Divergence in floral morphology and pollinator interactions in two sympatric Australian pea plants

Sarah Stock[1]

Abstract

The diversification of flowering plants is often attributed to plant-pollinator interactions. Plants often evolve combinations of traits (collectively termed 'pollinator syndromes') which attract and utilise specific groups of insects. *Oxylobium ellipticum* (Vent, R.Br) and *Hovea montana* (Hook.f.) J.H.Ross are two Australian pea plants found in sympatry in Kosciuszko National Park. These plants differ in floral colour, but exhibit what appear to be identical mechanisms for accepting and depositing pollen from/to a pollinator. Through the analysis of floral morphology and pollinator activity it was found that these species have diverged in floral traits further than previously thought. *Oxylobium ellipticum* has larger keel petals resulting in a requirement for greater force to open flowers and the pressure point is found further from the base of the flower. These differences may be the result of reproductive competition leading to differential pollinator filtering systems or potentially divergent exploitation of the same pollinators.

Key Words

Fabaceae, Kosciuszko, pollination syndromes, reproductive competition

Introduction

The association between flower morphology and pollinators has been studied for over 200 years, and the diversification of flowering plants species has been linked to pollinator presence and behaviour (Johnson and Steiner 2000; Johnson 2010). In Australia, little study has been conducted on the pollination biology of legumes with papilionate flowers despite being a diverse family in southern Australia (but see Gross *et al.* 2000; Gross 2001).

A central concept of pollination biology is pollination syndromes (Fenster *et al.* 2004). Pollination syndromes are defined as combinations of floral traits which are linked to attraction and exploitation of specific groups of pollinators (Fenster *et al.* 2004). These trait combinations are typically convergent among unrelated plants that utilise the same pollinators (Johnson 2010). Many aspects of flower morphology have been linked to the attraction of particular pollinator species including colour, odour and pollen production. For example, red flowers with dilute nectar are associated with bird pollination systems and this trait combination has evolved in at least 65 different plant families (Cronk and Ojeda 2008). Many plants rely on animals as vectors for pollination.

When considering sympatric species of flowering plants, analysing the difference in floral traits and pollinator behaviour provides insight into the selective forces which have led to diversification. One suggested driving force of divergence for sympatric species is reproductive interference. Reproductive interference includes competition for pollinators and seed dispersal agents as well as interspecific pollen transfer (Armbruster and Herzig 1984). Interspecific pollen transfer can lead to pollen wastage, stigma and style clogging, and the potential production of unfit or inviable offspring (Kephart and Theiss 2004). Sympatric plants can reduce reproductive interference by reducing interspecific pollen transfer or increasing conspecific pollination by exhibiting divergent floral traits (Kepart and Theiss 2004).

Competition for reproductive success in plants can lead to divergence in floral traits such as colour, and can lead to development of different pollination syndromes. Muchhala *et al.* (2014) suggest that novel floral colours evolve in cases of related sympatric plants. Flower colour impacts pollinator attraction as pollinators have preference for different colours. Certain colours are associated with specific functional groups of faunal pollinators (Fenster *et al.* 2004; Muchhala *et al.* 2014). Functional groups are

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[1] Corresponding author: u5808617@anu.edu.au, Research School of Biology, ANU, Canberra, ACT, Australia

commonly used in pollination biology rather than focusing on specific species of insects. Pollinators which exhibit similar behaviour or morphology are grouped together as they are likely to exert similar selection pressures (Fenster *et al.* 2004). Some functional groups may contain one species whilst others contain many species (Galloni *et al.* 2008). The study species' flowers are purple (*Hovea montana)* and yellow (*Oxylobium ellitpicum*). Purple and yellow are both associated with the attraction of bees (Fenster *et al.* 2004). Determining if only bees or whether other functional groups visit these species is required to attempt to understand whether these traits influence competition between the species.

Competition for pollinators may drive floral divergence in sympatric species for traits other than simply colour. The two study species have a very similar mechanism for accepting and depositing pollen, best described as a tripping mechanism. When pressure is placed on the petals by the floral visitor, the keel petals open and the style and stamen come forward. After the pressure on the petals is removed the style and stamen return to being hidden in between the keel petals. This mechanism distributes pollen on insects' abdomens which can then be deposited on other stamen (Raju and Rau 2006). While these species' pollen depositing and accepting mechanisms are similar, further analysis is required to distinguish subtle differences in, for example, floral morphology or the position of pollen placement. Differences in pollination mechanisms can reduce competition between species and pollen wastage (Kepart and Theiss 2004).

This study focuses on two species of pea plants that occur sympatrically in Kosciuszko National Park. It aims to understand ways these species reduce reproductive interference by examining 1) whether the flower morphologies of these species have diverged further than just colour differences, and 2) whether the pollinators of *H. montana* and *O. ellipticum* match the expected visitors predicted by their respective flower colour and form.

Methods

Study species

Oxylobium ellipticum (Vent, R.Br) and *Hovea montana* (Hook.f.) J.H.Ross are sympatric pea species from the family Fabaceae (Figure 1). Both species are native Australian plants and are found in southern New South Wales, Victoria and Tasmania. However, *O. ellipticum* has a greater range than *Hovea*. *Hovea montana* is typically found among graminoid open sedges, open woody trees and sparse woody trees (Atlas of Living Australia 2017a). It is a low spreading shrub with deep violet-blue or white flowers (Costin *et al.* 2000). *Oxylobium ellipticum* is primarily found among open and closed woody trees and sparse woody trees (Atlas of Living Australia 2017b). It is a variable shrub which can grow to over 2 m at lower elevations. Its flowers are orange-yellow with a reddish patch at the base of the banner petal. The keel of the flower is also a reddish colour (Costin *et al.* 2000). Both flowers consist of five petals: a banner, two wings and two small petals which form the keel. Apart from the difference in colour, the flowers show very little difference in morphology.

Field location

Field work was conducted in the Australian alpine region of Kosciuszko National Park from 4 to 17 December 2016. Two sites were selected within the park. One site was along the Rainbow Lake Walk and was located near the first creek crossing from the main road. The second site was located at Charlotte Pass Ski Village around the ski lift areas and behind the lodges. Each site has both study species present, however, at Charlotte Pass the *O. ellipticum* had yet to flower and the *H. montana* had almost finished flowering at Rainbow Lake. Therefore, the samples from Rainbow Lake are only *O. ellipticum* and the samples collected from Charlotte Pass are *H. montana.*

Figure 1: (A) *Oxylobium ellipticum* flowers and (B) *Hovea montana* flowers.

Flower morphology

To determine the area of each petal, five flowers of *H. montana* and four of *O. ellipticum* were dissected. The flower of both species has bilateral symmetry. Each flower has five petals; a banner, two wings and two keel petals. Petals were carefully plucked individually using forceps. For the keels it was necessary to use a dissecting microscope in order to not damage the petals. A total of 45 petals and a 10-cm ruler for reference were placed onto a scanner and scanned into ImageJ software (U.S. National Institute of Health, Bethesda, Maryland, USA). ImageJ was used to determine the area for each petal.

Samples collected were used to measure the force required to access the stigma and style. Fresh samples must be used and so testing was conducted on the same day as sample collection. A digital force gauge (STARR FDG-50) was placed in a test stand which had a digital ruler attached in a way that measured distance travelled. A chisel head attachment was used for all tests. To measure the amount of force required to open the flowers, a single flower was held in place under the attachment head and the gauge and rulers calibrated. Slowly increasing force was applied by the force gauge until the flower opened. The distance travelled and the peak force required was recorded. Ten flowers were tested for each species.

To further investigate the opening mechanism, the distance along the wing petals at which the style and stigma were fully exposed was measured. This point will be referred to as the pressure point. To determine the pressure point the banner petal was removed and the rest of the flower placed into a ball of blue tack. Removing the banner allows visualisation and greater access to wing and keel petals. The blue tack keeps the flower above the bench surface to prevent the petals hitting the bench when pushed down on. The flower in blue tack was placed on top of 1mm graph paper. The base of the flower and the end of the wing petals were marked on the paper. A glass cover slip was used to find the pressure point by sliding it from the base of the petals down towards the tip of the wings. The point at which the stigma and style were exposed was marked. The distance between the base of the flower and marked pressure point was measured. This process was repeated for 12 *O. ellipticum* flowers and 10 *H. montana* flowers.

Pollinators

To determine which insects pollinate the study species, insects were caught and swabbed for pollen. Time in the field was limited and so visitation data could not be directly determined. Prior to catching insects, samples of different sympatric flowering plants at the study sites were collected. These samples included the two study species as well as *Bossiaea foliosa* A.Cunn, *Nematolepis ovatifolia* (F.Muell.) Paul G.Wilson and *Olearia phlogopappa (*Labill) Benth. The *B. foliosa* has yellow flowers while *N. ovatifolia* and *O. phlogopappa* have white flowers.

For each flowering species collected, a pollen reference slide was created using the method described in Wooller *et al.* (1983) as a starting point. This method was adjusted by using a drying oven at 35°C for 3–5 minutes to melt the gel as opposed to the suggested lighter or match. Slides were left to cool before analysing them. Photographs were taken under a compound microscope using an iPhone 6. The photograph time and microscope magnification were recorded for each sample so photos could be matched to slides.

A total of 13 insects were collected. Two methods of collection were used. Some insects were caught using a butterfly net and were then transferred into a clean sample jar. Others were caught using only a clean sample jar. Insects were caught at both sites. The small sample size is due to limited time exacerbated by bad weather. Insects were placed into a fridge at 4°C overnight. If insects were still active after being in the fridge they were placed in the freezer for 2 minutes. Insects were swabbed and slides created, then samples were photographed using the same method as for the pollen reference slides. All insects were released at their collection site after being allowed to warm up. Although 13 insects were caught only 11 slides were usable due to methodology errors.

Photographs were analysed on a computer. For each insect, the total number of different pollen types was recorded. If the pollen matched any of the reference species this was recorded. Pollen that did not have a reference slide was recorded as unknown. Due to low collection rates of pollen grains, no minimum pollen grain threshold was applied. The limitations of this approach are addressed in the discussion.

Statistical analysis

For petal area measurements, an unpaired *t*-test was conducted for each petal type using RStudio v1.0 (R Core Team, 2016). *P*-values were adjusted using a Bonferroni adjustment. Unpaired *t*-tests were also used for the force gauge measurements and the pressure point measurements. No statistical analysis was conducted on the pollen carrier data due to sample size being too small for each insect group.

Results

The area of the banner and wing petals (left and right) did not differ between species. The *O. ellipticum* left and right keel petals had means of 0.219 cm^2 and 0.212 cm^2 respectively. This is significantly larger than the *H. montana* keel petals (left = 0.096 cm^2 and right = 0.090 cm^2, $p \leq 0.001$, Figure 2).

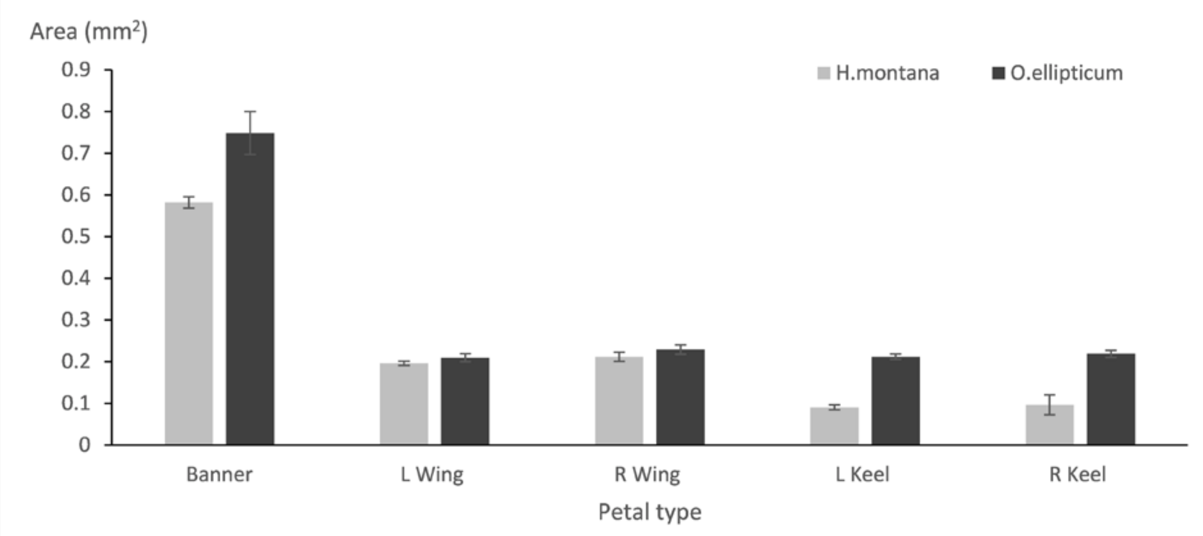

Figure 2: Average petal area for five different petals (banner, left and right wing, and left and right keel) for two species of Fabaceae, *Hovea montana* and *Oxylobium ellipticum.*

Significantly more force was required to open the *O. ellipticum* flowers (0.224 N) than the *H. montana* (0.150 N, $p = 0.004$, Figure 3). The pressure point location was also different ($p = 0.003$, Figure 4). The *H. montana* pressure point is closer to the base of the flower at an average of 3.50 mm ± 0.02 SE and the *O. ellipticum* pressure point is further down with an average of 5.00 mm ± 0.014 SE.

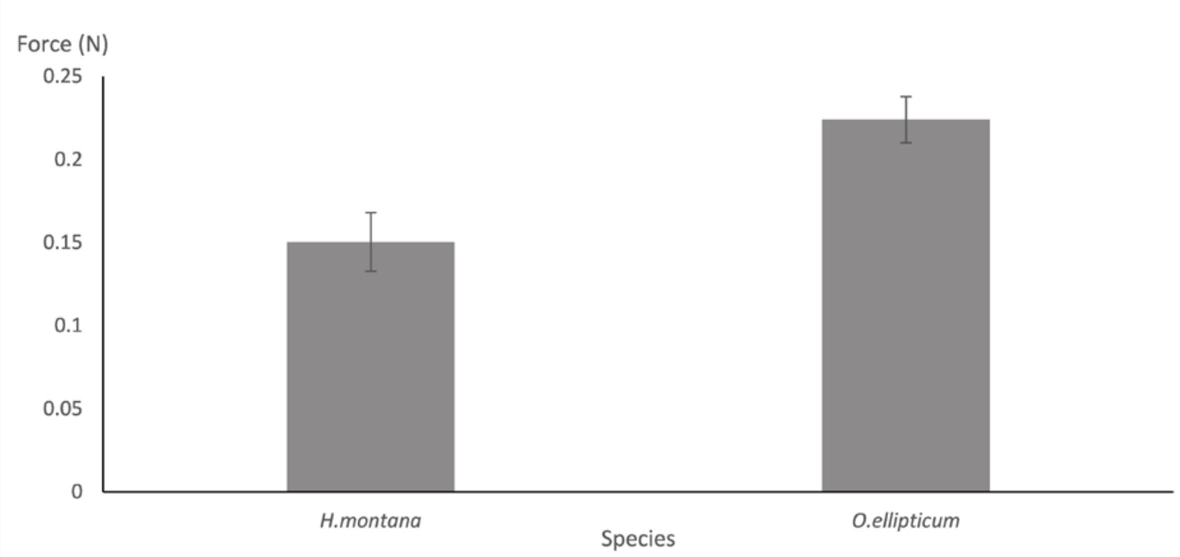

Figure 3: Average force (N) required to fully open flowers of *Hovea montana* and *Oxylobium ellipticum.*

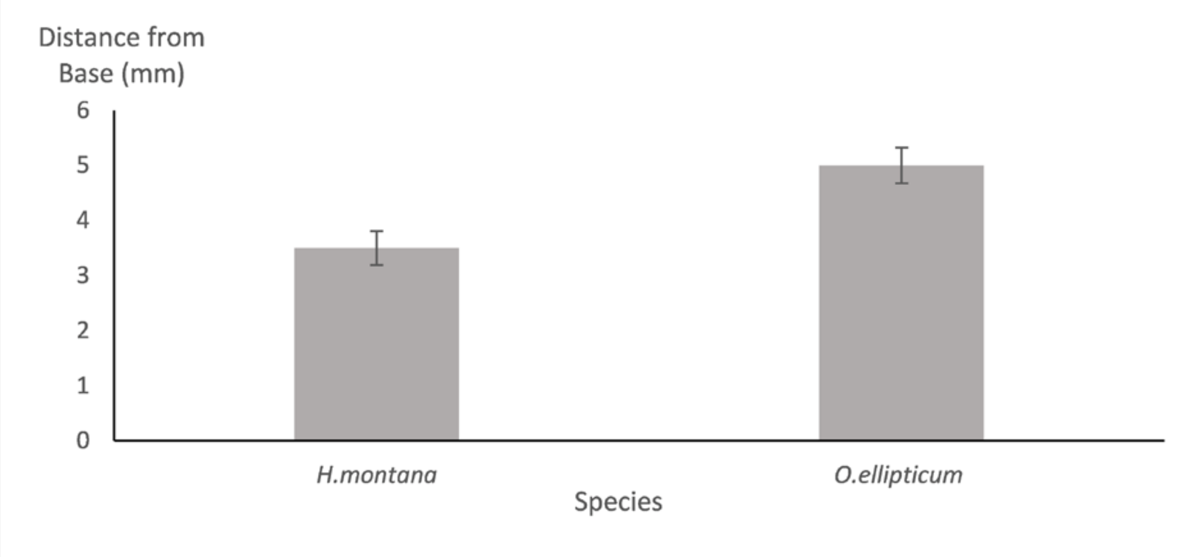

Figure 4: Average pressure point distance, the point at which flowers open fully from base of flower, for two species, *Hovea montana* and *Oxylobium ellipticum.*

The pollen reference slides (Figure 5) show that *H. montana* pollen have a triangular shape with protruding sections at each corner. The *O. ellipticum* pollen are round and solid in colour with a clear border. The *B. foliosa* have a very similar structure to the *O. ellipticum* and pollen of the two species are indistinguishable under the compound microscopes. The *O. phlogopappa* pollen are easy to distinguish from the study species as they have a spiky appearance. The *N. ovatifolia* pollen are similar to that of *H. montana* but are still distinguishable because of their more oval shape, three small protruding sections and darker stain.

Six different functional groups of pollinators were caught: large bees, beetles, hoverflies, wasps, butterflies and large flies. Two bees were caught and together they carried five types of pollen overall (Table 1). The bees carried all reference pollens except the *N. ovatifolia* as well as an unknown species. One solider beetle was caught and it carried three species of pollen including both the

Oxylobium/Bossiaea and *H. montana* along with an unknown species. None of the three hoverflies caught carried pollen from *H. montana*. All carried the *Oxylobium/Bossiaea* pollen. The hoverflies also carried three unknown pollen types as well as *Brachyscome* sp. and *N. ovatifolia* pollen. The wasp only carried pollen from *Oxylobium/Bossiaea*. The two butterflies and the large fly carried no pollen.

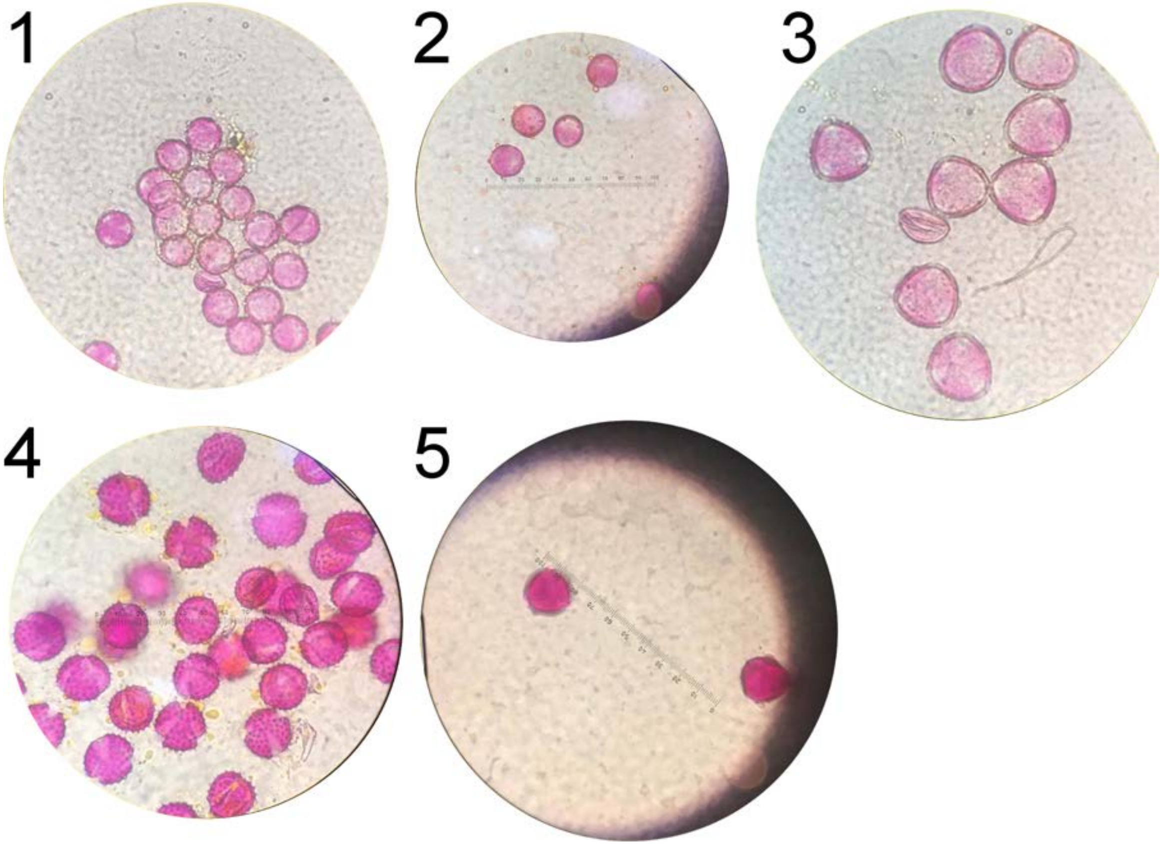

Figure 5: Pollen reference slides for five Australian alpine flowering plants: 1) *Oxylobium ellipticum*, 2) *Bossiaea foliosa*, 3) *Hovea montana*, 4) *Olearia phlogopappa*, 5) *Nematolepis ovatifolia.* Note: All species magnified 1000X.

Table 1: Pollen loads for five known species of flowering plants from Rainbow Lake and Charlotte Pass carried by six functional groups of pollinators.

Insect	Genera Oxy/Bos*	*Hovea*	*Nematolepis*	*Olearia*	Unknown	Total (yes)	n
Large bee	yes	yes	no	yes	yes	4	2
Beetle	yes	yes	no	no	yes	3	1
Hoverfly	yes	no	yes	yes	yes	4	3
Wasp	yes	no	no	no	no	1	1
Butterfly	no	no	no	no	no	0	2
Large fly	no	no	no	no	no	0	1
Total (yes)	4	2	1	2	3		

*** *Oxylobium* and *Bossiaea* pollen are indistiguishable under the compound microscopes.**

Discussion

Avoidance of reproductive interference appears to be reducing pollen wastage for two sympatric species of pea flowers in Kosciuszko National Park. *Hovea montana* and *Oxylobium ellipticum* have more differences in flower morphology than simply colour. Through the analysis of flower morphology and pollen transfer mechanisms it becomes evident that these species differ in petal area of the keels, force required to open flowers fully and the point at which the flower opens. These differences in the flower morphology may influence which pollinators are excluded from successful pollination.

Divergence of floral morphology

I suggest that the larger area of the keel in *O. ellipticum* is the underlying mechanical cause for the difference in pressure points and operative strength required to open the flowers. The larger keels require more mass to move them. More mass requires greater force to move it, therefore leading to a higher operative strength being required to open the flower. However, there are alternative explanations for the difference in force. For example, the difference in operative strength may instead be due to the keels in *O. ellipticum* being more tightly connected where they meet, meaning more force is required to separate the keels. The different pressure point location may be due to larger and longer keels in *O. ellipitcum*. As size increases the same point respective to morphology will be further away from the base of the flower. The tripping mechanism is likely tripped by placing force on the same relative part of the flower, but due to the larger size of the keel this location is further from the base of flower in *O. ellipticum* than in *H. montana*.

These differences in the pressure point's position may result in the divergent use of pollinators. Previous studies show that exploiting the same pollinators in different ways allows sympatric species to maintain reproductive isolation and reduce competition (Yang *et al.* 2007; Muchhala and Potts 2007). Sympatric species of flowers can reduce interspecific pollen transfer by the segregation of pollen placement. By placing pollen on a particular location on the pollinator's body which correlates to the location of the stigma for that same species, the likelihood of picking up incorrect pollen is reduced (Huang and Shi 2013). Yang *et al.* (2007) show that sympatric louseworts target different parts of bumblebee's bodies. This prevents both pollen from being wasted and the clogging of reproductive organs (Huang and Shi 2013). In our study, the difference in pressure point may mean that rather than the front legs tripping the mechanism, the back legs set it off and the pollen hits the insect further back for *O. ellipticum*. This would prevent pollen from *O. ellipticum* being transferred to *H. montana* or vice versa. An alternate possibility is that the primary pollinator of each species is a different size and so the difference in pressure point is due to targeting the same part of differently sized insect bodies. Studies on *Stylidium* found that a mean difference of 2 mm in pollen placement may effectively segregate pollen flow (Armbruster *et al.* 1994). Further study is required to test which of these theories is correct. Without knowing the accuracy and precision of pollen placement on insects and stigma contact for the two study plant species I was limited in knowledge of how different the mechanisms truly are.

As the study species were found in the same locations, but not flowering together, they may have different flower phenologies. This could be driven by either competition or natural selection. Divergence in flowering phenology allows for reduction in reproductive interference as there is less chance of pollen mixing (Gross *et al.* 2000). Inouye and Pyke (1988) provide the flowering phenology for *O. ellipticum*, but not *H. montana* due to *H. montana* having already started flowering by the time they started research. This further suggests that the flowering phenology may be different. However, these species both flower during the summer period and so there is likely to be some overlap. Overlap in flowering time occurs in most species in alpine regions due to restricted growing seasons (Totland 1993). To understand the exact ways these species interact it would be important to study the flowering phenologies over multiple summers to test if there is a pattern of flowering phenology in which the two species avoid having the peak of flowering at the same time.

Filtering of pollinator visitation

One of the main concerns with the results from the pollen carrier tests is that the *B. foliosa* and *O. ellipticum* had indistinguishable pollen grains when using this pollen visualisation method. *Bossiaea foliosa* was abundant in the area so it is possible that our caught insects were carrying its pollen rather than that of *O. ellipticum*, impacting the validity of the results. Direct observation of pollinator visitation is required. A second limitation of the approach used in this study is that the number of pollen grains on a pollinator was typically low and a cut-off was not used to score pollen presence. Typically, researchers apply a cut-off, such as 10 or more grains, to confirm visitation for swab methods (Wooller *et al.* 1983). Cut-offs reduce the likelihood of including pollen that is only present due to contamination by humans or other pollinators. Honey bees (*Apis mellifera*), for example, are generalist foragers that could spread pollen grains from one species to another; these 'foreign' pollen grains could then be picked up by more specialised pollinators. While the results are not conclusive, they are still worth discussing as it may help to direct further research, particularly in terms of understanding pollinator filtering.

Plants with specialised pollination systems should have the ability to use a subset of potential resources more effectively than generalised ones (Armbruster 2016). Filtering which groups of pollinators are attracted to a flower is a by-product of selection for particular pollinators. The four types of filtering process that can occur in pollination systems are attraction filters, reward chemistry filters, pollinator filters and mechanism filters (Armbruster 2016). The filters of most interest for this study are mechanism filters. These include pollen pick up and deposition, and other structural features which influence compatibility between pollinator and flower for successful pollination (Armbruster 2016).

Differences in the operative strength to open flowers may restrict which insects can pollinate the flowers. Cordoba and Cocucci (2011) conducted a study in which the operative strength to open six different species of pea flowers was measured. Many bees could not open flowers by their weight alone. Bees had to use strength as well as their own mass to open the flowers, with only one species unable to open the strongest flowers. This suggests that knowing the strength as well as the weight of visitors is important. Surprisingly, although the flowers of *H. montana* were easier to trip, this species appeared to have a greater diversity of visitors.

This study does not allow us to determine how effective as pollinators the visitors were. Typically, some pollinators are more effective than others (Galloni *et al.* 2008). Effective pollinators are those which deposit the most conspecific pollen on stigma per visit. This is impacted by various factors including body shape and size of pollinators (Adler 2005). Having a less sensitive mechanism potentially may be an adaption to reduce pollen wastage on ineffective pollinators. Adaption to a lighter or less strong primary pollinator may also explain the easier tripping mechanism of *H. montana*.

The overall diversity of pollinators visiting the study species was surprising to some extent as most studies on morphologically similar flowers with tripping mechanisms report that flowers are dependent on bees or wasps for pollination (Raju and Rao 2006; Palmer-Jones and Forster 1965; Davis 1987). However, for other studies conducted in this region, high pollinator diversity was common in comparison to other alpine regions and so these results are not unusual for the Australian alpine region (Inouye and Pyke 1988). No pollen was collected from butterflies or flies, suggesting they are do not pollinate the study species. This is unexpected as Inouye and Pyke (1988) reported that flies were the most common visitor at higher elevations. Whilst it was unexpected in the alpine region, research on other Australian peas report bees as the expected visitors rather than flies (Gross 2001).

To fully understand the filtering process, one must consider the relative role of all four types of filtering mechanisms. Whilst our study species may have evolved subtly different tripping mechanisms and different colours the data are too few to determine if they are effective filters. Therefore, additional studies that determine whether the *H. montana* and *O. ellipticum* have different, specialised combinations of floral traits are required, particularly for testing the effectiveness of both the tripping mechanisms and colour as filters (Johnson and Steiner 2000).

Conclusions

In summary, this study shows that *Oxylobium ellipticum* and *Hovea montana* differ in floral colour and tripping mechanism. The underlying selection pressures which have resulted in these differences remains unknown. I suggest that these differences are leading to alternative pollinator filtering pathways or divergent use of the same pollinators and thus reduce interspecific pollen transfer. Environmental factors and elevation may be placing selective pressures on these plants in addition to pollinators. Further study is required to truly understand what is driving the shifts in morphology.

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