Pollination ecology and the floral rewards of *Vaccinium myrtilloides* and *V. vitis-idaea* (Ericaceae)

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Abstract

The goals of this research project were to investigate aspects of pollination biology of two native boreal species: *Vaccinium myrtilloides* (Canadian blueberry) and *Vaccinium vitis-idaea* (lingonberry) in central Saskatchewan. Accordingly, surveys of insect taxa visiting the flowers were performed, and determination of the effectiveness of these insect taxa to serve as pollinators was measured through pollen deposition and pollen tube growth in the style. Accompanying fieldwork, and morphological and anatomical studies were done for the two food rewards offered by flowers of both *Vaccinium* species: pollen released through poricidal anthers and nectar secreted from the nectary atop the inferior ovary.

Pollen-ovule (P/O) ratios were determined for the two study species in Saskatchewan (*V. myrtilloides, V. vitis-idaea*) as well as for five other *Vaccinium* species from eastern Canada (Nova Scotia – *V. angustifolium, V. boreale, V. caespitosum, V. corymbosum,* and *V. uliginosum*). Pollen, released at maturity as tetrads, were converted to total pollen grains per flower to yield P/O ratios ranging from 238 (*V. caespitosum*) to 2,008 (*V. vitis-idaea*), but 736 for the latter in Saskatchewan. These P/O ratios are indicative of a breeding system ranging from facultative autogamy to facultative xenogamy. Additionally, the structure of mature stamens and pollen tetrads was studied in *V. myrtilloides* and *V. vitis-idaea*. Each anther was functionally bilocular; had a single-cell thickness (i.e., epidermis) with regularly occurring papillae; lacked an endothecium; and possessed two distal, hollow tubules each terminating in a pore. Overall pollen grain viability was 76-97% (*V. myrtilloides*) and 51-93% (*V. vitis-idaea*), with about 20% of tetrads having only 1-3 grains viable, and 12% and 27% of tetrads entirely non-viable in *V. myrtilloides* and *V. vitis-idaea*, respectively. Pollen tetrads occasionally were connected by a sticky substance resembling pollenkitt, but viscin threads were absent. One instance of precocious (*in situ*) germination of tetrads was recorded within anthers of *V. myrtilloides*.

The floral nectary was a disk of secretory tissue situated between the stamens and the style. The epidermis possessed solitary stomata that were variable in number, but not different between *V. myrtilloides* and *V. vitis-idaea*. The nectary was vascularized by phloem alone; many traces were found for *V. myrtilloides* throughout the nectary, whereas *V. vitis-idaea* had few traces at the nectary base, concentrated at the inner side of the disk closest to the style base. Young sclerenchyma cells were found throughout the nectary parenchyma. Nectar production started on the day of anthesis for both species, although many flowers of *V. vitis-idaea* appeared to have no measureable nectar at that time. *V. myrtilloides* produced a larger range of nectar solutes per flower (0 - 3684.1 µg), than *V. vitis-idaea*

(1.29 to 1147.62 µg) over both years; nectar volumes per flower never exceeded 5 µL. Nectar was measured daily in flowers aged 1 – 4 days in 2010 and 1 – 6 days in 2011, however, over the two years at the same study site there was no clear pattern of secretion and reabsorption throughout flower life for *V. myrtilloides*, and only a gradual increase for *V. vitis-idaea* as flowers aged.

Insect visitors to flowers surveyed in 2010 included a large proportion of honeybees (*Apis mellifera*) as visitors to both species, whereas in 2011 there were no honeybees present at the field site. There was a larger proportion of hoverflies (Syrphidae) found on the flowers of *V. vitis-idaea* than on *V. myrtilloides*. Other visitors to *V. myrtilloides* were bees (*Bombus, Andrena, Osmia, Colletes*) and wasps (Vespidae), whereas flowers of *V. vitis-idaea* were visited by bees (*Bombus, Andrena, Osmia, Lasioglossum, Colletes, Hylaeus*), an ant (Formicidae) and a butterfly (Lycaenidae). *Bombus* spp. were shown to be pollinators of *V. myrtilloides*. *Andrena* spp. were probable pollinators, whereas honeybees appeared to be poor pollinators. *Bombus* spp. seemed probable pollinators of *V. vitis-idaea* and hoverflies to be barely more than visitors, though small sample sizes did not allow for conclusive evidence. The time that an insect spent on a virgin flower had no relationship to the pollination result. Among individuals of various *Bombus* spp. that did or did not sonicate flowers of *V. myrtilloides*, the action of “buzz pollination” was shown to result in an increase in the number of pollen tetrads deposited and in pollen tube growth.

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Table of Contents

Table of Figures

List of Tables

1 Introduction to *Vaccinium* species

1.1 Taxonomy and characteristics of *Vaccinium* species

The genus *Vaccinium* belongs to the Ericaceae, a cosmopolitan family that contains approximately 4,100 species within 124 genera. An extensive review by Kron *et al.* (2002a) confirms the group is monophyletic and provides a thorough account of the clades within the family. Sub-families of Enkianthoideae, Monotropoideae, Arbutoideae, Ericoideae, Styphelioideae and Vaccinioideae are currently recognized. Sub-family Vaccinioideae in turn encompasses the tribes of Oxydendreae, Lyonieae, Andromedeae, Gauttherieae, and Vaccinieae, the lattermost of which includes *Vaccinium myrtilloides* Michx. and *V. vitis-idaea* L.

The tribe Vaccinieae is composed of 30-35 genera that are mostly found in tropical regions around the world, inhabiting all continents except Australia and Antarctica, and only the southern portion of Africa (including Madagascar). Within this tribe, flower morphology is typically used to separate genera but it often fails to delimit them; well- supported groups are not represented in the current taxonomical groupings. In the case of *Vaccinium*, though more morphological and molecular evidence is needed, the genus is thought to be polyphyletic and thus will likely need to be expanded or disbanded (Kron *et al.* 2002b).

Although primarily recognized for its edible temperate species of blueberry and cranberry, the genus *Vaccinium* is very tropical in its distribution. Current estimates (Judd *et al.* 2008) have the number of *Vaccinium* species worldwide at 740, positioning *Vaccinium* as the third largest genus in the Ericaceae after *Erica* (860 spp.) and *Rhododendron* (850 spp.).

Table 1.1 shows a comparison between the two study species: *V. myrtilloides* and *V. vitis-idaea* that are described below.

1.1.1 *Vaccinium myrtilloides*

Vaccinium myrtilloides Michx. is commonly known as velvet-leaf blueberry, Canadian blueberry, sour-top blueberry or sour-top bilberry. In taxonomic classification systems, it is also known by a variety of synonyms: *V. canadense* Kalm ex A. Rich., *V. pensylvanicum* var. *myrtilloides* Michx., *V. angustifolium* var. *myrtilloides* (Michx.) House, and *V. angustifolium* var. *inegrifolium* Legage. There is also a form of *V. myrtilloides* that possesses white fruit instead of the typical blue fruit and it is denoted as *V. myrtilloides* forma *chicoccum* (Deane). *Vaccinium*

Table 1.1 Comparison of previously reported characteristics of *Vaccinium myrtilloides* and *Vaccinium vitis-idaea.*

Character	*Vaccinium myrtilloides* Michx.	*Vaccinium vitis-idaea* L.
Common names	Velvet-leaf blueberry, Canadian blueberry, sour-top blueberry or sour-top bilberry[1]	Foxberry, cowberry, mountain cranberry, partridgeberry, dry ground cranberry, rock cranberry, ling berry, lingonberry, redberries[1]
Distribution	North America-transcontinental[1]	Circumpolar[1]
Habitat	Sandy well drained pine dominated sites[1]	Boreal taiga and tundra – Jack pine stands, muskegs, lichen woodlands[1]
Light conditions	light to moderate shaded areas[2]	moderate to heavy shaded areas[2]
Shrub type	Deciduous[1]	Evergreen[1]
Plant height	< 50 cm[3]	4-10 cm[4]
Twig description	May be verrucose, terete, pubescent or pilose[1]	Terete and puberulent[1]
Reproduction	Asexually by rhizomes Sexually through seed formation[3]	Asexually by rhizomes Sexually through seed formation[4]
Clone diameter	10 metres[3]	
Inflorescence	Raceme	Tight raceme
Flower symmetry	Actinomorphic	Actinomorphic
Flower orientation	Pendulous	Slightly pendulous
Flower formula	$*Ca^5Co^5A^{10}$	$*Ca^4Co^4A^8$
Petal fusion	Almost complete	Half to three quarters fused
Anther	Awnless, poricidal dehiscence from apical tubes	Awnless, poricidal dehiscence from apical tubes
Pollen grains	Tetrads	Tetrads
Tetrads per flower	2,400 ± 500[7]	11696 (multiplied from per anther)[9]
Tetrads per anther	240 (divided from total flower)[7]	1462 ± 227.3[9]
Stigma	Wet type, capitate with a lobed surface covered in papillate cells[5]	Dry stigma[6]
Style	elongate, hollow[5]	
Ovary	pentalocular inferior ovary with axile placentation[3]	Tetralocular inferior ovary[4]
Ovules		64.1 ± 5.5[9]
Nectar volume per flower	mean 0.3 ± 0.11 µL[7]	mean 0.63 ± 0.13 µL[8]

[1] Vander Kloet (1988); [2] Smith (1962); [3] Vander Kloet and Hall (1981); [4] Hall and Shay (1981); [5] Noormets and Olson (2006); [6] Guillaume and Jacquemart (1999); [7] Reader (1977); [8] Jacquemart (1992); [9] Jacquemart (1997)

myrtilloides belongs to the *Vaccinium* section *Cyanococcus*, along with nine other species (Vander Kloet 1983).

Vaccinium myrtilloides is found across Canada from central Labrador (excluding Newfoundland) to British Columbia and north throughout Canada's territories. It is found as far south as Virginia, and occurs in all the states of the U.S.A. bordering the Great Lakes. Generally it has not been found at heights greater than 1200 m above sea level. It is the only transcontinental species in *Vaccinium* section *Cyanococcus*; all other species occur exclusively in eastern North America (Vander Kloet and Hall 1981).

Shrubs of *V. myrtilloides* are less than 50 cm in height, and the leaves are deciduous, elliptical in shape and pubescent on both sides. Twigs are either brown or green and may be verrucose, terete, pubescent or pilose (Vander Kloet and Hall 1981; Vander Kloet 1988). Plants can spread asexually by rhizomes, as well as by seed dispersal, and Vander Kloet and Hall (1981) report that a single clone may eventually grow to a diameter of 10 m.

The flowers of this species are arranged in racemes; each flower is actinomorphic and pendulous with five green sepals, five white-pink fused petals, 10 stamens, and an inferior ovary. The fruit is an edible blue pseudo-ten-loculed berry, which ripens 49-68 days after flowering (Vander Kloet and Hall 1981). The average number of mature seeds per fruit is 12 ± 10 (Vander Kloet and Hall 1981) though the total number of mature and aborted seeds can be as high as 72 per berry (Hokanson and Hancock 2000).

1.1.2 *Vaccinium vitis-idaea*

Vaccinium vitis-idaea is known as the lingonberry, or bog cranberry, and produces an edible berry not unlike a cranberry. *Vaccinium vitis-idaea* is a circumpolar species, and is found in both North America and Eurasia through the northern boreal and tundra (Hall and Shay 1981). The North American *V. vitis-idaea* is often referred to as *V. vitis-idaea* L. var. *minus* Lodd (Hall and Shay 1981), but such distinctions will not be made throughout this text. *Vaccinium vitis-idaea* belongs to the *Vaccinium* section *Vitis-idaea* of which it is the sole member (Vander Kloet 1988). In North America, *V. vitis-idaea* is transcontinental, found from Newfoundland and Labrador across Canada within the boreal forest to British Columbia. It is recorded as far south as Connecticut and as far as north as 77ºN in Greenland (Vander Kloet 1988).

Vaccinium vitis-idaea is an evergreen species with leaves lasting 3 years (Hall and Shay 1981). It is quite small, 4-10 cm (Vander Kloet 1988) and forms a mat with its roots close to the surface. This shallow rooting means that *V. vitis-idaea* takes longer to recover after fire compared other *Vaccinium* spp. but will reemerge up to 6 years after (Hall and Shay 1981).

Work in Russia indicates that *V. vitis-idaea* plants do not flower until 14-20 years old, whereas work in England claims that in cultivation, flowers are not formed until the plants are 5-10 years old (Hall and Shay 1981). Flowers occur in tight racemes and are tetramerous. The corolla is bell shaped and fused for half its length; the stigma and style protrude past the tip of the corolla. There are 8 (usually) stamens in two whorls. The ovary is inferior and four-loculed, whereas berries are bright red and edible (Hall and Shay 1981; Vander Kloet 1988). Jacquemart and Thompson (1996) reported a mean fruit set of 34.0 ± 16.7 and 53.4 ± 25.3 % in natural stands for two respective years of data collection in Belgium. There were 8.7 ± 5.5 and 6.8 ± 5.8 seeds per berry per year (Jacquemart and Thompson 1996).

1.2 Pollination Biology

Interest in angiosperm pollination can take many forms. The focus of this thesis is on the rewards or enticements, that flowers possess to attract potential pollinators. The primary rewards offered by both *Vaccinium* species studied here are that of nectar secreted from a disk nectary at the base of the corolla, and pollen grains released through poricidal anthers.

1.2.1 Specialized arrangements of pollen and pollen delivery

Pollen grains are an important reward for insects visiting flowers, especially for bees. The pollen grains of *Vaccinium* species (and most of the Ericaceae) are released as tetrads of pollen grains at maturity. The occurrence of pollen tetrads at maturity is common to at least 52 plant families (including both monocots and dicots, Copenhaver 2005). The potential increase in number of pollen grains that will be transferred as a unit has implications for efficiency of pollen transfer to a receptive stigma, because in the case of pollen tetrads for every unit transferred there are now four potential fertilization events that could result instead of the potential for only one from a single pollen grain (Cruden 2000).

1.2.1.1 Poricidal anthers and buzz pollination

Most of the Ericaceae have a novel pollen dispensing mechanism: their anthers dehisce poricidally, rather than along longitudinal slits. This mechanism is thought to be a specialization for pollinators that participate in "vibratory pollen collection" (Proctor *et al.* 1996), having the ability to buzz pollinate flowers. The pollen grains are not out in the open and easily available to an insect seeking to collect them, nor is it likely that pollen grains will come to contact with an insect visiting the flower for the reward of nectar. Thus, to extract pollen from poricidal anthers, some bees have the ability to vibrate the anthers (or whole flowers) with their indirect flight muscles. At certain frequencies (<500 Hz, Harder and Barclay 1994; King and Buchmann 2003), pollen is resonated out of the anthers onto the bee's body. This vibration is often audible to the human ear as a loud buzz for larger bees such as bumblebees (*Bombus* species).

Approximately 72 plant families (both monocots and dicots) possess poricidal anthers, and it is likely that at least some of their species are buzz pollinated; however, field confirmation is still lacking for most examples (Buchmann 1983). In addition to possessing poricidal anthers, a buzz-pollinated flower typically has small pollen grains between 5-40 µm, far smaller in

comparison to the angiosperm range of 5-250 µm. The flowers usually do not produce nectaries, though the family Ericaceae is an exception. Androecia consisting of poricidal anthers have been noted to appear brightly coloured and thus "full" of pollen, therefore suggesting a trait of flower deceit.

The argument is made that poricidal anthers act as a dispensing mechanism, thereby controlling pollen release to some extent. This theory would seem to be true in that the optimum frequency (Hz) to remove pollen from anthers seems to be around 500 Hz, and bees have never been recorded to reach that frequency (Harder and Barclay 1994; King and Buchmann 2003). Harder and Barclay (1994) also found that with increasing flower age, more pollen grains were released from poricial anthers of *Dodecatheon* when bees visited previously unvisited flowers. Corbet *et al.* (1988) theorized that poricidal anthers gradually release pollen over many buzz pollination visits, thus dispensing pollen onto the bodies of multiple visitors. For each anther vibration, only the upper, dry layer of pollen will move out of the anther. After the buzz pollination visit, the top layer of the remaining pollen will slowly dry, permitting it to be removed by the next buzz visit.

Using vibrations from thoracic muscular contractions by bees is not unique to buzz pollination. Bees use vibrations for many purposes including to create heat (thermoregulation), as alarm buzzes to frighten predators, as copulation sounds, to aid in nest construction, and for thermoregulatory fanning of the hive and communication in *Apis* (Buchmann 1983). Although theoretically the muscles necessary for buzz pollination are present in all bee species, it has not been confirmed whether all bees engage in this particular foraging behaviour. Moreover, buzz pollination is not restricted to bees; one fly species, *Volucella mexicana* (Syrphidae), has been observed to vibrate poricidal flowers just as though it were the carpenter bee it mimics (Buchmann 1983).

Whether this ability to buzz pollinate is instinctive or learned in the species that exhibit this foraging behaviour, is still unknown. Buchmann (1983) reviewed reports of bees instantly buzzing upon their first contact with poricidal anthers, as well as reports of bees probing for nonexistent nectar in poricidal flowers and then buzzing at the non-poricidal-anthered flowers with nectaries.

Buzzing is not the only way that bees obtain pollen from poricidal anthers. Biting of the flower and anthers has been observed in *Xylocopa* species, even though they can also buzz

pollinate (Cane *et al.* 1985; Dedej and Delaplane 2005). In a behaviour described as “milking” the anthers, *Trigona* species will bite down on the anther and squeeze or pull the pollen down to the poricidal end to remove it. In addition, species such as *Apis mellifera* “glean” pollen grains left behind from previous visitors (Buchmann 1983).

A theoretical compromise appears to exist for tubular flowers with poricidal anthers (such as *V. myrtilloides* and *V. vitis-idaea*). It is proposed that although the micro-environment created by the corolla maintains a relative humidity high enough to reduce evaporation from the nectar, thereby keeping the concentration of nectar solutes at an acceptable level for bees, the relative humidity would not be ideal for drying within the poricidal anthers and thus may reduce the dispersal of pollen (Corbet *et al*. 1988).

1.2.2 Floral nectaries in Ericaceae

Floral nectar is the most important single reward offered by plants to entice potential pollinators (Simpson and Neff 1983). The floral nectar comes from floral nectaries, organs found throughout angiosperm species that vary widely in form. Due to their large diversity of positions they occupy on the flower and associated structures, nectaries are thought to have evolved independently many times in angiosperms (Bernardello 2007).

Nectaries are common in the Ericaceae, though there is no review indicating in which taxa they are present and absent, either within different subfamilies, tribes or genera. In the sub-family Ericoideae, Palser and Murty (1967) reported them in all but 4 of the 54 species of *Erica* examined, and they are found in more than 175 *Rhododendron* spp. (Feldhofen 1933; Palser *et al*. 1991).

Wallace (1977) found floral nectaries in all 12 species of the Monotropoideae studied, and later Wallace (1995) confirmed nectaries in Neotropical *Monotropa* and *Pterospora* as well as the single species of *Orthilia,* and noted an absence in *Pyrola*. In northern Europe, Knudsen and Olesen (1993) reported that *Moneses uniflora* and three species of *Pyrola* lacked nectaries, although they are present in their other two study species, *Orthilia secunda* and *Chimaphila umbellata*. The lack of a floral nectary in *Pyrola* has been referred to as the loss of the nectariferous disk (Freudenstein 1990 in Luteyn 1995), implying that nectaries are the plesiomorphic state, although this possibility has not been thoroughly investigated for Ericaceae.

Stevens (1971) mentions that the genus *Enkianthus* (Enkianthoideae) has floral nectaries. Floral nectaries also occurred in a number of ericaceaen species (*Ampelothamnus*, *Andromeda*,

Cassiope, *Chamaeaphne, Enkianthus, Epigaea*, *Harrimanella*, *Leucothoe, Lyonia, Neopieris, Oxydendrum, Pieris* and *Xolisma*) investigated by Palser (1951). These genera are in a number of different sub-families under the current classification of Kron *et al.* (2002a). Nectaries are present in *Arctostaphylos* and *Comarostyphylis* (Diggs 1995a; Diggs 1995b), both of which used to be in the subfamily Vaccinioideae, but now are considered separately in Arbutoideae (Kron *et al.* 2002a). Palser (1961) reported floral nectaries in all *Vaccinium* species (roughly 30, without including synonyms) and *Gaylussacia* (four species) studied, both of which are in the tribe Vaccinieae.

The floral nectaries in Ericaceae are most often described as forming a nectariferous "disk" (Luteyn 1995; Simpson 2010). In *Vaccinium* species, the nectary is located on top of the inferior ovary between the stamens and the style base. The floral nectaries of *Vaccinium* possess stomata on their epidermis (Palser 1961), which is the apparent method of secretion in most of the Ericaceae where authors have noted features of the nectary epidermis (Kerner and Oliver 1895; Feldhofen 1933; Palser 1961; Palser and Murty 1967; Wallace 1977).

1.2.2.1 Nectar attributes of *Vaccinium*

Floral nectar is a high-energy reward for potential pollinators. It is dominated by the sugars sucrose, fructose and glucose. The ratios of these sugars vary, sometimes even widely within a genus, but are fairly constant within species and over the life of the flower (Nicolson and Thornburg 2007). The different ratios of sugar have been suggested to have resulted from co-evolutionary relationships with different types of floral visitors, however the only general correlations found are that bees, butterflies, moths and hummingbirds tend to be pollinators of flowers having high sucrose nectar, whereas flowers with high hexose (fructose and glucose) nectars tend to be pollinated by unspecialized insects, passerine birds and Neotropical bats (Nicolson and Thornburg 2007).

Glucose and fructose predominated in the floral nectar of *V. corymbosum*, a cultivar of *V. corymbosum* 'Triumph" and *V. lamarcki* (synonym. to *V. angustifolium*), with only small amounts of sucrose present (Percival 1961). High amounts of glucose and fructose with only a trace of sucrose were also reported by Wood and Wood (1963) for the nectar of *V. angustifolium* and *V. myrtilloides*. However, there does seem to be variation within the genus: Jacquemart (1992) reported that the floral nectar of *V. myrtillus* was predominantly sucrose, *V. vitis-idaea* was almost 30% sucrose and *V. uliginosum* had less than 1% sucrose. Although different insect

visitor species were noted for those three latter species of *Vaccinium*, there was a great overlap of bees (bumblebees and honeybees were most predominant), and small flies visited all three species (Jacquemart 1992, 1993). Thus it would seem that nectar sugar composition gives no clear signal of pollinator assemblage in the case of *Vaccinium* species.

1.2.3 Breeding system

Although offering many insect rewards to ensure a level of cross pollination, *Vaccinium* species are not self-incompatible, and have shown low levels of self-compatibility in different studies. For *V. vitis-idaea* in Belgium, Jacquemart and Thompson (1996) compared hand-pollinated plants that were given self pollen, to that of hand-pollinated plants that were given crossed pollen, and found that selfing produced 84% of regular fruit set, though the number of seeds per fruit was only 36% of the hand-crossed flowers that produced berries. Likewise, autonomous selfing of flowers only produced 23% fruit set as compared to hand-crossed flowers, whereas seeds per fruit were only 22% relative to crossed flowers. Thus *Vaccinium vitis-idaea* is self compatible, though less so than some of its relatives (*Vaccinium myrtillus*, *V. uliginosum*, Jacquemart and Thompson 1996). Its flower morphology, in particular the relatively large stigma-anther distance, suggested more of an outcrossing tendency. In a later study, similar crosses to *V. vitis-idaea* were made in a greenhouse setting (Guillaume and Jacquemart 1999). Again, there were more fruit set in cross pollination than self pollinations, though this difference was not significant in the second year of study. Of the fruit produced, there were fewer plump seeds (their measurement of developed seeds) in the self-crossed flowers that produced berries. As there was no distinction made between aborted seeds and unfertilized ovules, the study was unable to answer their question of whether reduced seed formation was from inbreeding depression or gametophytic selection, though they favored the former.

Vaccinium myrtilloides is generally classified as an outbreeding species, however data from pollination studies (Reader 1977; Usui *et al.* 2005) indicated variable fruit set when pollen of the same genet and the same plant was applied by hand (40-80% and 31%, respectively). In selfing trials, Hokanson and Hancock (2000) found limited fruit set on some of the plants collected, giving a fruit set percentage of between 0-30%. Most of the seeds in these fruits were aborted in various stages of development, with only 0-10% reaching maturity. They concluded that *V. myrtilloides* exhibits signs of inbreeding depression, which is caused by the build up of deleterious alleles. It was also noted that seed set in *V. myrtilloides* was higher when flowers were

self-pollinated on the first day of anthesis and then cross-pollinated the following day, than if they were simply cross-pollinated on the days after flower opening. When Noormets and Olson (2006) investigated whether any deposition of self pollen on the stigma (autogamy) occurs before the flower has opened, they found that 13% of previously-unvisited open flowers had already experienced self pollen deposition, whereas 18% at petal opening, 3% at petal elongation and 1% in the young bud stages had at least one pollen tetrad present on the stigma. Noormets and Olson (2006) suggested that pollen grains being dislodged from anthers onto the stigma may be wind-promoted, as proposed for other *Vaccinium* species (Hagerup 1954). As their trials took place in greenhouses protected from wind and insects, the amount of autogamy is presumed greater in a natural setting. Studies by Noormets and Olson (2002) also indicate that the percent of stigmas testing positive for peroxidase activity (i.e., a measure of the stigma's receptivity) was 35% and 36% at the young bud and petal elongation stages, with an increase to 61% at petal spread. Consequently, at petal spread there could be a very small percentage of flowers with pollen already deposited on receptive stigmas; however, Noormets and Olson (2006) only found pollen tube growth on fully open flowers. Usui *et al*. (2005) reported very low fruit set with *V. myrtilloides* for flowers bagged with no pollination treatment (6 / 1794), indicating a low rate of natural autogamy in that case.

Although there is evidence for inbreeding depression in some *Vaccinium* species (Hokanson and Hancock 2000), there is also the possibility of gametophytic selection of pollen tubes. Cane (2009) reports than in *V. macrocarpon* most pollen grains were viable, but only one fourth of pollen tubes exited the base of the style in a semi-vivo stylar culture technique.

1.2.3.1 Implications for this study

The breeding system is important as it relates to the technique used to investigate pollinator potential among insect visitors to the *Vaccinium* species. As there is potential for wind-facilitated pollen deposition (Hagerup 1954; Noormets and Olson 2006), pollen grains on the stigma of a flower visited by only one insect do not necessarily represent a pollination contribution from only that visitor, but also self-deposited pollen grains, making the counting of pollen deposition on a stigma a proxy measurement to the true amount of pollen grains deposited by the insect. Furthermore, an insect visit can itself cause self pollen grain deposition through the insect's disturbance of the flower. As there may or may not be pollen tube selection occurring in

the style, the counting of any pollen tubes that reach the base of the style is also a proxy of pollinator effect.

1.3 Objectives

The goals of this project were to expand our knowledge about the pollination biology of *V. myrtilloides* and *V. vitis-idaea* in central Saskatchewan. Of particular interest were characteristics of flower morphology, insect visitor occurrence and behavior, and how those attributes influence overall reproductive success. As nectar and pollen are both offered as rewards to flower-visiting insects, their characteristics were of particular interest. Accordingly, there were three main objectives for this project:

1) to determine floral characteristics relevant to sexual reproductive biology such as pollen/ovule ratios, pollen viability, anther and filament structure, and function of poricidal anthers in both *Vaccinium* spp.;
2) to investigate nectar secretion dynamics at a native field site, and to describe the morphological and anatomical structure of the floral nectary in both *Vaccinium* spp.; and
3) to assay pollination biology in a native stand possessing both *V. myrtilloides* and *V. vitis-idaea*, including surveys and identification of insect visitors to flowers and an investigation of the pollination efficiency of these visitors to previously-unvisited flowers.

Pollen-ovule ratios in seven species of *Vaccinium* (Ericaceae) and stamen structure in *V. myrtilloides* and *V. vitis-idaea*

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2 Pollen-ovule ratios in seven species of *Vaccinium* (Ericaceae) and stamen structure in *V. myrtilloides* and *V. vitis-idaea*

2.1 Introduction

Vaccinium L. is a well recognized and widely distributed genus in the family Ericaceae. Although primarily known for its edible fruit-yielding temperate species such as blueberry and cranberry, the genus *Vaccinium* is very tropical in its distribution, inhabiting all continents including the southern portion of Africa plus Madagascar, except Australia and Antarctica (Kron *et al.* 2002b). With its 740 spp., *Vaccinium* is the third largest genus in the Ericaceae after *Erica* and *Rhododendron* (Judd *et al.* 2008). Flower morphology is typically used to separate *Vaccinium* from the other 30-35 genera in the tribe Vaccinieae but it often fails to delimit them, such that well-supported groups are not represented in current taxonomic treatments. Though further morphological and molecular research is needed, the genus *Vaccinium* is thought to be polyphyletic and thus will likely need to be expanded or disbanded (Kron *et al.* 2002b).

Despite the reduced number of *Vaccinium* species in North America versus that of the tropics, species delimitation is still difficult (Vander Kloet 1988). The genus is often divided into sections and in Canada, Vander Kloet (1988) recognized 17 species in six sections. We had access to seven of those for this study. *V. vitis-idaea* L. section *Vitis-idaea* and *V. uliginosum* L. section *Vaccinium* are both circumpolar, whereas *V. caespitosum* Michx. section *Myrtillus* is transcontinental in North America. The majority of species are in *Vaccinium* section *Cyanococcus* and like *V. boreale* I.V. Hall & Aalders occur in eastern Canada (North America); however, *V. myrtilloides* Michx. occurs across Canada, *V. angustifolium* Ait. is reported as far west as southern Manitoba (Vander Kloet 1988) to the eastern Saskatchewan border (R.G. St. Pierre, pers. comm.) and *V. corymbosum* L. is now also grown commercially in British Colombia.

Vaccinium is economically important for the annual harvest of its fleshy fruit in both commercial and wild settings. Selection and breeding efforts have led to cultivar development in horticulturally important species, which successfully spread vegetatively using rhizomes or suckers. *Vaccinium* floral structure is intricate and features a specialized mechanism of poricidal dehiscence of anthers. Increased knowledge of floral reproductive traits such as pollen (released at maturity as tetrads), ovule and nectar production that pertain to pollination requirements and the reliance on biotic vectors of pollination, are required to further understand the sexual

reproductive biology of *Vaccinium* with regard to mixed mating systems and reproductive isolation (Jacquemart 2003), given that many Canadian *Vaccinium* spp. are sympatric in the wild.

Determination of the total number of reproductive units (i.e., ovules and pollen grains) per flower enhances our understanding of the sexual reproductive effort and permits estimates of reproductive success in *Vaccinium*. Furthermore, calculation of pollen/ovule (P/O) ratios is a long standing method of estimating the breeding system of an individual plant species, such as its dependence on self- versus cross-pollination, for example (Cruden 1977, 2000; Erbar and Langlotz 2005). P/O ratios are available for some ericaceaen taxa (Erbar and Langlotz 2005) although, to date, only have been recorded for less than 1% of *Vaccinium* spp. (Jacquemart 1997; Brevis *et al.* 2006; Pereira 2008).

Accordingly, the goals of this study were i) to determine the comparative P/O ratios of seven *Vaccinium* spp. native to Canada, in order to add to the description of floral characteristics related to mating systems and to potentially assist delineation of these species taxonomically; and ii) for two of these species (*V. myrtilloides*, *V. vitis-idaea*) from Saskatchewan, to provide greater depth in understanding of *Vaccinium* flower structure in relation to (particularly male) function in pollination biology, by investigating the structure of poricidal anthers of mature stamens, plus the structure and viability of their pollen tetrads. These topics both pertain to an increased knowledge of reproductive biology in *Vaccinium*.

2.2 Materials and methods

2.2.1 Plant material and fixation

Inflorescences containing open flowers and buds of seven Canadian species of *Vaccinium* were collected during spring of 2009-2011 from four and three locations in Nova Scotia and Saskatchewan, respectively (Table 2.1). For the dense, living accessions at Irving Botanical Garden, Acadia University, multiple infloresences (typically 4-6) from different branches (but possibly still representing the same genet) were collected. On the other hand, individual samples received from wild-crafted settings of *V. angustifolium* consisted of a single multibranched sprig, thus representing one ramet but from each of three distinct clones per site (Diligent River, Glasgow Mountain and Pigeon Hill, NS). The flowering material from Debden, SK was taken at a natural setting (without maintenance or propagation) from multiple and distant transects believed to represent different

genets. Material was fixed with FAA (formaldehyde/acetic acid/ethanol; Jensen 1962) in labeled vials, then processed using different protocols (see below).

Pollen viability, however, was determined using fresh flowers. Owing to the absence of any loss in anther volume (size) in *Vaccinium* (Richter 1929) at anthesis, coupled with the stamens' relative obscurity (i.e., surrounded by the tubular corolla), anthesis was most accurately judged by examination for the distal separation of petal tips. Age of flowers *in situ* was monitored daily, thereafter.

2.2.2 Pollen/ovule (P/O) ratios

Indehiscent anthers from mature flower buds were removed and pollen grains (in tetrads) were dissected separately from each anther with the aid of an Olympus SZ40 dissecting microscope, onto small droplets of water on a labeled glass slide. Then a glass coverslip was applied and sealed with fast-drying fingernail polish. Thereafter, absolute counts of pollen tetrads were made using a compound microscope, viewed in a systematic across-down-back-down-across sequence with the aid of a hand counter. The total quantity of pollen tetrads per flower was determined by summation of the counts per individual anther. Tetrads were assumed to have remained intact during the counting procedure and thus the total quantity of pollen grains per flower was estimated by multiplying the tetrad number by four.

Similarly, all ovules from the inferior ovary of each of the same mature buds used above for pollen quantification were teased from the axillary placentae (Fig 2.5 a, b) using a dissecting scope into small droplets of water on a glass microslide, then counted. Significant differences in the number of tetrads between anthers, and the number of tetrads, ovules and the P/O ratios between flowers, were tested for *V. angustifolium*. An ANOVA and then pairwise comparisons were made for the normally distributed data using The R Project for statistical computing (R Core Development Team, 2012).

Table 2.1 Collection localities and data for seven species of *Vaccinium* examined.

Species	Location/Accession No.	Date	Collector
V. angustifolium Aiton	Diligent River, Nova Scotia (N45.40 564, W64.45345)	June 2, 2009	G.C. Cutler
V. angustifolium Aiton	Glasgow Mt., Nova Scotia (N45.45 428, W64.45804)	June 2, 2009	G.C. Cutler
V. angustifolium Aiton	Pigeon Hill, Nova Scotia (N45.5555, W63.36223)	June 9, 2009	G.C. Cutler
V. boreale Hall and Aalders	Acadia University, Wolfville, Nova Scotia (AU 01.00508)	May 21, 2009	A.R. Davis
V. caespitosum Michx.	Acadia University, Wolfville, Nova Scotia (AU 01.00518)	May 21, 2009	A.R. Davis
V. corymbosum L.	Acadia University, Wolfville, Nova Scotia (AU 02.00234)	May 15, 2009	R.C. Evans
V. myrtilloides Michx.	Besnard, Saskatchewan (N55.29839 W106.09563; N55.43441 W105.96463)	June 24, 2009	R.G. St. Pierre
V. myrtilloides Michx.	Debden, Saskatchewan (N53.69199 W106.96823)	June 15, 2009	R.G. St. Pierre
V. myrtilloides Michx.	Smoothstone, Saskatchewan (N54.53870 W106.92278)	June 15, 2009	R.G. St. Pierre
V. myrtilloides Michx.	Debden, Saskatchewan (N53.69193 W106.96706)	June 2010, 2011	D.T. Stephens
V. uliginosum L.	Acadia University, Wolfville, Nova Scotia (AU 01.00537)	May 21, 2009	A.R. Davis
V. vitis-idaea L.	Acadia University, Wolfville, Nova Scotia (AU 01.00547)	May 21, 2009	A.R. Davis
V. vitis-idaea L.	Debden, Saskatchewan (N53.69193 W106.96706)	June 2010, 2011	D.T. Stephens

2.2.3 Pollen viability

The cut ends of woody flowering branches from plants of *V. myrtilloides* and *V. vitis-idaea* from the site at Debden, SK (Table 2.1) were kept in tap water to keep the material fresh during the drive (2.5 hr) back to the lab. Once there, pollen was extracted from the poricidal anthers of mature flowers of known age (ranging from one day pre-anthesis to the third day of anthesis) onto glass microslides, via sonication using a tuning fork (Buchmann 1983). Pollen tetrads were immediately covered by a solution of fluorescein diacetate (FDA) and then pollen grain viability estimated using the fluorochromatic reaction test (Shivanna and Rangaswamy 1992). Intra-tetrad viability was scored only from tetrads in which all grains of the tetrad were clearly discernible. Over 330 total tetrads were examined per species.

2.2.4 Light microscopy

2.2.4.1 Paraffin embedding and sectioning

Fixed flowers and buds of *V. myrtilloides* and *V. vitis-idaea* collected in 2009 were rinsed in a buffer solution (25 mM sodium phosphate, pH 6.8) before transferring through a butanol dehydration series (Jensen 1962). Samples were incubated in vials at 60°C during which 100% butanol was gradually replaced by Paraplast® (Oxford Labware, St. Louis, MO, USA) with periodic swilling. Embedded samples were sectioned at a thickness of 7.5 µm using a Leitz-Wetzlar rotary microtome. Ribbons of wax sections were mounted on slides coated with Mayer's adhesive and stained for 10-12 minutes with 0.05% toluidine blue 0 in sodium benzoate buffer, pH 4 (O'Brien and McCully 1981), then dewaxed in xylene (overnight) after which coverslips were secured with Permount®. Images were taken using a Zeiss Universal photomicroscope.

2.2.4.2 Resin embedding and sectioning

Mature buds one day pre-anthesis and open flowers on the sixth day post-anthesis were fixed in spring 2010 from plants of *V. myrtilloides* and *V. vitis-idaea* at Debden, SK. Subsequently they were rinsed three times with 25 mM sodium phosphate buffer before three rinses with distilled water. After passing through a graded ethanol series, tissues in absolute ethanol were gradually infiltrated with LR White resin (London Resin Company, Basingstoke, Hampshire, UK). Tissues in pure resin were sealed in beem capsules to produce anaerobic conditions and placed in an oven at 60°C for 72 hours. Sections 1.5 µm thick were cut using glass

knives on a Reichert ultramicrotome, then heat fixed to glass slides before staining with 0.5% toluidine blue 0 in 0.1% sodium carbonate buffer (pH 11.1), 0.1% aqueous Calcofluor White (O'Brien and McCully 1981) or auramine O (Heslop-Harrison 1977). Photographs were taken with a Zeiss Axioplan photomicroscope equipped with epifluorescence.

2.2.4.3 Scanning electron microscopy (SEM)

Fixed flowers were processed for SEM (Murza and Davis 2003) after dissection to reveal features of stamens. Briefly, samples were rinsed with 25 mM sodium phosphate buffer, post-fixed in 1% OsO_4 in the same buffer, rinsed several times in distilled water and then transferred through an acetone dehydration series before critical-point drying (Polaron Instruments, Watford, U.K.). Specimens were secured to aluminum stubs, sputter coated with gold (Edwards S150B) and then viewed with a Philips 505 scanning electron microscope at 30 kV. Photographs were taken with Polaroid 665 film or Fuji FP-100B film.

2.3 Results

2.3.1 Pollen/ovule ratios in seven Canadian *Vaccinium* species

Of the seven species of *Vaccinium* examined, five (*V. angustifolium*, *V. boreale*, *V. cespitosum*, *V. corymbosum*, *V. myrtilloides*) are pentamerous (Palser 1961), normally producing 10 stamens per flower (Fig 2.2 d) and five ovule-containing locules per inferior ovary (Fig 2.5 b). Flowers of *V. vitis-idaea* are tetramerous (Fig 2.1 b; Fig 2.5 a), whereas those of *V. uliginosum* are usually tetramerous but occasionally pentamerous (Palser 1961; Vander Kloet 1988). Owing to this variability among species and the occasional abortion of single stamens per flower (Fig 2.1 c), the average number of stamens available for pollen quantification varied from 8.4 (*V. vitis-idaea*), 9.0 (*V. caespitosum, V. uliginosum*), 9.9 (*V. angustifolium, V. myrtilloides*), 10.0 (*V. boreale*) to 10.3 (*V. corymbosum*) per flower.

From wild plants at the three Nova Scotian locations surveyed in June, 2009, flowers of *V. angustifolium* at Pigeon Hill produced significantly lower quantities of total pollen grains per flower (Table 2.2). However, flowers at the three sites possessed equivalent mean numbers of ovules (61-75) per ovary (Table 2.2). When these reproductive characteristics are expressed as a P/O ratio, the Glasgow Mountain population (415) significantly exceeded both Diligent River (321) and Pigeon Hill (290) (Table 2.2).

Across our entire study, the highest mean numbers of pollen grains per flower were found from plants of *V. vitis-idaea* (64165), whereas the lowest quantities were produced by *V. uliginosum* (19160), both from Nova Scotian samples (Table 2.3). Overall, the P/O ratios ranged from 239 (*V. caespitosum*) to 2008 (*V. vitis-idaea*) in Nova Scotia (Table 2.3). Correspondingly, the highest and lowest mean total numbers of ovules per flower occurred in *V. caespitosum* (96) and *V. vitis-idaea* (32) from Nova Scotia (Table 2.3).

As with *V. angustifolium* in Nova Scotia (Table 2.2), differences in P/O ratios were recorded among different populations of *V. myrtilloides* in Saskatchewan (Table 2.3). Mean pollen quantities per flower (26515, 25850) were very similar, whereas mean ovule numbers per ovary (63, 81) varied owing to high quantities of ovules per ovary at the Debden field site in 2011 (Table 2.3).

Moreover, P/O ratios varied for the single species (*V. vitis-idaea*) sampled at two distant locations within Canada (Table 2.3). Flowers from plants at Debden, SK had lower and higher average quantities of pollen and ovules per flower (32661, 46), respectively, than at Acadia University in Wolfville, NS (64165, 32) (Table 2.3). As a result, the P/O ratio of *V. vitis-idaea* from Nova Scotia was almost 3 times that found in Saskatchewan.

Table 2.2 Mean values (± S.E) of pollen and ovules per flower of *Vaccinium angustifolium* from three wildcrafted blueberry sites in Nova Scotia in June, 2009.

	Diligent River	Glasgow Mountain	Pigeon Hill	F value	P value	df
Pollen tetrads per anther	595.4 ± 10.5 b	646.9 ± 12.2 a	488.9 ± 9.95 c	54.353	2.20E-16	175
Pollen grains per flower	23816 ± 758.3 a	25014 ± 1320 a	19554 ± 837.2 b	8.1861	0.003950	15
Ovules per flower	75 ± 3.3 a	61 ± 4.0 a	69 ± 3.5 a	3.4076	0.060260	15
P/O ratio	321.4 ± 14.6 b	414.9 ± 29.8 a	289.5 ± 27.4 b	6.8713	0.007616	15

Significant differences ($p < 0.05$) across rows are indicated with different letters.
Two flowers were sampled from three different clones per site ($n = 6$ per site).

Table 2.3 A review of comparative pollen data and pollen/ovule (P/O) ratios in 12 species of *Vaccinium* (Ericaceae)

	Mean tetrads per anther	Mean pollen grains per flower	Mean ovules per flower	Pollen/Ovule ratio	Tetrad/ Ovule ratio	Sample Size	Location	Source
V. ashei ¶		33736*	85 ± 7	402 ± 36	101 ± 9	100 flowers, 25 each, 4 cultivars	2 locations in Georgia, U.S.A.	Brevis et al. (2006)
V. augustifolium	576 ± 106	22794 ± 1614	64 ± 10	341.9 ± 79	85 ± 20	18 flowers, 6 per location §	3 locations in NS, Canada	Present study
V. boreale	671 ± 64	26872 ± 742	66 ± 3.4	410.2 ± 26	102 ± 6	4 flowers §	Acadia University, Wolfville, NS, Canada	Present study
V. caespitosum	638 ± 73	22986 ± 2427	96 ± 8.5	238.8 ± 38	60 ± 9	4 flowers §	Acadia University, Wolfville, NS, Canada	Present study
V. corymbosum	804 ± 84	32950 ± 252	61 ± 6.8	542.7 ± 56	134 ± 14	4 flowers §	Acadia University, Wolfville, NS, Canada	Present study
V. cylindraceum		119748*	55.4*	2225.4 ± 1215.4	556*	84 flowers, 12 flowers per island	7 islands of Azores archipelago, Portugal	Pereira (2008)
V. macrocarpon	704.3*	28172*				19 flowers	Southern New Jersey, U.S.A.	Cane *et al*. (1996)
V. myrtilloides	663 ± 96	26515 ± 3207	63 ± 5.0	417.5 ± 47	104 ± 28	4 flowers §	Various locations, Northern SK, Canada	Present study
V. myrtilloides	665 ± 90	25850 ± 3310	81 ± 16	323 ± 53	80 ± 13	4 flowers §	Near Debden, SK, Canada	Present study
V. myrtilloides		9600*				10 flowers	2 locations in Southern Ontario, Canada	Reader (1977)
V. myrtillus	1291.7 ± 290.2	52380*	90.8 ± 15.5	582.5 ± 48.4	145*	12 flowers	Upper Ardennes, Belgium	Jacquemart (1997)
V. oxycoccus	781.7 ± 397	34986*	21.6 ±6.2	1666.7 ± 989.2	416*	12 flowers	Upper Ardennes, Belgium	Jacquemart (1997)
V. uliginosum	532 ± 143	19160 ± 2421	77 ± 6.8	250.0 ± 16	62 ± 4	4 flowers §	Acadia University, Wolfville, NS, Canada	Present study
V. uliginosum	555 ± 86.6	24024*	84.9 ± 6.5	286.6 ± 31.1	72*	12 flowers	Upper Ardennes, Belgium	Jacquemart (1997)
V. vitis-idaea	895 ± 242	32661 ± 4747	46 ± 12	736.8 ± 187	184 ± 46	4 flowers §	Near Debden, SK, Canada	Present study
V. vitis-idaea	1462.3 ± 227.3	50112*	64.1 ± 5.5	783.8 ± 80.5	196*	12 flowers	Upper Ardennes, Belgium	Jacquemart (1997)
V. vitis-idaea	1944 ± 220	64165 ± 4325	32 ± 2.3	2008 ± 139	502 ± 34	4 flowers §	Acadia University, Wolfville, NS, Canada	Present study

¶ Authors did not report whether values of variation represent standard deviations or standard errors
* Value calculated from data reported
§ All 8 or 10 anthers per flower were analysed for their pollen content

2.3.2 Flower morphology and anatomy

2.3.2.1 *Vaccinium vitis-idaea*

The perfect tetramerous flowers occurred singly or most often in inflorescences. The four sepals were pale pink to green and the corolla light pink to white and campanulate, with petals fused for roughly 2/3 of their length. The eight stamens (rarely 7-9) had brown anthers above white filaments that attached interior to the corolla (Fig 2.1 a, b). Situated between the stamen attachment and around the style base was a slightly raised nectary disk atop the inferior ovary (Fig 2.1. a-c). At anthesis, the stamens did not protrude from the corolla. However, the style either remained within the corolla or extended beyond it (Fig 2.1 a), resulting in a variable distance between the stamen tips and stigma (Fig 2.1 a-c).

Deformed stamens (Fig 2.1 c, right) in which the vestigial anther and filament appeared to have ceased growth compared to normally-developed stamens (Fig 2.1 c, left), were common. Moreover, other stamens that developed to their full size frequently occurred fused to one or more stamens either along both their filaments and anthers, or at their anthers alone.

Each staminal filament was covered laterally, but often only sparsely on its abaxial and adaxial surfaces, with elongate, unicellular, non-secretory trichomes (Fig 2.1 d, g, k, n, p). Less commonly, these hairs were bicellular. Parenchyma and especially epidermal cells of the filament base possessed large plastids (Fig 2.1 l). A solitary vascular bundle, positioned slightly adaxial from centre (Fig 2.1 l), supplied the broad filament base and extended through the narrowing, dorsally-ventrally flattened filament (Fig 2.1 j) into the filament-anther junction (Fig 2.1 i). Each filament connected abaxially to about the middle of the anther (Fig 2.1 d, e). Atop the anther halves were two tubules that were the same length as the pollen sacs (Fig 2.1 b-d, g). These elongate, hollow appendages possessed apical pores of irregular margin (Fig 2.1 d, g; Fig 2.3 a). Flowers collected in Saskatchewan often had small appendages (spurs; Hermann and Palser 2000) of variable size, shape and orientation located on the abaxial side at the anther-tubule junction (Fig 2.1 d, e). Spurs had a papillate surface (Fig 2.1 d-f) and cells of the interior were thickened (Fig 2.1 f). Also located on the stamen's abaxial surface were two multicellular ridges of the connective tissue (which included the spurs, if present) that began as the filament's lateral edges (Fig 2.1 d, i) on each half of the anther and continued from the point of anther attachment (Fig 2.1 d, e, p) to beyond the anther-tubule junction (Fig 2.1 h, left). On the filament abaxial

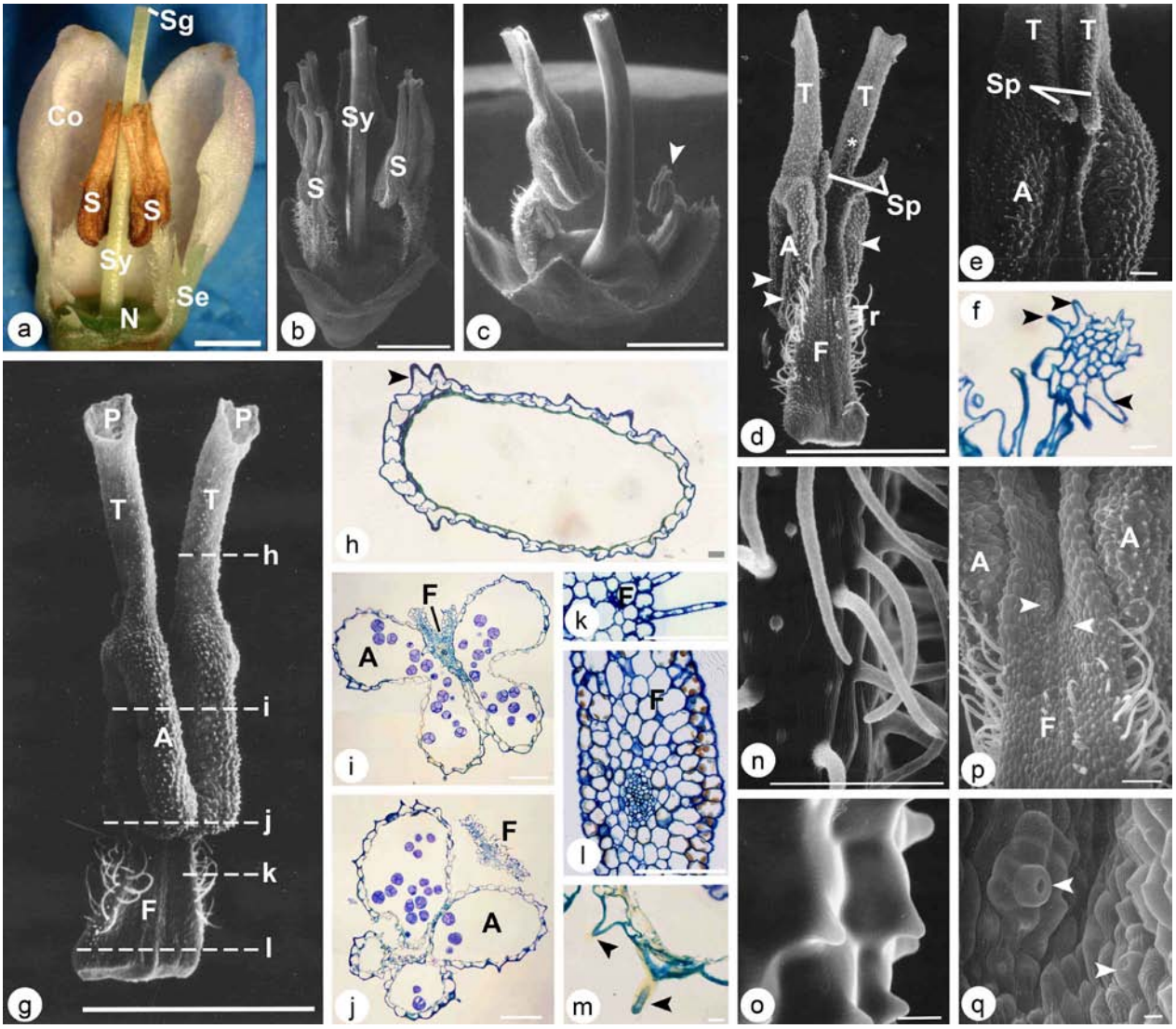
surface, immediately distal to the anther-filament junction, one or two raised stomata (Fig 2.1 p, q) were evident on some specimens.

At flower maturity, each anther was tetrasporangiate (four pollen sacs present) as was evident at its base (Fig 2.1 j). Functionally, however, each anther was bilocular, the septum between two lateral microsporangia being absent such that they shared one locule (Fig 2.1 i) that formed a continuum with the hollow tubule above (Fig 2.1 h), thus allowing pollen of each anther half to escape together from a single tubule (Fig 2.3 a).

The anther wall at post-dehiscence was reduced to a single, intact cell layer, namely the epidermis (Fig 2.1 h-j). Along most of the anther's length, several of the epidermal cells were unicellular papillae (Fig 2.1 e, g, h-j, m, o). Exceptions occurred between the lateral pollen sacs (Fig 2.1 i) and adjacent to the ridge and spur along the abaxial side (Fig 2.1 p). Papillae tips showed a bipartite nature in section when stained with TBO (Fig 2.1 m) but their staining with auramine O failed to display fluorescence indicative of a suspected cuticle. When sections were stained with Calcofluor White, the barest trace of fluorescence in the epidermis signaled the presence of cellulose (not shown). Non-papillate epidermal cells were abundant but sectional views along the anther's length (Fig 2.3 c, d) and extending into the tubule (Fig 2.1 h) never revealed secondary wall thickenings indicative of an endothecium. Deteriorated inner layers of anther (Fig 2.1. h, m), plus the suspected remnants of tapetum (Fig 2.3 c, d), sporadically lined the locule immediately below the epidermis.

Figure 2.1 Stucture of the flower and androecium in *Vaccinium vitis-idaea*.a) Partially dissected, fresh flower showing epigynous parts: sepal (Se), corolla (Co), stamens (S), and nectary disk (N) surrounding base of style (Sy) terminating in flattened stigma (Sg). b) Open flower with perianth and the eighth stamen removed to show arrangement of stamens (S) around central style (Sy). c) Comparison of typical stamen (left) and diminutive, vestigial stamen (arrowhead). d) Abaxial aspect of stamen, showing two tubules (T), two spurs (Sp), and three of four pollen sacs (arrowheads) of anther (A), and filament (F) with widening base possessing trichomes (Tr) mainly borne laterally. Asterisk corresponds to thickened side of anther tubule in cross section (see left side h). e) Abaxial view of two short spurs (Sp) directed toward filament, at the junction of tubules (T) and anther (A). f) Oblique section through spur, showing central region of cells with thickened walls, and unicellular papillae (arrowheads) in epidermis. g) Adaxial surface of isolated stamen, showing large pores (P) with irregular margins at distal end of each anther tubule (T) extending from the anther base (A), and filament (F). Dashed lines correspond to approximate regions of cross sections in Fig 2.1 h-l. h) Tubule showing its almost entirely unicellular thickness, slightly wider at the leftmost region (abaxial surface at asterisk in Fig 2.1 d) which bears papillae (arrowhead). i) Junction of filament (F) to anther (A) containing mature pollen tetrads. j) Four microsporangia of anther below attachment of anther (A) to filament (F). k) Non-secretory, linear trichomes at lateral edge of filament (F). l) Filament (F) showing solitary vascular bundle surrounded by parenchyma cells. Plastids evident, particularly in cells of the epidermis. m) Unicellular anther wall showing distinctive wall layers of papillae (arrowheads). n) Higher magnification of elongate, non-secretory trichomes on lateral edge of filament. o) Higher magnification of outer anther wall lining pollen sac, showing papillae. p) Abaxial surface at junction of filament (F) with pollen sacs of anther (A), showing filament extending proximally as two longitudinal ridges (corresponding to top of Fig 2.1 i). Two filament stomata are indicated (arrowheads). q) Higher magnification of two slightly elevated stomata of Fig 2.1 p on abaxial surface of filament.

Fresh material; Fig 2.1 b-e, g, n-q – Scanning electron micrographs; Fig 2.1 f, h-m – Light micrographs stained with toluidine blue 0. Scale bars: 1 mm – Fig 2.1 a-d, g; 0.1 mm – Figs. e, i-l, n, p; 10 μm – Figs. f, h, m, o, q.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2.3.2.2 *Vaccinium myrtilloides*

Each perfect, epigynous flower was pentamerous. The five sepals were green to dark pink whereas the petals ranged from white to dark pink, with the pink often occurring in streaks on each petal or primarily at the petal tips. The corolla was urceolate to campanulate, the petals fused for almost their entire length. Filaments of the 10 stamens were white and the anthers brown, the stamens inserting around a nectary disk (Fig 2.2 a) above the inferior ovary. The style did not protrude from the corolla, such that the stigma was often flush with the petal tips or just below.

Within a flower bud, stamens closely surrounded the central style and were compact within the corolla; anther lobes of adjacent stamens regularly interdigitated (Fig 2.2 b). At anthesis, the anthers were less tightly packed within the more spacious corolla tube (Fig 2.2 d), but stamens remained concealed by the latter.

Staminal filaments possessed elongate, unicellular, non-secretory trichomes (Fig 2.2 f, i) especially laterally, but less abundantly on their abaxial and adaxial surfaces (Fig 2.2 a, g, h, k, l, p). Each filament contained elongated cells (Fig 2.2 i) around a central vascular bundle (Fig 2.2 d, e) that continued until its junction abaxially, approximately at mid-anther (Fig 2.2 a left, h), whereupon the filament narrowed considerably (Fig k, p). Multicellular ridges, apparent extensions of connective tissue that continued distally on the abaxial tubule surface (Figs 2.2 j, p), bore papillae (Fig 2.2 m, p) and internally contained thick-walled cells (Fig 2.2 m). However, in *V. myrtilloides*, neither spurs nor stomata were detected on the ridges nor on the filament near its connection to the anther (Fig 2.2 p), respectively.

When immature, each anther was tetrasporangiate (Fig 2.2 b) and contained an epidermis, middle layers and tapetum. At maturity, four distinct pollen sacs remained evident only at the anther base (Fig 2.2 l, q), the anther having become functionally bilocular (Fig 2.2 d, e, k), with each lateral pair of microsporangia now confluent (Fig 2.2 k). Moreover, halves of each anther were narrowly attached (Fig 2.2 k), particularly in the absence of the filament's connective (Fig 2.2 d). At anther maturity, only remnants of middle layers and/or tapetum were evident (Fig 2.4 d), as anther thickness typically was reduced to a single cell layer, the epidermis (Fig 2.2 e, l). Sections of the remnants of middle layers and/or tapetum fluoresced after staining with Calcofluor White, signaling cellulose (Fig 2.2 o), but the epidermis itself showed only traces of fluorescence and staining with auramine O did not cause fluorescence indicative of cuticle (not

shown). Epidermal cells were often compressed (Fig 2.2 k, l; Fig 2.4 d), without any signs of an endothecium (Figs 2.2 n; Fig 2.4 d). The anther epidermis included numerous papillae (Fig 2.2 a, f, g, l, n-q) except in the anther's abaxial region above the filament-anther junction (Fig 2.2 p). Papillae had thin cuticular striations (Fig 2.2 r) not apparent in *V. vitis-idaea* (Fig 2.1 o).

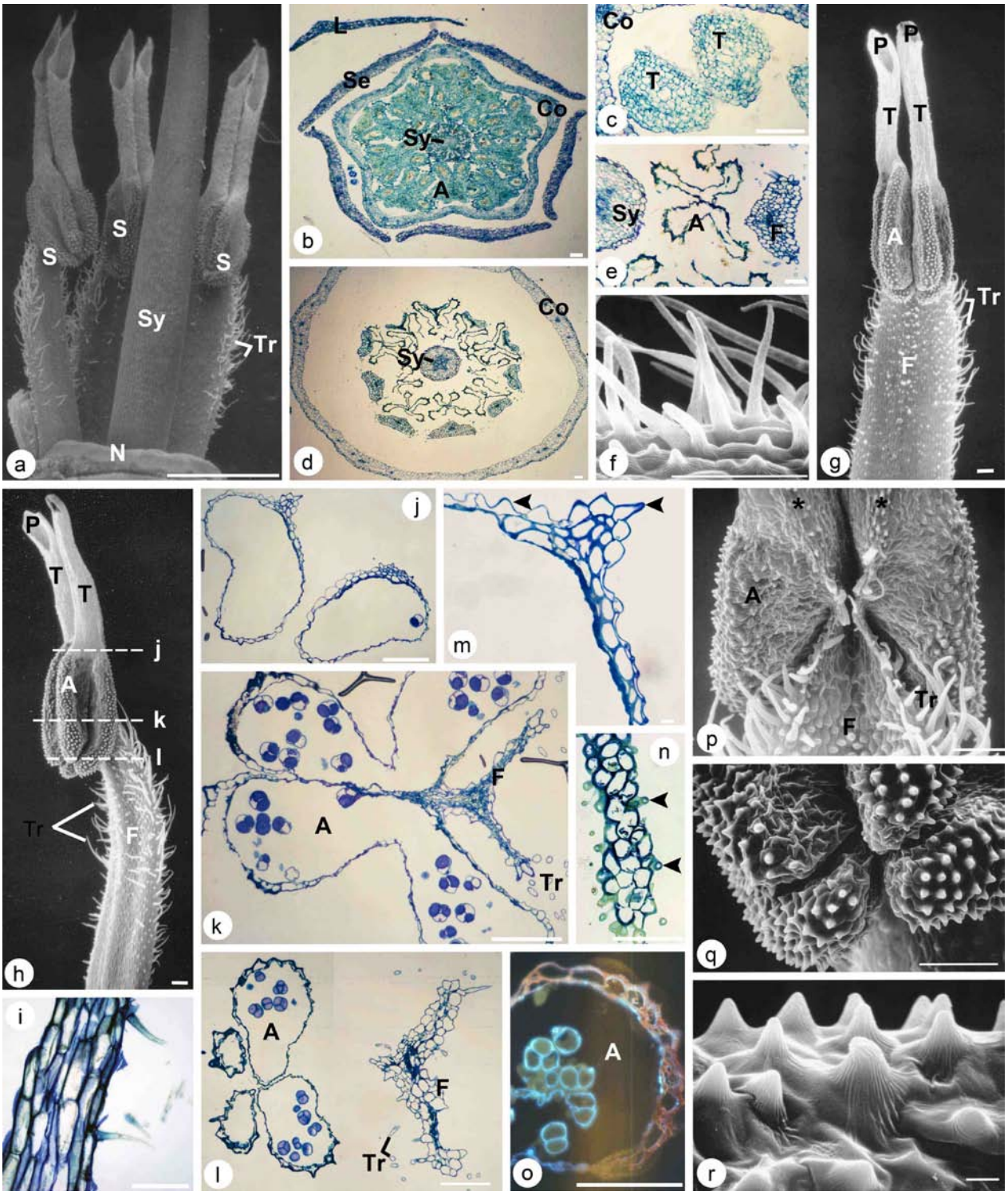
Pairs of tubules joined each anther at its apex and, at stamen maturity, generally exceeded anther length (Fig 2.2 a, g, h). Tubules within the pair were usually narrow and straight (Fig 2.2 a right), but sometimes tubules of the same stamen had different shapes (Fig 2.2 g, h) or were even twisted slightly (Fig 2.2 a left). At the bud stage, anther tubules were not yet hollow, being composed of small parenchyma cells (Fig 2.2 c). At stamen maturity, however, tubule thickness declined to a single cell width at the adaxial surface (Fig 2.2 j); however, along the abaxial surface, the number of layers ranged from 1-6 cells, tubules being reinforced (Fig 2.2 j, m) in the regions corresponding to the extended ridges of connective tissue (Fig 2.2 p).

Unlike the irregular, uneven margin of the apical pores in *V. vitis-idaea*, pores at the tubule apices in *V. myrtilloides* were tapered (Fig 2.2 g, h left; Fig 2.4 a) and slanted toward the style (Fig 2.2 a), although exceptions occurred (Fig 2.2 h right). At anther maturity, the cavity within each tubule connected with the shared locule in each half of the anther, thus providing an unrestricted path for pollen (Fig 2.2 j) to exit from each obliquely-oriented pore (Fig 2.4 a).

Figure 2.2 Structure of the flower and androecium in *Vaccinium myrtilloides.*

a) Lateral view of a dissected flower with all but three of the ten stamens (S) removed. The raised nectary disk (N) encircles the style base (Sy). Trichomes (Tr) are abundant along staminal filaments. b) Cross section of developing flower bud showing sympetalous, pentamerous corolla (Co) enveloping 10 tetrasporangiate anthers (A) that surround the style (Sy). Sepals (Se) and leaf (L). c) Cross section of developing flower bud through the two immature tubules (T) extending above the anther's pollen sacs. Parenchyma cells occupy tubule centre. Corolla (Co). d) Slightly oblique section through mature flower showing space between united corolla (Co) in circular profile surrounding mature anthers and abaxially-located filaments of 10 stamens encircling style (Sy). e) Style (Sy), mature anther (A) and filament (F) of a stamen from the same flower as d. f) High magnification of elongate, non-secretory trichomes along lateral edge of filament. g-h) Adaxial and lateral views, respectively, of isolated stamens showing tapered pores (P) at apex of elongate tubules (T) atop each half of the anther (A) connected abaxially to the filament (F) which possesses elongate, non-secretory trichomes (Tr); the longest trichomes occur laterally. Dashed lines in h show approximate location of anther cross sections illustrated in Fig 2.2 j-l. i) Longitudinal section of filament showing elongated cells plus trichomes in epidermis. j) Tubules of a mature anther. Note thin, unicellular thickness for most of tubule circumference in contrast to the pre-dehiscence tubules in Fig 2.2 c. Multiple wall layers exist in tubule regions corresponding to asterisks in Fig 2.2 p. k) Near mid-region of tetrasporangiate anther (A) connected abaxially to filament (F) that laterally possesses several trichomes (Tr) in section. Note unicellular thickness of anther containing tetrads in its two locules. l) Base of the anther's four pollen sacs distant from filament (F) attachment. Trichomes (Tr). m) Enlargement of region of Fig 2.2 j (top, centre) showing thickened cells in interior of longitudinal ridge running along tubule corresponding to location of asterisks in Fig 2.2 p. Papillae (arrowheads). Epidermal cells of rest of tubule are undulating or concave. n) Tangential longitudinal section of anther wall revealing papillae (arrowheads) and absence of endothecial thickenings in epidermal cells. o) Resin-embedded section demonstrating fluorescent cellulosic content of walls where pollen sacs adjoin (left) and beneath the epidermis of the papillae (right). Exine of pollen grains of tetrads also fluoresces. p) Abaxial view of filament (F) attachment to anther (A) with its tubule bases possessing longitudinally-oriented ridges (asterisks) corresponding to thickened regions in Fig 2.2 j, m. Abundance of elongated, non-secretory trichomes (Tr) on filament edges. q) Papillate nature of surface of a mature anther's four pollen sacs viewed basally. r) Higher magnification of papillae on anther wall. Note slightly-raised cuticular ridges on papillae.

Fig 2.2 a, f-h, p-r – Scanning electron micrographs; Fig 2.2 b-e, i-n – Light micrographs of sections stained with toluidine blue 0. Fig 2.2 o – Fluorescence micrograph of section stained with Calcofluor White. Scale bars: 1 mm – Fig 2.2 a; 0.1 mm – Fig 2.2 b-l, n-q; 10 μm – Fig 2.2 m, r.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2.3.3 Pollen tetrad morphology and anatomy

Primary observations by SEM were made by breaking the pollen sacs of anthers to expose the pollen tetrads within. However, tetrads commonly escaped the anther via its apical pores during the gold-coating process – presumably due to the instrument's vibrations – thus illustrating the accommodating diameter of the anther tubule cavity and terminal pore to facilitate tetrad release (Fig 2.3 a; Fig 2.4 a). SEM confirmed the tetrahedral arrangement, including alignment of colpi among all adjacent pollen grains within a tetrad (Fig 2.3 e; Fig 2.4 b). A sticky substance resembling pollenkitt stretched between adjacent pollen tetrads (Fig 2.3 b, Fig 2.4 c, e), whereas many tetrads had little sign of this material and barely adhered to one another (Fig 2.3 e; Fig 2.4 b). Viscin threads were completely absent on mature tetrads in both *Vaccinium* species (Fig 2.3 e; Fig 2.4 a, b). Pollen grains within an anther of *V. myrtilloides* had pollen tubes growing from several different tetrads (Fig 2.4 e), thus demonstrating that precocious germination is possible for this species, though observed only once, in one anther.

Semi-thin sections through resin-embedded anthers typically showed that cytoplasm of pollen grains within a tetrad stained densely and evenly with TBO in *V. vitis-idaea* (Fig 2.1 i, j, Fig 2.3 c) and *V. myrtilloides* (Fig 2.2 l). However, intra-tetrad variability in staining was a frequent occurrence in some anthers, the cytoplasm occupying the periphery of the vegetative cell in *V. vitis-idaea* (Fig 2.3 d) and *V. myrtilloides* (Fig 2.2 j, k; Fig 2.4 d). A putative, spindle-shaped generative cell was evident in Fig 2.3 d (right). Intact walls occurred between pollen grains of a tetrad (Fig 2.3 c, d; Fig 2.4 d), although thickness of the turquoise-staining exine covering entire tetrads was variable between anthers of the same species (Fig 2.3 c, d) and also could be variable within an anther (Fig 2.3 d). In some instances, a material staining similarly to the exine (peritapetal membrane) was found lining the mature anther's locule continuously and in abundance (Fig 2.3 d), whereas other sections showed material lining the locule intermittently (Fig 2.2 o).

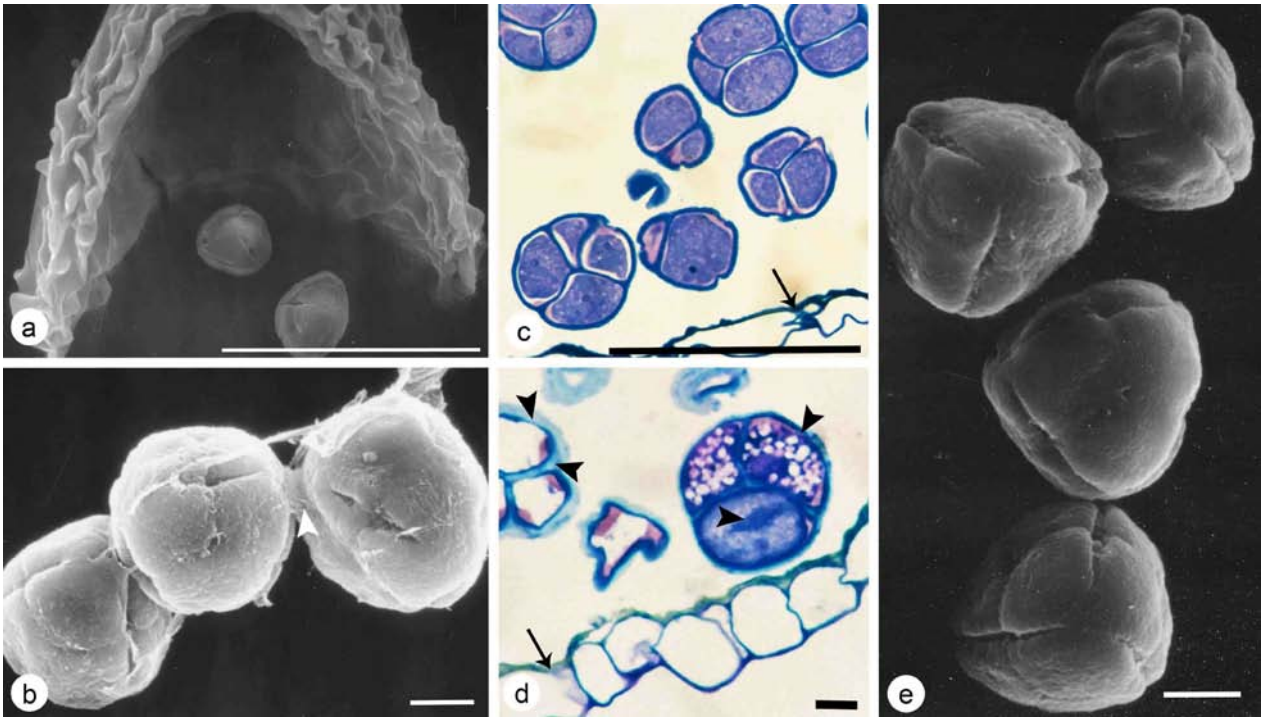


Figure 2.3 Mature tetrads of pollen of *Vaccinium vitis-idaea.*

a) Two liberated tetrads at apical pore of anther tubule. Note irregular surface and fluted contour of pore opening. b) Three adjacent tetrads connected by pollenkitt (arrowhead). c) Two to four densely-cytoplasmic pollen grains per tetrad in sectional view, within locule. Accumulated material (arrow) lining locule below unicellular thickness. d) Tetrad with sparse but clumped, peripheral cytoplasm (left), whereas tetrad at right contains two pollen grains (uppermost) with grainy appearance compared to lower grain with putative spindle-shaped generative cell (arrowhead) evident. Thick exine of sporopollenin (arrowheads) surrounds many grains; torquoise-staining material also lines the locule (arrow) surrounded by unicellular anther wall that lacks an endothecium. e) Sixteen pollen grains of four tetrads, showing absence of pollenkitt.

Fig 2.3 a-b, e – Scanning electron micrographs; Fig 2.3 c, d – Light micrographs stained with toluidine blue 0. Scale bars: 0.1 mm – Fig 2.3 a, c; 10 μm – Fig 2.3 b, d-e.

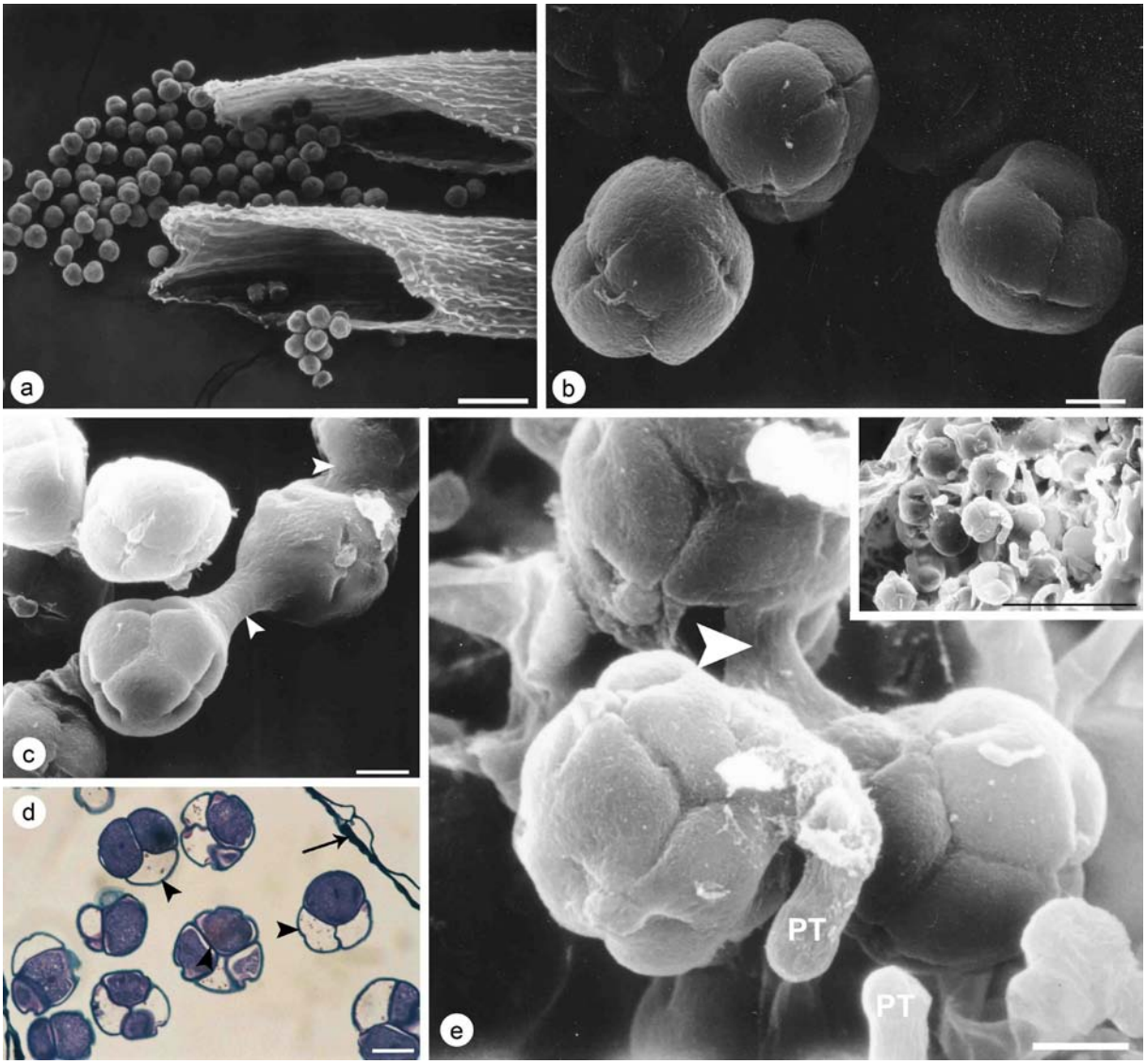


Figure 2.4 Mature tetrads of pollen of *Vaccinium myrtilloides*.

a) Many expelled tetrads at each of the apical pores of the anther's two tubules. b) Tetrads of pollen showing alignment of colpi between adjacent pollen grains within a tetrad. c) Pollenkitt (arrowheads) between several adjacent tetrads. d) Two to four pollen grains per tetrad in section, illustrating intra-tetrad viability. Densely-cytoplasmic grains adjacent to one or more grains with very sparse, often peripheral cytoplasm. Complete walls exist between individual grains within tetrads. Exine (arrowheads). Note material (arrow) lining the locule below the anther's unicellular wall (top right). e) Evidence of precocious germination by pollen grains residing within anther whose wall was dissected to reveal tetrads (inset, upper right). Higher magnification showing pollen tubes (PT) emerged from colpi of several grains in tetrads. Pollenkitt (arrowhead) also evident between adjacent tetrads.

Fig 2.4 a-c, e – Scanning electron micrographs; Fig 2.4 d – Light micrograph stained with toluidine blue 0. Scale bars: 0.1 mm – Fig 2.4 a, e inset; 10 μm – Fig 2.4 b-e.

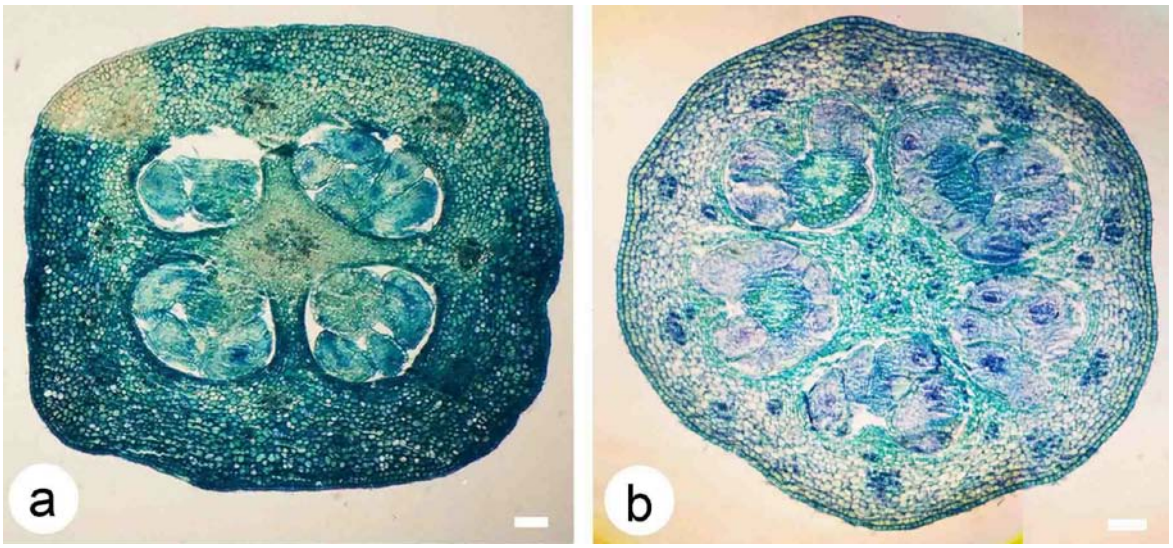


Figure 2.5 Cross sections through inferior ovaries of *Vaccinium vitis-idaea* (Fig 2.5 a) and *V. myrtilloides* (Fig 2.5 b), illustrating multiple ovules in axile placentation within four (Fig 2.5 a) or five (2.5 b) locules per ovary. Paraffin sections stained with toluidine blue 0. Scale bars – 0.1 mm.

2.3.4 Pollen viability

Pollen viability was found to be 51-93% in flowers of *V. vitis-idaea* and 76-97% in flowers of *V. myrtilloides*. Comparisons of intra-tetrad viability for the two species (Table 2.4) showed that although four viable grains per tetrad accounted for the highest percentage of all tetrads examined after FDA staining, tetrads that were completely non-viable were the second most frequent. Tetrads containing only 1-3 viable grains each accounted for 17% (*V. myrtilloides)* to 23% (*V. vitis-idaea*) of tetrads examined. *V. vitis-idaea* (27%) had over twice as many fully non-viable tetrads as *V. myrtilloides* (12%), and most of those tetrads were shrivelled in appearance. The poorly-staining pollen grains with peripheral cytoplasm observed in sectional view with TBO staining (Fig 2.2. j, k; Fig 2.3 d; Fig 2.4 d) probably represented non-viable grains as determined by the FDA test (Table 2.4).

Table 2.4 Percent viability within tetrads of two *Vaccinium* species studied in Saskatchewan.

No. grains viable per tetrad	*V. myrtilloides* (n = 401 tetrads)	*V. vitis-idaea* (n = 331 tetrads)
4	70%	51%
3	7%	2%
2	4%	7%
1	6%	14%
0	12%	27%

Flowers ranged from one day pre-anthesis to third day anthesis
V. myrtilloides represents data over two years (2010, 2011)
V. vitis-idaea represents data for 2011.

2.4 Discussion

2.4.1 Pollen-ovule ratios in *Vaccinium*

Knowledge of the total number of reproductive units (i.e., ovules and pollen grains) per flower assists our understanding of the sexual reproductive effort and permits estimates of reproductive success in horticulturally important species of *Vaccinium*. Furthermore, from the perspective of guiding our understanding of the mating system of individual plant species, these data allow determination of the P/O ratio, an index that generally estimates a species' reliance on self- versus cross-pollination, for example (Cruden 1977, 2000). In addition, comparing P/O ratios of species within a genus as we have done here should provide a relative comparison of breeding system (Erbar and Langlotz 2005).

All *Vaccinium* species in this study, with the single exception of *V. vitis-idaea* from Nova Scotia (discussed below), have P/O ratios that place them in between the categories of facultative autogamy (P/O ratio = 169) and facultative xenogamy (797) (Cruden 1977). Due to the fact that the pollen grains are released as tetrads (potentially representing a more efficient transfer of pollen), the P/O ratios obtained for *Vaccinium* may in fact rank lower than Cruden's original categories for breeding system (Cruden 2000). The alternative approach to P/O ratios would be to compare the number of pollen dispersal units (tetrads) to ovules, as shown in Table 2.3. This perspective would then put all species studied except *V. vitis-idaea* in the realm between obligate autogamy (28) and facultative autogamy (169), which we consider unlikely given the literature indicating lowered yields with self pollen for most species (Jacquemart and Thompson 1996; Hokanson and Hancock 2000) and the provision of nectar as a reward offered by the flowers. Pollen tetrads from *Vaccinium* flowers are mainly available to insects (certain species) capable of floral sonication that ejects pollen from the poricidal anthers leading to its accumulation on specific body sites, ventrally. This selective harvest and localized distribution of pollen tetrads evidently promotes pollination efficiency involving insects. Proctor *et al.* (1996) had also suggested the likelihood that vibratory collection from pendulous flowers leads to a greater proportional deposition of pollen on the insect itself. Therefore, we conclude that with their poricidal anthers, P/O ratios for these Canadian species of *Vaccinium* underestimate, to some extent, the entomophilous nature of their flowers.

That P/O ratios can vary intraspecifically with location has been revealed for three *Vaccinium* spp. Differences in P/O ratio for *V. angustifolium* sampled at three Nova Scotian sites was attributed to differences in numbers of pollen grains per flower. For two different populations of *V. myrtilloides* investigated in Saskatchewan, mean numbers of pollen grains (25850, 26515) per flower were essentially identical (and greatly exceeded the 9600 pollen grains per flower estimated by Reader [1977; see below]), such that differences in P/O ratio instead were driven by variability in numbers of ovules per flower. However the P/O ratios of *V. myrtilloides* in Saskatchewan were both similar to that found in *V. boreale* from Nova Scotia, a species closely related to *V. myrtilloides* and known to hybridize with it in the wild (Vander Kloet 1983). Previously, Brevis *et al*. (2006) reported a small location effect of P/O ratios for *V. ashei* between two sites of the same cultivar in Georgia, U.S.A., whereas differences between cultivars were highly significant, suggesting that genotype is very important.

The greatest intraspecific discrepancy in P/O ratio was encountered for *V. vitis-idaea*. Values obtained for Nova Scotia and Saskatchewan (this study) and Belgium (Jacquemart 1997) were 2008, 736 and 784, respectively (Table 2.3). Interestingly, variability in the total numbers of pollen grains and ovules per flower is relatively high in this species. The close correspondence in P/O ratio between locations in Saskatchewan and Belgium actually masks their reciprocal differences in pollen and ovule quantities per flower (Table 2.3). The *V. vitis-idaea* from Nova Scotia, however, had much higher and lower numbers of pollen grains and ovules, respectively, per flower. Barring any hybridization among the living accessions growing in close proximity at Acadia University, or mislabeling of the specimens, this dramatic difference in P/O ratio for *V. vitis-idaea* from Nova Scotia may be further evidence of suspected trade-offs between male and female function, for different locations within a species, for *V. angustifolium*, *V. myrtilloides* and *V. vitis-idaea*. Although the full resource allocation devoted to pollen tetrad formation is assured before ovule fertilization begins, it appears that at least for some *Vaccinium* spp., there may be plasticity in the process of resource supply.

There are other reports of P/O ratios for *Vaccinium* spp. growing outside of Canada (Table 2.3). For example, the P/O ratio (286.6 ± 31.1) of *V. uliginosum* in Belgium (Jacquemart 1997) closely matches that of Nova Scotia (250.0 ± 16). As Jacquemart and Thompson (1996) report that this species can both self- and cross-pollinate, this low P/O ratio fits with a breeding system that has "a tendency towards autogamy" (Jacquemart 1996). *V. caespitosum* shows a low P/O (239) that suggests it too would be closer to autogamy.

The commercial highbush blueberry, *V. corymbosum*, had a P/O ratio intermediate to most of the other species reported for this study and similar to that of *V. myrtillus* (Jacquemart 1997), though not through comparable numbers of pollen or ovules. *V. corymbosum*'s mean pollen grains per flower (32950) are closer to that of *V. ashei* (33736) and *V. vitis-idaea* (32661, Saskatchewan). Dogterom *et al*. (2000) found no difference in fruit set or seed viability between self and cross pollen in tetraploid cultivars of *V. corymbosum*, however Vander Kloet and Lyrene (1987) reported a reduced amount of fruit set following self pollination as compared to cross-pollination across all ploidy levels. Hokanson and Hancock (2000) gave evidence for inbreeding depression in *V. corymbosum*, *V. angustifolium* and *V. myrtilloides* and concluded that ploidy level as well as levels of previous inbreeding will determine the amount of deleterious recessives that become homozygous (and thus fatal) when selfing occurs.

Despite the wide difference in P/O ratios between *V. vitis-idaea* (784) and *V. oxycoccus* (1667), Jacquemart (2003) concluded that both Belgian spp. are facultatively xenogamous; Cruden's (1977) average P/O ratio for this designation (facultative xenogamy) is 797. However, based on other floral traits (stigma exertion from the corolla; stigma-anther distance), *V. vitis-idaea* functioned more as an outcrosser, contrary to its P/O ratio being doubled by *V. oxycoccus*. Possessing the highest P/O ratio (2225) reported to date (Table 2.3), but with the occurrence of autogamy still existing, Pereira (2008) concluded that the breeding system for *V. cylindraceum* on the Azores, is facultative xenogamy.

Although the P/O ratios within these seven species of *Vaccinium* provide preliminary data that allow us to rank the species (Erbar and Langlotz 2005) from least to greatest outcrossing, accompanying field data and broader representation of clearly separate genets of known age and populations within these same species across Canada and internationally will be required in future to test the ranking. The P/O ratios of these *Vaccinium* suggest an effect of the genotype of individual clones, or the selection pressure of local mating system conditions on populations within species on the number of ovules and pollen grains produced. However, it is interesting that the related species from *Vaccinium* section *Cyanococcus* consistently occupy the middle range (323-543) of P/O ratios whereas the Canadian species from other sections are either above (*V. vitis-idaea*) or below (*V. caespitosum, V. uliginosum*), suggestive that P/O ratios have some relevance taxonomically.

Pollen extraction techniques do have a bearing on reported numbers. Studies have shown that bees cannot reach (King and Buchmann 2003) the optimal sonication frequency of ~500 Hz that efficiently liberates pollen from poricidal anthers of various plant taxa (Buchmann and Hurley 1978; Corbet *et al*. 1988; Harder and Barclay 1994), thus allowing for a gradual dispersion of pollen from flowers (Harder and Barclay 1994). To extract pollen for complete counts, Brevis *et al.* (2006) hung flowers to dry and then repeatedly sonicated them with a tuning fork to remove residual pollen. When performing a pollen release experiment on two cultivars, they instead rolled the flowers between two fingers to release pollen. This rolling method released 90% of the pollen tetrads for one cultivar, but only 52% in the other, leading to speculation that this disparity was due to differences in tubule diameter and length and might also affect the process of anther dehydration (Brevis *et al.* 2006). This outcome may explain the low number of tetrads extracted from *V. myrtilloides* by Reader (1977), who tapped post-anthesis flowers with a

finger until no more pollen tetrads fell; likely there remained pollen in those anthers that were not recovered. As anther dehiscence for these pendulous flowers occurs before anthesis (observed for all species in this study) and bud autogamy has been reported for a low percentage in *V. myrtilloides* (Noormets and Olson 2006), we maintain that the most accurate method of pollen quantification is to use indehiscent anthers.

2.4.2 Stamen structure in relation to anther dehiscence and pollen removal by insects

The stamens of Ericaceae are novel in their combination of many characteristics such as anther inversion during development, presence of a secretory tapetum, lack of an endothecium, occurrence of poricidal dehiscence, presence of staminal appendages, and having mature pollen grains released as tetrads. Thus, many studies have examined stamens of Ericaceae, or specifically those of *Vaccinium*, during their ontogeny (Artopoeus 1903; Matthews and Knox 1926; Palser 1961; Stushnoff and Palser 1969; Venkateswarlu and Maheswari Devi 1972; Hermann and Palser 2000). *Vaccinium* anthers, including those of *V. vitis-idaea* specifically studied by Artopoeus (1903), invert during development. As a result, the anther apex whereupon the tubules arise from meristematic tissue following inversion, is therefore actually the anther base. However, throughout this article, Palser's (1961) instruction to utilize terms like "apex" and "base" in the positional context evident at stamen maturity, is followed. All authors reported that in *Vaccinium*, the epidermis alone remains at anther maturity, or sometimes with remnants of the anther's middle layers, but without any bands of secondary wall thickenings reminiscent of a true endothecium having formed in the epidermis or elsewhere. Our anatomical results from stamens of *V. myrtilloides* and *V. vitis-idaea* agree. The brown anther colour of both *Vaccinium* spp. probably indicates the presence of tannins (Hermann and Palser 2000). However, determination of the precise composition of the wall material of the anther epidermis was elusive; fluorescent-staining techniques (Calcoflour White, Auramine O) demonstrated the presence of cellulose, but apparently not cuticle.

Staminal appendages are characteristic of the Ericaceae, though their role is not fully understood. The terminology for these appendages used herein was reiterated clearly by Pedraza-Peñalosa (2008) based on the definitions of Matthews and Knox (1926), Palser (1961) and Hermann and Palser (2000). In this study of two *Vaccinium* spp., two types of sterile appendages were present: "tubules" and "spurs". Tubules are often referred to as "awns" (Matthews and Knox

1926) prior to dehiscence, however some anthers have awns above the pollen-releasing pore that do not become hollow.

Tubules are only found in the subfamily Vacciniodeae in current classifications of Ericaceae, in particular within Vaccinieae and some Gaultherieae (Kron *et al.* 2002a). Most often there is a pair of tubules per anther as seen in our *Vaccinium* spp., however in some genera of *Macleania* and *Agapetes,* Matthews and Knox (1926) report that only one tubule per anther provides the sole escape for pollen. The sterile tubules allow for anther extension without using the anther's sporogenous tissue (Matthews and Knox 1926).

Anther tubules in *Vaccinium* are very conspicuous and well adapted for pollen release in the Ericaceae (Artopoeus 1903; Matthews and Knox 1926), approximately matching the length of the fertile pollen sacs themselves (Artopoeus 1903). To achieve their role as pollen-dispensing appendages in the absence of any endothecium, the awns become hollow tubules soon before anthesis (Artopoeus 1903; Matthews and Knox 1926; Palser 1961; Hermann and Palser 2000) though the precise mechanisms leading to dehiscence are unknown. For taxa such as *Erica* (Matthews and Knox 1926) and *Kalmia* (D'Arcy *et al.* 1996), dehiscence is associated with calcium oxalate-accumulating tissue or 'granular pouches' (also known as "resorption tissue", of Artopoeus 1903) though they have not been proven to cause dehiscence (D'Arcy *et al.* 1996). Calcium oxalate crystals have only been reported for one species in the tribe Vaccineae: *Vaccinium albicans* (de Vallamil 1980, in D'Arcy *et al.* 1996), but were not seen in either *V. myrtilloides* or *V. vitis-idaea*. Eventually in *Vaccinium* spp., the tubule's epidermal layer remains alone and intact, unchanged in thickness and featuring papillae (Artopoeus 1903) though in our material, at lower densities than the pollen-sac walls themselves. Related to the hollowing process of tubules is the breakdown of the septum between the two originally distinct, laterally-adjacent microsporangia (e.g., *V. myrtillus* – Atropoeus 1903; Matthews and Knox 1926; *V. alaskaensis* and *V. reticulum* – Palser 1961) that yields the anther's functionally bilocular nature at maturity, wherein each pollen-ejecting tubule serves one anther half. The outcome of this process was verified for both *Vaccinium* spp. studied.

A structural comparison of anthers from both *Vaccinium* spp. suggests that tubule straightness plus the shape and orientation of its terminal pore at anther maturity, is influenced by sclerenchyma. Earlier studies of the tubule's abaxial surface referred to "sclerenchymatous development of the hypodermis" (Matthews and Knox 1926) and that thickened cells in *V.*

ovatum were stained red with safranin (Palser 1961). Similarly thickened cells residing below the abaxial epidermis were detected in anthers of both species investigated here. In *V. myrtilloides*, these lignified cells continued from the connective tissue ridge onto the abaxial surface of the tubule (Fig 2.2 j) uninterrupted into the tapered tubule's abaxial tip, apparently contributing to the typically straight, contiguous pair of tubules per stamen in that species (Fig 2.2 a). Moreover, the same abaxial position of the narrower but still sclerified ridge extending into the taper may confine the occurrence of the elongate, somewhat elliptical and highly slanted pore at the tubule tip to the adaxial surface, thereby leading to the introrse arrangement shown in Fig 2.2 a. In anthers of *V. vitis-idaea*, on the other hand, ridges spanning the connective tissue to the tubules themselves have intermittent sclerenchyma present throughout, with some sections of tubule not showing any (Fig 2.1 h) whereas others of the same anther did have sclerenchyma present. This reduction of reinforcement in *V. vitis-idaea* may allow the regular divergence among tubules of each pair (Figs 2.1 b left, d, g), plus the more irregular, sometimes fluted margins of the approximately horizontal (rather than slanted) apical pores, in close agreement to the line drawing for *V. vitis-idaea* by Richter (1929).

In addition to tubules, staminal spurs that originated from the connective tissue slightly above the filament-anther junction on the stamen's abaxial surface, were detected. Known as appendages of variable occurrence, position, orientation, shape and length (Artopoeus 1903; Matthews and Knox 1926; Palser 1961), these paired structures per anther were not encountered in *V. myrtilloides* of Saskatchewan, in agreement with Palser (1961) for material of *V. myrtilloides*/*V. canadense* Kalm from Michigan and New Hampshire, U.S.A. and Nova Scotia, Canada. In species with relatively spacious clearance between staminal filaments and corolla tube (Fig. 2.2 d), with the stamen tips extending close to or beyond the corolla mouth, spur absence follows the general trend in floral morphology outlined by Matthews and Knox (1926). However, short spurs (Fig 2.1 d-f) were detected in *V. vitis-idaea* of Saskatchewan, although specifically recorded as absent in that species examined from Nova Scotia (this study), Alaska and New Hampshire, U.S.A. and the Northwest Territories, Canada (Palser 1961), Germany (Artopoeus 1903; and not described or featured in the adaxial perspective of an otherwise entire, mature stamen drawn by Richter, 1929) and presumably Scotland (Matthews and Knox 1926). Like Palser (1961) described and illustrated for the short spurs of *V. ovatum* Pursh, several adjacent cells with thickened, lignified walls (sclerenchyma) occupied the centre of the spur in *V. vitis-*

idaea (Fig 2.1 f) and appears continuous with the sclerenchyma running into the ridges of connective tissue, as illustrated by Palser (1961) for *V. ovatum*.

In her survey of many *Vaccinium* spp., Palser (1961) remarked on the abundance and variety of floral trichomes encountered, ranging from papillate cells to highly elongated extensions. In stamens of both *Vaccinium* spp. studied here, the papillate outer walls of the anther's pollen sacs and tubules (and spurs themselves in *V. vitis-idaea*; Fig 2.1 d-f) may provide a rugose surface that contributes to the ability of pollinating insects to clasp these structures, during sonication leading to pollen ejection. On the other hand, the filaments in both *Vaccinium* spp. possessed elongate trichomes especially along their lateral edges, where adjacent stamens meet. These trichomes closely resembled those drawn for *V. ovatum* and agree with the written description of Palser (1961) for the same two species studied here.

Filament bases and staminal spurs of the Ericaceae already have received some attention with respect to their prospective role during flower visitation by insects. Apart from the relatively wide bases of the filaments (Fig 2.1 d, g) themselves, the abundance of elongated hairs that can intermingle with those of adjacent filaments surrounding the nectary disk probably assist the retention of nectar in these pendulous flowers. At the same time, these trichomes may contribute to partial obstruction from access to the nectar, potentially helping to guard against nectar robbing in concert with the generally narrowed corolla entrances that restrict passage to long tongues of insects having sufficient mass and appropriate foraging behaviour to vibrate the flowers when handled. Alternatively, the trichomes may ensure that an insect foraging for nectar must disturb the anthers, potentially getting pollen on their bodies, even without sonication. Obstruction by spurs to floral nectar (Artopoeus 1903) may be more important than these filament hairs, however.

Staminal spurs are usually absent in flowers of *Vaccinium* and *Erica* species wherein the stamens project beyond the corolla lobes (Matthews and Knox 1926; Palser and Murty 1967, in Hermann and Palser 2000), with the exception of *V. stamineum* (Hermann and Palser 2000). Spur presence is less predictable when stamen length more or less equals the corolla tube (Artopoeus 1903; Matthews and Knox 1926; Hermann and Palser 2000). In urceolate-type flowers such as *V. uliginosum* and *V. myrtillus* with its long spurs, the latter can occupy the space between the stamen whorl and the corolla, thus being encountered by insects forcing their way into the corolla tube or can be potentially held during vibration (Artopoeus 1903). In addition, Matthews and

Knox (1926) referred to contact of the corolla tube by the curved filaments themselves, and mention “a trigger mechanism” with the inside of the corolla in instances where the appendages are well developed in *Erica*, which we interpreted as a reference to causing liberation of pollen. We submit that the spur’s lignified interior and its continuum with the hypodermal sclerenchyma cells of the ridges of the filament’s connective tissue, may play an important functional role in the receipt and transmission of insect-generated vibrations of the stamens themselves or, when sonication of the corolla itself is performed, in cases where elongate spurs encounter the inner surface of the shaken corolla.

Although their presence is correlated with floral features in Ericaceae, spurs overall are not thought to be taxonomically important. Vander Kloet and Avery (2007) demonstrated the apparent plasticity of stamen features by crossing *V. stamineum* (spurs) and *V. darrowii* (no spurs) both from the section *Polycodium*, that yielded stamens in an F_1 hybrid that resemble those of *V. dentatum* in a completely different section. They also demonstrate that stamen morphology in general does not predict taxonomy as their cluster analysis failed to sort out by genus, but note that current taxonomy does not reflect DNA data in the literature either (Kron *et al.* 2002b). As Kron *et al.* (2002a) review: spurs, like many features traditionally used for taxonomic designations, are very homoplasious. This plasticity is seen in the inconsistency of spur presence in *V. vitis-idaea*.

2.4.3 Pollen structure and viability

Pollen tetrad characteristics of *Vaccinium* have been explored by several authors. In their key for pollen of the Ericales of Canada, Warner and Chinnappa (1986) described *Vaccinium* pollen as the type in which “individual grains within the tetrad are not clearly delimited when rolled and viewed in all positions”. Sarwar *et al*. (2006) described *V. vitis-idaea* (samples from Sweden) as having a compact tetrad, whereas *V. myrtilloides* (from the U.S.A.) possesses a lobed tetrad. These descriptions are in accordance with our findings: *V. vitis-idaea*’s pollen grains were more compact within their tetrads (Fig 2.3 e) whereas those of *V. myrtilloides* bulged slightly at each grain (Fig 2.4 b).

The presence of viscin threads – thin, sticky, acetolysis-resistant structures anchored to the pollen tetrads of *Rhododendron* spp. (Ericaceae) (Richter 1929; Bowers 1930; Waha 1984; Hesse 1979, 2010) and known by entanglement to permit total pollen extraction from individual anthers (Richter 1929; Bowers 1930) – generally leads to reduced P/O ratios based on an

increased efficiency in pollen adherence and delivery by flower-visiting insects (Cruden and Jensen 1979). However, our study of *Vaccinium* pollen did not detect viscin threads, in agreement with others (Richter 1929; Wallace 1975; Waha 1984; Sarwar *et al.* 2006).

Pollenkitt occurs in most angiosperms with entomophilous-related pollination syndromes (Pacini and Hesse 2005). With regard to possession of poricidal anthers, pollen escape is presumed to be hampered by adhesive pollenkitt (Buchmann and Hurley 1978) although pollenkitt exists in some taxa (Ericaceae) but not others (Solanaceae) (Pacini and Hesse 2005). A clear consensus about the existence and/or temporary nature of pollenkitt in the Ericaceae is lacking, except that pollenkitt is absent in tetrads possessing viscin threads (i.e., *Rhododendron*; Hesse 1979, 2010). Pollenkitt in *Andromeda japonica*, *Calluna vulgaris* and *Erica herbacea* may represent an aberrant "inaktivierten" form (Hesse 1979) in that it evidently dries out over time, such that the mode of pollination shifts from entomophily to anemophily (Hesse 1979, 2010). Indeed, transport of single tetrads of *V. vitis-idaea* and *C. vulgaris* have been recovered from air currents in northern Europe (Nilsson *et al.* 1977).

The substance found here between some *Vaccinium* tetrads has obvious elasticity and viscosity. It is unlike the "exinal connections" illustrated by SEM between acetolysed legume pollen grains (Cruden and Jensen 1979) but resembles the substance between pollen grains of *Cucurbita pepo* (Pacini 2000). The best descriptor for non-sporopollenin, pollenkitt-like material between the non-acetolysed tetrads in our study is that "ordinary pollenkitt may sometimes assume a rope-like habit" (Halbritter and Hesse, pers. obs.; in Hesse 2010). However, the strands of material we illustrate (Fig 2.3 b; Fig 2.4 c, e) are very unlike the "pollenkitt ropes" of the related *Notopora schmoburgkii* (Sarwar *et al.* 2005). Furthermore, we cannot exclude a temporary nature of pollenkitt. For example, 8-10 tetrads can form an aggregate such as at the bottom of Fig 2.4 a, whereas the majority of tetrads shown are singular. Obviously the question of pollenkitt in *Vaccinium* requires more attention, including whether it promotes the adherence and quantity of tetrads carried by flower-visiting bees.

Within shed tetrads of the Vacciniaceae, individual pollen grains can be binucleate or trinucleate (Davis 1966). Pollen viability in *Vaccinium* spp. has been examined with various methods and found to be variable, and most often higher than both species studied here. For *V. corymbosum*, Huang and Johnson (1996) also used fluorescein diacetate (FDA) staining and

found that pollen viability was not significantly different from their assessment of pollen germination.

Jacquemart and Thompson (1996) reported pollen viability of three *Vaccinium* spp. by recording tetrads and grains separately. For *V. vitis-idaea*, 90% of tetrads had all four grains viable, whereas individual grains scored 96%. Both *V. myrtillus* and *V. uliginosum* also scored over 90% for both measurements. Those data are markedly greater than in our study, in which tetrads with all pollen grains viable comprised only 51% of tetrads for *V. vitis-idaea* and 70% for *V. myrtilloides*. Numbers of tetrads with 0, 1, 2 or 3 pollen grains viable, were not provided in that study.

By analysing pollen-tube germination and growth in hybrids of *V. corymbosum* crossed with several other blueberry species, Lang and Parrie (1992) recorded intra-tetrad viability of grains. Most cultivars had a high level of multiple pollen-tube germination, but among cultivars, the quantity of tetrads with 2 (roughly 8-38%), 3 (roughly 8-35%), and 4 (roughly <1-49%) pollen tubes varied, possibly due to hybrid incompatibilities. Between 4-20% of tetrads were completely non-viable, slightly lower than the 12% (*V. myrtilloides*) and 27% (*V. vitis-idaea*) recorded here.

Ortiz *et al*. (1999) examined variability of pollen viability (acetocarmine staining) between years at different locations of natural populations of *V. elliottii*, *V. myrtilloides* and *V. tenellum*. Viability was highest in *V. myrtilloides* and varied with location between 82-92% in both years. There were no significant differences among populations between the two years tested, but significant differences between clones and location, suggesting that genotype plays the major role in pollen viability.

Finally, it is important to note that method of pollen liberation from anthers of *Vaccinium* spp., though not always reported, may influence viability characteristics of the pollen actually analysed. Stushnoff and Palser (1969) used pollen shed from anthers, but suggested that since these tetrads were heavier, their viability might not reflect overall viability owing to irregular tetrads still residing within the anther. They did report a high number of irregular tetrads in some of their complex hybrids; this factor might not have a bearing on wild diploid populations. *V. vitis-idaea* did appear to have a high number of completely non-viable tetrads which were usually small and shrunken, and our method of pollen extraction via vibration evidently included irregular pollen tetrads.

One instance of pollen-tube growth from tetrads residing within an anther of *Vaccinium myrtilloides* was recorded incidentally, using SEM. This phenomenon was reported in Ericaceae for *Enkianthus perulatus*, however anthers of that primitive genus release single pollen grains rather than tetrads (Safijowska 1960). Precocious germination of pollen within non-cleistogamous flowers was detected previously in permanent tetrads of the apocynacean, *Periploca graceae* (Dane 2000). Factors favouring pollen germination inside anthers include pollen asynchrony among monads, and water availability (Pacini and Franchi 1982). Events that triggered precocious germination in *V. myrtilloides* are unknown, but asynchrony in pollen development can exist among tetrads (Fig 2.3 d – *V. vitis-idaea*) and the flowering plant material collected at Debden, SK, generally grew in relatively high moisture conditions. The wider occurrence of this phenomenon plus its impact on successful reproductive biology in *Vaccinium*, remains to be explored.

3 Morphology and anatomy of the floral nectary, and nectar secretion dynamics of *Vaccinium myrtilloides* and *V. vitis-idaea*

3.1 Introduction

Floral nectar production is a common and important food reward that flowers offer potential pollinators to entice them to visit. Indeed, from the plant's perspective, nectar is a relatively inexpensive attractant compared to pollen or oil (Simpson and Neff 1983). Nectar is secreted by a floral nectary, the structure of which varies widely among plants, but consists of an epidermis that mediates nectar secretion, nectary parenchyma that produce and/or store pre-nectar constituents, and vascular tissue which supplies the nectary (Bernardello 2007).

In terms of the epidermis, there are many ways nectar can escape from the nectary but pores of modified stomata provide the most frequent method reported for dicots (Bernardello 2007). In fact, stomata have been detected on the nectary surfaces of more than 175 *Rhododendron* spp. (Feldhofen 1933; Palser *et al*. 1991), 50 species of *Erica* (Feldhofen 1933; Palser and Murty 1967), and 25 further non-*Vaccinium* Ericaceae (Kerner and Oliver 1895; Feldhofen 1933; Palser 1961), although are absent in *Pernettya rigida* (Anderson *et al*. 2000). Moreover, the presence of stomata on the disk nectary in *Vaccinium* has been reported previously for *V. corymbosum* (Feldhofen 1933; Palser 1961) and each of the additional 31 species of *Vaccinium* that Palser (1961) examined, including *V. myrtilloides* and *V. vitis-idaea*. However, apparently no scanning electron micrographs exist for floral nectaries of *Vaccinium*, and stomata on floral nectaries of *Vaccinium* were illustrated only once, by line drawings (*V. erythrocarpum*; Palser 1961). Furthermore, quantification of total stomata per nectary has not been found for any member of the Ericaceae. In the continuing search for floral nectary traits that are indicative of high nectar production, the interspecific relationship between nectary stomata and nectar-sugar production is mostly known from Fabaceae (Davis 2000) and warrants exploration in other angiosperm taxa.

The characteristics of nectar secretion are less well reported for *Vaccinium* species. Jacquemart (1992, 1993) reports floral nectar volumes for *V. myrtillus*, *V. vitis-idaea* and *V. uliginosum*, but no solute values were collected from *V. vitis-idaea*. Reader (1977) reports a pooled value of volume, sugar concentration and nectar solute for *V. myrtilloides*. Cane and Schiffhauer (1997) report nectar values for varieties of *V. macrocarpon*, Wood (1961) on *V.*

angustifolium, and Rajotte and Roberts (1979) on *V. corymbosum*. The range of nectar solutes secreted for *V. myrtilloides* and *V. vitis-idaea* has not been previously reported, nor is there information about the dynamics of secretion as the flower ages.

Accordingly, to increase our knowledge of *Vaccinium* floral nectar and nectaries, the aims of this chapter were i) to investigate the morphology and anatomy of the floral nectaries of *Vaccinium myrtilloides* and *V. vitis-idaea* using light and scanning electron microscopy, including quantification of total stomata on the nectary surfaces and documentation of the specific vascular tissue supplying these nectar-secreting glands; ii) to determine nectar volume, solute concentration and total nectar solutes per flower for both *Vaccinium* species at the same field site over two years, to investigate whether there was any pattern in nectar secretion dynamics with flower age and in relation to environmental variables such as temperature and relative humidity, and iii) to attempt to relate differences in nectar production per flower among the two species, with structural features of their flowers and nectaries.

3.2 Materials and methods

3.2.1 Light microscopy of resin embedded sectioned material

Nectary material used for resin sections was fixed with FAA (see section 2.2.1) in June 2011 for both *V. myrtilloides* and *V. vitis-idaea* from different clones of both species throughout the site at Debden, SK (see Table 2.1 for coordinates). Flowers collected were between one day pre-anthesis and six days post-anthesis. Following the removal of the floral whorls, samples were rinsed first with 25 mM sodium phosphate buffer, then with distilled water. Tissues underwent a graded ethanol series until they resided in absolute ethanol. Next they were gradually infiltrated with LR White resin (London Resin Company, Basingstoke, Hampshire, UK), sealed in beem capsules and subjected to 60°C for 72 hours. Sections 2 µm thick were cut using glass knives on a Reichert ultramicrotome, then heat fixed to glass slides before staining with 0.5% toluidine blue 0 in 0.1% aqueous sodium carbonate buffer (pH 11.1). Photographs were taken with a Zeiss Universal photomicroscope.

3.2.2 Scanning electron microscopy (SEM)

Flowers from the Debden field site were fixed in FAA in both 2010 and 2011. Samples were rinsed with 25 mM sodium phosphate buffer, post-fixed in 1% OsO_4 in the same buffer, rinsed several times in distilled water and then transferred through an acetone dehydration series

before critical-point drying (Polaron Instruments, Watford, U.K.). Specimens were dissected to remove the floral whorls, then secured to aluminum stubs, sputter coated with gold (Edwards S150B), and viewed with a Philips 505 scanning electron microscope at 30 kV. Photographs were taken with Polaroid 665 film or Fuji FP-100B film.

3.2.2.1 Quantification of nectary stomata

Mature flower buds of both species collected in 2011 at the Debden field site were processed for SEM. To obtain a count of all the stomata on a nectary surface, each nectary was surveyed by SEM in a systematic across-down-back across-down-across sequence while stomata were diagramed and labeled. Unique features such as a broken sepal, or pollen debris were used to orient on the nectary surface.

3.2.3 Nectar sampling from floral nectaries

Nectar was withdrawn from a flower by gently probing the nectary with a Drummond micropipette (Microcaps®) of 1.0 µL. The volume of nectar was calculated from the length of the micropipette(s) that filled with nectar. Solute concentration of the nectar was measured with a Bellingham and Stanley Pocket Refractometer, corrected for temperature, and converted from the nectar solute concentration by weight (NCW) to the nectar solute concentration based on volume (NCV), or grams sucrose/ml solution using the equation of Búrquez and Corbet (1991). Total nectar solute (µg) per flower was calculated as volume multiplied by the NCV. At least 10 flowers of each age were measured for nectar volume. If the flower had a 0 volume reading, or if the solute concentration could not be obtained from the refractometer's prisms due to low nectar volume, these measurements were not carried forward to calculations of solute. A range of 8 to 10 flowers for *V. myrtilloides* and 3 (Day 1 flowers) to 11 flowers for *V. vitis-idaea* provided solute values for each day of flower age considered.

Nectar measurements where taken once a day on multiple days of flower anthesis as well as on different dates. Sampling dates for *V. myrtilloides* were June 16th, 17th, 19th, 20th, and 22nd in 2010 and June 2nd, 6th, 7th, 8th, 9th, 10th, 11th, 12th and 22nd in 2011. Sampling dates for *V. vitis-idaea* were 15th, 16th, 17th, 18th, 19th, 20th, and 22nd in 2010 and June 16th, 17th, 18th, 20th, 21st, 22nd, and 23rd in 2011. Nectar was sampled from flowers aged 1-4 and 1-6 days post-anthesis in 2010 and 2011, respectively. Unopened flowers were bagged with a 5 cm by 4 cm bag made from grey nylon mesh with drawstring at the bottom (see Fig 4.1 d) before subsequent nectar sampling

to prevent withdrawal of nectar by flower visitors before measurements were taken. Flowers were not sampled for nectar more than once, as subsequent SEM of the nectary of nectar-sampled flowers revealed variable damage caused by the microcapillary tube; some flowers had large tissue damage from microcapillary contact, whereas others did not have any visible marks.

A Wireless Vantage Pro2^{TM}Plus weather station from Davis Instruments was set up at the field site during the sampling hours and was used to record site temperature (^{o}C) (also required for eventual corrections to nectar-solute concentrations), relative humidity (RH) and precipitation.

As sample sizes were small, sometimes unequal, and did not follow a normal curve, the non-parametric Kruskal-Wallis one-way analysis of variance and the Wilcoxon signed rank test were used to test differences between categories. The data between years were significantly different, thus the years are first considered separately, after which flowers aged 1-4 from both years are combined to seek common correlations. The pattern of nectar secretion and possible reabsorption of nectar with flower age was not apparent or consistent over the two years, thus correlations were used to test which variables were most closely tied with total nectar solute, volume and NCV. Variables included were flower age (FA), temperature (Temp), relative humidity (RH), previous precipitation (PP), date and transect. All statistical tests were run and figures created with The R Project for statistical computing (R Core Development Team, 2012).

3.3 Results

3.3.1 Nectary morphology and anatomy

The location of the floral nectary of both species was similar. The disk nectary was located between the filaments and the style, atop the flower's inferior ovary (Fig 3.1 a; Fig 3.2 a). In *V. myrtilloides*, the nectary disk remained confined to the margin of the inferior ovary (Fig 3.1 a), whereas the nectary tissue surrounded the style base in *V. vitis-idaea* (Fig. 3.2 a). Also, nectaries were variable in height within species, and nectaries of *V. vitis-idaea* appeared more flattened above the ovary overall than those of *V. myrtilloides*. Stomata, always solitary rather than occurring in pairs or groups, were found scattered on the surface of both nectaries (Fig 3.1 a, b, c; Fig 3.2 a, d). There was a large range in the number of stomata found from the sampled flowers (Fig 3.3); for *V. myrtilloides* (32-130) was greater than that of *V. vitis-idaea* (52-124), though this difference may be an artifact of the small sample size. Numbers of stomata were not

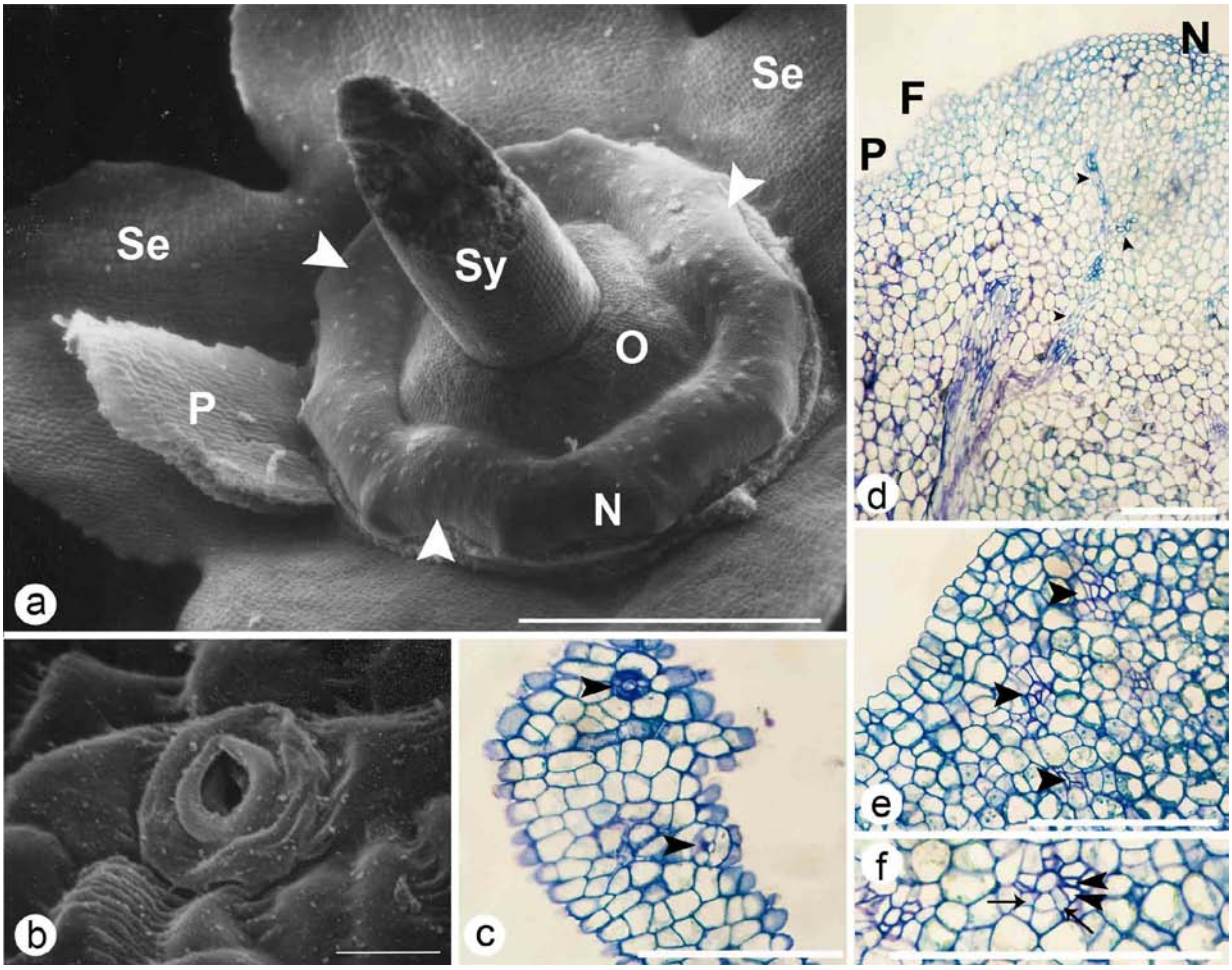
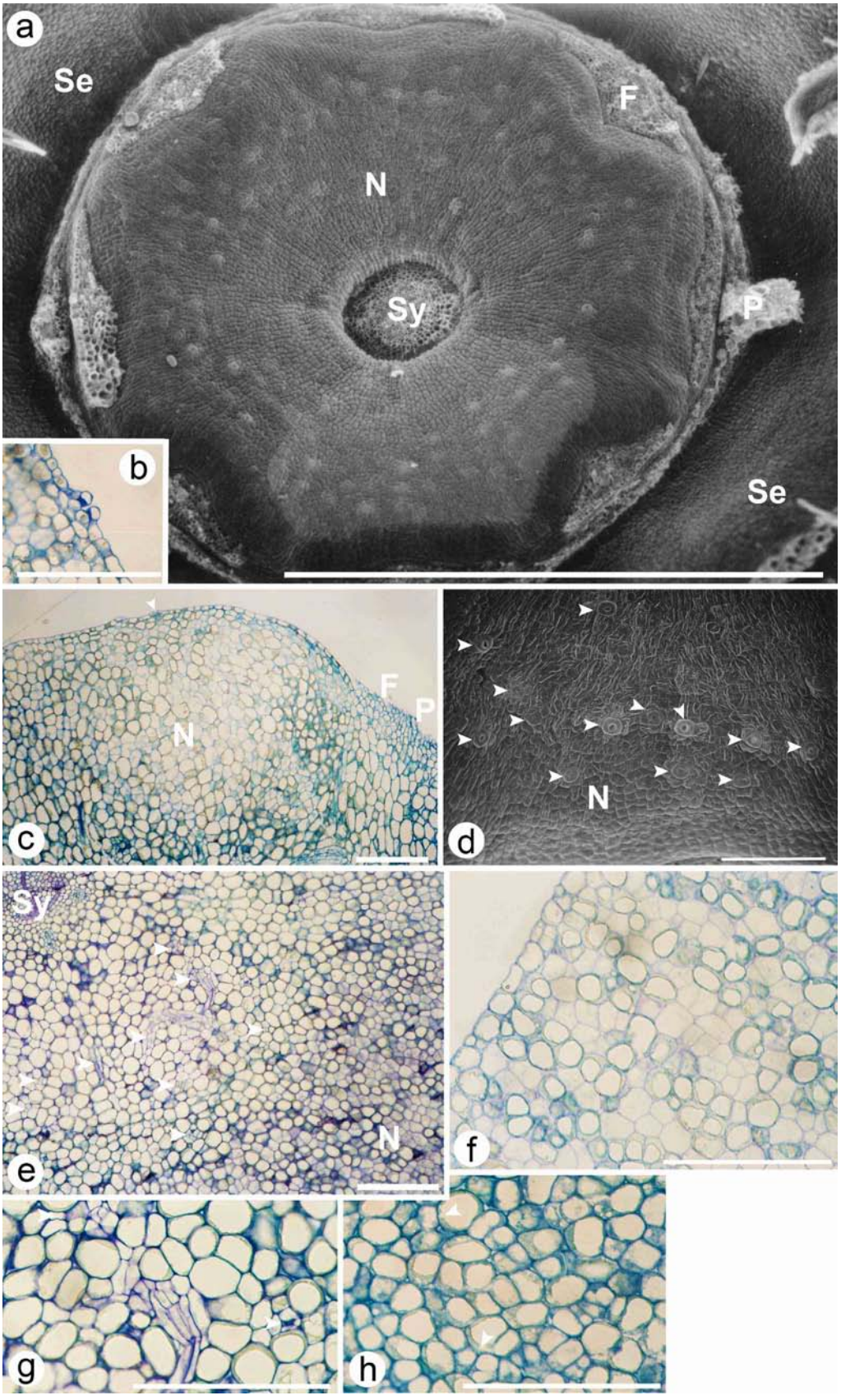


Figure 3.1 Morphology and anatomy of the floral nectary of *V. myrtilloides*.

a) The disk nectary (N, indicated by arrows) on the periphery of the inferior ovary (O) is located between the removed stamens and petals (part of a remaining petal indicated by P), and the style base (Sy); the sepals (Se) of the flower can still be seen. b & c) Nectar is released via stomata (arrowhead in c) on the nectary surface. d) A main trace that is destined for the attachment point of a filament (F) and petal (P) is visible at the bottom left. The vascular tissue (arrowheads) leading to the nectary is branching from this main trace towards the nectary (N). All vascular tissue found leading into the nectary appears to be phloem. e) Phloem in the lower portion on the nectary is shown in cross-section. Three bundles of phloem alone are indicated by arrowheads. f) Higher magnification of traces of phloem heading into the nectary. Small cells that are relatively clear with purple staining walls are sieve tube elements (arrowheads) and the more densely staining companion cells (arrows) are adjacent to them.

Figure 3.2 Morphology and anatomy of the floral nectary of *V. vitis-idaea*.

a) The disk nectary (N) is located between the filaments (F) and the dissected style (Sy). The petal whorl (P) is mostly removed, however the sepals (Se) can still be seen. b) Longitudinal section of the nectary surface showing raised guard cells of a stoma with an open pore but with a small substomatal space below the guard cells. c) A complete longitudinal section of the nectary (N) showing the attachment points of the removed filament (F) and corolla (P). A stoma on the epidermis is indicated by the arrowhead. d) Solitary stomata (arrowheads) on the surface of the nectary (N). e) Cross section through the ovary at the base of the nectary; the style (Sy) is located at the top left, whereas true nectary tissue (N) can be seen at the bottom right. Phloem alone (indicated by arrowheads) presumed to lead to the nectary was found on the inner side of the nectary. f) Cross section through the nectary showing the clear cell files of sclerenchyma with thickened walls staining a dark blue and no inner cell content, among nectary parenchyma with thin cell walls staining light purple and faint staining cell content. g) Higher magnification of Figure 3.2 e, showing the phloem in a section that cuts across the phloem bundles (centre) as well as two bundles in cross section (arrowheads). h) Cross section of the nectary focusing on sclerenchyma cells. Arrowheads indicate pits in the secondary cell wall typical of sclerenchyma.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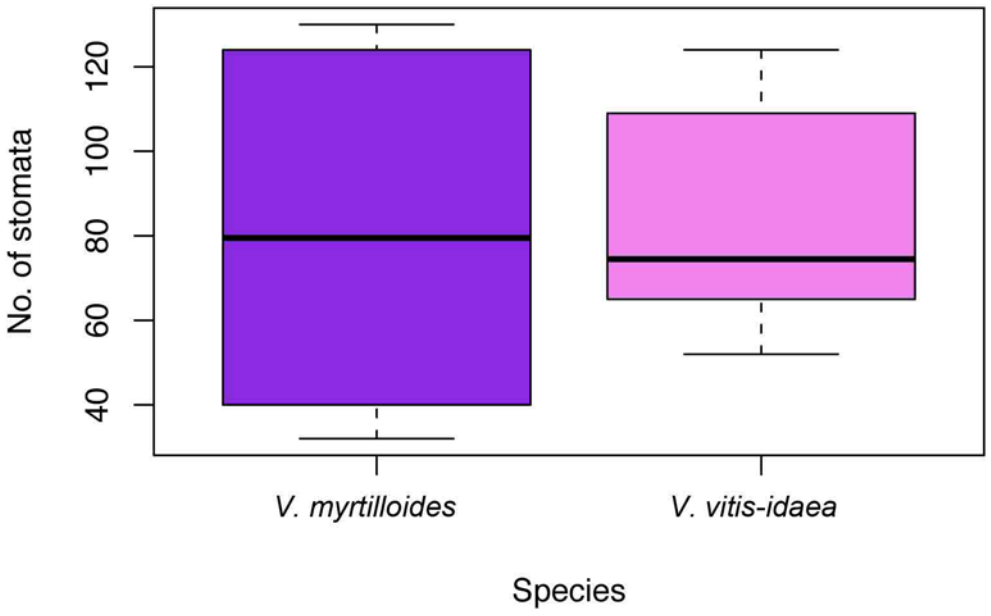


Figure 3.3 Number of stomata counted per floral nectary of *Vaccinium myrtilloides* and *V. vitis-idaea.* Six floral nectaries from the Debden field site were examined per species. The coloured boxes represent the upper and lower quartiles whereas the solid black line represents the median. Whiskers indicate the maximum and minimum values.

significantly different between species (data normal, T test: t = -0.1111, df = 8.463, p = 0.9141). Stomata frequently appeared raised above the adjacent epidermal cells (Fig 3.2 b, d), had only small substomatal chambers (Fig. 3.2 b), and stomatal pores appeared to be open in most cases.

Internally, the nectary was composed of parenchyma, cells of relatively small diameter (Fig 3.1d, e; Fig 3.2 c, e, f). These cells stained darker than the rest of the ovary tissue beneath them, characteristic of dense granular cytoplasm. The nectary also contained what appear to be stone cells, or sclerenchyma, in clear cell files seen in both longitudinal and cross section (Fig 3.2 f). The sclerenchyma cells were isodiametric and show characteristic secondary walls with uniform thickening, and had simple pits within these walls (Fig 3.2 h). Sclerenchyma cells were seen in nectaries of mature buds, as well as those of Day 6 flowers.

In *V. myrtilloides,* many traces of phloem alone entering the nectary were observed in cross section (Fig 3.1 e, f) throughout the base of the nectary, and in longitudinal section (Fig 3.1 d) branching toward the nectary. These phloem traces consisted of sieve tube elements and their adjacent companion cells (Fig 3.1 f). The distance that the phloem traces extended into the nectary tissue was greater in *V. myrtilloides*, often to within 8-10 layers of parenchyma cells below the epidermis. In *V. vitis-idaea*, however, the phloem traces that supplied the filaments and petals, did not enter the base of the nectary tissue as deeply (Fig. 3.2 c), but appeared to travel only as far as nectariferous parenchyma cells at the nectary base. Nectaries that were cross-sectioned at the level of the style base had fewer phloem traces that presumably entered the nectary and these had fewer sieve tube elements per trace (Fig 3.2 e, g), compared to *V. myrtilloides* (Fig 3.1 e, f).

3.3.2 Nectar secretion with flower age

Nectar secretion was measured in 2010 and 2011 at the Debden field site. Bagged flowers aged 1-4 days were sampled in 2010 and flowers aged 1-6 days were sampled in 2011 for both *Vaccinium myrtilloides* and *V. vitis-idaea*. Mature flower buds of both species were probed for nectar in 2011 but none was found in pre-anthesis flowers.

3.3.2.1 *V. myrtilloides*

3.3.2.1.1 Nectar production in 2010

In 2010, nectar was sampled from flowers aged 1-4 days. There appeared to be an overall pattern of increasing solute secretion from Day 1 to Day 3, with a decrease at Day 4, suggestive of the nectary reabsorbing nectar as flowers aged (Fig 3.4 e). The pattern observed for nectar solute per flower was driven by the nectar volume ($r = 0.751$, $p = 8.55e\text{-}08$), which showed a similar trend (Fig 3.4 a). After Day 1, nectar solute concentration remained constant, around 700 µg/µL (Fig 3.4 c). Total nectar solute per flower ranged from 50.6 to 3684.1 µg, nectar volume from 0 to 4.44µL, and nectar concentration by volume from 104.7 to 1441.9 µg/µL (Table 3.1). There were three flowers found to have no nectar when sampled: two that were Day 3 flowers and one Day 4 flower.

Nectar solute, nectar volume and nectar concentration by volume (NCV) were not correlated with the variable of flower age in 2010. Correlations run with variables such as temperature and relative humidity (RH), did not have any significant relationships. However the variable previous precipitation was found significantly correlated with both volume ($r=0.368$, $p = 0.0192$) and NCV ($r = -0.381$, $p = 0.0199$). There was only one day of measurements that had followed a day of precipitation. Three of the four data points from that date showed a high nectar volume and low NCV.

The measurements of nectar volume showed a correlation with the date it was sampled on ($r = 0.367$, $p = 0.0199$).

3.3.2.1.2 Nectar production in 2011

In 2011, the sampling of nectar over flower age was extended to 6 days because nectar was still present in Day 4 flowers and the pattern of 2010 suggested reabsorption of nectar starting on the fourth day of anthesis. The pattern for total nectar solute per flower in 2011,

however, did not show the same trend as in 2010. The median quantity of nectar solutes of Day 3 flowers (the highest recorded in 2010) was lower than that of Day 1 and Day 2 flowers. Day 4 flowers showed the largest range of nectar solute, with Days 5 and 6 comparable or lower than Days 1 and 2 (Fig 3.4 f). Daily nectar volumes (Fig. 3.4 b) followed the same trend as nectar solute quantity per flower (Fig 3.4 f), owing to the consistency in nectar solute concentration (NCV; Fig 3.4 d). Nectar solute ranged from 66.8 to 2142.9 µg, nectar volume from 0 to 4.69 µL, and nectar concentration by volume from 192.4 to 1164.2 µg/µL (Table 3.1). There were four flowers that were found to have no nectar when sampled: one was a Day 1 flower, another a Day 4 flower, and two were Day 6 flowers.

Nectar solute, nectar volume and nectar solute concentration by volume (NCV) were not correlated with the flower age in 2011. Correlations run with variables such as temperature, humidity, and if the previous day had received precipitation found significant relationships with nectar volume and humidity ($r=0.371$, 0.00298), and for the variable of previous precipitation, for both volume ($r=0.663$, $p = 4.4e-09$) and NCV ($r = -0.576$, $p = 2.272e-06$). Though there was only one day that a measurement was taken following a day that had received precipitation, the value does cluster with the previous values following rain in 2010 (see Fig 3.6 a).

The measurements of nectar volume and total nectar solute per flower showed a correlation with the date for which the flower was sampled ($r = 0.451$, $p = 0.000227$; $r = 0.287$, $p = 0.031$).

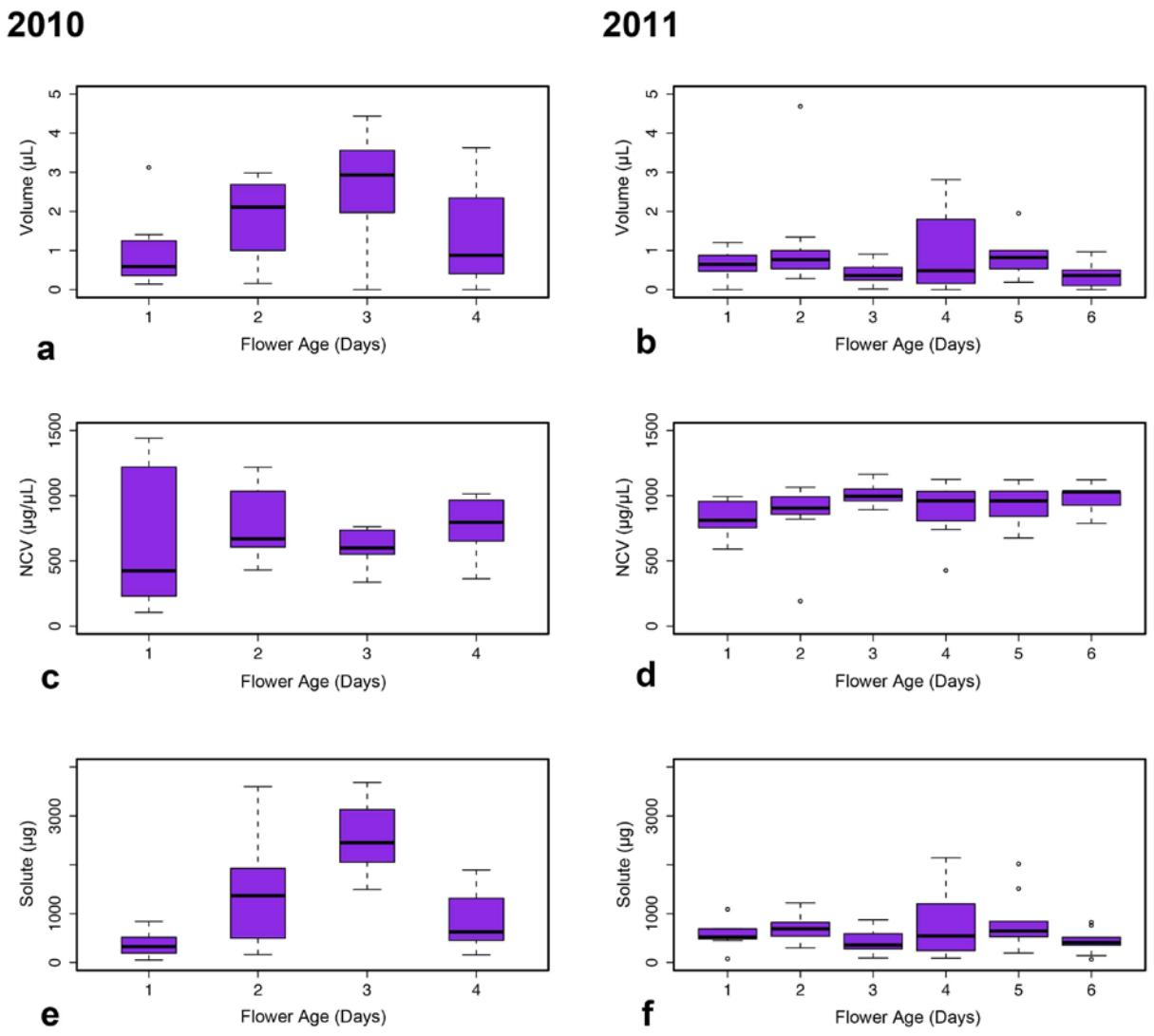


Figure 3.4 Total nectar volume (a, b), nectar solute concentration by volume (NCV; c, d), and total nectar solute produced per flower (e, f) by bagged flowers of *Vaccinium myrtilloides* of known age in 2010 (a, c, e) and 2011 (b, d, f). The coloured boxes represent the upper and lower quartiles whereas the solid black line represents the median. Whiskers indicate the maximum and minimum values and outliers are shown when 1 ½ times the upper or lower quartile.

Table 3.1 Descriptive statistics on the nectar solute (µg), volume (µL) and nectar concentration by volume (NCV; µg/µL) collected from *V. myrtilloides* over two years (2010, 2011) and multiple flower ages (Day).

		Mean	Range	Sample size
2010				
Day 1	Solute	373.85 ± 234.79	50.58-838.87	n = 10
	Volume	0.898 ± 0.881	0.141-3.125	n = 10
	NCV	660.78 ± 509.92	104.65-1441.86	n = 10
Day 2	Solute	1447.73 ± 1080.76	161.58-3598.78	n = 10
	Volume	1.834 ± 1.009	0.156-2.984	n = 10
	NCV	778.05 ± 276.36	429.96-1218.63	n = 10
Day 3	Solute	2554.3 ± 738.5	1493.4-3684.1	n = 8
	Volume	2.522 ± 1.480	0-4.4375	n = 10
	NCV	608.72 ± 141.55	336.54-762.67	n = 8
Day 4	Solute	896.5 ± 639.8	155.5-1893.7	n = 9
	Volume	1.262 ± 1.249	0-3.625	n = 10
	NCV	774.36 ± 208.13	362-1014.58	n = 9
2011				
Day 1	Solute	558.51 ± 267.78	77.31-1091.39	n = 9
	Volume	0.606 ± 0.362	0-1.203	n = 10
	NCV	829.67 ± 135.12	590.74-993.74	n = 9
Day 2	Solute	687.59 ± 255.04	299.48-1217.1	n = 10
	Volume	1.126 ± 1.285	0.281-4.687	n = 10
	NCV	856.41 ± 245.11	192.42-1064.81	n = 10
Day 3	Solute	429.55 ± 233.0	90.95-870.92	n = 10
	Volume	0.399 ± 0.270	0.0156-0.906	n = 11
	NCV	1007.52 ± 72.83	892.63-1164.20	n = 10
Day 4	Solute	777.05 ± 740.07	87.88-2142.91	n = 9
	Volume	0.928 ± 1.078	0-2.812	n = 10
	NCV	902.63 ± 220.66	426.96-1124.94	n = 9
Day 5	Solute	822.5 ± 540.88	193.9-2019.78	n = 10
	Volume	0.914 ± 0.596	0.187-1.953	n = 10
	NCV	927.26 ± 136.32	675.37-1121.94	n = 10
Day 6	Solute	433.84 ± 249.87	66.76-823.17	n = 9
	Volume	0.375 ± 0.327	0-0.969	n = 11
	NCV	979.05 ± 109.90	787.72-1122.13	n = 9
Combined years				
	Solute	872.76 ± 808.04	50.6-3684.1	n = 94
	Volume	1.073 ± 1.099	0-4.687	n = 102
	NCV	835.54 ± 260.20	104.6-1441.8	n = 94

3.3.2.1.3 Combined years of nectar data for *V. myrtilloides*

The data between the years were very different, however measurements from 2010 were combined with measurements on Day 1 to Day 4 flowers from 2011 to test if recorded variables such as temperature (T), relative humidity (RH) (both taken from the same time that flowers were sampled for nectar: 10:30 AM) and previous precipitation (PP) could explain the variation. The range of temperature experienced during the nectar collection trials on *V. myrtilloides* was different, with 2011 experiencing cooler mornings (15.8 ºC), whereas in 2010 the coolest temperature at 10:30 was 18.3 ºC. The range of RH experienced between the two years was similar; in 2010, there was a range of 42-74%, whereas in 2011 the range was 34-83%, but in both years there was an even distribution of RH. Each day in both years that had previous precipitation coincided with the only days the RH was greater than 70% during the entire period of nectar sampling in *V. myrtilloides*.

The nectar solute values per flower were driven by the changes in nectar volume rather than NCV. All three measurements of nectar solute, volume, and NCV appear to be weakly correlated with temperature ($r = 0.338$, $p = 0.0029$; $r = 0.275$, $p = 0.012$; $r = -0.262$, $p = 0.0231$; Fig 3.5). NCV and nectar volume are correlated with PP ($r = -0.463$, $p = 2.87e-05$; $r = 0.478$, $p = 6.38e-06$) and the red data points in Fig 3.6 a show the nectar to have high volume and diluted NCV due to rain. Nectar volume is correlated with RH ($r = 0.329$, $p = 0.00263$, Fig 3.6 b). Interestingly, the date the samples were taken correlated with nectar solute, volume and NCV ($r = 0.404$, $p = 0.000317$; $r = 0.492$, $p = 3.15e-06$; $r = -0.404$, $p = 0.000326$). This could represent a change in the secretion over the flowering season, or reflect increasing temperature. However, the dates sampled over both years barely overlap and so the effect of date is probably due to other differences between the two years that are unaccounted for. No transect stood out as significantly different from the others in terms of total nectar solute production per flower (chi-squared = 12.4513, df = 7, p-value = 0.08666).

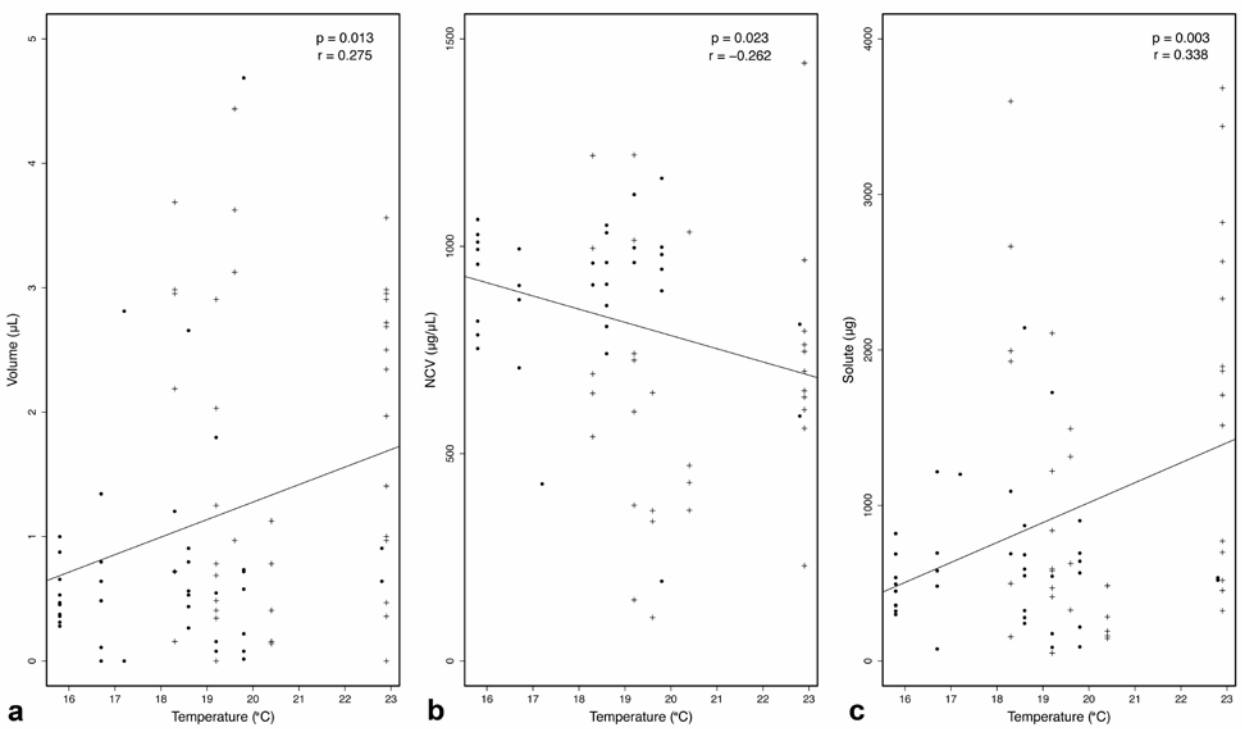


Figure 3.5 Total nectar volume (a), nectar concentration based on volume (NCV) (b), and nectar solute (c) per flower correlated with the temperature recorded at the time of nectar measurements for *V. myrtilloides* flowers aged 1-4 days anthesis. Symbols: 2010 (+), 2011 (•).

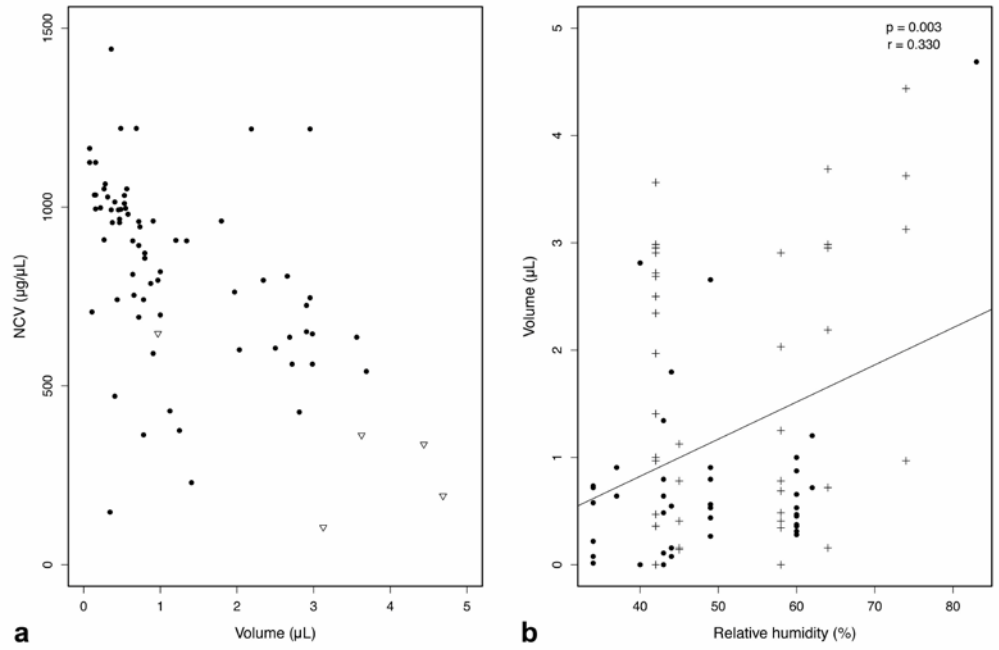


Figure 3.6 a) Nectar concentration based on volume (NCV) and corresponding nectar volume measurements for flowers of *V. myrtilloides* aged 1-4 days anthesis, from 2010 and 2011. Triangular data points were measurements taken on days that experienced precipitation the previous day, which also correspond to the only days that the RH was greater than 70%. b) Correlation of nectar volume with relative humidity in samples of flowers aged 1-4 days anthesis from 2010 (+) and 2011 (•).

3.3.2.2 V. vitis-idaea

3.3.2.2.1 Nectar production in 2010

In 2010, there seemed to be a slight increase in total nectar solutes per flower from Day 1 to Day 4, with Days 2 and 3 almost the same in data spread (Fig 3.7 e). The pattern of nectar solute was driven by nectar volume ($r = 0.390$, $p = 0.0207$) which increased with flower age (Fig 3.7 a) while nectar concentration by volume (NCV) remained consistent from Day 2 onward (Fig 3.7 c). Nectar solute per flower ranged from 8.07 to 1147.6 µg, nectar volume from 0 to 4.83 µL, and NCV from 54.7 to 1112.2 µg/µL (Table 3.2). Four flowers did not appear to have nectar when sampled: three that were Day 1 flowers and one Day 2 flower.

Nectar solute and NCV were correlated with the variable of flower age in 2010 ($r = 0.359$, $p = 0.034$), but nectar volume was not. Correlations run with variables such as temperature, relative humidity (RH), and whether the previous day had received precipitation, showed significant relationships with RH for both solute ($r = 0.389$, $p = 0.021$) and NCV ($r = -0.513$, $p = 0.00161$), and a significant relationship between nectar solute and temperature ($r = 0.428$, $p = 0.0104$). The correlation with previous precipitation was significant with both nectar volume ($r = 0.651$, $p = 1.69e\text{-}06$) and NCV ($r = -0.854$, $p = 6.96e\text{-}11$). There were two days of measurements taken following days of precipitation. All eight data points from those dates showed a low NCV and six represent nectar volumes higher than all other data points that year (Fig 3.8).

The measurements of NCV showed a negative correlation with the date it was sampled on ($r = -0.541$, $p = 0.000782$).

3.3.2.2.2 Nectar production in 2011

In 2011, the measurement of nectar over flower age was extended to 6 days post-anthesis as the pattern of 2010 suggested nectar solute might have been increasing with flower age. The total nectar solute per flower in 2011 showed an increase to Day 5 flowers with Day 6 flowers comparable to Day 4 (Fig 3.7 f). The range in nectar solute per flower (Fig 3.7 f) and its concentration (Fig 3.7 d) were much lower in 2011 than the range measured in 2010. However volumes of this dilute nectar were greater in 2011 (Fig 3.7 b) than in 2010 (Fig 3.7 a). Nectar solute ranged from 1.29 to 673.5 µg, nectar volume from 0 to 3.25 µL, and NCV from 13.84 to 984.45 µg/µL (Table 3.2). There were fourteen flowers that lacked any nectar when sampled:

eight were Day 1 flowers and two were Day 2 flowers, three were Day 5 flowers and one a Day 6 flower.

Nectar solute and nectar volume were correlated with the flower age in 2011 ($r = 0.311$, $p = 0.0263$; $r = 0.44$, $p = 0.000138$), the nectar solute driven by the nectar volume ($r = 0.756$, $p = 1.43e-10$). Correlations run with variables such as temperature, humidity, and if the previous day had received precipitation found significant relationships with nectar solute, volume and NCV with relative humidity ($r= -0.301$, $p = 0.031$; $r = 0.276$, $p = 0.0209$; $r = -0.741$, $p = 5.11e-10$). NCV was also correlated with temperature ($r = 0.647$, $p = 2.85e-07$) and nectar volume was correlated with previous precipitation ($r = 0.323$, $p = 0.00634$). There were three days of nectar sampling that followed a day that had previous precipitation. All data points from those dates showed a low NCV, however most of these points do not stand apart from all other data points that year (Fig 3.8).

The measurements of NCV showed a positive correlation with the date the flower was sampled on ($r = 0.279$, $p = 0.0475$), in an opposing trend to the previous year.

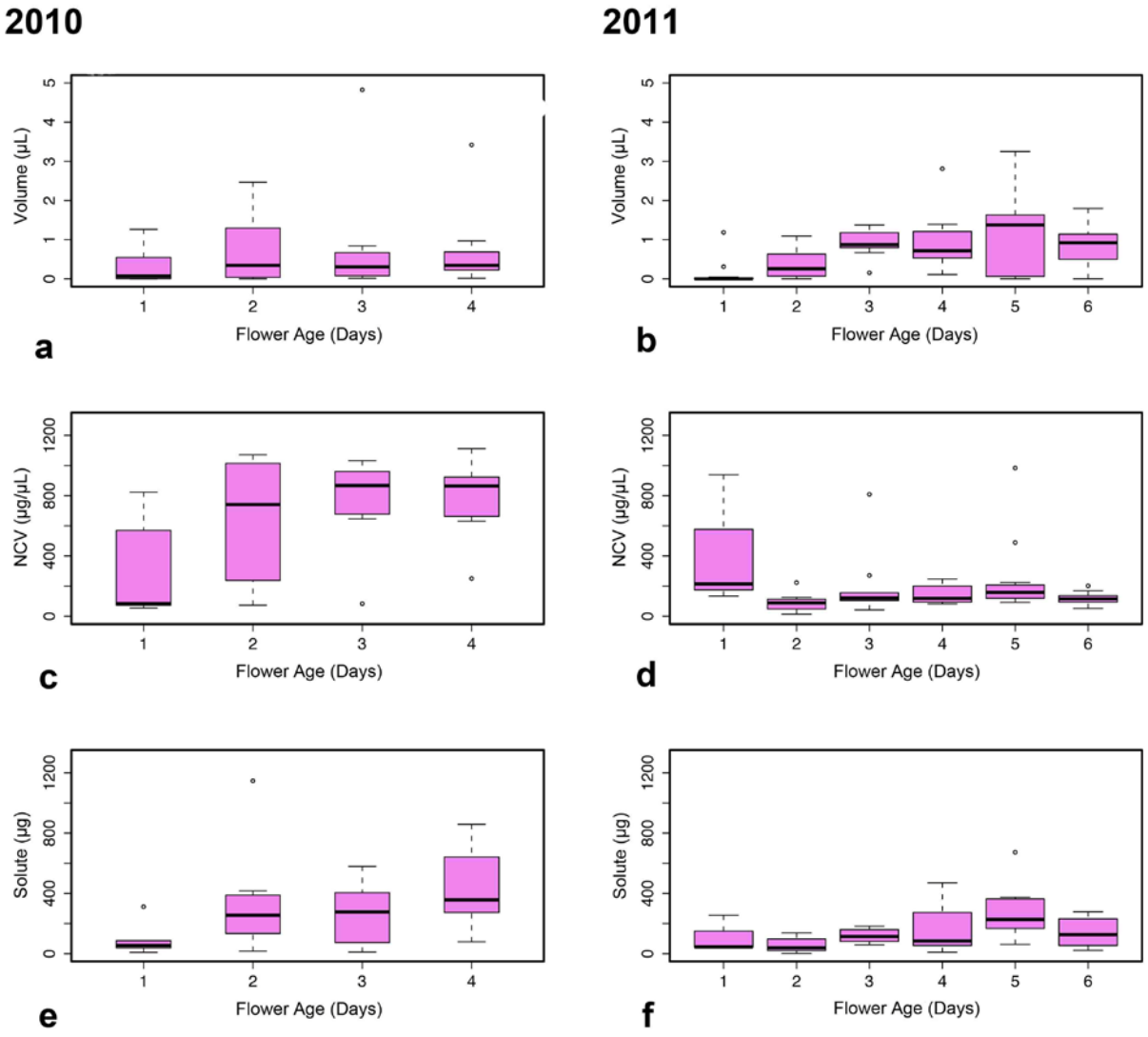


Figure 3.7 Total nectar volume (a, b), nectar solute concentration by volume (NCV; c, d), and total nectar solute produced per flower (e, f) by bagged flowers of *Vaccinium vitis-idaea* of known age in 2010 (a, c, e) and 2011 (b, d, f). The coloured boxes represent the upper and lower quartiles whereas the solid black line represents the median. Whiskers indicate the maximum and minimum values and outliers are shown when 1 ½ times the upper quartile.

Table 3.2 Descriptive statistics on the nectar solute (µg), volume (µL) and nectar concentration by volume (NCV; µg/µL) collected from *V. vitis-idaea* over two years (2010, 2011) and multiple flower ages (Day).

		Mean	Range	Sample size
2010				
Day 1	Solute	92.45 ± 110.81	8.07-311.74	n = 6
	Volume	0.298 ± 0.427	0-1.267	n = 12
	NCV	281.63 ± 331.6	54.69-823.41	n = 6
Day 2	Solute	327.16 ± 340.38	16.11-1147.62	n = 9
	Volume	0.777 ± 0.965	0-2.469	n = 11
	NCV	660.07 ± 401.54	73.76-1070.7	n = 9
Day 3	Solute	279.25 ± 209.51	10.10-579.51	n = 10
	Volume	0.775 ± 1.45	0.0156-4.828	n = 10
	NCV	786.6 ± 278.42	83.87-1032.68	n = 10
Day 4	Solute	424.82 ± 258.19	78.91-858.07	n = 10
	Volume	0.676 ± 0.954	0.0156-3.4218	n = 11
	NCV	798.42 ± 235.88	250.76-1112.22	n = 10
2011				
Day 1	Solute	113.55 ± 122.35	41.79-254.82	n = 3
	Volume	0.141 ± 0.359	0-1.187	n = 11
	NCV	429.17 ± 443.53	133.73-939.18	n = 3
Day 2	Solute	56.59 ± 51.5	1.29-137.5	n = 8
	Volume	0.358 ± 0.357	0-1.094	n = 12
	NCV	92.45 ± 64.11	13.84-223.91	n = 8
Day 3	Solute	119.69 ± 46.31	57.65-182.42	n = 10
	Volume	0.927 ± 0.344	0-0.156	n = 11
	NCV	195.67 ± 223.71	41.93-809.4	n = 10
Day 4	Solute	160.59 ± 153.63	8.97-469.78	n = 10
	Volume	0.946 ± 0.735	0.1094-2.812	n = 11
	NCV	144.86 ± 57.96	82.04-247.53	n = 10
Day 5	Solute	266.84 ± 171.55	61.53-673.52	n = 11
	Volume	1.143 ± 0.997	0-3.250	n = 15
	NCV	254.32 ± 265.72	91.74-984.45	n = 11
Day 6	Solute	134.29 ± 93.4	21.87-277.5	n = 9
	Volume	0.908 ± 0.555	0-1.797	n = 10
	NCV	119.53 ± 46.4	51.85-202.24	n = 9
Combined years				
	Solute	212.56 ± 210.19	1.29-1147.62	n = 86
	Volume	0.702 ± 0.824	0-4.828	n = 114
	NCV	381 ± 362.63	13.84-1112.21	n = 86

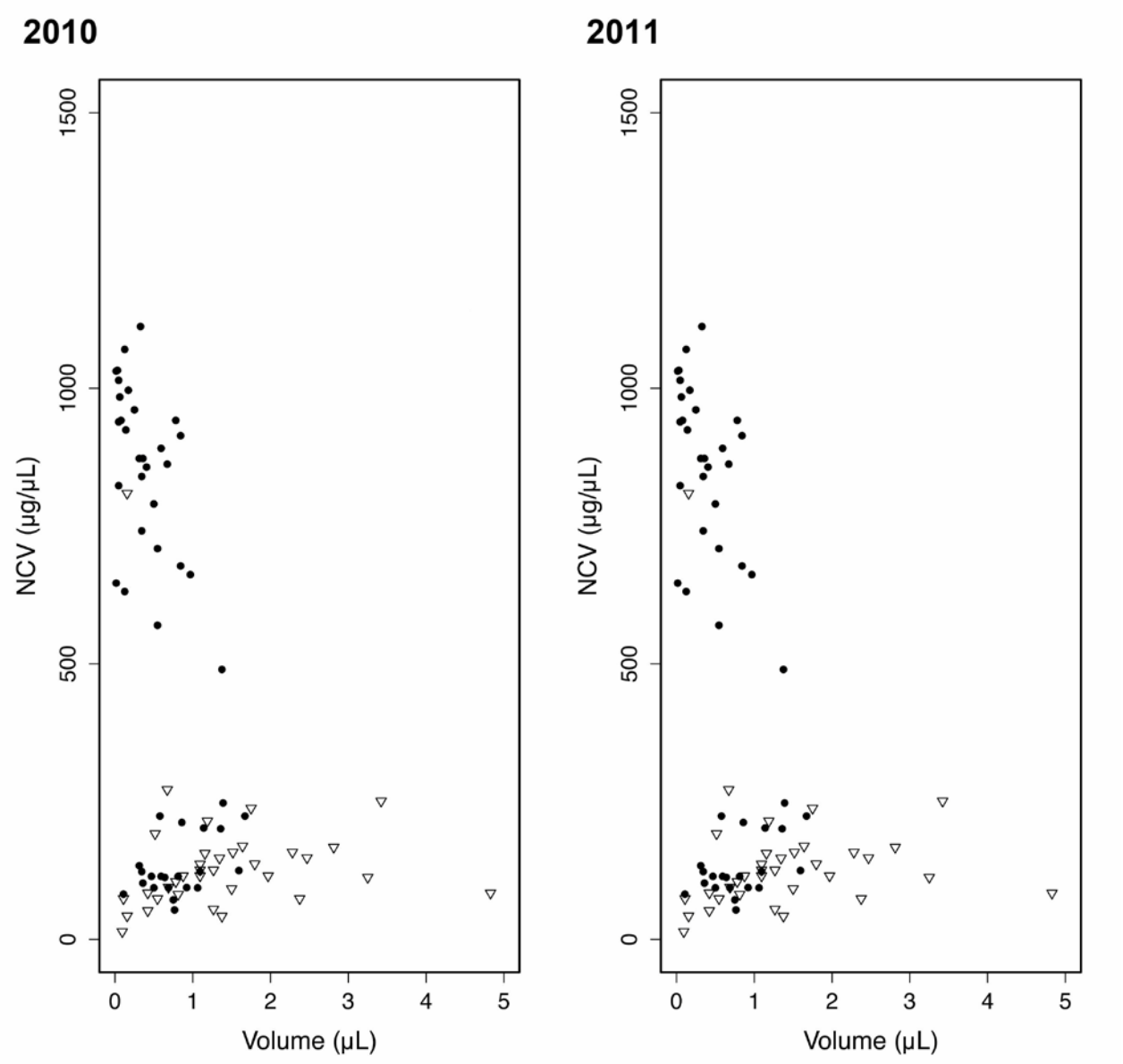


Figure 3.8 Nectar solute concentration by volume (NCV) and corresponding nectar volume measurements for flowers of *V. vitis-idaea* in 2010 and 2011. Triangular data points were measurements taken on days that experienced precipitation the previous day.

3.3.2.2.3 Combined years of nectar data for *V. vitis-idaea*

The data between the years were very different, however nectar characteristics from 2010 were combined with those on Day 1 to Day 4 flowers from 2011 to determine whether variables such as temperature (T), relative humidity (RH) and previous precipitation (PP) could explain the variation. The range of temperatures experienced at the sampling time during the dates measurements were taken on *V. vitis-idaea* were quite different; 2010 was warm, with temperatures between 18.3-22.9 ºC, whereas 2011 experienced lower temperatures from 13.7 – 23.8ºC. The RH between the two years was completely different: in 2010 there was a range from 42-74%, but in 2011 although the range was only slightly higher (58-97%), almost all days during which nectar sampling occurred had RH greater than 80%.

Both nectar volume and NCV were significantly correlated with the resulting nectar solute per flower ($r = 0.412$, $p = 0.000574$; $r = 0.324$, $p = 0.00786$). Solute was also weakly correlated with flower age ($r = 0.256$, $p = 0.038$) as in both years the quantities of nectar solute increased from flower ages 1-4 (Fig 3.9 a) and volume also showed a weakly positive relationship with flower age (Fig 3.9 b) for the combined data ($r = 0.292$, $p = 0.00548$). Solute and NCV were positively correlated with temperature ($r = 0.415$, $p = 0.000535$; $r = 0.509$, $p = 1.27e\text{-}05$; Fig 3.10 a, b) and negatively with RH ($r = -0.504$, $p = 1.56e\text{-}05$; $r = -0.774$, $p = 2.47e\text{-}14$; Fig 3.10 c, d). In particular the relationship with NCV and RH is very high, however there is very little overlap between the different year's data points and thus could also be due to an unknown variable that differed between years (Fig 3.10 d). Previous precipitation (PP) is correlated with both NCV ($r = -0.577$, $p = 3.91e\text{-}07$) and nectar volume ($r = 0.422$, $p = 3.72e\text{-}05$), and values of both NCV and volume tested significantly different between samples that had received PP and those that had not (NCV: $W = 173$, $p = 1.053e\text{-}05$; volume: $W = 1261.5$, $p = 0.001074$; Fig 3.11). There is an effect of date for NCV ($r = -0.276$, $p = 0.025$) however the pattern is not clear when the data were plotted and it seems to reflect the changes in temperature, rather than an increase in NCV as the season progresses. No transect stood out as different from the others in terms of total nectar solute per flower (chi-squared = 5.2351, df = 5, p-value = 0.3879) although transect 8 (high shade, likely high moisture) did have a consistently high NCV and low volume over both years (measurements were taken over a range of temperatures and different RH readings).

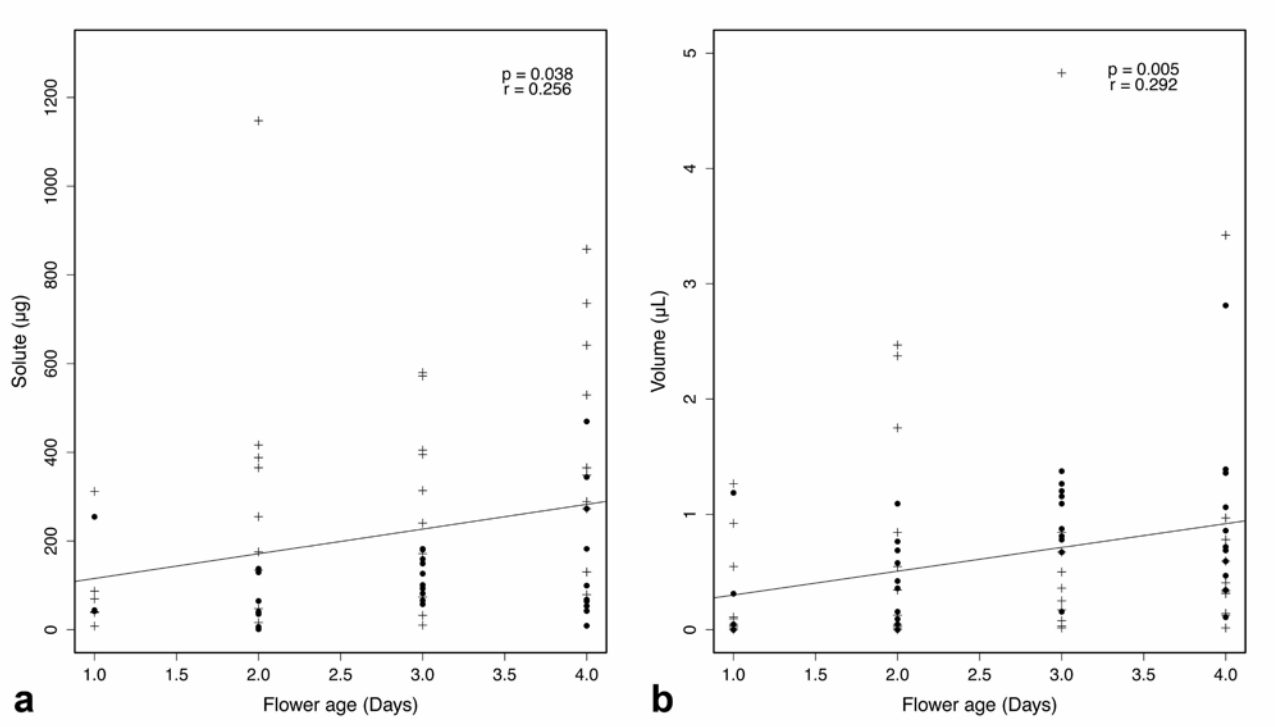


Figure 3.9 Correlations of nectar solute (a) and nectar volume (b) per flower of *V. vitis-idaea* with flower age (anthesis, days 1-4) for the combined data of 2010 (+) and 2011 (•).

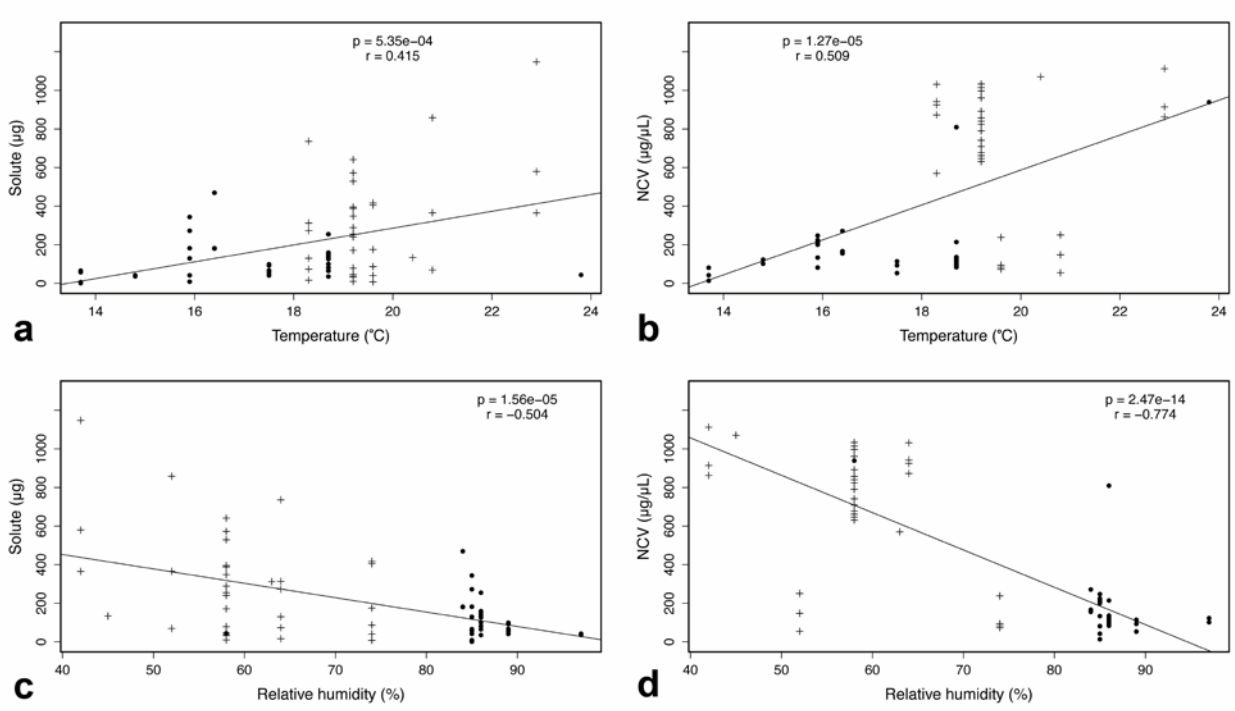


Figure 3.10 Correlations of nectar solute and nectar solute concentration by volume (NCV) with temperature and relative humidity for the flowers of *V. vitis-idaea* aged 1-4 days, after combining the data for 2010 (+) and 2011 (•).

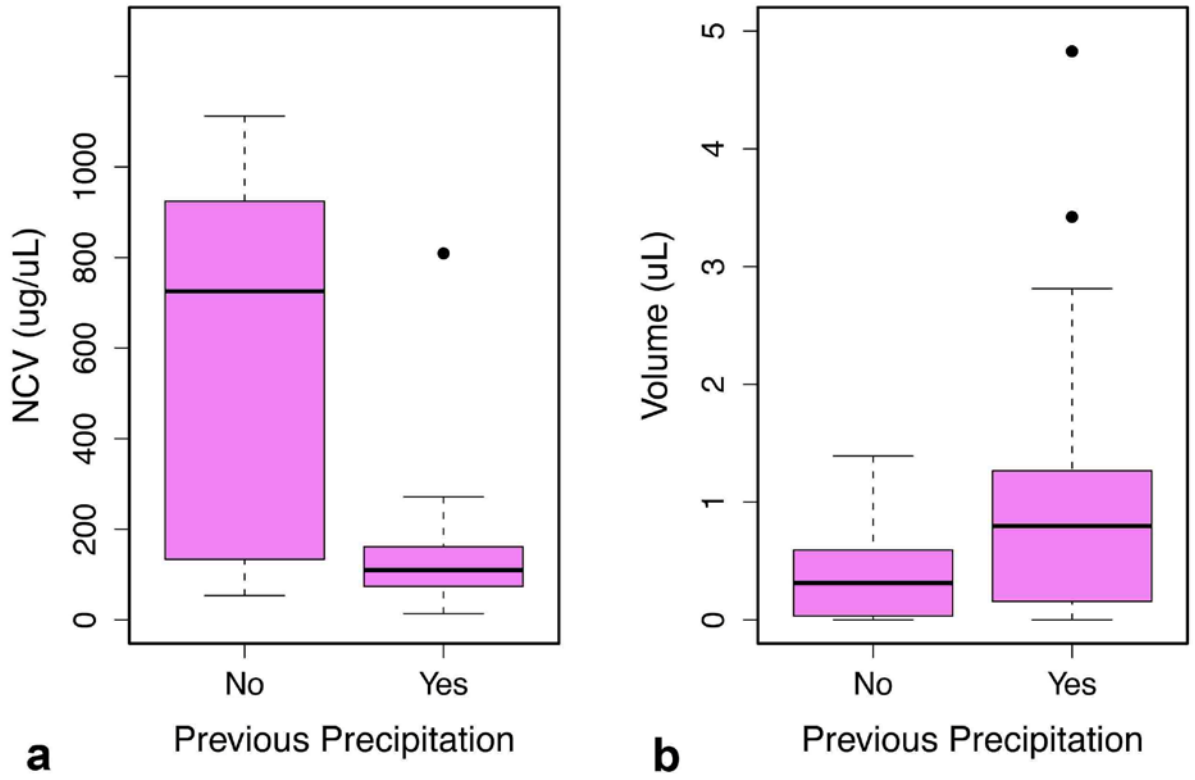


Figure 3.11 Values of nectar solute concentration by volume (NCV) and nectar volume from flowers of *V. vitis-idaea* aged 1-4 days from 2010 and 2011. Boxplots show data grouped by whether there had been precipitation on the previous day. There is a significant difference between groups for both NCV (W = 173, p-value = 1.053e-05) and nectar volume (W = 1261.5, p-value = 0.001074). The coloured boxes represent the upper and lower quartiles whereas the solid black line represents the median. Whiskers indicate the maximum and minimum values and outliers are shown when 1 ½ times the upper quartile.

3.4 Discussions and conclusions

3.4.1 Vascular supply to the nectary

This study's findings of many small traces of phloem entering the nectary tissue confirms previous findings on *Vaccinium* spp. by Frei (1955), Palser (1961) and Kartashova (1965). Frei (1955) reported phloem alone in *V. myrtillus* and *V. vitis-idaea*, and Kartashova (1965) stated the same for *V. myrtillus* and *V. uliginosum*. Although Palser (1961) did not state that only phloem was present, a later report on the genus *Erica* (Palser and Murty 1967) stated that for species with a nectary present, bundles supplying the nectary were "small and frequently entirely provascular". In this case the term "provascular" might be taken to refer to phloem, as at the time it may not have been known that xylem and phloem could be found separately from one another.

In the sections of flowers from the Debden field site, only phloem entered the nectary for *V. myrtilloides* and *V. vitis-idaea*, which confirms Frei's (1955) record for the latter species from Switzerland. These traces of phloem entering the nectary tissue appear to branch from major vascular bundles destined to the style and filament bases, in accordance with Frei (1955). These observations place the two *Vaccinium* species studied here within the majority of angiosperms surveyed by Frei (1995) and Kartashova (1965). From the 366 species examined, 175 (47.8%) had floral nectaries that receive phloem alone, 145 (39.6%) lacked any direct vascularization, whereas the minority (46; 12.6%) received both xylem and phloem (see Fahn 1979).

3.4.2 Nectary stomata

Nectary stomata on *Vaccinium* species have been previously reported by Feldhofen (1933) and Palser (1961). Palser (1961) noted that stomata "varied from a few scattered ones to many", and their occurrence on the nectary did seem restricted to specific areas for some species. In our study, nectary stomata in *Vaccinium* spp. were solitary and scattered over the surface of the nectary and stomatal pores were normally open, similar to observations by Palser (1961).

In other Ericaceae, Palser and Murty (1967) found stomata on all the *Erica* species that possessed a nectary. The stomata were described most often as common, which we take to mean in contact, in the different *Erica* species, although scattered in some. Palser *et al.* (1991) reported nectary stomata in all 177 *Rhododendron* species examined, some with large groups of connected stomata and had stomata consisting of just a solitary guard cell; stomata in contact with one another on the nectary surface was not uncommon, and they were most often open. For species in

the ericaceaen subfamily Monotropoideae, floral nectaries existed either with, or without, stomata occurring in pairs (Wallace 1977).

The numbers of nectary stomata are generally not correlated with the volume of nectar secreted (Davis 2000), and it is thought that not all stomata secrete nectar (as reviewed by Nepi 2007). Although Bernardello (2007) suggests that the features of numbers of stomata and their position on the nectary may have taxonomic significance and thus should be more thoroughly reported, our study (admittedly, of only two species) would suggest that the number of stomata is not taxonomically useful between the species of *Vaccinium*, but may have another correlation, perhaps with genotype or the environmental conditions the flower developed under.

That these two species have similar quantities of stomata on their nectary surfaces, yet individual flowers of *V. myrtilloides* produced more nectar volume and solutes overall than those of *V. vitis-idaea* in both years studied, suggests that number of stomata is not useful to distinguish high nectar-yielding species of *Vaccinium* (see Davis 2000). However, the greater quantity of phloem entering the floral nectary of *V. myrtilloides* than *V. vitis-idaea* may be an anatomical trait to consider further in this regard, similar to the situation in many floral nectaries of the Brassicaceae (Davis *et al.* 1998).

3.4.3 Nectary sclerenchyma

To our knowledge, sclerenchyma cells within nectary tissue have not been noted previously. However it is not completely surprising that they were found forming in the nectaries of *Vaccinium* species as they are present, though in varying amounts, in the fruit of *V. corymbosum* (Gough 1983), *V. formosum, V. pallidum,* and *V. tenellum* (Yarbrough and Morrow 1947) and closely related *Gaylussacia* species (Eames and McDaniels 1925 in Yarbrough and Morrow 1947). By light microscopy, Allan-Wojtas *et al.* (2000) illustrated sclerenchyma, or stone cells as they called them, throughout the fruit of different cultivars of *V. corymbosum.* Gough (1983) investigated the development of stone cells over fruit life in *V. corymbosum* and found them to have secondary wall thickening and pitting even in the youngest fruits. The nectary is located atop the ovary in *Vaccinium* species, thus does not abscise with the corolla and stamens at the end of flowering, instead it becomes part of the forming fruit. Thus the presence of developing sclerenchyma in the ovary of *V. myrtilloides* and *V. vitis-idaea* is logical, though not reported in other descriptive work on flowers of *Vaccinium* (Frei 1955; Palser 1961) Palser *et al.* (1991) noted sclereids in the ovary tissue of 11 species of *Rhodondendron* but there was no

mention of whether or not they were seen in the nectary. The presence of sclerenchyma in the nectary of *Vaccinium* is likely incidental and connected with aspects of the developing fruit rather than the functioning of the nectary during flower life.

3.4.4 Comparisons of floral nectar secretion among *Vaccinium* species

Despite its 740 species (Judd *et al.* 2008), floral nectar secretion in *Vaccinium* has not been widely investigated. Jacquemart (1992) recorded floral nectar production in *V. myrtillus*, *V. uliginosum* and *V. vitis-idaea* from Belgium. However, out of 100 flowers of *V. vitis-idaea* sampled, she was able to obtain nectar from only six of them. The range of nectar volume from those six flowers was 0 - 3.00 µL, which corresponds well to our *V. vitis-idaea* flowers (range 0-4.83 µL). In a later paper, Jacquemart (1993) reported that the mean nectar quantity per flower is 0.6 ± 0.3 µL for *V. vitis-idaea*, close to our mean of 0.702 ± 0.824 µL over the two years of sampling.

Contrasting *V. vitis-idaea* to the other two *Vaccinium* species she examined, Jacquemart (1992, 1993) reported relatively high nectar volumes for *V. myrtillus* and *V. uliginosum* with 0-23.38 µL (mean 3.4 ± 4.6 µL) and 0-9.37 µL (mean 1.6 ± 2.2 µL), respectively. Cane and Schiffhauer (1997) reported the mean volume of nectar for cultivar "Stevens" of *V. macrocarpon* to be 1.4 ± 0.5 µL. The mean found in these three *Vaccinium* species is higher than both our values for *V. vitis-idaea* (above) and for that of *V. myrtilloides*: mean 1.073 ± 1.099 µL; range 0-4.69 µL. In turn, the mean nectar volume per flower we found for *V. myrtilloides* is much higher than that of Reader (1977) of 0.30 ± 0.11 µL from Ontario, but closer to our median volume of 0.67 µL. For *V. angustifolium*, Wood (1961) recorded an average nectar volume of 141.8 ± 18.4 µL per 50 centrifuged flowers, or ~2.8 µL per flower, higher than our mean values for both *V. myrtilloides* and *V. vitis-idaea* but well within their ranges. Wood (1961) also stated the highest volume recorded was 835 µL per 50 flowers or ~16.7 µL per flower placing the value as the second highest volume recorded behind *V. myrtillus* (Jacquemart 1992, 1993).

Jacquemart (1992, 1993) was unable to obtain any % concentration readings for nectar from flowers of *V. vitis-idaea*, but reported means of 18.8 ± 9.7% for *V. myrtillus* and 16.4 ± 4.9% for *V. uliginosum*, respectively. These values are low and remarkably consistent compared to the values we obtained: raw % concentrations (before conversion into NCV) ranged from 0-79% for *V. vitis-idaea*, and 14-82% for *V. myrtilloides*. Wood (1961) measured concentrations of 3-58.5 % for *V. angustifolium* and Kartashova (1965) reported a single value (50%) for *V.*

myrtillus. On the other hand, Reader (1997) recorded a high value of 87.9 ± 6.8 (% dissolved solids in nectar) for 1-3 day old flowers of *V. myrtilloides* that opened in stable lab conditions (n was ≥ 30 flowers, 20-23°C, 50% RH). His high value might be partly explained by the lack of rain or dew for flowers indoors rather than in the elements. Cane and Schiffhauer (1997) found a value of 25 ± 4% for their cultivar of *V. macrocarpon*. Interestingly, varieties of cranberry (presumably all *Vaccinium macrocarpon***)** grown in Massachusetts gave a sugar concentration of 38-62%, however these values were obtained via extraction from the foregut (honey sac) of honey bees foraging on cranberry flowers, rather than direct measurements of floral nectar (Shaw *et al*. 1956).

The total nectar solutes per flower for the two species recorded by Jacquemart (1992,1993) were 45.8 ± 7.2 µg (*V. myrtillus*) and 27.0 ± 11.1 µg (*V. uliginosum*), much lower than our reported mean value for *V. vitis-idaea*: 212.56 ± 210.19 µg. Reader (1977) found a value of 260 µg for *V. myrtilloides*, lower than our mean of 872.76 ± 808.04 µg. For *V. macrocarpon*, Cane and Schiffhauer (1997) reported a mean of 301 ± 84 µg for the lowest cultivar ('Early Black') to 411 ± 89 µg for the highest cultivar ('Stevens'). Wood (1961) stated a mean of 27.3 ± 0.5 mg per 50 flowers of *V. angustifolium*, ~ 546 µg per flower for nectar collected through flower centrifuge. Rajotte and Roberts (1979) found the peak nectar solute of *V. corymbosum* to be 4000 µg for the cultivar 'Jersey' and 5000 µg for the cultivar 'Coville'; they used a technique of washing the flowers rather than collecting nectar with microcapillaries.

Initially the quantity of nectar solute per flower was hypothesized to increase with flower age. Although nectar solute production per flower was sometimes significantly different from others, neither species appears to have a consistent pattern of nectar secretion in relation to flower age that held for both years. One exception was the positive trend over both years in nectar solute with flower age to Day 4 flowers of *V. vitis-idaea*. In both cultivars of *V. corymbosum*, Rajotte and Roberts (1979) showed significant differences in mean solute values among flowers of different ages; they suggest there is an increase in total nectar solutes as the flower ages, followed by a reabsorption in the 8^{th} and 9^{th} day for one of the cultivars. However, the details of their statistical test are not given and testing the mean of the data collected, as they reported, would not account for the true variability of the data. There is also an increase in mean sugar content reported for *V. angustifolium* with flower age (Wood 1961). Both the nectar volume and mean

weight of nectar increased with age until about 10 days after anthesis, after which time flowers had wilted, or there was a decrease in nectar sugar.

It is important to note that we did not explore the full range of nectar secretion with flower age for *V. myrtilloides* and *V. vitis-idaea*: flowers excluded from insects by bagging (and hence were possibly unpollinated) seemed to last longer than their open counterparts. Thus floral nectar might have been present up until Day 8 of flower life as in *V. corymbosum* (Rajotte and Roberts 1979) or perhaps even until Day 10 of flowering as in *V. angustifolium* (Wood 1961). From personal observation, relatively shady conditions seemed to prolong anthesis but most flowers of *V. myrtilloides* and *V. vitis-idaea* had wilted by Day 8. Regardless, the question of whether uncollected nectar is reabsorbed within the flower life span was not answered for these two species.

3.4.4.1 Nectar extraction and nectar-less flowers

As described earlier, Jacquemart (1992, 1993) was unable to obtain nectar solute concentrations from flowers of *V. vitis-idaea*. The low rate of success she experienced in nectar sampling may have been due to sampling equipment. She used 10 µL microcapillaries, whereas ours (1 µL) likely provided a smaller diameter and the ability to have a greater capillary action. If low volumes of nectar with high NCV (from low RH conditions) were present, the viscosity of the nectar made extraction difficult and required multiple micropipettes to be used in the case of our study, to ensure maximum extraction. It is unlikely Jacquemart (1992, 1993) would have been able to extract nectar under those conditions, using the large micropipettes; indeed, she mentioned difficulties with nectar viscosity (1992).

There were flowers of *V. myrtilloides* and *V. vitis-idaea* from which we could not extract nectar, though more were recorded in *V. vitis-idaea*, and primarily in the first day of anthesis. It is hypothesized that *V. vitis-idaea* does not always start secretion of nectar on the first day of anthesis. In 2010, nine out of twelve flowers of *V. vitis-idaea* measured for nectar on the day of anthesis had a measureable amount of nectar, however three of those were too small a quantity to be carried forward for NCV measurements. In 2011, only three out of eleven flowers produced nectar measurable on the first day of anthesis. These flowers for which a nectar volume could not be obtained are noted in the Results Section 3.3.2.2, and are tentatively called nectar-less flowers. It is not known, however, whether these were truly nectar-less flowers, or whether it was the inability of the size of microcapillary to extract nectar at high viscosities and/or low volumes.

Thus the problems with nectar extraction reported by Jacquemart (1992, 1993) may have also been factors in our study to a lesser extent.

The technique of submerging flowers in a known quantity of water and using spectrophotometrical means to determine the nectar solute for *V. corymbosum* (Rajotte and Roberts 1979) resulted in a nectar solute peak that was almost 5 times the average solute we obtained in our study of *V. myrtilloides*. The maximum value obtained for *V. myrtilloides* over both years (3684.1 µg) is still below that of the mean values reported for both cultivars of *V. corymbosum* at their floral stage of maximum nectar production. Rajotte and Roberts (1979) did not list the exact mean, nor a standard deviation or range of nectar solute values obtained, but did state that there was "extreme variability of sugar content among flowers of the same age and seasonal disposition." Thus, whether the high values reported by Rajotte and Roberts (1979) reflect a difference between species or measuring techniques is not certain. Other work with small flowered ericacean species (*Acrotriche patula*) in which Petit *et al.* (2011) first extracted nectar with microcapillary tubes from flowers then subsequently washed them, suggests that microcapillaries fail to extract the majority of the nectar present in small flowers. The values of nectar solutes reported with washing flowers of *V. corymbosum* were high - higher than any other *Vaccinium* species reported - however it was also reported that there was an extreme variability of nectar solutes between flowers (Rajotte and Roberts 1979). This generalization could also be applied to our data, however, it is unfortunate that there is not enough information provided to make comparisons of variability.

3.4.4.2 Environmental factors and their effect on nectar production

Photosynthesis can be an important source of nectar for nectaries that photosynthesize (Pacini *et al*. 2003). Although the floral nectaries are green (Fig. 2.1a), it is unknown whether *Vaccinium* species rely on nectary photosynthesis, and to what extent. Nor was the factor of light exposure to individual plants sampled for nectar measured in this field study. However, the prediction that environmental factors such as temperature, RH and previous precipitation play an important role in determining the total nectar solute secreted by flowers would seem to be true to various extents. Lack of controls on any of these environmental factors makes for a difficult interpretation of the results, however.

Clearly rain affected the volume and NCV of the nectar in predictable ways: the larger volumes and lower NCV values for *Vaccinium* nectar likely reflect water vapour condensation

inside the corolla, or addition of water directly through rain deposition, despite the protection that the pendulous nature of the flowers might have provided. It must be mentioned that the bag surrounding the flowers may have contributed by holding more moisture around the flowers of the inflorescence than would be found naturally. Wood (1961) also mentioned an increase in volume and decrease in nectar concentration of *V. angustifolium* following days of precipitation, when plants were kept under large screened cages.

Less clear is the relationship of nectar with air temperature and RH. For *V. myrtilloides*, although there was no correlation between RH and nectar solute produced per flower, RH was correlated with nectar volume. However, this correlation was driven by the high RH only present after rain experienced in both years; and when these data points are not considered, the correlation is no longer significant ($r = 0.060$, $p = 0.605$). The effect of RH on *V. vitis-idaea* was more pronounced; high RH (not always tied with PP) seemed to result in low NCV values and lower nectar solute quantities per flower, likely due to reduced evaporation of the water component of nectar under these conditions (Shuel 1952; Corbet *et al.* 1979). Corbet *et al.* (1979) found the RH over the period of a day to be highly negatively correlated with the concentration of sugar in nectar during the day. The fact that this correlation is less strong in our case likely reflects the procedure of once a day measurements scattered over different dates that do not take into account previous RH and temperature history.

The temperature used for the correlations with nectar was the temperature at the time that nectar measurements were made (10:30 a.m. Central Standard Time). Other studies (Jakobsen and Kristjansson 1994; Kropácová and Haslbachová 1970 in Jakobsen and Kristjansson 1994) have found relationships between the previous night's low temperature and nectar production the next day (perhaps due to decreased night respiration) in white clover (*Trifolium repens*), however a night temperature effect was not found for the closely related *Trifolium pratense* (Shuel 1952). As we often did not leave the weatherstation out at night, the precise low is not available. At best, the temperature at 10:30 a.m. might indicate the relative low of the previous night. The fact that a correlation is only seen for *V. myrtilloides* might mean that the low temperatures experienced by *V. myrtilloides* affect this species more than the low temperatures experienced by *V. vitis-idaea*, or that the apparent temperature effect is actually due to another factor, such as RH (which is strongly correlated with temperature) shown to correlate with nectar volume for *V. myrtilloides*. Higher nectar volumes were found in plants of *V. myrtillus* in Russia (Kartashova 1965) when the

temperature was between 18 – 25°C and conditions were cloudy (3.56 ± 0.29 mg of nectar fluid per flower) versus 25 – 30°C with sunny conditions (0.85 ± 0.02 mg of nectar fluid per flower), although different conditions of sunlight confuses the matter. Similar favourable or unfavourable temperatures and conditions produced different amounts of nectar solutes (3.8 ± 0.2 mg and 0.14 ± 0.01 mg, respectively), however it is not certain whether these quantities are pooled amounts nor what technique was used to obtain the data.

Jacquemart (1992) studied the production pattern of nectar volume for *V. myrtillus* and *V. uliginosum* by the date sampled, indicating that the flower's nectar peak corresponded with the plant's peak period of flowering. She, however, cautioned that the results are too few and too variable, unable to take into account parameters of temperature and relative humidity. Rajotte and Roberts (1979) also reported an increase in the mean nectar levels of caged flowers of *V. corymbosum* over the flowering season. That *V. myrtilloides* in this study shows a pattern of increasing nectar solute with the season is not conclusively shown, although the nectar volume does show a positive correlation with date in individual years as well as with combined data.

The small volume of nectar available to insect visitors of *Vaccinium* flowers and other Ericaceae, though comparatively inexpensive for the plant to produce, is contrary to the majority of angiosperm taxa exhibiting the buzz pollination syndrome. The role that floral nectaries play in a system with poricidal anthers has not been investigated. Insects visiting flowers solely for nectar are unlikely to contribute to the pollination of the flower when the pollen is not in a position to make contact with the insect. Plant species with poricidal anthers generally do not possess a floral nectary in addition to offering a pollen reward; that is the point of a specialized system (Buchmann 1983). The only other exception is in some members of the family Melastomataceae where nectaries seem to have developed in a few species, but their poricidal anthers have been retained (Varassin *et al.* 2008). Among the Ericaceae however, both nectaries and poricidal anthers are common. This combination could be beneficial to ericacean plants if bees are able to obtain both the nectar they need for energy and to pack pollen onto corbiculae (for bees that have corbiculae), while gathering pollen, inadvertently pollinating the flowers. Offering both nectar and pollen may keep certain bees loyal to a single flower species for a period of time, increasing the chances that the pollen carried on their bodies will be of that particular species, and thus increase the chances that compatible pollen will successfully reach a flower's stigma.

4 Flower-visiting insects and their effectiveness as pollinators of *Vaccinium myrtilloides* and *V. vitis-idaea*

4.1 Introduction

Vaccinium species possess poricidal anthers, a mechanism thought to limit pollen release to those insects with the ability to buzz pollinate, or sonicate flowers. There are various reports in the literature as to which insect species have this ability; so far it has only been reported in bees and one fly which acts as a bee mimic. Among bee families there are many reports of buzzing ability in the Apidae (notably *Bombus*) but one of the world's most important pollinators, the honeybee (*Apis mellifera*), does not appear to be able to sonicate flowers (Buchmann 1983) though there is a contradictory report of sonication by the Africanized honeybee *Apis mellifera scutellata* in Brazil (Renner 1989). Other families such as Colletidae and Halictidae are reported to contain species that can buzz (Buchmann and Hurley 1978; Thorp 2000), whereas Megachilidae for the most part do not, though there are reports of buzzing in *Megachile* (in Thorp 2000). There is uncertainty as to buzzing ability for bees in Andrenidae (Cane and Buchmann 1989; buzz-milking in *Proandrena*, Thorp 2000) though Javorek et al. (2002, citing Sampson 1993) names *Andrena* as one of the genera that "routinely sonicate flowers".

During buzz pollination, pollen forcibly ejects onto the bee's body, leading to accumulation on specific body sites that may later come into contact with the stigma of another flower. Though the buzz pollination syndrome indicates that *Vaccinium* flowers will be most effectively pollinated by bees that can sonicate, the flowers also possess nectaries that serve as an alternative, or additional, reward to insect visitors. The role of nectaries in a buzz pollination system is unknown as the combination is rare (known from one other family, the Melastomataceae, in which most members do not have nectaries, but nectariferous Melastomataceae usually retain their poricidal anthers [Varassin *et al.* 2008]) and it is presumed that attracted alternative visitors may also act as pollinators.

Previous studies have reported flower visitors to both *Vaccinium myrtilloides* (Table 4.1), and *V. vitis-idaea* (Table 4.2) with various degrees of detail. The most comprehensive investigation of *V. myrtilloides* was done by Usui (1994), however there was no distinction made between visits to *V. myrtilloides* and intertwining *V. angustifolium*. Usui (1994) reported a total of 53 species of insect visitors – primarily bees, but also wasps, flies, and a few butterflies and

beetles. Potential insect pollinators were also surveyed for *V. vitis-idaea* (Davis *et al*. 2003; Jacquemart 1993) and reports include bees, wasps, hoverflies and a butterfly visitor.

The objective of this portion of the project was to observe and identify insect visitors to flowers of both *Vaccinium* species in a natural setting. Furthermore, the single insect visitation method was used to determine if floral visitation by different insect taxa resulted in pollination of a flower, thus allowing ranking of insects by their ability to serve as pollinators, and potentially by their action of flower sonication.

Table 4.1 Previous records of insect visitors to flowers of *Vaccinium myrtilloides*, all from Ontario, Canada.

Order/Family	Insect species	Author
Order Hymenoptera		
Andrenidae	*Andrena* spp.	Reader (1977)
	Andrena forbesii, A. w-scripta, A. bradleyi, A. sigmundii, A. vicina	Mohr and Kevan (1987)*
	Andrena bradleyi, A. regularis, A. rufosignator, A. vicina, (10 additional species)+	Usui (1994)*
Apidae	*Apis mellifera, Bombus* spp.	Reader (1977)
	Bombus frigidus couperi, B. terricola, B. vagans, Psithyrus ashtoni	Mohr and Kevan (1987)*
	Bombus ternarius, B. terricola, B. vagans, (4 additional species)+, *Psithyrus* sp. (1)	Usui (1994)*
	Anthophora sp. (1), *Nomada* spp. (2)	Usui (1994)*
Colletidae	*Colletes validus*	Mohr and Kevan (1987)*
	Colletes validus (1 additional species)+	Usui (1994)*
Halictidae	*Evylaeus rufitarsis, E. comagenensis, Halictus rubicundus, Lasioglossum acuminatum*	Mohr and Kevan (1987)*
	Halictus confusus, H. rubicundus	Usui (1994)*
	Lasioglossum sp. (*Evylaeus quebecensis, Dialictus pilosus pilosus,* 8 additional species)+, *Sphecodes* sp. (1)	
Megachilidae	*Hoplitis* spp. (2), *Megachile* spp. (3), *Osmia integra, O. tersula* (7 additional species)+	Usui (1994)*
Vespidae	*Dolichovespula arenaria, D. norvegicolides, D. maculata*	Usui (1994)*
Order Diptera		
Bombyliidae	*Bombylius* spp. (2)	Usui (1994)*
Syrphidae	*Blera confusa, Eristalis dimidiata, Eupeodes* sp., *Sphaerophoria philanthus*	Usui (1994)*
Other Diptera	*Crinurina* sp., *Cryptomeigenia* sp., *Epalpus signiferus, Gonia* sp., *Lauxania cylindricornis, Lypha* sp., *Myopa* sp., *Myospila meditabunda, Scathophaga furcata, Tachinomyia nigricans, Thecophora* sp., *Zodion* sp.	Usui (1994)*
Order Coleoptera	*Cicindela longilabris, Ctenicera morula, Trichiotinus assimilis*	Usui (1994)*
Order Lepidoptera	Family Lycaenidae and Sphingidae	Usui (1994)*

+ Includes bee species noted as most frequent followed by additional number of species recorded.

* Authors reported insects as visitors to lowbush blueberry (i.e. a combination of *Vaccinium myrtilloides* and *V. angustifolium*).

Table 4.2 Previous records of insect visitors to flowers of *Vaccinium vitis-idaea*.

Order/Family	Insect species	Author	Location
Order Hymenoptera			
Andrenidae	*Andrena lapponica*	Ritchie (1955)*	Great Britain
	Andrena spp.	Jacquemart (1993)	Belgium
	Andrena spp.	Davis *et al*. (2003)	USA, Alaska
Apidae	*Apis mellifera, Bombus occidentalis, B sandersonii, B. flavifrons flavifrons, B. fridigus, B. sylvicola, Psithyrus* sp.	Davis *et al*. (2003)	USA, Alaska
	Apis mellifera, Bombus lucorum, Megabombus pascuorum floralis, Psithyrus bohemicus, Psithyrus sylvestris, Pyrobombus pratorum	Jacquemart (1993)	Belgium
	Bombus lapponicus, B. terrestris, Nomada ruficornis	Willis and Burkill (1903)*	Great Britain
	Bombus hortorum, B. jonellus, B. lapidarius, B. muscorum, B. proteus	Knuth (1908)*	Germany
	Bombus agrorum, B. hortorum, B. lapponicus, B. lucorum	Ritchie (1955)*	Great Britain
	Bombus jonellus, B. lucorum, B. pratorum	Haslerud (1974)*	Norway
	Bombus alpinus, B. balteatus, B. hypnorum, B. jonellus, B. lapponicus, B. pascuorum, B. pratorum	Lundberg (1974)*	Sweden
	Bombus hortorum, B. hypnorum, B. jonellus, B. lucorum, B. pascuorum, B. practorum, B. soroeensis	Terräs (1985)*	Finland
	Bombus cryptarum, B. lucorum, B. magnus, Megabombus humilis, Pyrobombus hypnorum, P. pratorum	Rasmont (1988)*	France and Belgium
Megachilidae	*Osmia nigriventris*	Knuth (1908)*	Germany
Pteromalidae	pteromalid wasps	Davis *et al*. (2003)	USA, Alaska
Vespidae	*Dolichovespula arenaria, D. norvegicoides*	Davis *et al*. (2003)	USA, Alaska
	Vespula norvegica, V. sylvestris	Ritchie (1955)*	Great Britain
Order Diptera			
Empididae	*Empis lucida, E. livida*	Willis and Burkill (1903)*	Great Britian
Syrphidae	*Melangyna* sp., *Syrphus* sp.	Davis *et al*. (2003)	USA, Alaska
	Eristalis nemorum, Rhingia campestris, Sericomyia lappona, Sericomyia silentis	Jacquemart (1993)	Belgium
	Melanostoma quadrimaculatum	Willis and Burkill (1903)*	Great Britain
	Sericomyia lappona	Haslerud (1974)*	Norway
Order Lepidoptera			
Geometridae	*Rheumaptera* sp.	Davis *et al*. (2003)	USA, Alaska
?	*Triphaena* sp. current name not found.	Willis and Burkill (1903)*	Great Britain

*species list by authors compiled by, and copied from, Jacquemart (1993).

4.2 Materials and methods

4.2.1 Study site description

One field site located in central Saskatchewan near highway 55 between Debden and Big River at N 53.69193 W 106.96706 was used during the field seasons of 2010 and 2011. Within this study area, twelve patches of plants (*V. myrtilloides* Michx., *V. vitis-idaea* L.) were selected and one transect of twenty-five paces was staked out within each patch (Fig 4.1 a, b). Transects were in different areas of sun and shade and ran with gradients such as slope. Some patches contained both species, whereas others were predominantly *V. myrtilloides* or *V. vitis-idaea*. Transects were used for insect survey data collection, and the surrounding patches of flowers were used for nectar sampling and experiments involving single insect visitation.

Jack pine trees (*Pinus banksiana*) dominated the site, though there were open treeless areas also. During the bloom of *V. myrtilloides* and *V. vitis-idaea,* there were many other shrubs and herbs flowering. Proceeding and during *V. myrtilloides* bloom, bearberry (*Arctostaphlos uva-ursi*) attracted many visitors. Other angiosperms in the area noted as blooming concurrently with either of the *Vaccinium* species were: wild rose (*Rosa*), wild legume (*Lathyrus ochroleucus*), purple violets (*Viola* spp.), strawberries (*Fragaria* spp.), raspberries and dwarf raspberry (*Rubus* spp.), sarsaparilla (*Aralia nudicaulis*), prairie everlasting (*Antennaria neglecta*), star flower (*Trientalis borealis*), bunchberry (*Cornus canadensis*), false solomon's seal (*Smilacina stellata*), wintergreen (*Pyrola virens*), labrador tea (*Ledum groenlandicum*), honeysuckle (*Lonicera glaucescens*) and green alder (*Alnus crispa*).

A Wireless Vantage Pro2^{TM}Plus weather station (Davis Instruments, USA), equipped with an anemometer (speed and direction), UV, solar radiation, temperature and humidity sensors, and a rain collector, was set up at the field site (Fig 4.1 e).

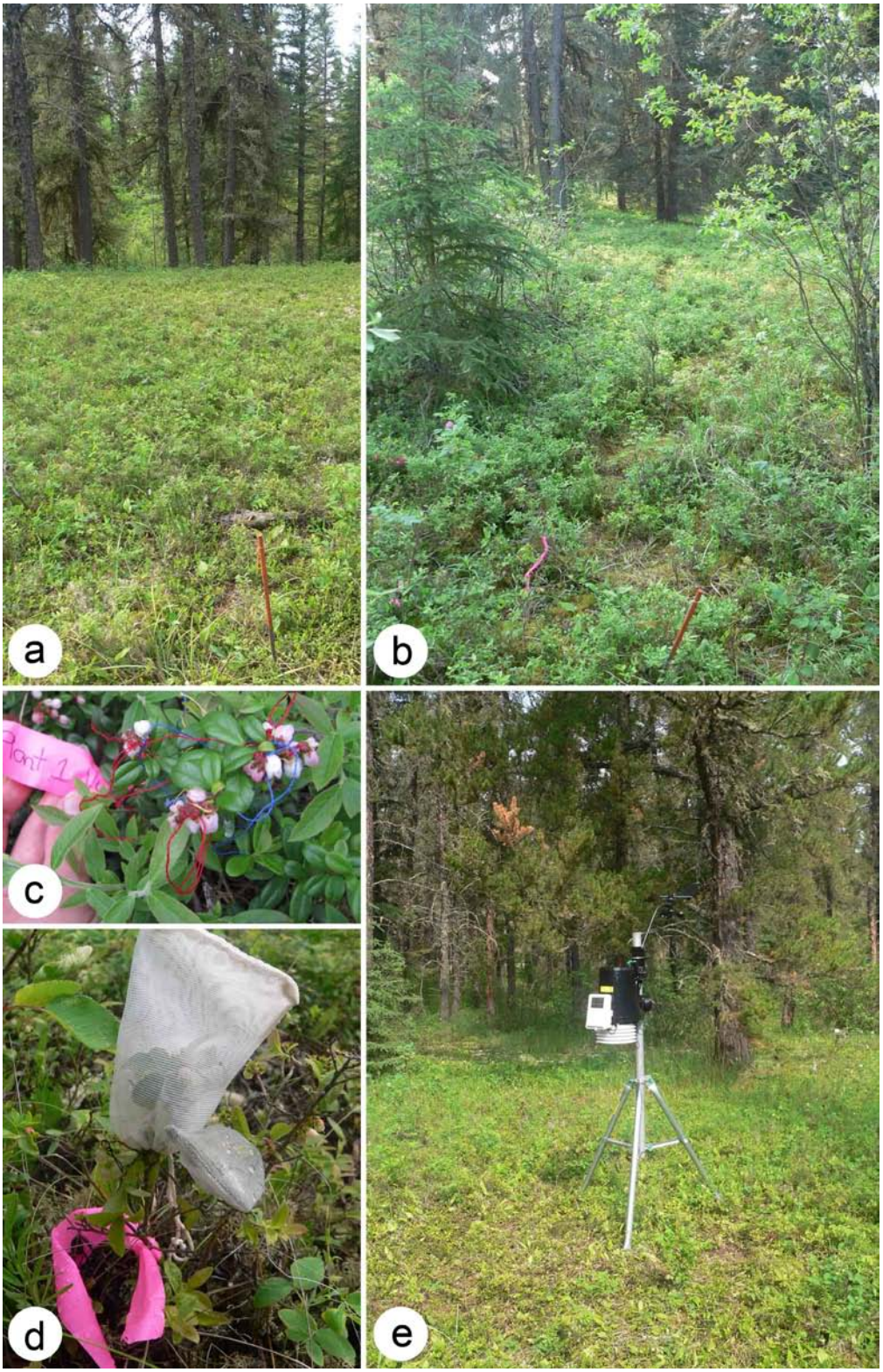


Figure 4.1 Study site used for field seasons 2010 and 2011. a) Patch 5: a fairly open patch containing both *V. myrtilloides* and *V. vitis-idaea*. b) Patch 4: a less open patch through shrubs containing primarily *V. myrtilloides*. c) Embroidery thread tagging system (nectar sample example shown here). d) Both sizes of bags employed on a plant of *V. myrtilloides*. e) Davis Instruments weather station used at the site.

4.2.2 Survey of insect visitors to flowers

Insect surveys sampled general insect visitor presence for the two study species, *V. myrtilloides* and *V. vitis-idaea*. They were taken roughly every other day on days without rain, though not necessarily both species of plant were surveyed every other day. A new transect was used for sampling each survey day with a few repeats of transects that were not consecutive. Designated sampling times were 09:00, 12:00, 15:00 and 18:00; earlier times of 06:00 and later times of 21:00 were initially attempted, however insects were not found to be visiting flowers at those times and so they were not continued. At survey times, a transect was walked slowly, from one end to the other, for a period of five minutes. During walking, all insect visitors to flowers of *V. myrtilloides* and/or *V. vitis-idaea* in that patch of flowers were recorded and capture of visitors was attempted (by my field assistant) for later identification. For *V. myrtilloides*, there were 6 survey days in 2010 and 7 days in 2011. For *V. vitis-idaea*, there were 4 survey days in 2010 and 6 days in 2011.

Insects were killed by confinement to jars containing ethyl acetate, and subsequently pinned. They were identified as closely as possible to their nearest known designation using keys of Packer et al. (2007) and from "The key to the genera of Syrphidae of North America North of Mexico" (Miranda *et al.*, in preparation). Identification of all Syrphidae to species was done by Dr. Jeff Skevington of the Canadian National Collection (CNC) of Insects, Arachnids, and Nematodes. All non-*Apis* bees were confirmed to genus and identified to species by Dr. Cory Sheffield at York University, Ontario. All Syrphidae are housed at the CNC in Ottawa, ON and all bees at the Royal Saskatchewan Museum collection in Regina, SK. Each insect visitor in both the insect survey and single insect visitations has its own number and corresponding information available to be added to a database.

Graphs of the flower-visiting insects surveyed for each of the *Vaccinium* species were produced with Microsoft Excel.

4.2.3 Single insect visitation to virgin flowers

4.2.3.1 Field collection

Single insect visitation (SIV) experiments where performed using virgin flowers of *V. myrtilloides* from June 1 to June 17 in 2010 and from May 30 to June 28 in 2011. Experiments

with *V. vitis-idaea* were not successful in 2010 but were performed from June 22 to June 29 in 2011.

Flower buds were selected if they showed signs of nearing anthesis (large, sometimes swelling). First, each bud was tagged with a single strand of coloured embroidery thread so that selected flowers would be distinguishable from other flowers on the branch (Fig 4.1 c). A bag 5 cm by 4 cm, or 10 cm by 8 cm made from grey nylon mesh was placed over the flower and the drawstring at the bottom tightened with a single knot (Fig 4.1 d). Mesh holes measured 0.25 mm by 0.6 mm (Langenberger 2000) allowing for the potential travel of windborne pollen through the bag. All bags were checked the morning of the following day and any with open flowers were available to be used for SIVs later that day. The bags were carefully removed from flowers, after which the observers would sit back at a distance of at least a metre from the flower, while still maintaining a clear view. When an insect landed on the previously unvisited flower, the timer was started and the insect's behavior was noted as far as possible (i.e., *Bombus* buzzing was distinct, whereas if *Andrena* buzzed it was not noticeable). When the insect left the flower, the timer was stopped. One person was poised with the insect net, and as soon as the insect flew clear from the SIV flower, capture was attempted. Insects were killed and identified as in section 4.2.2. The bag was placed carefully back over the SIV flower and left for 72 h to allow pollen tubes to grow to the base of the style. At that time, the bags were then carefully removed and the flower was cut from the plant. The corolla and androecium were removed first and then the stigma was cut off onto a fresh glass microscope slide. Fuchsin gel (see Javorek 2002, adapted from Beattie 1971) was placed on the stigma and a coverslip applied. The remainder of the flower was placed in a beem capsule filled roughly halfway with 3:1 ethanol: glacial acetic acid fixative for later examination of pollen tubes within the style using fluorescence microscopy (see below).

Flowers were also collected for two different types of controls to the SIVs. Control 1 flowers were collected to test for the possibility that we the observers inadvertently pollinated the flower with the removal/reapplication of the bag. Thus in a Control 1 flower, the flower was unbagged to simulate treatment then rebagged without allowing an insect to visit; often failed SIV flowers where no insect had visited, were used as Control 1 flowers. Control 2 flowers tested for autogamous self-pollination/wind pollination, thus these flowers were bagged before anthesis and were not touched until their collection on the third day of anthesis. The technique for harvesting the styles and stigmas was the same as described for SIVs.

4.2.3.2 Quantification of pollen tetrads per stigma

The Fuchsin gel stained pollen grains (tetrads) a deep pink. The same individual counted the pollen from all stigmas in 2010. Two people counted each slide in 2011 until a consensus was reached. For a number of stigmas, further dissection of the stigma was needed to determine the final quantity of pollen tetrads. Tetrads appeared to be intact groups of four pollen grains and thus the final quantity of pollen grains per stigma was the number of tetrads counted multiplied by four.

4.2.3.3 Quantification of pollen tubes per style

Collected styles were removed from 3:1 ethanol: glacial acetic acid after 24 h, rinsed with and then placed in 70% ethanol. At least 24 h before observation, the ethanol was replaced with 1% aqueous aniline blue to stain the callose plugs in pollen tubes evident by fluorescence (Martin 1959). The styles of each species varied in length, therefore a count at 40X magnification was done at the middle point of the style, and near the basal end. A count at the apical end did not reveal all the tubes found at the mid point. The epidermis of the style auto-fluoresced and blocked pollen tubes from view in the style squash. In 2011, removal of the epidermis was attempted by scraping with a razor blade, then peeling with insect pins and fine forceps. This procedure greatly improved the ease of counting when successful. Large numbers of pollen tubes were counted several times for each style with the aid of a hand counter.

4.2.3.4 Statistical analysis

Visitors to SIV flowers were grouped based on their taxonomy, either to genus or family. This designation was partly for convenience and partly due to the known ability of some groups to buzz flowers (*Bombus*) whereas others lack this behaviour (*Apis mellifera,* Syrphidae). The categories of *Andrena*, *Apis*, *Bombus* and Syrphidae all had multiple samples (for at least one of the flower species), whereas *Coelioxys*, *Colletes,* Halictidae, and *Osmia* only had single samples. The data was assessed by the Shirpo-Wilks test for normality, as well as visually by histograms and qqplots and found not to be normally distributed. Thus to test whether there was a significant difference between SIVs and Controls, the Kruskal-Wallis rank sum test was used separately on both quantities of pollen grains per stigma and pollen tubes per style. To determine which groups differed from one another, a post hoc nonparametric pairwise analysis was run with a

conservative Bonferroni correction. The results of the analysis are not defining for the groups with small sample sizes (groups with less than 10 visits) and are interpreted thusly.

The relationship between insect duration on flowers and number of grains deposited on stigmas and number of pollen tubes at the base of the style was fitted with a linear model (regression) in R. The same method was applied for duration of visitation by *Bombus* spp., those that audibly sonicated flowers, and those that did not. All boxplots and linear graphs were produced with The R Project for statistical computing (R Core Development Team, 2012).

4.3 Results

4.3.1 Survey of insect visitors to flowers

When all flower-visiting insects identified to species are considered overall, visitors to flowers of both species were primarily from Orders Hymenoptera and Diptera, with representatives from 5 families of bees, and Vespidae, as well as many Syrphidae (Table 4.3). One specimen from Order Lepidoptera (like Fig 4.3 h) was found on *V. vitis-idaea* in 2011. One specimen of ant (Formicidae) was recorded on *V. vitis-idaea* in 2011, but the specimen was not recovered. Likewise a species of beetle (Coleoptera, Fig 4.3 g) was found on a flower of *V. myrtilloides* during a non-survey time but was not recovered. There was quite a difference in the visitor composition found between years. The field season 2010 was less diverse in bee species, but had *Apis mellifera* present at the site. In 2011, *Apis mellifera* was absent, while more species of other families of bees were recorded. The field season in 2011 was also longer with fewer rainy days and thus more insect surveys were performed.

Table 4.3 Species confirmed from identified specimens as insect visitors to flowers of *V. myrtilloides* and *V. vitis-idaea.*

	V. myrtilloides	*V. vitis-idaea*
Order Hymenoptera		
Andrenidae	*Andrena* sp.[+] *A. rufosignata*, *A. nivalis*, *A. vicina*[+]†	*Andrena lupinorum?*[+]†, *A. nivalis*[+], *A. rufosignata*[+]
Apidae	*Apis mellifera**, *Bombus flavifrons**, *B. frigidus*, *B. melanopygus*[+], *B. ternarius*, *B. vagans*	*Apis mellifera**, *Bombus flavifrons*[+]†, *B. frigidus*, *B. griseocollis**, *B. melanopygus*, *B. ternarius*, *B. vagans*
Colletidae	*Colletes consors*[+]	*Colletes consors*[+], *Hylaeus basalis*[+]
Halictidae	*Lasioglossum* sp.[+]†, *L. prasinogaster**†	*Lasioglossum prasinogaster*[+]
Megachilidae	*Coelioxys sodalis*[+]†, *Osmia* sp., *Osmia inermis*[+]	*Osmia* sp.[+]
Vespidae	*Dolichovespula* spp.	
Order Diptera		
Syrphidae	*Helophilus hybridus*[+], *Helophilus lapponicus**, *Platycheirus* sp., *Platycheirus octavus*[+], *Platycheirus nearcticus*[+], *Syrphus ribesii*[+]	*Eristalis cryptarum**, *Eristalis stipator*[+], *Helophilus hybridus*[+], *Helophilus lapponicus*[+], *Platycheirus* sp.*, *Sericomyia chalcopyga*[+], *Sphaerophoria contigua*[+], *Volucella bombylans* 'yellow face'[+]
Order Lepidoptera		
Lycaenidae		Subfamily Polyommatinae[+]

* insect species only recorded for corresponding *Vaccinium* sp. in 2010

[+] insect species only recorded for corresponding *Vaccinium* sp. in 2011

† insect species only recorded for an SIV, not during insect surveys for corresponding *Vaccinium* sp.

4.3.1.1 Insect survey of floral visitors to *V. myrtilloides*

Over both years the insect surveys recorded, and later identification confirmed, 20 insect species (not including those of SIVs) as visitors to *V. myrtilloides* during survey times. In 2010, the confirmed number of species was 11, though likely there were at least two species of wasps and possibly multiple *Platycheirus* spp. (6 days, n = 55). The diversity of bees changed between the two years with *Api*s *mellifera* (56%, Fig 4.3 a) dominating the survey in 2010 (Fig 4.2), followed by *Bombus* spp. (25%). Otherwise no other bee species were recorded during complete survey days, though both an *Andrena* sp. and *Osmia* sp. were recorded on survey days that were not complete and so were not included here (all species data however, are still available in Table 4.3). Two genera of hoverflies (Syrphidae) were found visiting flowers: *Platycheirus* and *Helophilus*. Both were recorded during survey times however Fig 4.3 f shows another genus of hoverfly. Wasps (Vespidae: *Dolichovespula* spp., example Fig 4.3 b) were also visitors to *V. myrtilloides* flowers (Fig 4.2).

In 2011, there were 16 species confirmed as visitors to *V. myrtilloides* (7 days, n = 62). During that field season, *Apis mellifera* was not present at the site and *Bombus* spp. (60%, example Fig 4.3 c) dominated the visitations to flowers (Fig 4.2). Although not in large numbers, other bee genera also visited the flowers during survey times: *Andrena* (example Fig 4.3 d), *Colletes*, and *Osmia* plus a small Halictidae (not recovered). Once again wasps were present on flowers, as well as hoverflies that saw the addition of another genus, *Syrphus*.

In both years, visitor numbers were lower overall at the 9:00 sampling time and highest overall at the 18:00 sampling time. Visits by *Apis mellifera* increased over the day in 2010. In 2011, all the non-*Bombus* bees were found at 12:00 and 15:00 sampling times. Complete survey numbers can be seen in Table 4.4 (2010) and Table 4.5 (2011).

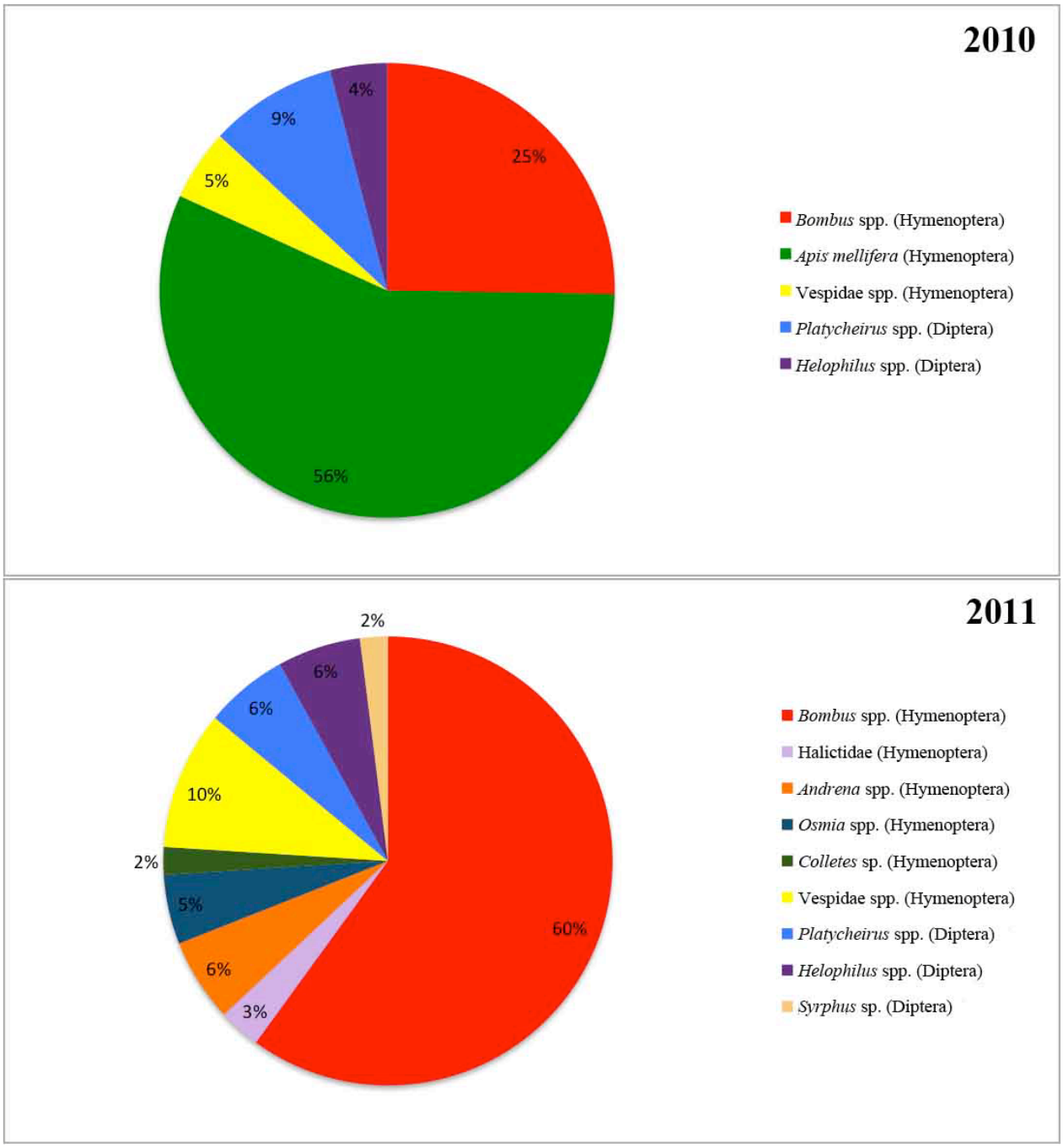


Figure 4.2 Relative proportions of different taxa recorded visiting flowers of *Vaccinium myrtilloides* during complete insect surveys in 2010 (n = 55) and 2011 (n = 62).

Table 4.4 Number of insect visitors per taxonomic group to flowers of *Vaccinium myrtilloides* according to survey date, time, and site transect, in 2010.

			No. of Visitors				
				Apidae			
Date	Transect	Time	Total	*Bombus* spp.	*Apis mellifera*	Vespidae	Syrphidae
02-Jun	1	9:00	0				
		12:00	1			1	
		15:00	2			1	1
		18:00	0				
12-Jun	8	9:00	2	1		1	
		12:00	1	1			
		15:00	3	1			2
		18:00	1		1		
13-Jun	5	9:00	0				
		12:00	1				1
		15:00	0				
		18:00	1	1			
14-Jun	4	9:00	2	1	1		
		12:00	3		2		1
		15:00	2		2		
		18:00	4		3		1
15-Jun	9	9:00	3	3			
		12:00	3	2	1		
		15:00	10		10		
		18:00	13	4	8		1
18-Jun	2	9:00	0				
		12:00	1		1		
		15:00	1		1		
		18:00	1		1		
		Totals	55	14	31	3	7

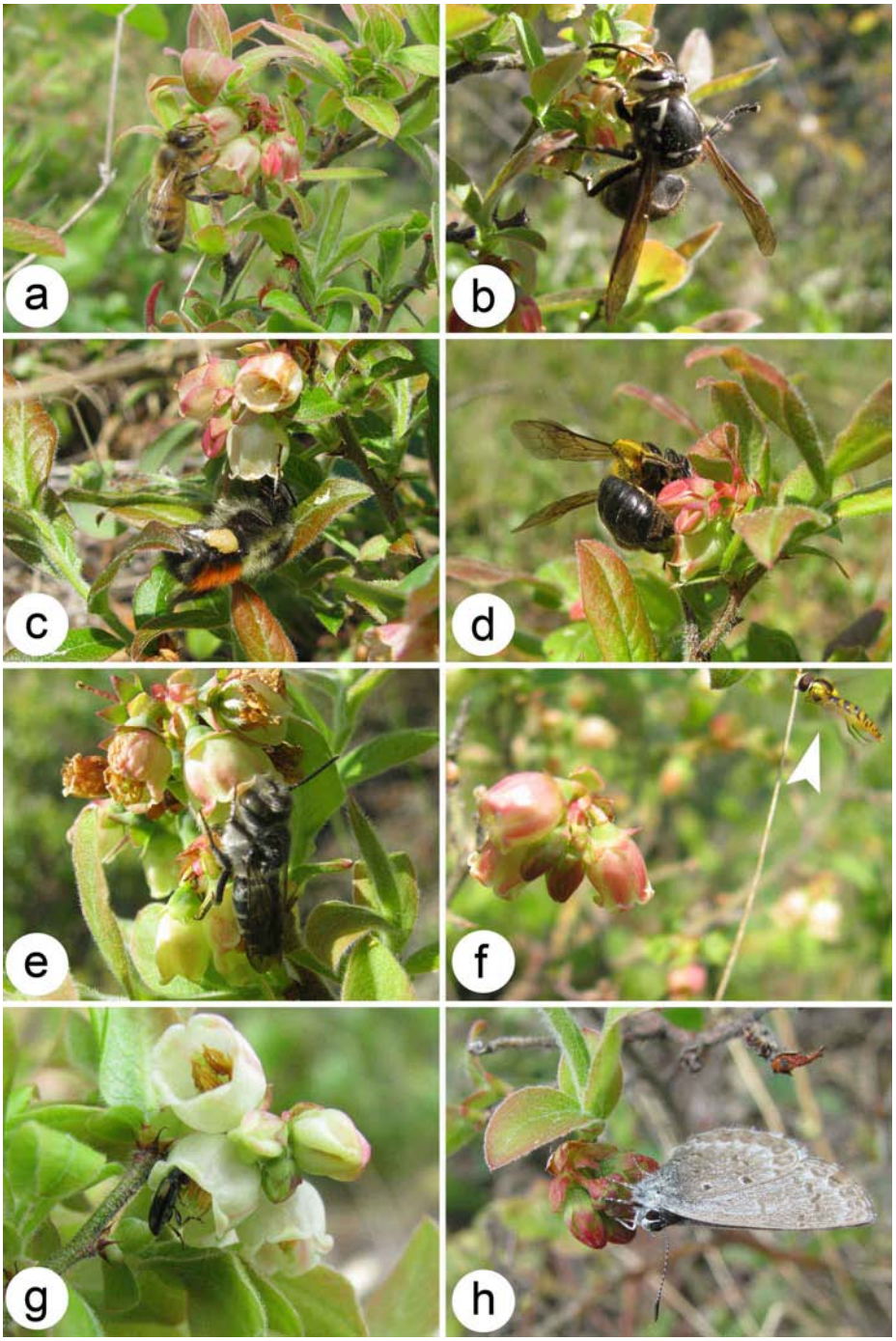


Figure 4.3 Insects on flowers of *Vaccinium myrtilloides*. a) *Apis mellifera* (honeybee). b) *Dolichovespula* sp. (wasp). c) *Bombus* sp. (bumblebee, worker caste). d) *Andrena* sp. (bee). e) *Coelioxys* sp. (bee). f) Syrphidae (hoverfly). g) Coleoptera (beetle). h) Lepidoptera, Lycaenidae, Subfamily Polyommatinae (blue).

Table 4.5 Number of insect visitors per taxonomic group to flowers of *Vaccinium myrtilloides* according to survey date, time, and site transect, in 2011.

			No. of Visitors							
Date	Transect	Time	Total	*Bombus* spp.	Halictidae	*Andrena* spp.	*Osmia* spp.	*Colletes* sp.	Vespidae	Syrphidae
31-May	7	9:00	0							
		12:00	1			1				
		15:00	1						1	
		18:00	1							1
02-Jun	3	9:00	0							
		12:00	1							1
		15:00	0							
		18:00	0							
06-Jun	9	9:00	1	1						
		12:00	7	3		1	1	1	1	
		15:00	4	1	2		1			
		18:00	10	6					1	3
08-Jun	6	9:00	3	3						
		12:00	5	2		1	1			1
		15:00	3	2		1				
		18:00	4	3						1
10-Jun	4	9:00	1	1						
		12:00	2	2						
		15:00	2	2						
		18:00	5	5						
12-Jun	1	9:00	1							1
		12:00	1	1						
		15:00	0							
		18:00	0							
16-Jun	8	9:00	1						1	
		12:00	1						1	
		15:00	2	1					1	
		18:00	5	4						1
		Totals	62	37	2	4	3	1	6	9

4.3.1.2 Insect survey of floral visitors to *V. vitis-idaea*

Surveys of *V. vitis-idaea* recorded 23 species of flower visiting insects over both years (not including those of SIVs) during complete insect surveys. In 2010, the confirmed number of species was eight (4 days, n = 26). The diversity of bees changed between the two years as again *Apis mellifera* (42%) dominated in 2010. There was, however, a greater proportion of Syrphidae (Fig 4.4, 35% both years) on the flowers of *V. vitis-idaea* than found with *V. myrtilloides* (Fig. 4.2, 13-14%). There was no evidence that non-hoverfly dipterans visited either *Vaccinium* species, and the example seen Fig 4.5 e was determined not to be a case of active foraging but rather a random landing site.

In 2011, there were 18 species confirmed as visitors to *V. vitis-idaea* (6 days, n = 27). During that field season, *Apis mellifera* (Fig 4.5 b) was not present at the site and *Bombus* spp. (37%, example Fig 4.5 a) had the greatest proportion of visitations to flowers. Although not in large numbers, other bee genera (*Andrena* [Fig 4.5 c], *Colletes, Hylaeus, Lasioglossum* [Fig 4.5 d] and *Osmia*) also visited the flowers during survey times. There was also a greater diversity of Syrphidae in 2011 with 6 genera (*Eristalis, Helophilus* [Fig 4.5 f], *Platycheirus, Sericomyia, Sphaerophoria,* and *Volucella*) whereas in 2010 there were only *Eristalis* and *Platycheirus*. Interestingly, there was one visit from a blue butterfly (Lycaenidae, as in Fig 4.3 h) and one from an ant (Formicidae), though the ant was not captured and thus does not appear on Table 4.3.

In 2010, overall insect visitors increased from 9:00 to 18:00. However, in 2011 both 9:00 and 18:00 tied for the lowest number of insect visitors at that time, though the 12:00 and 15:00 times show a gradual increase. Complete survey numbers can be seen in Table 4.6 (2010) and Table 4.7 (2011).

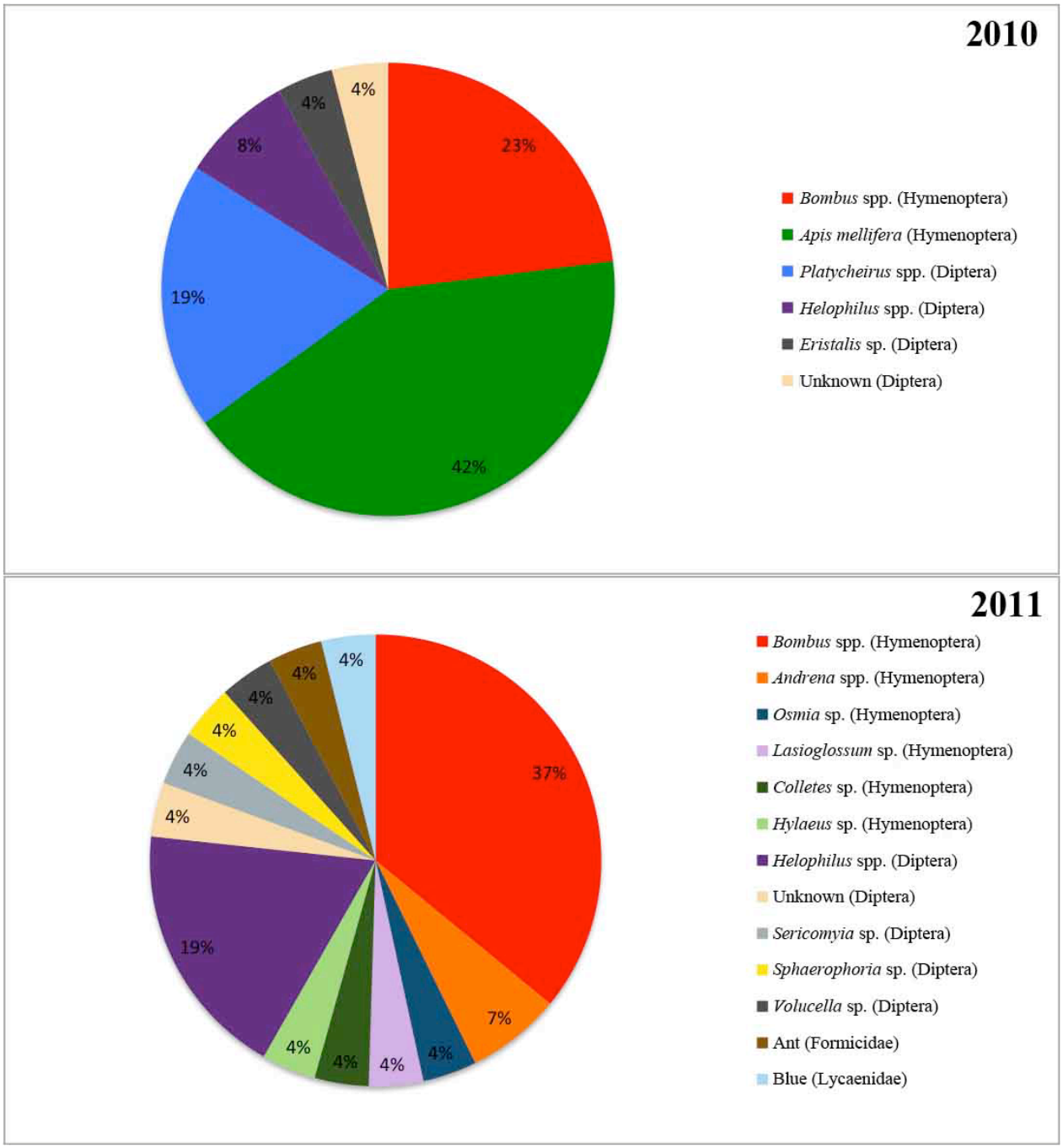


Figure 4.4 Relative proportions of different taxa recorded visiting flowers of *Vaccinium vitis-idaea* during complete insect surveys in 2010 (n = 26) and 2011 (n = 27).

Table 4.6 Number of insect visitors per taxonomic group to flowers of *Vaccinium vitis-idaea* according to survey date, time, and site transect in 2010.

			No. of Visitors			
				Apidae		
Date	Transect	Time	Total	*Bombus* spp	*Apis mellifera*	Syrphidae
12-Jun	8	9:00	0			
		12:00	3	1		2
		15:00	1	1		
		18:00	0			
13-Jun	5	9:00	2		1	1
		12:00	1		1	
		15:00	3		3	
		18:00	8		5	3
14-Jun	4	9:00	0			
		12:00	2			2
		15:00	0			
		18:00	0			
18-Jun	2	9:00	0			
		12:00	0			
		15:00	3	1	1	1
		18:00	3	3		
		Totals	26	6	11	9

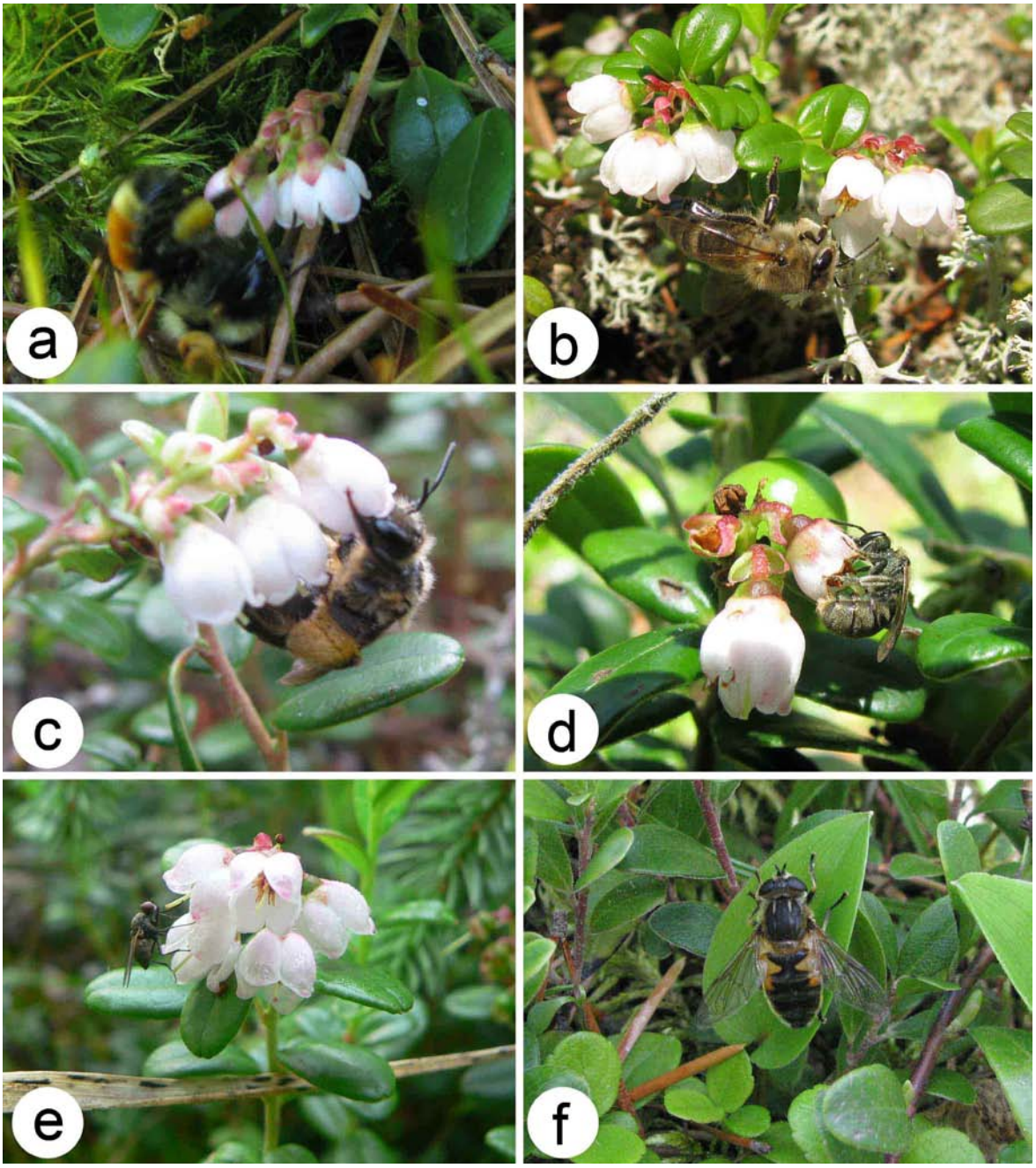


Figure 4.5 Insects on flowers of *Vaccinium vitis-idaea*. a) *Bombus* sp. (bumblebee, worker caste). b) *Apis mellifera* (honeybee). c) *Andrena* sp. d) *Lasioglossum* sp. e) Incidental fly (Diptera). f) *Helophilus* sp. on foliage.

Table 4.7 Number of insect visitors per taxonomic group to flowers of *Vaccinium vitis-idaea* according to survey date, time, and site transect in 2011.

			No. of Visitors								
Date	Transect	Time	Total	*Bombus* spp.	*Andrena* sp.	*Osmia* sp.	Halictidae	Colletidae	Syrphidae	Formicidae	Lycaenidae
16-Jun	8	9:00	0								
		12:00	0								
		15:00	3						2		1
		18:00	1	1							
22-Jun	8	9:00	0								
		12:00	0								
		15:00	1						1		
		18:00	2			1				1	
23-Jun	NT9	9:00	3	3							
		12:00	4	1	1				2		
		15:00	2	2							
		18:00	1	1							
25-Jun	8	9:00	0								
		12:00	0								
		15:00	2					1	1		
		18:00	0								
27-Jun	T9NW	9:00	1						1		
		12:00	2	1					1		
		15:00	2	1					1		
		18:00	0								
28-Jun	NT9	9:00	1					1			
		12:00	1				1				
		15:00	0								
		18:00	1		1						
		Totals	27	10	2	1	1	2	9	1	1

4.3.2 Single insect visitation to virgin flowers

4.3.2.1 *Vaccinium myrtilloides*

In 2010, there were nine SIVs in which the insect-visited flower was successfully recovered post-visitation. The greatest number of visits were by honeybees (*Apis mellifera*, 5), then bumblebees (*Bombus* spp., 3), and one visit from an *Andrena* sp. In 2011, there were 20 successful SIV flowers. Bumblebees made up the majority of these with 12 SIVs, followed by *Andrena* spp. with three SIVs, and the remaining were four visits by other bee taxa and one visit by a syrphid fly (*Helophilus hybridus*). As with insect survey data, bees from the family Apidae (*Apis, Bombus*) made up the largest proportion of flower visitors to *V. myrtilloides*. Data from two flowers could not be used: one in 2010 had the bag torn off by an unknown animal exposing the SIV flower overnight, and one in 2011 was visited by a *Bombus* species, collected and processed, however the pollen tubes failed to fluoresce and only callose plugs could be seen.

The SIV data were compared to those of the two Control types to assess whether an insect visit made a difference to the number of pollen grains deposited and number of pollen tubes that grew to the base of the style. The number of pollen grains and pollen tubes for Control 1 and Control 2 flowers did not differ significantly from one another (Fig 4.6 a, b), suggesting that the actions of unbagging and then rebagging of flowers during the SIVs were also not having an effect different from that which an untouched flower experienced: the researchers were not inadvertently acting as the pollinator. Regarding the number of pollen grains on the stigma, the SIVs as a whole differed significantly from both control types (see Fig 4.6 a, b). When controls were tested separately with individual taxonomic groups (*Bombus*, *Apis*, *Andrena*), quantities of pollen grains deposited by *Andrena* spp. were different from that of Control 2 but not Control 1 (Fig 4.6 c) and numbers of pollen tubes per style following visits by *Andrena* spp. were significantly different from both Controls (Fig 4.6 d). *Apis mellifera* deposited pollen grains were higher in number from those of Control 2 but not Control 1 (Fig 4.6 e) and no differences between Controls and visits by *Apis mellifera* were found for number of pollen tubes per style (Fig 4.6 f). Only *Bombus* spp. were found to be significantly different in the number of grains deposited on the stigma from both Control 1 and Control 2 flowers (Fig 4.6 g). The same occurred for number of pollen tubes per style (Fig 4.6 h). Thus the visiting insects deposited more pollen on the whole than was found in flowers left to avenues of wind and self pollination, but

when comparing the number of pollen tubes reaching the base of the style, only the SIVs as a whole group and *Andrena* and *Bombus* spp. differed significantly from the control types (Fig 4.6).

Bombus had the highest mean (± SD) pollen grain deposition per stigma of any taxonomic group (363 ± 299), followed by *Andrena* (243 ± 274), then *Apis* (105 ± 23). The same order was seen for pollen tubes at the base of the style with *Bombus* having the highest mean (24 ± 24), followed by *Andrena* (10 ± 8), then *Apis* (0.4 ± 0.9). For single sample taxa visits, *Colletes* had the highest pollen deposition (648) followed by *Osmia* (308). Neither of these visits had any pollen tubes at the base of the style. *Lasioglossum* and *Coelioxys* (example seen in Fig 4.3 e) both had many pollen grains on the stigma (204, 216), as well as a relatively high number of pollen tubes that reached the base of the style (18, 21). There were no pollen grains deposited on the stigma as a result of the single syrphid visit (Table 4.8).

Bombus spp. that were recorded as audibly buzzing the flowers of *V. myrtilloides* during SIV visits had a higher mean number of pollen grains (524 ± 302) deposited on stigmas ($W = 46$, $p = 0.0423$, Fig 4.7) and a higher number of pollen tubes (35.1 ± 26.2) found at the base of the style ($W = 46.5$, $p = 0.0364$, Fig 4.7) than *Bombus* spp. visiting flowers without sonicating them (178 ± 167; 11.8 ± 16.7). No comparisons between castes of *Bombus* could be done, as there was only one confirmed *Bombus* queen involved in SIV experiments.

There was no significant relationship between the period that the insects spent on the SIV flowers and the number of pollen grains deposited on the stigma ($p = 0.543$, Fig 4.8) or number of pollen tubes found in the base of the style ($p = 0.595$, Fig 4.8). There were also no significant differences between the visitation duration between the *Bombus* spp. that sonicated flowers and those that did not ($W = 21$, $p = 0.4634$). In addition, the relationship between duration of visit and pollen grain deposition and pollen tube growth was also considered separately for *Bombus* spp. (Fig 4.9 a, b), and for those that buzzed (Fig 4.9 b, c) and did not buzz (Fig 4.9 d, e); however no significant relationship was found.

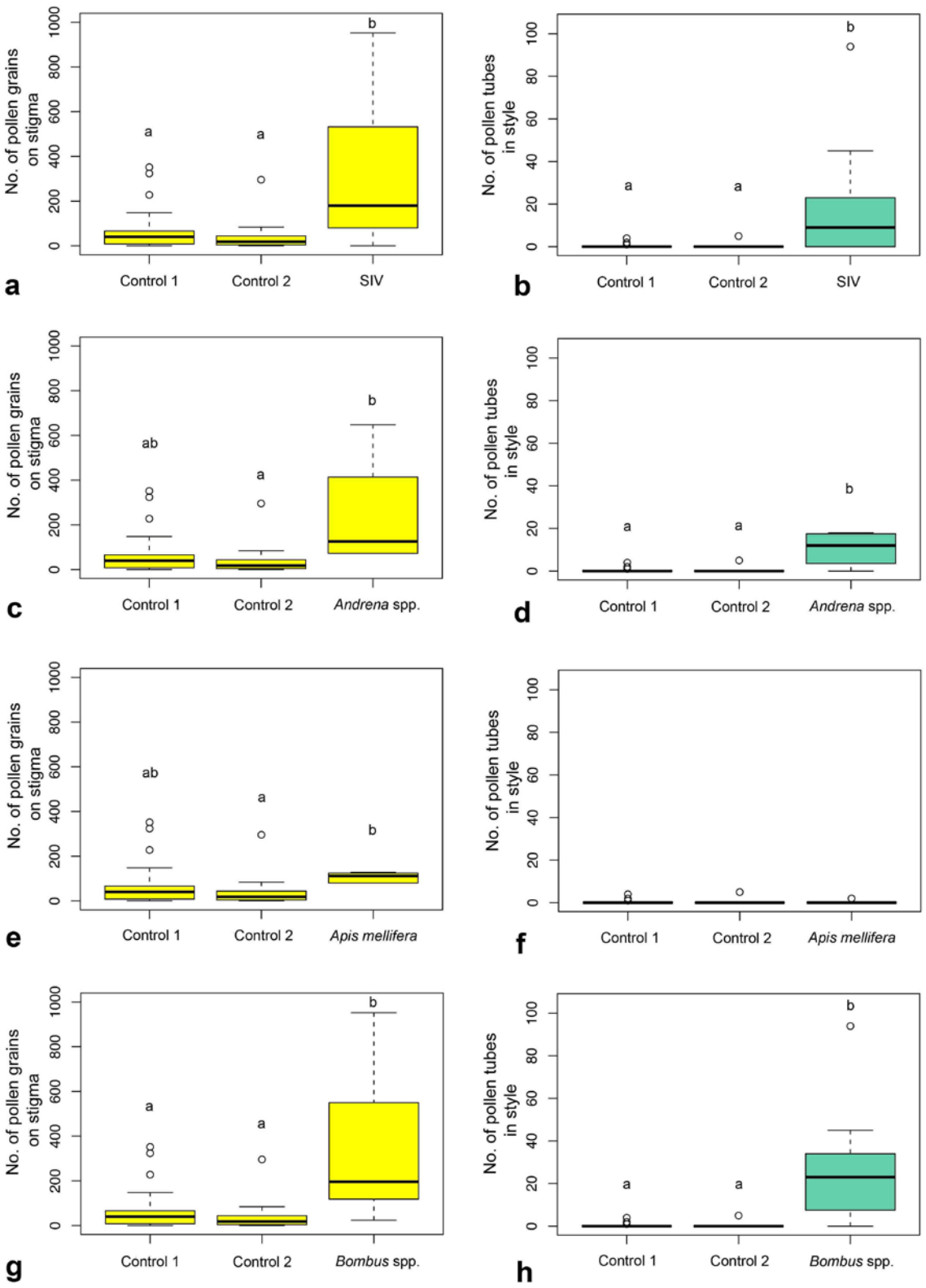
Table 4.8 Single insect visitation data for *Vaccinium myrtilloides* conducted during 2010 and 2011 (mean ± SD).

	Taxon	Family	No. pollen grains on stigma	No. pollen tubes in style base	Visit duration (sec)	Year	Sonication
Individual Insect Visits							
	Andrena nivalis	Andrenidae	72	17	0.45	2010	
	Andrena nivalis	Andrenidae	72	0	5.56	2011	
	Andrena rufosignata	Andrenidae	180	7	6.12	2011	
	Andrena vicina	Andrenidae	648	18	14.93	2011	
	Apis mellifera	Apidae	80	0	12.69	2010	
	Apis mellifera	Apidae	80	0	6.53	2010	
	Apis mellifera	Apidae	124	0	2.56	2010	
	Apis mellifera	Apidae	112	0	9.5	2010	
	Apis mellifera	Apidae	128	2	5.32	2010	
	Bombus frigidus	Apidae	656	40	2.17	2011	Buzzing
	Bombus frigidus	Apidae	24	0	5.63	2010	
	Bombus ternarius	Apidae	532	24	4.85	2011	Buzzing
	Bombus ternarius	Apidae	532	45	4.13	2011	
	Bombus ternarius	Apidae	136	23	6.69	2011	
	Bombus ternarius	Apidae	100	6	5.31	2011	
	Bombus vagans	Apidae	456	44	4.35	2011	Buzzing
	Bombus vagans	Apidae	144	18	5.47	2011	Buzzing
	Bombus vagans	Apidae	816	94	5.78	2011	Buzzing
	Bombus melanophygus	Apidae	568	10	5.28	2011	Buzzing
	Bombus sp.*	Apidae	72	0	1.3	2011	
	Bombus sp.§	Apidae	192	0	6.16	2011	
	Bombus sp.	Apidae	952	28	7.06	2011	Buzzing
	Bombus sp.	Apidae	72	23	2	2010	Buzzing
	Bombus sp.§	Apidae	196	9	8.31	2010	
	Osmia sp.	Megachilidae	308	0	5.88	2011	
	Coelioxys sodalis	Megachilidae	216	21	6.5	2011	
	Colletes consors	Colletidae	648	0	4.66	2011	
	Lasioglossum sp.	Halictidae	204	18	8.93	2011	
	Helophilus hybridus	Syrphidae	0	0	3.78	2011	
Total SIVs (n = 29)			286.9 ± 265	15.4 ± 20			
Control 1 (n = 33)			58 ± 88	0.72 ± 2.4			
Control 2 (n = 28)			47 ± 76	0.25 ± 1			

*Single *Bombus* queen recorded
§ *Bombus* caste unknown

Figure 4.6 Boxplots of number of pollen grains deposited on stigmas (a, c, e, g) and the number of pollen tubes which grew to the base of the style (b, d, f, h) in flowers of *Vaccinium myrtilloides* in two types of controls and all single insect visitations, and by repeated insect taxa of *Andrena* spp., *Apis mellifera* and *Bombus* spp. The coloured boxes represent the upper and lower quartiles whereas the solid black line represents the median. Whiskers indicate the maximum and minimum values and outliers are shown when 1 ½ times the upper quartile. Significant differences between groups are indicated by different letters where tested.

a) chi-squared = 28.0199, df = 2, p = 8.233e-07 b) chi-squared = 25.6569, df = 2, p = 2.683e-06 c) chi-squared = 7.4906, df = 2, p = 0.02363 d) chi-squared = 14.8244, df = 2, p = 0.0006038 e) chi-squared = 7.1832, df = 2, p = 0.02755 f) chi-squared = 2.4994, df = 2, p = 0.2866 g) chi-squared = 21.7836, df = 2, p = 1.861e-05 h) chi-squared = 31.9094, df = 2, p = 1.178e-07


No. of pollen grains on stigma
No. of pollen tubes in style
Control 1
Control 2
SIV
Andrena spp.
Apis mellifera
Bombus spp.
a
b
c
d
e
f
g
h

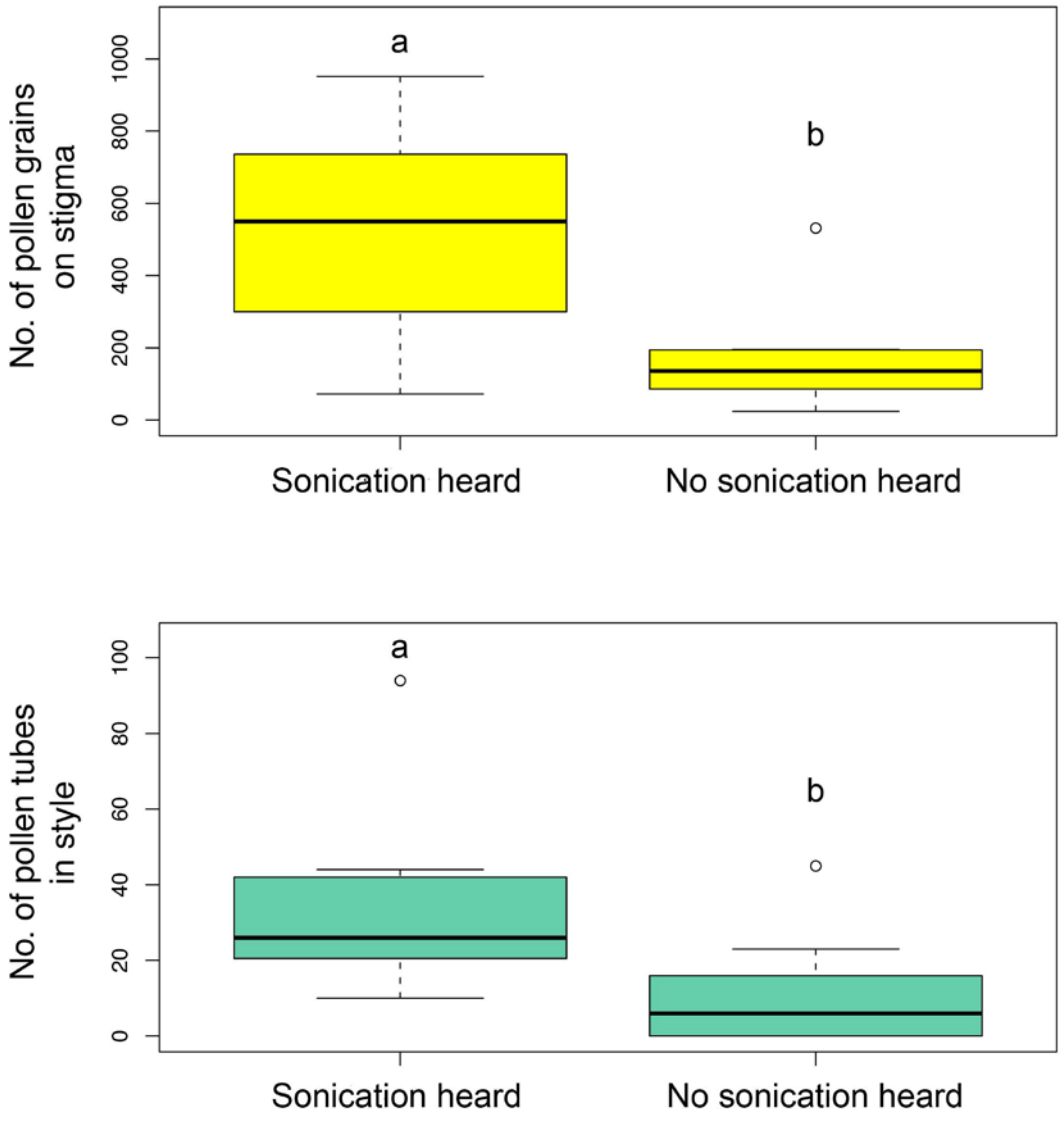


Figure 4.7 Comparison of buzz pollination by *Bombus* spp. visiting virgin flowers of *V. myrtilloides* recorded as audibly sonicating a flower (n=8) versus not sonicating the flower (n=7). Wilcoxon t test (Grains: W = 46, p = 0.0423; Tubes: W=46.5, p = 0.0364); p value for testing between tubes is approximate due to the presence of ties.

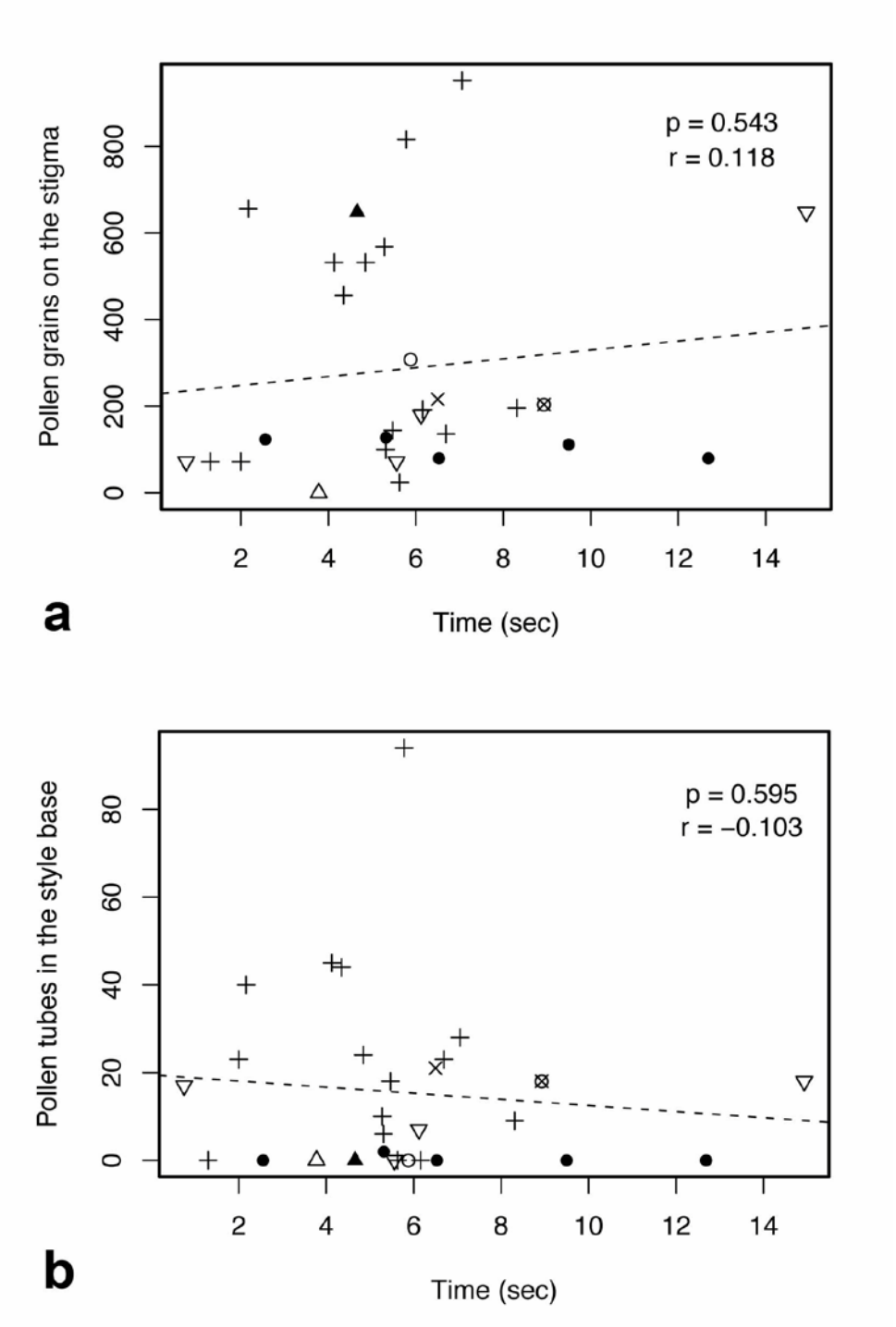


Figure 4.8 Insect visitation time has no significant relationship with pollen deposition on the stigma (a) or pollen tubes found in the style base (b) of single-visited flowers of *Vaccinium myrtilloides*. Symbols: *Andrena* = ▽, *Apis* = ●, *Bombus* = +, *Coelioxys* = x, *Colletes* = ▲, Halictidae = ⊗, *Osmia* = ❍, Syrphidae = Δ

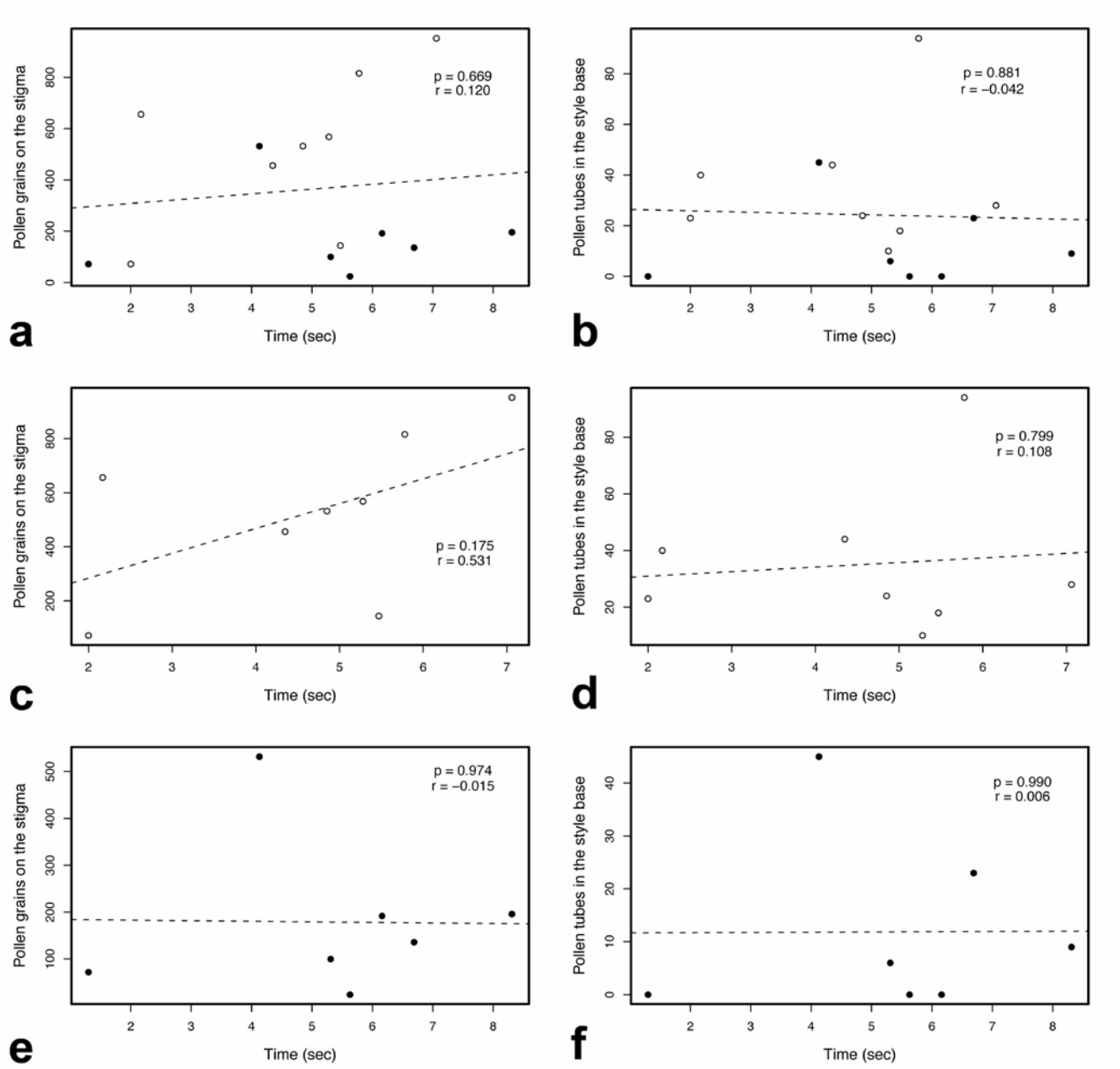


Figure 4.9 Plots of insect visitation time to previously-unvisited flowers versus pollen deposition on the stigma or pollen tubes found in the style base of single-visited flowers of *Vaccinium myrtilloides* by *Bombus* spp. (a, b); *Bombus* spp. visitors that soniciated flowers (c, d); and *Bombus* spp. visitors that did not sonicate flowers (e, f). Symbols: sonication = ❍, no sonication = ●.

4.3.2.1.1 Pollen viability investigation pertaining to *Apis mellifera*

For the SIVs by *Apis mellifera* in 2010, the low number of tubes to reach the style base out of the number of grains that had been deposited on stigmas (Table 4.8), warranted further investigation. In 2011, pollen tetrads of *V. myrtilloides* were subjected to a temperature of 34°C (hive temperature as in Kraai 1962, though in a sealed petri dish with high relative humidity rather than on a bee's body) for a period of 21 h (Dicklow *et al*. 1985) and the pollen grains subsequently tested for viability using the fluorochromatic reaction test (Shivanna and Rangaswamy 1992; see Section 2.1.3). After 21 h at this elevated temperature, all the pollen appeared non-viable irrespective of whether it was from a 1, 2, or 3 day old flower. Pollen grains from the same ramet that were kept at room temperature and subjected to the same pollen viability test scored between 74 and 80% viable. Thus it is suggested that pollen deposited by *Apis mellifera* might show poor germination if it had been collected on a previous day, and then resided on the honeybee's body overnight, inside the hive.

4.3.2.2 *Vaccinium vitis-idaea*

Single insect visits to virgin flowers of *V. vitis-idaea* were only successful in field season 2011. *Bombus* species represented the largest proportion of visits (4) followed closely by 3 Syrphidae spp., and lastly one *Andrena* sp. (Table 4.9). However, with a total of eight SIVs, the validity of performing tests on the data is questionable. There was no significant difference in pollen tubes found at the base of the style, nor in pollen grains found on the stigma, between the SIVs, Control 1 and Control 2 (Fig 4.10). When the groups of *Bombus* and Syrphidae are tested separately with the Controls, there was a difference between the number of pollen tubes at the base of the style that resulted from *Bombus* visitation versus the Control 2 treatment (Fig 4.10 d) but no differences were found for the Syrphidae visits (Fig 4.10 e, f).

For the four *Bombus* visits to flowers of *V. vitis-idaea*, no comparisons could be made of buzz vs non-buzzing as only one of the four *Bombus* visits did not buzz the flower. It was not the lowest in terms of pollen grains or pollen tubes (Table 4.9). All *Bombus* visits to *V. vitis-idaea* were by worker caste.

Of the three hoverfly visits to *V. vitis-idaea*, extremely low pollen deposition occurred as the two *Helophilus* spp. deposited one tetrad each and no stigmatic pollen was found on the flower visited by *Eristalis stipator* (Table 4.9). No pollen tubes were found (Fig 4.10). Likewise

the *Andrena* visit yielded only one tetrad and no tubes, however the visit was brief, without observation of foraging of any kind.

Control 1 flowers showed a much higher pollen deposition than that of the SIVs. There was a high number of pollen grains (33.2 ± 41) found on stigmas as well as a high number of pollen tubes at the style base (Table 4.9). Mean pollen tube number per style of these 10 flowers was driven by one flower which had 35 tubes and another with 11 tubes (Fig 4.10). Likewise the Control 2 flowers, that were never unbagged, showed a number of pollen grains on the stigma (9.2 ± 9.6), however no pollen tubes were found at the base of any of the 10 flowers (Table 4.9). The high number of pollen grains on the Control 1 flowers vs Control 2 flowers would suggest human error when flowers were either initially bagged, or when rebagged, as the mean number of pollen grains deposited on Control 1 flowers was higher than that of the SIVs (15.5 ± 17).

Table 4.9 Single insect visitation data for *Vaccinium vitis-idaea* conducted in 2011 (mean ± SD).

	Visitor	Family	No. pollen grains on stigma	No. pollen tubes at style base	Visit duration (sec)	Sonication
Individual Insects	*Andrena* sp.	Andrenidae	4	0	<1	
	Bombus frigidus	Apidae	44	12	2.56	Buzzed
	Bombus frigidus	Apidae	40	4	3.38	
	Bombus flavifrons	Apidae	8	2	8.93	Buzzed
	Bombus vagans	Apidae	20	3	5.22	Buzzed
	Eristalis stipator	Syrphidae	0	0	4.03	
	Helophilus lapponicus	Syrphidae	4	0	5.22	
	Helophilus sp.	Syrphidae	4	0	<1	
Total SIVs (n = 8)			15.5 ± 17	2.6 ± 4		
Control 1 (n = 10)			33.2 ± 41	4.9 ± 11		
Control 2 (n = 10)			9.2 ± 9.6	0		

All *Bombus* spp. were worker caste

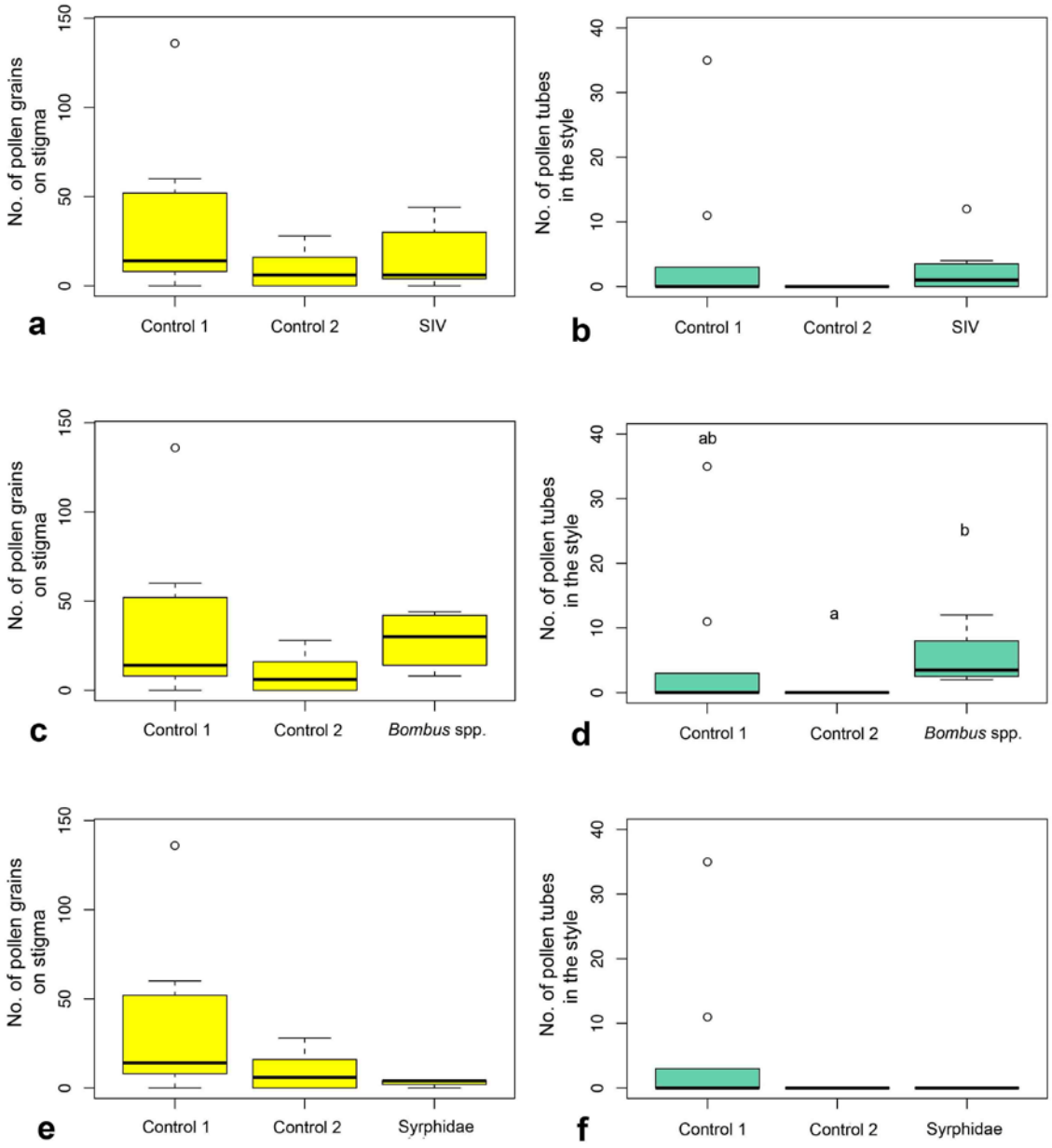


Figure 4.10 Boxplots of number of pollen grains deposited on stigmas (a, c, e) and the number of pollen tubes which grew to the base of the style (b, d, f) in flowers of *Vaccinium vitis-idaea* in two types of controls and all single insect visitations, and by repeated insect taxa of *Bombus* spp. and Syrphidae. The coloured boxes represent the upper and lower quartiles whereas the solid black line represents the median. Whiskers indicate the maximum and minimum values and outliers are shown when 1 ½ times the upper quartile. Significant differences are indicated by different letters. a) chi-squared = 2.3829, df = 2, p = 0.3038 b) chi-squared = 5.4385, df = 2, p = 0.06592 c) chi-squared = 4.1395, df = 2, p = 0.1262 d) chi-squared = 11.5322, df = 2, p = 0.003132 e) chi-squared = 4.0679, df = 2, p = 0.1308 f) chi-squared = 4.2653, df = 2, p = 0.1185.

4.4 Discussion and conclusions

Pollination is a difficult process to study due to the inability to examine all flowers at once, to observe all visitors, and then distinguish visitors from true pollinators. Insect surveys as done here, give a brief overview of which insects were visiting flowers, and using different but consistent survey times it was attempted to account for the varying foraging schedules of potential pollinations. However, there were insect species recorded for SIVs not found in the insect surveys (and vice versa), thus indicating the complexity of plant-insect interactions.

4.4.1 Species diversity of insect visitors

There was no difference in the number of insect species recorded as flower visitors between the plant species (*V. myrtilloides,* 23; *V. vitis-idaea,* 23, Table 4.3). Some of the insect species reported here have been reported as visitors to these flowers before. *Apis mellifera* visits flowers of both species when they occur at the same site (Jacquemart 1993; Reader 1977; Davis *et al.* 2003). For *V. myrtilloides,* Usui (1994) reported two overlapping *Bombus* species: *B. frigidus* and *B. ternarius*; an *Osmia* species: *O. inermis*; all three of our identified *Andrena*: *A. rufosignata* (called *A. rufosignator* by Usui 1994), *A. nivalis* (not listed individually in Table 4.1), and *A. vicina*; the oligolectic blueberry (Ericaceae) forager *Colletes validus*; and wasp species of the same genus *Dolichovespula* (species unconfirmed). Usui (1994) also reported Coleoptera visitors to flowers of *Vaccinium* (*angustifolium/myrtilloides*) and there was one beetle (Fig 4.3 g) observed in a flower of *V. myrtilloides* in 2010.

For *V. vitis-idaea* in Alaska, Davis *et al.* (2003) reported only two flower visiting species that were shared by this study: *Apis mellifera* and *Bombus frigidus*. However, their study shared many of the same groups of visitors: *Bombus* spp., *Andrena* spp. and Syrphidae (see Tables 4.2, 4.3). Interestingly, they reported *Dolichovespula* spp. (wasps) as visitors whereas these were only seen foraging on the earlier blooming *V. myrtilloides* at our field site. The one butterfly visitor we report to *V. vitis-idaea* is from the same family (Lycaenidae) as reported to lowbush blueberries by Usui (1994), whereas a moth (Geometridae) was reported by Davis *et al*. (2003). A species review by Hall and Shay (1981) cited bees and butterflies (Newfoundland; Torrey 1914) and bumblebees and bee flies (British Columbia; Pojar 1974). Though bee flies were not seen at our site, overall pollinator groups seem consistent with other reports in the North American boreal forest.

Jacquemart (1993) surveyed flower visitors to *V. vitis-idaea* in Belgium, and also reported *Apis mellifera*, bumblebees, *Andrena* spp. and Syrphidae. She made a note that ants did visit the flowers for nectar, but that ant visitation was more common in another study species, *V. myrtillus*. Beetles, thrips, butterflies and flies (other than Syrphidae) were rare and so excluded from their published data. Previous reports from Europe listed in Jacquemart (1993, see Table 4.2) also list primarily bumblebee species, though also *Andrena*, *Nomada*, *Osmia*, fly species (both Syrphidae and Empididae) and one undetermined moth. Warming 1908 (in Hall and Shay 1981) also reported *Bombus* species as pollinators in Greenland.

4.4.2 Insect survey of visitors to flowers

The largest change between the two consecutive years at the study site was the presence of *Apis mellifera* in 2010. The honeybees were found foraging first on bearberry, but then moved on to *V. myrtilloides* as the majority of these plants came into bloom, and then *V. vitis-idaea* as that species started blooming. Honeybees are generally flower loyal for the day of foraging and the small ericaceous plants provided a large amount of blossoms without far to move between. It is not known where the honeybees were coming from, however they were not present in 2011 making it likely that their hives were moved with a cropping system.

The presence of the honeybee is suggested to have an effect on visitation rates by native bees in some cases (reviewed by Paini, 2004), and may be the case at the study site. Out of 55 insects recorded in 2010 for *V. myrtilloides*, 56% were *Apis mellifera*. Out of 26 insects recorded in 2010 for *V. vitis-idaea*, 42% were *Apis mellifera*. Though the insect surveys done were not a visitation rate, the overall numbers of insects recorded in each of the two years are comparable for each plant species, and only in 2011 when *Apis mellifera* was absent were other bee species (besides *Bombus*) recorded in the surveys. Native bees were still seen at the study site in 2010, many *Bombus* were present, there were *Osmia* and *Andrena* species that were found in incomplete survey days, and a *Lasioglossum* SIV. However a decrease or change in the foraging behaviour of native bees due to the presence of *Apis mellifera* could explain the differences between the two years.

4.4.2.1 *V. myrtilloides*

That visitor number increased with survey time throughout the day is not unusual. Due to cooler morning temperatures (< 15°C) insects would be less likely to forage in the coolest

morning but increase as the temperature increases throughout the day. The hardy *Bombus* made up most of the early morning visitors, along with Vespidae, *Apis mellifera* and Syrphidae. Other bees were only recorded during the 12:00 and 15:00 survey times.

The primary visitors to flowers of *V. myrtilloides* were bees. *Apis mellifera* was the most common visitor in 2010, however over both years, *Bombus* spp. were the most common. As *V. myrtilloides* flowers early in the growing season, *Bombus* queen visits were common, as well as flower visits by *Bombus* workers. Thus it would seem that *Bombus* spp., which have excellent pollinator potential, are also the highest recorded visitor. Usui (1994) classified many bee species (Table 4.1, including all the *Bombus* recorded) as the most common visitors to lowbush blueberry. Interestingly, Usui (1994) postulated that wasps (*Dolichovespula*) may be important secondary pollinators of lowbush blueberry "due to their large numbers, fast moving flight behavior and their ability to forage during poor weather". Although wasps were flower visitors to *V. myrtilloides* at the field site, there was no indication they were foraging for pollen, which might predispose them to greater success as pollinators. Moreover, the possession of fewer and unbranched body hairs (Lukoschus 1957) makes wasps less likely candidates as pollinators compared to bees.

4.4.2.2 *V. vitis-idaea*

In our survey there was a gradual increase of visitors throughout the day in 2010 but in 2011, visitation increased until 15:00 and then numbers decreased for 18:00. This decline may have been due to overcast afternoons that often resulted in storms nearby or later that evening.

The survey times for flower visitors used in this study correspond well to the times that Davis *et al.* (2003) recorded the majority of their potential pollinators visiting flowers of *V. vitis-idaea*. Before 08:00 they recorded mostly wasp visits and after 18:00 only a few Halictidae. Their study was located in Alaska with near 24 hour light, however bees foraged at the same times as lower latitudes.

A study by Jacquemart (1993) done in Belgium found *Apis mellifera* to be the most common potential pollinator for *V. vitis-idaea* at their site. They also listed other bumblebee species (see Table 4.1) and commented on the lack of visits by queens due to the time of flowering. They did not comment on honeybee interactions with other bees, however noted that honeybees were not major visitors to their other two *Vaccinium* species studied, perhaps due to fewer honeybees being available during their early flowering.

4.4.3 Plant pollinators: a working definition for this study

The definition of a pollinator can have very general or very specific terms. A pollinator facilitates plant reproduction by transferring pollen to the stigma from either the individual's anthers, or that of another individual of the same species (the latter crucial for plants with self-incompatible systems). However, not every visit results in plant pollination for a number of reasons: the visitor does not carry pollen from the appropriate plant species; it carries pollen but fails to transfer it to the stigma because of where the pollen is situated on the visitor body; or there are limitations imposed by the visitor's size, tongue length, or behaviour on the flower; or the visitor may seek other rewards offered by the flower and circumvent contact with anthers or stigma for that reward. It can be difficult to distinguish when a flower visitor is acting as a pollinator, rather than a nectar thief or pollen thief – stealing the floral rewards without providing a pollination service (terminology, Inouye 1980).

There are many ways to measure the potential that different animal species have for pollinating specific plant species. For this study, the range of visitors to the flowers of both species at the field site was established, whereas pollinator potential was measured by what different insect taxa would deposit on a previously untouched flower during a single visit. The number of pollen grains counted on the stigma represents deposition attributable to a single visit, but also any wind-facilitated autogamy and potential human error while the flowers were handled. Because neither of these *Vaccinium* species is self incompatible, with small degrees of selfing reported (Hokanson and Hancock 2000; Usui *et al*. 2005; Guillaume and Jacquemart 1999; Nuortila *et al*. 2002), the effect of the potential pollinator on both pollen grains and pollen tubes can be compared to control flowers at the same site. Though potential wind borne pollen could still make it through the mesh of the bags used to exclude pollinators, it is likely that most pollen that is deposited on the stigmas through abiotic means was from the same flower or from flowers of the same inflorescence. This pollen deposition would occur through bud autogamy (Noormets and Olson 2006) or from wind-facilitated autogamy (Hagerup 1954). It is reasonable to assume that with a less sheltered site, the role of wind in *Vaccinium* pollination would be higher than our largely treed field site.

It is also important to keep in mind the perennial growth of both *Vaccinium* species may result in resource allocation among clones, flowers and years of flowering (Stephenson 1981). Thus the measurement of the number of grains and tubes reflects a potential pollination effect,

not necessarily answering questions about the possible effects of deleterious recessives on fertilization, fruit production, or the number of seeds produced from that insect visit. To be considered pollinators for this study the taxa must, for the most part, deposit more pollen grains that result in successive pollen tubes than can be found in bud or wind autogamy alone, thus demonstrating a greater effect than autogamy alone. Notwithstanding this ideal situation, there could be instances in which the visitor deposits pollen on a flower that has experienced no wind-facilitated pollination and the pollen deposited during a visit may not be greater than the bud or wind autogamy experienced by control flowers. The only correction possible is large numbers of samples, which are not available in this study.

4.4.3.1 Number of pollen grains on the stigma versus the number of pollen tubes at the style base

Whether a pollen tube selection process occurs in either *Vaccinium* species is not known. The varying ratio between the number of pollen grains found on the stigmas versus the number of pollen tubes recorded at the style base could suggest such a process, however non-viability of pollen may also be an important factor. Pollen viability was found to be 51-93% in flowers of *V. vitis-idaea* and 76-97% in flowers of *V. myrtilloides* at the field site, indicating that a portion of pollen deposited on the stigma would be non-viable. The duration of pollen viability is unknown and could decrease with the time the pollen spent on a bee's body, especially for the case of *Apis mellifera*, as the result of experiments performed in this study suggest.

The lack of pollen tubes may also be due to a lack of stigma receptivity when they were deposited. For *V. myrtilloides,* Noormets and Olson (2002) reported that at full petal spread, peroxidase activity was found in 69% (n = 227) of stigmas tested. Peroxidase activity is "associated with the success of pollen adhesion to the stigmatic surface and the level of peroxidase activity appears to be correlated with the success of pollen germination" (from Noormets and Olson citing Galen and Plowright, 1987). All flowers were open for an unknown period, possibly up to 15 h, before use as SIV flowers (i.e., it is not known when in the evening or morning an individual experimental flower actually opened). Nor is it known how long after full petal spread 100% of stigmas would have peroxidase activity, or if all stigmas become receptive. Thus it is possible that stigmas of some of the SIV flowers were not receptive at the time of visitation. Likewise, Noormets and Olson (2006) reported that 13% (n = 169) of flower buds of *V. myrtilloides* examined at the open flower stage had already undergone autogamy; most often

this pollen deposition would occur before stigma receptivity, therefore it is not known whether these pollen grains would germinate.

4.4.3.2 Pollinators

4.4.3.2.1 *V. myrtilloides*

Given the above criteria for a pollinator in this study, *Bombus* spp. clearly demonstrated pollination effectiveness, whereas *Andrena* spp. demonstrate a probable pollination effect that remains to be substantiated with increased numbers of observations. All *Bombus* SIVs possessed pollen grains on the stigma (almost all with higher numbers than the mean of Control 1 and Control 2), and more importantly, all but three visits resulted in pollen tubes at the base of the style. This is important if there is a selection process occurring among pollen tubes between self and cross pollination. The number of samples for this group creates confidence that *Bombus* can be called a pollinator with certainty.

The small number of samples for *Apis mellifera* and *Andrena* mean that we can not rule out the possibility that they are not different than the effect of self-pollination or even wind pollination from nearby flowers. Comparing the data collected from the two species, it is likely that *Andrena* provides better pollination service considering that three of the four visits resulted in 7-18 pollen tubes at the base of the style, whereas the five single visits by *Apis mellifera* had zero tubes in all but one style (which had two), not making the visit significantly different from the controls.

4.4.3.2.1.1 Buzz pollination by *Bombus* species

Buzz pollination was observed at the field site, and was assumed to increase the chance of pollination success: pollen grains ejecting onto the body of the bee would make it more likely that pollen be caught in safe sites of the bee body, or that not all pollen would be groomed into pollen pellets between flower visits. This theory would suggest buzzing bees to have more pollen on their bodies, however how the sonication act might affect the act of pollen transfer can only be speculated on. In this study, when a *Bombus* sp. sonicated a flower, it was curled around the corolla aperture (to maximize pollen collection), thus this area showered with pollen was also close to the stigma of the flower. When *Bombus* individuals used this foraging behaviour, their pollinator effectiveness increased from those *Bombus* that did not. This difference was

measurable between the two groups for both the number of pollen grains deposited on the stigma and pollen tubes that subsequently grew to the base of the style.

The contribution of buzz pollination by bees to the actual pollination of a flower in a poricidal anther species evidently has not been measured previously. Studies have investigated pollen removal from anthers by flower sonication (Harder and Barclay 1994; Buchmann and Cane 1989), however not the direct number of pollen grains deposited on stigmas in association with the action. The non-poridical anther, buzz-pollinated species *Pedicularis chamissonis* was assessed for pollen deposition after visitation by *Bombus* species; it was found that both the number of visitors and the length of the buzzing period positively affected pollen deposition (Kawai and Kudo 2009). Unfortunately, the length of visit to the flower was the measurement in our study, rather than the number and duration of buzzes by bees over the flower visit.

Duration of visitation was not correlated with pollination effectiveness here and has also been demonstrated for other studies (Davis 1992, Caswell 2008). This outcome is not unexpected considering that different foraging behaviours (nectar versus pollen) may take varying amounts of time without necessarily increasing the chance of pollen adhering to the stigma. Even during visits by *Bombus* spp. exhibiting buzz pollination, the time spent on the flowers did not equate to a greater or lesser amount of pollen grains deposited ($p = 0.175$) or pollen tubes that grew ($p = 0.799$), though the correlation between time spent on flowers and number of pollen grains deposited was $r = 0.531$ (Fig 4.9 c). It has been shown that handling times increased with "virgin" flowers of another poricidal species (*Solanum elaeagnifolium*) versus those with anthers that had been plugged or previously emptied with a tuning fork (Buchmann and Cane 1989), suggesting that if sonicating bees are obtaining pollen from the flower, they will continue to spend more time attempting to gather more pollen. Whether the flower is better pollinated may be incidental to the plant rewards and the method of obtaining them, rather than strictly measuring a visit's duration.

4.4.3.2.2 *V. vitis-idaea*

The SIV numbers obtained for *V. vitis-idaea* were low and consequently it is uncertain how accurately they demonstrate what is happening at this site. If generalities are made, it would seem that Syrphidae are not likely to be pollinators, whereas *Bombus* spp. visits had a number of pollen grains on the stigma (8-44) and pollen tubes at the base (2-12) making them the best pollinator candidate. Davis *et al.* (2003) inferred pollinator potential from the number of pollen grains removed from the bodies of flower visitors to *V. vitis-idaea*. No fly or wasp had more than

64 tetrads found on its body, whereas bees had from 2 to > 3000 tetrads. This result would also indicate that the potential of hoverflies as pollinators is low and that of bees (*Andrena* spp., *Apis mellifera* and *Bombus* spp., in their case) is high.

However, in the case of *V. vitis-idaea*, there seems to be an unknown quality at work. It would appear the investigators facilitated self pollination as the Control 1 flowers had a higher pollen deposition mean than the SIVs. The SIV flowers, however, were also carefully unbagged and rebagged, and so went through the same procedure as the Control 1 flowers, plus an insect visitation. The Control 2 flowers did not test differently from that of the SIV group, but at the same time do not show any pollen tubes at the base. Control 1 flowers and Control 2 flowers were sampled in the same areas and over the same time periods. They were generally in sheltered areas that would not be overexposed to the wind, nor was the wind measured by the weather station greater during that time period than what was experienced over the field season. In conclusion there is neither a large enough sample size, nor a clear understanding of the self-pollination capabilities of this species, to sufficiently understand the high stigmatic loads of pollen obtained in the controls of *V. vitis-idaea*.

4.4.3.3 Effectiveness of pollinators carried through to potential seed formation

4.4.3.3.1 *V. myrtilloides*

The number of ovules found for *V. myrtilloides* in Saskatchewan ranged from 60-104 (n=8). Therefore the mean *Bombus* visit (24 pollen tubes at the style base) would potentially fertilize 33% of the mean total number of ovules per flower (72.5; Table 2.3) found in *V. myrtilloides* from Saskatchewan. The mean number of pollen tubes at a *Bombus* spp. visit is higher than what Vander Kloet and Hall (1981) reported to be the mean seeds per berry: 12 ± 10 (no sample size reported). *Andrena* spp. had a mean of 10.5 (range 0-18, n = 4) pollen tubes reaching the base of the style, meaning roughly 7% of ovules could be fertilized after a single flower visit. For *Apis mellifera*, the numbers were so low (range 0-2, n = 5) that not even 1% of the ovules would be potentially fertilized. The single visits of both *Coelioxys sodalis* and the *Lasioglossum* sp. resulted in 21 and 18 tubes at the base, respectively. This result means that potentially both species show the same pollinator potential as the *Bombus* species. Interestingly, both the visit by an *Osmia* and a *Colletes* bee had no pollen tubes at the base, though both had a sizable amount of grains deposited on the stigma. From our results, it would take 3-4 visits by

Bombus spp. (and potentially *Colioxys sodalis* and the *Lasioglossum* sp.) to potentially fully fertilize the mean number ovules present in the flowers of *V. myrtilloides* (see Table 4.10).

Overall, in single insect visits that resulted in pollen tubes at the style base (n = 18), the numbers were 9% of the grains found on the stigma. For Control 1 flowers, only 5 had pollen tubes at the style base and this was a mean of 5% of the deposited grains on the stigma. For Control 2 flowers, there were only 2 that had tubes at the base, with a mean of 5% of the pollen grains on the stigma.

4.4.3.3.2 *V. vitis-idaea*

The mean ovules in *V. vitis-idaea* flowers are 46 (n=4, Table 2.3) for the Saskatchewan field site. The mean *Bombus* sp. visit (n =4) resulted in 5 pollen tubes at the base of the style. Therefore, one *Bombus* spp. visit could potentially fertilize 11% of the mean number of ovules per flower in *V. vitis-idaea* at the field site. It could theoretically take up to 9-10 visits by *Bombus* spp. bees to fully fertilize the mean ovule number in *V. vitis-idaea* (Table 4.10). The Syrphidae did not have any pollen tubes at the base of the style as a result of flower visitation, nor did the sole *Andrena* sp. visit.

Control 1 flowers had 3 flowers with pollen tubes at the base of the style, a mean of 4.6% of pollen grains deposited on corresponding stigmas. For Control 2 flowers, there were no pollen tubes found at the base of the style for any of the ten flowers.

The improbability of getting a fully fertilized fruit in a natural setting corresponds with work done with *V. vitis-idaea* in Finland. Nuortila *et al*. (2002) reported a mean ovule number per flower of 31.8 ± 1.3 (n = 20) for *V. vitis-idaea*. The seed set in open-pollination flowers at their site had a mean of 14.2 ± 2.3 seeds per fruit, or around 50%. If related to our *Bombus* spp. pollen tube numbers, those flowers would have had about 6 *Bombus* spp. visits, though sample size is low for *Bombus* spp. visiting *V. vitis-idaea* flowers.

Table 4.10 Ratios of pollen tubes that grew to the base of the style as related to both the pollen grains deposited on the stigma, and the mean number of ovules for that *Vaccinium* species, in single insect visitations by flower visitor groups and individuals.

	Pollen Tubes: Pollen Grains	Pollen Tubes: Ovules	Sample size
V. myrtilloides		Mean ovules: 72.5	
Apis mellifera	1:262	1:181	n = 5
Andrena spp.	1:23	1:7	n = 4
Bombus spp.	1:15	1:3	n = 15
Coelioxys sodalis	1:10	1:3.5	n = 1
Lasioglossum sp.	1:11	1:4	n = 1
V. vitis-idaea		Mean ovules: 46	
Bombus spp.	1:6	1:8.8	n = 4

Visitors groups or individuals that had no pollen tubes at the base of any of the flowers could not be compared and are not included in this table.

5 General discussion and conclusions

Vaccinium myrtilloides and *V. vitis-idaea* are two members of the very successful and widespread family Ericaceae. The pollination strategy of both species involves flowers offering two different rewards: pollen, through a specialized pollen release system, and nectar, at the flower base. Due to the campanulate nature of the corolla, observations about which reward was sought by different taxa was not always possible. Nonetheless, undoubtably some taxa could not buzz or otherwise remove pollen from the poricidal anthers and thus visited flowers solely for nectar without pollinating the flower. That there is a host of 'nectar thieves' in this system is likely. This larceny (Inouye 1980) has not seemed to be detrimental to these plant species however, considering their successful range, and the obvious success of other ericacean plants with the same rewards.

In particular, these *Vaccinium* species are interesting in that the bell shaped corolla acts as a microclimate, and create a higher humidity environment that while useful to regulate nectar sugar concentration, would likely have a detrimental effect on pollen release from poricidal anthers, which is critical for successful sexual reproduction. Interestingly, the presence of 'pollenkitt' seen on the pollen of *V. myrtilloides* and *V. vitis-idaea*, is not thought to be associated with poricidal anthers either (Buchmann 1983). Perhaps a dry microclimate is less critical for pollen release from poricidal anther than previously thought or that pollenkitt facilities more pollen release within a high humidity system.

Although this study has investigated basic morphology and anatomy of poricidal anthers and pollen, further work should be done to confirm pollenkitt in these species, and investigate the release of pollen under different temperature and humidity levels to determine the trade off between nectar and pollen release. Also the question of what the anther wall is composed of is still unanswered, and confirming either the presence or non-presence of cuticle, or other materials, may explain how the single – cell layered anther is strong enough to act as a vibratory receptacle while keeping pollen protected and viable.

Additional work is also needed on nectar secretion dynamics. If nectar production in these two *Vaccinium* species truly increases with flower age as demonstrated in other studies, it was not clearly seen amidst all the conflicting environmental factors that were not controlled for. Likewise, other studies (Jacquemart 1992; Rajotte and Roberts 1979) on *Vaccinium* state that nectar production per flower increases with date, or as the flowering season progresses. This

possibility would be very interesting to investigate for other *Vaccinium* species such as *V. myrtilloides* and *V. vitis-idaea*. Could increasing nectar production make the final flowers of the season the most attractive to increase their chances of pollination? Also in terms of nectar secretion, the technique of nectar sampling should be re-evaluated and other methods tested so that the amount of nectar solute is not grossly underestimated. This is work for the field of nectar study, not necessarily these species in particular, but for all small nectariferous flowers.

Relating floral nectar secretion to characteristics such as the number of stomata has not been studied often in the past (Davis 2000). The large range of stomata seen in the six flowers of both *V. myrtilloides* and *V. vitis-idaea*, combined with the large variation in nectar solutes measured per flower for both species, raises the question: is stomatal number on the nectary surface related to nectar production capacity by that particular flower?

Attempting to distinguish insect visitors from insect pollinators has not been previously attempted for these two species. Although time consuming, it is important to confirm that not all flower visitors are pollinators and indeed the visitors one may suspect as being pollinators, may not be pollinating. In the case of hoverflies (Syrphidae), it would seem that they are not dependable pollinators, even though they routinely visited flowers of both species, and in particular *V. vitis-idaea*. Honeybees (*Apis mellifera*) were shown to provide a poor pollination service to *V. myrtilloides* in terms of number of pollen tubes that grew to the base of the style. We suggest this poor pollen tube growth could be partly due to the effect of the warm hive conditions on any pollen previously on the bee's body, but is also likely due to their apparent inability to buzz poricidal anthers and extract large amounts of pollen easily. Honeybees are routinely used to insure fruit set of commercial blueberry operations, however fruit set does not necessary equal maximum pollination as even complete selfing has been shown to produce some fruit in both species (Guillaume and Jacquemart 1999; Hokanson and Hancock 2000). This study would indicate that visits by *Apis mellifera* - though perhaps facilitating adequate fruit set – might result in low seed set not likely to contain a large amount of genetic diversity.

Andrena spp. were likely a pollinator of *V. myrtilloides* although whether they can buzz pollinate is still unproven. In agreement with our result, *Andrena* spp. had the second highest mean pollen deposition after *Bombus* queens on closely related *V. angustifolium* in Nova Scotia (Javorek 2002). Other single visit bee taxa to *V. myrtilloides* (*Coelioxys, Colletes, Lasioglossum, Osmia*) showed high amounts of pollen deposition, though not necessarily pollen tube growth.

Without more samples it is difficult to tell if the lack of pollen tube growth was due to the source of the pollen, or simply non-receptivity of the particular flower. It may be that these small numbers of solitary bees are very successful pollinators of *V. myrtilloides* as the number of pollen grains deposited would suggest. Again it is not known whether these small bees buzz pollinated the flowers in questions. Future work should include a method of recording audio during a visitation to be analyzed for increased frequencies typical of buzzing.

Bumblebees (*Bombus* spp.) were frequent visitors, exhibited discernable buzzing behaviour, and were effective pollinators of *V. myrtilloides* and likely *V. vitis-idaea* in terms of effective pollen transfer. That bumblebees were pollinators was not unexpected for either *Vaccinium* species, but has now been confirmed. Interestingly, they did not always buzz the flowers they visited, even though all flowers used for SIV were first day anthesis flowers. It is assumed that they used these flowers for nectar alone. This difference in foraging behaviour allowed us to test if the behaviour of buzzing flowers had an effect on the pollination of the flower. That a significant difference in loads of tetrads deposited on stigma occurred between the flowers bumblebees buzzed and those they did not implies that the action of buzzing increases flower pollination or that past buzzing (or not) of the bumblebee increased its success as a pollinator.

One aspect of pollination that this study did not address was the possibility of nocturnal pollination. As nectar was present in the flowers at night, and the corollas do not close, it is possible that moths could visit the flowers for nectar. Davis *et al.* (2003) did not record nocturnal visitors to the flowers of *V. vitis-idaea*, however they did record moth visits, as we recorded a butterfly visit during our surveys. Thus although night visitations could occur, it is not expected that nocturnal visitors would facilitate a high amount of flower pollination.

The flowers of both *Vaccinium* species studied in Saskatchewan, and all other *Vaccinium* whose P/O ratios were calculated in this study, are entomophilous in nature, possessing the same two floral rewards. Further knowledge of the mating system of the plants would expect to come from P/O ratios as the index generally allows for an estimate of a species' tendency towards self- versus cross-pollination (Cruden 1977, 2000). There was a wide range in P/O ratios between *Vaccinium* species, as well as intraspecifically with location, as shown by *V. angustifolium* within Nova Scotia and with *V. vitis-idaea* between Nova Scotia and Saskatchewan, that also contrasts with the number of pollen grains and ovules reported in Belgium (Jacquemart 1997). Further P/O

ratio work is needed to determine if these changes in P/O ratios also correspond with changes in the breeding system of a specific species.

It would appear that the pollination of both *V. myrtilloides* and *V. vitis-idaea* is not as simple as most buzz pollination systems in other plant families, likely due to the presence of a nectary. The possible tradeoff between nectar production and the poricidal anther system deserves more attention. Why it persists in Ericaceae, but is found almost nowhere else in the plant kingdom, is interesting from an evolutionary perspective. The structure and dynamics of the anthers also require more investigation, including the role of anther spurs within *Vaccinium*, and tubules as that structure is unique to the subfamily Vaccinioideae. The compilation of P/O ratios done in this study on a number of *Vaccinium* taxa could indicate their limited usefulness in predicting the breeding system of the genus perhaps due to the increase in pollen units (tetrads). Contrary to this possibility, perhaps specific local adaptations that may drastically change this traditional indicator of breeding systems reflect a change in the breeding system of the plant itself, as it adapts to low or high availability of insect pollinators.

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