Sexual deception as a pollination strategy investigated in three *Pterostylis* greenhood orchids in New Zealand

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Liezl Thalwitzer

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Abstract

Background and Aims Sexual deception is a species-specific pollination strategy commonly found in Orchidaceae. Sexually deceptive orchids lure male insect pollinators by mimicking the sex pheromones and/or appearance of female insects, which elicit copulatory behaviour with the flower by the male insects. This specialised pollination strategy has recently been found in a *Pterostylis* species in Australia. *Pterostylis* orchids also occur in New Zealand, although very few studies have been done on this genus, and no such specialised insect pollination strategy has been documented in New Zealand.

Methods I investigated the breeding system and pollinators of three *Pterostylis* spp. to determine whether sexual deception may be operating in *P. oliveri*, *P. irsoniana* and *P. venosa* growing in native beech forests in Arthur's Pass. We also investigated the floral headspace volatiles of *P. oliveri* to determine which compounds are present, and which may be responsible for pollinator attraction.

Key Results Breeding system experiments suggest that *P. oliveri* and *P. irsoniana* are self compatible, but exclusively dependent on insects for pollination. Only male fungus gnats (Diptera: Mycetophilidae) were found carrying pollinia attached to their thoraxes in traps set up over the flowers. Insect identification and ITS DNA analysis of the pollinia showed that each orchid species was pollinated by a specific fungus gnat species; *Mycetophila latifascia* males found with pollen of *P. oliveri*; *Morganiella fusca* males found with pollen of *P. irsoniana*; and *Tetragoneura* sp. males found with pollen of *P. venosa.* Field tests of an unidentified compound found in headspace volatiles of *P. oliveri* did not attract any *Mycetophila latifascia* males.

Conclusions These results indicate that pollination via sexual deception may be operating in these three *Pterostylis* spp. However, further floral volatile analyses are required to confirm whether the flowers emit volatile compounds that resemble the sex pheromones of the specific pollinators.

Glossary

Column. Floral organ located in the interior of an orchid flower, where the stigma and anther regions are located. The anther is located at the top, and the stigma at the base of the column.

Pollinarium (pollinaria pl.). A pollinium with a sticky tag. Each orchid anther has 4 pollinaria.

Pollinium (pollinia pl.). A discrete mass of pollen. Each orchid anther has 4 pollinia.

Stigma. The stigmatic region of an orchid flower is located the base of the column and has a sticky surface (viscidium), to which pollen can adhere to.

Labellum. A modified flower petal, also known as the flower's lip, is associated with floral attraction (either producing scent of by its morphology)

Anthesis. The 'opening' of a flower, after which the flower parts are available for pollination.

Perianth. A collective term for the petal and sepals, or tepals of a flower.

Chapter 1

Introduction

Pollination by deception in Orchidaceae

It has been estimated that over a third of Orchidaceae, a plant family of over 26 000 species (WCoSPF, 2011), may achieve pollination by deceptive means; promising a false reward, whether it be food, brood site or sex (Cozzolino and Widmer, 2005; Schiestl, 2005; Peakall *et al.*, 2010; Xu *et al.*, 2012). Instead of providing rewards to attract pollinators, deceptive orchids exploit insect behaviour or other plant-pollinator relationships to achieve pollination (Jersakova *et al.*, 2006). Deceptive pollination strategies include brood-site selection (Li *et al.*, 2006), shelter imitation (Dafni *et al.*, 1981), territorial defence (pseudoantagonism) (Jersakova *et al.*, 2006), food deception (Cheng *et al.*, 2009), and sexual deception (Dafni and Ivri, 1981; Ackerman, 1986; Schiestl, 2005; Jersakova *et al.*, 2006; Gaskett, 2011). Deceptive pollination strategies outside of Orchidaceae are rare but has recently been found in *Oncocyclus* irises (Iridaceae) (Sapir *et al.*, 2005), and *Gorteria diffusa* (Asteraceae) (Ellis and Johnson, 2010; Schiestl, 2010a; Urru *et al.*, 2011).

Brood-site selection occurs when a female insect is attracted to orchid flowers which resemble a suitable site for oviposition (Jersakova *et al.*, 2006; Urru *et al.*, 2011). Brood-site mimicry coupled with food-deception may be the pollination strategy of the Chinese *Cypripedium tibeticum* (Li *et al.*, 2006). Li *et al*. (2006) postulated that *C. tibeticum*'s large dark purple flowers resemble potential nesting sites for fertilized *Bombus* spp. queens as they emerge from hibernation in the spring. They found that the flowers offer no rewards and are fertilized once the queen bee pushes her way past the column while exiting the pouched labellum (Li *et al.*, 2006).

Shelter imitation is a pollination strategy where the flower mimics insect resting or hiding places (Jersakova *et al.*, 2006). The dark red flowers of the Mediterranean genus *Serapias* are pollinated by solitary bees which take refuge in the flowers as they resemble nest entrances during bad weather (Dafni *et al.*, 1981). Four sympatric *Serapias* spp. have been found to have similar floral traits and flowering periods, and are all pollinated by solitary bees. Chemical analyses of the floral scents showed slight differences in the floral scent of the four *Serapias* spp. which Pellegrino *et al.* (2012) theorise may provide a pre-pollination barrier and prevent hybridisation Pellegrino *et al.* (2012). Deceptive orchids are known not to provide any rewards to the insects fooled into pollinating these orchids, however in the case where the orchid flowers offer shelter, in return for pollination, I would

argue that this is at least some form of mutualistic interaction (Bronstein *et al.*, 2006), as the insect is likely to gain from the interaction.

One of the least studied but most fascinating and rare forms of orchid deception is when the orchids exploit the territorial defence behaviour of hymenopterans, known as pseudoantagonism. This pollination strategy has only been documented in the South American *Oncidium* and *Tolumnia* species (Jersakova *et al.*, 2006). Such orchid flowers vibrate in the wind, which may resemble the hymenopterans' antagonist, and are pollinated as the insect attacks the flower (Jersakova *et al.*, 2006).

The largest portion of deceptive orchids are known as being 'food deceptive'. This pollination strategy involves mimicking the floral traits of food rewarding plants growing in the same area (Cozzolino and Widmer, 2005; Schiestl, 2005; Jersakova *et al.*, 2006). Food deceptive orchids offer no pollen or nectar, and are theorised to rely on foraging pollinators mistaking the non-rewarding orchid flowers for food rewarding flowers of the neighbouring plants. As expected, this pollination strategy is subject to negative density-dependent selection; where the smaller non-rewarding orchid populations benefit from higher pollination rates, as pollinators can learn to avoid non-rewarding flowers, especially when the non-rewarding orchids are more abundant (i.e. increase in population numbers) (Gumbert and Kunze, 2001; Jersakova *et al.*, 2006). Studies have shown that bee pollination and the fruit-set of *Cephalanthera rubra, Orchis israelitica,* and *Orchis boryi* increases when plants not only mimic the food-rewarding plants' colouration and reflectance spectra, but also with similar flowering time and close vicinity to the food-rewarding plants (Dafni and Ivri, 1981; Nilson, 1983; Gumbert and Kunze, 2001). In the Baltic Sea island of Gotland, *Cephalanthera rubra* orchids are mostly (but not exclusively) pollinated by male *Chelostoma fuliginosum* and *C. campanularum* solitary bees. These orchids mimic the floral colouration of *Campanula* flowers where the female bees forage for food. This is also where the male bees search for mates. By mimicking the colouration of the *Campanula* flowers, the *C. rubra* orchids are pollinated by male bees which mistake the flowers for sites where the females can be found (Nilsson 1983). Food deceptive orchids also attract pollinators via olfactory cues. *Coelogyne fimbriata* and *Steveniella satyrioides* flowers attract female worker *Vespula* wasps by producing the scent of nectar, but offer no actual nectar (Nazarov, 1995; Cheng *et al.*, 2009).

Pollination by deception outside of Orchidaceae

Deceptive pollination strategies outside of Orchidaceae are rare but, brood-site imitation has been found in other plant families such as Aristolochiaceae, Asclepiadaceae, and Araceae (Jersakova *et al.*, 2006); shelter imitation in *Oncocyclus* irises (Iridaceae) (Sapir *et al.*, 2005), and the 'intermediate form' of sexual deception in *Gorteria diffusa* (Asteraceae) (Ellis and Johnson, 2010; Schiestl, 2010a).

Shelter imitation has been found in *Oncocyclus* irises (Iridaceae) which grow throughout the Middle East (Sapir *et al.*, 2005). Their dark-coloured flowers offer no nectar and have not been seen to be pollinated during the day. These flowers are pollinated by solitary male bees (Apidea) which enter the flowers at dusk and stay inside the flowers overnight. The breeding system experiments from Sapir *et al*. (2005) showed that six *Oncocyclus* irises are self-incompatible and their fruit-set depends on the pollination of the night sheltering bees (Sapir *et al.*, 2005).

The South African Daisy *Gorteria diffusa* presents a novel form of sexual deception which most probably evolved from food reward (Schiestl, 2010a). Previously, sexual deception has only been found in orchids which don't offer any food, have their pollen packed in pollinia, and attract species-specific male insects with a modified labellum which look and/or smell like a female insect (Kullenberg, 1961; Peakall and Beattie, 1996; Schiestl *et al.*, 1999; Ayasse *et al.*, 2003). In contrast, the discovery of the pollination strategy of *G. diffusa* is unexpected and unique in that Daisies have granular pollen, produce nectar and are visited by generalist pollinators. These floral traits show that sexual deception can also evolve in a generalist pollination system. In *G. diffusa*, the black spots (each a modified petal) on the orange inflorescence mimic a sitting insect which attracts male bombyliids which to try copulate with the black petals (Ellis and Johnson, 2010; Schiestl, 2010a).

Sexual deception in Orchidaceae

Sexually deceptive orchid species produce no food reward, but lure species-specific male insect pollinators by mimicking the sex pheromones and/or appearance of female insects, which elicits some degree of copulatory behaviour (pseudocopulation) with the flower by the male insects (Kullenberg, 1961; Peakall, 1990; Schiestl, 2005; Ayasse *et al.*, 2010; Gaskett, 2011; Vereecken *et al.*, 2011).

Pollinators involved in sexual deception

The most common pollinators of sexually deceptive orchids are wasps and bees (Nilson, 1983; Peakall, 1990; Ayasse *et al.*, 2001; Gaskett and Herberstein, 2006; Gaskett, 2011), however ants (Peakall, 1989), beetles (Gaskett, 2011) and flies (Blanco and Barboza, 2005; Phillips *et al.*, 2014) have also been documented. Twelve orchid genera have been found to be sexually deceptive in Australia, including *Caladenia, Chiloglottis, Drakaea, Cryptostylis,* and recently *Pterostylis*. Each species of *Chiloglottis, Caladenia and Drakaea* are pollinated by different species of thynnine wasps (Peakall, 1990; Peakall and Handel, 1993; Peakall and Beattie, 1996; Mant *et al.*, 2005b; Hopper and Brown, 2007). While all five *Cryptostylis* species, which are morphologically distinct, share the same ichneumonid wasp pollinator, which is theorized to be as a result of the orchids all producing the same volatile compounds (Schiestl *et al.*, 2004; Gaskett and Herberstein, 2006; Gaskett and Herberstein, 2010; Gaskett, 2011). Hybridization has been confirmed to be prevented by post-pollination isolation in four of the *Cryptostylis* species, which have different chromosome numbers (Dawson *et al.*, 2007; Cozzolino and Scopece, 2008; Gaskett, 2011).

Eight genera in South and Central America have been studied, including *Lepanthes glicensteinii* which was the first confirmed case of a sexually deceptive orchid being pollinated by male fungus gnats (*Bradysia floribunda*, Sciaridae: Diptera). The tiny non-rewarding flowers of *L. glicensteinii* attract male fungus gnats which copulate with the miniscule labellum (**REF**). It is strongly expected that the labellum produce specific volatile compounds, which will be comparable with the female *B. floribunda* sex pheromones, to attract the *B. floribunda* males (Blanco and Barboza, 2005). Over 165 *Ophrys* spp. have been studied in Europe, which are pollinated by bees, wasps, and beetles (Devey *et al.*, 2008; Cortis *et al.*, 2009; Streinzer *et al.*, 2010; Vereecken *et al.*, 2011; Xu *et al.*, 2011; Breitkopf *et al.*, 2013). In South Africa, Steiner *et al.* (1994) found two sister *Disa* species, which don't offer any food reward, to be pollinated exclusively by male wasps. *Disa atricapilla* was pollinated by *Podalonia canescens* (Sphecidae), and *Disa bivalvata* was pollinated only by *Hemipepsis hilaris* (Pompilidae) male wasps (Steiner *et al.*, 1994).

Chemical cues of sexual deception

The chemical volatiles produced by the flowers are important in the attraction mechanism of sexually deceptive orchids. (Schiestl *et al.*, 2003; Franke *et al.*, 2009; Peakall *et al.*, 2010; Vereecken *et al.*, 2011). Chemical ecological studies on have shown that the orchid-pollinator interaction is strongly maintained by the specific compounds, in a variety of mixtures and their relative

concentrations. Peakall *et al.* (2010) found that Australian sympatric *Chiloglottis* spp. attract their specific pollinators by either emitting a single different compound or different concentrations of the same compounds (Peakall *et al.*, 2010). Ayasse *et al*. (2003) also found that the differences in *Ophrys* spp. volatiles are not as the result of producing a wide variety of compounds but rather, producing different concentrations of the same compounds (Ayasse *et al.*, 2003). As for the location of the scent production; Phillips *et al.* (2014) found that the labellum was the sole source of the attractant in *Pterostylis sanguinea*, which elicits copulatory behaviour from the insects (Phillips *et al.*, 2014). In *Ophrys* spp. the volatiles are produced by subcuticular cells in the plant epidermis (Samuels *et al.*, 2008). Peakall and Beattie (1996) have shown that the sexual pheromone mimic volatiles are produced by the calli (swollen structures on the labellum) of *Caladenia tentaculata* (Peakall and Beattie, 1996). It is possible that the subcuticular cells of the calli structures on the labellum are producing the same volatiles as the female insects.

The majority of studies on sexually deceptive orchids have found that the orchids attract their pollinators with olfactory cues. The most comprehensive studies which were able to confirm sexual deception as the pollination strategy in orchids use gas chromatography–mass spectrometry (GC-MS) to determine which floral volatile compounds are present, coupled with gas chromatography - electroantennographic detection (GC-EAD) to determine which of these floral compounds trigger a response from the male insect antennae, and a pseudocopulatory response in behavioural assays (Schiestl *et al.*, 1999; Ayasse *et al.*, 2003; Mant *et al.*, 2005a; Schiestl and Peakall, 2005).

The main function of cuticular hydrocarbons present on the bodies of insects is to prevent desiccation. However their composition has been shown to serve as the distinguishable sex pheromones among different insects (Ayasse *et al.*, 2001; Ayasse *et al.*, 2003; Schiestl, 2005; Peakall *et al.*, 2010; Ayasse *et al.*, 2011; Vereecken *et al.*, 2011; Pellegrino *et al.*, 2012). Schiestl *et al*. (1999) showed that *Ophrys sphegodes,* which are pollinated by *Andrena nigroaenea* solitary bees, attract the males by producing the same compounds in similar relative proportions as the female bees (Schiestl *et al.*, 1999), and *Ophrys speculum* has been found not only to mimic the sex pheromones of *Campsoscolia ciliate* wasps, but actually be more attractive to the male wasps than the females (Ayasse *et al.*, 2003; Ayasse *et al.*, 2010; Ayasse *et al.*, 2011). Mant *et al.* (2005) found that 22 active compounds in the cuticular extracts of the female *Colletes cuniculrius* bees were also produced by *Oprhys exaltata* which is pollinated by the males (Mant *et al.*, 2005a). Both *Chiloglottis trapeziformis* and *Chiloglottis valida* which are pollinated exclusively by male *Neozeleboria cryptoides* and *Neozeleboria monticola*, respectively, produce a single biologically active component called

'chiloglottone' which is also produced by the female wasps (Schiestl *et al.*, 2003; Schiestl and Peakall, 2005; Peakall *et al.*, 2010). Behavioural field experiments showed that the compound was highly attractive to both male wasp species, which means they could visit any of the two orchid species, yet the two orchid species are genetically distinct. This indicates that there has to be a reproductive barrier isolating these two orchid species. From field observations Schiestl and Peakall (2005) theorized that the difference of floral height (*C. trapeziformis* grows taller than *C. valida*) may be as a result of difference in height of each of the wasp species mate-searching flights (Schiestl and Peakall, 2005). And that the difference in the wasp species mate-search behaviour maintains the orchid species reproductive barrier, also known as pre-mating isolation (Schiestl and Peakall, 2005). The sympatric orchids *Cryptostylis erecta* and *Cryptostylis subulata* are morphologically distinct, yet share the same pollinator, male *Lissopimpla excels* wasps (Schiestl *et al.*, 2004). GC-MS analyses have not been able to detect the actual compound responsible for the wasps' attraction. However, GC-EAD analyses showed that the male *L. excels* wasps responded to the same compound (GC peak) in both orchid volatiles, which indicated that a single compound produced by the two *Cryptostylis* spp. is responsible for the male's attraction (Schiestl *et al.*, 2004). With no hybrids found in field among these two sympatric populations, Schiestl *et al.* (2004) theorizes that some form of genetic incompatibility is most likely to be the reproductive barrier.

Sexual behaviours of the pollinators triggered by floral chemical cues

Sexually deceptive orchids elicit different levels of sexual behaviour from their male pollinators (Gaskett, 2011; Phillips *et al.*, 2014). In the most extreme and rare cases the pollinator will ejaculate while attempting to copulate with the labellum of the flower. This level of sexual behaviour has only been observed in *Cryptostylis* spp. (Gaskett *et al.*, 2008; Gaskett, 2011), and *Lepanthes glicensteinii* (Blanco and Barboza, 2005). In most cases the pollinator will merely attempt to copulate with the labellum, as seen in *Ophrys* spp. (Schiestl, 2005). Less intense interactions include some form of pre-mating behaviour where the insect will fan their wings and/or grip the labellum (Blanco and Barboza, 2005; Phillips *et al.*, 2014). In *Pterostylis sanguinea*, the probing copulatory behaviour of a male fungus gnat triggers the labellum to trap the insects inside the flower. The orchid is pollinated as the fungus gnat escapes from the flower (Cheeseman, 1872; Johns and Molloy, 1983; Bernhardt, 1995; Proctor *et al.*, 1996; Gaskett, 2011; Phillips *et al.*, 2014).

Male insects are lured to the flowers of sexually deceptive orchids by volatile semiochemicals. The semiochemicals of each orchid species act as long range attractants, and mimic the sex pheromones

of a single insect species (Schiestl *et al.*, 1999; Ayasse *et al.*, 2001; Ayasse *et al.*, 2003; Schiestl *et al.*, 2003; Schiestl and Peakall, 2005; Bower and Brown, 2009; Peakall *et al.*, 2010). At close range, the mimicry of visual and/or tactile cues has been shown to stimulate copulatory behaviour in *Ophrys*, *Chiloglottis*, *Drakea* and *Caleana* species (Cortis *et al.*, 2009; Gaskett, 2011). The colour of the flower perianth can increase the detectability of the flower against their background (Gaskett and Herberstein, 2010; Streinzer *et al.*, 2010; Vereecken *et al.*, 2011), while the shape and pilosity (trichomes on the surface of the labellum which makes it appear as 'furry') of the labellum can direct the position of the pollinator during copulation, as seen in *Ophrys* spp. (Devey *et al.*, 2008; Cortis *et al.*, 2009; Vereecken *et al.*, 2011). *Drakeae* and *Caleana* orchids have intriguing insectiform (female-like) flowers. In *Drakeae livida* the labellum resemble the flightless females of the *Zaspilothynnus nigripes* (Thynnidae) wasps. The flowers are pollinated when a male wasp tries to pick up and fly away with the 'female-like' labellum (Hopper and Brown, 2007; Gaskett, 2011). Similarly in *Chiloglottis* spp. along with the odour attractant, the calli resemble the female body and encourage the male wasps to try and pick up the flightless females (Schiestl, 2004; Schiestl, 2005).

Can a specialised pollination strategy such as sexual deception be operating in Pterostylis greenhood orchids in New Zealand?

Several of the sexually deceptive orchid genera present in Australia are also present in New Zealand, including *Caladenia*, *Chiloglottis*, *Cryptostylis* and *Pterostylis* spp., among other genera (Hatch, 1946; Johns and Molloy, 1983; St. George, 1999). However, no cases of highly specialised plant-insect pollination systems, such as sexual deception in orchids, have been documented in New Zealand (Lehnebach *et al.*, 2005; Newstrom and Robertson, 2005; Gaskett, 2011). Currently, New Zealand has over 160 known orchid species within 35 genera (NZNOG, 2015). *Pterostylis* makes up the largest genus with 29 species in New Zealand (St. George, 2014), and one of the four genera (along with *Plumatichilos, Hymenochilus* and *Diplodium*) which are known as the 'greenhood' orchids (Clements *et al.*, 2011; NZNOG, 2015)

Pterostylis spp. have been thought to be sexually deceptive for many years, going back to Cheeseman (1872), and Darwin (1885). Although these orchids were generally known to be pollinated by fungus gnats (Diptera: Sciaridae and Mycetophilidae), evidence of the actual pollination strategy and pollinator species have been largely based upon anecdotal evidence. (Bates, 1977; Bernhardt, 1995; Jones and Clements, 2002; Lehnebach *et al.*, 2005; Gaskett, 2011). In a pollination study of four New Zealand orchids, Lehnebach *et al.* (2005) showed that *Pterostylis alobula* (Syn: *Diplodium alobulum*) and *P. patens* were self-compatible but dependent on insects for pollination. They found male *Zygomyia* (Mycetophilidae) fungus gnats inside the flowers of *P.*

alobula, but none were carrying pollen, which meant the insects were regarded as 'suggested pollinators' (Adams and Lawson, 1993; Lehnebach *et al.*, 2005). These results left open the question as to whether the pollination strategy of *Pterostylis* orchids in New Zealand involves sexual deception. Only recently, Phillips *et al.* (2014) confirmed sexual deception in *Pterostylis sanguinea* in Western Australia. The orchids were pollinated after male *Mycomya* (Mycetophilidae) fungus gnats were recorded attempting to copulate with flower labella (Phillips *et al.*, 2014).

The aim of this study was to determine whether sexual deception is operating as the pollination strategy in *P. oliveri*, *P. irsoniana,* and *P. venosa* growing in native beech forests in Arthur's Pass National Park (NP), New Zealand. The field experiments were carried out at three locations where the orchids were the most abundant; Greyney's Shelter, Cockayne Nature Walk, and Scott's Track. Four hypotheses were tested to determine whether the orchids are sexually deceptive: (a) the flowers of each of the three *Pterostylis* spp. depend on insects for pollination; (b) each of the three *Pterostylis* spp. flowers attract a different insect pollinator species; (c) the flowers attract only male insect pollinators; (d) the flowers emit volatile compounds which resemble the sex pheromones of the specific insects.

In Chapter 2, I will investigate the breeding system of the orchids, with pollination treatments in order to determine whether the orchids depend on insect for pollination. In chapter 3, I trap and identify the orchid pollinators. And finally I will attempt to find the floral volatiles of *P. oliveri* in Chapter 4. In this study, each of the chapters answers a very specific question. In chapter 5, I am able to combine the answers of the previous questions and make to conclusion of the study as a whole; are the three *Pterostylis* orchids sexually deceptive?

Chapter 2

Do the *Pterostylis* orchids depend on insects for pollination?

Introduction

In this chapter I will describe the floral trapping mechanism of *Pterostylis* flowers which have been the subject of speculation concerning the orchids' pollination system. I will introduce the three *Pterostylis* species included in the study, and the locations where the field experiments were carried out. I will describe the methods specific to the breeding system experiments, including the four pollination treatments.

Investigating whether the pollination strategy of *Pterostylis* orchids in New Zealand is by sexual deception we first have to understand these orchids' floral traits and breeding system. In New Zealand all *Pterostylis* spp. bear solitary flowers (Clements *et al.*, 2011; NZNOG, 2015), which trap their pollinators (Fig. 2.1) (Johns and Molloy, 1983; Proctor *et al.*, 1996; Lehnebach *et al.*, 2005). Most of the perianth is fused to form a floral chamber; the dorsal and ventral sepals form a floral tube around a small and narrow labellum in the centre of the flower. This distinct floral structure most likely evolved as trapping mechanism to promote out-crossing (Lehnebach *et al.*, 2005; Phillips *et al.*, 2014). The production of a floral scent, which resembles the sex pheromones of the female insect, is expected to be the reason why the male insect flies towards and lands on the labellum, as previous studies on sexually deceptive orchids have shown (Ayasse *et al.*, 2003; Schiestl *et al.*, 2003; Mant *et al.*, 2005c). Either the motion of the landing or attempted copulatory behaviour triggers the hinged labellum to catapult the insect into the base of floral tube, imprisoning the insect. The only passage for escape is past the stigma, where any pollinium the insect is carrying is deposited, and subsequently past the anthers, where a pollinium attaches to the thorax of the insect. Once the insect is trapped within another flower, the pollen is transferred to the adhesive stigmatic region on exit and cross pollination is achieved (Bernhardt, 1995; Lehnebach *et al.*, 2005; Gaskett, 2011; Phillips *et al.*, 2014). After fertilisation the inferior ovary will swell and once ripe, eventually dry out to become longitudinally dehiscent capsules which release the seeds at the end of the flowering season. Depending on the fruit-set number, thousands of minute dry seeds (< 1 mm long) will be dispersed by wind (Johns and Molloy, 1983).

In a previous pollination study of four New Zealand orchids, Lehnebach *et al.* (2005) conducted breeding system experiments on two *Pterostylis* spp. They found that both *P. patens* and *P. alobula* were self-compatible but non-autogamous, i.e. the plants depended on insects for pollination

(Lehnebach *et al.*, 2005). This study aims to document the breeding systems of *P. oliveri* and *P. irsoniana* growing in Arthur's Pass National Park, particularly to test for self-compatibility, pollen limitation and dependence on insects for pollination. To test for this, I carried out natural-, self- and cross-pollination treatments on the flowers in the field, and excluded insect pollinators in the control treatment for *P. oliveri* and *P. irsoniana*. At the end of each season, I counted the fruit-set of the flowers in each pollination treatment. I was not able to include the third species, *P. venosa*, in all the pollination treatments but did count the natural fruit-set.

The data analyses of the pollination treatments focused on; (1) the mean fruit-set difference among the four breeding system treatments of *P. oliveri* at Greyney's Shelter (2012/13 and 2013/14), and (2) *P. irsoniana* at Cockayne walk (2013/14), in order to compare their levels of self-compatibility and pollen limitation. I also looked at (3) the differences in the natural fruit-set of *P. oliveri*, *P. irsoniana*, and *P. venosa* among the three locations, as the three locations differed in vegetation composition, and different numbers of the three orchids species were found at each of the three study locations.

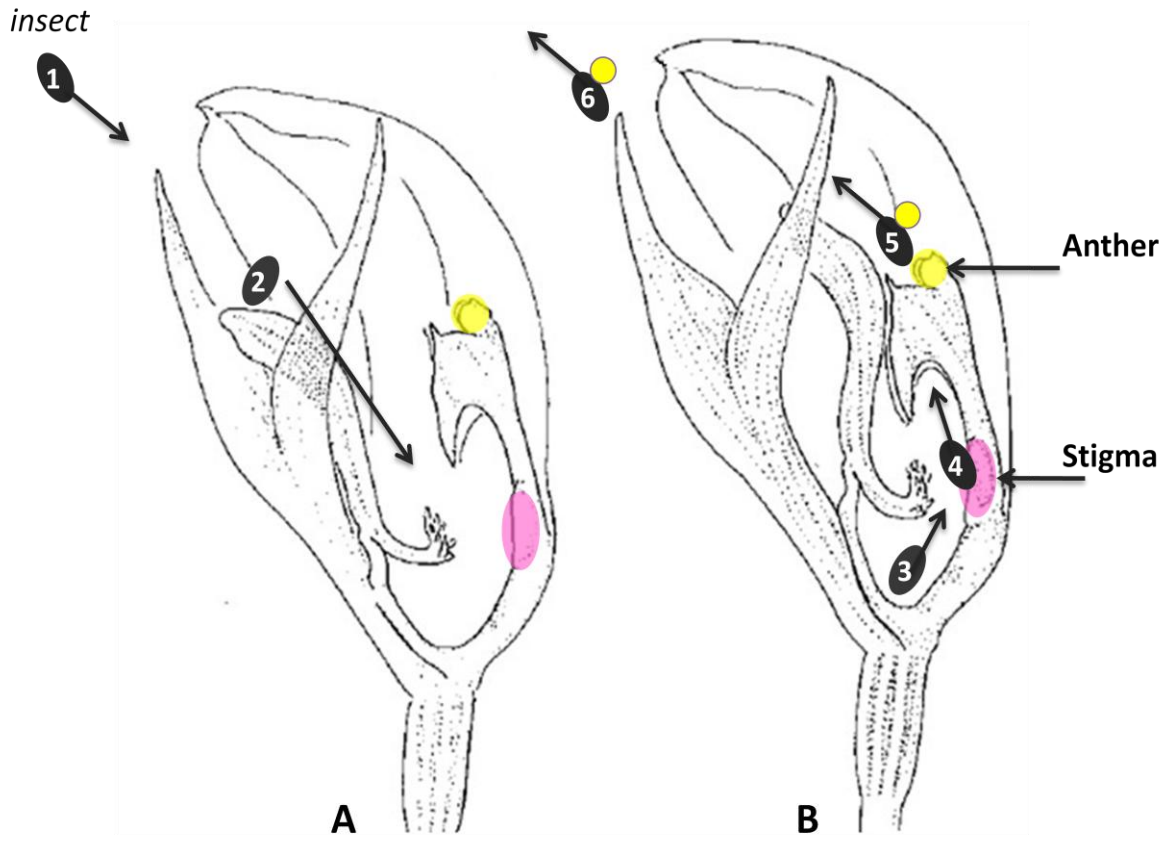


Figure 2.1 Illustration showing the interior of a *Pterostylis* flower trap. The anthers and pollinia are shown in yellow, and the stigma are shown in pink. **A** The labellum at rest; (1) insect flies towards the flower, (2) motion of insect on labellum triggers it to flick back into the flower, catapulting the insect into the base of the flower (3). **B** Labellum triggered; (3) insects tries to escape, (4) crawls past the stigma, through the column wings, and (5) anthers. (6) Insect escapes bearing a pollinium. Adapted from Fig. 3 in Bernhardt (1995)

Methods

Three Pterostylis species studied

Five different *Pterostylis* species; *P. oliveri, P. irsoniana, P. australis, P. montana, and P. venosa*, were found at different locations in Arthur's Pass NP. However, only three of the species; *P. oliveri, P. irsoniana, and P. venosa* were included in this study, as they had sufficient population numbers to carry out the breeding system and pollinator trap experiments for this study (Table 2.1).

The diagnostic features of each species are described below.

Pterostylis oliveri are relatively large (10 - 30 cm), entirely green plants (Fig. 2.2 A). The lance-ovate leaves are in alternate arrangement on the stem, with slight wavy margins. The solitary flower is relative large (3 - 6 cm tall). The dorsal sepal is green with white/translucent stripes at the base. Its long style curls downwards over the flower. The green petals are tucked in beneath the dorsal sepal. The lateral sepals end in very long styles (> 8 cm from base to tip). The labellum (3 - 4 cm long by 5-8 mm wide) is a yellow/brown to green. In Arthur's Pass *Pterostylis oliveri* flower from mid November to the end of January.

Pterostylis irsoniana plants are more delicate and grass-like (Fig. 2.2 A), ranging from 10 – 15 cm in size. The leaves are linear with entire margins, in alternate arrangement on the stem. Colouration on the leaves include light and dark green stripes along a pink/orange midvein. The stem and ovary can sometimes be the same colour as the mid-vein. The flowers are small (2.5 - 3 cm tall). The dorsal sepal is short as it bends over the flower at a right angle, and has green and white stripes which blend together at the orange tip. The petals have stripes ranging in colour from dark green to maroon, and are the same length as the dorsal sepal, tucked away beneath it. The lateral sepals are green at base with pink/orange tips, and extend backwards at right angles. The labellum is white with purple stripes, and has a curled black tip. In Arthur's Pass *Pterostylis irsoniana* flower about the same time as *P. oliveri,* from late November to the end of January.

Pterostylis venosa are very small plants (4 - 7 cm tall). It grows close to ground among moss patches (Fig. 2.2 B). The leaves are green, and broadly ovate with wavy margins. The plants have short stems and appear to have a basal leaf arrangement. The flowers (2 cm tall) are light in colour. The dorsal sepal and petals are largely white with green stripes, curved over the flower at a right angle. The lateral sepals are green at base with light pink styles, and extend upward past the dorsal sepal. The labellum is dark red/purple in colour. In Arthur's Pass *Pterostylis venosa* flower from October to end of November.

Figure 2.2 Study species; **A** *Pterostylis irsoniana* (left) and *Pterostylis oliveri* (right) growing in sympatry at Greyney's Shelter (Picture taken December 2012). **B** *Pterostylis venosa* at Scott's Track (Picture taken October 2013). Scale bars 1 cm. Pictures taken by Liezl Thalwitzer.

Study Sites

Three locations were included in this study; Greyney's Shelter, Cockayne Walk, and Scott's Track in Arthur's Pass NP. These locations had sufficient population numbers of one or two of the *Pterostylis* study species, and were easily accessible to allow for the pollination treatments and pollinator trapping experiments to be carried out. Arthur's Pass NP is situated along the Southern Alps, between Canterbury and the West Coast regions of the South Island. All study sites were located on the west side of Highway 73 within Arthur's Pass NP.

Old Coach Road at Greyney's Shelter (42°58'S, 171°35'E) is a walking track located 6 km south of Arthur's Pass Village. The area was dense in native mountain beech (*Nothofagus solandri*) forest, along with fern, lancewood (*Pseudopanax crassilofius*), kapuka (*Griselinia littoralis*), and marbleleaf (*Carpodetus serratus*) undergrowth. *P. oliveri* was present in a large number of colonies (together >400 plants across the site) which stretched about 200 meters along the foot path. A small number of *P. irsoniana* and *P. montana* colonies were also present in this area. The breeding system,

pollinator trapping experiments, and volatile headspace collections of *P. oliveri* were conducted at this site. Pollinator trapping experiments were also conducted on *P. irsoniana*.

Scott's Track (42°56'S, 171°33'E) is a tramping track which starts 1 km north of Arthur's Pass village. The vegetation changes from native beech forests to sub-alpine shrub with rising elevation of the track. Over 15 small colonies of *P. venosa* plants (2 to 5 plants per colony) were recorded along the first 300 meters of the walking track and surrounding area. Pollinator trapping experiments of *P. venosa* were conducted at this site.

Cockayne Nature Walk at Kelly's Creek (42°48'S, 171°34'E) is a walking track located 17 km north of Arthur's Pass in the West Coast region. This side of Arthur's Pass is a temperate rain forest with diverse podocarp-broadleaf plants including large fuchsia (*F. excorticata*) and totara (*Podocarpus totara)* trees, along with kapuka, lancewood, tutu (*Coriaria arborea*), and fern undergrowth. Large colonies of *P. oliveri* (> 150 plants) and *P. irsoniana* (> 80 plants), and small colonies of *P. australis* (> 5 plants) and *P. montana* (> 3 plants) were found along the entrance of the walking track. The breeding system of *P. irsoniana,* and pollinator trapping of *P. irsoniana* and *P. oliveri* were conducted at this site.

Study species population numbers at the study sites

Sufficient numbers of the three different *Pterostylis* spp. flowers were needed to carry out the pollination treatments and pollinator trapping experiments in the field. Both *P. oliv*eri and *P. irsoniana* were present at Greyney's Shelter and Cockayne Walk (Table 2.1). *P. oliveri* were present in large numbers at Greyney's Shelter, which made it possible to carry out all planned experiments on *P. oliveri* at this location. Overall, *P. oliveri* were more common at Greyney's Shelter and Cockayne Walk compared to *P. irsoniana*, except for the last recorded flowering season (Summer of 2014/15) in which only two *P. oliveri* plants were found at Cockayne Walk. *P. irsoniana* was present in relatively small numbers at Greyney's Shelter and Cockayne Walk. The 2013/14 flowering season at Cockayne Walk made it possible to carry out the pollination treatments on *P. irsoniana*, which was not possible the previous year when only 17 flowering plants were counted in total. *P. venosa* was only found at Scott's Track in 2013/14, and was present in many small colonies (2 - 5 plants) in the area of the track. *P. venosa* was not found at the other two locations. The plant size and scattered population distribution of *P. venosa* at Scott's Track made it impossible to carry out the all the pollination treatments (See *Pollination treatments in the field*, below).

Table 2.1 The three *Pterostylis* spp. population numbers at Greyney's Shelter, Cockayne Walk and Scott's Track for three flowering seasons (2012/13 to 2014/15). NP: orchid species not present at study area/location; NA: population number not measured.

Location	Year	Number of orchid flowering plants per location		
		P. oliveri	*P. irsoniana*	*P. venosa*
Greyney's Shelter	2014/15	430	23	NP
	2013/14	244	37	NP
	2012/13	302	27	NP
Cockayne Walk	2014/15	2	15	NP
	2013/14	157	87	NP
	2012/13	86	17	NP
Scott's track	2014/15	NP	NP	39
	2013/14	NP	NP	57
	2012/13	NP	NP	NA

Pollination treatments in the field

The breeding system of *P. oliveri* at Greyney's Shelter, and *P. irsoniana* at Cockayne Walk were investigated with treatments adapted from Newstrom and Robertson (2005). Both *Pterostylis* species were subjected to four different pollination treatments in the field; natural pollination (open), autogamy (bagged), self-pollinated (selfed) and cross-pollination (crossed) (Fig. 2.3 A, C). The short flowering stalks of *P. venosa* made it difficult to securely bag any of the plants, thus only the natural fruit-set was scored and included in this experiment. All plants included in the study were tagged (with various coloured flagging tape) and labelled at the base of the stems. In the treatments where the plants were bagged, the flowers were covered with fine nylon netting material, which was securely closed below the ovary, to exclude potential insect visitors. The bagged plants were checked weekly to ensure the plants were still alive. For the self- and cross-pollination treatments, the plants were hand pollinated once the stigma was sticky (i.e. receptive to pollen), which can take up to 2 weeks after anthesis (personal observation). For this reason the flowers were bagged before anthesis, and hand pollinated after 2 weeks, and bags replaced. All plants were scored in the field once the flowers dried up on the stalks (where a fruit was ripening) or the flower stalks started to wilt (when the fruit was not developing) (Fig. 2.3 B).

Figure 2.3 Breeding system experiments; **A**, Flowers of *P. oliveri* in bagged (rear) and natural (front) pollination treatments. **B**, Fruit-set: unfertilised wilted flower (left), and fertilised (right) fruit of *P. oliveri*. **C**, Flowers of *P. irsoniana* in bagged (left) and natural (right) pollination treatments. Pictures taken by Liezl Thalwitzer.

The four breeding system treatments are described below.

1. *Natural pollination* treatment establishes the natural percentage of fruit set of the orchids. Plants with developing floral buds were tagged at their base, and allowed to develop and set fruit under natural conditions without any manipulation. Fruit-set was scored after flowering was complete.

2. *Autogamy* treatment tests whether the plants are able to set fruit/self-fertilise without insect visitation. The floral buds were bagged before anthesis, to exclude all insect pollinators. Fruit-set was scored after flowering was complete.

3. *Self-pollination* treatment tests whether the plants are self-compatible, i.e. able to set fruit when fertilized with its own pollen. The floral buds were bagged before anthesis to prevent cross-pollination by insects. Once the stigmas of the flowers became receptive, flowers were hand-

pollinated with one of its own pollinia. Plants were again bagged after pollination and fruit-set was scored after flowering was complete.

4. *Cross-pollination* treatment tests whether the plants are able to set fruit when fertilized with pollen from another plant. The floral buds were bagged before anthesis. Once the stigmas of the flowers became receptive, flowers were hand-pollinated with a single pollinium from a flower of a neighbouring colony. Plants were again bagged after pollination and fruit-set was scored after flowering was complete.

Data analyses

The fruit-set means are presented as percentages in the tables. The degree of self-sterility was calculated using the Self-Compatibly Index (SCI: Self-pollinated fruit-set / Cross-pollinated fruit-set); and the degree of pollen limitation was calculated using the Pollination Limitation Index (PLI: 1 – Natural fruit-set / Cross-pollinated fruit-set, which was truncated at 0, Larson and Barrett, 2000) (Newstrom and Robertson, 2005).

The fruit-set means data were analysed using binomial GLMs in R version 3.1.2. The focus of the different datasets included; comparing the fruit-set means of the four treatments to determine the different levels of self-compatibility and pollen limitation of (1) *P. oliveri* at Greyney's Shelter (2012/13 and 2013/14), and (2) *P. irsoniana* at Cockayne walk (2013/14); and (3) comparing the natural fruit-set means of *P. oliveri* and *P. irsoniana* among Greyney's Shelter and Cockayne Walk, and *P. venosa* at Scott's Track.

Comparisons among treatment means were difficult to analyse in R when one of the treatments had no variation (e.g. the bagged treatments which all had 0% fruit-set, and the selfed and crossed treatments of *P. oliveri* at Greyney's Shelter in 2013/14 which all had 100% fruit-set) because this gave a "complete separation" error which inflated the standard errors. For this reason, I ran binomial GLMs with the bagged treatment to determine any significant treatment effect, and then ran another GLM without the bagged treatment for more reliable coefficient estimates of the other three treatments.

Comparisons of particular *a priori* interest were the self vs. crossed (to assess self-compatibility) and natural vs. crossed (to determine pollen limitation). Analyses of *P. oliveri* at Greyney's Shelter with two years of data available, included Treatment, Year, and the Treatment x Year interaction. If the interaction was non-significant, the GLM was run again without the interaction term to get the best

estimates of the coefficients for Treatment and Year. For analyses of the *P. irsoniana* (2013/14) dataset, I ran GLMs with the crossed, natural and selfed treatments, using crossed as the reference treatment (intercept) to compare with each of the other treatment means for the *a priori* tests mentioned above.

The natural fruit-set of *P. oliveri* was recorded for three years (2012/13 - 2014/15), and two years for *P. irsoniana* (2013/14 and 2014/15) at Greyney's Shelter and Cockayne Walk. The fruit-set of *P. venosa* was recorded in 2014/15 at Scott's Track. The natural fruit-set was compared among the three species and three locations, thus the analyses of the fruit-set means included Location, Species, and the Location x Species interaction term. If the interaction was non-significant, the GLM was run again without the interaction term to get the best estimates of the coefficients for Locations and Species.

Results

Data analyses of the pollination treatments in the field

There were significant differences among the four breeding system treatments of *P. oliveri* and *P. irsoniana*. For both species, none of the flowers in the bagged treatment set fruit. Hand self- and cross-fertilised plants had high fruit-set means (Table 2.2). These results indicate that *P. oliveri* and *P. irsoniana* flowers are self-compatible, however they are not able to autonomously self fertilise (non-autogamous), which shows that these two species are dependent on external pollinators for fertilisation. The natural fruit-set of both *P. oliveri* (80%) and *P. irsoniana* (92%) was higher at Cockayne Walk, when compared to the natural fruit-set of *P. oliveri* at Greyney's Shelter (Table 2.5). The natural fruit-set of *P. venosa* was also high at Scott's Track. Out of a total of 39 flowers, 29 set fruit (74%).

Table 2.2 The mean fruit set (in %, with number of flowers in brackets), and the self-compatibility (SCI) and pollination limitation (PLI) indices for *Pterostylis oliveri* (2012/12 & 2013/14) at Greyney's Shelter, and *P. irsoniana* (2013/14) at Cockayne Walk under four pollination treatments. The degree of self-sterility was calculated using the Self-Compatibly Index (SCI: Self-pollinated fruit-set / Cross-pollinated fruit-set); and the degree of pollen limitation was calculated using the Pollination Limitation Index (PLI: 1 – Natural fruit-set / Cross-pollinated fruit-set) (truncated at 0, Larson & Barrett 2000).

Treatment	***P. oliveri (2012/13)***	***P. oliveri (2013/14)***	***P. irsoniana (2013/14)***
Natural (open)	21.00 (24)	44.00 (25)	92.30 (13)
Bagged	0.0 (15)	0.0 (11)	0.0 (10)
Self-pollinated	91.00 (11)	100 (10)	80.0 (10)
Cross-pollinated	73.00 (11)	100 (10)	80.0 (10)
SCI	1.25	1	1
PLI	0.71	0.56	0

1. P. oliveri fruit-set at Greyney's Shelter (2012/13 and 2013/14)

There was a significant difference among the 4 treatments (Table 2.3a). There was a much lower fruit-set in the bagged treatment (none of the bagged flowers set fruit). The bagged treatment was excluded from subsequent analyses, as the lack of variation in the bagged treatment caused a "complete separation" error which inflated the standard errors in the R summary output. With the bagged treatment excluded from the dataset, there was still a highly significant difference among the 3 treatments and year (Table 2.3b). There was no interaction effect between the years and treatments; Table 2.2 shows that, apart from the bagged treatment, the natural, selfed and crossed treatments had higher fruit-set in the second year (2013/14). The selfed and crossed treatments were similar (Table 2.3c), and both were significantly higher than the natural treatment. The similarly high fruit-set means of the crossed and selfed treatments indicated that *P. oliveri* is highly self-compatible. The natural fruit-set was significantly lower than the crossed fruit-set, which indicates that *P. oliveri* is highly pollen limited at Greyney's Shelter.

Table 2.3 Analyses of the pollination treatments for *P. oliveri* fruit-set at Greyney's Shelter (2012/13 and 2013/14)

(a) Binomial GLM including all four treatments

	Df	Deviance	Residual Df	Residual Deviance	P (Chi)
Null			116	161.503	
Treatment	3	74.332	113	87.172	< 0.001

(b) Binomial GLM of three Treatments + Year

	Df	Deviance	Residual Df	Residual Deviance	P (Chi)
Null			90	122.958	
Treatment	2	35.787	88	87.172	< 0.001
Year	1	6.557	87	80.615	0.010

(c) Coefficients from (b) (Logit-transformed units)

Treatment	Coefficient	Std. Error	Z value	P (>|z|)
Crossed (Intercept)	-2894.4834	1199.4403	-2.413	0.015813
Natural	-2.8358	0.7555	-3.754	0.000174
Selfed	1.2421	1.2154	1.022	0.306810
Year	1.4392	0.5961	2.415	0.015755

2. P. irsoniana fruit-set at Cockayne walk (2013/14)

There was a significant difference among the 4 treatments (Table 2.4a). However, with the bagged treatment excluded from the data, the remaining 3 treatments did not differ (Table 2.4b). The natural fruit-set was 12% higher than the crossed treatment (Table 2.2). The crossed and selfed plants had identical fruit-set means (Table 2.2, 2.4c). The identical crossed and selfed fruit-set means showed that *P. irsoniana* was highly self-compatible (SCI: 1, Table 2.2). With the natural fruit-set being 12% higher than the crossed fruit-set, the population at Cockayne Walk was not pollen limited (PLI: 0, Table 2.2).

Table 2.4 Treatment analyses of *P. irsoniana* at Cockayne Walk (2013/14)

(a) Binomial GLM including all four treatments

	Df	Deviance	Residual Df	Residual Deviance	P (Chi)
Null			42	55.618	
Treatment	3	28.551	39	27.067	< 0.001

(b) Binomial GLM excluding the bagged treatment

	Df	Deviance	Residual Df	Residual Deviance	P (Chi)
Null			32	28.072	
Treatment	2	1.0047	30	27.067	0.61

(c) Coefficients from (b) (Logit-transformed units)

Treatment	Coefficient	Std. Error	Z value	P (>|z|)
Crossed (Intercept)	1.386	0.791	1.754	0.08
Natural	1.099	1.307	0.841	0.401
Selfed	0.0	1.118	0.0	1.0

3. Natural fruit-set of P. oliveri (2012/13-2014/15) and P. irsoniana (2013/14 & 2014/15) at Greyney's Shelter and Cockayne Walk, and P. venosa (2014/15) at Scott's Track

There was a significant difference in the overall natural fruit-set among locations, and species (Table 2.5, 2.6a), but no significant interaction effect. The natural fruit-set of both *P. oliveri* and *P. irsoniana* was significantly higher at Cockayne Walk (Table 2.5, 2.6b). The fruit-set mean of *P. oliveri* was 34.3% (2012/13-2014/15) at Greyney's Shelter, and 80% at Cockayne Walk (2013/14 and 2014/15); and the mean of *P. irsoniana* was 4.4% at Greyney's Shelter (2014/15) and 69.5% at Cockayne Walk (2013/14 and 2014/15) (Table 2.5). The overall fruit-set at Cockayne Walk was similar to Scott's Track (Table 2.5, 2.6b) as the combined mean of *P. oliveri* and *P. irsoniana* was 71% at Cockayne Walk and 74.4% for *P. venosa* at Scott's Track was 74.4% (Table 2.5).

Table 2.5 The mean percentage of natural fruit set of *Pterostylis oliveri, P. irsoniana* and *P. venosa* at the three study locations. The number of flowers per treatment is shown in brackets. NP: orchid species not present at study location. NA: natural fruit-set not scored.

Location	**Year**	**Mean percentage of natural fruit-set**		
		P. oliveri	*P. irsoniana*	*P. venosa*
Greyney's Shelter	2014/15	37.97 (79)	4.35 (23)	NP
	2013/14	44.00 (25)	NA	NP
	2012/13	20.83 (24)	NA	NP
Cockayne Walk	2014/15	NA	46.67 (15)	NP
	2013/14	80.00 (10)	92.31 (13)	NP
	2012/13	NA	NA	NP
Scott's track	2014/15	NP	NP	74.36 (39)
	2013/14	NP	NP	NA
	2012/13	NP	NP	NA

Table 2.6 Analyses of the natural fruit-set of *P. oliveri* and *P. irsoniana* at Greyney's Shelter and Cockayne Walk, and *P. venosa* at Scott's track.

(a) Binomial GLM of the three locations and three species

	Df	Deviance	Residual Df	Residual Deviance	P (Chi)
Null			227	313.95	
Location	2	36.547	225	277.40	< 0.001
species	1	10.407	224	267.00	0.001

(b) Coefficients (Logit-transformed units) with Cockayne and *P. irsoniana* as the intercept (1 not defined because of singularities)

Group	Coefficient	Std. Error	Z value	P (>|z|)
Cockayne (intercept)	0.5770	0.3774	1.529	0.126
Greyneys	-2.8911	0.6625	-4.364	< 0.001
Scotts	0.4877	0.5262	0.927	0.354
P. oliveri	1.6995	0.6304	2.696	0.007
P. venosa	NA	NA	NA	NA

Discussion

P. oliveri and *P. irsoniana* flowers were highly self-compatible, however they were not able to self fertilise (non-autogamous), which shows that these two species are dependent on insects for pollination. The high percentage of selfed fruit-set indicated a low degree of self-sterility (Newstrom and Robertson, 2005; Gaskett, 2011). A low degree of self-sterility can be the result of a lack of early acting inbreeding depression within a population (Newstrom and Robertson, 2005; Jersakova *et al.*, 2006). The lack of inbreeding could be the result of the efficiency of the trapping mechanism of the flower, which is promotes cross-fertilisation (Lehnebach *et al.*, 2005; Gaskett, 2011; Phillips *et al.*, 2014).

P. oliveri and *P. irsoniana* both had very high natural fruit-set rates at Cockayne Walk, relative to Greyney's Shelter. *P. oliveri* had an average natural fruit-set of 34% at Greyney's Shelter and was highly pollen limited (PLI: 0.71 in 2012/13, and 0.56 in 2013/14), whereas the natural fruit-set at Cockayne Walk was very high (80%) in 2013/14. Similarly with *P. irsoniana*, the natural fruit-set at Greyney's Shelter was extremely low (4.4%) in 2014/15, and high at Cockayne Walk (69% average for 2013/14 and 2014/15). *P. irsoniana* was not pollen limited (PLI: 0) at Cockayne Walk. These results indicate that the pollination rates are higher are Cockayne Walk.

P. venosa was included into the study in October 2013, but I was unable to include this species into the hand-pollination treatments, or bag the flowers as they were very small. However in October 2014, I tagged all the flowering plants I could find in the beginning of the flowering season. From a total of 39 flowers, 29 set fruit (74%). This gives *P. venosa* a high natural pollination rate.

There was a significant difference in fruit-set in both *P. oliveri* and *P. irsoniana* among the two study sites; *could the different habitats of Greyney's Shelter and Cockayne Walk be responsible for the difference in the natural fruit-set rates of P. oliveri and P. irsoniana?* The two locations are on opposite sides of 'the divide'. Greyney's Shelter is on the eastern side of the Southern Alps region of Canterbury, and Cockayne Walk is on the western side in the West Coast region. The climate on the west side certainly gets more rain, and the vegetation among the two locations was different. Cockayne Walk is in a temperate rain forest with diverse podocarp-broadleaf plants. While Greyney's Shelter is a walking path cut into a dense native mountain beech forest. If the differences among the locations are to be considered, a future study can document the ecological variables such as climate, elevation, vegetation, precipitation. However, I do not however expect that the habitat differences are directly responsible for the difference in the orchid population size or fruit set.

Janes *et al.* (2010) investigated the environmental variables of 9 *Pterostylis* spp. in Tasmania, in order to understand their ecological requirements and niche space, and to determine whether competitive exclusion may contribute their population range and sizes (Janes *et al.*, 2010). They included several ecological variables such as altitude, soil drainage and texture, precipitation, vegetation type, temperature, climate etc. However, they found no clear ecological separation between the orchid species ranges. Janes *et al*. (2010) concluded with the suggestion that further research into pollinator specificity is required, as they recognised that the niche partitioning may not result from ecological variables but from pollinator shifts, as these orchids may be sexually deceptive. According to the competitive exclusion theory (Hardin, 1960), two closely related species or two species with similar ecological requirements cannot coexist indefinitely, as competitive exclusion will result in each species either finding their own niche or go extinct. In this case however, if the orchids are sexually deceptive, they are attracting specific male pollinators. Most sexually deceptive orchids do not to compete for pollinators, as even closely related species produce different semiochemical compounds (or relative compositions of the same compounds) (Peakall *et al.*, 2010), or have different floral morphologies (Schiestl and Peakall, 2005; Schiestl and Schluter, 2009), to attract a different species of pollinator. These traits in turn also act as pre-zygotic isolation barriers to prevent hybridisation among the orchid species.

Another explanation for the difference in fruit-set of *P. oliveri* and *P. irsoniana* among the two study sites could be due the local abundance of each pollinator species. According to the breeding system results of *P. sanguinea* from Phillips *et al.* (2014), *P. alobula* and *P. patens* in Lehnebach *et al.* (2005), and *P. oliveri* and *P. irsoniana* in this study, the *Pterostylis* orchids fecundity (fruit-set success) is dependent on insects for pollination. Next, I will investigate the insect pollinators in Chapter 3, and determine whether the pollination system operating here is also sexually deceptive.

Chapter 3

Which insects pollinate the orchids?

Introduction

According to the results from the previous chapter, *P. oliveri* and *P. irsoniana* require insect pollinators for fertilisation. The next questions are: *do the flowers of each orchid species attract a different pollinator species*; and *are these pollinators all male*?

In this chapter I will discuss the investigation of the pollinators of the three *Pterostylis* species. In most cases sexually deceptive orchids attract only a single pollinator species, and in all reported cases the pollinators are male insects (Ayasse *et al.*, 2001; Schiestl *et al.*, 2003; Schiestl and Peakall, 2005; Bower and Brown, 2009; Peakall *et al.*, 2010; Gaskett, 2011). A review by Gaskett (2011) showed that all previous studies done on *Pterostylis* in Australia and New Zealand found fungus gnat (Diptera: Mycetophilidae) pollinators. Lehnebach *et al.* (2005) found dead and alive male fungus gnats, from the genus *Zygomyia*, trapped inside *P. alobula* flowers in the North Island of New Zealand. However, evidence for sexual deception as the pollination strategy in *Pterostylis* was not confirmed in that study (Lehnebach *et al.*, 2005). Only recently, Phillips *et al.* (2014) found that *Pterostylis sanguinea* was pollinated solely by male *Mycomya* sp. (Diptera: Mycetophilidae) in Western Australia, confirming sexual deception in *Pterostylis* for the first time. In that study they were able to gather observational and video recordings of the male fungus gnats attempting to copulate with the flower labellum (Phillips *et al.*, 2014).

Pollinator traps were set up over the three *Pterostylis* spp. to investigate whether these orchids attract species-specific male pollinators. This is the first empirical study to show species specificity in *Pterostylis* orchids in New Zealand. The results serve as circumstantial evidence for sexual deception operating as the pollination strategy, which has not been recorded in New Zealand. Here, insect traps were set up over *P. oliveri*, *P. irsoniana*, and *P. venosa*. Insects with pollinia stuck to their thoraxes were identified as pollinators, and the pollen was sent to Landcare Research for ITS DNA analyses to determine which orchid species the pollen originated from.

Methods

Pollinator traps and insect identification

The aim of this experiment was to catch the pollinators as they visit the flowers. Traps were set up over *P. oliveri* and *P. irsoniana* flowers for two flowering seasons (summer of 2012/13 and 2013/14) at Greyney's Shelter and Cockayne Walk; and *P. venosa* for one season (2013/14) at Scott's Track. Small sticky traps were designed to cover the individual flowers. Each trap consisted of a clear plastic tube (~10cm long, 7cm diameter) attached to a metal stand, with the interior lined with insect trapping glue ('Tanglefoot') (Fig. 3.1). The treatment traps were placed over individual plants, covering the flower. The control traps were set up within the orchid population, among the treatment traps, but not over any flowers. All traps were set up close to the ground, at the same height as the orchid flowers. Each set of traps were left out in the field for two week periods. The number of traps varied among species and sites (see Table 3.2). After the traps were collected from the field, they were inspected under a stereomicroscope. The identities of the pollinators were determined by their morphology and wing venation, following the Tonnoir & Edwards (1927) description key. The sexes of the insects were determined by studying their abdominal terminalia (Tonnoir and Edwards, 1927; Colles and McAlpine, 1991).

In the first field season (2012/13) I didn't know what to expect to find in the traps, or how I would determine which insects the pollinators were. For this reason I had both treatment and control traps set up in the field. The following season (2013/14) only treatment traps were set up over the flowers with the aim to increase the replication of the pollinators caught in the traps. In addition, treatment traps were also set up over *P. venosa* flowers in October 2013/14, after populations were found at Scott's Track.

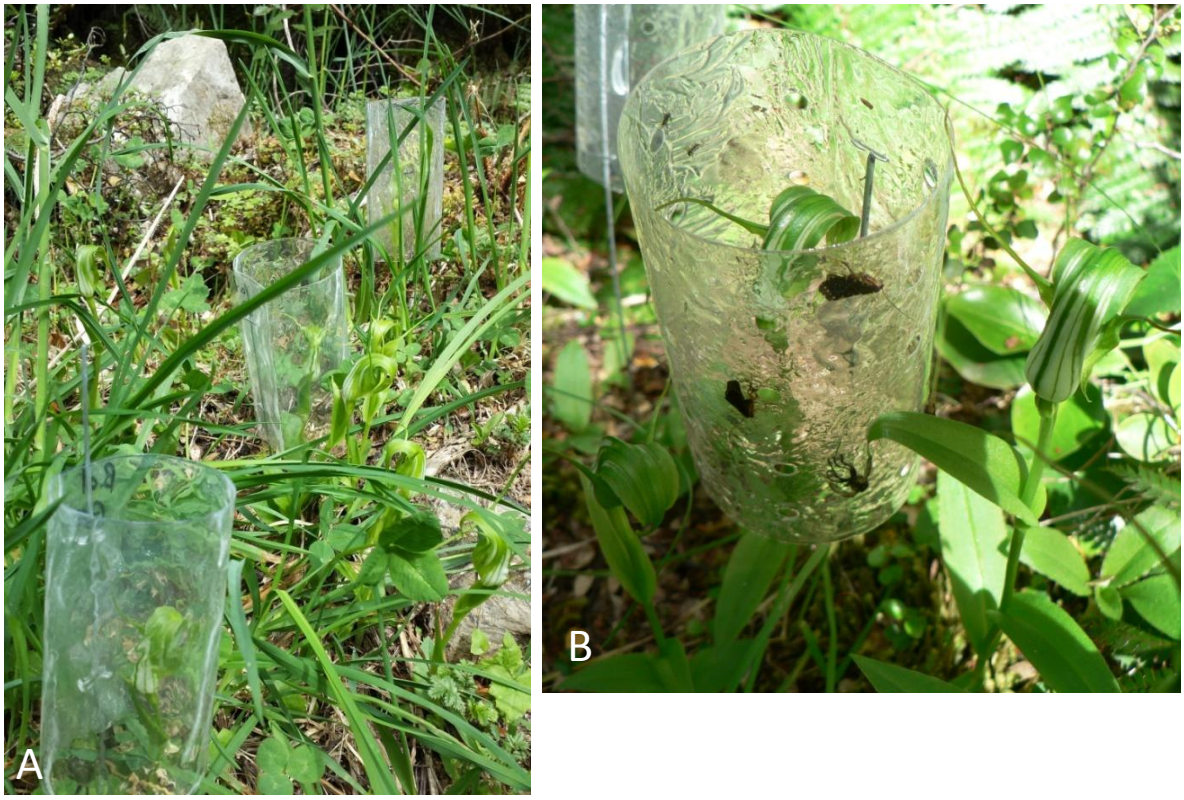


Figure 3.1 Pollinator treatment traps set up over *P. oliveri* flowers in the field. A, Treatment traps with the interior of the trap lined with insect trapping glue; B, Treatment trap with the exterior of the trap lined with insect trapping glue (initial experimental traps of the first field season, 2012/13). Pictures taken by Liezl Thalwitzer.

Plant and pollen DNA identification

DNA sequences from the nuclear ribosomal internal transcribed spacer (ITS) region can be amplified by standard PCR methods (Clements *et al.*, 2011). ITS primers are useful in phylogenetic studies of angiosperm families (Baldwin *et al.*, 1995; Álvarez and Wendel, 2003). ITS DNA analysis was performed for two reasons in this study; (a) to ensure that we were working with the same orchid species at the each of the study sites; and (b) to determine which orchid species the pollinia from the pollinators originated from. DNA was extracted and the ITS regions amplified from fresh plant tissue samples of *P. oliveri* and *P. irsoniana* at Greyney's Shelter and Cockayne Walk, *P. venosa* at Scott's Track; and the pollen samples from pollinators caught on the insect traps. All DNA analyses were done by Rob Smissen at Landcare in Lincoln.

DNA was extracted from plant tissue or pollinia using the Maxwell 16 system (Promega). The nuclear ribosomal DNA region containing Internal Transcribed Spacer (ITS) 1, 5.8S RNA and ITS2 was amplified by PCR using Roche FastStart*Taq*DNA polymerase and reagents and the primers ITS5 (White *et al.*, 1990) and ITS28cc (Glenny and Wagstaff, 1997). PCR products were sequenced using BDT 3.1 sequencing reagents with cycle sequencing products separated on an ABI 3500 Genetic

Analyser. The generated sequences were aligned with available GenBank sequences of New Zealand *Pterostylis* species *P. oliveri* (FJ473348, GQ866390), *P. irsoniana* (GQ866375), *P. venosa* (GQ866404), *P. australis* (GQ866352), *P. montana* (GQ866380) and as an outgroup the Australian species *P. cucullata* (GQ866362). Aligned sequences were analysed under maximum parsimony using PAUP*4.0b10 (Swofford, 1999).

Data analyses of the specific pollinators in the traps

The data from the insect traps were analysed to determine the significant differences in the specific pollinator presence among the three different orchid species and three different locations, as well as among the treatment and control traps. The pollinator trap data were analysed using a Poisson GLM in R version 3.1.2. None of the analyses were over-dispersed. The data were analysed by running each specific pollinator against 5 different trap groups; *P. oliveri* treatment-, *P. oliveri* control-, *P. irsoniana* treatment-, *P. irsoniana* control, and *P. venosa* treatment traps. Location was added as the second term in the analyses. Each of the three analyses were run with Groups + Location, after none of the analyses showed a significant Groups x Location interaction effect.

Results

The results from the pollinator traps indicate that *P. oliveri, P. irsoniana,* and *P. venosa* attract species-specific male pollinators. Three different fungus gnat (Diptera: Mycetophilidae) species were caught carrying pollen in the pollinator traps; *Mycetophila latifascia, Morganiella fusca, Tetragoneura* sp. A total of 43 gnats were caught carrying pollen, all of which were identified as male insects. ITS DNA analyses of the pollinia carried by the fungus gnats confirmed that *Mycetophila latifascia* only carried pollinia from *P. oliveri*; *Morganiella fusca* only carried pollinia from *P. irsoniana*; and *Tetragoneura* sp. fungus gnats only carried pollinia from *P. venosa*.

Pollinator traps and insect identification

Three different fungus gnat species (Diptera: Mycetophilidae) were caught carrying pollen on their thoraxes in the traps (Table 3.1). The fungus gnats were identified by their morphological characteristics and wing venation. Both *Mycetophila latifascia* Edwards and *Morganiella fusca* Tonnoir males were caught in the treatment and control traps of *P. oliveri* and *P. irsoniana* flowers at Greyney's Shelter and Cockayne Walk. Only a single species, from the genus *Tetragoneura* Winn., was found with pollinia in the traps set up over *P. venosa* at Scott's Track. Pollen from each of the three orchid species was found on three different fungus gnat species (Table 3.1, Fig. 3.2).

Only *Mycetophila latifascia* male fungus gnats were caught bearing *P. oliveri* pollinia. A total of thirteen *M. latifascia* males were confirmed (with ITS DNA analyses) to carry pollinia from *P. oliveri* at Greyney's Shelter (*n*=11) and Cockayne Walk (*n*=2). Only *Morganiella fusca* male gnats were caught bearing *P. irsoniana* pollinia. Twenty-five *M. fusca* males were caught with pollinia at Cockayne Walk, of which nine pollinia samples were confirmed to belong to *P. irsoniana.* No *M. fusca* males were caught with pollinia at Greyney's Shelter. Four *Tetragoneura* sp. male gnats were found with pollinia at Scott's Track, and three of the pollen samples were confirmed to belong to *P. venosa.*

Table 3.1 Pollinia bearing *Mycetophila latifascia*, *Morganiella fusca*, and a single *Tetragoneura* species caught in both treatment and control traps over *P. oliveri, P. irsoniana* and *P. venosa* at Greyney's Shelter, Cockayne Walk, and Scott's Track in Arthurs Pass. Origin of the pollinia was determined with ITS DNA analyses.

Orchid species	**Location**	**Total insects bearing pollinia**		**Orchid origin confirmed** (number of pollinia origin confirmed via ITS DNA analyses)
		n	Species (sex)	
P. oliveri	Greyney's Shelter	10	*M. latifascia* (m)	*P. oliveri* (10)
	Cockayne Walk	3	*M. latifascia* (m)	*P. oliveri* (2)
		2	*M. fusca* (m)	*P. irsoniana* (1)
P. irsoniana	Greyney's Shelter	1	*M. latifascia* (m)	*P. oliveri* (1)
	Cockayne Walk	23	*M. fusca* (m)	*P. irsoniana* (8)
P. venosa	Scott's Track	4	*Tetragoneura* sp. (m)	*P. venosa* (3)

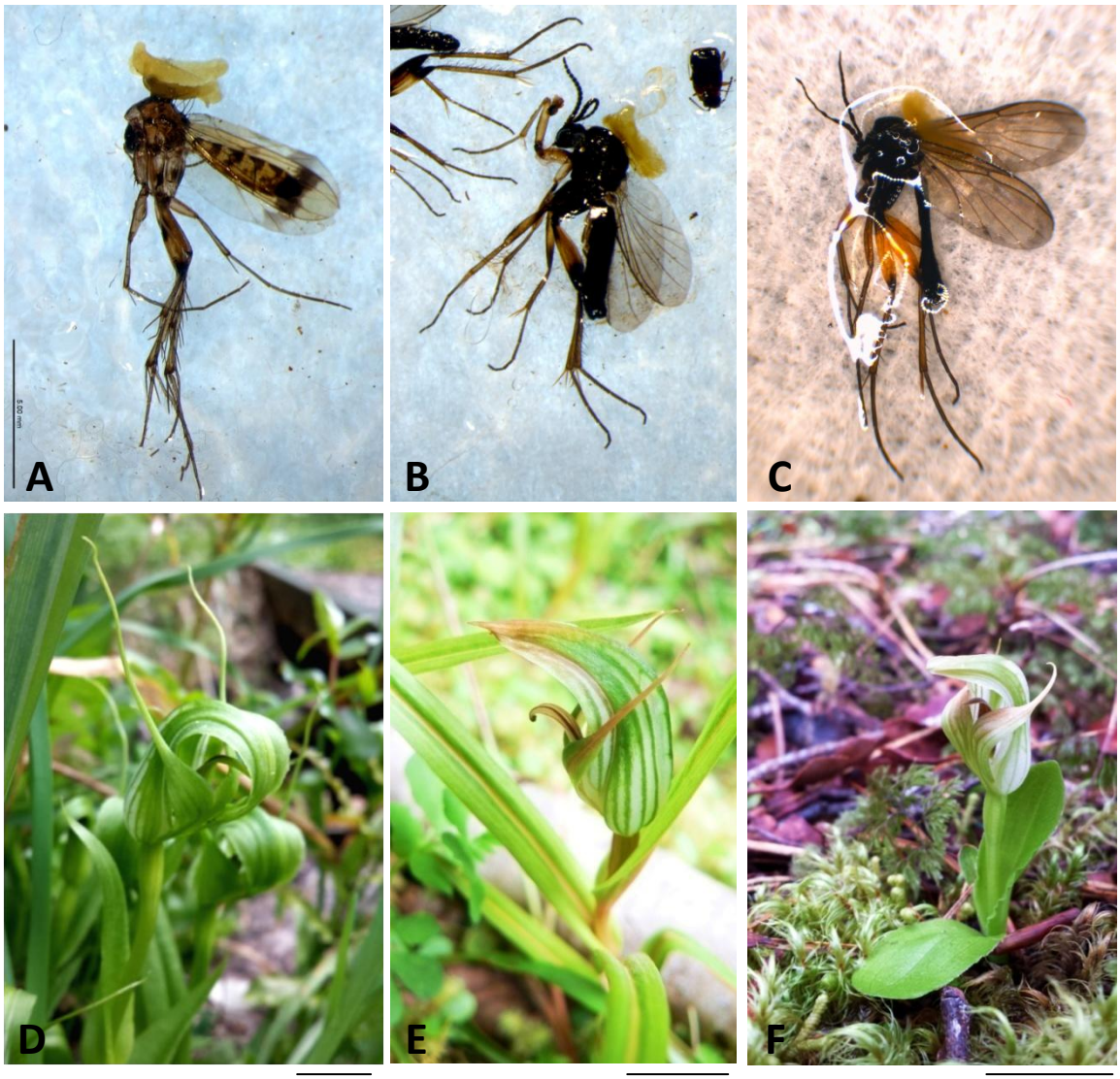


Figure 3.2 Three different fungus gnats (Mycetophilidae) caught with orchid pollinia in the pollinator traps. **A**, *Mycetophila latifascia* caught with *P. oliveri* pollinia (D). **B**, *Morganiella fusca* caught with *P. irsoniana* pollinia (E). **C**, *Tetragoneura* sp. caught with *P. venosa* pollinia (F). Scale bars; Insect scale bar 5mm (A-C); Flower scale bar 2cm (D-F). Pictures taken by Liezl Thalwitzer.

Plant and pollen DNA identification

We sequenced the ITS DNA region from fresh plant material of *P. oliveri*, *P. irsoniana*, and *P. venosa* and compared the sequences to those available in GenBank. In all cases there were differences between our sequences and those in GenBank which were generated from two previous studies; *P. oliveri* sequence FJ473348 was generated by (Alvarez-Molina and Cameron, 2009); and the other sequences were generated by (Clements *et al.*, 2011). The differences involved a number of insertions ("GC" or "GCG" motifs in the GenBank sequences, not found in our sequences) or deletions, but not any substitutions. The positions of these insertions/deletions were consistent across the three GenBank sequences. We interpret the differences observed between sequences

generated for our study and those in GenBank as resulting from errors in the GenBank sequences. These are likely to have arisen during the alignment of sequences in the studies of Alvarez-Molina and Cameron (2009) and Clements *et al.* (2011). Such errors often arise in phylogenetic studies and are not always corrected when they do not impact on the results, as is probably the case here.

What is important here is that all of our newly generated plant and pollinia sample sequences corresponded; all *P. oliveri* plant samples, from both study sites, and all pollinia recovered from *M. latifascia* fungus gnats, shared exactly the same sequence. The same was found with *P. irsoniana* plant samples and all pollinia recovered from *M. fusca* gnats. All plant samples of *P. venosa* at Scott's Track and pollinia from *Tetragoneura* sp. The sequences of the three species differed by at least 7 substitutions (*P. irsoniana* vs. *P. oliveri*), and as many as 18 (*P. irsoniana* vs. *P. venosa*).

The ITS sequences recovered from some samples had little similarity to those of orchids, but had significant similarity to fungal ITS sequences. The best match returned by a nucleotide Blast search on GenBank was to JN569114, an ITS sequence from a root associated Ceratobasidium (Ceratobasidiaceae) sample. Some Ceratobasidium species form mycorrhizal associations with orchids (Irwin *et al.*, 2007). Inadvertent amplification of fungal ITS sequences from green plant sample is not an uncommon occurrence. The fact that we found many of the pollinia samples infested with fungal DNA was initially not expected, but not at all surprising as the traps were left in the field for two week periods close to the ground in humid conditions. In two instances, when traps were not refrigerated after collection from the field, and left out in the laboratory, fungal mycelia and spores were seen around the pollinia on the fungus gnats.

Data analyses of the specific pollinator abundances in the traps

There was a significant difference in the presence of the specific pollinators among the three different *Pterostylis* spp. traps and the three locations. *M. latifascia* were caught in *P. oliveri* traps (treatment and control), and *P. irsoniana* control traps at both Greyney's Shelter and Cockayne Walk. There were significantly more *M. latifascia* gnats caught in traps over *P. oliveri* flowers (0.7/trap) at Cockayne Walk (Table 3.2, 3.3) than Greyney's Shelter. No *M. latifascia* gnats were caught in any of the traps set up over *P. irsoniana* or *P. venosa* flowers. *M. fusca* gnats were caught in both the treatment and control *P. irsoniana* traps at Cockayne Walk. There were significantly more *M. fusca* caught in traps set up over *P. irsoniana* flowers (3.91/trap) at Cockayne Walk (Table 3.2, 3.4) than the control traps, or any trap at Greyney's Shelter. A total of 157 *M. fusca* were caught at Cockayne Walk, while only one was caught at Greyney's Shelter. A single species of *Tetragoneura* fungus gnats were caught at Scott's Track in the traps set up over *P. venosa* flowers. These insects

were not recorded in any other traps, at any other location. The 12 treatment traps at Scott's track were sparse with insect number and diversity; a total of 23 insects were caught of which 19 were *Tetragoneura* sp. fungus gnats. Neither *M. latifascia* nor *M. fusca* were found in any of these traps.

Table 3.2 The average number of specific pollinator species (*M. latifascia, M. fusca,* and *Tetragoneura* sp.) caught per trap in the traps set up over (flower treatment) and among (control) flowering plants of *P. oliveri, P. irsoniana* and *P. venosa* at Greyney's Shelter, Cockayne Walk, and Scott's Track in Arthurs Pass.

Orchid species	**Location**	**Treatment**	**Number of traps**	**Average number of pollinators caught per trap (total insects caught)**		
				M. latifascia	*M. fusca*	*Tetragoneura* sp.
P. oliveri	Greyney's Shelter	Flower	82	0.35 (29)	0.01 (1)	0 (0)
		Control	60	0.1 (7)	0 (0)	0 (0)
	Cockayne Walk	Flower	26	0.70 (18)	0.15 (4)	0 (0)
		Control	17	0.18 (3)	0.29 (5)	0 (0)
P. irsoniana	Greyney's Shelter	Flower	21	0 (0)	0 (0)	0 (0)
		Control	21	0.05 (1)	0 (0)	0 (0)
	Cockayne Walk	Flower	34	0 (0)	3.91 (133)	0 (0)
		Control	18	0.17 (3)	0.83 (15)	0 (0)
P. venosa	Scott's Track	Flower	12	0 (0)	0 (0)	1.58 (19)

Table 3.3 Analyses of *M. latifascia* fungus gnat presence in traps over the three *Pterostylis* spp. and at the three locations.

(d) Poisson GLM Groups + Location

	Df	Deviance	Residual Df	Residual Deviance	P (Chi)
Null			290	242.52	
Groups	4	54.414	286	188.11	< 0.001
Location	1	6.368	285	181.74	0.012

(b) Coefficients (Log-transformed units) with *P. oliveri* treatment traps as the intercept. Scott's Track (location) not defined due to similarities to *P. venosa* (flower)

Treatment	Coefficient	Std. Error	Z value	P (>|z|)
P. oliveri (flower)	-0.3554	0.2185	-1.627	0.104
P. oliveri (control)	-1.2984	0.3639	-3.568	< 0.001
P. irsoniana (flower)	-18.6945	1245.1787	-0.015	0.988
P. irsoniana (control)	-1.6087	0.5248	-3.065	0.002
P. venosa (flower)	-18.9472	2721.2299	-0.007	0.994
Location Greyneys	-0.6917	0.2657	-2.603	0.009
Location Scotts	NA	NA	NA	NA

Table 3.4 Analyses of *M. fusca* fungus gnat presence in traps over the three *Pterostylis* spp. and at the three locations.

(a) Poisson GLM Group + Location

	Df	Deviance	Residual Df	Residual Deviance	P (Chi)
Null			290	786.31	
Species	4	341.14	287	445.18	< 0.001
Location	1	168.71	285	276.47	< 0.001

(b) Coefficients (Log-transformed units) with *P. irsoniana* treatment traps as the intercept. Scott's Track (location) not defined due to similarities to *P. venosa* (flower)

Treatment	Coefficient	Std. Error	Z value	P (>|z|)
P. irsoniana (flower)	1.35931	0.08684	15.653	< 0.001
P. irsoniana (control)	-1.55045	0.27240	-5.692	< 0.001
P. oliveri (flower)	-3.03163	0.45593	-6.649	< 0.001
P. oliveri (control)	-2.60953	0.45605	-5.722	< 0.001
P. venosa (flower)	-18.66189	1001.08453	-0.019	0.985
Location Greyneys	-4.88050	1.00514	-4.856	< 0.001
Location Scotts	NA	NA	NA	NA

Discussion

The results from the pollinator traps indicate that the pollination system of *P. oliveri, P. irsoniana,* and *P. venosa* are species-specific; pollen from each of the orchid species was found being carried by unique fungus gnat species. Hundreds of insects were caught in the traps set up of the flowers of each orchid species, in the three locations (Greyney's Shelter, Cockayne Walk and Scott's Track), yet only three fungus gnat species, all male, were caught carrying pollen. Only *Mycetophila latifascia* male fungus gnats were caught bearing pollinia in the traps set up over *P. oliveri* flowers at Greyney's Shelter (*n*=14) and Cockayne Walk (*n*=3). Fourteen of those pollinia samples were confirmed to belong to *P. oliveri* with ITS DNA analyses. Only *Morganiella fusca* males were caught bearing pollen in the traps set up over *P. irsoniana* flowers at Cockayne Walk (*n*=23). Nine of those pollinia samples were able to be confirmed to belong to *P. irsoniana*. Four fungus gnats of a single species, from the genus *Tetragoneura,* were found with pollen in the traps set up over *P. venosa* flowers at Scott's Track. Three of the pollinia samples were confirmed to belong to *P. venosa.*

The pollinators were found in different abundances at the different locations. *M. latifascia* were present at both Greyney's Shelter (37 insects) and Cockayne Walk (24 insects), but was most abundantly found in the traps over *P. oliveri* flowers at Cockayne Walk. Only one *M. fusca* was caught at Greyney's Shelter, while 157 of the insects were caught at Cockayne Walk, mostly over *P. irsoniana* flowers. Nineteen *Tetragoneura* sp. fungus gnats were caught at Scott's Track. These

insects were not present at the other two locations, and none of the other two pollinator species were present in the traps of *P. venosa* at Scott's Track.

These results of the pollinator traps along with the breeding system results from Chapter 2, are discussed in Chapter 5, as together these results indicate that sexual deception is operating as the pollination system in these *Pterostylis* orchids, and that the fruit-set of the orchids are dependent on the specific insects.

Next, I will investigate the semiochemical volatiles of *P. oliveri* at Greyney's Shelter, in an attempt to identify the compound/s responsible for attracting *M. latifascia* which were caught with the pollen of *P. oliveri* in the pollinator traps.

Chapter 4

Do the orchids attract their pollinators with volatile compounds which resemble the sex pheromone of the female insect?

Introduction

In this chapter, I will describe the methodology of collecting the semiochemical volatiles from sexually deceptive orchids, and how I collected the volatiles of *P. oliveri*. I will describe the methods of the volatile analyses, and testing compounds in the field.

Study methods of chemical ecology

Chemical ecology studies of sexually deceptive orchids investigate the compounds present in the floral volatiles which are responsible for attracting the male pollinators. Such studies start by collecting the floral volatiles. The dynamic headspace collection method involves collecting the headspace volatiles from the plants within a closed chamber around the plant part of interest such as the flowers (Fig. 4.1). Air is drawn through the chamber by a vacuum pump, and the extracted air is passed through a cartridge tube with adsorbent particles which traps any volatile compounds emitted from the plant (Raguso and Pellmyr, 1998). Any compounds present in the cartridge are subsequently eluted with a solvent, and sub-samples are analysed with gas chromatography–mass spectrometry (GC-MS) to identify the compounds present in the sample (Schiestl *et al.*, 2004; Salzmann *et al.*, 2006).

Solvent extraction is another method of volatile collection which involves the plant organ of interest being immersed in a solvent to extract any volatiles present in the wax layer on the surface of the plant (Ayasse *et al.*, 2003; Schiestl *et al.*, 2003; Mant *et al.*, 2005a; Schiestl and Peakall, 2005; Peakall *et al.*, 2010). Most chemical ecology studies of sexually deceptive orchids focus on the orchid labellum, which has been shown to be the site of emission (Ayasse *et al.*, 2001; Schiestl, 2005; Phillips *et al.*, 2014). Solvent extraction is also used to identify the actual sex pheromones of the female insects, by rinsing the female head and/or body with solvent to collect any cuticular hydrocarbons such as the insect specific pheromones (Ayasse *et al.*, 2003; Schiestl *et al.*, 2003; Mant *et al.*, 2005a).

Once the volatiles from the plant and female insects have been identified with GC-MS analyses, they can be compared to determine which compounds from the female insect are also present in the plant volatiles. The corresponding compounds can then be synthesised and used for gas

chromatography-electroantennal detection (GC-EAD). A GC-EAD records any responses from live but amputated male antennae, as the synthesised compounds are released to flow past the antennae. The compounds found to elicit a response from the male antennae, known as biologically active compounds, are then further investigated with behavioural bioassays to test the perceived signal function in the field. Plastic beads treated with the biologically active compound/s, along with plant labella and female insect cuticular extracts, are set up in the field for both quantitative and qualitative comparisons of the male insect attraction to the different volatiles, by counting the number of visitors to the plastic beads and recording their behaviour (Ayasse *et al.*, 2003; Schiestl *et al.*, 2003; Mant *et al.*, 2005a; Schiestl and Peakall, 2005; Peakall *et al.*, 2010). The compounds that elicit copulatory behaviour from the expected male insect species can then be confirmed as the sexual pheromone mimic compound which functions as the orchid attractant in its sexual deceptive pollination strategy. Schiestl *et al.* (2003) investigated the sexual deceptive orchid *Chiloglottis trapeziformis* which was known to attract male *Neozeleboria cryptoides* thynnine wasps. With GC-MS analyses of the floral and female insect body volatiles, they found a single unique compound, called 'chiloglotone', within the female sex pheromone volatiles that was also produced by the flower labella. The compound was then synthesised, and found to trigger a response in the male antennae with GC-EAD analyses. In the field experiments (bioassays), the male insects were found to be equally attracted (copulatory behaviour observed) to the synthesized compound, female extracts and floral extracts (Schiestl *et al.*, 2003).

No evidence has yet been provided that fungus gnats are lured to *Pterostylis* orchids with a sexual pheromone mimic volatile (Gaskett, 2011). However, a sexual chemical attractant is expected to be the reason why the species-specific males are visiting the flowers (Ayasse *et al.*, 2003; Schiestl *et al.*, 2003; Mant *et al.*, 2005c). Thus, it is useful to investigate the floral and female insect volatiles, along with pollinator behaviour. Here, I collected the volatiles of *P. oliveri*. The main purpose of this part of the study was to identify a compound or compounds in the volatile analyses that were present only the orchid volatile samples. And to test this compound in the field for its attractiveness to *Mycetophila latifascia*, the fungus gnat species found with pollinia from *P. oliveri* in chapter 3.

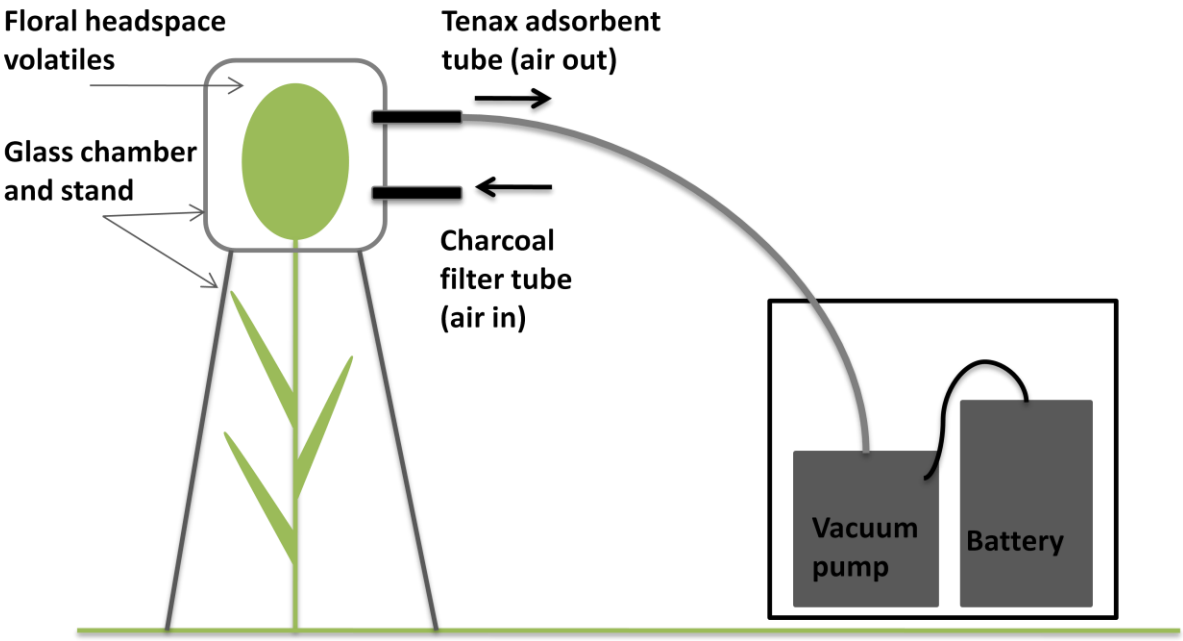


Figure 4.1 Diagram of the dynamic headspace volatile collection in the field, showing the flower enclosed by a glass chamber on stands; direction of air flow, in through charcoal glass tube, and out through a Tenax glass tube; connected via silicon tubing to the vacuum pump. Adapted from Fig.1 in Raguso & Pellmyr (1998)

Methods

Volatile collection in the field

Headspace volatiles were collected from *P. oliveri* at Greyney's Shelter in December 2012 (Fig. 4.2), using a dynamic headspace collection method (Raguso and Pellmyr, 1998). I tested which volatiles were coming from the flower (treatment), and which volatiles were background/field volatiles (control). For the treatment samples, five flowers were individually enclosed within small glass chambers to capture the headspace volatiles. Inflow air passed through a charcoal filter glass tube, while outflow air passed through a volatile adsorbent (Tenax) trap (a 60mm long x 6mm wide glass tube containing 60mg of Tenax-GR 35/60 from Sigma-Aldrich Co.). The control samples were taken from empty glass chambers set up in the same area as the flowers, to capture any background volatiles. Each volatile collection session lasted for 24 hours.

Figure 4.2 Headspace volatile collections of *P. oliveri* flowers at Greyney's Shelter. In the picture, the glass chambers are set up over the flowers. The charcoal tube (larger tube), and the Tenax trap (small tube) which is connected to the floral chambers and the battery powered vacuum pump (within the white plastic container) via silicon tubing. Picture taken by Liezl Thalwitzer.

Volatile analysis

The Tenax traps were eluted with 500 µl of n-hexane (AnalaR BDH, Laboratory Supplies, UK). Each sample was subsequently reduced to 50 µl under argon stream, and 1 µl of the concentrated sample was injected into a GC-MS (Varian 3800 GC coupled to a Varian 2200 MS; Varian, Inc., Walnut Creek, CA) for volatile compound analyses. Helium was used as the gas carrier (1 ml min^{-1}), in a VF-5 ms non-polar column (30 m × 0.25 mm inner diameter × 0.25 µm film; Varian, Inc., Walnut Creek, CA). The Kováts retention indexes (KI) were calculated for the compounds (Kovats, 1965). The unidentified compounds of interest were named for their structure by John Revell, Ashraf El-Sayed and Rikard Unelius, after structural assignments of the compounds were made by comparing their mass spectra with the MS library (NIST 2011), as well as by comparison to Kováts retention indices from *The Pherobase*, a database of known insect pheromones (El-Sayed, 2014)

Bioassays in the field

One of the compounds of interest, the lavender lactone that was identified, was synthesised by Rikard Unelius at Plant and Food Research (Lincoln) for field bioassays. After synthesis, the compound was injected onto small pieces of rubber (called septa, 1 cm x 2.5 cm) for slow volatile release in the field. Four different blends of the compound were prepared in the laboratory; 0.1 mg, 1 mg, and 10 mg of the lactone dissolved in hexane; and a hexane control. Five septa were prepared for each of the four treatments, and each group of septa were vacuum sealed in aluminium packets to prevent chemical contamination or odour loss. Twenty insects traps (five traps for each of the four treatments) were prepared to house the rubber septa in the field. The traps were constructed from plastic, in the shape of a 'bird-house'. Inside of the trap, a plastic sheet covered with 'Tanglefoot' glue was placed (~20 cm^2).

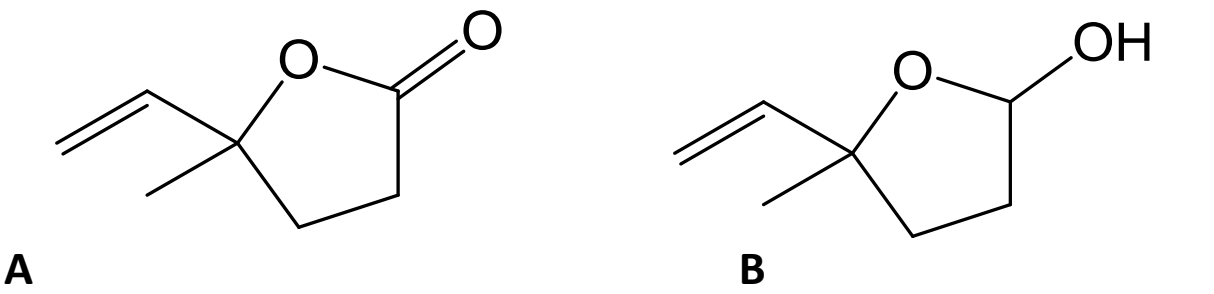
In the field, the traps were placed in the four groups, labelled and the rubber septa of each treatment placed inside of the 'bird-house' traps, on top of the sticky sheet. A trap from each of the treatments was placed in a location (total of four traps at five different locations) along the walking track at Greyney's Shelter where *P. oliveri* orchids are found. The traps were left out in the field for 3 weeks in December 2013. After the traps were collected, the plastic sheets were removed from the plastic housing and inspected under a stereomicroscope for the presence of *M. latifascia* male fungus gnats.

Results

Over 45 compounds were found in the treatment and control volatile samples taken from P. oliveri in December 2012. However, many more compounds in trace amounts may be present. Two unidentified isomeric compounds were consistently found in all five treatment volatile samples at extremely low but similar concentrations (Fig. 4.3). These compounds were identified by John Revell, Ashraf El-Sayed and Rikard Unelius from Plant and Food Research Ltd., after comparisons with known compound structures.

The compounds were identified as lavender lactone and lavender lactol, with Kovat Indices of 929 and 948, respectively. The lavender lactone was synthesized by Rikard Unelius. Unfortunately the attempts for synthesising lavender lactol repeatedly failed. For this reason, only the lavender lactone was tested in the field. No *M. latifascia* insects were present on any of the plastic sheets, in any of the four treatments.

These results show that the lavender lactone is not an attractant of *P. oliveri*. Further volatile analyses is required to determine whether *P. oliveri* attract their pollinators with volatile compound/s which resembles the sex pheromone of the female insect.



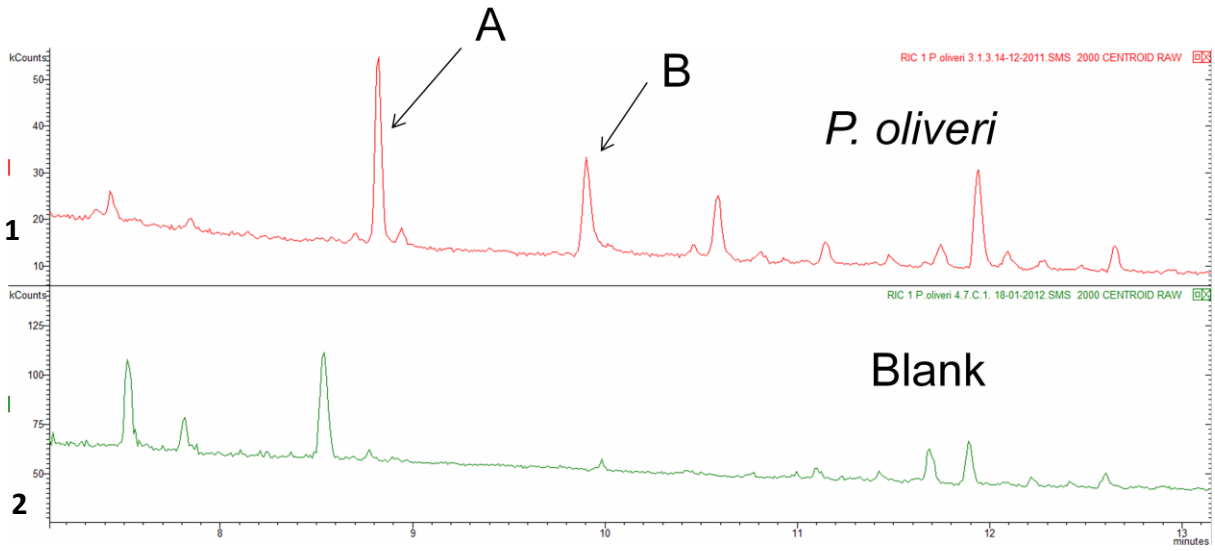


Figure 4.3 Chromatogram showing (**1**) the unidentified compounds (A and B) found in *P. oliveri* flower volatile, against (**2**) the control volatile sample. The two isomeric compounds found in *P. oliveri* volatile; **A** Lavender Lactone, and **B** Lavender Lactol.

Discussion

Two previously unidentified compounds, lavender lactone and lavender lactol, were found to be present in the volatiles of *P. oliveri* collected in the field. Only the lavender lactone was synthesised and tested in the field. This compound did not appear to be an attractant of *P. oliveri*, as none of their pollinators, *M. latifascia* fungus gnats, were caught in any of the traps with a blend of lavender lactone.

Investigating the volatile compounds of sexually deceptive orchids normally takes several years to elucidate, and several factors in this study have made finding the volatiles of *P. oliveri* even more challenging.

If the dynamic volatile collection method is used in a future study, the control headspace volatile collections should also include taking volatiles from the leaves of the orchid species, along with an empty chamber in the field. The method I followed tested which volatiles were coming from the flower (treatment), and which volatiles were background/field volatiles (control). The control samples should include being taken from a glass chamber enclosed around a leaf in order to determine which volatiles were coming from the leaves, i.e. the plant. The volatiles present on the leaf can then be compared to volatile present in the flower, which should eliminate a lot of compounds, and narrow down on possible compounds of interest. We also expect the attractant compound to be present at extremely low concentrations, which increases the difficulty for identifying the compound/s.

In studies that have found the floral attractant, researchers were able to compare compounds in the floral volatiles with those of the female insect of the pollinator species. The pollinators in these studies were relatively easy to catch or trap alive. The female bodies are rinsed in a solvent for GC-MS analyses, while the male insects are kept alive until their antennae are amputated for GC-EAD analyses (Ayasse *et al.*, 2003; Schiestl *et al.*, 2003; Mant *et al.*, 2005c).

Fungus gnats are very small and fragile insects, and hard to trap alive long enough to separate the males from the females, and to use the male antennae for GC-EAD analyses. Throughout my study, I was unable to catch these insects alive. Future studies of the volatile compounds of *Pterostylis* orchids and their fungus gnat pollinators will need to find a method of catching the insect in the field, or perhaps even methods of rearing the insects in the laboratory.

In this study we were unable to find the attractant of *P. oliveri*, or to determine which volatiles present in the flowers resembled the sex pheromones of the female pollinator species. Future

volatile collections should include a *P. oliveri* (a) flower, (b) leaf, (c) and background control. The volatiles produced by the plant (leaf) can serve as a control, which can be useful in determining and excluding the compounds which are present in both the leaf and the flower, and narrow down the amount of volatiles of interest.

Chapter 5

Discussion

In Chapter 2, I investigated (a) whether the flowers of each of the three *Pterostylis* spp. depend on insects for pollination. *P. oliveri* and *P. irsoniana* flowers were highly self-compatible, and non-autogamous, which shows that these two species are dependent on insects for pollination. The breeding system experiments also indicated a lack of early acting inbreeding depression within both species populations at Greyney's Shelter and Cockayne Walk, which is most likely the result of natural intra-specific cross-pollination promoted by the trapping mechanism of the flower (Lehnebach *et al.*, 2005; Newstrom and Robertson, 2005; Phillips *et al.*, 2014).

In Chapter 3, I determined (b) whether each of the three *Pterostylis* spp. flowers attract a different insect pollinator species, and (c) whether the flowers attract only male insect pollinators. Results from the pollinator traps indicated that sexual deception is operating as the pollination system in these three *Pterostylis* spp. Each of the three *Pterostylis* spp. flowers attracted only male species-specific fungus gnats. Only *Mycetophila latifascia* male fungus gnats were caught bearing pollinia of *P. oliveri* flowers. Only *Morganiella fusca* males were caught bearing pollen of *P. irsoniana* flowers. And only a single species, in the genus *Tetragoneura,* was found with pollen of *P. venosa* flowers.

In chapter 4, I investigated whether (d) the flowers emit volatile compounds which resemble the sex pheromones of the specific insects. The study species was limited to *P. oliveri* populations at Greyney's Shelter. The volatiles of *P. oliveri* were collected in the field, and after GC-MS analyses, two previously unidentified compounds, lavender lactone and a lavender lactol, were found to be present in the volatiles of *P. oliveri*. Only the lavender lactone was tested in the field for its attractiveness to the *P. oliveri* pollinator. However, the compound did not attract any of *P. oliveri*'s pollinators, *M. latifascia* fungus gnats, to the traps. Thus this compound is not an attractant of *P. oliveri*. I was unable to catch any live *M. latifascia* in the field, thus I was not able to perform any volatile extractions from the female insect bodies or perform any GC-EAD on the male antennae. It is highly expected that the orchids must attract their pollinators with sexual olfactory cues, as seen with other sexually deceptive orchid spp. (Ayasse *et al.*, 2003; Schiestl *et al.*, 2003; Mant *et al.*, 2005c), and further research is needed to identify the semiochemical compounds in the *P. oliveri* floral volatiles which resemble the sex pheromones of the female *M. latifascia* fungus gnats.

Phillips *et al.* (2014) investigated *Pterostylis sanguinea*, and found that the flowers were pollinated by a single male fungus gnat species. In that study Phillips *et al.* (2014) also introduced a list of the

'*Criteria for confirming pollination by sexual deception*'. Meeting one or more of the criteria is confirmation of sexual deception. The criteria includes; the observation of (1) any courtship or pre-mating behaviour by the insect to the flower, or (2) attempted copulation with the flower, or (3) ejaculation of the male insect during attempted copulation, or (4) the discovery of chemical mimicry by the flower (i.e. finding semiochemicals in the flower volatiles which mimic the sex pheromones of the female insects, to elicit a sexual response from the males in bioassays). According to this criteria the pollination system of the *Pterostylis sanguinea* could be confirmed as being sexually deceptive, as the male pollinators were observed (and recorded in the study) to attempt to copulate with the labellum (Phillips *et al.*, 2014).

The results from my study cannot confirm sexual deception. However, in the absence of behavioural observations or chemical volatile confirmation, other lines of evidence (circumstantial evidence) can indicate pollination by sexual deception; (1) finding only male pollinators, (2) flowers which lack any food reward, (3) only one, or two pollinator species (species-specificity), (4) finding the insects are attracted to a covered flower (where visual signals are reduced or eliminated), or artificially presented (baited) flower (chemical attractant), (5) the flower having an insect-like shape or shaped organ such as the labellum, and /or being dull in colour, inconspicuous flowers, or flowers with a large labellum (Phillips *et al.*, 2014). Thus according to the criteria; finding only male pollinators, and only one pollinator species for each of the three orchid species, indicates that *P. oliveri, P. irsoniana* and *P. venosa* may be sexually deceptive orchids.

We now know that the fecundity (fruit-set success) of the at least four *Pterostylis* orchids in New Zealand, including *P. oliveri* and *P. irsoniana* from this study, and *P. alobula* and *P. patens* from Lehnebach *et al.* (2005) are dependent on specific insect species, we have a deeper understanding that could aid in their future conservation. Pauw and Bond (2011) and Pauw and Hawkins (2011) theorised that specialised orchid pollination systems can make the orchid populations vulnerable, as their fecundity depends on their pollinators. For this reason it would be useful to understand whether the orchid fruit-set is related to the abundance of the specific pollinators.

Could the orchid population sizes affect their own fruit-set rate, assuming they are sexually deceptive?

The potential for negative frequency-dependent selection has been shown in non-rewarding deceptive orchids (Smithson and Macnair, 1997; Ayasse *et al.*, 2000; Schiestl, 2005; Schiestl and Schluter, 2009). In this case the fitness of the orchid (fruit-set rate) will decrease as it becomes more common in the plant community, as a result of the pollinators 'wising up' to the false promise of sex

and start to avoid the flowers. If this is the case with the *Pterostylis* spp. of my study, I would expect to see the smaller orchid populations have a higher fruit-set rate at the end of each flowering season. This could be the case with *P .oliveri,* which had a lower fruit-set (average 34%) at Greyney's Shelter where the orchid population size was large (average or 352 plants); and a high fruit-set (average 80%) at Cockayne Walk with a smaller population size (average of 82 plants). However, this doesn't explain why *P. irsoniana* found in small populations (average 29 plants) at Greyney's Shelter had such a low fruit-set (average 4.4%), when it had a higher fruit-set (average 69%) at Cockayne Walk, where an average of 40 plants were found from 2012-2015. The negative frequency-dependent selection does not appear to be operating here. Phillips *et al.* (2014) investigated the fruit-set of 19 populations of *Pterostylis sanguinea* in two separate years (2008 and 2012), where the population sizes ranged from 5 to 127 plants. They didn't find any relationship between the natural fruit-set and the population size or densities. There is also no current evidence that Diptera have the ability to learn to avoid non-rewarding flowers. The studies where the insects were fooled into being pollinators, and subsequently learned to avoid the non-rewarding flowers, were all hymenopterans (Smithson and Macnair, 1997; Ayasse *et al.*, 2000; Schiestl, 2005).

Could the pollinator population numbers be related to the natural pollination rates?

The simplest explanation for the orchid populations thriving or suffering from pollen limitation could simply be related to the number of insects present at each location. Perhaps there are more of the pollinator species (*M. latifascia* and *M. fusca*) present at Cockayne Walk. Future studies can focus on trapping insects for the purpose of surveying the number of each pollinator present per trap, per location, along with recording the natural fruit-set of the orchids at each location.

The current distribution and population ranges of New Zealand *Pterostylis* orchids may also depend on the fungi in the soil in the native forests. It has long been thought that the New Zealand *Pterostylis* species are vagrants from Australia, as seeds are carried by the west winds to the islands, populations can only establish where the right soil fungi is present (Hatch, 1946; Johns and Molloy, 1983; Irwin *et al.*, 2007). *Pterostylis* orchids are known to have mutual associations with soil fungi such as *Ceratobasidium* species (Irwin *et al.*, 2007). These associations have also been shown to be species-specific (Warcup, 1981). Irwin *et al.* (2007) sampled *Pterostylis nutans* from across south eastern Australia and found that the plants roots were colonised by only two closely related *Ceratobasidium* sp. of fungi. Knowledge of the orchid-mycorrhizae associations are important for the conservation of orchid populations, considering the orchid species distribution depends not only on whether the soil is colonized by mycorrhizal fungi (Feuerherdt *et al.*, 2005), but also whether the right species of fungi may be present.

Janes *et al.* (2010) considered many ecological variables (see Chapter 2 Discussion) as possible abiotic factors which could have some effect on the current distribution of the *Pterostylis* orchids in Tasmania. They found that none of the ecological (abiotic) variables could explain the current distribution of the orchids, which is interesting as the results from Phillips *et al*. (2014) and my study show that sexual deception is operating in *Pterostylis* orchids. This means biotic interactions, such as the pollinator dependency and root associated fungi, need to be considered as possible predictors of *Pterostylis* orchid population size and distribution ranges.

Conclusions

The results from this study indicate pollination via sexual deception may be operating in these three *Pterostylis* spp. The breeding system results showed that *P. oliveri* and *P. irsoniana* are self-compatible but depend on insects for pollination. The pollinator trapping experiments showed that each orchid species was pollinated by a unique fungus gnat species, all of which were male insects. Further floral volatile analyses are required to confirm whether the flowers emit volatile compounds which resemble the sex pheromones of the specific pollinators. This is the first empirical study to show species specificity of *Pterostylis* spp. in New Zealand, and serve as circumstantial evidence of sexual deception operating as the pollination strategy which has not been recorded in New Zealand.

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