POLLINATION AND COMPARATIVE REPRODUCTIVE SUCCESS OF LADY'S SLIPPER ORCHIDS *CYPRIPEDIUM CANDIDUM*, *C. PARVIFLORUM*, AND THEIR HYBRIDS IN SOUTHERN MANITOBA

by

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ABSTRACT

I investigated how orchid biology, floral morphology, and diversity of surrounding floral and pollinator communities affected reproductive success and hybridization of *Cypripedium candidum* and *C. parviflorum*. Floral dimensions, including pollinator exit routes were smallest in *C. candidum*, largest in *C. parviflorum*, with hybrids intermediate and overlapping with both. This pattern was mirrored in the number of insect visitors, fruit set, and seed set. Exit route size seemed to restrict potential pollinators to a subset of visiting insects, which is consistent with reports from other rewardless orchids. Overlap among orchid taxa in morphology, pollinators, flowering phenology, and spatial distribution, may affect the frequency and direction of pollen transfer and hybridization. The composition and abundance of co-flowering rewarding plants seems to be important for maintaining pollinators in orchid populations. Comparisons with orchid fruit set indicated that individual co-flowering species may be facilitators or competitors for pollinator attention, affecting orchid reproductive success.

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DEDICATION

To Mom and Dad. For everything.

To David. For your unending love, support, and encouragement. You've changed my life.

I love you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

1. Introduction and General Background

1.1 Pollination biology of rewardless flowers

Sexual reproduction and the transfer of male gametes (in the form of pollen) in flowering plants is complex due to several characteristics of angiosperm biology. Firstly, being mostly hermaphroditic, flowering plants have the potential to both outcross and self fertilize; secondly, their modular growth and construction enables complex and varied packaging and deployment of male and female gametes; and finally, they are sessile and thus require pollen vectors for the transfer of pollen (Barrett 1998; Barrett and Harder 1996). The mechanisms through which pollen is transferred between flowers include wind (anemophily) and water (hydrophily), but most commonly animals (biotic pollination). Animal pollination usually involves a mutualism between animals and plants where the plant provides a flood reward for the pollinator, and in return receives pollination services.

Not all plant-animal relationships are mutualistic, however. Some plant species have a deceptive (or rewardless) reproductive strategy. They produce no floral rewards, but because they still require the services of pollinators, their flowers falsely advertise the presence of these rewards (Ackerman 1986; Dafni 1984; Van der Pijl and Dodson 1966). Rewardless flowers are most common in the Orchidaceae, occurring in approximately one third of the 30,000 - 35,000 known species (Cozzolino and Widmer 2005a; Jersáková et al. 2006; Pellegrino et al. 2008; Schiestl 2005; Tremblay 1992; van der Pijl and Dodson 1966). By contrast, in other angiosperm groups, rewardless flowers occur in only a few species per family (Cozzolino and Widmer 2005a).

In the following sections, I discuss biotic pollination with a specific emphasis on rewardless flowers, particularly in Orchidaceae and *Cypripideum*. Section 1.1.1 gives an overview of the general features of biotic pollination, followed by an examination of the evolution of rewardlessness in section 1.1.2. Here I summarize some of the consequences, benefits and costs, and mechanisms that have promoted this syndrome in orchids, as well as the major types of deception. Section 1.1.3 discusses pollinator learning and the role of co-flowering species. Section 1.2 provides an overview of hybridization by outlining some of the outcomes of the process and its implications for deceptive plants. Section 1.3 gives a brief outline of my study system by describing both my study species (*Cypripedium parviflorum* and *C. candidum*) and study sites. Finally, section 1.4 outlines my project goals, objectives, and hypotheses.

1.1.1 General features of animal pollination

For animal pollinated plant species, there are several requirements that must be met for successful pollination. Firstly, plants must attract a pollen vector, which is usually accomplished via traits that advertise floral rewards, such as flower colour or scent (Proctor et al. 1996). Other characters such as flower shape, pattern (e.g. nectar guides), size, or floral display also play a role in attraction (Faegri and van der Pijl 1979). Secondly, the position of the sex organs (anthers and stigma) is critical for effective pollen dispersal (Barrett and Harder 1996; Faegri and van der Pijl 1979; Geber and Moeller 2006). Finally, floral rewards offer an incentive for pollinators to make return visits. These rewards may be essential to the survival of the pollinators, and include pollen (nutritionally rich), nectar, and special oils (Proctor et al. 1996).

Successful pollination depends not only on the attraction, but also on the effectiveness of pollinators when they visit flowers. In any biotic pollination system, only a subset of floral visitors acts as effective pollen vectors. The term "floral visitors" includes any insect that rests or alights on a flower, and visitors may or may not be actively foraging for rewards or searching for a nesting or hiding place (Nilsson 1979; Stoutamire 1967). Effective pollination on the other hand, is carried out by those insects able to successfully remove pollen from the male sex organs of one flower and transfer it to female sex organs of another flower (Faegri and van der Pijl 1979).

Species that bloom at the same time in a given community (hereafter co-flowering species) may indirectly affect one another by acting as facilitators or competitors for pollinator attention. Facilitation increases fitness of one or more species, and occurs when one species in a community attracts pollinators that then visit other less attractive species, or when the overall composition of a mixed community is more attractive to pollinators than when species are growing a single species population (Laverty 1992; Moeller 2004). By contrast, competition reduces pollination success (fitness) of one or more species in a mixed community relative to its fitness in isolation (Campbell 1985a, 1985b; Bell et al. 2005; Johnson et al. 2003; Juillet et al. 2007; Pelligrino et al. 2008). When pollinators visit many species, pollinator sharing may result in the receipt of pollen from another species or export of pollen to an incompatible taxon, both of which interfere with reproductive success (Bell et al. 2005; Johnson et al. 2003; Waser 1978). Pollinator sharing may also lead to hybridization and breakdown of species boundaries (Cozzolino and Widmer 2005b).

The degree of specialization in all plant-pollinator relationships is an important aspect of their evolutionary biology. These terms may apply to either member of the association; a generalist or specialist plant can have pollinators that are generalists or specialists, and there are well-documented examples of highly generalized and specialized interactions (Kay and Schemske 2004). Attempting to define these terms can be problematic however. For example, Waser et al. (1996) described generalization as "the use of several plant species by a pollinator and of several pollinator species by a plant," but what is meant by several? Additionally, at what point does generalization become specialization? It has been suggested that these terms be used in a relative sense (Olesen and Jordano 2002), and as a broad spectrum (from one to hundreds of pollinator species) rather than a dichotomy (Johnson and Steiner 2000; Waser et al. 1996). For example, many species in the Orchidaceae may be more specialized than those in the Ranunculaceae (Waser et al. 1996), but less specialized than other orchid species, such as those that mimic specific rewards (Li et al. 2008a).

The degree to which generalization or specialization occurs in pollination has implications for survival and reproductive success of plants and pollinators alike (Johnson and Steiner 2000). For example, generalized plants may be at a lower risk for, and more "resilient" to, loss of pollinator species than plants that are specialized to one pollinator species (Johnson and Steiner 2000). Additionally, specialized plants such as the Orchidaceae may rely on fewer, more effective pollinators, and can face substantial loss of reproductive effort when pollen is removed by inefficient pollinators (Tremblay 1992). Evolution of flower characteristics and diversity, and speciation can also be affected by the dynamics of plant-pollinator relationships (Johnson and Steiner 2000;

Waser et al. 1996). Tremblay (1992) also suggests that overspecialization can be unfavorable if the range of the plant is restricted to that of its pollinators. The distribution of plants along a spectrum of specialization within a community could also affect emergent properties of that community such as stability and resilience (Kay and Schemske 2004).

1.1.2 Evolution of rewardless flowers

Effective pollination in species with rewardless flowers is achieved by repeatedly attracting pollinators through the false advertisement of floral rewards (Ackerman 1986; Dafni 1984). An obvious advantage of rewardless flowers is that resources usually used for production of rewards (nectar, edible pollen or oils) may instead be reallocated and expended for other functions that could increase the fitness of deceptive plants (Ackerman 1986; Boyden 1982). This is a risky system for plants however, because reproductive success depends upon how well pollinators are deceived, and how quickly they learn. In the extreme case, if pollinators avoid rewardless flowers, the result may be reproductive failure (due to lower visitation/pollination rates and reduced fruit production) and local species extinction (Boyden 1982; Case and Bradford 2009). Rewardlessness has a variety of additional consequences that stem from altered pollinator behavior when rewards are absent. These include: low rates of visitation and fruit set (Ackerman 1986; Jersáková et al. 2006; Neiland and Wilcock 1998; Nilsson 1992; Tremblay et al. 2005), decreased number of flowers probed per plant promoting outcrossing and reducing selfing (Ackerman 1986; Dafni 1987; Jersáková et al. 2006; Johnson and Nilsson 1999), increased distance between mates (i.e., greater pollen

dispersal distances) due to increased likelihood of pollinators leaving a patch (Cozzolino and Widmer 2005a; Jersáková et al. 2006; Keasar 2000), and increased likelihood of pollinators visiting alternate phenotypes leading to potential receipt of foreign pollen and increased likelihood of hybridization (Cozzolino and Widmer 2005b; Neiland and Wilcock 2000).

The evolution and maintenance of rewardless flowers requires that the net fitness benefits associated with deception outweigh the costs. The high incidence of rewardless flowers in orchid species may be facilitated by several characteristics that reduce the costs of deceptive pollination. Firstly, orchids are morphologically adapted to promote efficient pollen transfer (Jersáková et al. 2006; Nilsson 1992). Their styles and stamens are fused into an organ called the column, and pollen grains are presented at the tip as coherent masses called pollinia (Nilsson 1992). Pollinia are either mass exported as a single unit or smeared onto the bodies of pollinators (Nilsson 1992). In this way, all or most of an individual plant's pollen is transferred in a single pollinator visit, making fewer visits necessary for successful pollination (Jersáková et al. 2006; Smithson and Gigord 2001). Secondly, prolonged stigma receptivity, delayed ovule development, pre-pollination floral longevity, and prolonged viability of excised pollen are other mechanisms that have also evolved within the Orchidaceae (Cozzolino and Widmer 2005b; Neiland and Wilcock 1995). They may promote pollination success by prolonging the opportunity for insect visitation and effective pollen deposition as well as reducing pollen wastage (Neiland and Wilcock 1995; Primack 1985). Lastly, population structure and life history traits may play a role in evolution of rewardlessness. For example, the tendency to occur in small scattered populations may create a diffuse pollinator source,

which in turn could make it difficult for plants to sustain pollinators, whether they offer rewards or not (Ackerman 1986; Jersáková et al. 2006). Small population size or rarity of orchids in a community may make it more difficult for pollinators to discriminate between rewarding and non-rewarding plants, and thus increasing visitation (Ferdy et al. 1999; Jersáková et al. 2006; Sabat and Ackerman 1996; Sun et al. 2009).

There is also some evidence for trade-offs involving fruit production. When fruit production in a plant is high one year, reduced vegetative growth and lower probability of flowering may be more likely in subsequent years (Calvo 1993; Cozzolino and Widmer 2005a; Primack and Hall 1990; Primack and Stacy 1998; Shefferson and Simms 2007). Deceptive orchids may maintain seed production with low fruit set and high seed production per capsule (Boyden 1982; Cozzolino and Widmer 2005a). Orchids produce ovules with hundreds to millions of seeds in one capsule, so that populations may be maintained even when only a few flowers in a given population are pollinated (Boyden 1982). Over evolutionary time, investment in pollinator attraction should be sufficient to ensure that seed set is equally limited by pollen and resource availability (Haig and Westoby 1988).

Multiple strategies of deception have evolved, which show varying levels of pollinator specialization. Deceptive species may employ specialized or highly generalized strategies to ensure their reproductive success. For example, specialist taxa, such as sexual mimics (pseudocopulation), produce flowers that mimic the female mating signals of a specific insect species and are pollinated by the males when they attempt copulation (Dafni 1984; Jersáková et al. 2006). In order to attract pollinators, these plants use sex pheromone-like odors, as well as visual and tactile cues that tend to be highly species

specific (Jersáková et al. 2006; Paulus and Gack 1988). Brood site mimicry involves the attraction of insects seeking a place to lay their eggs, by imitation of common oviposition sites or larval food substrates (Ackerman 1986; Jersáková et al. 2006). Batesian floral mimicry involves rewardless species (usually those that are rare in a community) that mimic a specific rewarding plant that is more common (Jersáková et al. 2006; Peter and Johnson 2008). This strategy is relatively specialized because it tends to exploit mutualisms between rewarding plants and their pollinators.

At the other end of the spectrum are generalist mimics that may be attractive to a variety of pollinators. For example, generalized food mimicry is employed by deceptive species that exploit the instinctive food seeking behavior of potential pollinators by exhibiting nonspecific resemblance to, and producing general floral signals of, rewarding species, but provide no food reward such as nectar (Dafni 1984; Nilsson 1992; Peter and Johnson 2008). Among the types of deception, generalized food mimicry is the most common in the Orchidaceae, and is thought to either be ancestral in the group or to have evolved early (Ackerman 1986; Cozzolino and Widmer 2005a; Jersáková et al. 2006).

The idea that apparently generalized plants may be specialists was proposed by Ollerton (1996), and *Cypripedium*, which is the focus of this thesis, is a very good candidate for exploring this hypothesis. *Cypripedium* exhibit generalized food deception, which may result in attraction of many potential pollinators, but may in fact be more specialized in terms of their effective pollinators due to restrictive floral architecture. *Cypripedium* orchids belong to the subfamily Cypripedioideae, whose members display a petal that has been modified to form a prominent pouch-like labellum (or slipper – terms that will be used interchangeably throughout the thesis), that varies in colour, shape, and

size among species (Figure 1.1, Stoutamire 1967). The labellum acts as a one-way trap (Ames et al. 2005; Li et al. 2006), and once floral visitors have entered they cannot re-emerge from the same aperture due to its infolded margins and smooth interior surface. Insects are guided towards a narrow passage at the base of the labellum by morphological features including colourful markings, hairs, and translucent windows (Figure 1.1, Catling and Knerer 1980; Catling and Catling 1991; Stoutamire 1967). Insects must move through this passage and out of the labellum via exit routes, which consist of constricted openings on either side of the column (Figure 1.1, Ames et al. 2005; Stoutamire 1967). This configuration leads insects past the stigma and then the anthers. If the pollinator has the correct general morphology this may result in the transfer of pollen on to the body of the pollinator (Stoutamire 1967). It is important to note that most orchids have pollen grains that are held together as a coherent mass called a pollinium (Nilsson 1979; Proctor and Harder 1994). In *Cypripedium* pollen from a pollinium is dispersed as a sticky smear on a number of pollinators. However in other orchids the entire pollinium is dispersed as a single unit on a single pollinator (Nilsson 1979; Proctor and Harder 1994; Stoutamire 1967).

Relatively few studies have investigated the relationship between floral morphology and effective pollination in *Cypripedium* orchids, in part because few insect visitations are observed in the field (Case and Bradford 2009). Most of the work carried out on various Asian and European *Cypripedium* species has shown effective pollination by only one to a few insect species, which were usually determined by the size of the entry and exit routes (Bänziger et al. 2005, 2008; Erneberg and Holm 1999; Li et al. 2006, 2008a, 2008b; Nilsson 1979). This is in contrast to some genera of non-orchid species

Figure 1.1: Floral morphology of a *Cypripedium candidum* x *C. parviflorum* hybrid growing in southern Manitoba. Stigma is concealed beneath the staminode and is not visible. Photo by Anne Worley.

with trap-like mechanisms, such as *Aristolochia* (Burgess et al. 2004; Murugan et al. 2006; Sakai 2002) and *Arisaema* (Nishizawa et al. 2005; Vogel and Martens 2000), which were generally found to have a broader spectrum of pollinators.

1.1.3 Pollinator learning and the role of co-flowering species

For successful pollination to occur, insects carrying pollen from one flower must come into contact with the stigma of another, and thus must be deceived into entering and exiting at least two flowers (Case and Bradford 2009). Pollinator learning is affected by the presence and abundance of co-flowering rewarding species in a variety of ways, including: density of rewarding and rewardless plants, ability of rewardless plants to mimic rewarding ones, spatial dispersion among rewarding and rewardless plants, and phenology (Internicola et al. 2007, 2008; Johnson et al. 2003; Juillet et al. 2007; Lammi and Kuitunen 1995; Thomson 1978; Waser 1978) Interactions among plant species (rewarding or not) and between plants and their pollinators may also in part depend on, and/or influence, the structure in a given community (Moeller 2004; Waser 1978). Within a community, pollinator foraging patterns play a role in determining the extent of competition for pollination (Bell et al 2005).

Facilitation may be an especially important process in rewardless pollination systems because co-flowering rewarding flowers may be collectively functioning to attract and maintain a local pool of pollinators (Johnson et al. 2003; Juillet et al. 2007; Peter and Johnson 2008; Thomson 1978). Flower colour is thought to play an important role in facilitation in that pollinators may be more likely to visit rewardless flowers when they are similar in colour to those of rewarding co-flowering species (Chitka and Raine

2006; Chitka et al. 1997, Johnson et al. 2003; Peter and Johnson 2008; Pellegrino et al. 2008). This type of facilitation is exemplified by Batesian floral mimicry and the magnet species effect. According to the magnet species theory, rewardless flowers receive more pollinator when growing close to rewarding plants (Lammi and Kuitunen 1995). In rewardless *Podophyllum peltatum*, *Anacamptis* (*Orchis*) *boryi* and *Traunsteinera globosa*, increased pollinator visitation and reproductive success has been linked to the density of magnet species and proximity of rewardless species to them (Gumbert and Kunze 2001; Juillet et al. 2007; Laverty 1992).

Pollinator learning and facilitation may be affected by the relative densities of rewardless and rewarding flowers in the community. When overall densities of rewarding and rewardless flowers are low, facilitation may be more likely to occur because deceptive species may benefit from the presence of co-flowering rewarding species due to greater pollinator availability (Moeller 2004). When rewarding flowers occur in high densities however, facilitation may be less likely because pollinators must divide their visits among co-flowering plants, and plants may then have to compete for a limited number of pollinators (Rathcke 1983; Thomson 1978). Deceptive species in high densities may experience reduced visitation (Nilsson 1980), and the more abundant a species is, the faster pollinators can learn to avoid it (Boyden 1982; Ferdy et al. 1998). Reproductive success in deceptive orchids then may thus be subject to density-dependent variation (Ferdy et al. 1998). In the deceptive orchid *Calypso bulbosa*, a high density of orchids in the study area reduced the effectiveness of deception because pollinators (bumblebee queens) encountered many flowers, which increased the rate of avoidance learning (Boyden 1982).

Polymorphic floral phenotypes within rewardless species may affect pollinator learning in a manner similar to low density because each phenotype may be "rare" in the population when compared with co-flowering rewarding species (Ferdy et al. 1998). Multiple floral variants have been observed in some deceptive orchid species such as *Dactylorhiza sambucina* (Nilsson 1980), *Orchis masculata* (Nilsson 1983), *Orchis morio* (Nilsson 1984), and *Tolumnia variegata* (Ackerman and Galarza-Pérez 1991). In such cases, pollinator learning may be slowed as it may be more difficult for foraging pollinators to learn to avoid the "rare" rewardless morphs when in low densities, than those morphs existing in high densities (Ferdy et al. 1998; Keasar 2000; Sun et al. 2009). Similarly, when rewardless flowers are growing with compatible congeners, pollinators may have more difficulty recognizing rewardless flowers, and if pollinator fidelity is low, this situation may promote hybridization.

Rewardless species must often compete with co-flowering species, because flowers with superior rewards may draw pollinators away (Johnson et al. 2003; Waser 1978). One strategy adopted by rewardless species is a shift towards early-season flowering, which helps them avoid or reduce competition from rewarding species, and also takes advantage of pollination services by insects that have not yet learned to avoid flowers without reward (Internicola et al. 2006, 2008; Johnston 1991; Rathcke 1983; Sun et al. 2009). Early in the flowering season, insects such as newly emerged queen bees have not yet learned to avoid deceptive species, nor have they established foraging routes to rewarding patches, and are thus considered "naive" (Boyden 1982; Nilsson 1980). One question that has been addressed by researchers is whether or not a reliance on naive pollinators selects for early flowering in rewardless plant species (Keasar 2000; Sun et al.

2009). For example, reproductive success in some early-flowering deceptive orchids, including *Calypso bulbosa* (Orchidaceae), was found to rely upon the deception of newly emerged naive bumblebee queens in late spring for pollination (Ackerman 1981, Boyden 1982). Much of the evidence for this idea comes from studies carried out on European species. Other orchids that have been found to depend on naive pollinators include *Anacamptis morio* (Nilsson 1984), *Orchis masculata* (Nilsson 1983), *Orchis spitzelli* (Fritz 1990) *Dactylorhiza sambucina* (Nilsson 1980), and *D. incarnata* (Lammi and Kuitunen 1995). Reliance on naive pollinators may increase reproductive success in deceptive species, but may also be detrimental once pollinators learn avoidance behavior (Ackerman 1986; Dafni 1984; Ferdy et al. 1999).

Trying to sort out the relationships between deceptive orchids and their pollinators is difficult due to low levels of pollinator visitation, the difficulty of observing pollination events, and the potential interactions among the factors involved (Bänziger et al. 2005, 2008; Case and Bradford 2009; Li et al. 2008a). Plant-pollinator relationships have been studied in relatively few slipper orchids (Cypripedioideae), and pollinators are only known for 9 to 15 of the ~45 *Cypripeium* species (Bänziger et al. 2008; Bernhardt and Edens-Meier 2010). There is also very little information available regarding how orchid density and co-flowering species affect reproduction in *Cypripedium* orchids. Reproductive success of *Cypripedium japonicum* was greater when plants were growing singly (as opposed to clustered), in individuals that flowered earlier, and when local conspecific flower density was lower (Sun et al. 2009). In *Cypripedium acaule*, there was also evidence for selection towards earlier flower opening, as well as higher reproductive success in plants growing close to Ericaceous shrubs (O'Connell and Johnston 1998).

Many of the remaining *Cypripedium* studies to date have focused on describing their biology, breeding systems, and reproductive success, but have not explored the relationship with pollinators, or in many cases the biological or environmental factors that affect reproduction in these species (e.g. Case and Bradford 2009; Catling 1990; Catling and Knerer 1980; Catling and Bennet 2007; Curtis 1954; Primack and Hall 1990; Primack and Stacy 1998; Shefferson 2006; Shefferson and Simms 2007; Stoutamire 1967).

1.2 Hybridization

Mating between inter-fertile congeners (i.e. hybridization) may result in the evolution of new species, formation of natural (stable) hybrid zones, or the extinction of one or both parental species (Allendorf et al. 2001; Campbell and Aldridge 2006; Rieseberg 1995, 1997). Additionally, hybridization may be a natural process in a given community, resulting in novel plant phenotypes and increased genetic diversity, or it may threaten the persistence and genetic integrity of native species when due to human activity (Vilà et al. 2000). Rates and outcomes of hybridization vary across populations and communities, and the causes of this variation are not always fully understood (Ramsey et al. 2003). The outcome of hybridization can impact community structure and depends on the frequency of inter-specific mating, compatibility of parental species, and the fitness of hybrids relative to their parents (Cozzolino et al. 2006; Rieseberg 1995, 1997; Vilà et al. 2000).

It has been proposed that pre-mating barriers are important in reproductive isolation in rewardless orchids (DeHert et al. 2011, 2012; van der Pijl and Dodson 1966). Pre-

mating barriers often involve factors affecting the opportunities for interspecific pollen transfer and may include pollinator specificity, the interaction between floral dimensions and floral visitors, density and distribution of parental species, and temporal and spatial overlap (Aldridge and Campbell 2007; Ramsey et al. 2003). These factors may depend on interactions with the community of potential pollinators and local environmental conditions. (Vilà et al. 2000).

The interaction between floral dimensions and floral visitor morphology is particularly important for hybridization in some orchid species, where these traits determine the effectiveness of a particular pollinator (Nilsson 1979). If plants are sharing pollinators, but their floral morphologies differ, pollinators may not be effectively transferring pollen between them. In these cases, improper fit between plants and pollinators could be preventing hybridization. Lack of flower sharing by pollinators in cases where two plant species are morphologically compatible with insects, could also prevent natural hybrid formation. This was reported for near-by or mixed stands of *Cypripedium yunnanense* and *C. flavum* that were both size-compatible with regard to pollinators, which were unshared between them (Bänziger et al. 2008). Ramsey et al. (2003) also found that this was the case for sympatric populations of *Mimulus lewisii* and *M. cardinalis*. If pre-mating barriers are incomplete, pollinator sharing and formation of hybrid bridges may be of particular importance in populations of rewardless plants. When growing with compatible congeners, low pollinator fidelity could promote hybridization. For example, in sympatric populations of the deceptive orchid *Orchis collina* and rewarding *O. coriophora* common pollinators were reported to contribute to the formation of hybrid offspring (Dafni and Ivri 1979).

Community and population structure also affect the probability of hybridization. In populations of rewardless species, low plant density, scattered distribution, and flower polymorphisms can slow pollinator learning, resulting in increased visitation to rare and/or alternate phenotypes (Cozzolino and Widmer 2005b; Ferdy et al. 1998; Neiland and Wilcock 2000). If pollinators are visiting a greater number of phenotypes or species in a population, an increased likelihood of hybridization may result. This may also be the case for populations of endangered species that are small with limited distribution, where events leading to hybridization may represent a larger proportion of the total reproductive success of species than in larger populations (Burgess et al. 2005). Wu and Campbell (2005) examined the role of spatial variation in environment-dependant natural selection of a hybrid zone (rewarding plants *Ipomopsis aggregata* and *I. tenuituba*), and found that both environment and pollinator-mediated selection may contribute to genetic structure of the populations. Additionally, physical changes to communities such as habitat loss and land-use changes may be responsible for breaking down pre-mating geographic barriers, and are thought to affect the extent of hybridization in *C. candidum* and *C. parviflorum* (Rhymer and Simberloff 1996; Vilà et al 2000).

Post-mating barriers can also affect hybridization in cases where pre-mating barriers break down or are incomplete. They often relate to relative fitness of hybrids and parents, in particular the reproductive inviability or sterility that results in many hybrid offspring (Mallet 2007; Ramsey et al. 2003 and references therein). When hybrid offspring are not viable or show lower fitness than parental species, there is usually little concern for populations undergoing inter-specific mating (Campbell and Aldridge 2006; Cozzolino and Scopece 2008; Rieseberg 1997). For example, strong karyotype

differences and chromosomal changes were important in maintaining reproductive isolation and reducing fitness of hybrids among sympatric, pollinator-sharing species of Mediterranean deceptive orchids (Cozzolino and Widmer 2005b). If on the other hand, parental taxa have lower fitness than hybrid offspring, one or both of them may become extinct (Hedge et al. 2006; Rieseberg 1997). Hybrid offspring that are more fit are more likely to produce fruit and viable seed than those with lower fitness. These individuals, if they survive to flowering, are more likely to mate with other hybrids or parental taxa and reproduce. In some cases this situation may be a conservation concern for rare or endangered species because increased rates of hybridization can lead to local extinction via genetic assimilation when backcrossing occurs between fertile hybrids and a more abundant parental species (Allendorf et al 2001; Burgess et al. 2005; Ellstrand 1992; Rhymer and Simberloff 1996). This was the case for *Raphanus sativus* and *R. raphanistrum*, two species of radish, which have been completely replaced by their hybrid descendants in California (Hedge et al. 2006). In other instances the impact on parental species can asymmetrical. Burgess et al. (2005) found that in two species of mulberry (the endangered *Morus rubra* and the more abundant *M. alba*), a significant proportion of hybrids contained more nuclear markers from *M. alba*.

1.3 Study system

1.3.1 Study species

Cypripedium is the most studied genus of the slipper orchids and is comprised of 45 known species (Bänziger et al. 2005). Plants are perennial and terrestrial with large, brightly coloured flowers with an enlarged lower petal (or labellum) that acts as a one-

way semi trap, subterranean rhizomes, and plicate leaves (Stoutamire 1967). *Cypripedium candidum* (the small white lady's slipper orchid) is a relatively small plant (~ 11 - 40 cm) that grows in compact genets of 3 - 60 ramets (Figure 1.2, Bowles 1983; Curtis 1946; Sheviak 2002). Its flowers have yellow-green to brown lateral sepals and petals, a white labellum with reddish purple flecks around the entrance and interior surface, and a sweet and pungent/spicy odor (Figure 1.2, Sheviak 2002; Stoutamire 1967). *Cypripedium candidum* emerge in early spring, and the flowers are short-lived, lasting only 10 - 14 days under favorable conditions (Bowles 1983). The plants grow mostly in tall-grass and mixed-grass prairie habitats in the central U.S. and adjacent Canada (Bowles 1983; Cribb 1997; Stoutamire 1967). *Cypripedium candidum* tends to be shade intolerant and disappears when woody plants replace the herbaceous vegetation (Curtis 1946; Stoutamire 1967). It is considered globally endangered, with Canadian populations restricted to only 15 populations in Manitoba and 7 in Ontario (Worley et al. 2009). However, it may be locally abundant, and some populations comprise thousands of stems (Environment Canada 2006; Worley at al 2009). Most populations occur syntopically with at least one variety of *C. parviflorum* (Worley et al. 2009).

Cypripedium parviflorum (the yellow lady slipper orchid) is a highly variable and morphologically complex species (Figure 1.2). There are three recognized varieties, two of which (var. *pubescens* and var. *makasin*) occur in Manitoba (Sheviak 2002; Worley et al 2009). These varieties tend to occur in woodland (var. *makasin*) or open habitats (var. *pubescens*), and are distributed across most of Canada and the eastern and western parts of the United States (Ames et al. 2005; Sheviak 2002). Floral morphology ranges from large, bright yellow labella with greenish-yellow sepals and petals (var. *pubescens*) to

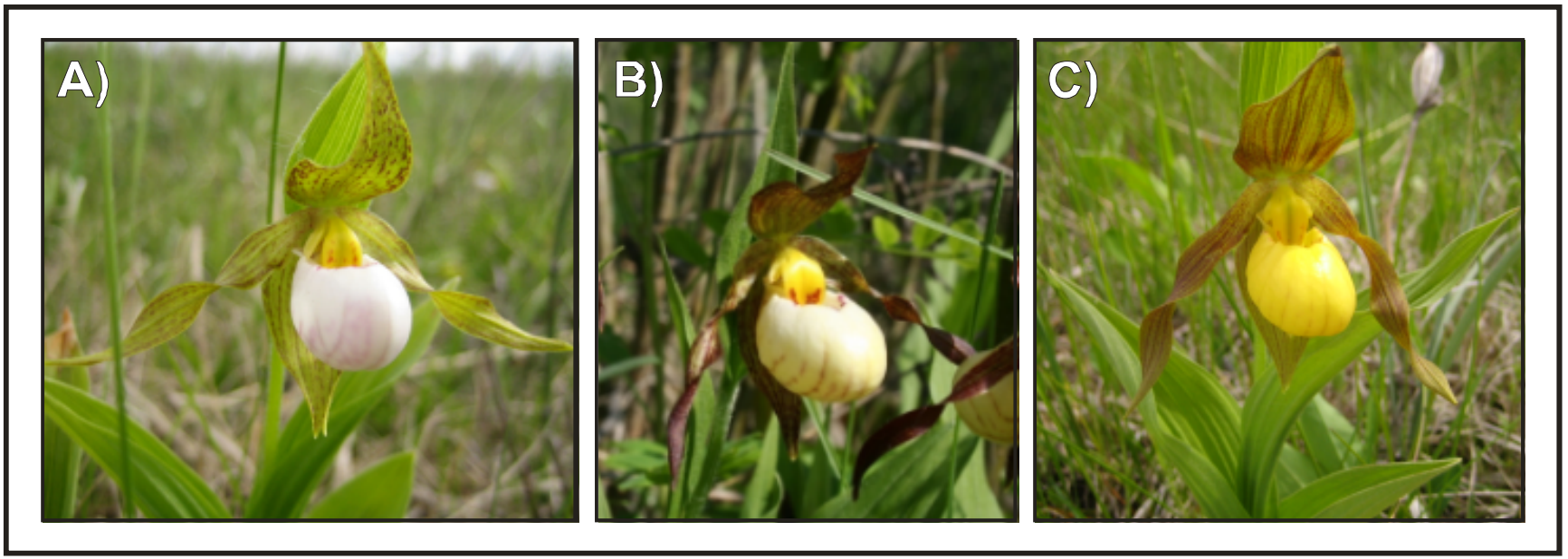

Figure 1.2: Lady's slipper orchids growing in Southern Manitoba. Shown are A) *Cypripedium candidum*, B) hybrids, and C) *C. parviflorum*. Photo B) by Anne Worley.

smaller bright yellow labella with brown-purple sepals and petals (var. *makasin*) *Cypripedium parviflorum* and *C. candidum* often overlap in their flowering. However, there is a tendency for *C. parviflorum* flower slightly later in the season.

The hybrids that occur between *C. candidum* and *C. parviflorum* have been referred to as *Cypripedium* x *favillianum* (Curtis 1932; Klier et al. 1991) and *Cypripedium* x *andrewsii* (Sheviak 2002), but will be referred to simply as "hybrids" in this thesis (Figures 1.1 and 1.2). They are highly variable, representing a continuum of morphologies between the two parents. Hybrids occur where *C. candidum* and *C. parviflorum* overlap, and are usually found in prairie-woodland ecotones. They are observed throughout the range of *C. candidum*, and have been reported from all Canadian populations (Worley et al. 2009). Fire suppression, habitat fragmentation, and woody encroachment have likely contributed to an increase in ecotonal habitats where both species can be found growing together, which in turn has increased the amount of hybridization between the taxa (Worley et al 2009).

1.3.2 Study sites

My research focused on four orchid populations in southern Manitoba, at the northern extremity of *C. candidum*'s range (Figure 1.3). Due to *C. candidum*'s status as an endangered species, the exact locations of the study sites will not be provided. Three of these are remnant prairie patches, two located west and one south of Winnipeg (hereafter Grossart, Manitoba Conservation [MBC], and Doyle), while the fourth is a roadside ditch also west of the city (hereafter McPherson). The species composition of *Cypripedium* orchids varies across the sites: Grossart and MBC are composed of all 3

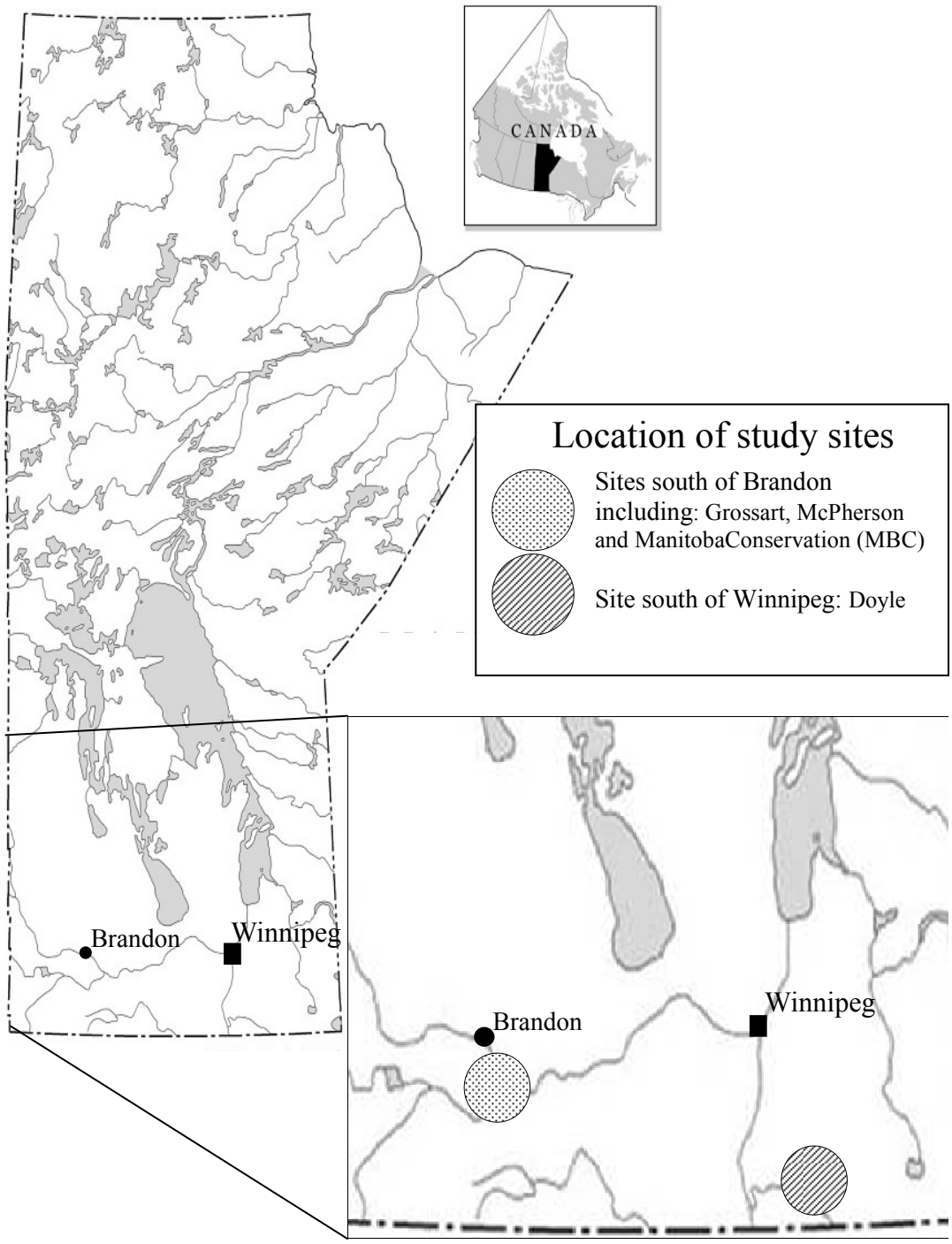

Figure 1.3: Map of Manitoba showing the location of the four study sites in Southern Manitoba: Grossart, McPherson, Manitoba Conservation (MBC) and Doyle.

taxa with the occurrence of extensive hybridization. At Grossart, genets of *C. candidum* and hybrids were the most clustered together of all the populations, with *C. parviflorum* sparsely dispersed across the site. This site also consisted of the densest *C. candidum* population. At MBC, the distribution of all three taxa was patchier, with orchids growing in more localized clumps. There seemed to be a larger population of *C. parviflorum* here in comparison to Grossart. At McPherson, only *C. parviflorum* was observed with genets consisting of one or two ramets growing interspersed with shrubby vegetation along the ditch edge. At Doyle orchid composition consisted mostly *C. candidum*, fewer *C. parviflorum*, and little to no evidence of introgression. The distribution of *C. candidum* was again more or less patchy, and *C. parviflorum* tended to grow along the ditch edge and at the edges of treed areas, but not interspersed with *C. candidum*. Doyle provided a good contrast to Grossart and MBC because of the seeming lack of hybrids and the clear distinction between the two parental species.

1.4 Research goals, objectives, and hypotheses

The main focus of this thesis is to examine the factors affecting the reproductive success of two species of lady's slipper orchids and their hybrids in southern Manitoba, by investigating relative fruit and seed set, differences in floral morphologies, and the relationship between reproductive success and the surrounding floral and insect communities. This project will contribute to the limited body of knowledge on the reproductive fitness and pollination ecology of the endangered *C. candidum* and its hybridization with the common *C. parviflorum*. My study will also provide valuable

background information for future studies of *Cypripedium* reproductive biology and ongoing conservation efforts.

The main goal of Chapter 2 is to investigate some of the pre-mating barriers that may affect orchid pollination and hybridization. My first objective is to determine the amount of variation in morphological traits within and among orchid taxa. Based on field observations and taxon descriptions in the literature, I hypothesize that floral features will be smaller in *C. candidum* than in *C. parviflorum*, and that hybrids will have spectrum of morphologies that are intermediate between parents. My second objective in Chapter 2 is to describe the number and variety of insects that are visiting the three orchid taxa, and overlap in floral visitors among orchid taxa. Based on preliminary field observation, I expect the greatest number of floral visitors to be associated with *C. parviflorum*. My third objective is to determine which of the floral visitors may be acting as potential pollinators, and whether orchid species are sharing these potential pollinators. Based on the relatively small number of effective pollinators usually associated with *Cypripedium* species, I expect to find a large number of floral visitors, but relatively few effective pollinators. In sites where hybrids occur, I expect to find some pollinator overlap between *C. candidum* and *C. parviflorum*.

In Chapter 3, my main objective is to compare the female reproductive success of *C. parviflorum*, *C. candidum*, and their hybrids, and to explore whether floral phenotypes influence fruit set. In accordance with the low reproductive success generally reported for rewardless species (including *C. candidum* and *C. parviflorum*), and the prediction that C. parviflorum may be visited more frequently by pollinators, I expect to find relatively low fruit set for all three orchid taxa, with the highest values found in *C. parviflorum*, and the

lowest in *C. candidum*. I additionally wanted to determine the amount of seed set in each population, and compare it among orchid taxa. Seed set values reported in other studies (e.g. Proctor and Harder 1994), varied among taxa. Accordingly, I expect seed set in this study to vary within and among *C. candidum*, *C. parviflorum*, and their hybrids. If there are any genetic incompatabilities between parental taxa, I expect hybrids to show evidence of sterility, and thus have lower seed set than both parental taxa. My final objective is to investigate how plant phenotypes (floral morphology, plant height, and number of flowers) may be associated with orchid fruit set. Due to *Cypripedium*'s restrictive floral architecture and its role in pollination, I expect to find that floral dimensions will be associated with the likelihood of setting fruit. I also expect that, based on field observations and taxon desciptions in the literature, the number of flowering stems per cluster will differ among orchid taxa, and that *C. candidum* clusters will consist of the most flowering stems.

In Chapter 4, I will describe the floral community associated with four southern Manitoba populations of *C. parviflorum* and *C. candidum* that seem to differ in the frequency of hybrids. My main objective is to determine if and how the presence of local rewarding flowering plants affects orchid fruit set, and whether certain co-flowering species may be considered more likely facilitators than others. I am exploring two alternative hypotheses. First, if co-flowering plants are acting as facilitators, I expect to find that orchids growing with a high density of rewarding species will have higher fruit set compared to those growing in less dense areas. Alternatively, if co-flowering plants are acting as competitors for pollinator attention, I expect to find that orchids growing in high densities of rewarding plants would have lower fruit set. I will also use non-orchid

pollen collected from inside orchid slippers to provide insight into which co-flowering species may be important in maintaining potential pollinators. I expect that the non-orchid pollen found on orchid flowers will reflect the composition and relative abundance of co-flowering species. Since *Cypripdium* spp. employ a generalized pollination strategy, I expect to find few differences in the non-orchid pollen collected from *C. parviflorum* vs. *C. candidum* vs. hybrids.

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2.0 Chapter Summary

In this chapter I examined the relationship between orchid and insect visitor morphology, and considered how this interaction may influence pollination success and rates of hybridization. I measured variation in 10 plant characters within and among orchid taxa. *Cypripedium candidum* plants were generally shorter with smaller labella, *C. parviflorum* were tallest with the largest flowers, and hybrids showed morphologies along a spectrum between the two parental taxa. I also compared insect visitation among orchid taxa and determined which of these visitors were potential pollinators. *Cypripedium parviflorum* had the greatest number and variety of visitors, *C. candidum* the fewest, and hybrids were intermediate with respect to numbers, but the diversity of visitors was similar to that of *C. candidum*. Comparisons between insect body measurements and orchid exit routes indicated that for all orchid taxa there were a greater number of floral visitors than potential pollinators. Orchid taxa also showed overlap in bloom time, in spatial distribution, and potential pollinators at MBC in 2011, which may be affecting the directionality of mating between parental taxa and with hybrid offspring. The dipteran *Odontomyia pubescens*, and hymenopterans of the genus *Andrena* seem to be the most likely pollinators contributing to gene flow and hybridization among orchid taxa. A greater number of visitors and potential pollinators were shared between hybrids and *C. parviflorum* than between hybrids and *C. candidum*, or the two parental taxa. This may indicate that mating occurs more frequently between hybrid offspring and *C. parviflorum* at this site. Variation in exit route size among orchid taxa, which could influence the

abundance and diversity of potential pollinators, as well as overlap in spatial distribution and blooming period might be contributing to differences in pollination, reproductive success, and hybridization rates among orchid taxa.

2.1 Introduction

Floral dimensions and architecture play an important role in pollinator attraction and effectiveness. Although many angiosperms species are successfully pollinated by a broad range of animals, not all pollinators are equally effective (Ollerton 1996; Renner & Feil 1983; Waser 1983; Waser et al. 1996). In many cases, specific flower shapes and arrangement of sex organs are more suited for particular groups of pollinators (Campbell and Aldridge 2006, Fenster et al. 2004; Waser at al. 1996). In tubular flowers of *Polymonium brandegeei* for example, a recessed arrangement of sex organs (stigma located below anthers) and long, narrow corolla tubes were better suited to pollination by hawkmoths, while an exserted arrangement (anthers located below stigma) and wider corolla tubes was better suited to hummingbird pollination (Kulbaba and Worley 2008, 2011).

Floral morphology is particularly important for plants such as those in the Cypripedioideae that have trap-like flowers. In order to be effective, a pollinator must be small enough to enter the opening in the labellum, and large enough to come into contact with both sets of floral sex organs upon exiting the rear of the labellum (Bänziger et al. 2008; Li et al. 2006, 2008a, 2008b; Nilsson 1979). Any size incompatibilities between orchid and insect morphology may lead to loss of reproductive effort by the plant, due to death of the insect or ineffective pollen transfer (Tremblay 1992).

Floral morphology also affects the occurrence and frequency of hybridization. Formation of first generation hybrids may be more likely if similar corolla dimensions and position of sex organs promote pollinator sharing among plant taxa. It is also important to keep in mind that morphological overlap between compatible species doesn't always result in pollinator sharing and hybridization events. Bänziger et al. (2008) found no hybrids between sympatric and compatible *Cypripedium yunnanense* and *C. flavum,* which was due to lack of pollinator sharing, despite morphological overlap.

Ongoing hybridization may increase the morphological overlap and opportunities for further hybridization. The morphology expressed by hybrids results from the combined genetic contributions of both parental taxa. Interactions with the environment and the nature of the genetic control of traits influence the expression of those traits in hybrid offspring (Rieseberg 1995; Wu and Campbell 2005). Backcrossing between hybrid offspring and parental taxa (or other hybrids) may allow transfer of certain traits via a "genetic bridge" (Cozzolino et al. 2006). This transfer of genetic material however, may not be equal from both parental taxa (Burgess et al. 2004). With increased frequency of hybridization and backcrossing between hybrids and parental taxa, one would expect greater variation in genetic combinations. Due to increased mutation rates and new combinations of normal alleles, among other factors, hybrids may exhibit not only intermediate phenotypes, but also parental and novel characters (Rieseberg 1995). For example, Worley et al. (2009) found that hybrids of *C. candidum* and *C. parviflorum* orchids expressed intermediate as well as more parental-type morphologies that were consistent with their genetic structure. If morphologies among parental taxa and hybrid

offspring become increasingly similar, especially with regard to corolla dimensions and sex organ positioning, pollinator sharing and rates of hybridization may increase.

The functional floral morphology in relation to effective pollination has been studied in relatively few (six to my knowledge) of the approximately 45 *Cypripedium* species (Bänziger et al. 2005, 2008; Edens-Meier et al. 2011; Li et al. 2006, 2008a, 2008b). Floral and pollinator measurements have been taken to quantify the potential fit between the two. For example, the distance between the stigma and the bottom of the labellum and between the pollinia and the bottom of the labellum in *C. tibeticum* were lower than bumblebee queen thorax height, (who could contact stigma and pollinia), but larger than that of workers, who would not remove pollen (Li et al. 2006). Because floral entry and exit routes are likely the most restrictive floral dimensions, they may be of particular importance. For example, Bänziger et al. (2005) suggested that some floral visitors were too large to enter the labellum of *C. guttatum*, and smaller species were better suited to pollination, though this was not quantified in their study.

Despite the fact that *Cypripedium* species exhibit generalized food mimicry, they tend to be relatively specialized with respect to their pollinators (Appendix 2.1, Bänziger et al. 2005, 2008). Within this genus, the most in-depth work relating to pollinators has been carried out on *C. calceolus* (Appendix 2.1, Darwin 1887; Guignard 1886; Müller 1883; Nilsson 1979; Robertson 1928; Stoutamire 1967). These studies showed that many species of insects were visiting flowers, while relatively few were acting as effective pollinators (Appendix 2.1). On the other hand, Edens-Meier et al. (2011) reported that in other *Cypripedium* species, flowers were visited exclusively by their pollinators.

Regardless of the level of specialization in rewardless orchids, pollinator fidelity tends to be low (Ferdy et al. 1998; Jersáková et al 2006; O'Connell and Johnston 1998), which may promote hybridization when rewardless plants are growing with compatible congeners. If hybrids are rare in the population, pollinators may have more difficulty avoiding these phenotypes than others existing in high densities (Ferdy et al. 1998; Keasar 2000; Sun et al. 2009), which may increase visitation to hybrids promoting further hybridization. The extent of effective pollinator sharing among plant taxa, and the degree of interspecies fertility, may also have direct bearing on the level of hybridization in a given population (Bänziger et al. 2008, Cozzolino and Widmer 2005b; Dafni and Ivri 1979).

The spatial distribution, density, and phenology of plants within sites or populations can also influence pollination, reproductive success and hybridization, as well as genetic structure of hybrid zones (Burgess et al. 2004; Campbell and Aldridge 2006; Wu and Campbell 2005). Dispersion of rewardless plants and taxa within populations, and proximity of those plants to one another can affect pollinator visitation, which will either facilitate or impede the occurrence of hybridization. Flowering phenology may also be important because there is a much greater likelihood that pollen will be transferred between species with substantial overlap in flowering times (Cozzolino and Widmer 2005a; Dafni and Ivri 1979; Vilà et al. 2000).

The main focus of this chapter is to investigate orchid morphological traits and insects that may act as floral visitors or potential pollinators. My first objective is to explore the floral morphology of *C. candidum*, *C. parviflorum* and their hybrids, to determine the extent of morphological variation within and among taxa. Particular

attention will be paid to pollinator entry and exit routes. Because flowers of *C. candidum* are generally smaller than those of *C. parviflorum*, I expect that individual floral features such as labellum size and entry and exit route dimensions will follow this pattern as well. Hybrids combine the genome of two parental species due to hybridization, and potential backcrossing of offspring with parental taxa. Therefore, I expect that hybrid floral morphology will span a continuum between that of *C. candidum* and of *C. parviflorum*.

My second objective is to document floral visitors for each orchid taxon. I am interested in comparing the pool of insects among the three orchid taxa, the relative abundance of insect visitors to each taxon, and the overlap in floral visitors among taxa. In sites where hybrids occur (MBC and Grossart), I expect to find some overlap in floral visitors among all orchid taxa. In sites where few hybrids exist (Doyle), I expect find less overlap between the visitors found on *C. candidum* and *C. parviflorum*.

My third objective is to determine which species of visitors may be acting as potential pollinators, based on comparisons of insect and floral dimensions. Because there are a relatively small number of potential pollinators usually associated with *Cypripedium* species, I expect to find a large number of floral visitors, but relatively few potential pollinators. Overlap in potential pollinators (i.e. potential pollinator sharing) was examined at MBC in 2011, a site where hybrids occur. At this site, I expect to find overlap in floral visitors between *C. candidum* and *C. parviflorum*. I also suspect that hybrids may be backcrossing with parental taxa, and therefore expect to find pollinator overlap between hybrids and both parental taxa.

Finally, I will consider the overlap in orchid spatial distribution and flowering phenology, and the possibility that these two factors may play a role in pollination and

hybridization of *Cypripedium* orchids. I expect to find differences in hybridization between sites that differ in the spatial distribution of orchid taxa.

2.2 Materials and methods

2.2.1 Quadrat setup

In the spring of 2010, 80 permanent sampling plots ($4m^2$ quadrats) were set up in areas of high orchid density in three sites near Brandon, Manitoba (Grossart, McPherson, and Manitoba Conservation [MBC]) and in one site at Tall Grass Prairie Preserve (Doyle). In 2011, 8 additional quadrats were set up in an area of lower orchid density at Grossart. All sampling was carried out within these 88 quadrats, with the exception of some *C. parviflorum* and hybrid individuals at Grossart and Doyle that were located in a ditch adjacent to the primary sampling area (Appendix 2.2). Within quadrats, I tagged all "clusters" of each orchid taxon (Appendix 2.2). Each tag consisted of a numbered copper label attached to a galvanized nail that was pushed into the ground near the base of a cluster. Clusters are groups of orchid stems that occurred within 10 cm of one another, and represent the best estimate of genetic individuals. Quadrat composition provided information on how orchids were distributed across the sites. *Cypripedium parviflorum* clusters that were sampled along the ditch at Grossart, but outside of quadrats, were not included in quadrat composition data. Each cluster was assigned a unique identification so that I could relate flowering, fruiting, and morphological data back to individuals for 2010 and 2011. The following information was recorded for each cluster: taxon name, number of vegetative stems (2010 only), number of flowering stems, number of stems in bud, and flower colour.

2.2.2 Orchid morphological measurements

In 2010 and 2011 subsets of tagged flowering stems of each orchid taxon were chosen at each site for morphological measurements (11-40 *C. candidum*, 7-39 *C.*

parviflorum, 20-23 hybrids; Appendix 2.2). In 2010 plants were chosen randomly. In 2011 the subset included plants of each taxon that had been measured in 2010, which were a balanced mix of those that fruited in 2010 and those that did not. Data from 2010 and 2011 were used for statistical analyses of year-to-year morphological consistency as outlined below. For each plant, I measured a total of 10 floral and vegetative traits. Using digital calipers (accurate to 0.01 mm), I measured labellum morphology (labellum length/width, entry route lengths/width, and exit route length/width), and using a standard 30 cm ruler (accurate to 1 mm) I measured plant height. Labellum colour was determined by comparison with paint colour charts (Glidden/C-I-L brand's "Clean and Playful" series, card numbers CP19 and CP20). I also calculated labellum entry and exit route diagonals to estimate the maximum expected size of pollinators. All measurements were made on fresh, fully opened flowers. Date of measurements provided indirect evidence of differences in flowering time among orchid taxa. Additional measurement data were also collected from untagged orchids at each site in 2009 and have been included in some analyses.

2.2.3 Insect collection

In 2010 and 2011 I collected any insects (living or dead) that were found inside orchid slippers during site walk-throughs. Because visiting insects were found infrequently, most individuals were sampled outside of quadrats. In 2011, I concentrated my insect collection at the MBC site and insects were collected in two ways: "the ribbon technique" and "random walks". For the ribbon technique, ribbons made out of light-weight tulle fabric (light grey in colour) were placed around the exit routes of 30 *C.*

parviflorum, 40 *C. candidum*, and 29 hybrid flowers at MBC to prevent any visiting insects from exiting flowers (modified from Case and Bradford (2009)). Ribbons were placed in the morning of the first day of sampling, and left on until the flowers had withered. Because I wanted to sample newly opened flowers, the placement of ribbons was staggered along with the bloom times of each orchid taxon. Checks were carried out at varying times during the day, during the course of the sampling period (June 9 to June 22, 2011). *Cypripedium candidum* was sampled 15 times over 7 sampling days, *C. parviflorum* 10 times over 6 days, and hybrids 9 times over 6 days. In cases where live insects were encountered, a mesh bag was placed over the entire flower and cinched closed. The ribbon was then removed, and the insects were allowed to exit into the mesh bag. I did not always directly observe the insects exiting. On some occasions insects appeared unable to exit on their own after about 10-20 minutes. In these cases, they were helped out by gently bending the labellum. This time frame was based on Nilsson's (1979) observation that insects usually emerged within about 10 minutes after imprisonment. Once insects were in the mesh bags, they were placed in a "kill jar" with ethyl acetate to euthanize them. They were then placed in 70% ethyl alcohol for storage until processing.

In addition to the ribbon technique, I conducted random walks through the site (MBC) where I checked flowers for the presence of insects. In these cases, the number of flowers checked was counted, and all insects (alive or dead) were collected. For living insects, mesh bags were placed over the flowers and insects collected as described above. Dead insects were removed from flowers using forceps. For all collections it was usually noted whether an insect was dead or alive. This information however, is missing for

approximately 30% of insects in 2010 and 6% of those collected in 2011. I also noted instances when an orchid pollen smear was visible on an insect after exiting the flower.

All insects were pinned and labeled according to standard entomological procedures and stored in an insect collection box. Identification was carried out to the family level, and often to the genus and species level (BugGuide 2012) with the assistance of Sarah Semmler, a graduate student in the Department of Biological Sciences, University of Manitoba with extensive experience in identifying prairie insects. All specimens will be deposited in the Wallis Roughley Museum of Entomology at the University of Manitoba in Winnipeg, Manitoba.

Insects were then measured in order to assess their potential as pollinators. Because very few insects were actually observed exiting orchid flowers, it was difficult to determine with certainty which insects were "effective" pollinators. In this study, orchid exit route dimensions were compared with the body size of individual visitors, along with presence/absence of visible orchid smears, to determine which taxa may be considered "potential" pollinators. All measurements were carried out using digital calipers (accurate to 0.01 mm) under a dissecting microscope. Following methods outlined in Li et al. (2006, 2008a, 2008b), I measured body length, body width (thorax and abdomen), and thorax height (Appendix 2.3). Some insects were too small to measure with the digital calipers and these were excluded from comparisons with orchid morphology. Other insects were excluded because of their unsuitability (e.g., Lepidopterans are very unlikely pollinators of *Cypripedium* orchids because they could not exit without damaging their wings). Additionally, the literature on *Cypripedium* pollinators suggests that pollination in mainly carried out by Hymenopterans (Appendix 2.1). Although Dipterans have not been

recognized as effective pollinators of *Cypripedium*, they have been noted as visitors (Appendix 2.1). Furthermore, the morphology and behavior of these insects suggests that they could be potential pollinators. Because little is known about *C. candidum* and *C. parviflorum* pollinators, I investigated the possibility that Dipterans may be important for these species.

Thorax height and maximum body width of Dipteran and Hymenopteran insects collected at all sites in 2010 and 2011 were compared with orchid floral measurements (5^{th} and 95^{th} percentiles of all measurements taken in 2010 and 2011 combined) to help determine potential pollinator status. Comparisons were made between body measurements of individual insects and the exit route dimensions of the orchid taxon from which they were collected. To determine whether there was any overlap of insect visitors or potential pollinators among orchid taxa I used only data collected at MBC in 2011. I chose to be conservative by exploring insect overlap at a single point in time and space, instead of pooling data from all sites and years together. This is because the insects collected at MBC in 2011 represent the greatest sampling effort. By contrast, I collected a much lower number of insects in other sites and years, which I did not believe to be comprehensive enough to use for making any conclusions regarding pollinator sharing.

2.2.4. Statistical analyses

As mentioned above, 10 floral and vegetative traits were measured on all orchid samples (see section 2.3.2). All measurement data were log transformed prior to statistical analyses to satisfy assumptions of normality.

In order to characterize morphological separation among orchid taxa, I explored patterns of variation using principal components analyses (PCA) and a correlation matrix (ORDIN module, SYN-TAX 5.02Mac, Podani 1995). Separate analyses were conducted on plants measured in each year (N = 209 in 2010, N = 160 in 2011). Individuals with missing values were removed from all PCA analyses (three in each year). Additionally, entry and exit route diagonal measurements were removed from the PCA data matrix, because they were highly correlated with entry and exit route length and width.

To determine if there were differences in morphological traits among orchid taxa and sites, two-way analyses of variance (ANOVA) were carried out using SAS v. 9.2 (PROC GLM, SAS Institute Inc. 2004). I carried out two sets of analyses because not all orchid taxa occurred at all sites. The first series compared *C. candidum* and *C. parviflorum* at Doyle, MBC, and Grossart, and the second compared all three taxa at MBC and Grossart. Because only *C. parviflorum* occurred at McPherson, no comparisons among taxa could be made, and this site was excluded. Plant height, entry route (diagonal, length, and width), and exit route (diagonal, length, and width) in each year (2009, 2010, and 2011) was analyzed separately. Orchid taxon, site, and the two-way interaction between them were included as effects in the model.

I also investigated the consistency across years of certain morphological traits that may be important for pollination. In order to determine if plant height, entry route dimensions, and exit route dimensions in 2010 were a reliable predictor of those traits in 2011, I used regression analyses in SAS v. 9.2 (PROC GLM, SAS Institute Inc. 2004). Separate analyses were carried out for each trait and orchid taxon combination. Traits in 2011 were included in the model as the dependant variable, and traits in 2010 as the

covariate. McPherson was again excluded because no *C. parviflorum* plants were measured at that site in both years.

2.3 Results

2.3.1 Orchid morphology

The PCA for orchid morphological characters in 2010 and 2011 showed that individuals of each orchid taxon generally clustered together based on eight floral and vegetative characters (Figure 2.1). In 2010, axes one and two combined explained 81.2% of the variation, while in 2011 it was slightly higher at 84.8%. In both years, separation among orchid taxa occurred along axis one, and all characters contributed approximately equally to this separation (Appendix 2.4). *Cypripedium parviflorum* were generally taller with larger labella than *C. candidum*, while hybrids were intermediate between parents and overlapped with both. The second axis was more important for variation among plants within taxa: in 2010 exit length had the highest loading, while in 2011 it was exit length, width, and plant height (Figure 2.1, Appendix 2.4).

The ANOVAs of plant height and entry and exit diagonals showed similar trends to the PCA. *Cypripedium candidum* and *C. parviflorum* were morphologically distinct from one another, and hybrids were intermediate and overlapped with both parents. Overall results were similar among years, and are summarized below for 2010. Taxon explained the most variation in all three traits, but significant taxon by site interactions also occurred (Table 2.1). Site effects were significant for plant height only (Table 2.1). Within all sites, dimensions of *C. parviflorum* were significantly larger than those of *C. candidum* (for all pairwise comparisons $t > 2.89$, $P < 0.01$; Figure 2.2). Hybrids always had intermediate dimensions; all three measures differed from both parental taxa at Grossarts (all $t > 2.81$, $P < 0.01$; Figure 2.2). At MBC, plant height and exit diagonal of

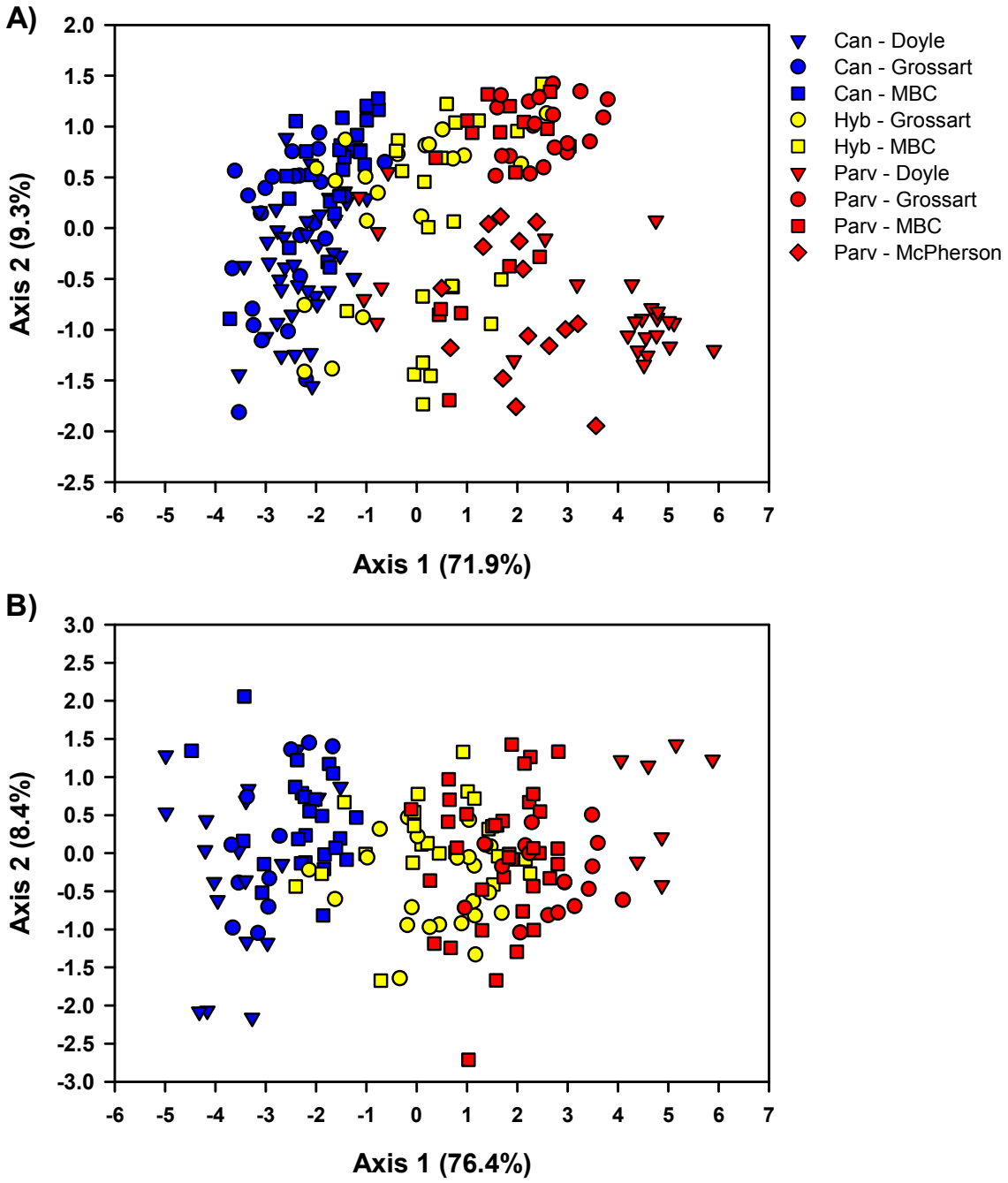

Figure 2.1: Principal components analysis of eight floral and vegetative characters for *Cypripedium candidum* (Can), *C. parviflorum* (Parv), and their hybrids (Hyb). Shown are results for characters measured in 2010 (A) and 2011 (B) at four sites in southern Manitoba (Doyle, Grossart, MBC, and McPherson).

Table 2.1: Analysis of variance of orchid morphological traits measured in 2010 on *Cypripedium candidum* (Can), *C. parviflorum* (Parv), and their hybrids (Hyb) at three sites in Southern Manitoba: Doyle (D), Manitoba Conservation (M), and Grossart (G).

Dependant Variable	Model Fit	Can vs Parv (D, M, G)	Can vs. Hyb vs. Parv (M, G)
Plant Height	Model	$R^2 = 0.6141$, $P < 0.0001$	$R^2 = 0.5093$ $P < 0.0001$
	Taxon	$\boldsymbol{F_{1,149} = 174.78}$ ***	$\boldsymbol{F_{2,124} = 44.23}$ ***
	Site	$\boldsymbol{F_{2,149} = 7.95}$ **	$F_{1,124} = 2.92$
	Taxon x Site	$\boldsymbol{F_{2,149} = 14.26}$ ***	$\boldsymbol{F_{2,124} = 16.25}$ ***
Entry Diagonal	Model	$R^2 = 0.7138$ $P < 0.0001$	$R^2 = 0.6426$ $P < 0.0001$
	Taxon	$\boldsymbol{F_{1,149} = 323.95}$ ***	$\boldsymbol{F_{2,123} = 101.21}$ ***
	Site	$F_{2,149} = 1.01$	$F_{1,123} = 2.40$
	Taxon x Site	$\boldsymbol{F_{2,149} = 7.03}$ **	$\boldsymbol{F_{2,123} = 6.37}$ **
Exit Diagonal	Model	$R^2 = 0.6063$ $P < 0.0001$	$R^2 = 0.5404$ $P < 0.0001$
	Taxon	$\boldsymbol{F_{1,149} = 197.53}$ ***	$\boldsymbol{F_{2,123} = 63.30}$ ***
	Site	$F_{2,149} = 2.68$	$F_{1,123} = 0.00$
	Taxon x Site	$\boldsymbol{F_{2,149} = 5.29}$ **	$\boldsymbol{F_{2,123} = 7.46}$ **

* = $P < 0.05$
** = $P < 0.01$
*** = $P < 0.001$

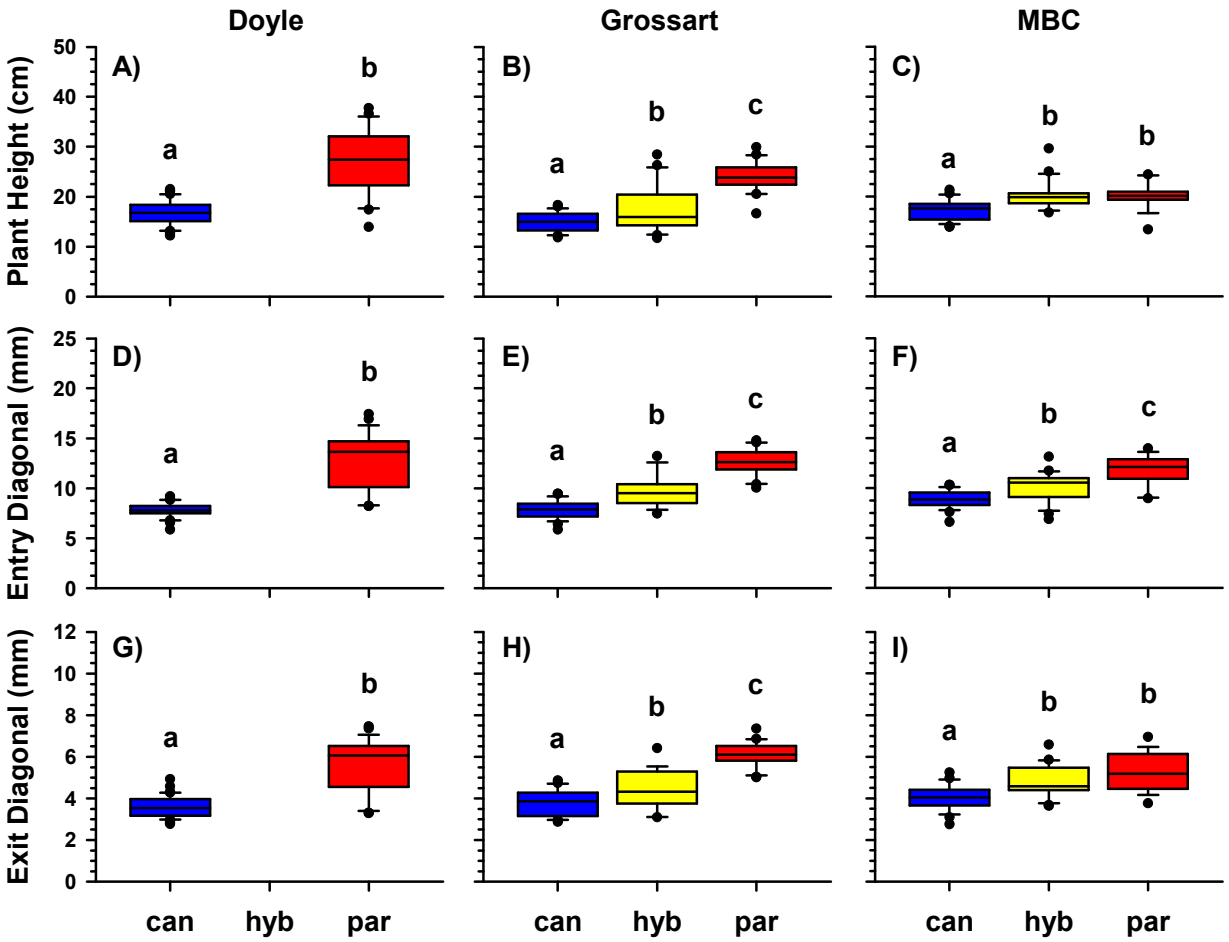

Figure 2.2: Orchid morphological traits measured for *Cypripedium candidum* (can), *C. parviflorum* (par) and their hybrids (hyb), at three sites in southern Manitoba (Doyle, Grossart, MBC) in 2010. Each box shows the median (line) and 25th and 75th percentiles (upper and lower box boundaries) for plant height (A, B, C), entry diagonal (D, E, F) and exit diagonal (G, H, I). Error bars represent the 10th and 90th percentiles, black dots represent outliers, and differences in lowercase letters indicate significant within-site differences among orchid taxa based on analyses of variance (Table 4.1).

hybrids did not differ from those dimensions of *C. parviflorum* (all $t > 0.15$, $P > 0.05$; Figure 2.2).

I also compared plant dimensions across sites within each taxon. *Cypripedium candidum* and hybrids were significantly shorter at Grossarts than they were at either MBC, or Doyle in the case of *C. candidum* (all $t > 2.43$, $P < 0.05$; Figure 2.2). By contrast, *C. parviflorum* was shorter at MBC than at two other sites (all $t > 3.33$, $P < 0.005$; Figure 2.2). Entry and exit diagonals differed across sites for *C. candidum*, with significantly larger values at MBC compared to entry routes at Doyle or Grossart, and exit routes at Doyle only (all $t > 2.67$, $P < 0.05$). *Cypripedium parviflorum* entry diagonals did not differ among sites, but exit diagonals were significantly larger at Grossart than either MBC or Doyle (all $t > 2.28$, $P < 0.05$). In hybrids, neither entry nor exit diagonals differed across sites. Separate analyses of exit length and width data showed similar trends to that found in the ANOVA of diagonal measurements.

Orchid morphology in 2010 was a significant predictor of morphology in 2011 for most taxa and traits measured. Individuals of all orchid taxa that had large values for plant height and entry diagonal in 2010, tended to also have large values in 2011 (Figure 2.3, Table 2.2). In contrast, exit route diagonal was consistent across both years in *C. parviflorum* only (Figure 2.3, Table 2.2). Although site was not included in analyses because it was confounded with 2010 floral dimensions, there did appear to be some effect of site. This was especially the case for plant height in *C. parviflorum* and entry diagonal for *C. candidum* and *C. parviflorum* (Figure 2.3). Across both years, some sites (Doyle and Grossart in particular) have taller *C. parviflorum* plants with larger entry routes and some have *C. candidum* plants with especially small entry routes (Figure 2.3).

Table 2.2: Analyses of covariance assessing the consistency of orchid plant height, entry diagonal, and exit diagonal across years (2010 and 2011). Shown are results for *Cypripedium candidum*, *C. parviflorum*, and their hybrids growing at four sites in southern Manitoba: Doyle (D), Manitoba Conservation (M), Grossart (G), and McPherson (Mc).

Dependant Variable	**Model Fit**	***C. candidum*** **(D, M, G)**	**Hybrids** **(M, G)**	***C. parviflorum*** **(D, M, G, Mc)**
Plant Height 2011	Model	$R^2 = 0.2795$, $P < 0.0001$	$R^2 = 0.4828$, $P < 0.0005$	$R^2 = 0.3595$, $P < 0.005$
	Plant Height 2010	$\mathbf{F_{1,50} = 19.40}$ ***	$\mathbf{F_{1,23} = 21.47}$ **	$\mathbf{F_{1,23} = 12.91}$ **
Entry Diagonal 2011	Model	$R^2 = 0.2870$, $P < 0.0001$	$R^2 = 0.1608$, $P < 0.05$	$R^2 = 0.2581$, $P < 0.01$
	Entry Diagonal 2010	$\mathbf{F_{1,50} = 20.12}$ ***	$\mathbf{F_{1,23} = 4.41}$ *	$\mathbf{F_{1,23} = 8.00}$ **
Exit Diagonal 2011	Model	$R^2 = 0.0131$, $P = 0.4190$	$R^2 = 0.0414$, $P = 0.3293$	$R^2 = 0.3438$, $P < 0.005$
	Exit Diagonal 2010	$F_{1,50} = 0.66$	$F_{1,23} = 0.99$	$\mathbf{F_{1,23} = 12.05}$ **

* = $P < 0.05$
** = $P < 0.01$
*** = $P < 0.001$

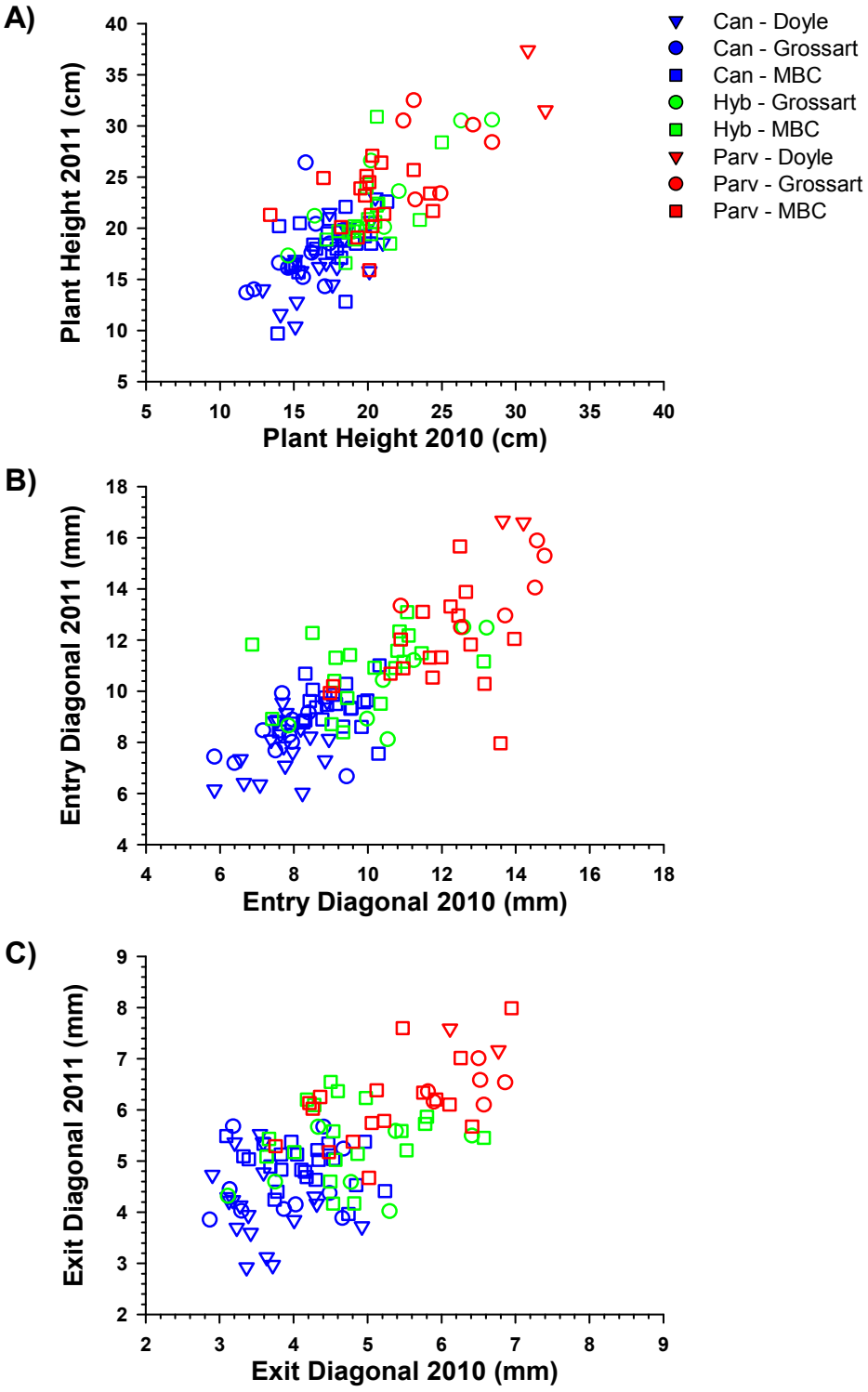

Figure 2.3: Comparison of orchid morphological characters measured in 2010 and 2011. Shown are: A) plant height, B) entry diagonal, and C) exit diagonal for *Cypripedium candidum* (Can), *C. parviflorum* (Parv), and their hybrids (Hyb) at three sites in southern Manitoba (Doyle, Grossart, and MBC).

2.3.2 Insect collection

Between 2010 and 2011, a total 132 insects were collected from orchids, 17 from *C. candidum*, 13 from hybrids, and 102 from *C. parviflorum*. Most of these (98 individuals) were collected from the MBC site in 2011. At MBC in 2011, the percentage of flowers that contained insects was relatively low for all three orchid taxa (Figure 2.4). The fewest insects were found in *C. candidum* (2.5% - 3.3% of flowers contained insects, Figure 2.4), the most in *C. parviflorum* (4.9% - 26.7%, Figure 2.4), and an intermediate number of insects were in hybrids (4.1% - 13.8%, Figure 2.4). For hybrdids and *C. parviflorum*, a greater percentage of insects were found in ribboned flowers than in those without ribbons (Figure 2.4). There were a greater number of *C. parviflorum* and hybrid flowers inspected during random flower checks than *C. candidum* (Figure 2.4). This was because the population of *C. parviflorum* was larger than that of *C. candidum* at MBC, and due to the prioritization of field activities, random walks were carried out past the peak bloom of *C. candidum*. Because of these reasons, I encountered more *C. parviflorum* flowers during random walks. Of the 98 insects collected at MBC in 2011, 33 were recorded as being alive and 58 as dead at the time of collection (data not shown). It was usually noted if and how live insects exited orchid flowers. Individuals crawled out on their own (with or without an observer present), exited with help from an observer, or died before they could exit (Table 2.3). Information is lacking for some individuals in both 2010 and 2011, regarding whether an insect was alive or dead at the time of collection (30% in 2010 and 6% in 2011), and whether or not they exited flowers (all but 3 individuals in 2010, 6% in 2011).

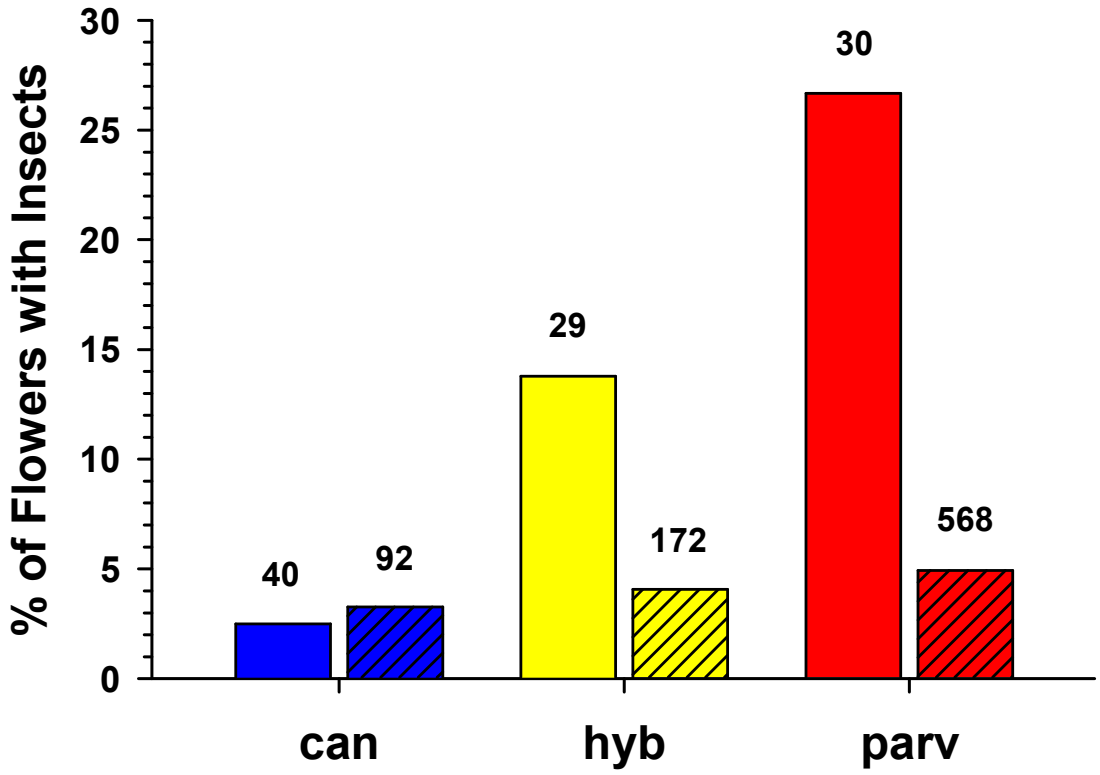

Figure 2.4: Percent of inspected *Cypripedium candidum* (can), *C. parviflorum* (parv), and hybrid (hyb), flowers that contained insects (alive or dead) in one population in southern Manitoba (Manitoba Conservation) in 2011. Solid bars represent flowers with ribbons, and cross-hatching represents flowers that were inspected randomly. N represents the total number of flowers inspected for each collection technique.

Table 2.3: Dipteran and hymenopteran visitors and potential pollinators of *Cypripedium candidum*, *C. parviflorum*, and their hybrids in 2010 and 2011 combined. Shown are insect taxa that were visitors (V) and determined to be likely potential pollinators (PP) for each orchid taxon. Potential pollinator status was determined using comparisons of insect body size with orchid exit route dimensions, and presence/absence of visible orchid smears. Question marks indicate questionable pollinator status. For live insects, exiting status (E) is noted. Numbers indicate: (1) exited with observation, (2) exited without observation, (3) were assisted in exiting, (4) were stuck to pollinia, (5) data is missing, and (6) live insect died before it could exit.

			C. candidum		**Hybrid**		***C. parviflorum***	
Order and Family	**Genus**	**Species**	V/PP	E	V/PP	E	V/PP	E
DIPTERA								
Ceratopogonidae			-	-	-	-	V	-
Conopidae	*Zodion*	*sp.*	-	-	-	-	V	-
Culicidae			V	-	V	-	V	3
Stratiomyidae	*Odontomyia*	*pubescens**	-	-	V, PP	-	V, PP	3
Syrphidae	*Eristalis*	*anthophorina**	-	-	-	-	V, PP	-
		*dimidiata**	-	-	-	-	V, PP	-
		*stipator**	-	-	-	-	V, PP	2, 3,6
	Eupeodes	*cf. americanus**	-	-	V, PP	-	-	-
		volucris	-	-	-	-	V, PP	-
	Helophilus	*latifrons**	-	-	-	-	V, PP	-
		*hybridus**	-	-	-	-	V, PP	3
	Lejops	*bilinearis**	-	-	-	-	V, PP	1
	Paragus	*sp.**	V, PP	-	-	-	-	-
	Syritta	*pipiens**	-	-	-	-	V	4
	Toxomerus	*geminatus**	V, PP	-	-	-	V	2
		*marginatus**	V, PP	4	V	-	V	4
HYMENOPTERA								
Andrenidae	*Andrena*	*carlini*	-	-	-	-	V, PP	-
		*erythrogaster**	V, PP	-	V, PP	-	V, PP	-
		prunorum	-	-	-	-	V, PP	5
		*sp.**	-	-	V, PP	-	V, PP	-
		vicina	-	-	-	-	V, PP	5
Apidae	*Apis*	*mellifera**	V	-	-	-	V, PP	1, 2
	Bombus	*ruficinctus*	-	-	-	-	V	3
Chalcidoidea^			-	-	V	-	-	-
Halictidae	*Halictus*	*rubicundus*	-	-	V, PP	5	-	-
		sp.	-	-	-	-	V, PP	5
	Lasioglossum	*leucozonium**	-	-	-	-	V	-
		paraforbesii	-	-	-	-	V	5
		*sp.**	-	-	V, ?	3	V	-
		*zonolum**	V, PP	-	-	-	V	1, 2
	Sphecodes	*sp.**	-	-	V, ?	1	-	-

^ Superfamily

* Taxon collected at MBC in 2011.

Across all sites and years (2010 and 2011), three insect orders (Diptera, Hymenoptera, Hemiptera), six families, 8 genera, and at least 9 species visited flowers of *C. candidum* (Appendix 2.5, BugGuide 2012). Only two species collected from *C. candidum* had orchid pollen smears: *Toxomerus marginatus*, and an unidentified dipteran (Appendix 2.5).

The diversity of insect visitors to hybrid flowers was comparable to *C. candidum*, and included three insect orders (Diptera, Hymenoptera, Arachnida) seven families, 10 genera, and at least 10 species (Appendix 2.5, BugGuide 2012). Five species collected from hybrids had orchid pollen smears, and these included: *Toxomerus marginatus*, *Andrena erythrogaster*, *Andrena sp*., *Halictus rubicundus*, and *Lasioglossum sp*. (sub-genus *Dialictus*; Appendix 2.5).

The greatest diversity of insects was found visiting *C. parviflorum* flowers, and represented six orders (Diptera, Hymenoptera, Coleoptera, Hemiptera, Lepidoptera, and Neuroptera), 12 families, 20 genera, and at least 32 species (Appendix 2.5, BugGuide 2012). Twelve species collected from *C. parviflorum* had orchid pollen smears: *Odontomyia pubescens*, *Eristalis stipator*, *Eupeodes volucris*, *Syritta pipiens*, *Toxomerus marginatus*, *Andrena* (four species), *Apis mellifera*, *Halictus sp.*, and *Lasioglossum zonolum* (Appendix 2.5).

2.3.3 Floral visitors and potential pollinators

Not all insects that visit a flower are able to carry out pollination. From the overall collection of insects from all sites in 2010 and 2011, only dipteran and hymenopteran species were compared with orchid exit routes and considered for potential pollinator

status (see methods section 2.2.3, Figures 2.5 and 2.6). *Cypripedium parviflorum* had the largest number of potential pollinators (8 dipterans and 7 hymenopterans), while *C. candidum* had the fewest (3 dipteran and 2 hymenopteran taxa), and hybrids only slightly more (2 dipterans and 3-5 hymenopterans; Table 2.3, Figures 2.5 and 2.6).

There were a number of dipteran visitors to all three orchid taxa. Many of the common visitors to *C. parviflorum* were likely too large to visit *C. candidum*, and perhaps also hybrids (Figure 2.5; Table 2.3). In particular, *Odontomyia pubescens* and *Eristalis stipator* appeared to be important potential pollinators of *C. parviflorum*, and some individuals also had orchid pollen smears (Appendix 2.5, Figure 2.5, Table 2.3). In both years, individuals of these two species were found alive in *C. parviflorum*, and some individuals exited on their own without observation (Table 2.3). *Odontomyia* was also an occasional visitor/potential pollinator to hybrids, but not to *C. candidum* (Figure 2.6, Table 2.3). The dipteran *Lejops bilinearis* was observed visiting and exiting *C. parviflorum*, but did not visit any other orchid taxon (Table 2.3). *Toxomerus spp*. were frequent dipteran visitors to the three orchid taxa, but appeared to be too small for pollination, even for *C. candidum* (Figure 2.5, Table 2.3). Several individuals had visible pollen smears (Appendix 2.5), however many were also found stuck to pollinia. This suggests that only individuals that are large and/or strong enough may be acting as potential pollinators, and likely only occasionally.

Hymenopternas were also visitors to all three orchid taxa, but were not considered potential pollinators in all cases (Figure 2.6, Table 2.3). Several species that frequented *C. parviflorum* flowers were not visiting *C. candidum* or hybrids at all (Figure 2.6, Table 2.3). *Apis mellifera*, a non-native species, was a frequent visitor/potential pollinator of

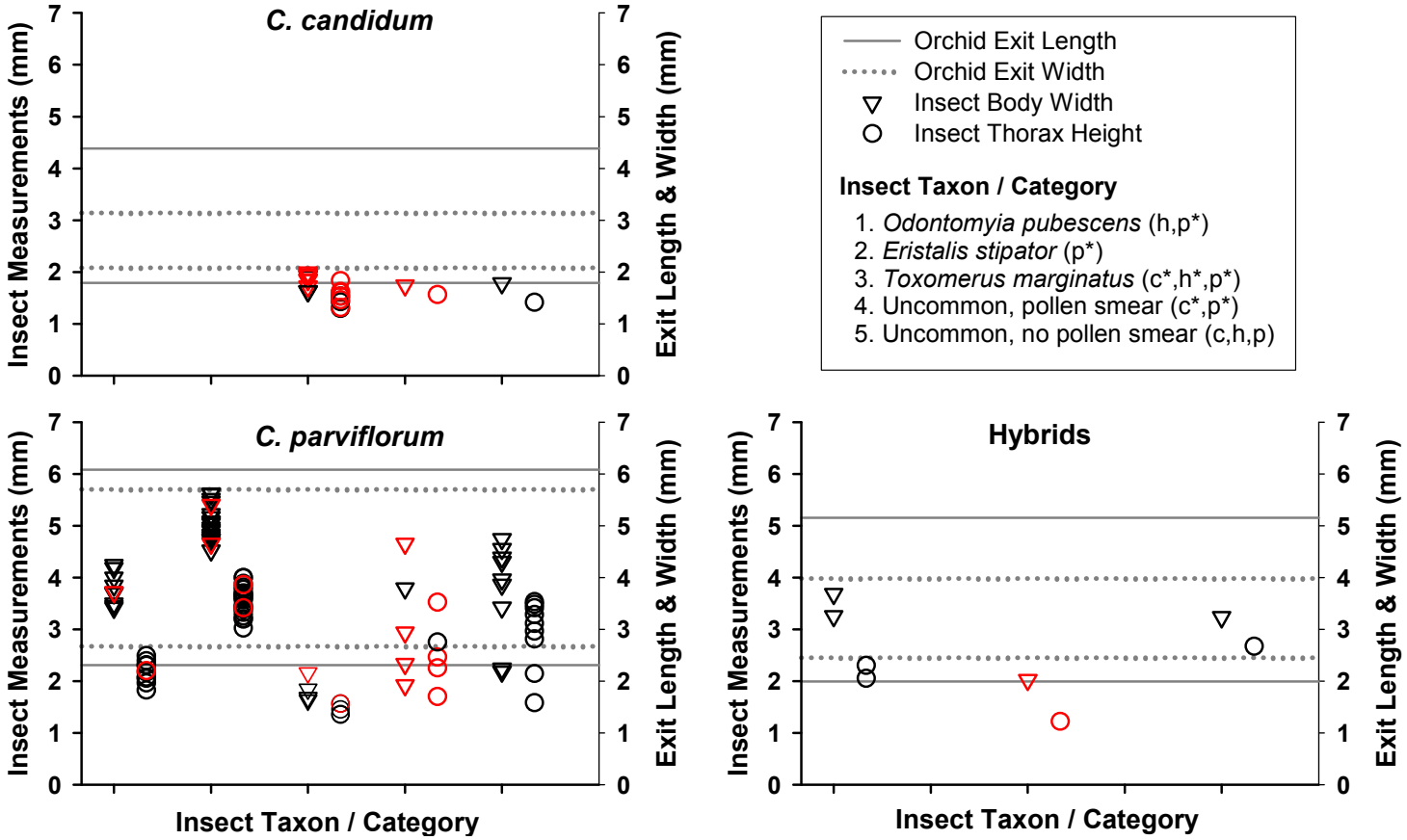

Figure 2.5: Dipteran visitors to *Cypripedium candidum*, *C. parviflorum*, and hybrid flowers at all sites in 2010 and 2011. Shown are body width and thorax height for 3 common taxa with pollen smears, uncommon taxa with pollen smears, and uncommon taxa without pollen smears. Letters in brackets represent orchid taxa in which insect taxa were found and asterisks show which orchids had insects with pollen smears. Red symbols indicate individuals with an orchid pollen smear, and black symbols had no pollen smears. Solid lines represent $5^{th}/95^{th}$ percentiles of orchid exit route length, and dotted lines represent $5^{th}/95^{th}$ percentiles of orchid exit route width measured at all sites over both years (2010 and 2011).

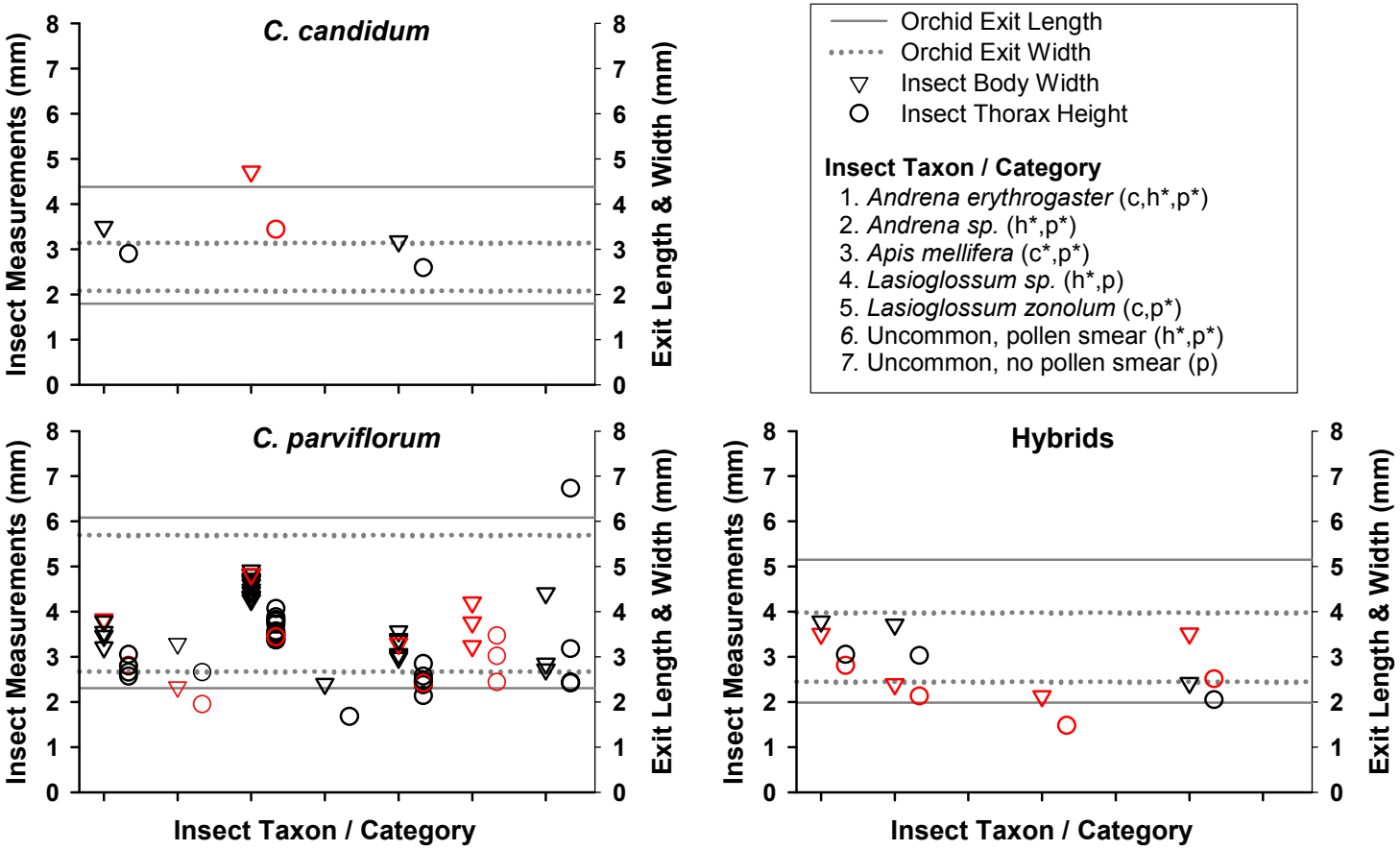

Figure 2.6: Hymenopteran visitors to *Cypripedium candidum*, *C. parviflorum*, and hybrid flowers at all sites in 2010 and 2011. Shown are body width and thorax height for three common taxa with pollen smears, uncommon taxa with pollen smears, and uncommon taxa without pollen smears. Letters in brackets represent orchid taxa in which insect taxa were found and asterisks show which orchid had insects with pollen smears. Red symbols indicate individuals with an orchid pollen smear, and black symbols had no pollen smears. Solid lines represent $5^{th}/95^{th}$ percentiles of orchid exit route length, and dotted lines represent $5^{th}/95^{th}$ percentiles of orchid exit route width measured at all sites over both years (2010 and 2011).

C. parviflorum, and two individuals exited though I only observed one of these occurrences (Figure 2.6, Table 2.3). A single individual also visited *C. candidum* and had a pollen smear, but seemed too large for this orchid (Figure 2.6, Appendix 2.5). Five *Lasioglossum zonolum* individuals exited from *C. parviflorum*, and three of these were observed, though I did not observe whether contact was made with sex organs (Table 2.3). This species however, appears too small to pollinate *C. parviflorum*, but may be a potential pollinator of *C. candidum* (Figure 2.6, Table 2.3). About half of the hymenopteran visitors/potential pollinators to hybrids were also visiting *C. parviflorum*, while the remaining taxa were found in hybrids only (Table 2.3). Pollinator status of *Lasioglossum sp.* (sub-genus *Dialictus*) and *Sphecodes sp.* was uncertain for hybrids because they are at the lower limit of exit route, but the presence of pollen smears suggests that they could be occasional pollinators (Figure 2.6, Table 2.3, Appendix 2.5). Additionally, the *Sphecodes* individual was observed using the flower's staminode to climb out of the entry route.

At MBC in 2011 there was some overlap in floral visitors and potential pollinators among orchid taxa (Table 2.3). All three orchid taxa shared only two visitors, *Toxomerus marginatus* (dipteran) and *Andrena erythrogaster* (hymenopteran), and only *A. erythrogaster* was a shared potential pollinator (Table 2.3). Between *C. candidum* and *C. parviflorum* only, *Toxomerus geminatus* (dipteran) and *Lasioglossum zonolum* (hymenopteran) were shared visitors, neither of which were shared potential pollinators (Table 2.3). Hybrids shared no visitors or potential pollinators with *C. candidum* only. However, the dipterans *Lasioglossum sp*. and *Odontomyia pubescens*, and hymenopteran

Andrena sp. were shared visitors between hybrids and *C. parviflorum*, with *O. pubescens* and *A. sp.* being shared potential pollinators (Table 2.3).

2.3.4 Orchid distribution and phenology

In addition to morphological data, I also recorded information on spatial distribution and flowering time of orchids. At Grossart, genets of *C. candidum* and hybrids had a more clustered distribution than *C. parviflorum* which was sparsely dispersed and widely spaced. Hybrids and *C. parviflorum* were also growing together along the ditch. Quadrats were mostly comprised of *C. candidum* and hybrids, or all three taxa (Figure 2.7). By contrast, the distribution of taxa At MBC was patchier with orchids growing in more localized clumps. There were a larger number of quadrats with *C. parviflorum* with either *C. candidum* or hybrids (Figure 2.7). Doyle and McPherson lacked hybrids, and provide a contrast to the highly introgressed populations at Grossart and MBC. At Doyle the distribution of *C. candidum* and *C. parviflorum* was patchy and parental taxa did not grow interspersed with one another (Figure 2.7). At McPherson, only *C. parviflorum* was observed (Figure 2.7).

All orchid taxa emerged from the ground in early spring (usually in May – personal observation). Orchid measurements were taken earlier in 2010 than in 2011 (Table 2.4). The time from emergence to flowering and the length of the flowering period varied among years. In both years, *C. candidum* flowered earlier and for a shorter period than *C. parviflorum* (Table 2.4). In 2010 most *C. candidum* flowers were measured before or concurrently with the bulk of *C. parviflorum* at all sites. Emergence also varied among sites, and in particular between Doyle and the three western sites. In both years the

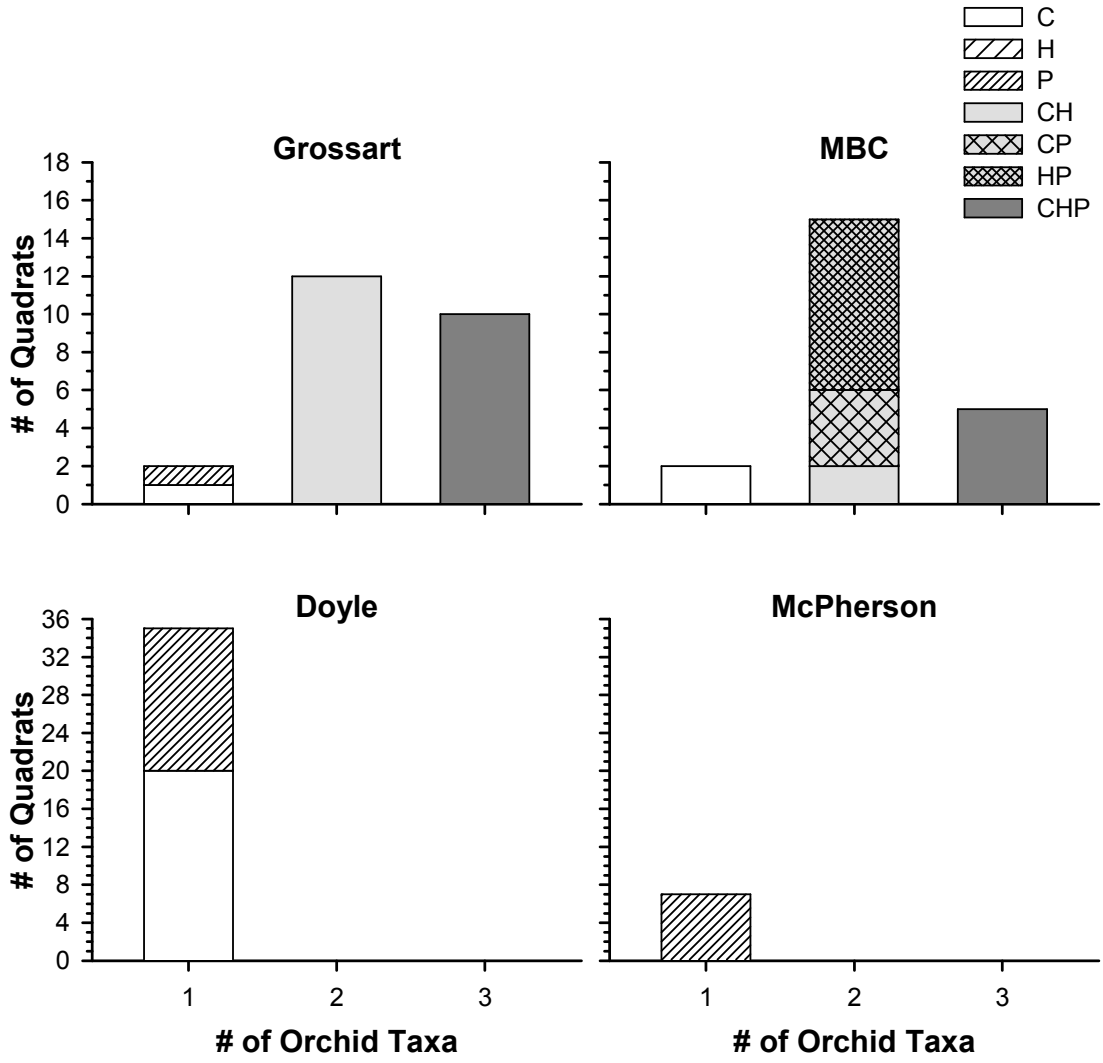

Figure 2.7: Distribution of orchid taxa among sampling quadrats at four sites in southern Manitoba (Grossart, MBC, Doyle, and McPherson). Shown are the number of quadrats containing one (C – *Cypripedium candidum* only; H – hybrids only; P – *C. parviflorum* only), two (CH – *C. candidum* and hybrids; CP - *C. candidum* and *C. parviflorum*; HP – hybrids and *C. candidum*), or three (CHP – *C. candidum*, hybrids, and *C. parviflorum*) orchid taxa. *Cypripedium parviflorum* clusters that were sampled in the ditch at Grossart, but were not in quadrats, are not included.

Table 2.4: Chronology of orchid measurements taken at four sites in southern Manitoba (Grossart, MBC, Doyle, and McPherson). Shown are the number of measurements taken on each sampling date, and the total number of measurements at each site for *Cypripedium candidum*, *C. parviflorum*, and hybrids.

	2010				**2011**			
	Date	***C. candidum***	**Hybrid**	***C. parviflorum***	**Date**	***C. candidum***	**Hybrid**	***C. parviflorum***
Doyle	May 26	28	-	4	June 3	19	-	-
	May 27	12	-	8	June 20	-	-	7
	June 9	-	-	15				
	Total	40	-	27		19	-	7
Grossart	May 20	7	2	-	June 9	5	1	-
	May 21	15	9	-	June 10	4	2	-
	May 23	3	6	3	June 11	-	1	-
	June 2	-	4	17	June 15	2	10	2
					June 16	-	7	15
					June 17	-	2	-
	Total	25	21	20		11	23	17
MBC	May 31	11	1	8	June 6	23	2	4
	June 1	14	12	10	June 10	2	14	19
	June 3	-	9	-	June 18	-	7	16
	Total	25	22	18		25	23	39
McPherson	June 2	-	-	15				
	Total			15				

earliest measurements were taken on *C. candidum* - at Grossart in 2010 and at Doyle in 2011 (Table 2.4). The latest measurements were taken on *C. parviflorum* at Doyle, and this was consistent in both years (Table 2.4). *Cypripedium parviflorum* continued to bloom past the last date on which flowers were measured. Hybrid flowering time and measurements overlapped with those of both parental species (i.e. there were early individuals with *C. candidum* and late individuals with *C. parviflorum*; Table 2.4).

2.4 Discussion

2.4.1 Comparative orchid morphology

The general patterns of orchid morphology (plant height, labellum size, entry route, and exit route) were similar between 2010 and 2011 and consistent with my hypothesis that floral features should be smaller in *C. candidum* than *C. parviflorum* with hybrids being intermediate between the parental taxa. Although this was generally the case, some aspects of hybrid morphology were more similar to *C. parviflorum* than to *C. candidum*. In particular, hybrids were more similar to *C. parviflorum* at MBC, and to *C. candidum* at Grossarts (Figure 2.2). Hybrid morphology is a direct result of gene flow between hybridizing taxa. If backcrossing and introgression is occurring more frequently with *C. parviflorum* at MBC and with *C. candidum* at Grossart, this could help explain morphological similarities between hybrids and parental species at these sites. Distribution of orchid taxa within each site may also be influencing patterns of gene flow among them. At Grossart, hybrids and *C. candidum* tend to grow intermingled in quadrats, and are morphologically more similar to one another (Figure 2.7). At MBC on the other hand, hybrids are growing with *C. parviflorum*, and these two taxa tend to have a greater morphological overlap (Figure 2.7). In contrast to Grossart and MBC, no substantial hybridization occurred at Doyle, and there was little morphological or spatial overlap between parental species (Figures 2.1 and 2.2).

The observed morphological overlap among orchid taxa and the formation of F1 hybrids may also be contributing to further introgression. Increased similarities in characters such as entry and exit routes may be facilitating increased pollinator sharing among taxa, which should in turn facilitate backcrossing to one or both parents. Orchid

flowering phenology may also be contributing to differences or similarities in morphology because hybridization can only occur when there is overlap in the bloom times between species (Vilà et al. 2000). At MBC and Grossart the morphological overlap, intermingled distribution, and phenological overlap of all three taxa, may help to explain the extensive hybridization at these sites. By contrast, *C. candidum* and *C. parviflorum* at Doyle were spatially separated within the site, had more staggered flowering times (*C. candidum* tended to flower earlier), and little morphological overlap, which could be contributing to the lack of observed hybrids. Spatial and temporal separation of *C. candidum* and *C. parviflorum* has been reported by other authors as a means of reproductive isolation between these species (Bowles 1983, Klier et al. 1991).

Among taxa, the general differences in orchid traits were consistent across years. The observed patterns of morphology, when considered with potential annual variation in the insect community, could be important for the "correct fit" between flowers and insects. *Cypripedium parviflorum* had the largest entry and exit routes, tended to start blooming later, and bloomed for the longest period. In combination, these traits could expose *C. parviflorum* to larger, and perhaps more abundant, insects.

For individual plants, morphology in 2010 was generally a predictor of morphology in 2011, with the exceptions of exit diagonal in *C. candidum* and hybrids. This may be in part because small variations in flower shape may cause measureable differences in entry and exit routes, and implies that the fit between individual plants and their pollinators may vary from year to year. Additionally, when measuring smaller spaces such as exit route in hybrids and *C. candidum*, small mis-measurements represent a larger error than when measuring larger spaces.

2.4.2 Floral visitors and potential pollinators

Observed variation in the number and diversity of floral visitors/potential pollinators among orchid taxa may be affecting opportunities for pollination, resulting in differential reproductive success. Overall, the fewest insect visitors were found on *C. candidum* flowers and the most on *C. parviflorum* (Figure 2.4), which is consistent with my preliminary field observations. There are several factors that could be contributing to this variation. Differences in flowering time and/or period could be exposing orchids to different complements of insects. Furthermore, smaller floral dimensions, entry and exit routes in particular, could be restricting the number of pollinators in *C. candidum* more than in the other two taxa. Additionally it is possible that other traits such as plant height, flower colour, and scent may be making *C. parviflorum* a more attractive species overall (see Faegri and van der Pijl 1979; Proctor et al. 1996). Lower visitation to *C. candidum* could be leading to reduced pollen removal and deposition in this species. If reproduction is pollen limited, as is the case for many rewardless orchids, a decrease in pollen transfer could result in reduced fruit and/or seed set (see Chapter 3, Neiland and Wilcock 1998; Smithson and Gigord 2001).

As expected, the number of insect taxa that may be considered potential pollinators was generally less than the number of floral visitors to each orchid taxon. However, I did not expect that dipterans would be an important group of potential pollinators. If my definition had been more restricted to effective pollinators that were directly observed exiting flowers, contacting sex organs, and carrying *Cypripedium* pollen, my results likely would have been more comparable to the number of pollinators reported by other authors (Figures 2.5 and 2.6, Table 2.3). Effective pollinators for *C. candidum* have been

reported to range from one bee species (Stoutamire 1967) to several (Catling and Knerer 1980), despite the observation of a larger number of floral visitors. Case and Bradford (2009) investigated the pollination system of *C. parviflorum* and found 10 species of hymenopteran floral visitors with only two species qualifying as effective pollinators (as these were the only individuals to remove pollen).

Several of the hymenopterans reported as pollinators of other *Cypripedium* species were important at MBC as well. *Andrena* species for example, were pollinators in other studies of *C. candidum*, *C. parviflorum*, and *C. flavum* (Table 2.3, Appendix 2.1). Halictine bees, such as *Lasioglossum* and *Halictus*, were also pollinators in other studies of *C. candidum*, *C. henryii*, *C. plectrochilum*, *C. yunnanense*, *C. arienatum*, and *C. guttatum* (Table 2.3, Appendix 2.1). The non-native hymenopteran *Apis mellifera* was likely contributing to pollination of *C. parviflorum*, and was also reported for *C. reginae* (Edens-Meier et al. 2011). *Bombus* species on the other hand, are pollinators of some *Cypripedium* species (Appendix 2.1), but seemed to be too large to pollinate even *C. parviflorum* at MBC (Table 2.3).

In this study, dipterans were also found to have a potentially important role in pollination of all orchid taxa at MBC, despite previous observations of hymenopterans as effective pollinators for *C. candidum* and *C. parviflorum* (Appendix 2.1). In Manitoba populations, dipterans have also been observed as common visitors to other spring co-flowering species (Semmler, in prep). This contrasts with the lack of reported dipterans in other *C. candidum* and *C. parviflorum* populations (Case and Bradford 2009; Catling and Knerer 1980). Additionally, this study was carried out at the edge of *C. candidum*'s range. It is possible that the complement of insects or the proportion of dipterans that are present

when orchids bloom, differs between Manitoban and other populations of *C. candidum* and *C. parviflorum* (e.g. Case and Bradford 2009, Catling and Knerer 1980). Direct observations of insect visitation and pollen removal/deposition would make a very valuable contribution to better understanding the relationships between these orchid species and their pollinators.

Differences in floral architecture (entry and exit routes in particular) may be restricting the number and variety of pollinators, which could translate into differences in pollination success (i.e. a larger entry/exit may allow for a greater variety of pollinators). Additionally, it may be that exit routes are the most important limiting factor in restricting effective pollinators, since they are smaller than entrance routes. In this study, *C. candidum* had the smallest entry and exit routes, which may be part of the reason that the number of potential pollinators was more limited (i.e. it may be more specialized in terms of its pollinators that the larger hybrids and *C. parviflorum*). The size of floral visitors relative to floral morphology has also been reported as a limiting factor to effectiveness of pollinators in other *Cypripedium* species. For example, Li et al. (2006) found that bumble bee workers were a poor fit with *C. tibeticum* floral functional morphology, but that bumblebee queens were suitable pollinators. These authors also found that floral morphology restricted which insects acted as pollinators in *C. henryi* and *C. plectrochilum* (Li et al. 2008a and b).

2.4.3 Floral morphology and pollinator sharing

At MBC in 2011, I found that floral visitors, potential pollinators, and entry/exit routes overlapped among the orchid taxa. Along with the observed hybridization

occurring at MBC, these findings confirm that pollinator sharing is occurring at this site. Among insects collected at MBC in 2011 there was overlap in floral visitors between *C. candidum* and *C. parviflorum* as well as between hybrids and both parental taxa. At this site, hybrids seemed to be sharing more visitors and pollinators with *C. parviflorum* than *C. candidum*. The dipteran *Odontomyia pubescens* and hymenopterans of the genus *Andrena* seemed to be contributing to hybridization between hybrids and *C. parviflorum*. These were common visitors to *C. parviflorum*, but less common to hybrid flowers. *Andrena erythrogaster* was the only pollinator potentially contributing to gene flow among all three orchid taxa, and most commonly visited *C. parviflorum*. The non-native hymenopteran *Apis mellifera* visited both *C. candidum* and *C. parviflorum*. From the single individual collected from *C. candidum*, which seemed to be too large for effective pollination but had a visible orchid pollen smear (which could have come from a previously visited *C. parviflorum*), it is unclear whether this species is contributing to hybridization between parental taxa. When taken into consideration with the observed morphological similarity, overlap in phenology, and spatial intermingling between *C. parviflorum* and hybrids, it is possible that there is greater gene flow between them at this site, than between hybrids and *C. candidum*. Worley et al. (2009) found that in other Manitoba populations, *C. candidum* and *C. parviflorum* plants remained genetically distinct, while for hybrids back-crossing and introgression were likely bi-directional.

2.4.4 Conclusions

In conclusion, I found that variation in morphology and insect visitation among orchid taxa could have implications for differences in pollination and reproductive

success (see Chapter 3). Exit routes may be especially significant for restricting the number and variety of floral visitors that can effect pollination. Overlap in morphology, phenology, and potential pollinators among orchid taxa are required for hybridization, and in this study I found evidence of this at MBC. Hymenopterans of the genus *Andrena* and the dipteran *Odontomyia pubescens* seemed the most likely contributors to hybridization among orchid taxa. Based on the observed hybridization at Grossart, I expect there to be pollinator sharing occurring at this site as well. At Doyle I observed that lack of hybridization may be partly due to differences in phenology and spatial separation between *C. candidum* and *C. parviflorum*. Although few pollinators were collected from Doyle, I expect that the two parental taxa share fewer potential pollinators, if any, than plants at MBC or Grossart.

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Appendix 2.1: Floral visitors and potential or effective pollinators reported in *Cypripedium* orchid species.

Cypripedium Species	Floral Visitors Observed/Reported	Potential/Effective Pollinators Observed/Reported	Reference
Cypripedium acaule	Diptera Hymenoptera (small bees) Aranaea (crab spider) Lepidoptera	Hymenoptera (2 species of *Bombus*)	Stoutamire 1967
Cypripedium arietinum	Hymenoptera	Hymenoptera (1 species of *Dialictus*, 2 species of *Lasioglossum*)	Stoutamire 1967
Cypripedium calceolus	Thysanoptera (1 species of *Taeniothrips*) Lepidoptera (2 genera) Coleoptera (7 genera) Diptera (18 genera) Hymenoptera (4 genera)	Hymenoptera (3 genera)	Nilsson 1979
	Hymenoptera (8 genera) Diptera (3 genera)	Hymenoptera (4 genera)	Stoutamire 1967
	Hymenoptera (5 species of *Andrena*) Diptera (4 genera)		Müller 1883
	Diptera (3 species)		Darwin 1887
	Coleoptera (1 species of *Anthaxia*) Hymenoptera (1 species of *Andrena*)	Hymenoptera (1 species of *Osmia*)	Guignard 1886
	Hymenoptera (species of *Ceratina*)		Robertson 1928
Cypripedium candidum		Hymenoptera (1 species of *Adrena*)	Stoutamire 1967
	Hymenoptera (4 genera) Elateridae (1 species)	Hymenoptera (3 genera: *Augochlorella*, *Halictus*, *Dialictus*)	Catling and Knerer 1980
Cypripedium flavum	Andrenidae (6 species)	Hymenoptera (2 species of *Adrena*)	Bänziger et al. 2008
Cypripedium guttatum	Hymenoptera (5 families)	Hymenoptera (3 species of *Lasioglossum*)	Bänziger et al. 2005

Appendix 2.1: Cont'd

Cypripedium Species	Floral Visitors Observed/Reported	Probable/Effective Pollinators Observed or Reported	Reference
Cypripedium henryii	Araneida (1 species) Coleoptera (7 genera) Hymenoptera (2 genera) Lepidoptera (2 genera)	Hymneoptera (3 species of *Lasioglossum*)	Li et al. 2008b
Cypripedium parviflorum	Hymenoptera (4 genera)	Hymenoptera (2 species of *Adrena*)	Case and Bradford 2009
Cypripedium plectrochilum	Diptera (6 genera) Hymenoptera (3 genera) Lepidoptera (1 genus)	Hymenoptera (3 species of *Lasioglossum*, 1 species of *Certina*)	Li et al. 2008a
Cypripedium tibeticum	Hymenoptera (3 genera) Diptera (4 genera) Coleoptera (3 genera) Orthoptera (1 species)	Hymenoptera (3 species of *Bombus*)	Li et al. 2006
Cypripedium reginae		Hymenoptera (1 species of *Bombus*)	Raffil 1913
	Lepidoptera (3 genera) Coleoptera (1 genus)	Hymenoptera (2 species of *Megachile*)	Guignard 1886, 1887
		Hymenoptera (one unidentified medium sized black bee)	Stoutamire 1967
	Coleoptera (2 genera) Diptera (1 species) Hymenoptera (11 genera) Lepidoptera (several genera)	Hymenoptera (1 species of *Apis*, 2 species of *Anthophora*, 1 species of *Hoplitis*, 1 species of *Megachile*)	Edens-Meier et al. 2011
Cypripedium. yunnanense	Halictidae (10 species)	Hymenoptera (1 species of *Lasioglossum*)	Bänziger et al. 2008

Appendix 2.2: Taxon sampling distributions in 2010 and 2011 for *Cypripedium candidum, C. parviflorum* and putative hybrids in four Manitoba populations. Included are: number of quadrats in which each taxon is present, number of tagged clusters, number of orchid morphological measurements, and number of capsules collected. Some orchids were not identified beyond the genus level, and are noted as "*C.* sp".

	Site							
	Grossart[h,c,p*]		**MBC**[h,c,p]		**McPherson**[p]		**Doyle**[c,p*]	
	2010	2011	2010	2011	2010	2011	2010	2011
# of Quadrats								
Total	16	24	22	22	7	7	35	34
C. candidum	15	23	11	12	-	-	20	20
C. parviflorum	6	9	17	18	7	7	15	14
Hybrids	14	22	16	16	-	-	-	-
C. sp	15	14	3	4	-	-	-	-
# of Tagged Clusters								
C. candidum	249	456	46	61	-	-	194	231
C. parviflorum	47	58	76	104	81	98	76	93
Hybrids	83	107	32	37	-	-	-	-
C. sp	47	48	1	8	-	-	-	-
# of Measurements								
C. candidum	25	11	25	25	-	-	40	19
C. parviflorum	20	17	18	39	15	-	27	7
Hybrids	20	23	22	23	-	-	-	-
C. sp	-	-	-	-	-	-	-	-
# of Capsules Collected								
C. candidum	6	12	3	10	-	-	6	10
C. parviflorum	11	12	12	12	14	12	12	15
Hybrids	6	12	11	12	-	-	-	-
C. sp	-	-	-	-	-	-	-	-

[h,c,p] = populations from which fruit and fruit set data were collected ([h]=hybrids, [c]=*C. candidum*, [p]=*C. parviflorum).*

* *C. parviflorum* were also sampled along the ditch (not in quadrats).

Appendix 2.3: Body measurements of insects collected at four sites in southern Manitoba over two years (2010 and 2011 combined). Shown are the range and mean for thorax height, body length, thorax width, and abdomen width. N represents the number of insects of each taxon that were measured. Only insect taxa that were found visiting orchid taxa are included.

Order and Family	Genus	Species	N	Body Length (mm)	Thorax Height (mm)	Thorax Width (mm)	Abdomen Width (mm)
DIPTERA							
Calliphoridae	*Lucilia*	*silvarum*	1	8.16	3.63	4.37	3.78
Ceratopogonidae			1	-	-	-	-
Conopidae	*Zodion*	*sp.*	1	6.39	2.14	2.24	1.63
Culicidae			3	-	-	-	-
Stratiomyidae	*Odontomyia*	*pubescens*	13	7.36-9.39 (x=8.32)	1.82-2.49 (x=2.18)	2.62-3.44 (x=3.01)	3.25-4.24 (x=3.75)
Syrphidae	*Cheilosia*	*sp.*	1	7.72	2.4	3.32	3.27
	Eristalis	*anthophorina*	1	9.64	3.53	4.48	4.74
		dimidiate	1	10.83	3.52	4.6	4.65
		stipator	24	9.40-12.34 (x=11.21)	3.02-4.00 (x=3.56)	3.99-5.36 (x=4.66)	4.52-5.62 (x=4.99)
	Eupeodes	*cf. americanus*	1	11.47	2.67	3.16	3.23
		volucris	2	8.51-10.42 (x=9.47)	2.46-2.75 (x=2.61)	2.53-3.2 (x=2.87)	2.94-3.79 (x=3.37)
	Helophilus	*latifrons*	3	11.46-12.53 (x=12.09)	2.81-3.48 (x=)3.19	3.41-4.32 (x=3.83)	3.42-4.56 (x=4.06)
		hybridus	2	11.26-12.33 (x=11.80)	2.96-3.41 (x=3.19)	3.88-4.30 (x=4.09)	3.79-4.38 (x=4.09)
	Lejops	*bilinearis*	1	8.88	3.11	3.86	3.26
	Paragus	*sp.*	1	4.47	1.56	1.74	1.66
	Syritta	*pipiens*	3	6.98-8.34 (x=7.84)	1.70-2.25 (x=1.97)	1.92-2.33 (x=2.16)	1.44-2.17 (x=1.74)
	Toxomerus	*geminatus*	2	4.87-6.14 (x=5.51)	1.41-1.58 (x=1.50)	1.78-1.87 (x=1.83)	1.72-2.18 (x=1.95)
		marginatus	17	4.41-6.30 (x=5.34)	1.22-1.83 (x=1.47)	1.36-1.92 (x=1.63)	1.46-2.16 (x=1.81)
Tachinidae	*Peleteria*	*sp.*	2	8.16-8.5 (x=8.33)	2.8-3.05 (2.93)	3.25-3.83 (x=3.54)	3.37-3.92 (x=3.65)
Dipterans			3	-	-	-	-
HYMENOPTERA							
Andrenidae	*Andrena*	*carlini*	1	11.17	3.47	3.49	4.20
		erythrogaster	9	9.79-11.26 (x=10.43)	2.51-3.05 (x=2.79)	2.73-3.36 (x=3.05)	3.21-3.89 (x=3.56)
		prunorum	1	12.23	3.02	3.62	3.76
		sp.	6	6.42-10.36 (x=8.26)	1.66-3.03 (x=2.35)	2.13-3.41 (x=2.72)	2.19-3.71 (x=2.90)
		vicina	1	12.04	3.18	3.78	4.41
Apidae	*Apis*	*mellifera*	14	10.32-12.15 (x=11.12)	3.37-4.07 (x=3.62)	3.74-4.33 (x=4.05)	4.26-4.91 (x=4.62)
Apidae	*Bombus*	*rufocinctus*	1	18.59	6.73	7.48	8.01

Appendix 2.3: Cont'd

Order and Family	Genus	Species	N	Body Length	Thorax Height	Thorax Width	Abdomen Width
Chalcidoidea (superfamily)			1	-	-	-	-
Halictidae	*Halictus*	*rubicundus*	1	9.9	2.51	3.1	3.52
		sp.	1	9.35	2.44	2.57	3.24
	Lasioglossum	*leucozonium*	1	9.02	2.44	2.84	2.85
		paraforbesii	1	8.33	2.42	2.72	2.71
		sp.	6	4.89-6.65 (x=5.64)	1.45-1.68 (x=1.53)	1.49-2.01 (x=1.82)	1.71-2.40 (x=1.98)
		zonolum	9	8.54-10.31 (x=9.23)	2.14-2.85 (x=2.51)	2.83-3.15 (x=2.96)	2.98-3.56 (x=3.22)
	Sphecodes	*sp.*	1	8.08	2.05	2.42	2.38
ARACHNIDA							
Thomisidae			1	-	-	-	-
COLEOPTERA							
Curaculionidae			1	2.79	1.23	-	1.5
HEMIPTERA							
Membracidae			2	-	-	-	-
Pentatomidae			3	11.26-12.19 (x=11.58)	3.67-4.17 (x=3.90)	-	6.46-7.06 (x=6.85)
Thyreocoridae			1	5.33	2.14	-	3.68
LEPIDOPTERA			1	-	-	-	-
NEUROPTERA							
Chrysopidae			1	-	-	-	-

Appendix 2.4: Principal components analysis of eight morphological traits measured on *Cypripedium candidum*, *C. parviflorum*, and their hybrids across all sites in 2010 and 2011. Shown are loadings of each trait on axes one and two.

	2010		2011	
Morphological Trait	Axis One	Axis Two	Axis One	Axis Two
Labellum Colour	0.3558	-0.0658	0.3581	-0.1026
Plant Height	0.3535	-0.1658	0.3234	-0.5260
Labellum Length	0.3774	-0.2198	0.3771	-0.1611
Labellum Width	0.3802	-0.2034	0.3806	-0.1847
Entry Length	0.3483	0.2340	0.3535	-0.0406
Entry Width	0.3838	0.0137	0.3783	-0.0450
Exit Length	0.2523	0.8873	0.3219	0.6170
Exit Width	0.3593	-0.1903	0.3293	0.5181

Appendix 2.5: Insects recorded as flower visitors to *Cypripedium candidum*, *C. parviflorum*, and their hybrids over two sampling years (2010 and 2011 combined). Shown are the numbers of each insect taxon found in each orchid taxon. Numbers in brackets represent the insects with visible pollen smears.

Order and Family	Genus	Species	*C. candidum*	Hybrid	*C. parviflorum*
DIPTERA					
Ceratopogonidae			-	-	1
Conopidae	*Zodion*	*sp.*	-	-	1*
Culicidae			1	1	1
Stratiomyidae	*Odontomyia*	*pubescens*	-	2	10 (1)
Syrphidae	*Eristalis*	*anthophorina*	-	-	1
		dimidiata	-	-	1
		stipator	-	-	22 (3)
	Eupeodes	*cf. americanus*	-	1	-
		volucris	-	-	2* (1)
	Helophilus	*latifrons*	-	-	3
		hybridus	-	-	2
	Lejops	*bilinearis*	-	-	1
	Paragus	*sp.*	1	-	-
	Syritta	*pipiens*	-	-	2 (2)
	Toxomerus	*geminatus*	1	-	1
		marginatus	9 (6)	1 (1)	5 (3)
Dipterans			1 (1)	-	2
HYMENOPTERA					
Andrenidae	*Andrena*	*carlini*	-	-	1 (1)
		erythrogaster	1	1 (1)	5 (1)
		prunorum	-	-	1 (1)
		sp.	-	2 (1)	2 (1)
		vicina	-	-	1
Apidae	*Apis*	*mellifera*	1*	-	13+ (1)
Apidae	*Bombus*	*rufocinctus*	-	-	1*
Chalcidoidea^			-	1	-
Halictidae	*Halictus*	*rubicundus*	-	1* (1)	-
		sp.	-	-	1* (1)
	Lasioglossum	*leucozonium*	-	-	1
		paraforbesii	-	-	1
		sp.#	-	1 (1)	1
		zonolum	1	-	7 (1)
	Sphecodes	*sp.*	-	1	-
ARACHNIDA					
Thomisidae			-	1	-
COLEOPTERA					
Curaculionidae			-	-	1*
HEMIPTERA					
Membracidae			-	-	2
Pentatomidae			-	-	3+

Appendix 2.5: Cont'd

Order and Family	Genus	Species	*C. candidum*	Hybrid	*C. parviflorum*
Thyreocoridae			1	-	-
LEPIDOPTERA			-	-	1
NEUROPTERA					
Chrysopidae			-	-	1

***** Insect species collected from site(s) other than MBC
+ Insect species collected from MBC and additional site(s)
^ Superfamily
# Sub-genus *Dialictus*

3.0 Chapter Summary

Fruit and seed set were surveyed in *Cypripedium candidum*, *C. parviflorum*, and their hybrids to assess female reproductive success and to determine which traits were correlated with variation in fruit set within taxa. Fruit set varied across years and sites, but was consistently lowest in *C. candidum*, highest in *C. parviflorum*, and intermediate in hybrids. The number of flowering stems per cluster was highest in *C. candidum*, lowest in *C. parviflorum* and again intermediate in hybrids. Analyses of morphological characters showed that hybrid plants with larger floral exit routes, and taller *C. parviflorum* plants were more likely to set fruit. Within each taxon, plants with a larger number of flowering stems were more likely to set fruit. The number of ovules, seeds with mature embryos, and dead embryos showed the same trend as fruit set (*C. parviflorum* > hybrids > *C. candidum*). However, when total number of ovules was included as a factor, the proportion of seeds with mature and dead embryos was similar in all three taxa. As female reproductive success in hybrids was intermediate between both parental taxa, I concluded that they are not showing any evidence of sterility. Differences in fruit set among orchid taxa seem to be influenced by several factors, including flowering phenology, floral architecture, variation in spring climate and pollinator communities, and spatial distribution of orchids.

3.1 Introduction

Reproductive success in orchids with rewardless flowers may be affected by many factors that may act together or separately across time and space (Ackerman and Montalvo 1990; Calvo 1993; Campbell and Halama 1990; Lipow et al. 2002; Primack et al. 1994). Orchids are often characterized by low fruit set, which is generally lower (11.5% to 41.4%) for rewardless orchids than rewarding species (24.9% to 74.4%) (Neiland and Wilcock 1998). Because visitation is low in deceptive orchids, and insects can learn to avoid them, factors that affect the visibility and attractiveness of individual plants to inexperienced pollinators may be of particular importance for reproductive success (Bell et al 2005; Internicola et al. 2007, 2008; Johnson et al. 2003; Juillet et al. 2007; Lammi and Kuitunen 1995; Thomson 1978; Waser 1978). For example, plants that are taller, or have more attractive flower colouration may be visited more often if these traits make them more visible to insects (O'Connell and Johnston 1998; Walsh and Michaels 2012a). The size of floral display (number of flowers) may also affect insect pollinator foraging behavior because larger inflorescences may be initially more attractive to pollinators (Lipow et al. 2002; Walsh and Michaels 2012b).

As described in Chapter 2, *Cypripedium* orchids have a restrictive floral architecture that may influence the pool of insect visitors that are able to act as effective pollinators (Bänziger et al. 2008; Li et al. 2006, 2008a, 2008b; Nilsson 1979; Stoutamire 1967). Variation in entry and exit routes could affect reproductive success if potential pollinators are either too large to maneuver through the flower to gain access to the sexual organs, or too small to contact the sex organs. Differences in fruit set among species may occur if the pool of potential pollinators is more limited due to floral

architecture in one taxon versus another, although this has not been previously explored in either *C. candidum* or *C. parviflorum*.

Fruit set, either fruit production per plant or per flower, has been reported for rewardless orchid taxa by many researchers (e.g. Ackerman and Montalvo 1990; Hogan 1983; Snow and Whigham 1989; Zimmerman and Aide 1989). Within the subfamily Cypripedioideae, fruit set has been measured in at least 15 of the approximately 47 known *Cypripedium* species, and varies within and among taxa (Appendix 3.3, Bernhardt and Edens-Meier 2010). Few studies, however, have investigated the relationship between plant traits and reproductive success in *Cypripedium* orchids. For example, flower height and labellum length, in addition to microhabitat, were associated with pollination and reproductive success in *C. acaule* (O'Connell and Johnston 1998). In Estonian populations of *C. calceolus*, clones consisting of a greater number of flowers set relatively fewer fruits (Kull 1998), while the number of flowers per inflorescence was unrelated to fruit set per flower in *Cypripedium fasciculatum* (Lipow et al.2002). To my knowledge Walsh and Michaels (2012a) is the only study that has looked at plant traits in relation to fruit set in *C. candidum*. In the investigation of a single population, they found that taller *C. candidum* plants had higher pollinia removal and fruit set, while the number of stems and flowering stems did not influence pollinia removal or fruit set.

Seed characteristics provide a more refined measure of female reproductive success, because they provide information about the number and viability of individual offspring. The two measures are complementary to one another, especially in plants that have multi-floral inflorescences or multiple flowering stems per plant. In those with solitary flowers seed set may more useful, because fruit set has only two possible outcomes: a plant either

successfully set fruit or did not (Proctor and Harder 1994). When used together, fruit and seed set can provide a more in depth understanding of reproductive success than either of the two measures alone.

The number of ovules, abnormal embryos, and of fully developed seeds (i.e. number of fertilized ovules) may be influenced by hybrid sterility, inbreeding depression, or self-incompatability (Bernhardt and Edens-Meier 2010, Tremblay et al. 2005). If hybrids are producing fruits with much fewer, or much less viable, seeds than parental taxa, then the risk for extensive hybridization, or extinction of parental taxa due to backcrossing may be much less than if hybrids are more fit than parents (Campbell and Aldridge 2006; Cozzolino and Scopece 2008; Rieseberg 1997).

The proportion of ovules that are fertilized and develop into seeds can also be influenced by the amount of pollen received (Proctor and Harder 1994; Tremblay et al. 2005). Most orchids package their pollen into masses called pollinia, which are connected to a sticky viscidium (Cozzolino and Widmer 2005; Nilsson 1992). This adaptation is thought to promote efficient pollen transfer when entire pollinia are removed by a single visitor, deposited directly on the stigma of another flower, and contain enough pollen to completely fertilize all ovules (Ardetti 1992; Cozzolino and Widmer 2005; van der Pijl and Dodson 1966). By contrast, *Cypripedium* orchids have pollinia that disperse pollen as a sticky smear on a number of pollinators (Nilsson 1979; Proctor and Harder 1994; Stoutamire 1967). This may result in pollen transfers that do not necessarily contain enough pollen to fertilize all ovules of a given flower, especially if visitation is low, and these orchids may then suffer from pollen limitation. Additionally, substantial ovule development does not begin in many orchids until after pollination has occurred, and

fertilization in some *Cypripedium* species has been reported to take place three or four months after pollination (Dressler 1990; Duncan and Curtis 1942). To my knowledge, Proctor and Harder (1994) is the only study that has looked at pollen load and seed set in *Cypripedium* orchids. The authors found that an increase in pollen deposition had no effect on the number of seeds produced in *Cypripedium calceolus* (= *C. parviflorum*), although it was noted that small sample sizes may have compromised statistical power.

The primary focus of this chapter is to investigate female reproductive success within and among orchid taxa and populations (sites), and to explore the influence of plant and floral traits on fruit set.

My first objective is to compare fruit set of *C. parviflorum*, *C. candidum*, and their hybrids. In accordance with the low reproductive success generally reported for rewardless plant species, I expect to find relatively low fruit set for all three orchid taxa. However, based on the observed patterns of insect visitation reported in Chapter 2 (see section 2.4.2), I expect that fruit set among orchid taxa will be highest in *C. parviflorum*, lowest in *C. candidum* and intermediate in hybrids.

My second objective is to determine seed set values in each population, and compare them among the three orchid taxa. Preliminary observations in the field suggest that *C. parviflorum* may receive more insect visitors, and thus more pollen than both *C. candidum* and hybrids (see section 2.4.2). Based on these observations, I expect to find comparatively higher rates of seed set in *C. parviflorum* than *C. candidum*. Additionally, hybrid offspring can be sterile or less fit than parental taxa (Rieseberg 1995). In this study, I expect to find evidence for hybrid sterility, with hybrid capsules having no seeds, fewer seeds, or more seeds with abnormal embryos than the two parental taxa.

My third objective is to investigate whether certain plant features (floral morphology, plant height, and number of flowers) are associated with orchid fruit set. Due to *Cypripedium*'s restrictive floral architecture and its role in pollination, I expect to find that floral dimensions will be associated with the likelihood of setting fruit. Data presented in Chapter 2 showed that entry and exit routes of *C. parviflorum* were largest, and should therefore be open to a wider pool of pollinators (see sections 2.4.1 and 2.5.3). Additionally, insect visitation was highest in this species. In light of this, I would expect that *C. parviflorum*, and those phenotypes closest to it would be more likely to set fruit. Based on personal observations, I also expect that the number of flowering stems per cluster will differ among orchid taxa, and that *C. candidum* clusters will consist of the most flowering stems. Because floral displays can affect insect foraging behavior, and larger displays may be more attractive, I also predict that, within each orchid taxon, the number of flowering stems will be significantly and positively associated with the probability that individuals will set fruit.

My research will contribute further to our general knowledge of *C. candidum* and *C. parviflorum* reproductive success for a number of reasons. Firstly, I investigated four populations over three sampling season, which will enable me to make comparisons between sites and across years. Secondly, three of my populations consisted of multiple orchid taxa, two of which included hybrid individuals. This will enable me to make comparisons not only between taxa at each sites, but among sites as well. And finally, the populations that I studied are at the edge of *C. candidum's* range, where very few studies have been carried out to date. By contrast, most other studies to date have been carried

out in more southerly populations of *C. candidum* and *C. parviflorum* (Appendix 3.3, e.g. Bowles 1983; Case and Bradford 2009; Shefferson and Simms 2007).

3.2 Materials and Methods

3.2.1 Fruit Set Data

All sampling in 2010 and 2011 was carried out within the permanent sampling plots set up at all four sites, as described in Chapter 2. Flowering and fruiting data were collected within quadrats, with the exception of some *C. parviflorum* individuals at Grossart and Doyle that were located in the ditch (see Appendix 2.2). For all tagged clusters of each orchid, the following information was recorded: taxon, number of vegetative stems (2010 only), number of flowering stems, and number of stems in bud. I revisited quadrats later in the season to record the number of fruiting stems for each cluster and collect between 3 and 15 mature capsules from each taxon at each site (see Appendix 2.2). For some individuals fruit set could not be recorded because of the inability to relocate individual plants, removal of plants during ditch maintenance, animal browsing, or plant dormancy. Fruit set data were assessed in three different ways: 1) percent clusters to set fruit (# fruiting clusters / # flowering cluster); 2) percent flowering stems to set fruit (# fruiting stems per cluster / # flowering stems per cluster), and 3) cluster fruiting status (each cluster designated as "1" if it set fruit and "0" if it did not).

During preliminary work in 2009, fruit set data for *C. candidum* was collected using a transect method at Doyle, MBC, and Grossart. At each site, quadrats were placed along transects at irregular intervals where orchids were most concentrated. Within each quadrat I recorded the number of *C. candidum* clusters, and for each cluster: the number of flowering stems, the total number of stems, and the number of fruiting stems (later in the season). Individual clusters were not tagged in 2009, so fruit set could not be correlated with flowering data for individual plants. Instead, *C. candidum* fruit set was

calculated as the percent of clusters to set fruit and the percent of flowering stems to set fruit (see above). Fruit set data outside of transects were also collected for each orchid taxon at each of the three sites. Stems were marked with acrylic paint to identify each taxon, and I recorded the total number of stems and the number of fruiting stems. In these cases, fruit set was calculated as the percent clusters to set fruit. Because fruit set data collected in 2009 was not directly comparable to the data collected in 2010 and 2011, it was used to describe fruit set (see Figure 3.2), but was not used in any of the statistical analyses outlined below. Additionally, the number of flowering stems was recorded for several unpainted clusters of each orchid taxon outside of quadrats, and used in statistical analyses comparing orchid cluster size, as outlined below.

3.2.2 Seed Set

The number of capsules that were sampled per taxon varied among years and taxa: for 2010, 18 of *C. parviflorum*, 12 of *C. candidum*, and 16 of hybrids; and for 2011 32 of *C. parviflorum*, 32 of *C. candidum*, and 24 of hybrids. All capsules collected in 2010 and 2011 were stored individually in glass vials, and these were allowed to air dry for a minimum of three weeks. To determine seed set, dried capsules were first broken apart inside a small glass vial (~ 15 mL), then vortexed to remove as many seeds from the placentae as possible. All remaining seeds and ovules were removed from the placentae using a static free paintbrush. Capsule remains were discarded and seeds were retained in vials.

To each vial of seeds, distilled water, TWEEN® 20, and safranin stain (0.1% aqueous solution) were added. Volumes varied for each of these depending on the

amount of seeds in each capsule. Distilled water (4 – 8 mL), TWEEN® 20 (2 - 3 mL), and safranin stain (1 mL) were measured into a centrifuge tube. The tube was gently mixed by hand and the contents were then poured back into the original glass vial, along with the seeds. A second amount of distilled water (2 – 4 mL) was added to the centrifuge tube to rinse out any mixture left behind, and this was also added back into the glass vial. The final volume in each vial was recorded. Vials containing seeds, water, TWEEN® 20, and stain were then placed in a vacuum chamber at approximately 675 mmHg for 10 minutes. They were then gently swirled and placed back in the chamber for another 10 minutes. Vials were swirled again, and left to sit for at least one hour before counting. This process removed air pockets from the seeds, and allowed them to absorb the stain (protocol modified from Proctor and Harder (1994), and from personal communication with Nina Hobbhahn, University of Calgary).

Because *Cypripedium* orchids produce many thousands of seeds per capsule, a sub-sampling technique was employed. For each capsule, 3 subsamples were taken, and each one "decanted" onto a filter paper inside of a 45 mm petri dish. Before each subsample was taken, the contents of the vial were thoroughly mixed for approximately 10 seconds to suspend the seeds in the liquid. Subsamples were always drawn from as close to the center of the vial as possible, and volumes of each subsample were recorded. A paper grid (0.5 cm) was taped to the underside of each petri dish to facilitate counting. Fully developed seeds were divided into three categories: 1) seeds with mature embryos (those with fat, round, embryo with a smooth surface); 2) seeds with dead embryos (those with small, shriveled up, black embryos); 3) empty seeds (those with no embryos), and each category was counted separately (Figure 3.1). It is important to note that chemical tests,

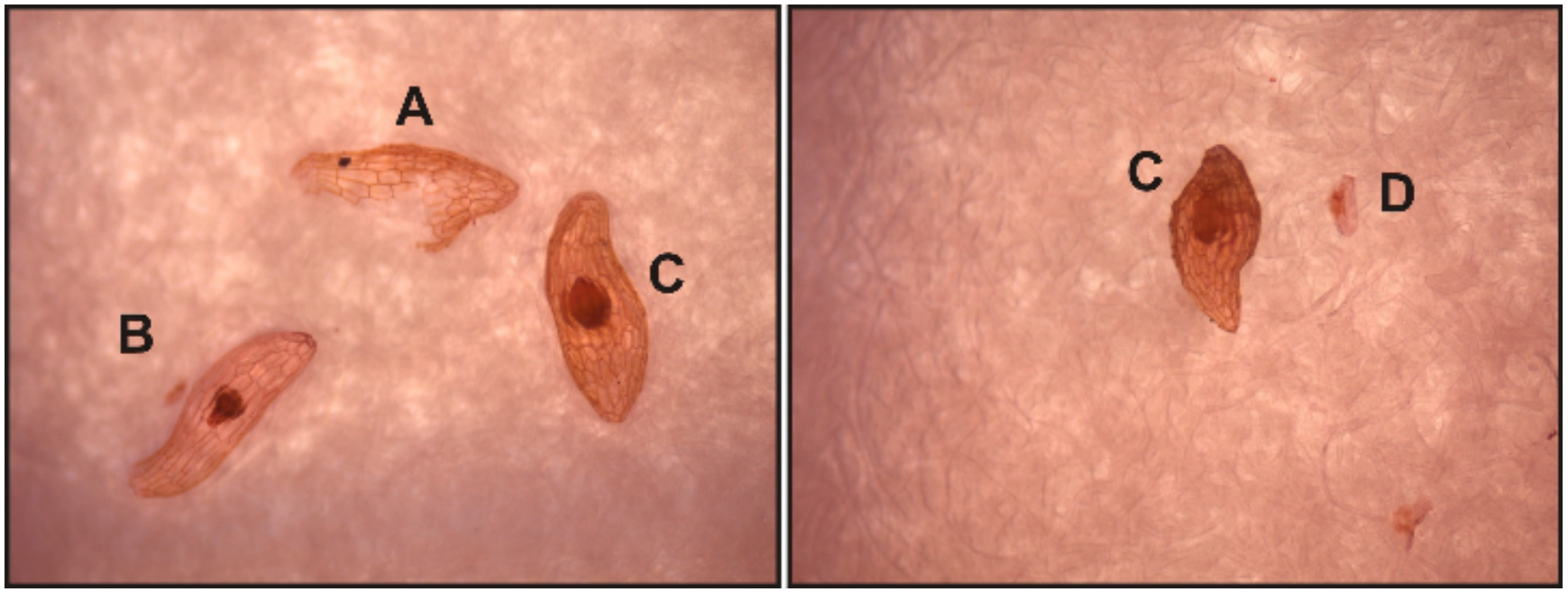

Figure 3.1: Seed and ovule counting categories. Shown for *Cypripedium candidum* are: A) damaged seed, B) seed with a dead embryo, C) seed with a mature embryo, and D) unfertilized ovule.

along with germination experiments, would be required to determine seed viability (e.g. see Vujanovic et al. 2000; Wood et al. 2004). Damaged seeds and unfertilized ovules were also counted (Figure 3.1). From these data, the total number of seeds (all categories of fully developed seeds were added together, including mature seeds with dead embryos), and the total number of ovules (i.e. total number of potential seeds – total number of seeds was added to the number of unfertilized ovules) were calculated. In addition, the proportion of ovules that developed to fully mature seeds (whether they were likely to be viable or not) was estimated. All seed and ovule categories for each subsample were then converted to "per capsule" values. It was these "per capsule" values that were used in statistical analyses.

3.2.3 Statistical Analyses

In order to investigate whether taxon, year, and site affected the probability of clusters setting fruit, I analyzed fruit set in 2010 and 2011 as a binary outcome ("fruiting status" - clusters either set fruit or did not) using a logistic transformation in SAS v. 9.2 (SAS Institute Inc. 2004). First, to determine within taxon differences in fruit set, one analysis was carried out for each taxon using year, site, and the interaction between year and site as fixed effects in the model. Secondly, to examine among taxon differences in fruit set, two analyses were carried out because not all orchid taxa occurred at every site. The first compared *C. candidum* and *C. parviflorum* at Doyle, MBC, and Grossart, and the second compared all three taxa at MBC and Grossart. Both analyses used taxon, site, year, and all two-way interaction terms as fixed effects in the model. All models were run using the "PROC LOGISTIC" command with the "descending" option.

In order to determine whether the number of flowers produced by individual plants (clusters) differed among orchid taxa, I carried out a series of analyses of variance (ANOVA) using the "PROC GLM" command in SAS v. 9.2 (SAS Institute Inc. 2004). The number of flowering stems per cluster in all three sampling years (2009, 2010, and 2011) was first square-root transformed to increase normality and stabilize variances, then entered into the ANOVA model as the dependant variable. I again carried out two sets of analyses because not all orchid taxa occurred at all sites. The first series compared *C. candidum* and *C. parviflorum* at Doyle, MBC, and Grossart, and the second compared all three taxa at MBC and Grossart. One analysis was carried out for each of the sampling years (total of six analyses), and each of these included orchid taxon, site, and an interaction between taxon and site in the model as effects.

Logistic regressions ("PROC LOGISTIC", SAS Institute Inc. 2004) were used to investigate whether plant phenotypes (morphological characters and number of flowering stems per cluster) affected the probability of clusters setting fruit. The first set of analyses was carried out for each orchid taxon in each of two years (2010 and 2011 – total of six analyses). Because not all of the 2232 sampled individuals were measured both years, and individuals with missing data were omitted from analyses, a subset of 318 plants was used. For each analysis, site was included as an effect and the following explanatory variables were included in the model: plant height, labellum length, labellum width, entry length, entry width, exit length, and exit width. The "descending" option, and additional options of "stepwise selection" and "single hierarchy" were also included in all analyses. All non-significant effects were eliminated from the final model.

The second set of analyses was used to determine, within orchid taxa, if cluster size (number of flowers per cluster) in 2010 and 2011 was associated the likelihood of clusters setting fruit. In these regressions, I was able to analyze 1872 sampled individuals (some of the 2232 individuals had missing data and were removed from analyses). I carried out one logistic regression for each orchid taxon separately. For each of these, the number of flowering stems per cluster was included as the explanatory variable, and the effects of year, site and year by site interaction were included in the model. Fruit set was analyzed as a binary outcome ("fruiting status" - clusters either set fruit or did not). For each analysis, the "descending" option, and additional options of "stepwise selection" and "single hierarchy" were also included.

I examined variation in orchid seed set by carrying out a series of ANOVAs using the "PROC GLM" command in SAS v. 9.2 (SAS Institute Inc. 2004). Seed count data per capsule were first square root transformed for the following categories: number of mature seeds, number of dead seeds, and total number of ovules. Analyses were carried out individually for each of the seed count variables. The first set of analyses for each analysis included the following effects in the model: orchid taxon, year, site, and all two-way interaction terms. The second set of analyses included the total number of ovules as a covariate (not included in the analysis of total number of ovules) as well as all effects from the first set of analyses and all two and three way interaction terms. Non-significant effects involving covariates were removed from the final model using backward elimination. Outliers were examined, and were left in because they did not significantly influence the results.

3.3 Results

3.3.1 Orchid fruit set and cluster size

Taxon and year had the strongest influence on variation in fruit set (Table 3.1). Among orchid taxa, fruit set was lowest in *C. candidum*, highest in *C. parviflorum*, and intermediate in hybrids (Figure 3.2). Higher fruit set in *C. parviflorum* than in *C. candidum* was consistent across all years, and all sites (Figure 3.2a, Appendix 3.1). Hybrid fruit set varied among sites, and was more similar to that of *C. candidum* at Grossart and to that of *C. parviflorum* at MBC (Figure 3.2a).

Within taxa, logistic analysis of *C. candidum* showed significant year, site, and year by site effects (Table 3.1). Among years, there was a higher percentage of examined clusters with at least one fruit in 2009 (14% - 35%) than in 2010 (11% - 13%) or 2011 (18% - 27%) at MBC and Grossart, while at Doyle fruit set was lower in 2009 (3%) than in 2010 (9%) or 2011 (8%; Figure 3.2a, Appendix 3.1). Among sites, fruit set was generally lowest at Doyle, intermediate at MBC, and highest at Grossart, and this trend was consistent across all three years (Figure 3.2a, Appendix 3.1).

Logistic analysis of fruit set in *C. parviflorum* showed significant year, and year by site effects (Table 3.1). Similar to *C. candidum*, the percentage of clusters to set fruit was higher in 2009 than 2010 and 2011, though values for *C. parviflorum* were much higher in all years (89% - 100%, 22%- 67%, and 50%-77% respectively; Figure 3.2a, Appendix 3.1). Among sites, trends were not consistent across years and the pattern was more variable than in the other two taxa (Figure 3.2a). In 2009, fruit set was lowest at Doyle and highest at Grossart, in 2010 lowest at Grossart and highest at McPherson, and in 2011 it was lowest at McPherson and highest at MBC (Figure 3.2a, Appendix 3.1).

Table 3.1: Logistic analyses of orchid fruit set (fruiting status – clusters either set fruit or did not). Shown are results for among and within taxa comparisons of fruit set measured in 2010 and 2011 for *Cypripedium candidum* (Can), *C. parviflorum* (Parv), and their hybrids (Hyb) at four sites in southern Manitoba (Doyle (D), Manitoba Conservation (M), Grossart (G), and McPherson (Mc)).

Effect	**Can vs Parv (D, M, G)**	**Can vs. Hyb vs. Parv (M, G)**	**Can (D, M, G)**	**Parv (D, M, G, Mc)**	**Hyb (M, G)**
Full Model	χ^2 = 329.23, P < 0.0001	χ^2 = 190.35, P < 0.0001	χ^2 = 64.32, P < 0.0001	χ^2 = 36.40, P < 0.0001	χ^2 = 18.95, P < 0.0005
Taxon	**$\chi^2_{1,1563}$ = 219.66 *****	**$\chi^2_{2,1284}$ = 93.87 *****	-	-	-
Can vs Hyb		**χ^2 = 102.77 *****	-	-	-
Can vs Parv		**χ^2 = 30.31 *****	-	-	-
Hyb vs Parv		**χ^2 = 24.48 *****	-	-	-
Year	**$\chi^2_{1,1563}$ = 35.25*****	**$\chi^2_{1,1284}$ = 49.63 *****	**$\chi^2_{1,1183}$ = 7.49 ****	**$\chi^2_{1,547}$ = 4.78 ***	**$\chi^2_{1,249}$ = 6.36 ***
Site	$\chi^2_{2,1563}$ = 0.15	**$\chi^2_{1,1284}$ = 3.96 ***	**$\chi^2_{2,1183}$ = 11.95 ****	$\chi^2_{3,547}$ = 5.18	**$\chi^2_{1,249}$ = 12.48 *****
D vs G	-	-	**χ^2 = 22.02 *****	-	-
D vs M	-	-	**χ^2 = 4.04 ***	-	-
G vs M	-	-	χ^2 = 1.03	-	-
Taxon x Year	$\chi^2_{1,1563}$ = 0.27	$\chi^2_{2,1284}$ = 1.78	-	-	-
Taxon x Site	**$\chi^2_{2,1563}$ = 14.13****	**$\chi^2_{2,1284}$ = 9.31 ****	-	-	-
Year x Site	**$\chi^2_{2,1563}$ = 13.30****	$\chi^2_{1,1284}$ = 0.21	**$\chi^2_{2,1183}$ = 10.19 ****	**$\chi^2_{3,547}$ = 27.90 *****	$\chi^2_{1,249}$ = 0.58

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

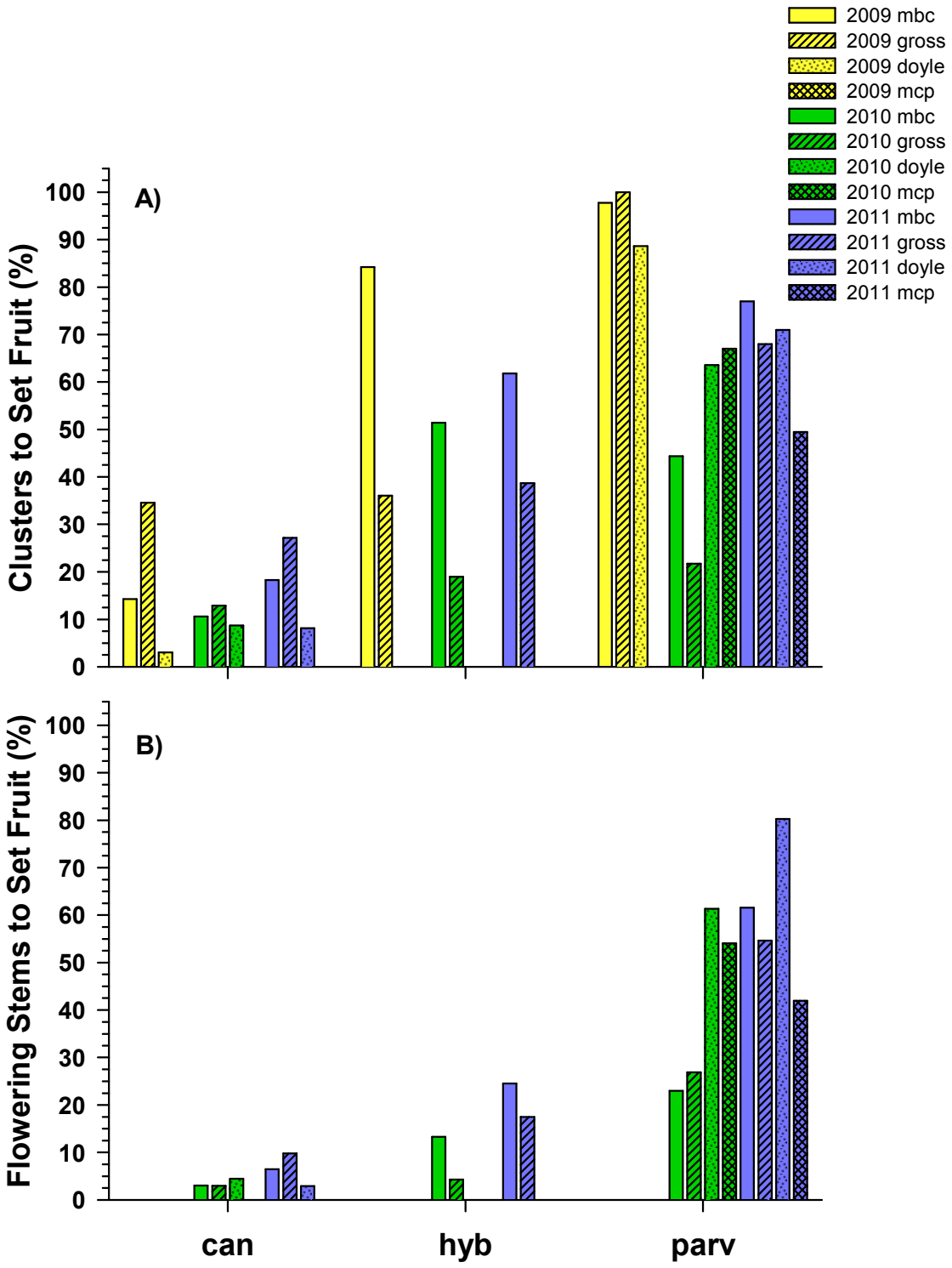

Figure 3.2: Fruit set in *Cypripedium candidum* (can), *C. parviflorum* (parv), and their putative hybrids (hyb) in four southern Manitoba populations (mbc, gross (Grossart), doyle, and mcp (McPherson)). Shown are A) percent clusters to set fruit in 2009, 2010, and 2011, and B) percent flowering stems to set fruit in 2010 and 2011.

Trends in hybrid fruit set were consistent among sites and years, and these were significant effects in logistic analysis (Figure 3.2a, Table 3.1). The percentage of clusters that set fruit was higher in 2009 (36% - 84%) than 2010 (19% - 51%) and 2011 (39% - 62%), and higher at MBC in all years than at Grossart (Figure 3.2a, Appendix 3.1).

Analysis of variance results comparing the two parental taxa showed that *C. candidum* had significantly more flowers per cluster than did *C. parviflorum*, and this was consistent across all years (for all pairwise comparisons $t > 4.93$, $P < 0.0001$; Table 3.2, Figure 3.3). There was also a significant site effect that was consistent across all years: plants at Grossart and MBC had more flowers per cluster than those at Doyle (all $t > 3.95$, $P < 0.0001$), but there was no significant difference between plants at Grossart and MBC (all $t > 0.08$, $P > 0.0821$; Table 3.2, Figure 3.3). The ANOVA comparing all three taxa showed that hybrids had more flowering stems than *C. parviflorum* in 2010 and 2011 (all $t > 3.53$, $P < 0.0004$), but the two taxa were not significantly different in 2009 ($t = 5.81$, $P = 0.5623$). Hybrids also had fewer flowers per cluster than *C. candidum* in 2009 ($t = 2.90$, $P = 0.0044$), but the two were not significantly different in 2010 or 2011 (all $t > 0.60$, $P > 0.5179$). There was also a significant site effect in 2011: plants at MBC had more flowers per cluster than those at Grossart ($t = 3.29$, $P = 0.0011$; Table 3.2, Figure 3.3).

3.3.2 Traits affecting orchid fruit set

Certain morphological characters were significant predictors of orchid fruit set, though these were not consistent across taxa or years (Table 3.3). In particular, hybrids showed a significant association with exit route characteristics. In 2010, hybrids with a

Table 3.2: Analyses of variance comparing cluster size among 3 taxa of lady's slipper orchids. Shown is the number of flowering stems per cluster for *C. candidum* (Can), *C. parviflorum* (Parv), and their hybrids for three years (2009, 2010, and 2011), and at three sites in southern Manitoba (Doyle (D), Manitoba Conservation (M), and Grossart (G)).

Dependant Variable	Effect	Can vs. Parv (D, G, M)	Can vs. Hyb vs. Parv (G, M)
2009			
# Flowers/Cluster	Model	$R^2 = 0.3053$, $P < 0.0001$	$R^2 = 0.1173$, $P < 0.05$
	Orchid taxon	$\boldsymbol{F_{1,155} = 24.27}$ ***	$\boldsymbol{F_{2,114} = 6.97}$ **
	Site	$\boldsymbol{F_{2,155} = 21.68}$***	$F_{1,114} = 0.13$
	Orchid Taxon * Site	$F_{2,155} = 1.26$	$F_{2,114} = 0.54$
2010			
# Flowers/Cluster	Model	$R^2 = 0.1930$, $P < 0.0001$	$R^2 = 0.0634$, $P < 0.0001$
	Orchid taxon	$\boldsymbol{F_{1,572} = 38.34}$ ***	$\boldsymbol{F_{2,406} = 12.20}$ ***
	Site	$\boldsymbol{F_{2,572} = 35.54}$***	$F_{1,406} = 1.06$
	Orchid Taxon * Site	$F_{2,572} = 2.33$	$F_{2,406} = 1.04$
2011			
# Flowers/Cluster	Model	$R^2 = 0.0578$, $P < 0.0001$	$R^2 = 0.0288$, $P < 0.0005$
	Orchid taxon	$\boldsymbol{F_{1,933} = 27.97}$ ***	$\boldsymbol{F_{2,798} = 8.17}$**
	Site	$\boldsymbol{F_{2,933} = 16.60}$***	$\boldsymbol{F_{1,798} = 10.82}$**
	Orchid Taxon * Site	$F_{2,933} = 1.27$	$F_{2,798} = 2.31$

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

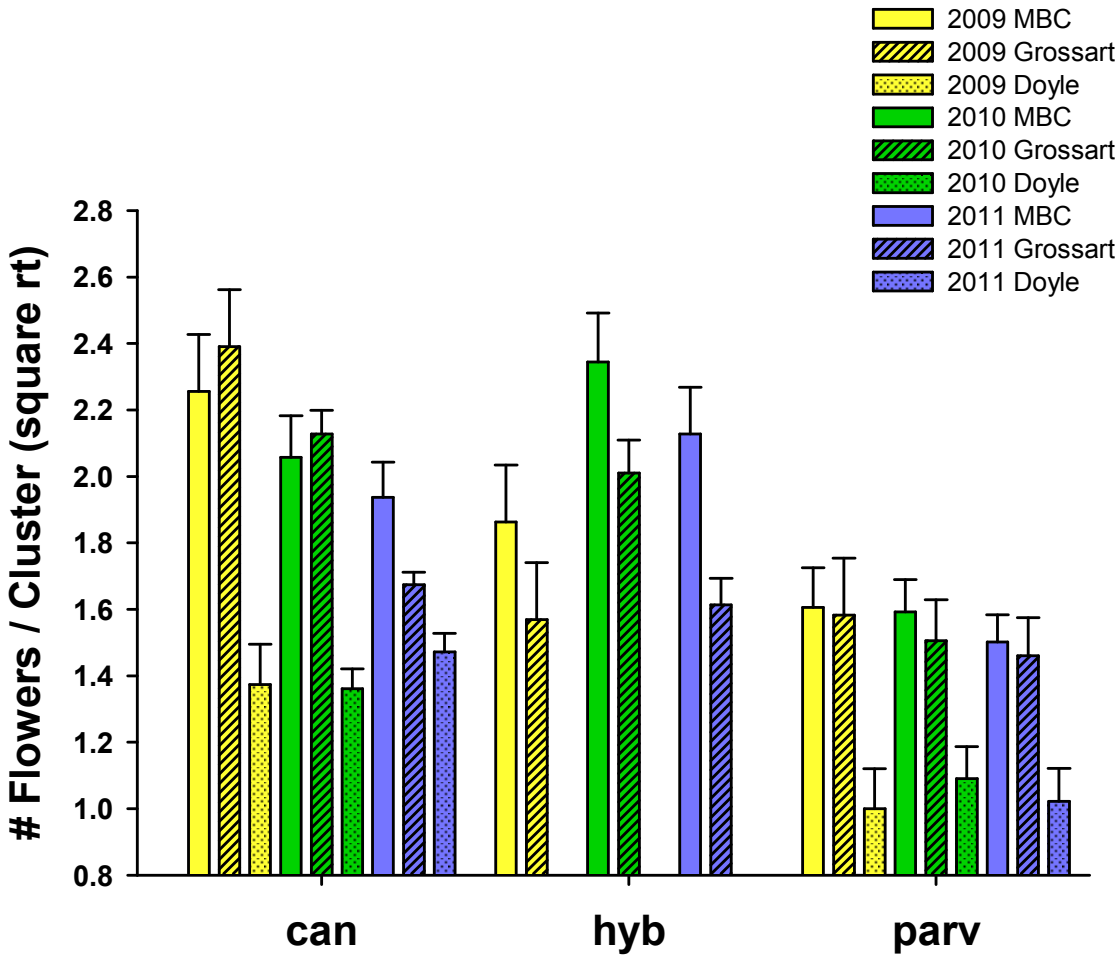

Figure 3.3: Cluster size for *Cypripedium candidum* (can), *C. parviflorum* (parv), and their hybrids. Shown is the square root of the mean number of flowers per clusters at three sites in southern Manitoba (Grossart, Doyle, and MBC) in two years (2010 and 2011). Black bars represent standard error.

Table 3.3: Logistic analyses comparing fruiting status (clusters either set fruit or did not) with floral morphological traits of three taxa of lady's slipper orchids (*C. candidum*, *C. parviflorum*, and their hybrids) over two years (2010 and 2011) at three sites in southern Manitoba (Doyle, Grossart, and MBC). Values in brackets beside significant results are estimate values plus or minus standard error.

Effect	***C. candidum***	**Hybrids**	***C. parviflorum***
2010			
	not significant	Wald $\chi^2 = 4.76$, $P < 0.05$	not significant
Plant height	$\chi^2_{1,80} = 0.14$	$\chi^2_{1,31} = 1.24$	$\chi^2_{1,44} = 2.49$
Exit length	$\chi^2_{1,80} = 1.84$	$\chi^2_{1,31} = 1.81$	$\chi^2_{1,44} = 0.03$
Exit width	$\chi^2_{1,80} = 0.99$	**$\chi^2_{1,31} = 4.76$* (1.93 ± 0.88)**	$\chi^2_{1,44} = 0.81$
Site	$\chi^2_{2,80} = 2.29$	$\chi^2_{1,31} = 0.02$	$\chi^2_{2,44} = 1.31$
2011			
	Wald $\chi^2 = 6.61$, $P < 0.05$	Wald $\chi^2 = 4.88$, $P < 0.05$	Wald $\chi^2 = 7.44$, $P = 0.0590$
Plant height	$\chi^2_{1,44} = 0.25$	$\chi^2_{1,35} = 0.09$	**$\chi^2_{1,51} = 6.47$ * (0.80 ± 0.32)**
Exit length	$\chi^2_{1,44} = 0.09$	**$\chi^2_{1,35} = 4.88$ * (1.34 ± 0.61)**	$\chi^2_{1,51} = 0.25$
Exit width	$\chi^2_{1,44} = 0.01$	$\chi^2_{1,35} = 0.00$	$\chi^2_{1,51} = 0.04$
Site	**$\chi^2_{2,44} = 7.50$ ***	$\chi^2_{2,35} = 2.85$	**$\chi^2_{2,51} = 6.65$ ***
MBC vs Grossart	$\chi^2 = 3.52$	-	**$\chi^2 = 5.18$ ***
MBC vs Doyle	$\chi^2 = 1.04$	-	**$\chi^2 = 5.70$ ***
Grossart vs Doyle	**$\chi^2 = 6.33$ ***	-	$\chi^2 = 4.50$

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

larger exit width and in 2011 those with a larger exit length were more likely to set fruit (Table 3.3). Fruit set in *C. parviflorum* in 2011 was associated with plant height and site – taller plants were more likely to set fruit (Table 3.3). Finally, *C. candidum* fruit set in 2011 showed only a significant site effect (Table 3.3). Labellum length and width as well as entry length and width showed no significant effect on fruit set for any taxon, and were not included in Table 3.3.

The number of flowering stems per cluster was a significant predictor of fruit set in all three taxa (Table 3.4). The logistic analysis of *C. candidum* showed significant effects of year as well as a year by site interaction (Table 3.4). Year was also a significant effect for hybrid fruit set, along with site, but unlike *C. candidum* there was no significant year by site interaction (Table 3.4). For *C. parviflorum*, significant effects included year, site, and a year by site interaction (Table 3.4).

3.3.3 Orchid seed set

Overall, seed set (total number of ovules, mature seeds, dead seeds per capsule) was highly variable among orchid taxa and across years (Appendix 3.2). *Cypripedium parviflorum* produced an average of 10,321 in 2010 to 24,931 in 2011 total seeds per capsule, while *C. candidum* produced between 4,015 (2010) and 8,513 (2011), and hybrids between 7,609 (2010) and 13,183 (2011, Appendix 3.2). Analyses of variance of ovules per capsule showed significant taxon and year effects (Table 3.5). *Cypripedium candidum* had significantly fewer ovules per capsule than either *C. parviflorum* or hybrids (for all pairwise comparisons, $t > 2.97$, $P < 0.005$), but there was no difference between the latter two ($t = 1.03$, $P = 0.3064$; Figure 3.4a, Appendix 3.2). Overall, all

Table 3.4: Logistic analyses comparing fruiting status (clusters set fruit or did not) with the number of flowering stems per cluster for three taxa of lady's slipper orchids (*C. candidum*, *C. parviflorum*, and their hybrids) over two years (2010 and 2011) at four sites (Doyle (D), MBC (M), Grossart (G), and McPherson (Mc) in southern Manitoba. Numbers in brackets are estimates values.

Effect	Candidum (D, M, G)	Hybrids (M, G)	Parviflorum (D, M, G, Mc)
	$\chi^2 = 95.78$, $P < 0.0001$	$\chi^2 = 23.83$, $P < 0.0001$	$\chi^2 = 58.73$, $P < 0.0001$
# Flowers/Cluster	$\chi^2_{1,1073} = 47.49$ ***	$\chi^2_{1,235} = 11.22$ **	$\chi^2_{1,543} = 26.59$ ***
Year	$\chi^2_{1,1073} = 10.60$ ** (-0.45)	$\chi^2_{1,235} = 8.06$ ** (-0.46)	$\chi^2_{1,543} = 7.67$ ** (-0.29)
Site	$\chi^2_{2,1073} = 1.98$	$\chi^2_{1,235} = 8.76$ **	$\chi^2_{3,543} = 14.32$ **
Year x Site	$\chi^2_{2,1073} = 16.16$ **	$\chi^2_{1,235} = 0.37$	$\chi^2_{3,543} = 30.70$ ***

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

Table 3.5: Analyses of variance of seed counts for three taxa of lady's slipper orchids. Shown are comparisons among *C. candidum* (Can), *C. parviflorum* (Parv), and their hybrids (Hyb) for the total number of ovules, number of seeds with mature and dead embryos per capsule. Shown are results for 2010 at three sites in southern Manitoba (Doyle (D), Manitoba Conservation (M), and Grossart (G)).

Dependant Variable	Effect	Can vs Parv (D, M, G)	Can vs. Hyb vs. Parv (M, G)
# Ovules/Capsule		R^2 = 0.4251, P < 0.0001	R^2 = 0.3489 P < 0.0001
	Taxon	$\boldsymbol{F_{1,68} = 19.42}$ ***	$\boldsymbol{F_{2,83} = 6.98}$ **
	Year	$\boldsymbol{F_{1,68} = 5.91}$ *	$\boldsymbol{F_{1,83} = 6.34}$ *
	Site	$F_{2,68}$ = 1.14	$F_{1,83}$ = 1.01
	Taxon x Year	$F_{1,68}$ = 2.79	$F_{2,83}$ = 0.64
	Taxon x Site	$F_{2,68}$ = 2.65	$F_{2,83}$ = 1.29
	Year x Site	$F_{2,68}$ = 1.49	$\boldsymbol{F_{1,83} = 9.06}$ **
# Mature Seeds/Capsule		R^2 = 0.3693 P < 0.0001	R^2 = 0.2541 P = 0.0039
	Taxon	$\boldsymbol{F_{1,68} = 14.16}$ ***	$\boldsymbol{F_{2,83} = 3.49}$ *
	Year	$\boldsymbol{F_{1,68} = 9.97}$ **	$\boldsymbol{F_{1,83} = 9.61}$ **
	Site	$F_{2,68}$ = 1.85	$F_{1,83}$ = 3.49
	Taxon x Year	$F_{1,68}$ = 0.98	$F_{2,83}$ = 0.09
	Taxon x Site	$F_{2,68}$ = 1.13	$F_{2,83}$ = 0.33
	Year x Site	$F_{2,68}$ = 0.89	$F_{1,83}$ = 1.86
# Dead Seeds/Capsule		R^2 = 0.2215 P=0.0367	R^2 = 0.0953 P = 0.4695
	Taxon	$\boldsymbol{F_{1,68} = 6.98}$ *	$F_{2,83}$ = 3.09
	Year	$\boldsymbol{F_{1,68} = 4.23}$ *	$F_{1,83}$ = 0.78
	Site	$F_{2,68}$ = 0.86	$F_{1,83}$ = 0.63
	Taxon x Year	$F_{1,68}$ = 0.77	$F_{2,83}$ = 0.32
	Taxon x Site	$F_{2,68}$ = 0.13	$F_{2,83}$ = 0.16
	Year x Site	$F_{2,68}$ = 1.79	$F_{1,83}$ = 0.44

* P < 0.05
** P < 0.01
*** P < 0.001

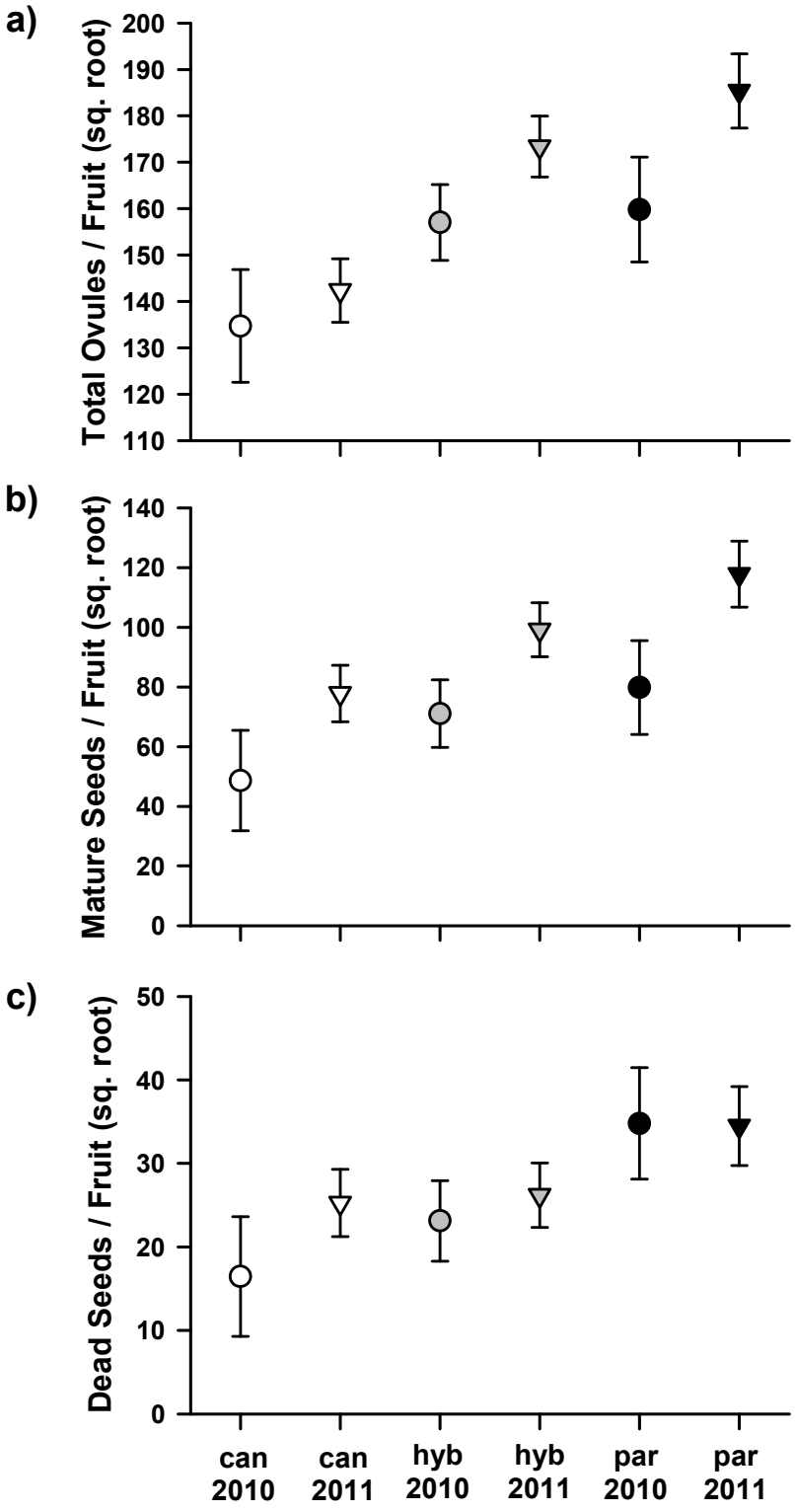

Figure 3.4: Square root values of seed counts for *Cypripedium candidum* (can), *C. parviflorum* (par), and their hybrids (hyb) at four populations in southern Manitoba. Shown are a) total ovules per fruit, b) the number of seeds with mature embryos per fruit, and c) the number of seeds with dead embryos per fruit for two years (2010 and 2011).

three taxa had a greater number of ovules per capsule in 2011 than 2010 (all $t > 2.43$, $P < 0.05$) (Table 3.5, Figure 3.4a). There was a significant year by site interaction in the analysis of all three orchid taxa (Table 3.5). The number of ovules per capsule at MBC in 2011 was higher than in 2010 and higher than at Grossart in both years (all $t > 2.47$, $P < 0.05$).

The proportion of ovules that developed into seeds with mature embryos was lowest in *C. candidum* (20% - 33%), slightly higher in hybrids (27% to 38%), and highest in *C. parviflorum* (34% to 50%; Appendix 3.2). The number of ovules that developed into seeds with mature embryos also showed taxon and year effects (Table 3.5). There were significantly fewer seeds with mature embryos in *C. candidum* than *C. parviflorum* (all $t > 2.61$, $P < 0.05$) but there were no differences between either parental taxon and hybrids (all $t > 1.14$, $P > 0.07$; Figure 3.4b, Appendix 3.2). For all taxa, the number of seeds with mature embryos was higher in 2011 than in 2010 (all $t > 3.10$, $P < 0.005$; Figure 3.4, Appendix 3.2). Although the increase in mature seeds in 2011 was consistent with an increase in the number of ovules, the difference between years was proportionally less (Figure 3.4, Appendix 3.2). However, when the total number of ovules per capsule was included in analyses, effects of taxon and year were no longer significant and the R^2 of the models changed from 0.37 to 0.76 for the analysis comparing *C. candidum* and *C. parviflorum*, and from 0.25 to 0.56 for the analysis comparing all three taxa (analyses not shown).

The number of ovules that developed into seeds with dead embryos was by contrast much smaller than then number of seeds with mature embryos (Figure 3.4, Appendix 3.2). The only significant effects were taxon and year for the comparison between *C.*

candidum and *C. parviflorum*. As for the above two variables, there were a greater number of dead seeds in 2011 than 2010 ($t = 2.06$, $P < 0.05$), as well as for *C. parviflorum* over *C. candidum* (all $t > 2.37$, $P < 0.05$; Figure 3.4c, Appendix 3.2). Again, when the total number of ovules per capsule was included in analyses, effects of taxon and year were no longer significant and the R^2 of the model changed from 0.22 to 0.35 for the analysis comparing *C. candidum* and *C. parviflorum*, and from 0.09 to 0.26 for the analysis comparing all three taxa (analyses not shown).

3.4 Discussion

3.4.1 Differences in fruit set among orchid taxa

Overall, the percent of flowering clusters to set fruit varied among orchid taxa, and was lowest in *C. candidum,* intermediate in hybrids and highest in *C. parviflorum*. This pattern of fruit set among orchid taxa was consistent with my expectation based on the pollinator observations reported in Chapter 2. The values observed in this study for *C. parviflorum* and *C. candidum* are generally within the range previously reported for these two taxa (Appendix 3.3, Bernhardt and Edens-Meier 2010; Case and Bradford 2009; Curtis 1954; Shefferson and Simms 2007). Many of the previous *C. candidum* studies have been carried out in more southern populations and near the center of its range (e.g. southwestern New York, Illinois, Virginia) where fruit set is often higher (5% to 54%; Appendix 3.3). In Manitoba, *C. candidum* is growing at the northern limit of its range. Most values reported in this study were below 15% of flowering clusters to set fruit, however the lowest value was 3%, and the highest was 35% (Figure 3.2, Appendix 3.1). Differences in latitude, as well as localized environmental variation may account for some of the variation in reproductive success reported among studies.

There are several factors that could be contributing to the differences in fruit set among taxa and the high fruit set in *C. parviflorum*. Firstly, *C. parviflorum* received greater insect visitation than either *C. candidum* or hybrids. Characteristics such as floral scent and plant height may be making *C. parviflorum* more attractive to pollinators. Field observations have suggested that *C. parviflorum* has a stronger scent than either *C. candidum* or hybrids, and this trait is thought to be a secondary pollinator attractant (Faegri and van der Pijl 1979). Secondly, within taxa, increased exit route size and plant

height were associated with higher fruit set, and both of these characters were largest in *C. parviflorum*. Finally, *C. parviflorum* had smaller clusters and higher fruit set than *C. candidum* that had the largest clusters and lowest fruit set. It is possible that other characteristics of *C. parviflorum* that make its flowers more attractive to a larger number of insect visitors (and potential pollinators) are more important for successful reproduction than the number of flowers per plant.

Some of the above factors were also found to vary within taxa, and hybrids ranged from *C. candidum*-like to *C. parviflorum*-like. The data collected, and the analyses carried out in this study, have allowed me to directly test the influences of cluster size and, for a subset of individuals, plant height and floral dimensions on orchid fruit set. These effects were examined within each taxon, and are discussed below.

3.4.2 Factors influencing orchid fruit set within orchid taxa

3.4.2.1 Orchid morphology

The low number of significant associations between fruit set and morphological characters within orchid taxa was not consistent with my predictions. For all taxa, exit route size was expected to limit insect visitation and pollinators, and therefore reproductive success, but was only significantly associated with fruit set in hybrids. It is possible that *C. candidum* flowers are too small for variation in exit or entry route size to matter (since visitation to this species is so low to begin with), or for effects on fruit set to be detectable given the sample size in this data set. In addition, other floral dimensions may influence pollination success. Li et al. (2006) used the distances between both the stigma/pollinia and the bottom of the labellum of cross-sectioned *C. tibeticum* flowers to

distinguish between the fit of queen and worker bumblebees. Unfortunately, the endangered status of *C. candidum* precluded destructive sampling. In the case of *C. parviflorum*, flowers can already accommodate a large number of potential pollinators, and variation in exit route size may not have enough of an effect on these to cause variation in reproductive success.

Hybrids have a variable morphology that approaches both *C. candidum* and *C. parviflorum*. Flowers with larger exit routes, approaching *C. parviflorum*, could have been more likely to set fruit than those with smaller exit routes because they can accommodate more potential pollinators. Within a given year, hybrids had fruit set values more similar to those of *C. candidum* at Grossart, and to *C. parviflorum* at MBC (Figure 3.2). This is consistent with findings (see Chapter 2) that at MBC, hybrids were morphologically similar to *C. parviflorum*, and that (in 2011 at least) they seemed to be sharing pollinators (Table 2.3). These morphological similarities provide further suggestion that hybrids may be receiving a greater genetic contribution from, and may be backcrossing more frequently with, *C. parviflorum* compared with *C. candidum* at MBC.

Variation in plant height was significantly associated with fruit set in *C. parviflorum*. In this study I found that taller plants of this taxon were more likely to set fruit in 2011. This trend was not detectable in *C. candidum* or hybrids. Interestingly, Walsh and Michaels (2012a) found that taller *C. candidum* plants in an Ohio population had significantly higher pollinia removal and fruit set. Similarly, O'Connell and Johnston (1998) found that taller *C. acaule* plants had higher male and female success in some populations. Data presented in Chapter 2 showed that overall, *C. parviflorum* had more insect visitors at MBC in 2011 than *C. candidum* or hybrids. It is possible that taller *C.*

parviflorum plants were more visible, and thus more attractive to insect visitors, which may have increased the probability of setting fruit.

3.4.2.2 Fruit set and floral display

Within taxa, orchid clusters with more flowers had a significantly greater likelihood of setting fruit than those with fewer flowers (Table 3.4), a trend similar to that found by Falb and Leopold (1993) in their study of *C. candidum*. The number of flowering stems in a cluster may be related to pollinator behavior in that a larger, more visible cluster may be more attractive to foraging pollinators (Walsh and Michaels 2012b). This in turn, may affect fruit set. Although I did not explore the possibility, it may be that if each flower in a cluster has the same probability of setting fruit, then larger flowering clusters of a given taxon would have higher fruit set simply because there are more flowers. On the other hand, in Estonian populations of *C. calceolus*, clones consisting of a greater number of flowers set relatively fewer fruits (Kull 1998). While larger clusters of flowers may increase the speed at which pollinators learn, decreasing visitation, and resulting in lower reproductive success, this did not appear to be a factor in this study.

The finding that cluster size is greatest in *C. candidum* also indicates that this species may be relying more heavily on vegetative propagation than sexual reproduction. In Manitoba, there is concern that low fruit set in *C. candidum* compared to *C. parviflorum* and hybrids could be a factor leading to an increased risk of extirpation through genertic assimilation in some populations (Worley et al. 2009). Reliance on vegetative reproduction would decrease the risk of extirpation. Additionally, the large

number of seeds produced by relatively few fruit (Boyden 1982; Cozzolino and Widmer 2005) may be sufficient to maintain populations of *C. candidum*.

3.4.2.3 Annual and among-site variation

Cypripedium candidum, *C. parviflorum*, and hybrids all showed variation in reproductive success and cluster size among taxa, populations and years, a trend similar to that reported in earlier studies of *C. candidum* and *C. parviflorum* (Curtis 1954; Falb and Leopold 1993), *C. acaule*, (Primack and Hall 1990; Primack and Stacy 1998), and *C. fasciculatum* (Lipow et al. 2002). Overall, I observed that fruit set was lowest in 2009, highest in 2010, and intermediate in 2011. There are many factors that could be affecting this observed variation, including differences in habitat, local environment, and climate. Annual variation in spring weather may influence fruit set by affecting the length of flowering time, or by damaging flowers. Frost may be of particular importance to *C. candidum*, especially in Manitoba at the periphery of its range. In their study of *C. candidum*, Falb and Leopold (1993) suggested that frost may affect variation in the number of flowering individuals. In this study, *C. candidum* was among the first plants to emerge and flower (see Chapter 2), which may put it at risk for lower reproductive success because growing conditions in the early spring may not always be favorable. In 2009, I observed frost at Doyle that damaged the first flush of *C. candidum* flowers so severely that they did not produce fruit. Some flowers from a later flush however did produce fruit. The later flowering *C. parviflorum* on the other hand, appeared not to be as severely affected by the frost. It could be that by flowering slightly later, this species is avoiding some of the potentially damaging or killing frost making it more likely to set

fruit than *C. candidum*. By comparison, populations of *C. candidum* and *C. parviflorum* in the more temperate southern populations are likely to experience a greater number of frost-free days than those here in Manitoba, and may be less susceptible to damage from adverse climatic conditions in early spring – which could be contributing to similar fruit set values for these species in more southerly populations. For example, Shefferson and Simms (2007) found that close to 34% of plants set fruit for both *C. candidum* and *C. parviflorum* (Appendix 3.3). Falb and Leopold (1993) also found rabbit herbivory of *C. candidum*, and suggested it may be related to low fruit production in a given year. In my study I saw evidence of herbivory to orchid plants – particularly at Doyle. However, observed damage was minimal, and affected only a few of my study plants.

Site management is also an important consideration when looking at variation in orchid reproductive success and cluster size. At the Grossart site, the landowners mow the ditch where the orchids grow on an annual basis. This seems to have resulted in a denser population with a greater number of plants, perhaps because mowing reduced competition by shrubby vegetation, and helped spread seeds and rhizomes. In a multi-year experiment, Curtis (1946) suggested that control of shrubs was essential to maintaining or increasing population size of *C. candidum*, and that mowing was a practical approach for management. Fruit set was highest at Grossart in all three years for *C. candidum*, but not for hybrids or *C. parviflorum*.Thus, mowing may not necessarily lead to increased fruit production.

In contrast to Grossart, Doyle is not managed by mowing, but has experienced a number of fires, the most relevant to my study being one that occurred in October, 2009 (Hamel et al. 2004). Burning is considered a key process in maintaining prairie habitats,

and has been shown to influence plant composition and productivity, as well as plant-plant interactions (Engel and Bidwell 2001; Hamel et al. 2004; Gibson and Hulbert 1987; Suding 2001). In the case of *C. candidum* and *C. parviflorum*, fires may be beneficial to these plants that do not thrive under competition, because they help keep down the thatch as well as reduce woody encroachment and competition from other plant species (Hamel et al. 2004). *Cypripedium candidum* did show greater fruit set at Doyle in 2010 and 2011 compared with pre-fire values in 2009, a trend which was not observed for *C. parviflorum* at this site. On the other hand, an intense fire could potentially kill rhizomes, or cause too much openness and not enough protection for the orchids in early spring when there is a risk of frost. For example, Primack et al. (1994) found that an unplanned fire decreased the percent of heavily burned *C. acaule* plants that re-emerge in the following year, compared with plants that were unburned or slightly burned.

3.4.3 Orchid seed set

As expected, *C. parviflorum* showed higher seed production than *C. candidum* and hybrids. Hybrid seed set was also higher than *C. candidum*, which was contrary to my prediction that hybrids would less fit than both parental taxa. This suggests that there is no evidence of hybrid sterility in these Manitoba populations. Observed variation in the number of mature and dead seeds was explained by the total number of ovules per fruit. This means that once plants set fruit, the proportion of ovules per fruit that developed into seeds was equivalent among orchid taxa. The differences in seed set among taxa then, are mainly due to the number of ovules produced per fruit, further evidence that hybrids do not seem to be showing any depressed reproductive ability. The idea that hybrids are

often sterile or less fit than parents is common in the literature (Campbell and Aldridge 2006; Mallet 2007; Ramsey et al. 2003 and references therein). In some cases however, hybrids may become more successful than parental taxa and may replace them in a population entirely (Hedge et al. 2006; Rieseberg 1997), however this does not appear to be an immediate outcome for *Cypripedium* populations in this study. If this were the case, I would have expected to observe an overall increase in hybrid population size over the course of my three sampling years. Additionally, I would have expected hybrid reproductive success to surpass that of both parental taxa. Neither of these situations occurred in my study populations. Long term monitoring of population size and reproductive success would help to track the long term status of these orchids, and determine any possible likelihood of extinction.

It has been suggested that many rewardless orchids are pollen limited, and that fruit set can be increased when the amount of pollen is increased (e.g. Falb and Leopold 1993; Lipow et al. 2002; Proctor and Harder 1994; Tremblay et al. 2005). This could help to explain the observed variation in *Cypripedium* ovule production among taxa. Although this study did not set out to address pollen limitation, I did observe that only a fraction of the ovules produced by all three orchid taxa developed into mature seeds. These values ranged from the lowest proportion, 20%, in *C. candidum* to the highest, 50%, in *C. parviflorum*, and indicate that all ovules are not being fertilized. Alex Hare, an undergraduate student in the Department of Biological Sciences, carried out hand pollination experiments on *C. candidum* and *C. parviflorum* at MBC in 2011. His results were suggestive that pollen limitation may be a factor affecting fruit set for *C. candidum* but not for *C. parviflorum*. Walsh and Michaels (2012a, 2012b) also showed pollen

limitation of fruit set in *C. candidum* in an Ohio population, while Proctor and Harder's (1994) study suggested that *C. parviflorum* may not be pollen limited. Further pollen supplementation experiments in conjunction with assessments of seed set and seed viability may provide insight into whether or not *C. candidum*, *C. parviflorum*, and their hybrids tend to be pollen limited in Manitoba populations.

3.4.4 Conclusions

This study investigated some of the factors that may influence orchid reproductive success, pollination, and hybridization. I found that fruit set and seed set varied within and among taxa, populations, and across years. These values were lowest in *C. candidum*, highest in *C. parviflorum*, and intermediate in hybrids. Cluster size also varied within and among taxa and populations, and was largest in *C. candidum*, smallest in *C. parviflorum*, and intermediate in hybrids. Within taxa, clusters with a larger number of flowers were more likely to set fruit. However, among taxa, *C. candidum* had the lowest fruit set. It could be that taxon effects are more important for orchid reproductive success than cluster size. Hybrid flowers with larger exit routes, and taller *C. parviflorum* plants were more likely to set fruit. Plant morphology, specifically exit route in hybrids and plant height in *C. parviflorum*, may affect reproductive success by affecting the number and variety of pollinators, or by playing a role in pollinator attraction. Other aspects of flower phenotypes such as scent and flower colour may also affect fruit set but were not examined in this study. Additionally, for species with low visitation rates, such as *C. candidum*, more effort into vegetative reproduction may be a good strategy for survival. Environmental factors such as climate or composition of the pollinator community, along

with orchid phenology (time of emergence/flowering) are also important because they may influence opportunities for pollination events.

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Appendix 3.1: Fruit set in 2009, 2010 and 2011 for four Manitoba populations of *Cypripedium candidum, C. parviflorum* and their putative hybrids. Included are number of clusters to set fruit, total number of clusters, percent of clusters to set fruit, and percent of flowering clusters to set fruit. Some orchids were not identified beyond the genus level, and are noted as "*C.* sp".

	Site										
	Grossart			**MBC**			**McPherson**		**Doyle**		
	2009	2010	2011	2009	2010	2011	2010	2011	2009	2010	2011
# of Clusters to Set Fruit											
C. candidum	19	8	123	10	5	11	-	-	2	17	17
C. parviflorum	2	10	34	87	32	77	55	45	39	49	49
Hybrids	22	12	41	16	18	21	-	-	-	-	-
C. sp	-	27	10	-	4	6	-	-	-	-	-
Total # of Clusters											
C. candidum	55	62	453	70	47	60	-	-	66	195	211
C. parviflorum	2	46	50	89	72	100	82	91	44	77	69
Hybrids	61	63	106	19	35	34	-	-	-	-	-
C. sp	-	256	37	-	4	8	-	-	-	-	-
% Clusters to Set Fruit											
C. candidum	34.6	12.9	27.2	14.3	10.6	18.3	-	-	3.0	8.7	8.1
C. parviflorum	100.0	21.7	68.0	97.8	44.4	77.0	67.0	49.5	88.6	63.6	71.0
Hybrids	36.1	19.0	38.7	84.2	51.4	61.8	-	-	-	-	-
C. sp	-	10.6	27.0	-	50.0	75.0	-	-	-	-	-

Appendix 3.2. Seed count data for *Cypripedium parviflorum*, *C. candidum*, and their hybrids from capsules collected at four sites in southern Manitoba in 2010 and 2011. All variables are presented as means ± standard deviations, and were calculated on a per capsule basis from all subsamples per year and taxon.

	C. parviflorum		***C. candidum***		**Hybrids**	
Sample Year	2010	2011	2010	2011	2010	2011
# Capsules	18	32	12	32	16	24
# Subsamples	54	96	36	96	48	72
# Mature Seeds	8 607.54 ± 7133.75	20 810.12 ± 15678.57	3 671.36 ± 3252.35	6 845.68 ± 5982.52	6 633.77 ± 6237.53	11 940.00 ± 11543.94
# Dead Seeds	1 336.27 ± 2164.16	2 989.83 ± 3659.97	297.19 ±347.48	1 258.98 ± 1642.38	748.48 ± 886.03	772.74 ± 627.73
# Empty Seeds	0.00 ±	0.00 ± 0.00	0.00 ±0.00	0.00 ± 0.00	0.00 ±0.00	0.00 ± 0.00
Total # Seeds	10 321.78 ± 8655.80	24 931.93 ± 18229.95	4 015.78 ±3362.68	8 513.18 ± 6818.67	7 608.98 ± 7040.84	13 182.81 ± 11845.10
# Unfertilized Ovules	15 008.20 ± 7947.98	16 587.75 ± 10798.49	14 158.69 ±6989.21	12 261.71 ± 5612.52	17 411.75 ± 7531.55	18 321.17 ± 10329.83
Total Ovules[1]	25 329.98 ± 10657.99	41 519.68 ± 21089.23	18 174.47 ± 8245.74	20 774.89 ± 6796.86	25 020.73 ± 8294.85	31 503.97 ± 13733.36
% Mature Seeds	83.39 ± 82.42	83.47 ± 86.00	91.42 ± 96.72	80.41 ± 87.74	87.18 ± 88.59	90.57 ± 97.46
% Dead Seeds	12.95 ± 25.00	11.99 ± 20.08	7.40 ± 10.33	14.79 ± 24.09	9.84 ± 12.58	5.86 ± 5.30
% Developed Seeds[2]	33.98 ± 66.93	50.12 ± 74.34	20.20 ± 39.44	32.95 ± 88.02	26.51 ± 75.20	37.90 ± 84.06
% Undeveloped Seeds[3]	5.28 ± 20.31	7.20 ± 17.35	1.64 ± 4.21	6.06 ± 24.16	2.99 ± 10.68	2.45 ± 4.57

[1]Total ovules = total # seeds + # of unfertilized ovules.
[2]This is the percent of total ovules that developed into seeds with mature embryos.
[3]This is the percent of total ovules that developed into seeds with dead embryo

Appendix 3.3: Fruit set previously reported in species of *Cypripedium* orchids.

Species	Location	Measure of Fruit Set	Value	Reference
C. candidum	New York	% ramets to set fruit	7.5 - 15.8%	Falb and Leopold (1993)
	Illinois	Fruiting success	4.6 - 54%	Bowles (1983)
	Illinois	% plants to set fruit	~ 34%	Shefferson and Simms (2007)
	Wisconsin	% flowers to set fruit	average 22%	Curtis (1954)
	Various (USA)	% capsule set	average 11.6%	Neiland and Wilcock (1998)
C. parviflorum	Illinois	% plants to set fruit	~ 34%	Shefferson and Simms (2007)
	Virginia	% flowers to set fruit	14.8%	Case and Bradford (2009)
	Various (USA)	% conversion flowers to fruit	10.5 – 40%	Bernhardt and Edens-Meier (2010)
	Wisconsin	% flowers to set fruit	average 42 – 70%	Curtis (1954)
C. reginae	Wisconsin	% flowers to set fruit	average 23%	Curtis (1954)
	Unsure	% conversion flowers to fruit	4.6 - 33%	Bernhardt and Edens-Meier (2010)
C. macanthros	Japan	% flowers to set fruit	1.2 - 8.3%	Sugiura et al. (2001)
C. fasciculatum	Various (USA)	% flowers to set fruit	18 - 69%	Lipow et al. (2002)
	Oregon	% conversion flowers to fruit	0 – 58%	Ferguson (2005)
C. calceolus	Estonia	% fruit set	average: 10.5%	Kull (1998)
	Sweden	% flowers to set fruit	0 – 25%	Nilsson (1979)
C. acaule	Massachussetts	% flowers to set fruit	0 - 25%	Primack and Stacy (1998)
	Nova Scotia	% flowers to set fruit	5 - 15%	O'Connell and Johnston (1998)
	USA	% conversion flowers to fruit	4.3 – 9.4%	Bernhardt and Edens-Meier (2010)
	Various (USA)	% capsule set	average 2.8%	Neiland and Wilcock (1998)
C. tibeticum	China	% flowers to set fruit	9.6 – 13.8%	Li et al. (2006)
C. jamponicum	China	% flowers to set fruit	5.2 – 7.7%	Sun et al. (2009)
C. henryi	China	% flowers to set fruit	17.0 – 19.4%	Li et al. (2008b)
C. plectrochilum	China	% flowers to set fruit	38.9 – 45.9%	Li et al. (2008a)

4.0 Chapter Summary

Interactions among rewarding and rewardless plants, and their pollinators in a community can influence plant survival and reproductive success. In this study, I examined the role of co-flowering rewarding plants across four southern Manitoba populations of *Cypripedium candidum*, *C. parviflorum*, and their hybrids. I investigated which plants, if any, were important for maintaining pollinators in orchid populations, and if the diversity of the surrounding floral communities affected orchid fruit set. The density and diversity of vegetation associated with orchids varied across sites and years. I examined non-orchid pollen found inside orchid flowers, and found that in general, abundant species within sites were visited by pollinators prior to orchids. No association however, was found between orchid fruit set and overall co-flowering plant abundance. Relationships between orchid fruit set and abundance of individual co-flowering plant species were also investigated. All three orchid taxa had greater fruit set when associated with lower abundance of at least one co-flowering plant species, including *Zizia spp.*, *Hypoxis hirsuta*, and *Fragaria virginiana*, but results varied across sites and years. When these species are growing in high densities and in close proximity to orchids, they may be drawing pollinators away, having a negative impact on orchid reproductive success. By contrast, some co-flowering rewarding species, such as *Agoseris glauca*, may be facilitating orchid pollination and fruit set. Additionally, in some sites and years, *C. candidum* and *C. parviflorum* may have greater likelihood of setting fruit when surrounded by more *Cypripedium* flowers. My results suggested that the composition,

and abundance of co-flowering species at a site are important for maintaining pollinators and influencing orchid reproductive success.

4.1 Introduction

Cypripedium parviflorum and *C. candidum* are generalized food mimics that provide no food reward, and may depend on rewarding flowers to attract and maintain a local pool of pollinators. The consequences of this deception are most influenced by pollinator learning, because insects can start to avoid the non-rewarding flowers (Bell et al. 2005; Internicola et al. 2007, 2008; Johnson et al. 2003; Juillet et al. 2007; Lammi and Kuitunen 1995; Thomson 1978; Waser 1978). Interactions between rewardless and rewarding co-flowering plants in a community may be affecting pollination and reproductive success in a number of ways. Firstly, rewarding plants may facilitate pollination. Pollination success and fitness can be increased in a species lacking rewards, by flowering close to one or more rewarding species, as these plants may be collectively functioning to attract and maintain pollinators (Johnson et al. 2003; Juillet et al. 2007; Peter and Johnson 2008; Thomson 1978). For example, O'Connell and Johnston (1998) found that pollination rates in *Cypripedium acaule* were higher for plants growing closer to ericaceous shrubs. Flower colour is thought to play an important role in facilitation, in that pollinators may be more likely to visit rewardless flowers when they are similar in colour to those of rewarding co-flowering species (Chitka et al. 1997, Chitka and Raine 2006; Johnson et al. 2003; Pellegrino et al. 2008; Peter and Johnson 2008). In a generalized system however, where no taxon specific mimicry is thought to occur, flower colour may not have as much importance.

Pollinator learning and facilitation may be affected by the relative density of rewardless and rewarding flowers in the community. Pollinator learning can be slowed when rewardless species are rare in a population or when they appear similar to rewarding plants, as it is more difficult for pollinators to avoid rare or similar phenotypes (Boyden 1982; Ferdy et al. 1998; Jersáková et al. 2006; Keasar 2000; Sabat and Ackerman 1996; Sun et al. 2009).

In some cases, co-flowering species with superior rewards may be functioning in a competitive capacity, as a result of pollinator preference. Plant species that differ in rewards may compete for the "attention" of pollinators, which may result in reduced visitation and pollination rates for the less attractive species because flowers with superior rewards may draw pollinators away (Nilsson 1980; Waser 1978). This is often the case with rewardless species, and has been previously reported in some rewardless orchids (Johnson et al. 2003; Lammi and Kuitunen 1995). Additionally, the more abundant a rewardless species is, the faster pollinators can learn to avoid it (Boyden 1982; Ferdy et al. 1998). This can lead to lower visitation rates and thus lower reproductive success in dense populations (Case and Bradford 2009; Sun et al. 2009). Reproductive success in deceptive orchids then, may be subject to density-dependent variation (Ferdy et al. 1998). In the deceptive orchid *Calypso bulbosa*, a high density of orchids in the study area reduced the effectiveness of deception because pollinators (bumblebee queens) encountered many flowers which increased the rate of avoidance learning (Boyden 1982). Additionally, Sun et al. (2009) found that male and female fitness was higher in *Cypripedium japonicum* plants growing singly than those growing in clusters.

In this chapter I will describe the floral community associated with four southern Manitoba populations of *C. parviflorum* and *C. candidum* that seem to differ in the frequency of hybrids. These populations were also investigated in Chapters 2 and 3. My first objective is to determine if and how the presence of local rewarding flowering plants affects orchid fruit set. I am interested in knowing if the overall density of co-flowering species and orchids are associated with orchid fruit set. Because there is very little data on the association between *C. candidum* and *C. parviflorum* fruit set and co-flowering species, I am exploring two alternative hypotheses. First, if co-flowering plants are acting as facilitators, I expect to find that orchids growing with a high density of rewarding species will have higher fruit set compared to those growing in less dense areas. Alternatively, if co-flowering plants are acting as competitors for pollinator attention, I expect to find that orchids growing in high densities of rewarding plants would have lower fruit set. Based on reports in other rewardless orchid species (e.g. Boyden 1982, Sun et al. 2009), I expect to find that fruit set in all three orchid taxa to be higher in quadrats where orchid density is lower.

My second objective is to determine whether particular co-flowering species are important in maintaining potential pollinators. I will use non-orchid pollen collected from inside orchid slippers to determine which co-flowering species insects are visiting before they visit orchids. As discussed in previous chapers, higher rates of visitation and fruit set in *C. parviflorum* lead me to expect a higher frequency of non-orchid pollen deposition in the flowers of this species, relative to *C.candidum* and hybrids. Observations of plant-pollinator interactions at the Doyle site suggest that many of the potential pollinators of *Cypripedium* visit multiple species of plants (Semmler, in prep). This leads me to expect

that the pollen from co-flowering species found on orchid flowers will be multi-species in origin, and reflect the composition of flowering species at each site. *Cypripedium* orchids are thought to have a generalized food mimicry system, and thus should not be mimicking any specific plant growing in the same community. I do not expect to find a pattern of similar flower colour between orchid taxa and those co-flowering species visited prior to them (i.e. species whose pollen was left behind in orchids). Since I expect pollinators to be visiting multiple, and likely many of the same, rewarding species within each site, I should find few differences in the origins of non-orchid pollen collected from *C. parviflorum* versus *C. candidum* versus hybrids.

4.2 Materials and Methods

4.2.1 Co-flowering vegetation surveys

Co-flowering vegetation was assessed in all sampling quadrats at all sites, in 2010 and 2011. See chapter 2 for number and distribution of quadrats. In each quadrat, I recorded the number of stems with flowers and buds for each non-orchid species (vegetative plants were not enumerated). Because vegetation surveys were done at a single point in time, bud and flower data were combined to reflect the fact that buds were likely to be open during the course of orchid flowering. Stem counts for each co-flowering plant species (including all orchid taxa) were summed across quadrats, and tabulated per site in each year (Appendices 4.2 to 4.5). These data were used to produce rank abundance plots that characterize the vegetation at each site and year.

Orchid stem density and fruit set were also estimated per quadrat. For each orchid taxon in each quadrat (as outlined above), the total number of flowering stems and fruiting stems was tallied. Fruit set per quadrat was calculated in two ways: 1) as the total number of fruiting stems, and 2) as fruit set per flower (# of fruiting stems / # of flowering stems). These data were used in statistical analyses, as outlined below.

4.2.2 Non-orchid pollen collection and pollen reference library

During my vegetation assessment I occasionally found non-orchid pollen on hairs near the exit routes inside the labella of orchids that was left behind by insects that had visited flowers. Across all study sites and orchid taxa, 21 samples were collected in 2009, 7 in 2010, and 10 in 2011. In 2010 and 2011“bee sticks”, constructed using the thorax of newly emerged *Apis mellifera*, were swabbed inside the labella of orchids when non-

orchid pollen was observed. Pollen from bee sticks was removed using small cubes of fuschin jelly, or rinsed with 70% ethanol which was then filtered, using 45 μm cellulose nitrate membrane filters (13 mm diameter), and collected on small cubes of fuschin jelly. These were mounted on slides with coverslips and sealed with clear nail polish. In 2009 orchid hairs were collected directly from orchids and stored in 70% ethanol. These samples were vortexed, to dislodge any pollen stuck to hairs, then filtered and mounted as above.

To create a pollen reference library, I collected flowers from all plants that bloomed during orchid flowering at each of my sites. I dissected the anthers and extracted pollen from each species. Pollen grains were mounted directly into fuschin jelly as above. Using a light microscope at 400x – 600x, photos were taken of each pollen type, and physical characteristics (such as shape and surface sculpturing) were recorded (Appendix 4.1). Reference plant specimens, pollen reference slides, and photographs will be deposited in the herbarium at the University of Manitoba, Winnipeg. Pollen grain size of for each species was recorded by taking measurements from 10 individual pollen grains using the program Image Pro Express 4.5 (Media Cybernetics Inc., Rockville, Maryland). Pollen morphological characteristics were then used to help determine which non-orchid species were present in the non-orchid pollen samples collected from orchids.

Using a light microscope at 400x – 600x, non-orchid pollen slides were scanned to determine the identity and relative abundance of each plant species. Unknown pollen grains were directly compared with species in the reference library. In some cases, a single pollen type was associated with more than one species (see e.g. Appendix 4.1). These samples were identified as one species “or” the other. For each slide, 15 non-

overlapping fields of view were then selected. Since pollen was not distributed evenly throughout the slide, fields were chosen from areas where pollen grains were concentrated. Within each field, species presence was noted. From this information, the percentage of fields in which each grain type appeared was calculated to provide an estimate of relative abundance within the sample.

4.2.3 Statistical analyses

To characterize the vegetation associated with orchids at my four sample sites, stem counts (per quadrat) for all co-flowering plant species were obtained for 80 and 88 quadrats in 2010 and 2011, respectively. A total of 32 species were observed to be flowering in 2010 while 30 species were enumerated in 2011. Data for each year was square-root transformed and subjected to a principal components analyses (PCA) using a co-variates matrix and the ORDIN program module in SYN-TAX 5.02Mac (Podani 1995). In order to characterize only the co-flowering vegetation surrounding the orchids themselves, orchid stem counts were removed from the data set for the PCA only.

I was also interested in whether orchid fruit set within quadrats was significantly correlated with the loadings associated with the first two axes of the PCAs of co-flowering species. One logistic analysis was run for each year – 2010 and 2011 using SAS 9.2 (SAS Institute Inc. 2004). For each of these analyses, orchid fruit set per flower (# fruiting stems / # flowering stems), for each orchid taxon, in each quadrat, was used as the dependant variable. The component scores of each co-flowering species along axes one and two, as well as an interaction term between them, were included as effects in the initial models. Any non-significant effects were not retained by SAS into the final model.

In addition, I investigated the possibility that within quadrats, orchid fruit set was significantly associated with the abundance of co-flowering vegetation, including orchids. Calculating orchid fruit set per flower required using flowering stems per quadrat as the denominator. However, the number of flowering orchid stems was found to be highly correlated with the total number of orchid stems in a quadrat. Because of this relationship, I could not explore the relationship between orchid fruit set per flower and total stem density using logistic regression. Instead, I examined the relationship between the number of fruiting orchid stems per quadrat and the total flowering stem density per quadrat of orchids, as well as all co-flowering stems together, using analysis of variance (ANOVA) in SAS 9.2 (SAS Institute Inc. 2004). Stem counts for all co-flowering species and all orchid taxa were separately summed for each quadrat and the totals were log transformed. One analysis was run for each orchid taxon (three) in each year (2010 and 2011), for a total of 6 analyses. The number of orchid fruiting stems per quadrat, of each orchid taxon, was used as the dependant variable. Site, orchid stem count per quadrat, co-flowering species stem count per quadrat, and all two-way interaction terms were included as effects in the initial models. Non-significant effects involving covariates were removed from the final model using backwards elimination.

A second set of analyses was used to investigate whether orchid fruit set per flower was significantly associated with any of the abundant rewarding co-flowering species. I used rank abundance plots of stem counts (per site) and the results from non-orchid pollen analysis (see Figure 4.1, Appendices 4.2 to 4.5, and Tables 4.1 to 4.3) to determine the most abundant and most frequently occurring co-flowering species at each site in each year (2010 and 2011). Stem counts per quadrat for each of these species were

then square-root transformed. One analysis was run for each orchid taxon (three) at each site in which each taxon occurs (two to four) and in each year (two), for a total of 17 analyses. There was no analysis carried out for *C. parviflorum* at Doyle in 2010 because the sample size was too low ($N < 5$ quadrats). Fruit set per flower, of each orchid taxon, was used as the dependant variable. For each analysis, stem counts for the most abundant species (between five and seven) at each site and year, and all two-way interaction terms were included as effects in the initial models. Any non-significant effects were not retained by SAS in the final model.

4.3 Results

4.3.1 Composition and density of orchids and co-flowering species

The species diversity, including orchids, was comparable between Grossart, 18 - 21 species, and MBC, 19 - 23 species, across years (Figure 4.1, Appendices 4.3 and 4.4). By comparison, species richness was slightly higher at Doyle, 24 - 26 species, and lower at McPherson, 6 - 10 species, in both years (Figure 4.1, Appendices 4.2 and 4.5). The density and distribution of orchids varied within and among sites. Since quadrats were placed in areas of high orchid density, it was not surprising that they were consistently among the most abundant species within sites. *Cypripedium candidum* had the highest stem counts at Doyle, Grossart, and MBC, while *C. parviflorum* was the most abundant in quadrats at McPherson, the second most abundant at MBC, and in the top ten at Doyle and Grossart (Figure 4.1, Appendices 4.2 to 4.5). Hybrids were in the top five at both Grossart and MBC (Appendices 4.3 and 4.4). In all sites, quadrats seemed to be dominated by a few key species, with the remainder present in relatively low numbers (Figure 4.1, Appendices 4.2 to 4.5). Within each site, the top five most abundant co-flowering species (not including orchids) was generally consistent across years, with a few exceptions. Among sites, only *Zizia spp.*, *Fragaria virginiana*, *Hypoxis hirsuta*, and *Polygala senega* were among the most abundant at more than one site in a given year.

The PCA showed that quadrats generally tended to cluster together by site, though sites did overlap with one another to some extent. This overlap was not attributable to the common presence of orchids among sites, because orchid stem counts were not included in the PCA. In 2010, the first two PCA axes explained about 39% of the variation in the data (Figure 4.2). Separation among sites was mainly along axis one, while separation of

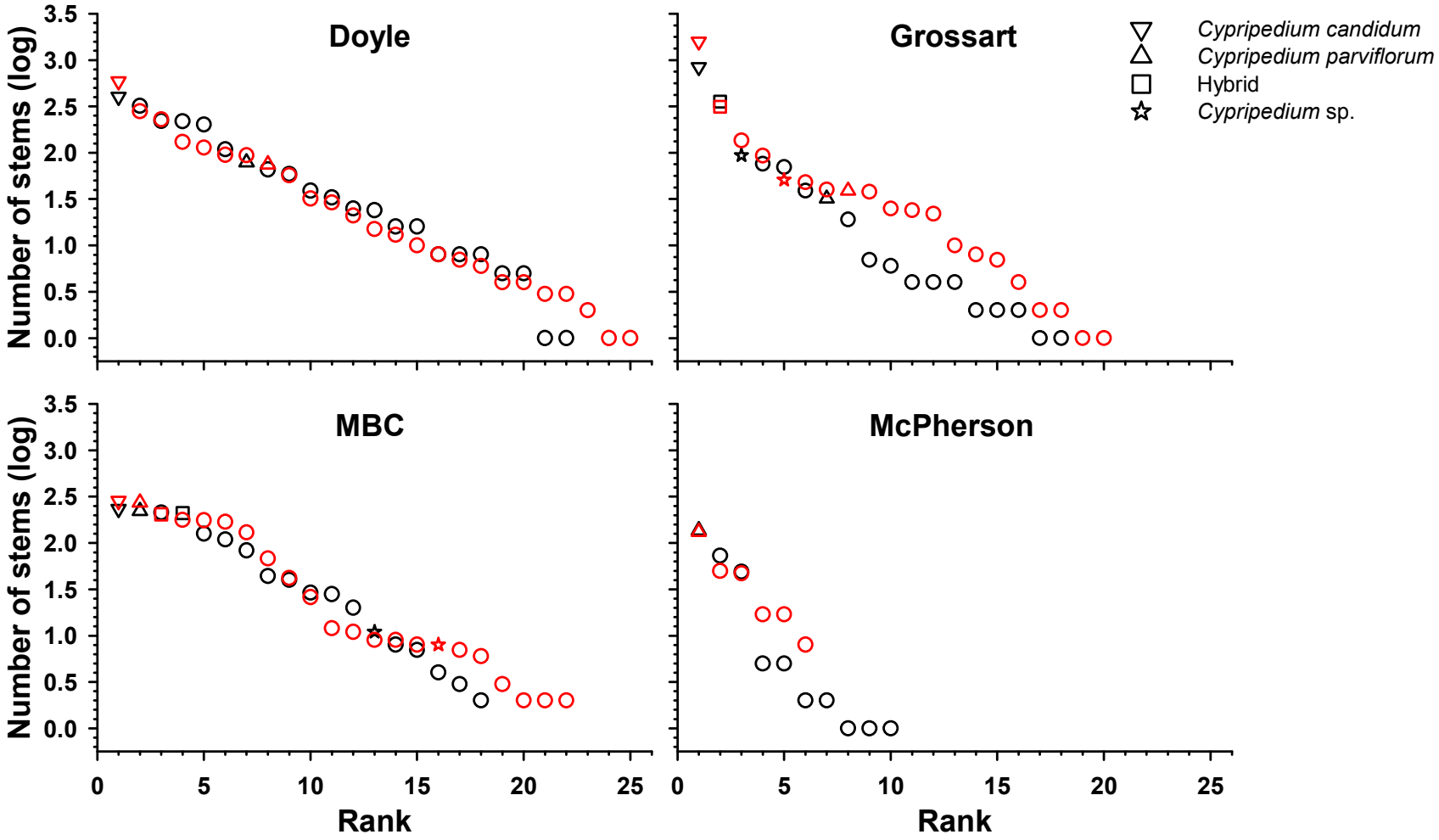

Figure 4.1: Rank abundance plots for all co-flowering vegetation occurring in quadrats at four sites in southern Manitoba: Doyle, Grossart, MBC, and McPherson in 2010 (black symbols) and 2011 (red symbols). Names of each taxon ranked can be found in Appendices 4.2 to 4.5.

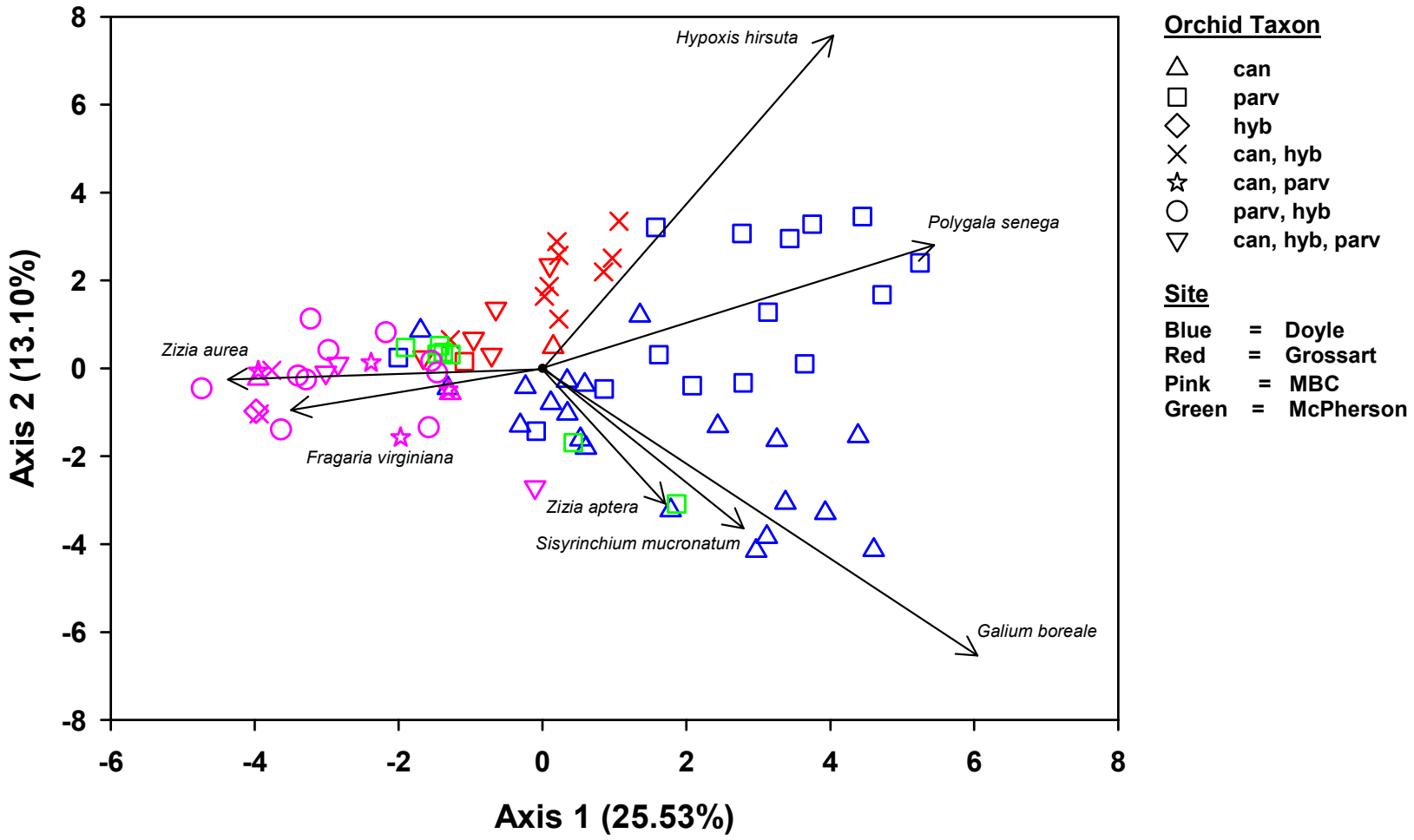

Figure 4.2: Principal components analysis of co-flowering vegetation occurring in sampling quadrats at four sites in southern Manitoba (Doyle, MBC, Grossart, and McPherson) in 2010. Symbols represent the *Cypripedium* orchid taxon mix of each quadrat, and colours represent sites. Arrows represent co-flowering species with the highest axis loadings (see Appendix 4.6).

quadrats within sites was along both axes. In 2011 the amount of variation explained by the two axes was comparable to 2010 (Figure 4.3). Although there was a change in the characteristic vegetation between years, separation among sites was still mainly along axis one and separation of quadrats within sites was along both axes. In both years, co-flowering species with the highest loadings ($\geq$ 0.30) along the first two axes were generally consistent with the most abundant species, though some species, such as *Fragaria virginiana*, were not present in quadrats at all sites (Figure 4.1, Appendices 4.2 to 4.5). Species with high loadings on axis one or two in the analysis of 2010 data included *Galium boreale*, *Polygala senega*, *Hypoxis hirsuta*, *Zizia spp.,* and *Sisyrinchium spp.* (Figure 4.2, Appendix 4.6). *Hypoxis hirsuta*, *Polygala senega*, and *Zizia spp*. also had high loadings in 2011, along with *Comandra umbellata*, *Fragaria virginiana, Senecio pauperculus,* and *Pedicularis canadensis* (Figure 4.3, Appendix 4.7).

In 2010, Doyle showed more variation in quadrat composition than any of the other sites (Figure 4.2). While *C. candidum* quadrats tended to be characterized by higher abundance of *Galium boreale*, *Sisyrinchium spp.*, and *Zizia spp.*, *C. parviflorum* quadrats were usually associated with *Hypoxis hirsuta* and *Polygala senega* (Figure 4.2, Appendix 4.6). By comparison, there was less distinction between quadrat composition of co-flowering species at MBC and Grossart, which contained different complements of orchid taxa. At Grossart quadrats containing *C. candidum* and hybrids were characterized by a greater abundance of *Hypoxis hirsuta* and *Polygala senega* and fewer *Galium boreale*, *Sisyrinchium spp.*, and *Zizia spp.* than those that contain all three orchid taxa (Figure 4.2, Appendix 4.6). At McPherson, most quadrats were tightly clustered together

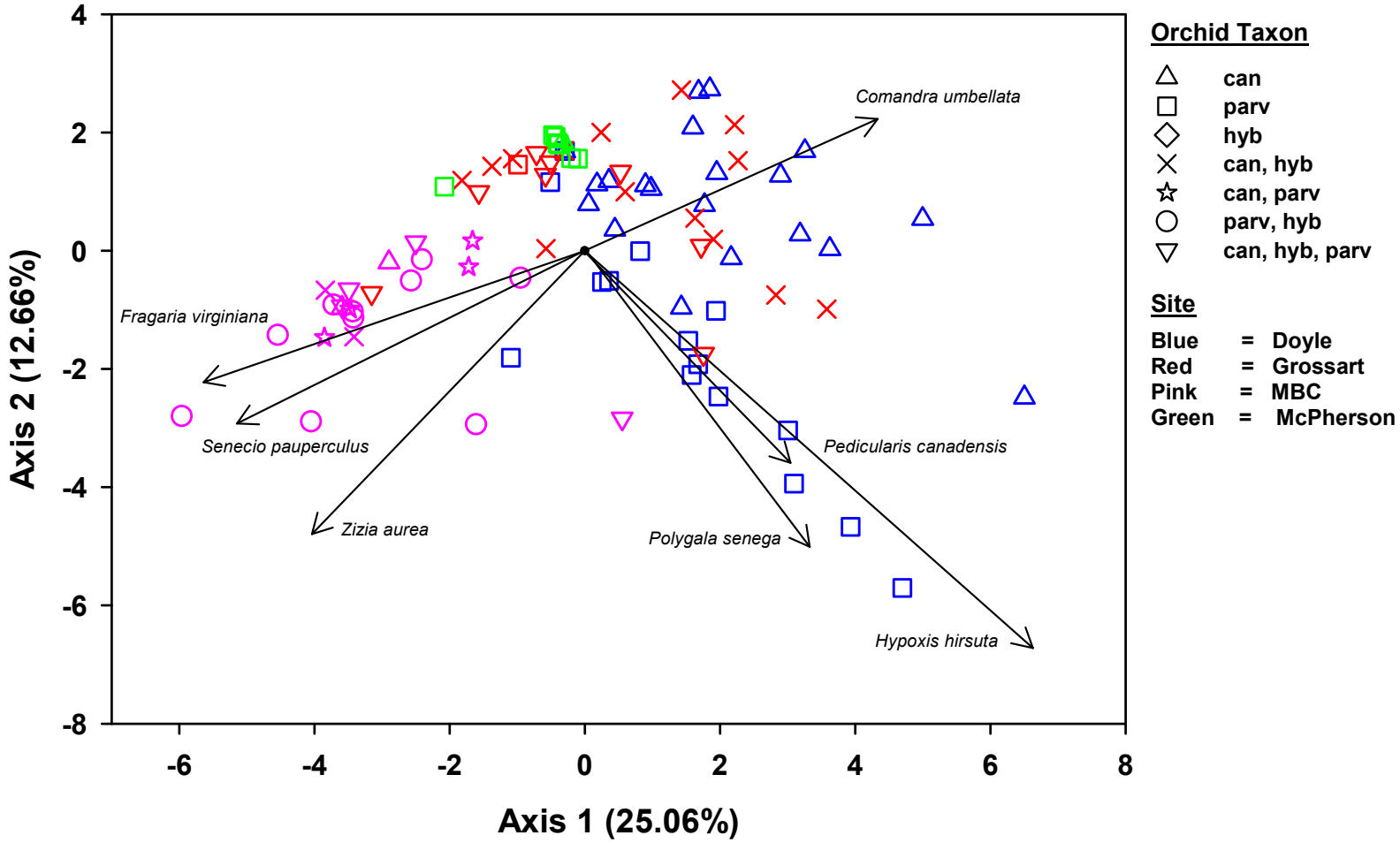

Figure 4.3: Principal components analysis of co-flowering vegetation occurring in sampling quadrats at four sites in southern Manitoba (Doyle, MBC, Grossart, and McPherson) in 2011. Symbols represent the *Cypripedium* orchid taxon mix of each quadrat, and colours represent sites. Arrows represent co-flowering species with the highest axis loadings (see Appendix 4.7).

and overlapped with the other three sites, while two quadrats were more similar to the *C. candidum* quadrats at Doyle (Figure 4.2).

Quadrats at all sites in 2011 were characterized by some combination of the most abundant co-flowering species (Figures 4.2 and 4.3). Because of annual changes in vegetation across sites, these combinations are not identical to those in 2010. However, many of the influential species coincide and the relative positioning of sites is somewhat similar. Quadrats at Grossart were less clustered together than in 2010 and overlapped with all other sites (Figure 4.3). This is likely due to the addition in 2011 of eight quadrats in the meadow portion of the site, whereas all quadrats in 2010 occurred in the ditch. At Doyle, characteristic vegetation in quadrats containing *C. candidum* was again distinct from those containing *C. parviflorum* (Figure 4.3). There did not, however, appear to be any clear pattern regarding the vegetation characterizing quadrats containing different complements of orchid taxa at either MBC or Grossart in 2011 (Figure 4.3).

4.3.2 Non-orchid pollen samples

Co-flowering species in non-orchid pollen samples varied across sites and years (Tables 4.1 to 4.3). Within a given year, there was some overlap between sites with regard to common co-flowering species identified in pollen samples (Tables 4.1 to 4.3). In 2010 for example, *Zizia spp.* and *Senecio pauperculus* pollen was found in *C. parviflorum* at both Doyle and MBC (Tables 4.1 and 4.2). Within sites, there were also some co-flowering species that appeared in subsequent years (Tables 4.1 to 4.3). At MBC for example, *Senecio* or *Erigeron asper*, *Zizia spp.*, and *Smilacina stellata* pollen all appeared in *C. parviflorum* in both 2010 and 2011 (Table 4.2). Among orchid taxa, most

Table 4.1: Non-orchid pollen collected from *Cypripedium candidum*, *C. parviflorum*, and hybrids at Doyle in 2009 and 2010. Shown are means and ranges of abundance (percent of fields of view each pollen type was present in) and the overall abundance rank (see Appendix 4.2). A value of "0" indicates that the pollen was in the sample, but not in any field of view that was sub-sampled. N is the number of samples in which each co-flowering species was observed, among those collected from each orchid taxon (N).

Co-Flowering Species	Collection Year	N	Abundance	2011 Rank
C. candidum	**2009**	**Total: 1**		
Comandra umbellata		1	6.7	7
Fragaria virginiana		1	6.7	21
Krigia biflora or *Agoseris glauca*		1	0.0	11 or 15
Pedicularis canadensis		1	0.0	5
Hybrids	**2009**	**Total: 1**		
Zizia sp.		1	100.0	6
Erisymum asperum *		1	53.3	-
Senecio pauperculus		1	6.7	-
Krigia biflora or *Agoseris glauca*		1	0.0	11 or 15
C. parviflorum	**2009**	**Total: 19**		
Hypoxis hirsuta or *Sisyrinchium sp.* #2		18	82.2 (40.0 – 100.0)	2 or 3
Zizia sp.		17	87.8 (20.0 – 100.0)	6
Hypoxis hirsuta or *Sisyrinchium sp.* #1		17	84.3 (33.3 – 100.0)	2 or 3
Comandra umbellata		17	44.3 (6.7 – 100.0)	7
Polygala senega		14	37.1 (6.7 – 73.3)	4
Krigia biflora or *Agoseris glauca*		13	51.8 (6.7 – 100.0)	11 or 15
Senecio pauperculus		13	24.1 (0.0 – 73.3)	-
Fragaria virginiana		7	31.4 (6.7 – 66.7)	21
Erisymum asperum *		4	28.3 (0.0 – 66.7)	-
Pedicularis canadensis		4	5.0 (0.0 – 20.0)	5
Cornus stolonifera *		2	20.0 (13.3 – 26.7)	-
Tofieldia glutinosa *		2	50.0 (33.3 – 66.7)	14
Lathyrus sp. *		1	20.0	18
Elaeagnus commutata *		1	6.7	24
	2010	**Total: 2**		
Hypoxis hirsuta *		1	100.0	2
Krigia biflora or *Agoseris glauca*		1	100.0	11 or 15
Fragaria virginiana		1	60.0	21
Zizia sp.		1	60.0	6
Cornus stolonifera *		1	33.3	-
Pedicularis canadensis		1	33.3	5
Ribes americanum *		1	20.0	-
Lithospermum canescens		1	6.7	10
Senecio pauperculus		1	6.7	-

Table 4.2: Non-orchid pollen collected from *Cypripedium candidum*, *C. parviflorum*, and hybrids at MBC in 2010 and 2011. Shown are means and ranges of abundance (percent of fields of view each pollen type was present in) and the overall abundance rank (see Appendix 4.4). A value of "0" indicates that the pollen was in the sample, but not in any field of view that was sub-sampled. N is the number of samples in which each co-flowering species was observed, among those collected from each orchid taxon (N).

Co-Flowering Species	Collection Year	N	Abundance	2011 Rank
C. candidum	**2011**	**Total: 1**		
Smilacina stellata *		1	100.0	13
Agoseris glauca or *Taraxacum officinale*		1	86.7	- or 19
Fragaria virginiana or *Potentilla sp.*		1	66.7	4 or -
Zizia spp.		1	13.3	5
Senecio pauperculus or *Erigeron asper*		1	6.7	6 or -
Hybrids	**2011**	**Total: 2**		
Fragaria virginiana or *Potentilla sp.*		2	100.0	4 or -
Agoseris glauca or *Taraxacum officinale*		1	26.7	- or 19
C. parviflorum	**2010**	**Total: 2**		
Senecio pauperculus or *Erigeron asper*		2	40.0 (0.0 – 80.0)	6 or -
Zizia spp.		1	100.0	5
Smilacina stellata *		1	6.7	13
Thalictrum spp. *		1	0.0	14 or 18
	2011	**Total: 6**		
Senecio pauperculus or *Erigeron asper*		5	89.3 (53.3 – 100.0)	6 or -
Agoseris glauca or *Taraxacum officinale*		5	29.3 (6.7 – 100.0)	- or 19
Zizia spp.		3	71.1 (13.3 – 100.0)	5
Anemone canadensis		3	57.8 (0.0 – 100.0)	20
Fragaria virginiana or *Potentilla sp.*		3	22.2 (6.7 – 40.0)	4 or -
Smilacina stellata *		2	60.0 (26.7 – 93.3)	13
Cornus stolonifera *		1	100.0	-
Lathyrus sp. *		1	100.0	-
Hypoxis hirsuta or *Sisyrinchium sp.*		1	80.0	9 or 10
Sisyrinchium sp. *		1	20.0	10
Elaeagnus commutata		1	13.3	17
Ribes americanum *		1	13.3	-

Table 4.3: Non-orchid pollen collected from *Cypripedium candidum* and hybrids at Grossart in 2010 and 2011. Shown are means and ranges of abundance (percent of fields of view each pollen type was present in) and the overall abundance rank (see Appendix 4.3). A value of "0" indicates that the pollen was in the sample, but not in any field of view that was sub-sampled. N is the number of samples in which each co-flowering species was observed, among those collected from each orchid taxon (N).

Co-Flowering Species	Collection Year	N	Abundance	2011 Rank
C. candidum	**2010**	**Total: 2**		
Fragaria virginiana or *Potentilla sp.*		2	71.7 (40.0 – 100.0)	9 or -
*Cornus stolonifera**		2	10.0 (6.7 – 13.3)	-
*Hypoxis hirsuta **		1	20.0	4
Hybrids	**2011**	**Total: 1**		
*Lathyrus sp.**		1	100.0	-

samples were collected from *C. parviflorum* (29), with only four from *C. candidum,* and three from hybrids. Among sites, most pollen samples were collected from Doyle in 2009 (21), one of which was collected from an orchid identified as a possible hybrid.

Co-flowering pollen in samples (on slides) varied from sparse to abundant. The number of species also varied between two and ten co-flowering taxa per sample. However, most samples with many species were dominated by the pollen from one to a few species. In some cases, taxa were distributed equally within the sample. Among orchid taxa, there did appear to be fewer species of pollen in hybrid (two species) and *C. candidum* (four to six species) flowers than in *C. parviflorum* (two to 10 species), although the small number of samples makes it difficult to draw any firm conclusions. Among sites, orchid flowers at Doyle had slightly more taxa per sample (three to 10 in 2009 and three to nine in 2010) than MBC (two to six in 2010 and 2011) and Grossart (four taxa in 2010 and two in 2011). The colour of the orchid being visited did not necessarily match with the colour of the co-flowering species represented in a pollen sample. For example, I did not find that only yellow flowered species were being visited prior to *C. parviflorum*.

The most common species found in non-orchid pollen samples, tended to coincide with the most abundant co-flowering species within sites, based on quadrat stem count data. Additionally, some species that appeared infrequently in pollen samples, *Cornus stolonifera*, *Ribes americanum*, *Lathyrus sp.* for example, were present at a site but not in quadrats (Appendices 4.2 to 4.4, Tables 4.1 to 4.3). At Doyle, the most common species of pollen found in orchid flowers included *Comandra umbellata*, *Hypoxis hirsuta* (or *Sisyrinchium sp.*), *Krigia biflora* (or *Agoseris glauca*), *Polygala senega*, *Senecio*

pauperculus, and *Zizia spp.*, and these were often abundant in quadrats (Table 4.1, Appendix 4.2). In 2010, several species were present in samples from both *C. candidum* and *C. parviflorum*, while only *Krigia biflora* (or *Agoseris glauca*) was found in all three taxa (Table 4.1). In 2010, all species present were found in one of the two pollen samples collected, but never in both (Table 4.1). At MBC, *Senecio* pauperculus (or *Erigeron asper*), *Zizia spp. Agoseris glauca* (or *Taraxacum officinale*) were among the most common, and were also abundant in quadrats (Table 4.2, Appendix 4.4). In 2010 All co-flowering species present were found in one to two of the samples, but never in all three (Table 4.2). In 2011, three species were found in samples from both *C. candidum* and *C. parviflorum*, while two species were present in samples from all three orchid taxa (Table 4.2). At Grossarts in 2010, *Cornus stolonifera* and *Fragaria virginiana* were present in both samples, while in 2011 *Lathyrus sp.* was in the single sample collected (Table 4.3). At this site, none of the co-flowering species were found in more than one orchid taxon. *Hypoxis hirsuta* was the only species that was also common in quadrats (Table 4.3, Appendix 4.3).

4.3.3 Orchid fruit set and co-flowering species

An analysis of variance showed no significant relationship between overall stem abundance of co-flowering plant species and orchid fruit number, even after accounting for variation in the number of orchid flowers (Table 4.4). An increased number of fruits of *C. candidum* in 2011 and *C. parviflorum* in 2010 and 2011 were significantly associated with greater overall abundance of orchid stems (Table 4.4). There was also a significant site by orchid stem interaction for *C. candidum* and *C. parviflorum* in 2011

Table 4.4: Analysis of variance comparing fruit set (# of fruiting stems per quadrat) for *Cypripedium candidum*, *C. parviflorum*, and their hybrids, with orchid flowering stem and co-flowering plant stem abundance per quadrat. Shown are results for 2010 and 2011 at four sites in southern Manitoba (Doyle, Grossart, MBC, and McPherson). N is the number of quadrats used in each analysis.

Effect	***C. candidum***	**Hybrids**	***C. parviflorum***
2010	N = 42	N = 29	N = 43
Model	$R^2 = 0.1656$, P = 0.1426	$R^2 = 0.1601$, P = 0.2171	$R^2 = 0.6686$, P < 0.0001
Site	$F_{2,37} = 0.86$	**$F_{1,25} = 4.75$ ***	**$F_{3,37} = 18.50$ *****
Co-flowering Stems	n.s.	$F_{1,25} = 1.71$	$F_{1,37} = 0.00$
Orchid Flowering Stems	$F_{1,37} = 0.99$	$F_{1,25} = 0.50$	**$F_{1,37} = 24.03$ *****
$b \pm s_b$	n.s.	n.s.	**0.54 ± 0.11**
2011	N = 54	N = 30	N = 47
Model	$R^2 = 0.7625$, P < 0.0001	$R^2 = 0.0385$, P = 0.7913	$R^2 = 0.5441$, P < 0.0001
Site	$F_{2,49} = 1.76$	$F_{1,26} = 0.92$	$F_{3,38} = 2.23$
Co-flowering Stems	n.s.	$F_{1,26} = 0.04$	$F_{1,38} = 0.00$
Orchid Flowering Stems	**$F_{1,49} = 21.44$ *****	$F_{1,26} = 0.47$	**$F_{1,38} = 8.12$ ****
$b \pm s_b$	**0.60 ± 0.13**	n.s.	**0.41 ± 0.14**
Site * Orchid Flowering Stems	**$F_{2,49} = 4.23$ ***	n.s.	**$F_{3,38} = 4.88$ ****
$b \pm s_b$ Doyle	**0.33 ± 0.14**	n.s.	**0.44 ± 0.22**
Grossart	**0.89 ± 0.12**	n.s.	n.s.
MBC	n.s.	-	**0.87 ± 0.27**
McPherson	-	-	n.s.

* P < 0.05
** P < 0.01
*** P < 0.001

(Table 4.4). The resulting slopes for these two taxa at Doyle in 2011 were substantially less than a value of one (Table 4.4), which indicates that the increase in fruit set is less than proportional to the increased number of orchid stems in quadrats at this site. Similar results were found for *C. candidum* at Grossart and *C. parviflorum* at MBC in 2011 (Table 4.4). These slopes were much closer to one however, suggesting that the likelihood of an orchid stem setting fruit is almost proportional to an increase in orchid stems.

Significant relationships between the abundance of individual co-flowering species and orchid fruit set varied across sites and years (Tables 4.5 to 4.7). However, some co-flowering species were not very abundant, and/or did not occur in all sites and years. In addition, the power of these analyses to detect significant relationships may be limited, as the number of quadrats included was often quite low. In most cases, orchids were more likely to set fruit when associated with lower densities of co-flowering species, though there were some exceptions to this.

Most co-flowering species, in low densities, were associated with fruit set by a single orchid taxon in a specific site and year. Lower stem counts of *Zizia* were associated with higher *C. candidum* fruit set at Doyle in 2011, and this species was present in all sites (except McPherson) and years (Table 4.5). Higher *C. parviflorum* fruit set was associated with lower stem counts of *Erigeron asper* and *Lithospermum canescens* in different sites and years, though these species were not always present or abundant (Table 4.6). Hybrid fruit set was also more likely with lower densities of *Hypoxis hirsuta* and *Fragaria virginiana*, also in different sites and years (Table 4.7). These species were mostly present but not always abundant (Table 4.7).

Table 4.5: Logistic analyses comparing *Cypripedium candidum* fruit set (proportion of flowering stems to set fruit) with co-flowering plant abundance over two years (2010 and 2011) at three sites in southern Manitoba (Doyle, Grossart, and MBC). N represents the number of quadrats, and the numbers in brackets are estimate values. Hyphens represent species that were present in quadrats, but not included in analyses, while np represents species not present in quadrats.

Effect	**Doyle**	**Grossart**	**MBC**
2010	N = 20	N = 12	N = 10
Model	Wald $\chi^2 = 4.77$ $P < 0.05$	Wald $\chi^2 = 16.55$ $P < 0.0001$	Wald $\chi^2 = 5.13$ $P < 0.05$
Galium boreale	$\chi^2_{1,18} = 0.04$	-	$\chi^2_{1,8} = 3.28$
Polygala senega	$\chi^2_{1,18} = 0.36$	$\chi^2_{1,10} = 0.78$	-
Sisyrinchium spp.	**$\chi^2_{1,18} = 4.77$* (-0.34)**	np	**$\chi^2_{1,8} = 5.13$ * (1.65)**
Hypoxis hirsuta	$\chi^2_{1,18} = 0.56$	$\chi^2_{1,10} = 3.50$	np
Zizia spp.	$\chi^2_{1,18} = 2.23$	-	$\chi^2_{1,8} = 0.75$
Pedicularis canadensis	$\chi^2_{1,18} = 1.42$	np	np
Comandra umbellata	-	$\chi^2_{1,10} = 2.37$	np
Elaeagnus commutata	np	$\chi^2_{1,10} = 1.41$	-
Agoseris glauca	$\chi^2_{1,18} = 0.26$	**$\chi^2_{1,10} = 16.55$*** (1.06)**	-
Fragaria virginiana	np	$\chi^2_{1,10} = 0.00$	$\chi^2_{1,8} = 0.40$
Senecio pauperculus	np	-	$\chi^2_{1,8} = 1.40$
Lithospermum canescens	-	-	$\chi^2_{1,8} = 0.61$
Viola sororia	-	np	$\chi^2_{1,8} = 3.28$
2011	N = 20	N = 23	N = 12
Model	Wald $\chi^2 = 7.80$ $P < 0.01$	Wald $\chi^2 = 5.39$ $P < 0.05$	Not Significant
Sisyrinchium spp.	$\chi^2_{1,19} = 0.03$	$\chi^2_{1,22} = 0.00$	$\chi^2_{1,12} = 0.57$
Hypoxis hirsuta	$\chi^2_{1,19} = 0.04$	$\chi^2_{1,22} = 1.99$	-
Zizia spp.	**$\chi^2_{1,19} = 7.80$* (-0.78)**	$\chi^2_{1,22} = 0.02$	$\chi^2_{1,12} = 0.38$
Comandra umbellata	$\chi^2_{1,19} = 0.63$	$\chi^2_{1,22} = 0.11$	-
Polygala senega	$\chi^2_{1,19} = 1.03$	-	-
Pedicularis canadensis	$\chi^2_{1,19} = 0.33$	np	-
Agoseris glauca	$\chi^2_{1,19} = 0.57$	$\chi^2_{1,22} = 0.98$	-
Fragaria virginiana	np	$\chi^2_{1,22} = 1.32$	$\chi^2_{1,12} = 0.10$
Senecio pauperculus	np	**$\chi^2_{1,22} = 5.39$* (-0.46)**	$\chi^2_{1,12} = 0.30$
Lithospermum canescens	-	-	$\chi^2_{1,12} = 1.33$
Viola sororia	-	-	$\chi^2_{1,12} = 1.28$

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

Table 4.6: Logistic analyses comparing *Cypripedium parviflorum* fruit set (proportion of flowering stems to set fruit) with co-flowering plant abundance over two years (2010 and 2011) at four sites in southern Manitoba (Doyle, Grossart, MBC, and McPherson). N represents the number of quadrats and numbers in brackets are estimate values. Hyphens represent species that were present in quadrats, but not included in analyses, while np represents species not present in quadrats.

Effect	Doyle	Grossart	MBC	McPherson
2010	N = 14		N = 17	N = 7
Model	Not Significant	n/a	Not Significant	Wald $\chi^2 = 4.55$ $P < 0.05$
Galium boreale	$\chi^2_{1,14} = 0.00$	-	$\chi^2_{1,17} = 0.21$	$\chi^2_{1,5} = 0.31$
Polygala senega	$\chi^2_{1,14} = 0.11$	-	np	np
Sisyrinchium spp.	$\chi^2_{1,14} = 0.61$	-	-	np
Hypoxis hirsuta	$\chi^2_{1,14} = 0.06$	-	-	np
Zizia spp.	$\chi^2_{1,14} = 0.75$	-	$\chi^2_{1,17} = 0.08$	np
Comandra umbellata	-	-	np	np
Polygala senega	$\chi^2_{1,14} = 0.11$	-	-	-
Elaeagnus commutata	np	-	-	$\chi^2_{1,5} = 1.28$
Agoseris glauca	-	-	np	np
Fragaria virginiana	np	-	$\chi^2_{1,17} = 2.68$	$\chi^2_{1,5} = 0.23$
Senecio pauperculus	np	-	$\chi^2_{1,17} = 0.79$	np
Lithospermum canescens	$\chi^2_{1,14} = 0.11$	-	$\chi^2_{1,17} = 1.46$	-
Viola sororia	-	-	$\chi^2_{1,17} = 0.08$	-
Antennaria sp.	np	-	np	$\chi^2_{1,5} = 0.00$
Erigeron asper	np	-	np	**$\chi^2_{1,5} = 4.55$*(-0.53)**
2011	N = 13	N = 9	N = 18	N = 7
Model	Not Significant	Wald $\chi^2 = 4.92$ $P < 0.05$	Wald $\chi^2 = 3.87$ $P < 0.05$	Not Significant
Polygala senega	$\chi^2_{1,13} = 0.25$	-	-	-
Sisyrinchium spp.	$\chi^2_{1,13} = 0.57$	$\chi^2_{1,7} = 0.96$	$\chi^2_{1,16} = 1.62$	-
Hypoxis hirsuta	$\chi^2_{1,13} = 1.16$	$\chi^2_{1,7} = 0.57$	$\chi^2_{1,16} = 0.00$	-
Zizia spp.	$\chi^2_{1,13} = 0.20$	-	$\chi^2_{1,16} = 0.38$	-
Comandra umbellata	-	$\chi^2_{1,7} = 0.14$	-	-
Pedicularis canadensis	$\chi^2_{1,13} = 0.28$	np	-	-
Agoseris glauca	np	**$\chi^2_{1,7} = 4.92$* (1.01)**	-	-
Fragaria virginiana	-	$\chi^2_{1,7} = 0.04$	$\chi^2_{1,16} = 0.91$	$\chi^2_{1,7} = 1.39$
Senecio pauperculus	np	$\chi^2_{1,7} = 0.99$	$\chi^2_{1,16} = 0.10$	-
Lithospermum canescens	$\chi^2_{1,13} = 0.35$	$\chi^2_{1,7} = 0.29$	**$\chi^2_{1,16} = 3.87$*(-0.14)**	-
Viola sororia	-	-	$\chi^2_{1,16} = 0.90$	-
Galium boreale	$\chi^2_{1,13} = 0.03$	-	-	$\chi^2_{1,7} = 0.37$
Elaeagnus commutata	-	np	-	$\chi^2_{1,7} = 1.80$
Erigeron asper	np	np	-	$\chi^2_{1,7} = 1.42$
Antennaria sp.	np	np	-	$\chi^2_{1,7} = 0.36$

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

Table 4.7: Logistic analyses comparing *Cypripedium* hybrid fruit set (proportion of flowering stems to set fruit) with co-flowering plant abundance over two years (2010 and 2011) at two sites in southern Manitoba (Grossart and MBC). N represents the number of quadrats and numbers in brackets are estimate values. Hyphens represent species that were present in quadrats, but not included in analyses, while np represents species not present in quadrats.

Effect	**Grossart**	**MBC**
2010	N = 13	N = 16
Model	Wald $\chi^2 = 4.11$, $P < 0.05$	Wald $\chi^2 = 11.43$, $P < 0.001$
Hypoxis hirsuta	$\chi^2_{1,11} = 0.19$	-
Zizia spp.	-	$\chi^2_{1,6} = 0.66$
Comandra umbellata	$\chi^2_{1,11} = 0.00$	np
Polygala senega	$\chi^2_{1,11} = 0.71$	np
Elaeagnus commutata	$\chi^2_{1,11} = 0.04$	$\chi^2_{1,6} = 0.11$
Agoseris glauca	$\chi^2_{1,11} = 0.33$	np
Fragaria virginiana	-	**$\chi^2_{1,6} = 11.43$** (-0.74)**
Senecio pauperculus	**$\chi^2_{1,11} = 4.11$ * (1.39)**	$\chi^2_{1,6} = 0.27$
Lithospermum canescens	-	$\chi^2_{1,6} = 1.63$
Viola sororia	np	$\chi^2_{1,6} = 2.40$
2011	N = 22	N = 16
Model	Wald $\chi^2 = 4.45$, $P < 0.05$	Not Significant
Hypoxis hirsuta	**$\chi^2_{1,13} = 4.45$ * (-0.22)**	$\chi^2_{1,16} = 2.58$
Zizia spp.	-	$\chi^2_{1,16} = 0.01$
Comandra umbellata	$\chi^2_{1,13} = 0.19$	np
Agoseris glauca	$\chi^2_{1,13} = 2.08$	np
Fragaria virginiana	$\chi^2_{1,13} = 0.08$	$\chi^2_{1,16} = 0.03$
Senecio pauperculus	$\chi^2_{1,13} = 1.02$	$\chi^2_{1,16} = 0.56$
Lithospermum canescens	-	$\chi^2_{1,16} = 0.31$
Viola sororia	-	$\chi^2_{1,16} = 0.71$
Sisyrinchium spp.	$\chi^2_{1,13} = 0.00$	$\chi^2_{1,16} = 0.95$

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

There were three co-flowering species that had significant relationships with more than one orchid taxon. Fruit set in both *C. candidum* in 2010 and *C. parviflorum* in 2011 was more likely with higher densities of *Agoseris glauca* at Grossart (Tables 4.6 and 4.7). *Senecio pauperculus* was also associated with higher orchid fruit set at Grossart. *Cypripedium candidum* fruit set was more likely with lower densities of this species in 2011 while hybrid fruit set on the other hand, was more likely with higher densities in 2010 (Tables 4.5 and 4.6).

Significant associations between orchid fruit set and loadings of co-flowering plants along PCA axes one and two (see Figures 4.2 and 4.3, Appendices 4.6 and 4.7) varied among orchid taxa and across years (Table 4.8). Species with high loadings on both axes were not the same across years, so it is important to note that the two years are not directly comparable. *Cypripedium candidum* fruit set was not associated with axis loadings in either 2010 or 2011 (Table 4.8). Hybrid fruit set by contrast was significantly associated with axis two in 2010 and with both axes in 2011, while *C. parviflorum* fruit set was associated with axis one in 2010 and axis two in 2011 (Table 4.8). This suggests that fruit set in these two orchids is associated with the co-flowering species with the highest loadings along the two PCA axes in both years, although site and axis scores were somewhat confounded. Many of these co-flowering species were also among the most abundant plants in quadrats among sites (Figures 4.2 and 4.3, Appendices 4.2 to 4.5). In 2010 these included *Galium boreale*, *Polygala senega*, *Zizia spp.*, *Hypoxis hirsuta*, and *Sisyrinchium spp.* (Figure 4.3, Appendix 4.6). *Hypoxis hirsuta*, *Zizia spp.*, and *Polygala senega* had high axis loadings in 2011. *Fragaria virginiana*, *Senecio pauperculus*, *Comandra umbellata*, and *Pedicularis canadensis* were more important in 2011 than

Table 4.8: Logistic analyses comparing fruit set per flower for three orchid taxa (*Cypripedium candidum*, *C. parviflorum*, and their hybrids) with principal components analysis component scores on axes one and two (see Figures 4.2 and 4.3, Appendices 4.6 and 4.7). Shown are results for two years (2010 and 2011) at four sites in southern Manitoba. N represents the number of quadrats.

Effect	***C. candidum***	**Hybrids**	***C. parviflorum***
2010	N = 42	N = 29	N = 42
Model	Not Significant	Wald $\chi^2 = 12.52$, $P < 0.001$	Wald $\chi^2 = 19.19$, $P < 0.0001$
Axis one	$\chi_{1,39} < 0.00$	$\chi_{1,26} = 0.32$	**$\chi_{1,39} = 19.19$*** (0.28, SE 0.06)**
Axis two	$\chi_{1,39} = 0.20$	**$\chi_{1,26} = 12.52$** (-0.48, SE 0.13)**	$\chi_{1,39} = 2.57$
Axis One * Axis Two	n.s.	n.s.	n.s.
2011	N = 55	N = 38	N = 47
Model	Not Significant	Wald $\chi^2 = 11.83$, $P < 0.05$	Wald $\chi^2 = 16.20$, $P < 0.0001$
Axis one	$\chi_{1,52} = 0.06$	**$\chi_{1,35} = 11.67$** (-0.19, SE 0.05)**	$\chi_{1,44} = 0.46$
Axis two	$\chi_{1,52} = 0.05$	**$\chi_{1,35} = 4.21$* (0.19, SE 0.09)**	**$\chi_{1,44} = 16.20$*** (-0.26, SE 0.07)**
Axis One * Axis Two	n.s.	n.s.	n.s.

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

2010 (Figure 4.4, Appendix 4.7). Some of these species also appeared in the non-orchid pollen samples (Tables 4.1 to 4.3). Among those with high loadings along axis one in 2010 and axis two in 2011, *Zizia spp.*, *Hypoxis hirsuta*, and *Pedicularis canadensis* appeared in *C. parviflorum* pollen samples. Hybrid pollen samples in 2011 included *Fragaria virginiana*, which had a high loading along axis one in that year.

4.4 Discussion

4.4.1 Composition of co-flowering rewarding species among sites

Among sites, there were differences in the composition of co-flowering vegetation surrounding orchid taxa within quadrats. This was most pronounced at Doyle. where there was significant spatial separation between *C. candidum* and *C. parviflorum*. *Cypripedium parviflorum* was found closer to existing treed areas and tended to have more woodland species shrubs in the quadrats. On the other hand, *C. candidum* was found in more open prairie habitats, with fewer woodland species. By contrast, taxa were growing closer together at MBC and Grossart where the composition of non-orchid species was more similar among orchid quadrats, because orchid taxa were more interspersed. At the Grossart site, the land owners mow the ditch where the orchids grow every fall, in order to keep down woody encroachment from shrubs also growing there. Across all sites, Grossart had the largest concentration of *C. candidum* stems. In conjunction with spring water levels in the ditch, the mowing could be affecting not only the surrounding vegetation, but the population of orchids themselves. By contrast, there is very little management of MBC and McPherson.

Across years, differences in composition of quadrats could be due in part to local conditions, which in some years may be affecting the growth of plants, including orchids. The use of fire for example, is a common management practice in prairie communities (Hamel et al. 2004; Howe 2000 and references therein). It can influence plant abundance, composition, and plant-plant interactions, which may depend in part on the season and intensity of the burn (Engel and Bidwell 2001; Howe 2000; Suding 2001). Doyle was

subject to an unplanned fire in the fall of 2009, which likely impacted the growth and abundance of vegetation in the spring of 2010.

4.4.2 Orchid reproductive success and co-flowering vegetation

Relationships between orchid fruit set and overall abundance of co-flowering vegetation were not significant. This is not consistent with my hypothesis that orchid fruit set would be lower when plants were growing in areas of high co-flowering plant density. Logistic regression results showed that *C. parviflorum* and hybrid fruit set was significantly associated with the PCA axis scores of co-flowering vegetation (see section 4.3.3), indicating that the particular combination of species must be important. In addition, the multi-species composition of pollen in the samples collected from orchids suggests that insects were visiting several different co-flowering species. There was some overlap between the co-flowering pollen found in orchids and abundant co-flowering species in quadrats. Of particular note were *Hypoxis hirsuta* (or *Sisyrinchium spp.*) and *Zizia spp.* at Doyle (Table 4.1); *Senecio pauperculus*, *Zizia spp.*, and *Fragaria virginiana* at MBC (Table 4.2); and *Fragaria virginiana* at Grossart (Table 4.3). This is generally consistent with my hypothesis that non-orchid pollen found on orchid flowers would reflect the species composition at a site. Considered together, these results suggest that the overall composition of plants at a site, or the presence of a combination of certain species, may be influencing orchid visitation and reproductive success.

Because *Cypripedium* species employ a generalized pollination system, I hypothesized that I would find few differences in pollen collected from the three orchid taxa. This suggests that pollinators visiting orchids may be more generalists than

specialists, and are supported by abundant species at a particular site. This conclusion is consistent with research carried out by at the Doyle site (Semmler, in prep). Her observations of plant-pollinator interactions indicate that some species, that in this study were found visiting orchid taxa, were visiting several flowering species and may be generalists (Semmler, in prep). For example, she observed that dipterans such as *Eristalis stipator*, *Odontomyia pubescens*, and *Toxomerus spp*. visited, among many others, abundant species at Doyle such as *Zizia spp.*, *Galium boreale*, and *Hypoxis hirsuta*. Her observed visits of hymenopterans were generally low for most species, but these insects appeared to be visiting fewer rewarding species than dipterans. Species such as *Andrena spp*., *Lasioglossum spp*. and *Sphecodes spp*. were seen visiting *Zizia* plants, among others.

I additionally sought to determine if the most abundant co-flowering species influenced *Cypripedium* pollination and reproductive success. I found that orchids were more likely to set fruit when growing with lower densities of *Zizia spp.*, *Lithospermum canescens*, *Erigeron asper*, *Hypoxis hirsuta*, *Fragaria virginiana*, and sometimes *Sisyrinchium spp.* and *Senecio pauperculus*. These species may be functioning in a competitive capacity, which is not uncommon for deceptive species as insects can learn to avoid plants once they realize there is no reward (Internicola et al. 2007, 2008; Johnson et al. 2003; Juillet et al. 2007; Lammi and Kuitunen 1995; Thomson 1978; Waser 1978). Competitive effects of co-flowering rewarding species have been reported in other rewardless orchid species. For example, Lammi and Kuitunen (1995) found that adding rewarding *Viola* x *wittrockiana* plants to populations of *Dactylorhiza incarnata* had a negative impact of fruit set.

On the other hand, orchid fruit set was also more likely when growing with higher densities of *Agoseris glauca,* and in some cases *Sisyrinchium spp.* and *Senecio pauperculus*. These species may be acting in a facilitative way, by drawing pollinators close to orchids, which may be subsequently visited. Evidence for facilitative interactions has been reported in other rewardless species as well. Juillet et al. (2007) found that reproductive success of *Traunsteinera globosa* was positively affected by co-occurrence of the rewarding plant *Trifolium pretense*. Johnson et al. (2003) found that pollination success in *Anacamptis morio* was greater for individuals growing alongside rewarding plants *Geum rivale* and *Allium schoenoprasum*. Higher pollination rates in *Cypripedium acaule* were associated with the presence of rewarding ericaceous shrubs (O'Connell and Johnston 1998). Reports for both competition and facilitation of co-flowering species in rewardless systems are widespread in the literature. However, a synthesis of the conditions under which each of these occurs is lacking, and would be very useful for future work examining these relationships.

In some cases, species whose pollen was commonly found in orchids had few significant relationships with orchid fruit set. *Zizia spp.* and *Hypoxis hirsuta* for example, were common in pollen samples from all three orchid taxa, indicating pollinators visited these species prior to visiting orchids (Tables 4.1 and 4.2). These species however, were rarely significantly associated with orchid fruit set in quadrats, and when they were significant, seemed to be acting in a competitive capacity. There were also species in the pollen samples (e.g. *Cornus stolonifera* and *Ribes americanum*) that were present in sites but not in quadrats. These observations suggest that it is important to consider that the scale at which vegetation is investigated may be important in such relationships. For

example, Johnson et al. (2003) compared interactions between pollination success of the rewardless orchid *Anacamptis morio* and two species of rewarding plants at two spatial scales: 1 m^2 and 100 m^2 plots. They found that there was no significant relationship at the smaller $1m^2$ scale with either rewarding plant, but there was a significant positive relationship with both species at the 100 m^2 scale. In this study we were able to capture a picture of the vegetation surrounding orchids at one specific point in time, and at a specific scale (multiple $4m^2$ quadrats). Future studies of larger scale patterns, such as those at the level of the whole site, in combination with smaller scale research, such as this study, could shed additional light on the relationships between *Cypripedium* orchids and co-flowering species.

There was little consistency in the significant associations between *Cypripedium* fruit set and abundant co-flowering species among taxa, sites, or years. The power to detect such relationships was limited in this study, because the sample size (# of quadrats) in some analyses was low. However, unlike a Batesian mimicry system where a rewardless orchid mimics a specific rewarding species, there may not be a consistent link in this system between any specific co-flowering species and *Cypripedium* orchid reproductive success. The rewarding plants and pollinators that are affecting orchid reproduction in one year may not be playing the same role in another year. This may also be occurring in conjunction with annual fluctuations in orchid and non-orchid populations, or with site management.

4.4.3 Conclusions

Communities of co-flowering vegetation varied across site and years in four southern Manitoba populations of *Cypripedium* orchids. The observed differences across years were most marked at Doyle, and at this site were likely due to a fire in fall of 2009. Across sites, there were greater differences among quadrats at Doyle than at any of the others, which could be due in part to differences in spatial separation among orchid taxa, as well as management practices.

Co-flowering vegetation may be acting to attract and maintain pollinators to orchid communities at the site level. However, when abundant and growing close to orchids, certain species such as *Zizia spp.*, *Lithospermum canescens*, *Erigeron asper*, *Hypoxis hirsuta*, *Fragaria virginiana*, and sometimes *Sisyrinchium spp.* and *Senecio pauperculus*, may be drawing pollinators away from orchids because of their floral rewards. On the other hand, there were also a few species that appeared to be acting as facilitators to orchid pollination. These included *Agoseris glauca* and sometimes *Sisyrinchium spp*. and *Senecio pauperculus*. Overlap between species found in non-orchid pollen samples, those that were abundant in quadrats, and those that had high loadings on PCA axes, indicates that it is likely composition and/or the presence of combinations of species that are important for pollination and reproductive success of orchids. It is also possible that the presence of some species, such as *Zizia spp.*, *Hypoxis hirsuta* (or *Sisyrinchium spp.*), *Pedicularis canadensis*, and *Fragaria virginiana*, may be more important than others.

This study has laid some basic groundwork for understanding the relationships between *C. candidum* and *C. parviflorum* and the local co-flowering community. The observation/correlation approach taken in this study is a good starting point to take this

kind of research further, and begin employing some experimental approaches. Future studies consisting of manipulation of co-flowering vegetation, and of individual species in particular, would be very useful for improving our understanding of the relationships between rewardless *Cypripedium* species and co-flowering vegetation.

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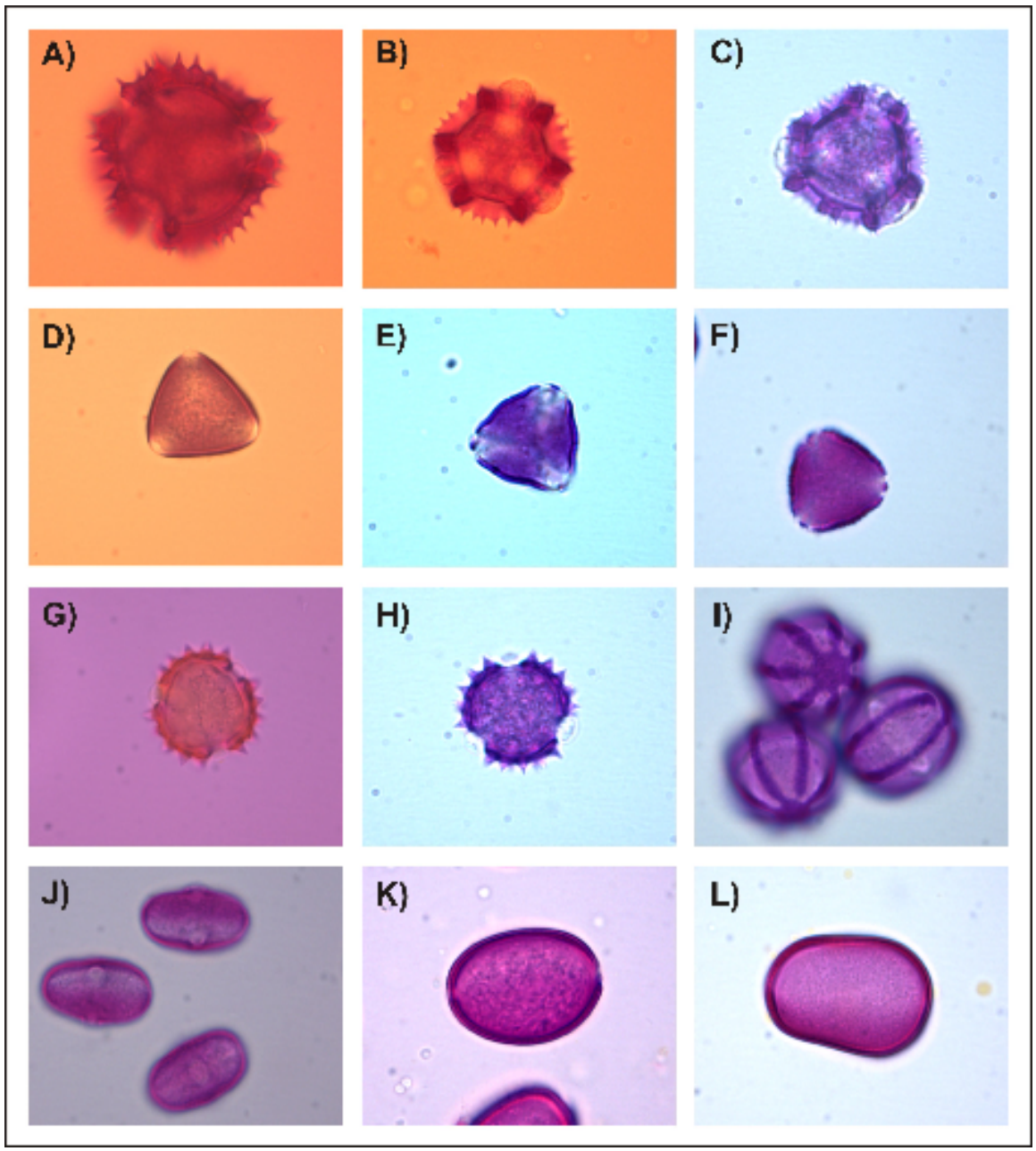
Appendix 4.1: Pollen grains from flowering, rewarding plants growing in association with *Cypripedium candidum* and *C. parviflorum*. A) *Agoseris glauca*, B) *Krigia biflora*, C) *Taraxacum officinale*, D) *Comandra umbellata*, E) *Potentilla anserina*, F) *Fragaria virginiana*, G) *Senecio pauperculus*, H) *Erigeron asper*, I) *Polygala senega*, J) *Zizia* sp., K) *Hypoxis hirsuta*, and L) *Sisyrinchium mucronatum*. Images were taken with a compound microscope at 600x.



Appendix 4.2: Total stem counts for all flowering species sampled in all quadrats at the Doyle site in 2010 and 2011. Shown are the abundance rank for each species and the number of quadrats (N).

	2010 N = 35 quadrats		**2011** N = 35 quadrats	
Rank	**Species**	**Stem Count**	**Species**	**Stem Count**
1	*Cypripedium candidum*	401	*Cypripedium candidum*	591
2	*Galium boreale*	321	*Hypoxis hirsuta*	281
3	*Polygala senega*	221	*Sisyrinchium spp.*	230
4	*Sisyrinchium spp.*	219	*Polygala senega*	131
5	*Hypoxis hirsuta*	202	*Pedicularis canadensis*	114
6	*Zizia spp.*	109	*Zizia spp.*	95
7	*Cypripedium parviflorum*	79	*Comandra umbellata*	94
8	*Pedicularis canadensis*	66	*Cypripedium parviflorum*	75
9	*Thalictrum dasycarpum*	59	*Galium boreale*	57
10	*Vicia americana*	39	*Lithospermum canescens*	32
11	*Viola sororia*	33	*Krigia biflora*	29
12	*Lithospermum canescens*	25	*Sanicula marilandica*	21
13	*Agoseris glauca*	24	*Vicia americana*	15
14	*Sanicula marilandica*	16	*Tolfieldia glutinosa*	13
15	*Tolfieldia glutinosa*	16	*Agoseris glauca*	10
16	*Comandra umbellata*	8	*Zygadenus elegans*	8
17	*Lathyrus palustris*	8	*Castilleja coccinea*	7
18	*Zygadenus elegans*	8	*Lathyrus palustris*	6
19	*Krigia biflora*	5	*Arenaria lateriflora*	4
20	*Thalictrum venulosum*	5	*Thalictrum dasycarpum*	4
21	*Anemone cylindrica*	1	*Fragaria virginiana*	3
22	*Arenaria lateriflora*	1	*Thalictrum venulosum*	3
23	*Anemone canadensis*	0	*Viola sororia*	2
24	*Antennaria sp.*	0	*Elaeagnus comutata*	1
25	*Cornus canadensis*	0	*Cypripedium sp.*	1
26	*Cypripedium* hybrids	0	*Anemone canadensis*	0
27	*Cypripedium sp.*	0	*Anemone cylindrica*	0
28	*Elaeagnus comutata*	0	*Antennaria sp.*	0
29	*Erigeron asper*	0	*Astralagus agreshis*	0
30	*Euphorbia esula*	0	*Cicuta maculata*	0
31	*Fragaria virginiana*	0	*Cornus canadensis*	0
32	*Senecio pauperculus*	0	*Cornus stolonifera*	0
33	*Smilacina stellata*	0	*Cypripedium* hybrids	0
34	*Taraxacum officinale*	0	*Erigeron asper*	0
35			*Euphorbia esula*	0
36			*Potentilla fruticosa*	0
37			*Senecio pauperculus*	0
38			*Smilacina stellata*	0
39			*Taraxacum officinale*	0

Appendix 4.3: Total stem counts for all flowering species sampled in all quadrats at the Grossart site in 2010 and 2011. Shown are the abundance rank for each species and the number of quadrats (N).

	2010 N = 16 quadrats		**2011** N = 24 quadrats	
Rank	**Species**	**# Stems**	**Species**	**# Stems**
1	*Cypripedium candidum*	840	*Cypripedium candidum*	1591
2	*Cypripedium* hybrids	357	*Cypripedium* hybrids	313
3	*Cypripedium sp.*	94	*Comandra umbellata*	136
4	*Comandra umbellata*	76	*Hypoxis hirsuta*	93
5	*Hypoxis hirsuta*	70	*Cypripedium sp.*	51
6	*Polygala senega*	39	*Agoseris glauca*	48
7	*Cypripedium parviflorum*	32	*Senecio pauperculus*	40
8	*Elaeagnus comutata*	19	*Cypripedium parviflorum*	39
9	*Agoseris glauca*	7	*Fragaria virginiana*	38
10	*Fragaria virginiana*	6	*Taraxacum officinale*	25
11	*Galium boreale*	4	*Polygala senega*	24
12	*Senecio pauperculus*	4	*Sisyrinchium spp.*	22
13	*Zizia spp.*	4	*Zizia spp*	10
14	*Euphorbia esula*	2	*Euphorbia esula*	8
15	*Lithospermum canescens*	2	*Galium boreale*	7
16	*Taraxacum officinale*	2	*Thalictrum dasycarpum*	4
17	*Lathyrus palustris*	1	*Elaeagnus comutata*	2
18	*Thalictrum dasycarpum*	1	*Lithospermum canescens*	2
19	*Anemone canadensis*	0	*Smilacina stellata*	1
20	*Anemone cylindrica*	0	*Viola sororia*	1
21	*Antennaria sp.*	0	*Anemone canadensis*	0
22	*Arenaria lateriflora*	0	*Anemone cylindrica*	0
23	*Cornus canadensis*	0	*Antennaria sp.*	0
24	*Erigeron asper*	0	*Arenaria lateriflora*	0
25	*Krigia biflora*	0	*Astralagus agreshis*	0
26	*Pedicularis canadensis*	0	*Castilleja coccinea*	0
27	*Sanicula marilandica*	0	*Cicuta maculata*	0
28	*Sisyrinchium spp.*	0	*Cornus canadensis*	0
29	*Smilacina stellata*	0	*Cornus stolonifera*	0
30	*Thalictrum venulosum*	0	*Erigeron asper*	0
31	*Tolfieldia glutinosa*	0	*Krigia biflora*	0
32	*Vicia americana*	0	*Lathyrus palustris*	0
33	*Viola sororia*	0	*Pedicularis canadensis*	0
34	*Zygadenus elegans*	0	*Potentilla fruticosa*	0
35			*Sanicula marilandica*	0
36			*Thalictrum venulosum*	0
37			*Tolfieldia glutinosa*	0
38			*Vicia americana*	0
39			*Zygadenus elegans*	0

Appendix 4.4: Total stem counts for all flowering species sampled in all quadrats at the Manitoba Conservation site in 2010 and 2011. Shown are the abundance rank for each species and the number of quadrats (N).

	2010 N = 22 quadrats		**2011** N = 22 quadrats	
Rank	**Species**	**Stem Count**	**Species**	**Stem Count**
1	*Cypripedium candidum*	232	*Cypripedium candidum*	284
2	*Cypripedium parviflorum*	222	*Cypripedium parviflorum*	274
3	*Zizia spp.*	213	*Cypripedium* hybrids	204
4	*Cypripedium* hybrids	210	*Fragaria virginiana*	177
5	*Fragaria virginiana*	126	*Zizia spp.*	176
6	*Senecio pauperculus*	109	*Senecio pauperculus*	169
7	*Lithospermum canescens*	83	*Lithospermum canescens*	130
8	*Viola sororia*	44	*Viola sororia*	68
9	*Thalictrum dasycarpum*	40	*Hypoxis hirsuta*	42
10	*Smilacina stellata*	29	*Sisyrinchium spp.*	26
11	*Anemone canadensis*	28	*Zygadenus elegans*	12
12	*Galium boreale*	20	*Galium boreale*	11
13	*Cypripedium sp.*	11	*Smilacina stellata*	9
14	*Taraxacum officinale*	8	*Thalictrum venulosum*	9
15	*Elaeagnus comutata*	7	*Arenaria lateriflora*	8
16	*Hypoxis hirsuta*	4	*Cypripedium sp.*	8
17	*Euphorbia esula*	3	*Elaeagnus comutata*	7
18	*Sisyrinchium spp.*	2	*Thalictrum dasycarpum*	6
19	*Agoseris glauca*	0	*Taraxacum officinale*	3
20	*Anemone cylindrica*	0	*Anemone canadensis*	2
21	*Antennaria sp.*	0	*Euphorbia esula*	2
22	*Arenaria lateriflora*	0	*Astralagus agreshis*	2
23	*Comandra umbellata*	0	*Agoseris glauca*	0
24	*Cornus canadensis*	0	*Anemone cylindrica*	0
25	*Erigeron asper*	0	*Antennaria sp.*	0
26	*Krigia biflora*	0	*Castilleja coccinea*	0
27	*Lathyrus palustris*	0	*Cicuta maculata*	0
28	*Pedicularis canadensis*	0	*Comandra umbellata*	0
29	*Polygala senega*	0	*Cornus canadensis*	0
30	*Sanicula marilandica*	0	*Cornus stolonifera*	0
31	*Thalictrum venulosum*	0	*Erigeron asper*	0
32	*Tolfieldia glutinosa*	0	*Krigia biflora*	0
33	*Vicia americana*	0	*Lathyrus palustris*	0
34	*Zygadenus elegans*	0	*Pedicularis canadensis*	0
35			*Polygala senega*	0
36			*Potentilla fruticosa*	0
37			*Sanicula marilandica*	0
38			*Tolfieldia glutinosa*	0
39			*Vicia americana*	0

Appendix 4.5: Total stem counts for all flowering species sampled in all quadrats at the McPherson site in 2010 and 2011. Shown are the abundance rank for each species and the number of quadrats (N).

	2010 N = 7 quadrats		**2011** N = 7 quadrats	
Rank	**Species**	**Stem Count**	**Species**	**Stem Count**
1	*Cypripedium parviflorum*	137	*Cypripedium parviflorum*	132
2	*Elaeagnus comutata*	73	*Galium boreale*	50
3	*Galium boreale*	49	*Eleagnus comutata*	47
4	*Antennaria sp.*	5	*Antennaria sp.*	17
5	*Erigeron asper*	5	*Fragaria virginiana*	17
6	*Fragaria virginiana*	2	*Erigeron asper*	8
7	*Taraxacum officinale*	2	*Agoseris glauca*	0
8	*Cornus canadensis*	1	*Anemone canadensis*	0
9	*Lithospermum canescens*	1	*Anemone cylindrica*	0
10	*Viola sororia*	1	*Arenaria lateriflora*	0
11	*Agoseris glauca*	0	*Astralagus agreshis*	0
12	*Anemone canadensis*	0	*Castilleja coccinea*	0
13	*Anemone cylindrica*	0	*Cicuta maculata*	0
14	*Arenaria lateriflora*	0	*Comandra umbellata*	0
15	*Comandra umbellata*	0	*Cornus canadensis*	0
16	*Cypripedium candidum*	0	*Cornus stolonifera*	0
17	*Cypripedium* hybrids	0	*Cypripedium candidum*	0
18	*Cypripedium sp.*	0	*Cypripedium* hybrids	0
19	*Euphorbia esula*	0	*Cypripedium sp.*	0
20	*Hypoxis hirsuta*	0	*Euphorbia esula*	0
21	*Krigia biflora*	0	*Hypoxis hirsuta*	0
22	*Lathyrus palustris*	0	*Krigia biflora*	0
23	*Pedicularis canadensis*	0	*Lathyrus palustris*	0
24	*Polygala senega*	0	*Lithospermum canescens*	0
25	*Sanicula marilandica*	0	*Pedicularis canadensis*	0
26	*Senecio pauperculus*	0	*Polygala senega*	0
27	*Sisyrinchium spp.*	0	*Potentilla fruticosa*	0
28	*Smilacina stellata*	0	*Sanicula marilandica*	0
29	*Thalictrum dasycarpum*	0	*Senecio pauperculus*	0
30	*Thalictrum venulosum*	0	*Sisyrinchium spp.*	0
31	*Tolfieldia glutinosa*	0	*Smilacina stellata*	0
32	*Vicia americana*	0	*Taraxacum officinale*	0
33	*Zizia spp.*	0	*Thalictrum dasycarpum*	0
34	*Zygadenus elegans*	0	*Thalictrum venulosum*	0
35			*Tolfieldia glutinosa*	0
36			*Vicia americana*	0
37			*Violoa sororia*	0
38			*Zizia spp.*	0
39			*Zygadenus elegans*	0

Appendix 4.6: Co-flowering species loadings on axes one and two of principal components analysis (PCA, see Figure 4.2). Shown are results for PCA carried out on all quadrats across all four sampling sites in 2010.

Axis One		Axis Two	
Taxon	Loading	Taxon	Loading
Galium boreale	0.50293	*Hypoxis hirsuta*	0.62983
Polygala senega	0.45387	*Galium boreale*	-0.54642
Zizia aurea	-0.36390	*Sisyrinchium mucronatum*	-0.30373
Hypoxis hirsuta	0.33837	*Zizia aptera*	-0.25709
Fragaria virginiana	-0.29182	*Polygala senega*	0.23330
Sisyrinchium mucronatum	0.23423	*Viola sororia*	-0.16681
Senecio pauperculus	-0.22023	*Comandra umbellata*	0.14197
Pedicularis canadensis	0.14845	*Eleagnus comutata*	0.09265
Zizia aptera	0.14150	*Fragaria virginiana*	-0.08101
Lithospermum canescens	-0.13787	*Agoseris glauca*	-0.07711
Thalictrum dasycarpum	0.09418	*Pedicularis canadensis*	0.06364
Vicia americana	0.08590	*Zygadenus elegans*	0.05532
Eleagnus comutata	-0.07609	*Tolfieldia glutinosa*	0.05447
Agoseris glauca	0.06328	*Thalictrum dasycarpum*	0.05394
Sanicula marilandica	0.05369	*Sanicula marilandica*	0.04161
Tolfieldia glutinosa	0.05217	*Vicia americana*	0.03833
Viola sororia	-0.05133	*Krigia biflora*	0.03467
Smilacina stellata	-0.04493	*Sisyrinchium montanum*	0.03322
Zygadenus elegans	0.04332	*Lathyrus palustris*	0.03008
Taraxacum officinale	-0.03881	*Lithospermum canescens*	0.02898
Comandra umbellata	0.03838	*Thalictrum venulosum*	0.02604
Anemone canadensis	-0.03260	*Senecio pauperculus*	-0.02602
Lathyrus palustris	0.02580	*Zizia aurea*	-0.02173
Thalictrum venulosum	0.02567	*Taraxacum officinale*	-0.01821
Krigia biflora	0.02348	*Antennaria sp.*	-0.01529
Euphorbia esula	-0.01319	*Arenaria lateriflora*	0.01296
Sisyrinchium montanum	-0.01159	*Anemone canadensis*	-0.00645
Erigeron asper	-0.00887	*Anemone cylindrica*	0.00516
Anemone cylindrica	0.00649	*Erigeron asper*	0.00406
Cornus canadensis	-0.00394	*Smilacina stellata*	0.00283
Arenaria lateriflora	0.00327	*Cornus canadensis*	0.00192
Antennaria sp.	0.00198	*Euphorbia esula*	-0.00021

Appendix 4.7: Co-flowering species loadings on axes one and two of principal components analysis (PCA, see Figure 4.3). Shown are results for PCA carried out on all quadrats across all four sampling sites in 2011.

Axis One		Axis Two	
Taxon	Loading	Taxon	Loading
Hypoxis hirsuta	0.47592	*Hypoxis hirsuta*	-0.56182
Fragaria virginiana.	-0.40142	*Polygala senega*	-0.41849
Senecio pauperculus	-0.36724	*Zizia aurea*	-0.39906
Comandra umbellata	0.30961	*Pedicularis canadensis*	-0.30019
Sisyrinchium mucronatum	0.29980	*Senecio pauperculus*	-0.24267
Zizia aurea	-0.28654	*Lithospermum canescens*	-0.20475
Polygala senega	0.23787	*Comandra umbellata*	0.18551
Pedicularis canadensis	0.21785	*Fragaria virginiana*	-0.18507
Lithospermum canescens	-0.20254	*Viola sororia*	-0.15718
Viola sororia.	-0.17172	*Krigia biflora*	-0.13758
Zizia aptera	0.12536	*Sisyrinchium mucronatum*	0.12879
Sisyrinchium montanum	-0.08170	*Agoseris glauca*	0.08757
Krigia biflora	0.06034	*Zygadenus elegans*	-0.0623
Eleagnus comutata	-0.04399	*Eleagnus comutata*	0.05647
Castilleja coccinea	0.04131	*Tolfieldia glutinosa*	-0.05616
Galium boreale	0.04039	*Galium boreale*	-0.05118
Tolfieldia glutinosa	0.03082	*Zizia aptera*	0.04317
Arenaria lateriflora	-0.03050	*Castilleja coccinea*	-0.04126
Thalictrum venulosum	-0.02710	*Sanicula marilandica*	-0.03967
Taraxacum officinale	-0.02387	*Antennaria sp.*	0.03617
Vicia americana	0.01332	*Vicia americana*	-0.02624
Smilacina stellata	-0.01250	*Arenaria lateriflora*	-0.02506
Sanicula marilandica	0.00982	*Erigeron asper*	0.02112
Agoseris glauca	-0.00893	*Thalictrum dasycarpum*	-0.01939
Astralagus agreshis	-0.00469	*Thalictrum venulosum*	-0.01906
Euphorbia esula	-0.00457	*Sisyrinchium montanum*	-0.01879
Anemone canadensis	-0.00453	*Lathyrus palustris*	0.01085
Thalictrum dasycarpum	0.00320	*Taraxacum officinale*	0.01051
Zygadenus elegans	-0.00256	*Euphorbia esula*	0.01040
Erigeron asper	-0.00255	*Astralagus agreshis*	-0.00148
Lathyrus palustris	-0.00242	*Anemone canadensis*	0.00090
Antennaria sp.	-0.00238	*Smilacina stellata*	-0.00029

5. Concluding Comments and Future Directions

This thesis explored the factors affecting the reproductive success of two species of lady's slipper orchids and their hybrids in southern Manitoba, by investigating relative fruit and seed set, differences in floral morphologies and the relationship between the attributes of orchid taxa and the surrounding floral and insect communities. I found that these aspects of orchid biology were affected by multiple factors, and more than one of these is likely acting at the same time. Orchid morphology, exit route in particular, is one factor influencing reproductive success among orchid taxa by differentially limiting the number and diversity of potential pollinators visiting each taxon. Across sites, *Cypripedium candidum* had the smallest exit routes, *C. parviflorum* the largest, and hybrids were intermediate. This pattern was reflected at MBC in the insect visitation to each taxon: *C. candidum* has the fewest visitors, *C. parviflorum* the most, and hybrid visitors were intermediate. Variation in exit routes may be affecting pollinator sharing between orchid taxa, which is likely influencing the frequency and directionality of hybridization. Hybrid exit routes at MBC overlapped more with *C. parviflorum* than with *C. candidum,* and the two species were also sharing more floral visitors and potential pollinators. The importance of morphology and pollinator sharing has also been investigated in other rewardless orchids. For example, Bänziger et al. (2008) found that despite morphological compatibility, hybridization between species was prevented by lack of pollinator sharing. In addition to influencing hybridization, morphological overlap among orchid taxa could be a consequence of genetic exchange among taxa resulting from hybridization. If hybrids are exchanging genes more frequently with one parent

versus another, those taxa may have greater morphological overlap than with the less frequent parent.

The co-flowering vegetation within a site also seems to be affecting orchid reproductive success. Based on the presence of non-orchid pollen, composition and density of co-flowering rewarding plants seems to be functioning in attracting and maintaining pollinators in orchid populations. Within sites, the overall abundance of co-flowering stems did not affect orchid fruit set. Orchids however do appear to be setting more fruit in some instances when growing with a greater number of orchid stems. Individual co-flowering species may be acting both in a facilitative and competitive capacity by either drawing pollinators towards or away from the rewardless *Cypripedium* orchids. These specific relationships seem to differ among sites and years, which could be a result of changes in the vegetation due to factors such as site management or climate. Both facilitative (e.g. O'Connell and Johnston 1998) and competitive interactions (e.g. Johnson et al. 2003; Lammi and Kuitunen 1995) have been previously reported for generalized food mimics.

Flowering phenology and distribution of rewarding and rewardless plants may also be impacting orchids. Differences in phenological overlap and spatial separation among orchid taxa could be influencing interactions with the pollinator community. If orchids are not blooming at the same time, or if they are too far away from each other within a site, the likelihood of pollinator sharing and hybridization may be decreased. Alternatively, orchids that were growing more interspersed with one another may have an increased likelihood of sharing pollinators. It would be interesting to collect data from more southerly populations of *C. candidum*, where reproductive success seems to be

higher than in Manitoba populations (e.g. Bowles 1983, Shefferson and Simms 2007). It would be possible to then investigate whether flowering early in the season helps these populations avoid an increased level of competition, which may occur with a greater number of rewarding flowers later in the season.

There is a relatively small body of literature regarding the factors involved in the reproductive success and hybridization of *C. candidum* and *C. parviflorum*. There are many aspects of *Cypripedium* orchid biology introduced in this study that require further exploration. Studies spanning various geographic areas would be helpful for making comparisons between populations of species throughout their individual ranges as well as between species in Asia, Europe, and North America. In populations where hybridization involves endangered species such as *C. candidum*, these types of studies would contribute important information regarding hybrid and parental population structure, which would be very useful for management and conservation of these species. In order gain a better understanding of reproductive success, studies looking at pollen viability would be a good complement to studies of fruit and seed set. In combination, these three measures could provide a more complete picture of orchid reproductive success. The differences in fruit set among taxa reported in this study are consistent with 2009 pollen viability results that were determined in a previous study on the same populations: average pollen viability was lowest in *C. candidum*, intermediate in hybrids and highest in *C. parviflorum* (Worley and Ford, unpubl. data).

More focused and detailed research is certainly needed to better understand the relationships between *C. candidum*, *C. parviflorum* and their pollinators, the factors that affect these relationships, and the impact these relationships have on reproductive success.

These could include for example, studies looking at changes in the pollinator community throughout the orchid flowering season, impacts of site management (such as mowing or fire regime), and effects of climatic conditions. Studies investigating orchid phenology (especially bloom dates) and spatial distribution in conjunction with the variation and composition of the local pollinator communities would help researchers gain a better understanding of the potential and realized interactions between plants and pollinators. Additionally, a more thorough sampling of non-orchid pollen collected from each orchid taxon over several years would be very helpful in carrying out a more robust analysis of the relationships with co-flowerins species. This type of data could potentially reveal annual and long-term patterns in floral visitation by pollinators. In conjunction with this, a collection of pollen from the bodies of insects observed visiting orchids would provide more direct evidence of floral visitation patterns, and would be complementary to the data provided by collecting pollen from orchids themselves.

There is also a need for more in depth and experimental research looking at the role played by rewarding co-flowering vegetation in populations of *C. candidum* and *C. parviflorum*. For example, once the abundant species at a site have been determined, and potentially important species for orchid reproductive success have been identified, these could be used in more targeted vegetation removal or addition experiments. These types of studies would help determine the impact of individual co-flowering species or orchid fruit set. Additionally, long-term monitoring of orchid populations, using a larger sample size, would help researchers track the annual fluctuations in orchid reproductive success and abundance of co-flowering vegetation. Various scales should also be investigated to

see if there are any differences in the importance of co-flowering vegetation at different levels of the landscape (e.g. $1m^2$ versus 100 m^2).

This project has contributed to the limited body of knowledge on the reproductive fitness and pollination ecology of the endangered *C. candidum* and its hybridization with the common *C. parviflorum*. My research will also provide valuable background information for future studies of *Cypripedium* reproductive biology and ongoing conservation efforts. In the future, the types of studies outlined above will enrich our understanding of the biology and reproduction of these orchid species, and will be especially important for addressing any conservation or management concerns regarding the endangered *C. candidum*.

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