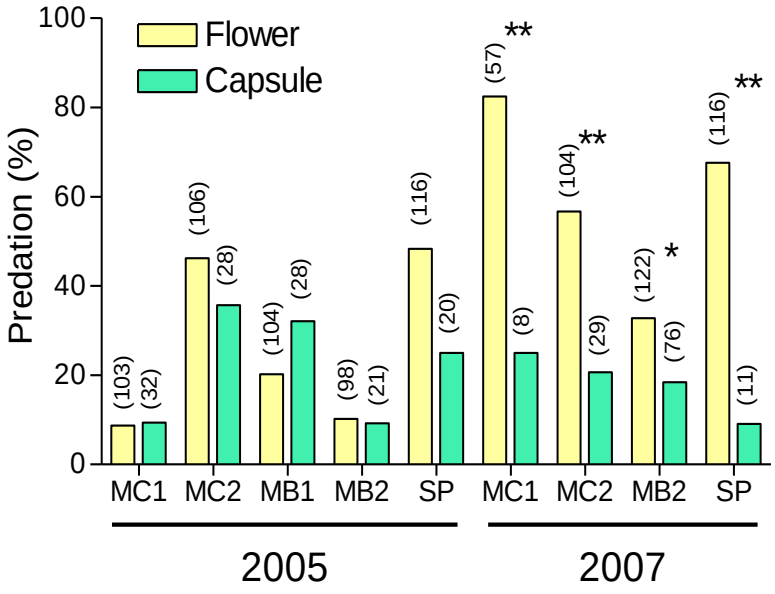

Fig. 6. 2 Predation of *Caladenia rigida* open flowers and capsules in 2005 and 2007. Pair-wise comparisons at each site were carried out using Fisher's exact tests. **P*<0.05; ***P*<0.01.

6.3.2 Vegetation removal experiments

Broad-scale slashing experiment

Extremely high rates of florivory at MC1 in 2007 meant that few flowers were available for pollination, and made meaningful comparisons between treatments difficult. We thus failed to detect an effect of slashing on pollination or successful seed release, regardless of the amount of vegetation that had been removed (Fig. 6.3A). The proportion of grazed flowers also did not differ between treatments; however, flowers within the slashed area were more likely to be browsed within the first 10 days of flowering than those surrounded by intact vegetation (Fisher's exact test, $P = 0.0025$, $n = 108$). At the start of monitoring, 59% of flowers were browsed in the slashed area, reaching 86% by Day 10 and 88% by Day 33. In contrast, 28% of flowers were browsed in the control area at Day 1, increasing to 60% at Day 10 and reaching 86% by Day 33. This was not due to differences in flowering phenology between the two treatments as the proportions of flowers and buds at Day 1 were the same (slashed: 67% flowers, 33% buds; control: 62% flowers, 29% buds;). When considering all plants, the emergence status and flowering of emergent plants did not differ between treatments in 2007 or in 2008. Comparison of only those plants with a VS >10 prior to pruning revealed marginally higher rates of emergence in 2008 in the slashed area (slashed: 85.7%, $n = 35$; control: 63.6%, $n = 22$; Fisher's exact test, $P = 0.1$);

however, the proportion of flowering and non-flowering plants did not differ between treatments (slashed: 65.7% flowering; control: 50.0% flowering). When considering only emergent plants, there was no difference in the proportion of flowering plants between treatments (slashed: 76.7% flowering, $n = 30$; control: 78.6% flowering, $n = 14$).

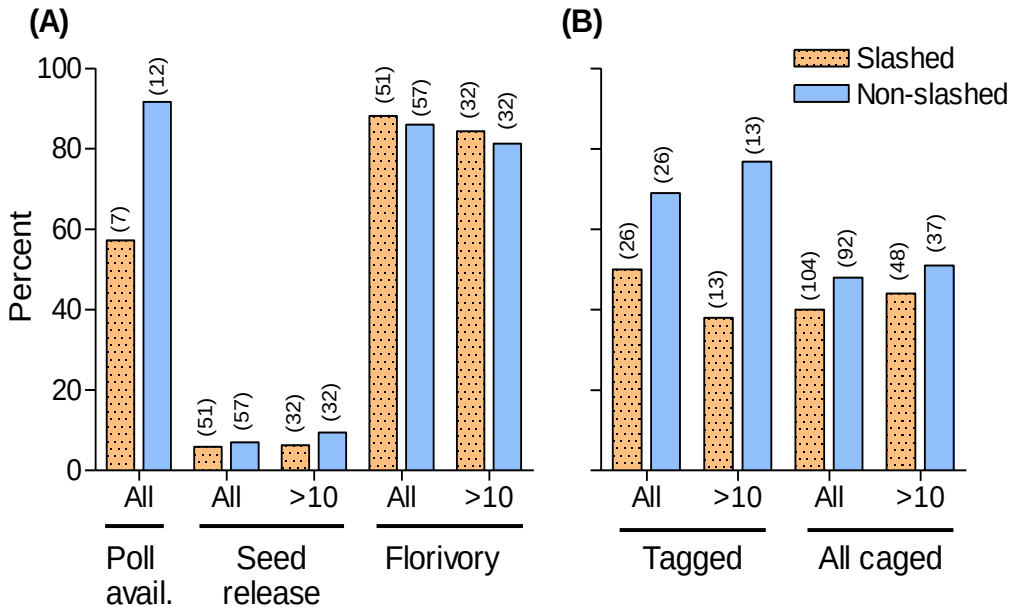

Fig. 6. 3 Effect of A) broad-scale slashing on pollination of available flowers, seed release and florivory of *Caladenia rigida* at site MC1 and B) localised vegetation removal on pollination success of tagged plants and of all flowers within guards at site MB1. All: all plants regardless of VS prior to removal; >10: plants with a pre-treatment VS >10. Numbers of flowers are shown in parentheses.

Localised vegetation removal

Regardless of the VS of plants prior to vegetation removal, the percentage of flowers that were pollinated did not differ between treatments (Fig. 6.3B). Increasing the statistical power of our analyses by including all *C. rigida* flowers within guards also failed to detect a treatment effect. As all of the capsules went on to dehisce, results for successful seed release and pollination success are the same. The width of capsules produced did not differ between treatments (slashed: 6.3 ± 0.2 mm (mean ± s.e.m.), $n = 25$; control: 6.6 ± 0.2 mm, $n = 27$). Removal of vegetation had no effect on the emergence status or flowering of plants in 2007 or in 2008. Similar results were obtained when considering only those plants with a VS >10 before removal.

6.4 Discussion

6.4.1 Influence of apparency on biotic interactions

The successful release of seeds by *C. rigida* is the outcome of interactions between pollination success, florivory and capsule predation. Flower height, local density and concealment were each found to influence final reproductive output; however, we found no strong evidence that any of these characteristics imposed a trade-off between pollination success and predation risk. This indicates that the pollinators and herbivores of this orchid respond to different stimuli. Our results suggest that although pollinator-mediated selection for flower height is not directly disrupted by conflicting interactions with antagonists, selection pressures may be complicated or dampened by temporal variability and extrinsic characteristics of the plant population, or of the ecological community as a whole.

In one study year, taller flowers were more likely to be pollinated, but were not at greater risk of being grazed. If the positive relationship between flower height and pollination success is a response to increased visual or olfactory prominence, then the same signals do not seem to be influencing the foraging decisions of herbivores. Alternatively, pollinator choice may be governed by an innate height preference independent of the flower's apparency (Handel and Peakall 1993). There are at least two possible explanations for the lack of response in 2007. Firstly, pollinator assemblages often vary spatially and temporally (Herrera 1988), and the response to flower height may change according to the behaviour of the dominant pollinators active at a particular site or in a particular year. Secondly, height-choice may operate at a much finer spatial scale than that considered in this study. For example, Dickson and Petit (2006) demonstrated that the pollinators of a closely related orchid, *Caladenia behrii*, chose the tallest amongst local pairs of flowers; whereas at the population level, flowers of all heights were pollinated.

The positive effect of flower height on successful seed release in 2005 is evidently a direct result of the benefit for pollination. In 2007, this effect was at least partly due to a reduction in florivory at one site; however, the strength of the relationship between flower height and capsule dehiscence suggests that other factors acting on successful seed release (including pollination) also respond to flower height. There was no relationship between predation risk and flower height in populations where white-winged choughs are the primary florivore. In the only population at which choughs have not been recorded (MB2), shorter flowers were at greater risk of being browsed, suggesting that other species of

florivores displaying different foraging behaviour may have been active at this site (see Faast and Facelli 2009).

Both flowers and capsules were more likely to escape predation when they were obscured by neighbouring vegetation. As concealment had no effect on pollination success, this translated into an increase in successful seed release in 2005. Although exposed flowers suffered from higher rates of florivory in 2007, we did not detect a relationship between concealment and capsule predation or capsule dehiscence, most likely because there were few well-concealed plants in that year (19 in total). Our results support observations that exposed plants are at greater risk of vertebrate predation than those growing amongst vegetation (Gregg 2004; Petit and Dickson 2005; Miller *et al.* 2006). However, in contrast to findings for *C. behrii* (Petit and Dickson 2005), the presence of neighbouring vegetation had no detrimental consequences for fruit set. The different response observed for these congeneric species is likely to stem from differences in their pollination strategy. The male thynnine wasp pollinators of *C. behrii* (Dickson and Petit 2006) may be deterred by structural obstacles when they are patrolling for females, while the generalist pollinators of *C. rigida* are probably accustomed to foraging amongst vegetation. Given the broad spectrum of insects that pollinate *C. rigida*, it is also possible that flowers with different degrees of concealment are pollinated by different insects. Furthermore, the visual and/or olfactory cues used by food-seeking pollinators are likely to be quite distinct to those of insects searching for mates (Schiestl 2005; Salzmann *et al.* 2006).

Neighbouring vegetation can offer protection against grazing by operating as a biotic refuge through direct impediment of herbivores or interference with their search process (McAuliffe 1984; Milchunas and Noy-Meir 2002), or as an associational refuge whereby unpalatable vegetation discourages grazing of nearby plants (Hjältén *et al.* 1993; Callaway *et al.* 2000). Given the highly selective behaviour of white-winged choughs when consuming orchid flowers (Faast and Facelli 2009), the palatability of surrounding vegetation is unlikely to have affected the foraging decisions of these birds. As none of the neighbourhood plants in the present study have pronounced structural defenses, protection against predation is more likely to have been afforded through visual interference by neighbouring plants. However, we cannot rule out the possibility that neighbourhood plants also offer associational refuge in populations where generalist herbivores are responsible for orchid predation. More detailed investigation of the protection offered by

different species of plants may help to determine whether structure or palatability play an important role in providing protection against grazing.

In general, flowers were at greater risk of being browsed when they were aggregated rather than sparsely distributed, suggesting that dense floral displays are more easily detected by vertebrate florivores. The opposite situation occurred in the SP population in 2007 (Table 6.5), when local density was at its highest (Table 6.4), lending support to the hypothesis that individuals within large aggregations suffer proportionately lower rates of consumption than solitary plants (Vine 1973). Pollination of *C. rigida* was independent of the local density of conspecific flowers, indicating that floral aggregates do not attract more pollinators to this orchid species. Experiments manipulating orchid density also demonstrated density-independence in *Listera cordata* (Meléndez-Ackerman and Ackerman 2001). It is possible, however, that the spatial scale used in the current study was insufficient to detect a density effect, as scale-dependent responses have been demonstrated in other species (Spigler and Chang 2008). Furthermore, local densities in natural populations may simply not be high enough to elicit a pollinator-mediated response. As expected, the increase in florivory at high density observed in two of the populations in 2007 led to a concomitant decrease in the proportion of flowers releasing seed. Interestingly, we detected no such effect in 2005, suggesting that other factors can sometimes counteract the negative consequences of florivory.

Our results indicate that under intense grazing pressure, plants are at greatest risk of being browsed when they are flowering. Differences between the probabilities of predation for pollinated capsules and open flowers have important implications when considering the timing of fertilisation in *C. rigida*. Flowers that are pollinated soon after anthesis are more likely to escape predation and hence release seed, than those that remain open. Several studies have demonstrated the importance of flowering phenology on plant reproductive success, usually with regard to temporal variation in the abundance of, or competition with, pollinators and seed predators (O'Connell and Johnston 1998; Pilson 2000; Mahoro 2002). Our results present a different situation, whereby the timing of pollination for an individual flower, rather than the timing of flowering within a population, determines final reproductive output by influencing predation risk. However, the magnitude of this effect appears to be related to the intensity of grazing, as there was no difference in predation rates between flowers and capsules in 2005. Clearly, further studies are required to assess

whether pollination phenology determines the likelihood of successful seed release in *C. rigida*.

6.4.2 Vegetation removal experiments

Extremely high rates of florivory in both the slashed and control areas made it impossible to detect any effects on pollination success, but made it clear that under intense grazing pressure large-scale removal of vegetation offers no benefit with respect to the final reproductive output of *C. rigida*. Indeed, in the undisturbed area, herbivores took longer to locate flowers, but final levels of florivory were the same as those in the slashed area. Other studies have also detected a delay in the onset of browsing associated with the presence of neighbouring vegetation (Pietrzykowsi *et al.* 2003; Miller *et al.* 2006). In the absence of grazing, removal of vegetation in the immediate vicinity of orchid plants did not affect their probability of pollination or successful seed release. Together, these results lend further support to the role of concealment in alleviating predation risk without impeding the activity of pollinators.

While other studies have demonstrated an indirect effect of vegetation removal through increases in light availability or below-ground resources (Ågren *et al.* 2006; Jacquemyn *et al.* 2008), we did not find any evidence for higher seed output (as assessed by capsule width) in response to the removal of neighbouring vegetation. This suggests that either light is not limiting in this system, or that reproductive development relies on previously stored underground reserves. In the year following the slashing treatment, emergence was marginally higher for plants that had a considerable biomass of surrounding vegetation removed. However, we found no evidence for increased flowering of *C. rigida* in response to vegetation removal at either spatial scale, indicating that emergence and flowering respond to different cues. Mowing has been shown to promote the emergence and/or flowering of some terrestrial orchid species (Jersáková *et al.* 2002; Wotavova *et al.* 2004), but not others (Janeckova *et al.* 2006), and the only study to assess fruit set revealed a beneficial effect of mowing (Wake 2007). Some of this variation is likely to be species-specific and will depend on the intensity of competition within the study system. However, studies assessing the impact of disturbances often make comparisons among geographically separated populations, potentially confounding results with site-specific characteristics such as population size or density, and microclimate. We have eliminated this caveat by directly comparing the impact of vegetation removal within the same orchid

population. *Caladenia rigida* has been reported to flower profusely following fire (Bates 1984a), and our data suggests that such a response is unlikely to stem simply from increased light availability via the removal of above-ground vegetation. Fire is likely to produce a more severe reduction in the amount of nutrients and water extracted by competing vegetation, compared to slashing. In addition, orchids may respond specifically to fire-induced signals or nutrient enrichment. Alternatively, responses to vegetation clearance may only become evident when the biomass of undergrowth is higher than that removed in our populations.

Conclusions

This is the only study that we are aware of that simultaneously assesses the response of pollinators, florivores and seed predators, to both intrinsic and extrinsic characteristics affecting the apparency of flowers and fruit. We have shown that maternal fecundity of *C. rigida* relies on the complex interplay between these opposing interactions, and that the relative strength of these interactions varies both spatially and temporally. The large disparity between pollination and successful seed release in our study populations clearly demonstrates the detrimental consequences of herbivory. Conservation programs aiming to maximise the reproductive output of threatened species should strive to achieve a balance between mutualistic and antagonistic interactions, and understanding factors that affect the nature of these interactions is crucial for achieving this goal. For example, the reintroduction or translocation of plants into areas where the risk of herbivory is high may be more successful if the target plants are sparsely distributed, or located in close proximity to neighbourhood vegetation. Under intense grazing pressure, the large-scale removal of vegetation does not benefit the reproductive success of *C. rigida*, and indeed may place orchid populations at a disadvantage by drawing the attention of herbivores.

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CHAPTER 7

Hemispherical photograph taken at the MC2 site, Mount Crawford Forest
(Photo by author)

Chapter 7: Preamble

CHAPTER 7 evaluates the extent of variation in seed viability among populations of *Caladenia rigida*, and attempts to relate differences between declining and stable populations with plant attributes as well as population and habitat characteristics. To provide an estimate of the recruitment potential and hence long-term prospects of small populations, data for seed viability of *C. rigida* is combined with data for seed germination and seedling survival for two other *Caladenia* species.

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Contributions and signatures of authors:

Renate Faast

Designed experiments, collected and analysed all data and prepared manuscript as principle and corresponding author.

Signed..... ..... Date..29th June 2010

José M. Facelli

Sought and won funding, supervised development of research and evaluated manuscript.

Signed..... ..... Date..15/06/2010

Andrew D. Austin

Sought and won funding, advised on aspects of research and evaluated manuscript.

Signed..... ..... Date..30th June 2010

SEED VIABILITY IN DECLINING POPULATIONS OF *CALADENIA RIGIDA* (ORCHIDACEAE): ARE SMALL POPULATIONS DOOMED?

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Abstract

Despite comparatively good rates of pollination and seed production, some populations of the endangered terrestrial orchid, *Caladenia rigida*, continue to decline. To determine whether seed quality may be limiting reproductive potential, we assessed seed viability among declining populations of *C. rigida* (in the southern part of its distribution) and among populations that are regarded as stable (in the northern part of its distribution). We also compared differences in seed viability to plant traits, population size and habitat characteristics (soil properties, canopy cover, presence of proximate vegetation). Seed capsules from southern populations were significantly smaller, with only 9% of seeds being viable, compared to 36% in capsules from northern populations. Soil phosphorous concentrations differed between regions, but other habitat characteristics did not correlate with seed viability. Using calculations based on seedling recruitment data from other *Caladenia* species, we predict that seed output is insufficient to ensure the long-term persistence of the smallest *C. rigida* populations.

7.1 Introduction

While numerous processes can determine the persistence and growth of plant populations, the replacement of senescent individuals via establishment from seed is fundamental for most angiosperms. The number of seeds reaching a particular microsite can be limited by factors that act directly on seed production (eg. pollination success, florivory, resource limitation) or those that affect seed dispersal or seed predation. Most studies of rare or declining plant populations measure reproductive success based on the output of fruits or seeds (Cunningham 2000; Wolf and Harrison 2001; Ward and Johnson 2005). Similarly, investigations of the relationship between population attributes (size, density, isolation) and reproductive success, usually record fruit or seed production (Aizen and Feinsinger 1994; Ågren 1996; Alexandersson and Ågren 1996; Hackney and McGraw 2001; Ehlers *et al.* 2002). However, the ability of seeds to contribute to subsequent generations is often overlooked. Although the recruitment and establishment of offspring relies on the availability of safe sites for germination and growth (Eriksson and Ehrlén 1992), this becomes of minor importance if the production of viable seeds is limiting.

Both intrinsic and extrinsic factors can affect seed production or germinability. Pollination characteristics as well as population size or density can have important implications for fertilisation by influencing the attraction and behaviour of pollinators (Peakall and Beattie 1996; Knight *et al.* 2005). Self-pollination, and mating between closely related individuals, increases the risk of homozygosity and inbreeding depression, which have been linked to reductions in seed number (Paschke *et al.* 2002), seed viability (Ferdy *et al.* 2001; Wallace 2003) and germination (Menges 1991; Heschel and Paige 1995). Resource availability and habitat characteristics such as the presence of co-flowering species, have also been shown to influence seed output and seed viability (Helenurm and Schaal 1996; Oostermeijer *et al.* 1998).

The current study follows on from earlier findings that pollination success and seed release of an endangered terrestrial orchid, *Caladenia rigida* R.S.Rogers, varies among populations and among years (CHAPTER 5). This species is endemic to the Mount Lofty Ranges of South Australia where it occurs in several large (more than 200 flowering individuals) but discrete populations in the northern part of its range. Five small populations (less than 100 flowering plants), thought to be remnants of formerly larger populations (J. Quarmby, pers. comm.), are located approximately 30 km to the southeast.

Although these small populations exist within large areas of native vegetation (200 – 350 ha), they are continuing to decline. Conservation management currently consists of threat abatement (herbivore exclusion and weed removal) and augmentation of reproductive success through hand pollination (Quarmby 2006).

A three-year study assessing spatial and temporal variation in the reproductive success of 11 *C. rigida* populations revealed that during favourable years, southern populations had pollination success comparable to those in the north (CHAPTER 5). Furthermore, southern plants experienced lower rates of florivory and capsule predation (Faast and Facelli 2009). While the availability of pollinators and herbivore abundance do not appear to limit the production and release of seeds in southern populations (during favourable years), the quality of the seeds produced has never been assessed.

Ideally, assessment of the recruitment potential of seeds should also examine seed germinability; however, the requirement of mycorrhizal fungi for orchid seed germination imposes significant complexity for the design of such experiments. Mycorrhizal associations are often locally diverse, leading to large variations in the seed germination ability of fungal isolates, both among and within populations, and even among plant life-stages (Ochora *et al.* 2001; Sharma *et al.* 2003; Wright 2007). Given this variability, and the fact that the production of viable seeds is a prerequisite for germination, we focus our comparisons on seed viability. Population size differs widely between the two regions (7 – 100 flowering individuals in the southern populations, and 200 – 1500 in the northern populations). However, the regions also differ with respect to rainfall and habitat characteristics (soil properties, vegetation composition and structure), making comparisons based purely on population size uninformative. We therefore present results based on regions rather than population size.

In situ rates of germination and/or seedling recruitment are quite low for several taxa of terrestrial orchids including *Caladenia* (Batty *et al.* 2001a; Wright 2007; Oien *et al.* 2008; Coates and Duncan 2009). We were therefore also interested in estimating the minimum number of capsules required to sustain populations of *C. rigida*, particularly when population sizes are small. To this end, we used our seed viability data together with germination and seedling survival data from two other *Caladenia* species to estimate the recruitment potential of *C. rigida*.

The objectives of the present study were to evaluate the reproductive potential of declining populations of *C. rigida*, as measured by seed viability, and to compare this with that of more stable populations. We assessed variation in the viability of seeds collected from four northern and five southern populations and relate this to habitat and population characteristics. Specifically, our questions were: 1) does poor seed viability contribute to the decline of this species in southern populations? 2) can differences in seed viability be explained by habitat characteristics, population size or plant traits? 3) is the rate of capsule and hence seed production sufficient for small populations to remain viable?

7.2 Methods

Study species and site locations

Caladenia rigida, the rigid white spider orchid, is a tuberous perennial species, producing a single leaf in autumn and typically one flower early in spring. Flowers are self-compatible but rely on a diverse range of food-seeking insects for cross-pollination (Faast *et al.* 2009). The species is non-clonal, replacing its single tuber annually and thus reproduces only from seed. Seed capsules take six to eight weeks to mature and release thousands of dust-like seeds. *Caladenia rigida* usually occurs in *Eucalyptus* woodlands dominated by *E. obliqua* L'Her, *E. leucoxylon* F.Muell. or *E. fasciculosa* F.Muell. with understorey vegetation including *Acacia pycnantha* Benth., *Pultenaea daphnoides* J.C.Wendl., *Leptospermum myrsinoides* Schltdl., *Hibbertia* spp., *Platylobium obtusangulum* Hook and *Lepidosperma* spp. Reductions in the range and size of populations over recent decades have been attributed to habitat destruction and fragmentation, leading to the species being listed as Endangered under IUCN criteria (Quarmby 2006).

Long-term monitoring is being carried out on several populations of *C. rigida* throughout the Mount Lofty Ranges. A subset of these populations was selected for the present study, consisting of four populations from the northern region of its distribution, at Mount Crawford Native Forest Reserve (MC2), Millbrook Reservoir (MB1 and MB2) and South Para Reservoir (SP), and five populations from the southern region, at Ironbank (IB1, IB2 and IB3) and in Scott Creek Conservation Park (SC1 and SC2) (Fig.1). The area experiences a Mediterranean type climate, with wet winters and dry summers.

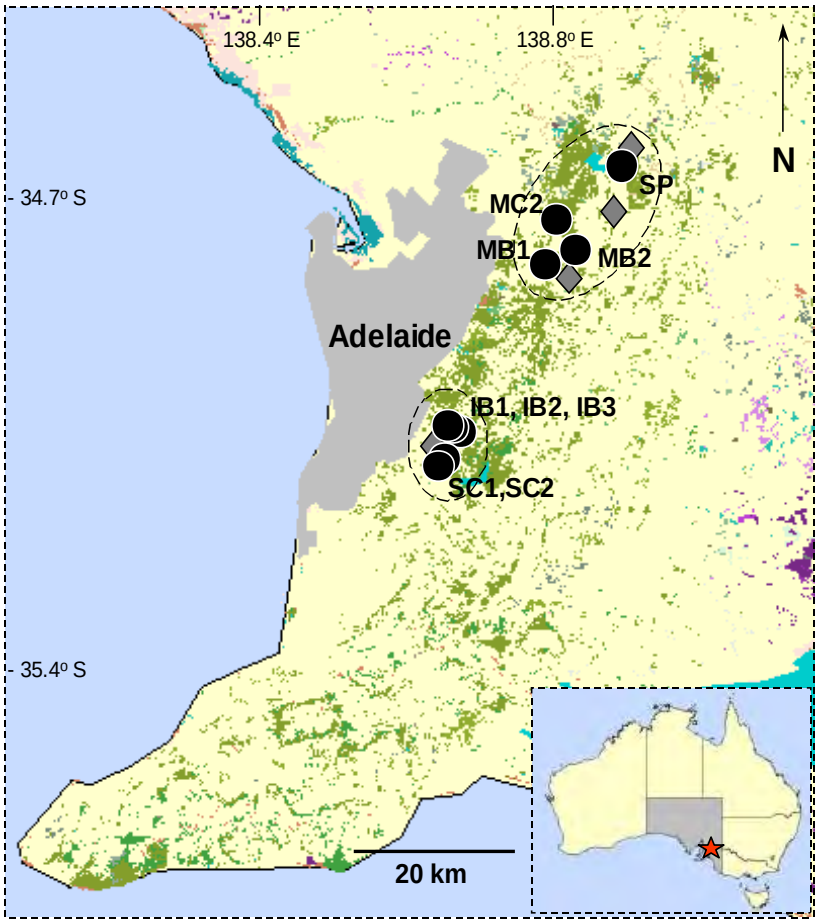

Fig. 7. 1 Map showing locations of *Caladenia rigida* populations (●) and weather stations (◆) within northern and southern regions (circled). Light grey shading shows the urban area of the city of Adelaide, green represents areas of extant native vegetation. Map source: Australian National Resources Atlas.

Seed number

In a preliminary study (2005) we assessed whether capsule size can be used as a non-invasive means of predicting seed output, by assessing seed number in 16 randomly selected *C. rigida* capsules from the northern populations. We used vernier callipers to measure the width of capsules just prior to dehiscence. Seed was removed from air-dried capsules and cleaned of debris. For seed counting, we immersed the total seed content from each capsule overnight in 50 ml of 0.002% Tween 20. We dispensed a 1 ml aliquot (maintained as an even suspension on a magnetic stirrer) onto a 55 mm filter paper disc, and used digital photographs to count the total number of seeds on each disc as well as the number of seeds with embryos. We confirmed the accuracy of this technique by analysing a subset of filters using a dissecting microscope to count seeds. Eight aliquots were counted for each capsule and the average of these was calculated to provide seed number per capsule.

The mean number of seeds produced per capsule was 7,901 ± 857 (s.e.m.) (range = 887 to 14000). Capsule width was positively related both to the total number of seeds (r_s = 0.56, n = 16, P = 0.023) and to the number of seeds with embryos (r_s = 0.52, n = 16, P = 0.038), and therefore provides an adequate estimate of seed output in *C. rigida* while avoiding destructive sampling.

Capsule production and plant traits

At each site, tagged *C. rigida* plants (up to 100 flowering plants per population) were monitored on a regular basis throughout the flowering season in 2007, recording capsule initiation and dehiscence and the maximum capsule width attained prior to dehiscence. To maintain consistency among plants, capsule size was always measured across the width with the dorsal sepal at the back. A concurrent study showed that natural fruit set is highly variable among populations and years, and in 2007 capsule production ranged from 25% of flowers at SC1 to 81% at MB2 (CHAPTER 5). For each plant, we also recorded leaf length and leaf width, as well as the length of the flower stalk to the base of the ovary. Leaf traits were not recorded at sites SP and IB2. Since the width of capsules may be influenced by plant size, we calculated the leaf area of maternal *C. rigida* plants based on measurements of leaf length and leaf width. Actual leaf area was determined for 15 *C. rigida* leaves, using a computer program developed by Grant Williamson (The University of Adelaide). A regression equation was then fitted to relate actual leaf area (A) to leaf width (W) and leaf length (L): A (cm^2) = 0.044 + 0.896 x L (cm) x W (cm), (R^2 = 0.95).

Seed viability

In 2007, we harvested mature seeds from dehiscing capsules of eight to 11 randomly selected plants at each site, except for SC1 and SC2 where seeds were collected from all of the capsules produced (three at each site). Seeds were air dried for one week at room temperature, and stored at 4°C with silicon beads until use. We tested seed viability using a modification of Pritchard (1985) whereby seeds were pre-treated overnight in deionised water containing 0.002% Tween 20. Seeds were stained for 15 - 30 min in a solution of 0.25% fluorescein diacetate (FDA) and viewed with UV-fluorescence microscopy. For each sample we counted three replicates of 100 – 150 seeds, and recorded the number of viable (FDA positive), non-viable (FDA negative embryo) and empty (lacking an embryo) seeds (Fig. 7.2). Removal of the testa did not increase the proportion of FDA-positive embryos (data not shown), confirming that the stain was able to permeate the seed coat. While seed viability ascertained by FDA-staining has been shown to overestimate

germination slightly and consistently, it correlates positively with germination in other *Caladenia* species (Batty *et al.* 2001b; Wright 2007). For example, Wright (2007) demonstrated that 55% of seeds collected from *C. tentaculata* stained positively with FDA, whereas 41 - 44% of seeds germinated in the presence of appropriate mycorrhizal isolates.

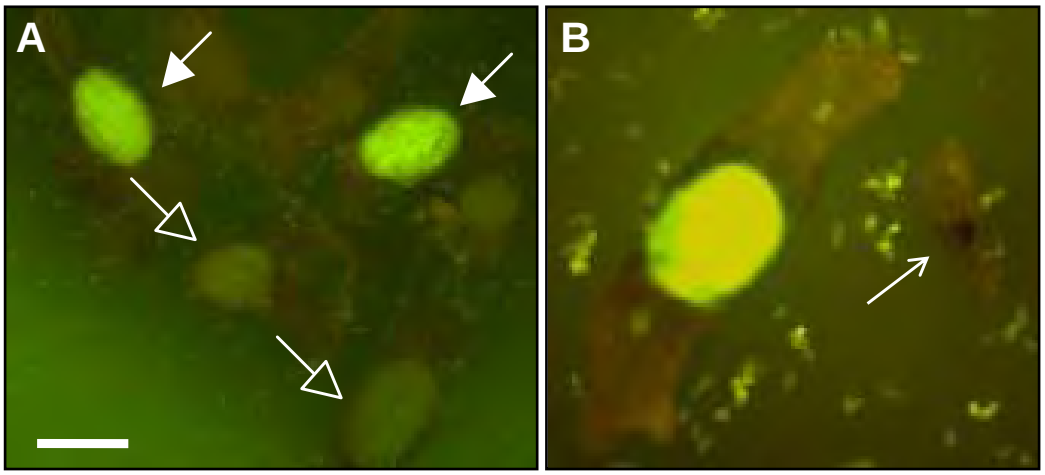

Fig. 7. 2 Viability tests of *Caladenia rigida* seeds. (A) Seeds containing FDA positive (solid arrow) and FDA negative (open arrow) embryos, (B) empty seed (plain arrow). Bar = 100 μm.

Habitat and population characteristics

At each site we used a 5 cm wide corer to collect soil samples to a depth of 10 cm, from within 50 cm of six randomly selected orchid plants. We pooled 20 g of dried soil from each sample, to provide a representative sample for each site. Soil was sent to CSBP Soil and Plant Analysis Service, Western Australia, and analysed for macronutrients: total nitrogen (N), available phosphorous (P) and potassium (K); micronutrients: zinc (Zn), manganese (Mn), iron (Fe) and copper (Cu); and exchangeable cations: calcium (Ca), magnesium (Mg), sodium (Na), potassium (K) and aluminium (Al). To measure pH, 10 g of soil was mixed with 50 ml of Milli Q water for 2 days and allowed to settle before testing with a pH meter. Soil texture was classified using the subjective test described by Northecote (1979). Rainfall data were obtained from the Australian Government Bureau of Meteorology from three weather stations within the northern region and one station in the south (Fig. 7.1). We calculated long-term annual averages based on data collected over 40 to 147 years. In addition, we obtained average rainfall within each region for 2007, as well as the September to November (2007) average to coincide with the period during which *C. rigida* capsules develop and mature.

To determine whether the amount of understorey and canopy vegetation (and hence light availability) differed between northern and southern populations, we calculated percent canopy cover using hemispherical photography. At each site, up to 30 photographs were taken at ground level adjacent to randomly selected orchid plants (Frazer *et al.* 2001). When orchids were clumped, only images at least 1 m apart were included in the analysis. We used an FC-F8 fisheye lens converter mounted onto a Nikon Coolpix 4500 digital camera, placed on a levelled board on top of a sandbag. To provide even backlighting, all photographs were taken at dusk or on evenly overcast days using the camera's self-timer. Photographs were analysed using the program Gap Light Analyzer version 2.0 (Frazer *et al.* 1999) and measurements were averaged across all photographs to provide an estimate of percent canopy cover per site. To estimate the degree to which orchid plants are surrounded by competing understorey vegetation, we used the same randomly selected plants as for the canopy analysis and recorded the presence or absence of vegetation within a 20 cm radius of the plant at two height classes (0 - 0.5 m and 0.5 - 1 m). We then calculated the proportion of plants with proximate vegetation at each site.

Data analysis

Relationships between capsule width and seed number, and between seed viability and plant traits (capsule width, leaf area, stalk length) were assessed using Spearman's rho measure of association. Due to a positive relationship between leaf area and stalk length ($r_s = 0.78$, n = 43, $P < 0.0001$), we used first-order Spearman's rho partial correlations to examine the association between capsule width and leaf area or stalk length, while holding the third variable constant. Similarly, we used second-order Spearman's rho partial correlations to examine the relationship between seed viability and each plant trait separately, keeping the remaining traits constant.

We assessed variation in capsule width and stalk length among populations using ANOVA. To allow for unequal sample sizes we calculated the *F* statistic using type III sum of squares, and used Hochberg's GT2 pairwise comparisons. For leaf area we used the non-parametric Kruskal-Wallis (KW) test, with Mann-Whitney U tests (Bonferroni corrected) for post-hoc comparisons. We assessed homoscedasticity using Levene's test for equal variances. All proportional data (eg. seed viability) were arcsine transformed prior to analysis. We compared seed viability among populations using Welch-corrected ANOVA followed by Games-Howell post-hoc comparisons, which are recommended when sample sizes and variances are unequal. To account for the skewed distribution of

these data, we also performed Kruskal-Wallis tests and present the results for both. Percent canopy cover was compared among populations using ANOVA.

We used Welch-corrected *t*-tests (heteroscedastic data) and Mann-Whitney U tests (non-parametric data) to assess differences in mean seed viability between the northern and southern populations, treating populations as replicates within each region. Likewise, we compared capsule width and stalk length between northern and southern regions using *t*-tests, while leaf area was compared using Mann-Whitney U tests. To maintain a consistent measure of comparison, we analysed regional differences in habitat characteristics (pH, soil nutrient concentrations, canopy cover and understorey vegetation), using unpaired *t*-tests on the mean values obtained for each region. Where significant differences in nutrient concentrations between the northern and southern regions were identified, we performed Spearman's rho correlation analyses of these factors against the average seed viability for each population. We used the statistical package R for Windows (2.9.0) for correlation analyses, and SPSS 15.0 for all other tests.

Estimate of seed limitation

Seedling recruitment and survival data are not available for *C. rigida*. To estimate how many of the seeds produced by small populations of *C. rigida* are likely to germinate and survive, we used published data available for two other *Caladenia* species, both of which typically produce a single capsule per plant. In an *in situ* seed baiting experiment, Batty *et al.* (2001a) reported that less than 1% of seeds produced by *C. arenicola* germinated and reached tuber stage. Using their estimate of 0.4 seedlings surviving per parent plant and the production of approximately 30,000 seeds per capsule, about 0.0013% of seeds actually recruit and survive for at least one year (ie. 0.13% of seedlings survive). The second study we employed, involved a direct seeding experiment in which several treatments were tested to optimise seedling recruitment of *C. tentaculata* (Wright 2007). In this case, seeds had also been inoculated with a mycorrhizal fungus required for germination and, under the best combination of treatments (soil disturbance, supplemental watering and the addition of organic mulch), 3.2 ± 1.3 (mean ± s.e.m.) seeds germinated from 20,368 seeds and 0.7 ± 0.5 seedlings survived summer dormancy. This translates into 0.016% germination success and 21.9% seedling survival. Therefore, under optimal conditions, 0.0034% of *C. tentaculata* seeds can be expected to produce a seedling that survives for at least a year.

7.3 Results

Seed number, capsule width and plant traits

The width of *C. rigida* capsules differed among populations (ANOVA: $F_{8,215} = 7.1$, MS = 5.0, $P < 0.0001$, Fig. 7.3A) and mean capsule width was smaller in populations from the southern region ($t = 3.7$, $P = 0.007$, Table 7.1). Although stalk length and leaf area also varied among populations (stalk length: ANOVA, $F_{8,468} = 10.2$, MS = 262.0, $P < 0.0001$; leaf area: KW, $\chi^2_6 = 38.3$, $P < 0.0001$, Figs. 7.3B, C), they did not differ significantly between regions. However, the small sample size (n = 7) and relatively large plant traits at one site (SC2) may have biased these comparisons (Figs. 7.3B, C); exclusion of this site results in a significant difference in leaf area between northern and southern populations (Table 7.1).

Capsule width was positively associated with the leaf area of maternal plants ($r_s = 0.24$, n = 125, $P = 0.005$, stalk length held constant) and with their stalk length ($r_s = 0.32$, n = 125, $P = 0.0002$, leaf area held constant). These results indicate that capsule width, and hence seed number, are positively related to the size of the maternal plant. The rate of capsule abortion was very low (< 4%) in all populations (Table 7.3) and did not differ between the two regions (Fisher's exact test, $P = 0.33$).

Seed viability

Staining with FDA revealed three types of seeds within *C. rigida* capsules: those containing FDA-positive (viable) embryos; those with unstained (non-viable) embryos; and those without an embryo (empty) (Fig. 7.2). The percentage of viable seeds differed among populations (Welch ANOVA: $F_{8,19} = 10.6$, $P < 0.0001$; KW: $\chi^2_8 = 38.4$, $P < 0.0001$, Fig. 7.3D). The proportion of seeds with non-viable embryos varied among populations (Welch ANOVA: $F_{8,17} = 3.6$, $P = 0.012$; KW: $\chi^2_8 = 19.6$, $P = 0.012$, Fig. 7.3E), as did the proportion of seeds lacking embryos (Welch ANOVA: $F_{8,19} = 5.6$, $P = 0.001$; KW: $\chi^2_8 = 26.0$, $P = 0.001$, Fig. 7.3F). We found that average seed viability was lower in the southern populations than in the northern populations (Welch-corrected $t = 10.1$, $P = 0.0002$; Mann Whitney $U = 0.0$, $P = 0.02$, Table 7.1) and that southern populations tended to have a greater number of empty seeds (Welch-corrected $t = 2.06$, $P = 0.086$; Mann Whitney $U = 3.0$, $P = 0.1$). The proportion of seeds with non-viable embryos did not differ between regions (Table 7.1).

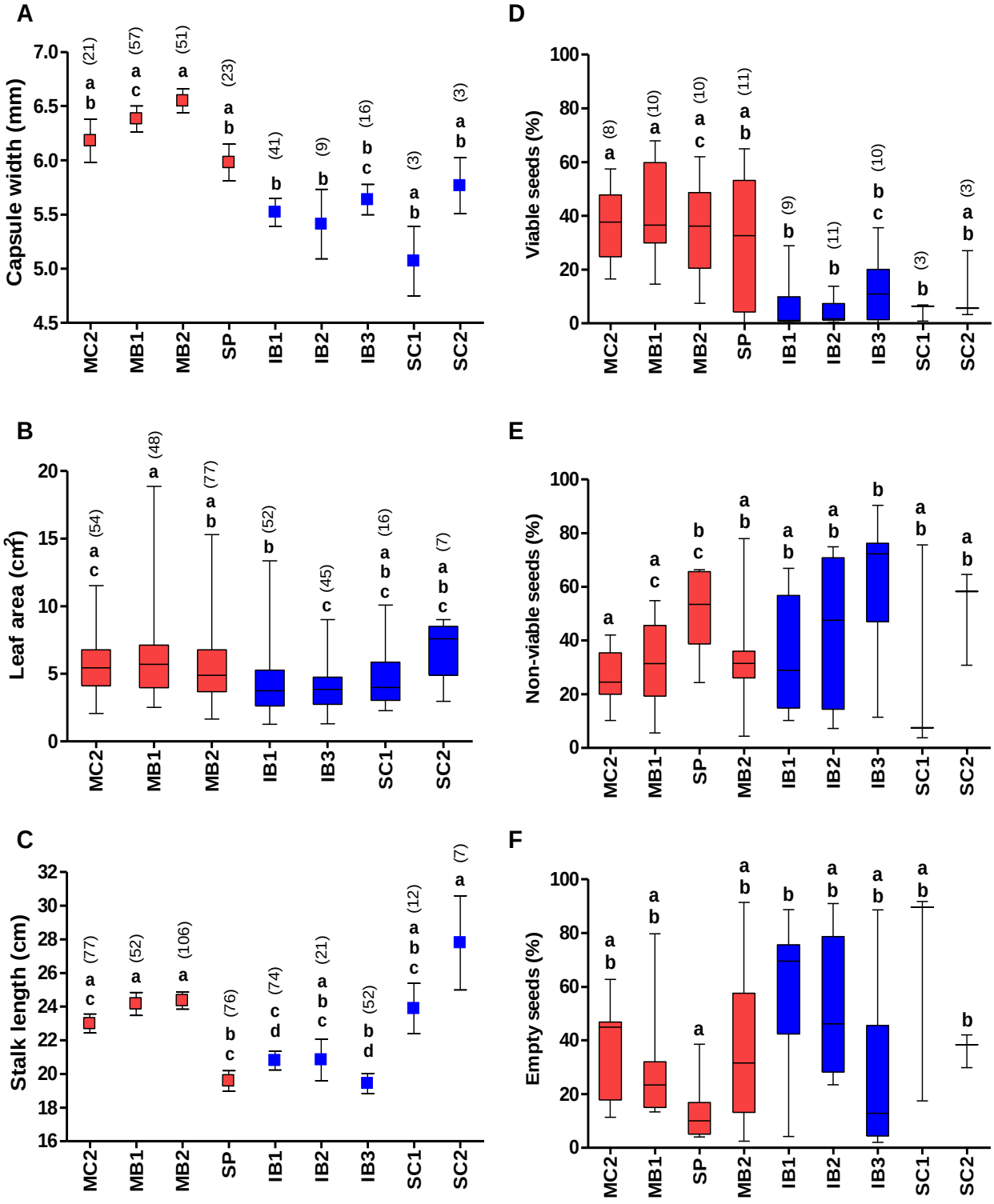

Fig. 7. 3 Plant traits and seed viability among northern populations (red symbols) and southern populations (blue symbols) of *Caladenia rigida*. (A) Capsule width (mean ± s.e.m), (B) leaf area (median + quartiles), (C) stalk length (mean ± s.e.m), (D) percentage of viable seeds, (E) seeds with non-viable embryos, and (F) empty seeds (median + quartiles). Sample sizes are shown in parentheses. Analyses of seed viability were performed on arcsine-transformed data. Populations with the same letter are not significantly different ($P > 0.05$).

Seed viability was positively correlated with capsule width (r_s = 0.41, n = 43, P = 0.005, leaf area and stalk length held constant), but was not related to leaf area (r_s = -0.014, n = 43, P = 0.93, capsule width and stalk length held constant) or stalk length (r_s = 0.075, n = 43, P = 0.64, capsule width and leaf area held constant). There was no relationship between the proportion of empty seeds or seeds with non-viable embryos, and any of the plant traits.

Table 7. 1 Comparison of seed viability and plant traits between northern and southern populations (mean ± s.e.m.).

	Region		*P* value (test statistic)
	Northern	Southern	
Viable seeds (%)	36.3 ± 2.2 (4)	9.0 ± 1.6 (5)	**0.0002** (Welch t = 10.1) **0.016** (U = 0.0)
Non-viable seeds (%)	36.2 ± 5.8 (4)	43.4 ± 6.8 (5)	0.45 (Welch t = 0.8) 0.73 (U = 8.0)
Empty seeds (%)	27.5 ± 6.2 (4)	47.6 ± 7.6 (5)	0.09 (Welch t = 2.1) 0.11 (U = 3.0)
Capsule width (mm)	6.2 ± 0.2 (4)	5.6 ± 0.1 (5)	**0.007** (t_7 = 3.7)
Leaf area (cm^2)	5.8 ± 0.2 (3)	4.9 ± 0.6 (4) 4.3 ± 0.2 (3)*	0.36 (t_5 = 1.0) **0.007** (t_4 = 5.0)
Stalk length (cm)	23.1 ± 0.9 (4)	22.7 ± 1.5 (5) 21.4 ± 0.9 (4)*	0.91 (t_7 = 0.1) 0.33 (t_6 = 1.1)

Number of replicate populations is indicated in parentheses. Percentage data were arcsine transformed. *Excluding site SC2 (see text); U: Mann-Whitney U tests; degrees of freedom are shown as subscripts. Significant values are in bold.

Habitat and population characteristics

Comparison of soil properties between regions revealed that concentrations of phosphorous were lower in the south (t = 2.4, P = 0.048, Table 7.2). We detected a marginally significant positive correlation (r_s = 0.65, n = 9, P = 0.066) between phosphorous concentration and average seed viability, however there was no relationship with the proportion of non-viable embryos (r_s = -0.52, n = 9, P = 0.16), or empty seeds (r_s = -0.13, n = 9, P = 0.74). The concentration of exchangeable potassium was marginally lower in the south (t = 2.2, P = 0.068, Table 2), and we detected a marginally significant positive correlation between exchangeable potassium concentration and average seed viability (r_s = 0.64, n = 9, P = 0.076), a negative correlation with the proportion of seeds with non-viable embryos (r_s = -0.76, n = 9, P = 0.021), but no correlation with empty seeds (r_s = -0.06, n = 9, P = 0.88). None of the other soil attributes differed between regions (Table 7.2). Soils from northern populations were all classified as sandy-clay-loam, whereas soils from the south were clay-loam (except at SC2 which had sandy-loam soils).

Table 7. 2 Soil properties at each site, comparing means between northern and southern sites.

Site	pH	Tot N	P	Tot K	Cu	Zn	Mn	Fe	Ca	Mg	Na	K	Al
Northern													
MC2	5.3	0.3	3	165	0.62	2.44	16.8	94.9	7.31	2.72	0.47	0.35	0.58
MB1	5.0	0.17	3	142	0.38	1.05	11.5	121.8	3.88	1.56	0.18	0.34	1.10
MB2	5.9	0.19	2	153	0.72	0.81	5.4	90.4	7.54	1.78	0.56	0.27	0.04
SP	5.2	0.11	2	99	0.3	0.48	8.6	82.4	2.01	1.18	0.16	0.23	0.43
Mean	*5.4*	*0.19*	*2.5*	*140*	*0.51*	*1.2*	*10.6*	*97.4*	*5.2*	*1.81*	*0.34*	*0.3*	*0.54*
s.e.m.	*0.2*	*0.04*	*0.3*	*14*	*0.1*	*0.4*	*2.4*	*8.6*	*1.3*	*0.33*	*0.1*	*0.03*	*0.22*
Southern													
IB1	5.2	0.14	1	125	0.43	0.98	8.2	87.7	2.54	1.51	0.2	0.22	0.83
IB2	5.2	0.16	2	142	0.35	0.65	3.0	151.4	2.37	1.5	0.33	0.21	1.16
IB3	5.5	0.2	1	122	0.58	1.49	6.7	136.6	4.47	1.97	0.33	0.21	0.34
SC1	5.3	0.21	2	170	0.71	1.8	31.1	79.1	4.62	2.53	0.24	0.3	1.00
SC2	5.1	0.21	2	96	0.47	1.08	9.9	114.3	4.19	1.69	0.24	0.18	0.68
Mean	*5.3*	*0.18*	*1.6*	*131*	*0.51*	*1.2*	*11.8*	*113.8*	*3.6*	*1.84*	*0.27*	*0.22*	*0.8*
s.e.m.	*0.1*	*0.01*	*0.2*	*12*	*0.06*	*0.2*	*5.0*	*13.8*	*0.5*	*0.19*	*0.03*	*0.02*	*0.14*
P	0.82	0.83	**0.048**	0.65	0.98	0.99	0.85	0.38	0.28	0.94	0.52	0.07	0.33

Total N expressed as %; P, Total K, Cu, Zn, Mn, Fe shown in mg/kg; Exchangeable cations Ca, Mg, Na, K, Al shown as meq/100g. All means were compared using un-paired *t*-tests, except Na (Welch-corrected *t*-test).

Table 7. 3 *Caladenia rigida* population attributes and habitat characteristics at each site.

Site	Pop. size [a]	Aborted capsules (%)	No. capsules releasing seed	Canopy cover (%)	Veg. 0 – 0.5 m (%)[c]	Veg. 0.5 – 1 m (%)[c]
Northern						
MC2	200	3.4 (29)	~ 40 [b]	24.7 ± 0.8 (26)	92.6	11.1
MB1	200	0 (63)	NA	26.7 ± 0.6 (29)	77.8	3.7
MB2	500	3.9 (76)	~ 200 [b]	29.8 ± 0.5 (30)	85.7	8.6
SP	1500	3.4 (29)	~ 50 [b]	32.2 ± 1.1 (21)	44.8	6.9
Mean ± s.e.m.				28.4 ± 1.7	75.2 ± 10.6	7.6 ± 1.6
Southern						
IB1	100	0 (43)	~ 50 [b]	27.4 ± 0.7 (15)	96.7	13.3
IB2	23	0 (9)	9	24.7 ± 1.0 (15)	93.3	53.3
IB3	60	0 (16)	16	28.2 ± 0.8 (10)	63.6	0
SC1	19	0 (3)	3	28.8 ± 1.6 (6)	81.3	0
SC2	7	0 (3)	3	28.3 ± 0.3 (2)	57.1	28.6
Mean ± s.e.m.				27.5 ± 0.7	78.4 ± 7.9	19.0 ± 10.1
P				0.61	0.81	0.33

[a] Number of flowering plants recorded in 2007 were based on exact counts for populations of up to 60 flowers, and estimates for larger populations. [b] Estimate extrapolated from data of population size and the percentage of capsules releasing seed (CHAPTER 5). [c] Percentage of orchid plants with proximate vegetation (see methods). Sample sizes are indicated in parentheses. Means were compared using *t*-tests or Welch-corrected *t*-tests (Veg. 0.5-1m).

While percent canopy cover was highly variable among populations (ANOVA: $F_{8,145} = 9.3$, MS = 120.8, $P < 0.0001$), it was not different between regions (Table 7.3). Similarly, the proportion of orchid plants with vegetation in close proximity varied among populations, but not between regions (Table 7.3). The long-term average annual rainfall was 925 mm in the south (one weather station) and 761 ± 52 mm in the north (averaged across three stations). In 2007, the southern region received more rainfall over the entire year (southern: 833 mm; northern: 706 ± 52 mm) as well as from September to November, when capsules develop and mature (southern: 46.2 mm; northern: 40.4 ± 3.6 mm).

Estimate of seed limitation

First, applying calculations from Batty *et al.* (2001a) to a single capsule of *C. rigida*, we can expect that 79 (1%) out of the 7,900 seeds produced will germinate and 0.1 seedlings (0.0013% of seeds) will survive. Alternatively, using calculations based on the optimal conditions described by Wright (2007), only 1.2 (0.016%) seeds will germinate but 0.27 seedlings (0.0034% of seeds) will survive. We wish to estimate whether small populations produce enough seeds to replace senescing adults. In 2007, the two smallest populations, (SC1 and SC2) consisted of 19 and 7 flowering plants, respectively, and each produced three capsules that released seed (Table 7.3). Therefore, under conditions similar to those experienced in the above studies, we can expect that the total number of seeds produced by either of these populations could potentially result in the recruitment of 0.3 to 0.8 seedlings. Depending on germination success and seedling survival rates, a population would therefore need to produce at least two to four capsules to ensure the replacement of just one individual. This approach relies on the supposition that closely related species will behave in a similar way and we acknowledge this may not necessarily be true.

The above scenarios do not take into consideration the low quality of seeds from the small populations assessed in this study. For example, calculations from Wright (2007) are based on seed viability (FDA staining) of 55%. Incorporating our results, average seed viability was only 5 or 12% at SC1 and SC2, respectively, thus reducing the expected number of surviving seedlings by up to eleven-fold (0.03 - 0.07 seedlings at SC1; 0.07 - 0.17 seedlings at SC2). In this case, between six and 34 capsules may be required to ensure recruitment of just one individual. Replacement of all seven mature individuals in SC2 would require 42 – 238 capsules over the lifetime of these orchids. Assuming that *C. rigida* plants live for 20 years (based on cultivated plants and so likely to be an overestimation), this translates to 2 - 12 capsules per year.

7.4 Discussion

7.4.1 Regional variation in seed viability

Our results demonstrate that seed viability varies substantially among populations of *C. rigida*. In particular, seed viability was considerably lower in plants from southern populations. Capsules with a low proportion of viable seeds had either more seeds with non-viable embryos, more empty seeds, or both. The relative proportions of non-viable versus empty seeds varied among populations but there was no clear pattern between regions. This suggests that several factors affect seed quality throughout seed development and that their importance varies among populations. Alternatively, non-viable embryos and ovule and/or seed abortion may be the result of different factors acting in combination, but with varying intensity, at each site.

Plants in the southern populations produced smaller capsules, correlating with a decrease in both the number of seeds produced and the proportion of viable seeds. Since the southern populations had considerably fewer flowering plants (~ 7 - 100) than those in the north (> 200), this might reflect a negative relationship between population size and seed number or quality (Menges 1991; Heschel and Paige 1995; Fischer and Matthies 1998; Oostermeijer *et al.* 1998). However, as population attributes are confounded with geographic separation, we cannot attribute differences in fecundity solely to population size. Most likely, final reproductive output is determined by a combination of factors.

There are at least three possible explanations for variation in seed viability among populations: differences in (1) amount or quality of pollen received, (2) resource limitation, or (3) genetic load (Haig and Westoby 1988a; Lee 1988; Charlesworth 1989). Henceforth, we assess the relevance of these with respect to seed viability in *C. rigida*.

<u>*Pollen limitation*</u>. Unfertilised ovules are unlikely to be the cause of the empty *C. rigida* seeds observed in our study, as the solid pollinia are transferred as a single unit, delivering relatively constant amounts of pollen grains. Furthermore, a recent study in *Caladenia behrii* demonstrated that seed number and seed size are not affected by pollen load (Petit *et al.* 2009). In some species, heterospecific pollen can reduce seed quality (Neiland and Wilcock 1998) and this requires further investigation in *C. rigida*. However, the risk of receiving foreign pollen is likely to be low for *C. rigida*, as the species flowers earlier than most heterospecifics (APPENDIX B).

Resource limitation. In our study sites, soil phosphorous differed significantly between the two regions and was related to seed viability. There was also a negative relationship between exchangeable potassium concentration and the proportion of seeds with non-viable embryos. Nutrient analyses of epiphytic orchids have demonstrated that 18 - 26% of plant phosphorous and around 30% of potassium is incorporated into maturing capsules (Benzing and Ott 1981; Zotz 2000). Although there is no empirical evidence linking these nutrients with seed viability, and it is not known what levels of soil nutrients are required for reproductive allocation, it is conceivable that nutrient concentrations may be affecting seed development in *C. rigida*.

The smaller size of *C. rigida* plants in most of the southern populations could reflect differences in resource availability. Larger *C. rigida* plants produced larger capsules (and hence more seeds), and the proportion of viable seeds within capsules increased with capsule size. However, the sampling intensity used may have been insufficient to detect a direct correlation between seed viability and plant size. The relationship between plant size and seed production is often used as an indirect measure of resource limitation (Colas *et al.* 2001; Griffin and Barrett 2002), as plants with a higher photosynthetic area and storage capacity are expected to allocate more resources to reproduction. In *C. rigida*, fruit maturation is unlikely to be limited by resources as capsule abortion was rare in all populations, even under conditions of supplemental pollination or drought (CHAPTER 5). However, reductions in capsule size and seed viability may reflect an increase in the seed abortion in response to resource constraints (Casper 1984; Helenurm and Schaal 1996). Water availability is unlikely to have contributed directly to seed viability as populations in the south received higher rainfall than those in the north. Additionally, we found no evidence for indirect consequences of rainfall, such as greater shading due to increases in canopy or understorey cover.

Genetic load. In plants that are predominantly outcrossing, the expression of lethal mutations during early seed development can lead to embryonic abortion (Wiens *et al.* 1987; Charlesworth 1989), and may account for at least some proportion of empty seeds recorded in populations of *C. rigida*. Additionally, competitive interactions amongst developing embryos can select against seeds with inferior genotypes (Wiens *et al.* 1987). Our data show that a considerable number of seeds contained non-viable embryos, regardless of the population or region they came from. One possible explanation for this is

that deleterious alleles that are expressed during later stages of embryo development can reduce seed viability; however, empirical support for this is lacking.

An increase in the incidence of selfing and/or bi-parental mating could explain the low proportion of viable seeds in the southern *C. rigida* populations. Pollinators are more likely to revisit flowers or move between closely related individuals when flower numbers are low (Maad and Reinhammar 2004; Waites and Ågren 2004; Johnson *et al.* 2009), and reductions in the mass and/or viability of seeds produced by self-pollination have been demonstrated for several orchid species, including the closely related *C. behrii* (Wallace 2003; Jersáková *et al.* 2006a; Jersáková *et al.* 2006b; Smithson 2006; Petit *et al.* 2009). Selfing is unlikely to be the cause of empty seeds in *Caladenia*, as other studies have found that capsules from artificial self- and cross-pollinations in *C. tentaculata* and *C. rigida* do not differ with respect to the proportion of seeds with embryos (Peakall and Beattie 1996; Bickerton 1998). However, as these studies did not distinguish between seeds containing viable and non-viable embryos, additional experiments are required to determine whether self-pollination affects the viability of *C. rigida* seeds.

In a concurrent study, microsatellite analysis detected inbreeding in several *C. rigida* populations; but the average inbreeding coefficient of adult plants did not differ between the northern and southern regions (L. Farrington, unpubl. data), and the magnitude of inbreeding was not particularly high in comparison with studies on congeneric species (Phillips *et al.* 2009b). Furthermore, there was no relationship between the average inbreeding coefficient and seed viability (data not shown). The potential longevity of *Caladenia* plants (up to 20 years, P. McCauley, pers. comm.) and low recruitment rates, are factors that are likely to delay the expression of inbreeding in adult plants. Genetic sampling of seeds or seedlings is therefore required to determine whether differences in seed viability can be attributed to mating patterns and inbreeding in the current generation.

7.4.2 Estimate of recruitment potential

Combining our seed viability data with measures of germination success and seedling survival based on two other *Caladenia* species, we estimate that between six and 34 capsules are required to replace a single *C. rigida* individual in the southern populations. Replacement of all seven mature individuals in SC2 would require 42 – 238 capsules over the lifetime of these orchids. Assuming that *C. rigida* plants live for 20 years (based on cultivated plants and so likely to be an overestimation), this would translate to 2 - 12

capsules required per year. Although this falls within the natural seed set (three capsules) observed in the two smallest populations in 2007, the number of emergent flowers and subsequent reproductive success of *C. rigida* is highly variable among years (CHAPTER 5). For example, in 2006 there were no flowering plants in SC1 and SC2, and no capsules produced in IB2 and IB3, whereas 2007 (the year of the current study) was a particularly favourable year for flowering and pollination in all populations.

Given the above estimates, rates of capsule production required to sustain the smallest populations are unlikely to occur under natural conditions. Furthermore, our calculations are probably conservative as they are based on seed viability ascertained by FDA staining, which has been shown to over-estimate the actual germinability of seeds (Batty *et al.* 2001b; Wright 2007). Yearly variation in seed viability or seedling survival may also influence the above predictions (Ouborg and Van Treuren 1995; Batty *et al.* 2001a; Oien *et al.* 2008), and multiple year studies are required to obtain a more complete understanding of patterns of seed viability and recruitment. Ideally, field germination trials, which were beyond the scope of this study, should also be conducted to assess actual seedling recruitment within each population. Nevertheless, based on reasonable assumptions, our findings indicate that poor seed quality places a major constraint on the recruitment potential of some populations, regardless of the availability of safe sites.

Conclusions

Clearly, long-term persistence of populations is contingent on the production of viable seeds that can contribute to subsequent generations. Most studies of threatened plant populations assess reproductive success based solely on measures of fruit or seed production. The results presented here, highlight the need to incorporate an assessment of seed quality and recruitment potential. From a management perspective, conducting germination trials on every threatened species is obviously resource exhaustive and impractical. Therefore, the use of seed viability data from the target species, combined with germination and seedling survival data from congeneric species, offers a more realistic approach for assessing the viability of threatened plant populations.

In a concurrent study we did not detect a consistent Allee effect (ie. reduced reproductive success in small populations) with respect to capsule production and seed release in *C. rigida* (CHAPTER 5). However, results from the present investigation suggest that small populations of *C. rigida* may indeed suffer from reduced fitness when considering the

output of potential recruits. As mentioned above, confirmation of this relies on disentangling the degree to which population size and geographic location influence seed quality.

Despite good rates of fruit set in some years, relative to larger northern populations, the outlook for the long-term persistence of southern populations seems grim unless intervention takes place. Supplemental pollination within populations will do little to augment reproductive output if seed viability is poor. Future management strategies might need to consider the introduction of new genetic material in the form of pollen, seed, or translocated plants from other (viable) populations. However, we stress the importance of further research employing controlled crossing experiments both within and between populations and regions, to determine whether reductions in seed quality are indeed due to genetic factors. The potential influence of soil nutrients on the viability of orchid seeds also needs to be tested empirically. Given the low rates of germination and recruitment observed for other *Caladenia* species, providing conditions that favour the establishment and survival of new recruits and adult plants may be crucial for ensuring the long-term persistence of populations, provided that these populations have first been shown to produce viable seeds.

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An unusual colour form of Caladenia rigida, at the South Para (SP) site (Photo by author)

GENERAL DISCUSSION

The objective of this thesis was to investigate multispecies interactions that influence the reproductive ecology of two *Caladenia* species. This final chapter draws together the main conclusions from my study, and discusses their contribution to plant science and plant conservation.

Three key interactions were identified as playing a major role in determining maternal fecundity, namely those with pollinators, florivores and frugivores. In the following sections, I discuss factors that affect the strength of these interactions, focusing first on the mutualistic relationship between orchids and pollinators, and then on antagonistic interactions with predators. This is followed by a section that summarises the implications of seed quality on the maintenance and growth of orchid populations. Finally, I relate the relevance of my findings to the management of declining plant populations, and suggest further areas of research, which can build on the results of this project.

8.1 Plant-pollinator interactions

Through the capture and identification of several food-seeking insects, I demonstrated that *C. rigida* employs a generalised pollination strategy (CHAPTER 3). At the onset of this study, the species was assumed to be rewardless; however, the results presented here reveal that the flowers secrete sugars at levels comparable to some nectar-producing orchids. Whether this nectar plays an important role in attracting or sustaining insect visitors requires further investigation, but strategies to achieve this are likely to be complex. I have therefore referred to *C. rigida* as a food-advertising orchid, as this term reflects the attraction of food-seeking insects, without dictating whether or not a food reward is actually offered. This term may also be useful to describe other *Caladenia* species that are currently regarded as food-deceptive, awaiting confirmation of the absence of nectar using more sensitive analyses. Regardless of the importance of nectar, findings that *C. rigida* utilises a generalised pollination strategy provided a unique opportunity for comparisons with a highly specialised congener, *C. tentaculata* (CHAPTER 5).

Supplemental pollination confirmed that both *C. rigida* and *C. tentaculata* are primarily limited by the availability of pollen. By comparing patterns of pollination success within the same geographic region and during the same flowering season, I showed that the generalist species, *C. rigida* and *C. carnea,* were less pollen limited than the specialist species, *C. tentaculata.* Plants that are pollinated by taxonomically diverse insects may be buffered against spatial and temporal fluctuations in pollinator availability, whereas those relying on species-specific pollination are at greatest risk of pollination failure when pollinators are scarce (reviewed in Wilcock and Neiland 2003). However, the infidelity associated with generalist strategies can have undesirable consequences such as an increased probability of pollen loss during transport or an increased likelihood of receiving heterospecific pollen (Wilcock and Neiland 2003). The timing of flowering may be an important strategy to reduce these risks, and my results demonstrate that the peak flowering time of *C. rigida* occurs just prior to the main heterospecific spring flush (APPENDIX B). In contrast, one could expect that the later-flowering generalist, *C. carnea*, is exposed to greater rates of pollen wastage and interspecific pollen transfer, possibly reducing pollination success. Although this area awaits further investigation, capsule production by *C. carnea* was still considerably higher than that of the specialist *C. tentaculata*, suggesting that the advantages of generalist strategies compensate for most disadvantages, at least with respect to successful pollination. Clearly, evaluation of the relative importance of pollination specificity requires a more comprehensive study that encompasses several generalist and specialist species across similar spatial and temporal scales. Nevertheless, the results presented here provide an important foundation and impetus for such research.

The seemingly inefficient pollination strategy of *C. tentaculata* raises the question as to how such extreme specialisation has evolved. One of the theories commonly used to explain the evolution of food-deception is that the advantages of outcrossing outweigh both the resource and genetic costs incurred by producing a nectar reward (Peakall and Beattie 1996; Schiestl 2005; Jersáková *et al.* 2006a). In a similar way, sexually deceptive specialists may have taken this one step further, optimising the benefits of outcrossing at the expense of pollen quantity. Furthermore, orchids exploiting the reproductive behaviour of insects may benefit from increased pollinator fidelity, thus reducing the transfer of interspecific pollen (Schiestl and Schlüter 2009). When attempting to understand the evolution of pollination strategies, it is important to keep in mind that current

environmental and habitat conditions are likely to be very different to those that have shaped evolutionary pathways. For example, *C. tentaculata* may once have enjoyed much greater pollination success, but is now vulnerable to changes in the abundance and distribution of thynnine wasps brought about by landscape modification and a changing climate. Drought conditions and land clearance have been implicated as factors that limit thynnine wasp egg production through declines in flowering food plants (Ridsdill Smith 1970), and through competition with introduced honey bees (Denny 1992; Paini and Roberts 2005).

One of the strengths of this study is that it monitored variability in reproductive performance over multiple years and sites. For *C. rigida*, pollination success was highly variable among populations and among flowering seasons (CHAPTER 5). Several factors were identified as potentially contributing to this variation. Spatio-temporal differences in pollinator assemblages are likely to play a major role but confirmation of this requires detailed characterisation of pollinator abundance and diversity among sites and years. Investigation of pollinator efficiency may also be valuable, as several studies have shown that different groups of insect pollinators vary in their behaviour and can influence not only pollination intensity, but also patterns of gene flow within and between populations (Mahy *et al.* 1998; Gómez and Zamora 1999; de la Bandera and Traveset 2006).

Even though pollination success for *C. tentaculata* was considerably lower in all study years, temporal and spatial variability, relative to average capsule production, was greater for *C. tentaculata* than for *C. rigida*. This is consistent with predictions that specialists are more prone to fluctuations in reproductive success than generalist species. Indeed, the specialist may enjoy much higher rates of pollination success in certain years, as reported for *C. tentaculata* populations within the eastern part of Australia (Peakall and Beattie 1996). A longer-term study of the populations examined in this project is therefore required to determine whether the pollination specialist is also subject to large fluctuations in reproductive success within the Mount Lofty region of South Australia.

The continued decline of *C. rigida* populations within the southern region of the species' distribution is of particular concern. Since rates of capsule production were comparable to those of northern populations, pollinator availability does not appear to be a limiting factor when conditions are favourable. However, the implications of small population size were shown to be important in a drought year (2006), providing one of the first examples of

temporal variation in an Allee effect. As hand pollination demonstrated that *C. rigida* was pollen limited during this drought season, it can be inferred that resources can indirectly constrain capsule production through the availability of pollinators. Hence, although orchids are primarily pollen limited (Tremblay *et al.* 2005), my results suggest that under some circumstances, resource constraints may drive pollen limitation.

Unfavourable conditions are not necessarily restricted to years of low rainfall. For example, despite good rates of emergence and flowering of *C. tentaculata* in 2005, the peak flowering time coincided with an extended period of cool and rainy weather (personal observation). This could partly explain the low rates of pollination success recorded for the species in 2005, as thynnine wasps are unlikely to be active during such conditions (Stoutamire 1983). With predictions of dramatic changes to climatic conditions, increases in the number of years with extreme weather may lead to substantial declines in reproductive success as the number of favourable years declines, or the time between favourable years increases. Population viability analyses predict that the compensatory effects of reproductively successful years are eliminated when poor years become too frequent, hence increasing the risk of extinction (Oostermeijer *et al.* 2003).

8.2 Antagonistic interactions

Factors that influence plant reproductive success are usually characterised based on a single response variable, such as pollination or fruit set. This project also assessed the proportion of plants that actually released seed and, thus, had the potential to contribute to further generations. Seed release by *C. rigida* and *C. tentaculata* was significantly lower than rates of pollination success at several sites and this difference was largely attributable to the predation of flowers and capsules. Whether the orchids have always been subjected to intense grazing pressure, or whether this is a relatively recent threat remains debatable. The latter is more likely to apply, as the abundance and distribution of the predominant florivore, the white-winged chough (*Corcorax melanorhamphos*), has changed in response to habitat fragmentation, with the birds now being restricted to large habitat remnants (Cox and Bauer 1997; Watson *et al.* 2003). In the current study, choughs appeared to be associated with sites containing nearby stands of *Pinus radiata* (personal observation), supporting observations that the birds also make use of this modified habitat (Higgins *et al.* 2006). Undoubtedly, further studies into the ecology and foraging behaviour of white-winged choughs would benefit both the birds and orchids.

The final reproductive output of *C. rigida* was clearly the outcome of interactions with both mutualists and antagonists, emphasising the importance of measuring the combined effects of pollinators and predators. The scale at which these interactions are assessed is also important, as their strength and direction varied substantially across time, among populations and within populations. For example, rates of florivory within populations differed between patches of plants and also according to their distance from habitat edges (CHAPTER 4). At the finer spatial scale, variation occurred in response to a phenotypic characteristic, floral height, as well as to the local environmental context, namely the density of conspecifics and concealment amongst neighbouring vegetation (CHAPTER 6 & APPENDIX C). Interestingly, none of these factors imposed a compromise between opposing interactions, suggesting that pollinators and predators respond to different cues when detecting these flowers. The height of flowers had a positive effect on pollination success but not predation risk. On the other hand, the local density of conspecifics and concealment among neighbouring vegetation both influenced a flower's risk of being browsed, but not its likelihood of being pollinated. Assuming that flower height is at least partly genetically determined, one might expect that the selective pressures imposed by pollinators would result in evolution towards taller *C. rigida* plants. However, spatio-temporal variation, such as that observed in this study, is likely to weaken or disrupt any evolutionary effects of pollinator-mediated selection (Ehrlén *et al.* 2002; Gómez 2003; Kolb *et al.* 2007). Furthermore, flower height may also be constrained by other genetic and environmental factors.

8.3 Seed viability and recruitment potential

Reduced seed quality appears to be a major cause for the poor status of southern populations of *C. rigida* (CHAPTER 7). Although a concurrent study did not detect differences in inbreeding coefficients among populations (L. Farrington, pers. comm.), it is possible that the reductions in the size of southern populations have occurred too recently for the expression of inbreeding to be detected. An important follow up to this research is therefore to identify factors affecting variation in seed viability. The contribution of genetic components could be elucidated through a series of self- and cross-pollination experiments with pollen donors from within populations, within regions, and between regions. Although most of the habitat characteristics assessed in this study did not differ between the two regions, environmental factors cannot be completely ruled out as a factor contributing to variation in seed quality. Differences in the concentration of soil

phosphorous may account for some of the variation; however, this needs to be tested empirically.

Calculations incorporating my results for *C. rigida* seed viability, with germination and seedling recruitment data from two other *Caladenia* species, indicate that current rates of seed production are unlikely to sustain the smallest of the southern populations. Clearly, this warrants urgent research into the causes of poor seed quality, striving for strategies that boost the production of viable seeds in these populations.

8.4 Implications for the management of threatened plant populations

Many of the findings of this project have relevance to the conservation and management of plants. Management plans usually address threats to species at the landscape level; however, my results suggest that in some cases, a more fine-scale approach may be necessary. All of the responses measured, namely pollination success, florivory, capsule predation and seed viability, varied significantly among populations, among years or both. Such spatio-temporal variation highlights the importance of monitoring multiple populations over several years in order to obtain an accurate perspective of population dynamics. This not only has implications for basic ecological research, but also is vital when obtaining baseline data for the design and implementation of conservation and management strategies. For example, identifying the populations that are at greatest risk of reproductive failure and, within these, the plants that are most likely to succeed, may help to initiate a more targeted approach to the successful conservation and recovery of rare plants.

Conservation strategies that also integrate the habitat requirements of pollinators first require knowledge of the pollination strategy employed by the target species and, ideally, identification of the pollinating vectors. The use of pan traps to capture insects carrying pollinia provides a simple and cost-effective way of identifying pollinators of food-advertising orchids, and has since been used successfully in the field by management officers (G. Nevill, pers. comm.). Although genetic analysis offers the most accurate method of pollinia identification, morphological characterisation of pollinia may be a simpler and less-expensive alternative, provided that the target orchid species does not have co-flowering congeners (with morphologically similar pollinia) in close proximity.

Grazing posed a major threat for several orchid populations in the northern region, and identification of herbivores provides an important first step towards alleviating this pressure. Threat abatement in these populations becomes a complex issue because the predominant florivore, the white-winged chough, is itself listed as Rare in South Australia (National Parks and Wildlife Act 1972). Caging plants to exclude florivores is only feasible for small populations, and mesh sizes need to be chosen carefully to minimise their impact on pollinator movement. Protection of larger orchid populations from florivores may require more innovative solutions. For example, visual and acoustic scaring devices or the supply of a decoy food source have been successfully used to reduce the impact of some bird species on economically important crops (Bomford and Sinclair 2002).

The greatest threat facing *C. rigida* populations in the southern region appears to be poor seed quality, and my findings emphasise the value of assessing seed viability in threatened plants. Current management strategies (protection from herbivores and hand-pollination) are unlikely to be successful unless the causes of low seed viability are addressed. This is probably one of the most urgently required areas of research as the size of some of these populations was demonstrated to be perilously low.

Site amelioration such as the removal of competing vegetation did not appear to benefit *C. rigida* populations and may indeed impose a greater risk of predation. My findings that low density and proximity to neighbouring vegetation offers some protection against grazing, without affecting pollination success, could be incorporated into the design of programs aimed at reintroducing or translocating threatened species back into the field. To be successful, such initiatives must also consider the ecological requirements of pollinators while keeping in mind the relationship between the timing of orchid flowering and that of heterospecific plants. For example, the flowering phenology of *C. rigida* suggests that this species may benefit from a lack of competition for pollinators (APPENDIX B).

Funding constraints impose that conservation programs are only applied to threatened species, and consequently the population dynamics of species that are currently considered to be widespread and common are not monitored. The results presented in this thesis, show that sexually deceptive species such as *C. tentaculata* may be particularly vulnerable to pollinator loss. Without ongoing monitoring, the long-term persistence of *C. tentaculata* populations cannot be predicted, and, given the potential longevity of some orchids,

declines are likely to remain undetected for some time. Slowing the rate of species loss may therefore require a major shift in the way we think about conservation; focusing our efforts on protecting species long before signs of degradation and decline become obvious.

8.5 Further research

Several areas requiring further investigation were identified throughout this study, and are summarised below.

- Characterising pollinator abundance and diversity among populations and among years.
- Comparing pollination efficiency among different pollinator taxa.
- Investigating the importance of nectar production for the pollination success of *C. rigida.*
- Analysing the scent emission of *C. rigida* using gas chromatography, and comparing this with kairomones released by sexually deceptive *Caladenia*.
- Investigating the possible production of nectar in other food-advertising *Caladenia*.
- Assessing the potential of cages with different mesh sizes to impede pollinators of *C. tentaculata* or other sexually deceptive species.
- Trials of mechanisms to alleviate grazing, such as scaring devices or the supply of a decoy food source.
- Investigating the long-term consequences of florivory on orchid population dynamics.
- Continuing the long-term monitoring of *C. tentaculata* across several populations to determine the frequency with which this species experiences more favourable years for pollination and reproductive success, and to assess whether it is also subject to the consequences of small population size.
- Further large-scale slashing experiments with herbivore exclusion and sampling of insects inside and outside of treatment areas, to determine whether vegetation removal affects the abundance, diversity and distribution of pollinators.
- Examining whether seed viability in *C. rigida* varies among years, and also assessing the germination potential of seeds.
- Assessing the importance of genetic contribution to seed viability by performing a series of self- and cross-pollination experiments with donor pollinia from within, and between, populations and regions.
- Elucidating the extent to which resources influence seed quality using nutrient (eg. phosphorous) addition experiments on cultivated plants.
- Investigating the degree to which orchid populations are seed or microsite limited through seed addition experiments.
- Investigating the identity and distribution of mycorrhizal fungi associated with *C. rigida*, and their potential role in seed production and seed quality.

8.6 Conclusions

Maternal fecundity of *C. rigida* and *C. tentaculata* relies on a number of counterbalancing interactions and final reproductive output is determined by the ecological context in which these interactions are embedded. At the plant level, phenotypical characteristics such as pollination strategy and flower height affected relationships between plants and pollinators. Interactions with florivores and capsule predators were influenced by variables operating at the population level (ie. density) and at the habitat level (ie. concealment amongst neighbouring vegetation). The smallest populations failed to reproduce during stressful environmental conditions, and reduced population size may also have contributed to poor seed quality in some populations. The findings of this project have provided valuable scientific data on which to base management decisions.

Priority areas requiring further research include investigating the underlying causes for poor seed quality in declining populations of *C. rigida*, alleviating grazing pressure in northern populations with poor reproductive output, and ongoing monitoring of populations of threatened as well as common orchid species.

IDENTIFICATION OF POLLINATORS OF *CALADENIA CARNEA*

During this study, insects bearing pollinia from *Caladenia carnea* were captured (in addition to those identified for *C. rigida* in CHAPTER 3). Identification of these insects is provided in this section.

Using the method described in CHAPTER 3, pantraps were placed amongst patches of *C. carnea* flowers at the CCP site (see CHAPTER 5 for site location and description). This involved setting up 15 yellow and 15 blue pantraps on 18th September 2007, and 10 of each colour on 4th October 2007. Two native bees bearing pollinia were captured in one of the yellow traps on 18th September. These were identified as females of *Lasioglossum* (*Ctenonomia*) *semipolitum* Cockerell and *L.* (*Chilalictus*) *clelandi* Cockerell (Ken Walker, pers. comm.). In both cases, pollinia were loosely attached to the dorsal part of the thorax and were dislodged when the insect was retrieved from the traps, leaving a small remnant of pollinia adhering to the (Fig. A.1). DNA sequencing confirmed that the pollinia were from *C. carnea* (Farrington *et al.* 2009).

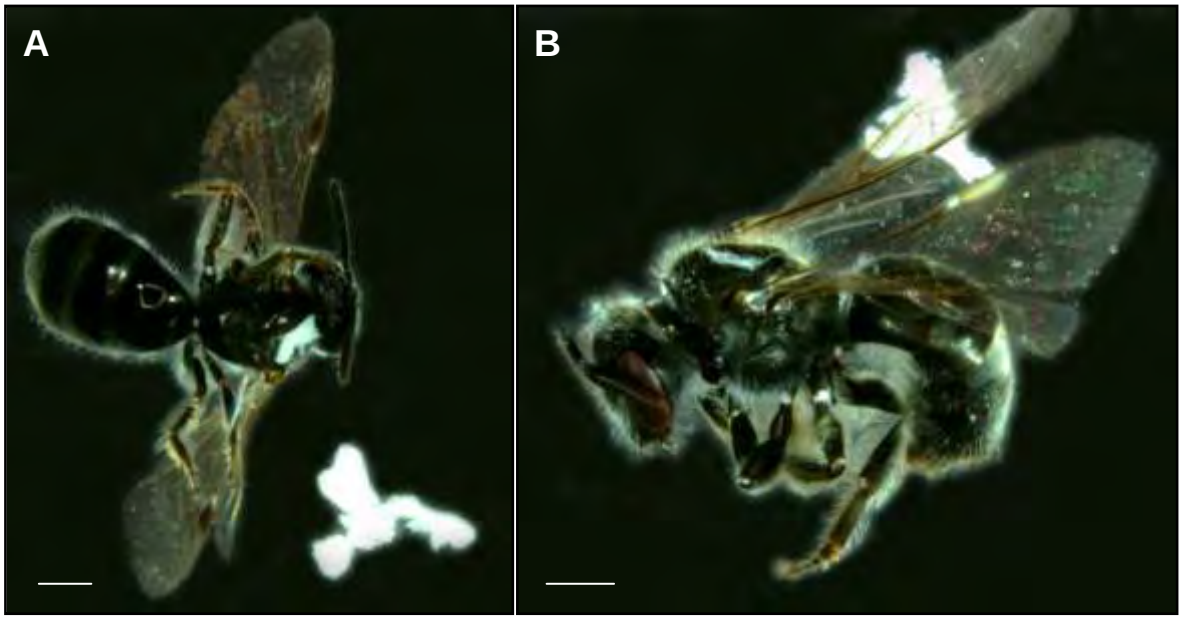

Fig. A. 1 Insects captured in pantraps, carrying *C. carnea* pollinia. A) *Lasioglossum semipolitum.* B) *Lasioglossum clelandi.* Pollinia were dislodged from the insect, but remnants can be seen attached to the dorsal thorax. Scale bar = 1 mm.

These findings complement a number of other studies that have reported native bees as pollinators of *C. carnea* (Bates 1984b; Adams *et al.* 1992; van der Cingel 2001). Of these studies, only *Trigona* spp. can be considered as confirmed pollinators of *C. carnea* in northern Queensland, on the basis of the observed transfer of pollinia (Adams *et al.* 1992). As discussed in CHAPTER 3, insects carrying orchid pollinia can also be inferred to be pollinators and, as such, the results presented here have added two more species of native bees as confirmed pollinators of *C. carnea*. In addition, native bees belonging to the genus *Homalictus* have been observed visiting *C. carnea* (van der Cingel 2001). Taken together, these findings confirm that *C. carnea* utilises a generalist pollination strategy, attracting a broad suite of food-seeking native bees. However, detailed nectar analyses are required to determine whether this species offers any reward.

ASSESSMENT OF THE ABUNDANCE OF CO-FLOWERING SPECIES RELATIVE TO ORCHID FLOWERING PHENOLOGY

B.1 Introduction

As mentioned in CHAPTERS 5 and 7, changes in the floristic community across space and time, may be an important source of spatio-temporal variation in pollination success (by affecting pollinator visitation and behaviour), as well as reproductive output (by affecting seed quality). In some cases, pollination of both food-deceptive and rewarding species can be facilitated by the presence of co-flowering plants that attract and/or sustain pollinators (Laverty 1992; Oostermeijer *et al.* 1998; Johnson *et al.* 2003; Juillet *et al.* 2007). This is often referred to as the 'magnet species hypothesis'. Conversely, the 'remote habitat hypothesis' predicts that heterospecific flowers are a source of competition for pollinator services, particularly for deceptive species (Lammi and Kuitunen 1995; Internicola *et al.* 2006). Evidence for competition has also been provided for plants that offer a reward. For example, experimental manipulations by Sih and Baltus (1987) demonstrated that pollinator visits to catnip (*Nepita cataria*) flowers were lowest when co-flowering species were at their peak. Similarly, Brown *et al*. (2002) demonstrated that the presence of an invasive co-flowering species, *Lythrum salicaria,* not only reduced pollinator visitation and seed set in its native congener, *L. alatum*, but also increased the risk of heterospecific pollen transfer. Theoretical modeling predicts that the timing of flowering may be one strategy that allows for the co-existence of species (Ishii and Higashi 2001). Rewarding orchids may therefore do best when competition for pollinators is minimised.

This section assesses spatio-temporal variation in the floristic composition of understorey vegetation among sites, and relates this to the phenology of orchid flowering and pollination, for both *Caladenia rigida* and *C. tentaculata*.

B.2 Methods

Abundance of co-flowering species

In 2005, I assessed the abundance and degree of flowering of co-flowering understorey species at each of the sites MC1, MC2, MB1, MB2, SP, MC4 and BR (site locations and descriptions are provided in Fig. 5.1 and Table 5.1). The MC and MB sites are characterised by *Eucalyptus* woodlands *(E. obliqua*, *E. leucoxylon*, *E. fasciculosa*) with understorey vegetation dominated by *Acacia, Leptospermum*, *Hibbertia, Pultenea* and *Lepidosperma* species. The SP site has a more open canopy of *Eucalyptus fasciculosa* and *Allocasuarina verticillata* and a sparser understorey including *Acacia paradoxa*, *Wurmbea dioica*, *Gonocarpus elatus*, *Hibbertia* spp. and *Lepidosperma* spp. Because data collection in 2005 began after the majority of *C. rigida* flowers had already opened, the surveys of co-flowering species were repeated in 2007 at sites MC2, MB1, MB2 and SP (MC1 was excluded due to the slashing experiment conducted there).

At each site, I set up three (50 m x 10 m) belt transects, divided into 20 (5 m x 5 m) quadrats. Transects started at the edge of the orchid population, usually along a fire break or track, and ran parallel to each other through the orchid population. Distances between transects were randomly assigned, but were at least 25 m and no more than 50 m apart, to ensure that they remained within the boundaries of the population.

Within each quadrat I recorded the number of co-flowering species. To obtain a better representation of the abundance of actual flowers, rather than entire plants, I subjectively estimated both the Abundance of the Flowering Species (AFS), i.e. the percentage of the quadrat occupied by the plant, and its Degree of Flowering (DF), i.e. the percentage of the plant covered by open flowers. I assigned a code value to each of five categories (1 = less than 5%; 2 = 5 to 25%; 3 = 26 to 50%; 4 = 51 to 75%; 5 = greater than 75%). An AFS of 1 therefore represents a flowering species occupying less than 5% of a quadrat, while an AFS of 5, represents a flowering species that occupies more than 75% of the quadrat. Similarly, a DFS of 1 corresponds to a species in which less than 5% of the plant was covered by open flowers, while a species with a DFS of 5 had more than 75% covered. The AFS and DF were monitored at two to three week intervals throughout the orchid flowering season. These two values were multiplied to obtain the Abundance of Co-flowers (ACF): for example, a species with AFS = 2 and DF = 3 has an ACF of 6. The sum of ACFs then provided the Total Abundance of Co-flowers for each transect. For

graphical representation and statistical analysis, I treated each transect as a replicate to provide the Average (Total) Abundance of Co-flowers for each time point at each site.

Phenology of Orchid Flowering and Pollination

As described in CHAPTER 5 (section 5.2), the flowering and pollination status of tagged orchid plants was recorded every one to two weeks, at each site. At each monitoring time point I calculated the percentage of open flowers and pollinated flowers, relative to the total number of flowering plants. Grazed flowers were excluded. The Average Abundance of Co-flowers was then overlaid graphically onto the orchids' flowering and pollination phenology at each site. Due to the low rates of pollination success for *C. tentaculata*, I have not shown the pollination phenology for this species.

Data analysis

I assessed variation in the Average Abundance of Co-flowers across monitoring time points using ANOVA followed by Tukey-Kramer HSD pairwise comparisons. Homoscedasticity was tested with Levene's test for equal variance. When variances were heterogeneous, I used Welch-corrected ANOVA with Games-Howell post-hoc comparisons. The statistical package SPSS 15.0 was used for all analyses.

B.3 Results

C. rigida

Most *C. rigida* flowers remained open for approximately 20 days, but a small number lasted for as long as 30 - 40 days. In both study years, the majority of pollinations (greater than 70%) occurred within the first two to three weeks of opening; the exception being at the SP population in 2007, where approximately 50% of pollinations occurred after this time (Fig. B.1 and Fig. B.2). In 2005, the Average Abundance of Co-flowers differed throughout the flowering season at sites MC1, MC2 and MB2, and this difference was also marginally significant at sites MB1 and SP (Fig. B.1). At all sites, except SP, there were fewer heterospecific flowers at the beginning of the orchid's flowering season, than towards the end. At SP, the Average Abundance of Co-flowers did not change during the orchid's peak period of flowering and pollination. In 2007, the Average Abundance of Co-flowers varied across the flowering season at all sites, although this difference was only marginally significant at MC2 (Fig. B.2). At each site, the abundance of heterospecific

flowers was lower at the beginning of the orchid's flowering season. In general, the increase in the Average Abundance of Co-flowers as the season progressed was due to an increase in the number of flowering species, combined with an increase in their degree of flowering (Table B.1).

C. tentaculata

The majority of *C. tentaculata* flowers remained open for at least three to four weeks. The Average Abundance of Co-flowers did not differ between time points at site MC4 and varied only marginally at sites SP and BR (Fig. B.3). At SP, heterospecific flowers were most abundant at the beginning of the orchid's flowering season, while the reverse situation occurred at BR.

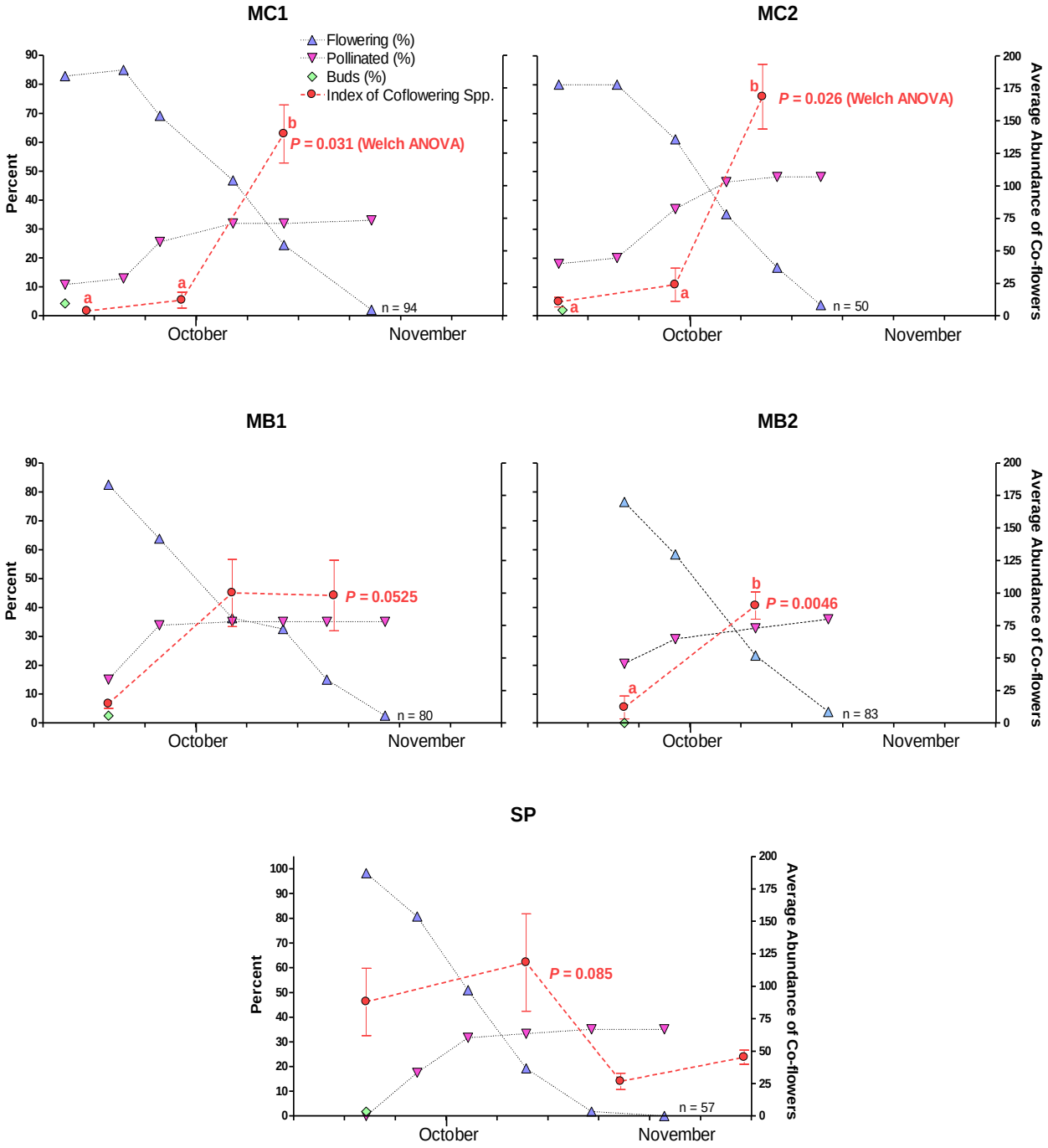

Fig. B. 1 2005 phenology of flowering (percent flowering plants) and pollination (percent pollinated plants) of *Caladenia rigida* (left axis), overlayed with the Average Abundance of Co-flowers (average of transects) (right axis). Note that pollination success is cumulative whereas flowering is non-cumulative. Error bars indicate the standard error of the mean (s.e.m.). Percentage of flowers still in bud are shown for the first monitoring time point. Results for ANOVA and Welch-corrected ANOVA, testing for significant variation among monitoring time points, are indicated. Time points at which the Average Abundance of Co-flowers is significantly different are indicated by different lowercase letters ($P < 0.05$; Tukey-Kramer HSD or Games-Howell pairwise comparisons).

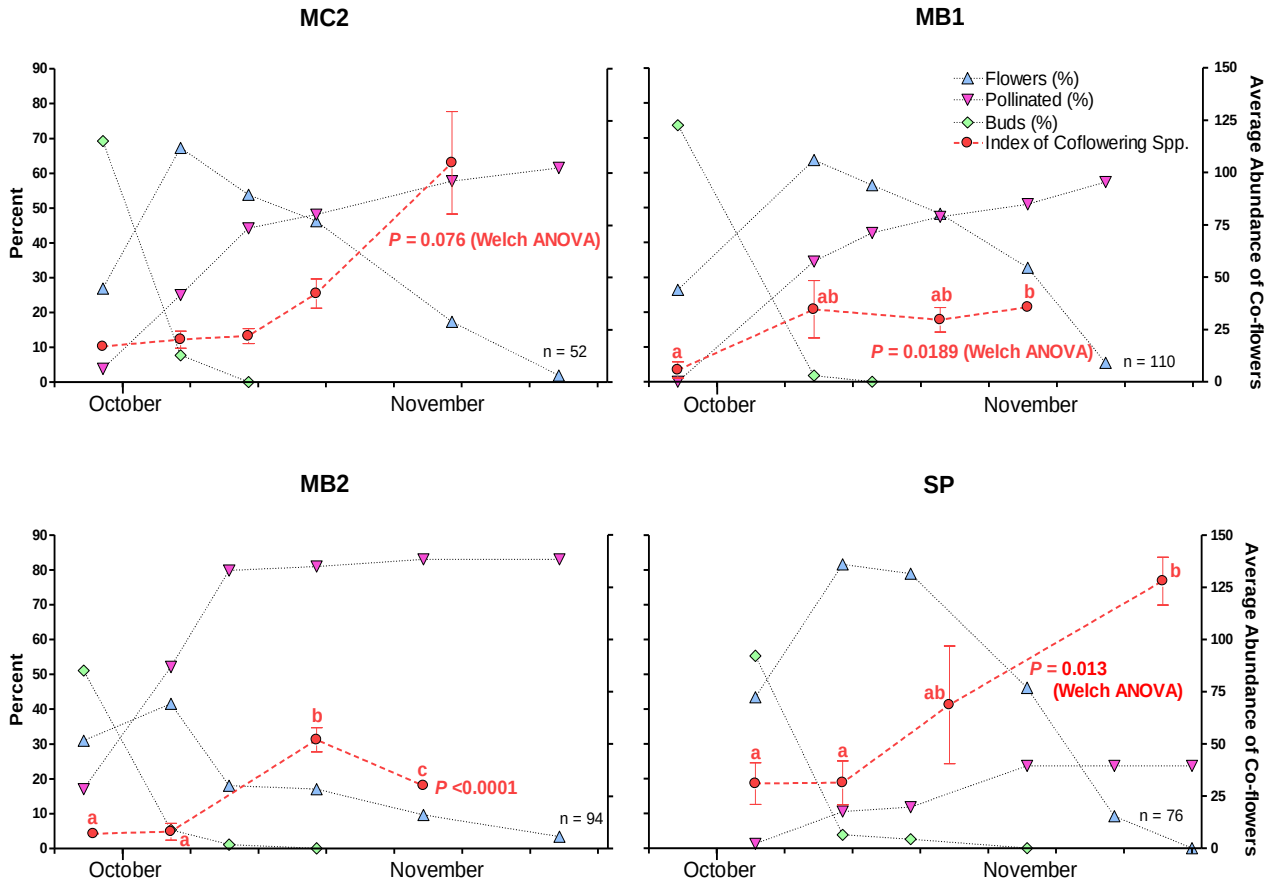

Fig. B. 2 2007 phenology of flowering (percent flowering plants) and pollination (percent pollinated plants) of *Caladenia rigida* (left axis), overlayed with the Average Abundance of Co-flowers (right axis). Note that pollination success is cumulative whereas flowering is non-cumulative. Error bars indicate the standard error of the mean (s.e.m.). Results for ANOVA and Welch-corrected ANOVA, testing for significant variation among monitoring time points, are indicated. Time points at which the Average Abundance of Co-flowers is significantly different are indicated by different lowercase letters ($P < 0.05$; Tukey-Kramer HSD or Games-Howell pairwise comparisons).

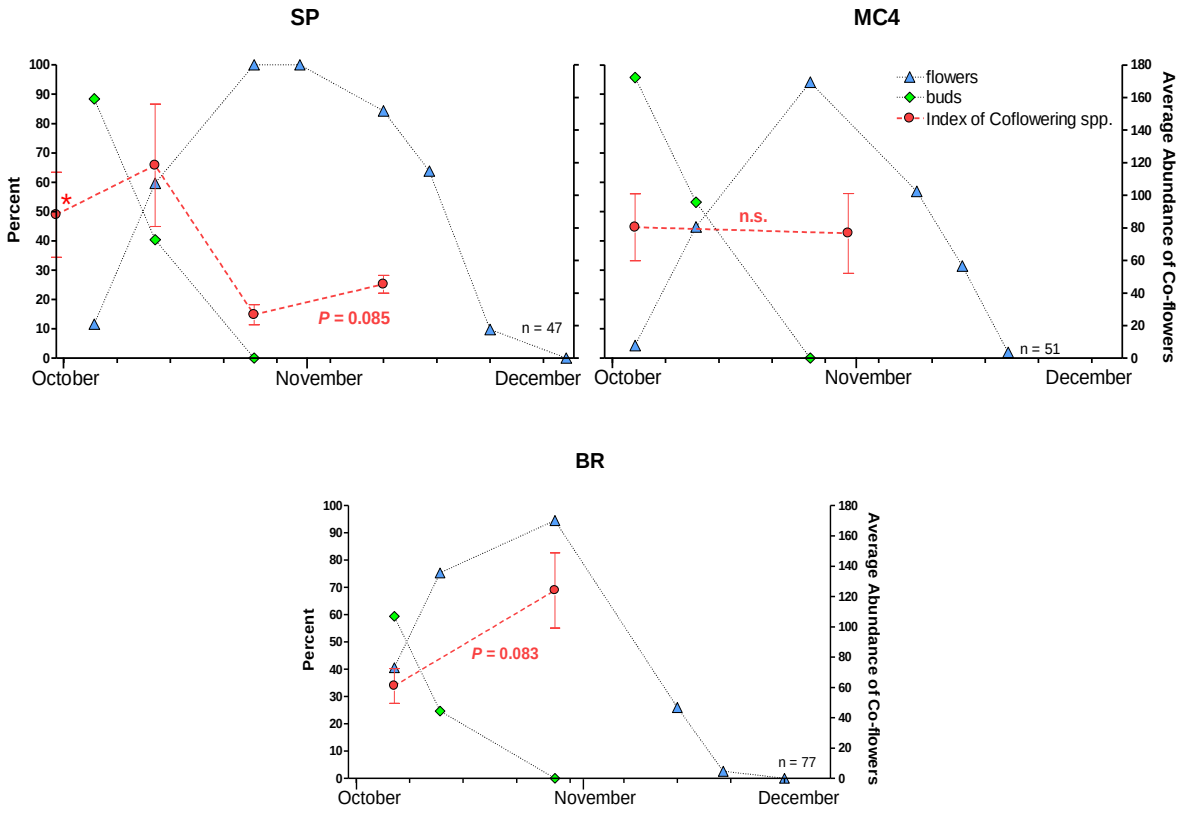

Fig. B. 3 2005 phenology of flowering (percent flowering plants) of *Caladenia tentaculata* (left axis), overlayed with the Average Abundance of Co-flowers (right axis). Error bars indicate the standard error of the mean (s.e.m.). Results for ANOVA, testing for significant variation among monitoring time points, are indicated. *Refers to data collected 10 days earlier (see SP site in Fig. B.1).

B.4 Discussion

These results demonstrate that at the majority of sites, flowering and pollination of *C. rigida* was at its peak before the main spring bloom of neighbourhood plants, whereas *C. tentaculata* flowered later, when the abundance of heterospecific flowers was considerably higher. In the case of *C. rigida*, competition may be particularly important if the amount of reward offered is relatively small compared to other nectar-producing species. Although the reward status of the plant community has not been determined, the scarcity of heterospecific flowers during the peak of *C. rigida* flowering and pollination suggests that this food-advertising orchid is more likely to benefit from a lack of competition rather than facilitation by co-flowering species. In contrast, the pollination strategy of the sexually deceptive *C. tentaculata* is unlikely to promote inter-specific competition for pollinator services. Rather, this orchid may benefit from the presence of

nectariferous plants that provide food resources for thynnine wasps (Phillips *et al.* 2009b). Due to practical reasons, the flowering status of the Eucalyptus overstorey was not formally assessed but may have contributed to the dynamics of orchid pollination. It was noted, however, that *E. obliqua* (the dominant tree at most *C. rigida* sites) began flowering towards the end of the orchid's flowering phenology.

The initial Average Abundance of Co-flowers was considerably higher at SP than at the remaining *C. rigida* sites (at least five-fold in 2005 and at least two-fold in 2007), perhaps partly explaining the lower pollination success observed at this site (CHAPTER 5). The composition of the vegetation community at SP is quite distinctive to that found within the other *C. rigida* populations (see Methods) and may support a different spectrum of pollinators. In particular, differences in the dominant flowering species account for most of the observed variation in the Average Abundance of Co-flowers among sites (Table B.1).

As discussed in CHAPTER 7, the composition of the floristic community has implications for seed quality by affecting pollinator constancy and hence the transfer of heterospecific pollen. The results presented here suggest that the deposition of foreign pollen is unlikely to be the cause of poor seed viability in *C. rigida*, particularly for capsules produced during the orchid's peak flowering period. It is possible that the risk of heterospecific pollen transfer increases later in the flowering season; however, this is not supported by the data collected at the SP site, where a higher initial Average Abundance of Co-flowers did not coincide with reductions in seed viability (CHAPTER 7). Nevertheless, there is an opportunity for further research into the relationship between seed quality and timing of pollination in *C. rigida*. Due to the pollinator specificity of *C. tentaculata*, the risk of heterospecific pollen deposition is expected to be negligible for this species.

Table B. 1 List of species co-flowering at each site during the orchid flowering season. The abundance of co-flowers (ACF) refers to the total of all transects for each species and the total ACF refers to the total of all transects at each time point. * Introduced species.

Site	Date	Dominant Co-flowering spp.	Family	Abundance of Co-flowers (ACF)	Total ACF per time point
2005					
***Caladenia rigida* populations**					
MC1	16/09/05	*Acacia myrtifolia*	Leguminosae	8	
		Lissanthe strigosa	Epacridaceae	3	11
	29/09/05	*Daviesia ulicifolia*	Leguminosae	12	
		Tetratheca pilosa	Tremandraceae	8	
		Acacia myrtifolia	Leguminosae	6	
		Hibbertia exutiacies	Dilleniaceae	4	
		Acacia paradoxa	Leguminosae	4	
		Pultenea daphnoides	Leguminosae	2	36
	13/10/05	*Hibbertia exutiacies*	Dilleniaceae	154	
		Pultenea daphnoides	Leguminosae	92	
		Pultenea largiflorens	Leguminosae	68	
		Tetratheca pilosa	Tremandraceae	44	
		Acacia paradoxa	Leguminosae	26	
		Daviesia ulicifolia	Leguminosae	18	
		Craspedia variabilis	Compositae	10	
		Daviesia leptophylla	Leguminosae	6	
		Acacia myrtifolia	Leguminosae	2	420
MC2	14/09/05	*Acacia myrtifolia*	Leguminosae	20	
		Spyridium parvifolium	Rhamnaceae	10	
		Lissanthe strigosa	Epacridaceae	2	32
	29/09/05	*Spyridium parvifolium*	Rhamnaceae	30	
		Hibbertia sericea	Dilleniaceae	28	
		Acacia myrtifolia	Leguminosae	12	
		Pultenea daphnoides	Leguminosae	2	72
	11/10/05	*Hibbertia exutiacies*	Dilleniaceae	322	
		Pultenea daphnoides	Leguminosae	170	
		Tetratheca pilosa	Tremandraceae	10	
		Daviesia ulicifolia	Leguminosae	2	504
MB1	19/09/05	*Acacia myrtifolia*	Leguminosae	19	
		Pultenea daphnoides	Leguminosae	16	
		Hakea rostrata	Proteaceae	9	44
	6/10/05	*Pultenea daphnoides*	Leguminosae	184	
		Hibbertia exutiacies	Dilleniaceae	92	
		Hakea rostrata	Proteaceae	16	
		Tetratheca pilosa	Tremandraceae	6	
		Caesia calliantha	Liliaceae	2	300
	20/10/05	*Hibbertia exutiacies*	Dilleniaceae	205	
		Pultenea daphnoides	Leguminosae	46	
		Hakea rostrata	Proteaceae	22	
		Pimelea stricta	Thymelaeaceae	6	
		Tetratheca pilosa	Tremandraceae	8	
		Caesia calliantha	Liliaceae	2	
		Leptospermum myrsinoides	Myrtaceae	1	290
MB2	23/09/05	*Pultenea daphnoides*	Leguminosae	12	
		Acacia myrtifolia	Leguminosae	8	
		Hakea rostrata	Proteaceae	8	
		Tetratheca pilosa	Tremandraceae	6	
		Hibbertia exutiacies	Dilleniaceae	2	36

Site	Date	Dominant Co-flowering spp.	Family	Abundance of Co-flowers (ACF)	Total ACF per time point
	10/10/05	*Hibbertia exutiacies*	Dilleniaceae	62	
		Hakea rostrata	Proteaceae	56	
		Pultenea largiflorens	Leguminosae	54	
		Pultenea daphnoides	Leguminosae	53	
		Tetratheca pilosa	Tremandraceae	46	271
***Caladenia rigida* and *C. tentaculata* populations**					
SP	20/09/05	*Acacia paradoxa*	Leguminosae	236	
		Astroloma humifens	Epacridaceae	10	
		Hibbertia riparia	Dilleniaceae	12	
		Oxalis perennans	Oxalidaceae	6	264
	12/10/09	*Hibbertia exutiacies*	Dilleniaceae	134	
		Acacia paradoxa	Leguminosae	110	
		Hibbertia sericea	Dilleniaceae	49	
		Caesia calliantha	Liliaceae	44	
		Craspedia variabilis	Compositae	15	
		Oxalis perennans	Oxalidaceae	3	355
	25/10/05	*Hibbertia riparia*	Dilleniaceae	60	
		Goodenia blackiana	Goodeniaceae	6	
		Craspedia variabilis	Compositae	4	
		Hibbertia exutiacies	Dilleniaceae	3	
		Oxalis perennans	Oxalidaceae	3	
		Arthropodium fimbriatus	Liliaceae	2	
		Caesia calliantha	Liliaceae	2	80
	11/11/05	*Hibbertia riparia*	Dilleniaceae	124	
		Scaevola albida	Goodeniaceae	4	
		Brunonia australis	Goodeniaceae	2	
		*Echium plantagineum**	Boraginaceae	2	
		Goodenia blackiana	Goodeniaceae	2	
		Burchardia umbellata	Liliaceae	1	
		Hibbertia exutiacies	Dilleniaceae	1	136
***C. tentaculata* populations**					
MC4	3/10/05	*Pultenea daphnoides*	Leguminosae	100	
		Hibbertia exutiacies	Dilleniaceae	91	
		Hakea carinata	Proteaceae	16	
		Caesia calliantha	Liliaceae	12	
		Craspedia variabilis	Compositae	6	
		Astroloma humifens	Epacridaceae	4	
		Acacia myrtifolia	Leguminosae	2	231
	31/10/05	*Hibbertia exutiacies*	Dilleniaceae	128	
		Caesia calliantha	Liliaceae	62	
		Hibbertia sericea	Dilleniaceae	24	
		Leptospermum myrsinoides	Myrtaceae	6	
		Pimelea stricta	Thymelaeaceae	6	
		Goodenia blackiana	Goodeniaceae	2	
		Platylobium obtusangulum	Leguminosae	2	230
BR	5/10/05	*Acacia paradoxa*	Leguminosae	37	
		Hibbertia exutiacies	Dilleniaceae	24	
		Caesia calliantha	Liliaceae	18	
		Hibbertia sericea	Dilleniaceae	18	
		Hibbertia riparia	Dilleniaceae	16	
		Pultenea daphnoides	Leguminosae	16	
		Calytrix tetragona	Myrtaceae	14	
		Hakea rostrata	Proteaceae	12	
		Pimelea stricta	Thymelaeaceae	10	
		Freesia sp.*	Iridaceae	6	
		Hakea rugosa	Proteaceae	6	
		*Chrysanthemoides monilifera**	Compositae	4	181

Site	Date	Dominant Co-flowering spp.	Family	Abundance of Co-flowers (ACF)	Total ACF per time point
	26/10/05	*Calytrix tetragona*	Myrtaceae	224	
		Hibbertia riparia	Dilleniaceae	132	
		Goodenia blackiana	Goodeniaceae	8	
		Leptospermum myrsinoides	Myrtaceae	8	372
2007					
***Caladenia rigida* populations**					
MC1	30/08/07	*Hibbertia exutiacies*	Dilleniaceae	26	
		Spyridium parvifolium	Rhamnaceae	11	
		Tetratheca pilosa	Tremandraceae	9	
		Acacia myrtifolia	Leguminosae	4	
		Daviesia ulicifolia	Leguminosae	1	51
	7/09/07	*Hibbertia exutiacies*	Dilleniaceae	26	
		Spyridium parvifolium	Rhamnaceae	13	
		Craspedia variabilis	Compositae	6	
		Tetratheca pilosa	Tremandraceae	5	
		Acacia myrtifolia	Leguminosae	4	
		Daviesia ulicifolia	Leguminosae	2	
		Drosera macrantha	Droseraceae	2	
		Hibbertia sericea	Dilleniaceae	2	
		Goodenia blackiana	Goodeniaceae	1	61
MC1	14/09/07	*Hibbertia exutiacies*	Dilleniaceae	49	
		Craspedia variabilis	Compositae	9	
		Daviesia ulicifolia	Leguminosae	3	
		Tetratheca pilosa	Tremandraceae	2	
		Goodenia blackiana	Goodeniaceae	1	
		Hakea rostrata	Proteaceae	1	
		Hibbertia sericea	Dilleniaceae	1	66
	21/09/07	*Hibbertia exutiacies*	Dilleniaceae	44	
		Caesia calliantha	Liliaceae	37	
		Pultenea daphnoides	Leguminosae	15	
		Hibbertia riparia	Dilleniaceae	11	
		Craspedia variabilis	Compositae	8	
		Daviesia ulicifolia	Leguminosae	4	
		Acacia paradoxa	Leguminosae	3	
		Goodenia blackiana	Goodeniaceae	3	
		Tetratheca pilosa	Tremandraceae	2	
		Hibbertia sericea	Dilleniaceae	1	128
	5/10/07	*Caesia calliantha*	Liliaceae	194	
		Hibbertia exutiacies	Dilleniaceae	34	
		Pultenea daphnoides	Leguminosae	30	
		Pultenea largiflorens	Leguminosae	22	
		Hibbertia riparia	Dilleniaceae	11	
		Thysanotus patersonii	Liliaceae	10	
		Platylobium obtusangulum	Leguminosae	6	
		Goodenia blackiana	Goodeniaceae	3	310
MB1	28/08/07	*Hybanthus floribundus*	Violaceae	8	
		Acacia myrtifolia	Leguminosae	6	14
	11/09/07	*Drosera auriculata*	Droseraceae	53	
		Hakea rostrata	Proteaceae	22	
		Hibbertia exutiacies	Dilleniaceae	18	
		Acacia myrtifolia	Leguminosae	2	
		Acacia retinoides	Leguminosae	2	
		Hybanthus floribundus	Violaceae	2	
		Pultenea daphnoides	Leguminosae	1	
		Tetratheca pilosa	Tremandraceae	1	101

Site	Date	Dominant Co-flowering spp.	Family	Abundance of Co-flowers (ACF)	Total ACF per time point
	24/09/07	*Drosera auriculata*	Droseraceae	59	
		Hibbertia exutiacies	Dilleniaceae	17	
		Pultenea daphnoides	Leguminosae	3	
		Hakea rostrata	Proteaceae	2	
		Hibbertia sericea	Dilleniaceae	2	
		Hybanthus floribundus	Violaceae	1	84
	3/10/07	*Drosera auriculata*	Droseraceae	29	
		Caesia calliantha	Liliaceae	20	
		Stackhousia sp.	Stackhousiaceae	19	
		Hibbertia exutiacies	Dilleniaceae	15	
		Pultenea largiflorens	Leguminosae	12	
		Hakea rostrata	Proteaceae	2	
		Hibbertia sericea	Dilleniaceae	2	
		Hybanthus floribundus	Violaceae	1	100
MB2	39/08/07	*Drosera auriculata*	Droseraceae	10	
		Chamaescilla corymbosa	Liliaceae	8	
		Hybanthus floribundus	Violaceae	4	22
	6/09/07	*Acacia myrtifolia*	Leguminosae	7	
		Hakea rostrata	Proteaceae	7	
		Hibbertia exutiacies	Dilleniaceae	3	
		Hybanthus floribundus	Violaceae	3	
		Tetratheca pilosa	Tremandraceae	2	
		Drosera auriculata	Droseraceae	1	
		Hibbertia sericea	Dilleniaceae	1	24
	21/09/07	*Drosera auriculata*	Droseraceae	79	
		Hakea rostrata	Proteaceae	18	
		Hibbertia exutiacies	Dilleniaceae	12	
		Hibbertia sericea	Dilleniaceae	6	115
	2/10/07	*Drosera auriculata*	Droseraceae	36	
		Hibbertia exutiacies	Dilleniaceae	12	
		Goodenia blackiana	Goodeniaceae	11	
		Hibbertia sericea	Dilleniaceae	6	
		Hakea rostrata	Proteaceae	1	66
SP	5/09/07	*Wurmbea dioica*	Liliaceae	44	
		Hibbertia sericea	Dilleniaceae	44	
		Hypoxis vaginata	Hypoxidaceae	2	
		Craspedia variabilis	Compositae	1	
	13/09/07	*Hibbertia sericea*	Dilleniaceae	34	
		Wurmbea dioica	Liliaceae	30	
		Caesia calliantha	Liliaceae	14	
		Chamaescilla corymbosa	Liliaceae	6	
		Acacia paradoxa	Leguminosae	4	
		Craspedia variabilis	Compositae	4	
		Hibbertia exutiacies	Dilleniaceae	2	94
	24/09/07	*Caesia calliantha*	Liliaceae	72	
		Chamaescilla corymbosa	Liliaceae	65	
		Hibbertia sericea	Dilleniaceae	51	
		Wurmbea dioica	Liliaceae	11	
		Craspedia variabilis	Compositae	4	
		Thysanotus patersonii	Liliaceae	3	206
	16/10/07	*Hibbertia sericea*	Dilleniaceae	198	
		Arthropodium fimbriatus	Liliaceae	58	
		Brunonia australis	Goodeniaceae	30	
		*Echium plantagineum**	Boraginaceae	30	
		Burchardia umbellata	Liliaceae	28	
		Goodenia blackiana	Goodeniaceae	24	
		Bulbine bulbosa	Liliaceae	16	384

INFLUENCE OF APPARENCY ON FLORIVORY OF *CALADENIA TENTACULATA*

C.1 Introduction

The implications of three factors (flower height, concealment and local density) potentially affecting the apparency of *C. rigida* flowers were investigated in CHAPTER 6, with respect to the orchid's pollination success, risk of florivory and successful seed release. Space constraints in the version submitted for publication prevented the inclusion of data collected from the congeneric species, *C. tentaculata*; however, in support of my findings for *C. rigida*, I present these additional results here.

Caladenia flowers that advertise the presence of food usually attract foraging insects via brightly coloured visual displays with or without the production of scent. In contrast, sexually deceptive species are typically dull in colour and produce a different scent profile to specifically lure their male thynnine wasp pollinator (Salzmann *et al.* 2006; Phillips *et al.* 2009b). Differences in the visual and/or olfactory cues employed by food-advertising versus sexually deceptive species may therefore alter the way in which the flowers are perceived by both pollinators and herbivores. For example, from the perspective of the human eye, the white flowers of the food-advertising *C. rigida* contrast starkly against the green and brown background of the environs, whereas the green and maroon flowers of the sexually deceptive *C. tentaculata* are more difficult to discern, particularly from a distance.

Like *C. rigida*, *C. tentaculata* suffers from intense grazing pressure with up to 87% of flowers browsed at some sites (CHAPTER 4), suggesting that both species of orchid are readily detected by herbivores. For the food-advertising *C. rigida,* flower height had a positive effect on pollination and successful seed release, but had only a minor negative effect on the risk of florivory in one year of the study (CHAPTER 6). In contrast, concealment amongst neighbouring vegetation consistently afforded protection against browsing, without impeding pollination success. I also showed that in most cases, the risk of florivory rose as the local density of conspecific flowers increased. Here, I have investigated whether interactions with the sexually deceptive, *C. tentaculata*, show similar responses to these measures of apparency. Unfortunately, the low rates of capsule

production for this species precluded me from assessing interactions with pollinators, and as such I have shown only those results pertaining to florivory.

Specifically, the questions addressed here are: 1. Do factors potentially influencing the apparency of *C. tentaculata* flowers (floral height, concealment and local density) affect their risk of florivory?; and, 2. Do responses to these factors differ to those observed for *C. rigida*?

C.2 Methods

Monitoring plant status and local environs

The relationship between florivory and factors potentially affecting the apparency of flowers was assessed in three populations of *C. tentaculata* in 2005 (MC4, BR and SP) and two populations in 2007 (MC4 and BR) (site locations and descriptions are provided in Fig. 5.1 and Table 5.1). The SP population was excluded in 2007 because the majority of plants formed part of the herbivore exclusion experiment (CHAPTER 4). Due to the low rates of flowering in 2006, data from this year was not included. Monitoring of tagged plants was carried out as described in CHAPTER 6, recording florivory, flower height, and floral concealment amongst neighbouring vegetation (categorised as well-concealed, concealed or exposed). There was no association between orchid flowers and any particular neighbourhood species (personal observation). Local density was measured using a snapshot approach, recording the number of conspecifics within a 35 cm radius at the monitoring time point with the highest rate of florivory.

Data analysis

The effect of concealment and population on flower height was tested using two-way ANOVA. Separate logit models were employed to evaluate the response of florivory (binary response variable) to each of the explanatory factors (flower height, concealment or local density) and their interaction with population. Likelihood ratio tests (G^2) were used to assess the contribution of individual explanatory factors within each model. Non-significant interaction terms were removed to retain the most parsimonious model (Underwood 1997). The direction of significant effects was determined using values of the coefficient estimate (B) and the corresponding odds ratio. Where interactions between population and flower height or density were significant ($P < 0.05$), single-factor analyses were performed for each population using logistic regression. The statistical package JMP

4.0 (SAS Institute) was employed for ANOVA and SPSS 15.0 was employed for logit models.

C.3 Results

Flower height varied among populations but not among categories of concealment in 2005 (ANOVA, population: $F_{2,199} = 4.3$, $P = 0.015$; concealment: $F_{2,199} = 2.3$, $P = 0.1$) and in 2007 (population: $F_{1,94} = 7.0$, $P = 0.01$; concealment: $F_{2,94} = 0.9$, $P = 0.4$). The effects of flower height and concealment could therefore be tested in separate analyses. In 2005, taller flowers were at greater risk of being browsed than shorter flowers (Table C.1). In 2007, the response of florivory to flower height varied among populations (significant population x flower height interaction, Table C.1) and analysis of separate populations revealed a positive relationship between flower height and the risk of browsing at the MC4 site. Concealment amongst neighbouring vegetation afforded protection against browsing in 2005, but not in 2007 (Table C.1).

The local density of conspecifics ranged from one to seven flowers (Table C.2) in most populations; however, the effect of local density on the florivory of *C. tentaculata* varied among populations in 2005, as indicated by a significant population x local density interaction (Table C.1). Single factor tests identified a positive effect of density at the MC4 site and a marginally negative effect at SP (Table C.1). Florivory was not affected by the local density of conspecifics in 2007.

Table C. 1 Effect of flower height, concealment, density and population on the florivory of *Caladenia tentaculata* in 2005 and 2007.

	Year	Population x Factor	Population	Factor	Individual sites [a]	N
Flower height						
	2005	1.2_2	**15.2_2****	**3.7_1*** [b]		240
	2007	**3.6_1**[†]	**8.9_1****	2.3_1	MC4*[b]	121
Concealment						
	2005	3.4_3	**31.2_2*****	**33.1_2***** [c]		386
	2007	0.17_2	**7.7_1****	0.55_2		118
Local density						
	2005	**21.8_2*****	**24.2_2*****	**3.6_1**[†]	SP [†c], MC4***[b]	307
	2007	1.4_1	0.27_1	0.008_1		89

Likelihood ratios (G^2) are shown for each factor, with degrees of freedom as subscripts. Non-significant interactions were removed from the model and the analysis repeated. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$; [†] $P = 0.073$. [a] Significant population by factor interactions were followed by single-factor analyses at each site. [b] positive relationship; [c] negative relationship.

Table C. 2 Number of flowering plants and the range of local density (number of conspecifics within 35 cm of target plant) recorded within populations of *Caladenia tentaculata* in 2005 and 2007.

	No. flowering individuals		Local density of conspecifics	
Population	2005	2007	2005	2007
MC4	120	300	1 – 7 (2.0)	1 – 7 (1.8)
BR	150	200	1 – 7 (2.6)	1 – 4 (1.9)
SP	120	300	1 – 7 (2.2)	

Mean values for local density are shown in parentheses.

C.4 Discussion

All three measures of apparency influenced the risk of *C. tentaculata* florivory to some extent; however, the strength and direction of these responses varied among populations and among years. In 2005, concealment amongst neighbouring vegetation protected flowers from being browsed, similar to what was observed for *C. rigida* (CHAPTER 6). Also coinciding with measurements for *C. rigida*, the direction of the relationship between florivory and the local density of conspecifics differed among populations. Such variation could arise if the identity of the predominant herbivores and their behavioural response to floral density differs among populations. The lack of response observed in 2007 for both concealment and local density could stem from the smaller sample sizes in that year. Unlike *C. rigida*, the height of *C. tentaculata* flowers was positively related to their risk of being browsed, suggesting that taller flowers are more conspicuous to herbivores or that herbivores have some innate height preference.

These results demonstrate that colour, as perceived by humans, does not play a major role in determining the response of herbivores to either concealment or local density, as both measures of apparency had similar effects on the florivory of *C. rigida* and *C. tentaculata*. However, colour may explain the different responses observed for flower height. Brightly coloured flowers, such as those of *C. rigida*, are likely to be conspicuous to florivores regardless of their height, whereas the more discrete flowers of *C. tentaculata* may be easier to locate when they are taller.

REPRINTS OF PUBLICATIONS PRESENTED AS CHAPTERS IN THIS THESIS

The following section contains reprints of the publications presented in CHAPTERS 3 and 4, as they appear in *Australian Journal of Botany* (2009), Volume 59.

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Farrington L, Macgillivray P, Faast R, Austin AD (2009) Evaluating molecular tools for *Caladenia* (Orchidaceae) species identification. *Australian Journal of Botany* 57:276-286

Phillips RD, Faast R, Bower CC, Brown GR, Peakall R (2009) Implications of pollination by food and sexual deception for pollinator specificity, fruit set, population genetics and conservation of *Caladenia* (Orchidaceae). *Australian Journal of Botany* 57:287-306

The first two articles will be presented as thesis chapters (with appropriate reformatting), and reprints of all four articles will be included as appendices. Full acknowledgement will be given to Australian Journal of Botany as the source of the material.

Thank you for your time,

Regards,
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Faast, R., Farrington, L., Facelli, J.M., Austin, A.D. (2009) Bees and white spiders: unravelling the pollination syndrome of Caladenia rigida (Orchidaceae).
Australian Journal of Botany, v.57 (4), pp. 315-325, 2009

Faast, R., Farrington, L., Facelli, J.M., Austin, A.D. (2009) *Grazing orchids: impact of florivory on two species of* Caladenia *(Orchidaceae).*
Australian Journal of Botany, v.57 (4), pp. 361-371, 2009

REPRINTS OF ADDITIONAL PUBLICATIONS

During the course of this project, I also contributed to two other publications that are related to this research but do not address the specific aims of this thesis. Reprints of these manuscripts, as they appear in *Australian Journal of Botany* (2009), Volume 59, are included in the following section.

Documentation from the publisher providing consent to reproduce published manuscripts in this thesis, is provided at the end of APPENDIX D.

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