AN INVESTIGATION OF HIGHBUSH BLUEBERRY FLORAL BIOLOGY AND REPRODUCTIVE SUCCESS IN BRITISH COLUMBIA

by

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Abstract

Vaccinium corymbosum (highbush blueberry) is and economically important crop in British Columbia and suffers from inconsistent and often inadequate pollination by the managed pollinator, the honeybee. The outcrossing strategy of blueberry requires a pollen vector; however honeybees are inefficient and choosey. One goal of this research was to investigate the variable attractiveness of blueberry flowers to pollinators, specifically focusing on odour, and the possibilities for improved pollination. Highbush blueberry flowers emit a wide range of volatile compounds that show heritability in the broad-sense. However, determining which compounds are important to pollinators is a challenging task and remains poorly understood. Pollinator choice was examined through monitoring of pollinators as well as determination of pollen movement through paternity analysis. There was no agreement between the analyses, which show that the genotypes Duke and Reka appear to attract more pollinators but the most common fathers are Bluecrop and Elliott.

A second goal of this research was to examine reproductive success; and the discrepancy between attraction and reproduction could be due in part to the range in fertility observed among highbush blueberry genotypes. Inbreeding depression due to the use of a narrow breeding pool to develop genotypes is a potential cause of the observed range in fertility and offspring vigour. The degree to which inbreeding will increase homozygosity depends on the inheritance pattern of the tetraploid highbush blueberry; however, the current levels of inbreeding limit an accurate description of the inheritance pattern.

Preface

The blueberry floral volatile samples were run on the Gas Chromatograph Mass Spectrometer using a protocol developed by Lina Madilao in the Mass Spectrometry Laboratory established by the Michael Smith Laboratories and the Wine Research Center at UBC. The blueberry microsatellite markers were amplified and genotyped in the Genetic Data Centre at UBC under the guidance of Carol Ritland.

I completed experimental design, sample collection, and data analysis. As of yet, no part or combination of this research has been published. However, manuscripts are planned as following:

1.) Chapter 2 plus Chapter 3 (part 1)
2.) Chapter 4 plus Chapter 3 (part 2 monitoring experiments)
3.) Chapter 5

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Of course, a special thanks are owed to my family and friends, who have been a constant source of support throughout my years of education.

Dedication

I dedicate my dissertation work to Ron Greven for his incredible support and encouragement during this process.

Chapter 1: Literature Review

1.1   The Agroecological Approach

Agroecology as an approach, uses ecological methods and theories to investigate agricultural questions. The recognition that agricultural systems are in fact ecosystems took hold in the early 20th century. Since then, Agroecology has shifted toward a focus on sustainable agriculture based on the assumption that natural ecosystems are sustainable (Gliessman, 1998). This paradigm is important because it may, very simply, make agricultural improvements easier and more cost effective. Altieri (1989) argues that focusing on only the technological aspects limiting sustainability in agriculture is problematic and agroecology can provide the guidance for appropriate technology development. Considering technology as support for the ecosystem features of the agricultural system can blend technology and the environment versus setting them as opposing forces. I have addressed the agricultural issue of inconsistent pollination using an ecological context by integrating life-history characteristics of *Vaccinium*, section *Cyanoccocus*.

The agricultural issue explored by this research is the inconsistent pollination and resulting inconsistent fruit set seen in highbush blueberry (*Vaccinium corymbosum, Ericaceae*). Bumblebees are the most effective pollinators of blueberry in British Columbia due to the crops' early flowering and poricidal anthers that are more effectively foraged through sonication (Stubbs & Drummond, 2001; Cane & Schiffhauer, 2003; Javorek *et al.*, 2002; Ratti *et al.*, 2008). However, large blueberry fields cannot rely on wild/native pollinators and require the use of managed honey bees (Issacs & Kirk, 2010). Achievable pollination improvements are limited due to floral phenology and morphology; therefore, it is important to improve honeybee activity on days when the weather is suitable. Blueberry genetic characteristics - polyploidy, and inbreeding – as well as floral phenotypic characteristics – floral morphology and odour - will be discussed below by detailing how these may impact reproductive biology and therefore agricultural production.

1.2 The Subgenus: Blueberry

The genus *Vaccinium* contains hundreds of species distributed worldwide, many of which have edible fruits. The most edible *Vaccinium* found in North America include blueberry, cranberry and huckleberry and belong to the sections *Cyanococcus*, *Oxycoccus*, and *Myrtillus*, respectively (Camp, 1942). Fruits from these sections are popular and have high levels of antioxidants (Prior *et al*., 1998) which have been shown to impart health benefits (*reviewed in* Tsuda, 2012). *Vaccinium* fruit production serves an additional economic benefit in some areas where soils are unfit for other agricultural production due to high acidity and poor drainage (Draper, 1977; Vander Kloet, 1980; Lyrene & Ballington, 1986).

The section *Cyanococcus* includes cluster-fruited blueberries and species from this section constitute the majority of agricultural production, followed by the section *Oxycoccus* (cranberry). Species in *Cyanococcus* are poorly delineated, with the total number of recognized species ranging from 9 to 15 species (Camp, 1945; Vander Kloet, 1988; USDA, ARS, National Genetic Resources Program, 2014), and a complete species classification may not be possible (Luby *et al*., 1991). The distribution of the blueberry section includes much of eastern North America with one diploid species (*V. myrtilloides*) overlapping the Northern interior boreal forest range (Vander Kloet, 1981; Vander Kloet, 1988). *V. corymbosum* (largely highbush blueberry) is distributed along the east coast of North America from Florida to Quebec and as far west as Texas and Illinois (Vander Kloet, 1980). *Cyanococcus* spp. exist at three ploidy levels, diploid, tetraploid, and hexaploid with some variation in distribution among the ploidy levels such that the tetraploid species' range overlaps most of the diploid range except for an extension north, whereas the hexaploid species are limited to the southeastern US (Vander Kloet, 1980; Luby *et al*., 1991; Hancock *et al*., 1995).

Vaccinium spp. in the *Cyanococcus* section take mainly two growth forms including the crown forming blueberries and the low-growing vine-like blueberries (Vander Kloet, 1988). For the most part, blueberries are very long-lived, with some colonies estimated to be over 1000 years old (Darrow & Camp, 1945). Thus, all commercially grown blueberries can generate long-lived plantings with vegetative reproduction occurring through rhizomatous spread or suckering

(Vander Kloet, 1988). For low growing blueberries, this rhizomatous spread allows for the common cultivation technique of using biannual burns to regenerate the crop. For the crown forming varieties, crop cultivation requires the pruning of older canes in an orchard-like system.

1.3   **Hybridization in Blueberry**

A particularly interesting life-history characteristic of blueberry is that within a sub-genus/section, hybridization among species of the same ploidy level is common such that in areas where species overlap 'hybrid swarms' are present (Camp, 1942). Ballington & Galletta (1978) found the intraspecific crossability of four diploid *Vaccinium* species to be significantly higher than interspecific crossability, however both interspecific and intraspecific crossability were higher than selfing. Vander Kloet (1980; 1988) proposed that *Vaccinium corymbosum* (highbush blueberry) is a genetically aggressive 'compilo-species' (Harlan & de Wet, 1963), made up of the diploid progenitors, *V. darrowii*, *V. tenellum*, *V. pallidum*, and *V. myrtilloides*. Vander Kloet based this theory on the phenotypic traits of *V. corymbosum* which include traits from each of the progenitor species. The long-lived nature of blueberries could also facilitate their hybridization as it would increase the number of contact points among different species (Darrow & Camp, 1945). Darrow and Camp (1945) theorized that the degree of hybridization of blueberry species seen today occurred after the habitat disruption of white settlers in North America. The six species in *Cyanococcus* recognized by Vander Kloet and their ploidy levels are listed in Table 1.1.

The lack of reproductive barriers among species of blueberries has led to the use and success of wide hybridization in blueberry breeding programs (*reviewed in* Hancock *et al*., 1995). More than half of the species in *Cyanococcus* contribute, in varying degrees, to five different commercially grown fruits: highbush, southern highbush, half-high, lowbush, and rabbiteye blueberries (Hancock *et al*., 1995). In fact, Hancock & Goulart (1993) have noted that breeders have 'obliterated genetic distinctions among species'. Unfortunately, the capacity for wide hybridization, ongoing taxonomic uncertainty, and open pollinations has led to gaps in cultivar pedigrees (Brevis *et al*., 2008). Highbush blueberry, derived from *Vaccinium corymbosum*,

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forms two cultivated products, northern and southern highbush groups. Although interspecific hybridization is possible, *V. corymbosum* provides the vast majority of genetic contribution to northern highbush cultivars 97%; while, for southern highbush cultivars, *V. corymbosum* is estimated to provide 72% with contributions from five to six other species (Brevis *et al*., 2008). *Vaccinium corymbosum* and *V. angustifolium* both contribute to the half-high blueberry cultivars which, as the name describes, are a shorter version of the highbush blueberry (Rabaey & Luby, 1988). Far fewer breeding programs involve lowbush blueberry cultivars, the majority of which are made up of *V. angustifolium*, with a small amount of *V. myrtilloides* (Lyrene & Ballington, 1986). Lastly the rabbiteye cultivars, the most southern blueberry, come from a single species; *V. corymbosum* as determined by Vander Kloet (1988) or *V. virgatum* (syn *V. ashei*) as determined by Draper (1977). Although some blueberry genotypes incorporate a diversity of species (Hancock *et al*., 1995), there is underused diversity in natural populations that could greatly enhance future breeding programs (Brevis *et al.*, 2008).

Table 1-1: List of species and their ploidy levels in Vaccinium section Cyanococcus (blueberries) as described by Vander Kloet (1988).

Species	Ploidy Level
V. darrowii	diploid
V. tenellum	diploid
V. pallidum	diploid, some tetraploid
V. myrtilloides	diploid
V. boreale	diploid
V. myrsinites	tetraploid
V. hirsutum	tetraploid
V. angustifolium	tetraploid
V. corymbosum	diploid, tetraploid, hexaploid

1.4   Polyploidy and Inheritance Pattern in Blueberry

The existence of natural polyploids in the blueberry section, *Cyanococcus*, is another interesting life-history characteristic and has been useful for breeding enrichment through hybridization (Darrow & Camp, 1945; Draper, 1977; Brevis *et al*., 2008). Camp (1942) theorized that primary

speciation occurs within the diploid level of the blueberries however, polyploids form a secondary tier of evolution. Unlike many polyploids, Vander Kloet (1988) reported no discernable phenotypic differences among ploidy levels of *V. corymbosum*. However, the existence of a diploid *V. corymbosum* is a point of disagreement in the literature, with some indicating that *V. corymbosum* is strictly a tetraploid, separate from the diploid progenitor species (Hancock *et al*., 1995), that *V. corymbosum* was a diploid progenitor of the tetraploid *V. australe* (Camp, 1945), or that both diploid and tetraploid are *V. corymbosum* (Vander Kloet, 1988). Commercial production relies on naturally occurring diploids and tetraploids for lowbush blueberry, tetraploids for highbush blueberry (including half-high types), and hexaploids for rabbiteye blueberry plantings.

Polyploidy complicates blueberry breeding due to uncertainty in the inheritance pattern which impacts both trait segregation and inbreeding. An intermediate inheritance pattern becomes a strong possibility when considering the compilo-species origin of *V. corymbosum* alongside the non-random behaviour of chromosomes. An intermediate inheritance pattern would have ramifications for breeding programs, making the use of traditional tetrasomic inheritance models inappropriate (Stift *et al*., 2008). Evidence of tetrasomic inheritance exists in blueberries but this evidence does not exclude the possibility of an intermediate inheritance pattern, where chromosomes do not behave consistently at meiosis and may show preferential pairing (disomic inheritance) (Stift, 2008). Qu and Hancock (1995) used RAPD markers to assess inheritance pattern in a *V. darowii* and *V. corymbosum* hybrid population and found 31 of the 101 loci examined conclusively tetrasomic. Using isoenzyme analysis with six loci, Krebs and Hancock (1989) found that highbush blueberry showed a tetrasomic inheritance pattern conclusively at three loci and they found no evidence for the fixed heterozygosity that indicates disomic inheritance. However, they did not find double-reduction which would conclusively indicate tetrasomic inheritance. Double reduction, a key indicator of tetrasomic inheritance, results in both sister chromatids transferring to the same gamete, and can only result from multivalent formation. Previous *V. corymbosum* karyotype analysis supports the evidence of very low multivalent formation where primarily bivalent formations were seen with few tetravalents and no univalents, trivalents, or pentavalents (Jelenkovic & Harrington, 1971).

1.5 Genetic Diversity and Inbreeding in Blueberry

Inheritance pattern influences the results of breeding including genetic diversity (*reviewed in* Bever & Felber, 1992). Breeding in blueberry is relatively recent beginning with highbush blueberry in 1909, followed by rabbiteye in the 1940s, and later by lowbush in the 1960s (Hancock *et al*., 1995). Highbush blueberry breeding began with wild selections collected by Frederick Vernon Coville of the United States Department of Agriculture and just three of these, Brooks, Soy, and Rubel, have contributed the majority to early breeding programs (Hancock & Siefker, 1982). Pedigree analysis shows widely varying inbreeding coefficients among blueberry genotypes, from F = 0 for wild accessions to as high as F = 0.25 for bred genotypes (Hancock & Siefker, 1982). Inbreeding coefficients have been calculated with diploid and tetraploid methods; however, an intermediate inheritance pattern would mean that both of these methods are incorrect.

Inbreeding research has largely focused on cultivated forms of blueberries; however, some researchers have surveyed natural populations. Bruederle *et al*. (1991) examined three diploid species within *Cyanococcus* one of which, *V. elliottii*, was thought to belong to the highbush blueberry complex. The four populations of *V. elliottii*, had observed heterozygosities from 0.066 ± 0.020 to 0.126 ± 0.042 for 18 allozyme loci. Further research by Bruederle and Vorsa (1994) on diploid blueberry populations included *V. elliottii* as well as two other species, *V. atrococcum* and *V. caesariense*, which have all been recognized as *V. corymbosum* by Vander Kloet (1983), who recognized diploid and tetraploid levels within highbush blueberry. Genetic identity analysis in this study showed *V. elliottii* as being distinct from *V. corymbosum*, which includes *V. atrococcum* and *V. caesariense*, and suggests a hybrid origin for *V. corymbosum*. The three diploid *V. corymbosum* populations investigated by Bruederle and Vorsa (1994) had observed heterozygosities ranging from 0.244 ± 0.066 to 0.308 ± 0.068, for 11 of the allozyme loci used by Bruederle *et al*. (1991). Both studies note that the populations were in Hardy-Weinberg equilibrium with a slight heterozygous excess, as expected from an outcrossing species.

Experimental self-pollinations in blueberries have shown reduced seed count, fruit weight, and seedling vigour. Hellman and Moore (1983) found that self-pollinations of five highbush blueberry cultivars resulted in decreased fruit size in one out of five crosses and reduced seed set in three of five crosses. El-Agamy *et al.* (1981) also found that fruit set was reduced from 82 to 67% and seed counts from 11.2 to 3.9 per fruit for cross- versus self-pollination. Self-pollination in half-high and rabbiteye blueberry cultivars also resulted in a reduced seed count (Rabaey & Luby, 1988; Hellman & Moore, 1983; El-Agamy *et al.*, 1981). Krebs and Hancock (1990) described the reduced seed number seen for inbred pairings in *Vaccinium* as early-acting inbreeding depression. Hokanson and Hancock (2000) further described the early-acting inbreeding depression by showing that self-pollen does fertilize ovules, but they are aborted before reaching maturity. Genetic load has been suggested as the reason for reduced inbred fertility by within individual correlations between seed set and pollen viability (Vander Kloet, 1983) and between self- and outcross-fertility (Krebs & Hancock, 1991). Most research has focused on early life-stage metrics, such as seed size, seed count, and germination rates; however, Lyrene (1983) examined seedling survival and vigour (height and shoot diameter) in rabbiteye blueberry cultivars and reported that inbreeding reduced both metrics. Given the evidence that blueberries exhibit at least some inbreeding depression, it is especially important to clarify the inheritance pattern in polyploids (tetrasomic, disomic, or a mixture of these). An intermediate inheritance pattern where the genetic effects of inbreeding (homozygosity increase) are variable among loci and/or individuals may mean that pedigree inbreeding coefficients should be estimated by a mixture of diploid and tetraploid coefficients.

1.6   Blueberry Mating System

The mating system of a plant population is roughly defined as the degree of outcrossing and is shaped by a balance of selection pressures for outcrossing versus selfing. Although many studies have examined fruit production using self- versus outcross-pollen; outcrossing rates, actual or functional, have not been reported for natural populations of blueberry. However, life-history characteristics of blueberry predict that it should be a predominantly outcrossing species with

mixed mating (including geitonomous self-fertilization) as a consequence of clonal structure and high flower number (Goodwillie *et al*., 2005).

Many floral features act on mating systems by limiting self-pollination and increasing outcrossing, such as protandry (Dai & Galloway, 2011), poricidal anthers (Harder & Wilson, 1994), and floral volatiles (Kessler & Baldwin, 2006). In blueberries, autogamous self-pollination is discouraged by the downward facing direction of the flower, the flute shape of the style, the protandrous phenology of the flower, and poricidal anthers (Vander Kloet, 1988). Blueberries do not have self-incompatibility mechanisms but some individuals do show reduced self-fertility, which is proposed to be due to genetic load (Hokanson & Hancock, 2000). Specialization of male versus female sexual function has been seen in lowbush blueberry, with negative association within clones (genotypes) in terms of pollen versus ovule production (Myra *et al*., 2004). However, the same specialization has not been found in highbush blueberry (Vander Kloet, 1983).

Genetic factors, such as hybridization, polyploidy, and inbreeding, can also influence the mating system. A complex relationship exists among these three genetic factors as they are not independent of each other, nor are they independent of the mating system. Polyploid development through hybridization or genome duplication has been seen to impact mating systems (Husband *et al*., 2008). Inbreeding can also impact mating systems through loss of heterozygosity and the rate of this loss depends on the inheritance pattern of the polyploid (*reviewed in* Bever & Felber, 1992). Inbreeding has been shown to effect a wide variety of plant traits important to pollination including a reduction in green-leafy plant volatiles in horse-nettle (Delphia *et al*., 2009), a reduction in the floral volatiles of *Cucurbia pepo* (Ferrari *et al*., 2006), and has been shown both to influence (Takebayashi & Delph, 2000) and not to influence (Thiele *et al*., 2010) floral morphology.

1.7   The Phenotype of Blueberry Flowers

Vander Kloet (1988) detailed blueberry floral morphology in 'The genus *Vaccinium* in North America'. Blueberry flowers are perfect, fairly small, and clustered on racemes, with fused, light-pink or white, corolla lobes that open downwards. *Vaccinium corymbosum* has 10 stamens with filaments attached at the base of the flower around the nectary. The fliaments carry a two-part poricidal anther with two tubules for pollen. The poricidal anthers of *Vaccinium* have especially important consequences for pollination as they moderate the dispensing of pollen through required sonication (Harder & Barclay, 1994). *Vaccinium* anthers hold on average 100-300 pollen tetrads per anther and therefore approximately 4000 to 12000 pollen grains per flower (Vander Kloet, 1988). *Vaccinium corymbosum* ovaries are divided into five locules that each contain approximately 25 ovules and the style grows straight from the centre of the nectary (Palser, 1961). Therefore, the pollen-ovule ratio of highbush blueberry is approximately 8 to 24 (1000/125 to 3000/125). Blueberries are protandrous with pollen release occurring before stigma receptivity (Vander Kloet, 1988).

Pollen provides the main source of protein for flower foragers; however, knowledge regarding dietary requirements of foragers is limited to honeybees. The protein content of blueberry pollen is 13.9% and below the recommended 20% for a healthy honey bee colony (Somerville, 2001). Pollen can have an odouriferous external lipid and protein coating called pollenkitt. If the pollenkitt has an odour that differs from that of the whole flower it may represent a signal of pollen reward (Dobson *et al*.; 1996 Dobson, 1991). Some Ericaceae have pollenkitt (Pacini & Hesse, 2005); however, it has not yet been described in blueberry.

Nectar represents a significant part of total floral odour in that it advertises sugar content, the energy source for flower foragers. Nectar is produced by the nectary found at the base of the blueberry flower with a reported maximum amount of 1.45 ± 0.39 mg per flower (Rodriquez-Saona *et al*., 2011). In order to make the production of nectar cost-effective, the plant must optimize pollinator visitation length and pollinator visitation number and reduce the incidence of nectar theft. Optimizing nectar production can take many forms, such as production pattern through the day (Rodriquez-Saona *et al*. 2011, Pleasants, 1983) or season (Pleasants, 1983), in

response to the weather (Pleasants, 1983), and in terms of the blend of compounds found in post-pollination nectar (Schiestl & Ayasse, 2001) and after herbivore damage (Kessler & Halitschke, 2009). Rodriquez-Saona *et al.* (2011) measured nectar production in highbush blueberry at two hour intervals throughout the day and found that nectar production was highest in the morning (9 and 11 am).

Pollinators are attracted to flowers by visual and odour cues. The flowers of blueberry genotypes are mostly white but differ by slight pink hues at the base of the flower and by flower size (there are no substantial UV patterns). Floral odour includes all scents produced by the corolla as well as those in the nectar and pollen, and therefore encompasses an array of signals communicated by the plant. Highbush blueberry floral volatiles have been described by Szendrei *et al.* (2009) and Rodriquez-Saona *et al.* (2011) who reported 34 and 28 compounds, respectively. The compounds belong to a range of chemical classes including: alcohols, esters, ketones, monoterpenes, sesquiterpenes, phenyl propanoid derivatives, and hydrocarbons.

1.8   **Blueberry Pollination**

The majority of the literature shows a benefit in increased seed set, fruit size, and/or decreased ripening time with outcrossed pollination (Gupton & Spiers, 1994; Harrison et al., 1993; Krebs & Hancock, 1991; Lang & Danka, 1991; Krebs & Hancock, 1990, Lyrene, 1989, Rabaey & Luby, 1988; Gupton, 1984; El-Agamy et al., 1981). Regardless of the pollen source (outcrossed or selfed), pollinators are important to the movement of pollen in both wild and cultivated blueberry systems because of the flower morphology. Attempts to categorize flower/pollinator relationships, such as the concept of pollination syndromes, have generally failed to adequately predict pollinators. However, some floral traits limit potential pollinators (Ollerton *et al.*, 2009). Blueberries fulfill the stereotype of bee-pollination (melittophily) due to the size of the flowers and the size, timing, and locations of the nectar and pollen rewards (*reviewed in* Waser, 2006).

Pollinators are attracted to flowers for many reasons, including pollen, nectar, oils, and by deceptive advertising of food sources or mating. Blueberry flowers offer honest rewards of

pollen and nectar, both of which contribute to the floral odour that communicates the reward. The down-turned flowers and the poricidal anthers of *Vaccinium* flowers create a challenge for self-pollination without vectors and for pollination by vectors that do not perform buzz pollination. Not all bee visits are equal in terms of success; a number of studies have compared pollinator effectiveness in *Vaccinium* systems. Of bees visiting *Vaccinium*, bumblebees (*Bombus* spp.) deposit the most pollen per visit (Cane & Schiffhauer, 2003; Javorek *et al*., 2002) and result in larger fruits (Ratti *et al*., 2008). In fact, Stubbs and Drummond (2001) found over a five year study that bumblebees were more effective pollinators of lowbush blueberry than honeybees in terms of percentage fruit set, percentage yield of harvested berries, berry weight, seeds per berry, and flower handling time. Investigations have also included wild pollinators in cranberry, lowbush and highbush blueberry production and found a significant pollination potential from the wild species (Cane & Schiffhauer, 2003; Javorek *et al*., 2002; Tuell *et al*., 2009). Issacs & Kirk (2010) demonstrated the necessity of commercial blueberry growers to manage large populations of pollinators and thus a dependence on honeybees and the need to incorporate wild bee conservation strategies.

1.9   **My Thesis Research Questions**

Increasing pollination services in an agricultural system requires an exploration of how and why pollinators are attracted to flowers. The relative importance of morphological and developmental traits to pollination and fertilization depend on the context surrounding the flower. The focus of this research thesis is *to investigate opportunities through which blueberry pollination might be improved by incorporating information on floral phenotype traits and pollinator behaviour as well as blueberry reproductive biology into blueberry breeding and management programs*. To address this broad research idea, the four research chapters of the research thesis focus on 1) variation in floral volatile production, 2) pollinator response to floral volatiles, 3) pollen movement in the field as an indicator of pollinator choice and genotype fertility, and 4) genotypic factors that influence breeding programs.

Whole flower odour is a complex volatile assemblage of chemistry from nectar, pollen, and floral organs that provides long-distance and localized information to pollinators. Chapter 2 aims to investigate the differences among the flowers of highbush blueberry genotypes and lays foundations for future research. An improved collection method was used to compare floral volatiles among genotypes and the broad-sense heritability of floral odour was investigated. The presence of heritable phenotypic variation provides evidence of breeding potential for these traits; however, the significance of the variation must be addressed. The third chapter of this thesis surveys previous bee bioassays for the compounds of interest identified in Chapter 2. As well, monitoring and bioassay techniques are used to examine pollinator choice. Pollinator choice is also investigated in Chapter 3 using paternity assessment, an indirect method to gather information about pollen movement. Fertility of genotypes is investigated to compare male and female fertility. Most common pollen donors and aspects that might guide pollen movement are discussed. The final chapter of the research thesis investigates genetic architecture issues that are important to blueberry production and breeding programs including the pattern of inheritance and levels of inbreeding in highbush blueberry.

Chapter 2: Variation and Heritability of Highbush Blueberry Floral Volatiles

2.1 Introduction

Pollinator attraction and activity are necessary for production of many food crops, especially those that have morphological, developmental, or genetic constraints limiting self-pollination and fertilization. *Vaccinium corymbosum*, highbush blueberry, has constraints to autogamous self-pollination in the form of morphological characteristics including poricidal anthers, flower orientation, and herkogamy, and the developmental constraint of protandry (Vander Kloet, 1988). Highbush blueberry also has a genetic constraint to self-fertilization in the form of early-acting inbreeding depression (Hokanson & Hancock, 2000). Pollinators are an important part of the blueberry agricultural system due to these constraints and managed pollinators are recommended for large production areas (Eaton & Murray, 1997; Isaacs & Kirk, 2010). Pollinator attraction is a concern in highbush blueberry production systems because the most commonly managed pollinator, the honeybee, has been shown to be a comparably inefficient pollinator of blueberry (Javorek *et al*., 2002) and exhibits varietal preferences within blueberry (Brewer & Dobson, 1969; Courcelles *et al*., 2013). Pollinator attraction has not been considered thus far in blueberry breeding programs, likely due to the complexity of answering the following questions: (1) is there heritable variation among genotypes, and (2) is this variation important to pollinators?

In this chapter, I endeavour to answer the first question regarding the potential to incorporate selection for floral volatiles in highbush blueberry breeding programs. Pollinators are attracted to flowers by both visual cues, including colour, shape, and pattern, as well as odour cues. Blueberry flowers present 'honest rewards' of both nectar and pollen and floral odours could cue those rewards. Pollen odour has been shown to drive pollinator selection of flowers (Dobson *et al*., 1996; Pernal & Currie, 2002; Ashman *et al*., 2005) and pollen nectar has been shown to absorb hydrophilic compounds produced by flowers, however less is known about the corresponding response of pollinators (Raguso, 2004). Recently, Knauer and Schiestl (2015) reported on a relationship between a floral volatile (phenylacetaldehyde) and the amount of floral

reward (nectar and pollen). They also demonstrated the possibility to develop a corresponding preference for that compound by the pollinator (*Bombus terrestris* (L.)).

In order to assess the breeding potential of highbush blueberry floral volatiles, the mixture must be described and variation characterized among individuals. Szendrei *et al*. (2009) and Rodriquez-Saona *et al*. (2011) conducted investigations of blueberry floral volatiles and collected complex mixtures of 34 and 28 compounds, respectively, from a range of chemical classes including: aliphatics, monoterpenes, sesquiterpenes, phenyl propanoid derivatives, and other hydrocarbons. Damaged and undamaged highbush blueberry plant material have also been investigated and a variety of aliphatic compounds, monoterpenes, sesquiterpenes, and phenyl propanoid derivatives were reported (Rodriquez-Saona *et al*., 2009). Interestingly, the floral studies reported compounds not found in the damage-induced studies; the key compounds from the floral studies being the aliphatics, 2-heptanone and 2-undecanone, the monoterpenoids, limonene and ocimene, and the phenyl propanoid derivative, cinnamyl alcohol (Szendrei *et al*., 2009; Rodriquez-Saona *et al*., 2011).

The diversity of volatile compounds depends on the machinery of several biosynthetic pathways and therefore the action of many genes (*reviewed in* Dudareva *et al*., 2013). The monoterpene and sesquiterpene compounds are derivatives of the isoprenoid pathway; the phenyl propanoid (benzenoid compounds) contain a benzene ring which is derived from the amino acid phenylalanine through the shikimic acid pathway; and the aliphatic compounds are derived through a less-understood system involving fatty acids and the lipoxygenase pathway. Although information is growing rapidly, studies of metabolic pathways or genetic control of plant volatile pathways are restricted to relatively few species and are not currently useful for multiple trait cost-effective breeding programs.

Estimates of heritability have limited applications because each estimate is specific to a population in time and location (Allard, 1960); however, it is a primary step towards understanding the genetic control of a trait. For example, if repeated studies of heritability in different populations and environments consistently find significant heritability in a trait, this suggests that the trait has the potential to respond to selection in a breeding program. Family-

level variation in volatile profiles have been described in rose flowers (Cherri-Martin *et al.*, 2007), wild gourd flowers (Ferrari, 2006), blackberry fruit (Du *et al.*, 2010), blueberry fruit (Hirvi & Honkanen, 1983), strawberry fruit (Olbricht *et al.*, 2008), and green-plant material in horsenettle (Delphia *et al.*, 2009). For the fruit assessments, offspring often have levels of compounds intermediate to parents but this is variable among compounds (Du *et al.*, 2010; Hirvi & Honkanen, 1983; and Olbricht *et al.*, 2008). In addition, Rowan *et al.* (2009) reported high narrow-sense heritability estimates for esters and alcohols in apple fruit.

Breeders aim to find improvements to yield, both in fruit volume and consistency, across all aspects of crop life history; the presence of genetic variation provides the possibility for this improvement. Highbush blueberries, *Vaccinium corymbosum*, are bred in a traditional pedigree format with vegetative propagation of each cultivar (genotype) for commercial use. The purpose of this study was to examine the profile of volatile compounds in ten genotypes of blueberry and to estimate the environmental versus genetic effects that form the basis for estimating broad-sense heritability.

2.2 Methods

LOCATION OF COLLECTIONS: The majority of blueberry sample collections took place at the Agriculture, Agri-Food Canada (AAFC) research station located in Abbotsford, BC. The Abbotsford plot held fifteen genotypes, a mix of breeding and commercialized stocks, aged approximately seven years at the time of sampling. Ten of the 15 genotypes were planted with five replicates and those genotypes were used for this study. The replicated genotypes included: A-287, A-246B, A-98, Aurora, Bluecrop, Duke, Elliott, Liberty, MSU-60, and Reka. The analysis also included floral collections obtained from offspring of selected blueberry crosses located at the AAFC Agassiz, BC research station. The third location of floral collections, 'Onnink's Blueberry Farm' in Abbotsford, held the cultivar Brigitta. In 2011, volatile method

optimization was performed at the University of British Columbia (UBC) farm using the cultivar Reka.

FLORAL MEASUREMENTS: Floral size measurements were made in order to examine factors that are known to impact pollinator choice in blueberry (Courcelles *et al*., 2013). Highbush blueberry flower clusters were collected haphazardly from both sides of the shrub at mid-height, the flowers were removed from the branches, and a subset of flowers were randomly selected from the group for measurements. Blueberry floral measurements recorded in 2010, 2012, and 2013 included corolla length (n=10), diameter of corolla at widest area (n=10), and diameter of corolla opening (flower throat) (n=15 and n=6). Floral cylinder volume was calculated using the length of the corolla and the diameter at the widest portion. Approximate volume and flower throat size were compared using an Analysis of Variance and a Post-hoc Tukey test for multiple means comparison using the statistical program 'R' (R core team, 2014).

PLANT TISSUE EXTRACTIONS: In both 2011 and 2012, plant tissue extractions were performed using, hexanes:diethyl ether (50:50) as the solvent (Fisher Scientific and Sigma Aldrich). In 2011, dissection of blueberry flowers in the field was completed using forceps and a razor blade and the organs were submerged in 1 mL of the solvent for 24 hours at room temperature. Five flowers were pooled for the extractions of the floral organs, anther, stigma, nectary, and one flower was used for the corolla extraction. Also in 2011, whole flowers at three growth stages (unopened bud, recently opened flower (day 1), and mature flower (stigma receptive)) were extracted in 2 mL of the solvent for 24 hours at room temperature, after which tissues were removed for dry weight determination. In 2012, I performed leaf extractions with 2 mL of the hexanes:diethyl ether (50:50) solvent including isobutylbenzene as an internal standard (17 µg/ml). The weighed leaf tissue was carefully rolled to prevent excessive damage into 4 mL amber vials with the solvent mixture and rotationally extracted for 48 hours at room temperature. All plant extracts were analyzed using the same Gas-Chromatography Mass Spectrometry (GC-MS) protocol as the plant volatile elutions described below. An injection volume of 1 µL was used for the floral organ and flower extracts, whereas an injection volume of 0.5 µL was used for the blueberry leaf extracts.

OPTIMIZATION OF VOLATILE SAMPLING: Development of the highbush blueberry floral volatile collection method spanned the two years prior to the reported collection. The first sampling trial in 2010, involved flowers cut into headspace vials with clamped lids and subsequent headspace analysis using Gas-Chromatography coupled to Mass Spectrometry (GC-MS) (Aglient Technologies) at the Mass Spectrometry Core Facility jointly run by the Micheal Smith Laboratories and the Wine Research Centre at UBC. This method detected a large amount of variation among genotypes but was not used further because the destruction of plant tissue through cutting could cause false positive results (Raguso, 2004).

In 2011, short and long-term volatile collections were made following the floral scent analysis primer, Raguso (2004), which consists of a flow-through system with oven bags (LOOK!, Terinex, England), low-flow vacuum pumps (SKC Inc.), and Poropak Q filters (SKC Inc.). Prior to anthesis, pollinators were excluded from the flowers by fixing mesh bags on the blueberry branches. When the majority of flowers opened on the branch, sampling took place by removing the mesh bag and fitting the branch with an oven bag, vacuum, and filter.

Short-term qualitative collections in 2011 involved leaving the oven bag securely tied around the flower branch for three hours then quickly collecting the accumulated volatiles onto the filter with a higher vacuum flow-rate. For long-term flow-through collections, the vacuum was set to a low flow-rate to ensure that air was gently pulled over the flowers, to maintain a constant pressure, onto the filter for several hours. At UBC farm, long-term flow through collection trials ran from 30 min to 8 hours in order to determine the optimum collection time.   The short-term collections yielded few compounds, whereas long-term collections of more than 5 hours yielded a wider diversity of compounds, with no difference between 7 and 8 hours. I eluted the filters with hexanes:diethylether (50:50) and analyzed the eluate using GC-MS. Quantitative determination was not possible in 2011 due to solvent evaporation while eluting filters.

VOLATILE SAMPLING: Floral volatile profiles of the three genotypes analyzed by Rodriquez-Saona *et al*. (2011) could not be attributed solely to genotype because volatile collections were separated by some days due to flowering phenology, thereby leaving the effects of 'sampling day' unaccounted for. To avoid this problem, the five replicate volatile samples for

each genotype were not collected on the same day and a staggered collection program was used to encompass effects of 'sampling day' (i.e., weather). Brigitta was an exception because that genotype had a different location than the other 10 and collection occurred on a single day. Two controls were collected each sampling day; a 'solvent control' to monitor potential contamination of the elution solvent and an 'environmental control' to monitor interfering volatiles at the sampling location. Because the floral samples contained both green plant material and flowers (as there are some leaves on the floral branch), it is impossible to describe compounds only found in the floral tissue without cutting the flowers. A 'vegetation only' control was collected from each genotype to distinguish volatiles produced only in flowers.

Volatile collections followed the primer provided in Raguso (2004) with some adaptations as follows. The selection of blueberry branches took place before bloom, at the red-tip stage, for mid-height branches with the highest number of flowers. After branch selection, I used exclusion bags made of polyester tulle fabric fixed to the branch with twist ties to prevent pollinator visits. Sampling occurred when a maximum number of flowers opened on the branch, at approximately 90% bloom. Volatile collection chambers were fixed around the flowering branch using 45 x 55 cm oven bags (LOOK!, Terinex, England) with a narrow opening around the branch at the bottom of the bag to allow air to flow through the bag. An opening at the distal end of the oven bag was secured around the filter and the filter attached to the pump, thus creating the air flow over the flowers and through the filter. The pumps (SKC Inc.) were calibrated each day to a flow rate of 5 ml/min and ran for seven hours between 10 am to 6 pm. This time period was chosen because few floral visitors were observed before 10 am or after 6 pm (G. Huber, data not shown). After the 7 hour collection period, the Porapak Q filters (SKC Inc.) were closed with caps and transported to the laboratory. To prevent solvent losses and provide accurate volumes for quantitation, 1 mL of the elution solvent was added directly to the Poropak beads in 2 mL autosample vials (Agilent Technologies). As in previous years, the elution solvent was made up of an equal mixture of hexanes (Fisher Scientific) and diethylether (Sigma Aldrich) prepared with an internal standard of isobutylbenzene (Sigma Aldrich) at approximately 2.3×10^{-5} g/mL. The eluant samples were stored at -20 °C until analysis with GC-MS.

GAS-CHROMOTOGRAPHY MASS-SPECTROMETRY: Sample analysis was conducted using GC-MS (6890/5973N Gas Chromatograph Mass Spectrometer, Agilent Technologies). An auto-sampler (7683, Agilent Technologies) delivered 1 µL of each sample (0.5 µL leaf extraction) to the GC in splitless mode which was then carried onto the 30 m x 0.25 mm x 0.25 µm DB-WAX column (Agilent Technologies) with helium as the carrier gas at a flow rate of 54.3 mL min^{-1}. Each, 43.17 min analytical run, included 3 temperature ramps as follows: from 40 °C to 85 °C at 3 °C min^{-1} , from 40 °C to 180 °C at 10 °C min^{-1}, and from 40 °C to 250 °C at 15 °C min^{-1} where the temperature was held for 10 min. All samples were ordered by sampling day and analyzed continuously in sequence.

A mixed set of standards was run before and at three points during the floral volatile sample sequence, including: caryophyllene (Aldrich), 1,8 cineole (Aldrich), alpha-pinene (Aldrich), beta-pinene (Aldrich), limonene (Aldrich), linalool (Fluka), myrcene (Aldrich), and cinnamyl alcohol (Fluka). The standard compounds were diluted in the same solvent mixture used for the samples then combined in a mixture to approximately 0.1 mM concentration for each. The standard compounds aided in identification of peaks separated by the chromatography of the floral samples and allowed monitoring of potential changes to the chromatography conditions over the course of the analyses. The NIST library identifications were recorded for the remaining peaks, with a criterion of over 90% certainty and a continuity of peak identification among the samples. The GC/MSD ChemStation software (Agilent Technologies) was used to quantitate all peak areas.

DATA PROCESSING AND ANALYSIS: The quantitated peak areas of the compounds were first normalized to the internal standard, isobutylbenzene, and the normalized values were multiplied by 1×10^{-3}. Compound peak areas were then eliminated from the analysis if the compounds were also found in the solvent controls samples. As a third step, to remove environmental background, the 'environmental control' peak areas were subtracted from the sample peak areas. Finally, the relative non-environmental peak areas were divided by the number of flowers in that sample to obtain an estimate of the per flower peak area for each compound. The equation used was as follows:

Relative Peak Area = (volatile peak / internal standard peak) x 1000 / # flowers in sample.

The resulting compound list included 55 compounds, 32 of which were present in the vegetative control samples. The compounds were sorted into six classes based on their main structural features as follows: aliphatic, branched, monoterpenoid, sesquiterpenoid, benzenoid/phenylpropanoid, and unknown compounds. Data analysis was performed on total peak area, an addition of relative peak areas, as well as on numbers and ratios of compounds per group.

An analysis of variance for individual compounds would have been inappropriate due to the lack of independence among volatiles with overlapping biosynthetic pathways, therefore total volatiles and volatile number were examined. The log-transformed total relative peak area (total volatiles emission) and total compound number were compared among genotypes using an analysis of variance (ANOVA) with a post-hoc Tukey test for multiple means comparison in the statistical program 'R' (R core team, 2014). To examine the effect of daily temperature (obtained from Environment Canada) on total volatile emission, a paired t-test was employed. Regression analyses were conducted in 'R' to examine relationships among floral characteristics including volatiles, floral volume and opening size, and number of flowers per branch.

Estimates of broad-sense heritability were calculated using the equation $H^2 = \sigma^2 \text{ genetic} / \sigma^2$ phenotypic, where phenotypic variation is the total variation within the population and the genetic variation is estimated by the difference between the environmental (clonal) variation and the total variation. Principle Component Analyses (PCA) have been used to investigate the similarity of plant volatile profiles within familial groups (Cherri-Martin *et al*., 2007; Delphia *et al*., 2009; Lee *et al*., 2010). I completed PCAs to look for family relationships and underlying patterns of variation among the blueberry genotypes using the statistical program 'R' ('R' core team, 2014). The PCAs included several of the volatile metrics: ratios of grouped compounds, numbers in compound groups, and peak area per group.

2.3   **Results**

VARIATION IN FLORAL SCENT PROFILES

Complex blends of floral volatiles consisting of 55 compounds from across all 11 highbush

blueberry genotypes were collected (Table 2.1). The total volatiles amount found to be variable

both within genotype (average $\sigma^2 = 48$) and across all genotypes. Variation was also seen in the

richness of volatile profiles (number of compounds), with genotype averages ranging from 7 to

30 compounds. Only 13 % (7) of the volatile compounds were common to all genotypes, these

being: 3-hexen-1-ol acetate, linalool, an isomer of bourbonene, benzaldehyde, cinnamaldehyde,

and both isomers of cinnamyl alcohol. Geometric isomers are common in floral odours

(Knudsen & Gershenzon, 2006) and in this study there were many volatiles for which two

isomers were detected: terpinene, ocimene, bourbonene, caryophyllene, cubebene,

cinnamaldehyde, cinnamyl alcohol, and cinnamyl alcohol acetate.

The volatiles were grouped by biosynthetic origin as mentioned above and the relative peak areas

for these groups (Table 2.1) were summarized in Figure 2.1. All compound groups were variable

among genotypes; however, the monoterpenoid group of compounds was interesting because for

some genotypes, it made up the majority of the volatile output. This was due to one

monoterpene compound in particular, myrcene which had strikingly high peak areas in some

genotypes. Myrcene made up the vast majority of the total volatile measure, contributing 47% of

the total peak area for all genotypes.

FLORAL-ONLY VOLATILES

One 'vegetation-only' sample was collected for each genotype and the combined profiles of

these samples included 33 of the 55 compounds recorded for the floral samples (Table 2.1). The

compounds unique to the floral samples (22) were distributed among the chemical groups as

follows: 1 aliphatic compound, 1 branched compound, 7 monoterpenes, 1 sesquiterpene, 9

benzenoids, and 3 unknown compounds (shown in bold, Table 2.1. Total peak area for floral-

only volatiles was related to total peak area of volatiles ($R^2 = 0.45$) and there were also

significant differences among genotypes for floral-only volatile production ($p = 2.6 \times 10^{-04}$).

Nonetheless, there are differences in the relative ratio of floral only to total volatiles: Duke had the highest floral-only volatile production, while Liberty and A 246 had the highest total volatile production (Table 2.2).

Table 2-1: Blueberry floral scent profiles of genotypes at the Agriculture Canada Station, Abbotsford, BC.
Compounds grouped by common structural characteristics shown as averaged (n=4 or 5) volatile peak area relative to the internal standard and the number of flowers per vacuum sample. Table includes '+' for volatiles observed in vegetation control samples as a group; compounds found only in floral samples are in bold.

RT (min)	NAME	A 98	A 246	A 287	Aurora	Blue-crop	Duke	Elliott	Liberty	MSU 60	Reka	Brigitta	Veg. Control
Aliphatic Compounds													
4.9	Hexanal*			0.02	0.01		0.01		0.08		0.02		+
8.3	**2-Heptanone***		0.01	0.05		0.06	0.66	0.03	0.07				
13.7	3-Hexen-1-ol, acetate*	0.09	0.09	0.11	0.13	0.04	0.21	0.17	0.19	0.07	0.22	0.19	+
16.6	3-Hexen-1-ol*			0.03	0.02	0.02	0.36	0.05	0.04		0.02	0.04	+
19.6	Butanoic acid, 3-hexenyl ester*								0.05				+
22.9	2-Undecanone*		0.05		0.02		0.06		0.08			0.09	+
Branched Compounds													
6.3	3-Methyl-1-butanol, acetate*	0.04					0.05						+
8.8	3-Methyl-3-butenyl, acetate*				0.05			0.07					+
12.8	**1-(3,3-Dimethyl-oxiranyl) ethanone***			0.01		0.01			0.03				
21.8	2-Methyl-6-methylene-1,7-octadien-3-one *		0.12	0.03	0.08				0.14	0.07		0.27	+
Monoterpenoid													
3.5	.alpha.-Pinene	0.14	0.18	0.18	0.05	0.20	0.94		0.25	0.09	0.09	2.60	+
3.6	**Thujene***		0.17				0.03						
4.4	Camphene*	0.05				0.05	0.09				0.01	0.04	+
5.6	.beta.-Pinene	0.06	0.11	0.13	0.08	0.08	0.44		0.17	0.18	0.03	0.46	+
6.1	Sabinene*	0.40	0.65	0.67	0.03	0.82	4.40		1.15	0.31	0.32	4.48	+
7.7	.beta.-Myrcene		11.00	4.36	8.12	0.04	0.32		10.37	7.81		17.96	+
8.0	**Terpinene isomer 1***						0.08				0.01	0.05	

Table 2-1: cont'd

RT (min)	NAME	A 98	A 246	A 287	Aurora	Blue-crop	Duke	Elliott	Liberty	MSU 60	Reka	Brigitta	Veg. Control
Monoterpenoid cont'd													
8.7	dl-Limonene	0.06	0.14	0.04	0.04	0.08	0.33		0.12	0.04	0.01	5.98	+
9.0	**Phellandrene***		0.30	0.10	0.21				0.17	0.19		0.51	
9.1	Eucalyptol	0.40		0.64		0.59	3.46		1.09	0.02	0.43	0.78	+
10.6	Terpinene isomer 2*		0.16	0.02		0.03	0.19		0.03			0.36	+
11.1	Ocimene isomer 1*		0.10		0.25	0.01	0.02	0.01	0.27	0.18		0.19	+
11.5	**Cymene isomer***		0.21				0.09					0.19	
13.4	4,8-Dimethyl-1,3,7-nonatriene*			0.03	0.16	0.09	0.39	0.07	0.28	0.08	0.02	0.19	+
17.5	**Ocimene isomer 2***		0.01	0.01	0.09				0.12	0.04		0.08	
21.6	Lilac aldehyde*			0.02					0.01			0.18	+
22.0	L-Linalool	0.04	0.12	0.09	0.06	0.03	0.30	0.07	0.12	0.05	0.01	0.18	+
24.3	**1-8-menthadien-4-ol***		0.10										
25.1	**2,6-dimethyl-1,5,7-Octatrien-3-ol***		0.14	0.02	0.09				0.13	0.07		0.21	
Sesquiterpenoid													
20.3	Copaene*	0.07				0.02			0.06				+
20.7	Bourbonene isomer 1*		0.01	0.01	0.01	0.04					0.01		+
21.0	Bourbonene isomer 2*	0.15	0.57	0.21	0.17	0.65	0.37	0.09	0.65	0.22	0.37	0.22	+
22.2	Caryophyllene isomer 1*		0.04	0.02	0.03	0.05	0.07		0.13	0.01	0.01	0.03	+
22.5	Cubebene isomer 1*		0.10	0.02	0.01	0.11	0.01		0.08	0.01	0.02		+
22.6	Caryophyllene		0.03	0.06	0.07	0.02	0.38	0.04	0.38	0.06		0.16	+
23.4	**Farnesene isomer 1***		0.29	0.08	0.11				0.25	0.12	0.01	0.40	
24.0	Farnesene isomer 2*						0.13						+
24.5	Cubebene isomer 2*		0.02		0.01				0.02	0.04		0.02	+
27.8	Caryophyllene oxide isomer 2*	0.03		0.01	0.02	0.04	0.09		0.07	0.01		0.02	+

Table 2-1: cont'd

RT (min)	NAME	A 98	A 246	A 287	Aurora	Blue-crop	Duke	Elliott	Liberty	MSU 60	Reka	Brigitta	Veg. Control
Benzenoid													
20.8	**Benzaldehyde***	0.17	0.10	0.21	0.07	0.15	0.29	0.22	0.12	0.07	0.34	0.29	
23.5	**1-Phenylethanone***				0.04	0.04							
24.7	**Acetic acid, phenylmethyl ester***	0.02			0.02	0.03	0.08	0.15	0.08		0.16		
25.3	Methyl salicylate*		0.03		0.00					0.00		0.02	+
26.8	**Cinnamaldehyde isomer 1***			0.04	0.01	0.06	0.05	0.03		0.04	0.02		
27.1	Benzyl nitrile*				0.03	0.01	0.17	0.02	0.10	0.01			+
28.1	**Cinnamyl alcohol, acetate isomer 1***	0.09	0.04		0.05	0.08	0.14	0.12	0.06		0.07	0.13	
28.3	**Cinnamaldehyde isomer 2***	0.05	0.01	0.02	0.03	0.14	0.26	0.14	0.06	0.13	0.11	0.09	
28.4	Benzenepropanol*	0.01		0.07	0.00	0.05	0.15	0.01	0.05	0.01	0.01		+
29.4	**Cinnamyl alcohol, acetate isomer 2***	0.02			0.02	0.06	0.12	0.08	0.04		0.02	0.02	
29.6	**Cinnamyl alcohol isomer 1***	0.11	0.05	0.14	0.07	0.20	0.50	0.16	0.08	0.10	0.14	0.25	
30.5	**Cinnamyl alcohol**	0.10	0.08	0.13	0.09	0.36	1.10	0.35	0.23	0.25	0.25	0.33	
Unknown													
18.3	unknown **		0.08										+
25.9	**unknown** **		0.01		0.01			0.01	0.01				
26.3	**unknown** **		0.08				0.01						
27.3	**unknown** **	0.02					0.01	0.01			0.00		

* tentatively identified using the NIST library and comparison of mass spectrum
** unable to identify based on mass spectrum and libraries
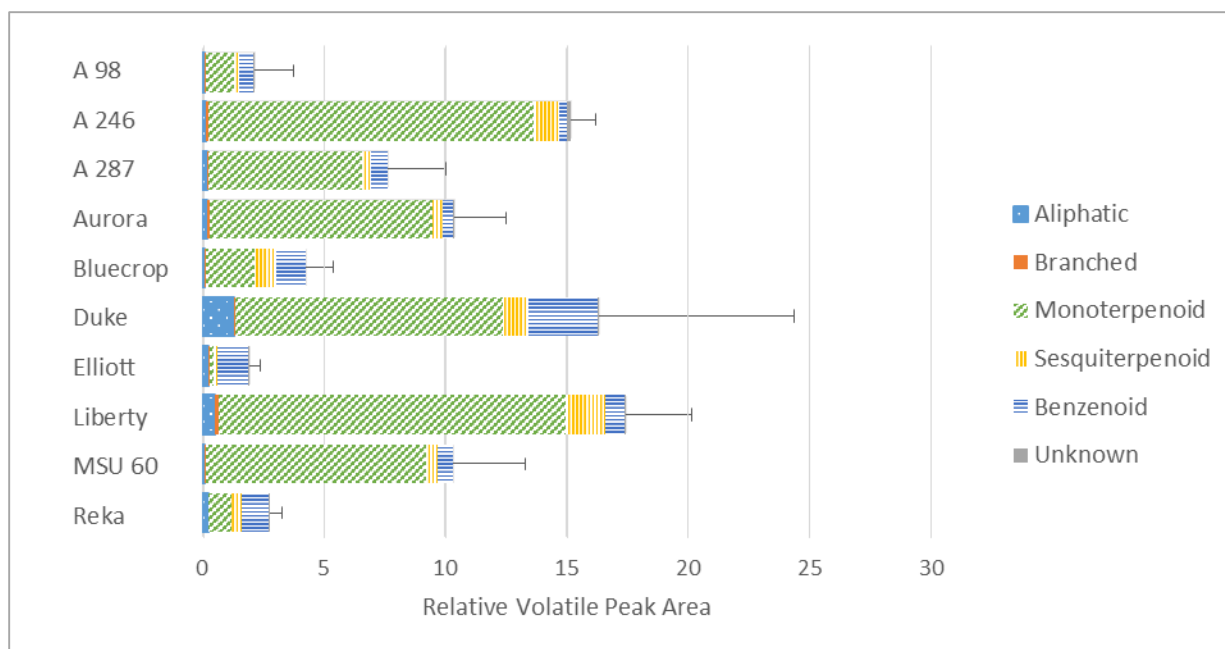
Figure 2-1: Average relative volatile peak area (total volatiles) including standard error for each genotype, total volatile bars are divided by compound class to summarize Table 2.1.

RELATIONSHIP AMONG FLORAL CHARACTERISTICS

Additional floral characteristics and statistical analyses are summarized in Table 2.2. The genotype Brigitta was excluded from some analyses due to missing data and because the replicates were collected on one sampling day and from a separate location. For the characteristics examined, the genotypes had overlapping ranges and the letters describing these overlaps were assigned using the Tukey analyses. Despite the fact that peak areas are adjusted for number of flowers per branch, flower number can impact the amount of volatiles collected during the analysis because volatiles must reach a critical volume before they are detectable. However, no relationship was found between number of flowers and total volatile peak area nor between number of flowers and number of volatile compounds. Aurora and MSU 60 had higher numbers of flowers per branch while A 98 and Brigitta had much lower numbers ($p = 1.2 \times 10^{-03}$). The genotype Duke had larger flowers on average and MSU 60 had smaller flowers in terms of both flower volume and throat diameter (flower size, $p = 8.4 \times 10^{-14}$, and flower throat opening, $p = 8.9 \times 10^{-16}$), Table 2.2. Regression analyses showed there are no relationships among the floral characteristics of number of flowers, floral size, and floral throat opening.

When compounds were grouped by biosynthetic origin, there were some relationships found among the groups. The significant adjusted R-square statistic values were: monoterpene and sesquiterpene ($R^2 = 0.30$, p = 1.2 x 10^{-5}), monoterpene / sequiterpene and branched ($R^2 = 0.66$, p = 6.9 x 10^{-14} / $R^2 = 0.26$, p = 5.6 x 10^{-5}), sequiterpene and aliphatic ($R^2 = 0.34$, 2.3 x 10^{-6}), and benzenoid and aliphatic ($R^2 = 0.63$, p = 7.6 x 10^{-13}). These relationships are reinforced by the relationship found between total volatiles and number of volatiles, $R^2 = 0.36$, p = 1.2 x 10^{-6}. This suggests that volatile metrics (total peak area, number of compounds, grouped peak areas) are all somewhat genotype dependent. There were some deviations from the assumptions required for the ANOVA including slight heteroscedasity seen in the residual versus fitted plots for total volatile peak area and floral-only volatile peak area showed some slight. As well, the samples sizes were too small for thorough assessments of normality. However, analyses of variance revealed significant differences among the genotypes in terms of total volatile peak areas, p = 1.4 x 10^{-06} and number of volatiles, p = 2.0 x 10^{-05}, with Liberty being among the highest and A 98 among the lowest for both metrics.

Table 2-2: Highbush blueberry floral characteristics of genotypes at the Agriculture Canada Station, Abbotsford, BC.
Traits measured included flowers per branch, flower size estimated by cylindrical volume (mm³, n=10), diameter of flower opening (throat) (mm, n=15 or 6*), genotype averages for grouped volatiles and total volatiles (measured as peak area relative to the internal standard and the number of flowers per vacuum sample) and number of volatile compounds (n=4 or 5). Shared letters indicate no significant differences based on the post-hoc Tukey analyses. Family relationships were obtained from Agriculture Canada and bloom time based on three years of observation (G. Huber).

	A 246	A 98	Reka	A 287	Duke	Bluecrop	Liberty	Aurora	MSU 60	Elliott	Brigitta
Family Relationship	US 239 x bluegold	G 1561 x US 75	E 118[1] x bluecrop	276-5 x G 303	G 100[1] x 192-8	GM 37[2] x CU 5	brigitta x elliott	brigitta x elliott	brigitta x elliott	burlington[2] x US 1	late blue x NA
Bloom Time	late April – May	early May	early May	mid May	early May	mid May	mid May	late May	late May	late May	late May – June
Flowers per Branch ± SE	64 ± 14 ab	34 ± 4.6 b	92 ± 16 ab	72 ± 6.6 ab	86 ± 20 ab	123 ± 18 ab	81 ± 6.1 ab	131 ± 11 a	126 ± 13 a	103 ± 38 ab	35 ± 3.5 b
Flower Vol. (mm3 ± SE)	422 ± 12 cdef	386 ± 15 cdefg	465 ± 22 bcde	451 ± 16 cde	630 ± 50 a	398 ± 35 cdefg	359 ± 21 cdefg	331 ± 20 defg	294 ± 17 efg	351 ± 16 cdefg	N/A
Flower Throat (mm ± SE)	4.36 ± 0.20# bc	N/A	4.19 ± 0.11 c	3.94 ± 0.07# c	5.03 ± 0.14 ab	4.31 ± 0.10 c	4.80 ± 0.08 ab	5.25 ± 0.11 a	3.75 ± 0.16# c	4.25 ± 0.09 c	N/A
Floral Only Volatiles	1.58 ± 0.12 ab	0.58 ± 0.34 b	1.13 ± 0.32 b	0.85 ± 0.28 b	3.40 ± 0.90 a	1.20 ± 0.20 b	1.45 ± 0.29 b	0.90 ± 0.17 b	1.02 ± 0.27 b	1.30 ± 0.32 b	N/A
Total Volatiles	15.1 ± 1.0 a	2.13 ± 1.6 c	2.73 ± 0.53 abc	7.62 ± 2.4 ab	16.3 ± 8.0 ab	4.24 ± 1.1 abc	17.4 ± 2.7 a	10.4 ± 2.1 ab	10.3 ± 3.0 ab	1.90 ± 0.42 bc	37.0 ± 7.6 N/A
Number of Volatiles	27.3 ± 1.4 ab	7.4 ± 3.3 c	16.4 ± 2.1 bc	17.4 ± 2.6 bc	25.6 ± 4.2 ab	22.8 ± 2.9 ab	30.6 ± 3.0 a	24.8 ± 3.4 ab	22.6 ± 1.8 ab	15 ± 1.5 bc	24.3 ± 1.9 ab

Note: 1 = individuals with shared parent, Earliblue, and 2 = individuals with shared parent, Pioneer.
         * = flower opening (throat) measured in 2010, others measured in 2013

ENVIRONMENTAL EFFECT ON FLORAL SCENT VARIATION

Some variation seen in the floral scent profile data is expected due to daily differences in environmental conditions. This environmental sampling effect can be approximated from the variation within genotype because there should be few, if any, genetic differences among clones. The Agriculture Canada station provided a relatively homogenous environment where the 10 blueberry genotypes were replicated in a blocked design with no apparent differences in soil type or moisture conditions. Over the sampling period, the weather information shows that temperature varied widely, from daily highs of 11 °C to 26 °C, and with precipitation, cloud cover, and wind speed also varying between days. Eleven out of the 14 sample days were cloudy, five of these had traces of rain, and the three clear days were not the warmest sample days. There was a positive relationship between temperature and both total volatiles ($R^2 = 0.23$, $p = 3.2 \times 10^{-4}$) and number of volatiles ($R^2 = 0.35$, $p = 4.7 \times 10^{-6}$).

The effect of temperature on total volatiles and number of volatiles detected was further examined using one-tailed, paired t-tests that showed a significant effect for total volatiles ($p = 3 \times 10^{-04}$) and for number of volatiles ($p = 9.5 \times 10^{-06}$). The genotype pairs included in the analysis were collected on days that had an approximately 5 °C spread in temperature, with the higher temperature collections having $3 \times$ greater total volatile peak area and $1.5 \times$ more compounds than the cooler weather collections. The genotypes with suitable pairs included, A 98, A 287, Aurora, Bluecrop, Duke, MSU 60, and Reka. An analysis of variance showed that there was a genotypic difference in temperature ($p = 7.5 \times 10^{-4}$) with genotypes flowering later in the season experiencing higher temperatures. This creates a bias towards higher volatile values for later flowering genotypes and prevents the use of temperature as a covariate in analyses of variance.

GENETIC EFFECT ON FLORAL SCENT VARIATION

Clonal replicates of each genotype allow for a partitioning of the environmental / experimental variation from the population variation. For all floral metrics examined, there is greater among-genotype variance than within-genotype variance, including the number of flowers per branch ($1.7 \times$), floral size ($2.2 \times$), floral throat size ($2.2 \times$), total volatile peak area ($1.5 \times$) and number of volatiles ($2 \times$). The broad-sense heritability (H^2) estimates below are calculated using the

within-genotype and among-genotype variances and are consistently moderate for the different volatile measures.

H^2 ratio of volatiles = (σ^2 total – average σ^2 clone) / σ^2 total
= (0.198 – 0.071) / 0.198
= 0.5

H^2 number of volatiles = (71 - 36) / 71
= 0.5

H^2 number of floral only volatiles = (8.6 – 4.2) / 8.6
= 0.5

Three PCAs were performed and they revealed a similar pattern in the variation of floral scent profiles among genotypes. Figure 2.1 is a plot of PC 1 and PC 2 from a PCA using ratios of volatile categories. Appendices A.1 and A.2 show the plots of PCs 1 and 2 from the analysis of volatile peak areas and of volatile compound numbers, respectively. For all PCAs, principal components 1 and 2 explain more than half of the variation among genotypes and were plotted to provide a visual description of the underlying pattern (Figure 2.1, Appendix A.1, and Appendix A.2). There were clearly evident groups in Figure 1.1 as follows: A 98, Bluecrop, Duke, and Reka versus Aurora, Brigitta, Liberty, MSU 60, and A 287 with Elliott and A 246 standing independently. As expected, the PC plot for number of compounds (Appendix A.2) showed clustering similar to the ratio analysis with two groups of genotypes as follows: Bluecrop, Duke, Elliott, and Reka versus Aurora, Brigitta, Liberty, MSU 60, and A 287 with A 98 and A 246 standing independently. These two PC plots show that the full-sibs, Aurora, Liberty, and MSU 60 grouped with the maternal parent Brigitta. The PC plot for the peak areas in each category (Appendix A.1) is more influenced by daily effects due to weather and was less similar to the above mentioned plots but has a higher amount of variation explained by PC1 and PC 2 (76%). Appendix A.1 does not include the genotype Brigitta and shows A 246, Liberty and Duke as a group separated from the other genotypes.
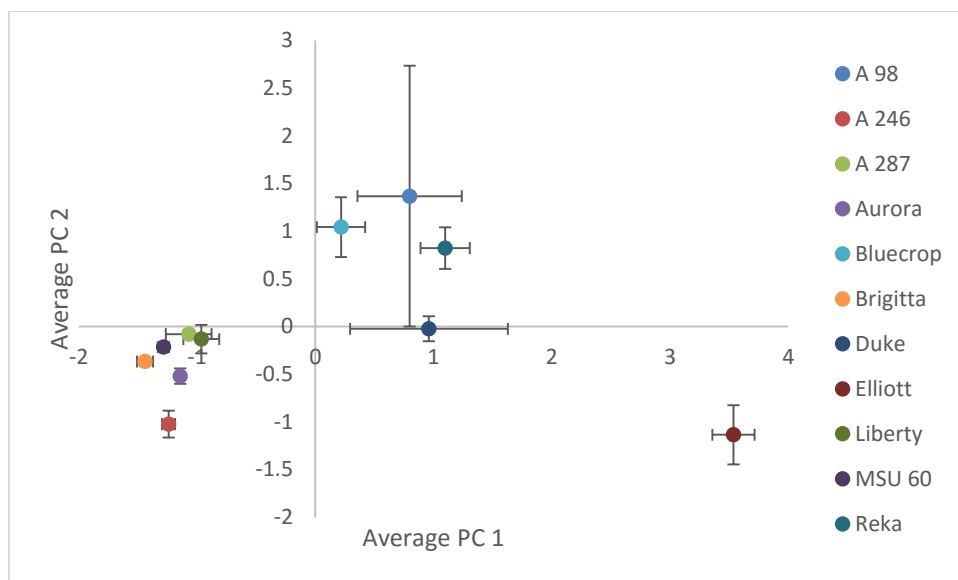
Figure 2-2: Plot of PC 1 and 2 from the PCA of ratio of grouped floral volatiles for the highbush blueberry genotypes sampled.
Standard error is shown with error bars for each genotype. PCs 1 and 2 explain 43% and 20% of variance, respectively.

2.4 **Discussion**

The purpose of this study was to look for evidence that floral volatiles have heritable variation that would allow the volatiles to be candidates for selective breeding. Large variation was found among genotypes in terms of floral volatiles, however, the heritability of the traits is not easily discerned due to high variability within genotype. The variability found among studies, among genotypes, and within genotypes is discussed with particular emphasis on a terpenoid, myrcene, as well as the floral-only volatiles.

HIGHBUSH BLUEBERRY SCENT PROFILES

Despite the fact that highbush blueberry flowers do not have a particularly strong odour to the human nose, they have a complex blend of floral volatiles. In the present study, more highbush blueberry floral volatiles were observed, 55, than in previous blueberry research by Szendrei *et*

al. (2009) and Rodriquez-Saona *et al*. (2011) who reported 34 and 28 volatiles, respectively (see Appendix 1.3). However, the prior analyses involved fewer genotypes, Duke, Bluecrop, and Elliott, and in the case of Rodriquez-Saona *et al*. (2011), less than half the collection time. The most substantial difference between the two previous studies and the present research is the reduced diversity of ester compounds reported here. The previous studies reported more than double the number of ester compounds, none of which were found in this study. Szendrei *et al*. (2009) and Rodriquez-Saona *et al*. (2011) reported only one benzenoid compound, cinnamyl alcohol; whereas 12, including cinnamyl alcohol, were found in the current study. The monoterpene and sequiterpene compounds recorded here were similar to those reported previously. The reason for the differences among studies is unknown but could be due to many factors including growing environment, sampling methods and elution solvent, as well as the GC-MS program.

THE CASE OF MYRCENE

The exceptionally high peak areas that I observed for myrcene, which made up more than half of the total volatiles for some genotypes, is particularly intriguing. High levels of myrcene were also observed during the two years of floral volatile method development (data not shown) and in vegetation-only control samples. The genotypes with disproportionate peak areas of myrcene included: A 246, A 287, Brigitta, Aurora, Liberty, and MSU 60. Relatively moderate levels of myrcene were recovered from Duke collections, low levels from Bluecrop collections, and no myrcene was found in volatile collections from Reka, Elliott, or A 98. Rodriquez-Saona *et al*. (2011) found moderate levels of myrcenone in the three genotypes that they studied (Duke, Bluecrop and Elliott). Myrcenone is structurally different from myrcene such that the methylene group has been replaced by oxygen. The difference in production of myrcene and myrcenone exemplifies the difficulty associated with assessing volatile production as a fingerprint, in that the genotype Elliott produced no myrcene in this study location during the three years examined but produced myrcenone in the previous work. Clones must be grown in a range of environments to determine how much plasticity there is around these highly variable traits and the biosynthetic machinery required to produce the volatiles.

ENVIRONMENTAL IMPACT ON GENOTYPIC VARIATION

Along with the variation among studies and research sites, the production of floral volatiles is variable among replicates within each experimental situation. Many plant traits are known to show plasticity with changing environmental conditions. As found in previous research (Gouinguene & Turlings, 2002), I also found that temperature impacted both the number and amount of highbush blueberry floral volatiles produced. Increased volatile production and/or release with temperature creates a bias towards collecting more volatiles from later flowering genotypes. The effect of temperature at bloom time has biological implications in that honey bees are known to be less active and not reach full foraging potential at temperatures less than 20 ℃ (Tan *et al*., 2012). Aside from temperature during collection, there could be minor differences in soil nutrient and water conditions among the replicates of the genotypes that contributed to the variance seen within genotype. Soil fertilization has been shown to increase production of some herbivore-induced plant volatiles (HIPVs) in maize (Gouinguene & Turlings, 2002) and phenyl-propanoid derivatives in woody plants (Koricheva *et al*., 1998). However, these results are not consistent across all plants, chemical groups, or fertilization regimes as fertilization was also seen to have no effect or to decrease volatile production (Gouinguene & Turlings, 2002; Chen *et al*., 2008).

Although no heavy insect infestations were seen during the study, herbivore activity could also impact floral volatile production. In fact, much of the plant volatile research to date has focused on HIPVs and these should be considered when looking at floral volatiles. Rodriquez-Saona *et al*., (2009) tested caterpillar damage, mechanical damage, and methyl jasmonate treatment (a common component of caterpillar saliva) on blueberry leaves and found that the production of 17, 5, and 11 volatile compounds increased, respectively. However, green-plant material may respond differently than floral material. For example, Szendrei *et al*. (2009) found that after herbivore damage of highbush blueberry flowers, volatile production of the flowers was generally reduced. However, the risk of false positives in floral samples resulting from herbivore damage should be considered in future as it could cause variation among and within genotypes.

HERITABILITY OF VOLATILES

Variation in floral volatiles among the ten highbush blueberry genotypes was seen in this study and the broad-sense heritability estimate for the volatile compounds was moderate, at $H^2 = 0.5$. This means, for this population, time, and place, approximately half of the variation among genotypes is not explained by the environmental factors that cause the within genotype variance (Allard, 1960). The broad-sense heritability estimate is a good starting point and indicates that it would be worthwhile to estimate narrow-sense heritability if there was an interest to include floral volatiles in blueberry breeding programs. Narrow-sense heritability estimates utilize the additive genetic variance while excluding other components of genetic variance and can therefore estimate a response to trait selection (Allard, 1960).

The Principal Component Analysis was used to examine and visualize genetic / family relationships within the volatile data. Cherri-Martin *et al*. (2007) used PCA to assess heritability of hybrid tea rose floral odour and found that monoterpene volatiles best described the family groups. Lee *et al*. (2010) used PCA to compare floral volatiles of genotypes from two species, as well as some hybrids, of the genus *Gentiana* and found a clear separation between the species. As well, Principle Coordinate Analysis was used by Delphia *et al*. (2009) to show that inbreeding impacts the volatile profile of *Solanum carolinense* (horsenettle). In my results, the plot of PC 1 and 2 (Figure 2.1) reveals two main groups of genotypes divided by monoterpene production; with the high monoterpene/myrcene genotype collections in the bottom left quadrant, the moderate monterpene collections towards the upper middle, and the genotype Elliott with very low levels of monoterpenes to the far right. This was not surprising because monoterpenes, myrcene in particular, represent an overwhelming percentage of the total volatile output. However, variation in myrcene production is not a sufficient explanation for the pattern, for example the variation in branched compounds may cause some genotypes to be in the upper vs the lower half of the plot. A larger number of blueberry genotypes could clarify the categories of floral odour type or simply fill in the gaps to create a continuum of odour types.

My sampling included a set of closely related genotypes: parents Brigitta and Elliott and three offspring Aurora, Liberty, and MSU 60. Sampling of this family group allowed me to question more specifically the appearance of compounds in the parents and offspring. Brigitta and the

offspring were high producers of myrcene, whereas Elliott was not found to produce the monoterpene. While many more offspring, and an F2 generation, would be needed to examine the trait transmission, my results suggest that the production of myrcene could be dominant. The expectation was that the offspring would only produce volatiles that the parents also produce, however this was not the case. Aurora had four, Liberty had five, and MSU 60 had one extra compounds, a total of seven compounds not found in either parent, which makes a substantial proportion (24%) of the compounds found in the family group. The production of these seven compounds that were not found in either parent could be related to the great degree of plasticity around floral volatile production.

SIGNIFICANCE TO POLLINATORS

Highbush blueberry volatiles were found to have both variation among genotypes and broad-sense heritability of these traits. However, the variation was limited such that genotypes with a larger variety of volatile compounds also had a larger total output of volatiles and there were only three genotypes with independently unique compounds. In addition, volatile traits as a group appear to have great plasticity and further investigations should focus on the impact that climate and growing conditions may have on the production of these compounds. The important question remains as to whether these volatiles influence pollinator behaviour to a degree that could impact yield. Research has shown the precise blend of volatile compounds (Wright *et al*., 2005) and even the ratio of the blend's components (Cha *et al*., 2011) to be important for insect discrimination and choice. Pollinators are attracted to flowers by olfactory cues but the relative importance of compounds within the volatile blend is unknown. Much more must be learned about the impact of certain compounds within blends, the impact of diversity, and tolerance to opposing volatiles.

Chapter 3: Bee Response to Highbush Blueberry Floral Volatiles

3.1   Introduction

Flowers advertise rewards with colour, shape, pattern, and odour, and pollinators are attracted and selective based on these advertisements. Pollinator-mediated selection in a pollinator-dependent agricultural population could impact yield for fruit crops as well as impact breeding programs and seed producers. Due to pollinator decline and it's significance to agriculture (*reviewed in* Potts *et al*., 2010), there is an interest in breeding programs adopting goals to improve pollination. Pollinator-mediated selection among conspecific plants has been seen to impact floral display, size, colour, and shape (*reviewed in* Parachnowitsch & Kessler, 2010) as well as odour (Parachnowitsch *et al*., 2012). However, past and present agents of selection acting on floral traits should be considered, including competition, heat and water stress, pathogen attack, and herbivory (*reviewed in* Strauss & Whittall, 2006). Because of the potentially antagonistic selection on floral traits, flowers may reflect a mixture of attractive, neutral, and deterrent traits relating to pollinators. This knowledge is critical for a breeding program.

Bees are the pollinators of interest in this study because they are the dominant pollinator of highbush blueberry. In fact, economical yields of cultivated highbush blueberry currently depend on managed honeybees (Issacs & Kirk, 2010). Blueberry varietal preference by honeybees and wild bees has been noted by growers and breeders, and two studies have examined this preference in detail. Brewer and Dobson (1969) investigated the preference for the genotype Rubel over Jersey. None of the floral traits studied were found to explain the genotype preference, including nectar volume, nectar sugar content and composition, flower size (including size of opening and flower length), UV patterns, and pollen quantity. More recently, Courcelles *et al*. (2013) investigated honeybee preference among four blueberry genotypes by comparing floral morphology and visitation rates. They found that Duke was visited more often than Bluecrop and Draper, but not more than Liberty, and that the larger size of the flower

opening, or throat, of Duke allowed the honey bee greater access and opportunity to deposit pollen.

Floral volatiles are an important aspect of pollinator attraction that could explain some of the varietal preference displayed by bees. Genotype specific or genotype absent blueberry floral volatiles have been described previously (Chapter 2; Rodriquez-Saona *et al.*, 2011) and provide a good starting point from which to examine bee preference among genotypes. In Chapter 2, most of the genotypes had variable volatile profiles such that 48 volatiles were absent in at least one genotype and only seven volatiles were found to be ubiquitous in odour samples. However, there were very few compounds, only three, that were found to be genotype specific. Rodriquez-Saona *et al.* (2011) identified eight blueberry volatiles that were found to be variable in a present/absent fashion with 20 volatiles shared by the genotypes.

The goal of this study was to investigate what role, if any, floral volatiles play in genotype preference of bees, based on both previous research and new experiments. Part 1 of this study is a literature survey of bee response research, to highlight highbush blueberry volatiles and preface the following experimental studies (Part 2). I also discuss studies examining floral blends, as it is known that bees respond more accurately to floral blends (Rodriquez-Saona *et al.*, 2011). The experimental studies involve monitoring for a pollinator preference and the development of a floral volatile bioassay with bumblebees.

3.2 **Part 1: Literature Survey**

A systematic review of literature investigating the response of bees (clade: Anthophila) to plant volatiles was conducted to collate such reports and review for blueberry volatiles. The literature search up to 2013 included the keywords: bee, Anthophila, Andrenidae, Apidae, Colletidae, Dasupodaidae, Halictidae, Megachilidae, Meganomiidae, Melittidae, Stenotritidae, bioassay, floral, and volatile. A variety of pollinators have been utilized in bioassay studies with floral

volatiles; for this literature survey, experiments using bees were included because they are the chief pollinators in an agricultural context. I divided the literature into experimental papers using single volatile compounds versus those using volatile blends. The single volatile research was organized into compound categories as in the previous chapter (Chapter 2): aliphatic (straight-chain), benzenoid, branched, and terpenoid compounds, recorded in Table 3.1. Experiments used a variety of methods from in field flower augmentation or simulated flowers where the type of response was measured as attractant, '+', or deterrent, '-', to the more simple electro-anntenogram where only a response is noted as '*' (could be attractant or deterrent). The literature review table, Table 3.1, includes the compound name, the response type (+, -, or *), the genus or species name, and the reference.

The most common method used to determine bee response to volatiles is electroantennography (EAG) (Table 3.1) which can be coupled with gas chromatography for floral extracts (GC-EAD and GC-EAG) or used with synthetic volatiles (Ayasse *et al*., 2007; Dotterl *et al*., 2005; Henning & Teuber, 1992; Klatt *et al*., 2013; Patricio *et al*., 2004; Salzmann *et al*., 2007). For this method, electrical responses in the detached bee antennae are monitored while volatiles elute from the GC and are simultaneously being identified (Schiestl & Marion-Poll, 2002). The electro-antennogram method allows an observation of response but not a more detailed observation of attractant or deterrent effects. The vast majority of volatiles tested in the EAG bioassay studies showed a response (Table 3.1), some with variable results between studies. The EAG measures provide limited information as most volatiles show a positive result and the response does not necessarily relate to a relevant action on the part of the insect.

The proboscis extension method (PE) comprises a live bee secured in a brace as volatiles flow over the bee and extension of the proboscis is monitored (Blight *et al*., 1997; Wadhams *et al*., 1994). The PE method involves a more complex relationship between the bee and the volatiles because the method uses a live bee and can include conditioning (CPE) (Schiestl & Marion-Poll, 2002). For example, Wadhams *et al*. (1994) showed through a honeybee study using both EAG and CPE that the EAG exhibited responses to all compounds tested, whereas the CPE exhibited responses to half.

The next step in research complexity is behavioural tests carried out under field or flight cage environments with fake or augmented flowers (Ackermann, 1989; Andrews *et al*., 2007; Hagler & Buchmann, 1993; Henning *et al*., 1992). One third of the research studies in Table 3.2 used flight experiments, which allow for determination of attraction versus deterrence, or even aggressive behaviour as was seen for the response to cis-3-hexenyl acetate (Henning *et al*., 1992). Response to volatiles can differ among species of bees. An example is the attraction of euglossine bees (Ackerman, 1989) versus the deterrence of honey bees (Henning *et al*., 1992) to the volatile, methyl salicylate.

Volatile bioassays can also increase in complexity with the number of compounds tested. For many insects, single compound lures are a sufficient attractant and can be used in pest monitoring, e.g. for the striped cucumber beetle and cranberry weevil (Andrews *et al*., 2007; Szendrei *et al*., 2009). However, it is known that insects respond more accurately to blends of compounds and a variety of blending methods have been used to test bee response to floral volatiles including whole flower extracts (Grajales-Conesa *et al*., 2012), volatile addition to flowers (Dobson *et al*., 1999; Odell *et al*., 1999; Adler & Irwin, 2005), and synthetic blends (Roy & Raguso, 1997; Rodriquez-Saona *et al*., 2011). Rodriquez-Saona *et al*. (2011) tested a simplified blend of highbush blueberry volatiles in a field experiment and found that the blend was attractive to honey bees, but saw no effect for individual compounds.

Volatiles that have been determined to be present in highbush blueberry floral odour (Chapter 2; Rodriquez-Saona *et al*., 2009 and 2011) are highlighted in Table 3.1. The volatiles that were found in all of the genotype volatile collections in Chapter 2 include: 3-hexen-1-ol acetate, linalool, an isomer of bourbonene, benzaldehyde, an isomer of cinnamaldehyde, and two isomers of cinnamyl alcohol. Among these, the benzenoid compounds (benzaldehyde, cinnamaldehyde, and cinnamyl alcohol) were also found to be specific to floral samples. One particular compound, myrcene, was found to have a very high relative peak area for some genotypes while much lower for others.

Table 3-1: Literature review of single volatile compound bioassays using species in the bee clade, Anthophila. Methods as follows: GC- gas chromatography, EAG/EAD- electroantennogram/ electroantennographic detection, CPE- conditioned proboscis extension, flight- flight chamber test. Volatiles found in highbush blueberry are in bold. Response denoted, *, no response, NS.

Volatile Compound	Bee: family, genus, or species	Response	Method	Reference
Aliphatic				
decyl acetate	Apis mellifera	NS	GC-EAD	8
docosane	Andrena nigroaenea	*	GC-EAD	3
1-dodecene	Apis mellifera	*	GC-CPE	4
dodecyl acetate	Apis mellifera	NS	GC-EAD	8
dodecyl tetradecanoate	Andrena nigroaenea	*	GC-EAD	3
eicosanal	Andrena nigroaenea	*	GC-EAD	3
(E, E)-farnesol	Andrena nigroaenea	*	GC-EAD	3
(E, E)-farnesyl hexanoate	Andrena nigroaenea	*	GC-EAD	3
heneicosane	Andrena nigroaenea	*	GC-EAD	3
heptacosane	Andrena nigroaenea	*	GC-EAD	3
(Z)-9-heptacosene	Andrena nigroaenea	*	GC-EAD	3
hexacosane	Andrena nigroaenea	*	GC-EAD	3
(Z)-2-hexenal	Apis mellifera	*	GC-EAD	12
(Z)-2-hexenal	Apis mellifera	NS	GC-CPE	12
cis-3-hexenyl acetate*	Apis mellifera	Defensive behaviour	flight	7
cis-3-hexenyl acetate*	Apis mellifera	*	GC-EAD	8
(Z)-3-hexenyl acetate*	Osmia bicornis	*	EAG	9
linolenic acid ethylester	Andrena nigroaenea	*	GC-EAD	3
(Z)-9-nonacosene	Andrena nigroaenea	*	GC-EAD	3
2-nonanal	Frieseomelitta silverstri	*	EAG	10
nonanal	Osmia bicornis	*	EAG	9
nonanoic acid	Andrena nigroaenea	*	GC-EAD	3
2-nonanone	Apis mellifera	*	GC-CPE	4
2-nonyl dodecanoate	Andrena nigroaenea	*	GC-EAD	3
2-nonyl tetradecanoate	Andrena nigroaenea	*	GC-EAD	3
octadecanal	Andrena nigroaenea	*	GC-EAD	3
3-octanone	Apis mellifera	Deterrent	flight	7
3-octanone	Apis mellifera	NS	EAG	8
1-octen-3-ol	Apis mellifera	*	GC-EAD	12
1-octen-3-ol	Apis mellifera	NS	GC-CPE	12
pentacosane	Andrena nigroaenea	*	GC-EAD	3
(Z)-9-pentacosene	Andrena nigroaenea	*	GC-EAD	3
tetracosane	Andrena nigroaenea	*	GC-EAD	3
tricosane	Andrena nigroaenea	*	GC-EAD	3
2-undecanol	Frieseomelitta silverstri	*	EAG	10
1-undecene	Apis mellifera	*	GC-CPE	4

Volatile Compound	Bee: family, genus, or species	Response	Method	Reference
Benzenoid				
ρ-anisaldehyde	Osmia bicornis	*	EAG	9
anisaldehyde	Apis mellifera & Bombus terrestris	*	GC-EAD	11
benzaldehyde	Apis mellifera	*	GC-CPE	4
benzaldehyde	Osmia bicornis	*	EAG	9
benzyl alcohol	Apis mellifera	*	GC-CPE	4
benzyl alcohol	Apis mellifera	*	GC-EAD	12
benzyl alcohol	Apis mellifera	NS	GC-CPE	12
caffeic acid	Apis mellifera	Attractant	flight	6
(E)-cinnamaldehyde	Squash bee	Attractant	flight	2
p-cymene	Apis mellifera	*	GC-CPE	4
dihydro-beta-ionone	Osmia bicornis	*	EAG	9
1,4-dimethoxybenzene	Andrena vaga	*	GC-EAG	5
1,4-dimethoxybenzene	Apis mellifera & Bombus terrestris	*	GC-EAD	11
eugenol	Andrena vaga	*	GC-EAG	5
genistic acid	Apis mellifera	Attractant	flight	6
β-ionone	Osmia bicornis	*	EAG	9
phenylacetaldehyde	Apis mellifera	*	GC-CPE	4
2-phenylethanol	Apis mellifera	*	GC-CPE	4
2-phenylethanol	Andrena vaga	*	GC-EAG	5
linalool & 2-phenylethanol	Apis mellifera	*	GC-EAD	12
linalool & 2-phenylethanol	Apis mellifera	*	GC-CPE	12
methyl cinnamate	Euglossine bees	Attractant	flight	1
methyl salicylate	Euglossine bees	Attractant	flight	1
methyl salicylate	Apis mellifera	*	GC-CPE	4
methyl salicylate	Andrena vaga	*	GC-EAG	5
methyl salicylate	Apis mellifera	Deterrent	flight	7
methyl salicylate	Apis mellifera	*	GC-EAD	8
methyl salicylate	Osmia bicornis	*	EAG	9
methyl salicylate	Apis mellifera	*	GC-EAD	12
methyl salicylate	Apis mellifera	*	GC-CPE	12
4-oxoisophorone/benzyl nitrile	Andrena vaga	*	GC-EAG	5
1,2,4 – trimethoxy-benzene	Squash bee	Attractant	flight	2
vanillin	Euglossine bees	Attractant	flight	1
Indole (benzenoid)				
indole	Squash bee	NS	flight	2
indole	Andrena vaga	*	GC-EAG	5
skatole	Euglossine bees	Attractant	flight	1

Volatile Compound	Bee: family, genus, or species	Response	Method	Reference
Branched				
2-methylbutanol	Apis mellifera	NS	GC-EAD	8
6-methyl-5-hepten-2-one	Osmia bicornis	*	EAG	9
Terpenoid				
β-caryophyllene	Frieseomelitta silverstrii	*	EAG	10
cineole	Euglossine bees	Attractant	flight	1
1,8-cineole	Apis mellifera	*	GC-CPE	4
α -cubebene	Frieseomelitta silverstrii	*	EAG	10
4,8-dimethyl-1,3,7-nonatriene	Andrena vaga	*	GC-EAG	5
(E,E)-α-farnesene	Apis mellifera	*	GC-CPE	4
cis-α-farnesene	Andrena vaga	*	GC-EAG	5
α-farnescense	Osmia bicornis	*	EAG	9
geranyl acetatone	Osmia bicornis	*	EAG	9
geranylfarnesol	Frieseomelitta silverstrii	NS	EAG	10
humulene	Frieseomelitta silverstrii	*	EAG	10
lilac alcohol isomer	Andrena vaga	*	GC-EAG	5
dl-limonene*	Osmia bicornis	*	EAG	9
limonene*	Apis mellifera	*	GC-CPE	4
limonene*	Apis mellifera	NS	GC-EAD	8
linalool	Apis mellifera	*	GC-CPE	4
linalool	Andrena vaga	*	GC-EAG	5
linalool	Apis mellifera	Attractant	flight	7
linalool	Apis mellifera	*	GC-EAD	8
monoterpene oxides	Andrena vaga	*	GC-EAG	5
myrcene	Apis mellifera	NS	GC-EAD	8
cis-nerolidol	Andrena vaga	*	GC-EAG	5
ocimene	Apis mellifera	NS	EAG	8
β-ocimene	Andrena vaga	*	GC-EAG	5
α-pinene	Apis mellifera	*	GC-CPE	4
sesquiterpene oxide	Andrena vaga	*	GC-EAG	5
sesquiterpene?	Andrena vaga	*	GC-EAG	5
α-terpinene	Apis mellifera	*	GC-CPE	4

References for table as follows:

1   Ackerman, 1989

2   Andrews *et al*., 2007

3   Ayasse *et al*., 2001

4   Blight *et al*., 1997

5   Dotterl *et al*., 2005

6   Hagler & Buchmann, 1993

7   Henning *et al*., 1992

8   Henning & Teuber, 1992

9   Klatt *et al*., 2013

10   Patricio *et al*., 2004

11   Salzmann *et al*., 2007

12   Wadhams *et al*., 1994

3.3 Part 2: Experimental Studies

3.4 Methods

FLORAL VISITOR MONITORING: At the Agricultural Canada research sub-station in Abbotsford BC, floral visitors of highbush blueberry genotypes were monitored in-field to compare the number of visitors and assess genotype preference. The number of floral visits has been used as an assessment of pollinator choice, in experiments comparing pollinated versus un-pollinated flowers (Larson & Barrett, 1999), floral morphology (Anton *et al*., 2013), and multiple trait combinations (Gegear & Laverty, 2005; Gegear, 2005). Ten genotypes were included in the study, each with five biological replicates throughout the field. Six monitoring counts were conducted during the period May 20 to June 6 (2012) between 10 am and 12 pm on sunny days. Monitoring was conducted while walking north to examine the west side of the shrubs and south to count on the east side, spending approximately 30 seconds at each shrub. Floral visit counts were recorded when the pollinator landed on an open flower and was observed to insert its head in the flower or proboscis in the side of the flower (nectar robbing). Floral visitor numbers were analyzed using an analysis of variance (ANOVA) and post-hoc Tukey analysis in the statistical program 'R' ('R' core team, 2014).

BIOASSAY METHOD DEVELOPMENT: A bioassay was developed in spring 2013 to test *Bombus impatiens* response to blueberry floral volatiles present or absent in a blend. The bioassay was designed for a small glass house at Simon Fraser University, Burnaby, BC with the assistance of Dr. Gerhard Gries. The following compounds were diluted in pentane (Sigma Aldrich) at various concentrations: alpha-pinene (Aldrich), sabinene (chromadex), myrcene (Aldrich), and eucalyptol (Aldrich). The bumblebee, *Bombus impatiens* Cresson (Biobest Biological Systems Canada, Leamington, Ontario) was used for the bioassay due to the high efficiency of bumblebees to pollinate blueberry and other crops with poricidal anthers (Javorek *et al*., 2002). In the greenhouse, the bioassay was conducted from April 11th to April 29th, and by April 29th the hive was mature such that activity had decreased and queens were seen blocking the exit doors in an attempt to leave the hive. Testing and observations were recorded for 12 days during that period when bee activity was highest, between 9 am and 2 pm.

Figure 3-1: Picture of bioassay set-up including Bombus impatiens hive at the south end of the table and food or choice test at the north end in a small glass house at Simon Fraser University.

The bioassay set-up is shown in Figure 3.1. The glasshouse was cleaned and a long table set in the centre with the bumblebee hive box at the south end and the food, bee pollen and sugar water, or choice tests at the north end, separated by a distance of approximately 1.5 m. The experimental choices were always placed in the same position on the table as the food so the bees would be trained to visit that location. The choice tests involved inverted white plastic lids (2.5 cm diameter) filled with sugar water and a natural colour rubber septum in the centre. The larger cup of the septum (1 cm diameter) was loaded with 100 µl of the pentane control or a diluted volatile. Each testing day, the pollen and sugar water were removed and the table was cleaned with water to remove residue 1.5 to 2 hours before the testing began. The previously diluted compounds and the control were loaded into the septum and were left to evaporate for 1 hour before use. A choice was recorded when a bumblebee drank sugar water from the lid, most often the bees were observed to hover back and forth between the options before making a choice. After a choice was made, the sugar water, septum, and the bumblebee were removed from the greenhouse. The bee was removed with an aspirator and euthanized in order to eliminate the possibility of information being transferred by volatile residue or a bias from bee

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learning. For each choice, clean lids with sugar water and new septa were used if they were

handled by bees and the septa were used for a maximum of 2.5 hours of testing (3.5 hours from

filling).

BIOASSAY SEPTA TEST: The septa test was performed in order to determine the length of

time that volatile compounds were emitted from the septa as was used in the bioassay detailed

above. The mixed compounds were loaded onto the septa and were allowed to evaporate for 1,

2, 3, and 3.5 hours. For analysis, the septa were placed into headspace autosample vials with

clamped lids and were sampled immediately using gas chromatography coupled to mass

spectrometry with a head space analyzer at the Mass Spectrometry Core Facility jointly run by

the Micheal Smith Laboratories and the Wine Research Centre at UBC (Agilent 6890/5973N

Gas Chromatograph Mass Spectrometer and Agilent 7694 Headspace Autosampler, Agilent

Technologies). Each timed sample was performed in triplicate with a control triplicate.

Statistical analyses were done using the software 'R' ('R' core team, 2014).

3.5   **Results**

BLUEBERRY FLOWER VISITORS:

Blueberry floral visitors were monitored to compare genotypes and assess varietal preference

noted by previous research. Figure 3.2 shows the average count of floral visitors for the

genotypes at each sampling day. Lines are the linear approximation of those averages over the

duration of sampling. The data is approximately normal as standardized residuals versus fitted

values were close to random; however, the sample size was too small to assess this conclusively.

The linear model fits well for most of the genotypes (Table 3.2), but not for Bluecrop, Duke, and

Reka, for which R^2 values were less than 0.2. The earlier flowering genotypes A-246 and Reka

show an expected decrease in floral visitors over time as bloom matures and the genotypes loose

flowers. Whereas Duke, another early flowering genotype, shows consistent activity over the

sampling period and therefore a longer period of pollinator attraction. The majority of other genotypes show an increase in floral visitors over time.

The floral visitor monitoring was conducted from when the early genotypes reached 50% bloom until all genotypes reached greater than 90% bloom; however, the sampling period should have begun earlier and continued later in order to capture the visits throughout each genotype's bloom period. Figure 3.2 shows the floral visitor numbers over time and the slope of the corresponding line of best fit clearly separates the three early blooming genotypes, Duke, Reka, and A-246. Although it appears as though the genotypes Duke and Reka attracted a higher number of visits, the day with the highest number of floral visits for each genotype was compared and found to be the same among the genotypes ($p = 0.86$). The highest number of visits was compared to avoid bias due to earlier blooming genotypes attracting greater visits in total. Bloom phenology is not a discreet measurement for the blueberry genotypes because bloom overlaps and lasts longer for some genotypes. It is important to standardize by the number of flowers produced and the longevity of those flowers for true a comparison of pollinator preference and eliminate the bias of different flowering phenologies.
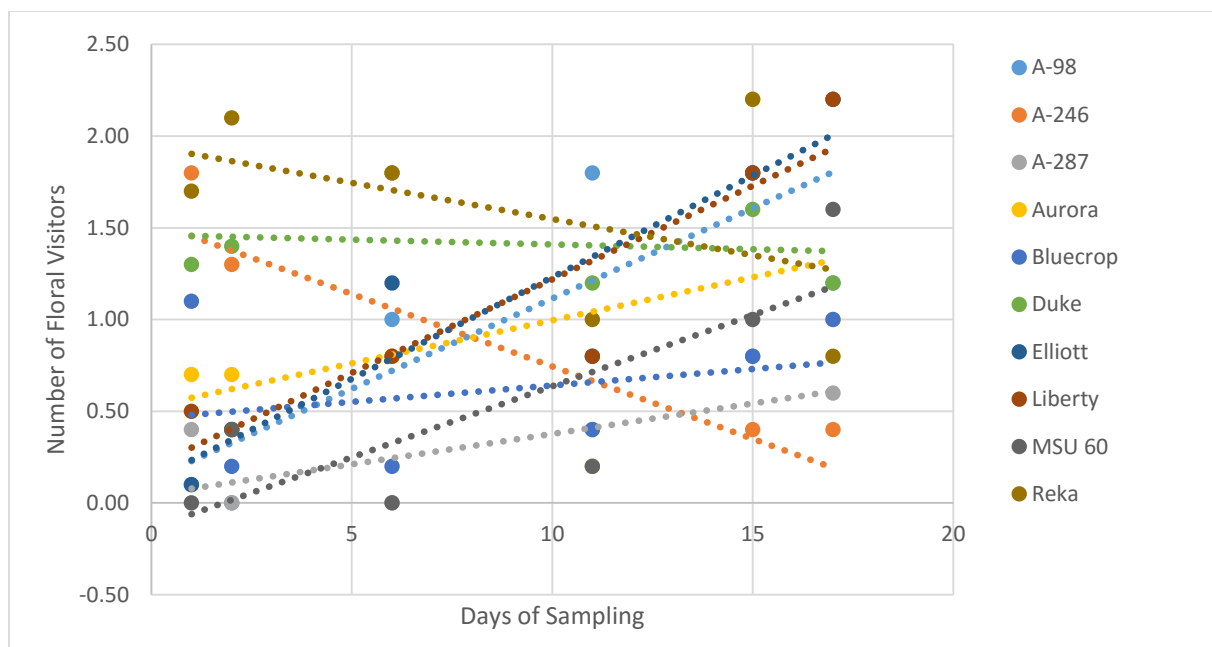
Figure 3-2: Average number of floral visits to each highbush blueberry genotype per sampling day. Sampling days numbered over the sampling period of 17 days. Average floral visitors are calculated by number of visitors relative to number of genotype replicates. Lines of best fit for the average floral visitors are shown for each genotype and the R^2 values for best fit lines are in Table 3.1.

Table 3-2: Monitoring statistics for highbush blueberry floral visits
Including R^2 values for the line of best fit of the number of floral visitors over time, and the highest number of visitors, the total number of visitors, and the relative bloom period based on cultivar documentation.

Genotype	Bloom Period	Highest # Visitors	Total # Visitors		R^2 values for linear fit
A-98	Mid	1.8 ± 0.7	7.0 ± 1.0	ab	0.70
A-246	Early	1.8 ± 0.5	6.0 ± 1.5	ab	0.82
A-287	Mid	0.8 ± 0.5	1.6 ± 0.5	b	0.46
Aurora	Late	2.2 ± 0.7	7.0 ± 1.5	ab	0.22
Bluecrop	Mid	1.1 ± 0.7	3.2 ± 0.9	b	0.09
Duke	Early	1.8 ± 0.6	12.6 ± 2.0	a	0.02
Elliott	Late	1.8 ± 0.6	5.0 ± 0.6	b	0.84
Liberty	Mid	1.8 ± 0.6	7.8 ± 2.0	ab	0.86
MSU 60	Late	1.6 ± 0.7	3.6 ± 0.7	b	0.66
Reka	Early	2.2 ± 0.6	12.4 ± 2.6	a	0.21
ρ-values		**$\rho = 0.86$**	**$\rho = 2.9 \times 10^{-05}$**		

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BIOASSAY METHOD DEVELOPMENT:

The result presented here are from a bioassay in development and are preliminary. The bumblebees (*Bombus impatiens* Cresson) from Biobest Biological Systems were ideal for experimentation because they can be easily transported in contained hives with easily closed doors and a manageable size. There were several stages to the bioassay method development. The first was to test a single compound of interest, myrcene, over several concentrations (Figure 2.2). At 1 mg of myrcene per septum loading, the compound may have been a deterrent to the bumblebees, however, there was no observable difference in choice between the control (pentane) and myrcene (diluted in pentane) at 0.6, 0.3, or 0.1 mg. The second stage involved a simple mixture of the monoterpenoid compounds, α-pinene, sabinene, myrcene, and eucalyptol, at 0.1 mg each. Again, there was no observable difference in choice between the sugar water displays with the control or the mixture of compounds with 10 of the 26 choices made for the mixture (38%). Finally, the third stage involved cut individual flowers of the genotypes Elliott and Aurora, floating in the sugar water. Once again there was no observable difference in the choice of the bumblebees with the 22 choices being split evenly among the Elliott and Aurora flowers.
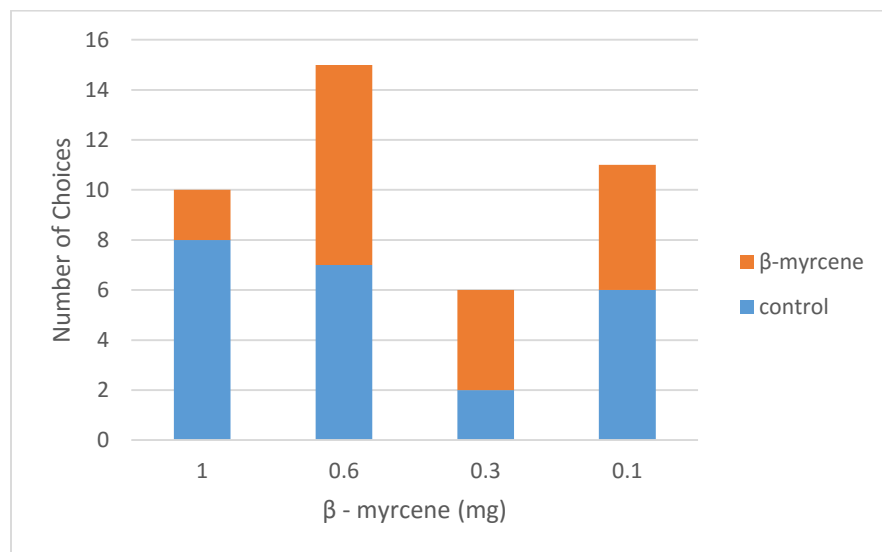
Figure 3-3: Choices made by *Bombus impatiens* between sugar water feeding displays with control (pentane) versus β-myrcene diluted in pentane; four levels of β-myrcene were tested.

BIOASSAY SEPTA TEST:

This experimental test showed that the septa did indeed hold the volatile compounds for sometime. However, there was a substantial decline in volatile output over three hours (Figure 3.4). The most significant result from the septa experiment was that the levels of pentane emitted were much higher than expected, as they were expected to evaporate within 1 hour (the peak area of pentane was nearly 100 fold higher than the terpene compounds). In addition, the terpenes were added to the mixture at the same concentration, but myrcene was seen to impregnate the septa less. Myrcene has the lowest density of the compounds included, which could lead to a faster evaporation as well as error during weighing.
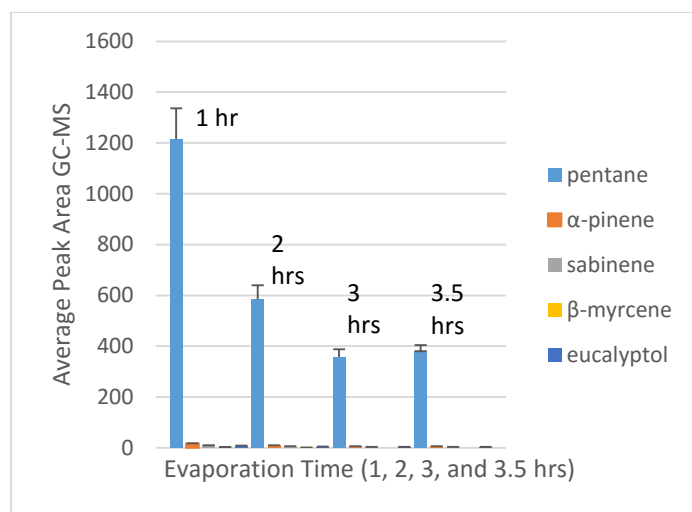
a.)
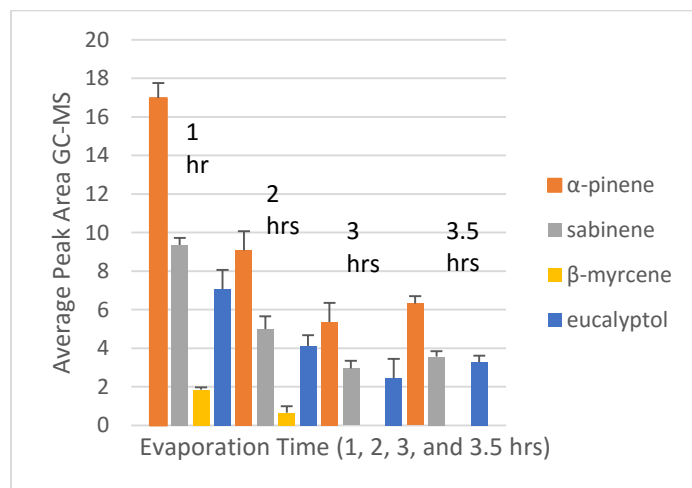
b.)

Figure 3-4 a & b: Headspace analysis using GC-MS to determine emission of volatiles from septa at 4 evaporation times (1, 2, 3, and 3.5 hrs).
(a.) shows emission of the dilution solvent, pentane, versus the monoterpenes and (b.) shows the monoterpene mixture alone.

3.5 **Discussion**

The purpose of this study was to explore the response of pollinators to the genotypic differences in highbush blueberry floral odours in order to investigate the genotypic preference observed in bees. Current literature detailing pollinator response to floral odours was collated and monitoring and bioassay techniques were developed to address this question further. In-field monitoring did not reveal a genotype preference by bees and method improvements are suggested. Method development and early bioassay results are discussed as they relate to the bee bioassay literature that was surveyed.

ASSESSING PREFERENCE

Although I failed to show pollinator preference among genotypes of blueberry in the field due to experimental shortfalls, others have reported this. Simply monitoring the number of floral visitors on a particular shrub can be erroneous because it is difficult to tease out preference from other factors such as number of flowers, bloom period, and the persistence of flowers (including post-pollination or fertilization changes). For example, in this study there was no evidence of preference among genotypes when the day with the highest number of floral visitors was considered for each genotype, despite the significant difference in total number of visitors (Table 3.2). Instead, assessments should be made based on the number of visits per flower to control for difference in flower number. Standardizing the number of flowers through removal is not recommended because damage-induced volatiles may alter floral chemistry (Raguso, 2004; Rodriquez-Saona *et al*., 2009).

Monitoring can be adjusted over the bloom period to evenly sample genotypes, as was done by Courcelles *et al*. (2013); however, the sampling days should also be standardized by percent bloom. For example, the number of visitors on the genotype Duke, although variable, was similar at the beginning and end of the monitoring period despite it being an early flowering genotype (Figure 3.2). Duke was also found to be the most attractive among the four genotypes examined by Courcelles *et al*. (2013) which may explain why, in my study, floral visitors persisted on Duke despite there being later flowering genotypes with more flowers available. An

increased duration of monitoring to encompass for the bloom period of each genotype would improve the assessment of pollinator preference.

Another more complex factor to consider is the non-random foraging behaviour and learning capacity of bees, in that they show fidelity toward a particular pollen and/or nectar source (Waser, 1986). It is possible that early flowering genotypes could influence bee preference such that they would continue to visit the same genotype, even though there are more flowers available elsewhere. It follows from this known behaviour that conditioning bees before analysis improves the response of simple EAG and PE experiments (Wadhams *et al*., 1994) and has also been used in flight cage and field experiments (Henning *et al*., 1992). Conditioning in the natural environment can also modify foraging behaviour, as shown by Dobson (1987) who compared foraging of experienced and inexperienced wild bees and found that bees learned the odours associated with pollen. Although it is clear that there must be a mechanism of choice, it is unclear how different the flower types must be. For example, Gegear and Laverty (2005) tested flower constancy in bumblebees and found that as the number of traits increased from one (colour only) to three (colour, size, and morphology), the bees showed more preference for the flower type they were first exposed too. Additionally, Gegear (2005) examined size and odour and again found that the bumblebees were more selective when two traits were used. The authors attributed these results to limits in the ability of the bees to remember multiple trait groups. Due to the possibility of a learning of multiple trait groups it would be better to combine volatile preference assessments with ancillary traits including flower and inflorescence characters to serve as covariates.

HIGHBUSH BLUEBERRY VOLATILES

Highbush blueberry flowers produce many of the terpenoid and some of the benzenoid volatiles previously used in bee bioassay studies (found in bold, Table 3.1); however, none of the aliphatic nor branched compounds were included. Most of the blueberry volatiles previously used in bee choice assessments involved EAG bioassays and it is therefore not possible to determine the attractiveness vs deterrence of the volatiles. However, benzenoid compounds may be key to pollinator attraction because a large proportion of them are found only in floral samples (Chapter

2). More benzenoid compounds have been used in flight bioassay experiments, and those studied were attractive to bees.

There were many volatile compounds from previous highbush blueberry floral collections that could represent genotypic differences in a present/absent fashion (Chapter 2; Rodriquez-Saona *et al*., 2009 and 2011). However, only four of those compounds have been included in previous bee bioassays. The aliphatic compound cis-3-hexenyl acetate was shown to elicit a response using EAG for *Apis mellifera* (Henning & Teuber, 1992) and *Osmia bicornis* (Klatt *et al*., 2013) and more definitively cause a defensive behaviour in *A. mellifera* (Henning *et al*., 1992). The monoterpenoids, myrcene and limonene were found to show no significant response using an *A. mellifera* EAG (Henning & Teuber, 1992); in contrast, limonene was also found to show an EAG response with *O. bicornis* (Klatt *et al*., 2013) and a CPE response with *A. mellifera* (Blight *et al*., 1997). The sesquiterpene, farnesene was found to show responses using EAG with three genera of bee, *A. mellifera* (Blight *et al*., 1997), *Andrena vaga* (Dotterl *et al*., 2005), and *O. bicornis* (Klatt *et al*., 2013). From Chapter 2, around half of the volatiles common to all genotypes were found only in floral samples and were benzenoid compounds. Two of these, benzaldehyde and cinnamaldehyde, were used in previous bioassays. Benzaldehyde was found to show a response through GC-CPE (*A. mellifera*) and EAG (*O. bicornis*) (Blight *et al.*, 1997 and Klatt *et al.*, 2013, respectively) and cinnamaldehyde was shown to be an attractant of *Peponapis pruinosa* (Andrews *et al.*, 2007).

Consistent with these previous studies, myrcene was not seen to have an impact of pollinator choice through my preliminary examination (Figure 3.2). However, future work should investigate the possibility that the compound is a deterrent at high levels, given the high levels of myrcene seen in some floral collections from highbush blueberry and the preliminary results of the bumblebee bioassay at levels of 1 mg (Figure 3.2). The GC-MS analysis of the septa and compound mixture used in the bioassay showed that large amounts of pentane were emitted from each septum at the time that the bioassay would have been carried out. The pentane was used as a dilution solvent, which is common in volatile studies. For example Roy and Raguso (1997) used hexane to dilute compounds and create a blend of floral volatiles that was found to be more attractive to bees than the hexane control; however, the hexane control itself was attractive and

received more visits than the unscented control. Pentane has not been used to dilute or extract volatile compounds for bee bioassays, so more information is required before using pentane in this way. A control of sugar water with an empty septa should have been included in the bioassay to determine the impact of pentane.

ATTRACTION VERSUS DETERRENCE

Rodriquez-Saona *et al*. (2011) compared bee attraction to two blends; one based on floral odour (16 compounds) and the other on leaf odour (12 compounds). The floral blend used by Rodriquez-Saona *et al*. (2011) contained a mixture of compound classes including the floral-only benzenoid, cinnamyl alcohol. Whereas, the leaf blend contained mainly terpenoid compounds. More honeybees were attracted to the floral blend than the leaf blend or single compounds, although bees were attracted to the single compounds and the leaf blend. It would be interesting to know if the leaf blend was less attractive due to the presence of deterrent compounds or due to the absence of attractive compounds.

A consideration must be made to the relative importance of floral-only volatiles in genotypic profiles and variation. The benzenoid group was made up of mostly floral-only volatiles and those volatiles were not found to differ widely among genotypes with half of them being present in collections from all genotypes. This could suggest that the 'attractiveness' of blueberry genotypes is fairly consistent; however, the relative deterrence of some compounds is a key area requiring further investigation. The floral odour of blueberry is influenced by aspects of the environment such as temperature and herbivore damage, all of which interact with genotype (Chapter 2). Herbivore-induced volatile compounds have been identified in highbush blueberry plant tissue with the following compounds showing a large increase in production; linalool, myrcenone, benzene acetonitrile, caryophyllene, humulene, and γ–cadinene, (Rodriquez-Saona *et al*., 2009). Breeding for floral odours, or any plant chemistry, requires an understanding of how the herbivore-plant-pollinator dynamic works within the context of volatiles. The fact that many of the compounds tested were attractive to bees, including those that are deterrent to herbivores, is a good sign for plant breeding efforts that endeavour to increase the anti-feedant response.

Pollinator-mediated selection has long been thought to be a major driving force in the diversification of angiosperms (Stebbins, 1970), and the impact of this selection on floral traits has been confirmed experimentally (*reviewed in* Parachnowitsch & Kessler, 2010; Parachnowitsch *et al*., 2012). However, the degree and impact of pollinator choice in the agricultural context is largely unexplored and is an exciting avenue from which to consider sustainability as well as yield improvements. In order to determine if the fine-scale choices that pollinators make impact yield, efforts must be made to ensure choice is being assessed correctly so that the choices presented to pollinators are accurate for the biological system under investigation.

Chapter 4: Pollen Movement and Reproductive Success in Highbush Blueberry

4.1 Introduction

Several research papers have discussed the importance of outcross pollen for complete seed and fruit development in *Vaccinium* species and much of this literature also shows a range in self-compatibility for *Vaccinium* genotypes/individuals. El-Agamy *et al*. (1981), Hellman & Moore (1983), Vander Kloet (1984), Gorchov (1985), Rabey & Luby (1988), Lyrene (1989), Krebs & Hancock (1990), Lang & Danka (1991), Krebs & Hancock (1991), Harrison *et al*. (1993), and Dogterom (2000) have examined various *Vaccinium* reproductive traits including fruit set, days to maturity, fruit weight, seed number, and seed weight; and all have reported greater reproductive success with outcross versus self- pollen fertilization. However, this is not consistent across all genotypes, as there is a range of self-fertility and even improved selfed fruit set with some *Vaccinium* hybrids (Gupton, 1984). Because of this genotype-specific direct impact to yield, knowledge about mating systems and what constitutes adequate pollination with outcross pollen is important to blueberry producers.

There are many mechanisms, morphological, developmental, chemical, and genetic, by which plants discourage self-pollination/fertilization and promote pollination and fertilization with outcrossed pollen. Morphological features including herkogamy (where stigmas and anthers are spatially separated) and poricidal anthers (which require sonication to dispense pollen) as well as developmental features such as dichogamy (where male and female functions are temporarily separated) all discourage autogamous self-pollination (Lloyd & Webb, 1986; Webb & Lloyd, 1986; Harder & Wilson, 1994). Deterrent compounds in floral chemistry have been shown to decrease the length of pollinator visits, thereby increasing pollinator movement and outcrossing (Kessler & Baldwin, 2006). Sexual specialization is another mechanism to encourage outcrossing where some individuals can be more functionally male or female (Devlin & Stephenson, 1987), possibly changing this trait between seasons (Gonzalez *et al*., 2005). If self-pollination does occur, there are genetic based mechanisms to prevent or limit self-fertilization of

ovules such as self-incompatibility and post-zygotic mechanisms involving early abortion of seeds due to insufficient levels of heterosis (Seavey & Bawa, 1986).

Blueberries have many of the morphological and developmental features listed above to aid in the transfer of outcross pollen, including approach herkogamy (bee contacts stigma before reaching the anthers), protandry (anthers mature before stigma), and poricidal anthers (Vander Kloet, 1988). Sexual specialization has been seen in other *Vaccinium* (Myra *et al.*, 2004), but not in highbush blueberry (Vander Kloet, 1983). Blueberries are self-compatible, in that pollen-tube formation occurs and ovules can be fertilized by self-pollen (Vander Kloet, 1991). However, flowers pollinated with outcross pollen produce more seeds, and researchers have proposed this is due to high genetic load and early acting inbreeding depression (Krebs & Hancock, 1990; Krebs & Hancock, 1991; Hokanson & Hancock, 2000). In fact, pollination by more than one outcrossed pollen donor has been suggested to further increase seed number (Vander Kloet, 1984).

There are two reproductive factors important to economical yields in highbush blueberry production, the transfer of pollen and the outcrossed origin of the pollen. The transfer of pollen requires a vector due to the floral characteristics mentioned above. However the honeybee, the main pollinator in blueberry, has been observed to be inefficient (Javorek *et al.*, 2002) and choosey in blueberry fields (Brewer & Dobson, 1969; Courcelles *et al.*, 2013). In an agricultural context, where planting consist of large blocks of clonally replicated genotypes, the movement of outcross pollen is also a challenge. Pollinizer genotypes positioned to donate outcross pollen are used in orchard systems such as apple; however, this practice has not been widely investigated for highbush blueberry. The goal of this research is to investigate both female and male reproductive success across the selected commercial genotypes in order to explore their relative fertility and tolerance to inbreeding. As well, a discussion of fertility implications for both blueberry breeders and growers is included.

4.2 **Methods**

FIELD SITES: The materials for this study were collected in Abbotsford, BC at the Agriculture Canada substation. Six commercial cultivars of highbush blueberry, Aurora, Bluecrop, Duke, Elliott, Liberty, and Reka comprised the maternal study subjects for this analysis of paternity (pollen flow). The six commercial cultivars were chosen as they were replicated throughout the research plot and randomized within the row. Paternal study subjects included all the genotypes at the research station, those six listed above and the following cultivars: A-12, A-98, A-246B, A-287, Chippewa, Chandler, Draper, MSU 36, MSU 60, ORUS 5-1, ORUS 10-1, ORUS 10-3, and US 645. Figure 4.1 is a representation of the Agriculture Canada station blueberry plantation to show locations of blueberry genotypes relative to each other. Highbush blueberry fruit collection from maternal subjects occurred between July 27 and September 7, 2012 when each blueberry cultivar presented a level of 50% ripe fruit. Twenty ripe fruits were collected from each side of the genotype block, East and West.

PLANTING SYSTEM: Because the focus of this study was to evaluate the performance of different paternal genotypes, sampling was designed to favour collection of outcrossed seeds and fruits. For fruit collection, earlier ripening and most distal fruit in the cluster (inflorescence) were harvested because previous research has shown that flowers pollinated with outcrossed pollen develop fruit earlier (Dogterom *et al*., 2000) and the most distal flower in a blueberry cluster is more available to pollinators and the first to bloom (Vander Kloet, 1988).

Five fruits were haphazardly chosen from the 20 collected and the seeds from these were planted. The fruit was weighed and the seeds were removed and cleaned then sorted by maturity to exclude aborted seeds. Mature seeds appear plump with no sunken sides and are dark in colour according to Hokanson & Hancock (2000). Seeds that were light in colour or shrunken/concave on one side were not included in the count (Figure 4.2). Seeds were pooled by berry and planted together in the compartments of a planting tray using potting soil with a thin layer of ground peat moss to cover the seeds and watering using a spray bottle to avoid disturbing the seeds and overwatering. Thus, the planting system included the seeds from five berries from each side (East and West) of five cultivar replicates, equaling a total of 50 berries per cultivar. The seeds

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were germinated in the laboratory at room temperature near natural light and, after approximately two months, were moved to a growth chamber with 16 hours of light and 8 hours of darkness at 20 $^{\circ}$C and 15 $^{\circ}$C, respectively. Seeds from thirteen genotypes in total (Aurora, Bluecrop, Duke, Elliott, Liberty as well as A 12, A 98, A 246, A 287, Draper, MSU 36, and MSU 60) were planted.

ANALYSIS OF SEED SET & GERMINATION DATA: I recorded blueberry seed germination at two and four months and harvested plant tissue for DNA isolation at four months. A second set of seeds was cleaned from blueberry fruits for the purpose of calculating the ratio of mature seed to aborted seed and to measure seed length using calipers. Fruit weights, seed number, seed size, and germination rate of blueberry genotypes were all compared among genotypes using Analyses of Variance (ANOVA) and a post-hoc Tukey analysis to compare means. Fruit weights, seed number, and germination rate were analyzed for correlation. Statistical analyses, ANOVA and regression, were completed using 'R' ('R' core team, 2014).

DNA ISOLATION & GENOTYPING: For genotyping analysis, two offspring from each berry were selected, the largest blueberry progeny and a second progeny (sibling) at random. The largest offspring was chosen to create a bias for outcrossing and increase the probability of selecting an offspring with an outcrossed father. Collection of highbush blueberry leaf tissue from the mothers and potential fathers took place in the spring of 2013 to ensure collection of younger leaves. Isolation of DNA from leaf tissue followed the plant protocol established by Doyle & Doyle (1987) with some modifications as follows. The procedure volumes were reduced to accommodate 1.5 ml isolation volumes, DNA was re-suspended in water, and additional steps included the addition of RNase A treatment followed by a Proteinase K treatment and a phenol:chloroform:isoamyl (50:48:2) wash to further clean the DNA. DNA isolations were conducted for offspring of the commercial cultivars: Aurora, Bluecrop, Duke, Elliott, Liberty, and Reka. DNA was isolated from two siblings per berry and 8 out of 10 berries (four out of five from each of east and west), for a total of 40 sibling pairs (80 progeny) per maternal parent. In addition to the offspring, DNA of all individuals from the agriculture Canada station was isolated (excepting two failures for the individuals, Chippewa and ORUS 10-1), for a total of 17 genotypes. Seven microsatellite markers developed by the United States Department

of Agriculture for highbush blueberry (Boches *et al.*, 2005; Table 4.1) were used to genotype all individuals, parents and offspring, using acrylamide gels on the LI-COR system (LI-COR Biosciences, Inc.). Amplification of the SSR markers followed the protocols set out in Boches *et al.* (2005). Scoring of genotypes was completed using the SAGA program for sizing of alleles and transferring data in a binary format to Excel. For each of the six mother genotypes, a clone check was performed by comparing two randomly chosen replicates of each genotype. All progeny samples were removed from the analysis if there were discrepancies with genotype.

Table 4-1: Primers developed by the USDA used to genotype and assess paternity for highbush blueberry. Published primers, CA190R to NA961 (Boches *et al.*, 2005) and unpublished primers (USDA), contigs 324F to 722F.

Locus	# bp in Repeat	Sequence	Size Range	# Alleles in Sample
CA190R	3	F: TTATGCTTGCATGGTGGTA R: TTGCGAAGGGACCTAGTAGC	250 - 280	3
CA344F	3	F: TTACCAAAACGCCTCTCCAC R: GTTTCTTCCTTACGCCCCTGAAAT	170 - 190	5
CA787F	3	F: TCCTCGTTCTCTCCCTCTCA R: GTTTCGCTGAAGTTGGAGTCCTT	270 - 300	6
NA961	3	F: TCAGACATGATTGGGGAGGT R: GTTTGGAATAATAGAGGCGGTGGA	205 - 220	4
contig 324F	3	F: AAGGAAGGGGGAGGGTTTAT R:TTTCCCCCACTTATTTGCAG	256	5
contig 588F	3	F: GGGGACGATCAAGAAGACAA R: CATTACGCCCCTGATTCACT	194	4
contig 722F	3	F: AAGTGGATTTCGATTCGGTG R:TAATCCCCATCACCGTCATT	204	4

PATERNITY ANALYSIS: A paternity analysis was conducted on the offspring from six maternal parents, Aurora, Bluecrop, Duke, Elliott, and Reka. A macro written by Riday *et al.* (2013) for use in the statistical program SAS (SAS Institute Inc.) was used to analyze paternity. The program works on the basis of exclusion analysis, where the choice of father is narrowed by presence of non-maternal alleles. For offspring with non-maternal alleles (outcrossed) fathers were selected and in those cases without consensus, fractional (probabilistic) paternity was calculated. In cases where all fathers are excluded, offspring were removed from the analyses. In cases where progeny have only maternal alleles, self-pollination is proposed as a default. Although the program is robust to null alleles, outcrossing can be underestimated by the presence

of nulls. Outcrossing can also be underestimated by inbreeding or close relations between parents due to the resulting shared alleles between mothers and potential fathers.

N ↑

Ch IV				Sd I	
O-1 IV	O-3 III	O-5 II		Sd I	
Ell IV	Blu III	98 II			
M60 IV	246 III	287 II		Ell I	
Blu IV	Aur III	Du II		Blu I	
287 IV	M36 III	Ell II		Re I	
	98 III	246 II		287 I	
246 IV	Du III	Re II		M36 I	
Lib IV	Ell III	Lib II		246 I	
98 IV	Re III	M60 II		Aur I	
12 IV	Dra III	Aur II		M60 I	
Aur IV	Lib III	Blu II		Du I	
Du IV	M60 III	12 II		98 I	
645 IV	287 III	Cha II		Lib I	
Re IV	Ell V	Aur V		287 V	
M60 V	Du V	98 V		Blu V	
Dra V	Lib V	Re V		246 V	

12	A-12
98	A-98
246	A-246
287	A-287
Aur	Aurora
Blu	Bluecrop
Ch	Chandler
Dra	Draper
Du	Duke
Ell	Elliott
Lib	Liberty
M36	MSU 36
M60	MSU 60
O-1	ORUS 10-1
O-3	ORUS 10-3
O-5	ORUS 10-5
Re	Reka
Sd	Seedlings
645	US 645

Figure 4-1: Field Design of highbush blueberry genotype collection at Agriculture Canada's research site in Abbotsford, BC.
Genotype name and replicate number with a representation by dots of the number of shrubs in each section.

61
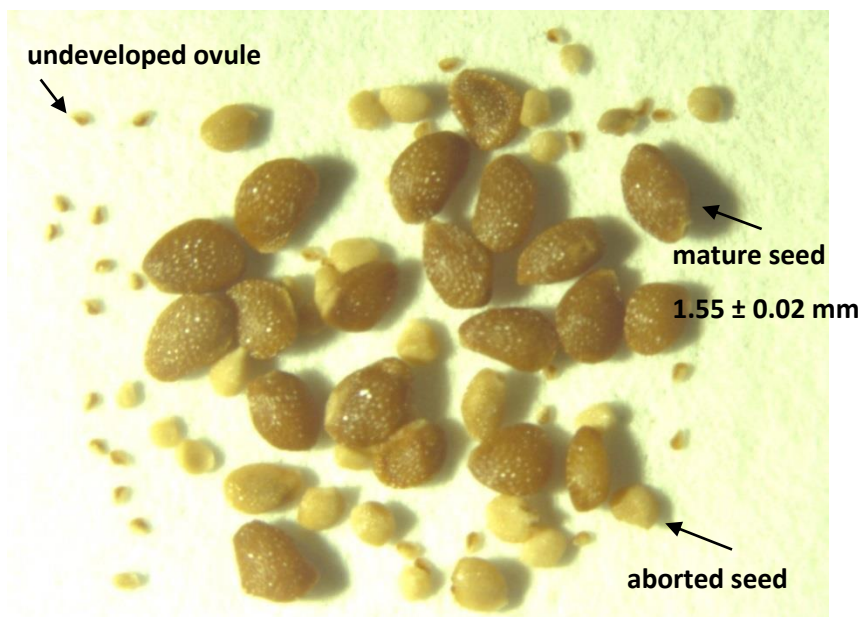
Figure 4-2: Seeds of one highbush blueberry fruit (maternal genotype, Liberty) including undeveloped ovule, early aborted seeds, and mature seeds.

4.3 Results

FRUIT AND SEED PARAMETERS:

Highbush blueberry maternal reproductive success was compared among genotypes by examining several fruit and seed characteristics. All factors considered, fruit weight, seed number, seed size, and seed germination rate, were significantly different at the genotype level using ANOVAs (Table 4.2). However, regression analyses showed there were no significant correlations among any of the measurements. Genotype relationships between mature seed number and fruit weight were expected due to previous reports; however, only two genotypes had R^2 values over 0.2, Liberty ($R^2 = 0.4$, slope = 0.05) and Reka ($R^2 = 0.2$, slope = 0.05). When the genotype seed numbers and fruit weights were pooled, the R^2 value was only 0.01 (slope = 0.008) and, in fact, Duke had a slightly negative relationship (Figure 4.3). There were significant differences found among genotypes for each parameter, although the variation among genotypes

may not have been large enough to show correlations especially as environmental factors impacting fruit and seed production could contribute to noise.

Table 4-2: Highbush blueberry maternal reproductive success as indicated by fruit and seed parameters. Letters represent Tukey multiple mean comparisons, with a being the largest, to show significant differences among genotypes.

|	Berry Weight (g)		Mature Seed #		Mature Seed Size (mm)		Germination Rate	
Aurora	1.38 ± 0.05	b	7.72 ± 0.58	c	1.52 ± 0.02	bc	0.61 ± 0.03	b
Bluecrop	1.39 ± 0.06	b	13.02 ± 0.88	ab	1.44 ± 0.02	c	0.77 ± 0.02	a
Duke	1.58 ± 0.06	ab	11.42 ± 0.54	bc	1.47 ± 0.02	bc	0.72 ± 0.02	ab
Elliott	0.97 ± 0.04	c	14.22 ± 0.76	a	1.54 ± 0.02	ab	0.46 ± 0.03	c
Liberty	1.64 ± 0.06	a	9.83 ± 0.78	c	1.55 ± 0.02	ab	0.75 ± 0.03	a
Reka	1.48 ± 0.05	ab	10.28 ± 0.43	c	1.45 ± 0.02	c	0.71 ± 0.03	ab
p-values	7.3×10^{-16}		1.8×10^{-10}		5.3×10^{-4}		3.4×10^{-15}	
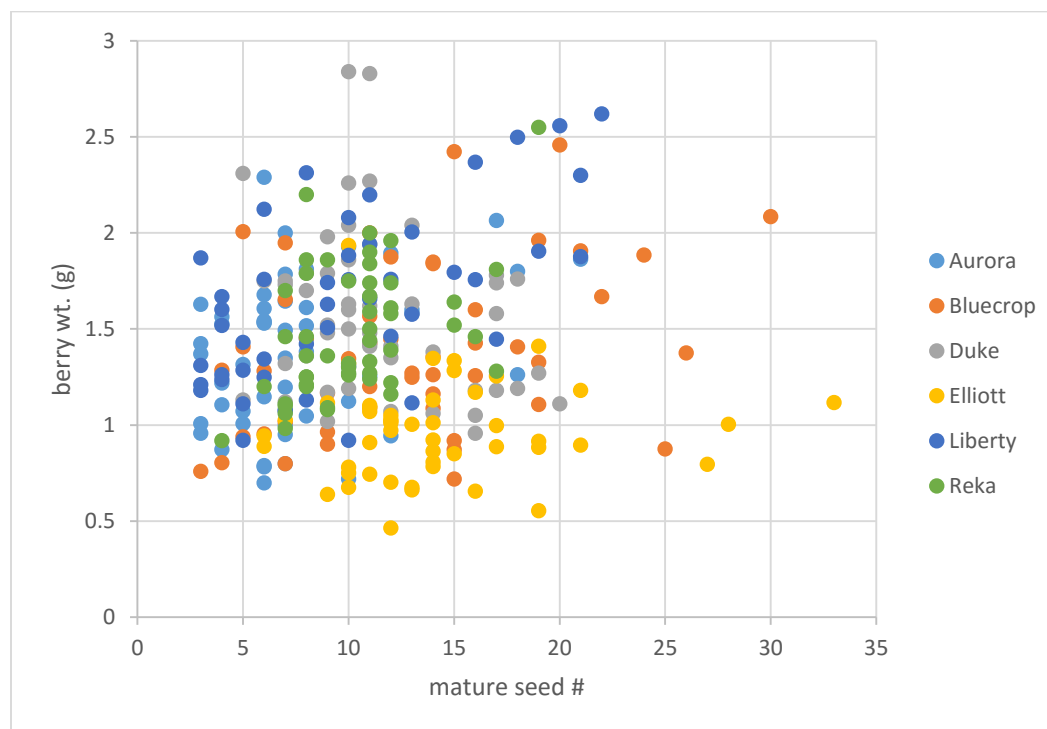
Figure 4-3: Mature seed count versus fresh weight of berry for berries from highbush blueberry genotypes.

PATTERN OF PATERNITY:

Conclusive paternity was difficult to assess for the offspring, likely due to the genetic similarity among the parents such that there were only three to five distinct alleles per locus in the population (average 4.4 alleles/locus). In addition, two potential fathers, Chippewa and ORUS 10-1, were not genotyped due to a failure to isolate DNA after several attempts. Table 4.3 shows the summary of results from the paternity assignment analysis using the macro developed by Riday *et al*. (2013). The number of possible self-pollinations for each maternal genotype is shown in Table 4.3 and in Table 4.4 in brackets. These are assigned as self-pollination due to the absence of a non-maternal allele and non-exclusion of fathers. Fathers are not excluded based on the absence of matching alleles, due to the possibility of null alleles. Because dosage cannot be reliably determined, null alleles could be hidden in a genotype in which fewer than four alleles are counted, and these could match with the father. The ratio of offspring with only maternal alleles (proposed selfed) to those with at least one non-maternal allele (outcrossed) varies widely among the progeny populations. This variation suggests there is a difference in tolerance to inbreeding among the mothers, i.e. Aurora and Liberty have higher numbers of outcrossed offspring.

There were forty-eight progeny (12%) overall mothers for which a father was unambiguously assigned. Bluecrop and Elliott were the most common fathers each siring 25% of the 48 progeny. Bluecrop and Elliott remained the most common fathers when the number of offspring was adjusted to the number of shrub replicates planted at the research station (Table 4.4). The identified father in each of these 48 cases was examined in terms of proximity to the mother genotype and bloom timing. Proximity was assessed based on concentric rings of genotypes in any direction from the mother (Figure 4.1). Bloom phenology roughly divides the genotypes into three groups, early, mid, and late. Half of the assigned fathers (49%) were a near neighbour of the mother, within the first ring of genotypes, and nearly half (40%) initiated flowering after the mother. Figure 4.4 shows the relationship ($R^2 = 0.59$) between a measure of maternal reproductive success (mature seed number) and paternal reproductive success (conclusive paternity relative to number of father replicates).

For all offspring with a non-maternal allele, where paternity could not be assigned conclusively and more than one father was implicated, a fractional paternity was calculated (Appendix B). The fractional paternity was estimated by summation of all the partial contributions across mothers and dividing by the number of field replicates of that father. For example, if an offspring had three potential fathers each of those fathers would be assigned a value of 1/3 and these fractions were averaged across the offspring.

Table 4-3: Summary of paternity results for highbush blueberry offspring including no assigned paternity, possible selfing (maternal alleles only), and conclusive and inconclusive paternity (non-maternal allele).

	Number of Offspring	No Assigned Paternity	Maternal Alleles Only	Non-Maternal Alleles	
				Conclusive Paternity	Inconclusive Paternity
Aurora	64	4	11	7	42
Bluecrop	72	0	47	8	17
Duke	72	3	41	10	18
Elliott	60	0	35	1	24
Liberty	62	5	7	17	33
Reka	68	7	31	5	25

Table 4-4: Conclusive paternity of highbush blueberry offspring including self-pollinations in brackets. The relative total shows the number of fathers assigned relative to the number of field replicates of that father, excluding self-pollinations. The most common fathers are in bold.

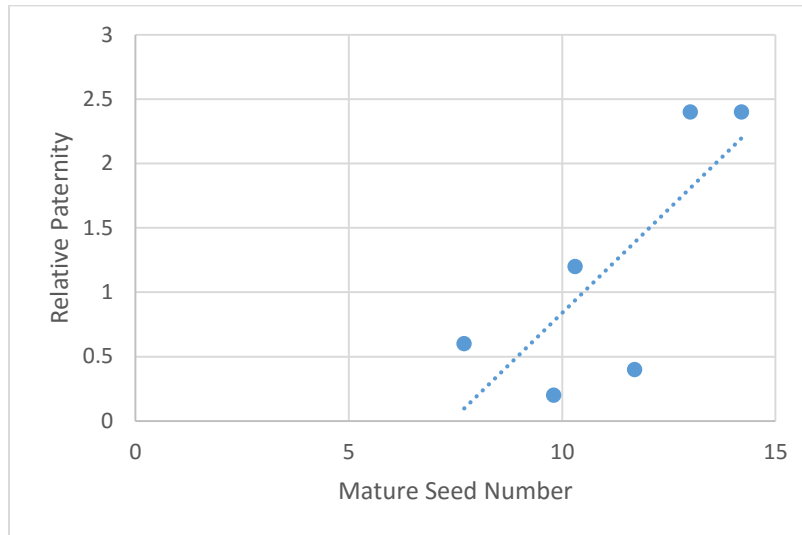|	Mothers						Relative Paternity
Fathers	**Aurora**	**Bluecrop**	**Duke**	**Elliott**	**Liberty**	**Reka**	**Paternity**
A 12							0
A 98		1					0.2
A 246			1		3		0.8
A 287							0
Aurora	(11)				3		0.6
Bluecrop	1	(47)	3	1	7		**2.4**
Chandler							0
Draper	1						0.5
Duke	2		(41)				0.4
Elliott		7	2	(35)		3	**2.4**
Liberty					(7)	1	0.2
MSU 36							0
MSU 60					1		0.2
ORUS 5							0
ORUS 10	3						1.5
Reka			4		2	(31)	1.2
US 645					1	1	1
Total	*18*	*55*	*51*	*36*	*24*	*36*	

Figure 4-4: Mature seed number versus relative paternity shows a relationship between female and male reproductive success ($R^2 = 0.59$).

ALLELIC RICHNESS:

Allele statistics were calculated for the seven microsatellite loci used in this analysis and comparisons made between the offspring groups that were harvested, the large sibling and the randomly selected sibling. Allelic richness was used as a proxy for measures of diversity since allele frequencies are required for these calculations and cannot be accurately calculated without dosage information. Unknown dosage of alleles at loci is a complication in tetraploid genotype data where an individual with three alleles, ABC, could have 3 different genotypes, AABC, ABBC, or ABCC (assuming no null alleles). Table 4.5 displays the allele statistics organized by maternal parent and shows significant differences among the siblings, the largest versus the randomly chosen. Mature seed number and germination rate were compared to allelic richness in Figure 4.5. However, neither of the reproductive factors, mature seed number nor paternity, appear to be related to allelic richness across these seven microsatellite markers.

Table 4-5: Comparison of averages for allelic richness, presence of non-maternal alleles (outcrossed), and number of non-maternal alleles based on 7 SSR primers.
The largest progeny and the randomly chosen progeny groups are shown, displayed by mother. Between-sibling paired t-tests performed on all progeny for allelic richness, number of non-maternal alleles, and genotype averages for % outcrossed.

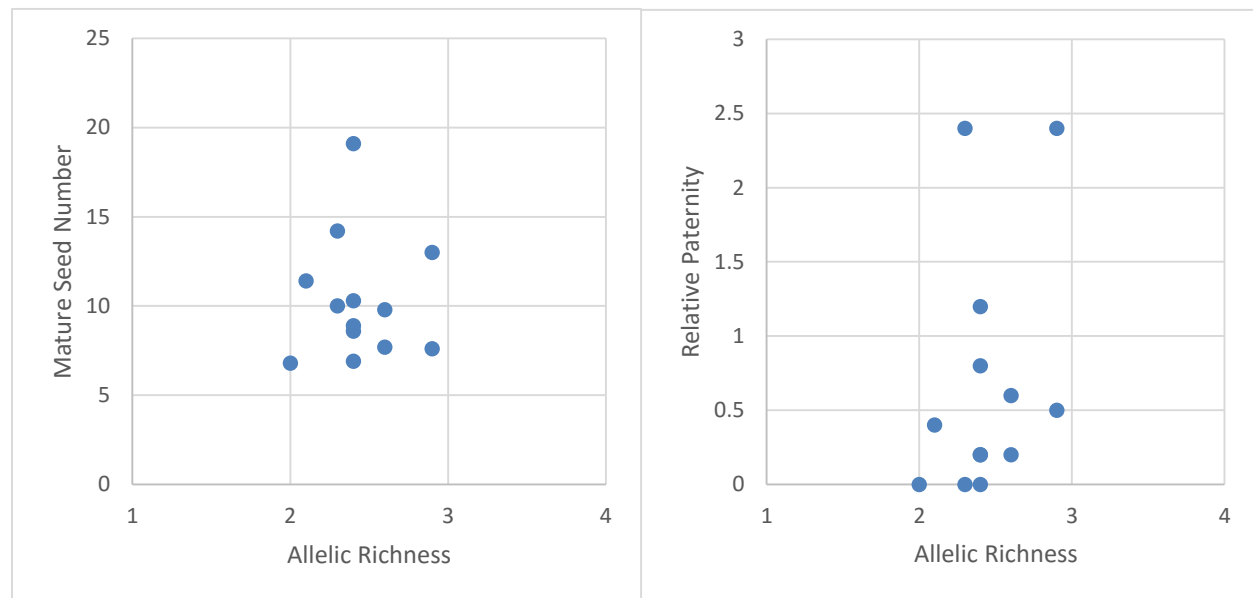|	Maternal Allelic Richness	Progeny Allelic Richness		% Outcrossed		# Non-Maternal Alleles per Progeny	
		Large	Random	Large	Random	Large	Random
Aurora	2.6	2.3	2.3	86	78	2.1	2.0
Bluecrop	2.9	2.5	2.3	39	31	1.1	1.1
Duke	2.1	2.0	1.9	58	33	1.8	0.9
Elliott	2.3	2.0	2.0	43	40	1.3	1.3
Liberty	2.6	2.2	2.2	90	84	1.9	2.0
Reka	2.4	2.2	2.1	71	38	1.6	1.0
	p-value	0.024		0.031		0.038	

Figure 4-5: For each blueberry genotype, allelic richness vs mature seed number ($R^2 = 5 \times 10^{-05}$) and vs relative paternity (excluding self-pollinations) ($R^2 = 0.1$) showing no relationships between the factors.

4.4 **Discussion**

The purpose of this study was to investigate the fertility of highbush blueberry genotypes grown in British Columbia. Fertility is an important part of blueberry production because fruit size has been correlated with seed set; however, the same correlation was not found here likely due to the fruit selection bias employed. Genotypic variation in fertility and reduced inbred offspring vigour were consistent with previous literature. Genotypic variation in fertility is discussed in relation to implications for blueberry growers and breeders.

THE RELATIONSHIP BETWEEN SEEDS AND FRUIT

Many researchers have cited a relationship between *Vaccinium* seed number and fruit weight; however, a correlation was only seen here for the genotypes, Liberty and Reka. This could be due to the fruit collection bias for outcrossing that was employed (most distal fruit); such that the fruits may have fulfilled the minimum required seed number for a fully developed fruit. Dogterom *et al*. (2000) compared four pollen tetrad application levels in highbush blueberry, 10, 25, 125, and 300 pollen grains, and found that the number of mature seeds increased with amount of pollen applied, however the fruit mass was only greater at the 125 and 300 pollen levels. Cane & Schiffhauer (2003), in a similar dose-response study in cranberry, reported that there is a minimum threshold and maximum asymptote (approximately 30 seeds) for cranberry fruit size. Both of these studies were conducted using one genotype of *Vaccinium*, and the genotype differences in fruit size observed in this study, Table 4.1, are most likely due to genotype characteristics.

RATES OF SELF-POLLINATIONS

There are many modes by which self-fertilization can occur but these can be organized into two broad categories, autonomous, in which there is no pollen vector, and facilitated, which involves pollen vectors (Lloyd & Schoen, 1992). Due to the morphology of the blueberry flower, downturned with a flute-shaped style, it is unlikely that autogamous pollen transfer can occur

(Vander Kloet, 1988). In fact, Cutler *et al*. (2012) found that fruit set on pollinator-excluded lowbush blueberry flowers was only 7%.

These offspring populations are not the result of random sampling because of the selection of early and most distal fruit and should not be interpreted to give outcrossing or selfing rates as would apply to mating system calculation. I estimated self-pollination levels for the progeny to be 42% (167 out of 398), but this would likely be higher if fruit was collected randomly throughout the season. The SAS macro developed by Riday *et al*., (2013) determines 'possible self-pollinations' as the presence of maternal alleles only and could overestimate selfing in two ways. First, outcrossed-pollinations could be underestimated if there are null alleles shared between the offspring and father. We know from other work that there are likely null alleles present in two of the seven primers used for this study (Chapter 5). Second, self-pollinations assigned through counting only maternal alleles could underestimate the number of outcrossed offspring in this data because so many of the fathers share alleles with the mothers. Highbush blueberry genotypes have been bred from a narrow pool of genetic resources (Ehlenfeldt, 1994) and because of this breeding history, many of the genotypes have shared alleles that are identical by descent. Despite the potential over-estimate of actual self-pollinations, the presence of large numbers of shared alleles can functionally represents self-pollination at those loci and similarly reduced heterozygosities (Ritland, 1984).

GENETIC LOAD: REPRODUCTIVE SUCCESS AND OFFSPRING VIGOUR

Genetic load and inbreeding depression in *Vaccinium* have been discussed as they relate to a decrease in seed survival with an increase in the relatedness of parent pairs (Hellman & Moore, 1983; Krebs & Hancock, 1990; Krebs & Hancock, 1991; Hokanson & Hancock, 2000). Vander Kloet (1983) observed a range in highbush blueberry fertility, with a strong relationship between female and male reproductive success; berry size and number of plump seeds per berry were related to pollen viability. A similar relationship, $R^2 = 0.59$, was observed in this data set between number of mature seeds (female reproductive success) and paternity (success as a pollen donor). In addition, Hellman & Moore (1983) investigated fresh weight of first-year seedlings and found a negative correlation between seedling vigour and increased inbreeding as described

by inbreeding coefficients. In this study, the largest offspring per berry is more likely to have been outcrossed and showed higher allelic richness than the randomly selected offspring from each berry (p=0.03 and p=0.02, respectively) (Table 4.4). On average, both the large and randomly collected offspring groups had lower allelic richness than the maternal genotype. However, in cases where the maternal genotype had only one or two alleles at a locus, the offspring, on average, had more alleles.

Allelic richness was not a good predictor of measures of reproductive success, mature seed number nor paternity (Figure 4.5). Heterozygosity- and mean squared distance-fitness correlations have been used by many to estimate the impact of inbreeding, reduced allelic heterozygosity, on fitness, i.e. inbreeding depression, with mixed and inconsistent results (Hansson, 2010; Slate *et al*., 2004). The variable utility of the allelic data- an association with offspring vigour but not with reproductive success -may have been improved with more loci, but it is possible that the high number of shared alleles among the parents may limit inference gains made from using more loci.

POLLINATOR CHOICE AND PARENT PAIRS

The early fruits were collected to increase the probability of observing non-geitonogamous pairings and therefore outcross pollen donors. However, only 12% of the offspring had outcrossed paternity confirmed (48 out of 398) while another 40 % (159) were outcrossed with unconfirmed paternity. The pattern of parentage is therefore based on relatively few cases for each maternal genotype and conclusions about the pattern are not possible. It was expected that paternity would most often belong to neighbouring plants due to the patch-type, resource efficient, foraging seen by bees (Waser, 1986). In addition, paternity was expected to belong more often to genotypes that bloom at the same time or slightly after the maternal genotype due to the dichogamous phenology of blueberry flowers. However, general observations were not clear in that only 50% of confirmed father genotypes were neighbouring the mother and 40% of the father genotypes bloomed slightly after the maternal genotype. As mentioned earlier, more loci would help with father identification; however, this would be at diminishing returns due to high numbers of shared alleles. Bluecrop and Elliott were the most common fathers here

partially because they were more easily resolved using this combination of microsatellite loci. More loci may reveal other fathers to be more common. However, Bluecrop and Elliott had high numbers of mature seed counts and a correlation between male and female fertility is consistent with previous literature.

Reproductive success is economically significant in a highbush blueberry agricultural context; however, there are many factors that influence the divide between the reproductive capacity and the reproductive success. Pollen movement is necessary for fruit development in blueberry but depending on the fertility (or tolerance to inbreeding) this may not need to be outcrossed pollen. In terms of preliminary recommendations for blueberry growers, Elliott and Bluecrop appear to be more self-fertile than the other genotypes examined and may be better choices for large planting blocks. More comprehensive assessments are needed to explore fertility variation among genotypes and this information would provide opportunities for cultivar improvement through breeding. Mixed planting have been recommended for half-high blueberry cultivars (Harrison *et al*., 1993(b)) and this may be the solution for less self-fertile highbush blueberry genotypes. Mixed plantings of compatible genotypes with similar bloom times, could increase the flow of outcross pollen and yield; however, field studies must demonstrate that yield increases outweigh the additional harvesting and cultivation costs that large monocrop systems minimize.

Chapter 5: Inheritance Pattern and Inbreeding in Highbush Blueberry

5.1   **Introduction**

Polyploidy is common in plants and impacts breeding programs in terms of interpreting trait segregation patterns, size of offspring populations required, and the effect of inbreeding (Acquaah, 2007). Polyploid inheritance patterns can fit along a spectrum, the opposing ends of which are disomic and polysomic inheritance. Disomic inheritance dictates that crossover during gamete formation is limited within grandparent genotypes, such that gametes will have 50% of their chromosomes from each grandparent. In contrast, polysomic inheritance dictates that gametes will have a random mixture of parental DNA (Allard, 1960). Historically, these patterns were thought to correspond to the mode of creation of the polyploid; disomic and polysomic inheritance for allo- and auto-polyploids, respectively. However, there are many processes and constraints acting on the genome that affect inheritance patterns, and more species than previously thought have been shown to exhibit inheritance that is a mixture of disomic and polysomic (Stift *et al*., 2008). Evidence for polysomic inheritance can be the random pairing of genes and the presence of double reduction, when the gamete receives two copies of a gene that the parent has only one copy of as a result of chromosome multivalent formation. In contrast, evidence for disomic inheritance can be the non-random pairing of genes (fixed heterozygosity) due to preferential pairing of chromosomes.

Cultivated highbush blueberry, *Vaccinium corymbosum*, is tetraploid but belongs in wild settings to a polyploid complex that includes diploid, tetraploid, and hexaploid populations (Vander Kloet, 1988). It is known that *Vaccinium* species can hybridize easily within ploidy levels (Ballington & Galletta, 1978) and the origins of the higher ploidy blueberries, whether auto- or allo-polyploids, are still uncertain and likely vary in cause (Camp, 1945; Vander Kloet, 1980). However, an autopolyploid origin for *V. corymbosum* has been suggested due to evidence of tetrasomic inheritance (polysomic inheritance for tetraploids). Draper & Scott (1971), Krebs & Hancock (1989), and Qu & Hancock (1995) have all found evidence leaning towards a

tetrasomic inheritance pattern for blueberry; however, these studies do not exclude the possibility of an inheritance pattern that is intermediate or mixed between tetrasomic and disomic.

The interspecific crossability and therefore likely hybridization events of the past and the interspecific contributions to genotypes that have been made by breeders (*reviewed in* Lobos & Hancock, 2015) suggest there could be significant deviation between homeologous chromosomes. In addition, the observations of predominant bivalent pairing of chromosomes (Jelenkovic & Hough, 1970; Jelenkovic & Harrington, 1971; Qu *et al.*, 1998) suggest intermediate inheritance as a possibility in highbush blueberry. More specifically, the type of inheritance could be genotype-specific as chromosome behaviour was seen to vary among genotypes with some displaying higher numbers of multivalents than others (Jelenkovic & Harrington, 1971). Combining knowledge about genotypic variation in trait segregation with breeding history including interspecific contributions to those genotypes could help breeders anticipate trait segregation.

Inbreeding impacts polyploids and diploids differently, in that the progress to a homozygosity is around three times slower for a tetraploid compared to a diploid and in tetraploids more than one generation of random mating is required to reach Hardy-Weinberg equilibrium (*reviewed in* Bever & Felber, 1992). Cultivated blueberries, highbush and lowbush, are known to suffer from early-acting inbreeding depression that results in reduced fruit and seed set when inbred (Hellman & Moore, 1983; Krebs & Hancock, 1990; Krebs & Hancock, 1991; Hokanson & Hancock, 2000). Pedigree inbreeding coefficients have been calculated for highbush blueberry using a disomic interpretation of pedigrees (Hancock & Siefker, 1982) and more recently a tetrasomic interpretation (Krebs & Hancock, 1990; Ehlenfeldt, 1994). However, there are many gaps in blueberry pedigrees due to a history of open pollination, which create challenges for determining inbreeding coefficients for some genotypes (Brevis *et al*., 2008). Microsatellite markers can be used to estimate inbreeding based on levels of heterozygosity; but for populations experiencing non-random mating, pedigree inbreeding coefficients have been found to more accurately reflect inbreeding (Baumung & Solkner, 2003). Measures such as heterozygosity and mean squared distances among microsatellites show variable correlations with pedigree inbreeding coefficients (Hendrick *et al*., 2001; Curik *et al*., 2003). In addition, calculating

heterozygosity for non-random mating polyploid individuals is more error-prone when the dosage of alleles is unknown, and must be estimated.

The goal of this research was to address two important breeding questions; is there genotypic variation in inheritance pattern, and what are the levels of inbreeding in cultivars used in the Agriculture Canada breeding program in British Columbia? Knowledge of inheritance pattern is essential to monitor the segregation of traits and the impacts of inbreeding. With this information, blueberry breeders would be able to better predict trait segregation and to incorporate information about the inbreeding levels to ensure vigorous cultivar development.

5.2 **Methods**

LOCATION: The study sites for collection of highbush blueberry samples were the Agriculture Agri-Food Canada blueberry plots in Abbotsford and in Agassiz, BC. The controlled crosses used to determine inheritance pattern were made by Chaim Kempler in 2007 and 2008. The offspring of the controlled crosses are located in Agassiz, BC. The parent material, eight-year old shrubs, is located in Abbotsford, BC. Five controlled crosses each with approximately 30 offspring, for a total of 150 individuals, were used for the inheritance and inbreeding analyses (Table 5.1). The Abottsford blueberry plot has approximately fifteen cultivars, both research and commercialized. Partial pedigrees were available for ten of these genotypes: A-98, Aurora, Bluecrop, Chippewa, Draper, Duke, Elliot, Liberty, MSU-36, Reka, and US-645. However, full pedigree information was available for only one of the crosses included in the study. Additional plant material was obtained from the National Clonal Germplasm Repository in Corvallis, Oregon to examine other family relationships such as full and half sibs, and grandparents.

Table 5-1: Controlled crosses performed by Chaim Kempler through Agriculture and Agri-Food Canada with number of progeny used in analyses.

Cross	Mother	Father	# of Progeny
1005	A12	Elliott	30
1009	A246	MSU36	29
1103	A246	Duke	25
1112	Bluecrop	Elliott	29
1114	Draper	A12	30

DNA ISOLATION: Leaf tissue of the blueberry genotypes was collected in Abbotsford and Agassiz, stored on dry ice, and then transported back to the University of British Columbia where it was stored at -80 ^{o}C until DNA isolation. The CTAB procedure of Doyle and Doyle (1987) was used to isolate DNA as follows. Leaf tissue was ground while frozen using liquid nitrogen and mortar and pestle. Leaf tissue obtained from the National Clonal Germplasm Repository in Corvallis, Oregon, arrived lyophilized and was ground from this dried state. The tissue was incubated with the CTAB buffer at 65 ^{o}C before washing with chloroform:isoamyl (25:1) then precipitated using 70% isoamyl. The re-suspended precipitate was incubated with RNAse and Proteinase then washed once with phenol:cholorform:isoamyl (26:25:1) and once with chloroform:isoamyl (25:1). The samples were precipitated using 100% ethanol and rinsed with 70% ethanol before re-suspension in water and storage at -20 ^{o}C. DNA quantity and quality was assessed using a NanoDrop 2000 and associated software (Thermo Fisher Scientific Inc.)

GENOTYPING: Researchers at the United States Department of Agriculture developed and evaluated more than 250 microsatellite markers for *Vaccinium* using highbush blueberry expressed sequence tags and genomic libraries, 30 of which have been published (Boches *et al*., 2005). All collected individuals were genotyped using 15 of these short sequence repeat (SSR) markers developed by the United States Department of Agriculture (Table 5.2). The PCR reactions were performed following protocols outlined by Boches *et al*. (2005) and the product was run using acrylamide gels on the LI-COR system (LI-COR Biosciences, Inc.). All gels were scored using Saga[GT] Software (LI-COR Biosciences Inc) to call base pair-length which was

recorded in Excel 2013 (Microsoft Office 2013, Microsoft) because Saga[GT] Software cannot process polyploids. All gels were scored twice to ensure consistent calling of alleles across each primer and 10% of samples were re-analyzed from PCR for error analysis.

Table 5-2: Primers developed by the USDA used to genotype and assess inheritance pattern for highbush blueberry.
Published primers, CA190R to VCC_K4 (Boches *et al*., 2005) and unpublished primers, contigs 78F to 722F.

Locus	# bp in Repeat	Sequence	Size Range	# Alleles in Sample
CA190R	3	F: TTATGCTTGCATGGTGGTA R: TTGCGAAGGGACCTAGTAGC	250 - 280	3
CA344F	3	F: TTACCAAAACGCCTCTCCAC R: GTTTCTTCCTTACGCCCCTGAAAT	170 - 190	5
CA787F	3	F: TCCTCGTTCTCTCCCTCTCA R: GTTTCGCTGAAGTTGGAGTCCTT	270 - 300	6
CA855F	2 x 3	F: CGCGTGAAAAACGACCTAAT R: GTTTACTCGATCCCTCCACCTG	250 - 300	9
NA398	4	F: TCCTTGCTCCAGTCCTATGC R: GTTTCCTTCCACTCCAAGATGC	210 - 240	5
NA741	2	F: GCCGTCGCCTAGTTGTTG R: GTTGATTTTGGGGGTTAAGTTTGC	240 - 290	11
NA961	3	F: TCAGACATGATTGGGGAGGT R: GTTTGGAATAATAGAGGCGGTGGA	205 - 220	4
NA1040	2	F: GCAACTCCCAGACTTTCTCC R: GTTTAGTCAGCAGGGTGCACAA	180 - 270	9
VCC_J3	3	F: TGATTACATTGCCAGGGTCA R: TGGAAACAACCGGGTTACAT	150 - 200	5
VCC_K4	2 x 2	F: CCTCCACCCCACTTTCATTA R: GCACACAGGTCCAGTTTTTG	150 - 300	11
contig 78F	3	F: AAAGCCAAAGTATTGGGTGG R: CAAACCTTTAGCCAGTCCCA	280	4
contig 324F	3	F: AAGGAAGGGGGAGGGTTTAT R:TTTCCCCCACTTATTTGCAG	256	5
contig 563F	2	F: CCTCCAAAAAGGACAGCAGA R: GAAGACCAGAGGCTGTTTCG	228	3
contig 588F	3	F: GGGGACGATCAAGAAGACAA R: CATTACGCCCCTGATTCACT	194	4
contig 722F	3	F: AAGTGGATTTCGATTCGGTG R:TAATCCCCATCACCGTCATT	204	4

INHERITANCE PATTERN ANALYSIS: Microsatellite markers can be used to determine the inheritance pattern when parent(s) and offspring are genotyped. For polyploids, genotyping with microsatellites can be greatly complicated by the problem of unknown allele dosage of heterozygotes, in that if there are fewer than four alleles it is not possible to know which allele(s) have more than one copy. As well, there is a challenge with this unknown dosage masking the

presence of null alleles, where one cannot distinguish a homozygote from a heterozygote that includes a null allele. The term geno-phenotype is used to describe the partial genotype, which gives information about the number of different alleles, but does not include dosage and therefore does not describe the genotype. The SSR data was used to both examine the inheritance pattern of highbush blueberry (*V. corymbosum*) and examine allelic richness. Due to the complications of unknown allele dosage and the inability to detect null alleles, options for inferring inheritance patterns and levels of inbreeding are limited.

There are two points of evidence for polysomic inheritance, random allele assortment among offspring and the presence of double reduction. Double reduction is where both sister chromatids (i.e. the same allele copy from the same grand-parental chromosome copy) are found in a gamete and can only result when multivalents (groupings of more than two chromosomes), are formed in meiosis (Levings & Alexander, 1966). The clearest evidence of disomic inheritance is fixed heterozygosity, where allele pairings are limited and some alleles may not be found in the same gamete. I evaluated evidence for tetrasomic and disomic inheritance by predicting allele distributions under tetrasomic and disomic conditions (Table 5.3 and 5.4, respectively) and comparing these to the allele distributions observed at each locus in the offspring populations. Fixed heterozygosity can be explored using the linkage disequilibrium equation within each locus, where the frequency of finding any two alleles together does not equal the multiplied frequencies of the alleles. I also examined each locus within populations for indications of fixed heterozygosity using the following equation for linkage disequilibrium to assess the potential deviation from expectation:

Linkage Disequilibrium $D = f(AB) - [f(A) * f(B)]$, where alleles A and B are two of a four allele geno-phenotype and are **unshared** between parents

Table 5-3: Expected tetraploid parental genotypes and gamete production based on a tetrasomic inheritance pattern and random chromosome assortment with no multivalent production (no double reduction) and the resulting proportion of offspring with at least one allele copy.

Genotype	Gametes						Proportion of Offspring with Allele							
AAAA	AA	AA	AA	AA	AA	AA	A	1.00						
AAAB	AA	AA	AA	AB	AB	AB	A	1.00	B	0.50				
AABB	AA	AB	AB	AB	AB	BB	A	0.83	B	0.83				
ABBB	AB	AB	AB	BB	BB	BB	A	0.50	B	1.00				
AABC	AA	AB	AB	AC	AC	BC	A	0.83	B	0.50	C	0.50		
ABBC	AB	AB	BB	AC	BC	BC	A	0.50	B	0.83	C	0.50		
ABCC	AB	AC	AC	BC	BC	CC	A	0.50	B	0.50	C	0.83		
ABCD	AB	AC	AD	BC	BD	CD	A	0.50	B	0.50	C	0.50	D	0.50

0.50 = 12, 0.83 = 5, 1.00 = 3

Table 5-4: Expected tetraploid parental genotypes and gamete production based on a disomic inheritance pattern and preferential pairing of chromosomes and the resulting proportion of offspring with at least one allele.

Genotype	Gametes						Proportion of Offspring with Allele											
	Situation 1		Situation 2		Situation 3													
AAAA	AA	AA					A	1.00										
AAAB	AA	AB					A	1.00	B	0.50								
AABB	AA	BB	AB	AB			A	0.50	B	0.50	**or**	A	1.00	B	1.00			
ABBB	AB	BB					A	0.50	B	1.00								
AABC	AA	BC	AB	AC			A	0.50	B	0.50	C	0.50	**or**	A	1.00	B	0.50	C	0.50
ABBC	AB	BC	AC	BB			A	0.50	B	1.00	C	0.50	**or**	A	0.50	B	0.50	C	0.50
ABCC	AB	CC	AC	BC			A	0.50	B	0.50	C	0.50	**or**	A	0.50	B	0.50	C	1.00
ABCD	AB	CD	AC	BD	AD	BC	A	0.50	B	0.50	C	0.50	D	0.50				

0.50 = 23, 1.00 = 8

INBREEDING ANALYSES: Pedigree inbreeding coefficients, the earliest calculation of inbreeding, has been argued to be the most accurate measure for assessing inbreeding (Baumung & Solkner, 2003). Generally, the pedigree inbreeding coefficient is a summation of the probability that the offspring will inherit two of the same genes due to common ancestors within the family tree (Falconer, 1960). The pedigree inbreeding coefficients were calculated with the equation used by Krebs & Hancock (1990), Ehlenfeldt (1994), and Brevis *et al*. (2008) to calculate blueberry inbreeding coefficients. The pedigree inbreeding coefficient was modified by the addition of the inbreeding coefficients of the parents and the factor which accounts for the probability of transferring an allele as follows:

$F_{offspring} = 1/6 \, (F_{parent \, y} + F_{parent \, z} + 4 \, R_{yz})$, where the relationship coefficient of the parents is R_{yz} $= \sum [\, (1/4)^{n1 + n2 + 1} \, (1 + 3F_{ancestor})$

Parental assessments of inbreeding through an estimate of pairwise relatedness were completed using the microsatellite marker data in the PolyRelatedness program developed by Huang *et al*. (2014). The markers were also used to assess various measures of diversity within and among the families based on the well explored concept of heterozygosity-fitness correlations. Statistical analyses comparing these allelic measures included paired t-tests and were performed using the functions available in Excel 2013. Allelic richness and proportion of loci that are polymorphic were calculated for the parents and offspring from each cross, as was calculated in Chapter 4. Mean squared distance (d^2) of alleles is a measure of the amount of variation within locus and estimates individual allelic variation based on the stepwise mutation model; however, this method has been criticized for a lack of correlation with inbreeding (Hansson, 2010). Mean d^2 was calculated using a modified equation to accommodate polyploidy by simply examining the distance between the largest and smallest alleles at each locus, in order to capture the spread of allele sizes. The mean squared distance of alleles equation introduced by Coulson *et al*. (1998) is as follows:

mean $d^2 = 1 \, / \, n \sum (i_a - i_b)^2$, where the equation was modified such that mean d^2 is summed over all loci (n), i_a and i_b were the length in repeat units of the largest allele a and the smallest allele b at locus i, and n is the number of loci.

Relative population size, is an examination of the amount of heterozygosity relative to the maximum possible heterozygosity where the number of alleles at each locus is considered relative to the maximum possible number of alleles (4) in an equation as follows:

Relative Population Size (RPS) = $\sum$ (# alleles i / 4 n) / # individuals, where RPS was summed over all loci, i, and divided by the maximum number of alleles for a tetraploid (4) and the number of loci, n, then made relative to number of individuals in the population.

5.3   Results

INHERITANCE ANALYSIS:

The analysis of inheritance pattern was complicated by the issue of unknown allele dosage (unknown allele copy number). Where the individual has two or three different alleles presenting, the most common case in my data, there are three potential genotypes. For example, an individual with an AB geno-phenotype could have a genotype of AAAB, AABB, or ABBB, or an individual showing ABC could have a genotype of AABC, ABBC, or ABCC. If these two individuals are crossed, there are nine possibilities of offspring ratios (3 genotypes X 3 genotypes). Unknown dosage is further complicated in this data set because highbush blueberry is derived from a narrow breeding pool (Lyrene & Ballington, 1986) and many of the parent pairs share alleles at the loci.

The method used to assess inheritance was therefore modified to focus on situations where a deviation from tetrasomic inheritance would be visible, for alleles that are **unshared** between parents. For the four parental geno-phenotypes possible, one to four alleles, there are eight different possible genotypes. For those genotypes, Table 5.3 outlines the gametes that are expected under a tetrasomic pattern without any multivalent formation or double reduction and the proportional occurrence of those alleles in an offspring population. There are three possible proportions in the offspring, 0.50, 0.83, and 1. Table 5.4 outlines the gamete proportions that are expected under a disomic pattern including the options for preferential pairing, as situation 1

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through 3. Over all disomic cases, just like diploid inheritance, there are two possible proportions of alleles, 0.5 and 1.00.

The description above can be compared against the actual distribution of the proportion of offspring alleles, separating the alleles into those that are and those that are not shared between the parents (Figure 5.1). As expected, the frequency of proportions for the **shared** alleles (107 allele cases) diminishes from 1 to the lowest being 0.47 (around 0.5). In contrast, the shape of the frequency graph of **unshared** allele proportions has a centre around the proportion, 0.50. Out of the 168 **unshared** allele cases, 101 of the alleles fell within the proportions 0.41 to 0.60 in the offspring populations. This is not surprising since the very nature of the **unshared** alleles means they are less common and likely to be present with only one copy. As well, the shape of the distribution is roughly symmetrical, with 21 offspring found to have proportions between 0.31 to 0.40 and 17 offspring with allele proportions between 0.61 to 0.70. The same symmetrical trend centred around proportions of 0.50 was found when the range of allele proportions were categorized by cross, Figure 5.2, and by loci, Figure 5.3.

Figures 5.1, 5.2, and 5.3 show a slight deviance from the symmetrical trend with an increase in allele proportions around 0.8 and 0.9 that could indicate tetrasomic inheritance. Specifically, the crosses 1005, 1103, and 1114 as well as the loci NA 961, ct 324, and ct 722 have an increase in allele proportions in this range and suggest there may be some **unshared** alleles present in more than one copy. Generally, the lack of allele proportions near 0.83 (i.e. a 5:1 ratio) muddies the distinction between disomy and tetrasomy since that proportion is not possible with disomy and is a key indicator of tetrasomic inheritance.

Thirteen percent of the 168 **unshared** allele cases were present in proportions between 0.24 and 0.40 which could be the result of variation and relatively small offspring populations. Otherwise, these lower allele proportions could be due to the formation of multivalents during meiosis as double reduction would result in ratios different from those in Table 5.3. Multivalent formation can be variable and can result in allele proportions ranging from 0.25 to 0.50; in addition, multivalents could also limit the number of allele proportions that approach 1.00.

A commonly used line of evidence for disomic inheritance is fixed heterozygosity, where pairing among alleles is limited. **unshared** alleles from fully heterozygous parental geno-phenotypes (4 alleles) were used to examine limited pairing. These situations were necessary in order to ensure the actual frequency of the allele versus the proportion of appearance. No evidence of fixed heterozygosity was found for any of these 15 loci.
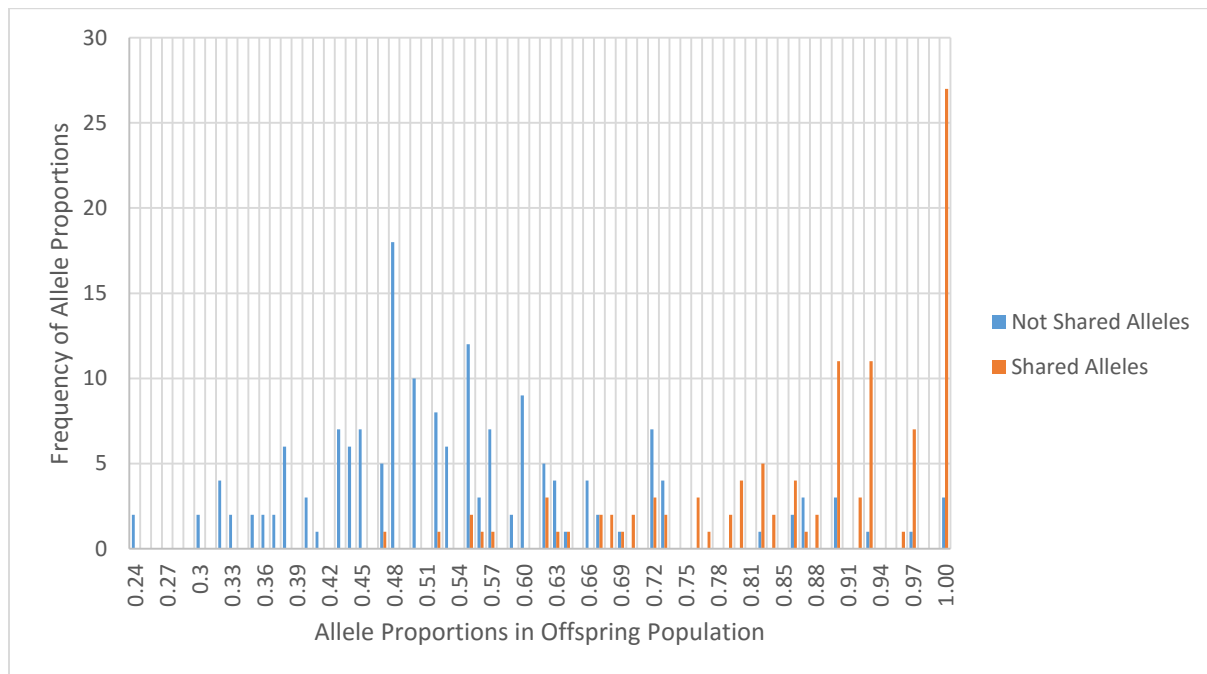

Figure 5-1: Analysis of the proportions of parental alleles in the offspring population.
Including alleles that are unshared (168) and shared (107) between parents with the allele by population conditions considered independently. Expected population proportions are: 0.50, 0.83, and 1.00 for unshared, and a range from 0.5 to 1.00 for the shared alleles.
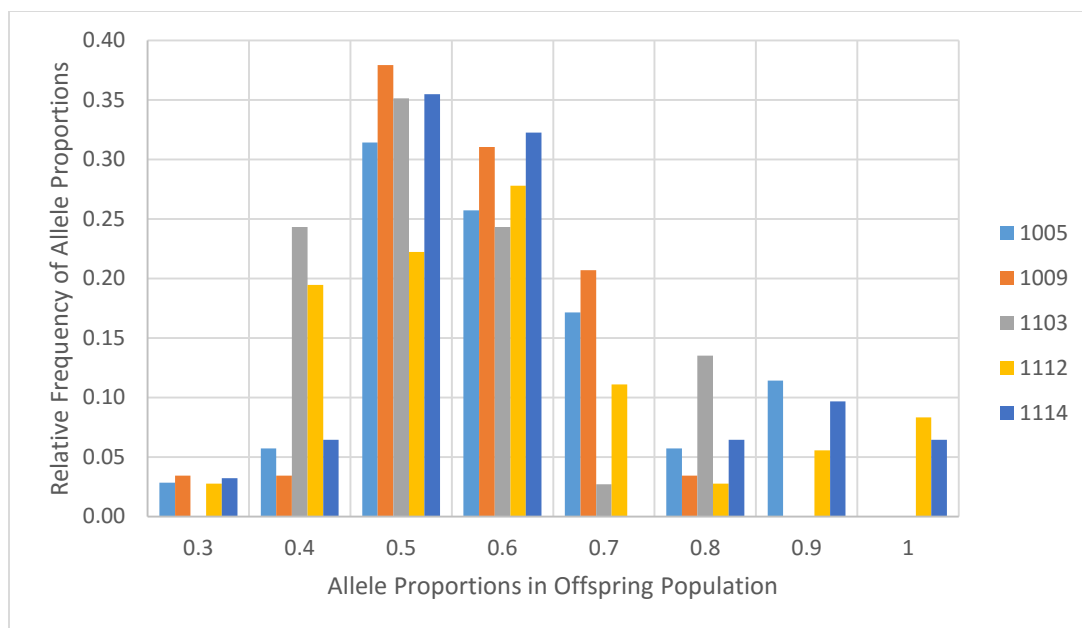
Figure 5-2: Proportions of unshared parental alleles in the offspring population separated by cross.
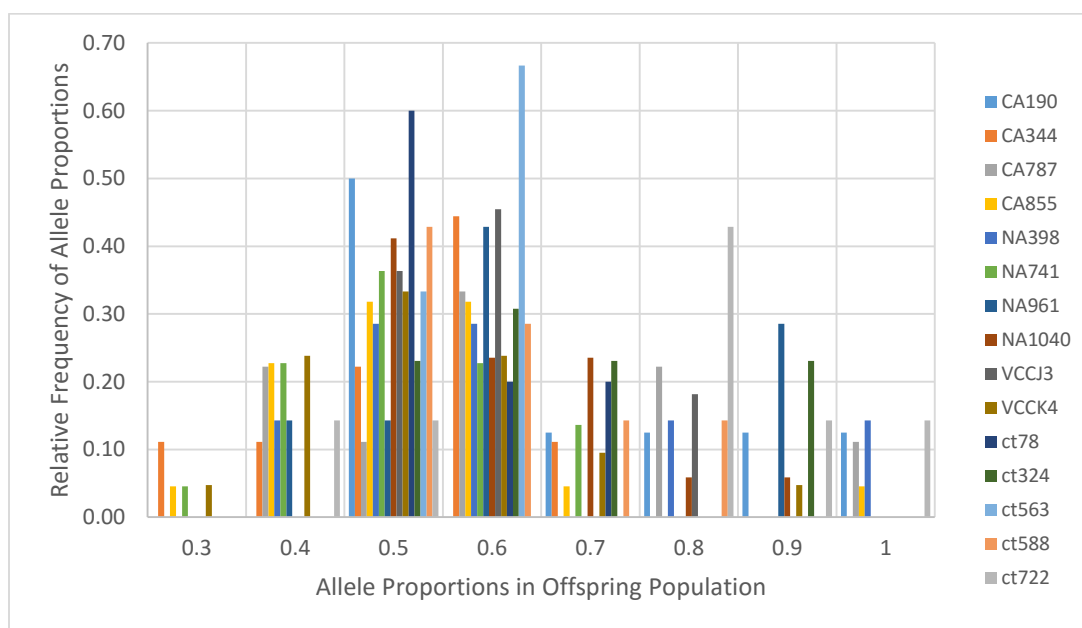

Figure 5-3: Proportions of unshared parental alleles in the offspring population separated by loci.

NULL ALLELES:

Six combinations of highbush blueberry parents and primers had some offspring with apparent null alleles, i.e., no parental allele was detected. This occurred for the primers CA 190 and contig 324 (Table 5.5). For primer CA 190, missing alleles were noted for crosses involving the genotypes A 246 and Elliott. For primer contig 324, missing alleles were noted for crosses involving the genotypes Duke and Draper. Missing parental alleles were detected for both mothers and fathers and were consistent among crosses, thereby reducing the possibility that they were caused by a mistaken pollination. In addition, Duke is the maternal parent of Draper and both produced offspring with discrepancies for the primer contig 324. Null alleles can impact some genotypic analyses and therefore these primers have been excluded from relevant analyses.

Table 5-5: Evidence of null alleles for two loci, CA 190F and contig 324F (ct 324F).
Including the number of offspring that do not have an allele matching the parent shown above.

cross	1005		1009		1103		1112		1114	
	mother	father	mother	father	mother	father	mother	father	mother	father
Primer	A12	Elliott	A246	MSU36	A246	Duke	Bluecrop	Elliott	Draper	A12
CA 190F	---	5	3	---	5	---	---	1	---	---
ct 324F	---	---	---	---	---	3	---	---	3	---

PEDIGREE INBREEDING COEFFICIENT:

Highbush blueberry breeding has followed a pedigree format. However, there are many gaps in the pedigree data, because of open pollinations and unknown proprietary information. Due to gaps in pedigrees, a full inbreeding coefficient calculation was only possible for one offspring population, cross 1112, where $F = 0.0038$.

PAIRWISE RELATEDNESS:

The 'Ritland estimator' within the PolyRelatedness program (Huang *et al.*, 2014) was used to estimate pairwise relatedness among the parents because it gave the highest relatedness value for a genotype against itself. However, the relatedness value was on average was 0.85 for the same individual which suggests more loci and/or less ambiguous loci are needed to refine this

analysis. Despite the high number of shared alleles, all relatedness coefficients among parental combinations used in this study were found to be negative, other than A 246 and MSU 36 (cross 1009) which had a relatedness coefficient of 0.11.

ALLELIC VARIATION:

For the allelic analyses, the 13 markers free of evidence of null alleles were used (CA344F, CA787F, CA855F, NA398, NA741, NA961, NA1040, VCC_J3, VCC_K4, as well as the contigs 78F, 563F, 588F, and 722F). The proportion of shared alleles between parents was calculated to be around 40% for the crosses (Table 5.6). The shared alleles are assumed to be identical by decent due to the narrow breeding pool of highbush blueberry, the largest portions of genetic material coming from only three wild accessions (Ehlenfeldt, 1994). The allelic richness of the offspring population was found on average to be 2.4 alleles per individual and 2.6 for the parent genotypes, with 87% of loci typed to have more than one allele. A similar measure, the proportion of heterozygous loci, was also included. All individuals, parents and offspring, had high numbers of heterozygous loci and for many of the parent genotypes all loci were heterozygous. A modified mean squared distance (d^2) of alleles was calculated in order to examine variation within the loci and no difference was seen between parental averages and offspring averages. The final column on Table 5.6 are the relative population sizes based on the allelic richness relative to the total richness possible, 4. As expected, allelic richness, proportion of heterozygous alleles, and relative population sizes of the offspring populations were lower than the parental average using paired t-tests (p = 2.9 x 10^{-03}, p = 9.4 x 10^{-04}, and p = 5.8 x 10^{-03}, respectively).

Table 5-6: Measures of allelic diversity across and within loci for parents and offspring of controlled crosses. Including: the proportion of shared alleles between parents, allelic richness, proportion of loci with more than 1 allele, mean squared distance among alleles, and the relative population size as compared to maximum heterozygosity (4 alleles per locus), and paired t-test comparisons between the parental average and the offspring average.

Cross		Prop. Shared Alleles	Allelic Richness	Prop. Heterozygous	mean d^2	Relative Population Size
1005	parents	0.370	2.420	1.000	387	0.615
	offspring		2.370	0.908	374	0.608
1009	parents	0.413	2.460	0.846	535	0.616
	offspring		2.350	0.790	463	0.588
1103	parents	0.360	2.577	0.923	371	0.644
	offspring		2.390	0.828	400	0.598
1112	parents	0.392	2.731	1.000	626	0.673
	offspring		2.580	0.918	440	0.646
1114	parents	0.438	2.654	0.962	322	0.663
	offspring		2.540	0.918	395	0.635
paired t-test			2.9×10^{-03}	9.4×10^{-04}	0.25	5.8×10^{-03}.

5.4 Discussion

The purpose of this study was to expand on previous highbush blueberry inheritance studies that have reported a tetrasomic inheritance pattern by detailing potential genotype variation in segregation patterns using microsatellite markers. In addition, to expand on reported inbreeding coefficients of blueberry genotypes with estimates using the microsatellite markers. Limitations in data analysis due to the current levels of inbreeding in blueberry genotypes will be discussed.

INHERITANCE PATTERN

Table 5.3 outlines the simplest case of tetrasomic inheritance involving random assortment of homologous chromosomes into bivalents, often called random chromosome assortment. However for loci on chromosomes that form multivalents, there is a wider range of allele proportions possible due to double reduction, often called random chromatid segregation. The

amount of double reduction depends on many factors including the probability of multivalent formation, the probability of equational division, the number of chiasmata, and the probability of the sister chromatids moving to the same gamete (*reviewed in* Bever & Felber, 1992). Crossing-over during the first half of meiosis has been discussed as it relates to tetraploids (Haldane, 1930; Little, 1945). However, in the simplest case of tetrasomic inheritance with no multivalent formation, crossing over will not impact the final allele proportions. In disomic inheritance models, chiasma formation is restricted by preferential pairs of chromosomes, which creates a sort of linkage disequilibrium within the locus and only two allele proportions (0.5 and 1.0).

Highbush blueberry cytotype research reaches back more than 40 years and was first published by Jelenkovic & Hough (1970). They found that between 93 and 96% of the chromosomes paired into bivalents. Similarly, Jelenkovic and Harrington (1971), found low numbers of multivalents, between one and five, in around three-quarters of the pollen mother cells examined. Marker-based research on inheritance pattern of blueberry such as the single gene recessive trait, seedling albinism, displayed a ratio close to expected for tetrasomic inheritance (Draper & Scott, 1971). In addition, markers segregated closest to a tetrasomic model in studies using isoenzyme loci (Krebs & Hancock, 1989) and RAPD markers (Qu & Hancock, 1995; Vorsa & Novy, 1995; and Qu & Hancock, 1997). Four out of six isoenzyme markers showed segregation patterns that were closest to tetrasomic segregation with random chromosome assortment and no double reduction (Krebs & Hancock, 1989). For the RAPD marker analyses, tetrasomy was described as segregation in a ratio of 5:1 (or a proportion of 0.83 as described in this study) since disomic ratios are either 1:1 or 1. In these analyses, variable amounts of tetrasomic loci segregating at 5:1 were observed including 28% (31/109), 50% (7/14), and 76% (65/85) (Qu & Hancock, 1995; Vorsa & Novy, 1995; Qu & Hancock, 1997, respectively).

The patterns of inherited allele proportions in this study show no strongly distinguishing evidence for tetrasomic or disomic inheritance. This is because Figures 5.1, 5.2, and 5.3 show highest frequencies of **unshared** allele proportions around 0.50 and both inheritance models are likely to produce this result. Very few alleles were found to produce the tetrasomic distinguishing ratio, 5:1; in fact, only 8% of alleles were in the range of 3:1 to 1. However, when the data are organized by cross and by loci, the frequencies of allele proportions show some

indication that there may be tetrasomic segregation patterns for some loci. The nature of examining **unshared** alleles confounds these results because if the allele is present in only one copy it will show a ratio of 1:1. Although allele proportions of 0.5 were expected for **unshared** alleles due to their likelihood of being less common, there is a wide range of allele proportions around 0.5. There are many factors that could impact these proportions such as multivalent formation and linkage between loci that are under selection; however, larger offspring population sizes could reduce this error or elucidate the impacting factors. Preferential pairing in disomic inheritance models can cause fixed heterozygosity (or within locus linkage) of which there was no evidence in this data. By a reasoning of exclusion, these results corroborate the previous work that has been done on highbush blueberry, suggesting a mainly tetrasomic inheritance model dominated by bivalent pairing, random chromosome assortment, with a small number of multivalents (Krebs & Hancock, 1989; Qu & Hancock, 1995; Vorsa & Novy, 1995; and Qu & Hancock, 1997).

INBREEDING AND ALLELIC VARIETY

Inbreeding affects polyploids differently than diploids and also has variable effects within polyploids such that the increase in homozygosity due to inbreeding is slower for polyploids than for diploids. Generally, it has been proposed that homozygosity increase is 2.9 times slower for polyploids that exhibit multivalent formation and double reduction and 3.8 times slower for polyploids that exhibit random chromosome assortment (Parsons, 1959a). Due to this difference, deleterious alleles can be maintained in higher frequencies in polyploid populations, because there is slower purging of alleles.

Highbush blueberry, as well as other species of *Vaccinium*, has been noted to show inbreeding depression in the form of lowered fertility, including reports of both genotype fertility and immediate impacts on seed production. Vander Kloet (1983) reported a relationship between male and female fertility, and Krebs & Hancock (1990) showed female fertility to decrease more rapidly than male fertility under inbreeding. The general reduction in fertility was found to be associated with lower heterozygosity and therefore expected to be a result of the genetic load of the genotype (Krebs & Hancock, 1991). In addition, many authors have reported decreasing

mature seed production as inbreeding increases between parents, and this decrease was deemed to be early-acting inbreeding depression (Hellman & Moore, 1983; Krebs & Hancock, 1990; Krebs & Hancock, 1991; Hokanson & Hancock, 2000).

Highbush blueberry pedigree inbreeding coefficients have been calculated using diploid (Hancock & Siefker) and tetraploid methods (Krebs & Hancock, 1990; Ehlenfeldt, 1994) but considering the evidence showing predominantly tetrasomic inheritance, the latter is likely more accurate. For the genotypes examined here, the range of inbreeding coefficients was quite large, with the wild accessions considered to be $F = 0$ and the most inbred genotypes around $F = 0.02$ (Ehlenfeldt, 1994). For this analysis, a traditional pedigree inbreeding coefficient was only calculated for one offspring population due to the incomplete pedigree information available. The inbreeding coefficient for the offspring in cross 1112 was $F = 0.0038$, with parent coefficients being Bluecrop – $F = 0.0034$ and Elliott- $F = 0.0059$ and these coefficients were similar to most of the inbreeding coefficients calculated by Ehlenfeldt (1994). Brevis *et al*. (2008) found a correlation between pedigree inbreeding coefficients and allelic richness for northern highbush blueberry genotypes but not for southern highbush genotypes. Although there are fewer resources for polyploid data than diploid data, there are some programs that can be used to estimate population genetics parameters. Specifically, a new program developed by Huang *et al*. (2014) can be used to estimate relationships between individuals with less bias due to assignment of missing alleles. However, the results are less biased when a higher number of loci are used and when there is less genotype ambiguity (Huang *et al*., 2014), which are both limitations of my study.

Heterozygosity has been used to estimate inbreeding and potential for inbreeding depression due to well-known correlations between heterozygosity and fitness (HFCs). However without knowledge of the dosage of alleles in the genotypes, it is difficult to assess heterozygosity and other measures related to genotype diversity. These measures can be biased if they do not fill in gaps due to unknown dosage. A bias can also be created when gaps in the pedigree are filled in and assumptions are made that the known alleles have an equal likelihood of being the extra allele copies. The analyses in Table 5.6 do not fill in gaps in the pedigree, and look at richness as opposed to diversity.

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For my data, shared alleles were assumed to be identical by decent due to the narrow breeding pool of highbush blueberry, with the largest portions of genetic material coming from only three wild accessions (Ehlenfeldt, 1994). Table 5.6 shows the percentage of shared alleles between the parents of each cross to be around 40%, which is a fairly high degree of inbreeding in that the parents are essentially close relatives. Table 5.6 also shows several measures of allelic diversity; including richness, proportion of loci that have more than one allele, mean distance between the smallest and largest alleles, and the size of the population as measured by the number of alleles relative to the total number possible (four). For each interpretation, the values were not significantly different from each other, but show the same trend in that offspring populations have lower values than the parental average. The measures of allelic variation are similar but could have different meaning in terms of inbreeding and the blueberry population.

IMPLICATIONS FOR BREEDING

The method of inheritance pattern analysis described here does not give a clear pattern but due to the variable nature of chromosome behaviour, such a description may be individual-specific and not transferrable to the whole population. Chromosome behaviour could depend on an individual by chromosome interaction (Soltis & Soltis, 2000), or be influenced by the environment (Soltis & Soltis, 2000), and could also depend on the evolutionary march of the chromosomes towards a more disomic mode (Wolfe, 2001). In fact, two studies examining inheritance pattern using RAPD markers and the same interspecific cross, *V. darowii* and *V. corymbosum*, found three-quarters (Qu & Hancock, 1997) versus one-half (Vorsa & Novy, 1995) behaved in a tetrasomic pattern. For marker-assisted breeding, if the SSR marker is close enough to the gene of interest it maybe worth doing a controlled cross to determine the inheritance pattern for that locus. The method used in this study could also be used to estimate the allele dosage in the parental genotypes from the offspring population.

Work to develop markers for assisted breeding in blueberry is underway, but determining which markers are closely linked to a desired adaptation is a time and resource-consuming process (Lobos & Hancock, 2015). However, there is much that the markers can be used for in the

interim such as detailing the inheritance pattern and developing knowledge of chromosome behaviour. Knowledge of the inheritance pattern can assist breeders to anticipate the segregation of traits in subsequent generations, i.e. fixed heterozygosity under disomic inheritance will limit this segregation (Allard, 1960). As well, breeders would know the size of population that must be planted out to achieve the required number of progeny with the desired trait. And finally, inheritance pattern can help breeders determine the effect of inbreeding on the rate of homozygosity increase (Bever & Felber, 1992). As mentioned above, inbreeding depression has already been seen in many *Vaccinium*, and several authors have cautioned against the increasing inbreeding that has occurred over the past decade of blueberry breeding.

Chapter 6: Conclusions

6.1 Synthesis of Research

The life history of *Vaccinium* sect. *Cyanococcus* (cluster fruited blueberries) has been extensively studied due to their economic importance as fruit crops. While all life-history traits are important to consider in an agroecosystem, those that relate most closely to fruit production and breeding are considered here: mode of reproduction, pollination mechanism, mating system and tolerance to inbreeding, hybridization, and ploidy. Blueberries are long-lived species that reproduce both clonally and sexually with biotic pollination and have a mainly outcrossing mating system (Vander Kloet, 1988). Selfing is usually the result of geitonomous, not autogamous, pollen transfer due to blueberries' poricidal anthers and dichogamous phenology and the most effective pollinator of blueberries so far examined is the bumblebee (Javorek *et al*., 2002). Blueberries exist in polyploid complexes including diploid, tetraploid and hexaploid levels (Camp, 1942; Palser, 1961; Vander Kloet 1988). There is potential for genetic transfer and hybridization from diploid upward through unreduced gametes as well as among species within the same ploidy level, as there are few barriers to hybridization (Draper, 1977; Ballington & Galletta, 1978).

Three fruit crops including highbush, lowbush and rabbiteye have been developed from the blueberry species *Vaccinium corymbosum*, *V. angustifolium*, and *V. ashei*, respectively. However, these divisions are weak as hybridization through breeding has muddied taxonomic and species distinctions (Hancock & Goulart, 1993). Highbush blueberry has the oldest breeding program, however it is still a relatively young crop at just over a century old. Inbreeding has occurred in all blueberry breeding programs and associated inbreeding depression has been observed (Ehlenfeldt, 1994). Blueberries show inbreeding depression in the form of lowered fertility with early abortion of zygotes (Hokanson & Hancock, 2000; Krebs & Hancock, 1991; Vander Kloet, 1983).

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Highbush blueberry, along with other blueberry crops, suffers from inconsistent pollination due to the relative ineffectiveness of honeybees, the crop's chief pollinator (Javorek *et al*., 2002). Honeybees are the crop's chief pollinator and necessary for large scale production due to their ease of management in colonies (Issacs & Kirk, 2010). However, they are poor pollinators of blueberry because they are unable to sonicate blueberry flowers, which greatly limits the release of pollen, and they have been observed to have a preference among genotypes (Brewer & Dobson, 1969; Courcelles *et al*., 2013). The goal of my research was to examine factors of flower phenotype, particularly flower odour, that might elucidate pollinator choice and to examine the breeding potential of floral odour. Pollinator choice was observed as well as placed in the context of previous studies regarding bee response to volatile compounds. Pollinator choice was also examined in the context of the agricultural system using a paternity test to determine pollen movement. Finally, highbush blueberry life-history factors that are important for breeding, including inheritance pattern and levels of inbreeding, were investigated.

An improved method of volatile collection was employed in order to observe accurate genotype-level differences. The emission of highbush blueberry floral odour is weather dependent, reinforcing the need to collect volatiles over a variety of conditions. The floral odour contains 55 different compounds, more than previously noted, from several biosynthetic origins. There is also a diversity in odour among genotypes. However, the diversity is not sufficient to describe a floral odour fingerprint per genotype as there were very few genotype-unique compounds. Floral odours are heritable in the broad-sense for this population of highbush blueberries, and with further investigation and determination of a narrow-sense heritability there could be opportunity to include floral odour in breeding programs.

The inclusion of floral odour in a breeding program requires strong evidence that altering the phenotype would improve pollination services. Many of the highbush blueberry floral compounds of interest have not been previously studied for bee response. Therefore, the literature survey did not adequately address the question. However, in the case of myrcene, which was identified to be highly variable among cultivars, there appeared to be no evidence of response in the literature, the preliminary bioassay, or field monitoring. The compounds in the benzenoid class are likely to be the most attractive to bees and they appear to be consistent

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among genotypes; in contrast, the compounds in the aliphatic and terpenoid classes show more variation among genotypes and could be attractants or deterrents. Further study might describe a push-pull mechanism inherent in blueberry floral odour, as has been observed in other species (Adler *et al*. 2001), which could explain the observed genotype choice by bees.

A paternity test can be used to examine pollinator choice, particularly for an outcrossing species with floral morphologies that limit self-pollination. Successful pollen donation is also accompanied by information about the ecosystem and biological limitations such as the early-acting inbreeding depression seen in blueberries. The most common pollen donors in the controlled array of the agricultural research station were Bluecrop and Elliott. Paternity was correlated with mature seed number, a correlation that has been seen in previous research of blueberry genotypes. Bloom time and neighbourhood explain some of the patterns of paternity. It is therefore proposed that alternating rows of compatible genotypes would be an ideal planting arrangement to increase the potential for cross-pollination as long as yield gains outweigh the extra cost of production.

Inheritance pattern is an important consideration in breeding programs as it impacts the segregation of traits, the rate of homozygosity increase due to inbreeding and the offspring population size needed to capture traits of interest (Acquaah, 2007). The inbreeding present in the highbush blueberry parents obscured the potential to examine segregation pattern variation among individuals and this analysis may have been more achievable using wild ancestors. The parental combinations shared alleles at most loci, and each individual had unknown dosage of alleles at most loci. Tetrasomic inheritance was present as evidenced by a small number of allele ratios fitting a tetrasomic segregation pattern as well as a lack of fixed heterozygosity. However, the degree of tetrasomy and difference among parents was not determined because the majority of allele cases were indistinguishable from disomic inheritance. Pedigree inbreeding coefficients have been found to correlate with estimates of heterozygosity but a similar comparison was not possible in this study due to gaps in the pedigrees. Heterozygosity analysis requires the estimation allele frequency, which can be biased in situations of unknown dosage; however, levels of variation, or lack of variation, were similar among the genotypes.

6.2 Contributions

Highbush blueberry is a high-valued crop important to British Columbia's economy and local blueberry breeding and cultivar testing is essential to maintaining this status (Lobos & Hancock, 2015). Breeders have many traits to consider including fruit characteristics, disease and pest resistance, and drought tolerance to name a few. However, my research suggests that reproductive biology, central to seed and therefore fruit production, could also be considered in breeding programs, namely pollinator attraction and fertility of genotypes. Pollinator attraction is a complex interaction between floral traits, including floral display, morphology, colour, pattern, and odour, and pollinator response to those traits. Regardless of which floral traits are more significant to pollinator choice, pollinator choice itself could be considered in breeding programs. I found floral odour traits to be variable among genotypes and show broad-sense heritability, which is a first step towards further examination.

Decreased fertility, both self and outcrossed, of highbush blueberry genotypes is associated with an increase in inbreeding. Since blueberry genotypes are substantially inbred, addressing the issue of inbreeding for future genotypes may improve fertility and thereby yield. Self-fertile genotypes are the most useful in current production systems where large monoculture blocks are planted.

New highbush blueberry growers entering the industry should consider pollinator attraction and fertility of the genotypes available when planning a production system. If the desirable attributes belong to a less self-fertile genotype, growers could consider alternating rows of genotypes to facilitate outcross pollen movement. All blueberry growers, regardless of production stage, should consider planting and constructing bee habitat for wild/native bee species to support pollination of the crop as well as for bee conservation.

6.3 **Future Directions**

The relationship between plants and pollinators shapes plant communities in an evolutionary context, but in an agricultural context it is just a slice of the relationship repeated year after year. The biological limitations of honeybee foraging cannot be changed but increasing the attractiveness of blueberry flowers could improve yields. More knowledge about what floral traits are most attractive or even deterrent to bees must be developed along with the fine scale methods required to examine bee selection. Future research that would be interesting from both an evolutionary and agricultural context could include a multiple floral trait model that incorporates pollinator preference through monitoring. More thorough monitoring would also help breeders and growers make informed decisions about genotype selection.

Highbush blueberry shows evidence of tetrasomic inheritance pattern; however, the degree of tetrasomic inheritance and the variation among individuals is still unknown. Considering the high number of shared alleles and unknown dosage at most loci, it is difficult to address this question using SSR markers. In addition, the non-random mating nature of the breeding population limits the accuracy of methods to estimate dosage of alleles. To my knowledge, variation among individuals in terms of allele segregation pattern has not been investigated however could have very interesting relationships with past hybridization and ploidy events. Agricultural crops with known dosage are a good choice for this investigation where the pedigree of each individual genotype can be used to include breeding history.

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Appendices

Appendix A
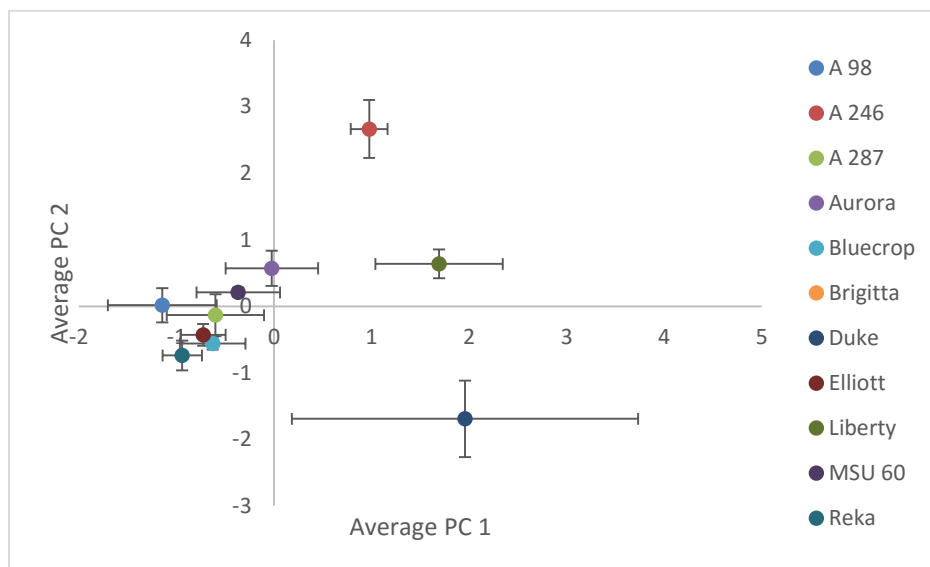
Appendix A 1: Principle Component Analysis (PCA) of relative peak areas for floral scent volatiles. Compound categories include: aliphatic, branched, monoterpenoid, sesquiterpenoid, benzenoid, and the unknowns. Standard error around genotypes shown, principle components 1 and 2 explain 52% and 24% of variance, respectively.
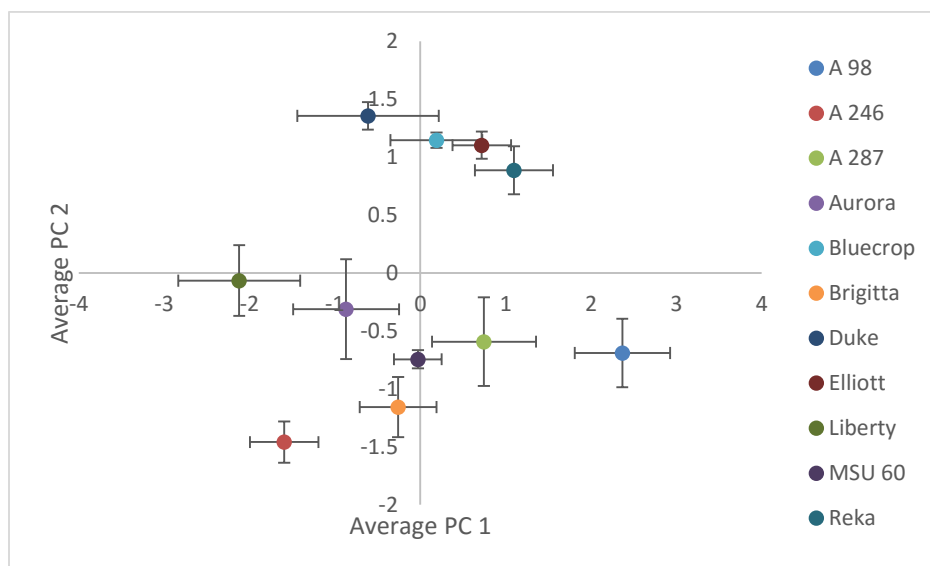
Appendix A 2: Principle Component Analysis (PCA) of number of floral scent volatiles. Categories include: aliphatic, branched, monoterpenoid, sesquiterpenoid, benzenoid, and the unknowns. Standard error for genotype shown with error bars. Principle components 1 and 2 explain 45% and 19% of variance, respectively.

Appendix A 3: List of floral volatile compounds described by Szendrei *et al*. (2009) and Rodriquez-Saona *et al*. (2011).

Volatile Group	Szendrei *et al*. (2009)	Rodriquez-Saona *et al*. (2011)
Alcohols	cis-3-Hexen-1-ol	cis-3-Hexen-1-ol
	Hexanol	Hexanol
Esters	Ethyl pentanoate	Ethyl pentanoate
	Ethyl-3-hexenoate	Ethyl-3-hexenoate
	cis-3-hexenyl acetate	cis-3-hexenyl acetate
	Hexyl acetate	Hexyl acetate
	trans-2-hexenyl acetate	Hexenyl acetate isomer
		cis-3-hexenyl propionate
		Hexyl propionate
	cis-3-hexenyl butyrate	cis-3-hexenyl butyrate
	Hexyl butyrate	Hexyl butyrate
	cis-3-hexenyl methylbutyrate	cis-3-hexenyl methylbutyrate
	Hexyl methylbutyrate	Hexyl methylbutyrate
	cis-3-hexenyl hexanoate	cis-3-hexenyl hexanoate
	cis-2-hexenyl acetate	
	Hexenyl butyrate	
	Hexyl ester	
	Hexenyl ester	
Ketones	2-Heptanone	2-Heptanone
	2-Undecanone	2-Undecanone
Monoterpenes	α-Pinene	α-Pinene
	β-Pinene	β-Pinene
	Limonene	Limonene
	Eucalyptol	Eucalyptol
	Myrcenone	Myrcenone
	Linalool	Linalool
	Ocimene	
Sesquiterpenes	Copaene	
	β-Bourbenene	β-Bourbenene
	Caryophyllene	Caryophyllene
	γ-Cadinene	γ-Cadinene
	Farnesene	Farnesene
	Humulene	
Phenyl propanoid derivatives	Cinnamyl alcohol	Cinnamyl alcohol
Hydrocarbons	n-Tridecane	n-Tridecane

Appendix B

Appendix B 1: Summation of fractional paternity where more than 1 father is not excluded.
Showing the most common father for each maternal genotype as well as the total relative contribution of each father in the research plot.

Fathers	Mothers Aurora	Bluecrop	Duke	Elliott	Liberty	Reka	Relative Paternity
A 12	0.01	0.01	0.01	0.04	0.03	0.01	6
A 98	0.06	0.05	0.03	0.04	0.05	0.08	6
A 246	0.07	0.00	0.09	0.06	0.10	0.10	8
A 287	0.05	0.04	0.01	0.02	0.05	0.05	4
Aurora	0.00	0.06	0.10	0.05	0.09	0.02	6
Bluecrop	0.11	0.00	0.13	0.23	0.23	0.05	15
Chandler	0.04	0.00	0.03	0.06	0.01	0.04	18
Draper	0.12	0.06	0.02	0.16	0.10	0.13	30
Duke	0.15	0.08	0.02	0.15	0.05	0.06	10
Elliott	0.05	0.49	0.15	0.01	0.02	0.15	17
Liberty	0.01	0.08	0.05	0.03	0.00	0.06	5
MSU 36	0.03	0.02	0.02	0.02	0.02	0.04	8
MSU 60	0.03	0.05	0.05	0.02	0.06	0.04	5
ORUS 5	0.01	0.00	0.06	0.04	0.02	0.02	7
ORUS 10	0.16	0.05	0.03	0.01	0.03	0.03	15
Reka	0.02	0.01	0.19	0.04	0.06	0.00	7
US 645	0.07	0.00	0.00	0.01	0.08	0.14	16