Effect of Altitude and Co-occurring Species on Sequential Reproductive Stages in a Grassland Harebell.

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A thesis submitted in partial fulfilment for the degree of

Master of Science in Botany

at the University of Otago, Dunedin, New Zealand

October 2012

“WHY IS A RAVEN LIKE A WRITING DESK?”

(CARROLL 1865)

Abstract

Understanding reproductive capabilities of individual species along altitudinal gradients is increasingly important in light of changes in habitat that are predicted with climate change. Alpine plants can be considered particularly at risk from climate-change as they occupy what are in effect habitat islands. Up until recently New Zealand's alpine flora has been assumed to be largely self compatible with high levels of generalised pollinator relationships. However recent evidence has found this not to be the case suggesting that reproduction in New Zealand alpine plants may be more sensitive to changes in climate and pollinator availability than previously thought. Of particular interest is an alpine bellflower, which is self-incompatible and has a short duration of stigma receptivity. This plant, *Wahlenbergia albomarginata* Hook*.,* ranges in flower size and petal colour can vary from light blue to white, with extreme blue flowers being more attractive to pollinators *en masse*. The central aim of this study was to discover if changes in environment over *W. albomarginata's* wide altitudinal range affect its reproductive characteristics namely: flower size, colour, pollen delivery and seed set.

The effect of environmental factors associated with altitude and soil chemistry on flower colour (reflectance 450:550 nm ratio) and flower size were compared in over three hundred flowers among three locations: the Blue Mountains(with sites ranging from 650m-1000m), The Remarkables (with sites ranging from 990m-1650m) and the Pisa Range (with sites ranging from 886m-1705m). Additional work tested for changes in flower colour over time and in restricted soil moisture. The effect of altitude on pollen load at the Blue Mountains was determined. Forty thousand pollen grains were identified on sixty stigmas. The proportion of hetero-specific pollen was compared with immediate flowering neighbourhood and with previous work carried out at The Remarkables. Finally, the effect of altitude on seed set and mass was tested at The Remarkables. Fifteen thousand seeds were counted from seventy two capsules to test the effect of location between the Blue Mountains and Remarkables.

Results showed environmental factors associated with altitude affected flower size at one of three locations. At the other two, significant variation in flower size between sites did not coincide with an altitudinal effect. This suggests flower size changes with environmental factors that do not always change with altitude. Flower colour was bluest in younger flowers, which is likely to enhance male fitness, by increasing pollen export by pollinators prior to gender phase change in this protandrous species. On average, eighty percent of pollen load on stigmas was exotic at the Blue Mountains. This was in stark contrast with previous findings at higher altitude at The Remarkables. Pollen load was not affected by altitude or immediate neighbourhood at the Blue Mountains, because the exotics, whose pollen was present on stigmas, were present at all sites. Seed set was

not affected by altitude at Tithe Remarkables, despite this being the largest gradient, and the only location where flower size was affected.

It can be concluded from this study that changes in flower size due to environment do not affect reproductive outcomes in the field. Changes in temperature associated with elevating altitude do not affect insect activity sufficiently to change pollen type or amount delivered to *W. albomarginata,* or seed set. Instead, location and different co-occurring species greatly affect the type of pollen delivered, but not overall seed set. This study shows that *W. albomarginata* has a high level of plasticity in floral characteristics that are dependent on abiotic conditions, and is able to maintain a consistent reproductive output, despite inhabiting areas with a variety of soil pH, soil moistures, soil nitrogen levels, temperatures, shelter and plant mosaics throughout its wide altitudinal range.

Acknowledgements

Thank you to the E.L Hellaby Indigenous Grasslands Research Trust and The University of Otago Botany Department for funding this research.

Thank you to my supervisor Dr. Janice Lord for her support with experimental design, data collection and statistics.

Dana Dudle, Dianne Campbell and Mascha Bischoff provided a fresh perspective and valuable advice. Ella Hayman, Vicky McGimpsey, Lorna Little, Lars Ludwig, Dr. Margaret Stanley and Christa Miller helped collect samples and make observations in the field.

I'd like to thank the staff and students of the Otago University Botany Department who were generous with their thoughts, time and expertise.

Finally, thank you to my family Stephen, Sarah and Robert Sawrey for their support throughout my years at university.

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1

Introduction to Alpine Pollination Ecology and *Wahlenbergia albomarginata*

"DOWN THE RABBIT HOLE"

(CARROLL 1865)

1.1 Floral Biology and the influence of Altitude.

Production of attractive flowers to entice pollinators is a fundamental process in the life history of many plants, because of their inability to move. Flower colour and size are major components of pollination syndromes, which describe flower features in the angiosperms that are thought to relate to different types of pollinators (Fenster et al. 2004). Yet the majority of plant pollinator relationships are increasingly acknowledged as more generalised than this theory would suggest (Chittka et al. 1996). Often complex networks with multiple plant species and more limited pollinator types make generalisation inevitable. Environmental factors associated with altitude and geographic location may affect flower characteristics and consequently, a plant's ability to attract and reward insects. This means plants develop reproductive strategies to suit the biotic and abiotic factors associated with the geographic range they inhabit (Fabbro and Körner 2004, Tian et al. 2011, Wig et al. 2011); yet many studies on this subject have neglected to take a holistic view on the way altitude and its associated stressors affect floral characteristics, pollination success and seed set (Körner 2004). To truly understand evolutionary pressures on floral characteristics, the reproductive consequences of environmental variation must be taken into account. This thesis explores the reproductive consequences of variation in floral characteristics of a single species in a variety of sites throughout its wide altitudinal range.

Attractive flowers are essential structures in biotic pollination, and the key feature of flower size is thought to be driven primarily by pollinator selection (Galen and Newport 1987, Conner et al. 1996). Pollinators have been shown to prefer large flowers to small either because they associate them with greater rewards(Stanton and Preston 1988, Cresswell and Galen 1991) or they are more visible, and easier to visit (Totland et al. 1998). However, plastic changes in flower size in response to abiotic environmental factors are also known to occur. High soil moisture is associated with relatively larger flowers in a variety of American taxa including the alpine plant *Polemonium viscosum* Nutt. (Galen et al. 1999, Galen 2000, Lambrecht and Dawson 2007). Water use efficiency tends to be greater in plants with larger floral size, leading to the suggestion that water loss from flowers influences leaf responses to the environment (Lambrecht and Dawson 2007).

At higher altitudes, temperature and atmospheric pressure decrease while solar radiation under a cloudless sky and UV-B radiation increase (Körner 2007). This in turn affects a host of other factors, such as relative humidity, soil moisture, ambient CO_2, duration of snow cover at temperate latitudes and co-occurring species (Körner 2007). Alpine plants have various phenological strategies to adapt to the short growing season with limited resources and pollinators (Zhang et al. 2010). Low stature vegetation has the advantage of experiencing solar warming in low ambient temperatures, however this causes the driving forces for vapour loss to increase (Smith and Geller 1979, Körner 2003a).

Flower size thresholds are known to vary depending on habitat (Wesselingh et al. 1997) and previous studies have highlighted an increase in allocation to flower size with increasing altitude among taxa, and attributed this to pollinator scarcity (Fabbro and Körner 2004). Yet, in *Ranunculus acris* L.*,* environmental factors interact with pollen availability to determine reproductive success, disrupting the causal pathway connecting low pollinator availability and plant traits (Totland 2001). Flower size variation over an altitudinal gradient within a species could aid understanding of the importance of abiotic environment in determining flower size in unstressed plants and is hitherto insufficiently researched.

Flowers often invest resources in producing petal pigments that are particularly attractive to their pollinators, yet plastic and temporal variation in floral colour are fairly common (Vaknin et al. 2005, Stiles et al. 2007). Plant pigments can act as "Swiss army knives" in plants, not only responsible for pigmentation in fruit and petals, which attract dispersers of pollen and seeds, but also for minimising the effects of a variety of stressors,. Petal colour can be altered by the configuration or amount of pigment, which can be dependent on environmental factors such as soil pH and soil nutrient availability (Markham et al. 2000). Variation in flower colour with age can also occur: for example the New Zealand alpine plant *Euphrasia dyerii* Wettest. is known to switch from white to purple as gender phase changes (McGimpsey and Lord in prep.). Quantifying patterns of variation in flower colour of wild flowering species is important in determining the effect flower colour has on reproductive success.

Evolutionary consequences of environmental factors changing flower characteristics cannot be understood without examining pollination success and seed set (Campbell 1991). Many studies have shown pollinators to be attracted to individuals with large flowers (Galen and Newport 1987, Campbell et al. 1991, Eckhart 1991, Campbell et al. 1996, Conner et al. 1996), but flowering species exist in complex communities, and pollinator choices may be affected by a plethora of other factors. Community composition changes dramatically over environmental gradients, in terms of both insect pollinator types and co-flowering species. Abundance, distribution and persistence of many high altitude species can be influenced by positive and negative interactions with their neighbours (Choler et al. 2001). Insect visitation choices may be more strongly influenced by marked differences between species than subtle differences within species.

The cold/short growing season of temperate alpine environments constrains plant growth and reproduction, so a limited number of life history strategies are employed (Pellissier et al. 2010). Investment in one stage of a plant's life history will often come at a cost to another. Large female flower size can be detrimental to fruits (Galen 2000). Seed and fruit properties are known to

change with increasing altitude (Pluess et al. 2005, Tian et al. 2011, Pato and Obeso 2012). Seed weight can differ between related species and increase within species with altitude (Pluess et al. 2005, Tian et al. 2011), or else investment in dispersal can often be limited by producing capsules with numerous small seeds (Pellissier et al. 2010). Simultaneous constraints on seed production resulting from low pollen availability and other environmental factors are common in natural populations (Haig and Westoby 1988, Corbet 1998). If plants are constrained by environmental factors, it is likely that high pollen loads will not translate to high seed set, so flower investment in size may be relaxed where environmental variables constrain plant growth (Campbell 1991, Totland and Eide 1999).

1.2 *W. albomarginata*'s Environment: Setting the Scene

The trade-off between physiological constraints associated with abiotic environment and production of showy flowers is given a unique twist in the New Zealand flora, by the globally unique network of pollinators amongst which its flowering plants have evolved. New Zealand is an island archipelago, which has been geographically isolated since its separation from Gondwana eighty five million years ago (McLoughlin 2001). This separation occurred prior to many significant evolutionary divergences in the world's fauna, so New Zealand has no native land mammals or social bees. This has had a marked effect on floral structure with simple open-access flowers and pale or white petal colour being favoured over vibrant, showy flowers with sophisticated access requirements (Wardle 1978, Campbell et al. 2010). So New Zealand plant pollinator networks are relatively generalised, with solitary bees and flies showing low fidelity to plant species (Primack 1978).

European settlement two hundred years ago coincided with a rapid insurgence of exotic plants and animals that pollinate, predate and compete with the pre-existing biota (Brockerhoff et al. 2010). Exotic plants are known to compete with native plants for pollinators (Chittka and Schurkens 2001) and generalised pollinators are known to assist exotic plants in integrating into new communities (Padrón et al. 2009). Flowers from exotic plants can also help to facilitate native pollination (Moragues and Traveset 2005). Exotic invertebrates are also known to impact indigenous plant species and their ecosystems (Simberloff 2005). Invasive species can undergo lag phases, where detrimental effects on new habitats may not be immediately apparent (Brockerhoff et al. 2010).

The temperate alpine environment is one of daily and seasonal temperature fluctuations and low nutrient availability (Streb et al. 1998, Körner 2007). Alpine plants have evolved particular life histories, morphology and physiology to suit this environment. Flowering occurs either in spring, right after snow has melted or later on in summer when temperatures are high and insect activity is optimal in longer days (Molau et al. 2005). Globally, alpine plants tend to have open access flowers that are long lived (Primack 1983), because conditions can be very changeable (Molau et al. 2005), and exclusive plant pollinator relationships do not sustain insect life. Instead pollinators behave in a generalised way. Flowers in the New Zealand alpine differ from others in that they are generally much paler (Swenson and Bremer 1997).

Over half of New Zealand's land area is grassland, which extends from high altitude to low (Wardle 1991). The diversity of New Zealand's grassland flora is known to affect native weevil diversity (Crisp et al. 1998), however the importance of native and exotic pollinators for even the most common exotic and native flowering plants in this environment is poorly understood (But see Huryn 1997).

W. albomarginata has been shown to be visited by species with relatively more loyalty than its native alpine neighbours (Bischoff 2008).

1.3 Study Species: *Wahlenbergia albomarginata* Hook.

This project will examine the reproductive success of a southern grassland herb, *Wahlenbergia albomarginata* Hook., in relation to its mountain environment. *W. albomarginata* is a rhizoatmous rosette-forming herb, which inhabits tussock grasslands of the South Island, New Zealand (Allan 1961). A member of the Campanulaceae or "bluebell" family, it has a single pale blue bell-shaped blossom with directed access (Bischoff 2008) that is upheld by a pedicel 10cm-30cm in length. Flowers are 1-3cm in display width and 1-2cm corolla height. *W. albomarginata* inhabits alpine and subalpinegrasslands 500m-1800m altitude, often in disturbed sites such as mountain roadside banks. In the course of this study a small red-leaved coriaceous growth form was observed in dry exposed sites, while a longer greener growth form was observed in moist, shelterred sites amongst the leaves of large tussocks and exotic grasses.

Wahlenbergia is a large Campanulaceae genus, comprising 260 species (Lammers 2007), most of which are from the Southern hemisphere (Lammers 1996). *Wahlenbergia* is thought to have diverged in South Africa aproximately 29.6 mya, and dispersed to Australia 4.8 mya (Prebble et al. 2011). New Zealand *Wahlenbergia* most likely radiated after two dispersal events from Australia, approximately 80 million years after New Zealand separated from Gondwana (Prebble et al. 2011, Prebble et al. 2012). Species delimitations are not always clear within New Zealand *Wahlenbergia;* studies of ribosomal ITS and chloroplast trnL-F and trnK-psbA show incomplete resolution, indicating *W. pygmaea* and *W. albomarginata* may be conspecific (Prebble et al. 2012).

While formal study of New Zealand species is more recent, the pollen and style presentation of the Campanulaceae family was among the first discussed as early as the eighteenth centuary by Sprengel and Linnaeus (Lloyd and Yates 1982b). *W. albomarginata* flowers have a two week longevity and are protandrous (Thomson 1881). As with all Campanulaceae, pollen presentation occurs indirectly on hairs on the style before the style branches diverge, exposing the receptive stigmas (Carolin 1960, Shelter 1979), a phenomenon first discussed by Linneaus in 1738 (Shelter 1979). In *W. albomarginata,* this change in gender phase is thought to be triggered by insect visitation (Bischoff 2008).

Protracted flowering can begin as early as November, peak in January and February, and carry on at a reduced rate as late as May (Lloyd and Yates 1982b). Nectar at the base of *W. albomarginata* corollas attracts Syrphid flies, Hallictid and Colletid bees (Lloyd and Yates 1982a), while primarily *Hylaeus* bees but also Syrphid and Tachinid flies visit *W. albomarginata* at The Remarkables Ski Field (Bischoff 2008). Visiting insects remove pollen from the uppermost part of the pollen cylinder at first, with subsequent visitors removing the lower pollen until none remains (Lloyd and Yates 1982a).

Male phase has been reported as four days and female phase two days (Lloyd and Yates 1982a), however, tagged flowers in this study remained in male phase over a five day period, and it is believed flower longevity, and male phase in particular, is prolonged when insect visitation rate is low (Bischoff 2008) . When petals senesce, sepals are retained and the carpels develop into seed capsules approximately 1cm x 0.4 cm. Seed capsules mature in February, March and April. Seed dispersal occurs over short distances by wind and gravity when capsules are damaged.

W. *albomarginata* petal colour ranges from blue to white (Lloyd and Yates 1982a), and is often paler than congeners such as New Zealand native *Wahlenbergia violacea* (Campbell et al. 2012) . Flower colour "blueness" is caused by the anthocyanin group cyanidin (Campbell et al. 2012), and may be a plastic environmental response or heritable trait. Anthocyanins are well known for the blue, purple or red appearance they give to plant parts (Revilla et al. 1998) and are often up-regulated as a response to physiological stress because they function as free radicle scavengers in the shoots and leaves of many plants (Christie et al. 1994, Gould et al. 2009, Gould et al. 2010). However this response has not been investigated in temporary organs such as petals. The evolutionary pressure supporting costly blue petal colour in this white/yellow-flower dominated community remains unclear. While *Hylaeus* is indifferent to flower colour variation between individual *W. albomarginata* flowers, it is more attracted to arrays of thirty blue flowers than thirty white flowers (Campbell et al. 2012), however arrays such as this do not appear to occur frequently in nature.

While some information is available about pollination of *W. albomarginata* at high altitude, where exotic invasion is uncommon (Bischoff 2008), little is known about its pollination network at the middle and bottom of its range, where exotic social bees and the flowers they co-evolved with have recently invaded. Recent exotic invaders at lower altitudes may be beneficial to *W. albomarginata* by attracting pollinators to the area: this is termed facilitation (Moeller 2004). However it can also be detrimental through stigma clogging by heterospecific pollen and interspecific competition for pollinators (Jakobsson et al. 2008, Cariveau and Norton 2009, Muchhala and Thomson 2012).

1.4 Aims

This study aimed to show the affect of diverse environmental conditions on the reproductive success of a Mountain New Zealand herb. This was done by A) Determining whether there was an effect of abiotic environment on investment in floral display and whether this affects reproductive success via seed set and B) Determining the extent to which co-occurring exotic plants and altitude affect pollen deposition in this self- incompatible (Bischoff 2006) native.

A) Floral display was quantified as three floral characteristics: display width, corolla height and 450:550 (blue: yellow) reflectance ratio of petals. This study aimed to demonstrate the degree to which the effect of altitude, soil nitrogen and soil pH on *W. albomarginata* flower size is consistent among locations. This study also aimed to illustrate if A) long-term environmental clines and B) plasticity affect petal "blueness" in *W. albomarginata* by observing the effect of soil nitrogen, soil pH, altitude, flower age and short-term differences in water regime on flower colour. Environmental factors that affected flower size were examined for concomitant effects on allocation to seed weight and number.

B) The second aim was to determine the effect of altitude and surrounding species on pollen deposition to *W. albomarginata* on the Blue Mountains, a location dominated by exotics. This study aimed to determine the effect of density and proximity of four co-occurring species on pollen type and amount delivered to *W. albomarginata* stigmas. The amount and ratio of heterospecific to conspecific pollen deposited on stigmas at the Blue Mountains was compared to the pre-existing knowledge of *W. albomarginata* pollination at a native dominated site on The Remarkables. Differences in seed set and mass between these two locations were tested for to illustrate the affect of invasions on reproductive success in this native species. Finally, this study aimed to illustrate *W. albomarginata's* plant-pollinator network at a lower altitude site than previously described by observing by observing visits by all pollinators to all flowers nearby.

1.5 Thesis Structure

This is the introduction to a six chapter thesis, which shall illustrate the effect of altitude on allocation to floral display, pollen deposition and seed set in *W. albomarginata.*

Chapter two describes the three locations, the Blue Mountains, Pisa Range and Remarkables, which were used for field work and the sites at each location.

Chapter three explores the relationship between abiotic environment and investment in floral display. Specifically, the effect of soil nitrogen, soil pH, soil moisture and altitude on display width, corolla height and petal colour is investigated. Additionally, the degree to which petal colour is plastic is illustrated, both as individual flowers age and in plants with different water regimes.

Chapter four focused on pollination in *W. albomarginata* both through insect visitation and pollen load on stigmas. This chapter describes how the proximity and density of surrounding flowers affect the type and amount of pollen deposited on *W. albomarginata.*

Chapter five describes the affect of altitude and location on seed size and mass. It also tests the consistency of mass allocation to each seed by determining whether seed capsule mass is relative to the number of seeds it contains.

Chapter six integrates and discusses the findings described in chapters three, four and five. The tradeoffs that occur between abiotic environmental constraints and biotic selective pressures for flower size and the consequences of this for reproduction are discussed.

2

Study Locations

"READ THE DIRECTIONS AND DIRECTLY YOU WILL BE DIRECTED IN THE RIGHT DIRECTION"

(CARROLL 1865)

2.1 Background

W. albomarginata occurs in montane to low alpine of New Zealand's South Island and Stewart Island. It tends to inhabit disturbed sites such as roadsides, steep slopes and river banks. In the course of this study it was found in loose gravel, tussock grassland and moist weedy areas. *W. albomarginata* is generally considered to have smaller leaves in drier sites and larger leaves in moist ones. Its affinity with disturbed sites means it is often found near weedy exotic species and some native subalpine species.

Although a small part of the data from this study came from a growth chamber experiment, most of the data came from three Otago locations over the course of two field seasons. These were The Pisa Range, the Blue Mountains and The Remarkables Range. Different locations were selected for different branches of this study. The effect of environment on *W. albomarginata* reproductive features was measured across an altitudinal gradient at three isolated locations. This complements and widens the scope of previous studies on *W. albomarginata* reproduction that took place at The Remarkables Ski Field (Bischoff 2008, Campbell et al. 2012). .

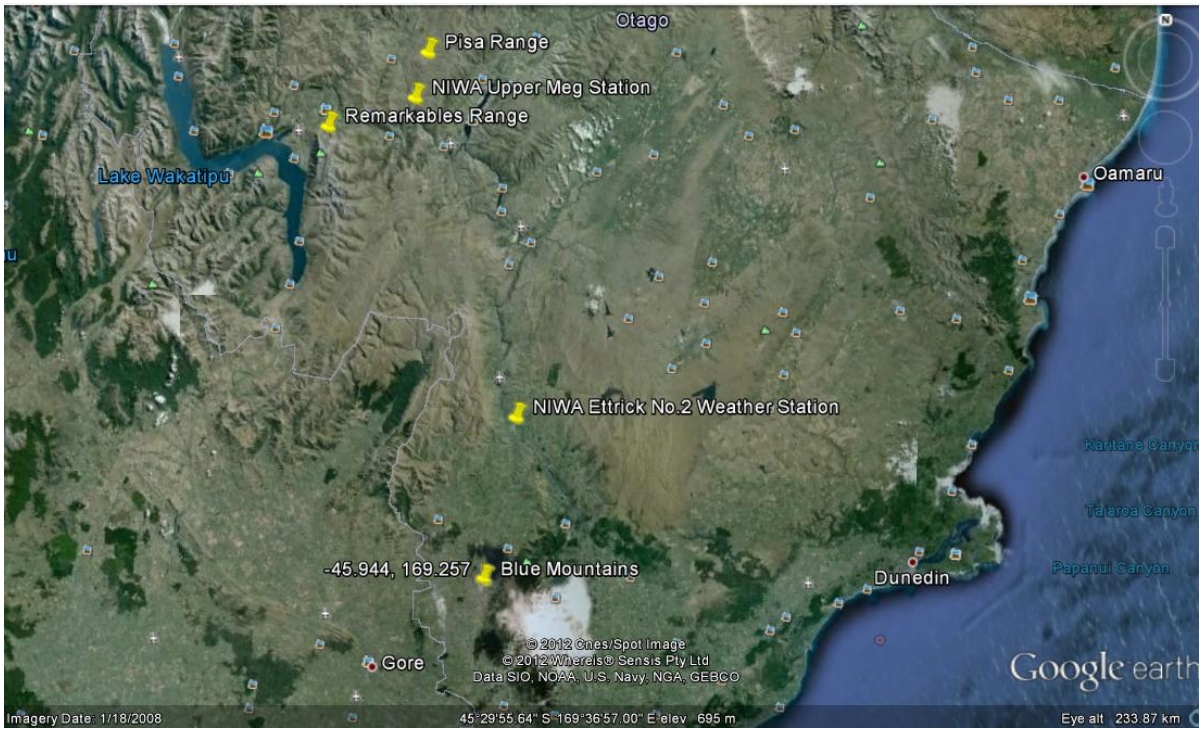


Fig 2.1.1 Map of Otago showing The Blue Mountains, The Remarkables Range, The Pisa Range and nearby NIWA weather stations Ettrick no.2 and Upper Meg.

Otago is one of the driest parts of New Zealand with the hottest summers and the coolest winters. Two National Institute of Water and Atmosphere (NIWA) research station records were used to give an indication of climate at these locations (NIWA 1980-2012):

The NIWA station Upper Meg is at an altitude of 500m and is about 110km North West of The Blue Mountains, 20 km north east of The Remarkables and 10 km south of the Pisa Range. It is a good indicator of rainfall in The Remarkables and The Pisa Range. However, temperature data was not recorded at this station. The Ettrick No. 2 station is 91m above sea level and is about 35km north of the Blue Mountains, 75km south east of The Remarkables and 80km South South East of The Pisa Range. This station's records indicated that the weather was different between Season One (January 2011-March 2011) of this study and Season Two (December 2011 – March 2012). Of particular note, December 2011 was unusually dry.

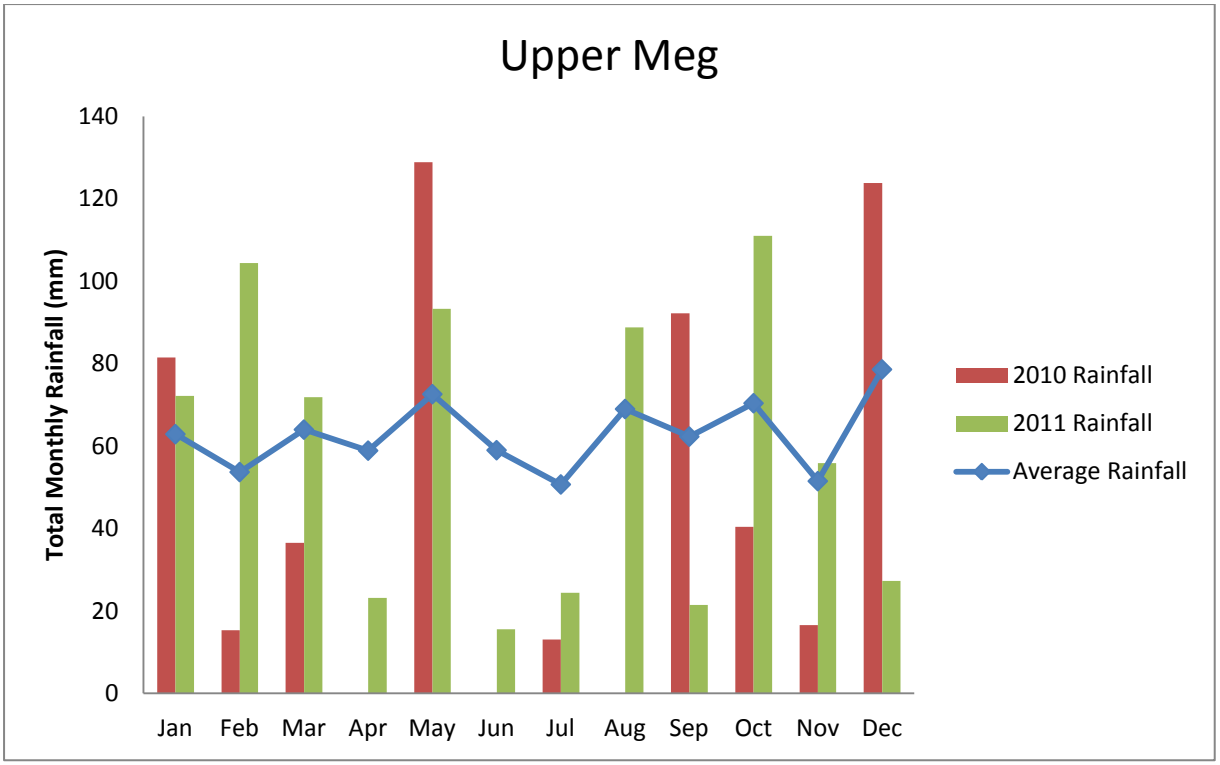


Fig 2.1.2 Total monthly rainfall at Upper Meg station during 2010 and 2011 compared with average over the past ten years.

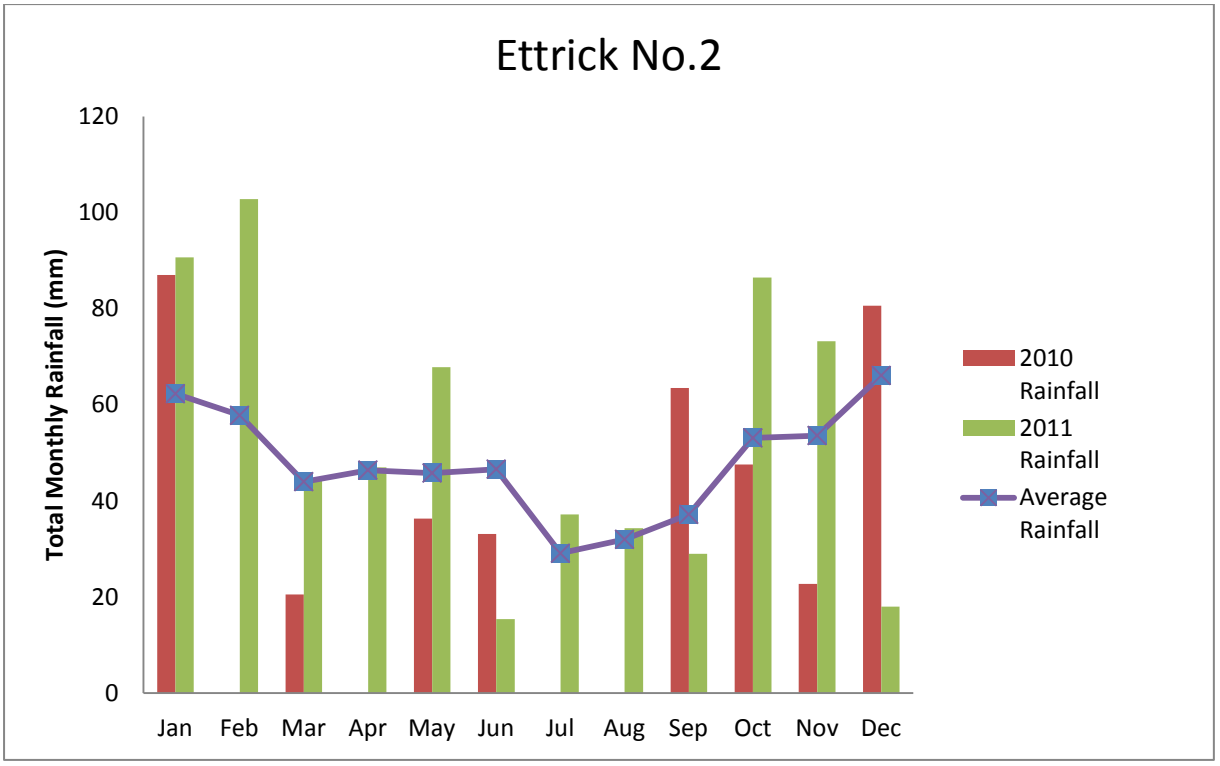


Fig 2.1.3. Total monthly rainfall in at Ettrick No. 2 weather station during 2010 and 2011 compared with average over the past ten years.

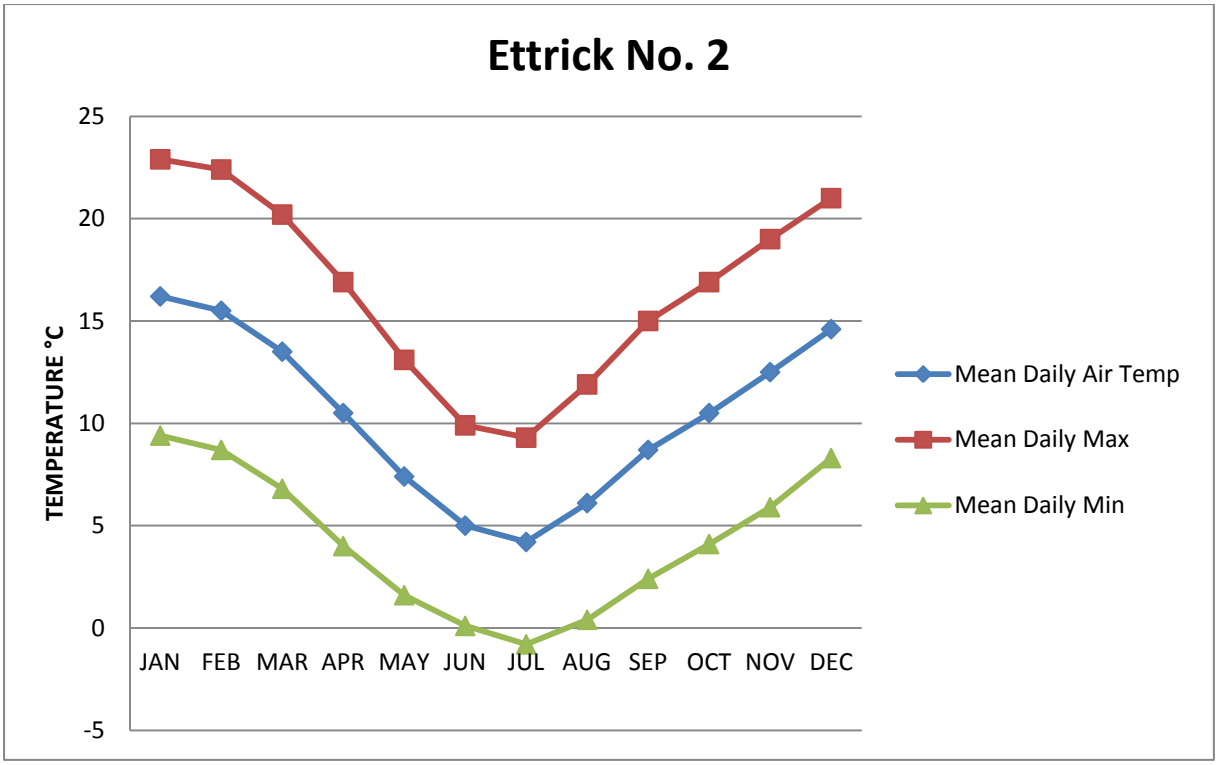


Fig 2.1.4 Mean, max mean and true mean air temperature at Ettrick No. 2 climate station.

2.2 The Blue Mountains

The Blue Mountains are situated between the towns of Lawrence and Tapanui in Otago, New Zealand (45°55'S 169°22'E). On the Blue Mountains there is a *Pinus radiata* D.Don plantation below the tree line and tussock-shrubland above. A raised cushion bog exists near the summit (1000m) containing *Sphagnum cristiatum* Hampe*., Euphraisia dyerii and Raoulia subsericea* Hook. f. . Unlike the other mountain sites in this study, elevation does not increase constantly (See Fig 2). Being the smallest range in this study, The Blue Mountains provided a contrast with other sites for studying the effect of altitude gradients, on *W. albomarginata.* Their proximity to Dunedin meant they were useful for studying the influence of biotic interactions and soil chemistry.

W. albomarginata occurs between 600m-1000m in exposed roadside grassy areas at the Blue Mountains. While *W. albomarginata's* presence in this area has been generally known, no in-depth study of this species has been carried out there before. Co-existing species vary with altitude and include *Hieracium pilosella* L*., Trifolium repens* L*., Lotus corniculatus* L*., Hypochaeris radicata* L*., Aciphylla horrid* R. Forst. & G. Forst. *, Celmisia gracilenta* Hook. f*.,* and *Dracophyllum pearsonii* Kirk.

For this study, all Blue Mountain sites were used for observations in early 2011. The effect of a variety of environmental factors on insect visitation, pollen on stigmas, flower dimensions, flower colour and seed count of *W. albomarginata* were examined at the Blue Mountains (Refer Chapters3-5). The Blue Mountains were revisited in early 2012 and *W. albomarginata* flowers were collected for further examination of flower colour and size.



Fig 2.2.1 *Aciphylla aurea* at the Blue Mountains Dip site, facing north.

2.2.1 Blue Mountains Sites

Five key sites were used to examine *W. albomarginata leaves, flowers and stigma pollen loads* in this study (see Fig 2.2.1.1 below)

- The Top Site (45°53’01.94”S, 169°23’36.23”E, 1001)
- The Dip Site (45°52’22.70”S, 169°24’38.89”E, 800m)
- The Bank Site (45°52’02.87”S, 169°24’40.12”, 907m)
- The Bottom Site. (45°52’01.94”S 169°25’43.08”E, 650m).
- The Tower Site (45°52'30.50"S 169°25'07.18"E, 913m).

The Top Site (45°53’01.94”S, 169°23’36.23”E, 1001m)

The Top Site was a flat dry roadside site, partially shaded by a west facing bank. The dominant species at this site included *Veronica* sp.*, Raoulia* sp.*, Poa colensoi, Hieracium pilosella, Hypochaeris radicata* and *Trifolium repens*.

Methodology: This site was visited frequently over two field seasons. Flower and leaf dimensions, flower colour and some pollinator observations were made at this site. Stigmas from flowers at this site were excised pollen loads scored for analysis in relation to altitude and nearby flowering species (Detailed in Chapter Four). The roadside from this site to the Dip site was used to look at the effect of *W. albomarginata* patch size and density on capsule mass and the number of seeds per capsule.

The Dip Site (45°52’22.70”S, 169°24’38.89”E, 800m)

The Dip site was a moderately moist saddle between the Top site and the Bank site. It is somewhat exposed, with an adjacent West facing bank providing some shelter in parts. This site has a slope of 0-10°. Dominant species include *Lotus corniculatus, Trifolium repens, Hypochaeris radicata, Phormium tennax, Poa colensoi, Agrostis capilaris* L. and *Euphrasia* sp.

Methodology: This site was visited frequently over two field seasons. Flower and leaf dimensions, flower colour and some pollinator observations were taken at this site. Stigmas from flowers at this site were excised and examined for pollen type for later analysis in relation to altitude and nearby flowering species.

The Bank Site (45°52'02.87"S, 169°24'40.12", 907m)

This site was a fairly sheltered west-facing roadside. It has an elevation of 900m above sea level. Dominant species include *Hieracium pilosella, Hypochaeris radicata, Lotus corniculatus and Trifolium repens.*

Methodology- This site was used in Season One for floral measurement and stigma excision.

The Bank site was omitted from soil manipulation experiments in late 2011 and early 2012 because of its steep gradient.

The Bottom Site. (45°52'01.94"S 169°25'43.08"E, 650m).

This site was selected because no suitable site existed at 700m. This site was sheltered by shrubland, a bank to the north and the mountains to the south. It is a dry bank of 0-20 degrees east facing. This site is populated by *Dracophyllum sp., Phormium tennax*, *Hieracium pilosella*, H*ypochaeris radicata*, *Lotus corniculatus and Anthoxanthum odoratum.*

Methodology: This site was used over two field seasons. Flower and leaf dimensions, flower colour and pollinator observations were made at this site. Stigmas from flowers were excised and examined for pollen type for analysis in relation to altitude and nearby flowering species. The roadside between this site and the Dip site was used to look at the effect of *W. albomarginata* patch size and density on capsule mass and the numbers of seeds per capsule.

The Tower Site (45°52'30.50"S 169°25'07.18"E, 913m).

This site was flat, elevated and reasonably moist. There is a small telecommunications tower structure at this site, and plants are subject to disturbance from occasional cars. Prominent species were *Aciphylla, Trifolium repens, Hieracium pilosella,* and *Raoulia subsericea*. *W. albomarginata* flowers here appeared to be much bluer than the other Blue Mountains sites. This site was used for pollinator observations.



Fig 2.2.1.1 Google Map showing Blue Mountains locations Top, Dip, Bank, Tower and Bottom Sites

2.3 The Remarkables Range

The Remarkables (45°09'S 168°50'E) is a range of glaciated schist mountains situated south east of Lake Wakatipu. Farm land exists at low altitudes and there is a ski field at an elevation of 1600m-1800m. The habitat ranges from wetland to loose gravel. This area is subject to more disturbance from humans than the other two locations because there is not gated access and there has been extensive maintenance of the ski field area undertaken in recent summers. The Remarkables have been the primary site for all recent work on *W. albomarginata* breeding systems (Bischoff 2008, Dobbie 2009). *W. albomarginata* occurs throughout the area from the farmland (800m) to just below Lake Alta (1750m). During this study it was found in dry grassland, roadside gravel and occasionally on boggy banks. Three native taxa: primarily *Hylaeus* bees but also Syrphid and Tachinid flies have been identified as pollinators of *W. albomarginata* at high altitude here (Bischoff 2008).

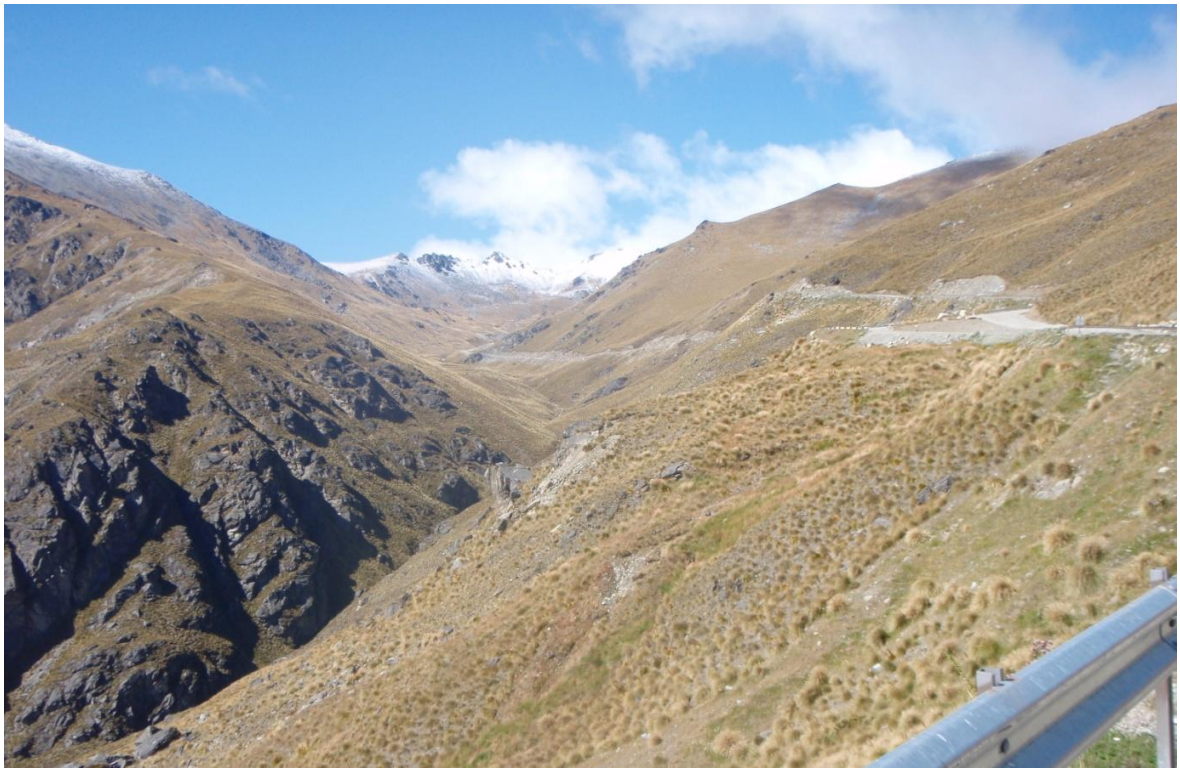


Figure 2.3.1 The Remarkables facing east looking towards Ski Lodge from Two Rocks

2.3.1 Remarkables sites

The bulk of the work for this study at The Remarkables was intended to be carried out at four sites:

- Ski lodge,
- Two Rocks,
- The Bend and
- The Radio Shack.

Eight study sites were used in March 2011 to look at the effect of altitude on flower dimensions and flower colour. Roadside sites were picked at the following altitudes.

- 1774m,
- 1633m (Ski lodge),
- 1488m,
- 1329m,
- 1245m,
- 1165m,
- 1025m,
- 922m.

The Ski Lodge (45°52'02.87"S, 169°24'40.12"E, 907m)

This was a west-facing tussock bank next to The Remarkables Ski Field Lodge. Dominant genera include *Donatia* J.R Forst & G. Forst*, Dracophyllum* Labill*., Celmisia* Cass. and *Chionochloa* Zotov. At this site, *W. albomarginata* occurred either in a small, leathery growth form amongst gravel on banks, or in a larger growth form sheltered by tussocks.

Methodology: Flowers were collected and their dimensions and colour were measured.

Two Rocks (168 48.042 S45"E, 1416 02.000"S,)

This was a dry gravelly area beneath a small roadside bank with a soil pH of 5.5. The area is dry, with loose rocks and an abundance of Nitrogen fixers. Common species at this site included *Trifolium repens, Lotus corniculatus* and *Aciphylla aurea, Poa colensoi* and *Hypochaeris radicata*.

Methodology: Flowers were collected for dimensions and colour. Rosettes and corollas were collected for anthocyanins.

The Bend (S45 01.677, E168 48.171, 1148m).

This is a north east facing 30° sloping bank with moist patches and dry pebbly patches densely populated by *Chionochloa rigida* and *Aciphylla aurea*. Common plant species also included *Lotus corniculatus, Trifolium repens, Hieracium pilosella*, *Hypochaeris radicata*, and *Agrostis capilaris.*

Methodology: Flowers were collected for dimensions and colour. Rosettes and corollas were collected for anthocyanins.

The Radio Shack (45°01'39.69"S, 168°46'54.56"E, 942m).

Common genera at this site included *Agrostis, Festuca* L.*, Raoulia* and *Aciphylla. W. albomarginata* was present at this site. It was intended to be used as a site for floral colour and dimensions in field Season Two, however flowering did not occur. This may be attributed to the unusually dry December prior to the 2012 flowering season.

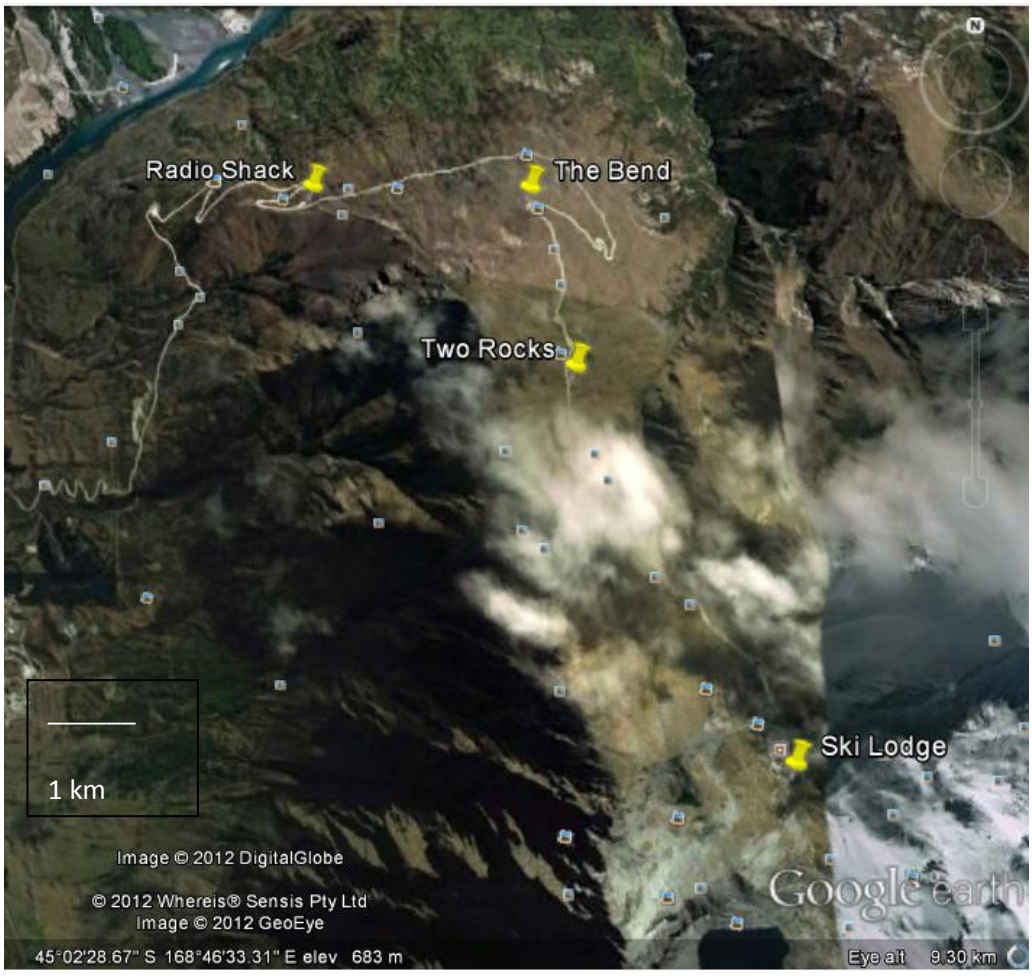


Fig. 2.3.1.1 Remarkables Range field sites Ski Lodge, Two Rocks, The Bend and Radio Shack sites.

2.4 The Pisa Range

The Pisa Range (44°53’41”S, 169°05’29”E) is situated thirty kilometres north of The Remarkables. Road access was from Cadrona. On the lower parts of the range there are extensively grazed sheep and cow farms. The Snow Farm ski field base is at 1500m altitude. On the Pisa Range *W. albomarginata’s* roadside habitat is dry, with loose rocks and the habitat is populated by many exotic weedy species such as thistle, *Digitalis purpurea* and *Hypochaeris radicata*. Beyond the snow farm is gated conservation wetland and tussock grassland frequented by trampers. The rock type is chlorite schist. In this study, sites on this Range were used in 2012 to examine floral characteristics at different altitudes.

W. albomarginata has not been studied at the Pisa Range before. However it is generally known that it inhabits this area. The highest it was observed during this study was at 1700m, and the lowest was at 886m. It was found in a number of different habitats including exposed dry, extensively farmed paddocks, road side gravel banks, elevated rocky terrain and moist, sheltered banks bordering wetlands.

Methodology: Flowers and rosettes were collected from all Pisa sites. Flowers were measured for dimensions, blueness and anthocyanin content. Rosettes were measured for leaf length and anthocyanin content.

2.4.1 Pisa Range Sites

The sites at the Pisa Range were:

- Tom's Creek,
- Possum,
- Aciphylla,
- Snow Farm, and
- Euphrasia Island.

Euphrasia Island (S44°54'14.20" E169°9'37.94", 1705m).

This site was a west facing 20° sloped stream bank on the very edge of a cushion wetland near The Kirtleburn Hut. The dominant species at this site were *W. albomarginata, Chionochloa macra, Raoulia subsericea,* and *Hieracium pilosella.*

Snow Farm (S44°52,792 E169°03,144 1550m).

This site is exposed, flat and dry with 30% gravel and 5% rock. The dominant species at this site were *Chionochloa macra, Poa colensoi, Raoulia grandifolia, Raoulia subsericea,* and *Pilosella officinalis*.

Aciphylla (S44°52,284 E169°04,435, 1400m).

This site is on a 20**°** north-facing slope with 5% gravel and 5% rock. The dominant species at this site were *Trifolium* sp.*, Anthoxanthum odoratum, Hieracium pilosella,* and *Aciphylla*.

Possum Bourne (S44°52'142" E169°03'144" 1150m).

This site is on a 15**°** North West facing slope with 10% slope. The dominant species at this site were *Anthoxanthum odoratum, Raoulia subsicaera, Aciphylla, Hieracium pilosella, Agrostis capilaris,* native broom *and Festuca novae-zelandiae.*

Tom's Creek (S44°55,044, E169°03,144, 886m).

This site is a 10° west-facing slope. The dominant species at this site were *Lupinis arboreus* L.*, Agrostis capilaris, Anthoxanthum odoratum, Hieracium pilosella, Hypochaeris radicata, Crepis capilaris* L.*, Trifolium repens* L.*, Discaria toumato* Raoulia subsericea.*, Festuca* sp.*, Poa colensoi* and *Aciphylla horrida* W. R. B. Oliv.

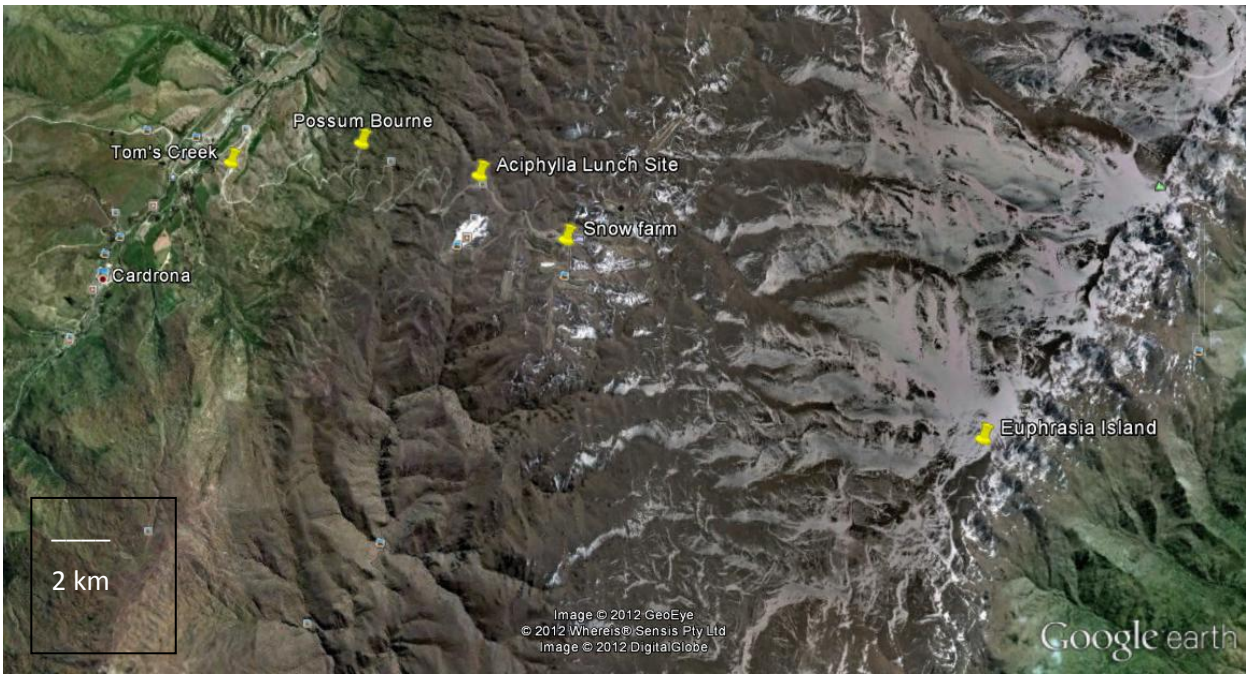


Fig 2.4.2.1 Google Map of Pisa Range Sites showing Tom's Creek, Possum Bourne, Aciphylla, Snow Farm and Euphrasia Island sites

3

Effect of Abiotic Environment on Investment in Floral Display

"AND CONTRARY-WISE WHAT IT IS IT WOULDN'T BE,
AND WHAT IT WOULDN'T BE IT WOULD. YOU SEE?"

(CARROLL 1865)

3.1 Introduction

Flower size and colour can greatly influence reproductive success within species as these traits are not only heritable and subject to long-term selective pressure, but are also subject to selection via short-term variation in environmental factors (Delgado et al. 2010). Flowers are subject to conflicting selection to maximise pollinator visitation and efficiency but minimise resource expenditure(Gadgil and Bossert 1970). Resource allocation to various floral characteristics including colour and size differs across the angiosperms and in many cases has been shown to vary with pollinator and habitat (Armbruster et al. 2004). Many plant species exhibit strong plasticity in floral characteristics (Galen 1999, Quinn and Wetherington 2002, Caño et al. 2008, Franks 2011). Previous studies have noted *W. albomarginata* appears to vary in size and colour (Bischoff 2008, Campbell et al. 2012), yet the contribution of environmental factors to this variation and its consequences for reproductive outcomes remain a mystery.

Plant reproductive characteristics are known to respond to environmental stressors. Temperature affects timing of flowering as well as flower size, mass and number in *W. albomarginata's* co-familial *Campanula carpatica* (Niu et al. 2001). Altitude is generally associated with a decrease in size of vegetative parts and decreased growth rate in order to maximise nitrogen use efficiency (Zhang et al. 2012), and flower mass is greater relative to leaf mass in a variety of European taxa at higher altitudes (Körner 2004).

Soil chemistry is also known to influence flower size. Lavender flower characteristics are known to be influenced by temperature and altitude as well as soil pH (Delgado et al. 2010). Short-term nitrogen deprivation improves flower production in *Ranunculus* sp. (Bernstein et al. 2011) where as low soil moisture favours the production of smaller sized *Polemonium viscosum* flowers (Galen 1999).

W. albomarginata flowers are generally known to range in colour from white to pale blue, with flowers of different shades occurring on the same plant, as well as on different plants. There is a weak relationship between petal colour and pollinator visitation, with *Hylaeus* bees preferring large groups of painted blue flowers to large or small groups of flowers painted white, however they are indifferent to natural flower colour variation among individual plants (Campbell et al. 2012). So, it is not clear if flower colour variation in *W. albomarginata* relates to pollinator attraction. The possibility of environmental factors other than selective pressure from pollinators influencing flower colour is understudied in plants and hitherto unexplored in *W. albomarginata*.

The blue pigment of *W. albomarginata* petals is due to the presence of anthocyanin; a flavinoid with a functional anthocyanidin structure known as cyanidin (Campbell et al. 2012). Anthocyanins are not

only red / blue / purple pigments in flowers, but are also produced as free-radical scavengers in leaves in response to stress (Gould et al. 2010). The depth and shade of petal colour can be affected by the amount of anthocyanin present and the functional anthocyanidin group as well as a variety of co-pigments and vacuolar pH (Markham et al. 2000, Sasaki and Ohmiya 2008). Anthocyanidins are redder at pH<3, colourless at pH3-6 and bluer at ph >6 (Sasaki and Ohmiya 2008).

Many physiological stressors are known to trigger phenolic expression in plants (Gould et al. 2000, Gould et al. 2010). *W. albomarginata* petal blueness may be caused by anthocyanin concentration, and regulated as part of plastic responses to physiological stress. It is possible that elevated anthocyanin levels in *W. albomarginata* leaves coincide with elevated levels in the pedicels and petals, causing physiologically stressed *W. albomarginata* plants to produce bluer flowers. A similar phenomenon has been documented in horticultural varieties but not naturally occurring plants. Short-term nitrogen deprivation, low temperature and low soil moisture are known to increase tomato fruit and leaf anthocyanin concentration, however the precise mechanisms for this are poorly understood (Reid 2001, Hatier and Gould 2008, Olsen et al. 2010, Olsen et al. 2012).
Altitudinal gradients are useful for studying ecological variation due to long-term processes associated with change in temperature (Fukami and Wardle 2005, Primack et al. 2009); however, short-term adaptations to environment, otherwise known as plastic responses, can be equally as important in determining plant phenotype.

An additional possible explanation for variation in *W. albomarginata* flower colour and size is flower age. Colour change in flowers was first observed some 220 years ago (Sprengel 1793), and has since been noted in many taxa (Eisikowitch and Rotem 1987, Weiss 1991). *W. albomarginata* flowers switch from male gender phase to female after insect visitation (Bischoff 2008) so it is important to distinguish between the effect of flower aging and flower gender when measuring petal colour change over time.

This study aimed to test the effect of age, altitude, temperature, soil moisture and soil nitrogen on *W. albomarginata* flower size and colour. It was planned that field observations within and between geographic locations combined with short-term growth chamber manipulations would elucidate whether responses to environment were caused by plasticity or natural selection.

3.2 Effect of Altitude on Floral Allocation

3.2.1 Methods

Locations

The effect of altitude on flower characteristics was measured in the Blue Mountains and Remarkables Range in 2011 and the Pisa Range in 2012 (detailed in Chapter Two). The Remarkables sites ranged in altitude from 922m to 1774m. The Blue Mountains sites ranged in altitude from 650m to 1000m and were the Top Site, Dip Site, Bank Site and Bottom Site. The Pisa sites ranged in altitude from 886m to 1550m and were the Euphrasia Island, DOC site, Snow Farm, Aciphylla, Possum and Tom's Creek.

Collection and Measurements

Whole flowering rosettes were collected complete with pedicel and flower to prolong longevity after collection. All rosettes were stored in 100mL plastic tubes with damp cotton wool and placed in coolers for no more than twenty four hours before measurements were taken. All floral dimensions were measured with a digital Vernier Calliper to an accuracy of 0.1mm and flower colour was measured using an Ocean Optics USB4000 UV-Vis reflectance spectrometer with a PX-2 pulsed Xenon light source and an RS-200-7-Vis fibre optic probe. Reflectance was standardised against a white reflectance standard (Ocean Optics WS-1-SL). Flower collection methodology was adjusted slightly between the three locations to suit the size and shape of *W. albomarginata* distributions at different sites.

At The Remarkables, 15 male phase rosettes were selected in groups of five using a random number sampling plan for three 7m x 2m areas. The rosettes were stored in upright plastic containers on ice for up to one hour before being measured. This collection was carried out in March 2011. At the Pisa Range, 12 rosettes with male flowers were collected in random groups of four by walking in a straight line and collecting one flower every seven paces. Flowers were stored for up to four hours then measured. This collection was carried out in January 2012. At the Blue Mountains, nine rosettes from each gender phase were collected using random co-ordinates within a 20m x 4m area at four sites. Flowers were measured within sixty minutes of collection. These were collected in January 2011. A second batch of flowers were collected from the same sites in the same manner in February 2011 and measured for flower colour after 12 hours. Gender phase was recorded and female phase flowers were not used in analyses because their longevity after being picked was shorter than for male phase flowers, and in order to keep measurements consistent. Only flowers in

the male phase were collected at The Remarkables and the Pisa Range because of their longevity, and measuring taking hours.

The floral characteristics measured varied slightly among the three locations (see Table 3.2.1.1). At The Pisa Range, corolla tube height, display width, pedicel length, the length of the longest leaf per rosette, the number of leaves per rosette and petal colour were measured. The ratio between flower diameter and leaf length was used as an indicator of resource allocation to flowers at The Pisa Range. Pedicel length, leaves per rosette and leaf length were omitted at The Remarkables and The Blue Mountains measurements. Corolla tube height was measured from the base to the tip of base of the corolla lobes to the point where the sepals fuse. Display width was the distance between the corolla lobe tips when flat against a surface. Flower colour was measured by placing a single petal on a dark background and measured as detailed above. The ratio between blue reflectance (450nm) and yellow reflectance (550nm) was then calculated to give flower "blueness" following Campbell et al. (2012).

Site	Corolla tube height	Display Width	Pedicel Length	Leaf Length	Petal Colour	Gender Phase
Blue Mountains	Yes	Yes	No	No	No	Female and Male
Remarkables Range	Yes	Yes	No	No	Yes	Male only
Pisa Range	Yes	Yes	Yes	Yes	Yes	Male only

Table 3.2.1.1 Floral characteristics recorded at the Blue Mountains, Remarkables and Pisa Range.

Analysis

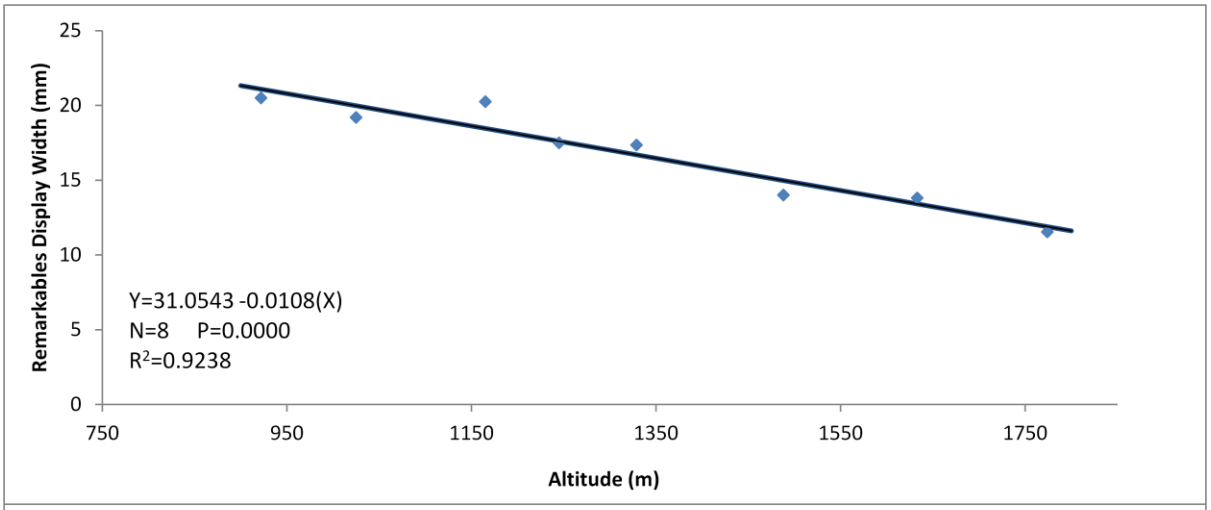
Analyses were conducted to test the difference in floral characteristics between sites, and the extent to which these differences could be explained by the difference in altitude. Statistical tests were performed using the program Statistix 9 (Analytical Software 2008).

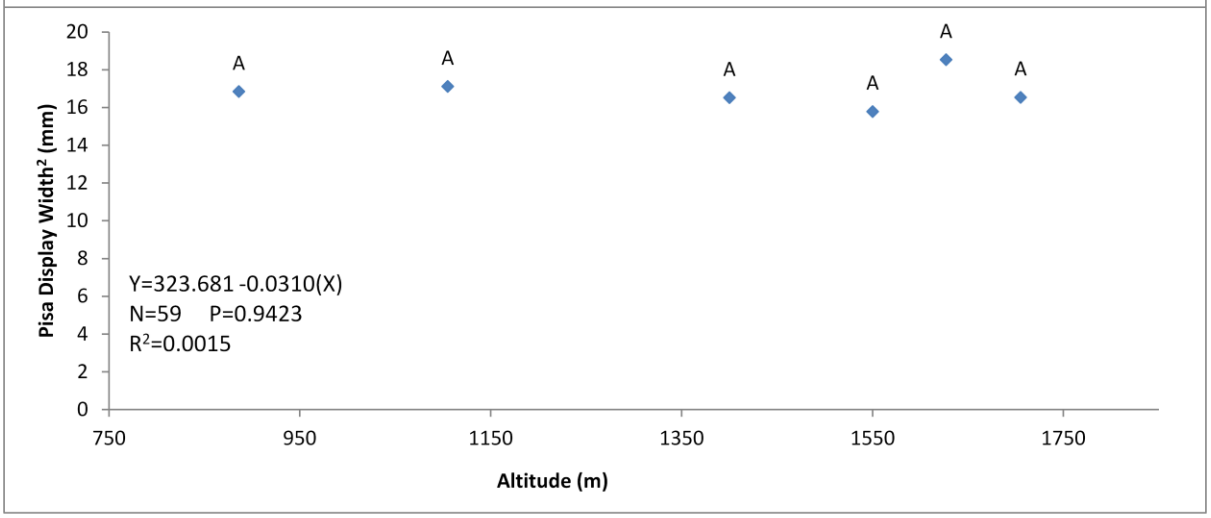
Data sets were tested for normality with the Shapiro-Wilk test, then log or square root transformed if they were not normal. Analysis of variance was used to determine whether there was a significant difference in flower dimensions or colour between the three locations. Analysis of variance was also used to analyse the difference in flower and vegetative characteristics between sites in each location. Linear regressions were used to analyse the effect of altitude on flower dimensions and colour at the three locations. The effect of altitude on leaf length and the ratio of display width to leaf length at The Pisa Range was also analysed using regressions.

3.2.2 Effect of Altitude on Floral Allocation: Results

Within The Pisa Range, significant variation in some characteristics between sites could not always be explained by altitude. There was no significant difference in display width between sites at The Pisa Range, however site display width did vary among sites at The Remarkables and Blue Mountains (Table 3.2.2.1). Altitude did not affect display width or corolla height at The Blue Mountains and Pisa Range (Figs 3.2.2.1 & 3.2.2.2). Display width and corolla height were both affected by altitude at The Remarkables (Figs 3.2.2.1 & 3.2.2.2). The corolla height to leaf length ratio significantly differed between the DOC site (second highest altitude) and the two bottom sites: Possum and Tom's Creek at the Pisa Range (Fig 3.2.2.5). The effect of altitude on the ratio corolla height to leaf length was narrowly non-significant (P=0.0932). Leaves per rosette significantly differed between the Aciphylla and *Euphrasia* Island sites at the Pisa Range, but there was no effect of altitude (Fig 3.2.2.4). Pedicel height differed significantly between a number of sites at the Pisa Range, and was significantly affected by altitude. Finally, leaf length significantly differed between most Pisa Range sites and was affected by altitude (Fig3.2.2.3).

There was a significant difference in corolla height and display width between the four Blue Mountains sites; however, this could not be explained by increasing altitude (Figs 3.2.2.1 & 3.2.2.2). There was a significant difference in size between the locations with The Remarkables having significantly smaller corolla height and pedicel length than the Pisa Range and Blue Mountains. While there was a marginal overall significant difference in display width between the three locations, this could not be accounted for by Tukey's test. The Remarkables flowers were also significantly whiter than the other two locations. The leaf length did not differ between the Blue Mountains and The Pisa Range and was not measured at The Remarkables Range.





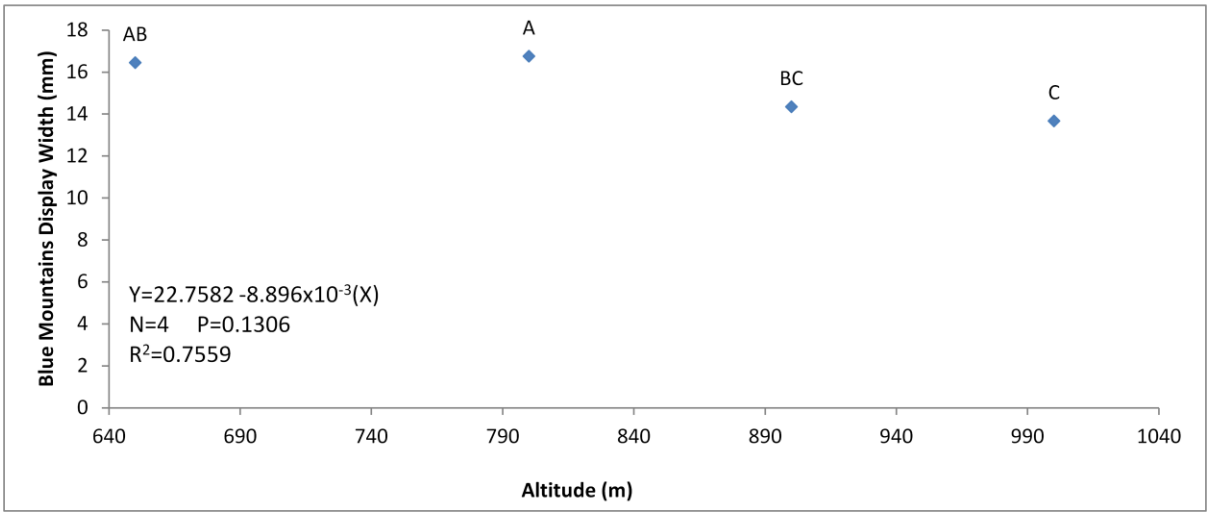
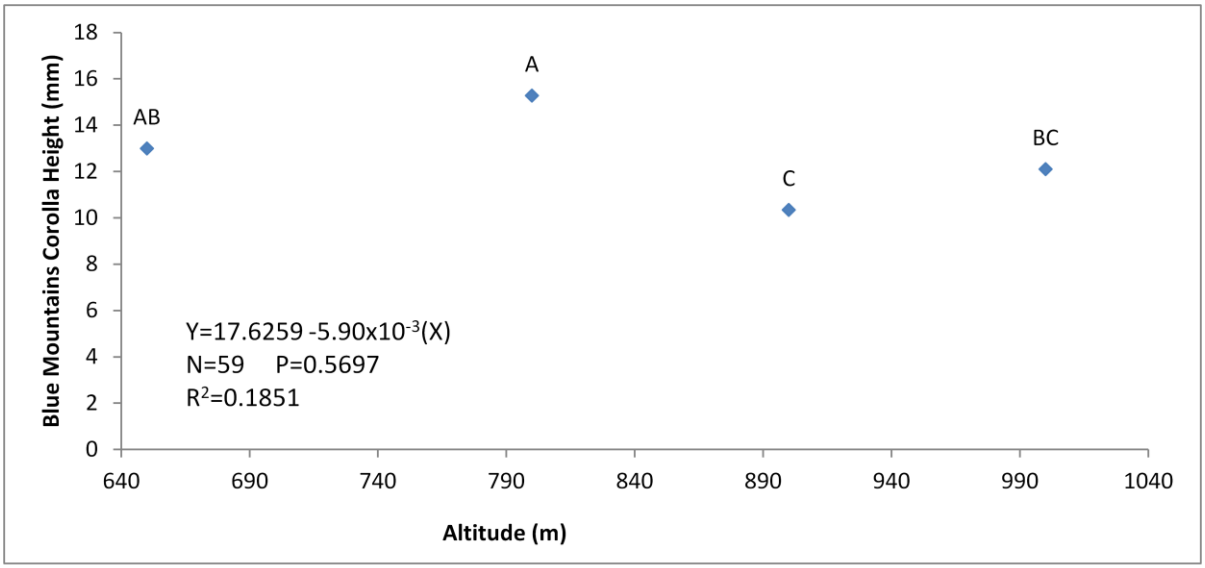
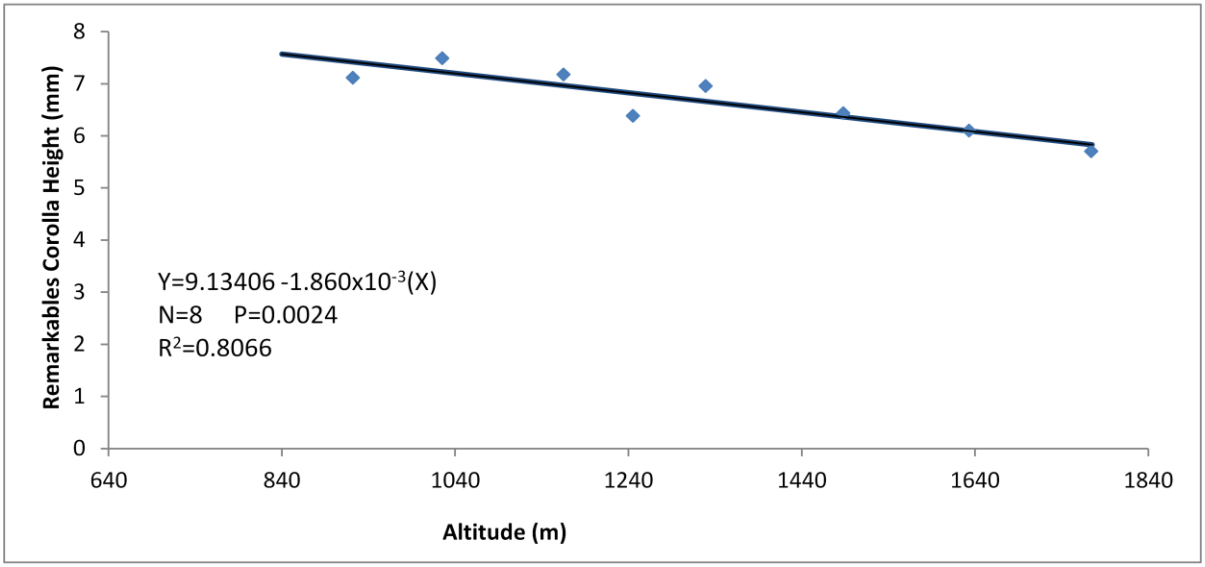


Fig 3.2.2.1 Mean Display Width at each site at The Remarkables, Pisa Range and Blue Mountains. Data labels (A,B,C) indicate groups from Tukey's test result where the linear regression was not significant. Least squares linear regression equations show the effect of altitude on display width (if P is less than 0.05, the result is significant, and a trend line is included).

Table 3.2.2.1 ANOVA for differences in display width between sites at each location

Difference in Display width between Sites	DF	SS	F	P
Blue Mountains Sites	3	70.004	5.87	0.0023
Error	36	143.169		
Total	39	213.173		
Remarkables Sites	7	1110.80	20.32	0.0000
Error	115	898.02		
Total	122	2008.82		
Pisa Sites	5	50.241	1.30	0.2742
Error	66	509.648		
Total	71	559.888		





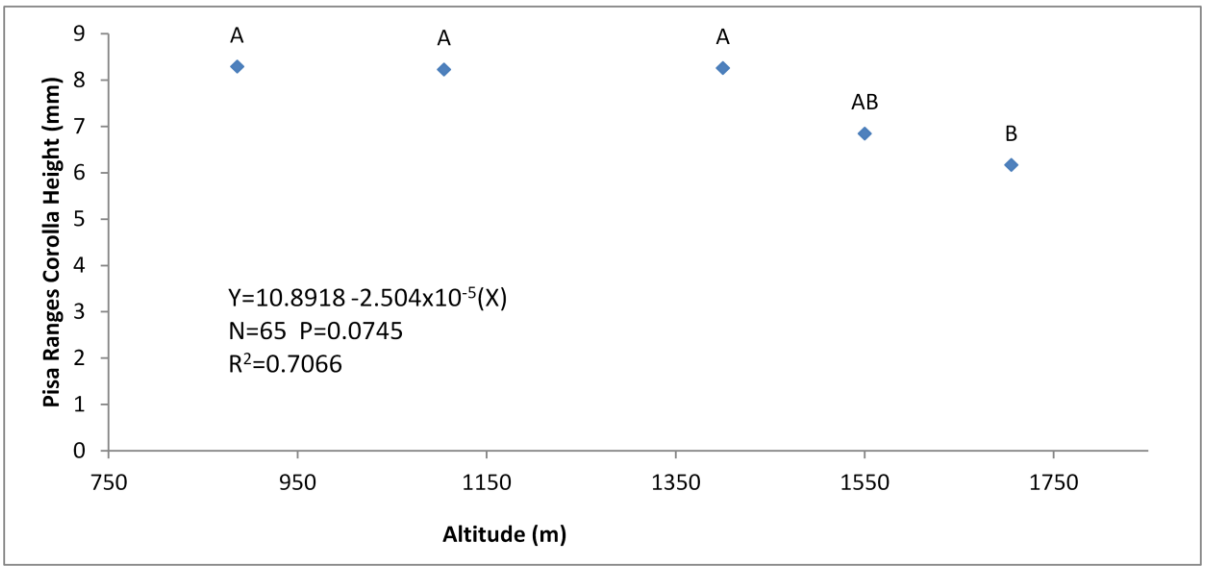


Fig 3.2.2.2 Mean Corolla Height at each site at the Blue Mountains, The Remarkables, and the Pisa Range . Data labels indicate Tukey's test result. Least squares linear regression equations show the effect of altitude on corolla height.

Table 3.2.2.2 ANOVA for differences in corolla height between sites at each location.

Difference in Corolla Height between Sites	**DF**	**SS**	**F**	**P**
Pisa Sites	**5**	**47.024**	**4.36**	**0.0039**
Error	**66**	**148.302**		
Total	**71**	**195.326**		
Remarkables Sites	7	73.763	13.43	0.0000
Error	115	90.265		
Total	122	164.028		
Blue Mountains Sites	3	125.928	10.58	0.0000
Error	36	142.839		
Total	39	268.767		

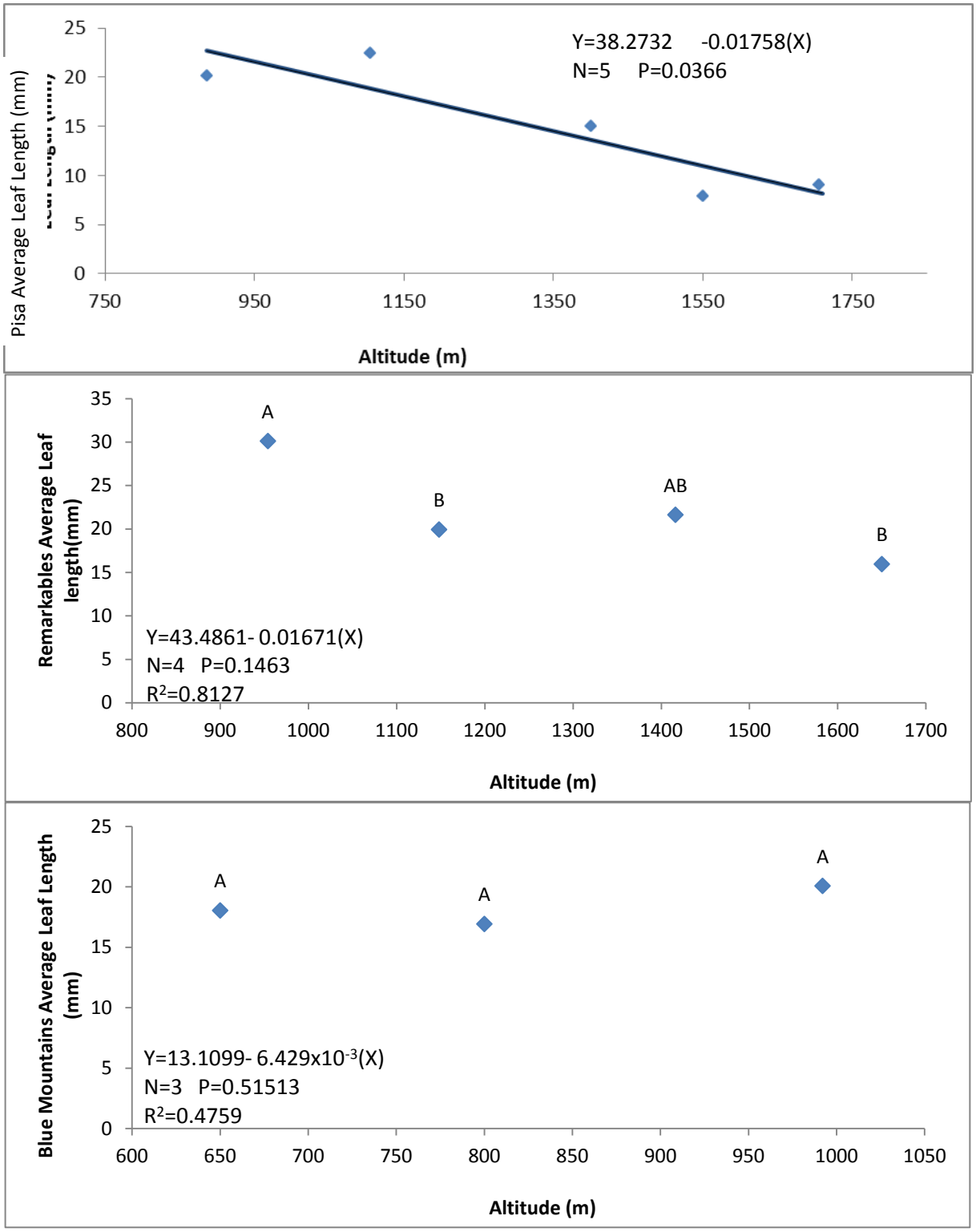


Fig 3.2.2.3 Mean length of longest leaf at each site at Pisa Range, The Remarkables and Blue Mountains. Data labels indicate Tukey's test result. Least squares linear regression equations show the effect of altitude on average leaf length.

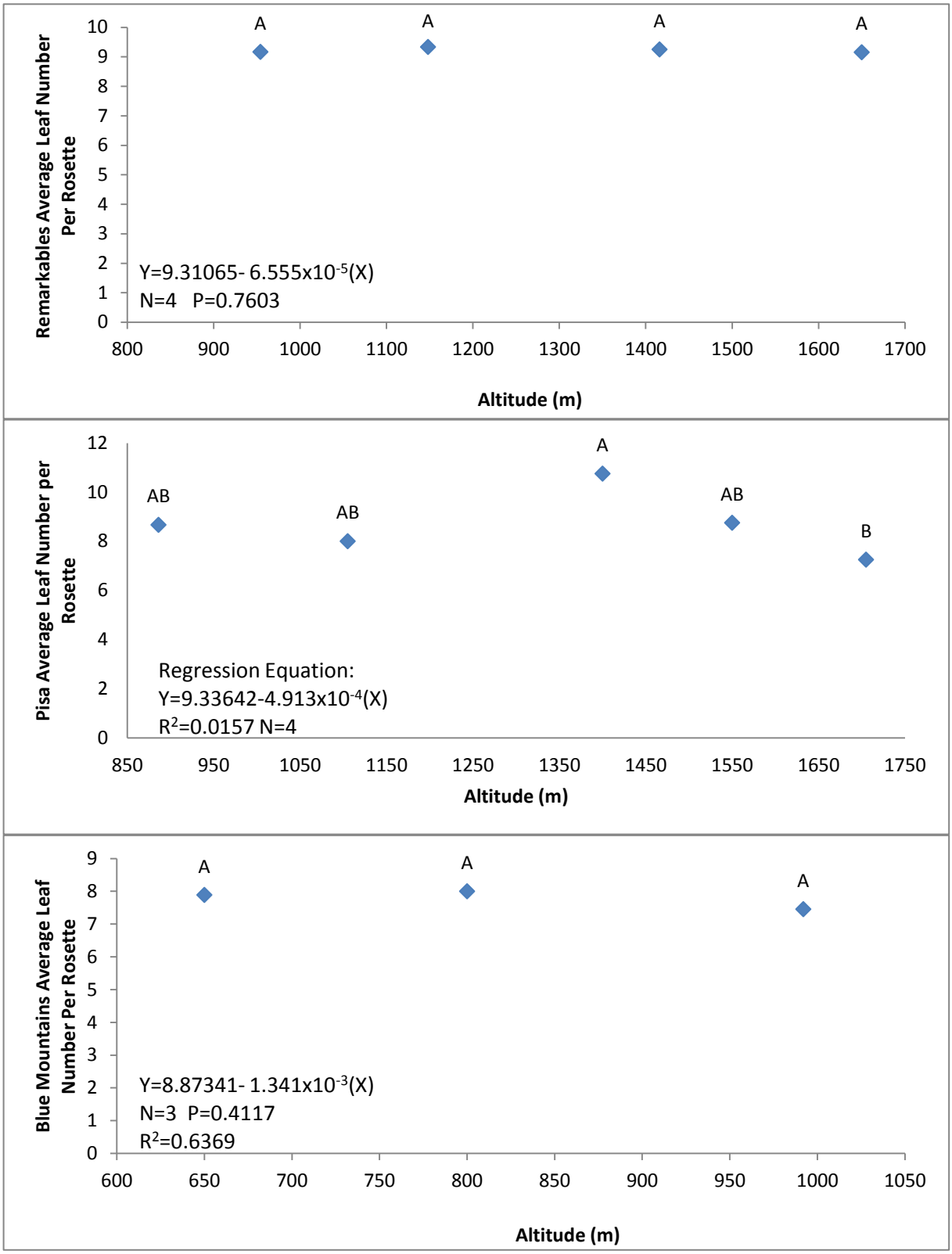


Fig3.2.2.4. Mean leaves per rosette at each site at The Remarkables, Pisa Range and Blue Mountains. Data labels indicate Tukey's test result. Least squares linear regressions equations show the effect of altitude on leaves per rosette.

Table 3.2.2.3 ANOVA for the differences in leaf number per rosette and length of the longest leaf between sites.

Variable		DF	SS	F	P
Pisa Leaf Number	Altitude	5	84.776	2.53	0.0384
	Error	58	38.083		
	Total	63	472.859		
Remarkables Leaf Number	Altitude	3	0.255	0.01	0.9979
	Error	45	292.276		
	Total	48	292.531		
Blue Mountains Leaf Number	Altitude	2	1.751	0.24	0.7904
	Error	27	99.616		
	Total	29	101.367		
Pisa Leaf Length	Altitude	4	2016.66	13.18	0.0000
	Error	55	2104.47		
	Total	59	4121.14		
Remarkables Leaf Length (mm)	Altitude	3	1315.93	6.09	0.0014
	Error	45	3240.60		
	Total	48	4556.53		
Blue Mountains Leaf Length (mm)	Altitude	2	53.80	0.68	0.5140
	Error	27	1064.80		
	Total	29	1118.60		

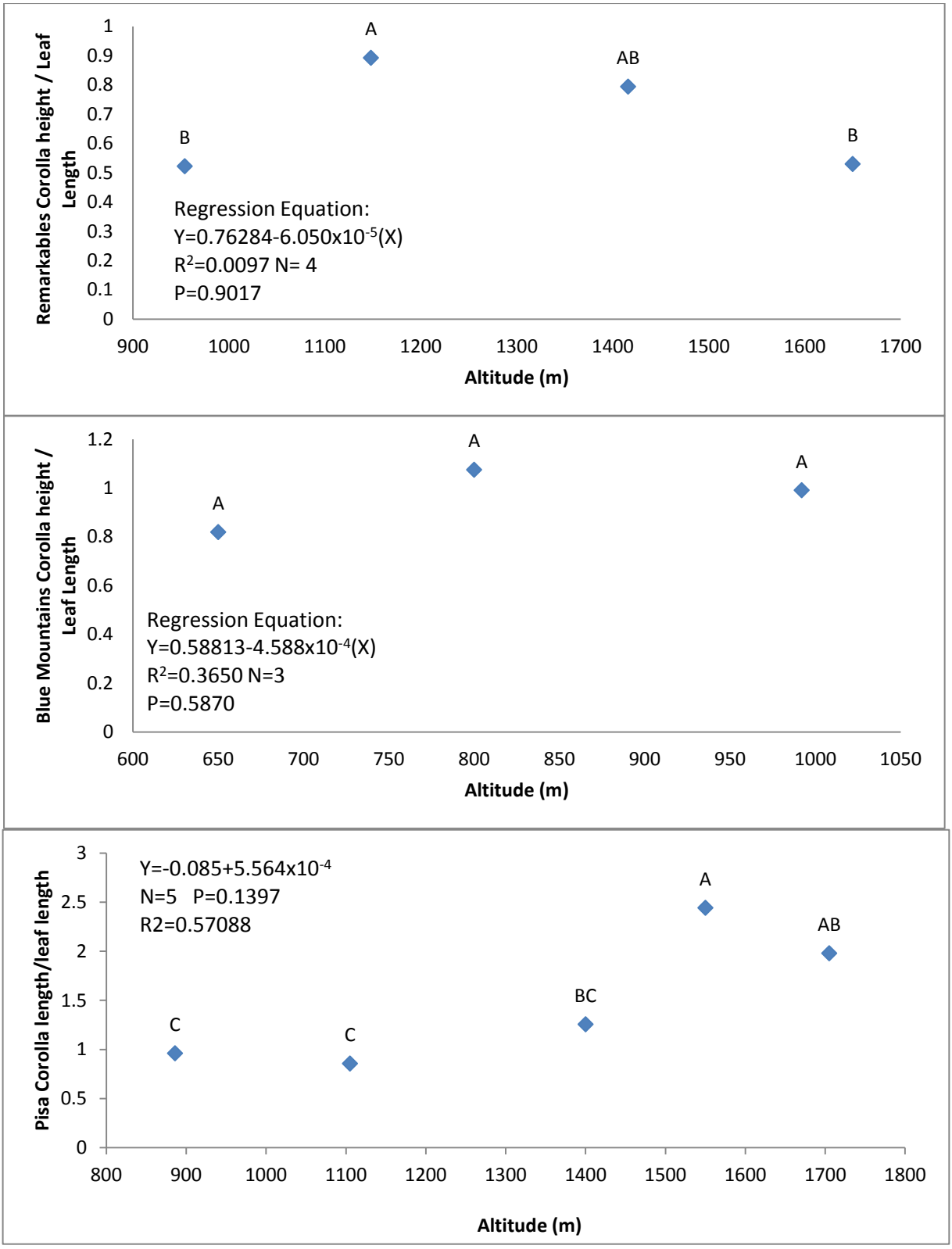


Fig 3.2.2.5 Mean Corolla height: Length of longest leaf ratio at each site at The Remarkables, Pisa Range and Blue Mountains. Data labels indicate Tukey's test result. Least squares linear regression equations show the effect of altitude on the ratio between corolla length and leaf length.

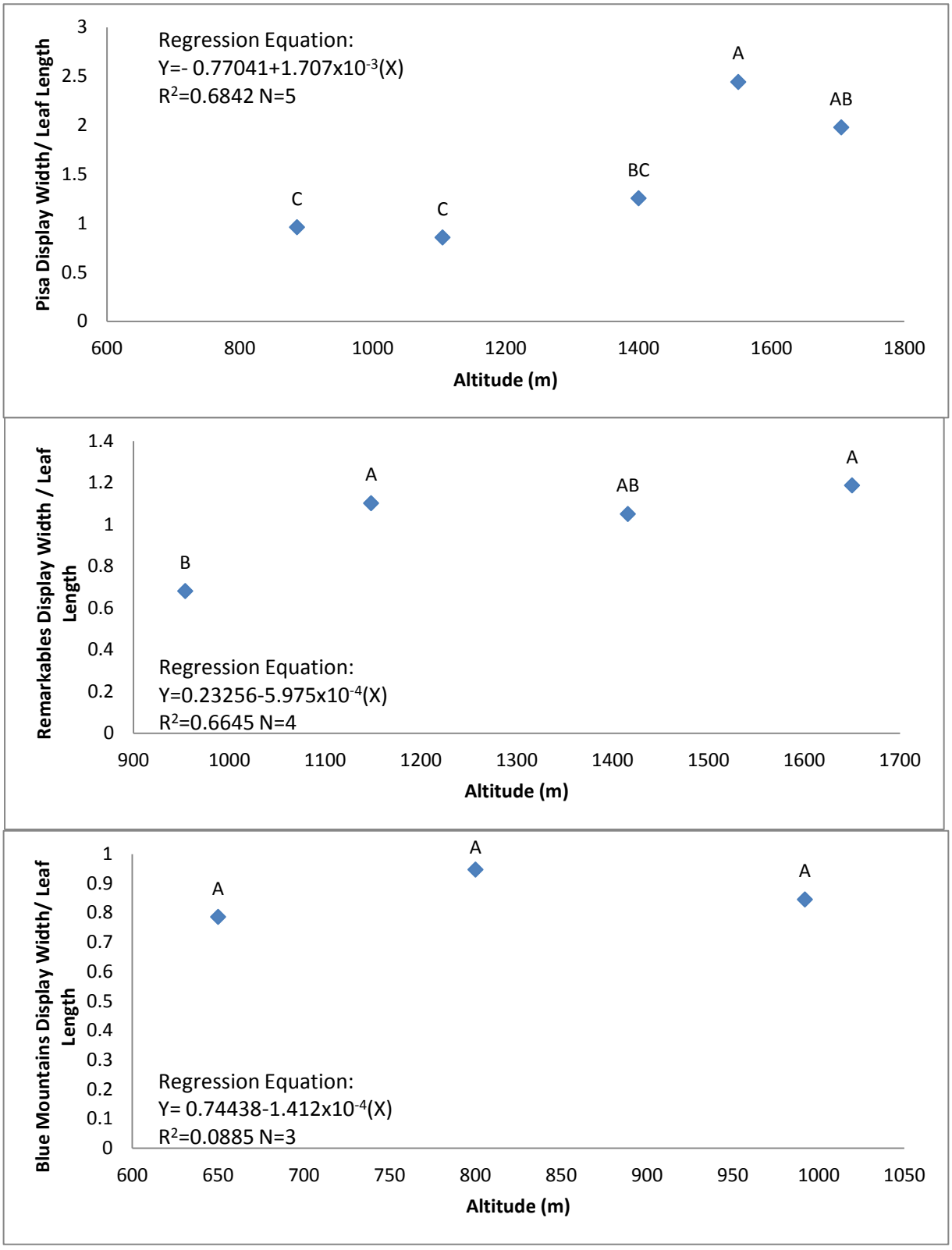


Fig 3.2.2.6 Mean Display Width: length of longest leaf ratio at each site at The Remarkables, Pisa Range and Blue Mountains. Data labels indicate Tukey's test result. Least squares linear regression equations show the effect of altitude on the ratio between display width and leaf length.

Table.3.2.2.4 ANOVA for differences in display width/ length of longest leaf and corolla height/ length of longest leaf between sites at each location.

		DF	SS	F	P
Pisa Corolla height /leaf length	Site	4	2.87562	7.22	0.0001
	Error	55	5.47784		
	Total	59	8.35346		
Remarkables Corolla height/leaf length	Site	3	1.28939	5.96	0.0016
	Error	45	3.24256		
	Total	48	4.53195		
Pisa Display Width/Leaf height	Site	4	22.5727	7.75	0.0001
	Error	55	40.0639		
	Total	59	62.6367		
Blue Mountains Corolla Height/ Leaf height	Site	2	0.07935	0.50	0.6125
	Error	27	2.14577		
	Total	29	2.22512		
Blue Mountains Display Height/Leaf Length	Site	2	0.31860	0.96	0.3960
	Error	27	4.48583		
	Total	29	4.80443		
Remarkables Display Width /leaf length	Site	3	1.82805	4.21	0.0105
	Error	45	6.52049		
	Total	48	8.34854		

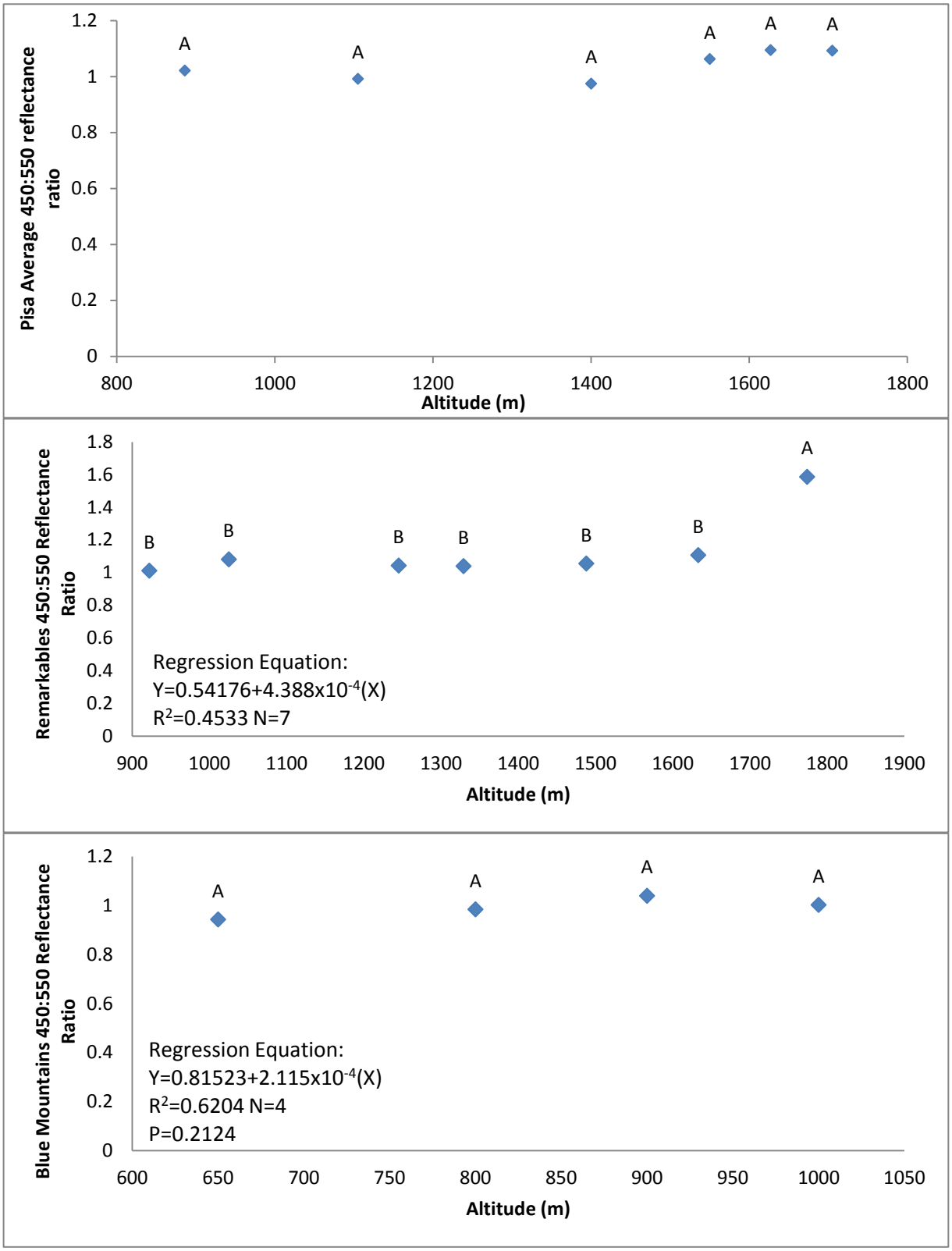


Fig 3.2.2.7 Mean 450:550 reflectance ratio at each site at The Remarkables, Pisa Range and Blue Mountains. Data labels indicate Tukey's test result. Least squares linear regression equations show the effect of altitude on the reflectance ratio.

Table 3.2.2.5 ANOVA for differences in petal reflectance ratio between sites at each location

Location	Category	DF	SS	F	P
Pisa ratio	Site	4	0.12505	2.40	0.0607
	Error	55	0.71559		
	Total	59	0.84065		
Blue Mountains ratio	Site	3	0.01928	0.332	0.8102
	Error	12	0.24034		
	Total	15	0.25962		
Remarkables ratio	Site	7	2.13120	16.08	0.0000
	Error	107	2.02643		
	Total	114	4.15763		

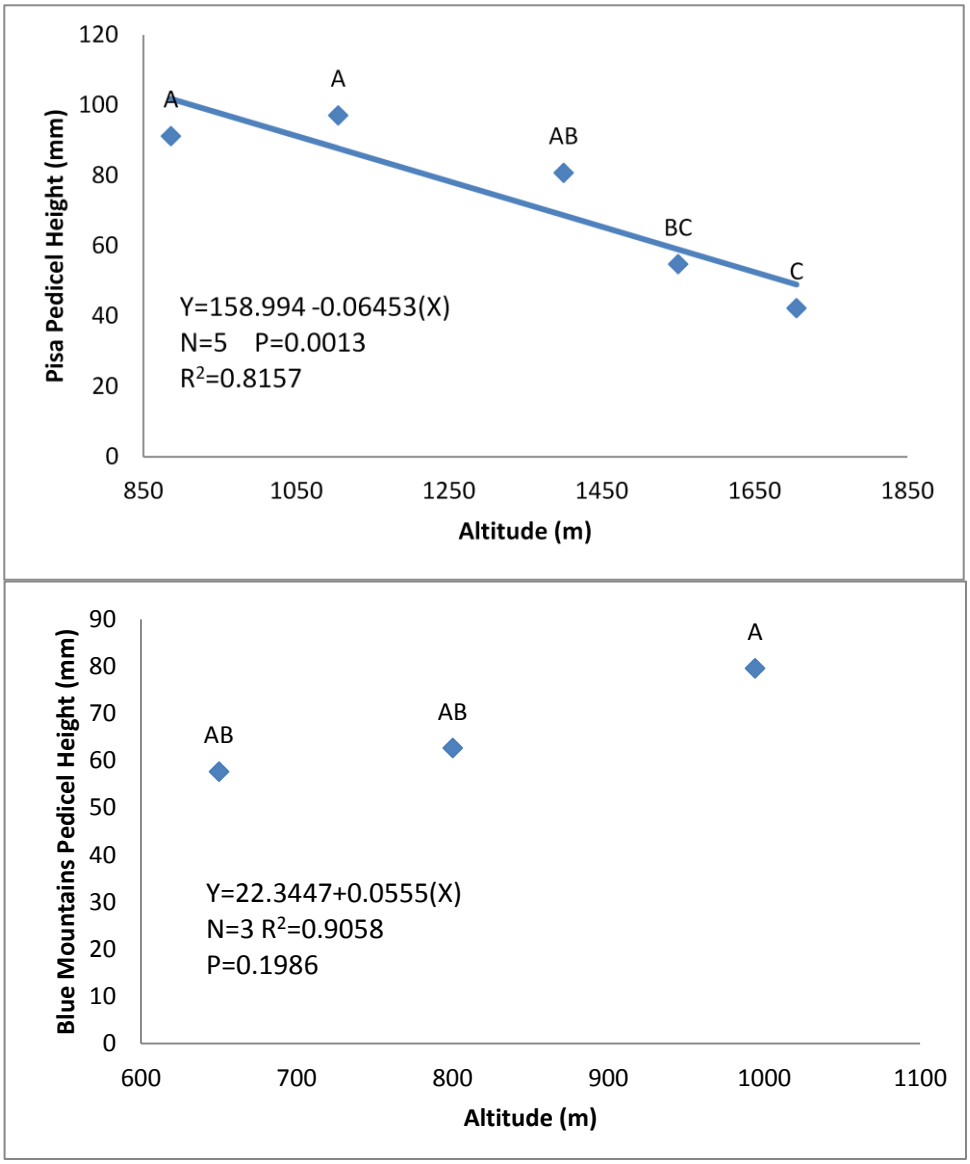


Fig 3.2.2.8 Mean pedicel height at each altitude at the Pisa Range and The Remarkables. Least squares linear regression equations show affect of altitude on pedicel height. Data labels denote site groupings by Tukey's Test (corresponding ANOVA below).

Table 3.2.2.6 ANOVA for differences in pedicel height between sites at the Blue Mountains and the Pisa Range.

Location	Category	DF	SS	F	P
Blue Mountains Pedicel Height		2	4939.8	6.03	0.0041
		60	24575.4		
		62	29515.2		
Pisa Range Pedicel Height	**Altitude**	4	26996.1	11.19	0.0000
	Error	55	33159.4		
	Total	59	26996.1		

3.3 Effect of Soil Chemistry on Floral Characteristics in the Field

3.3.1 Methods: Glass house pilot study

An experiment was attempted to test the effect of soil pH, soil Nitrogen and a combination of both in a glasshouse. An increase of soil pH caused by urea (Cabrera et al. 1991) combined with the effect of vacuolar pH on anthocyanin colour necessitated an examination of the effect of soil pH on flower size. Initially, eight pots were filled with unfertilised soil, and eight were filled with unfertilised soil and treated with 1.5g urea. It was found that samples treated with urea had consistently higher pH than controls, so a third treatment was introduced.

Plants of uncertain provenance but raised from the same seed lot were obtained from Ribbonwood Nurseries, Dunedin. They were removed from pots containing fertilised potting mix and roots were rinsed in water to remove as much soil as possible. Plants were then placed in unfertilised potting mix with pH, Nitrogen or control treatments. For the Nitrogen addition treatment, 1.5 g of urea was added to half of the 10 x 10 cm pots, as per packet instructions. Half of the nitrogen treatment pots and half of the non Nitrogen treatment pots had 10g of aluminium phosphate applied as per packet instructions to decrease pH. Soil samples were taken four days after each treatment and the pH was checked. Lime was used to elevate pH as a control for the pH effect caused by urea.

Table 3.3.1.1 *W. albomarginata* mortality in the glasshouse Nitrogen/pH experiment (15/10/2011)

		Urea (Nitrogen)	
		No treatment	2g
Aluminium phosphate (pH)	No Treatment	3/6 pots died	4/6 pots died
	10g	6/6 pots died	5/6 pots died

High mortality among plants in this experiment (Table 3.3.1.1), probably due to transplantation shock, made statistically viable data on flower characteristics impossible to collect. In view of time constraints, Nitrogen, Lime and control treatments were applied to quadrats in the field during spring rather than repeating the glasshouse experiment. Aluminium phosphate was replaced with lime for the field experiment because lime has a similar affect on soil pH to urea.

3.3.2 Methods: Effect of Soil Chemistry in the field

Locations and Setup

The soil chemistry experiment was carried out in the field at the Blue Mountains and Remarkables Range in October 2011. Three sites were chosen at different altitudes at both locations. The sites at the Blue Mountains were Top Site, The Dip and Bottom site. The sites at The Remarkables were Radio Shack, The Bend and Two Rocks (See Chapter Two for details). There were two treatment types: urea (N), Lime (pH) and a control with no additions. There were three replicates of each treatment at each site. In early November, Quadrats were selected that contained at least three *W. albomarginata* rosettes. The quadrats were 1m x 1m in area and at least two metres apart to avoid treatment overlap. Approximately 200g soil was taken from each quadrat prior to treatment. For the N treatment, 15g of urea was dissolved in 200mL water and applied with a syringe evenly over the quadrats. For the pH treatment, 200mL of distilled water was mixed with 200g of Lime to make a slurry, which was applied evenly over quadrats. Three weeks after both The Remarkables and Blue Mountains treatments, at least 200g soil was collected from each site and put on ice for transport back to the University of Otago. Soil was frozen for four weeks then tested for pH and moisture content in the Botany Department and bio-available nitrogen (NH_4-N and NO_3) was tested for in a University of Otago Geography Department laboratory.

Soil chemical analyses

Each soil sample was measured for pH by filling a beaker with soil up to 20mL, then with distilled water up to 80mL. The mixture was stirred with a glass rod prior to measurement with an Orion model 420A pH meter. Soil moisture was measured by weighing approximately 20g of soil into a tin tray, heating at 100º C over night, then weighing again. The percent of moisture was calculated by dividing the difference between the initial and final weight by the initial weight (less the weight of the container). NH4-N and NO3-N content was determined using a Tecator Flow Injection Analyser "FIASTAR 5000" system Flow Injection Analyser with a cadmium reduction column (part number 50003139) for NO3-N using standard methodology (Tecator 2000b, a).

Response variables measured

Flowering began at both sites in January 2012. Up to five male flowers were collected from each quadrat on two visits. Flowers were collected from the Blue Mountains on January seventeenth, 2012 and February the 5th, 2012 and The Remarkables 23rd January 2012 and February the 22nd 2012. The entire pedicel was collected and flowers remained fresh stored upright with a small amount of water and cotton wool in a fridge or chilly bin overnight. The pedicel length, corolla height and display width leaf length were measured with digital Vernier callipers to the nearest

0.1mm. Flower colour was the 450:550 reflectance ratio measured with an Ocean Optics USB4000 UV-Vis reflectance spectrometer with a PX-2 pulsed Xenon light source and an RS-200-7-Vis fibre optic probe.

A survey of the representation of different functional groups of plants in each experimental quadrat was also carried out to allow for the fact that in some areas a high cover of *Trifolium repens* may contribute to elevated soil nitrogen levels. For this, quadrats were divided into twenty five 20cm x 20cm squares. Functional groups were: Nitrogen fixers, herbs, shrubs, short grass (under 10cm high) and tall grass (over 10cm high). Bare rock, animal waste and bare soil were also recorded when present. Shoot presence or absence was recorded per square per quadrat for each functional group. Percentage cover was calculated from number of squares with the functional group present divided by total number of squares (twenty five).

Analysis

An Analysis of variance was used to determine whether the Nitrogen and pH treated quadrats had significantly different soil pH, NH4-N or soil moisture compared with non-treated quadrats. The effect of location (Blue Mountains or Remarkables) and site within location (One, two and three) on soil chemistry was also checked to see whether pooling data across site or location was appropriate. It was then intended to use analysis of variance to test for effect of treatment on the floral characters measured.

Linear regressions were instead carried out to test the effect of soil pH and soil NH4-N on mean pedicel height, flower colour, corolla height and display width per quadrat. Quadrats were pooled into two groups for regressions; those from The Blue Mountains and those from The Remarkables.

A co-variance model tested the effect of moisture on flower colour, pedicel height, corolla height and display width independent of site at The Blue Mountains.

All variables were measured for normality using a Shapiro-Wilk normality test, and log or rank transformed when appropriate. Outlying data points that more than doubled the next highest point or less than halved the next lowest point were omitted from analyses. All analyses were carried out using the statistical program Statistix 9 © (Analytical Software 2008).

3.3.3 Effect of Soil Chemistry on floral characteristics in the field: Results

The spring treatments of urea and lime did not have the predicted effect on soil nitrogen of the quadrats relative to the controls (Tables 3.3.2.3 & 3.3.2.4). Soil moisture was not significantly higher at The Remarkables than the Blue Mountains despite the higher altitude, but had a narrower range from 36% to 10% versus 49% to 4.4% (Table 3.3.2.1(A)). There was a significant effect of site on soil moisture within both locations, but no significant effect of location on soil moisture (Table 3.3.2.1). NH4-N varied from less than 1 mgL^{-1} to about 60 mgL^{-1} in the thirty two plots measured. NO_3 was measured but was negligible in every plot. Soil Nitrogen (NH_4-N) was higher in The Blue Mountains than The Remarkables but not by a significant margin (Table3.3.2.2 A) and did not differ among sites at both locations (Table 3.3.2.1B). Soil pH was significantly more acidic (P=0.000) at The Blue Mountains than The Remarkables (Table 3.3.2.4) and ranged from 5.68 to 4.04 versus 6.28 to 4.75 (Table 3.2.2.4). It did not vary among the sites at either of the two locations (Table 3.3.2.4).

Sixty two individual flowers were collected at The Blue Mountains and sixty four at The Remarkables. Flowering did not occur at The Remarkables Radio shack site, most likely due to the exceptionally dry summer (see Chapter Two), and was limited to four quadrats at The Bottom Site at The Blue Mountains on the first of the two collection trips. The Remarkables flowers were larger on average than the Blue Mountains but had shorter stalks (Fig 3.2.2.1).

Display width, reflectance ratio, corolla height and pedicel height were not significantly affected by soil Nitrogen (Figs 2.3.3.2 and 2.3.2.3) across both locations. Soil pH did not have a significant effect on display width, corolla height, reflectance ratio or pedicel length at either The Remarkables or The Blue Mountains (Fig 3.3.2.4, 3.3.2.5 and 3.3.2.6) and neither did soil moisture (Tables 3.3.2.6-3.3.2.10).

Table 3.3.3.1. One-Way Analysis of variance tests for a difference in soil moisture among quadrats containing *W. albomarginata* flowers between A) locations (Remarkables and Blue Mountains) and B) Sites (Blue Mountains Top Site, Blue Mountains Saddle Site, Blue Mountains Bottom Site, Remarkables Two Rocks and Remarkables Bend Site).

A)	DF	SS	F	P	B)	DF	SS	F	P
Locations	1	0.01389	1.36	0.2527	**Sites**	4	0.11268	3.61	0.0170
Error	31	0.31703			**Error**	28	0.21825		
Total	32	0.33092			**Total**	32	0.33092		

Table 3.3.3.2 One-way Analysis of variance tests of differences in Soil Nitrogen among quadrats containing *W. albomarginata* flowers between A) locations (Remarkables and Blue Mountains) and B) Sites (Blue Mountains Top Site, Blue Mountains Saddle Site, Blue Mountains Bottom Site, Remarkables Two Rocks and Remarkables Bend Site).

A)	DF	SS	F	P	B)	DF	SS	F	P
Locations	1	579.51	2.64	0.1135	**Sites**	2	1206.98	1.37	0.2677
Error	34	7465.19			**Error**	33	6837.72		
Total	35	8044.69			**Total**	35	8044.69		

Table3.3.3.3 One-way analysis of variance tests of differences in (A) Soil Nitrogen across treatment categories and (B) Soil moisture across treatment categories.

Soil Nitrogen	DF	SS	F	P	Soil Moisture	DF	SS	F	P
Treatment	2	350.14	0.75	0.4799	**Treatment**	2	0.03682	1.07	0.3554
Error	33	7694.55			**Error**	32	0.55131		
Total	35	8044.69			**Total**	34	0.58814		

Table 3.3.3.4 Mean and standard error of soil Nitrogen and moisture for number of quadrats from each treatment (Control, Lime and Nitrogen), where flowering occurred (N).

	Soil Nitrogen			**Soil Moisture**		
Treatment	Mean	SE	N	Mean	SE	N
Control	11.751	3.9472	15	0.3122	0.0351	15
Lime	11.379	4.6040	11	0.2372	0.0396	11
Nitrogen	18.548	4.8288	10	0.2617	0.0415	10

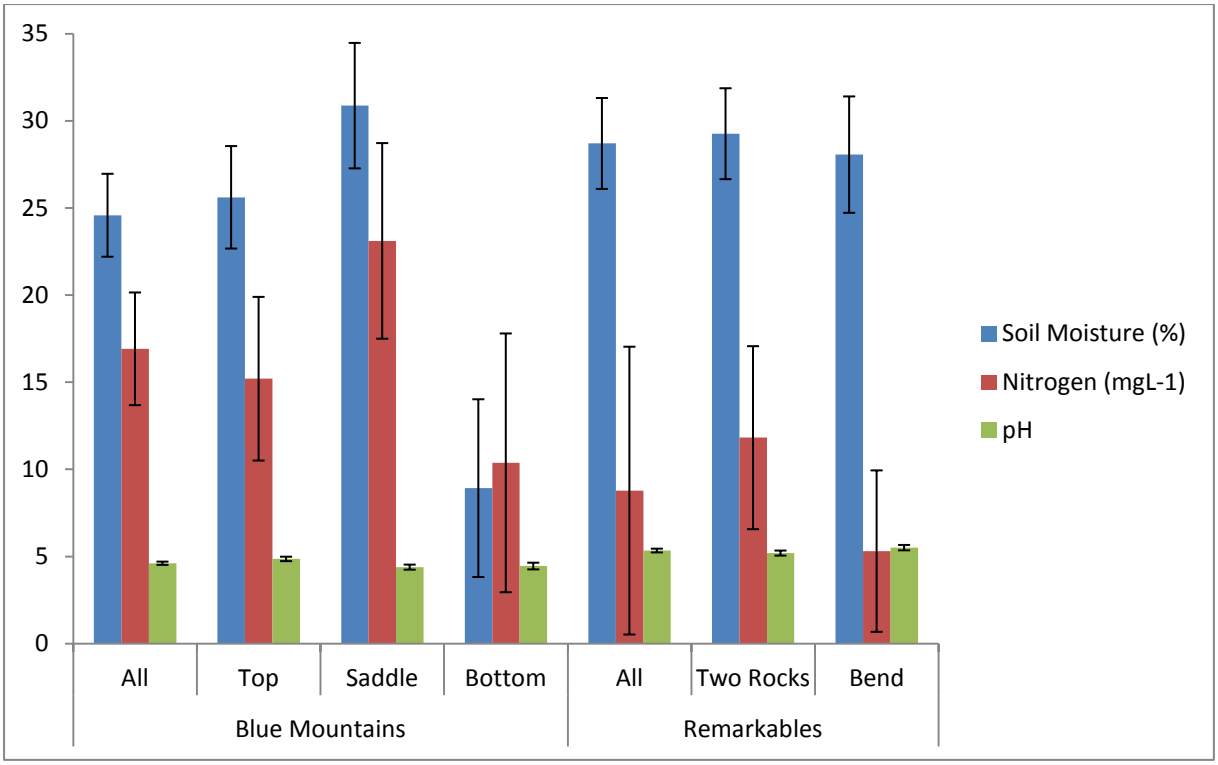


Fig 3.3.3.1 Mean soil moisture, N and pH at sites from both locations. Error bars denote standard error.

Table 3.3.3.5. Number of quadrats in which flowering occurred at each site

	Blue Mountains				Remarkables			
Site	All	Top	Saddle	Bottom	All	Two Rocks	Bend	Radio
Number Flowering Quadrats	18	9	6	3	15	8	7	0

Table 3.3.3.6 Two-way Analysis of variance tests for differences in soil pH between location (Blue Mountains and Remarkables), treatment (Control, Lime and Nitrogen) and treatment within location

pH by Location & Treatment	DF	SS	F-Value	P-Value
Location	1	4.5594	25.95	0.0000
Treatment	2	0.7005	0.21	0.8088
Location*Treatment	2	0.89632	0.274	0.0816
Error	33	5.7977		
Total	34	10.3571		

Table 3.3.3.7 Mean, standard error, and number of quadrats with flowering measured for soil pH from each treatment (Control, Lime, Nitrogen) and across all treatments; and each location (Blue Mountains and Remarkables) and across both locations.

	Locations								
	Blue Mountains			Remarkables			Both Ranges		
Treatment	Mean	SE	N	Mean	SE	N	Mean	SE	N
Control	4.5413	0.1431	8	5.2633	0.1652	6	4.9023	0.1093	14
Lime	4.88400	0.1530	7	5.1675	0.2024	4	5.0038	0.1268	11
Nitrogen	4.3980	0.1810	5	5.5680	0.1810	5	4.9830	0.1280	10
All	4.6100	0.0937	20	5.3393	0.1082	15	4.9747	0.1421	35

Table3.3.3 8 One-way Analysis of variance tests for differences in pH among sites for each location at The Blue Mountains and The Remarkables.

	Blue Mountains				**Remarkables**			
	DF	**SS**	**F**	**P**	**DF**	**SS**	**F**	**P**
Treatment	2	1.00749	3.42	0.0564	1	0.36333	2.16	0.1654
Error	17	2.50343			13	2.18596		
Total	19	3.51092			14	2.54929		

Table 3.3.3.9 Mean, standard error, and number of quadrats with flowering measured for soil pH at each site.

	Soil Nitrogen				**Soil Moisture**		
Blue Mountains	Mean	SE	N	**Remarkables**	Mean	SE	N
Top	4.8589	0.1279	9	**Two Rocks**	5.1937	0.1450	8
The Dip	4.3871	0.1450	7	**The Bend**	5.5057	0.1550	7
Bottom	4.4500	0.1919	4	**Radio Shack**	-	-	0

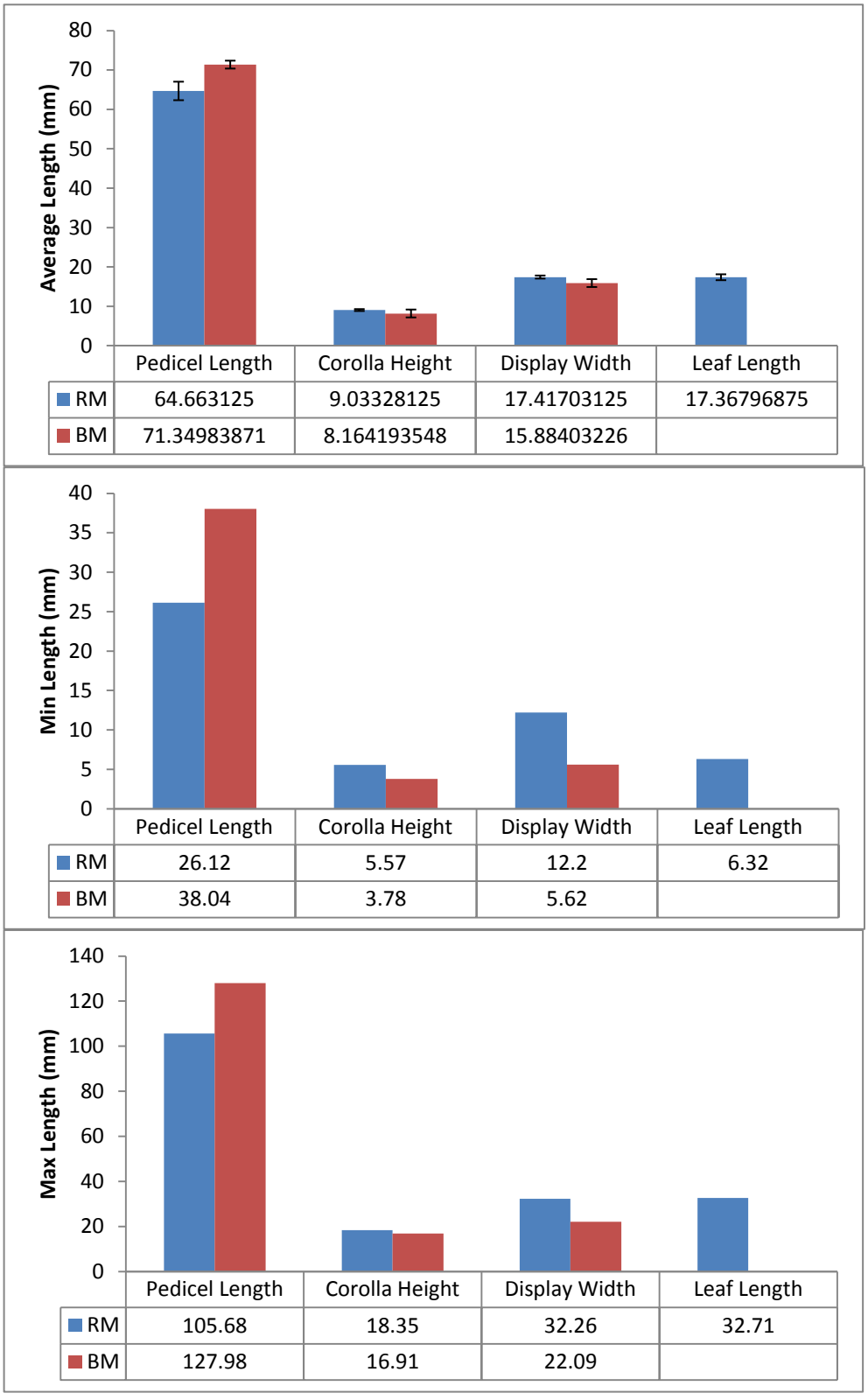


Fig 3.3.3.2 Average plant dimensions measured at The Remarkables (RM) and the Blue Mountains (BM) in experimental soil chemistry plots with error bars showing standard error (top), minimum plant dimensions measured at each location (Middle) and maximum plant dimensions measured from each location (bottom).

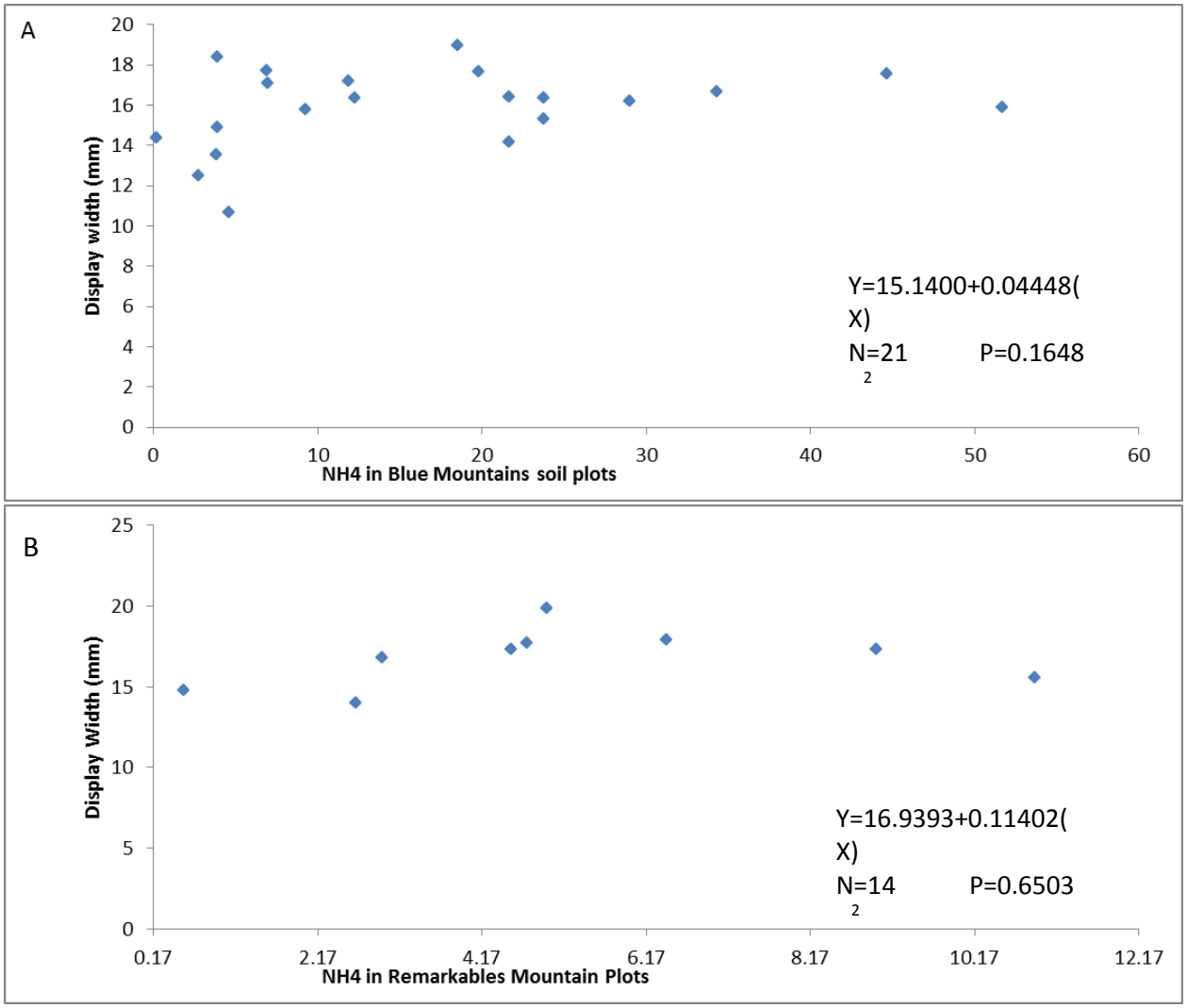


Fig 3.3.3.3 Least-wise linear regression showing relationship between average flower display width and soil nitrogen level of each quadrat at (A) the Blue Mountains and (B) The Remarkables.

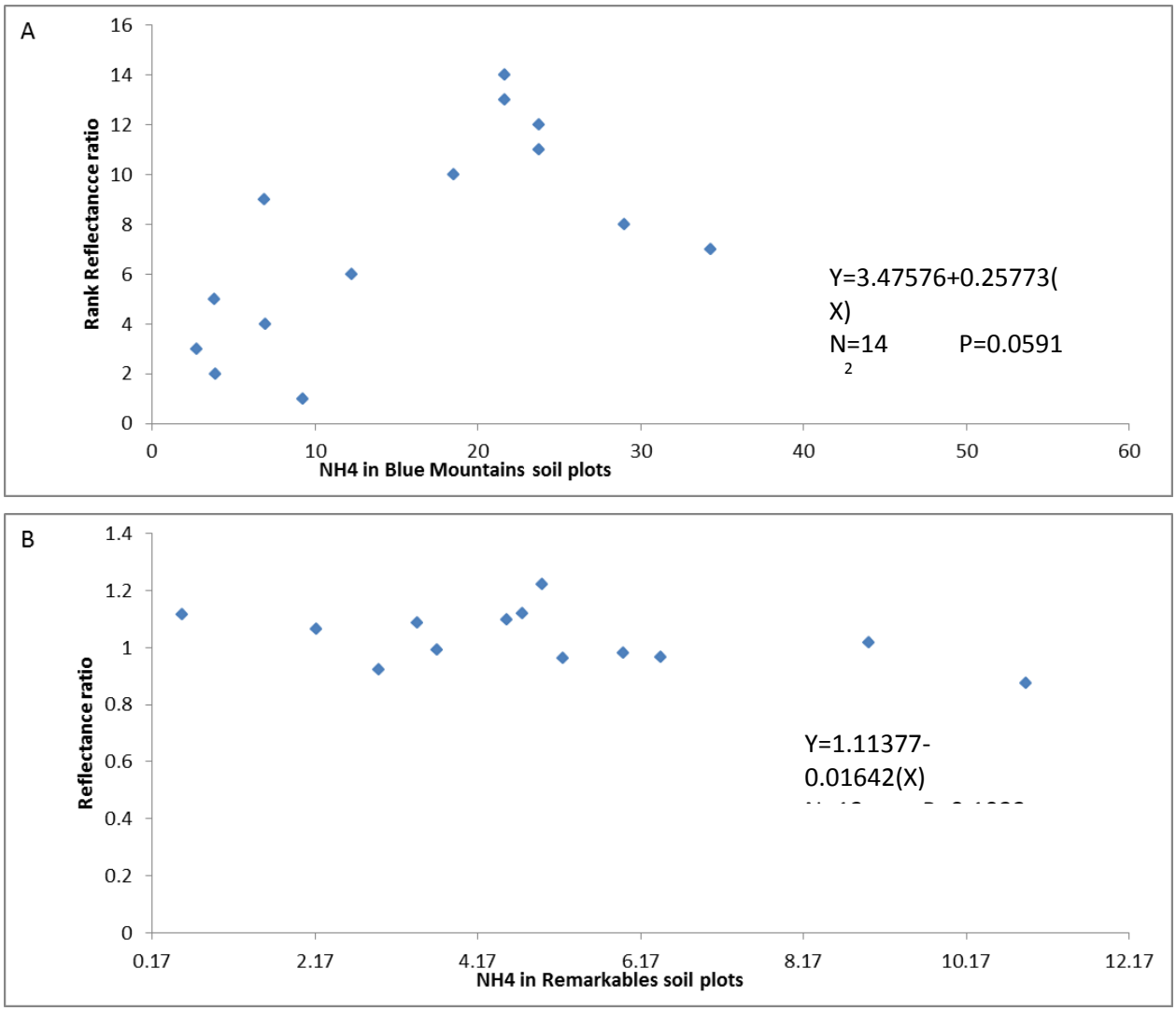


Fig 3.3.3.4 Least-wise linear regression showing relationship between average 450:550 reflectance ratio and soil nitrogen level of each quadrat at (A) the Blue Mountains and (B) The Remarkables.

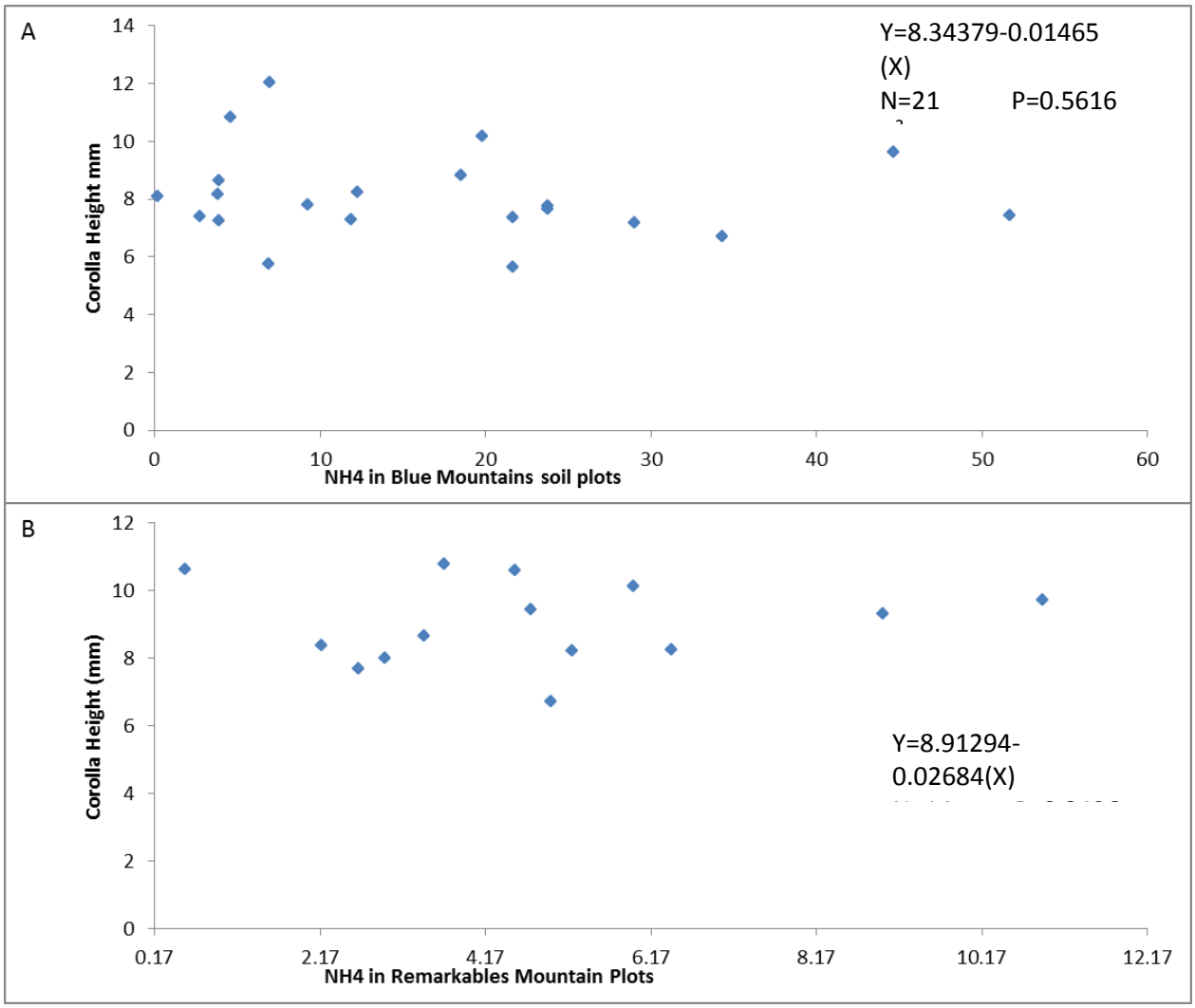


Fig 3.3.3.5 Least-wise linear regression showing relationship between average flower corolla height and soil nitrogen level of each quadrat at (A) the Blue Mountains and (B) The Remarkables.

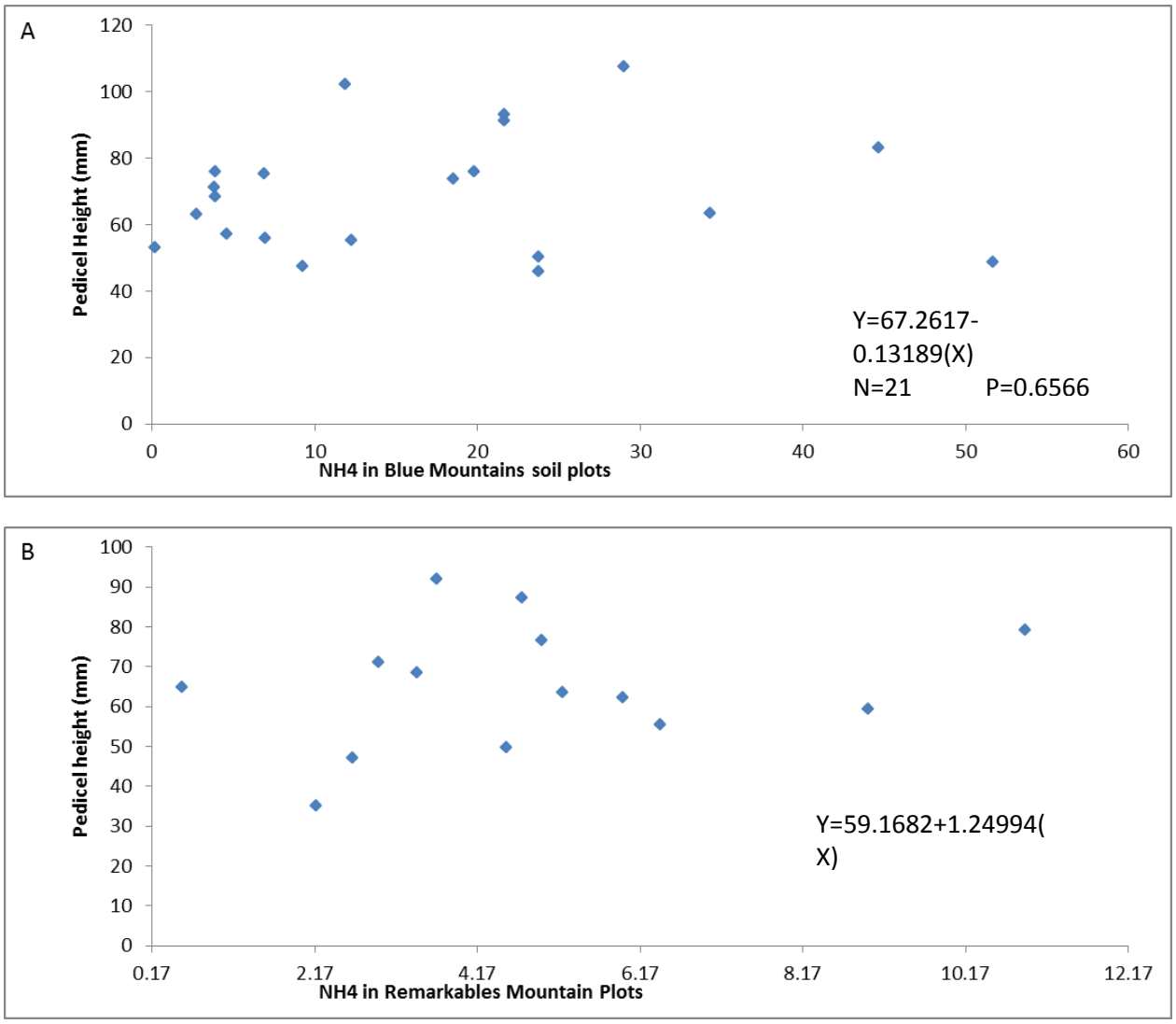


Fig 3.3.3.6 Least-wise linear regression showing relationship between average pedicel height and soil nitrogen level of each quadrat at (A) the Blue Mountains and (B) The Remarkables.

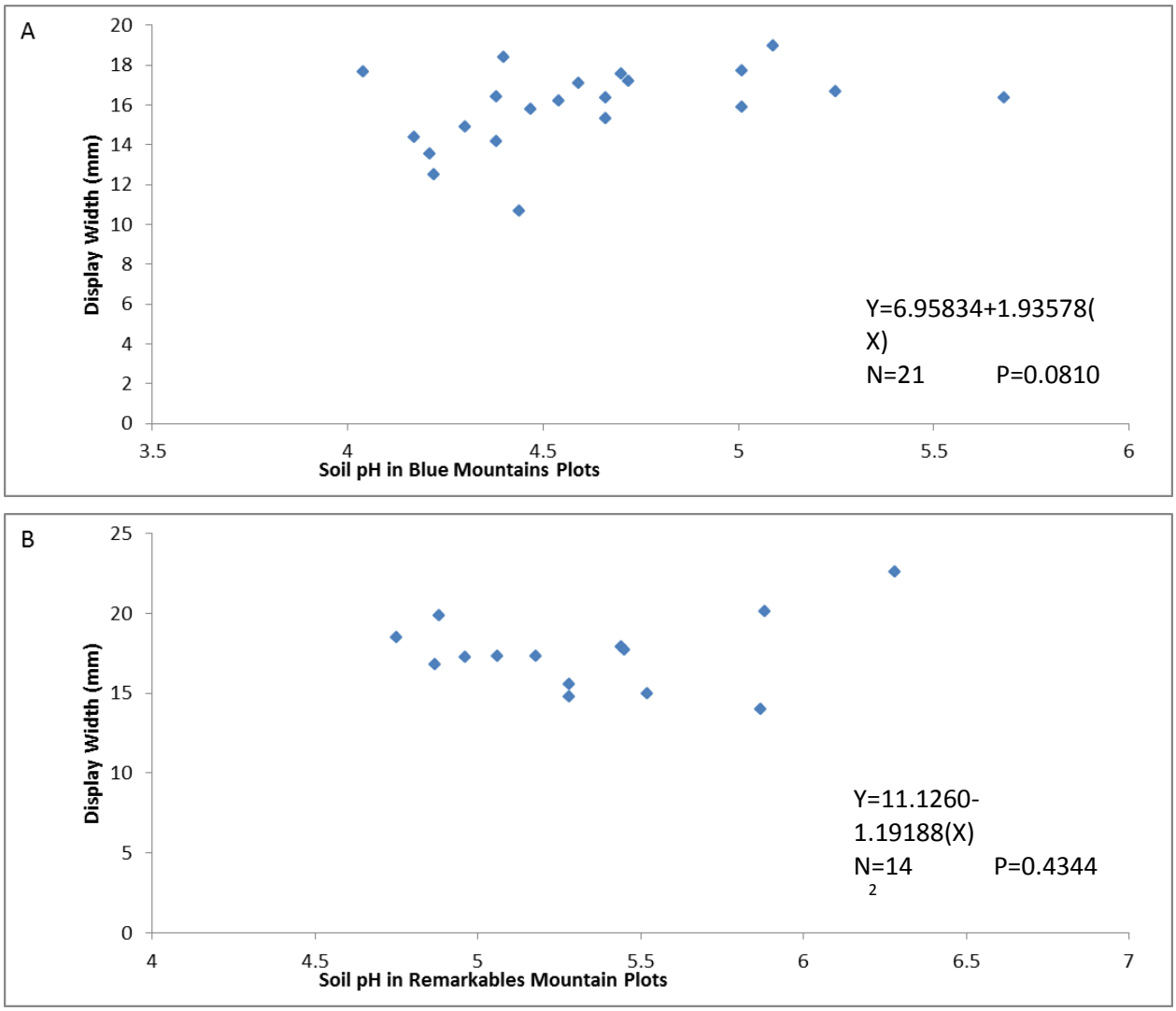


Fig 3.3.3.7 Least-wise linear regression showing relationship between average flower display width and soil pH level of each quadrat at (A) the Blue Mountains and (B) The Remarkables.

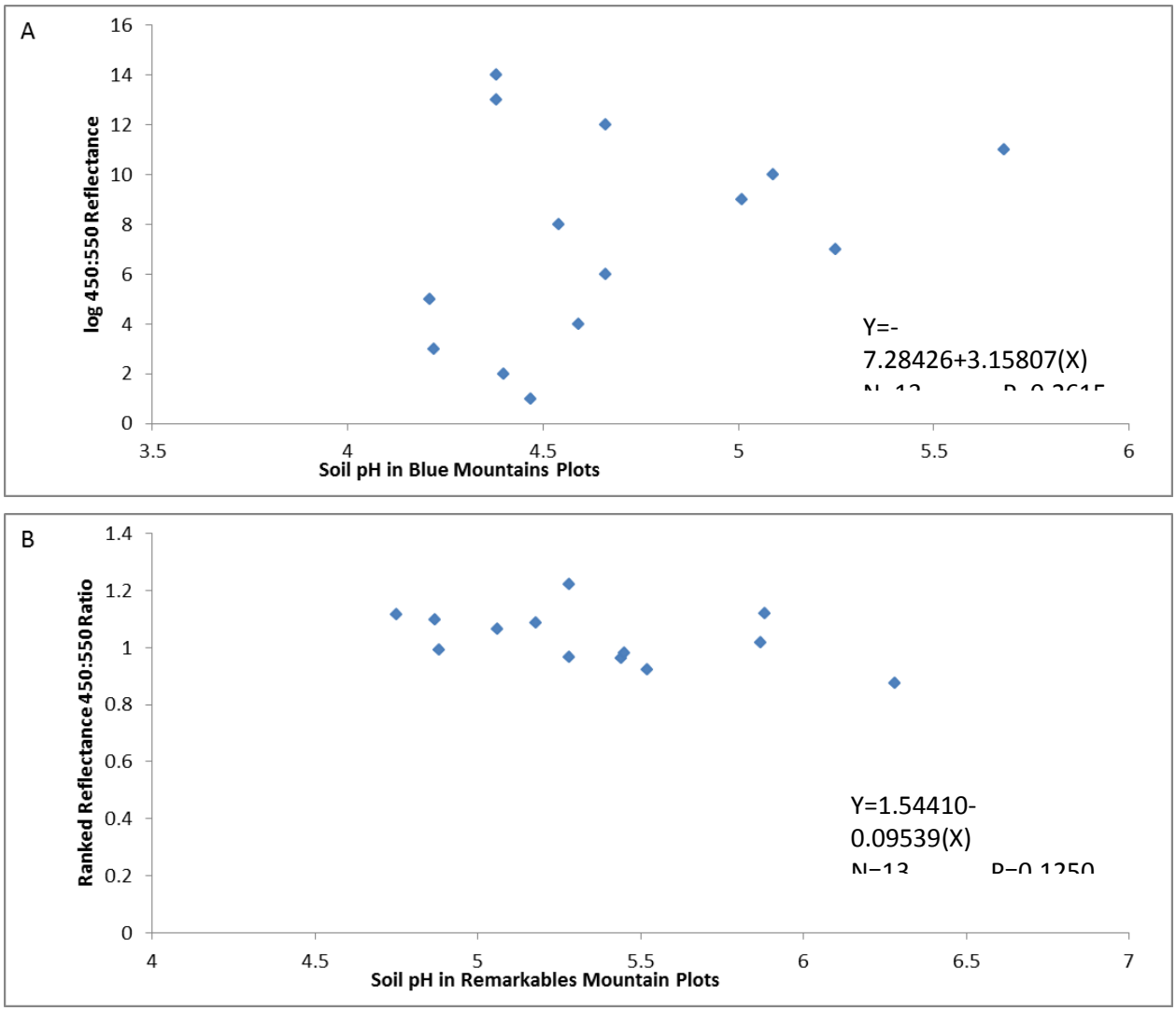


Fig 3.3.3.8 Least-wise linear regression showing relationship between average reflectance ratio and soil pH level of each quadrat at (A) the Blue Mountains and (B) The Remarkables.

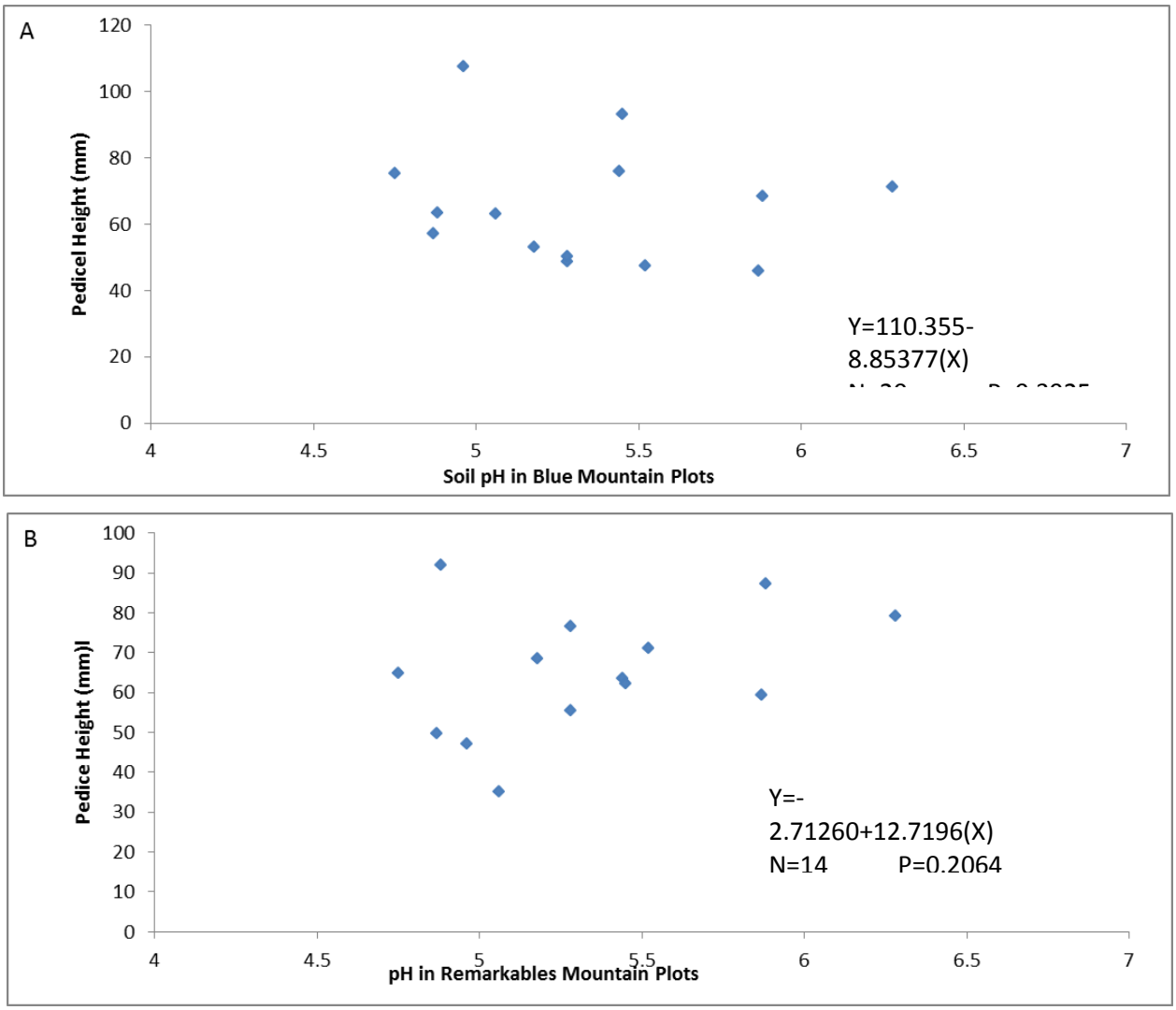


Fig 3.3.3.9 Least-wise linear regression showing relationship between average pedicel height and soil pH level of each quadrat at (A) the Blue Mountains and (B) The Remarkables.

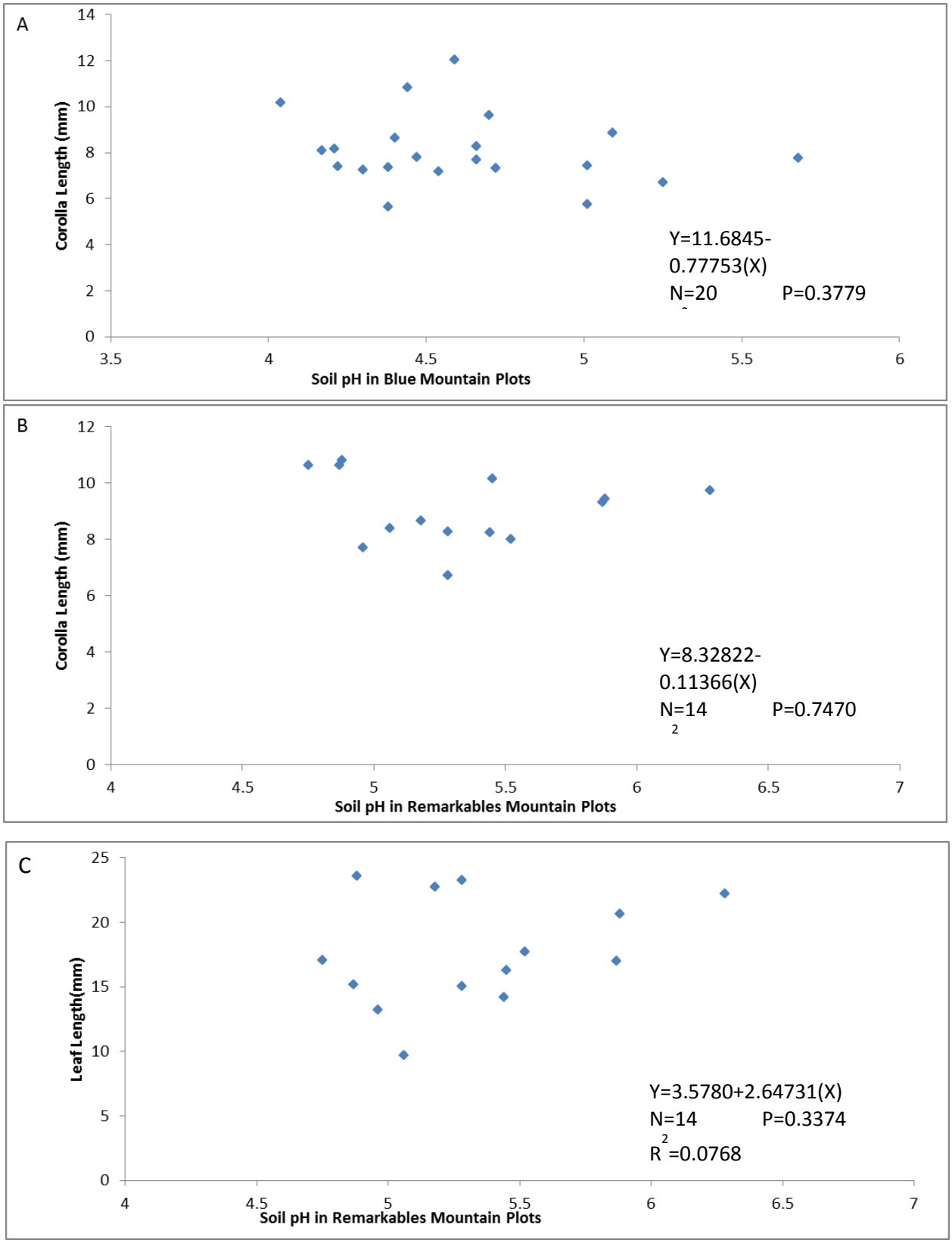


Fig 3.3.3.10 Least-wise linear regression showing relationship between average flower corolla height and soil pH level of each quadrat at (A) the Blue Mountains and (B) The Remarkables.

3.4 Effect of temperature and water availability on flower colour

3.4.1 Methods

Using purely field based data can make it difficult to tell which environmental factors are affecting a plant's phenotype. There are many factors to be considered in the field. Altitude alone can be correlated with gradients in soil moisture, nutrients, pH, temperature, humidity, competition from other species, exposure to UV irradiation and wind. In light of this, I utilised controlled climate experiments to see if specific environmental factors associated with altitude affect the characteristics of *W. albomarginata* flowers.

Temperature and Water

The following was designed to test the effect of temperature, water regime and the interaction of the two on flower characteristics. Thirty two *W. albomarginata* plants of unknown provenance, but from the same seed lot, were sourced from Ribbonwood Nursery in January 2010 then potted up in 10cm x 10cm x 18cm pots with 50% fertilised potting mix, 50% sand in December 2010. Five months later, sixteen plants were randomly allocated to one of two Sanyo MLR 350 growth chambers. One growth chamber was set to a cooler temperature treatment of 6°C "night" and 10°C "day" and the other set to a warmer treatment of 10°C "night" and 14°C "day". Half of the plants in each temperature treatment were given a water regime of 60mL per week and the other half was given 120mL per week. The 60mL watering regime had previously been shown in a glasshouse pilot trial, on plants from the same seed lot in the same sized pots, to trigger slight wilting without killing plants. All plants were initially given a spring treatment of 10 hours of light per day.

Table 3.4.1.1 Water and temperature treatments for potted *W. albomarginata* in growth chamber experiment

		Water treatments per week	
		60 mL	120 mL
Temperature **°C**	6°-10°	8	8
	10°-14°	8	8

After 15 weeks, a summer treatment of 14 hours of daylight per day was initiated. After a further eight weeks because no plants had begun to flower, chamber temperatures were increased to

temperatures 10° “night” to 14° “day” and 12° “night” to 16° “day. Water allowances were increased by 20 mL per week. As flowering was still poor in the growth chambers, pots were transferred outside on January 6th 2012. Petal colour of the first six flowers produced in each pot was recorded with half from male phase flowers and half from female phase flowers. Flowers were removed in this process so the same flower was never measured twice. The petal colour was recorded and “blueness” calculated as described previously. The difference in flower colour between temperature and water regimes, gender phases, plant of origin (for plants that produced three flowers or more) and months of collection were calculated using analysis of variance tests implemented in Statistix 9 (Analytical Software 2008).

3.4.2 Effect of temperature and water regime on flower colour- Results

Of the 16 plants in each chamber, eight cool treated plants produced flowers, while only three warm treated, well-watered, plants produced flowers. No warm treatment low-water plants survived. The rate of flowering among the other treatments did not increase when day length and temperature of potted plants increased. Flowering was sporadic with only one of the 32 pots placed outside producing six or more flowers over the course of nine weeks from January 6th 2012. It was therefore not possible to compare temperature treatments statistically and analyses of the effect of water regime were conducted using the cool-treatment plants only.

Within the cool treated flowers, the low water treated plants produced significantly bluer flowers (Table 3.4.2.2). Male phase flowers had a significantly higher 450:550 reflectance ratio than older female phase flowers. The month flowers were collected and plant of origin did not affect blueness (Table 3.4.2.1).

Male phase flowers produced by dry plants in the growth chamber were significantly bluer than those from plants with higher water regime (3.4.2.2). There was also a significant effect of gender phase on petal colour of these flowers (Table 3.4.2.2), with male flowers being bluer (Fig. 3.4.2.1 B) and no significant interaction between water regime and gender phase in determining blueness (Table 3.4.2.2).

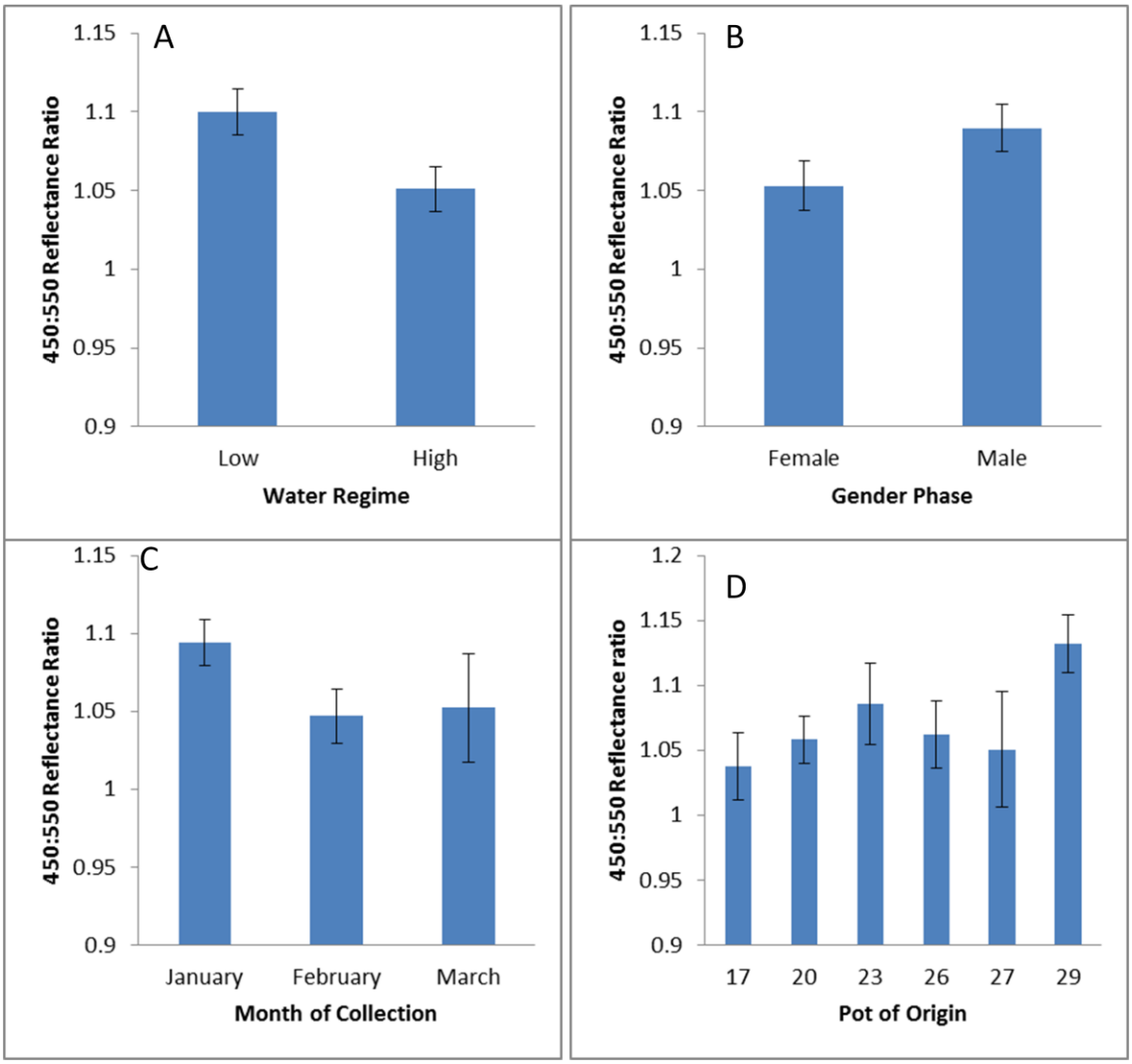


Figure 3.4.2.1 Distribution of mean 450:550 reflectance ratio across (A) water regime, (B)gender phase, (C), Month of Collection and (D) plant of origin in growth chamber plants

Table 3.4.2.1 One-Way Analyses of variance tests for the effect of Month of Collection and plant of origin on 450:550 reflectance ratio of flowers from cool growth chamber.

	df	SS	F	P
Month of collection	2	0.00102	0.18	0.8362
Error	28	0.06772		
Total	20	0.06873		
Plant of Origin	6	0.02005	1.68	0.1898
Error	16	0.03182		
Total	22	0.05187		

Table 3.4.2.2. Two-Way Analysis of variance showing the effect of water regime, gender phase and the interaction between the two on the 450:550 reflectance ratio in *W. albomarginata* grown in cool-treatment.

Source	**DF**	**SS**	**F**	**P**
Water regime	1	0.0139201	7.61	0.017
Gender phase	1	0.0125220	6.85	0.023
Interaction	1	0.0039105	2.14	0.169
Error	12	0.0219498		
Total	15	0.0523024		

3.5 Effect of Age on flower colour

3.5.1 Effect of Age on flower colour- Methods

The effect of flower age on petal colour was measured at The Remarkables in March 2011. At The Remarkables Ski lodge site 16 partially opened, intact male flowers which were separated by approximately two metres were tagged with coloured wire on 3rd March, 2011. One petal from each flower was carefully removed and mounted on black tape within 30 minutes of collection and measured for reflectance as described in section 3.2. Four days later, a second petal was removed from each of the tagged flowers and measured in the same way. The 450:550 reflectance ratios were calculated for each petal. A one-way analysis of variance was used to determine the difference in reflectance ratio between the two age groups.

3.5.2 Effect of Age on flower colour- Results

Overall, flowers were significantly bluer at the beginning of the five day period than the end when flowers appeared paler to the naked eye and this was confirmed by the quantified results. 7 of the samples measured on 7th March had to be discounted due to incorrect software calibration by another user of the equipment.

Table 3.5.2.1. One-Way Analysis of variance in 450:550 reflectance ratio between newly opened flowers and the same flowers after four days.

Source	df	SS	F	P
Between	1	0.36524	6.66	0.0167
Within	23	1.26205		
Total	24	1.62729		

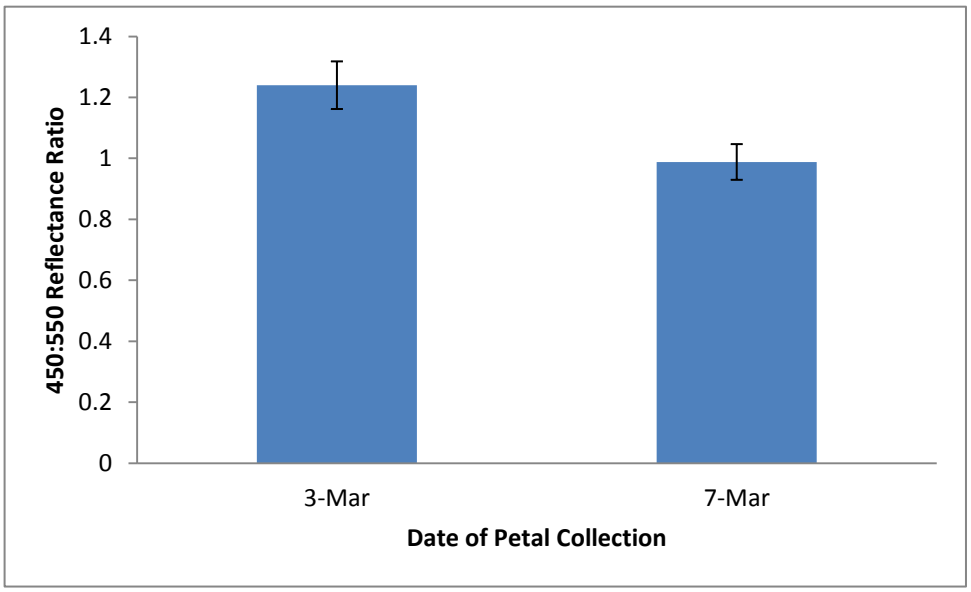


Fig 3.5.2.1. Mean and standard error 450:550 reflectance ratio of petals collected from flowers on March 3 (N=16) and March 7 (N=9) 2011.

3.6 Effect of Gender on flower colour

3.6.1 Effect of Gender on flower colour- Methods

In February 2011, flowers in both male phase and female phase were collected to be compared for their 450:550 reflectance ratio. For this experiment, five males and five females were selected from the Top Site, the Saddle Site, the Bank Site and the Bottom Site at the Blue Mountains. The closest male or female to random co-ordinates within a 15m x 2m area at each site was picked. Only completely intact, fully opened flowers that were not transitioning between male phase and female phase were selected. Flowers were chilled in plastic tubes, transported to Otago University and stored overnight. The reflectance ratio was measured as described (Section 3.5) between 16 and 18 hours after flowers had been picked.

3.6.2 Effect of Gender on flower colour- Results

On average, females were slightly yellower than males (Table 3.5.2.2); however, this result was not significant (Table 3.5.2.1).

Table 3.6.2.1. One way analysis of variance of 450:550 reflectance ratio between male and female flowers at the Blue Mountains, February 2011.

Source	df	SS	F	P
Between	1	0.03816	2.57	0.1166
Within	40	0.59316		
Total	41	0.63132		

Table 3.6.2.2. Mean, standard error and number of male and female phase flowers collected for 450:550 reflectance ratio on the Blue Mountains, February 2011.

	Replicates	Mean	Standard Error
Female	20	0.9245	0.0272
Male	22	0.9849	0.0260

3.7 Discussion

3.7.1 Effect of altitude on plant characteristics

W. albomarginata flower colour and size are affected by environmental factors in different ways. In the field, corolla height differed significantly between sites at all locations, whereas only one location showed significant variation in flower colour between a single site and the rest. There was significant variation in *W. albomarginata* for all the size characteristics quantified among the sites. Past studies have found greater variation in leaf size within species on south-facing slopes than north-facing one(Ackerly et al. 2002). The majority of study sites were west-facing (chapter two), so this is an unlikely explanation for the poor relationship between altitude and *W. albomarginata* dimensions. The effect of altitude on many of the characteristics measured was not consistent among locations, with The Remarkables alone showing a significant trend. The inconsistency of these results indicates that the explanation for variation in flower and leaf characteristics is complex. The Remarkables was the only location with snow making technology at the upper-most site. This may have enhanced the changes occurring over altitude in this characteristically dry region.

Leaf number per rosette was only measured at the Pisa Range, and did not change significantly with altitude, however leaf size significantly decreased. Decrease in vegetative size with altitude (Körner et al. 1986, Ackerly et al. 2002, Fabbro and Körner 2004, Zhang et al. 2012) is well documented. Display width, corolla height and leaf length all decreased with altitude in at least one location, yet the ratios between floral dimensions and leaf length did not alter with altitude. The lack of correlation between vegetative and floral measurements is partially supportive of the idea that allocation to reproduction increases with altitude (Fabbro and Körner 2004), but between-site factors in most locations appear to be more important.

3.7.2 Flower Size

Flower size (display width) differed significantly between sites at The Remarkables and the Blue Mountains, but was only affected by altitude at The Remarkables. This suggests environment does play a role in determining flower size, however the relationship between altitude and flower size is highly inconsistent. It is likely that a multitude of environmental factors contribute to flower size and these are sometimes, but not always, associated with altitude. This weak relationship with altitude contrasts with many taxa from the European Alps, which do not produce significantly different sized flowers at different altitudes (Blionis et al. 2001, Körner 2004). I suggest variation in flower size is not caused by altitude *per se*, but a number of interacting environmental factors that are more strongly related to altitude at The Remarkables, but not the Pisa Range or the Blue Mountains.

Quantitative data for soil pH and Nitrogen was collected at the Blue Mountains and The Remarkables, and qualitative site characteristics of aspect, shading and proximity to a road did not predict differences in how sites deviated from any altitudinal trend. Microclimates at specific sites cannot always be predicted based on nearby weather stations (Graae et al. 2012). The sites in this study may be unique in factors such as precipitation, sunlight, snow melt, herbivores, pollinators, disturbance and a raft of other factors not tested for. The main environmental factor affected by altitude is temperature, which triggers a web of changes in ecosystem structure (Körner 2003b). The Pisa Range soil properties were not measured, nor were weather patterns specific to individual sites or insect populations and visitation at any of the three locations. Future work at the sites used in this study is expected to explore these points (Miller et al., unpublished data).

W. albomarginata vegetative dimensions were consistently smaller at high altitude but only leaf length on the Pisa Range was significantly related to altitude. A reduction in the size of vegetative parts is a universally consistent trend among alpine taxa, and is attributed to slower growth rate and resource uptake (Zinderen Bakker and Werger 1974, Körner 2003a, Fischer et al. 2011).

It has been suggested that flower size is maintained at altitude because of low pollinator availability (Körner 2004). Low pollinator visitation rates have also been shown to be associated with flower longevity (Totland 2006). Taller floral appendages are known to attract more pollinators than short ones . Corolla height decreased with altitude on The Remarkables and differed among sites on the Blue Mountains and Pisa Range but not consistently with altitude. Casual observations suggest that corolla height is more affected by the height of the surrounding vegetation due to an etiolation response by the entire ramet. Alpine vegetation is often of low stature (Körner 2003a), however, this response may be triggered at more exposed sites as much as of altitude(Chittka et al. 1996).

Prioritising maintaining floral size at altitude possibly illustrates the importance of *W. albomarginata* attracting pollinators for out-crossing.

I suggest that *W .albomarginata* flowers are large relative to leaves at high altitude to be visible and attractive to pollinators, but not necessarily because pollinators are less frequent at altitude. Future studies on New Zealand alpine pollination ecology should quantify pollinator activity on a large altitude gradient.

While flower size was not affected by altitude at the Pisa Range, length of the longest leaf was. The fact that the ratio between flower size and leaf length did not differ significantly is indicative of subtle changes in flower size relative to leaf size, i.e. relative reproductive allocation. This result contrasts with a trend of greater mass allocation to flowers over shoot matter at high altitude at The Swiss Alps among a number of taxa (Körner 2004). While factors associated with altitude clearly can influence the relationship between floral and vegetative size characteristics of *W. albomarginata* they are not the only, or always the most influential, environmental variables. Similarly, within species variation in snow tussock size, growth and behaviour are more pronounced on the Old Man Range than Coronet Peak (Mark 1965). The Pisa Range may be an atypical example of environmental clines, because they are in a rain shadow, so experiences low soil moisture, even at higher altitude. These results show it is likely that a raft of associated factors such as high soil moisture, consistent temperatures due to shade and shelter from the wind culminate in causing a different, larger leaved growth form in this herb. To be sure of this, glasshouse experiments comparing different temperatures, levels of shade and water regimes are necessary. Enhanced understanding of growth responses in this species hinge on the ability to grow it successfully in experimental conditions. Results of this study indicate a sensitivity to root disturbance in established plants, which needs to be taken into account in future work.

Pedicel height was smaller at high altitude. There may be a reproductive advantage in having smaller pedicels at high altitude. There is speculation that this causes flowers to be warmer than their more visible, higher stalked counterparts, and consequently more attractive to pollinators (Chittka et al. 1996). Colder temperatures at high altitude generally contribute to smaller sized plants (Körner 2003a), so this result was expected.

3.7.3 Effect of Location on plant characteristics

The difference between The Remarkables and the other two sites in most floral characteristics was unexpected because geographically and attitudinally, the Blue Mountains is the outlier. The Blue Mountains is geographically isolated from the other two because it is slightly east of Central Otago whereas The Pisa Range and Remarkables are both in the Lakes District and have much higher summits. The difference in flower characteristics at The Remarkables compared with the other two sites cannot be explained by the data collected. Of the three study locations, snow cover is highest at The Remarkables, due to the use of snow machines at the higher sites. Snow cover is patchy at the Pisa Range and more so at The Blue Mountains. An alternative explanation that cannot yet be ruled out is that selection pressure due to insect preference has led to different flower characteristics at The Remarkables. Insect visitation has been shown to be affected by colour of arrays of flowers, rather than individuals, at The Remarkables ski lodge site (Campbell et al. 2012), however, this concept has not been tested at the Pisa Range and Blue Mountains. A detailed comparison of *W. albomarginata* pollinator behaviour at the three sites would be a useful avenue of enquiry for future work.

Flower size decreases significantly between sites at The Remarkables but remains constant at the Pisa Range. This highlights the importance of conducting research in as many sites as possible. It is likely that attractiveness to insects is a driver for large flower size and physiological stressors are drivers for smaller flower size. I suggest that pollinator behaviour at the Pisa Range may be maintaining larger flowers even at higher altitudes. The threshold size for attractiveness there may be higher that The Remarkables. The higher altitude Pisa Range sites were the only south-facing bank sites of the study. This means they did not experience as wide a temperature range as the other sites due to shading from the sun. Absence of this stressor in the summer months could be an alternate explanation for the difference in trends from the other sites.

3.7.4 Effect of soil properties on plant characteristics.

The results suggest that neither soil pH nor soil nitrogen status are important factors in determining floral characteristics in *W. albomarginata*. Soil nitrogen, pH and moisture did not affect flower size or colour in the field experiments. The high mortality rate of the low-nitrogen treated plants in the glasshouse experiment may have been caused by the harshness of the treatment, or disturbance of roots when plants were being re-potted.

W. albomarginata is not alone in displaying little variation in flower size in relation to plant nutrition. The effect of nitrogen application on flower size in *Paphiopedilum armeniacum* S.C Chen & F.Y. Liu. was also minor (Yan et al. 2012). It is unfortunate that short-term nitrogen application proved ineffective in the field, as this result could have been compared to phenolic expression triggered in tomato by a similar treatment (Olsen et al. 2010, Olsen et al. 2012). However the experiment does support the conclusion that naturally occurring variation in soil nitrogen and pH does not affect leaf size or flower size or colour in *W. albomarginata*.

3.7.5 Flower Colour Variation: Age and Plasticity

Flower colour did not differ between sites or locations to the same extent as floral and vegetative dimensions and neither altitude nor soil chemistry affected flower colour. In addition to this, the pot from which the flower had been collected did not influence flower colour, though water regime and age did, in plants raised from the same seed lot. This indicates that flower colour cannot be explained by simple Mendelian inheritance in *W. albomarginata,* but rather water availability and age. The possibility of flower colour being partially heritable from parents, but obscured by a high level of plasticity, cannot yet be eliminated. Elevation in anthocyanin content is a likely explanation for significantly bluer petals in long-term stress treated plants. While plasticity of fruit phenolic content has been documented in crop plants(Olsen et al. 2012), and vegetative parts of naturally occurring plants(Gould et al. 2000, Hatier and Gould 2008, Gould et al. 2010), the possibility of naturally occurring floral anthocyanin plasticity is novel, and warrants further study.

Results indicate that a significant change in flower colour occurs with age in the days after flower opening and as flowers transition from male phase to female phase. Flowers from The Remarkables ski lodge site, which were significantly bluer than all other Remarkables sites, decreased significantly in blueness over a four day period. The differing relationships which petal colour and flower size have with different sites does not support the hypothesis of flower size predicting "blueness", however an experiment tracking the flower age, size and colour of individual flowers in controlled conditions is necessary to rule this out with complete confidence. Based on the evidence from this study, it can be concluded that the main driver for *W. albomarginata* flower colour change is pigment depletion over the first few days following petal opening. The functional explanation for this can only be guessed at. It is possible anthocyanins are quickly allocated to long-term organs such as leaves once flowers have opened. Colour in *W. albomarginata* affects pollinator preference to flower arrays (Campbell et al. 2012). Allocating pigments to male phase flowers then transporting them to long-term vegetative parts from female phase flowers is consistent with theory surrounding male and female reproductive fitness (Bateman 1948). In hermaphroditic flowers, male fitness is maximised by the number of repeated visits exporting pollen to different stigmas whereas female fitness can be maximised in a single visit if pollen of sufficient quality and quantity is delivered (Bateman 1948, Lloyd and Yates 1982b).

3.7.6 Conclusions

W. albomarginata flower size undoubtedly varied among sites, however variation with altitude was inconsistent among locations. It is likely the environmental factors which trigger variation in flower size changed with altitude at The Remarkables, but not the Pisa Range and Blue Mountains. The snow farm site on the Pisa Range had unexpectedly large *W. albomarginata* flowers. Future studies could examine the effect of microclimates on expected altitudinal trends in vegetation and physical properties of The Remarkables and Pisa Range. Flower age appears to best explain "blueness", with allocation to pollinator-attracting colour coinciding with male phase. Physiological stress due to low soil moisture appears to be an additional contributor to flower colour. Future work should quantify the effect of physiological stressors on vegetative and floral anthocyanin content.

4

Effect of Altitude and Co-existing species on Pollen Delivery to *Wahlenbergia albomarginata*

"CURIOUSER AND CURIOUSER!"

(CARROLL 1865)

4.1 Introduction

The effect of exotic plants on native species through pollinator networks has received much attention as of late (Elle et al. 2012, King and Sargent 2012, Montero-Castaño and Vilà 2012, Muchhala and Thomson 2012, Rauschert and Shea 2012). Exotic plants can compete with native plants for pollinators, especially when the exotic plants have more attractive or rewarding flowers (Chittka and Schurkens 2001, Moragues and Traveset 2005, Jakobsson et al. 2008, King and Sargent 2012, Woods et al. 2012). Introduction of new plant species to an ecosystem can decrease the rate of visitation and proportion of conspecific pollen deposited per visit. Floral size can be an important determining factor in attracting pollinators (Galen and Newport 1987, Nakamura et al. 1989), but there are also many other traits that plants may evolve to exploit or counteract their environment to ensure effective pollination occurs (Waser and Price 1983, Charlesworth and Charlesworth 1987, Fenster et al. 2004). In this study, *Wahlenbergia albomarginata* Hook. varied in flower size among sites and in one instance, across altitudes (as described in Chapter 3). *W. albomarginata* flowers are unique in their environment because of their pale blue colour and having a single flower per rosette. Low pollination rates could significantly impede reproductive success in *W. albomarginata* because it is a self-incompatible, species with a short period of stigma receptivity (Bischoff 2008). The evolutionary significance of environmental effects on flower characteristics needs to be elucidated in the context of pollinator preference and competition with interspecific flowers. In this study, investigating the extent to which site, altitude and flower size affect successful visitation by pollinators has been examined to shed light on the relevance of flower size variation in *W. albomarginata* on reproductive fitness.

Investment in flower size has long been acknowledged as an important determinant in pollination success (Galen and Newport 1987, Nakamura et al. 1989, Eckhart 1991). However some insects make choices about which flowers they visit based on other criteria. It is thought that social bees are most likely to visit large flowers when they are likely to contain more nectar than smaller ones (Herrera 1995). In this study, the relationship between altitude and *W. albomarginata* flower size and colour was inconsistent among locations (as described in Chapter 3). The exact environmental factors affecting flower size remain unresolved; however, variation in display width and corolla height among sites was clearly shown in Chapter Three. The sites selected had different biotic and abiotic properties. The question of whether different sites would support delivery of different pollen mosaics to stigmas lead to examining the relevance of different site-related environmental factors in overall *W. albomarginata* reproductive success. Of particular interest was the effect of neighbouring exotic flowers on *W. albomarginata* pollen loads at different sites.

The population density of *W. albomarginata*, along with the types of plants and insects that live in proximity to it, may have an effect on reproductive success. Pollinators of native species often visit alien plants (King and Sargent 2012) and invaders are likely to have indirect effects on ecosystem processes such as complex food webs, decomposition and pollination (Brockerhoff et al. 2010). Shifts in ecosystem dynamics when new species are introduced can impact pre-existing species positively by increasing the rate of insect visitation (Herrera 1995) or negatively by competing for pollinators with longer established species. Do exotic species merely co-occur with *W. albomarginata* and its pollinators, or do they occupy the system in a more integrated way?

Hieracium pilosella L. is known to influence the soil pH, organic matter and nitrogen through free living microbes in New Zealand tussock grasslands (Saggar et al. 1999) and the invasive *Hieracium lepidulum* has been shown to flourish in subalpine areas of New Zealand that have low amounts of available plant nutrients and low pH (Radford et al. 2006, 2010). Changes in physical habitat due to exotic invaders are more likely to cause permanent changes in the structure of an ecosystem than competition alone. In addition to this, invaders are likely to have indirect effects on ecosystem processes such as complex food webs, decomposition and pollination (Brockerhoff et al. 2010).

This chapter aimed to determine the ecological interaction between *W. albomarginata* and neighbouring exotic species by examining stigmas for heterospecific pollen and relating pollen loads in relation to the density and proximity of other flowering species. In this study, insect observations were carried out to account for how heterospecific and conspecific pollen was delivered to *W. albomarginata* stigmas. In addition, the functional significance of large *W. albomarginata* flowers was tested across multiple sites to examine pollen loads associated with sites with plants having large flowers and those with small flowers.

4.2 Method

4.2.1 Stigma Pollen Loads

Five sites were selected along an altitude gradient at The Blue Mountains. At each site, twenty *W. albomarginata* plants that had fully matured female phase flowers (from here on referred to as female *W. albomarginata)* with neighbouring flowers within 7 metres were selected for excision. The distance between each female *W. albomarginata* and the nearest *Hypochaeris radicata* L.*, Hieracium pilosella, Trifolium repens* and *Lotus corniculatus* L. plant was measured within a 7m radius. *H. radicata* and *H. pilosella* were subsequently grouped into the Asteraceae category as their pollen couldn't be distinguished. The nearest *W. albomarginata* was deemed to be the plant itself, so this distance was always zero centimetres. The number of inflorescences from species with similar floral presentation within a 50cm radius was recorded. Similar floral presentation was deemed to be a display width of 10mm-50mm, and stand 50mm-200mm from the ground and any petal colour or florets per inflorescence. Species such as *Raoulia subsericea* that were in bloom, but significantly larger or smaller than *W. albomarginata* were disregarded. Pollinator activity was observed at each quadrat for is minutes. Pollination events of inflorescences were counted as one, even if multiple florets were visited.

Excised stigmas were stored fresh in eppendorf tubes, transported to the Botany Department at the University of Otago, and mounted on microscope slides. Stigmas were not preserved in ethanol to prevent pollen displacement so some had experienced significant decay before microscope mounting could be carried out. Slide mounting was done by slicing away the stamen with a scalpel, then placing the receptive part of the stigma upright in 60°C melted fuchsin jelly. Care was taken to make sure the section was completely covered with the fuchsin stain before the cover slip was applied. Slides made in this manner were permanent and were able to be viewed and reviewed throughout the period of the study.

During the six month period after collection, pollen types on stigmas collected were identified using reference slides held at The Botany Department, Otago University and from anthers removed from plants in the Blue Mountains. Four pollen types were identified to genus or family level, with a fifth pollen type comprising all other pollen grains. These were:

1. Asteraceae - *Hypochaeris* and *Hieracium* pollen were grouped into Asteraceae because they were too similar to distinguish while counting with x40 magnification,
2. *Wahlenbergia*- The only *Wahlenbergia* species at the Blue Mountains was *W. albomarginata*
3. *Trifolium*- The only *Trifolium* species observed was *T. repens*
4. *Lotus*- The only *Lotus* species present was *L. corniculatus*

5. Other- Pollen that was not Asteraceae, *Wahlenbergia, Trifolium* or *Lotus.*

For each stigma collected, the amount of each pollen type was counted.

Table 4.2.1.1 Most common flowering species and pollen types present on *W. albomarginata* across all five sites at the Blue Mountains

Wahlenbergia pollen type	Asteraceae pollen type		*Trifolium* pollen type	*Lotus* pollen type

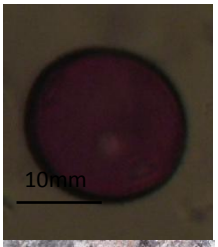
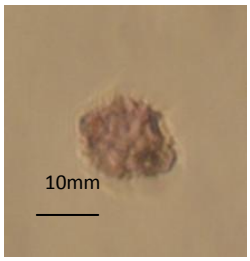
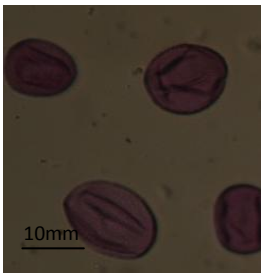
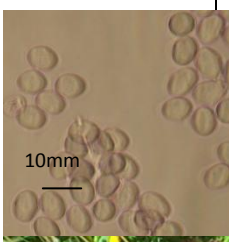

 	  		 	

Fig 4.2.1.1 *Wahlenbergia* pollen type (above), *W. albomarginata* flowers (below)	Fig 4.2.1.2 Asteraceae pollen type (above), *H. radicata* flowers (below, left) and *H. pilosella* flower (below, right)		Fig 4.2.1.3 *Trifolium* pollen type (above), *T. repens* flowers (below)	Fig 4.2.1.4 *Lotus* pollen type (above), *L. corniculatus* flowers (below)
Wahlenbergia albomarginata	*Hypochaeris radicata*	*Hieracium pilosella*	*Trifolium repens*	*Lotus officinalis*

**T. repens* pollen courtesy of University of Otago Botany Department herbarium

4.2.2 Analysis

Statistical analyses were used to determine the relationship between immediate flower neighbourhood, altitude, flower size and pollen load. Analyses of variance were run to determine whether pollen types varied among sites. The effect of altitude on each pollen type, the conspecific: heterospecific pollen ratio and total pollen load per stigma at each site was assessed with a linear regression using mean values for stigmas collected from each site.

The effect of proximity of flowering species to the presence of corresponding pollen type on stigmas was measured using linear regression in some cases. However, this was complicated by non-normal data. The distance to *Lotus* sp. and *Trifolium* sp. was subdivided into three categories:

- less than 100cm,
- 100cm-700cm and
- greater than 700cm,

The distance to the closest Asteraceae was subdivided into less than 100cm and greater than 100cm. An analysis of variance of the abundance of each pollen type on each target *W. albomarginata* stigma among these three categories was then performed.

Finally, four sites at the Blue Mountains were sorted into two categories: those with larger flowers and those with smaller flowers (as described in Chapter Three). The Bank Site (at 900m elevation) and the Top Site (at 1000m elevation) the small flower group while the Dip Site (800m) and the Bottom Site (650m) sites formed the large flower category (based on the results detailed in Chapter Three, Part Two). One way ANOVAs were performed to test the difference in pollen type and total load as well as the ratio of conspecific pollen to heterospecific pollen between the larger-flower and smaller-flower site categories.

All necessary homogeneity and Shapiro-Wilk normality tests were carried out and log or ranked data transformations were performed where appropriate. Statistix 9.0 © (Analytical Software 2008) was used to perform all statistical analyses.

4.2.3 Insect Observations

Forty circular quadrats with a 50cm radius were placed with a *W. albomarginata* female at the centre at The Tower Site and The Bottom Site at the Blue Mountains.

Quadrat placement was selected by *W. albomarginata* presence. Quadrats were chosen by walking seven paces in a predetermined direction, then finding the nearest *W. albomarginata* female which was designated to be the centre of the quadrat.

The number of flowers from each species in the quadrat was recorded. A 15 minute insect observation period took place for each quadrat. Insect type and flower choice was recorded for each insect visit within the quadrat. Overall, these observations were recorded on three days in February 2011, by four people. There was a total of ten hours observation time in warm conditions with no wind.

4.3 Results

4.3.1 Pollen load on stigmas

A total of 39,333 pollen grains were counted on stigmas across the five sites. Pollen types scored were *Wahlenbergia, Trifolium, Lotus,* Asteraceae and Other (Table 4.2.1.1). The three most common pollen types present on stigmas were *Wahlenbergia, Trifolium* and Asteraceae. *W. albomarginata* accounted for 14% of total pollen, Asteraceae accounted for 40%, *Trifolium* 45%, *Lotus* accounted for <1% and Other accounted for < 1%. (Fig 4.3.1.1). The weather was overcast and windy when *W. albomarginata* at the quadrats where stigmas were excised and no pollinator activity occurred.

The proportions of *Lotus, Trifolium, Wahlenbergia* Asteraceae and Other pollen types remained fairly consistent across the five sites, although numbers of grains counted differed. The most common flowers within a 50cm radius of each *W. albomarginata* stigma (excluding the flower it was collected from) were *W. albomarginata* and Asteraceae at all sites except for the site at 900m. *Trifolium* and other flower types occurred at the 650m site. *Lotus* occurred at the 850m and 900m sites and Other flower types were common at 900m as well (Fig 4.3.1.2).

The amount of *Wahlenbergia* and *Trifolium* pollen present on *W. albomarginata* stigmas was not significantly affected by the density or proximity of nearby flowers; however, Asteraceae pollen was more common in quadrats with *Trifolium* within 100cm (Tables 4.3.1.4 - 4.3.1.9).

No pollen type, total, or the ratio of conspecific to heterospecific pollen was significantly affected by the altitudinal gradient (Figs 4.3.1.4 & 4.3.1.5). There was no significant difference in conspecific or ratio of conspecific to heterospecific pollen presence among sites, but total pollen and Asteraceae pollen counts were significantly higher on stigmas from the 900m site (Tables 4.3.1.1 &4.3.1.2).

There was a significant positive correlation between the amount of Asteraceae pollen and the amount of *Trifolium* pollen on *W. albomarginata* stigmas but not between *Wahlenbergia* pollen and the other two (Table 4.3.1.3). Proximity within 700cm of flower types to the stigma in question did not affect the amount or type of pollen present on it with the exception of distance to *Trifolium* flowers affecting amount of Asteraceae pollen present (Figure 4.3.1.3).

The sites which had the largest male phase flowers in the Chapter Three study had significantly more conspecific and heterospecific pollen grain counts than the sites with smaller male flowers, but there was no difference in the conspecific and heterospecific pollen ratio between the two groups of sites (Tables 4.3.1.10 and 4.3.1.11).

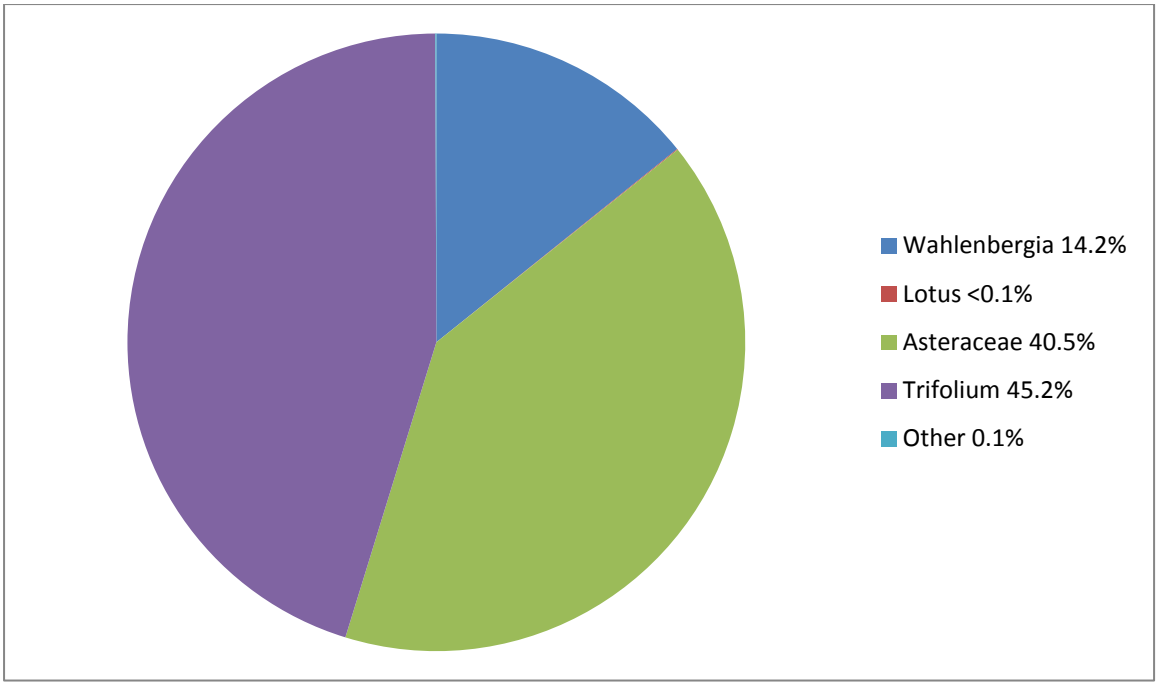


Fig.4.3.1.1 Percentage pollen present from each type on stigmas from all sites.

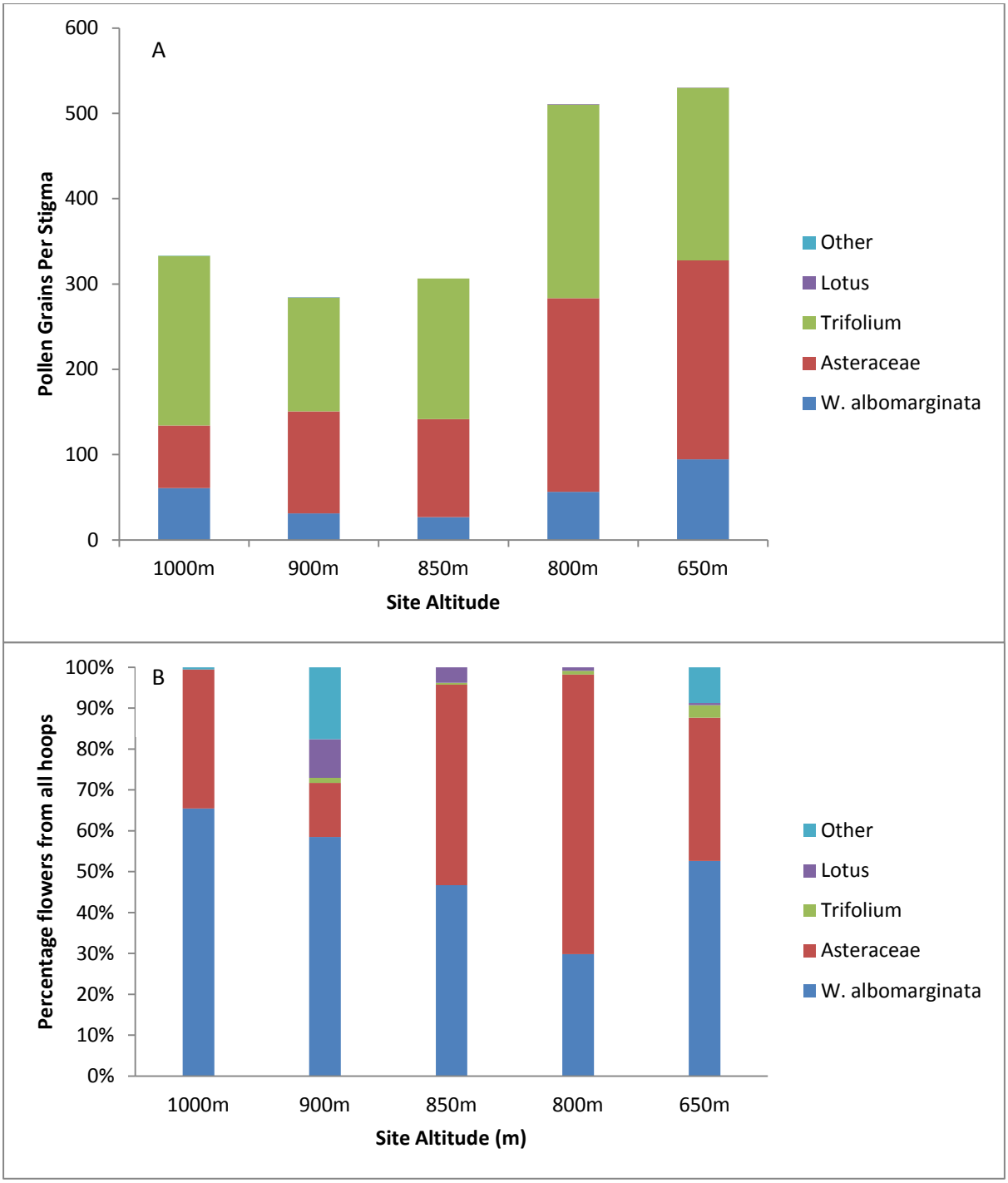


Fig.4.3.1.2A) Number pollen grains per stigma for each type at each site. B) Percentage of each flower type across all hoops at each site.

Table 4.3.1.1 One Way Analyses of variance in pollen types and conspecific: heterospecific pollen ratio among five study sites.

site	DF	SS	F	p
***W. albomarginata* pollen**	4	41598	2.03	0.103
	54	276502		
	58	318100		
Asteraceae pollen	4	316457	5.43	0.001
	54	786443		
	58	1102900		
***Trifolium* pollen**	4	55588	1.06	0.383
	54	705209		
	58	760797		
Total Pollen	4	845178	5.98	0.000
	54	1907277		
	58	2752455		
Heterospecific Pollen	4	552274	3.86	0.008
	54	1930408		
	58	2482682		
Conspecific:	4	0.648	0.70	0.594
Heterospecific Pollen	54	12.461		
	58	13.108		

Table 4.3.1.2 Replicates, Mean Standard Deviation and Tukey's grouping of pollen types from each site.

Pollen Type	Site	N	Mean	Standard Deviation	Tukey's Grouping
W. albomarginata pollen	**Top**	**11**	**59.43**	**48.03**	**A**
	Bank	**12**	**56.33**	**30.49**	**A**
	Rise	**10**	**28.45**	**22.19**	**A**
	Dip	**7**	**36.23**	**25.26**	**A**
	Bottom	**19**	**59.43**	**114.25**	**A**
Asteraceae pollen	**Top**	**11**	**79.3**	**70.9**	**A**
	Bank	**12**	**227.0**	**112.0**	**AB**
	Rise	**10**	**112.1**	**73.8**	**A**
	Dip	**7**	**90.7**	**86.3**	**A**
	Bottom	**19**	**245.9**	**167.4**	**B**
Trifolium pollen	**Top**	**11**	**199.9**	**141.0**	**A**
	Bank	**12**	**199.8**	**114.0**	**A**
	Rise	**10**	**158.9**	**102.7**	**A**
	Dip	**7**	**126.7**	**80.1**	**A**
	Bottom	**19**	**218.7**	**113.0**	**A**
Total pollen	**Top**	**11**	**338.6**	**164.2**	**AB**
	Bank	**12**	**483.1**	**158.6**	**B**
	Rise	**10**	**299.5**	**137.5**	**AB**
	Dip	**7**	**254.9**	**153.3**	**A**
	Bottom	**19**	**563.2**	**241.5**	**B**
Heterospecific pollen	**Top**	**11**	**279.2**	**167.9**	**AB**
	Bank	**12**	**426.8**	**153.1**	**B**
	Rise	**10**	**271.1**	**126.4**	**AB**
	Dip	**7**	**218.7**	**150.3**	**A**
	Bottom	**19**	**464.7**	**248.2**	**B**
Conspecific: Heterospecific pollen	**Top**	**11**	**0.2880**	**0.2631**	**A**
	Bank	**12**	**0.1454**	**0.0844**	**A**
	Rise	**10**	**0.1254**	**0.1441**	**A**
	Dip	**7**	**0.1946**	**0.1541**	**A**
	Bottom	**19**	**0.3805**	**0.7945**	**A**

Table 4.3.1.3 Pearson's correlation values (above) and corresponding P values (below) showing between pollen types present on the same stigma across all five sites at The Blue Mountains. DF=74 Significant*

	Wahlenbergia pollen	*Trifolium* pollen	Asteraceae pollen
***Lotus* pollen**	-0.029		
	0.807		
Asteraceae pollen	0.127	-0.065	
	0.279	0.579	
***Trifolium* pollen**	0.198	-0.071	0.510
	0.088	0.547	0.000*

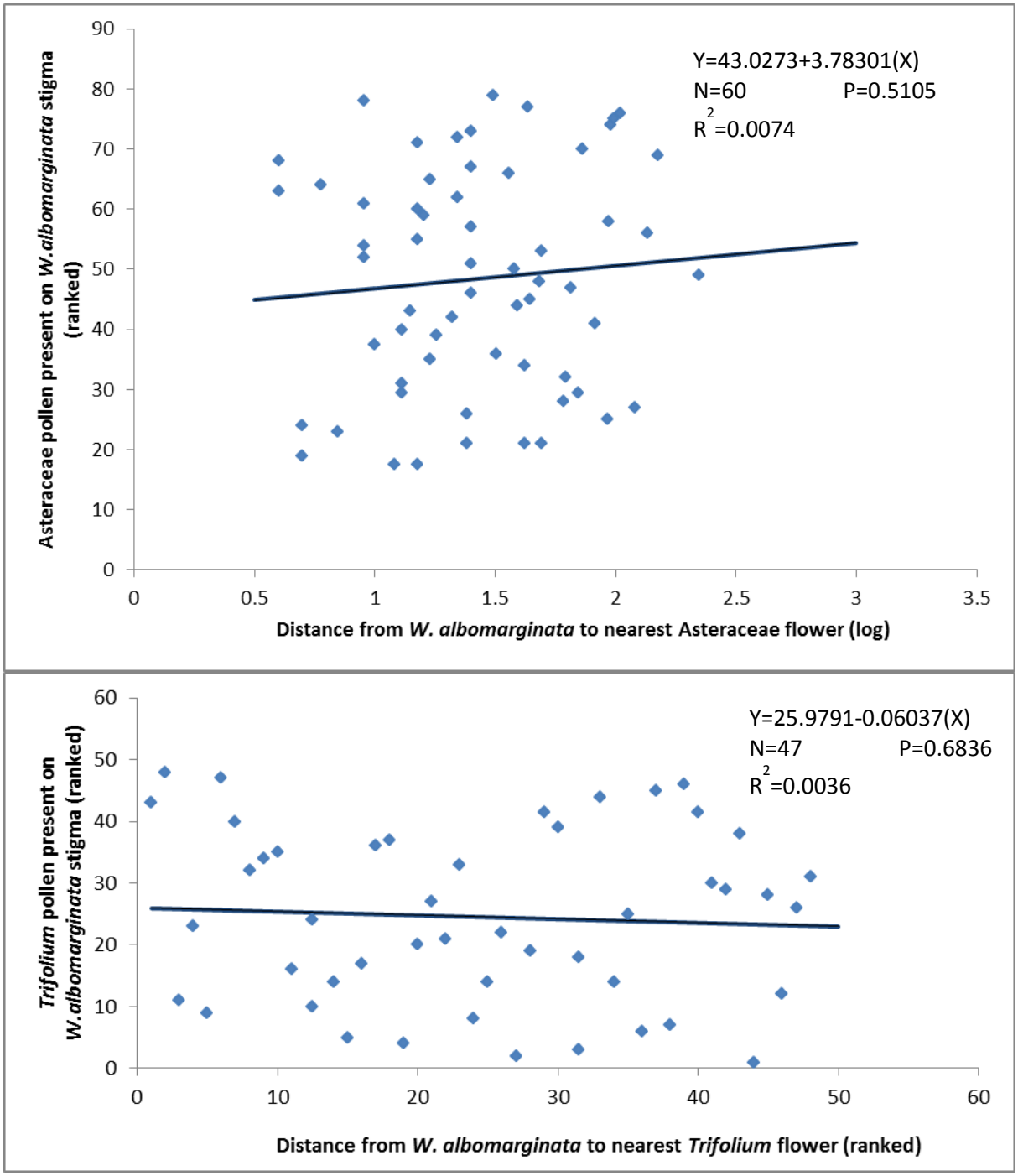


Fig 4.3.1.3. Leastwise linear regression showing the effect of proximity of the nearest *Trifolium* or Asteraceae flower within 7m to the amount of corresponding pollen present on the *W. albomarginata* stigma.

Table 4.3.1.4 Difference in Asteraceae, *Wahlenbergia* and *Trifolium* pollen loads caused by proximity to nearest *Trifolium* flower

Trifolium	DF	SS	F	P
Asteraceae pollen	2	144182	4.51	0.015
	59	942490		
	61	1086672		
***Wahlenbergia* pollen**	2	1398	0.13	0.881
	59	324282		
	61	325680		
***Trifolium* pollen**	2	2218	0.09	0.914
	59	729362		
	61	731580		

Table 4.3.1.5 Number of replicates, Mean and standard deviation of Asteraceae, *Wahlenbergia* and *Trifolium* pollen on *W. albomarginata* stigmas with three distance classes to the nearest *Trifolium* flower.

Trifolium	Distance Class	N	Mean	Standard Deviation
Asteraceae pollen	>700cm	14	79.7	61.2
	100-700cm	15	183.6	110.4
	<100cm	33	198.9	150.3
***Wahlenbergia* pollen**	>700cm	14	34.72	43.64
	100-700cm	15	68.39	60.84
	<100cm	33	60.09	88.04
***Trifolium* pollen**	>700cm	14	176.6	136.4
	100-700cm	15	194.1	95.1
	<100cm	33	184.9	106.2

Table 4.3.1.6 Difference in Asteraceae, *Wahlenbergia* and *Trifolium* pollen loads caused by proximity to nearest *Lotus* flower

Lotus	DF	SS	F	P
Asteraceae pollen	2	79530	2.33	0.106
	59	1007142		
	61	1086672		
***Wahlenbergia* pollen**	2	12509	1.18	0.315
	59	313171		
	61	325680		
***Trifolium* pollen**	2	525	0.02	0.979
	59	731055		
	61	731580		

Table 4.3.1.7 Number of replicates, Mean and standard deviation of Asteraceae, *Wahlenbergia* and *Trifolium* pollen on *W. albomarginata* stigmas with three distance classes to the nearest *Lotus* flower.

Lotus	Distance Class	N	Mean	Standard Deviation
Asteraceae pollen	>700cm	25	143.1	131.4
	100-700cm	12	130.7	112.0
	<100cm	25	211.5	137.6
***Wahlenbergia* pollen**	>700cm	25	55.43	52.01
	100-700cm	12	89.75	139.18
	<100cm	25	52.48	38.28
***Trifolium* pollen**	>700cm	25	181.8	120.2
	100-700cm	12	188.7	120.4
	<100cm	25	187.1	96.8

Table 4.3.1.8 Difference in Asteraceae, *Wahlenbergia* and *Trifolium* pollen loads caused by proximity to nearest Asteraceae flower

Asteraceae Distance	DF	SS	F	P
Asteraceae pollen	1	35502	2.03	0.160
	60	1051170		
	61	1086672		
***Wahlenbergia* pollen**	1	9042	0.34	0.559
	60	316639		
	61	325680		
***Trifolium* pollen**	1	72	0.01	0.939
	60	731508		
	61	731580		

Table 4.3.1.9 Number of replicates, Mean and standard deviation of Asteraceae, *Wahlenbergia* and *Trifolium* pollen on *W. albomarginata* stigmas with two distance classes to the nearest Asteraceae flower.

Asteraceae Distance	Distance Class	N	Mean	Standard Deviation
Asteraceae pollen	**>100cm**	**54**	**159.1**	**129.4**
	<100cm	**8**	**230.5**	**153.1**
***Wahlenbergia* pollen**	**>100cm**	**54**	**62.99**	**77.66**
	<100cm	**8**	**46.66**	**24.37**
***Trifolium* pollen**	**>100cm**	**54**	**184.9**	**108.4**
	<100cm	**8**	**188.1**	**124.4**

Table 4.3.1.10 One-Way Analyses of Variance showing the difference in pollen load in stigmas from sites characterised by large flowers and sites characterised by small flowers.

	Source	df	ss	F	P
***Trifolium* pollen load**	Size class	1	2185	0.17	0.682
	Error	385	4991138		
	Total	386	4993322		
***Wahlenbergia* pollen (Conspecific) load**	Size class	1	43837	7.19	0.008
	Error	385	2246771		
	Total	386	2390609		
Asteraceae pollen load	Size class	1	196330	9.83	0.002
	Error	385	7685721		
	Total	386	7882051		
Total pollen load	Size class	1	370419	7.90	0.005
	Error	385	18058447		
	Total	386	18428865		
Heterospecific pollen load	Size class	1	159398	3.59	0.059
	Error	385	17077526		
	Total	386	17236924		
Ratio Conspecific: Heterospecific pollen load	Size class	1	0.095	0.47	0.492
	Error	385	7.7371		
	Total	386	7.7466		

Table 4.3.1.11 Mean, standard deviation and number of replicates of pollen types, total pollen load and the ratio of conspecific: heterospecific from large flowered sites and small flowered sites

	Size	Mean	Standard Deviation	N
***Trifolium* pollen load**	**1**	**195**	**107.1**	**215**
	2	**199.8**	**121.8**	**172**
***Wahlenbergia* pollen (Conspecific) load**	**1**	**79.20**	**99.14**	**215**
	2	**57.78**	**37.74**	**172**
Asteraceae pollen load	**1**	**206.4**	**157.4**	**215**
	2	**161.1**	**118.1**	**172**
Total pollen load	**1**	**481.0**	**247.1**	**215**
	2	**418.7**	**170.9**	**172**
Heterospecific pollen load	**1**	**401.8**	**237.9**	**215**
	2	**360.9**	**170.5**	**172**
Ratio Conspecific: Heterospecific pollen load	**1**	**0.1666**	**0.1644**	**215**
	2	**0.1566**	**0.1068**	**172**

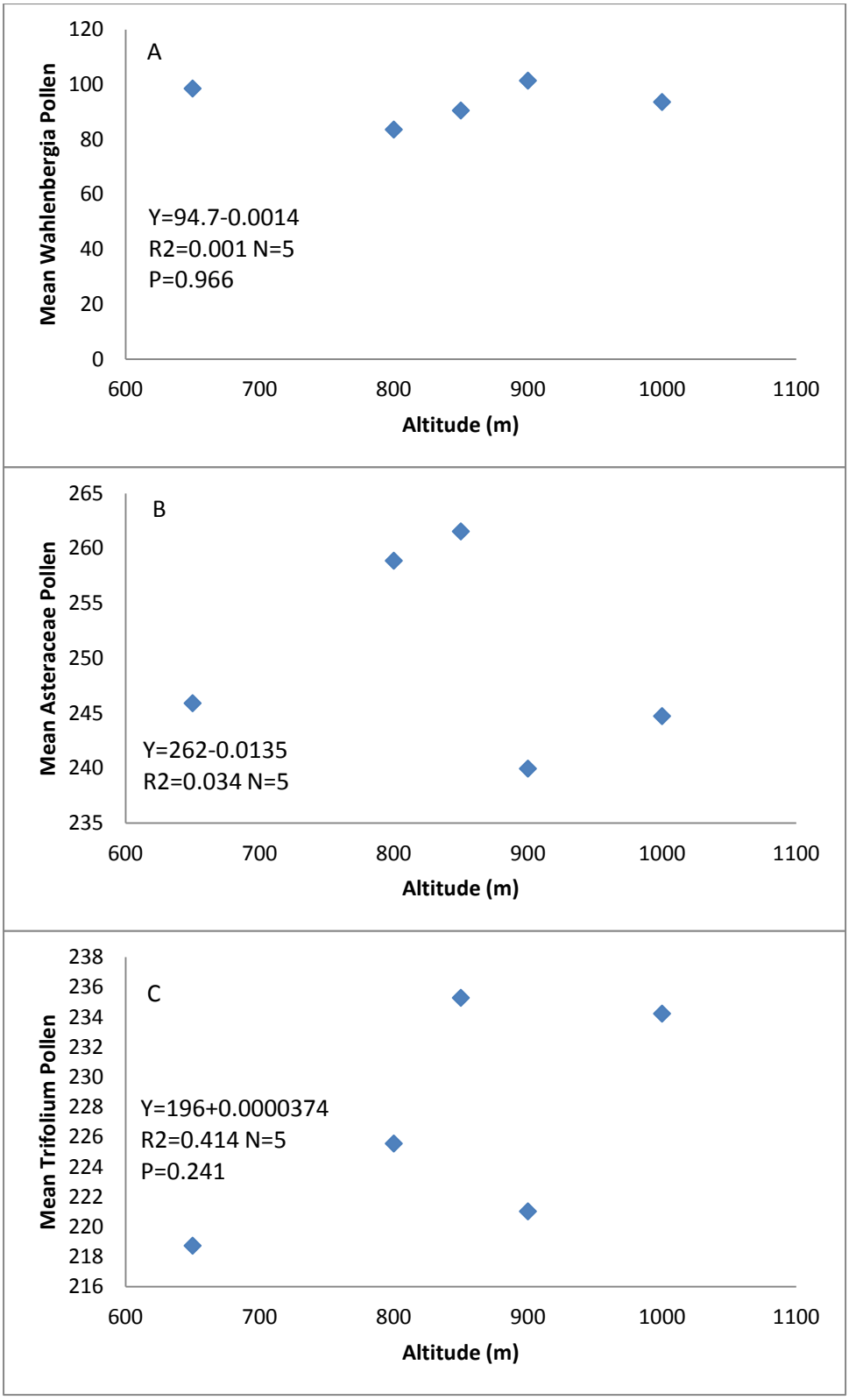


Fig 4.3.1.4 Least-Wise linear regression showing effect of site altitude on site mean (A) *Wahlenbergia* (B) Asteraceae and (C) *Trifolium* pollen present on *W. albomarginata* stigmas.

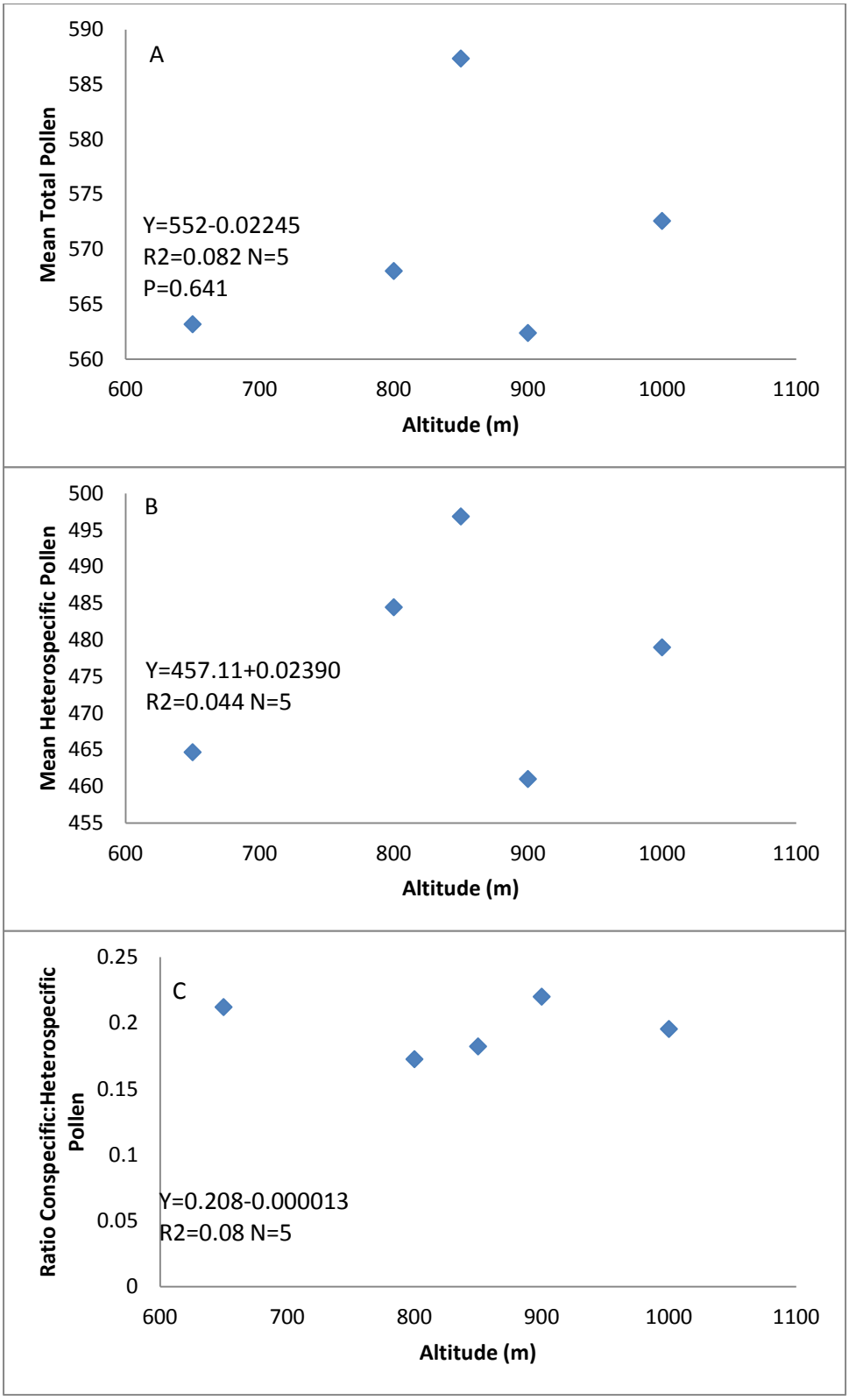


Fig 4.3.1.5 Least-Squares Linear regressions showing effect of site altitude on mean site (A) total pollen, (B) heterospecific pollen and (C) the ratio between conspecific and heterospecific pollen present on *W. albomarginata* stigmas at each.

4.3.2 Insect Visitation

The weather was overcast when stigma excision was undertaken at all of the five Blue Mountains sites. There was no pollinator activity at this time, however, pollinators were observed working flowers at the Tower and the Bottom sites on a separate occasion.

The most common flower present in the pollinator observation hoops was *H. radicata* with *H. pilosella* being the least common. Each flowering species had a unique distribution of pollinator types with a mixture of exotic and native pollinators servicing each plant species. Thrips observed on *W. albomarginata* would often stay at the base of the corolla tube for the entire 15 minute observation period. Syrphids were more attracted to the yellow dish shaped inflorescences of *H. pilosella* and *H. radicata* than they were to *W. albomarginata* flowers.

Native New Zealand bees (*Leioproctus* spp.) visited *W. albomarginata* and *H. radicata* the most, although they also visited *T. repens, L. corniculatus* and *Euphrasia* sp. The second most common airborne visitor of *W. albomarginata* was the introduced honeybee *Apis mellifera* L., which also visited *T. repens.*

Though present in the area, bumblebees (*Bombus* spp. L.) and *Argyrophenga antipodum* Doubleday seldom visited *W. albomarginata.*

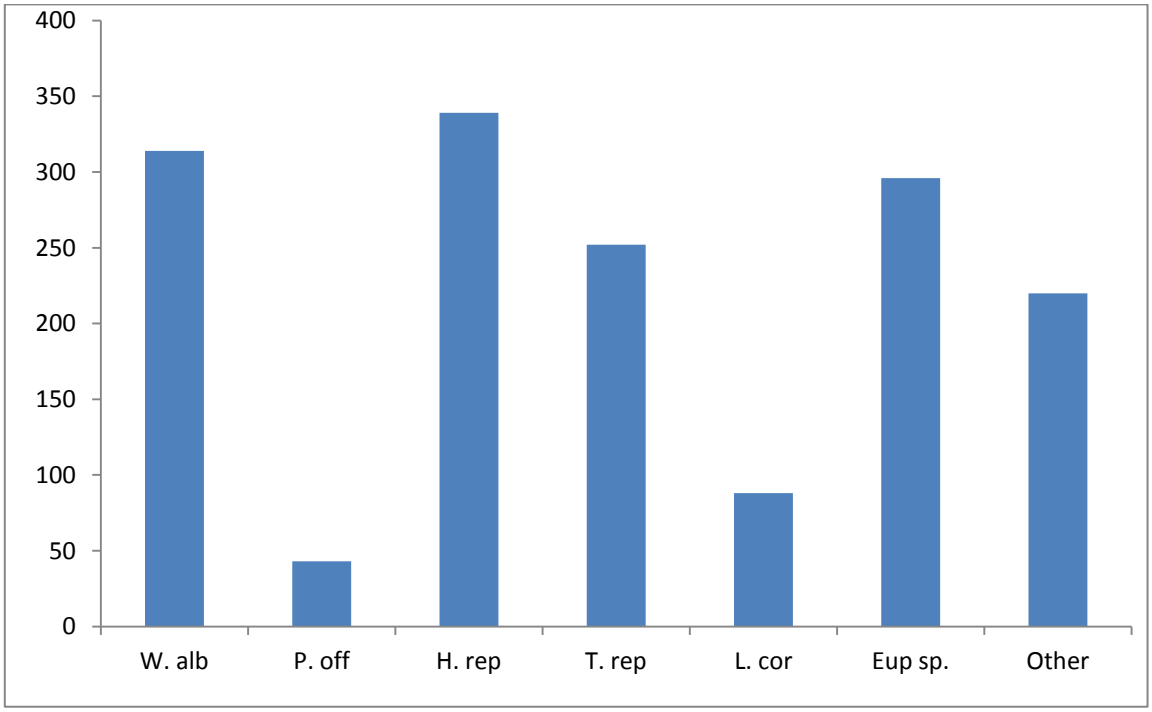


Fig. 4.3.2.1 Total number of *W. albomarginata, H. pilosella, H. radicata, T. repens L. corniculatus* and other flowers to occur in forty quadrats in both study sites.

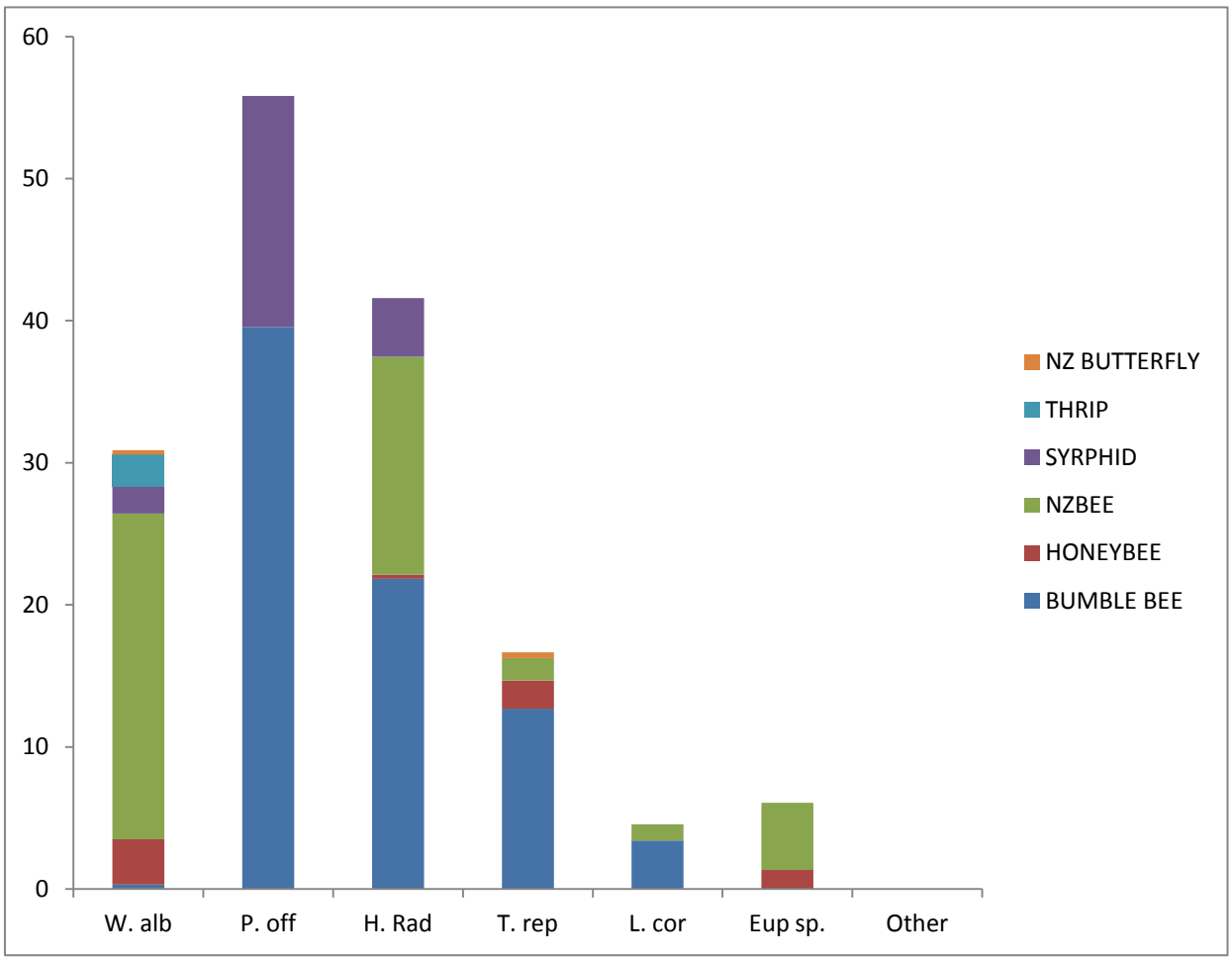


Fig. 4.3.2.2 Number visits per flower by each pollinator type to *W. albomarginata, H. pilosella, H. radicata, T. repens L. corniculatus* and other flowers over forty quadrats in both study sites.

4.4 Discussion

Despite an array of flowering species within a 100m radius, almost all stigmas excised had Asteraceae, *Trifolium* and *W. albomarginata* pollen only. Of these three main pollen types present, *W. albomarginata* was the least common. The most common pollen type present on *W. albomarginata* stigmas was Asteraceae. Nearby Asteraceae flowering species included *H. radicata* and *H. pilosella* in the immediate flowering neighbourhood and *Celmisia gracilenta w*ithin a 100m radius. It is likely that the majority of the Asteraceae pollen originated from *H. radicata* because this was the second most visited species by *W. albomarginata's* most common pollinator type, *Leioproctus* spp., while *H. pilosella* was the least visited by *Leioproctus spp*. *Trifolium* pollen was far more common than *Wahlenbergia* pollen, despite the fact that flowers only occurred within 50cm of *W. albomarginata* in 12% of quadrats. Although some native bees were recorded on *T. repens* flowers, it is not likely they made contact with anthers and collected pollen as they are short tongued. Given that the main *W. albomarginata* and *T. repens* pollinators observed did not significantly overlap, it may be that fewer *W. albomarginata* pollen grains adhere to exotic insects per visit than those of *T. repens,* so relatively more *T. repens* pollen grains are delivered to stigmas per visit than *W. albomarginata* pollen grains.

Overall, the strongest determinant of *W. albomarginata* pollen load was flower size. This affected the total pollen delivered, but not the ratio between conspecific and heterospecific pollen. With *Polemonium viscosum,* it was found that insects prefer larger flowers, probably because they contain more nectar than smaller flowers: reward/advertisement combinations are important determinants of insect behaviour, particularly in sophisticated insects like bumblebees (Cresswell and Galen 1991). Flower size was not measured directly for the flowers from which stigmas were excised as individual flower size was suspected to be a function of age. Instead, sites were pooled into two groups that had supplied the largest and smallest flowers (Table 4.3.1.10). It remains unclear whether the conditions that support larger flowers also support greater insect activity or whether larger *W. albomarginata* flowers are superior pollinator attractors.

The results show that *W. albomarginata* pollen is evenly deposited throughout its range at the Blue Mountains. While the primary pollinator observed at the Blue Mountains in January and February 2011 was a New Zealand solitary bee, it is unclear whether this pollinator was accountable for the majority of *W. albomarginata* pollination through-out its altitudinal range (Fig 4.3.2.2). It is important to note that flower characteristics were not affected by altitude in a consistent way among the locations (see Chapter Three). While examination of pollen on stigmas from The Remarkables and Pisa Range was outside the scope of this study, it may prove interesting to contrast pollen loads on an altitudinal gradient from these larger mountains with the Blue Mountains results

at hand. The Remarkables, which exhibited the only altitudinal flower size trends of the study, and had the widest variation in flower size and colour, would be of particular value to revisit to examine pollen loads (Chapter Three). Contaminating pollen from *Ourisia caespitosa* Hook. f., Asteraceae, and *Gaultheria nubicola* D.J. Middleton was found on *W. albomarginata* stigmas near the Ski Lodge site at The Remarkables (Bischoff 2008). The effect of greater variation in flower networks over *W. albomarginata's* range at the Pisa Range and Remarkables could be compared with the contrasting flower size response to altitude.

Significantly less conspecific pollen was delivered to *W. albomarginata* stigmas at the 850m site than the 1000m site, but no difference in heterospecific pollen deposition. This was despite a similar density of *W. albomarginata* flowers among sites. It would appear that the pollinators at this site were more generalised. There was significantly less Asteraceae pollen delivered to stigmas at the top site than the bottom two sites. At the 800m site, this could be explained by the high percentage of Asteraceae flowers at that site. However this explanation cannot be used to explain the significant difference between the sites at 1000m and 650m as very similar population based percentages of Asteraceae plants were recorded at both sites. It is likely that different pollinator behaviour at the two sites has caused this difference; however, there is insufficient data on pollinator behaviour at each site to comment further. It is likely that these site specific differences in pollination will reduce the likelihood of selective pressure by animals on plant traits (Herrera 1995).

Hieracium is known to influence the soil pH, organic matter and nitrogen through biological nitrogen fixation in New Zealand tussock grasslands (Saggar et al. 1999). Changes in habitat resulting from such components of an ecosystem are more likely to cause a permanent change in the structure of an ecosystem than competition alone. In addition, invaders are likely to have indirect effects on ecosystem processes such as complex food webs, decomposition and pollination (Brockerhoff et al. 2010)

W. albomarginata's most common visitor appears to be the native solitary bee (*Hylaeus* sp. at The Remarkables (Bischoff 2008) and *Leioproctus* sp. at the Blue Mountains). *Leioproctus* is a New Zealand native insect and was observed visiting complex exotic inflorescences such as *H. radicata* and *T. repens* as well as the relatively simple flowers it co-evolved with. Observed visits to *T. repens* are particularly interesting as native bees theoretically should not be able to reach rewards inside *T. repens* flowers. This requires further investigation. Native flowers were attracting incidental visits from exotic social insects such as honeybees. Such a high percentage of exotic pollen per stigma does beg the question: are exotics impeding successful pollination events in *W. albomarginata?*

The high proportion of exotic pollen on *W. albomarginata* stigmas could cause stigma clogging. This is where heterospecific or selfing pollen occurs on stigmas, and consequently impedes seed set (Jakobsson et al. 2008). In future, this could be investigated in *W. albomarginata* using artificial pollination techniques.

The results from this study indicate that neither the number of immediately neighbouring Asteraceae, *W. albomarginata* or *T. repens* flowers nor the different floral mosaics associated with different sites significantly affected pollen amount, diversity or conspecificity on stigmas. Half of New Zealand *Hylaeus* bees' bodies do not have pollen after flower visitation at The Remarkables (Bischoff 2008). There was no relationship between frequently occurring pollen types on stigmas and the distance to the nearest corresponding flower. This study's data shows that the most recently visited plants do not strongly affect the type of pollen delivered to the stigma, which indicates pollen adheres equally well to insects over medium and longer range distances as it does to insects over shorter distances. This is a positive result for *W. albomarginata* fitness, because it means the chances of inbreeding depression are reduced.

Proximity to Asteraceae and *Lotus* plants did not significantly affect the pollen loads present on *W. albomarginata* stigmas. This indicates that insects are active over a greater distance than 7m and do not visit plants sequentially. The increase of Asteraceae pollen in flowers adjacent to *Trifolium* plants was unexpected but may indicate that the presence of *T. repens* is drawing exotic pollinators from a wider area which have a mix of exotic pollen on their bodies.

Small *Hylaeus* bees are thought to habitually travel 100m – 200m per day, and sometimes as much as 1000m (Zurbuchen et al. 2010). Even outside the Asteraceae and *W. albomarginata* dominated immediate floral neighbourhood and defined sites in this study, *W. albomarginata* pollinators almost exclusively deliver *W. albomarginata, Trifolium* and Asteraceae pollen. This was despite the blossoming of exotic *Euphrasia* sp*. Digitalis purpurea* and *Lotus corniculatus that was* observed throughout the Blue Mountains at the time of collection. Therefore, insect visitation patterns of *W. albomarginata* pollinators are not completely random and unspecialised at The Blue Mountains. This is in agreement with findings that New Zealand alpine pollinator- flower networks are not random assemblages as previously thought (Bischoff 2008).

The most common pollinator type in the Blue Mountains was NZ native bee. This is also the case at The Remarkables, where *Hylaeus* is responsible for the vast majority of *W. albomarginata* pollination events, with some visitation from Syrphids (Bischoff 2008). *W. albomarginata* had the least pollinator types of that study; however my results show exotic social bees did make occasional visits

to *W. albomarginata* when exotic flowers were present nearby. While attempts at pollinator observations on an altitudinal gradient were not possible due to unfavourable weather conditions, this would be a valuable topic for future investigation.

While the vast amount of pollen observed on *W. albomarginata* came from neighbouring exotic species, the type of plants *W. albomarginata* is growing near to does not appear to strongly affect total pollen delivery or the ratio of conspecific to heterospecific pollen. This supports findings that pollinators do not discriminate against exotics that have been present in a system for a long time (King and Sargent 2012). However, the high rate of interspecific pollination in this solitary bee-dominated system contrasts with the low rate of interspecific pollination by bumblebees between a long-term invasive species and its native congener (King and Sargent 2012). Contrary to previous findings, surrounding flowers did not appear to affect a flower's chance of being visited successfully (Ghazoul 2006). It is important to stress that these studies concentrated on insect visitation whereas this Blue Mountain study concentrated on the final result of pollen load. It is possible that *W. albomarginata* female phase flowers are more likely to be visited in species diverse arrays, however, the fact that pollen load is not affected means this has no impact on selective pressures.

To summarise, sites that support large flower size also support larger total pollen delivery than sites that have smaller flower size. The sites that support large flower size either house more insects, or the insects at these sites find *W. albomarginata* more attractive to visit. Surrounding vegetation patterns and altitude held little bearing on pollination outcomes; however, this should also be investigated on a range of sites on larger mountains, where these factors would be more variable.

5

Effect of Altitude and Location on Seed Production

"LET ME SEE: FOUR TIMES FIVE IS TWELVE, AND FOUR TIMES SIX IS THIRTEEN, AND FOUR TIMES SEVEN IS- OH DEAR! I SHALL NEVER GET TO TWENTY AT THAT RATE!"

(CARROLL 1865)

5.1 Introduction

The environmental constraints associated with increasing altitude undoubtedly affect the evolution of reproductive strategies in plants (Fabbro and Körner 2004, Tian et al. 2011, Wig et al. 2011). Many environmental factors vary with altitude including temperature, UVB exposure, cloud and fog cover, human activity, plant community composition and pollinator types, however, some environmental factors such as precipitation and soil nutrient availability may vary from site to site but are not necessarily linked to altitude (Körner 2007). Plant growth rates are slow at high altitude so different trade-offs occur in biomass allocation than at low altitude (Fabbro and Körner 2004, Körner 2004, Falster et al. 2006).

Allocation of resources to seed number and mass is affected by a vast array of environmental factors linked to physiological stress including drought, sunlight availability (Westoby et al. 1992) growing season (Baker 1972, Wang and Smith 2002) and latitude (Ackerly et al. 2006, Wright et al. 2009). Studies of taxa across a wide altitude range have found an negative relationship between seed mass and number, where seeds decrease in size at high altitude, in favour of retaining high seed output (Baker 1972). Heavier seeds within a species have more nutritional reserves so seedlings are more likely to be viable (Vera 1997), therefore reduction in seed size with increasing altitude is likely to come at a cost to viability. Seed number per capsule is highly plastic and can be affected by pollen limitation, drought (Duthion and Turc 1994), temperature(Wilen and Bonham-Smith 2004) and wind (Ortega-Olivencia et al. 2010). Accessory costs to reproduction have been shown to be proportional to seed mass across a number of species (Lord and Westoby 2006). The question remains whether changes in investment in flowers and rewards translate into equal seed set and capsule mass across the altitude gradient.

In theory, biotically pollinated plants in cold temperate regions should invest relatively more in floral displays with increasing altitude (Fabbro and Körner 2004) and produce smaller seeds (Molau 1993). The ratio of floral mass to shoot mass is greater at higher elevation across a number of European taxa, possibly to compensate for lower pollinator abundance and diversity (Fabbro and Körner 2004). *W. albomarginata* flower size can decrease at higher altitude (Chapter 3), however, pollen delivery to stigmas is not affected (Chapter 4). *W. albomarginata* seldom reaches a full seed set of 380 per capsule at 1650m at The Remarkables (Bischoff 2008). Environmental pressures associated with high altitude, as well as different geographic locations, may affect seed set by triggering seed abortion or reduced allocation to capsule mass.

Geographic isolation between mountain ranges can result in a widespread alpine species being exposed to an array of differing environmental factors such as soil type, climate, disturbance, plant community composition, predators and pollinator species. Plant gene flow between mountains can be limited where pollination and seed dispersal modes are localized. Isolated selection pressures are more likely to differentiate genotypes suited to a certain area. A continuous gradient such as altitude with no geographical isolation would be more likely to exhibit phenotypic plasticity. The environmental factors that vary between different mountain ranges could affect the quantity and mass allocation to seed production, effectively selecting for phenotypic differences between locations.

The primary aim of this chapter was to determine whether the number of seeds per capsule and/or capsule mass relate to altitude in *W. albomarginata.* There are many possible explanations for trends in plant morphological characteristics along altitudinal gradients. This chapter investigates how soil moisture and solar irradiation change with altitude at The Remarkables to determine whether these factors can explain variation in seed production.

The second aim of this chapter was to quantify how much capsule mass and/or seeds per capsule varying between distinct populations, by comparing seeds from the Blue Mountains and The Remarkables.

The final aim of this chapter was to determine whether capsule mass can be used as an indicator of seed number in *W. albomarginata*.

5.2 Methods

Fresh, mature undamaged capsules were selected from a range of altitude across six sites from The Remarkables and 20 patches at the Blue Mountains in March 2011. Mature capsules were identified as being over three times wider at the top than the base, often with visible indentations caused by the inside. Capsules were fleshy and ranged from green to brown with dark red vertical stripe-like pattern. At The Remarkables, 24 capsules were collected at six roadside north-east facing gravelly sites on an elevation gradient. The sites were 1632m, 1484m, 1365m, 1182m, 1092m and 999m. All sites on steep faces ranging in slope from 30º-55º. Solar irradiation was measured approximately 10cm from the ground at each of The Remarkables sites with a LI-COR LI-250 solar irradiance meter. Five measurements were taken with an Oakton soil temperature probe at each site at The Remarkables. Suitable capsules were selected randomly at each site by walking seven paces then picking the nearest one. At the Blue Mountains, up to five seed capsules were collected per patch from twenty patches between 1001m and 814m. Patches were grouped into two altitudinal classes: 814m – 830m (lower) and 990-1001m (upper). Patches over 7m apart were deemed to be distinct because it was assumed rhizomes would not reach further beyond the central root system than this. When there were more than five suitable capsules in a patch, capsules were assigned numbers, and selected at random. All patches were on level ground next to an infrequently used dirt road.

Capsules were stored separately in numbered eppendorf tubes for up to three days then dried in separate labelled paper bags in a 40°C oven for a fortnight. Capsule biomass was recorded and seeds per capsule were counted by tipping the contents of the capsule and tube into a Petri dish with a grid at the base and inspecting with the naked eye. Aborted seeds occurred only occasionally and were easy to identify by their distinctive dark shrivelled coats and because they were less than half the size of filled seeds. These were not included in the final total. Twenty individual seeds were weighed to an accuracy of 0.001g

The effect of site on capsule mass and seeds per capsule was analysed using one-way analysis of variance and Tukey's Test for The Remarkables Range because sites were distinct. An outlier capsule of 0.065g and 416 seeds at The Remarkables 1632m site was omitted from statistical analyses. Effect of site on capsule mass and seed set was measured at the Blue Mountains. A one way analysis of variance was used to test the effect of locality on seed set, capsule mass and the relationship between the two. Standardised major axis regression was used to determine if there was a functional relationship between the number of seeds per capsule and capsule mass. This was done for each location and both data sets combined. All variables were tested for normality and homoscedasticity prior to analysis and transformed when not normal. Regressions and ANOVAs

were performed using the analytical software Statistix 9.0 © (Analytical Software 2008). The standardised major axis regression was performed using SMATR 2.0 © 2006 (Falster et al. 2006).

5.3 Results

A total of 47 capsules were collected and weighed from the Blue Mountains and twenty five from The Remarkables. 9776 and 5571 seeds were counted at the Blue Mountains and Remarkables respectively. All seeds weighed approximately 0.002g. Scales were not sufficiently sensitive to weigh more accurately than this. The average capsule mass and seed number was similar between the two locations.

Table 5.3.1 Mean, SD (standard deviation), maximum and minimum of mass and number of seeds contained in each capsule, as well as the capsule mass per seed.

Location	Variable	Mean	SD	Maximum	Minimum
Remarkables	Seed Capsule mass(g)	0.0387	0.01299	0.065	0.019
	Capsule mass per Seed (g)	1.8×10^{-4}	4.35×10^{-5}	3.243×10^{-4}	1.186×10^{-4}
	Seed Number	222.84	85.4165	416	85
Blue Mountains	Seed Capsule mass(g)	0.0386	0.01565	0.095	0.01
	Capsule mass per Seed (g)	2.047×10^{-4}	9.37×10^{-5}	5.893×10^{-4}	9.565×10^{-5}
	Seed Number	208	90.5349	410	19

At The Remarkables, there were significantly more seeds per capsule and higher capsule mass at 1365m than most of the other sites, however, allocation to capsule mass per seed did not differ significantly between sites (Table 5.3.2). There was no significant difference in capsule weight, seed number or capsule weight per seed between the two locations (Tables 5.3.3). Capsule and seed characteristics did not differ between the two sites at the Blue Mountains (Fig 5.3.3).

Both of the quantified environmental factors altered with altitude. Soil temperature decreased significantly with increased altitude at The Remarkables (R^2=0.9501). Sunlight also decreased significantly with altitude; however, solar irradiance at the uppermost site did not follow the trend (Fig 5.3.2). Solar irradiance and soil temperature of site 1365 did not differ significantly from the other points.

There was a functional relationship between capsule weight and seed number for both sites, fitting a slope of 6.117 (Figure 5.3.4). There were no significant shifts between data from the two locations, in elevation or along the common slope (Figure 5.3.4; Table 5.3.4)

Table 5.3.2 One-way ANOVAs showing difference in capsule mass, mass allocation per seed and seeds per capsule between the different altitudes at The Remarkables. A) Capsule mass per site B) Capsule mass allocation per seed site. C) Seed number per capsule by site comparing log seed number of each Remarkables site.

		DF	SS	F	P
A)	**Capsule Mass**	5	2.286×10^{-3}	7.95	0.0004
Error		18	1.035×10^{-3}		
Total		23	3.322×10^{-3}		
B)	**Mass per seed**	5	0.04970	1.02	0.4362
Error		18	0.17580		
Total		23	0.22550		
C)	Seeds per Capsule	5	0.39244	9.39	0.0002
Error		18	0.15042		
Total		23	0.54286		

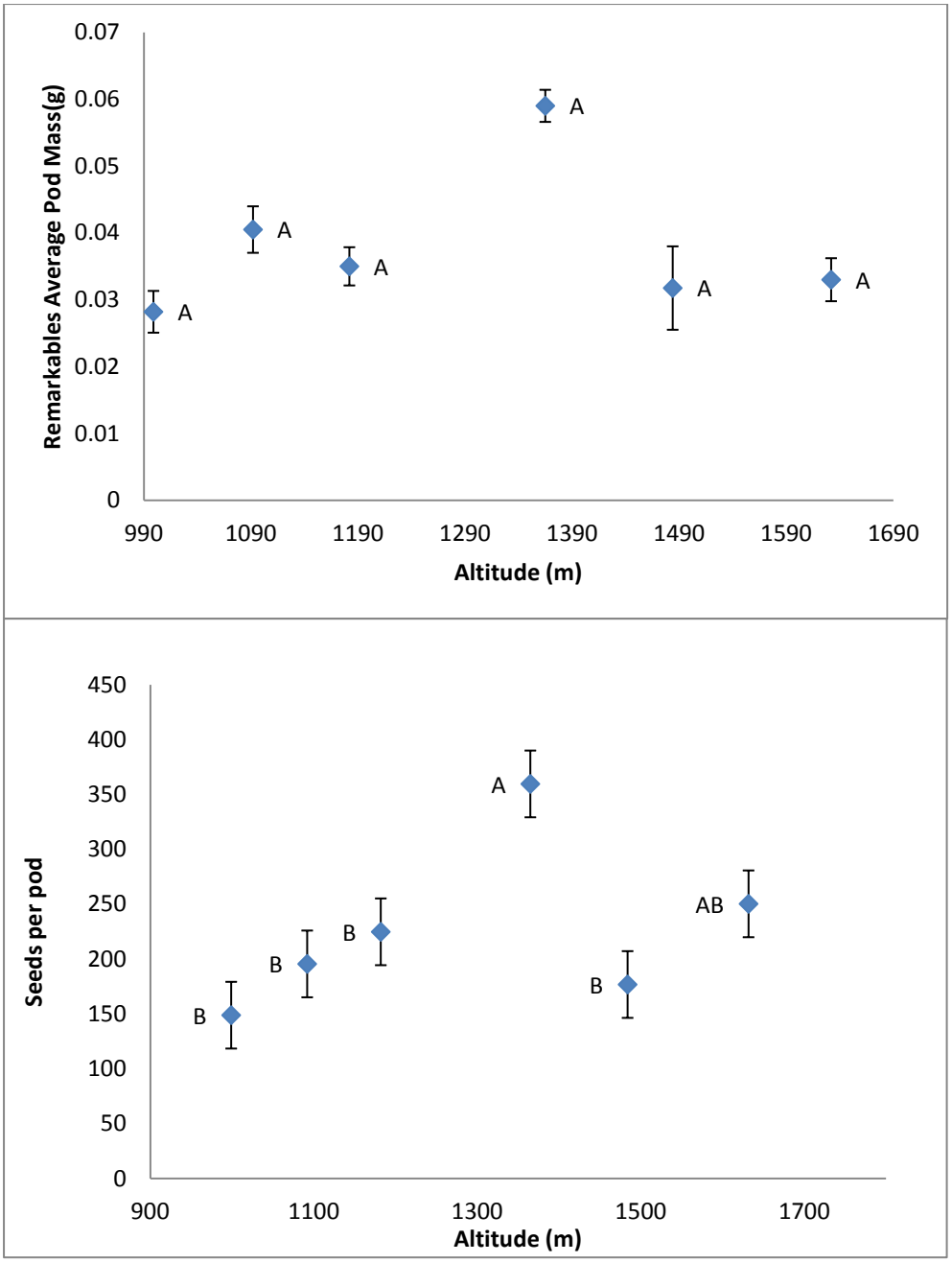


Fig 5.3.1. Effect of altitude on capsule mass (above) and seeds per capsule (below) at The Remarkables Range with standard error (Outlier omitted).

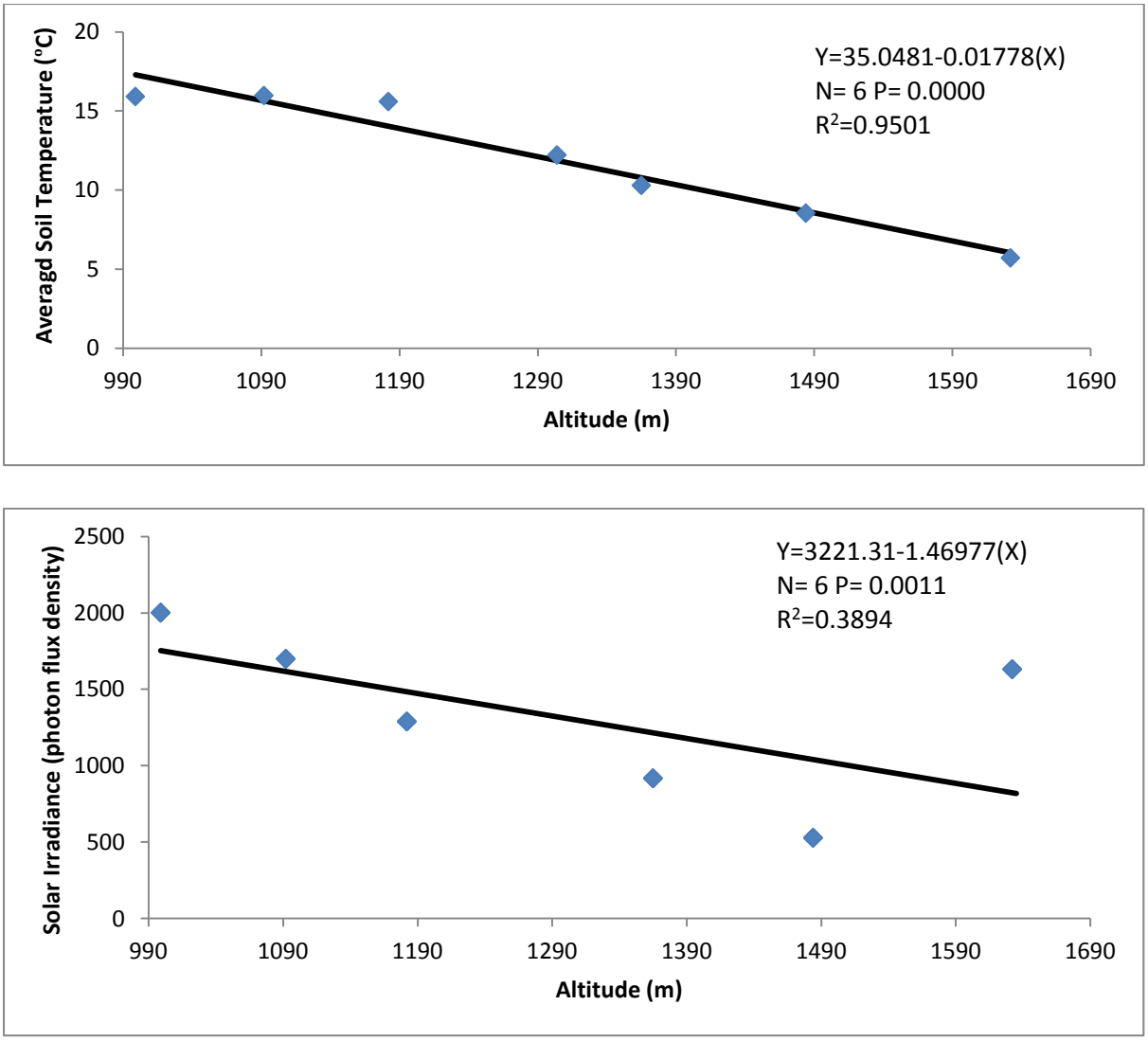


Fig 5.3.2. Linear regression showing affect of altitude on averaged soil temperature (above) and solar irradiance at The Remarkables.

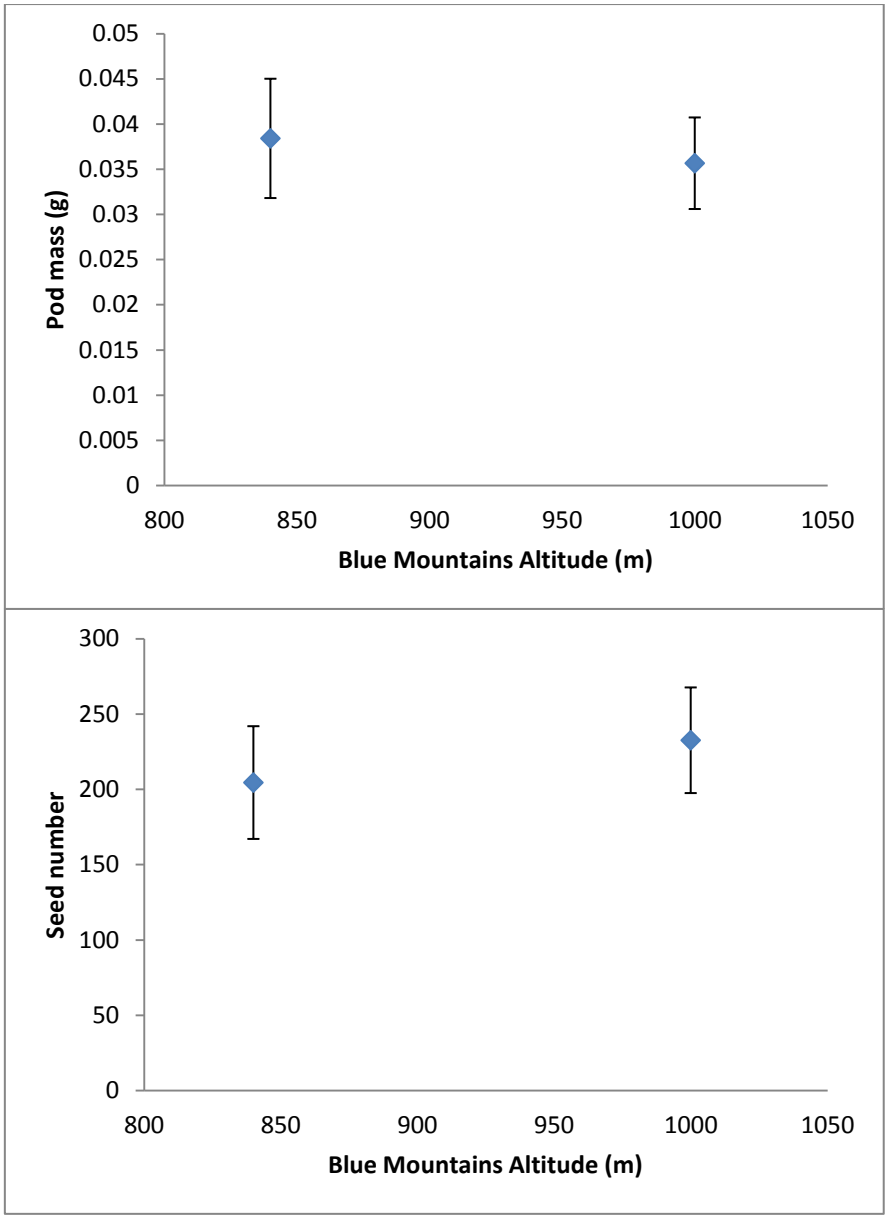


Fig 5.3.3. Effect of altitude on Capsule mass (above) and seeds per capsule (below), at The Blue Mountains.

Table5.3.3 One-way ANOVA showing difference in seed number, capsule mass and capsule mass per seed between The Remarkables and The Blue Mountains

Variable		DF	SS	MS	F	P
Seed Number	Between	1	733	732.83	0.10	0.7546
	Within	69	513280	7438.84		
	Total	70	514013			
Capsule Mass	Between	1	0.00001	5.82 x10-6	0.03	0.8686
	Within	69	0.01458	2.11 x10-4		
	Total	70	0.01459			
Capsule Mass per Seed	Between	1	9.119 x10-9	9.119 x10-9	1.40	0.2404
	Within	69	4.487 x10-7	6.502 x10-9		
	Total	70	4.578 x10-7			

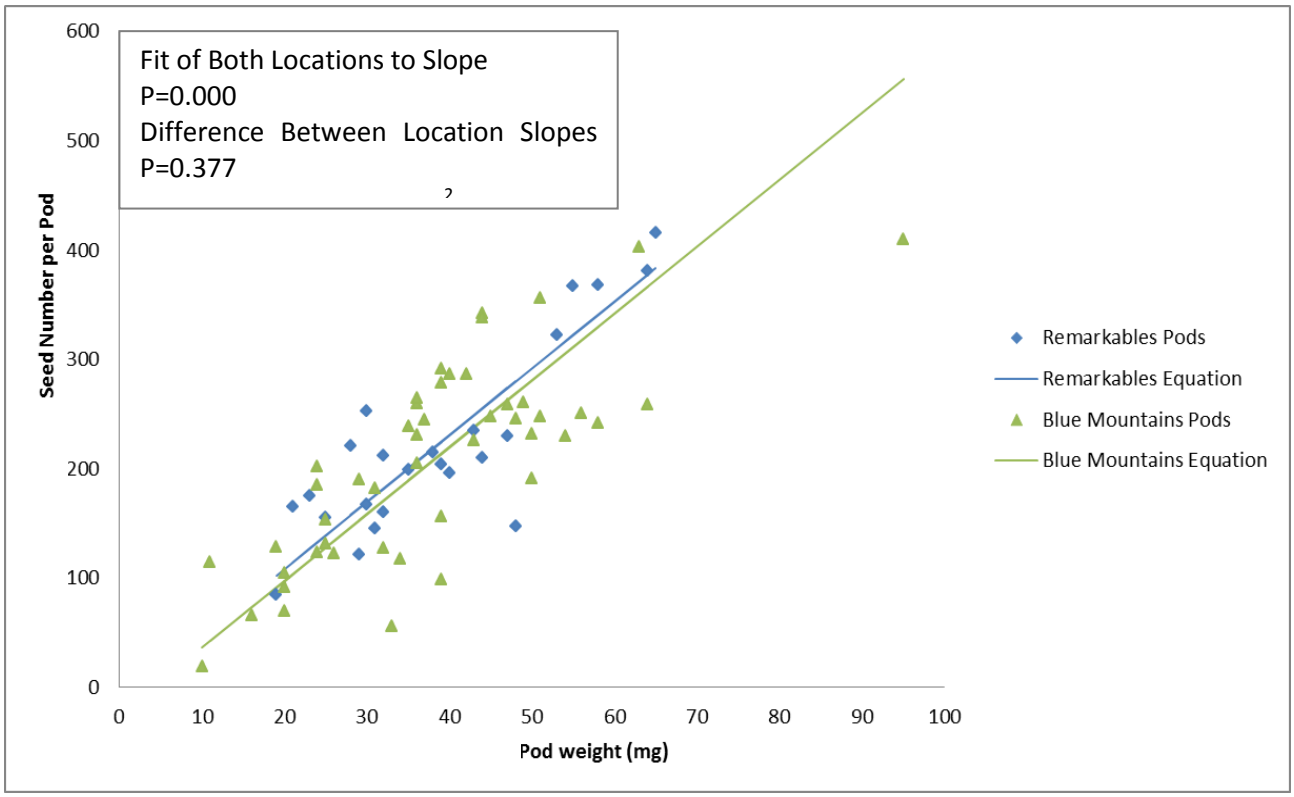


Figure 5.3.4 Model II standard major axis regression test for common slope across combined location datasets. Locations have unique intercepts.

Table 5.3.4. WALD statistic test for shifts between locations in elevation and along common slope.

Shift	Degrees freedom	Stat	P	A com
Elevation	1	0.700	0.403	-19.776
Common Slope	1	0.198	0.657	447.789

5.4 Discussion

W. albomarginata is found in a wide range of environments, across which many environmental factors change. Reproductive output at The Remarkables varied between sites, but this did not relate to altitude, or to any of the measured environmental variables. Maximum seed production was instead found at one mid altitude site (1365m at The Remarkables) which had significantly higher seed set and capsule mass than the other Remarkables sites. The data for soil temperature, soil type, slope, aspect and sunlight collected in this study did not predict for this point to be an outlier, however, there could be further differences in the effectiveness of pollination or other abiotic factors that may have led to this site being optimal. The altitudinal range and optimal altitude for plant species can be predicted by the balance between physiological stressors associated with high altitude and rapidly growing competitors associated with lower altitude (Theurillat and Kienast 1998). A number of factors may be contributing to a less stressful environment at 1365m and consequently, highest seed set occurred at mid range. For example, it is likely that mid altitude sites on The Remarkables have higher soil moisture than the drought-prone lower altitudes, but experience higher average temperature than higher altitudes.

Capsule mass per seed was not affected by altitude at The Remarkables (Table 5.3.2) because capsule mass was a predictor for seed number (Fig 5.3.4). Seed mass often decreases with altitude in a variety of taxa (Graf, Gugerli et al. 2010; Tian, Ren et al. 2011; Wig, Pico et al. 2011). *W. albomarginata* seed mass was not analysed because they were too small to be measured accurately. This study appears to be unique in measuring capsule mass allocation per seed instead. This alpine species prioritises plasticity in vegetative parts (See Chapter Three) and maintains reproductive allocation across multiple stages of reproduction from flowering to seed set.

Both soil temperature and solar irradiation decreased significantly with altitude. The strongly significant decrease in soil temperature as altitude increased was consistent with expectations from the literature (Körner 2007). The decrease in solar irradiation at higher altitude could be expected because clouds and fog tend to occur more at higher elevation(Körner 2007). Cloud cover has a much greater effect on sunlight experienced by plants than altitude. On a completely clear day, higher sunlight dose would be expected at higher altitude. The sunlight data collected were single data points recorded over the course of an hour. It would be beneficial for future work on The Remarkables to include a more robust solar irradiance data set, with repeats on different days and times of day, consistent across the gradient.

Pollen delivery to *W. albomarginata* stigmas was very different between The Remarkables Ski Lodge site, which had mostly conspecific pollen and only native heterospecific pollen, and the Blue

Mountains, where a small proportion of pollen delivered was conspecific, and the rest came from exotic co-occurring species. Also, soil chemistry, elevation and human disturbance differed between the study sites on the Blue Mountains and Remarkables (Chapter 3). Yet there was no difference in total capsule mass or seed number between the two locations. There was also no significant difference in capsule mass per seed between the two locations. This illustrates a flexible reproductive strategy that maintains seed set despite exotic invasion. Small-seeded plants with flat rosettes are known to be good colonists of disturbed sites (Lavorel and Tremont 1995); such as the roadside banks *W. albomarginata* inhabits. While large shifts are occurring in New Zealand tussock grasslands due to domestication and extensive introduction of exotics in recent years, this finding suggests at least some native plants can integrate well into systems with exotic species.

It should be noted that seed set is only one of a number of ways to measure the affect of environment on reproductive output. Reproductive fitness is a plant's ability to produce viable offspring; the quantifiable variables that can influence this span from pollination of the parent to flower production of the offspring. Variables such as germination rate, survival rate, progeny biomass, and progeny flower production to quantify reproductive success combine to give an accurate indication of plant fitness (Sakai et al. 1989). Dispersal of seeds to viable habitats is also an influential factor in reproductive fitness. This is now quantifiable through the use of isotope ratio and molecular genetic marker techniques (Wang and Smith 2002). Examination of seed viability and dispersal effectiveness across an altitudinal gradient would be an interesting line of enquiry for future work, to see if physiologically stressed seedlings suffer a greater rate of mortality. This may involve studies of the effect of altitude and different site factors on overall reproductive output.

To conclude, *W. albomarginata's* capsule mass and seed set are equally good at the top and bottom of its range, which shows the different set of stressors exist at each are equally obstructive to successful reproduction. Seed set is best at the middle of its range, possibly because there is less physiological stress than high altitude and competition than low altitude. The environmental factors that favour seed set at mid altitude are unclear from the environmental data collected. Future work should examine how seed properties are affected by low altitude stressors such as allocation to vegetative parts due to weedy competitors and different predator types as well as abiotic stressors associated the effects of with high altitude.

6

Discussion and conclusions

'TUT, TUT, CHILD!' SAID THE DUCHESS. EVERYTHING'S GOT A MORAL, IF YOU CAN ONLY FIND IT. "

(CARROLL 1865)

6.1 Summary

This thesis aimed to investigate the affect of altitude on floral characteristics, and illustrate whether this coincides with changes in pollen deposition and seed set within a species. *Wahlenbergia albomarginata* Hook. was used as a model system because of its variation in floral size and colour, and wide altitudinal range. A second aim was to explain whether abiotic environmental factors, or flower age, could influence *W. albomarginata* flower colour.

W. albomarginata the size of male phase flowers is affected by uncertain environmental factors, which sometimes from a cline with altitude. Flower colour is not significantly affected by altitude but is bluer in younger flowers with restricted water availability. Changes in flower size with altitude do not coincide with a change in seed set or capsule mass. There is a linear relationship between the mass of a *W. albomarginata* capsule and the number of seeds it contains. Most pollen observed on stigmas at five sites 1000m and lower at the Blue Mountains was from exotic *Trifolium* and Asteraceae species. This is in stark contrast with findings of Bischoff (2008) at a higher altitude Remarkables site. While the type of neighbouring species clearly influence pollen type present on *W. albomarginata* stigmas, their density, and exact proximity to *W. albomarginata* bears no relationship to the proportion of their pollen present on stigmas. There is clearly potential for stigma clogging in *W. albomarginata* at lower altitude where its pollinators are attracted to *Trifolium* and Asteraceae flowers

Table 6.1.1 Summary of findings in Chapters Three, Four and Five. Affect of increase in environmental factors on reproductive characteristics at The Remarkables (Rem), the Pisa Range (Pisa), the Blue Mountains (Blue) and in the growth chamber (GC) represented by: < (significant decrease), > (significant increase), - (no significant change) and X (not tested).

	Altitude			**Soil Nitrogen**	**Soil pH**	**Water Regime**	**Flower Age**	**Gender phase**
	Rem	Pisa	Blue	Rem & Blue	Rem & Blue	GC	Rem	GC
Display Width	<	-	-	-	-	-	-	-
Corolla Height	<	-	-	-	-	-	-	-
Leaf Length	X	<	X	-	-	-	-	-
Display width/Leaf length	X	-	X	-	-	-	-	-
Corolla Height/Leaf Length	X	-	X	-	-	-	-	-
Petal 450:550 reflectance ratio	-	-	-	-	-	<	>	>
Total Pollen	-	-	-	-	-	-	-	-
Conspecific: Heterospecific Pollen	-	-	-	-	-	-	-	-
Seed Set	-	X	-	-	-	-	-	-
Capsule Mass	-	X	-	-	-	-	-	-

6.2 Effect of Altitude on Flower Characteristics, Pollen Delivery and Seed Set

Too often, ecologists generalise with respect to physical properties and ecological trends over altitudinal gradients (Körner 2007) . In chapter three, flower size, leaf size and the ratio between them was shown to vary within a single species in different ways on two neighbouring, similar sized mountains, decreasing with altitude on The Remarkables and not changing with altitude on the Pisa Range. It is important to recognise that topography, soil chemistry, shade and a host of other environmental factors vary between sites of equal altitude, so some mountains may affect plant characteristics in different ways as altitude increases. This study illustrates clearly, the importance of replication for studies of alpine gradients over various mountains, and that there are exceptions to many rules. Ultimately, data collected along altitudinal gradients will always reflect regional peculiarities as well as general altitudinal trends (Körner 2007).

W. albomarginata flower size significantly decreased with altitude at The Remarkables but not at The Blue Mountains or the Pisa Range. It is possible that microclimates exist some of the sites selected on the Pisa Range, obscuring the altitudinal trend, or alternatively that low precipitation at the Pisa Range means variation of soil moisture with altitude does not occur. Despite the lack of correlation with altitude for most of the variables tested, significant differences among sites within locations were still apparent. This suggests that flower size in W. albomarginata as well as other factors measured are affected by environmental factors, which are sometimes but not always linked with altitude. The most consistent pattern found in this thesis was that leaf length significantly decreased with altitude, yet contrary to findings for species on the European Alps, allocation to flower size relative to leaves did not significantly increase with altitude (Fabbro and Körner 2004). This suggests that *W*. *albomarginata* is not under more intense selection to attract pollinators at higher altitudes, as has been suggested to explain the pattern of variation seen in European alpine species.

Many studies have illustrated that flower size influences insect choices (Galen and Newport 1987, Nakamura et al. 1989, Eckhart 1991, Herrera 1995). However, insect choice alone is not sufficient to result in selective pressure on flower size. The evidence from chapter four of insect selection based on flower size targeted to outcome of insect visitation more directly, because it looks at between-site effects on pollen loads on stigmas rather just insect visitation in relation to flower size. The fact that seed set was not significantly affected by an altitudinal gradient where flower size was, indicates there is not a reproductive advantage to *W. albomarginata* producing large flowers, unless it is necessitated by competition with different flowers for pollinators at lower altitudes (Chapter five). A future study of the flower size choices of insects at the top and bottom of *W.*

albomarginata's range at The Remarkables may help illustrate the selective pressure for flowers to be smaller at high altitude, in order to maintain seed set.

Changes in altitude from 1000m to 650m on the Blue Mountains did not affect conspecific or total pollen loads. This result was unexpected because insect pollinator population size and activity levels are generally thought to reduce with altitude (Arroyo et al. 1982, Arroyo et al. 1985) and *W. albomarginata* does not possess characteristics such as long stigma receptivity, which can cancel this out (Bischoff 2008). Exotic pollinators such as bumblebees have been observed at the uppermost parts of the Blue Mountains, so it could be that this gradient was not sufficiently large to affect pollinator type. The fact that altitude did not affect seed set over a much wider altitudinal gradient can perhaps be explained by a combination of decreasing exotic species presence and insect activity with altitude. The fact that exotic pollen is frequently delivered to *W. albomarginata* stigmas at sites with increased insect activity could mean assumed advantages of inhabiting lower altitude sites for visitation frequency do not occur.

Responses of floral characteristics to environment did not significantly affect reproductive output via seed set. Seed capsules collected from the same Remarkables sites did not vary significantly in weight over the same altitudinal range. Given that *W. albomarginata* is pollen limited (Bischoff 2008), one would expect this to be an indication of pollinator service under ideal growth conditions. The fact that seed set did not change significantly with altitude suggests pollination success is equally good at high altitude with smaller flowers in more native-dominated habitats and at low-altitude exotic-dominated roadside sites with larger flowers. This result is consistent with a study of an alpine co-familial species, whose seed set was affected by topographical isolation but not altitude (Gugerli et al. 2009).

Seed set was greatest at the mid-altitude site on The Remarkables (1365m). This may have been because pollinator services were most favourable at this site. Higher pollinator activity or population at this site may have been contributed to by warmer temperatures that the upper most sites and moister conditions than the lower sites. Results showed that seed number can be predicted by capsule mass, meaning that although seed weight was too small to be measured directly at all sites, it was also most likely fairly uniform. This suggests allocation to seeds is limited not by pollen delivery to stigmas, but abiotic conditions that support or restrict seed development.

6.3 Effect of Abiotic Environment on Flower Colour

Overall, experiments in this study indicated that flower blueness can vary in response to A) flower age and B) soil moisture at the time of bud formation. Natural variation in soil nitrogen and pH did not appear to affect flower colour, however manipulated growth cabinet results indicate a link between moisture and flower "blueness". The effect of temperature on flower colour, and cumulative effect of temperature and water regime was not able to be quantified, due to plant fatalities; however a restricted water regime prior to and during the time of bud formation, was shown to significantly increase the petal blueness of individuals, despite ample soil moisture during actual flowering. The fact that this difference occurred between plants with previously similarly coloured flowers raised from the same seed lot is highly indicative of plastic changes in flower colour within individuals dependent on physical environmental factors. A similar phenomenon has been noted in *Plantago lanceolata* L. which is also anthocyanin pigmented. *P. lanceolata* produces a higher quantity of petal anthocyanin in the petals of cool-treated flowers than warm treated ones, with genotype controlling the degree to which flower colour changes (Stiles et al. 2007). The mechanism by which this might occur in *W. albomarginata* is uncertain, but it is known that anthocyanins, which behave as floral pigments in *W. albomarginata* (Campbell et al. 2012), are also expressed in response to physiological and mechanical stress in vegetative tissues (Gould et al. 2000, Gould et al. 2009, Gould et al. 2010). Future tests should evaluate the possibility that up-regulated leaf anthocyanin in response to physiological stress coincides with up-regulated anthocyanin in the buds of forming flowers, resulting in flowers that are bluer. Re-allocation of anthocyanin to vegetative tissue may occur as a flower ages because a) it is expensive to produce, so is retained in permanent vegetative structures and B) as a cue to larger flowers with more pollen rewards (because male fitness is increased by multiple visits from different insects going to different places while female fitness is not). Unpublished initial results with respect to correlations between leaf, pedicel and petal anthocyanin concentration do not appear to support this hypothesis. Further work is required to determine the effect of physiological stress on floral and vegetative structures in *W. albomarginata* and the reproductive consequences (if any) of this.

The second factor influencing flower colour shown in this study was flower age, with significantly bluer flowers at the beginning of a five-day period than the end, and significantly bluer male phase flowers than the following female phase. Changes in anthocyanin content in plant organs have been shown with age (Karageorgou and Manetas 2006, Kytridis et al. 2008), and anthocyanins are known to degrade in flowers of *Brunfelsia calcynia* Benth independent of flower senescence (Vaknin et al. 2005), but *W. albomarginata's* co-familial *Campanula isophylla* has constant anthocyanin levels throughout anthesis, and a low level of anthocyanin during bud formation (Justesen et al. 1997).

Although anthocyanin transience has not been investigated in petals of *Wahlenbergia* species, it could conceivably be tested as an explanation for the decrease in petal blueness observed with age in *W. albomarginata*. Arrays of blue *W. albomarginata* flowers tend to be visited more than arrays of white flowers (Campbell et al. 2012). This means being blue should increase visitation of pollinators to younger male phase flowers, optimising pollen exportation and thus male fitness as per Bateman's Principle (Bateman 1948). It also means the method of quantifying colour in similar aged flowers employed to determine the effect of water regime here, should be used in future studies of flower colour in this species.

6.4 Pollination Network: The Effect of Surrounding Exotic Species on Pollination and Seed Set.

Nearby vegetation patterns and changes in altitude between 650m and 1000m had little bearing on pollination outcomes but pollen present on *W. albomarginata* stigmas was 85% exotic below 1000m (See Chapter Four). This contrasts starkly with pollination at The Remarkables at 1650m, where 90% of pollen delivered was conspecific (Bischoff 2008). The high visitation rate of New Zealand Solitary bees to *Hypochaeris radicata* combined with the high levels of Asteraceae and *Trifolium* pollen types on *W. albomarginata* stigmas suggests exotics are very much integrated into its Blue Mountains ecosystem. The number of conspecific pollen grains deposited on a receptive stigma indicates the effectiveness of pollinator service as well as pollen tube competition during fertilisation. *W. albomarginata* is one of a few known pollen limited alpine species at high altitude at The Remarkables, meaning an insufficient number of con-specific pollen grains are deposited per ovule to achieve full seed set (Bischoff 2008). The high rate of heterospecific pollen present on *W. albomarginata* stigmas may cause stigma clogging. This is when heterospecific or self-incompatible pollen is deposited on stigmas and either physically blocks space for legitimate pollen or chemically interferes with pollen tube growth and fertilisation (Jakobsson et al. 2008). The fact that seed set did not significantly differ between native-dominated sites and exotic-dominated sites (chapter five) suggests deposition of exotic pollen did not impede fertilisation.

Low pollination rates could significantly impede reproductive success in *W. albomarginata* because it is a self-incompatible, species with a short period of stigma receptivity which rarely achieves a full seed set of 380 (Bischoff 2008). Reliance on pollinators due to self incompatibility and short periods of stigma receptivity are rare in alpine and subalpine plants because changeable conditions mean successful pollination can be inconsistent (Richards 1997, Bingham and Ort 1998, Zhang and Li 2008). This high rate of visitation to exotic competitors by its pollinators could cause not only lower visitation frequency for *W. albomarginata* flowers, but clogging of stigmas by heterospecific pollen, which can physically block viable pollen, prevent fertilisation and ultimately decrease seed set (Jakobsson et al. 2008, Muchhala and Thomson 2012). A high proportion of heterospecific pollen does not occur on stigmas in areas dominated by native species (Bischoff 2008), but living amongst exotic *Trifolium* and Asteraceae species may be detrimental to the intergenerational persistence of *W. albomarginata* in lower altitude sites.

Results from Chapter Four show sites that support large flower size also support greater total pollen delivery. Large flowers are often more attractive to insects than smaller ones either because they are more visually striking or are likely to contain more nectar (Galen and Newport 1987, Nakamura

et al. 1989, Eckhart 1991, Herrera 1995). However, in this case it could be the abiotic conditions associated with site either support or limit flower size and insect activity and/or population.

The three main pollen types present on *W. albomarginata* were *Wahlenbergia, Trifolium* and Asteraceae despite the presence of a wide variety of insect pollinated plants nearby including *Raoulia subsericea* Hook. f*., Euphrasia* sp*., Lotus corniculatus* L. and *Digitalis purpurea* L. The fact that stigmas of *W. albomarginata,* a pollen-limited self-incompatible species, did not have a random mix of nearby species' pollen supports recent assertions that pollination in the New Zealand alpine is not completely generalised as previously thought (Bischoff 2008).

6.5 Conclusions

This study has shown the ability of a New Zealand alpine herb's reproductive strategy to withstand diverse abiotic conditions and the integration of competitive exotics into its pollinator network. Change in flower size is caused by environmental factors, which sometimes change on an altitudinal gradient. While altitude was not identified as a determinant of flower colour, water regime was. This opens many questions regarding the plastic regulation and allocation of anthocyanin compounds to petals in *W. albomarginata* and other Campanulaceae species in physiologically stressful conditions. The possibility that *W. albomarginata* maximises anthocyanin allocation to flowers when they are young to increase male fitness, and then lose these pigments over time is a specialisation not traditionally attributed to alpine natives. Allocation to floral display is clearly affected by the abiotic environment.

Despite changes in floral size with altitude, seed set remains consistent. In addition to this, changes in temperature associated with elevating altitude do not affect insect activity sufficiently to significantly change pollen type or amount delivered to *W. albomarginata* stigmas. Instead, stigmas in an area with predominantly native co-occurring species received mostly con-specific pollen, while the vast majority of pollen at the Blue Mountains came from locally abundant *Hieracium pilosella* L. and *Trifolium repens* exotics. Despite this, there was no significant difference in seed set between the two areas, suggesting exotic pollen does not clog stigmas or impede seed production.

The generalised reproductive systems are a global trend in alpine zones because they are variable and unpredictable. The robust reproductive system of this model species between natural sites which vary in co-occurring species, soil pH, soil moistures, soil nitrogen levels, temperatures and shelter is encouraging for native alpine and grassland conservation in light of New Zealand's unusually high presence of exotics and future changes in climate that are expected to occur globally.

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