

Latitudinal variation in pollinator and co-flowering communities, and their effects on reproductive success in a rewardless orchid, *Cypripedium candidum*

by

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ABSTRACT

Rewardless flowering plants may bloom early to take advantage of naïve pollinators and avoid competition with rewarding species. However, at high latitudes, this strategy may be constrained by the shorter growing season. I surveyed *Cypripedium candidum* populations to determine if reproductive success is correlated with latitude. At 21 sites, in four regions (Iowa, southern Minnesota, northern Minnesota, and Manitoba), I assessed the composition of the co-flowering community and *C. candidum*'s fruiting success. At 15 sites, I surveyed the insect community and quantified orchid flower morphology. As latitude increased, so did the diversity of plants and insects, but all regions shared many common taxa. Pollinating insects at higher latitudes were often too large to pollinate *C. candidum*, and more appropriately-sized insects were found further south. Fruiting success was highest in Iowa and was associated with a warmer growing season, a decrease in potential co-flowering competitors and an increase in facilitators.

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DEDICATION

In loving memory of my grandmother, Winnie Anderson. For her incredible dedication to my education and always making sure I was studying, even in the middle of summer vacation.

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1. INTRODUCTION AND GENERAL BACKGROUND

1.1 Introduction

Due to their sessile state, plants must endure a range of biotic and abiotic conditions. Species with a large geographical range experience wide variation in abiotic conditions, which may also alter the biotic community across their range (Whittaker et al. 2001). These changes may result in latitudinal variation in the reproductive success of a species (De Frenne et al. 2009, Brown and McNeil 2009, Thomson 2010). A species' reproductive success depends on mutualistic interactions with pollinators, which may be affected by competition and facilitation for pollination between co-flowering species (Thomson 1978, Moeller 2004). Changes in both the composition of these communities and the timing of life events introduce challenges for wide-ranging species to achieve successful reproduction.

Species that produce no food rewards (nectar or edible pollen) for foraging pollinators face additional challenges to achieve reproduction, as they often receive a low number of pollinator visits (Cozzolino and Widmer 2005). Pollinators can learn to avoid rewardless species (Cozzolino and Widmer 2005, Harder and Aizen 2010, Duffy and Johnson 2011), which results in decreased pollen load and reduces the number of ovules that can be successfully fertilized, a situation termed pollen limitation (Burd 1994). Researchers have theorized that a rewardless strategy may allow for conservation of resources without the need for energetically expensive pollen, or an increase in genetic outcrossing, as pollinators will typically fly further between visits to a rewardless species (Johnson 2000, Cozzolino and Widmer 2005, Jersáková et al. 2006, Walsh and Michaels 2017).

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To achieve reproduction under pollen limitation, rewardless species may mimic a rewarding species to slow pollinator learning (Neiland and Wilcock 1995, Cozzolino and Widmer 2005). The most common form of mimicry is generalized food mimicry, when a rewardless species produces attractive flowers but does not resemble a particular rewarding species (Neiland and Wilcock 1995). Thus, rewardless species are highly reliant on the co-flowering community's ability to attract foraging pollinators, but this may result in competition for insect visits with co-flowering species (Johnson et al. 2003, Internicola et al. 2007, 2008, Duffy and Johnson 2011).

My project focuses on the reproductive biology of the wide-ranging rewardless orchid, *Cypripedium candidum*. This species likely experiences variation in reproductive success in different parts of its range (Shefferson and Simms 2007, Pearn 2012, Walsh et al. 2014). This variation may be due to changes in several factors including: the phenology of flowers and insects; the composition of the floral and insect community; the role of co-flowering species; and phenotypic variation within both floral and insect species. In order to fully understand how these factors influence reproduction in a rewardless species, I will first discuss how they affect reproduction within plant communities, and how they may vary with latitude.

1.1.1 Phenology of plant communities

In order to achieve optimal reproductive success, a plant should flower during times of high pollinator abundance (Forrest and Miller-Rushing 2010). The best general predictor of floral and insect phenology is temperature, particularly in early season species at temperate latitudes (De Frenne et al. 2011, Diez et al. 2014, Wolkovich et al. 2014, Pearce-Higgins et al. 2015). At equatorial latitudes, or in later season species, precipitation may also have a significant

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influence on phenology as temperature is generally less limiting in these areas or time periods (De Frenne et al. 2011, Pearce-Higgins et al. 2015). A decrease in latitude results in earlier onset and increased length of the growing period (Reich and Oleksyn 2004). At more equatorial latitudes, species can flower for longer periods or throughout periods of high insect abundance. This strategy may maximize their pollination success, provided other environmental conditions remain optimal. In contrast, species at high latitudes may be constrained by the total length of the growing period (Forrest and Miller-Rushing 2010). Early flowering species with a wide geographical range may therefore show the most variation in their timing of flowering relative to the rest of the community and provide an ideal opportunity to study the effects of phenology on reproductive success along a latitudinal gradient.

Studying the effect of latitude on reproductive success requires both a wide geographical area and a large time commitment. Therefore, studies of this kind are relatively few. However, one study spanning from France to Sweden ($\sim 17^\circ$ latitude) did show a decrease in reproductive success of the early season species, *Anemone nemorosa*, *Paris quadrifolia* and *Oxalis acetosella*, in association with lower temperatures at higher latitudes (De Frenne et al. 2009). In addition, several studies have investigated the effect of elevation on reproductive success. These studies differ from latitudinal studies in that sites at different elevations would not differ in photoperiod, but changes in temperature and season length would be similar. Several studies have shown lower reproductive success at high elevations, associated with lower insect abundance (Totland 1993, Alonso 2005, Ramos-Jiliberto et al. 2010, Fischer et al. 2012). In contrast, Gugerli (1998) found neither a reduction in pollination success in *Saxifraga oppositifolia* at higher altitudes, nor higher rates of selfing, which might have explained this result. Weather conditions during his

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study may have optimized insect activity at high elevations, which obscured the expected trend in fruit set (Gugerli 1998).

An earlier and longer growing season may not always enhance reproduction, particularly in light of recent climate change (Thomson 2010, Robbirt et al. 2014, Willmer 2014). Thomson (2010) documented increasing pollen limitation over time in the alpine perennial herb, *Erythronium grandiflorum*, particularly in the earlier flowering individuals due to both frost damage and low pollinator abundances in warmer years. Furthermore, many studies have documented a mismatch in the timing of flowering and insect emergence over time have been documented in a wide range of early-season Mediterranean herbs, and two studies on deceptive orchids (Petanidou et al. 2014, Robbirt et al. 2014, Willmer 2014). It appears that plants and insects may not respond to climate change in the same ways, or are responding to different cues for their phenology (Petanidou et al. 2014). As plants and insects begin emerging at different times, both of the mutualism partners may suffer, leading to potential extirpation or extinction of a species (Thomson 2010).

To maximise reproductive success under pollen limitation, some rewardless species flower earlier than sympatric rewarding species to take advantage of naïve pollinators (Internicola et al. 2008), and bloom for a longer period to increase their chances of being visited (Internicola and Harder 2012). However, earlier flowering may subject rewardless species to an increased chance of frost damage, a common occurrence in years with early snow-melt (Inouye 2008). At lower latitudes, rewardless species may have the opportunity to flower earlier without risking damage by frost, allowing them to take full advantage of naïve pollinators (Internicola et al. 2008, Pellissier et al. 2010, Internicola and Harder 2012). No studies have examined pollination of rewardless species along a latitudinal gradient, leaving a large gap in the literature.

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One study of herbarium records confirmed a higher diversity of rewardless species at lower altitudes, and earlier flowering at low latitudes in rewardless taxa compared to related rewarding taxa (Pellissier et al. 2010). However, no research has confirmed whether growing season changes correspond to increased reproductive success within a rewardless species.

1.1.2 Community Composition and Diversity

In addition to influencing flowering phenology, abiotic conditions such as temperature, moisture, and nutrient availability determine the biotic community that can occur at a given location, through physiological constraints on species occurrence. Globally, species diversity increases with decreasing latitude (Rohde 1992), increasing the number of species a plant may interact with. However, at smaller scales (different sites within a latitudinal region), changes in species diversity may be obscured by local environmental features such as topography or available resources (e.g., soil resources); disturbance regimes such as fire and grazing; or biotic interactions such as competition (Whittaker et al. 2001, Rivadeneira et al. 2002). Therefore, there may be decreased diversity of flowering species early in the season at lower latitudes (Elzinga et al. 2007), even if these regions typically have higher diversity across the entire season (Fischer 1960, Rohde 1992). An increase in the synchronicity of flowering has not been demonstrated with increasing latitude, but has been with increasing altitude (Körner 2003, Pellissier et al. 2010). Changes in both the pollinator and co-flowering community diversity may cause variation in reproductive success throughout a species' range. For example, an increase in generalist pollinator diversity along land use gradients increases the reproductive output of the agricultural crops, *Coffea caniphora* and *Cucurbita moschata* (Klein et al. 2003, Hoehn et al. 2008)

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For generalist plant species, decreased biotic diversity may mean a decrease in the number of potential pollinators to facilitate reproduction. One trend that has been noted is the transition from a bee-dominated to fly-dominated system as elevation increased in the Wasatch mountains of Utah (Warren et al. 1988), and as latitude increased over $\sim 70^\circ$ in Europe (Olesen, unpubl. data in: Elberling and Olesen 1999). The effect of this transition still remains in question, as flies (Order: Diptera) have not had their pollination efficiency quantified in many systems. Flies have frequently been described as “pollen thieves” (flower visitors that consume more pollen than they transfer to other flowers) in certain systems, while bees have been documented as thieves less frequently (Hargreaves et al. 2009). Furthermore, fly taxa may lack the physical strength to successfully pollinate taxa with mechanically difficult pollination (Li et al. 2006). If these patterns are consistent, a transition from bee-dominated to fly-dominated system may result in more pollen limitation within the plant community.

Changes in diversity at lower latitudes may alter the variety of pollinators that visit a rewardless species across its range. A variety of pollinators may contain species that have innate preferences for traits possessed by a rewardless species (flower colour, shape, scent, etc.). This will increase the likelihood of pollinators visiting a rewardless species by mistake (Internicola et al. 2007). However, a rewardless species reproductive success may be decreased by both a decrease in the abundance of pollinators, or an increased rate of pollinator learning. This latter effect usually occurs if a rewardless taxa is easily distinguished from rewarding taxa (Internicola et al. 2007). A diverse flowering community may serve to attract a wide range of pollinators (Ghazoul 2006), preventing pollinators from learning to avoid specific flower characters (Gigord et al. 2001, Duffy and Stout 2011), and increasing a rewardless species' chances of reproductive success.

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1.1.3 Roles of Co-Flowering Plants

If multiple species flower during peak insect abundance, synchronized flowering within a plant community could either lead to facilitative or competitive interactions among plant species (Ghazoul 2006, Mitchell et al. 2009). In a facilitative relationship, two species that flower together will increase floral diversity, thus increasing insect attraction and fruiting success of both species (Moeller 2004, Ghazoul 2006, Lázaro et al. 2009). Facilitation may occur when co-flowering species occur at low densities or if there is a large pool of pollinators in a region. In contrast, competition occurs when plants begin to compete for pollinator visits, and lower each other's reproductive success (Thomson 1978). This situation is typical when co-flowering species are at high densities or when the pollinator pool is limited. The timing of flowering also determines which types of co-flowering interactions occur. Since flowering synchrony tends to increase with latitude (Wolkovich et al. 2014), higher latitude sites may experience more biotic interactions than lower latitudes due to an increase in co-flowering abundance.

Rewardless species have complex interactions with not only co-flowering species but also between individuals of their own species. While a large number of individuals grouped together will probably serve to attract pollinators (Pearn 2012), flowering in high densities may allow foraging pollinators to quickly learn which floral traits indicate a rewardless species (Sun et al. 2009). Studies have shown that if an individual produces a higher number of flowers, it has a higher chance of setting fruit (Pearn 2012, Anderson 2015). However, other studies have shown that pollinators learn to avoid rewardless species faster when they are presented in high density (Internicola et al. 2006, Sun et al. 2009). There is evidence for both of these effects of

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density and flowers per individual, creating the need for additional studies to examine overall trends between floral density and reproductive success.

1.1.4 Phenotypic Variation within Species

A pollinator's preference for certain floral traits imposes selection pressures on the species it visits. In a population, there is phenotypic variation that pollinators may select from, affecting plant reproductive success. Pollinators may show preferences for certain scents or colours (Galen et al. 1987, Jones and Reithel 2001). For example in *Polemonium viscosum*, Galen et al. (1987) saw that bumblebees selected for sweet-smelling flowers, while flies selected for a more skunk-like smell. In *Antirrhinum majus*, Jones and Reithel (2001) reported significant significantly higher pollinator visits to either yellow or white flowers depending on the year of research. Pollinators may also show a preference for the physical size of a flower (Li et al. 2008, Pearn 2012). Flowers with restrictive morphology may prevent certain sizes or types of pollinators from accessing their reproductive organs (Li et al. 2008, Hargreaves et al. 2009). Individuals with less restrictive morphology may be pollinated more readily (Pearn 2012; Anderson 2015), unless pollen is removed but not transferred effectively (Wilson and Thomson 1991, Hargreaves et al. 2009).

Bergmann's rule (1847) predicts that as environments become colder, an animal's body size will also increase to prevent heat loss, while Allen (1877) theorized that an increase in a body's roundness could create the same effect. These rules have been shown in some pollinator communities (Stone and Willmer 1989, Malo and Baonza 2002), but not others (Loder 1997). In their altitudinal survey, Maloe and Baonza (2002) found reproductive success was higher in *Cytisus scoparius* populations that have larger flowers in colder areas due to the increase in

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Bombus body size. Therefore, it may also be true that, in warmer areas, smaller flowers have higher pollination success due to environmental conditions supporting smaller bodied pollinators. These effect of Bergmann's and Allen's rule have not yet been investigated in depth in flower-visiting insects.

1.1.5 *Cypripedium*

This study focuses on the reproductive biology of the rewardless orchid, *Cypripedium candidum* (small white lady's slipper). Like other *Cypripedium* species, *C. candidum* is a rewardless species that relies on floral cues such as colour and scent in order to be successfully pollinated (Argue 2012). *Cypripedium candidum* is endemic to tall grass prairie habitats, and spans a large latitude in the western part of its range from southern Manitoban to southern Iowa. This orchid is globally threatened due to the destruction of tall grass prairie habitat (Rankou 2014) and often suffers from low reproductive success. In Manitoba, fruiting success in most years is between 10% and 15% (Pearn 2012), but some studies have reported fruiting success higher than 30% further south, in Illinois and Ohio (Shefferson and Simms 2007, Walsh et al. 2014).

As *C. candidum* is one of the earliest flowering plants in tall grass prairies, the longer growing season in southern regions of this species range (Iowa and southern Minnesota) may allow it to flower early without being damaged by frost (Internicola and Harder 2012). By flowering earlier, these populations may be able to avoid competition with other co-flowering species (e.g., *Zizia*, *Packera*, *Viola*, *Lithospermum* or *Sisyrinchium*) and have access to an exclusive pool of naïve pollinators (Internicola et al. 2007, Internicola and Harder 2012). The variation in fruiting success along *C. candidum*'s range may also result from a more facilitative

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rather than competitive co-flowering community in southern regions (Johnson et al. 2003, Internicola et al. 2007). Alternatively, the pollinator community may contain more species that naively visit *C. candidum*, or that have an innate preference for *C. candidum*'s floral traits. In Manitoba, many pollinators are too big for *C. candidum*'s pollination route (Anderson 2015). If Bergmann's rule applies to insect communities in the tall grass prairie, there could be more appropriately sized pollinators further south in *C. candidum*'s range, contributing to higher reproductive success.

1.2 General Objectives and Hypotheses

This study examined the potential effect of latitude on reproductive success in *C. candidum* across its western latitudinal range from Iowa to Manitoba, and overall changes in the tall grass prairie community. I hypothesized that the phenology of the tall grass prairie's co-flowering and pollinator community should be extended at lower latitudes and more compressed at higher latitudes. Therefore, at an early period in the growing season, I predicted that (1) the diversity and abundance of plants and pollinators at a site would be higher in northern regions compared to southern regions due to an increased overlap in flowering/emergence time. I tested this hypothesis by determining which flowering plant and insect pollinator species at each site were the most abundant over a latitudinal gradient, and recording changes in species' presence/absence across *C. candidum*'s range. Chapter 2 addresses the changes in the composition of the flowering community during *C. candidum*'s flowering period, and Chapter 3 addresses changes in the insect community composition.

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I also investigated how the changes discussed above affected biotic interactions and the reproductive biology of *C. candidum* across its latitudinal range. I predicted that the pollinator community further south was more effective at pollinating *C. candidum* and this may be due to (2) more appropriately sized pollinator species or a higher abundance of these species or (3) more pollinator species with an innate preference to *C. candidum*'s flower colour (white). I tested these latter two hypotheses using an insect collection created from pan trap surveys during my study. Finally, I hypothesized that (4) fruiting success would be higher at lower latitudes, due to the earlier and longer growing season and potential for more beneficial species interactions. Chapter 3 addresses questions regarding pollinator size and colour preference across *C. candidum*'s range as well as changes in orchid morphology, while Chapter 4 examines predictions regarding orchid reproduction.

1.3 Significance of Study

Approximately one third of all orchids (> 6,000 spp.) possess no food reward for pollinators (Cozzolino and Widmer 2005), suggesting that genetic outcrossing and resource saving outweigh the risk of low fruiting success (Cozzolino and Widmer 2005). However, little is known about how this strategy is affected by changes in biotic interactions across a species' range. This study is the first to examine if a rewardless species' reproduction is associated with latitudinal variation, potentially providing confirmation of long-standing predictions in the literature (Neiland and Wilcock 1995, Cozzolino and Widmer 2005, Pellissier et al. 2010, Argue 2012, Internicola and Harder 2012).

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In order to effectively manage *C. candidum* populations, more information is needed on how the insect community and co-flowering species affect reproduction in this orchid species (Environment Canada 2006). Research is also needed to determine whether the low reproductive success observed in Manitoban populations is a widespread phenomenon or is unique and the result of the shorter growing season. Interpreting these factors in the light of rapidly increasing climate change will provide background information for long-term conservation strategies for this threatened species.

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2. LATITUDINAL CHANGES IN THE CO-FLOWERING COMMUNITY OF *CYPRIPEDIUM CANDIDUM*

2.0 Chapter Summary

The patterns in diversity and composition of the co-flowering community surrounding *C. candidum* were examined in this chapter. I expected that earlier flowering in southern populations would result in fewer co-flowering taxa present at the time of *C. candidum* flowering. This prediction was confirmed through surveys along transects established in each *C. candidum* populations, separated into latitudinal study regions (Iowa, southern Minnesota, northern Minnesota, and Manitoba). There were a number of co-flowering genera that were common to all study regions, but additional genera were observed at more northerly locations. Overall, the richness and diversity of the co-flowering community was significantly higher in northern *C. candidum* populations. Ordination techniques also revealed that sites from different regions significantly varied in the co-flowering community composition. A resurvey of the flowering plant community later in the growing season, when *C. candidum* was in fruit showed that study regions were not significantly different in their co-flowering diversity, suggesting that community composition and diversity evens out among study regions as the season progresses.

2.1 Introduction

The tall grass prairie of North America is found in the centre of the continent, to the east of the short and mixed-grass prairies. Historically, this ecoregion ranged latitudinally from southern Manitoba to central Texas and the central area of the region extended west to Nebraska and east to Illinois and southern Ontario (for maps see Chaplin et al. 1999 and Sims et al. 1999).

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The tall grass prairie experiences a temperate climate with hot summers (mean annual summer temperature: 16°C) and cold winters (mean annual winter temperature: -12.5°C; Chaplin et al. 1999, Sims et al. 1999). Precipitation ranges from 450mm-700mm annually, with drier conditions occurring further west (Chaplin et al. 1999, Sims et al. 1999). The openness of the tall grass prairie was historically maintained by a short fire cycle (~2-10 years) and grazing by bison and elk (Hulbert 1972, Knapp et al. 1999). These disturbance regimes prevented tree encroachment and created conditions favourable for the tall grass prairie's characteristic vegetation (Gibson and Hulbert 1987).

The tall-grass prairie is differentiated from other prairies due to the dominance of grasses such as *Andropogon gerardii*, *Sorghastrum nutans*, *Schizachyrium scoparium*, and *Panicum virgatum* (Knapp and Hulbert 1986). The tall grass prairie is also known for its diversity of non-graminoid taxa, including *Anemone patens*, *Geum triflorum*, *Viola* spp., *Erigeron glabellus*, *Sisyrinchium* spp., and *Zizia aurea* in the early growing season; *Gaillardia aristata*, *Monarda fistulosa*, *Lilium philadelphicum*, *Rudbeckia hirta*, *Allium stellatum*, and *Dalea purpurea* in the middle of the growing season; and *Liatris ligulistylis*, *Helianthus maximiliani*, *Symphotrichum* spp., *Solidago* spp.; and *Gentiana* spp. in the late growing season (this study; <http://www.naturenorth.com/summer/wildflwr/wldflF2.html>). Because of the fertile soils in these areas, much of the tall grass prairie has been converted into agriculture. As a result, less than 5% of the original 68 million hectares of this ecoregion remains (Samson and Knopf 1994, Chaplin et al. 1999, Sims et al. 1999). To aid conservation efforts, more information is needed on patterns of diversity, phenology, and species interactions in the tall grass prairie.

2. Latitudinal changes in the co-flowering community across *C. candidum*'s range

The diversity of a community defines the ecological interactions that can occur within it. As all species have different physiological and environmental requirements, the biodiversity that an area can support depends heavily on abiotic factors associated with the area. Typically, areas with higher levels of a certain limiting factor will support more species than areas with lower levels (Fischer 1960, Peet 1978, Rohde 1992). For example, in a desert habitat, areas with higher moisture levels will usually have a higher diversity than drier areas (Peet 1978). Across a global scale, the factor that tends to be most limiting is temperature, which decreases at more polar latitudes, resulting in higher diversity at equatorial latitudes (Fischer 1960, Rohde 1992). At this scale many other abiotic variables can also influence the diversity, such as precipitation, soil nutrients, and land use (Peet 1978). Due to these confounding variables, large scale studies tend to use the primary productivity of an area (usually measured in biomass) as a proximate measure that should reflect the combined effects of all abiotic variables on the growth conditions of an area (Leigh 1965, Waide et al. 1999, Mittlebach et al. 2001).

With increasing productivity, two general patterns with species diversity tend to emerge (Waide et al. 1999, Mittlebach et al. 2001). At large latitudinal scales (i.e., along a continent), a linear relationship between productivity and diversity is typically observed, with more productive areas able to support a larger diversity of species (Francis and Currie 2003, Hawkins et al. 2003, Harrison et al. 2006). At smaller scales (i.e., along the United States), a unimodal relationship between species diversity and productivity is more common (Tilman and Pacala 1993, Leibold 1999, Waide et al. 1999, Dodson et al. 2000). Typically, as productivity increases, the number of species also increase, until a point when species begin to compete and take advantage of the high productivity. Species that can outcompete others under these conditions

2. Latitudinal changes in the co-flowering community across *C. candidum*'s range

tend to increase in proportion, leading to a decrease in diversity as some species become dominant (Mittlebach et al. 2001).

Studies examining diversity shifts at scales from the local (i.e., within a site) to ecoregion (i.e., among site within the tall grass prairie) level are lacking, leading to some disparities in the literature. Furthermore, patterns in diversity at smaller scales become less consistent and more difficult to interpret due to confounding factors such as nutrients, disturbance, etc. (Gross et al. 2000). Some studies have noted diversity increases with productivity were not evident at smaller scales (between plots in nearby grasslands), but were seen at the level of plant communities (between different grasslands across North America) (Grime 1973, Grace 1999, Waide et al. 1999). In contrast to these studies, a meta-analysis by Perelman et al. (2001) was able to quantify diversity changes in Argentina's Flooding Pampa grasslands associated with fine-scale environmental variables at the local scale, but there was no general pattern detected with latitude.

In addition to diversity changes, a latitudinal gradient should also produce a shift in the phenology of co-flowering species. The change in growing season length typically shifts the flowering period of species that use temperature as a flowering cue (Inouye 2008, Dunnell and Travers 2011, Mazer et al. 2013). With a longer growing season in more equatorial latitudes, there is potential for species to flower asynchronously with each other to avoid competition (Bengtson et al. 1994, Elzinga et al. 2007; Internicola et al. 2008). This may offset competitive effects that could be brought on by increases in diversity (Bengtson et al. 1994). If species diversity increases in the south, but flowering times are synchronous in northern regions, similar or even lower levels of diversity may be present in the south at any one point in time (Elzinga et al. 2007). Similarly, at the onset of spring emergence the north, there may be very few species in

2. Latitudinal changes in the co-flowering community across *C. candidum*'s range

flower, but diversity should rapidly increase when conditions become favourable in this region, as flowers need to quickly emerge to take advantage of the later growing season (Elzinga et al. 2007). While this theory has not been demonstrated empirically in the literature across latitudes, it has been shown across altitudes (Körner 2003). The combined effects of growing season and phenology patterns have not been examined in detail across latitudes.

Other drivers of patterns in diversity operate at a much more local scale than continents or ecoregions. Site-specific features can play a large role in changing diversity within an ecological region. Fire practices can change the amount of nutrients available, the amount of thatch cover, and in turn the productivity of a site (Hulbert 1972). Site drainage has a marked effect on moisture levels and will thus affect which species can survive and establish at a site (Peet 1978). Even the size and shape of site can affect the density of taxa and species interactions that can occur there (Jokimäki et al. 1998).

While many researchers have examined broad scale changes in diversity across a number of environmental variables, research is lacking in some areas. For example, many ecoregions, such as the tall grass prairie, do not span an entire continent, so additional studies on how diversity changes across the area of an ecoregion may consolidate findings from contrasting studies. Furthermore, almost all studies examine diversity across an entire growing season. While this may explain changes in overall diversity, the diversity at any given point in time may be drastically different across a geographic area due to varying phenologies and local or seasonal environmental conditions. Therefore, studies looking at diversity at a single point in the growing season may reveal important patterns that may not be evident when examined across a season.

2. Latitudinal changes in the co-flowering community across *C. candidum*'s range

2.1.1 Chapter Objectives

This chapter identified the latitudinal changes in the co-flowering community present at the time of *Cypripedium candidum* flowering. As an early spring-flowering species, *C. candidum* is an ideal focal species to record latitudinal changes in diversity in a short period of the growing season. Furthermore, rewardless flowers are highly dependent on co-flowering forb species to attract and support the pollinator community in an area (Lammi and Kuitunen 1995, Johnson et al. 2003, Molina-Montenegro et al. 2008), so these co-flowering taxa were my focus for diversity analyses and models of *C. candidum* reproductive success (chapter 4). This was the first study, to my knowledge, to examine both diversity patterns at the beginning of the growing season, as well as rewardless pollination across latitudes.

I expected that community diversity would increase at lower latitudes across the growing season, given that species diversity has been shown to increase with productivity, and productivity should be higher with the warmer areas in the south. However, at an early point in the growing season, diversity may be higher further north due to compressed phenology. Therefore, at the time of *C. candidum* flowering, I predicted to see the highest diversity of co-flowering taxa further north in its species range. This trend would be evident in species richness, Shannon's diversity indices, and species evenness from each study region. I also predicted that the differences in growing season length would create a greater disparity between the spring and summer community composition in the south than in the north. Ordination techniques such as principal components and redundancy analyses would show clear differences in the vegetative community between study regions, emphasizing the latitudinal increase in diversity. If phenology was creating the trends in the community composition, ordinations and indices using data collected during summer flowering period would differ compared to spring surveys.

2. Latitudinal changes in the co-flowering community across *C. candidum*'s range

2.2 Methods

2.2.1 Study Regions and Sites

The study area for this project consisted of 21 sites located in four latitudinal regions with remnant tall grass prairie habitat: southern Manitoba (6 sites), northern Minnesota (6 sites), southern Minnesota (6 sites), and northern Iowa (3 sites), representing the northern and southern extremes in *C. candidum*'s western range (Fig. 2.1; Appendix 2.1). All surveys were conducted during the orchid flowering season from 18 May to 08 June, 2016 ("spring surveys") and when fruits were visible (20-30 June, 2016; "summer surveys"). I can be reasonably sure that I visited sites at comparable points in *C. candidum*'s growing period, since most sites were at a similar orchid flowering stage (Iowa sites were a little before peak flowering, and both Manitoba and Northern Minnesota had two sites that were a little after peak flowering). All study sites neighbored agricultural fields, so the tall grass prairie patches were comparable in their surrounding land use. Given *C. candidum*'s status as a threatened species, exact study site locations are not provided in this thesis but have been kept on file by me and my supervisors (A. Worley, B. Ford).

2. Latitudinal changes in the co-flowering community across *C. candidum*'s range

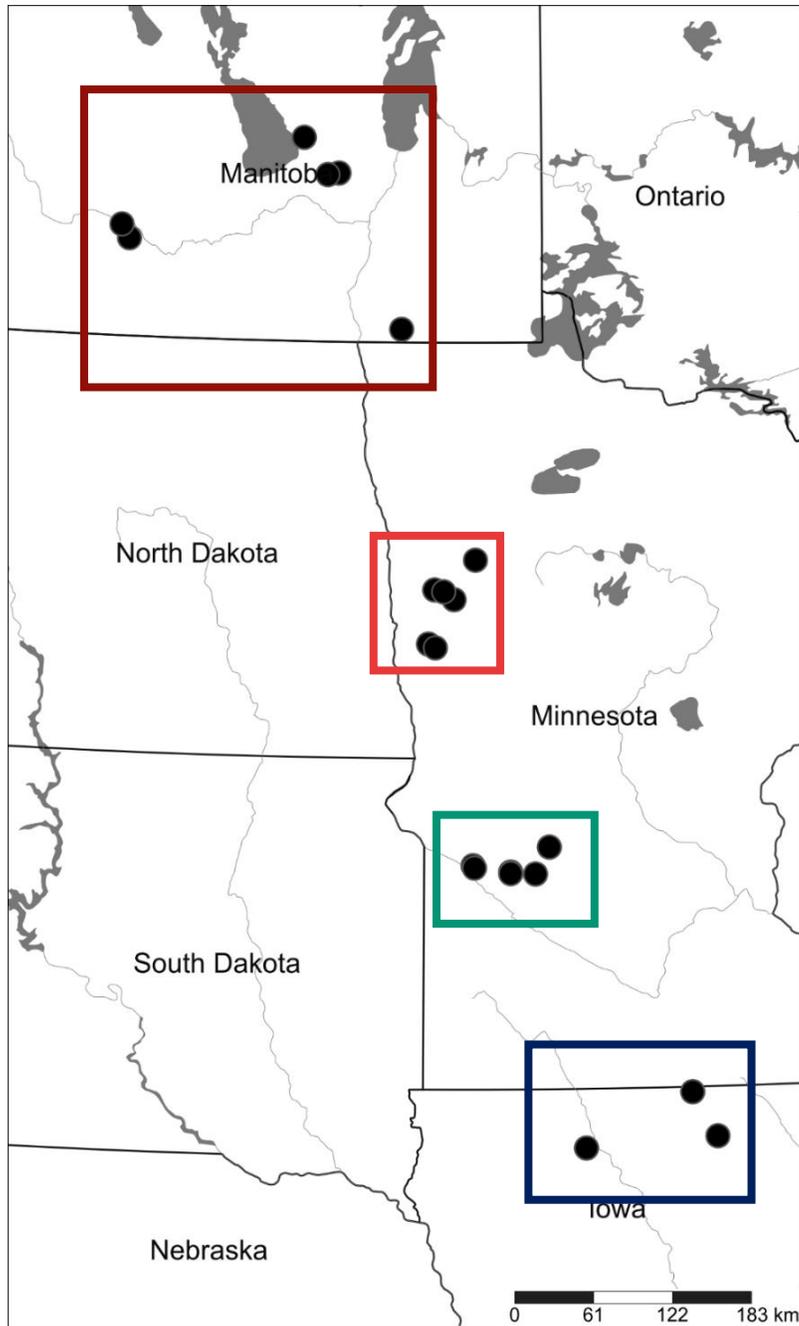


Figure 2.1. Map of *Cyripedium candidum* study sites in the 2016 flowering period. Boxes indicate study region (from bottom to top: dark blue=Iowa, light blue=southern Minnesota, light red=northern Minnesota, dark red=Manitoba).

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2.2.2 Transect Establishment and Vegetation Surveys

Cypripedium individuals produce clusters of genetically identical flowering stems called genets, with each flowering stem called a ramet. Upon arrival, each site was first surveyed to locate patches of orchid genets. I used these genets for a variety of studies including the measurement of floral features (Chapter 3) and assessment of fruit set (Chapter 4). I established two, 25 m transects at each site with individual transects separated by at least 4 m. Between these two transects, 30-50 orchid genets were identified with their distance along the transect recorded using a tape measure, so that they could be relocated in fruiting season to record reproductive success. To facilitate relocation, I marked transect ends with wooden stakes and georeferenced them using GPS (Appendix. 1). These transects also served as the focal point for surveying the co-flowering plant community and for pollination studies (Chapters 3 and 4).

Along orchid transects, I enumerated the number of flowering genets for all co-flowering species (including *C. candidum*) within 2 m of the centre of each transect on either side (Appendix 2.3). I counted stems if they were in bud or in flower, as plants that were in bud were likely to co-flower with orchids. All flowering plants were counted up to a maximum of 50 per species, to reduce the time needed to count very abundant species. To account for taxon differences in floral display, I averaged the number of flowers in ten genets for species with multiple flowers per genet. If flowers were less than 5 mm wide and were in a single cluster or head, I recorded them as one flower. If a plant had many clusters of small flowers arranged in heads and umbels (e.g., *Zizia* and *Senecio* spp.), I counted the number of clusters on ten flowering stems so that I could calculate an average cluster number for each species at each site. One site, S-MN11, was unique with respect to its co-flowering community (Appendix 2.1). This

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site had only three *Zizia* stems along the two surveying transects. Furthermore, the site MB3 was only large enough to establish one transect (Appendix 2.1).

In addition to transect-level surveys, I also conducted site-level surveys to account for off transect species. I ranked co-flowering species on a 4-point abundance scale within 100 m of orchid patches to capture the presence of any co-flowering species that may not have been present along the transects. To standardize surveying time at each site, I allotted fifteen minutes to walk around the site, but avoided areas with obviously different ecological conditions to where the orchids were found (e.g., higher elevation, changes in moisture levels, or increased woody vegetation), in order to avoid surveying additional diversity due to changes in habitats, and to ensure that the taxa I surveyed were associated with *C. candidum*.

When I surveyed transects later in the season for orchid fruit, I also resurveyed the co-flowering vegetation within 2 m of transects. Stems were not counted during this survey but rather each co-flowering genus was assigned a rank from 0 to 3, using the same abundance criteria adopted for site-level surveys during the spring (above). I also enumerated any stems of species in fruit, as these likely would have overlapped with orchid flowering.

For vegetation studies conducted during orchid flowering and fruiting (spring and summer surveys), I collected voucher specimens for each new species encountered, or when species identification was difficult. All voucher specimens will be deposited in the University of Manitoba Vascular Plant Herbarium.

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2.2.3 Statistical Analyses

Overall Co-flowering Community Patterns

Initial investigation of co-flowering data consisted of identifying trends in overall abundance and commonality of co-flowering genera. I first organized co-flowering taxa into groups based on which regions they were encountered in. This showed which genera were common and which were unique to certain study regions. Secondly, I ranked the co-flowering genera at each site to determine the top ten most abundant taxa per site. Then, I graphed these taxa using the number of times they were found in each of the top ten categories.

Vegetation Diversity Indices

All statistical analyses were completed using R version 3.6.3 (R Core Team 2017) using an alpha value of 0.05. I first identified the number of genera encountered, and then calculated Shannon's Diversity Index (H' ; Shannon 1948), and Pielou's Evenness Index (J' ; Pielou 1966) for each site using my site level survey data (within 100m of transects). See Appendix 2.2 for formulas for all indices used in this chapter I chose to analyze genera rather than species because the majority of the genera found in my study were monotypic (only had one species present at a site) and genera that were not monotypic at a site had species with very similar flowers (e.g., *Zizia aurea* vs. *Z. aptera*, *Viola sororia* vs. *V. nephrophylla*, *Sisyrinchium mucronatum* vs. *S. albidum* vs. *S. montanum*; Appendix 2.3).

As my site level data were collected on a four-point scale, I converted these to pseudovalues (rank 0=0, 1=10, 2=40, 3=80) before calculating diversity indices. These pseudovalues were then compared between each region using boxplots, and analyzed using a

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Kruskal-Wallis test since these variables were not normally distributed, even after transformation. To confirm that any trends observed were not caused by rare, off-transect genera, I calculated the same indices using genera only found directly on transects and tested them in a similar manner.

Finally, I calculated these same diversity indices in the same manner using data from the co-flowering survey conducted during the summer. Indices calculated from these surveys only used data from this time period, which were not combined with data from spring surveys. I made this choice because indices calculated from spring surveys used co-flowering data collected on a four-point scale in a 100m radius around transects, while data used for indices from summer surveys was only collected on a four-point scale within 2m of transects.

To compare beta diversity among study sites, Sørensen similarity indices (Sørensen 1948) were calculated to produce pairwise similarity tables for all study sites, using the same data sets described above. This index is calculated from the shared number of individuals from all genera at two sites, per the total number of individuals at each site (see Appendix 2.2 for calculations). After tables were constructed, I averaged the indices for each regional comparison, to compare similarity among and between study regions.

Vegetation Community Ordinations

To visualize how genera and sites were associated, I used principal components analysis (PCA) and redundancy analysis (RDA), using the “vegan” package in R (Oksanen et al. 2017). Stem count data from both transects at a site were averaged to produce site averages for each co-flowering genus. Analyzing transects separately would violate assumptions of independence for

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a RDA. I used transect averages for these ordinations as they captured a greater range of variation than site level (4-point scale) surveys. Since site MB3 was only large enough to establish one transect within it, I chose to average rather than sum the two transects from other co-flowering surveys, as this would control for the smaller area surveyed at this site.

These data were then log-transformed and analyzed with a PCA using a covariance distance matrix using the R-package “vegan” (Oksanen et al 2016). A covariance matrix is most appropriate for data measured on the same scale (Quinn and Keough 2002a). In addition to a PCA, I conducted a RDA to determine if the co-flowering composition of each site varied significantly by study region. While PCA characterizes the total variation in a data set and allowed for identification of contributing variables, RDA allows one to constrain the variation by certain variables to determine how much of the total variation is explained by them. I conducted redundancy analysis on my summer survey co-flowering data as well, using a data set of pseudo values (see above section) calculated from the 4-point ranks of species within two meters of transects during this time period.

Differences in overall genera richness and composition could be obscured on a PCA due to large differences in abundance. To determine how sites and genera were associated through the presence and absence of genera, I used my transect average data from spring surveys to obtain a data set of presence/absence of each flowering genus along either transect at a site and then analyzed this using a principal coordinates analysis (PCoA) using a Jaccard's distance matrix, again with the “vegan” package. Jaccard's distance was chosen because it does not emphasize mutual genera absences, which were common in my data set (Cheetham and Hazel 1969).

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Weather and Site Data

Climate data were obtained for U.S. locations from Weather Underground (www.wunderground.com/history) and Canadian locations from The Weather Network (www.theweathernetwork.com). These websites were chosen because they allowed for collection of historical data from a specific range of dates. Data were recorded for the average, maximum, and minimum temperatures; the maximum and sum of growing degree days; and the sum of precipitation recorded between the beginning of spring (April 1st, 2016) to the date I surveyed a site. This start date was based on Dunnell and Travers (2011), who found April temperature was the best predictor of floral emergence date in spring flowering species in the tall grass prairie of North Dakota.

I also collected these same data from the beginning of spring to the first day of my summer surveys (June 20, 2016), which were used in *C. candidum* fruit set analyses in Chapter 4. Total precipitation from the beginning of November to the each of these end dates was also summed, to represent the snow-melt and precipitation experienced at a site. I collected precipitation data for a year before orchid flowering began (May 15th, 2015 to May 15th, 2016) as well, to get an estimate of the amount of water populations had received prior to my field season. Finally, I collected data on the area of a site by using the polygon function of Google Earth.

Correlations were investigated among weather and spatial variables using a scatterplot matrix as well as testing to see if the Spearman's rank order correlation of variables was significant. Many variables were non-normally distributed, even after log transformation, so I chose this non-parametric test to investigate correlations. I then conducted an additional

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redundancy analysis to determine if any of these variables explained a significant amount of variation in the co-flowering community, using my data set from spring surveys. Those with significant Monte-Carlo permutation tests, were included in an analysis with study region to determine how much additional variation they explained.

2.3 Results

2.3.1 Patterns in Genera Dominance

Across the four study regions, the number of recorded genera increased in the more northern study regions (Table 2.1). The total number of genera encountered in this study was 31, and the total number of genera encountered in Iowa, southern Minnesota, northern Minnesota, and Manitoba were 13, 16, 20, and 29 respectively. Manitoba had seven taxa that were unique to the region, while Manitoba and northern Minnesota shared six additional unique taxa that were not encountered in southern Minnesota or Iowa (Table 2.1, Fig 2.2). A total of ten flowering taxa were common to all study regions (Table 2.1, Fig 2.2). When the ten most abundant genera at each study site were ranked, *Zizia* was found to be the most commonly encountered genus and was usually the most abundant genus at a site (Fig. 2.2). However, *Hypoxis*, *Senecio*, *Viola*, *Fragaria* and *Commandra* were all common and fairly abundant genera.

2. Latitudinal changes in the co-flowering community across *C. candidum*'s range

Table 2.1. All co-flowering genera observed in the 2016 spring flowering surveys, across tall grass prairie populations of *Cypripedium*. Genera are separated based on the number of study regions in which they were found (Iowa, southern Minnesota, northern Minnesota, or Manitoba; Fig 2.1). Genera found in high abundance in a region are marked with an “X”, while low abundance genera are marked with an “x”. Genera not observed in a region are symbolized with “(L)” if they were observed during summer surveys or “(X)” if *The Atlas of the Flora of the Great Plains* (Great Plains Flora Association 1977) or *Vascular Plants of Minnesota* (Ownbey and Morley 1991) indicated their presence in a region.

Genus	Iowa	Southern MN	Northern MN	Manitoba
Genera observed in all regions				
<i>Zizia</i>	X	X	X	X
<i>Packera</i>	X	X	X	X
<i>Commandra</i>	X	X	X	X
<i>Hypoxis</i>	X	X	X	X
<i>Pedicularis</i>	X	X	X	X
<i>Sisyrinchium</i>	X	X	X	X
<i>Fragaria</i>	X	X	X	X
<i>Lithospermum</i>	X	X	X	X
<i>Viola</i>	X	X	X	X
<i>Agoseris</i>	X	X	X	X
Genera observed in three regions				
<i>Polygala</i>		X	X	X
<i>Castilleja</i>	X	(X)	X	X
<i>Geum</i>	X	(X)	X	X
<i>Potentilla</i>	(X)	X	X	X
<i>Vicia</i>	(X)	x	X	x
Genera observed in two regions				
<i>Taraxacum</i>	(X)	X	(X)	X
<i>Maianthemum</i>		(X)	X	X
<i>Erigeron</i>		(X)	x	x
<i>Gallium</i>	(L)	(L)	x	x
<i>Lathyrus</i>	(X)	(X)	x	x
<i>Euphorbia</i>		x	(X)	x
<i>Coptis</i>			x	x
Genera observed in one region				
<i>Eleagnus</i>			(X)	X
<i>Anemone</i>	(X)	(X)	(X)	x
<i>Cypripedium</i> hybrids	(X)	(X)	(X)	x
<i>Cypripedium parviflorum</i>	(X)	(X)	(X)	x
<i>Rosa</i>	(L)	(L)	(L)	x
<i>Trifolium</i>	(L)	(L)	(L)	x
<i>Thalictrum</i>	(L)	(L)	(L)	x
<i>Lonicera</i>		x		
<i>Baptisia</i>	x			

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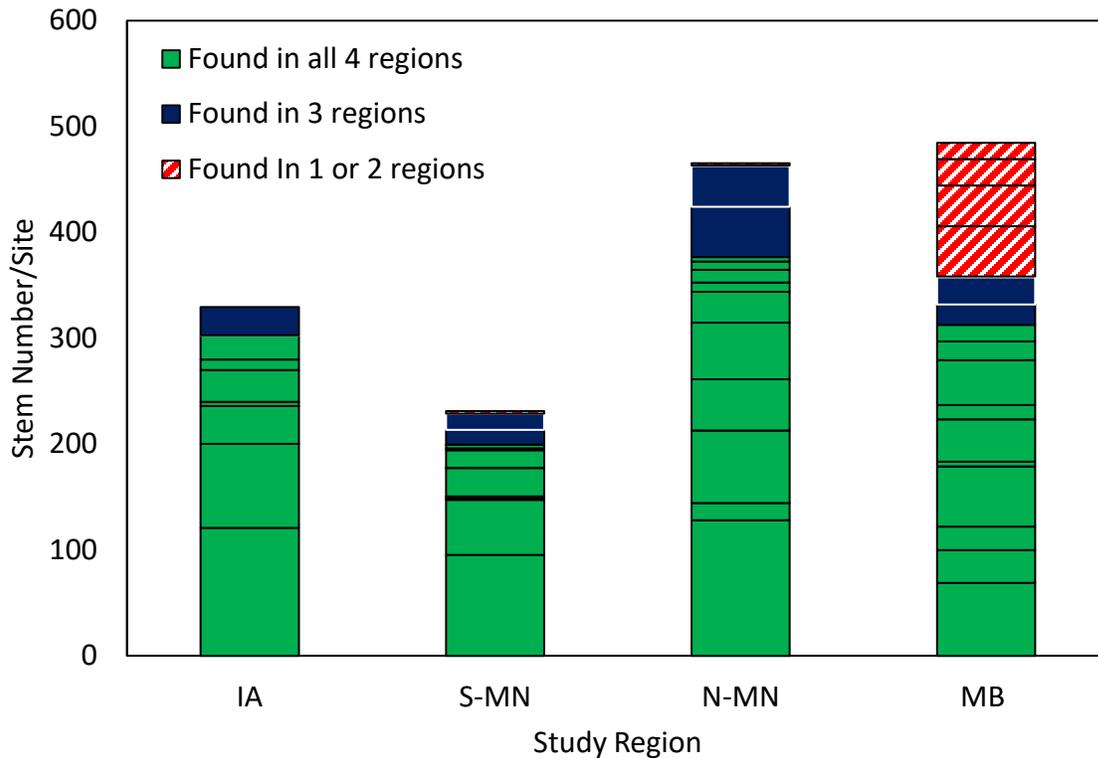


Figure 2.2. The number of co-flowering stems per site of all co-flowering genera observed during the *Cypripedium candidum* flowering period at each tall grass prairie population in 2016 (spring surveys). Genera are coloured by regional occurrence (see legend; Iowa: “IA”, Southern Minnesota “S-MN”, Northern Minnesota: “N-MN”, Manitoba: “MB”; Fig. 2.1). Each bar represents a plant genus and bars are ordered in decreasing abundance from bottom to top within each regional category. The most common genera encountered across regions were *Zizia*, *Packera*, and *Commandra* (bottom three green bars).

The genera *Anemone*, *Galium*, *Rosa*, and *Trifolium* were not found in southern Minnesota or Iowa during the flowering season of *C. candidum* (spring surveys) but were located during the fruiting season (summer surveys). Other genera were not found in either survey in southern regions. To determine if these taxa were actually native to southern study regions, but perhaps just not found at my study sites, I used range maps from *The Atlas of the Flora of the Great Plains* (Great Plains Flora Association 1977) and *Vascular Plants of Minnesota* (Ownbey and

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Morley 1991). This revealed a mixture of plants that are not found in southern regions and those that just were not observed in my study. The species I observed in the genera *Maianthemum*, *Erigeron*, and *Polygala* in this study are all absent from northwestern Iowa, but other species in these genera that flower later than my study period occur there. *Taraxacum* and *Vicia* were also not observed at study site in Iowa, but these genera do occur in the region (Great Plains Flora Association 1977, Ownbey and Morley 1991). Most of the taxa that were only found in Manitoba during spring surveys, also occur further south (e.g., *Cypripedium parviflorum*, *Anemone*) with the exception of *Eleagnus* which is only found as far south as northern Minnesota (Great Plains Flora Association 1977, Ownbey and Morley 1991). *Baptisia* occurs as far north as southeastern Minnesota, so this taxon would not occur at my study sites in Minnesota or Manitoba (Great Plains Flora Association 1977, Ownbey and Morley 1991).

2.3.2 Spring Survey Vegetation Diversity Indices

The number of genera present in the co-flowering community was variable across this study, the lowest number of genera observed at a site (including plants found away from transects) was four, not including *C. candidum*, while the highest was 16. Genera richness was significantly different among regions ($\chi^2_3=11.7$, $p<0.01$), with the highest mean being observed in Manitoba, and the lowest in Southern Minnesota (Fig. 2.3). Similar patterns were seen for Shannon's Diversity, with the highest diversity index observed at 2.07 in Manitoba and the lowest at 1.2 in southern Minnesota. Shannon's diversity differences were also significant ($\chi^2_3=11.5$, $p<0.01$, Fig. 2.3). Pielou's evenness was high in all regions, with no significant differences among them ($\chi^2_3=6.0$, $p>0.05$, Fig. 2.3. This value can range 0 to 1, but all sites had a

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value of over 0.7. Similar patterns in diversity indices were calculated using stem counts averages from genera just along the transects (excluding additional off-transect co-flowering genera).

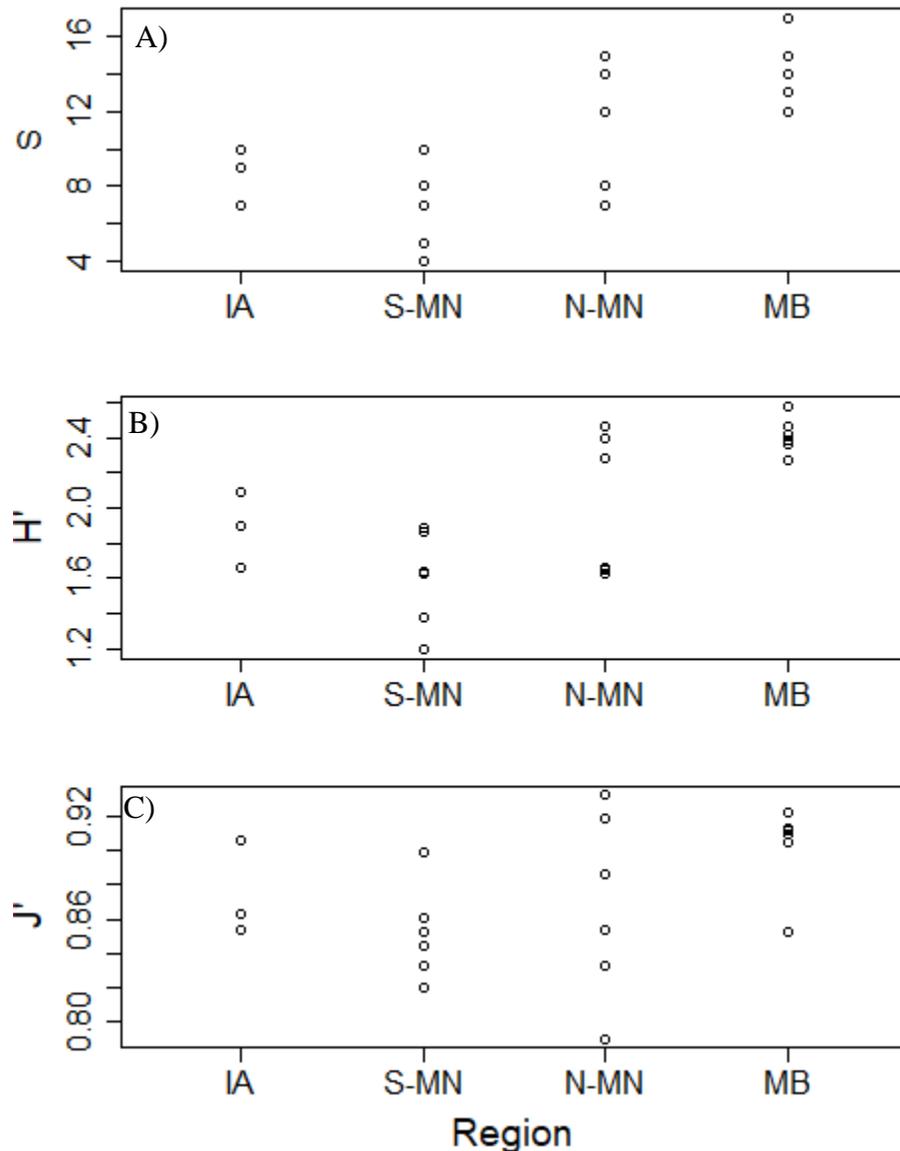


Figure 2.3. The number of genera (S, A), Shannon's diversity index (H', B), and Pielou's evenness index (J', C) of the spring flowering forb community in tall grass prairie populations of *Cyripedium candidum* (spring surveys), for each study region (Iowa= "IA", southern Minnesota= "S-MN", northern Minnesota= "N-MN", Manitoba= "MB"; Fig. 2.1) in 2016.

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Sørensen similarity indices showed moderate values among and between study regions (Table. 2.2). This index can range from zero to one, with larger values indicating higher similarity. The highest similarities were seen within Iowa (0.54) and between southern Minnesota and Iowa (0.50). In contrast, the lowest similarities were observed within Manitoba (0.29) and between southern Minnesota and Manitoba (0.30). Other regional comparisons ranged between 0.3 and 0.5, with more northern regions generally showing greater dissimilarity in the co-flowering community compared to other study regions (Table 2.2). Examination of raw data revealed that the increase in the number of genera further north created greater dissimilarity among sites due to the presence and absence of rare genera, which is typical for Sørensen's index (Colwell and Coddington 1994, Gotelli and Colwell 2001, Magurran 2004, Choa et al. 2005). In contrast, with a lower diversity in southern regions, sites had a more similar beta diversity simply because there was less opportunity for variation with fewer genera. Raw data also showed that there was a large amount of variation in the abundance common species in Manitoba, particularly *Zizia*, which had stem counts ranging from 3-80 stems along transects.

Table 2.2 Sørensen indices averages for all study regions (IA=Iowa, S-MN=Southern Minnesota, N-MN=Northern Minnesota, MB=Manitoba; Fig 2.1), calculated from surveys of the spring flowering forb community in tall grass prairie populations of *Cypripedium candidum*, for each study region in 2016 (spring surveys). Values with moderately high similarity (>.5) are highlighted in blue, and values with low similarity (<.3) are highlighted in red.

Region	IA	S-MN	N-MN	MB
IA	0.54	0.50	0.46	0.37
S-MN		0.43	0.41	0.29
N-MN			0.47	0.36
MB				0.30

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2.3.3 Spring Survey Vegetation Community Ordinations

All ordinations were conducted with a dataset of site averages of co-flowering stem counts from the two transect surveys at a site. Ordinations conducted with pseudovalues from ranked surveys at a site produced similar results, so only transect survey data are presented here due to their greater variation among co-flowering taxa. Between the first two principal components axes, 44.3% of the variation in the co-flowering community was explained (Fig. 2.4). Overall patterns indicate that the positive side of PC1 is most associated with an overall increase in co-flowering abundance, a common finding in ordination techniques (Jensen and Meyer 2001, Klimek et al. 2007, Marini et al. 2007). PC1 accounts for 30.4% of the variation and is strongly associated with the abundance of *Pedicularis*, *Polygala*, *Castilleja*, *Commandra*, and *Sisyrinchium*. Many northern Minnesota and some Manitoban sites are associated with these genera. The second axis was more strongly associated with *Cypripedium* spp., *Taraxacum*, *Maianthemum*, *Euphorbia*, *Eleagnus*, *Fragaria*, *Anemone*, and *Lithospermum* and accounts for 13.3% of the variation in the co-flowering community. *Lithospermum* is the highest weighted genus along PC2, with two Manitoban sites associated with an abundance of this taxon. Sites in Iowa and southern Minnesota are typically found towards the negative ends of both PCA axes, indicating that they are characterized by a low abundance of the aforementioned taxa. Axes 3 and 4 of this PCA explained an additional 19% of the variation in the co-flowering community and were associated with either an increased abundance of common taxa (PCA axis 3) or Manitoban taxa (PCA axis 4). No strong regional associations were seen along these latter axes.

2. Latitudinal changes in the co-flowering community across *C. candidum*'s range

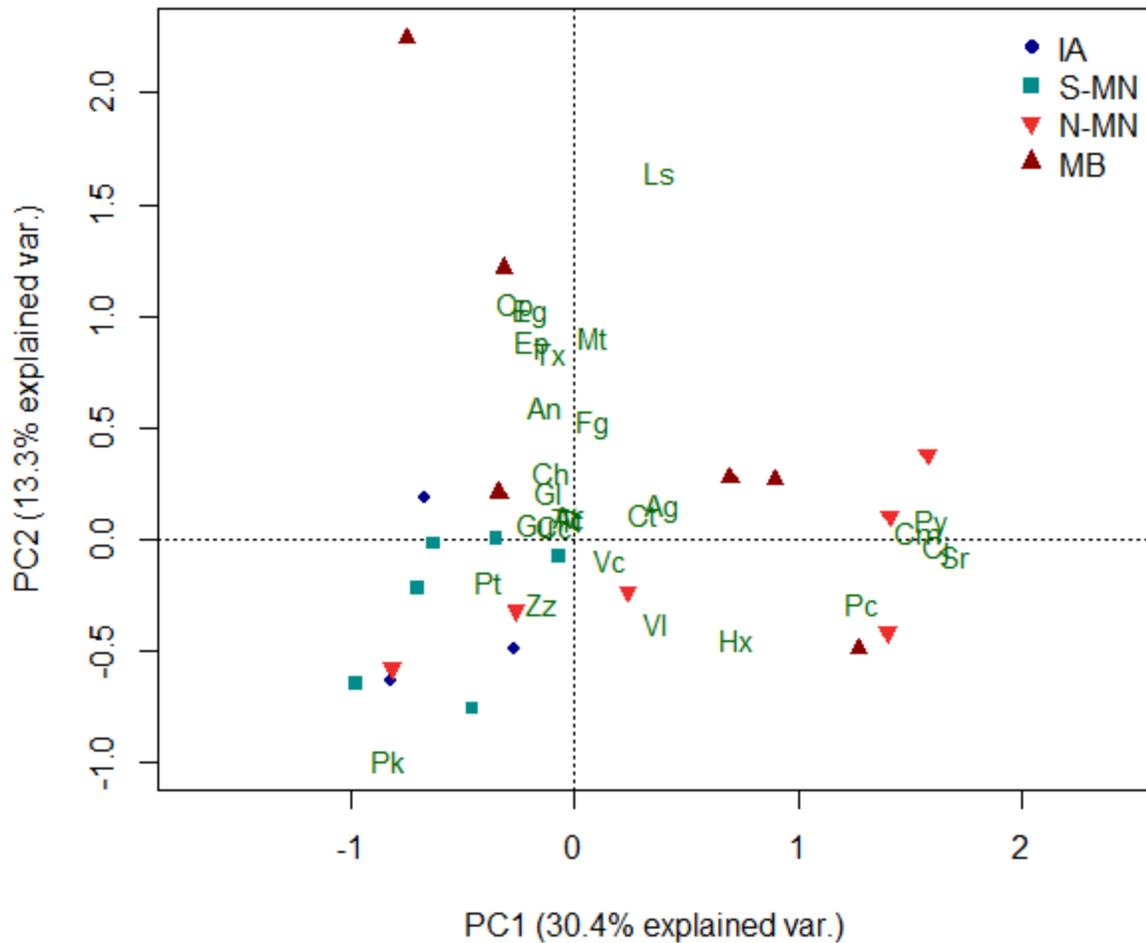


Figure 2.4. Principal components analysis of the co-flowering vegetation within each tall grass prairie population of *Cypripedium candidum* during its flowering period in 2016 (spring surveys), using a covariance matrix. Genera scores are plotted in green, and labelled based on a two-letter genus code (*Cypripedium candidum* “Cc”, *Agoseris* “Ag”, *Anenome* “An”, *Astragalus*, “At”, *Castilleja* “Cj” *Commandra* “Cm”, *Coptis* “Ct”, *Cypripedium* hybrids “Ch”, *Cypripedium parviflorum* “Cp”, *Taraxacum* “Tx”, *Eleagnus* “Eg”, *Maianthemum* “Mt”, *Fragaria* “Fg”, *Galium* “Gl”, *Geum* “Gu”, *Hypoxis* “Hx”, *Euphorbia* “Ep”, *Lithospermum* “Ls”, *Packera* “Pk”, *Pedicularis* “Pc”, *Polygala* “Py”, *Potentilla* “Pt”, *Sisyrinchium* “Sr”, *Trifolium* “Tf”, *Vicia* “Vc”, *Viola* “VI”, *Thalictrum* “Tc”, *Zizia* “Zz”). Sites within regions are plotted with different symbols, and colour coded by region (Iowa: “IA”, Southern Minnesota “S-MN”, Northern Minnesota: “N-MN”, Manitoba: “MB”; see legend and Fig. 2.1).

After constraining the variation in my PCA by the factor variable of study region in a redundancy analysis, permutation tests found that regions significantly differed in their co-flowering composition ($F_{3,17}=1.81$, $p<0.026$; Fig. 2.5). Weights for each co-flowering genus

2. Latitudinal changes in the co-flowering community across *C. candidum*'s range

along each RDA axis are given in Appendix 2.3. Study regions in Iowa and southern Minnesota did not differ from each other, and were both characterized by low co-flowering abundance and diversity, but these sites were associated with increased abundance of *Senecio*. The study regions, northern Minnesota and Manitoba were both different from Iowa and southern Minnesota, as well as different from each other. Northern Minnesota was associated with increased abundance of *Sisyrinchium*, *Commandra*, *Pedicularis*, *Hypoxis*, *Polygala*, and *Castilleja*. Higher abundance of *Taraxacum*, *Maianthemum*, *Cypripedium parviflorum*, *Eleagnus*, *Agoseris*, and *Lithospermum* characterized sites in Manitoba (Appendix 2.3). An outlier in Manitoba was obvious in both my PCA and RDA from spring surveys (Figs. 2.4 and 2.5), this site (MB6) was weighted so highly due to the mutual abundance of both *Lithospermum* and *Maianthemum*, which was an uncommon combination among other sites.

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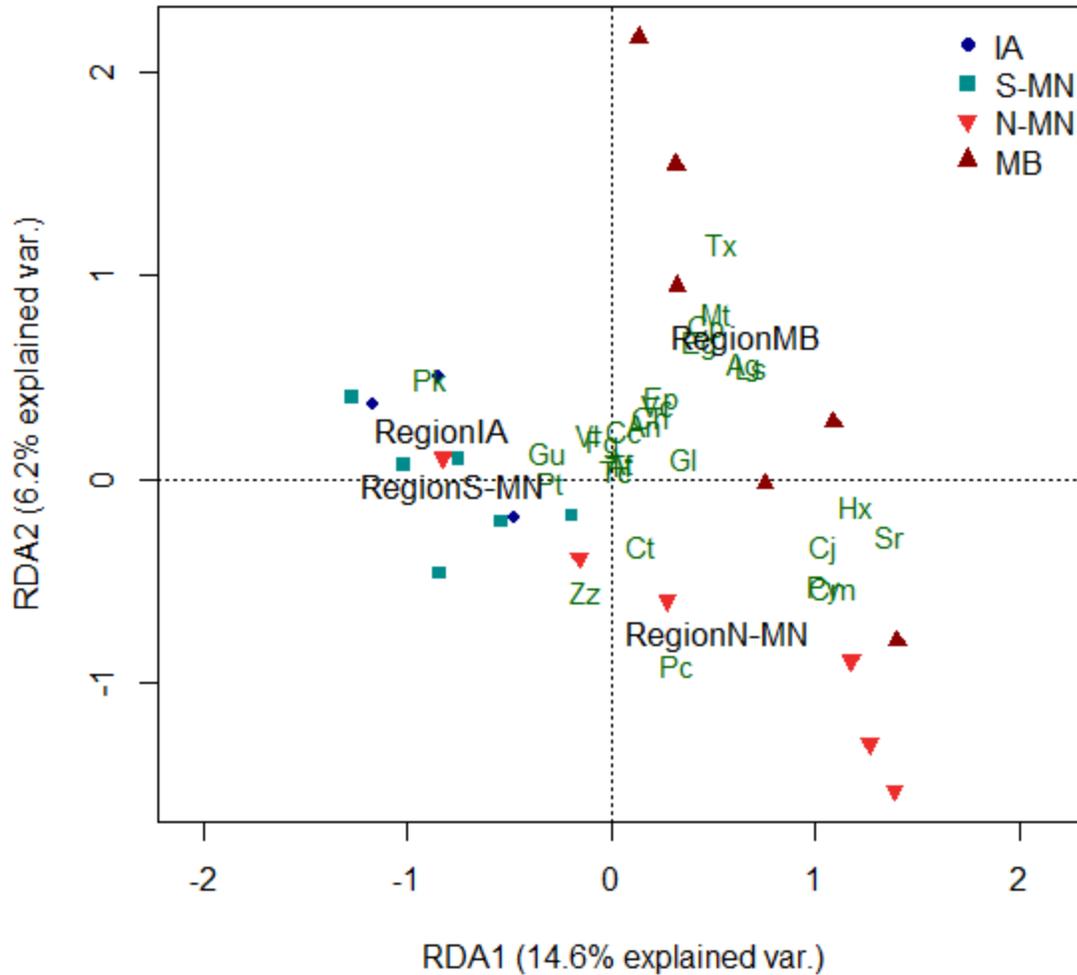


Figure 2.5. Redundancy analysis of the co-flowering vegetation within each tall grass prairie population of *Cyripedium candidum* during its flowering period in 2016 (spring surveys), using a covariance matrix and constrained by study region. Genera scores are plotted in green, and labelled based on a two-letter genus code (see Fig. 2.4 for full list of codes). Study regions (Iowa: “IA”, Southern Minnesota “S-MN”, Northern Minnesota: “N-MN”, Manitoba: “MB”; Fig. 2.1) are plotted in black, and indicate the associated co-flowering composition for each region. Sites within regions are plotted with different symbols, and colour coded by region (see legend).

I also conducted all of the above analyses using my stem count transect averages, multiplied by each species average number of floral units at that site. This was done to determine if the floral displays of certain species were associated with particular study regions. These

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analyses were almost identical to analyses conducted with stem count data alone, so they are not presented. The only noticeable difference between the two analyses was that *Zizia* floral display was higher in southern Minnesota and Iowa than more northern study regions. This trend was not detected using stem count data.

Principal co-ordinates analysis, based on a presence/absence matrix of species along transects, showed a similar pattern to that recovered using principal components analysis of the taxon frequency dataset. The first two axes accounted for 44.5% of the variation in the data (Fig. 2.6) with PCoA1 depicting an increase in the number of taxa, similar to my PCA. Site assemblages show similar patterns to principal components analysis (Fig. 2.4), except that Iowa sites group away from other regions, indicating the diversity of sites in this region more species-poor than other regions. Northern and southern Minnesota are more closely associated with each other in this analysis. Manitoba sites commonly fall further along the first PCoA axis, likely due to the higher diversity of genera.

2. Latitudinal changes in the co-flowering community across *C. candidum*'s range

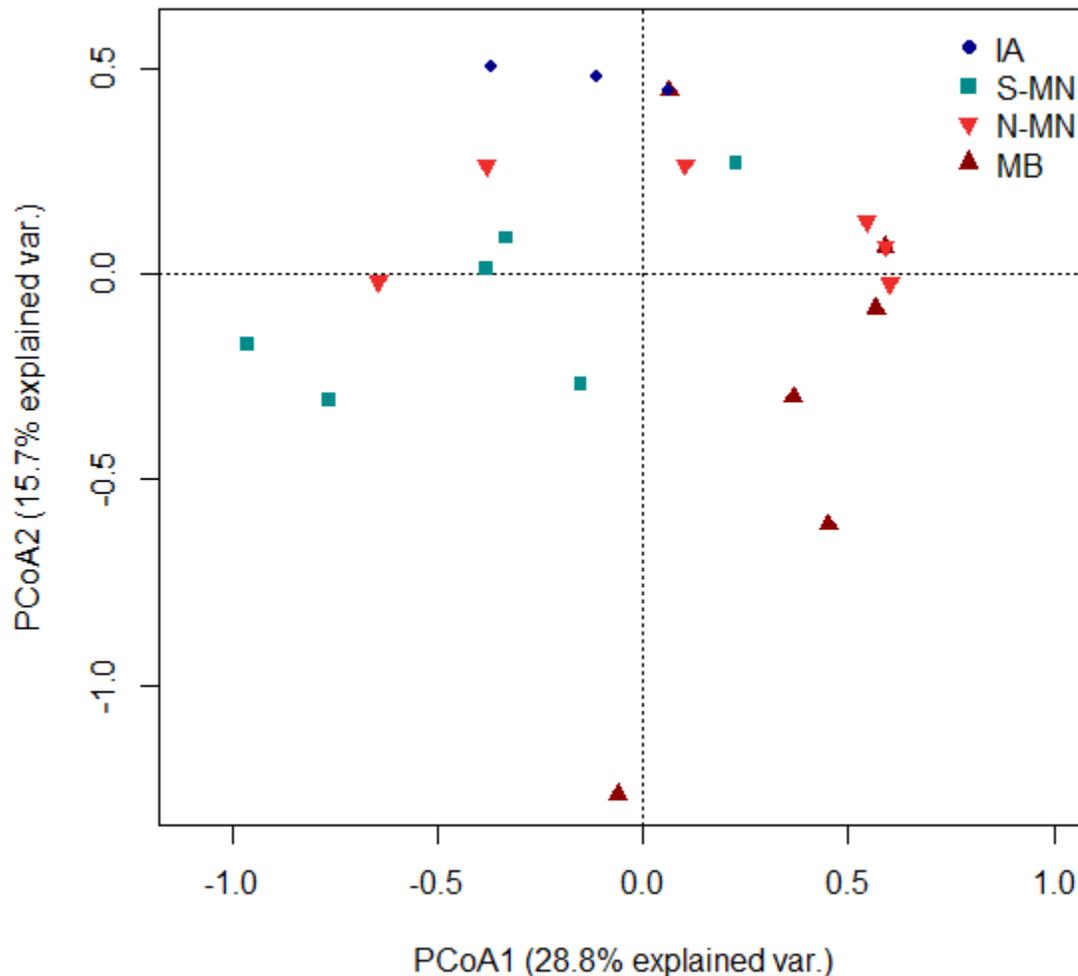


Figure 2.6. Principal co-ordinates analysis of the co-flowering vegetation within each tall grass prairie population of *Cypripedium candidum* during its flowering period in 2016 (spring surveys), using a Jaccard's distance matrix. Sites within study regions are plotted with different symbols, and colour coded by region (Iowa: "IA", Southern Minnesota "S-MN", Northern Minnesota: "N-MN", Manitoba: "MB"; see legend and Fig. 2.1).

2.3.4 Patterns in Environmental Variables during Spring Surveys

Scatterplot matrices of spatial and environmental variables showed that almost all temperature-related variables were highly correlated (all $r > 0.62$, all $p < 0.003$). As I had most

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confidence in the average temperature variable (growing degree day calculations seemed to vary among data sources), I used this in a scatterplot matrix with the other spatial and environmental variables. This analysis revealed a strong correlation between spatial variables (latitude and longitude) and both temperature and precipitation (all $p < 0.05$; Fig. 2.7).

When I included spatial and environmental variables in redundancy analyses, latitude, longitude, and temperature during the flowering season all explained a significant proportion of the variation in the co-flowering data (10% ,13.5%, and 15.4% respectively, $p < 0.05$ for each). However, as these data were highly correlated with each other and collinear with study region, it was not appropriate to include them all in a single redundancy analysis. Study region alone accounted for more of the variation in co-flowering community than any of these other variables, making it the preferred variable in the RDA.

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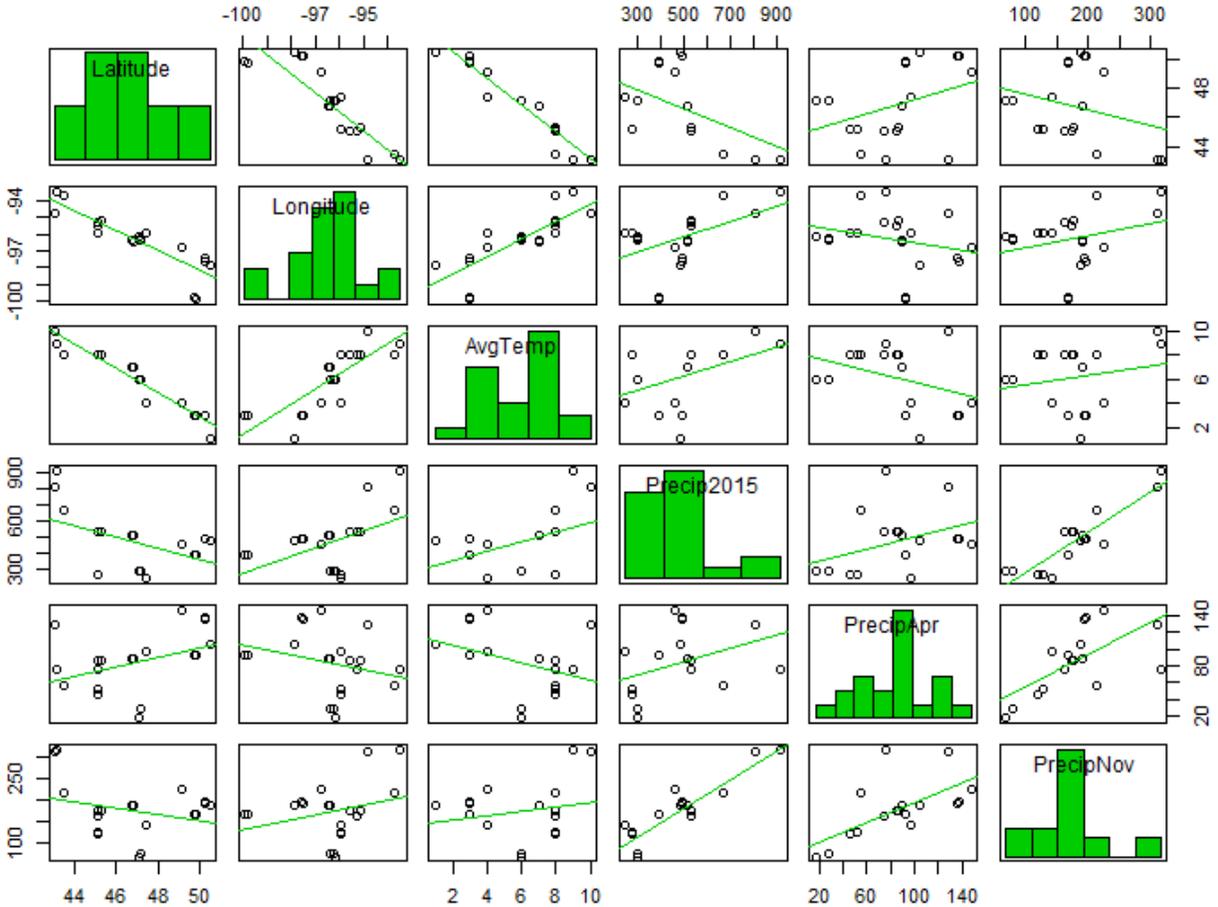


Figure 2.7. Scatterplot matrix of latitude ($^{\circ}$), longitude ($^{\circ}$), average temperature over the *Cyripedium candidum* flowering season ($^{\circ}\text{C}$), precipitation in the 12 months before *C. candidum* flowering season began (mm), precipitation from November 2015 to my arrival at a site (mm) and precipitation during the *C. candidum* flowering season (mm) for each study site in 2016. Histograms of each variable are plotted on the diagonal.

2.3.5 Summer Vegetation Surveys

Data collected during the summer surveys, during the fruiting period of *C. candidum*, showed similar patterns to those collected during spring surveys. As discussed in the methods, these data were not combined with data from spring surveys due to the differences in sampling scale. The number of plant genera in fruit or flower during these summer surveys did not vary significantly between study regions ($\chi^2_3=7.1$, $p>0.06$, Fig. 2.8a), nor did Shannon's diversity and

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evenness (respectively: $\chi^2_3=6.1$, $p>0.1$, Fig. 2.8b; $\chi^2_3=7.8$, $p>0.05$, Fig. 2.8c). Evenness was much higher in summer surveys, with a value greater than 0.7 for all sites (Fig. 2.8c). In total, 46 genera were recorded during summer surveys, in contrast to the 27 recorded during the spring. Eighteen genera were observed in both sets of floral surveys.

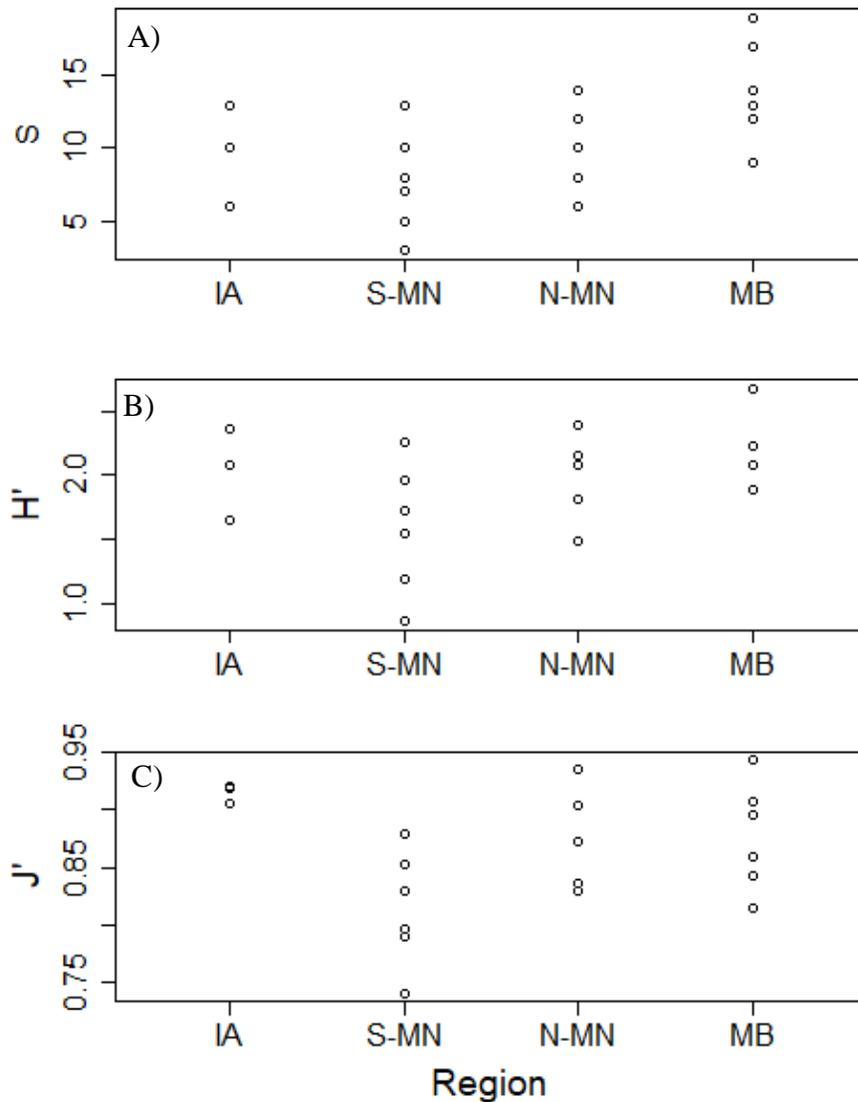


Figure 2.8. The number of genera (S, A), Shannon's diversity index (H', B), and Pielou's evenness index (J', C) of the late spring flowering forb community (summer surveys) in tall grass prairie populations of *Cypripedium candidum*, for each study region (Iowa= "IA", southern Minnesota= "S-MN", northern Minnesota= "N-MN", Manitoba= "MB"; Fig 2.1) in 2016. These data were collected during the fruiting period of *C. candidum*, see Fig. 2.2 for indices from the flowering period.

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A redundancy analysis showed that study region accounted for 20.9% of the variation in the plant community (Fig. 2.9). The first RDA axis explained 14.0% of the plant community's variation, and, similar to the spring survey RDA, is associated with the total number of genera at a site. Genera diversity increased towards the negative side, and more northern study regions were also found on this side. Highly associated genera with this axis included *Pycnathemum* and *Liatris* on the positive (lower diversity) side, and *Galium* and *Medicago* on the negative side. The second axis accounted for 6.9% of the variation in the vegetative community, with both Minnesota regions on its positive side and Manitoba and Iowa on its negative side. Genera associated with the positive side of this axis included *Dalea* and *Zigadenus*, and *Pycnanthemum* and *Galium* found towards the negative side of this axis. Unlike the spring RDA, both Minnesota regions separated from the other study regions in the summer survey RDA.

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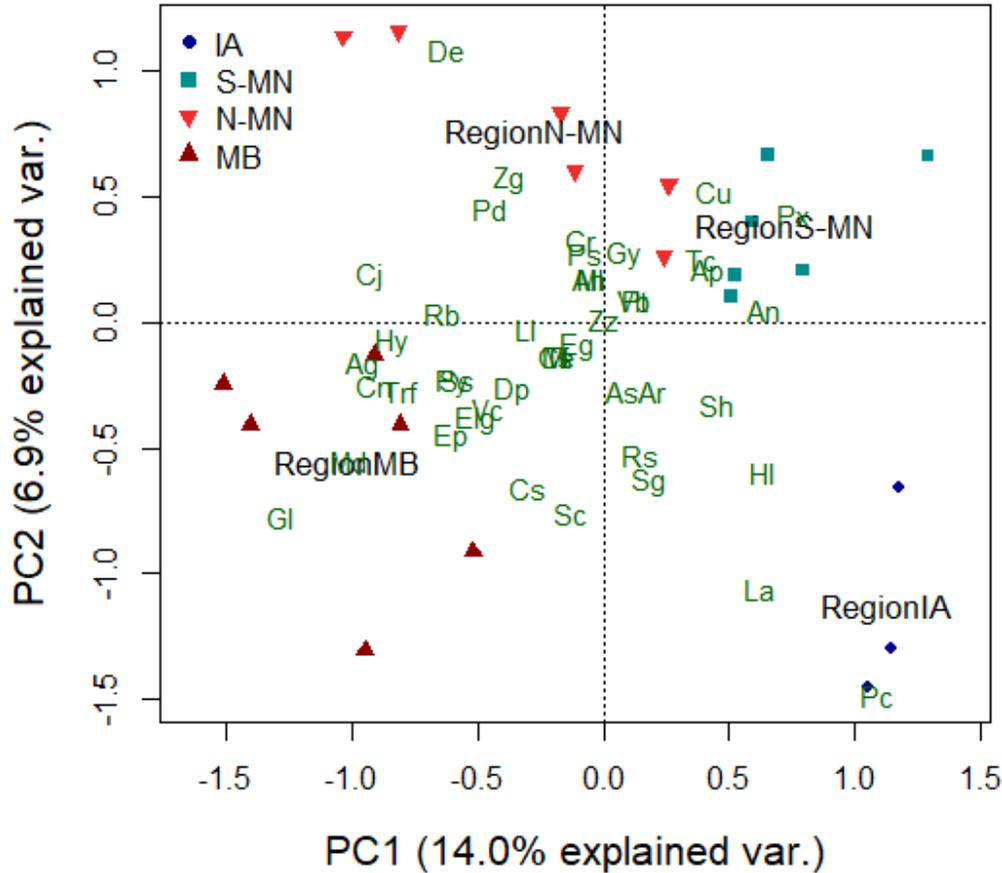


Figure 2.9. Redundancy analysis of the co-flowering vegetation within each tall grass prairie population of *Cyripedium candidum* during its fruiting season in 2016 (summer surveys), using a covariance matrix and constrained by study region. Genera scores are plotted in green, and labelled based on an abbreviated genus code (see Appendix 2.3 for full list of genera and abbreviations). Study regions (Iowa: “IA”, Southern Minnesota “S-MN”, Northern Minnesota: “N-MN”, Manitoba: “MB”; Fig. 2.1) are plotted in black, and indicate the associated co-flowering composition for each region. Sites within regions are plotted with different symbols, and colour coded by region (see legend).

2.4 Discussion

2.4.1 Overall Similarity of the Co-flowering Community

Despite significant changes between regions in genera dominance and diversity, ten of the 31 genera recorded were found in all four regions (Table 2.1), which combined to form over

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64% of the spring flowering community in all sites (Fig. 2.2). Some common genera (e.g., *Zizia*, *Fragaria*, *Viola*) were not highly weighted variables in any community ordinations, because these methods rely on a covariance matrix for computation (Quinn and Keough 2002a) and all sites had a similar proportion of these genera. These common genera, while not useful for distinguishing regions, probably play an important ecological role in supporting the pollinator community (Robson 2008) and may affect the pollination success of *C. candidum* (Chapter 4).

Site evenness was moderately high (0.8-0.92) across all study sites, likely due to the similar abundance of *Zizia*. Since this genus was often far more abundant than any other co-flowering genus at all sites, this would likely produce similar evenness values across my study. The higher evenness values in summer surveys likely reflect that other co-flowering genera had become more abundant, matching the abundance of *Zizia*. While *Zizia* was still the most dominant genus in summer surveys, *Galium*, *Anemone*, and *Thalictrum* were all quite abundant as well, likely driving the increase in evenness values from this time period.

My research indicates that *C. candidum* experiences a fairly similar spring community across its western range, which has not been noted in the literature. Patterns of genera dominance across the broad geographic area included in this study were consistent with field work conducted in Manitoba from 2011-2015 (Pearn 2012, Anderson 2015, Semmler 2016, unpubl. data). In these studies, *Zizia*, *Hypoxis*, *Packera*, *Commandra*, *Lithospermum*, etc were dominant genera during the *C. candidum* flower season, a pattern that was similar to that found in this study. Robson (2008) found that *Zizia* was the third most visited insect-pollinated plant, after two later season *Solidago* species, in a tall grass prairie site also used in the previously mentioned studies. Other studies of tall grass prairie vegetation do not identify flowering forb genera or

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species that dominate the community, making it difficult to compare results to my study (Mitchell et al. 1996, Coppedge et al. 1998).

2.4.2 Variation in the Co-flowering Community

Fourteen of 31 genera recorded during spring surveys were only found in the northern portion of *C. candidum*'s range (Northern Minnesota and Manitoba; Table 2.1), and seven of these were found only in Manitoba. Many of these genera were fairly rare, with fewer than five stems per site in each study region. Range maps of co-flowering genera revealed that four of these genera are absent from Iowa, although ten occur across my study area. These patterns in the co-flowering community confirm my prediction that diversity would increase with latitude during the flowering period of *C. candidum*. This finding was shown in both the Shannon's diversity, and number of co-flowering genera for each study region.

Trends in beta diversity across this study were strongly influenced by the presence or absence of rare taxa, with study sites becoming less similar as latitude increased. Sørensen values were highest among southern regions, but decreased both between and within study regions further north. This pattern was likely due to higher number of rare genera in Manitoba, which Sørensen indices are sensitive to (Colwell and Coddington 1994, Gotelli and Colwell 2001, Magurran 2004, Chao et al. 2005). The effects of rare genera were not as pronounced in my ordinations, but some of these taxa were highly weighted on both my redundancy and principal components analyses, differentiating northern regions from southern (Appendix 2.3). Out of the 12 genera with a weighting over 0.4 on both RDA axes, seven were found in all regions, while five were found in 3 or fewer regions (Table 2.1 and Appendix 2.3). These results indicate that

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significant changes in diversity among study regions is driven by both changes in the presence/absence of sparse genera as well as large scale changes in the abundance of common genera.

The extent to which rare genera may affect *C. candidum* pollination is unclear, especially if they exist at low floral densities. These rare genera are unlikely to affect pollination unless they attract novel pollinators to an area or share pollinators with *C. candidum*, two traits that are normally possessed by influential co-flowering taxa (Ghazoul 2006, Internicola et al. 2007, Sakata et al. 2014a, Seifan et al. 2014). The effects of these low-density taxa on reproduction are not examined in Chapter 4, as they were not commonly visited by insects (see Chapter 3). However, these taxa may show an effect at more local scales within a site, and this avenue of research should be explored in future studies.

Species occurrence showed similar patterns between spring and summer survey ordinations. The RDA using summer survey data showed more of a separation between Iowa and southern Minnesota, and less separation between the two Minnesota regions. This indicates that diversity and community composition is similar in the two Minnesota regions, but the co-flowering taxon abundance was lower during spring surveys in southern Minnesota. This trend is reinforced by the separation between Iowa and southern Minnesota in my PCoA from spring surveys as well. The PCoA was particularly good for illustrating the higher diversity in Manitoba sites, which separated from all other study regions. Despite certain differences within the patterns from each ordination, the overall increase of co-flowering genera in northern regions is consistent for all techniques, indicating that my data were robust and the patterns observed are reliable.

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The patterns revealed in this study are consistent with hypotheses regarding the effect of growing season on flowering phenology. The increase in diversity with latitude is consistent with predictions that in the early season, northern latitudes have a more compressed flowering season resulting in a higher density of co-flowering genera than southern regions (Elzinga et al. 2007, Wolkovich et al. 2014). Furthermore, since this study took place during the flowering season of *C. candidum*, the patterns described also match the predictions from the rewardless pollination literature, which predict that rewardless flowers should flower earlier than rewarding genera to avoid competition and take advantage of naïve pollinators (Internicola et al. 2008, Pellissier et al. 2010, Internicola and Harder 2012). My research indicates that *C. candidum* does flower with fewer co-flowering genera further south in its range, but this strategy may be limited in northern regions, as predicted by Pellissier et al. (2010).

The higher diversity encountered further north could also be due to the later survey date for northern populations. However, summer surveys no longer showed significant differences in diversity among study regions, indicating that more flowering genera had emerged further south to begin to match the total diversity of the northern regions. If this trend continued later into the season, perhaps southern regions would begin to exceed the diversity of northern regions, as predicted by the literature (Fischer 1960, Rohde 1992, Francis and Currie 2003, Hawkins et al. 2003), but this hypothesis would need to be investigated with later season plant surveys.

2.4.3 Environmental Factors Influencing Diversity

The increase in diversity with latitude found in this study contrasts with previous research, which generally shows a decrease in diversity with decreasing productivity associated

2. Latitudinal changes in the co-flowering community across *C. candidum*'s range with latitude (Waide et al. 1999, Gross et al. 2000, Mittlebach et al. 2001, Perelman et al. 2001, Willis and Whittaker 2002). However, many of these studies measured species diversity across a season, and included all genera, not just the flowering forbs, in their calculations. As most of the graminoids and forbs in the tall grass prairie reach peak flowering later in the season (Dunnell and Travers 2011, Mazer et al. 2013), it is likely that the time frame of my study did not capture the period of maximum diversity in this region.

Temperature and precipitation between April 1st and my spring surveys each explained a significant portion of the variation in co-flowering composition. Greater precipitation at northern sites in 2016 could explain their higher diversity. Precipitation influences phenology in desert vegetation, with some species delaying their emergence until after heavy rainfall (Beatley 1974, Kemp 1983, Kimball et al. 2011), while other species experiencing drought flower earlier to avoid excess stress (Kemp 1983, Kigel et al. 2011). Temperature showed a negative correlation with latitude, with the warmest conditions further south over the course of the growing period, and is an important phenology predictor in both Arctic (Chapin et al. 1995, Thórhallsdóttir 1998) and Mediterranean habitats (Kramer et al. 2000, Gordo and Sanz 2010). The warmer conditions in the spring in southern regions likely allowed early flowering species, like *C. candidum*, to flower earlier relative to other spring-emerging species, reducing co-flowering overlap as discussed above.

Other variables that I was not able to measure could differ between study regions, and explain more of the variation in co-flowering competition. These could include productivity, the depth and persistence of snow cover, the disturbance regime of a site, as well as nutrient availability. A more direct measure of productivity, such as vegetative biomass, would have

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allowed me to determine if latitude and productivity were as strongly correlated as I had assumed. Vegetative biomass is a common proximate measure of productivity in studies of diversity across large geographical areas (Waide et al. 1999, Dodson et al. 2000, Gross et al. 2000, Mittlebach et al. 2001, Chase and Leibold 2002). Snow depth and date of snow melt are important predictors of phenology in Arctic (Thórhallsdóttir 1998, Borner et al. 2008) and alpine habitats (Kudo 1991, Inouye 2008, Wipf and Rixen 2010, Kudo et al. 2014, Kameyama and Kudo 2015), as are the dates of last frost (Inouye 2000, 2008). In Mediterranean communities, diversity shows a decrease with longer periods between disturbances such as fire (Cowling et al. 1996). Finally, an addition of soil nutrients has been shown to accelerate phenology in Arctic areas (Chapin et al. 1995, Borner et al. 2008), and may have played a role in this study. Further research into drivers of floral emergence in the early flowering season would help explain what factors may be driving variation in diversity with latitude in the tall grass prairie.

This research is important for increasing our knowledge about the patterns of diversity that result from a variety of environmental conditions. Due to the dramatic decrease in native tall grass prairie in North America, research such as this will help us establish baseline knowledge of ecological processes that occur in this habitat. This knowledge will be important for understanding the potential impact human-induced climate change may have on these sensitive habitats. My study is the first, to my knowledge, to examine patterns in diversity of the co-flowering community across a latitudinal gradient at both the beginning of the growing season, as well as in the context of rewardless species pollination. The increase in co-flowering diversity with latitude at this point in the growing season has not been reported before, providing much needed support to some rewardless pollination literature. To strengthen the findings of this study, additional examination of other environmental drivers of diversity would be beneficial. More

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research on fire history, nutrient availability, snowfall, and a more detailed survey of the surrounding land use would all fill in gaps that I was unable to address.

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2.6 Appendices

Appendix 2.1. Location information and orchid numbers for each transect in my 21 sites located in my four main study regions: Iowa, Southern Minnesota, Northern Minnesota, and Manitoba (“IA”, “S-MN”, “N-MN”, and “MB” respectively). Asterisks indicate that the site was also used for insect surveys in Chapter 3.

Region	Site/ County/ Municipality	Date	Transect #	Genet #
IA	IW6* Palo Alto	May 19	1	27
			2	23
	IW4* Cerro Gordo	May 20	3	30
			4	20
	IW1* Winnebago	May 21	5	23
			6	20
S-MN	SM8* Swift	May 22	7	30
			8	20
	SM13* Chippewa	May 24	9	24
			10	23
	SM10* Chippewa	May 25	11	25
			12	15
	SM11* Chippewa	May 25	13	21
			14	25
	SM9 Chippewa	May 26	15	20
			16	17
SM4 Kandiyohi	May 27	17	16	
		18	26	
N-MN	NM10* Norman	May 27	19	23
			20	17
	NM8* Norman	May 28	21	22
			22	16
	NM9* Norman	May 28	23	29
			24	21
	NM2* Mahnomen	May 29	25	27
			26	18
	NM13 Clay	May 31	27	21
			28	13
	NM12 Clay	May 31	29	30
			30	20
MB	MB10* Stuartburn	June 02	31	29
			32	21
	MB3 Woodlands	June 03	33	32
	MB1* St. Laurent	June 05	34	36
			35	14

2. Latitudinal changes in the co-flowering community across *C. candidum*'s range

	MB4 Woodlands	June 05	36	30
			37	20
	MB7* Cornwallis	June 06	38	25
			39	25
	MB6* Cornwallis	June 06	40	30
			41	17

2. Latitudinal changes in the co-flowering community across *C. candidum*'s range

Appendix 2.2. Diversity Index Formulas

Calculation of Shannon's Diversity index (Shannon 1948):

$$H' = - \sum_{i=1}^S p_i \ln p_i$$

Where p_i is the proportion of individuals belonging to the i th species in a population and S is the total number of species.

Calculation of Pielou's Evenness Index (Pielou 1966):

$$J' = \frac{H'}{\ln S}$$

Where H' is the Shannon's diversity index for a population and S is the total number of species.

Calculation of Sørensen's index (Sorensen 1948):

This index compares presence/absence data collected from two sites. A higher index indicates similar beta diversity between sites.

$$C_s = \frac{2a}{2a + b + c}$$

Where a is the total number of genera present at both sites, b is the number of genera present only at site 1, and c is the number of genera present only at site 2.

2. Latitudinal changes in the co-flowering community across *C. candidum*'s range

Appendix 2.3. List of genera recorded in spring surveys of tall grass prairie *Cypripedium candidum* populations, along with abbreviations used in redundancy analysis (Fig. 2.5). Weights of each genus on each RDA axis are also listed, and data are sorted from highest to lowest weighting on RDA1.

Genus	Species	Abbreviation	RDA1	RDA2	RDA3
<i>Sisyrinchium</i>	<i>S. campestre</i> , <i>S. montanum</i> , <i>S. mucronatum</i>	Sr	0.848	-0.137	-0.022
<i>Hypoxis</i>	<i>H. hirsuta</i>	Hx	0.742	-0.063	-0.180
<i>Commandra</i>	<i>C. umbellata</i>	Cm	0.668	-0.265	-0.355
<i>Polygala</i>	<i>P. senega</i>	Py	0.648	-0.266	0.120
<i>Castilleja</i>	<i>C. coccinea</i>	Cj	0.641	-0.168	-0.079
<i>Packera</i>	<i>P. aurea</i>	Pk	-0.547	0.249	-0.278
<i>Lithospermum</i>	<i>L. canescens</i>	Ls	0.423	0.277	-0.136
<i>Agoseris</i>	<i>A. glauca</i>	Ag	0.403	0.281	0.052
<i>Taraxacum</i>	<i>T. officinale</i>	Tx	0.330	0.578	0.206
<i>Maianthemum</i>	<i>M. racemosum</i>	Mt	0.320	0.408	0.054
<i>Cypripedium parviflorum</i>		Cp	0.293	0.373	0.049
<i>Elaeagnus</i>	<i>E. commutata</i>	Eg	0.263	0.335	0.044
<i>Galium</i>	<i>G. boreale</i>	Gl	0.219	0.051	0.002
<i>Pedicularis</i>	<i>P. canadensis</i>	Pc	0.198	-0.452	0.309
<i>Geum</i>	<i>G. trifolium</i>	Gu	-0.191	0.066	-0.332
<i>Potentilla</i>	<i>P. anserina</i>	Pt	-0.188	-0.008	0.244
<i>Euphorbia</i>	<i>E. esula</i>	Ep	0.154	0.196	0.026
<i>Vicia</i>	<i>V. americana</i>	Vc	0.145	0.185	0.025
<i>Cypripedium</i> hybrids		Ch	0.123	0.157	0.021
<i>Anenome</i>	<i>A. canadensis</i>	An	0.105	0.134	0.018
<i>Coptis</i>		Ct	0.089	-0.162	-0.027
<i>Zizia</i>	<i>Z. aurea</i> , <i>Z. aptera</i>	Zz	-0.079	-0.272	-0.212
<i>Viola</i>	<i>V. nephrophylla</i> , <i>V. sororia</i>	Vl	-0.074	0.111	-0.404
<i>Astragalus</i>	<i>A. agrestis</i>	At	0.034	0.043	0.006
<i>Trifolium</i>	<i>T. pratense</i>	Tf	0.029	0.037	0.005
<i>Fragaria</i>	<i>F. virginiana</i>	Fg	-0.020	0.090	-0.283
<i>Thalictrum</i>	<i>T. dasycarpum</i>	Tc	0.017	0.021	0.003

2. Latitudinal changes in the co-flowering community across *C. candidum*'s range

Appendix 2.4. List of genera recorded in fruiting surveys of tall grass prairie *Cypripedium candidum* populations, along with abbreviations used in Fig. 2.9.

Genus	Species	Abbreviation
<i>Agoseris</i>	<i>A. glauca</i>	Ag
<i>Achillea</i>	<i>A. millefolium</i>	Ah
<i>Anemone</i>	<i>A. canadensis</i>	An
	<i>A. cylindrica</i>	
<i>Apocynum</i>	<i>A. cannabinum</i>	Ap
<i>Arnoglossum</i>	<i>A. plantagineum</i>	Ar
<i>Asclepias</i>	<i>A. speciosa</i>	As
	<i>A. ovalifolia</i>	
	<i>A. tuberosa</i>	
<i>Cerastium</i>	<i>C. arvense</i>	Ce
<i>Castilleja</i>	<i>C. coccinea</i>	Cj
<i>Companula</i>	<i>C. rotundifolia</i>	Cn
<i>Cornus</i>	<i>C. stolonifera</i>	Cr
<i>Cirsium</i>	<i>C. arvense</i>	Cs
	<i>C. flodmanii</i>	
<i>Cicuta</i>	<i>C. maculata</i>	Cu
<i>Dalea</i>	<i>D. pupurea</i>	De
<i>Dasiphora</i>	<i>D. fruticosa</i>	Dp
<i>Erigeron</i>	<i>E. philadelphicus</i>	Eg
	<i>E. strigosus</i>	
<i>Elaeagnus</i>	<i>E. commutata</i>	Elg
<i>Euphorbia</i>	<i>E. esula</i>	Ep
<i>Galium</i>	<i>G. boreale</i>	Gl
<i>Glycyrrhiza</i>	<i>G. lepidota</i>	Gy
<i>Heliopsis</i>	<i>H. helianthoides</i>	Hl
<i>Hypoxis</i>	<i>H. hirsuta</i>	Hy
<i>Lilium</i>	<i>L. philadelphicum</i>	Ll
<i>Lithospermum</i>	<i>L. canescens</i>	Ls
<i>Lathyrus</i>	<i>L. palustris</i>	La
<i>Medicago</i>	<i>M. lupulina</i>	Md
	<i>M. sativa</i>	
<i>Melilotus</i>	<i>M. alba</i>	Ml
	<i>M. officinalis</i>	
<i>Pycnanthemum</i>	<i>P. virginianum</i>	Pc
<i>Pedicularis</i>	<i>P. canadensis</i>	Pd
<i>Psoralea</i>	<i>P. argophylla</i>	Ps
<i>Potentilla</i>	<i>P. anserina</i>	Pt
<i>Phlox</i>	<i>P. pilosa</i>	Px
	<i>P. longifolia</i>	
<i>Polygala</i>	<i>P. senega</i>	Py
<i>Rudbeckia</i>	<i>R. cirocina</i>	Rb
<i>Rosa</i>	<i>R. acicularis</i>	Rs

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<i>Senecio</i>	<i>P. aureus</i>	Sc
(<i>Packera</i>)	<i>P. pauperculus</i>	
<i>Solidago</i>	<i>S. nemoralis</i>	Sg
<i>Stachys</i>	<i>S. palustris</i>	Sh
<i>Sisyrinchium</i>	<i>S. campestre,</i>	Ss
	<i>S. montanum,</i>	
	<i>S. mucronatum</i>	
<i>Thalictrum</i>	<i>T. dasycarpum</i>	Tc
<i>Tofieldia</i>	<i>T. glutinosa</i>	Tf
<i>Trifolium</i>	<i>T. repens</i>	Trf
<i>Verbena</i>	<i>V. hastata</i>	Vb
<i>Vicia</i>	<i>V. americana</i>	Vc
<i>Viola</i>	<i>V. nephrophylla,</i>	VI
	<i>V. sororia</i>	
<i>Zigadenis</i>	<i>Z. elegans</i>	Zg
<i>Zizia</i>	<i>Z. aurea</i>	Zz
	<i>Z. aptera</i>	

3. LATITUDINAL CHANGES IN INSECT COMMUNITY COMPOSITION, AND FLOWER AND INSECT MORPHOLOGY

3.0 Chapter Summary

The patterns in diversity, composition, and morphology of the insect community at *C. candidum* populations were examined. First, I expected that lower flowering diversity present in southern populations would result in fewer insect taxa present at the time of *C. candidum* flowering. Similar to the plant community, many insect genera were found in every study region, but additional genera were observed as my study progressed further north, resulting in significantly increased richness and diversity. Ordination techniques also reflected these patterns, and regions significantly varied in their insect community composition. Second, I conducted insect measurements on insect captured from pan traps and compared these to measurements of *C. candidum* flowers to determine if morphology of plants and insects shifted across latitudes. Northern study region had more insects from genera too large for pollination, and some genera also showed a significant increase in body size with latitude. The morphology of *C. candidum* flowers remained consistent in most study regions, but flowers in Iowa were significantly smaller. Finally, I also conducted timed pollinator surveys at each study site to compare visitor patterns across latitudes. Communities all showed a low number of connections between plants and pollinators, and the most abundant flowering plants at a site had the most insect visits. Overall, my results indicate that insect morphology may be more suitable for pollination of *C. candidum* in southern regions, although insects are less diverse.

3. Latitudinal changes in the insect community across *C. candidum*'s range

3.1 Introduction

The ecosystem service provided by foraging insects is crucial to the function of ecological communities. As over 87% of flowering plant species rely on insect vectors to achieve pollination (Ollerton et al. 2011), pollinators are essential for both the effective reproduction of flowering species and the maintenance of the food resources provided by fruit and seeds. Changes in the composition of the pollinator community can have dramatic effects on the reproduction of flowering plants within an area, particularly if these plants are adapted to pollination by a particular insect taxa (Devoto et al. 2005, Gómez et al. 2007, 2010). Insect diversity, morphology, and behaviour may all be affected by environmental gradients and the pollination success of the plant community may vary as a result.

As discussed in Chapter 2, total species diversity typically increases as latitudes become more equatorial (Rohde 1992), but, early in the season, lower latitudes should experience lower floral diversity due to asynchronous flowering over a longer growing season. If this pattern holds true, it could mean additional and more diversified early-season forage for insects further north, also promoting their diversity. The latitudinal trend should result in a more diverse pollinator population, with varied morphologies and innate colour preferences. An increased pollinator diversity typically increases the reproductive success of generalist flowers, which produce flowers that can be successfully pollinated by a variety of pollinator taxa (Albrecht et al. 2007, Gómez et al. 2007). It is unclear how this pattern may affect the reproduction of more specialized plants. The effects of increased diversity on pollination in these taxa may depend on the ability of additional taxa to effectively pollinate their flowers (Johnson and Steiner 2000, Armbruster and Muchhala 2009).

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Connectance, a pollination matrix measure, can provide some information on the likelihood of pollination in a community. This measurement comes from network analysis and is obtained by calculating “the proportion of potential interspecific interactions that are actually realized” (Vazquez et al. 2009). A highly connected community should show similar levels of reproduction among flowering plants, while a less connected community could indicate more specialization between plants and pollinators, or uneven pollination rates (Waser et al. 1996, Johnson and Steiner 2000, Dunne et al. 2002, Gilbert 2009). Increased diversity can provide redundancy in a network (Waser et al. 1996), with multiple species filling similar pollinator roles; however, higher diversity could also include more specialist relationships (when an insect species only feeds on one type of plant species) which would result in decreased connectance in a community (Johnson and Steiner 2000, Blüthgen et al. 2006, Armbruster and Muchhala 2009).

In addition to differences in connectance, latitude may have a strong effect on insect morphology. Bergmann's rule (1847) predicts that in colder environments the body size of an animal should increase. Increased body size in colder environments decreases the surface area to volume ratio of an animal and thus reduce heat loss to the environment. This theory has much supporting evidence from studies on endotherms such as mammals and birds (Ashton et al 2000, Ashton 2002, Freckle et al 2003). However, little research has been conducted on ectothermic species, such as pollinating insects. Furthermore, the effect of changes in insect body size on the reproductive success of flowering species has been the subject of only a few studies. One study of *Bombus* pollinators of *Cytisus scoparius* in an alpine habitat showed that as altitude increased, there were larger species of *Bombus* present in the area (Malo and Baonza 2002). This trend also corresponded to a parallel increase in the size of *C. scoparius* flowers, and reproduction was highest in flowers that were of similar size to the most abundant *Bombus* species.

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Malo and Baonza (2002) showed an increase of body size within the genus, *Bombus* (Family: Apidae), along an altitudinal gradient. A similar pattern could occur in the proportion of different genera present along a clinal gradient, if larger insect genera become more abundant in colder areas. An increase in the abundance of large-bodied genera with latitude has been demonstrated in ants in the British Isles (Cushman et al. 1993) and some butterfly species in North America (Hawkins and Lawton 1995); but Hawkins (1995) found few latitudinal trends in the proportion of differently sized bee genera across the eastern United States.

In addition to an increase in body size with latitude, Allen's rule (1877) predicts that surface area to volume ratios should also decrease along with a shortening of an individual's extremities relative to its body. Insects have not been the subject of many studies of Allen's rule, but it was supported in one study of *Bombus* bees (Peat et al. 2005). However, *Bombus* are facultative endotherms (Heinrich 1972), and as a result may follow Bergmann's and Allen's rule more closely than most insects, which are usually ectothermic. Differences in thermal regulation among taxa may account for the lack of a clear relationship between body morphology and latitude in insects and other ectotherms (Hawkins 1995, Hawkins and Lawton 1995, Mousseau 1997).

Another latitudinal trend that may affect plant reproduction is a transition from high Hymenopteran (bees) pollinator abundance to high Dipteran (flies) pollinator abundance as latitude increases (Elberling and Olesen 1999). This trend may lead to a decrease in pollination, as the pollination efficiency of flies is often lower than bees, with flies often consuming more pollen than they transfer (Bischoff et al. 2013, Hargreaves et al. 2014). Therefore, a greater

3. Latitudinal changes in the insect community across *C. candidum*'s range

abundance of bees should increase the reproductive success of a plant community, potentially increasing pollination success in the south.

Latitudinal shifts in pollinator diversity may result in shifts in the overall colour preference of the pollinator community. Different insect genera may show differences in colour preference (Lunau and Maier 1995, Leong and Thorp 1999, Lunau 2014). These preferences seem to be similar between genera from the same order; with hymenopterans often demonstrating a preference for blue or yellow (Lunau and Maier 1995, Leong and Thorp 1999, Semmler 2015), and dipterans demonstrating a preference for yellow (Lunau and Maier 1995, Lunau 2014). If the composition of insect community changes across an ecoregion, plants that have flowers that match the colour preferences of the dominant taxa may experience increased pollination success.

This study focuses on the changes in the insect community that may affect the pollination biology of the slipper orchid, *Cypripedium candidum*, across its range. Slipper orchids are characterized by pollination through a one-way semi-trap, where insects enter a large hole on the dorsal side of a large modified petal called a labellum, and then are forced to exit through a smaller hole at the back of the flower, passing by the orchid's sex organs and effecting pollination (Argue 2012). The ability of insects to exit this smaller hole may determine if they can effectively pollinate a flower (Li et al. 2008). Many solitary bees, which are the most common visitors to these orchids in Manitoba (*Lasioglossum* and *Andrena*), are too large to exit a *C. candidum* flower, as determined by Pearn (2012) and Anderson (2015). This is a potential factor contributing to the low fruit set in this species in Manitoba (see Chapter 4 for further

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discussion). If predictions from the literature are supported, and smaller insects are more common at lower latitudes, reproductive success of *C. candidum* in these areas may increase.

3.1.1 Chapter Objectives

My first objective in this chapter was to record changes in the pollinator community throughout *C. candidum*'s latitudinal range. As my study took place only during the flowering season of *C. candidum*, I predicted that insect diversity would be highest in the north during this period as insects in the south would be able to emerge asynchronously. Furthermore, the diversity and abundance of plants was highest in the north during *C. candidum* flowering (Chapter 2), which would provide more forage for insects, also leading to an increase in diversity. Besides overall changes in diversity of insects, I also expected the proportion of Dipterans to increase from the south to the north.

My second objective was to record changes in the average size of foraging insects across latitudes. Given Bergmann's and Allen's rules, I predicted larger insect genera would be more abundant further north, while smaller insect genera would be more abundant further south. I also predicted that the size of individuals within an insect genus would increase as latitudes become more polar.

My third objective was to observe the foraging patterns of pollinating insects across latitudes. As many solitary bees and flower flies are generalist foragers (Chambers 1968, Bernhardt and Burns-Balogh 1986, Giurfa et al. 1995, Waser et al. 1996, Chittka et al. 1997, Dicks et al. 2002, Shi et al. 2009, Song and Feldman 2014), I expected the colour preferences of foraging insects to reflect the overall colour of the co-flowering community. Therefore, I

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predicted that the proportions of insects captured in blue, yellow, and white pan traps would reflect the abundance of flowering plants belonging to each of these floral colours. I also predicted that pollination networks in higher latitudes would show lower connectance. This prediction is due to my earlier prediction that diversity will be higher at higher latitudes. With higher diversity, there would be more potential interactions between plants and pollinators. It is unlikely that all of these would be realized (Vazquez et al. 2009) which would decrease overall connectance of the network.

My final objective for chapter 3 was to determine if the size of *C. candidum* flowers changed across latitudes. As discussed above, insect size may increase with latitude, resulting in larger insects in northern regions. I predicted that flower size in *C. candidum* would increase further north in order to accommodate larger bodied insects.

3.2 Methods

3.2.1 Pollinator surveys

Pollinator surveys were conducted in four latitudinal regions within remnant tall grass prairie habitat: southern Manitoba (4 sites), northern Minnesota (4 sites), southern Minnesota (3 sites), and northern Iowa (3 sites), representing the northern and southern extremes in *C. candidum*'s western range (see Fig. 2.1, Appendix 2.1, Chapter 2) and represent a subset of the sites used for vegetation studies. This strategy was used to maximize the efficiency of field work, and to visit as many sites as possible within the short flowering period of *C. candidum*. Two study sites were visited on a given day with all surveys conducted on days with the warmest temperatures and the lowest chances of precipitation during the period I was in a study region.

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These are ideal conditions for pollinator activity (McCall and Primack 1992). I recorded average temperature and wind speed using a Kestrel © 3000 handheld weather meter at the beginning and end of each pollinator survey.

Pan traps surveys

The pan trap methodology developed by the Canadian Pollination Initiative (CANPOLIN, <http://www.uoguelph.ca/canpolin/Sampling/protocols.html>) was used to survey the insect community at each site. Pan traps consisted of brightly colored bowls (blue, yellow or white) to attract insects and were filled with water and dish soap (8 drops/L) to reduce the water tension and thus trap insects. I placed a 90 m transect at each site and alternated the bowl colors every 3 m (10 bowls of each colour). Pan traps were set out before 10 A.M. for a minimum of 6 hours. I ensured that pan trap transects were located in the same habitat with similar vegetation types as the orchids, but a minimum of 30 m away from orchid transects to prevent the pans from drawing insects away during pollinator monitoring surveys (see below). At the end of each day insects were collected from pan traps and stored in 70% ethyl alcohol.

Flower visitor surveys

On the same day as pan trap surveys, I conducted timed pollinator observations once the temperature had warmed up and pollinator activity had begun. This was typically just before noon for one site, and then early afternoon for the second. Along the two-25m transects established at each site, I conducted two 25-minute surveys (= 50 minutes of flower visitor observations at each site). During this time, all insect visits to orchid and co-flowering species

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within 2m of transects were recorded and voucher insect specimens were collected using a sweep net whenever possible. I did not discriminate among insect visitations and pollination events, as most plant species within this community had small flowers with accessible sex organs.

Therefore, if an insect was seen landing on a flower, it would likely pick up or transfer pollen. If an insect was encountered in *C. candidum*, a mesh bag was placed over the flower and the insect was allowed to exit naturally before collection. This was the only situation where I recorded if the insect exited a flower properly, and thus picked up pollen. Captured insects were placed in a kill jar with ethyl acetate to euthanize them and were stored in 70% ethyl alcohol.

Insect Processing

After returning to the lab, insects were separated into Hymenopterans, Dipterans, and “other” orders. Hymenopterans were dried with paper towel and then pinned and dried in insect pinning boxes. To protect their soft bodies, Dipterans were first soaked in 50:50 ethyl acetate and ethanol, followed by a second soak in 100% ethyl acetate with 2 drops of glycerin to maintain limb pliability (CANPOLIN, <http://www.uoguelph.ca/canpolin/Sampling/pinning%20flies.html>). Following soaking, Dipterans were pinned and dried similarly to Hymenopterans. All Hymenopterans and flower visiting Dipterans were identified to the genus, while orchid visitors were identified to genus using *The Bee Genera of Eastern Canada* (Packer et al. 2007), *The Manual of Nearctic Diptera* (McAlpine et al. 1981), and a reference list created by Sarah Semmler (unpl. data). Voucher specimens of all collected insects will be deposited in the Wallis Roughley Museum of Entomology at the University of Manitoba in Winnipeg, Manitoba.

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For each study region, up to ten individuals were measured from every genus captured in pan traps, for a maximum of 40 measurements for every genus. The only genus that was not measured was *Bombus*, as this genus is too large to be an effective *C. candidum* pollinator. The genus *Lasioglossum* was split into the subgenera of *Lasioglossum* and *Dialictus* prior to measuring, due to the fact that *Dialictus* are significantly smaller than the rest of this genus (Michener 2000). Similarly, the genus *Andrena* and the family Megachilidae (for which genera identification was difficult) were split into large and small size classes before they were measured. All measurements were done using digital calipers (accurate to 0.01 mm). Measurements were taken of the thorax height, abdomen width, as well as the sum of thorax and abdomen length. This proxy of body length did not include the head length, as the position of the head was often quite variable, making consistent measurements difficult.

3.2.2 Orchid Morphology Measurements

As described in Chapter 2, I established two 25m transects in high-density orchid patches at every study site, along which I recorded orchid location data for fruiting surveys (Chapter 4). In addition to recording orchid genet locations, I measured floral features on one flower from each of 15 genets at two sites in each region. I recorded measurements on slipper length/width, the distance from the base of the flower to the entry route, the entry route length/width, and the exit route length/width (Fig.3.1) using digital calipers (accurate to 0.01mm).

3. Latitudinal changes in the insect community across *C. candidum*'s range

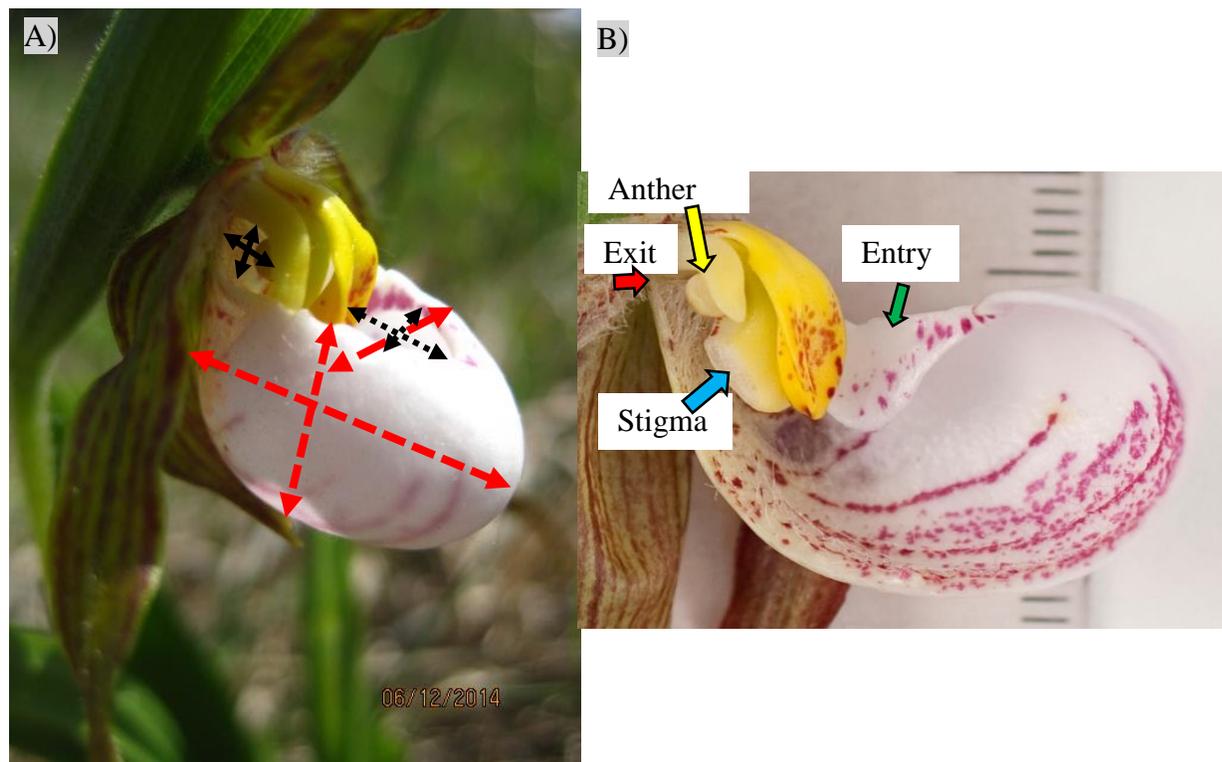


Figure 3.1. A) Floral morphology of a *Cypripedium candidum* flower, with measured features indicated with arrows (solid black: exit route length/width; dotted black: entry route length/width; dashed red: slipper length/width/height). B) Internal view of a *Cypripedium candidum* flower, illustrating an insect's path through the flower. Insects enter through the large dorsal orifice, and are forced to exit through the smaller orifice at the back of the flower, passing the flower's stigma and anther in the process.

3.2.3 Statistical Methods

In total, 2060 bees plus flower-visiting flies were captured in pan traps. Of the 1801 bees, 629 were captured at one study site in southern Minnesota (SM11). Due to the high number of captures at this site, all community composition analyses were conducted with and without SM11 to determine if trends observed were obscured by patterns at a single site.

3. Latitudinal changes in the insect community across *C. candidum*'s range

Insect Community Composition

Similar to indices used to characterize plant species diversity (Chapter 2), I calculated the number of insect genera observed, Shannon's diversity index (Shannon 1948), and the Pielou's evenness index (Pielou 1966) for all pollinator survey sites (Appendix 2.2). These values were then compared between regions using Kruskal-Wallis tests. Following this, I tested for significant-correlation between the number of plant and insect genera using Spearman's rank correlations. I also conducted a Chi-square analysis to determine whether the proportion of bees and flies varied significantly among study regions. I then examined the residuals from each cell of the resulting contingency table, to determine which contributed the most to any significant results.

To visualize how species and sites were associated, I used principal components analysis (PCA) and redundancy analysis (RDA). Count data for each insect genus captured in pan traps at each study site were tabulated. These data were then log-transformed and analyzed with a PCA using a covariance distance matrix using the R-package "vegan" (Oksanen et al 2016). A covariance matrix was used as it is most appropriate for data measured on the same scale (Quinn and Keough 2002). I also conducted a RDA to determine if the insect community composition of each site varied significantly by study region. I also used the same methodology described in chapter 2, to examine the effects of various environmental variables on the composition of the insect community. Additional variables that were included in redundancy analyses were average temperature, precipitation, and wind speed on the days that pan trap surveys were conducted.

3. Latitudinal changes in the insect community across *C. candidum*'s range

Insect body size changes across latitudes

To determine if genera of different sizes were more abundant in certain regions, I assigned insect genera to size class. This was done through boxplots, comparing insect body width to the exit route width of *C. candidum*. This measurement was determined by many authors to be the most likely orchid flower dimension to limit pollination (Li et al. 2008, Pearn 2012, Anderson 2015). Insects with an average body width larger than the exit route of *C. candidum* were classified as “too large”, while those that were not significantly different from exit route width were considered “large potential pollinators”. Insects significantly smaller than the exit route of *C. candidum* may still pollinate under certain circumstances, so they were classified as “small potential pollinators”. Following this step, I conducted a chi-square analysis to determine if the number of insects captured in each size class per site differed significantly among regions. I determined which squares in the contingency tables had the highest residuals, which would indicate the greatest contributors to any significant analyses based on their study wide proportions.

To examine variation in body size *within* each insect genus, a MANOVA was conducted on log-transformed measurement data. *Post-hoc* analyses of variance determined which body size measurements were significantly different, and which regions contributed to a significant size difference.

Patterns of Insect Visitation

I conducted a chi-square analysis to examine if insects appeared to show a preference for different pan trap colours in different regions. I analyzed the proportion of insects captured in

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blue, white, and yellow pan traps in each region to find whether the variation in these proportions between regions was significant. Once again, I determined which squares in the contingency tables had the highest residuals, and thus were the greatest contributors to any significant analyses. I used the same method to compare the number of flower stems of each colour per site between my study regions.

Using my observations from transect insect visitor surveys, I constructed insect visitation matrices for each site, each region, and the overall study. A visitation matrix contain all flowering plant species along one side of a table, and all pollinating species along the other (Vazquez 2009). All observed interactions between plants and pollinators are counted and tabulated in the cells of the matrix. Using these matrices, I determined which insect genera were the most common visitors in each region, and which plant genera were the most commonly visited. I also examined correlations between daily weather data and the number of insect visitations observed during pollinator surveys. In addition, I calculated overall community connectance (the number of realized connections divided by the number of possible connections) for each site. These values were then compared across regions using a Kruskal-Wallis test, as data were not normally distributed.

Latitudinal changes in orchid size

A multiple analysis of variance (MANOVA) was used to determine whether the response variables of flower number, slipper length/width, exit route length/width, and entry route length/width differed among study regions. Data were log transformed to meet assumptions of normality and heterogeneity of variances. Entry-to-base measurements were still non-normal following transformation, but were retained in analyses as MANOVA is robust to deviations

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from normality (Quinn and Keough 2002a), and the assumption of homogeneity of variance was still met. The number of flowers per orchid genet could not be included in MANOVA analyses, due to this variable's non-normal distribution and unequal variance among study region. Neither of these issues were solved by log-transformation so differences among study regions for this variable were tested using a Kruskal-Wallis test and visualized with boxplots. Following the significant MANOVA result (see below), *post-hoc* analyses of variance with subsequent Tukey HSD (honest significant difference) tests were used to determine which variables were significantly different among study regions, and which regions contributed to this difference. To visualize patterns of variation in my dataset, a principal components analysis (PCA) was performed using a covariance distance matrix and the R-package "vegan" (Oksanen et al 2016).

3.3 Results

3.3.1 Insect Community Composition

Most insects (eight of 15 taxa) captured in pan traps were seen in all study regions (Table 3.1; Fig. 3.2). Three taxa occurred in all regions but Iowa, and two taxa were in all regions but southern Minnesota. The flower fly, *Helophilus*, was only seen in northern Minnesota and Manitoba, and the bee genus, *Anthophora*, was only seen in Manitoba (Table 3.1; Fig. 3.2). Five of the seven taxa not seen further south have records in the corresponding regions (*Bombus*, *Hylaeus*, *Andrena*, *Agapostemon*, *Anthophora*; Ascher and Pickering 2017). *Helophilus* as a genus becomes more abundant and diverse at higher latitudes in North America, but some species in this genus have a range that extends as far south as Mexico (Stone 1965).

3. Latitudinal changes in the insect community across *C. candidum*'s range

Table 3.1. All insect genera captured in pan trap surveys, across tall grass prairie populations of *Cypridium* in 2016. Genera are separated based on the number of study regions in which they were found (Iowa, southern Minnesota, northern Minnesota, or Manitoba; Fig 2.1). Genera found in high abundance in a region are marked with an “X”, while low abundance genera are marked with an “x”. Genera not seen in a region are symbolized with “(X)” if Ascher and Pickering (2017) or Stone (1965) indicated their presence in a region.

Genus	Iowa	Southern MN	Northern MN	Manitoba
Genera observed in all regions				
<i>Dialictus</i>	X	X	X	X
<i>Augochlorella</i>	X	X	X	X
<i>Halictus</i>	X	X	X	X
<i>Toxomerus</i>	X	X	X	X
<i>Odontomyia</i>	x	x	x	X
<i>Lasioglossum</i>	x	x	x	X
<i>Ceratina</i>	x	x	x	X
<i>Nomada</i>	x	x	x	x
Genera observed in three regions				
<i>Bombus</i>	(X)	x	X	X
Megachilidae	(X)	X	X	X
<i>Hylaeus</i>	(X)	X	X	X
<i>Andrena</i>	x	(X)	x	x
<i>Agapostemon</i>	x	(X)	x	x
Genera observed in two regions				
<i>Helophilus</i> ¹	(X)	(X)	x	x
Genera observed in one region				
<i>Anthophora</i>	(X)	(X)	(X)	x

3. Latitudinal changes in the insect community across *C. candidum*'s range

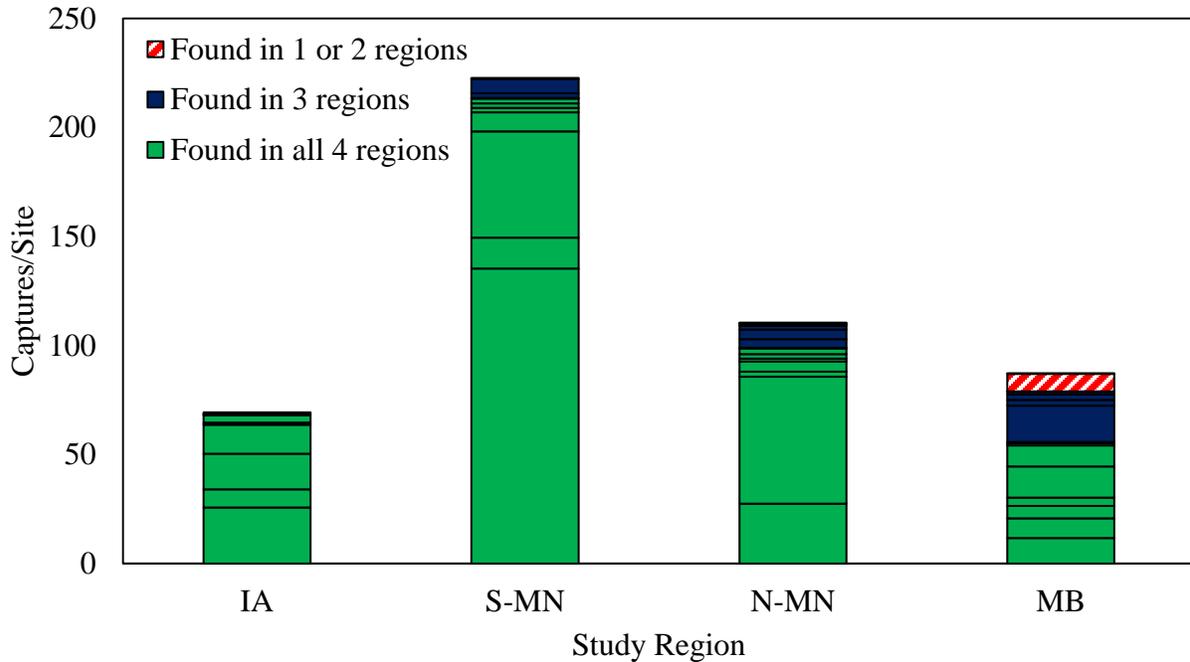


Figure 3.2. The number of captures per site of all insect genera for each tall grass prairie population of *Cyripedium candidum*, captured in pan traps during the 2016 flowering season. Genera are coloured by regional occurrence (see legend; Iowa: “IA”, Southern Minnesota “S-MN”, Northern Minnesota: “N-MN”, Manitoba: “MB”; Fig. 2.1). Each bar represents an insect genus and bars are ordered in decreasing abundance from bottom to top within each regional category. The most common genera encountered across regions were *Dialictus*, *Augochlorella*, and *Halictus* (bottom three green bars).

Both species richness and Shannon’s diversity varied significantly between study regions (Kruskal-Wallis tests, Richness: $\chi^2_3=10.6$, $p<0.02$; Diversity: $\chi^2_3=8.9$, $p<0.04$; Fig. 3.3) with the lowest values recorded in the southern study regions (Iowa and southern Minnesota) and the largest in the northern regions (northern Minnesota and Manitoba). Evenness did not vary significantly among study regions ($\chi^2_3=2.5$, $p>0.4$; Fig. 3.3) and ranged from medium levels of evenness (0.55) to moderately high (0.8). When I investigated correlation between the number of plant and insect genera, the result was significant ($r=0.68$, $p<0.01$).

3. Latitudinal changes in the insect community across *C. candidum*'s range

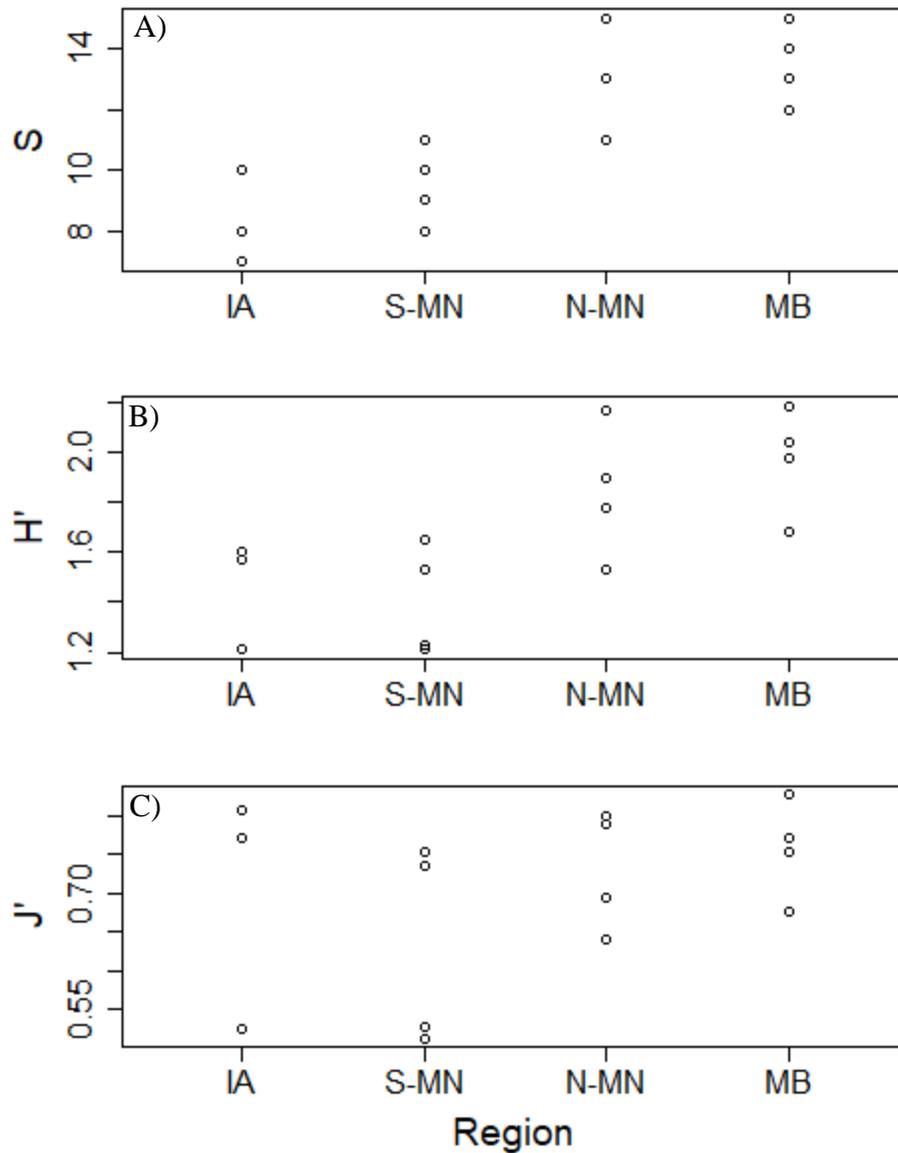


Figure 3.3. The number of genera (S, A), Shannon's diversity index (H', B), and Pielou's evenness index (J', C) of the early spring emerging insect community in tall grass prairie populations of *Cyripedium candidum*, for each study region (Iowa= "IA", southern Minnesota= "S-MN", northern Minnesota= "N-MN", Manitoba= "MB"; Fig. 2.1) in 2016.

Overall trends in pan trap data showed that the abundance of bees and flies varied significantly by region, with flies being more abundant in Manitoba than other regions, and less

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abundant in southern Minnesota than other regions ($\chi^2_3=26.36$, $p<0.0001$; Fig. 3.4). This pattern was also significant with the exclusion of site SM11 ($\chi^2_3=10.11$, $p<0.02$).

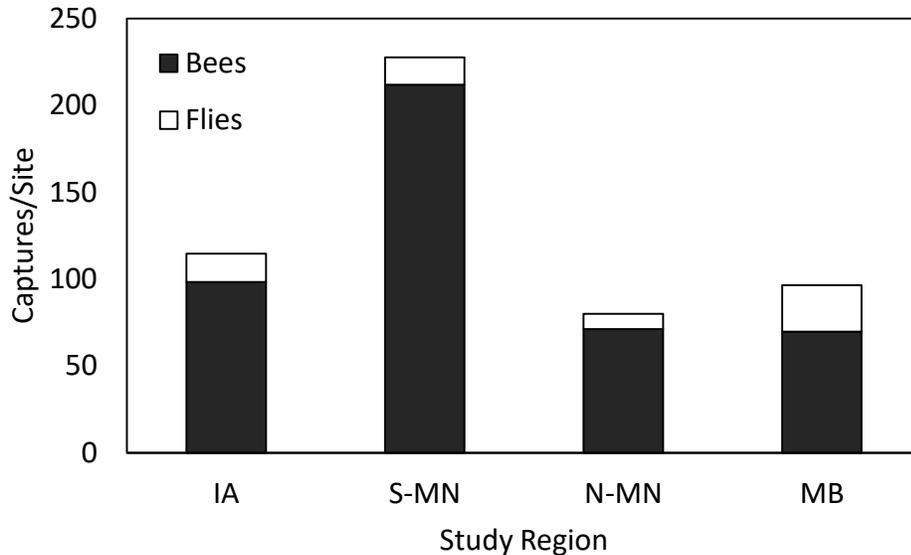


Figure 3.4. The number of pan trap captures from each order of pollinating insects (Hymenoptera=bees, Diptera=flies) per tall grass prairie population of *Cypripedium candidum* for each study region (Iowa: “IW”, Southern Minnesota “SM”, Northern Minnesota: “NM”, Manitoba: “MB”; Fig. 2.1). Insect were captured during the *C. candidum* flowering period in 2016 (see methods for description of pan trap methods).

Principal components analysis revealed that 46.2% of the variation in the insect community composition was represented by the first two principal components axes (Fig. 3.5). The first axis explained the most variation (29%) and was associated with the abundance of different sizes of insect taxa. Small taxa, such as *Halictus*, *Dialictus*, and *Toxomerus*, were associated with the negative side of this axis, while large taxa, such as *Bombus*, *Odontomyia*, and *Lasioglossum* were found toward the positive side. The second axis explained 17.2% of the variation, and was associated with the abundance of particular taxa. *Augochlorella* and wasps were weighted strongly along the positive side, while *Megachile* and *Lasioglossum* had a weak

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weighting along the negative side. Axes 3 and 4 explained an additional 23% of the variation in the insect community. Axis 3 represented a transition of fly to bee taxa, while axis 4 showed the greatest separation between the bee genus *Halictus* and the fly genus *Helophilus*. No strong regional associations were observed along these latter axes.

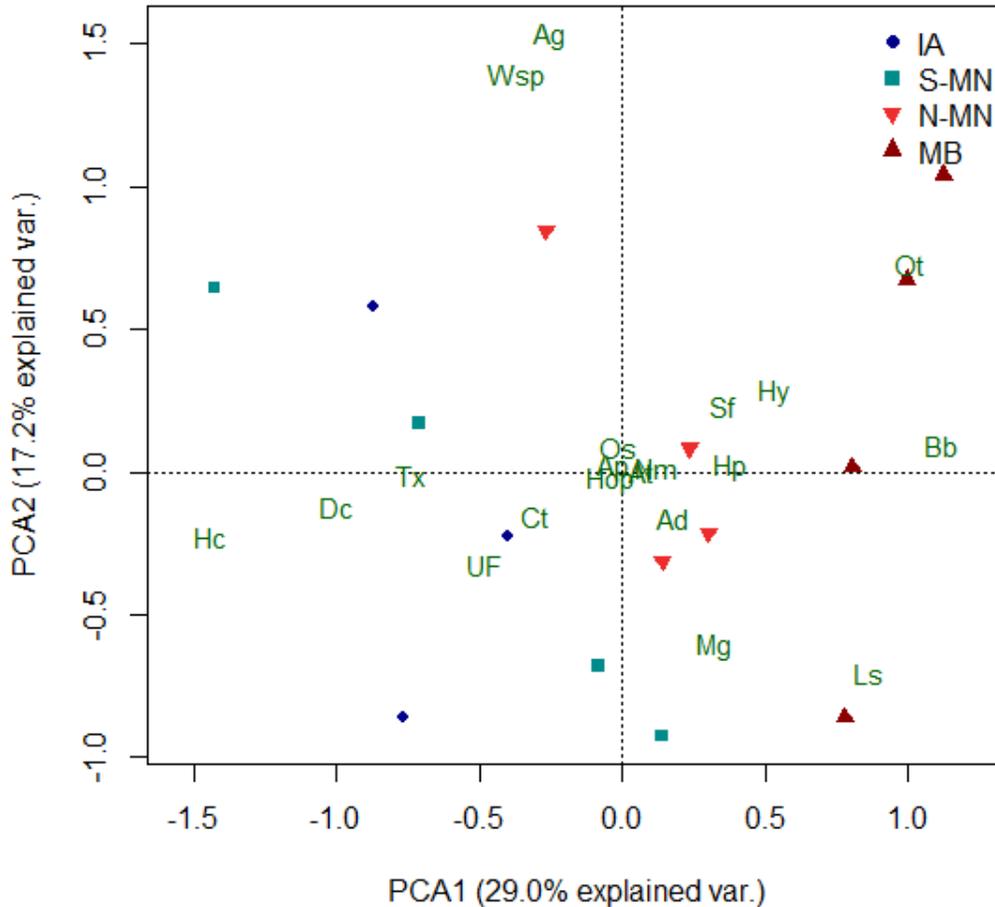


Figure 3.5. Principal components analysis of the pollinator genera captured in pan traps in each tall grass prairie population of *Cypripedium candidum* during its flowering period in 2016, using a covariance matrix. Genera scores are plotted in red, and labelled based on an abbreviated genus code (*Agapostemon* “Ap”, *Andrena* “Ad”, *Anthophora* “At”, *Augochlorella* “Ag”, *Bombus* “Bb”, *Ceratina* “Ct”, *Dialictus* “Dc”, *Halictus* “Hc”, *Helophilus* “Hp”, *Hoplitis* “Hop”, *Hylaeus* “Hy”, *Nomada* “Nm”, *Lasioglossum* “Ls”, medium-sized Megachilidae “Mg”, *Odontomyia* “Ot”, *Osmia* “Os”, sawflies “Sf”, *Toxomerus* “Tx”, unknown flies “UF”, wasps “Wsp”). Sites within regions are plotted with different symbols, and colour coded by region (Iowa: “IW”, Southern Minnesota “SM”, Northern Minnesota: “NM”, Manitoba: “MB”; see legend and Fig. 2.1).

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Redundancy analysis determined that study region had a significant effect on the insect community composition at a site ($F_{3, 17}=2.09$, $p<0.003$; Fig. 3.6). When I constrained the variation in my PCA by the factor variable of study region, the first two axes explained 32% of the variation in my data. The first axis explained 24.4% of the variation and was associated with a transition from smaller bodied insects to larger bodied genera. Genera associated with the positive side of this axis were *Toxomerus*, *Halictus*, *Dialictus*, and a few unknown fly taxa (Family Syrphidae, further identification pending). The study regions of Iowa and Southern Minnesota were also associated with this side of the axis. Higher numbers of *Bombus*, *Odontomyia*, *Lasioglossum*, *Hylaeus* and *Helophilus*, were all associated with the negative side of RDA1. Manitoban sites were also highly associated with this axis, also falling on its negative side. The second RDA axis seemed to be associated with the proportion of each small bodied genus, and separated Iowa and Southern Minnesota sites. Iowa was associated with higher levels of *Toxomerus* and *Augochlorella*, while southern Minnesota was associated with high abundances of *Dialictus*, *Halictus*, *Hoplitis*, *Megachile*, and wasp species.

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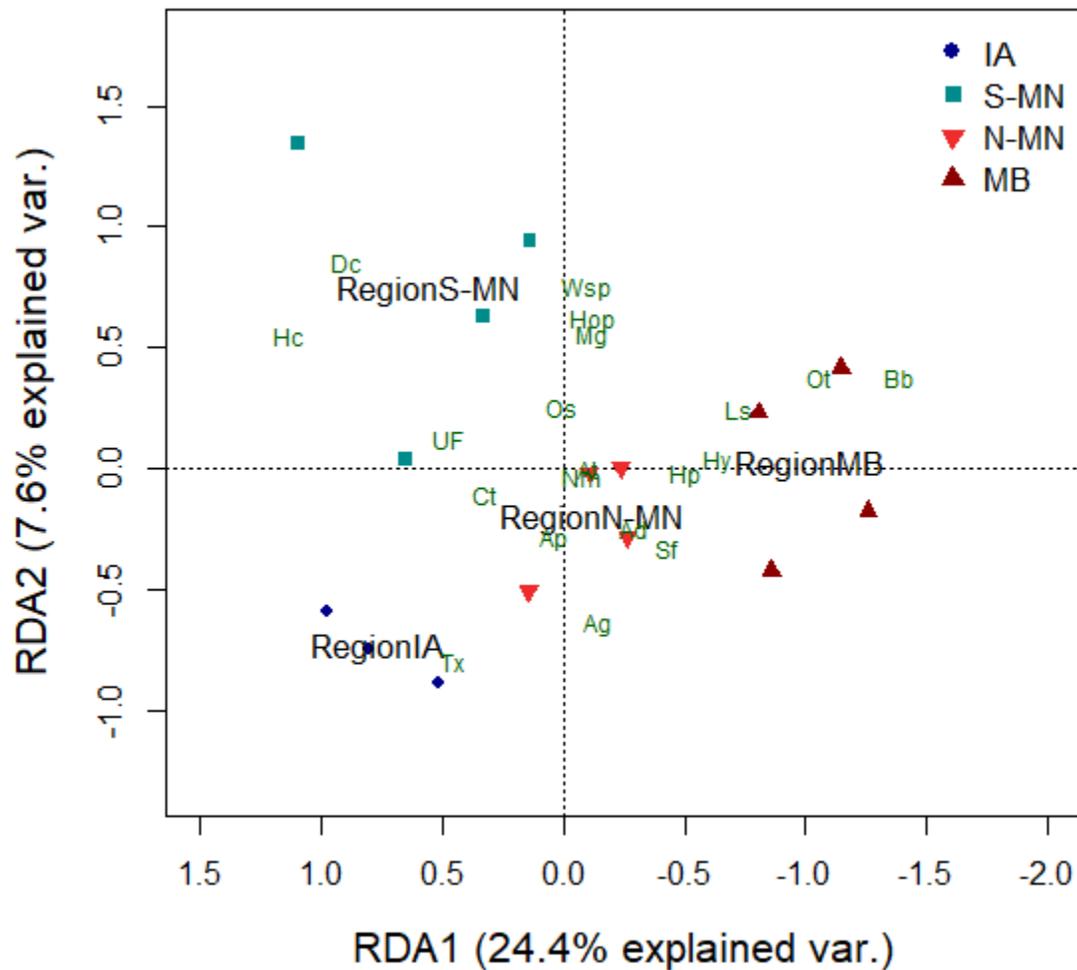


Figure 3.6. Redundancy analysis of the pollinator genera captured in pan traps in each tall grass prairie population of *Cyripedium candidum* during its flowering period in 2016, using a covariance matrix and constrained by study region. Genera scores are plotted in red, and labelled based on an abbreviated genus code (see Fig. 3.5 for abbreviations). Sites within regions are plotted with different symbols, and colour coded by region (Iowa: “IA”, Southern Minnesota “S-MN”, Northern Minnesota: “N-MN”, Manitoba: “MB”; see legend and Fig. 2.1).

Note: RDA axis 1 was flipped to reflect a south to north pattern, to match similar figures in this thesis.

Similar to Chapter 2, I examined the effect of all of the environmental variables outlined there on the composition of the insect community. Latitude, longitude, and average temperature

3. Latitudinal changes in the insect community across *C. candidum*'s range

from April 1st until the date of surveys all had significant effects on the insect community composition when included in the RDA alone, and explained 23%, 23%, and 14% of the variation in the insect community respectively (all $p < 0.05$). However, all of these variables varied significantly with study region (see Chapter 2), and co-varied with the other variables in the list. Therefore, I concluded that it was not appropriate to include multiple variables in my RDA, and chose to present just my RDA with study region, as this analysis explained the most variation in the insect community. Weather conditions on the date of pan trap surveys (average temperature, precipitation, and wind speed) did not explain a significant proportion of the variation in the insect community.

3.3.2 Insect Size Variation among Regions

Comparison of insect body width to the exit route width of *C. candidum* revealed three categories that I assigned insects to (Fig. 3.7). Insects larger than the exit width were called “too large” and included, *Lasioglossum*, *Odontomyia*, *Helophilus*, and *Bombus*. Insects that were about the same size as the exit width of *C. candidum* were classified as “large potential pollinators” and included *Augochlorella*, *Halictus*, and most insect captures from the family Megachilidae (identification pending). Finally, insects that were smaller than the exit width of *C. candidum* were classified as “small potential pollinators” and included *Dialictus*, *Toxomerus*, *Ceratina*, and *Hylaeus*.

A chi-square analysis comparing the proportion of these categories in each study regions was significant ($\chi^2_{6} = 199.2$, $p < 0.001$; Fig 3.8) and the largest contributors to this result were the higher than expected abundance of too large insects in Manitoba, the high abundance of potential

3. Latitudinal changes in the insect community across *C. candidum*'s range

pollinators in northern Minnesota, and the low abundance of too large pollinators in southern Minnesota. As discussed above, these expected values were based on the study wide proportion of each category. This analysis was still significant when I excluded site SM11 ($\chi^2_6=143.9$, $p<0.001$), though the low abundance of large pollinators in southern Minnesota had a smaller residual (and thus a reduced effect).

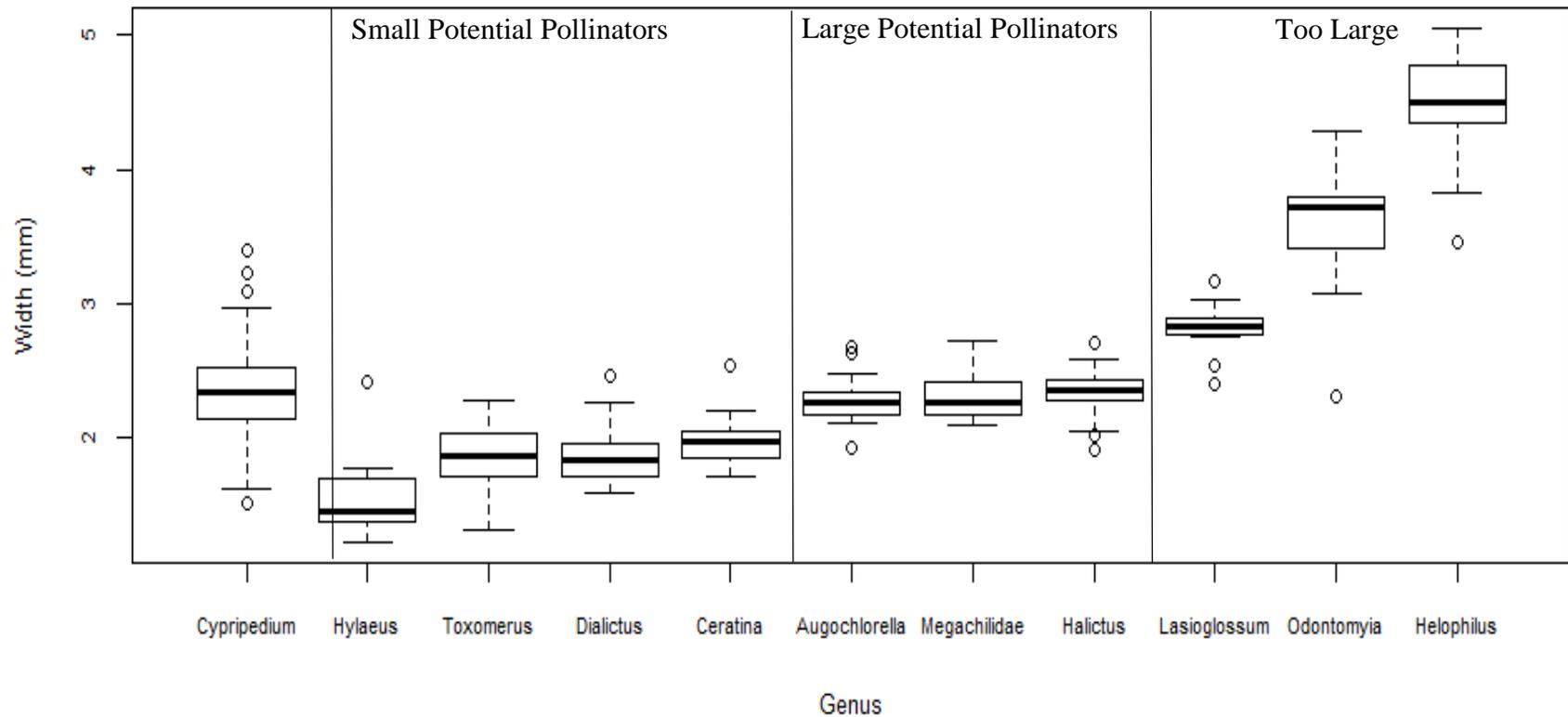


Figure 3.7. The exit route width of *Cypripedium candidum* compared to the body width of common insect taxa captured in pan traps in 2016. Boxplots display the median (middle line), the 25th and 75th percentile (top and bottom of the box, respectively), and 1.5x the inter quartile range (whiskers). These boxplots were used to organize captured insects into size classes (see text for description of methods and classes).

3. Latitudinal changes in the insect community across *C. candidum*'s range

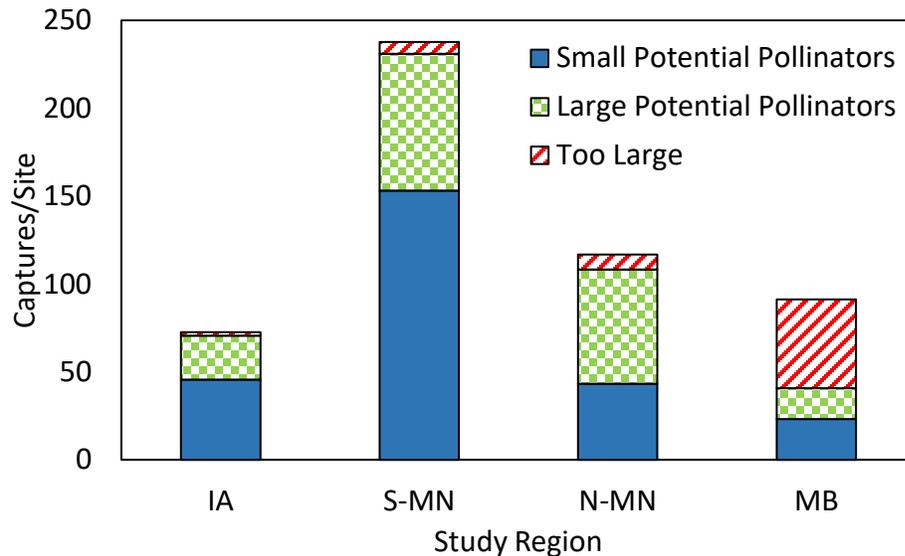


Figure 3.8. The number of captures of individuals from each size class (outlined in above text and Fig. 3.7) of insects per tall grass prairie population of *Cypripedium candidum* for each study region (Iowa: “IW”, Southern Minnesota “SM”, Northern Minnesota: “NM”, Manitoba: “MB”; Fig. 2.1). Insects were captured in pan traps during the flowering period of *C. candidum* (see methods for description of methods for pan trap surveys).

Multiple analyses of variance (MANOVA) results indicated that the bee genera *Halictus*, *Dialictus*, and *Augochlorella* had significant size differences between study regions (Table 3.2). The other bee taxa that were abundant enough to analyze (*Lasioglossum*, *Hylaeus*, *Ceratina*, and Megachilidae spp.) did not differ significantly (Table 3.2). Subsequent analyses of variance on taxa with significant differences indicated that body height was always significantly different between regions (*Halictus*: $F_{3,36}=8.5$, $p<0.001$; *Dialictus*: $F_{3,36}=3.3$, $p<0.05$; *Augochlorella*: $F_{3,43}=3.1$, $p<0.05$; Fig. 3.9), and body width was significant for *Dialictus* ($F_{3,36}=4.3$, $p<0.05$; Fig. 3.9) and *Augochlorella* ($F_{3,43}=6.6$ $p<0.001$; Fig. 3.9).

3. Latitudinal changes in the insect community across *C. candidum*'s range

Tukey *post hoc* tests showed that for all taxa, body height was smaller in Iowa than in Manitoba, and for *Halictus*, body height in Iowa was smaller than in all other study regions (Table 3.2; Fig. 3.9). Tukey *post hoc* tests on body width showed that, in *Dialictus* body width was larger in Manitoba than in southern Minnesota, and in *Augochlorella*, body width was larger in Manitoba than northern Minnesota and Iowa (Table 3.2; Fig. 3.9).

Multiple analysis of variance also indicated that fly genera, *Toxomerus* and *Odontomyia*, body size also varied significantly among regions. However, subsequent ANOVA's did not show a significant trend for the body measures individually, but graphical comparisons showed that body height was lowest in Manitoba for both genera, and body width was highest in Manitoba for *Odontomyia* (Table 3.2, Fig. 3.9).

Table 3.2. Multiple analysis of variance results for abundant insect taxa captured in pan traps during the 2016 *Cypridium candidum* flowering period. For each test, the approximate F-statistic and degrees of freedom are displayed. Significance is indicated with asterisks (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$) Taxa with significant MANOVA results are colour coded and their body width and height are compared in Fig. 3.9 to determine which regions contributed to the significant results.

Genus	approx. F	Genus	approx. F
<i>Halictus</i>	$F_{3,34}=3.33^{**}$	<i>Lasioglossum</i>	$F_{1,10}=2.87$
<i>Dialictus</i>	$F_{3,36}=2.59^{**}$	<i>Hylaeus</i>	$F_{1,6}=2.42$
<i>Augochlorella</i>	$F_{3,43}=2.56^{**}$	<i>Ceratina</i>	$F_{3,28}=1.22$
<i>Toxomerus</i>	$F_{3,35}=2.54^*$	Megachilidae*	$F_{2,22}=0.8$
<i>Odontomyia</i>	$F_{3,19}=2.16^*$		

3. Latitudinal changes in the insect community across *C. candidum*'s range

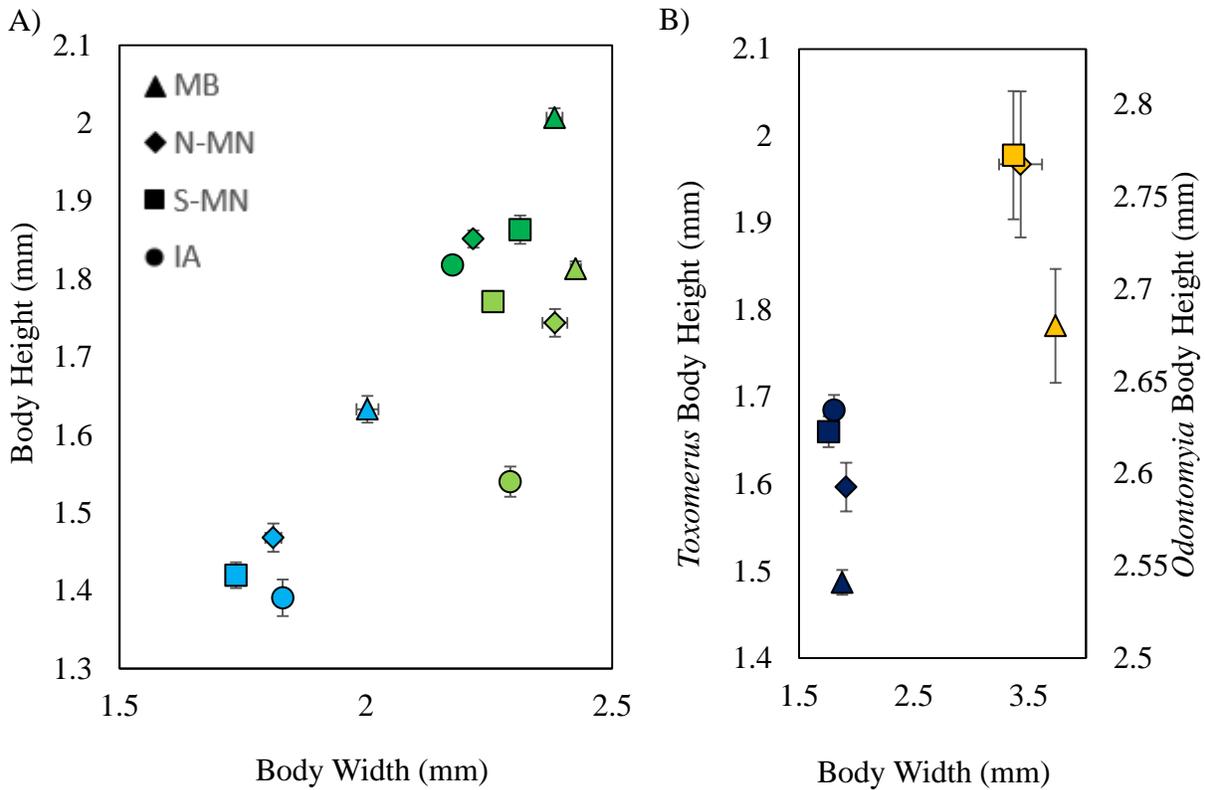


Figure 3.9. The body width (mm) compared to the body height for all bee genera (A) and fly genera (B) with significant size differences among study regions (Iowa: “IW”, Southern Minnesota “SM”, Northern Minnesota: “NM”, Manitoba: “MB”; Fig. 2.1), determined by MANOVA (results presented in Table 3.2). The regional averages for each genus are differentiated by different shapes that correspond to the study region (see legend) and different colours were used for each genus (see Table 3.2). Error bars indicate ± 1 standard error for each data point. The two fly genera (*Toxomerus* and *Odontomyia*) are plotted on separate vertical axes for clarity in regional patterns. All insects displayed were captured in pan traps in the 2016 *Cypripedium candidum* flowering period and measured in laboratory with digital calipers.

3.3.3 Insect Visitation Patterns

Colour preferences of Diptera did not vary significantly among study regions ($\chi^2_6=6.9$, $p>0.3$), but the colour preference of bees in southern Minnesota differed from the other study regions ($\chi^2_6=28.4$, $p<0.0001$; Fig. 3.10). This region had more bee captures in blue pan traps compared to yellow pan traps than would be predicted based on their study-wide proportion of

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captures in each pan colour. This result was still significant when site S-MN11 was excluded ($\chi^2_3=16.6, p<0.02$). In contrast, this study region had lower than expected number of blue co-flowering stems. This was the largest contributor to the significant result of a chi-square analysis comparing the proportion of stem counts of each flower colour between regions ($\chi^2_6=57.7, p<0.0001$; Fig. 3.11). One universal pattern was that yellow co-flowering stems formed the majority of flowering stems (65-85%) in every study region.

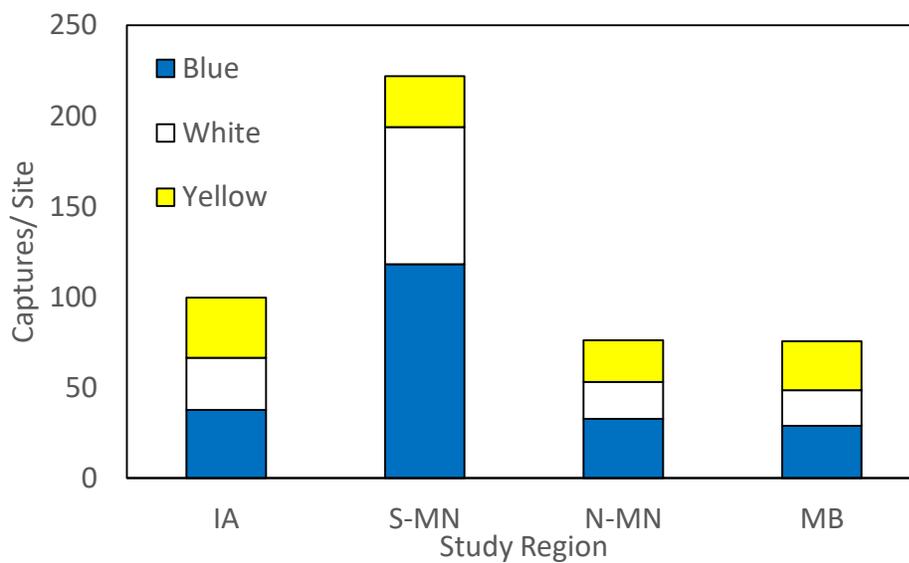


Figure 3.10. The number of captures of bee taxa from each colour of pan trap per tall grass prairie population of *Cyripedium candidum* for each study region (Iowa: “IW”, Southern Minnesota “SM”, Northern Minnesota: “NM”, Manitoba: “MB”; Fig. 2.1). Pan trap surveys were conducted in the 2016 *C. candidum* flowering period.

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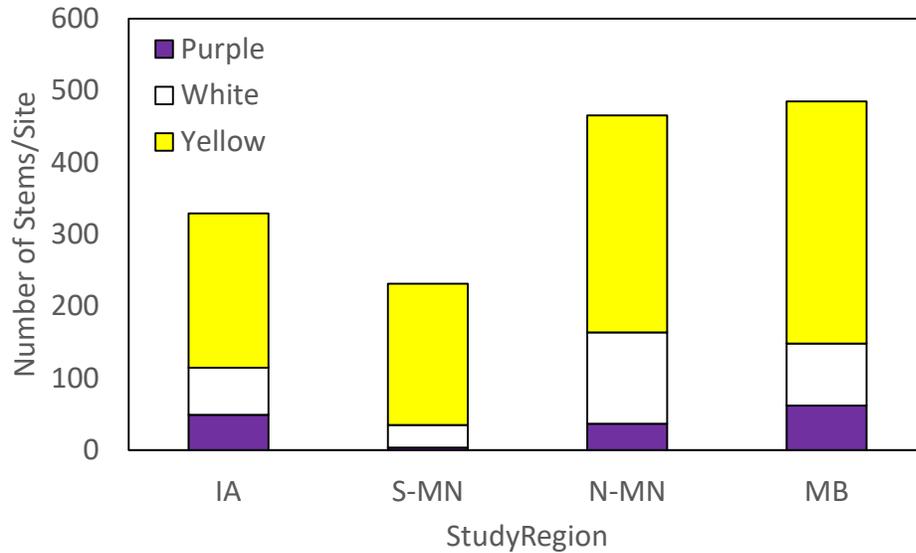


Figure 3.11. The number of stems of each flower colour per tall grass prairie population of *Cypripedium candidum* for each study region (Iowa: “IW”, Southern Minnesota “SM”, Northern Minnesota: “NM”, Manitoba: “MB”; Fig. 2.1). Flowering surveys were conducted during the 2016 *C. candidum* flowering period.

Pollinator matrices were quite sparse during this study (Table 3.3, Appendices 3.1, 3.2, 3.3, 3.4). All connectance measures were below 0.5, and there were no significant differences in connectance among study regions (Kruskal-Wallis Test: $\chi^2_3=6.6$, $p>0.08$). I also did not detect a significant correlation between daily weather variables (average temperature, precipitation, and wind speed) and the number of insect visitations observed during surveys at a site (all $r<|.3|$, all $p>0.25$).

In every study region, the most commonly visited co-flowering species was *Zizia* followed by *Packera* and *Agoseris* (see Appendices for each study region’s pollination matrix). *Pedicularis* was also highly visited by *Bombus*, but this plant genus was most frequent in northern Minnesota. The most common insect visitors observed during pollinator surveys were: *Toxomerus* and *Dialictus* in Iowa; *Odontomyia* and *Lasioglossum* in southern Minnesota;

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Toxomerus, *Bombus*, and *Augochlorella* in northern Minnesota; and *Bombus* and *Odontomyia* in Manitoba. The potential pollinators outlined above (both potential pollinators and small potential pollinators) were all seen foraging primarily on *Zizia*. The most common additional forage species for each potential pollinator included *Sisyrinchium* for *Toxomerus*, *Packera* for *Dialictus*, and *Agoseris* for *Augochlorella* (Table 3.3). *Halictus* was only found foraging on *Zizia*, though some observation of *Dialictus* may have been *Halictus* as these two genera are very difficult to tell apart in the field.

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Table 3.3. Combined pollination matrix obtained from surveys of pollinator activity at 15 study population of *Cyripedium candidum* across three study regions (see methods). Each population was surveyed for 50 minutes and all insect visitations were recorded and both the plant and insect were identified. These data were then combined across all sites to produce this matrix (see Appendices 3.1, 3.2, 3.3, and 3.4 to see each individual region's pollination matrix).

Order	Genus/Family	Zizia	Packera	Pedicularis	Agoseris	Fragaria	Castilleja	Cyripedium	Sisyrinchium	Lithospermum	Potentilla	Hypoxis	Euphorbia	Polygala	Total
Diptera	<i>Toxomerus</i>	80	2		1	2		2	3						90
Hymenoptera	<i>Bombus</i>	2	4	31	2	1	6			3					49
Hymenoptera	<i>Augochlorella/Agopostemon</i>	18	1		13	1	1							1	35
Hymenoptera	<i>Dialictus</i>	25	3		2	2		1*							33
Diptera	<i>Odontomyia</i>	23	4		2	1		1							31
Diptera	Muscidae/Calliphoridae/Tachinidae	20	4			1	1				1				27
Hymenoptera	<i>Lasioglossum</i>	12	6		1				1		1				21
Hymenoptera	<i>Apis</i>	9	2												11
Diptera	Syrphidae	3	6		1										10
Hymenoptera	Halictidae	5													5
Hymenoptera	Megachilidae	2										2			4
Hymenoptera	<i>Hylaeus</i>	2	1												3
Coleoptera		1	1			1									3
Hymenoptera	Wasps-Vespidae/Ichneumonidae	1											2		3
Hymenoptera	<i>Andrena</i>	1	1												2
Hemiptera		1	1												2
Hymenoptera		1													1
Hymenoptera	Apidae			1											1
Total		206	34	32	22	9	8	4	4	3	2	2	2	1	

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3.3.4 Variation in Orchid Measurements

MANOVA revealed that orchid flower size measurements varied significantly between study regions ($F_3=3.6$, $p<0.0001$; Fig. 3.12). *Post-hoc* analyses of variance and subsequent Tukey HSD tests determined that slipper width, entry route width, and exit route width all contributed to this significant result. Most of these variables averaged highest in Manitoba and lowest in Iowa. Subsequent analyses did not detect a significant difference in orchid flower size measurements among plants from Manitoba, southern Minnesota, or northern Minnesota (Fig. 3.12). A PCA of all flower measurement data depicted trends similar to those detected using MANOVA (Figure 3.12). Principal component one captured 39.9% of the variation in the data. This axis was associated with an overall decrease in orchid measurements from the negative to positive end of principal component one, with many of the orchids from Iowa found on the negative side of this axis. Principal component two captured 21.4% of the variation in the data set with entry route length and exit route length and width having high loadings on this axis. No geographic patterning was evident on this axis.

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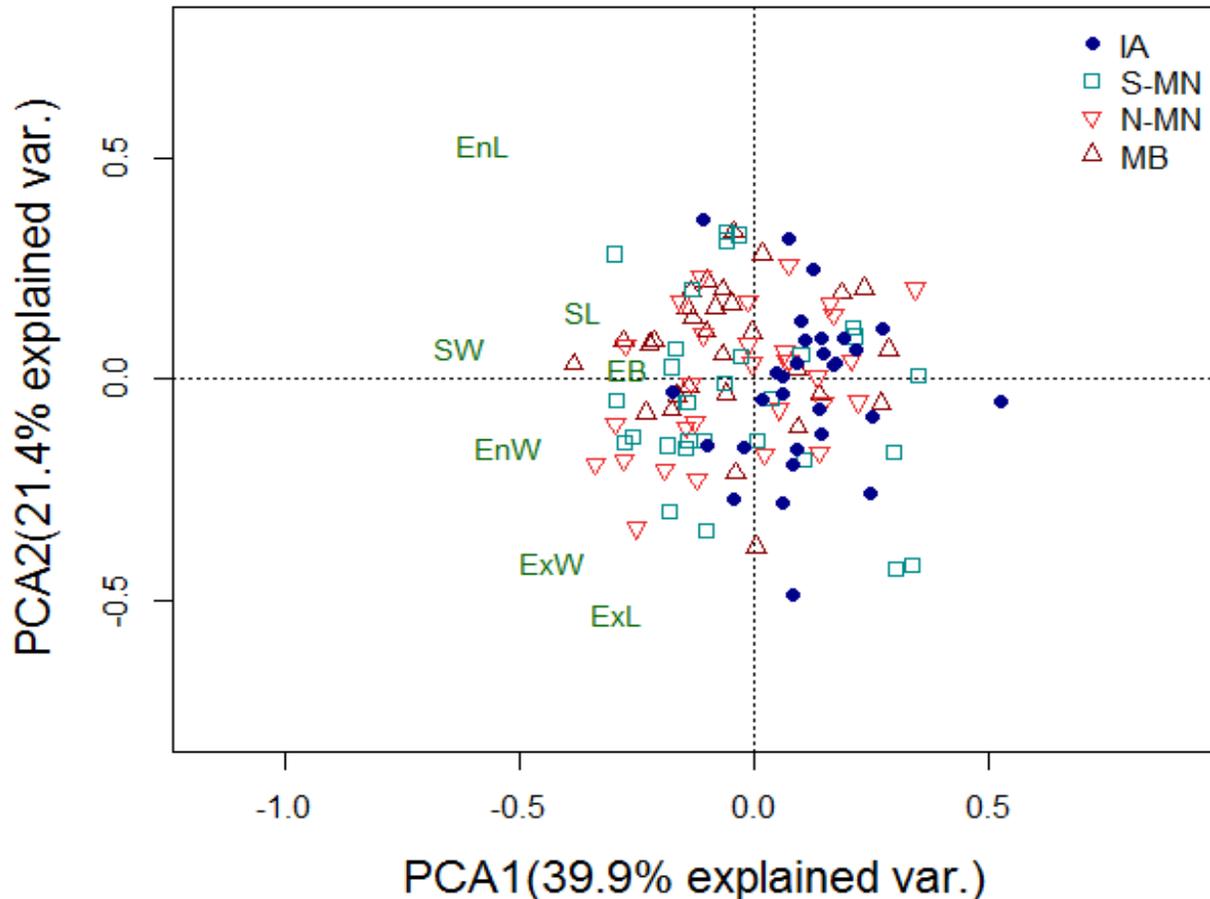


Figure 3.12. Principal component analysis of *Cyripedium candidum* floral measurements for each study region, plotted with different colours (see legend, “IA”=Iowa, “S-MN”= southern Minnesota, “N-MN”= northern Minnesota, “MB”= Manitoba; Fig. 2.1). Thirty orchids from two populations (15 orchids/site) from each study region were used for measurements. Eigenvectors of orchid measurements are plotted in green (“SL”=slipper length, “SW”= slipper width, “EnL”=Entry route length, “EnW”=Entry route width, “ExL”=Exit route length, “ExW”=Exit route width, “EB”=distance from entry route to base of labellum). MANOVA analyses determined that the orchid measurements taken were significantly lower in Iowa sites while other regions did not differ in orchid size, therefore Iowa sites are plotted with filled symbols while the other regions are plotted with open symbols.

Despite log transformation of the morphological data, the number of flowers per genet could not be included in the MANOVA due to its non-normal distribution and unequal variances among study regions. A Kruskal-Wallis test found significant differences between study regions

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for average number of flowers produced by *C. candidum* genets ($\chi^2_3=20.4, p<0.001$). Box-plots showed that orchids in Iowa produced more flowers than the other study regions (Fig. 3.13).

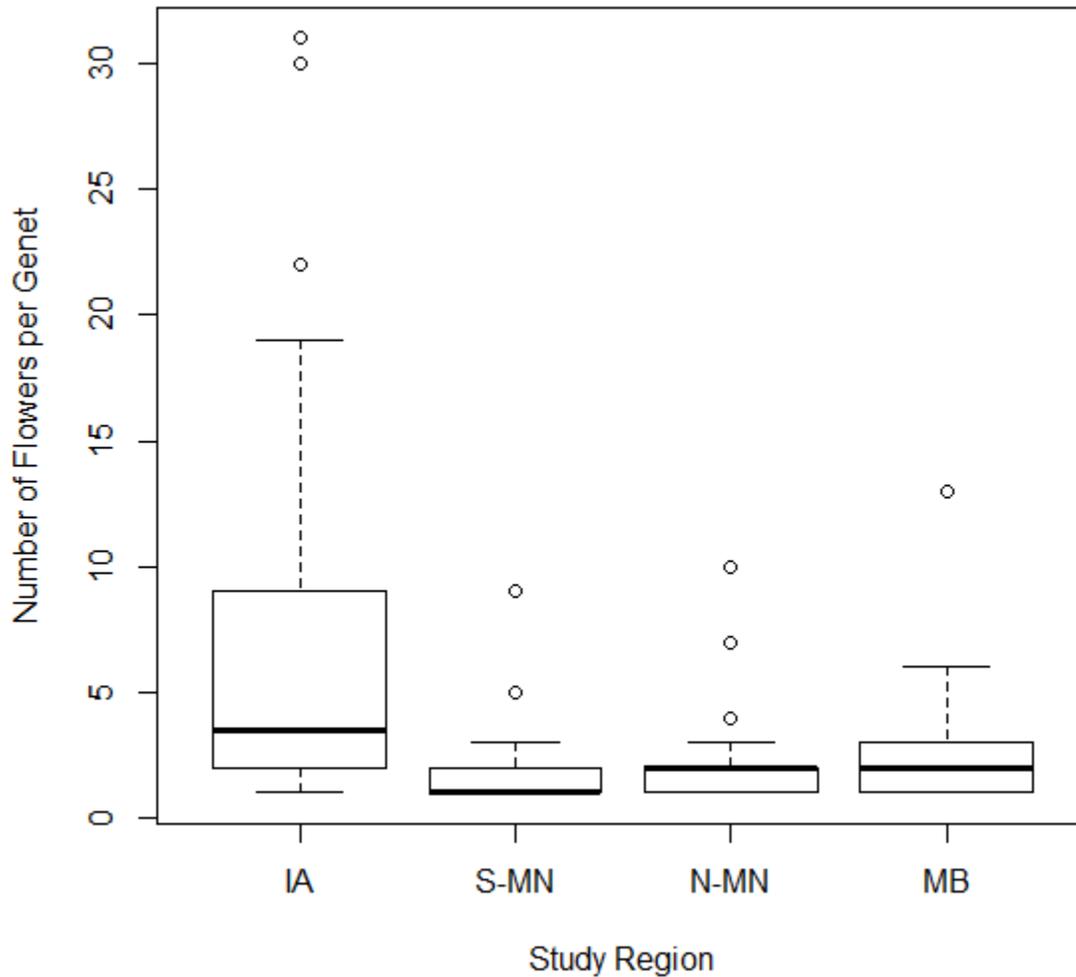


Figure 3.13. Boxplots of the average number of flowers per *Cypripedium candidum* genet for each tall grass prairie population of *C. candidum* in 2016, grouped by study region (IA=Iowa, S-MN=southern Minnesota, N-MN=northern Minnesota, and MB=Manitoba; Fig 2.1). Boxes represent the 25th to 75th percentile of the data, while the dark line represents the median. The tails represent the 5th and 95th percentile of the data, and outliers are plotted as points.

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3.4 Discussion

3.4.1 Patterns in Insect Community Composition

As was observed with flowering plants (see Chapter 2), the highest insect diversity was seen in northern study regions, while the lowest diversity was seen in the south. In addition, there were a number of insect taxa that occurred in every study region, while others were restricted to the north. These findings confirmed my prediction that more insect taxa would be found in northern regions, matching the diversity of plant taxa. It is difficult to compare these findings to the literature, because many studies of bee diversity examine diversity in an urban/agricultural setting. Studies by Kearns and Oliveras (2009) and Kennedy et al. (2013) have reported bee diversity increasing with distance from urbanization and with diversified agriculture respectively, both of which should be associated with an increase in flowering diversity. This trend has some parallels to the findings of this study, which also detected a significant correlation between plant and insect diversity. One study by Classen et al. (2015) was conducted along an elevational gradient and found that bee diversity significantly decreased with increasing altitude and a corresponding decrease in plant diversity. This study contradicts my findings; however, it was conducted across the growing season. My findings may have been similar if I had also conducted insect surveys across the growing season, but, these insects would not have represented potential pollinators of *C. candidum*.

Once again, the patterns that I observed in this study may be a result of a compressed phenology further north in *C. candidum*'s range. The abundance of *Bombus* in Manitoba seems to support this possibility. Because of their ability to heat regulate by uncoupling their wing muscles, *Bombus* is one of the first bee genera to emerge in the spring (Heinrich 1972, Petersen 1996). If insect emergence was simply at a later stage in the north compared to the south, there

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should have been a lower proportion of *Bombus* relative to other bee genera in northern regions. This was not the case, and the fact that diversity was highest in northern regions, and *Bombus* was one of the most abundant insect genera in this area is consistent with my hypothesis that higher early season diversity in the north is due to compressed phenology.

A notable result from my insect collections, is that many of the insects that were more abundant in the north were from genera with a large body size (e.g. *Bombus*, *Lasioglossum*, and *Helophilus*). This pattern suggests that conditions in southern communities may support an abundance of smaller insect taxa because of a plant community that supports smaller insects and/or because of climatic conditions which allow these smaller taxa to persist, in comparison to more northerly populations in Manitoba. It is unlikely that smaller-bodied taxa could outcompete larger-bodied insect, as most studies of competition among pollinating insects show that larger insects (particularly the domesticated honey bee, *Apis*) tend to outcompete smaller ones (Roubik 1978, 1980, Dupont et al. 2002, Thomson 2014). Overall, the transition from a high to low abundance of small-bodied insect genera may negatively affect, *C. candidum* reproduction, see Chapter 4 for further discussion.

As predicted, the total number of individuals and the diversity of fly taxa was highest in the north. Furthermore, the fly genera found in the north were larger than those observed in the south. These results match the findings of Elberling and Olesen (1999) who observed an increase in the dominance of fly genera in a subarctic-alpine site compared to lower latitude communities. Flies are known for low pollination efficiency, usually due to consuming more pollen than they transfer to a plants stigma (Bischoff et al. 2013, Hargreaves et al. 2014). *Cypripedium* pollen cannot be consumed because it is a sticky mass that adheres to insects that crawl through the exit

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route (Argue 2012). While flies may contact the anthers of *Cypripedium*, Li et al. (2008) suggested that fly visitors to *Cypripedium plectrochilum* would be too weak to remove themselves from the sticky mass of pollen or squeeze through the exit route. While some flies were confirmed to pick up pollen from *C. parviflorum* during a study by Pearn (2012), *Toxomerus* were observed stuck to the pollinia and unable to exit the flower. Furthermore, the vast majority of recorded visitors to *Cypripedium* flowers that managed to successfully remove pollen have been bees (Argue 2012). Therefore, increased fly abundance coupled with larger body size of insect taxa in general could decrease the pollination success of *C. candidum* in northern study regions (see Chapter 4).

3.4.2 Evidence for Bergmann's Rule in Pollinating Insects

As predicted by Bergmann's rule, larger genera were much more abundant in northern regions, particularly Manitoba, while Iowa had the highest proportion of small potential pollinators (though absolute values were highest in southern Minnesota, due to my survey at S-MN11). Furthermore, when body size varied significantly within genera, the largest individuals were in northern regions and the smallest in southern regions. Size differences in bee taxa have not been shown this clearly in any other studies. One study by Hawkins (1995) found no consistent patterns in the body size of native bee families across a 19° latitudinal gradient in the eastern United States, and additional studies of solitary bees are lacking in the literature. This study covered a 7.5° range of latitude, but focused on one ecological region. Perhaps the consistency in habitat allowed me to observe patterns that were obscured by large differences in environmental conditions in Hawkins' study.

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While the patterns observed in my study are clear, and are consistent with an advantage to larger bees in cold areas, the physiological mechanism that might explain this trend is unclear. While *Bombus* has been shown to match Bergmann's rule across latitudes, this species is a facultative endotherm (Heinrich 1972), and a larger body size in this genus would be adaptive in cold environments. For ectothermic taxa, like all other bee genera, a larger body size should not impart such a large ecological advantage, since a large body size prevents heat gain as well as heat loss. A thorough literature search (search terms: Hymenoptera, solitary bee, body size, morphology, latitude, clinal variation) found a single study on alkali bees (*Nomia* spp.; Rust 2006). Rust (2006) found that bees from higher latitudes gained more weight than those from lower latitudes when raised in the same conditions, which is similar to my findings.

The general abundance of small dipterans in the arctic is thought to refute Bergmann's rule in ectothermic insects (Kevan 1972, Elberling and Olesen 1999). Dipterans in my study did not show the predicted trends either. Although the larger fly genera were more common further north, size differences within fly genera did not follow a latitudinal trend. Smaller individuals were found in Manitoba for both *Toxomerus* and *Odontomyia*. Moseau (1997) also did not find any latitudinal patterns when he examined body size changes within fly genera. More research should be conducted on both flower-visiting fly and solitary bee physiology and demography to determine what mechanism may explain their contrasting patterns in body size observed in this study.

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3.4.3 Insect foraging remains consistent across regions

Despite the large changes in the insect community observed in this study, there was little change in insect foraging preferences. While southern Minnesota showed a higher proportion of bee captures in blue bowls, compared to yellow or white, the other study regions did not show a strong colour preference among bees. There are two potential explanations to this trend in southern Minnesota. First, since many of the bees in this study are generalist foragers (Chambers 1968, Bernhardt and Burns-Balogh 1986, Giurfa et al. 1995, Waser et al. 1996, Chittka et al. 1997, Dicks et al. 2002, Shi et al. 2009, Song and Feldman 2014), perhaps the low abundance of purple flowers in southern Minnesota made them more attracted to the novel visual stimulus presented by blue pan traps. Visiting new potential food sources is essential to a generalist foraging strategy, allowing foragers to take advantage of new and short-lived food resources (Chittka et al. 1997).

Alternatively, perhaps the dominant taxa in southern Minnesota have an innate preference for blue. As I only looked at the order level for colour preference, the family composition of the region may have been biased towards a bee family that prefers blue more than other families. Other researchers have documented that insects in the family Apidae (*Anthophora*, *Apis*, *Bombus*) demonstrate a preference for blue, while Andrenidae (*Andrena*) prefer yellow (Leong and Thorp 1999, Toler et al. 2005, Heneberg and Bogusch 2014). Insect from the families Megachilidae and Halictidae (*Agapostemon*, *Augochlorella*, *Dialictus*, *Halictus*, *Lasioglossum*) often do not show a colour preference (Leong and Thorp 1999, Toler et al. 2005, Heneberg and Bogusch 2014), though some species within Halictidae have shown a preference for white (*Lasioglossum lusorius*, Toler et al. 2005), yellow (*Lasioglossum glabriusculum*, Heneberg and Bogusch 2014), or blue (*Agapostemon angelicus*, Toler et al. 2005). Since most insects from

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southern Minnesota were from Halictidae, this region was likely higher in generalist foragers. Therefore, it is more likely that they were exploring the novel colour and did not have an innate preference to blue.

The connectance of the pollination networks observed in this study did not vary among study regions, due to low connectance values in every study region resulting from most insects foraging on the same yellow-flowered species (*Zizia*, *Packera* and *Agoseris*). All of these genera are tall, have showy flowers, and were the most abundant taxa in *C. candidum* communities. Since the majority of bees captured in this study are noted generalist species (Chambers 1968, Bernhardt and Burns-Balogh 1986, Giurfa et al. 1995, Waser et al. 1996, Chittka et al. 1997, Dicks et al. 2002, Shi et al. 2009, Song and Feldman 2014), they may have simply foraged on the most abundant species in a community, creating sparse pollinator matrices. These sparse networks could also be a reflection of the early spring community, with relatively few flowers and insects present to interact, or simply due to the relatively short, 50-minute survey period at a site. Nevertheless, the matrices do provide a fair indication of the most commonly visited plants and active pollinators when I summed all matrices across my study, which I used to identify co-flowering taxa that may influence *C. candidum* reproduction in Chapter 4.

I did not detect a relationship between the weather conditions at the time of my surveys and the number of insect visits observed, despite temperature during the spring having a significant effect on my redundancy analysis of pan trap data. By selecting dates for insect surveys that had conditions favourable for insect activity, I likely was able to reduce the effects on weather on insect activity during the day of surveys, since most of my dates of pan trap surveys had similar conditions. Weather conditions from the beginning of April would still affect

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insect emergence, so this is likely why spring temperature showed a significant effect on insect community composition among study sites.

3.4.4 Variation in Orchid size across *C. candidum*'s Range

Orchid flower size varied significantly among study regions, due to the smaller flowers found in plants in Iowa. Orchid size did not differ among other study regions. The difference between Iowa and other regions matches my predictions, but the trend in orchid size with latitude seems to be more bimodal than linear. The potential consequences of changes in flower size for fruiting will depend on the abundance of appropriately sized insects in a region.

Although orchids north of Iowa did have larger flowers, flowers in these regions were still not of a size large enough for pollination by “too large” insect genera. *Bombus*, *Helophilus*, *Odontomyia*, and *Lasioglossum* would all be unable to pollinate *C. candidum* across its range (this study, Anderson 2015). The abundance of these large insect genera in Manitoba could limit orchid reproduction in this study region. In addition, large individuals within the “potential pollinator” size class may not be able to pollinate the smallest *C. candidum* flowers since size ranges were overlapping.

This study is the first to document within species changes in *Cypripedium* flower size across a latitudinal gradient. Other studies have determined that reproduction in this genus is limited by the size of the exit route (Li et al. 2008, Case and Bradford 2009, Pearn 2012, Anderson 2015), but most have focused on the potential of various insects to fit through the route. None have examined how orchid size changes across the range of a species. Repeating this portion of my study on wider-ranging species, such as *C. parviflorum* could corroborate the

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findings in this study, as a wider species range could result in a more extreme shift in orchid morphology.

This chapter showed that insect diversity increased with latitude in the early spring community of the tall grass prairie. Body size of pollinating bees also increased with latitude, both through the proportion of genera with different body sizes, and the sizes of individuals within some genera. This is the first study to recover such a trend, especially in solitary bees. More research on the physiology of these understudied bees would provide information on what is causing this trend. Interestingly, orchid size did not match this trend in insect body size, with the only detectable size differences occurring between small orchid flowers in Iowa and larger flowers further north. If *C. candidum* flowers are mostly uniform across its range, and insect size increases, this may explain the low reproductive success in the northern portion of *C. candidum*'s range. Finally, despite changes in the dominant foraging insects, the overall colour preferences of insects remained consistent across this study. Their foraging patterns seemed to reflect the abundance of the same dominant, showy species in every study region, and provides evidence to determine which co-flowering species are the most likely to affect reproduction in *C. candidum*.

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3. Latitudinal changes in the insect community across *C. candidum*'s range

3.6. Appendices

Appendix 3.1. Combined pollination matrix obtained from surveys of pollinator activity at 3 study populations of *Cypripedium candidum* in Iowa. Each population was surveyed for 50 minutes and all insect visitations were recorded and both the plant and insect were identified. These data were then combined across all sites to produce this matrix.

Order	Family/Genus	<i>Zizia</i>	<i>Senecio</i>	<i>Cypripedium candidum</i>	<i>Fragaria</i>	Total
Diptera	<i>Toxomerus</i>	14	2	1		17
Hymenoptera	<i>Dialictus</i>	11	2	1		14
Diptera	Muscidae/Calliphoridae/Tachinidae	6	3			9
Hymenoptera	<i>Bombus</i>	2	1		1	4
Hymenoptera	Halictidae	3				3
Hymenoptera	<i>Lasioglossum</i>		2			2
Hymenoptera	<i>Augochlorella</i>				1	1
Hemiptera			1			1
Total		36	11	2	2	

3. Latitudinal changes in the insect community across *C. candidum*'s range

Appendix 3.2. Combined pollination matrix obtained from surveys of pollinator activity at 4 study populations of *Cypripedium candidum* in southern Minnesota. Each population was surveyed for 50 minutes and all insect visitations were recorded and both the plant and insect were identified. These data were then combined across all sites to produce this matrix.

Order	Genus/Family	<i>Zizia</i>	<i>Senecio</i>	<i>Potentilla</i>	<i>Cypripedium candidum</i>	<i>Fragaria</i>	Total
Diptera	<i>Odontomyia</i>	12	2				14
Hymenoptera	<i>Lasioglossum</i>	6	4	1			11
Diptera	<i>Toxomerus</i>	3			1		4
Hymenoptera	<i>Augochlorella</i>	4					4
Diptera	Muscidae/Calliphoridae/Tachinidae	1		1		1	3
Hymenoptera	Halictidae	2					2
Hymenoptera	<i>Bombus</i>		1				1
Hymenoptera	<i>Dialictus</i>	1					1
Total		29	7	2	1	1	

3. Latitudinal changes in the insect community across *C. candidum*'s range

Appendix 3.3. Combined pollination matrix obtained from surveys of pollinator activity at 4 study populations of *Cypripedium candidum* in northern Minnesota. Each population was surveyed for 50 minutes and all insect visitations were recorded and both the plant and insect were identified. These data were then combined across all sites to produce this matrix.

Order	Genus/Family	<i>Zizia</i>	<i>Pedicularis</i>	<i>Agoseris</i>	<i>Fragaria</i>	<i>Sisyrinchium</i>	<i>Senecio</i>	<i>Castilleja</i>	<i>Polygola</i>	Total
Diptera	<i>Toxomerus</i>	61			1	2				64
Hymenoptera	<i>Bombus</i>		31							31
Hymenoptera	<i>Augochlorella</i>	13		12				1	1	27
Diptera	Muscidae/Calliphoridae/Tachinidae	11								11
Hymenoptera	<i>Dialictus</i>	10								10
Hymenoptera	<i>Apis</i>	7					1			8
Diptera	<i>Odontomyia</i>	3			1					4
Hymenoptera	<i>Lasioglossum</i>	2								2
Diptera	Syrphidae	2								2
Coleoptera		1								1
Hymenoptera	<i>Andrena</i>	1								1
Hymenoptera	Apidae		1							1
Total		111	32	12	2	2	1	1	1	

3. Latitudinal changes in the insect community across *C. candidum*'s range

Appendix 3.4. Combined pollination matrix obtained from surveys of pollinator activity at 4 study populations of *Cypripedium candidum* in Manitoba. Each population was surveyed for 50 minutes and all insect visitations were recorded and both the plant and insect were identified. These data were then combined across all sites to produce this matrix.

Order	Genus/Family	<i>Zizia</i>	<i>Senecio</i>	<i>Agoseris</i>	<i>Castilleja</i>	<i>Fragaria</i>	<i>Lithospermum</i>	<i>Sisyrinchium</i>	<i>Hypoxis</i>	<i>Euphorbia</i>	<i>Cypripedium candidum</i>	Total
Hymenoptera	<i>Bombus</i>		2	2	6		3					13
Diptera	<i>Odontomyia</i>	8	2	2							1	13
Diptera	Syrphidae	2	6	1								9
Hymenoptera	<i>Dialictus</i>	3	1	2		2						8
Hymenoptera	<i>Lasioglossum</i>	4		1				1				6
Diptera	<i>Toxomerus</i>	2		1		1		1				5
Hymenoptera	Megachilidae	2							2			4
Diptera	Muscidae/Calliphoridae/Tachinidae	2	1		1							4
Hymenoptera	<i>Augochlorella</i>	1	1	1								3
Hymenoptera	<i>Apis</i>	2	1									3
Hymenoptera	<i>Hylaeus</i>	2	1									3
Hymenoptera		2								1		3
Hymenoptera	<i>Andrena</i>	1	1									2
Coleoptera			1			1						2
Total		30	17	10	7	4	3	2	2	1	1	

4. LATITUDINAL PATTERNS AND POTENTIAL DRIVERS OF *C. CANDIDUM* REPRODUCTION

4.0 Chapter Summary

This chapter examined whether fruit set by *Cypripedium candidum* in four study regions along a latitudinal gradient was associated with variation in the co-flowering community composition, insect community composition, or environmental conditions. Negative binomial regression was used to examine the effect of each of these sets of variables, along with variables of study region and *C. candidum* flower number, on both the number of fruit observed at each site, and the number of fruiting genets. Study region had a significant effect on fruiting success, with sites in Iowa having higher fruiting success than other study regions ($55\pm 16\%$ of genets successfully set fruit in Iowa vs. $22\pm 4\%$ in more northern regions). This pattern supported my hypothesis that reproductive success in this species would increase further south. Co-flowering variables revealed both facilitative (*Packera* and *Zizia*) and competitive (*Pedicularis* and *Sisyrinchium*) taxa that significantly affected fruiting success. As seen in Chapter 2, the facilitative taxa had higher abundance in southern study regions, while the competitive taxa became more abundant further north. Average temperature and growing degree days over the flowering season were environmental variables that had significant and positive effects on reproduction. No relationship was found between the insect community and fruit set in *C. candidum*, but sample size may have limited the power of analyses. The findings from this chapter provide potential explanations of latitudinal trends in *C. candidum* reproduction, and identify avenues of research to further examine the effects of environmental and co-flowering factors on reproduction in this rewardless species.

4. Latitudinal patterns in *Cypripedium candidum* reproduction

4.1 Introduction

Pollination by deceit has evolved in a few species in many plant families, but over one-third of species in the Orchidaceae employ this strategy (Cozzolino and Widmer 2005). Deceitful pollination occurs when a flowering species does not produce a food reward of nectar or edible pollen, although they provide cues normally associated with a reward or mating opportunity (Jersáková et al. 2006). Many rewardless species have low reproductive success, but such a widespread strategy (Cozzolino and Widmer 2005, Jersáková et al. 2006) should impart an evolutionary benefit. As discussed in Chapter 1, potential benefits may include an increase in genetic outcrossing or conservation of floral resources (Johnson 2000, Cozzolino and Widmer 2005, Jersáková et al. 2006). Rewardless species are reliant on the co-flowering community to attract and support the pollinator populations (Internicola et al. 2007), yet may suffer from pollen limitation if pollinators learn to avoid the rewardless species in favour of rewarding co-flowering species (Internicola et al. 2007, 2008).

Latitudinal patterns that may affect rewardless species' pollination are changes in biological diversity, and the composition of the floral community. In general, angiosperms that flower together may experience either facilitative or competitive interactions when attracting pollinators. With an abundant pool of pollinators, two species will typically act as facilitators, as the presence of each should increase the overall floral display and bring pollinators to the area (Moeller 2004, Ghazoul 2006, Lázaro et al. 2009). However, a limited number of pollinators may not be able to visit all the flowers that are present, and species may begin to compete with each other for insect visits (Waser 1983).

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A potential strategy to offset low pollinator visits in a rewardless species is to flower earlier than most rewarding species (Internicola et al. 2008, Pellissier et al. 2010). This strategy should allow rewardless species to avoid competition with rewarding species, and take advantage of pollinators that have not yet learned which floral traits are associated with a food reward (Internicola and Harder 2012). At lower latitudes, snowmelt should occur earlier in the year, which may provide the opportunity for greater asynchrony in flowering (Körner 2003, Elzinga et al. 2007, Pellissier et al. 2010). In addition, frost damage associated with early flowering should be reduced in areas with a longer growing period (Inouye 2008), potentially allowing rewardless species to flower earlier in the south than they could further north. Northern populations should be more limited by temperature, and thus not able to receive the benefits of flowering early (Munguía-Rosas et al. 2011a). The theoretical advantages of flowering early have been shown in both controlled insect flight rooms and in natural populations of *Calypso bulbosa* (Internicola et al. 2006, 2007, Internicola and Harder 2012), but no research has yet been done to confirm that this strategy is limited by latitude in natural populations.

As discussed in Chapter 2, species diversity generally increases in more equatorial latitudes across a growing season (Fischer 1960, Rohde 1992). For a flowering community, this could result in an increased potential for facilitation or competition at lower latitude. An increased species richness of co-flowering plants has been shown to increase pollination in *Raphanus raphanistrum* (Ghazoul 2006). While no studies have shown evidence that species richness increased competitive effects, competition for pollinator visits occurs between pairs of species, resulting in lower reproductive success (Flanagan et al. 2010, Dietzsch et al. 2011). The overall effect of increasing floral diversity on reproductive success has not been confirmed in the rewardless species' literature at this point, providing motivation for further research.

4. Latitudinal patterns in *Cypripedium candidum* reproduction

This study is focused on the reproductive success of the rewardless orchid, *Cypripedium candidum*. *Cypripedium candidum*, or “the small white lady’s slipper” is a tall grass prairie endemic, spring flowering species. Across its range, *C. candidum* is classified as threatened due to habitat loss through the conversion of tall grass prairie habitat to agriculture (Rankou 2014). In Manitoba, *C. candidum* has been shown to have low reproductive success, particularly in comparison to its sister species, *C. parviflorum* (Pearn 2012, Anderson 2015). In a typical year, between 10-15% of *C. candidum* individuals successfully setting fruit, while *C. parviflorum* usually range from 50-90% of its flowers (Pearn 2012, Anderson 2015). Research conducted in Illinois and Ohio, further south in *C. candidum*’s range, have reported higher fruiting success in some years than that observed in Manitoba (>30%, Shefferson and Simms 2007; >45%, Walsh et al. 2014; >30%, Walsh and Michaels 2017), raising the possibility of a latitudinal trend in pollination success in this orchid species.

4.1.1 Chapter Objectives

The main objective of this study was to determine if reproductive success changed across the latitudinal range of *C. candidum*. Given the potential for earlier flowering time in southern regions, I predicted that orchids further south would have a reproductive advantage, and have higher fruiting success than northern regions. Other studies further south in *C. candidum*’s range have reported higher fruit set has been recorded in Manitoba, suggesting a potential latitudinal trend in reproduction.

I also expected *C. candidum* to show the highest levels of reproduction in areas with a higher abundance of appropriately sized insects for pollination. My findings in chapter 3 show that southern regions of *C. candidum*’s range have a higher proportion of small bodied

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pollinators. Therefore, I predicted that reproductive success would increase in the south, or in study sites with the highest proportion of potential *C. candidum* pollinators.

Finally, I wanted to determine if the co-flowering community had an effect on the pollination rates of *C. candidum*. Overall, I predicted that *C. candidum* would have low reproductive success when surrounded by a high abundance of rewarding species. My findings in Chapter 2 showed that there is a lower abundance of co-flowering species further south in *C. candidum*'s range, possibly due to the longer growing season, which allows for an earlier flowering time in rewardless species. Therefore, I predicted that the lower co-flowering density in these regions would correspond to a higher rate of orchid pollination.

4.2 Methods

4.2.1 Orchid surveys

Transect Establishment and Orchid Monitoring

As described in chapter 2, each study site had two floral transects established in high density orchid patches. Along each transect established for floral monitoring, the location of orchids that occurred within 2m of each transect was recorded (30-50 genets between the two transects) so that they could be assessed later in the season for fruit set. To ensure that fruit set could be accurately recorded, orchid patches that were too dense to distinguish individual genets (<10cm between genets) were avoided when recording orchid locations. If orchid density was so sparse that the minimum of 30 genets could not be obtained between the two transects, orchid locations greater than 2m from the transects were recorded. These genets were marked with a

4. Latitudinal patterns in *Cypripedium candidum* reproduction

galvanized nail attached to a copper tag so that they could be easily relocated using a metal detector later in the season.

Composition of the Biotic Community and Weather Data

Methods for surveying the community composition of both the plant and insect community are described in detail in chapters 2 and 3 respectively, but I present a summary of methods here. Along the previously mentioned orchid transects, I recorded the number of stems of every co-flowering forb genus, up to a maximum of 50 stems for each genus. For flowers with multiple floral units (either flowers, or groupings of small flowers), I counted the number of units on ten flowers from each of these genera at a site. By multiplying the number of stems by the average number of floral units, I was able to obtain an estimate of floral density/display size for each co-flowering genus at a site.

The composition of the insect community was surveyed using pan trap surveys at 15 of my 21 study sites. Pan traps were laid along a 90m transect for 8 hours at every study site. At the end of the day, I collected all insects and preserved them in 70% ethyl alcohol until the Hymenoptera and pollinating Diptera could be identified in the laboratory. I also conducted 50-minute pollination surveys along my orchid transects at every study site. The number of visits of each observed insect genus to each co-flowering species was recorded. I used these pollination rates to determine which co-flowering species to include in models of fruiting success as potential influencers of *C. candidum* pollination.

Climate data were obtained for U.S. locations from Weather Underground (www.wunderground.com/history) and Canadian locations from The Weather Network

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(www.theweathernetwork.com). These websites were chosen because they allowed for collection of historical data from a specific range of dates. I recorded data for the average temperature; the sum of growing degree days; and the sum of precipitation recorded between the beginning of orchid flowering in Iowa (May 15th, 2016) to the first day of my fruiting surveys (June 20, 2016). Precipitation data was also recorded from the beginning of November 2015 until the orchid fruiting season. I also collected site data on the area of a site by using the polygon function of Google Earth.

Fruiting Success

To determine fruiting success, we revisited sites in the same order as they were studied during the flowering season, starting with Iowa. These surveys were conducted from June 20-30, 2016. At each site, orchid genets were relocated along each transect using previously recorded location data. For each genet, the number of flowering stems that had successfully set fruit was documented along with the number of genets that produced at least one fruiting stems. Previous work by Pearn (2012) found that all fruit that were successfully set in this species had fully-formed seeds. I also recorded whether the stems had been subjected to herbivory. Herbivory rates were quite low in 2016, with rates in Iowa, southern Minnesota, northern Minnesota, and Manitoba showing herbivory of 0%, 3%, 1%, and 1% of flowering stems respectively.

4.2.2 Statistical Analyses

Initial analyses of *C. candidum* fruiting success consisted of Kruskal-Wallis tests of the difference in the proportion of flowers to set fruit at each study site between study regions, as

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well as the proportion of successfully fruiting genets at each site between study regions. This non-parametric test was chosen since the response variables (proportions of genets and flowers at a site to set fruit) were highly skewed with many zero values. I had two transects at every study site except for site MB3 which only had space for one. For these, and all subsequent analyses, I summed the number of fruit or fruiting genets between the two transects for each study site. Based on preliminary analyses, using an average of stems rather than a sum would not change my findings.

Following these initial tests, I moved on to more powerful multiple regression analyses to explore additional factors that could be associated with variation in fruiting success (Quinn and Keough 2002b, 2002c). Similar to my Kruskal-Wallis tests, my response variables were either the number of fruiting stems or the number of successfully fruiting genets at each study site. Ideally, these count data would be analyzed with binomial regression using the proportion of successfully fruiting stems/genets. However, initial data exploration revealed that my data were overdispersed. Overdispersion occurs when the variation observed in a non-linear model is higher than predicted based on that particular model, and thus results in incorrect calculations of standard errors and increases the likelihood of a Type 1 error (see Quinn and Keough 2002d). Therefore, a negative binomial regression was used, as this analysis accounts for overdispersion in count-based models. I used the R package “MASS” to conduct these regression analyses (Venables and Ripley 2002).

The wide range of factors that could affect fruiting necessitated multiple stages of analysis. Table 4.1 summarizes my general approach. All analyses of fruit number included the total number of *C. candidum* flowers displayed by focal genets as an explanatory variable (Table 4.1). This was done because sites with more flowers would have more fruit even if the

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probability of individual flowers producing fruit remained similar. By comparison, analyses of genets to set fruit did not require a genet count because a similar number (32-50) of *C. candidum* genets were examined at each site.

Table 4.1. AICc Methodology to determine the effects of different sets of explanatory variables (co-flowering taxa, insect taxa, environmental variables) on either the number of *Cypripedium candidum* fruit at a study site in 2016, or the number of fruiting *C. candidum* genets. See text in methods for a full description of methods.

1. Select Dependant Variable

- a) Number of Fruiting Stems
- b) Number of Fruiting Genets

2. Explore sets of Explanatory Variables¹

- a) Study Region (n=21)
- b) Flower Number (n=21; only in analyses of fruit number)
- c) Co-flowering Taxa² (n=21)
 - i. *Packera*
 - ii. *Pedicularis*
 - iii. *Sisyrinchium*
 - iv. *Zizia*
 - v. (*Agoseris*)
 - vi. (*Fragaria*)
 - vii. (*C. candidum* floral display)^{*3}
- d) Insect Size Classes (n=15)
 - i. Small potential pollinators
 - ii. Potential pollinators
 - iii. Too large*
- e) Environmental Variables (n=21)
 - i. Average Temperature (May 15-June 20, 2016)*
 - ii. Growing degree days (May 15-June 20, 2016)*
 - iii. Precipitation (May 15-June 20, 2016)
 - iv. (SiteArea)
 - v. (Number of Plant Genera)*

3. Combine sets of explanatory variables, based on most commonly selected variables in AICc analyses for each set (excluding variables in brackets above)

1. Asterisks indicate predictor variables collinear with Region (VIF>5); these were only included in analyses excluding region.

2. Models of co-flowering taxa were conducted twice. Once using stem counts for each genus, and once using my estimates of floral display (stem count x the average flower number at each site)

3. *C. candidum* floral display was only using in analyses of the number of fruiting genets, with floral display estimates of co-flowering species.

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Study region was included in all initial models to account for variation due to spatial location of study sites. Models that included region as a significant predictor, were subjected to Tukey post hoc tests in order to determine which regions differed in fruiting success. Significant regional effects could reflect variation in the biotic community or environment (Chapters 2 and 3), or additional unmeasured variables that were associated with region (e.g., soil conditions, thatch cover).

Initial models that included study region could not include all potential explanatory variables, due to their regional patterns. I measured three groups of explanatory variables that could explain variation in fruiting success in *C. candidum*: composition of the co-flowering community, composition of the insect community, and weather variables. Within these groups, a few variables also showed changes between study regions. If these trends aligned with regional changes in fruiting success, the coefficients of the variables in my model would be incorrect, a phenomenon known as multicollinearity (Quinn and Keough 2002b). To determine if this was occurring in my models, I calculated variance-inflation factors (a common procedure to detect multicollinearity) prior to analyses and used these to select which variables could be included with study region in initial models (see Table 4.1 for which variables showed collinearity with study region). After initial analyses with study region included, I conducted analyses without study region and included these collinear variables to determine if the most parsimonious set of predictive variables changed. Variables that had an effect after the exclusion of study region may represent regional changes in predictor variables that affected *C. candidum* fruiting success (Quinn and Keough 2002b).

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For co-flowering genera, there were many recorded taxa that may have affected both the number of fruit at a site as well as the number of genets that successfully set fruit. With only 21 study sites, it would not be appropriate to include all co-flowering taxa in my list of candidate variables. To narrow down my list of potential variables, I sorted all co-flowering species by the number of insect visits recorded to each across my entire study. I picked the six most visited co-flowering genera to examine in my analyses. These were *Agoseris*, *Fragaria*, *Packera*, *Pedicularis*, *Sisyrinchium*, and *Zizia* (Chapter 3), none of which were collinear with region.

At the conclusion of these analyses, I repeated analyses with co-flowering species as predictors, but used my estimate of floral display (the average number of flowers of each co-flowering genus along transects at a site), rather than the average number of flowering stems. In these models, when the response variable was the number of successfully fruiting *C. candidum* genets, I also included a predictor variable of the estimate of *C. candidum* floral display between the two transects at a site. This variable was found to be collinear with study region, so I did not include it in initial analyses, to avoid the effects of collinearity.

I also wanted to examine the effect of environmental variables at study site on *C. candidum* fruiting success. I used a candidate list of the following list of environmental variables: study region, the number of co-flowering genera, the number of growing degree days during the flowering season, the average temperature during the flowering season, the total precipitation during the flowering season, and the area of each site. However, study region, growing degree days, and average temperature were all collinear with each other and could not be included in a model together. Therefore, I conducted all analyses with study region first, and then I replaced this variable with either average temperature or growing degree days for

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subsequent analyses. This was done for both analyses of the number of *C. candidum* fruit at a site, and the number of fruiting genets.

After examining the effects of the co-flowering community and environmental variables, I examined the potential effects of the three insect size classes as described in Chapter 3. The abundance of insects in the “too large” size class was collinear with study region, so this variable was not included in initial analyses, but replaced study region in subsequent analyses.

All models examined in this study were then subjected to a corrected Akaike’s Information Criteria (AIC) process to find the model that explained the highest amount of variation in fruiting success with the fewest number of variables. Corrected AIC (AICc) values were used given that the number of variables ($k=3-8$) was large in relation to the sample size ($n=15$ or 21 ; $n/k < 40$; Hurvich and Tsai 1989). This technique revealed the most parsimonious models of fruiting success. AICc tables were constructed by determining a list of candidate models, made up of every combination of predictor variables (study region and the number of *C. candidum* flowers were included in all models of the number of fruit at a site, and study region was included in all models of the number of fruiting genets), using the R package “MuMIn” (Barton 2017). Each model had its AICc value calculated, and models were arranged from smallest to highest values. Differences between the AICc values of all models and the smallest AICc value were then calculated. A difference in AICc greater than two indicated that the model with the smaller value of AICc explained more variation with a smaller set of predictor variables than a model with the larger AICc value, and thus was more parsimonious (Burnham and Anderson 2002). Models with a difference in AICc less than two are considered equally informative for modelling the response variable (Burnham and Anderson 2002). Following model generation, I also calculated approximate R^2 values for each candidate model by dividing

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the amount of variance explained by a particular model by the null variance (the amount of variance present in the response variable alone; Quinn and Keough 2002b). While these values can give an approximation of the amount of information a model explains, they can be inflated by additional variables in a model, hence my use of AIC methods (Quinn and Keough 2002a).

The procedures described above identified significant variables within each category of predictors (co-flowering, environmental, insect). As a final AICc procedure, I wanted to examine the effects of all of these potential explanatory variables in the same model to determine if combinations of these variables explained more variation in fruiting success than a single data set alone (Table 4.1). I combined all significant explanatory variables from initial AICc methods in new sets of candidate variables and once again conducted AICc model selection. This was done using data from all 21 study sites to examine the combined effects of co-flowering taxa and environmental variables. As I only conducted pan trap surveys at 15 of these sites, I conducted a separate AICc model selection using insect, co-flowering, and environmental data collected from these 15 sites.

4.3 Results

4.3.1 Fruiting Success in Latitudinal Study Regions

Average fruiting success for *C. candidum* genets and ramets in each study region was highest in Iowa and lowest in northern Minnesota and Manitoba (Fig. 4.1). The average fruiting success in Iowa was $55 \pm 16\%$ of genets successfully setting fruit, and $32 \pm 19\%$ of flowers. Southern and northern Minnesota showed fruiting success in $31 \pm 12\%$ and $17 \pm 5\%$ of genets, respectively, and $20 \pm 10\%$ and $11 \pm 3\%$ of flowers. Fruiting success in Manitoba was $18 \pm 4\%$ of

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genets, and $9 \pm 4\%$ of flowers. Kruskal-Wallis tests did not find that these differences were significant (genet fruiting success: $\chi^2_3=5.1$, $p>0.1$; ramet fruiting success: $\chi^2_3=2.5$, $p>0.4$).

However, these tests do not take into account variation in the biotic community or environmental variables within study regions, hence my use of regression analyses (see below).

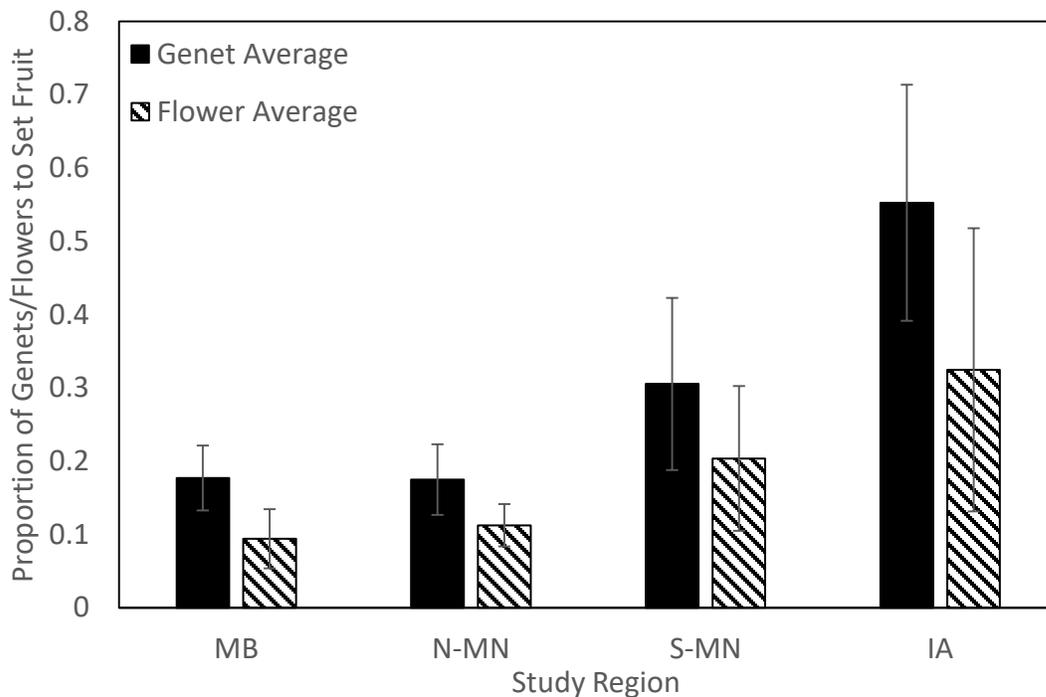


Figure 4.1. The average proportion of *Cypripedium candidum* genets (black bars) or flowers (hatched bars) to set fruit at each tall grass prairie population of *C. candidum* in 2016. Error bars represent standard error for each study region (IA=Iowa, S-MN=southern Minnesota, N-MN=northern Minnesota, and MB=Manitoba; Fig 2.1).

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4.3.2 Factors Affecting Orchid Fruiting Success

Significant Explanatory Variables

Following all AICc procedures outlined above, there were certain variables from the candidate data sets of co-flowering genera and environmental variables that frequently showed up in my best models and had a significant relationship with the number of fruit observed at a study site or the number of successfully fruiting genets (Table 4.2). Study region always had a significant effect on fruiting success, with the number of fruit at a site higher in Iowa compared to all other study regions (Tukey *post-hoc* tests: all $z < -3.1$, all $p < 0.01$). While the effect of study region was still significant when I examined the number of fruiting genets at a site, the difference between Iowa and southern Minnesota sites was no longer significant in models, nor in Tukey *post hoc* tests ($z = -1.9$, $p > 0.2$). Differences between Iowa and Manitoba and Iowa and northern Minnesota sites were still significant in negative binomial models of genet fruiting success ($p < 0.05$); but not in Tukey *post-hoc* tests ($p > 0.05$ and $p > 0.06$ respectively). As Tukey *post hoc* tests use an adjusted *p*-value for calculating contrasts in generalized linear models, this likely produced the non-significant results.

When conducting model selection with the exclusion of study region, using the number of fruit as my response variable, the number of *C. candidum* flowering stems recorded at a site showed a positive effect on fruiting success. This result justifies my inclusion of this variable in all models of fruit number, to control for variation in *C. candidum* flower density.

Co-flowering taxa that had a significant effect on fruiting success were *Packera*, *Pedicularis*, *Sisyrinchium*, and *Zizia* (Table 4.2). *Packera* always had a significant and positive effect, while *Pedicularis* had a negative effect in some models, but was non-significant in others. *Sisyrinchium* only showed a significant and negative effect when region was not included in

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models. *Zizia* did not show a significant effect when I examined co-flowering stem counts along my transects, but had a significant and positive effect when I examined the effects of my estimates of floral display for co-flowering taxa. Finally, I also found a significant and negative effect of the interaction between *Zizia* and *Sisyrinchium* floral display estimates (Table 4.2).

Of my environmental variables, both average temperature and the sum of growing degree days during the orchid flowering period (May 15th-June 20th, 2016) showed significant and positive effects on *C. candidum* reproduction (Table 4.2). These two variables were both collinear with region and each other so all AICc analyses were conducted with only one of these three variables. The only other environmental variables that had a detectable effect was precipitation during the orchid flowering period, which had a significant and negative effect when region was included in the model (Table 4.2).

When examining the number of insects belonging to the three size classes described in chapter 3 (small potential pollinators, potential pollinators, and too large insects) captured at my fifteen insect study sites on *C. candidum* fruiting success, I did not detect any significant effects.

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Table 4.2. A summary of all the significant predictor variables resulting from corrected Akaike's information criterion (AICc) model selection procedures comparing negative binomial regression models examining the relationship between *Cypripedium candidum* fruiting success at each tall grass prairie population and each set of candidate variables. This summary lists includes significant variables from both analyses of total fruit number, as well as the number of fruiting genets. See text above for a full description of negative binomial regression and AICc methods.

Significant Variable	Effect on Fruit Set
Study Region ¹	IA<S-MN=N-MN=MB
Flower Number ²	positive
Co-flowering Genera:	
<i>Packera</i>	positive
<i>Pedicularis</i>	negative
<i>Sisyrinchium</i>	negative
<i>Zizia</i> ³	positive
<i>Zizia</i> * <i>Sisyrinchium</i>	negative
Environmental Variables	
Average Temperature	positive
Growing Degree Days	positive
Precipitation ⁴	negative
1. Study regions: Iowa (IA), southern Minnesota (S-MN), northern Minnesota (N-MN), Manitoba (MB)	
2. Flower number only showed a significant effect in models without study region.	
3. <i>Zizia</i> only showed a significant effect when I used my estimate of its floral display, rather than stem count.	
4. Precipitation only showed a significant effect in models including study region.	

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Combined AICc Model Selection

After obtaining a list of variables that showed a significant effect on reproduction, I combined these variables into one list of potential variables that I used in a combined AICc model selection procedure. When I examined the effects of my combined data set of co-flowering and environmental variables on the number of *C. candidum* fruit at a site, I found consistent results in all models including region. The best model in these analyses included study region, *C. candidum* flower number, and *Packera* regardless of whether I used co-flowering stem counts or floral display (Table 4.3, procedures 1 and 3). When region was not included, the model with the lowest AICc included *C. candidum* flower number and *Sisyrinchium* when I used co-flowering stem count data (Table 4.3, procedure 2), or *C. candidum* flower number and *Zizia* and *Pedicularis* when I used co-flowering floral display estimates (Table 4.3, procedure 4). AICc model selection did not include average temperature or precipitation in any of the best models, but all previously identified significant co-flowering taxa appeared in my best models (with a difference of AICc less than two compared to the lowest AICc model). The model that explained the most variation in fruit number at a site included the variables of study region, *C. candidum* flower number, and *Packera* stem counts. When I did not include study region in my list of candidate variables, the model including *C. candidum* flower number, *Sisyrinchium* floral display, *Zizia* floral display, and the interaction between the two co-flowering taxa explained the most variation in fruit number.

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Table 4.3. AICc selection table for negative binomial models analyzing *Cypripedium candidum* fruit number at each tall grass prairie population (n=21) in 2016.

1) Initial models included the variables of *C. candidum* flower number and study region, as well as every combination of the candidate variables of the average number of *Packera*, *Pedicularis*, *Sisyrinchium* and *Zizia* stems as well as precipitation (Precip) during the flowering season.

2) Following initial models, study region was dropped from the list of candidate models to examine the effects of average temperature during the co-flowering season (AvgT), which was collinear with region.

3 and 4) These methods were then repeated, using my estimate of floral display for each co-flowering genus.

Models displayed include all models with a Δ AICc less than two, and the first model with a Δ AICc value over two. Significance of all co-flowering taxa and environmental variables is indicated by bold text (see Table 4.2 for direction of their effects).

Model Variables	AICc	Δ AICc	R ²
1) Models including study region and co-flowering stem counts and environmental variables			
Flower#, Region, <i>Packera</i>	164.3	0	0.62
Flower#, Region	166.8	2.47	0.47
2) Models excluding regions, and all co-flowering stem counts and environmental variables			
Flower#, <i>Sisyrinchium</i>	164.22	0	0.33
Flower#, AvgT	165.65	1.43	0.29
3) Models including study region and co-flowering floral display estimates and environmental variables			
Flower#, Region, <i>Packera</i>	166	0	0.59
Flower#, Region	166.8	0.8	0.47
4) Models excluding region, and all co-flowering floral display estimates and environmental variables			
Flower#, <i>Pedicularis</i> , <i>Zizia</i>	163.45	0	0.45
Flower#, <i>Sisyrinchium</i> , Zizia , Zizia * <i>Sisyrinchium</i>	163.61	0.16	0.54
Flower#, <i>Sisyrinchium</i>	164.23	0.77	0.33
Flower#, <i>Packera</i> , Pedicularis , Zizia	165.14	1.68	0.51
Flower#, Zizia	165.63	2.18	0.27

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When my response variable was the number of successfully fruiting *C. candidum* genets, study region and *Packera* were again the best combination of explanatory variables for both stem count data and floral display estimates (Table 4.4, procedures 1 and 3). When region was excluded from these analyses, the best models included *Packera* and *Pedicularis* for both stem counts and floral display estimates (Table 4.4, 2 and 4); however, *Pedicularis* only had a significant effect when it was included as a floral display estimate. The environmental variable of average temperature during the flowering season appeared in a few of my best models in this procedure, but only showed a significant and positive effect when *Packera* or *Zizia* were not in the candidate model. Once again, precipitation did not appear in any of these best models. The model that explained the most variation in the number of *C. candidum* fruiting genets included study region and *Packera* as the only variables. When region was excluded from models, the two models that explained the most variation included the candidate variables of: *Sisyrinchium* floral display, *Zizia* floral display, and their interaction; and *Packera* floral display, *Pedicularis* floral display and *Zizia* floral display. Both of these models explained approximately equal amounts of the variation in fruiting success.

I did conduct a second combined AICc procedure using the 15 study sites where I measured co-flowering, insect, and environmental variables, but the addition of insect variables did not change my best AICc models, and the sample size reduced my power of detection greatly. Therefore, I have only shown the previous combined procedure in this chapter, but present my combined AICc procedure with all set of predictor variables in Appendix 4.6 and 4.7. In one model, “too large” insects had a significant and negative effect on *C. candidum* reproduction, but this model had a difference in AICc of greater than two compared to the best model, so was not the most parsimonious (Appendix 4.6).

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Table 4.4. AICc selection table for negative binomial models analyzing the **number of successfully fruiting *Cypripedium candidum* genets** at each tall grass prairie population (n=21) in 2016.

1) Initial models included the variable of study region, as well as every combination of the candidate variables of the average number of *Packera*, *Pedicularis*, *Sisyrinchium* and *Zizia* stems as well as precipitation (Precip) during the flowering season.

2) Following initial models, study region was dropped from the list of candidate models to examine the effects of average temperature during the co-flowering season (AvgT), which was collinear with region.

3 and 4) These methods were then repeated, using my estimate of floral display for each co-flowering genus.

Models displayed include all models with a Δ AICc less than two, and the first model with a Δ AICc value over two. Significance of all co-flowering taxa and environmental variables is indicated by bold text (see Table 4.2 for direction of their effects).

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Model Variables	AICc	Δ AICc	R ²
1) Models including study region and co-flowering stem counts and environmental variables			
Region, <i>Packera</i>	149.11	0	0.48
Region	150.6	1.5	0.33
Region, <i>Packera</i>, <i>Pedicularis</i>	151.25	2.14	0.55
2) Models excluding regions, and all co-flowering stem counts and environmental variables			
<i>Packera</i> , <i>Pedicularis</i>	145.97	0	0.36
<i>Packera</i>	146.14	0.17	0.27
<i>Packera</i> , AvgT	146.23	0.26	0.35
<i>Pedicularis</i> , AvgT	146.46	0.49	0.35
AvgT	146.53	0.56	0.24
<i>Packera</i> , <i>Pedicularis</i> , AvgT	146.86	0.89	0.44
<i>Packera</i> , <i>Sisyrinchium</i>	147.06	1.08	0.33
<i>Packera</i> , <i>Pedicularis</i> , <i>Sisyrinchium</i>	147.98	2.01	0.41
3) Models including study region and co-flowering floral display estimates and environmental variables			
Region, <i>Packera</i>	150.5	0	0.45
Region	150.6	0.1	0.33
Region, <i>Pedicularis</i>	151.75	1.25	0.41
4) Models excluding region, and all co-flowering floral display estimates and environmental variables			
<i>Packera</i> , <i>Pedicularis</i>	146.42	0	0.35
<i>Pedicularis</i> , AvgT	146.46	0.03	0.35
AvgT	146.53	0.11	0.24
<i>Sisyrinchium</i> , <i>Zizia</i> , <i>Sisyrinchium</i> * <i>Zizia</i>	146.57	0.15	0.44
<i>Packera</i> , <i>Pedicularis</i> , <i>Zizia</i>	146.59	0.16	0.44
<i>Pedicularis</i> , <i>Zizia</i>	147.02	0.6	0.33
<i>Packera</i> , AvgT	147.12	0.7	0.33
<i>Packera</i>	147.29	0.86	0.22
<i>Packera</i> , <i>Pedicularis</i> , AvgT	147.34	0.91	0.42
<i>Pedicularis</i> , <i>Zizia</i> , AvgT	147.53	1.1	0.42
<i>Packera</i> , <i>Sisyrinchium</i>	148.04	1.62	0.32

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4.4 Discussion

4.4.1 Regional Patterns in *C. candidum* Reproduction

The observational nature of this study meant that I was able to document factors associated with variation in *C. candidum* reproduction, but I am not able to ascribe cause and effect. Latitudinal patterns in a rewardless species reproduction has not been investigated prior to this study, so a large number of factors that could vary across latitude remain to be explored. This study identified potential variables that may be important drivers of reproduction, but further research should investigate these variables with experimental study designs.

Across all negative binomial analyses, a consistent variable that significantly affected both the number of fruit at a site, as well as the number of fruiting genets, was study region. Tukey *post hoc* tests determined that the high fruiting success in Iowa sites differed significantly from sites in other regions. Overall, this lends support to my hypothesis that reproductive success would increase further south in *C. candidum*'s range. The range of fruiting success in Iowa ($55\pm 16\%$ of genets) is similar to that found in *C. candidum* populations in Illinois ($33.6\pm 10\%$, Shefferson and Simms 2007) and in some years in Ohio ($46\pm 6\%$, Walsh et al. 2014; $\sim 35\%$, Walsh and Michaels 2017), which are further east but at a similar latitude to sites in Iowa. Even the fruiting success of genets in southern Minnesota ($32\pm 19\%$) is comparable to these studies. In contrast, the average fruiting success of *C. candidum* genets observed in Manitoba by Pearn (2012) over three field seasons was $15\pm 3\%$ of *C. candidum* genets, similar to the $18\pm 4\%$ of genets successfully setting fruit in Manitoba in this study. This study, along with others conducted in different locations in *C. candidum*'s range, seem to confirm that lower latitudes benefit reproduction in this rewardless species, possibly due to earlier flowering time relative to other spring-flowering species. These findings indicate that the optimal factors for fruiting

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success routinely occur in the southern part of *C. candidum*'s range. In contrast, these same set of conditions may rarely, if ever, occur in the northern part of this species range. Environmental and biological factors, which showed an effect when combined with study region, or when analyzed alone, are discussed in more detail below.

4.4.2 *The influence of the co-flowering community on C. candidum reproduction*

One factor that showed an effect when combined with study region was the abundance of *Packera*, which showed a significant positive effect on fruiting success in all analyses. While encountered in all study regions, this taxon was more abundant in Iowa and southern Minnesota (see Chapter 2) compared to other co-flowering genera. In study sites further north, the proportion of co-flowering stems that belonged to this genus decreased as other taxa became more abundant.

There were a few factors in negative binomial models that showed an effect when study region was not included. Factors in this category may reflect the environmental and biological changes represented by a change in study region, creating these fruiting trends. One of these factors, which had a positive effect on *C. candidum* fruiting success, was *Zizia* floral display. *Zizia* was the most abundant co-flowering genus across my study, and did not vary among study region, as evidenced by its low weighting in the ordinations (PCAs and RDAs) presented in Chapter 2. In contrast, when I multiplied the number of stems by the average number of flowers to obtain an estimate of floral abundance, I found a higher abundance of *Zizia* flowers in Iowa. The statistical difference in *Zizia* flowers was not significant (ANOVA: $F=2.699$, $p>0.75$) but may have contributed to the significant effect on fruit number in my analyses including floral abundance.

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Other studies on pollination of rewardless flowers have documented facilitation by co-occurring “magnet” rewarding species, that serve to attract pollinators to an area (Lavery 1992, Johnson et al. 2003, Molina-Montenegro et al. 2008, Catling 2015). Both *Zizia* and *Packera* were highly visited during pollinator surveys, suggesting they may fill this role. It could be that as latitude increases, the proportion of facilitative co-flowering species decreases, while the proportion of competitive species increases (see below), which may contribute to the significant effect of study region on *C. candidum* fruiting success.

Sisyrinchium and *Pedicularis* were also included in models that excluded region, and had a significant and negative effect on fruiting. As discussed in Chapter 2, both of these genera were most abundant in northern study regions. The negative effects of the abundance of these taxa on fruiting success lends support to my hypothesis that competition among *C. candidum* and other co-flowering taxa may increase in northern regions, due to more synchronous phenology of species (Elzinga et al. 2007). This should increase competitive effects on reproduction in a natural system, particularly in rewardless taxa (Lammi and Kuitunen 1995, Internicola et al. 2006, 2007, 2008, Pellissier et al. 2010). The increase in facilitative taxa abundance further south was surprising as earlier phenology should reduce co-flowering abundance as a whole (Internicola et al. 2008, Pellissier et al. 2010, Internicola and Harder 2012). Perhaps *C. candidum* experiences selection to match the phenology of *Zizia* and *Packera*, or to flower when the proportion of facilitative to competitive co-flowering abundance is maximized. Since latitude limits selection on early flowering times (Munguía-Rosas et al. 2011), this may force *C. candidum* to flower with more competitive taxa further north.

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4.4.3 *The influence of environmental variables on C. candidum reproduction*

Environmental variables also showed significant effects on the fruiting success of *C. candidum*. Both the sum of growing degree days and the average temperature during the flowering season were collinear with study region, and had significant effects on *C. candidum* fruit set when study region was excluded. Warmer sites in the southern part of *C. candidum*'s range had higher reproductive success than those further north. Warmer temperature should increase pollinator activity (McCall and Primack 1992), which could increase pollination rates, but this was not directly examined in my study.

The only additional environmental variable that had a significant effect on *C. candidum* fruit set was precipitation during the flowering season, which had a significant and negative effect on fruiting success when study region was included in models. A long period of precipitation may have limited insect foraging and thus lead to lower pollination rates in some areas (McCall and Primack 1992). All sites with less than 50 mm of rainfall had over 20% of *C. candidum* genets set fruit, while areas with higher amounts of rainfall showed lower average fruiting success. Precipitation did not show up in models without study region, likely due to the fact that the more direct measures of temperature (average temperature and growing degree days) also reflect the variation in precipitation, with warmer regions experiencing less cooling due to increased precipitation.

4.4.4 *Other factors affecting C. candidum reproduction*

Despite regional changes in the abundance of certain insect taxa, insects did not show a significant effect on *C. candidum* fruit set in negative binomial models. However, pollinator activity observed in Chapter 3 does provide potential explanations behind the effects of

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facilitative and competitive co-flowering taxa. Both *Packera* and *Zizia* had the highest proportion of visits from *Toxomerus* and *Dialictus*. While *Toxomerus* may be too weak to effectively pollinate *C. candidum*, *Dialictus* is of appropriate size (see discussion in Chapter 3). These findings indicate that *Packera* and *Zizia* may attract a higher proportion of potential pollinators to a population. The competitive effects of *Pedicularis* and *Sisyrinchium* are more difficult to interpret. *Sisyrinchium* was mostly visited by *Toxomerus*, which could indicate that this species attracts ineffectual pollinators to a population. On the other hand, *Pedicularis* was almost entirely visited by *Bombus*, which may dissuade smaller bee from foraging in an area, decreasing the abundance of appropriately sized pollinators. This bee behaviour has been observed when foraging insects avoid areas the honey bee, *Apis mellifera*, forages, and other studies have shown smaller bees displaced by *Apis* in some cases (Roubik 1978, 1980).

Measurements of orchids in Chapter 3 showed that flowers in Iowa were smaller than the other study regions. In theory, this could limit reproductive success by preventing certain insects from fitting through *C. candidum*'s pollination route (Li et al. 2008, Case and Bradford 2009, Pearn 2012, Anderson 2015), but this region showed the highest fruiting success. Two findings from this study may indicate compensation for smaller orchid size in Iowa: the insect community composition and the number of flowers per orchid genet. As discussed in Chapter 3, Iowa had a significantly lower abundance of “too large” pollinators, compared to Manitoba and the highest proportion of “small potential pollinators” and “potential pollinators” (though this did not vary significantly from northern or southern Minnesota), which, in theory, should have shown an effect of the insect community composition on reproduction. The lack of detected effects of insect taxa on *C. candidum* fruiting success could be the result of small sample size (only 15 divided amongst 4 study regions), which may have limited my ability to detect effects. A more

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focused study on the insect community and *C. candidum* reproductive success may be able to detect results with a larger sample size. Other studies have shown that reproduction in *Cypripedium* is limited by insect size and the size of the orchid's exit route (Pearn et al. in prep, Li et al. 2008, Pearn 2012, Anderson 2015), so the make-up of the insect community may have an effect on pollination rates that could not be detected in this study.

While orchids in Iowa were significantly smaller than other regions, they also had a significantly higher number of flowers per individual. Therefore, this would increase the likelihood of at least one flower being successfully pollinated, and may increase the overall fruiting success of the orchid genet. A higher number of flowers per orchid has been shown to increase the chances of fruiting success for both *C. candidum* (Pearn 2012), *C. parviflorum* (Pearn 2012, Anderson 2015), and their hybrids (Pearn 2012). An increase in clonal propagation has been shown to increase the probability of survival in these taxa as well (Shefferson 2006), and has been shown to maintain genetic variation in the critical endangered *C. calceolus* (Brzosko et al. 2002). The effects of this clonal propagation and genet size were not directly examined in this study, as most data were collected at the site level, rather than for individual orchids.

As discussed above, both Iowa and southern Minnesota had sites with fruiting success greater than 50% of orchid genets. I would recommend further research at these sites, as their conditions may maximize orchid reproduction. While the potential competitive taxa outlined in this study were absent from these sites, their *Packera* and *Zizia* abundance was comparable with other sites that did not show such high fruiting success. Additional factors not measured in this study may be similar among these sites, and contribute to fruiting success, such as the amount of thatch cover. Many of the sites with high reproduction had anecdotally low thatch cover,

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indicating a higher rate of disturbance, but this was not a factor that I measured. Determining what factors may increase the reproductive success of this threatened species could greatly benefit conservation efforts (Environment Canada 2006).

Examining a wider ranging congener, such as *C. parviflorum*, may provide additional insights into the latitudinal trends observed in this study. More research should also be conducted on the density effects of potential facilitative and competitive co-flowering taxa, as well as the effects of orchid density, on fruit set. Rewardless flower density has been shown to increase initial pollination rates, but increases pollinator avoidance learning if co-flowering rewarding species density is low (Internicola et al. 2006, Sun et al. 2009, Sakata et al. 2014b). More transects per study site (>3) would allow for the use of mixed-effect models, and would be able to determine the extent of both local and regional floral density effects on rewardless species reproduction. Transect level measurements of environmental variables such as soil depth, moisture content, thatch cover, and nutrient availability would allow for examination of local environmental variables that may also affect reproduction at smaller scales.

This study was the first to document latitudinal changes in reproduction with a species of rewardless orchid. While significantly increased fruiting success was only seen in one study region, the region with the highest reproductive success was the most southern, which matches predictions in the literature (Internicola et al. 2008, Pellissier et al. 2010). Increased *C. candidum* reproduction was also associated with an increase in the abundance of facilitative co-flowering taxa, a decrease in the abundance of competitive taxa, and an increase in temperature during the growing season, all of which occurred in southern regions. These potential factors affecting *C. candidum* reproduction inspire future research into the mechanisms that drive their effects, and the scale at which they operate.

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4.6 Appendices

Appendix 4.1 AICc selection table for negative binomial models of *Cypripedium candidum* **fruit number** at each tall grass prairie population.

1) Initial models included the variables of *C. candidum* flower number and study region, as well as every combination of the candidate variables of the average number of *Agoseris*, *Fragaria*, *Packera*, *Pedicularis* and *Zizia* stems.

2) Following initial models, study region was dropped from the list of candidate models to determine the most parsimonious set of predictor variables without study region.

3 and 4) These methods were then repeated, using my estimate of floral display for each co-flowering genus.

Models displayed include all models with a Δ AICc less than two, and the first model with a Δ AICc value over two. Significance of all variables is indicated by bold text (see Table 4.2 for direction of their effects).

4. Latitudinal patterns in *Cypripedium candidum* reproduction

Model Variables	AICc	ΔAICc	R²
1) Models including study region and co-flowering stem counts			
Flower#, Region, <i>Packera</i>	164.3	0	0.62
Flower#, Region	166.8	2.47	0.47
2) Models including co-flowering stem counts only			
Flower#, <i>Sisyrinchium</i>	164.2	0	0.33
Flower#, <i>Sisyrinchium</i> , <i>Pedicularis</i>	166	1.78	0.38
Flower#, <i>Sisyrinchium</i> , <i>Packera</i>	166.5	2.29	0.37
3) Models including study region and co-flowering floral display estimates			
Flower#, Region, <i>Packera</i>	166	0	0.59
Flower#, Region	166.8	0.8	0.47
Flower#, Region, <i>Pedicularis</i>	168.4	2.39	0.54
4) Models including co-flowering floral display estimates only			
Flower#, <i>Pedicularis</i> , <i>Zizia</i>	163.5	0	0.54
Flower#, <i>Sisyrinchium</i> , <i>Zizia</i> , <i>Sisyrinchium</i> * <i>Zizia</i>	163.6	0.16	0.54
Flower#, <i>Sisyrinchium</i>	164.2	0.77	0.33
Flower#, <i>Packera</i> , <i>Pedicularis</i> , <i>Zizia</i>	165.1	1.68	0.51
Flower#, <i>Zizia</i>	165.6	2.18	0.27

4. Latitudinal patterns in *Cyripedium candidum* reproduction

Appendix 4.2 AICc selection table for negative binomial models of the **number of successfully fruiting *Cyripedium candidum* genets** at each tall grass prairie population.

1) Initial models included the variable of study region, as well as every combination of the candidate variables of the average number of *Agoseris*, *Fragaria*, *Packera*, *Pedicularis* and *Zizia* stems.

2) Following initial models, study region was dropped from the list of candidate models to examine the effects of co-flowering genus, *Lithospermum*, which was collinear with region.

3 and 4) These methods were then repeated, using my estimate of floral display for each co-flowering genus.

Models displayed include all models with a ΔAICc less than two, and the first model with a ΔAICc value over two. Significance of all variables is indicated by bold text (see Table 4.2 for direction of their effects).

4. Latitudinal patterns in *Cypripedium candidum* reproduction

Model Variables	AICc	ΔAICc	R²
1) Models including study region and co-flowering stem counts			
Region, <i>Packera</i>	149.1	0	0.48
Region	150.6	1.5	0.33
Region, <i>Packera</i>, <i>Pedicularis</i>	151.2	2.14	0.54
2) Models including co-flowering stem counts only			
<i>Packera</i> , <i>Pedicularis</i>	146	0	0.36
<i>Packera</i>	146.1	0.17	0.26
<i>Packera</i> , <i>Agoseris</i>	146.9	0.9	0.33
<i>Packera</i> , <i>Sisyrinchium</i>	147.1	1.08	0.33
<i>Packera</i> , <i>Pedicularis</i> , <i>Agoseris</i>	147.2	1.18	0.43
<i>Packera</i> , <i>Pedicularis</i> , <i>Sisyrinchium</i>	148	2.01	0.41
3) Models including study region and co-flowering floral display estimates			
Region, <i>Packera</i>	150.5	0	0.45
Region	150.6	0.1	0.33
Region, <i>Pedicularis</i>	151.7	1.25	0.41
Region, <i>Packera</i>, <i>Pedicularis</i>	152	1.48	0.52
Region, <i>Sisyrinchium</i>	153.1	2.61	0.37
4) Models including co-flowering floral display estimates only			
<i>Packera</i> , <i>Pedicularis</i>	146.4	0	0.35
<i>Packera</i> , <i>Pedicularis</i> , <i>Zizia</i>	146.6	0.16	0.44
<i>Pedicularis</i> , <i>Zizia</i>	147	0.6	0.33
<i>Packera</i>	147.3	0.86	0.22
<i>Packera</i> , <i>Pedicularis</i> , <i>Agoseris</i>	148	1.53	0.41
<i>Packera</i> , <i>Sisyrinchium</i>	148	1.62	0.3
<i>Sisyrinchium</i>	148.2	1.74	0.18
<i>Packera</i> , <i>Agoseris</i>	148.5	2.03	0.28

4. Latitudinal patterns in *Cypripedium candidum* reproduction

Appendix 4.3. AICc selection table for negative binomial models of *Cypripedium candidum* fruit number at each tall grass prairie population.

1) Initial models included the variables of *C. candidum* flower number and study region, as well as every combination of the candidate variables of the number of co-flowering genera at a site, precipitation over the course of the orchid flowering period (Precip), and site area.

2 and 3) Following initial models, study region was dropped from the list of candidate models to examine the effects of the average temperature, and growing degree day which were collinear with study region and each other.

Models displayed include all models with a Δ AICc less than two, and the first model with a Δ AICc value over two. Significance of all variables is indicated by bold text (see Table 4.2 for direction of their effects).

Model Variables	AICc	Δ AICc	R ²
1) Models including study region and non-collinear environmental variables			
Flower#, Region	166.8	0	0.47
Flower#, Region, Precip	168.5	1.65	0.54
Flower#, Region , SiteArea	171.2	4.42	0.47
2) Models including average temperature and non-collinear environmental variables			
Flower#, AvgT	165.6	0	0.29
Flower#	166.8	1.16	0.14
Flower#, NumGen	168.4	2.76	0.19
3) Models including growing degree days and non-collinear environmental variables			
Flower#, GDD	166.4	0	0.26
Flower#	166.8	0.42	0.14
Flower#, NumGen (n.s.)	168.4	2.02	0.19

4. Latitudinal patterns in *Cypripedium candidum* reproduction

Appendix 4.4. AICc selection table for negative binomial models of the **number of successfully fruiting *Cypripedium candidum* genets** at each tall grass prairie population.

1) Initial models included the variable of study region, as well as every combination of the candidate variables of the number of co-flowering genera at a site, precipitation over the course of the orchid flowering period (Precip), and site area.

2 and 3) Following initial models, study region was dropped from the list of candidate models to examine the effects of the average temperature, and growing degree days which were collinear with study region and each other.

Models displayed include all models with a ΔAICc less than two, and the first model with a ΔAICc value over two. Significance of all variables is indicated by bold text (see Table 4.2 for direction of their effects).

Model Variables	AICc	ΔAICc	R²
1) Models including study region and non-collinear environmental variables			
Region	150.6	0	0.33
Region , Precip	152.3	1.7	0.4
Region , SiteArea	154.3	3.68	0.34
2) Models including average temperature and non-collinear environmental variables			
AvgT	146.5	0	0.24
AvgT , NumGen	148.3	1.77	0.29
AvgT , SiteArea	149	2.44	0.27
3) Models including growing degree days and non-collinear environmental variables			
GDD	148	0	0.19
Null	149.9	1.93	NA
GDD , NumGen	149.9	1.94	0.23
GDD , SiteArea	150.2	2.24	0.22

4. Latitudinal patterns in *Cypripedium candidum* reproduction

Appendix 4.5. AICc selection table for negative binomial models of *Cypripedium candidum* **fruit number** at each tall grass prairie population (**1 and 2**) and of the number of **successfully fruiting *C. candidum* genets** at each population (**3 and 4**).

1 and 3) Initial models included the variables of *C. candidum* flower number and study region, as well as every combination of the candidate variables of the number of insects caught in pan traps from the size classes outlined in Chapter 3, small potential pollinators and potential pollinators.

2 and 4) Too large insects were substituted for study region in subsequent analyses due to collinearity.

Models displayed include all models with a ΔAICc less than two, and the first model with a ΔAICc value over two. Significance of all variables is indicated by bold text (see Table 4.2 for direction of their effects).

4. Latitudinal patterns in *Cypripedium candidum* reproduction

Model Variables	AICc	ΔAICc	R²
1) Fruit number models including study region and insect size class counts			
Flower #, Region	128.3	0	0.54
Flower #, Region , PP	134.8	6.49	0.57
2) Fruit number models including insect size class counts only			
Flower #	121.7	0	0.25
Flower #, TL	123.5	1.73	0.34
Flower #, SPP	125.2	3.47	0.27
3) Fruiting genet models including study region and insect size class counts			
Region	112.7	0	0.45
Region , PP	118.1	5.41	0.47
4) Fruiting genet models including insect size class counts only			
null	110.3	0	NA
TL	112.4	2.06	0.07

4. Latitudinal patterns in *Cypripedium candidum* reproduction

Appendix 4.6. AICc selection table for negative binomial models of *Cypripedium candidum* **fruit number** at each tall grass prairie population that was used for both co-flowering and insect surveys (n=15).

1) Initial models included the variables of *C. candidum* flower number and study region, as well as every combination of the candidate variables of the average number of *Packera*, *Pedicularis*, *Sisyrinchium* and *Zizia* stems, precipitation (Precip) during the flowering season, and the insect size classes: small potential pollinators (SPP) and potential pollinators (PP).

2) Following initial models, study region was dropped from the list of candidate models to examine the effects of average temperature during the co-flowering season (AvgT), and the insect size class “too large” (TL) which were collinear with region.

3 and 4) These methods were then repeated, using my estimate of floral display for each co-flowering genus.

Models displayed include all models with a Δ AICc less than two, and the first model with a Δ AICc value over two. Significance of all variables is indicated by bold text (see Table 4.2 for direction of their effects).

4. Latitudinal patterns in *Cypripedium candidum* reproduction

Model Variables	AICc	ΔAICc	R²
1) Models including study region and co-flowering stem counts, insect size classes, and environmental variables			
Flower#, Region	128.34	0	0.54
Flower#, Region, <i>Packera</i>	130.21	1.87	0.69
Flower#, Region, Precip	132.05	3.71	0.65
2) Models excluding regions, and all co-flowering stem counts, insect size classes, and environmental variables			
Flower#	121.72	0	0.25
Flower#, Precip	122.49	0.77	0.38
Flower#, AvgT	122.77	1.05	0.38
Flower# , <i>Packera</i>	122.98	1.26	0.36
Flower# , <i>Sisyrinchium</i>	123.34	1.62	0.35
Flower#, TL	123.45	1.73	0.34
Flower# , <i>Packera</i> , TL	123.93	2.21	0.5
3) Models including study region and co-flowering floral display estimates, insect size classes, and environmental variables			
Flower#, Region	128.34	0	0.54
Flower#, Region, <i>Packera</i>	130.88	2.53	0.67
4) Models excluding region, and all co-flowering floral display estimates, insect size classes, and environmental variables			
Flower#	121.72	0	0.25
Flower#, <i>Zizia</i>	122.41	0.69	0.38
Flower# , Precip	122.49	0.77	0.38
Flower#, AvgT	122.77	1.05	0.38
Flower# , <i>Packera</i>	123.27	1.54	0.36
Flower# , <i>Sisyrinchium</i>	123.34	1.62	0.35
Flower #, TL	123.45	1.73	0.34
Flower#, <i>Packera</i> , <i>Zizia</i>	124.06	2.34	0.49

4. Latitudinal patterns in *Cypripedium candidum* reproduction

Appendix 4.7. AICc selection table for negative binomial models of the **number of successfully fruiting *Cypripedium candidum* genets** at each tall grass prairie population that were used for both co-flowering and insect surveys (n=15).

1) Initial models included the variable of study region, as well as every combination of the candidate variables of the average number of *Packera*, *Pedicularis*, *Sisyrinchium* and *Zizia* stems, precipitation (Precip) during the flowering season, and the insect size classes: small potential pollinators (SPP) and potential pollinators (PP).

2) Following initial models, study region was dropped from the list of candidate models to examine the effects of average temperature during the co-flowering season (AvgT), and the insect size class “too large” (TL) which were collinear with region.

3 and 4) These methods were then repeated, using my estimate of floral display for each co-flowering genus.

Models displayed include all models with a ΔAICc less than two, and the first model with a ΔAICc value over two. Significance of all variables is indicated by bold text (see Table 4.2 for direction of their effects).

4. Latitudinal patterns in *Cypripedium candidum* reproduction

Model Variables	AICc	ΔAICc	R²
1) Models including study region and co-flowering stem counts, insect size classes, and environmental variables			
Region	112.69	0	0.45
Region, <i>Packera</i>	113.11	0.43	0.62
Region , Precip (n.s.)	115.47	2.79	0.55
2) Models excluding regions, and all co-flowering stem counts, insect size classes, and environmental variables			
<i>Packera</i>	107.97	0	0.3
AvgT	108.47	0.51	0.27
<i>Packera</i> , TL	108.94	0.97	0.42
<i>Packera</i> , AvgT	109.41	1.45	0.4
<i>Sisyrinchium</i>	110.08	2.12	0.19
3) Models including study region and co-flowering floral display estimates, insect size classes, and environmental variables			
Region	112.69	0	0.45
Region, <i>Packera</i>	113.81	1.11	0.6
Region , Precip	115.47	2.79	0.55
4) Models excluding region, and all co-flowering floral display estimates, insect size classes, and environmental variables			
<i>Packera</i>	108.28	0	0.28
AvgT	108.47	0.19	0.27
<i>Packera</i> , <i>Zizia</i>	109.08	0.8	0.41
<i>Packera</i> , TL	109.41	1.12	0.4
<i>Packera</i> , AvgT	109.68	1.4	0.39
<i>Sisyrinchium</i> , <i>Zizia</i> , <i>Zizia</i> * <i>Sisyrinchium</i>	109.92	1.64	0.55
<i>Sisyrinchium</i>	110.08	1.8	0.19
<i>Pedicularis</i>	110.19	1.91	0.19
<i>Zizia</i> , AvgT	110.19	1.91	0.37
null	110.32	2.04	n/a

5. CONCLUDING THOUGHTS AND FUTURE RESEARCH

This thesis was the first pollination study across the latitudinal range of a rewardless orchid, and as such has contributed to many gaps in the literature. Through my surveys of tall-grass prairie populations of *Cypripedium candidum*, I determined that patterns in the co-flowering and insect communities were consistent with the hypothesis that phenology is compressed further north, due to the short growing season. I was also able to detect patterns in diversity at a single point in the growing season (“instantaneous diversity”) that most studies conducted across a growing season do not detect, which may be particularly important in light of climate change. I documented latitudinal changes in bee size, which have not been previously considered in pollination studies across a species’ range. In addition, through negative binomial analyses, I was able to document that *C. candidum* fruiting success is highest in Iowa, at the furthest point south in its range. Increased fruiting success was also associated with higher average growing season temperatures. I also determined that certain co-flowering taxa have either facilitative or competitive effects on reproduction in this species. These findings are an important first step for testing hypotheses regarding rewardless pollination in a large-scale study on natural populations, and also provide opportunities for additional research on potential drivers of reproduction in *C. candidum* (discussed below), which could be useful for the development of management strategies for this threatened species.

As this study progressed from Iowa to Manitoba, the number of species that flowered in synchrony with *Cypripedium candidum* increased. While this finding could be considered to contradict other studies of diversity across latitude in North America (Francis and Currie 2003, Hawkins et al. 2003, Harrison et al. 2006), it is likely the result of examining only the instantaneous diversity early in the growing season. Phenology is predicted to be extended

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further south in this period, as there is opportunity for plants to flower more asynchronously with a longer growing season (Körner 2003, Elzinga et al. 2007). In the light of rapidly increasing climate change, this consideration of instantaneous diversity is particularly important. Sites further south in this study had higher average temperature during the growing season. If climate change results in higher average temperature further north, the instantaneous diversity in those regions could reflect southern regions in this study. Since southern regions had the highest reproductive success in this study (see below), this could lead to an increase in *C. candidum*'s reproduction further north, though this may be limited by other environmental factors such as precipitation. Reproduction in this species should be monitored carefully in light of climate change, as other studies have noted reproductive success decreasing in a region as the phenology of plants and pollinators is decoupled with increasing temperature (Thomson 2010, Petanidou et al. 2014, Robbirt et al. 2014, Willmer 2014).

The observed decrease in diversity further south in *C. candidum*'s range is also consistent with the hypothesis that rewardless species will flower before rewarding species when possible, perhaps to take advantage of naïve pollinators and avoid competition with rewarding species (Internicola et al. 2008, Pellissier et al. 2010, Juillet et al. 2011, Internicola and Harder 2012). Some uncertainty remains if the lower diversity further south is a result of phenology, as only five out of the nine taxa absent from southern regions have records there. To determine that phenology is indeed advanced relative to the co-flowering community in southern regions, a similar study should be conducted across *C. candidum*'s range. Parallel surveys could be conducted during the same week in all regions by separate teams of researchers, and repeated throughout *C. candidum*'s growing season to allow researchers to survey all populations

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simultaneously. This sort of project would allow for a more precise record of *C. candidum* life stage over the growing season, and would confirm that southern populations experienced a lower co-flowering diversity than northern populations at the same life stage.

An important limitation to early phenology is the effect of frost across a species range. The ability of species to flower early in the season may be limited by the frost period at high latitudes (Pellissier et al. 2010, Munguía-Rosas et al. 2011), as the risk of frost damage should increase in northern regions (Inouye 2000). However, the summer of 2016 did not support this hypothesis. This summer was a particularly warm spring, and snowmelt began early in the season. In this season, the only region where I detected frost damage to orchids was in Iowa, where early flowering *C. candidum* plants had been damaged prior to my arrival. All of the other study regions had been frost free for a while before orchid emergence, and thus I detected no frost damage here. Additional years of study of these populations may show different patterns of frost damage based on latitudinal location.

Despite the research and theory on latitudinal changes in reproduction, little attention has been given to changes in pollinator biology. The morphology of insects is critical to the successful pollination of many species (Malo and Baonza 2002, Kimball 2008, Bischoff et al. 2013). In slipper orchids, such as *C. candidum*, the floral architecture in their flowers restricts pollination by large insects (Li et al. 2008, Pearn 2012, Anderson 2015). Using the predictions of Bergmann's and Allen's rule (Bergmann 1847, Allen 1877), I formulated my hypothesis that as latitude increases, the size of insects should also increase. This hypothesis was supported by my findings, with a higher insect abundance further north due to additional taxa from larger-bodied genera, compared those encountered further south. While many studies attribute low

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reproduction at high latitudes or altitudes to a lower insect abundance (Totland 1993, Alonso 2005, Ramos-Jiliberto et al. 2010, Fischer et al. 2012), my findings indicate that the morphology of insects is also an important factor to be considered.

The increase in both the number of plant genera that flowered synchronously with *C. candidum* and the number of insect genera that were too large for pollination, along with hypotheses from the literature (Pellissier et al. 2010), contributed to my hypothesis that reproductive success in this orchid species would decrease with higher latitude. Negative binomial regression analyses supported this hypothesis, as study region had a significant effect on both the number of fruit at a site and the number of successfully fruiting *C. candidum* genets. Sites in Iowa had higher fruiting success than sites further north in *C. candidum*'s range, contributing to the significance of this effect. Though orchid reproductive success in southern Minnesota was not significantly higher than more northern regions, this region also had two sites with reproductive success greater than 50% of orchid plants, a success rate higher than what we have observed in Manitoba. Additional years of study at these sites with particularly high fruiting success would allow researchers to examine if these sites always have such high reproductive rates, or if 2016 was an unusual year. Nevertheless, given the incredible range of fruiting success in southern populations, it seems that the potential for fruiting success is much higher than in northern regions.

When included in models with region, the only variable that showed a significant effect on *C. candidum* reproductive success was the co-flowering genus *Packera*. This genus had a significant positive effect on both the number of fruit at a site as well as the number of

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successfully fruiting genets. This result suggest that *Packera* may be a facilitative co-flowering genus, increasing the reproductive success of *C. candidum* when they co-occur.

Due to the large number of variables collected in this study, and changes in them associated with study region, some variables could not be included in models with region due to multicollinearity. As such, after initial models were created with study region included, I conducted additional analyses without this variable to determine if other explanatory variables had an effect associated with region. Variables that had a significant effect after this procedure were the co-flowering genera *Zizia*, *Pedicularis*, and *Sisyrinchium* and the average temperature during the flowering season. Because these variables only showed an effect when region was not included, this may indicate collinearity with region that was not detected with variance inflation factors. Both *Pedicularis* and *Sisyrinchium* increased in abundance further north, while *Zizia* floral display increased further south. If the effect of these variables is consistent and not an artifact of collinearity with region, the findings of this study suggest that *C. candidum* experiences a decrease in facilitative taxa and increase in competitive taxa abundance as its range becomes more northerly. Additional research on the effects of these co-flowering taxa at more sites within a region, as well as manipulation of their density within a site could allow researchers to determine if their effect on *C. candidum* reproduction is independent of region, since smaller scale studies would not require as many spatial variables to meet the assumption of independence.

One avenue of research that I did not have time to pursue for this study was the effect of both orchid and co-flowering density on orchid reproduction. The density of co-flowering species can affect whether a certain taxon acts as a facilitator or competitor (Flanagan et al. 2010,

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Seifan et al. 2014). As all of my research was conducted at the site level, these small scales changes in density of co-flowering species could not be examined in detail. Additional surveys within a site at smaller spatial scales could identify co-flowering taxa that show varying effects on *C. candidum* reproduction at more local scales. I could not measure the effects of orchid density on reproduction in this study, because all transects were established in high density orchid patches. This allowed me to obtain an efficient and accurate estimate of fruiting success at a site, but prevented me from examining how variation in orchid density affect reproductive success in orchid patches. A smaller scale study at fewer study sites would also allow for investigation of this factor.

As the significant effect of average temperature suggests, there are likely other environmental variables that affect *C. candidum* reproduction. The focus of this study was to identify potential biological factors that affect reproduction, but further research should be conducted to examine abiotic factors. Variables such as site drainage, distance to woody vegetation, surrounding land usage, the extent of frost damage, site management practices such as fire or mowing, etc. could all be other potential factors that affect both the phenology and reproduction in *C. candidum*. The trends in reproduction identified in this study are fascinating and warrant further research to explore all of the explanations for latitudinal variation in reproductive success.

Beyond contributions to the rewardless pollination literature, this study also identified potential avenues to *C. candidum* conservation. This threatened species often has low reproductive output, and threats of introgression through hybridization with *C. parviflorum* are still a point of concern to this species' conservation (Environment Canada 2006, Worley et al.

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2009). Therefore, research into factors that increase or limit reproduction are important to develop potential management strategies to ensure the continuance of the remaining populations of this orchid. My research identifies some co-flowering genera that may facilitate or compete for pollination in *C. candidum*. Determining the most effective densities of these genera could lead to new strategies to increase or possibly maximize reproduction of *C. candidum* across its species range.

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