FACTORS CONTROLLING FRUIT SET OF RABBITEYE BLUEBERRY (*VACCINIUM ASHEI* READE)

by

PATRICIO ALEJANDRO BREVIS

(Under the Direction of D. Scott NeSmith)

ABSTRACT

Rabbiteye blueberry (REB) is the primary blueberry species cultivated in Georgia and other southern states. Poor fruit set is one of the most important problems of the REB industry, and the failure to set adequate fruit loads has been attributed, among other factors, to insufficient pollination. The research conducted as part of this dissertation addressed selected aspects of the pollination biology of REB in an effort to identify limiting factors for fruit set and yield.

Pollen grain production per ovule averaged 402 ± 35, which is very low for a xenogamous species. REB is a buzz-pollinated plant, and its low pollen-ovule ratio may be an indicator of the high efficiency of its pollen dispensing mechanism. Total pollen production and release varied among commercial cultivars. High percentages of in vitro tetrad germination (>80%) suggest that pollen viability does not contribute to REB reproductive failure.

REB requires cross-pollination for optimum fruit yield. Transport of cross-pollen by bumblebees was assessed in the field using a novel technique based on frequency distributions of pollen diameter, measured with a particle counter. The study was conducted in 2003 and 2004 in a blueberry planting with alternating rows of ‘Brightwell’ and ‘Climax’ plants. Bumblebees carried low proportions of cross-pollen, which indicated that these pollinators visited

‘Brightwell’ and ‘Climax’ flowers in a non-random fashion. The likelihood for cross-pollination was low and limited to the period of maximum bloom overlap. Specimens collected from ‘Climax’ in 2004 carried more cross-pollen than those from ‘Brightwell’, which may be related to the difference in pollen release between these cultivars.

The length of flower receptivity or effective pollination period (EPP) was established for the cultivars Brightwell and Tifblue. The EPP was 7 days at day/night temperatures of 23/10°C. Although low stigmatic receptivity limited tetrad germination in flowers pollinated on the day of anthesis, this variable and fruit set were not positively associated. Therefore, age-related factors other than stigmatic receptivity (ovule longevity and/or pollen tube growth rate) likely limit the EPP of REB. This study provided the first quantitative evidence of late stigma maturation in blueberry.

INDEX WORDS: Ericaceae, section *Cyanococcus*, pollen dispersion, self-pollination, tetrad size, pollen limitation, Coulter counter.

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PATRICIO ALEJANDRO BREVIS

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PATRICIO ALEJANDRO BREVIS

Major Professor: D. Scott NeSmith

Committee: Patrick Conner
Keith Delaplane
Harald Scherm
Hazel Wetzstein

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2005

DEDICATION

I dedicate this dissertation to my parents Clara and Juan, for their unconditional love and support. I also dedicate this dissertation to Gloria and Juan Carlos, for their love, advice and encouragement.

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TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

There has been considerable interest in blueberries over the last few years, given the increased awareness of their health benefits. Among fresh fruits and vegetables, blueberries are one of the richest sources of antioxidants (Prior et al., 1998). Blueberries and other antioxidant-rich fruit contain phytochemicals that are proven to retard age-related declines in neuronal and cognitive function of laboratory animals (Joseph et al., 1999). With the rising interest in blueberry nutraceutical value has come increased demand and a gradual increase in production. The world's blueberry cultivated area increased by 51% during the period 1995-2003 (Villata, 2005). North America is the leading production region in the world, with a cultivated area of 26,600 hectares and a total production of 122,561 tons.

Georgia is the fifth-largest producer of cultivated blueberries in the nation, behind the states of Michigan, New Jersey, Oregon, and North Carolina (U.S. Dept. Agr., 2004). Blueberry is Georgia's second most important fruit crop, with a cultivated area of >3200 hectares and a farm gate value of $26.7 million in 2003 (Boatright and McKissick, 2004). The Georgia blueberry industry is concentrated in the southeastern part of the state, with Bacon, Appling, Clinch, Wayne, and Ware Counties containing 76% of the current acreage.

Rabbiteye blueberry (*Vaccinium ashei* Reade) is native to south Georgia, north Florida, and southeast Alabama (Camp, 1945). Cultivation of the rabbiteye blueberry began in the southeastern United States during the late nineteenth century (Krewer and NeSmith, 2002).

Currently, rabbiteye cultivars represent most of Georgia's commercial blueberry production, occupying about 90% of the total acreage (Scherm and Krewer, 2003).

In Georgia, poor fruit set is one of the most important horticultural problems of the rabbiteye blueberry industry. According to a recent report, two-thirds of producers surveyed declared poor fruit set as a major to moderate constraint for blueberry production (Scherm et al., 2001). Since inadequate fruit set has been attributed to factors such as insufficient pollination (El-Agamy et al., 1981), blueberry producers in Georgia lease honeybee colonies to aid pollination and improve fruit set (Scherm et al., 2001).

Blueberries are capable of setting almost 100% of their flowers, and fruit set levels as high as 80% are required for adequate commercial production (Shutak and Marucci, 1966). However, estimates of fruit set as low as 8-10% have been recorded for the widely planted rabbiteye blueberry cultivar Tifblue under field conditions (Lyrene and Crocker, 1983; NeSmith and Adair, 2004). Rabbiteye cultivars have a limited degree of self-fertility and require cross-pollination with another rabbiteye cultivar (El-Agamy et al., 1981). In general, flowering plants with the capacity to self-fertilize exhibit less pollen limitation than those that cannot self-fertilize, because an additional pollen source (self-pollen) can contribute to reproductive success (Burd, 1994; Larson and Barret, 2000). Therefore, it is likely that fruit set of rabbiteye blueberries can be pollen-limited, rather than resource-limited, under certain conditions.

Although fruit set of rabbiteye blueberry may often be pollen-limited, there are important knowledge gaps regarding its pollination biology. The ultimate goal of this research was to investigate critical aspects of the pollination biology of rabbiteye blueberry in an effort to identify potential limiting factors for fruit set and yield. The specific objectives were:

1. Assess production, release, and viability of pollen in commercial rabbiteye blueberry cultivars.
2. Develop a reliable method to quantify pollen dispersion in blueberry plantings.
3. Quantify pollen dispersion of rabbiteye blueberry cultivars under field conditions.
4. Establish the effective pollination period in rabbiteye blueberry.

Dissertation outline

Chapter 2 describes the blueberry breeding system and reviews what is currently known about 1) pollen number and viability, 2) pollen dispersion, and 3) the effective pollination period for rabbiteye blueberry.

Chapter 3 describes a study conducted to quantify production, release, and viability of pollen in rabbiteye blueberry cultivars. Pollen-ovule ratios were also estimated so that pollen production per flower could be related to that of other flowering plants with similar breeding systems.

Chapter 4 describes a novel method developed to estimate potential pollen dispersion in rabbiteye blueberry plantings. Chapter 5 describes a study where transport of cross-pollen by bumblebees was assessed in a rabbiteye blueberry plot using the technique developed.

Chapter 6 describes an experiment conducted to establish the effective pollination period of the rabbiteye blueberry cultivars Brightwell and Tifblue.

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CHAPTER 2

LITERATURE REVIEW

Breeding system and reproductive biology of blueberries

Blueberries (*Vaccinium* section *Cyanococcus*) are xenogamous (outcrossed), bee-pollinated flowering plants. Several studies have demonstrated their inability to set fruit without pollination by bees (Mainland et al., 1979; Payne et al., 1989). Blueberry flowers are perfect (hermaphroditic) and morphologically designed to prevent autogamy (selfing). Self-pollination is unlikely due to the pendant habit of open flowers and the angled sides of the stigma (Coville, 1910). Blueberry flowers display homomorphic approach herkogamy, in which male and female functions are spatially separated. This feature favors outcrossing, since pollinators contact stigmas first upon their entry into the flower (Webb and Lloyd, 1986).

Although Vander Kloet (1988) and Drummond and Groden (2000) indicate that blueberry flowers are generally protandrous, quantitative data supporting this statement is lacking. In protandrous flowers, autogamy is unlikely because stigma maturation occurs after anther dehiscence. Protandry is the predominant kind of dichogamy among members of the Ericaceae family (Bertin and Newman, 1993).

Cross-pollination is beneficial for fruit set, berry size, and ripening rate in all blueberry species (El-Agamy et al., 1981; Meader and Darrow, 1944; Meader and Darrow, 1947; Morrow, 1943). Self-infertility in *Vaccinium* has been described as “self-incompatibility” in many instances (Garvey and Lyrene, 1987; Vander Kloet and Lyrene, 1987). This is actually a

conceptual misuse because blueberry pistils do not reject tetrads or pollen tubes following autogamous crosses (El-Agamy et al., 1982; Garvey and Lyrene, 1987; Krebs and Hancock, 1988; Vander Kloet, 1991). All the available information indicates that low self-fertility is due to early-acting inbreeding depression and not to conventional self-incompatibility mechanisms (Hokanson and Hancock, 2000; Krebs and Hancock, 1990; Krebs and Hancock, 1991). Reduced self-fertility in *Vaccinium* has been attributed to homozygosity for sublethal mutations at loci controlling embryo development, or to loss of heterotic interactions at these loci (Krebs and Hancock, 1991).

Blueberry flowers exhibit a buzz-pollination syndrome. Anthers are poridically dehiscent and sonication is required for efficient pollen collection by bees (Buchmann, 1983). Blueberries are exceptional among buzz-pollinated plants, because their flowers produce nectar in addition to pollen as reward for pollinators.

Pollen number and viability

Fruit set of blueberries is often pollen-limited. Some studies have examined the role of pollen quality in blueberry reproductive success. Pollen germination is known to be affected by genotype (Eaton, 1966; Goldy and Lyrene, 1983; Lang and Parrie, 1992), and poor fruit set has been linked to low pollen viability in some highbush blueberry (*V. corymbosum* L.) cultivars (Brewer and Dobson, 1969; Stushnoff and Hough, 1968). Moreover, Vander Kloet (1983) reported a significant correlation between pollen viability and fruit quality parameters such as fruit size and number of seeds per berry. Pollen viability studies in rabbiteye blueberry are limited (Cockerham and Galleta, 1976), so the role of pollen quality on fruit set remains unclear.

High pollen production is desirable for insect-pollinated plants. Since bees constantly groom their bodies and pack pollen into scopal transport devices, most of the pollen collected by these pollinators is unavailable for pollination (Buchmann, 1983). Pollen must thus be produced in abundance to compensate for its inefficient transfer by pollen vectors. In fact, xenogamous plants produce more pollen (on a per ovule basis) than cleistogamous or autogamous plants (Cruden, 1977). Despite these considerations, few studies have attempted to quantify total pollen production (Reader, 1977) and release (Brewer and Dobson, 1969) in *Vaccinium* section *Cyanococcus*, and there is no information available for rabbiteye blueberries. Knowledge of pollen production and release could help to identify rabbiteye cultivars with outstanding performance as pollinizers.

Pollen dispersion

Bee-mediated pollen dispersion in fruit crops can be quite limited. Research has shown that fruit set declines with increasing distance from pollinizer trees (Free, 1962; Free and Spencer-Booth, 1964; Milutinovic et al., 1996). Gene flow studies have furthered our understanding of the extent of cross-pollination in orchard crops. Genetic markers have been used to identify the pollen-contributing parent in embryos of seeds after fruit set (Jackson and Clarke, 1991; Kron et al., 2001; Wertheim, 1991). These studies indicate that the distance of gene dispersal is short and leptokurtic, with most of the gene flow occurring between adjacent cross-compatible trees.

Blueberries are pollinated by bees, and these insects are expected to transfer pollen from one cultivar to another. The information available for blueberries, although scarce, suggests that pollen dispersion between cultivars may not be optimal. Vander Kloet and Lyrene (1987)

indicated that geitonogamous selfing is likely to result as a consequence of the foraging behavior of blueberry pollinators. Moreover, Hancock et al. (1989) established that fruit size and seed number per berry declined with increasing distance from the source of cross-pollen. Pollen dispersion in rabbiteye blueberries has not been quantified previously, even though cross-pollination is critical for optimum yields. Knowledge of pollen dispersal is essential for maximizing cross-pollination and achieving optimal planting designs (Kron et al., 2001).

Although genetic markers have been used successfully to infer patterns of pollen dispersion in orchard crops, this approach may not be practical for blueberries. In *Vaccinium*, the potential number of seeds per fruit is high, ranging from 50 to 125 ovules per ovary (Palser, 1961). Therefore, paternity analyses would be quite labor-intensive because the development of a single berry is triggered by multiple independent fertilization events. Patterns of pollen dispersal would also be difficult to infer from the analysis of embryos in fully developed seeds, because self-pollination in blueberry can lead to fertilization and subsequent seed abortion. In other words, the paternal contribution of self-pollen would be difficult to detect, even if there was a high extent of selfing and thus ovule discount.

Pollen dispersion in entomophilous flowering plants can also be inferred by the flight distance of pollen vectors. However, pollinator flight distances may underestimate the extent of pollen dispersion because of the effects of pollen carryover (Waser and Price, 1982).

Effective pollination period

Effective pollination period (EPP) is defined as the number of days during which pollination is effective to produce a fruit (Williams, 1965). Research has shown that the EPP plays an important role in controlling fruit set of various fruit crops (Sanzol and Herrero, 2001).

The EPP of *Vaccinium* section *Cyanococcus* has received little attention. Blueberry flowers remain receptive up to 8 days after anthesis (DAA) in highbush blueberry (*Vaccinium corymbosum* L.) (Moore, 1964), 6 DAA in lowbush blueberry (*V. angustifolium* Ait.) (Wood, 1962), and at least 5 DAA in rabbiteye blueberry (*V. ashei*) (Young and Sherman, 1978). According to these studies, cultivar differences within a species also occur. Young and Sherman (1978) evaluated pollen tube penetration two days after pollination in styles pollinated at different time intervals from anthesis. However, this variable is not a good estimate for either pollen tube growth rate or stigmatic receptivity. Therefore, there is no information available on the parameters determining the EPP in blueberry species.

The EPP is determined by length of stigmatic receptivity, pollen tube growth rate, and ovule longevity (Sanzol and Herrero, 2001). The role that these parameters play in determining the EPP of blueberries remains unknown. Although the EPP of many fruit crops (mostly Rosaceae) is well studied, the reproductive biology of these species is too different relative to that of blueberry, so little or no generalization can be made from the literature available.

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CHAPTER 3

PRODUCTION AND VIABILITY OF POLLEN AND POLLEN-OVULE RATIOS IN FOUR RABBITEYE BLUEBERRY CULTIVARS[1]

[1]Brevis, P.A., D.S. NeSmith, H.Y. Wetzstein, and D.B. Hausman. To be submitted to the *Journal of the American Society for Horticultural Science.*

Abstract

Fruit set of rabbiteye blueberries (*Vaccinium ashei* Reade) is often pollen-limited. The objective of this study was to determine production, release, and viability of pollen, as well as pollen-ovule ratios in the rabbiteye blueberry cultivars Austin, Brightwell, Climax and Tifblue. In vitro tetrad germination varied among genotypes, although, values were high (>80%) in all cultivars. Pollen viability does not seem to contribute to reproductive failure in the cultivars studied. Total pollen production per flower averaged 8434 ± 604 tetrads across all cultivars. On a per ovule basis, pollen production of the xenogamous *V. ashei* is very low relative to other species with similar breeding systems. The low pollen-ovule ratio of rabbiteye blueberry (402 ± 35) may be an indicator of the high efficiency of its pollen dispensing mechanism. Total pollen production varied among cultivars. Moreover, a significant difference in pollen release was found between two cultivars with similar total pollen production per flower. The possible mechanism regulating pollen release in these cultivars is discussed.

It is common for xenogamous (outcrossed), hermaphroditic flowering plants to produce more flowers and ovules than fruits and seeds (Bawa and Webb, 1984). Two explanations for this pattern are 1) maternal resources available for fruit and seed maturation are limited, or 2) pollen delivery to the stigma is insufficient (Sutherland, 1987). Pollen limitation of seed set is widely reported in angiosperms (Burd, 1994; Larson and Barret, 2000).

According to Shutak and Marucci (1966), blueberries (*Vaccinium* section *Cyanococcus*) are capable of setting fruit for almost 100% of their flowers, and fruit set levels as high as 80% are required for adequate commercial production. However, estimates of fruit set as low as 8-10% have been recorded for the widely planted rabbiteye blueberry (*Vaccinium ashei*) cultivar Tifblue under field conditions (Lyrene and Croker, 1983; NeSmith and Adair, 2004). Poor fruit set is one of the most important horticultural problems of the rabbiteye blueberry industry in the southeastern United States (Scherm et al., 2001). Rabbiteye blueberries have a limited degree of self-fertility and require cross-pollination with another rabbiteye cultivar (El-Agamy et al., 1981). In general, flowering plants with the capacity to self-fertilize exhibit less pollen limitation than those that cannot self-fertilize, because an additional pollen source (self-pollen) can contribute to reproductive success (Burd, 1994; Larson and Barret, 2000). Therefore, it is likely that fruit set of rabbiteye blueberries can be pollen-limited, rather than resource-limited, under certain conditions.

Several studies have examined the role of pollen quality on the fruiting capacity of blueberry. Pollen germination is affected by genotype (Eaton, 1966; Goldy and Lyrene, 1983; Lang and Parrie, 1992), and low pollen viability has been linked to poor fruit set in some highbush blueberry (*Vaccinium corymbosum* L.) cultivars (Brewer and Dobson, 1969; Stushnoff and Hough, 1968). Moreover, Vander Kloet (1983) reported significant correlation between

pollen viability and fruit quality parameters such as fruit size. Pollen viability studies in rabbiteye blueberry are quite limited (Cockerham and Galleta, 1976), so the role of pollen quality on fruit set remains unclear.

High pollen production is desirable for bee-pollinated plants. Since bees constantly groom their bodies and pack pollen into scopal transport devices, most of the pollen collected by these pollinators is unavailable for pollination (Harder and Wilson, 1997; Thorp, 2000). Therefore, pollen must be produced in abundance to compensate for its inefficient transfer by bees, and in fact, xenogamous flowers produce more pollen (on a per ovule basis) than those of autogamous (self-pollinated) plants (Cruden, 1977). Blueberry is a bee-pollinated crop, and cross-pollination is beneficial for fruit quality and yield. Although knowledge of pollen production and release could help to identify cultivars with outstanding performance as pollinizers, these variables have received little attention in *Vaccinium* section *Cyanococcus*. Brewer and Dobson (1969) reported that 'Jersey', a highbush blueberry cultivar with problems of low fruit set, released less pollen than 'Rubel'. Reader (1977) quantified total pollen production per flower of *Vaccinium myrtilloides* Michx. growing in a peat bog. To our knowledge, there is no such information available for rabbiteye blueberries. The objective of this research was to determine viability, production, and release of pollen in selected rabbiteye blueberry cultivars. Additionally, the pollen-ovule ratio of rabbiteye blueberry was estimated to relate its pollen production to that of other flowering plants with similar breeding systems.

Materials and methods

Plant material and locations. Three-year-old blueberry plants of the rabbiteye cultivars Austin, Brightwell, Climax, and Tifblue were obtained from the blueberry breeding program at

the University of Georgia Griffin Campus. Plants were grown in 19 L containers using pine bark as a growing medium. Seven plants of each cultivar were kept from 6 Nov. 2001 until near the beginning of bloom in the following spring at Griffin and Alapaha in order to provide varied pre-bloom environments. Griffin is located in central Georgia, where the USDA plant hardiness zone is 7b. Alapaha (hardiness zone is 8a) is located in southern Georgia and is more representative of the blueberry producing area of the state.

Pollen production and viability. Near the beginning of bloom, plants were brought into a greenhouse at the University of Georgia Athens Campus to evaluate total pollen production and viability. Plants from Alapaha were moved into the greenhouse on 7 Mar. 2002, and those from Griffin were moved two weeks later. Plants were fertilized with a slow-release fertilizer (Osmocote Plus® 15-9-12) and watered as needed.

Pollen viability was assessed by determining germination of tetrads in vitro. Germination tests were conducted for each of the cultivars using pollen from flowers collected on the same day. Two samples of 15 flowers at the stage of anthesis were collected at each sampling day from the seven plants of each location × cultivar combination. Pollen was extracted in the laboratory by rolling a flower between the thumb and index finger over a petri dish. Pollen from each sample was suspended in a germination medium as described by Garvey and Lyrene (1987) at a rate of 100 $mg \cdot L^{-1}$, and 200-µl aliquots of stirring suspension were added to six wells of a culture plate (Falcon Microtest III, Lincoln Park, N.J.). After two hours of incubation in a dark growth chamber at 24°C, pollen germination was examined using an inverted microscope (Nikon, Garden City, N.Y.). Pollen grains were considered germinated if pollen tube extension was greater than the diameter of the tetrad (Lang and Parrie, 1992). The number of tetrads containing 0, 1, 2, 3, and 4 pollen tubes was recorded. Pollen viability was expressed as tetrad

germination (percentage of tetrads with at least one pollen tube). The mean number of pollen tubes per germinated tetrad was also calculated.

In order to determine total pollen production, flowers just prior to anthesis were collected. Experimental units of 30 flowers were sampled from the seven plants of each cultivar for each location. Flowers were detached with forceps and placed upright on the wells of a tissue culture plate. Plates were carried to the laboratory and stored in a refrigerator before flower dissection. The distal half of the tubular corolla and the style were removed so that pollen could be released directly into a 150-ml sample container without interference from flower parts. A pair of toothpicks and a slice of double-coated adhesive tape where used to hang multiple flowers from their pedicels. Thus, flowers were stored at room temperature in a hanging position inside the sample container for several weeks. Blueberry anthers are poricidally dehiscent and require vibration for efficient pollen release (Buchmann, 1983; Cane and Payne, 1988); therefore, flowers were sonicated with a 512 Hz tuning fork (Indigo Instruments, Waterloo, ON). Sonication was repeated several times, until little or no pollen appeared to remain inside the dry anthers. The number of tetrads in the container was measured electronically using a particle counter. Tetrads were suspended in 10 mL of 70% ethanol, and then 130 mL of Isoton II (Coulter Electronics, Hialeah, Fla.) were added. Repeated 0.5-ml aliquots of continuously stirred pollen suspension were counted with a Coulter (model ZB1, Coulter Electronics) counter using a 140-μm aperture.

The experimental design was a split-plot with location as the main plot and cultivar as the subplot, with repeated measurements of tetrad production and germination. Experimental units for both evaluations were sampled on three separate days, with two replications per treatment

combination per day. Data were analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, N.C.).

Ovule number and pollen release. Ovule number and pollen release were estimated in 2004 using containerized blueberry plants similar to those described previously. Three plants of each cultivar received their chilling requirement under natural conditions and then were brought into a greenhouse at the University of Georgia Griffin Campus to force bloom. Samples of 25 flowers per cultivar were collected and fixed in 7:3 95% ethanol:acetic acid. Ovaries were dissected and ovules were counted under a dissecting microscope.

Pollen release was evaluated under greenhouse conditions for 'Brightwell' and 'Climax' only. Twenty five flowers of each cultivar were collected one day after anthesis. Pollen was extracted by rolling a flower between the thumb and index finger until no additional pollen was released. Tetrads from individual flowers were collected into a 20-ml blood cell-counter vial (Labcon, Petaluma, Calif.) and counted using the previously described particle counter. Ovule number and pollen release data were compared among cultivars using one-way analysis of variance.

Results and discussion

Pollen viability. Cultivar was the only significant main effect for in vitro tetrad germination ($P \leq 0.05$; Table 3.1). Location had no effect on in vitro tetrad germination, nor did it interact with cultivar ($P > 0.05$). Although tetrad germination varied among genotypes (Table 3.2), values were greater than 80% in all four cultivars. These results were similar to the mean percentage of stainable pollen previously reported for rabbiteye blueberry (Cockerham and Galleta, 1976). The viability results from this study were also similar to the level of pollen

viability reported for southern highbush blueberry (*Vaccinium corymbosum* L. ×spp) cultivars (Lang and Parrie, 1992). The number of pollen tubes observed in germinated tetrads ranged from one to four. On average, germinated tetrads of all cultivars produced two pollen tubes per tetrad. These findings contrast with previous reports indicating that multiple pollen tubes per tetrad are infrequent in blueberry (Brewer and Dobson, 1969). According to Lang and Parrie (1992), the average number of pollen tubes per germinated tetrad ranged from 1.7 to 3.4 among southern highbush blueberry cultivars.

Total pollen production and pollen-ovule ratios. Mean (± SE) tetrad production per flower for rabbiteye blueberry cultivars overall was 8434 ± 604 (Table 3.3). This is about three times higher than for *V. myrtilloides* Michx. (Reader, 1977). Pollen numbers in 'Brightwell', 'Climax' and 'Tifblue' were significantly higher than in 'Austin'.

Pollen numbers were also affected by location ($P \leq 0.05$; Table 3.1). Plants from Alapaha and Griffin averaged 7613 and 9254 tetrads per flower, respectively. Additional research would be needed to confirm this location effect and to identify the environmental factors that may have an influence on pollen production. There was no location × cultivar interaction ($P > 0.05$).

Rabbiteye blueberry cultivars had an overall mean (± SE) pollen-ovule ratio of 402 ± 35. According to Cruden (1977), the pollen-ovule ratio of xenogamous species is 5859 ± 937. Clearly, pollen-ovule ratios in rabbiteye blueberry are much lower than in other flowering plants with similar breeding systems. It has been proposed that pollen-ovule ratios are inversely related to the likelihood of a pollen grain reaching the stigma (Cruden, 1977; Cruden and Jensen, 1979; Cruden and Lyon, 1989). Low pollen-ovule ratios in rabbiteye blueberry flowers indicate that pollen transfer might be more efficient than in other xenogamous entomophilous plants. Several aspects of the reproductive biology of blueberries support this idea. Blueberry flowers are

adapted to buzz-pollination (Buchmann, 1983; Cane and Payne, 1988). Research has shown that poricidal anthers act as an efficient pollen dispensing mechanism, restricting removal per pollinator and controlling release according to the visitation rate (Harder and Barclay, 1994). Moreover, pollen grains in poricidal flowers are well protected from environmental hazards such as exposure to ultraviolet light and rain (Buchmann, 1983; Michener, 2000). The high efficiency of the pollen dispensing mechanism would justify a reduction in the pollen-ovule ratio of rabbiteye blueberries, relative to other xenogamous non-buzz pollinated species. Pollen-ovule ratios reported in other buzz-pollinated plants are also low, which further support this hypothesis (Fig. 3.1).

Previous studies have shown that species with pollen transporting mechanisms such as tetrads, polyads, and viscin threads have lower pollen-ovule ratios than species that simply release pollen as monads (Cruden, 1977; Cruden and Jensen, 1979; Vasek and Weng, 1988). The pollen dispersal unit for *Vaccinium* is a tetrad (Cockerham and Galleta, 1976; Stushnoff and Hough, 1968). This strategy also helps to explain the low pollen-ovule ratio of rabbiteye blueberry.

Pollen release. 'Brightwell' flowers released almost twice as many tetrads as those of 'Climax', even though total pollen production per flower was the same in both cultivars. Brewer and Dobson (1969) reported a similar difference between two highbush cultivars, although the regulatory mechanism responsible for this variation was not identified. In 'Brightwell' and 'Climax', the length and diameter of the anther tubes may have a direct or indirect effect on pollen release. Examination of 'Climax' stamens revealed longer, narrower anther tubes, relative to 'Brightwell' (Fig. 3.2). Anther tubes with such characteristics will likely offer a higher resistance to the flow of pollen, thus restricting its release. Pollen grains from poricidal anthers

tend to form clumps due to either electrostatic interactions (Buchmann, 1983) or the presence of tapel fluid (King and Ferguson, 1994). Therefore, narrow tubes would tend to clog more easily with these clumps. Alternatively, tubal length and diameter may have an effect on the dehydration rate of pollen grains inside the anther locules. Shorter, wider tubes would allow better gas exchange, which would increase the rate of pollen dehydration. In poricidally dehiscent plants, pollen grains must be dry, smooth, and non-sticky for efficient release following sonication (Buchmann and Hurley, 1978). Hence, it is suggested that these anther characteristics can account for differences in pollen release observed among cultivars.

In summary, in vitro tetrad germination was high (>80%) in all cultivars studied. Pollen viability does not seem to be a limiting factor for fruit set in rabbiteye blueberry. The low pollen-ovule ratio of rabbiteye blueberry is apparently an indicator of the high efficiency of its pollen dispensing mechanism, characterized by poricidally dehiscent anthers. A significant difference in pollen release was found between flowers of two cultivars with similar pollen production. Additional research is needed to establish if different propensities for pollen release can influence cultivar preferences by pollen-collecting bees.

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Table 3.1. Type III tests of fixed effects for in vitro tetrad germination, number of pollen tubes per germinated tetrad, and tetrad production per flower.

Effect	df	Tetrad germination		Pollen tubes per germinated tetrad		Tetrad production per flower	
		F-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Location (L)	1	1.2	0.3859	1.1	0.4111	37.2	0.0258
Cultivar (C)	3	16.7	0.0026	1.7	0.2597	69.5	<.0001
Day (D)	2	1.8	0.1983	1.6	0.2283	16.1	0.0001
L × C	3	4.0	0.0692	2.9	0.1230	2.4	0.1624
L × D	2	4.7	0.0256	4.4	0.0305	1.0	0.4002
C × D	6	3.1	0.0333	1.7	0.1979	2.8	0.0484
L × C × D	6	2.5	0.0634	1.2	0.3428	1.1	0.4081

Table 3.2. In vitro tetrad germination and mean number of pollen tubes per germinated tetrad in four rabbiteye blueberry cultivars.

Cultivar	Tetrad germination (%)	Pollen tubes per germinated tetrad
Austin	80.8 ± 2.9 b[z]	2.0 ± 0.05 a
Brightwell	90.2 ± 1.7 a	2.1 ± 0.05 a
Climax	88.4 ± 2.1 a	2.0 ± 0.05 a
Tifblue	81.5 ± 2.4 b	2.0 ± 0.04 a

[z]Mean separation in columns by LSD test at $P \leq 0.05$ (n = 12).

Table 3.3. Pollen production and release, ovule number per flower, tetrad-to-ovule ratio and pollen grain-to-ovule ratio in four rabbiteye blueberry cultivars.

Cultivar	Total pollen production	Pollen release	Ovules per flower	Tetrad:ovule ratio	Pollen:ovule ratio[z]
	Tetrads per flower				
Austin	6,638 ± 703 b[y]	-	78 ± 1.7 c	85	342
Brightwell	9,213 ± 267 a	8,339 ± 249 a	88 ± 1.3 b	105	421
Climax	9,065 ± 405 a	4,771 ± 482 b	103 ± 2.7 a	88	352
Tifblue	8,818 ± 454 a	-	71 ± 1.2 d	124	495
Overall mean	8,434 ± 604		85 ± 7	101 ± 9	402 ± 35

[z]The number of pollen grains per flower was assumed to be four times the number of tetrads.

[y]Values are means ± SE. Mean separation in columns by LSD test at $P \leq 0.05$. Sample sizes were n = 12 for total pollen production per flower, and n = 25 for pollen release and ovule number per flower.

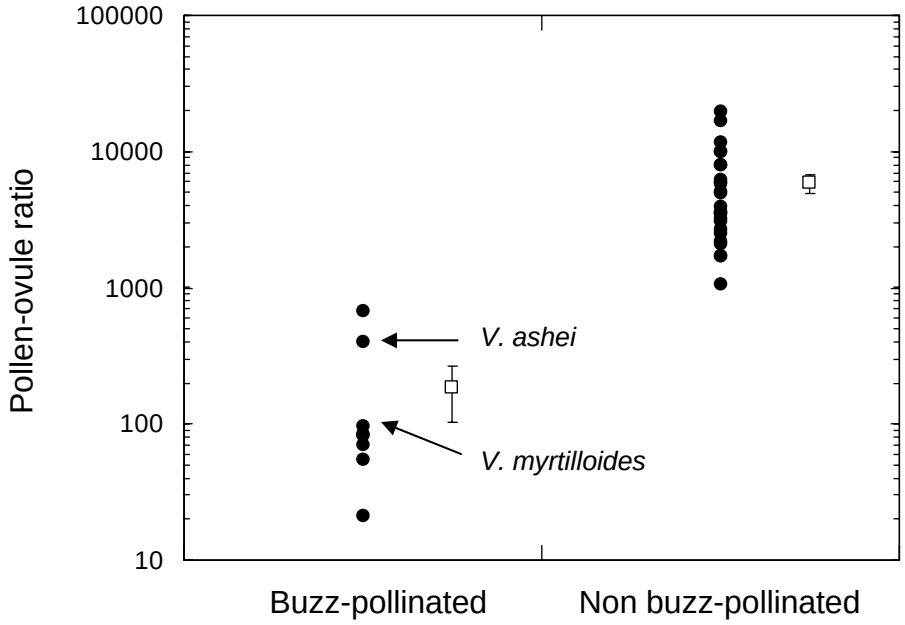

Fig. 3.1. Pollen-ovule ratios (●) in xenogamous species that are buzz-pollinated (n = 8) and non buzz-pollinated (n = 24), based on a literature review. Mean (± SE) pollen-ovule ratios for each group (□) are also presented. Buzz-pollinated species: *Moneses uniflora*, *Orthilia secunda*, *Pyrola minor*, *P. rotundifolia*, *P. chlorantha* (Knudsen and Olesen, 1993), *Rhexia virginica* (Larson and Barrett, 1999), *Vaccinium myrtilloides* [based on pollen and ovule numbers from Reader (1977) and Palser (1961)], and *V. ashei* (this study). Non buzz-pollinated species: 24 species from 10 families listed as xenogamous by Cruden (1977). These taxa are excluded from the list of poricidally dehiscent plants given by Buchmann (1983).

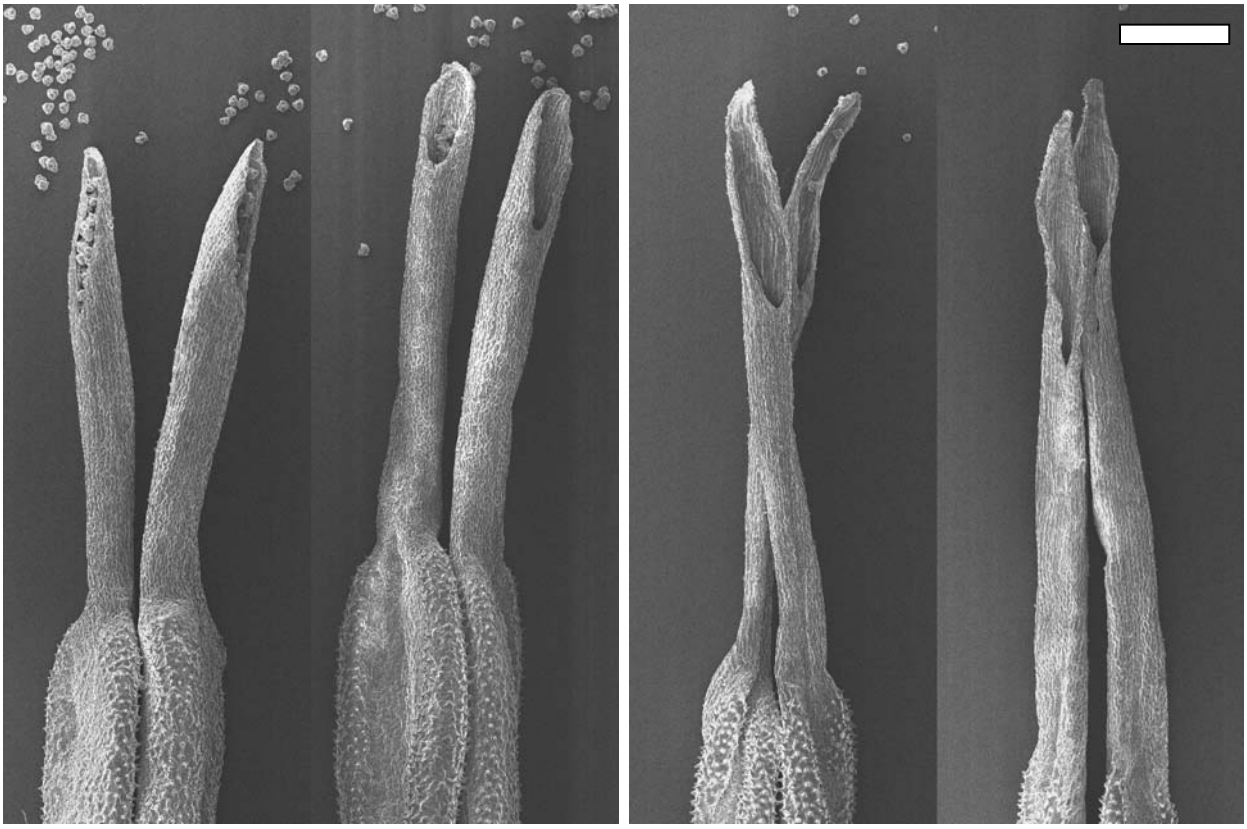

Fig. 3.2. Scanning electron micrographs of anther tubes from the rabbiteye blueberry cultivars Brightwell (left) and Climax (right). All micrographs are at the same scale. Scale bar: 0.5 mm.

CHAPTER 4

A NOVEL METHOD TO QUANTIFY POLLEN TRANSPORT BY BEES IN BLUEBERRY PLANTINGS[1]

[1]Brevis, P.A., D.S. NeSmith, L. Seymour, and D.B. Hausman. To be submitted to *HortScience.*

Abstract

Cross-pollination is beneficial or may be required for optimum fruit yields. All blueberry species (*Vaccinium* section *Cyanococcus*) grown commercially benefit from cross-pollination. Outcrossing increases fruit set, berry size, and rate of ripening. Although knowledge of pollen dispersal is essential for maximizing cross-pollination and achieving optimal planting designs, reliable methods to quantify this process in blueberry plantings are required. A novel method to quantify pollen transport by blueberry pollinators was developed based on frequency distributions of pollen tetrad diameter, measured with a particle counter. *Vaccinium ashei* Reade cvs. Brightwell and Climax were chosen for this study because they produce pollen of different size. The approach was used successfully to make unbiased predictions of the proportion of each cultivar in pollen mixtures with known composition. Subsequently, pollen extracts from bumblebees visiting a mixed 'Brightwell' and 'Climax' blueberry plot were analyzed. Cultivar proportions carried by bumblebees changed with the phenology of the crop following an expected pattern, indicating that the method performed well under field conditions.

Cross-pollination is beneficial or may be required for optimum fruit yields, because many fruit crops are self-incompatible. Although blueberry species (*Vaccinium* section *Cyanococcus*) do not have conventional self-incompatibility mechanisms (El-Agamy et al., 1982; Garvey and Lyrene, 1987; Krebs and Hancock, 1988; Vander Kloet, 1991), low self-fertility can occur as a consequence of early-acting inbreeding depression (Hokanson and Hancock, 2000; Krebs and Hancock, 1990; Krebs and Hancock, 1991). Cross-pollination is especially critical for adequate fruit set of *Vaccinium ashei* (rabbiteye blueberry) due to its low degree of self-fertility (El-Agamy et al., 1981; Garvey and Lyrene, 1987; Meader and Darrow, 1944). In blueberry species that are more self-fertile, such as *V. corymbosum* L. (highbush blueberry), cross-pollination is still beneficial, resulting in larger fruit size and faster ripening rate (Meader and Darrow, 1947; Morrow, 1943).

Research has shown that bee-mediated pollen dispersion in fruit crops (mostly members of the Rosaceae subfamilies Pomoideae and Prunoideae) can be quite limited. Fruit set tends to decline rapidly with increasing distance from pollinizer trees (Free, 1962; Free and Spencer-Booth, 1964; Milutinovic et al., 1996). Gene flow studies have furthered our understanding of the extent of cross-pollination in fruit orchards. Genetic markers have been used to identify the pollen-contributing parent in embryos of seeds after fruit set (Jackson and Clarke, 1991; Kron et al., 2001; Wertheim, 1991). These studies indicate that the distance of pollen-mediated gene dispersal is short and leptokurtic, with most of the gene flow occurring between adjacent cross-compatible trees.

Blueberries are bee-pollinated small fruit crops, and bee visitation is expected to result in cross-pollination. The information available, although scarce, suggests that pollen dispersion between cultivars may not be optimal. Vander Kloet and Lyrene (1987) indicated that

geitonogamous selfing is likely to result as a consequence of the foraging behavior of blueberry pollinators. Moreover, Hancock et al. (1989) established that fruit size and seed number per berry declined with increasing distance from the source of cross-pollen. Pollen dispersion in rabbiteye blueberry has not been quantified previously, even though cross-pollination is critical for optimum yields.

Although genetic markers have been used successfully to infer patterns of pollen dispersion in self-incompatible species of the Rosaceae family, this approach may not be as practical for blueberries. On the one hand, the potential number of seeds per fruit in *Vaccinium* section *Cyanococcus* is high, ranging from 50 to 125 ovules per ovary (Palser, 1961). Therefore, paternity analyses would be labor-intensive. On the other hand, the blueberry self-infertility mechanism offers another limitation. Since there is no rejection of the microgametophyte at the pistil level following autogamous crosses, self-pollination can lead to fertilization and subsequent embryo abortion. Problems of low fruit set in blueberry crops could thus be caused by deposition of pollen loads that are rich in self-pollen. The ideal technique to study pollen dispersion in blueberry should allow quantification of the extent of selfing, but genetic marker-based techniques will underestimate the paternal contribution of self-pollen.

Pollen dispersion in entomophilous plants can also be inferred by the flight distance of pollinators. However, this approach may underestimate the extent of pollen dispersion because of the effects of pollen carryover (Waser and Price, 1982). Knowledge of pollen dispersal is essential for maximizing cross-pollination and achieving optimal planting designs (Kron et al., 2001). Clearly, research is needed to understand the patterns of pollen dispersal in blueberry plantings, and a reliable method is required.

In the present study, we describe a novel method, based on frequency distributions of pollen diameter, to estimate the proportion of self- and cross-pollen transported by bees as they forage on blueberry bushes. In this technique, only the fraction of pollen carried dry and loose on the bees' bodies was extracted and analyzed (i.e., fraction available for pollination) (Harder and Wilson, 1997; Thorp, 2000). Therefore, estimates of proportions of self- and cross-pollen transported by bees are an indirect assessment of bee-mediated pollen dispersion in blueberry plantings.

Materials and methods

The technique described below discriminates between pollen grains of two blueberry cultivars based on frequency distributions of pollen diameter, measured with a particle counter. 'Brightwell' and 'Climax' rabbiteye blueberry plants were chosen for this study because their flowers produce pollen of different size (Fig. 4.1).

General procedure for pollen size analysis. Pollen samples extracted from either blueberry flowers or pollinators were stored at room temperature in a solution containing 25% ethanol, 5% formalin, 4% acetic acid, and 66% dH_2O per volume. The pollen dispersal unit for *Vaccinium* species is a tetrad (Cockerham and Galleta, 1976). Tetrad diameter and number were measured with a Coulter counter (Multisizer II; Beckman Coulter, Fullerton, Calif.) using a 140-μm aperture. Pollen suspensions were diluted in Isoton II diluent (Beckman Coulter) just prior to particle analysis. A magnetic stirrer was used to maintain uniform distribution of tetrads within the saline solution. Pollen grains from five to six 0.5-ml aliquots of continuously stirring suspension were sized and counted per sample. Raw data consisted of absolute accumulated frequencies of tetrads in classes of diameter at 0.38-μm intervals. The precision of the

measurements was monitored by adding 20-μm polystyrene latex particles (Beckman Coulter) to every analyzed sample.

Prediction of cultivar proportions in pollen mixtures. Proportions of 'Brightwell' and 'Climax' tetrads in pollen mixtures were predicted using the observed frequencies of pollen diameter. A smooth approximation to the probability density function for 'Brightwell' and 'Climax' pollen diameter was generated using kernel density estimation. The kernel density estimator was:

$$\hat{f}_h(x) = \frac{1}{h\sum Y_i} \sum Y_i K\left(\frac{x - X_i}{h}\right)$$

where h is the bandwidth, K is the kernel function, X_i is the midpoint of the ith diameter class, and Y_i is the number of observations in the ith diameter class. The general form of the kernel function is given by Härdle (1991). Appropriate kernel functions are symmetric about the vertical axis, positive, and integrate to 1. The kernel function chosen for this analysis was the normal kernel:

$$K(x) = \frac{\exp\left(\frac{-x^2}{2}\right)}{\sqrt{2\pi}}$$

Estimated probability density functions based on this method gave a good fit to the data (Fig. 4.2).

The proportion of 'Brightwell' in the pollen mixture was estimated using the maximum likelihood method. The likelihood function is the density function based on the observed data (assuming that the data are independent) but viewed as a function of the density's parameters. The likelihood function is maximized over the parameters to produce a maximum likelihood estimate of those parameters, based on the data. In practice, it is easier to maximize the log of the likelihood, which is equivalent to maximizing the likelihood function itself. See Wackerly et al. (2002) for a more in-depth discussion of elementary maximum likelihood estimation. The log-likelihood function for the pollen mixture was:

$$\sum_{i=1}^{n} Y_i * \log\left[(1-\beta) * f_c(X_i) + \beta * f_b(X_i)\right]$$

where $f_b(X)$ and $f_c(X)$ are the probability density functions for 'Brightwell' and 'Climax', respectively, β is the proportion of 'Brightwell' in the pollen mixture, and X_i and Y_i are as defined above. Then, the proportion of 'Climax' in the pollen mixture was estimated by the difference $1-\beta$.

The program to construct the kernel density estimates of the distributions of 'Brightwell' and 'Climax' pollen diameters, as well as to compute the maximum likelihood estimate of β, was written in the language F (Brainerd et al., 1996), a cheap-ware subset of Fortran (Fortran Co., Tucson, Ariz.), which can be compiled under most modern Fortran environments. The numerical optimization algorithm used to compute the maximum likelihood estimate of β was the quasi-Newton algorithm (Nash, 1990).

Validation of the model. The model was validated by predicting the proportion of 'Brightwell' in pollen mixtures where the cultivar composition was known.

Flowers were collected from field-grown 'Brightwell' and 'Climax' plants (the study site is described later). Pollen samples were extracted from 50 flowers of either cultivar. In 2003, six replications of each cultivar were collected on 4 Apr. In 2004, one replication per cultivar was collected on five different dates (24 Mar., 27 Mar., 2 Apr., 5 Apr. and 12 Apr). Pollen samples for each replicate were stored in vials with 20 ml of fixative solution as described earlier. The absolute tetrad concentration in each 20-ml suspension was estimated by counting the number of pollen grains in four aliquots using the previously described particle counter. Then, aliquots from a pair of 'Brightwell' and 'Climax' pollen samples (with a known tetrad concentration) were combined to achieve five pollen mixtures containing 20%, 40%, 50%, 60% and 80% of 'Brightwell'. Pollen mixtures with a known cultivar composition were analyzed with the particle counter following the procedure described previously.

The probability density function for diameter of pure pollen was estimated by kernel density estimation from observed frequency distributions as described above. Data from mixed pollen samples were analyzed using the density functions obtained from the pair of 'Brightwell' and 'Climax' samples used to assemble the mixture. The percentage of 'Brightwell' in the mixture was predicted by the maximum likelihood method.

Analysis of pollen extracted from bees. Bumblebees (*Bombus* sp.) and honeybees (*Apis mellifera* L.) were collected in 2003 and 2004 in a mixed 'Brightwell' and 'Climax' plot located at the Georgia Experiment Station in Griffin. The blueberry plot was 33 m × 29 m in size. Plants were spaced at 3.7 m across rows and 1.5 m within rows. Plants of each cultivar were arranged in alternating rows. The study site was isolated from other sources of blueberry pollen. The closest

blueberry plantings were > 800 m away. A honeybee colony was placed near the plot to ensure adequate visitation by *A. mellifera*. Native bumblebee queens were common at the study site during the entire blueberry flowering season. Although carpenter bees [*Xylocopa virginica* (L.)] were also present, they were not included in this study because of their questionable value as blueberry pollinators (Cane and Payne, 1991).

Bumblebee queens and honeybees were collected as they visited 'Brightwell' and 'Climax' bushes. Bees were caught with an insect net or directly using killing jars containing chloroform. The short exposure to chloroform was effective enough to quickly anesthetize or kill the specimens. Bees were then transferred individually to 20-ml blood cell counter vials (Labcon, Petaluma, Calif.) using forceps. All sample vials were stored inside of a cooler with ice until the specimens were brought to the laboratory. About 5 ml of the fixative described earlier was added to the vial, pouring the solution over the insect. If the specimen carried pollen in the corbicula, the corbicular pollen loads were removed from the hind legs using fine-pointed forceps before adding the solution to the vial. This was done because pollen carried in these pollen-transporting areas, moistened with nectar, is unavailable for pollination (Harder and Wilson, 1997; Thorp, 2000). Pollen tetrads were extracted from the bodies of bees by shaking the vials for 30 min at 270 rpm using a G10 Gyrotory shaker (New Brunswick Scientific; Edison, N.J.). Bees were then taken out of the solution and pollen was kept inside of the vial until analysis with the particle counter. In addition to extracting pollen from bees, pollen samples were also collected directly from flowers to be used as standard. A single pollen sample per cultivar was extracted on each sampling day from 50 flowers randomly collected in the experimental plot.

The diameter of blueberry tetrads carried by the bees was used to predict the proportion of each cultivar in the pollen extract, in a way similar to that described for pollen mixtures in the validation of the model. Probability density functions were estimated from pure pollen samples extracted from flowers of each cultivar. These densities were used to analyze the data from all the bees collected on that particular sampling day.

Results and discussion

Validation of the model. The predicted proportion of 'Brightwell' was linearly related to the actual proportion in the pollen mixture ($r = 0.995$, $P < 0.0001$, $n = 55$; Fig. 4.3). At $\alpha = 0.05$, the Y-axis intercept and the slope were not statistically different from 0 and 1, respectively. These results indicate that unbiased predictions of the proportion of each cultivar in the pollen mixture can be made using diameter frequency distributions.

Analysis of pollen extracted from bees. The presence of pollen from sources other than blueberry represented the major potential problem for applying this method in the field. Observations of pollen extracts under the microscope confirmed the presence of pollen from other plant species, which was consistent with previous reports (Cane and Payne, 1988; Delaplane, 1995; Dogterom and Winston, 1999; Vander Kloet, 1976). Two monads from unidentified plant species were the most common "foreign pollen" found in the collected specimens. However, these two pollen types were smaller than blueberry tetrads, with a mean diameter ($\pm$ SD) of 18 ± 1 μm and 30 ± 2 μm. No interference from foreign pollen was expected within the range of diameter of blueberry tetrads.

The average number of blueberry tetrads extracted per pollinator is shown in Table 4.1. Tetrad numbers per bumblebee and honeybee were significantly different. Bumblebee pollen

numbers were 11 to 28 times those of honeybees. The actual number of tetrads sized and counted in a sample extracted from the body of an average bumblebee was about 100-120 (n = 800 tetrads suspended in 20 ml; five to six 0.5-ml aliquots counted per sample). Bumblebees were considered to be a good model species for studying pollinator activity in blueberry plantings using this methodology. In contrast, the population of pollen tetrads sized and counted in samples from honeybees was too small to obtain accurate estimates based on diameter frequency distributions. It has been reported previously that honeybees can carry pollen grains in numbers ranging from several thousands to a few million (Free, 1970). The small number of blueberry tetrads extracted per honeybee in this study may be due to their incapacity to sonicate the poricidally dehiscent anthers of blueberry flowers (Buchmann, 1983). Another important contributing factor was the presence of carpenter bees at the study. These bees cut slits at the base of the tubular corolla to imbibe nectar, and honeybees readily visit the "robbery holes" made by carpenter bees (Dedej and Delaplane, 2004; Delaplane and Mayer, 2000). Honeybees acting as secondary nectar thieves avoid the opening of the flower, limiting the amount of pollen that they can collect from and transfer to the flower. Pierced corollas were available at the study during the entire flowering season of 2003 and 2004, and most of the flower visitations by honeybees were "illegitimate".

Frequency distributions of pollen diameter and predicted proportions of 'Brightwell' for selected bumblebees are shown in Fig. 4.4. Curves estimated with the maximum likelihood method gave a good fit to the data.

'Climax' plants bloom earlier than those of 'Brightwell', which gave an opportunity to determine if the predicted cultivar proportions changed as the season progressed. The proportion of 'Brightwell' carried by bumblebees collected in Spring 2003 is shown in Fig. 4.5. Specimens

collected at the beginning of the season carried small proportions of 'Brightwell', which was expected considering that very few 'Brightwell' flowers were open at that time. Conversely, all bees collected on the last sampling day carried almost a pure load of 'Brightwell' pollen. Bees collected between 29 Mar. and 2 Apr. carried mostly pollen from the cultivar where the specimen was foraging. These results indicate that the method worked well under field conditions.

Previous attempts have been made to identify pollen grains by cultivar in pollen loads carried by bees (Jackson and Clarke, 1992; Mittler, 1962; Vezvaei and Jackson, 1997). The pollen extracting procedure used in this study differed from previous work in that corbicular pollen loads were excluded. Therefore, pollen samples extracted from bumblebees were representative of the fraction of blueberry tetrads available for pollination. Pollen cultivar proportions can thus be considered as an estimate of the likelihood for 'Brightwell' or 'Climax' pollen to be deposited on the stigma.

In summary, frequency distributions of pollen diameter were used successfully to make unbiased predictions of the cultivar composition of a pollen mixture. When pollen extracts from bumblebees were analyzed, predicted cultivar proportions changed with the phenology of the crop following an expected pattern, indicating that the method performed well under field conditions. This method can be used to assess, indirectly, bee-mediated pollen dispersion between two plant populations or cultivars, provided the following conditions hold: 1) there is a consistent difference in pollen diameter between the two plant types, 2) there is no interference from foreign pollen, and 3) enough pollen can be extracted from the body of individual bees to obtain a meaningful sample.

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Table 4.1. Mean number of blueberry pollen tetrads extracted from the bodies of bumblebees and honeybees foraging on a mixed ‘Brightwell’ and ‘Climax’ rabbiteye blueberry plot.

	Year	
	2003	2004
Pollinator	Total number of tetrads per individual	
Bumblebee (queen)	4,595 ± 1,032 (51) a[z]	797 ± 67 (126) a
Honeybee (worker)	167 ± 41 (22) b	70 ± 10 (60) b

[z]Values are means ± SE, with sample sizes (individuals collected) in parentheses. Mean separation in columns by t-test at $P \leq 0.05$.

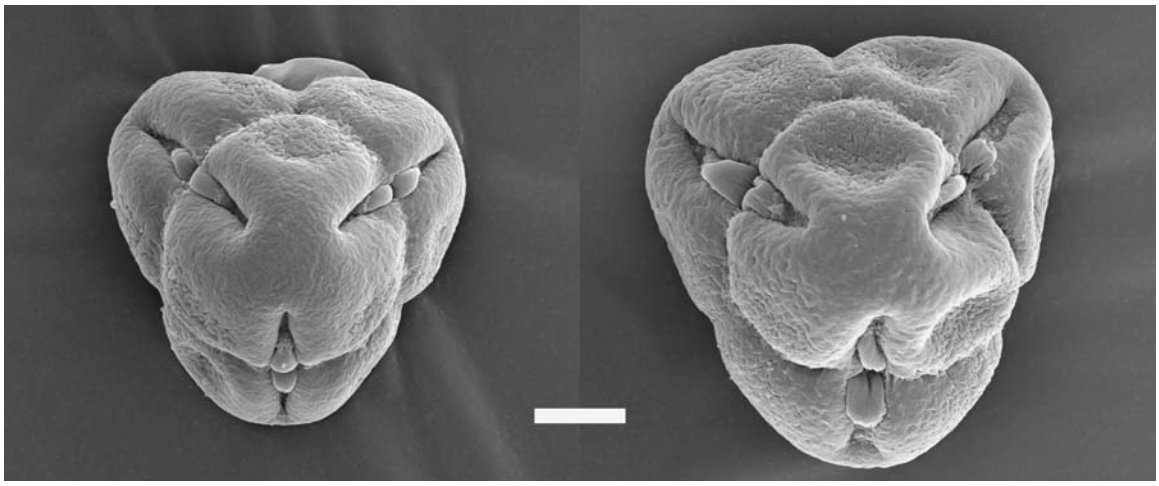

Fig. 4.1. Scanning electron micrographs of rabbiteye blueberry pollen tetrads from cultivars Climax (left) and Brightwell (right). Scale bar: 10 μm.

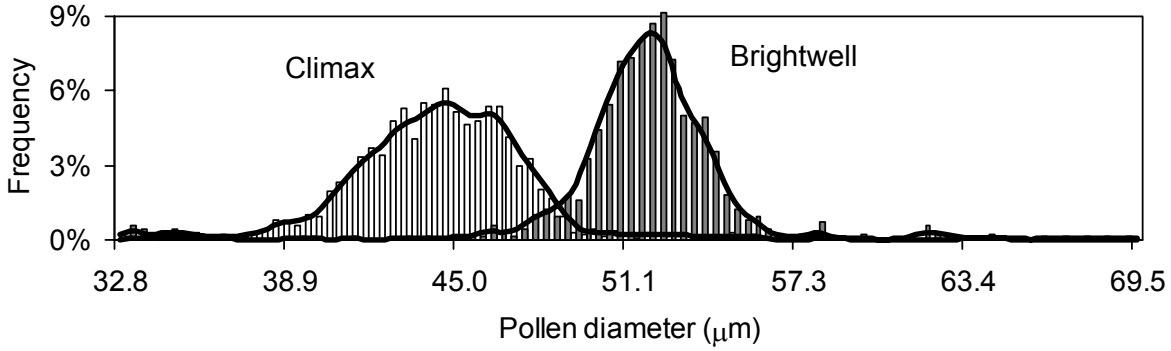

Fig. 4.2. Frequency distribution of 'Climax' and 'Brightwell' tetrad diameters. Pollen samples of each cultivar were extracted from 50 flowers collected at the study site (see text). Bars represent observed frequencies. Curves, fitted with kernel density estimation are smooth approximations of the probability density functions.

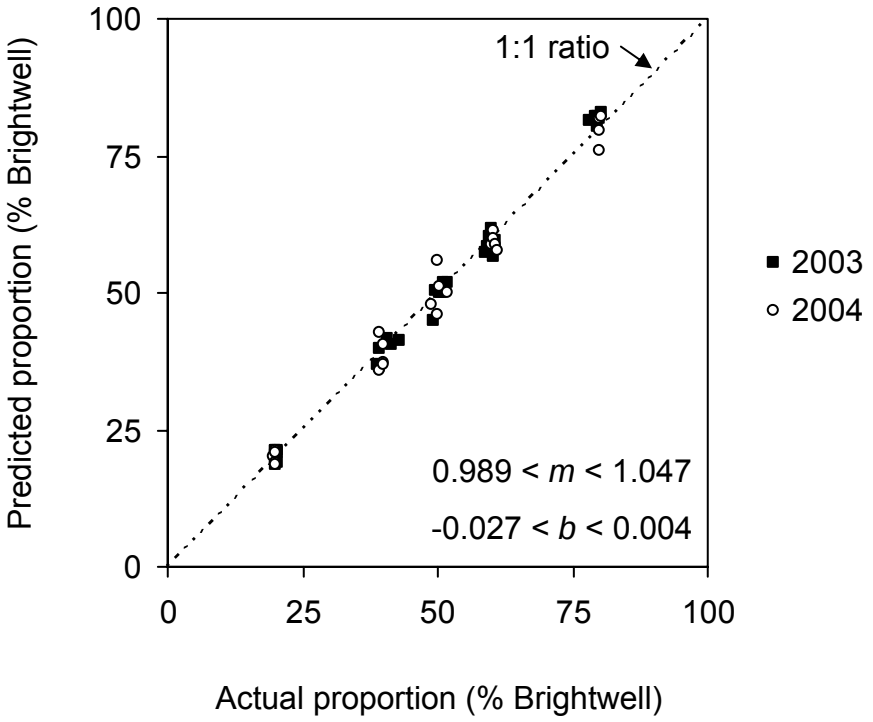

Fig. 4.3. Predicted versus actual cultivar proportions in 'Brightwell' 'Climax' pollen mixtures of known proportion (n = 55). Confidence intervals for the slope (*m*) and the Y-axis intercept (*b*) are also presented (α = 0.05).

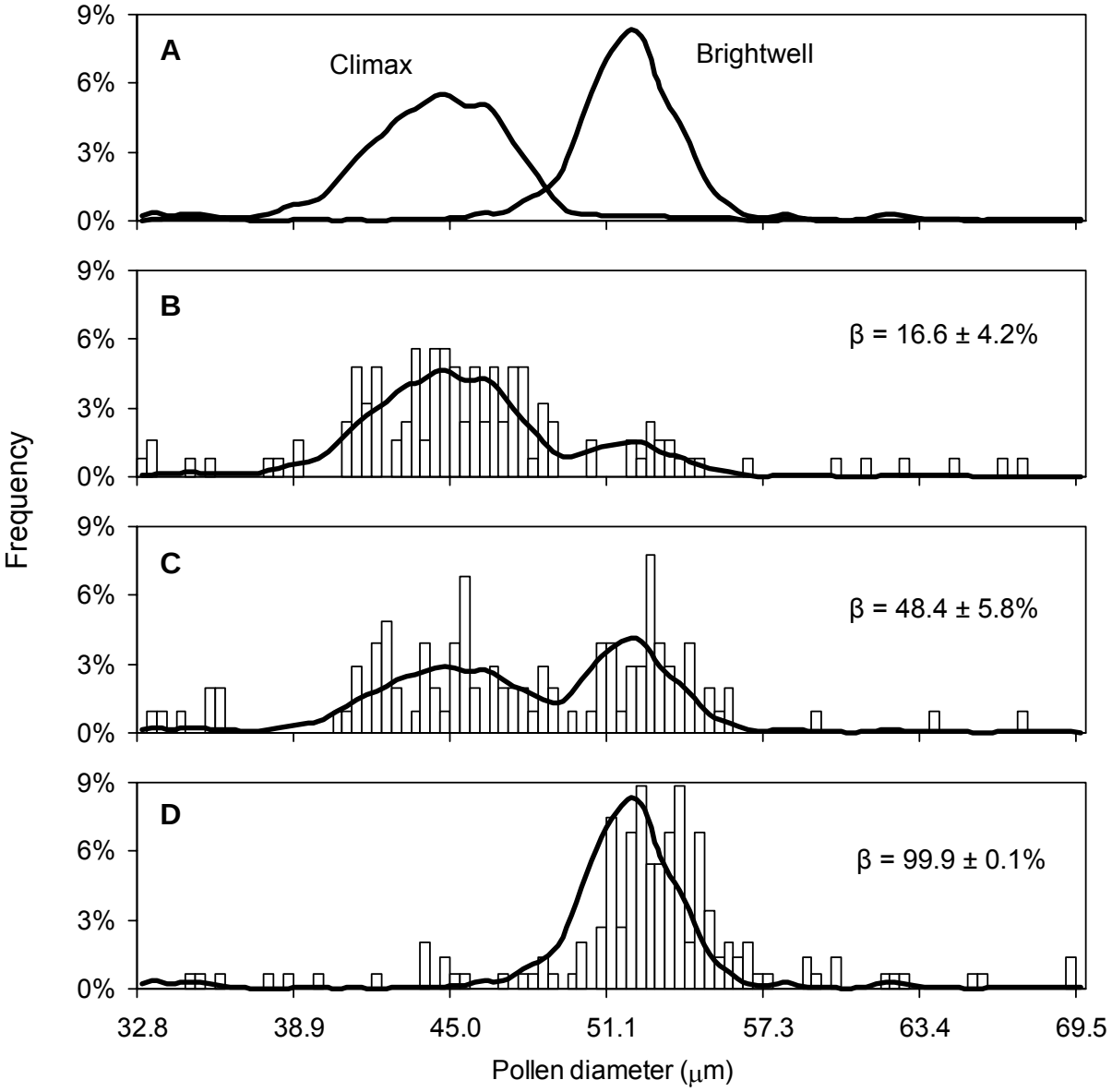

Fig. 4.4. A. Probability density functions for diameters of 'Climax' and 'Brightwell' blueberry tetrads. B-D. Diameter frequency distributions of blueberry tetrads extracted from individual bumblebees collected from 'Climax' (B-C) and 'Brightwell' (D) bushes. Curves fitted with the maximum likelihood method and predicted percentages of 'Brightwell' (β ± SE) are also presented.

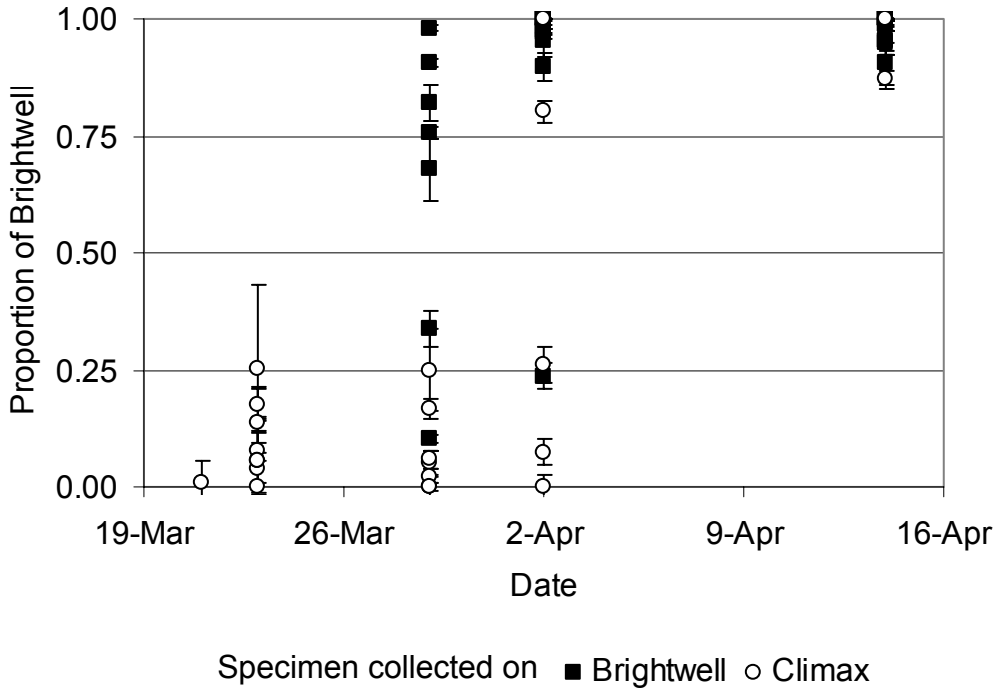

Fig. 4.5. Pollen cultivar proportions carried by individual bumblebees (n = 51) collected from 'Brightwell' or 'Climax' blueberry plants. Data points are estimated proportions of 'Brightwell' pollen (± SE) carried by individual bees collected in Spring 2003. Dates of cumulative 50% bloom: 'Climax', 29 Mar.; 'Brightwell', 4 Apr.

CHAPTER 5

TRANSPORT OF CROSS-POLLEN BY BUMBLEBEES IN A RABBITEYE BLUEBERRY PLANTING[1]

[1]Brevis, P.A., and D.S. NeSmith. To be submitted to *HortScience*.

Abstract

Rabbiteye blueberry (*Vaccinium ashei* Reade) is a bee-pollinated small fruit crop that often exhibits poor fruit set. Mixed cultivar plantings are recommended because cross-pollination is required for optimum yields, and bees are expected to transfer pollen from one cultivar to another. The objective of this study was to assess transport of cross-pollen by bumblebees in a rabbiteye blueberry planting. Experiments were conducted in 2003 and 2004 in a plot composed of 'Brightwell' and 'Climax' plants arranged in alternating rows. The proportion of 'Brightwell' and 'Climax' pollen carried on the bodies of bumblebees was estimated based on frequency distributions of pollen diameter, measured with a particle counter. About 75% of bumblebees collected in 2003 carried < 20% cross-pollen. Proportions of cross-pollen in 2004 were higher than in 2003, but still, 85% of bumblebees collected carried less than 40% cross-pollen. The low likelihood for cross-pollination was surprising, considering that all rows were adjacent to plants of the other cultivar (1:1 planting). The proportion of cross-pollen carried by bumblebees changed during the flowering season. The greatest likelihood for cross-pollination occurred during the time of maximum bloom overlap, although median proportion of cross-pollen carried by bumblebees was < 30% in all sampling days of 2004. The results from this study emphasize the need to select more self-fertile rabbiteye blueberry cultivars and to maximize bloom overlap in blueberry plantings.

The degree of self-fertility in many flowering plants is often limited by mechanisms such as conventional or late-acting self-incompatibility, and early-acting inbreeding depression (Klekowski, 1988; De Nettancourt, 1977; Seavey and Bawa, 1986). Outcrossing is thus beneficial or even required in such species for fruit set and seed maturation. In blueberries (*Vaccinium* section *Cyanococcus*), cross-pollination normally results in higher fruit set, larger fruit size, and faster ripening rate relative to self-pollination (El-Agamy et al., 1981; Meader and Darrow, 1944; Meader and Darrow, 1947; Morrow, 1943). Although highbush blueberries (*Vaccinium corymbosum* L.) can be planted in large monoclonal blocks, yields obtained under these conditions may not be as great as those from mixed cultivar plantings (Hanson and Hancock, 1988). In rabbiteye blueberry (*Vaccinium ashei*), cross-pollination is particularly critical for adequate fruit set (El-Agamy et al., 1981). Therefore, interplanting two or more cultivars is recommended to facilitate outcrossing and improve yields.

Blueberries are pollinated by several species of bees (Hymenoptera:Apiformes), and these insects are expected to transfer pollen from one cultivar to another. The information available for blueberries, although scarce, suggests that bee-assisted pollen dispersion between cultivars may not be optimal. Vander Kloet and Lyrene (1987) indicated that geitonogamous selfing is likely to result as a consequence of the foraging behavior of blueberry pollinators. Moreover, Hancock et al. (1989) established that fruit size and seed number per berry declined with increasing distance from the source of cross-pollen.

One of the most important horticultural problems of the rabbiteye blueberry industry is poor fruit set (Scherm et al., 2001). Although knowledge of pollen dispersal is essential for maximizing cross-pollination and achieving optimal planting designs (Kron et al., 2001), pollen

dispersion between rabbiteye cultivars has not been quantified previosuly. The objective of this research was to assess transport of cross-pollen by bumblebees in a rabbiteye blueberry planting.

Materials and methods

Study site. Experiments were conducted in 2003 and 2004 in a mixed 'Brightwell' 'Climax' rabbiteye blueberry plot located at the Georgia Experiment Station in Griffin. The plot was 33 m × 29 m in size. Plants were spaced at 3.7 m across rows and 1.5 m within rows. 'Brightwell' and 'Climax' plants were arranged in alternating rows (i.e. 1:1 planting). The study site was isolated from other sources of blueberry pollen. The closest blueberry plantings were >800 m away.

Proportion of cross-pollen carried by bumblebees. Bumblebees (*Bombus* spp.) were chosen as a model to study pollinator activity in blueberry plantings. The proportion of cross-pollen carried by bumblebees was used as an indirect measurement of pollen dispersion between 'Brightwell' and 'Climax' plants. Cross-pollen is defined here as 'Brightwell' pollen transported by bumblebees to 'Climax' plants, and vice versa. Frequency distributions of pollen tetrad diameter, measured with a particle counter, were used to predict the proportion of 'Brightwell' and 'Climax' pollen carried on the bodies of bumblebees. Brevis et al. (2005b) described the technique and statistical analysis used in this study, so only a brief summary is given here. Tetrad size and number were analyzed with a Coulter counter (Multisizer II; Beckman Coulter, Fullerton, Calif.). Cultivar proportions in pollen mixtures carried by bumblebees were predicted using the maximum likelihood method. Essentially, the statistical analysis used estimated the proportions of 'Brightwell' and 'Climax' pollen that would be necessary to obtain the frequency

distributions of tetrad diameter observed in pollen mixtures extracted from the bodies of bumblebees.

Sampling of bumblebees. Naturally occurring bumblebee queens were collected randomly from 'Brightwell' and 'Climax' bushes. *Bombus impatiens* Cresson was the most abundant, but *B. griseocollis* (Degeer), *B. bimaculatus* Cresson and *B. nevadensis auricomus* (Robertson) were also collected. A detailed description of the sampling procedure used in this study is given by Brevis et al. (2005b). Sampling dates and number of specimens collected are summarized in Table 5.1.

Flowering phenology. 'Climax' plants generally bloom earlier than 'Brightwell' (Krewer and NeSmith, 2000). In order to identify dates of cumulative 50% bloom and the period of maximum bloom overlap, flowering phenology data were collected at the study site in both years. Three twigs were marked in each of five representative plants of each cultivar. Open flowers were counted in tagged twigs at 1 to 3-day intervals during the flowering season. Open flowers were removed after recording data, so that individual flowers could not be counted more than once. A nonlinear logistic model of the form $y = N / [N + (1-N)*\exp(-r * t)]$ was fitted to the accumulated bloom data using NLIN procedure of SAS (SAS Institute, Cary, N.C.), where y was cumulative bloom, t was time in days, and N and r were parameters. The model gave a good fit to the data ($P < 0.0001$ for each cultivar and year). Fitted models were used to determine dates of cumulative 50% bloom and to predict the daily percentage of open flowers. Curves of open flowers (≤ 5 days after anthesis) were simulated for 'Brightwell' and 'Climax' in 2004.

Results

The proportion of cross-pollen carried by bumblebees (mostly *Bombus impatiens*) visiting 'Brightwell' and 'Climax' flowers in a mixed blueberry plot is summarized in Fig. 5.1. In 2003, proportions of cross-pollen for bumblebees collected in both cultivars followed the same distribution ($\chi^2 = 1.11$; df = 1, $P = 0.29$). About 75% of the specimens collected carried < 20% cross-pollen, and bumblebees with a 50:50 pollen mix from each cultivar were not found. In 2004, proportions of cross-pollen for specimens collected from 'Brightwell' and 'Climax' did not follow the same distribution ($\chi^2 = 11.80$; df = 2, $P \leq 0.01$). Most of the bumblebees collected from 'Brightwell' carried < 20% cross-pollen, while almost half of the catch from 'Climax' carried between 20% and 40% cross-pollen.

In 2004, cumulative 50% bloom for 'Brightwell' occurred 5 days later than for 'Climax' (3 Apr. and 29 Mar., respectively). The daily percentage of open bloom and the median proportion of cross-pollen carried by bumblebees are shown in Fig. 5.2. On 24 Mar., bumblebees collected from 'Climax' carried on average about 10% cross-pollen (that is, about 90% 'Climax' pollen). This was expected since 'Climax' was virtually the only cultivar with open flowers on that day. The proportion of cross-pollen carried by bumblebees foraging on 'Climax' increased as more 'Brightwell' flowers become available. The highest likelihood for cross-pollination of 'Climax' flowers was recorded on 5 Apr. Specimens were not collected on this cultivar later than 5 Apr. because of the relatively low availability of open bloom.

Bumblebees collected from 'Brightwell' plants on 27 Mar. carried on average only 5% cross-pollen. This was intriguing since there was a relatively high availability of 'Climax' flowers at the study site. The median proportion of cross-pollen for pollinators in 'Brightwell' followed the same pattern observed for those in 'Climax', and the maximum value was also

recorded on 5 Apr. On the last sampling day, bumblebees visiting ‘Brightwell’ flowers carried about 10% cross-pollen. When sampling was done simultaneously in both cultivars (from 27 Mar. to 5 Apr.), bumblebees from ‘Brightwell’ always carried less cross-pollen than those from ‘Climax’.

Bumblebees foraging on ‘Brightwell’ carried more blueberry tetrads on their bodies than those on ‘Climax’. In 2004, the mean number of tetrads (± SE) extracted per bumblebee was 917 ± 93 and 539 ± 49 for specimens collected from ‘Brightwell’ and ‘Climax’, respectively (significantly different at $P < 0.01$). These results confirmed the trend observed in 2003. The total number of tetrads and the proportion of ‘Brightwell’ pollen carried by bumblebees in 2004 were weakly but positively associated (Fig. 5.3). The association between the log-transformed number of tetrads and the proportion of ‘Brightwell’ was linear ($r = 0.24$, $P \leq 0.01$; $n = 126$).

Discussion

Bumblebees carried low proportions of cross-pollen in both years. Although actual pollen deposition on blueberry stigmas was not measured, it is expected that most of the flower visitations by bumblebees resulted in self-pollination. The low likelihood for cross-pollination was surprising, considering that all rows were adjacent to plants of the other cultivar (1:1 planting). Data indicate that insufficient cross-pollination is likely to play a major role in the failure of rabbiteye blueberry to set adequate commercial crops. Breeding efforts should be made to select more self-fertile rabbiteye blueberry cultivars.

Bumblebees with a 50:50 pollen mix from each cultivar were rare in 2004 and absent in 2003. These results clearly indicate that bumblebees visited ‘Brightwell’ and ‘Climax’ flowers in a non-random fashion. Waser and Price (1983) reported that 90% or more of bumblebees’

flower-to-flower flights are shorter than 1 m. Low proportions of cross-pollen carried by bumblebees at the study site might be explained by their tendency to visit many flowers within a plant (Vander Kloet and Lyrene, 1987) and to fly between adjacent monotypic plants within the same row.

Proportions of cross-pollen carried by bumblebees changed during the flowering season. The greatest likelihood for cross-pollination occurred around the date of maximum bloom overlap. Therefore, the chances of cross-pollination at the study site were not only low, but also limited to a narrow time window. The results from this study emphasize the need to maximize bloom overlap in blueberry plantings.

The low proportion of cross-pollen carried by bumblebees collected from 'Brightwell' early in the season was not consistent with the availability of open flowers of 'Climax'. Selectivity toward virgin 'Brightwell' flowers could explain these results, although no data was collected in order to determine cultivar preferences by bumblebees.

Brevis et al. (2005a) established that 'Brightwell', on a per flower basis, releases more tetrads than 'Climax'. This difference seems to have an effect on bumblebee-assisted pollen carryover, and thus, on the extent of cross-pollination. Data from 2004 indicate that bumblebees harvested more tetrads from 'Brightwell' than from 'Climax'. Bumblebee foraging behavior is such that when they assess high pollen returns during a flower visit, both the frequency of grooming episodes and the handling duration per flower increase (Buchmann and Cane, 1989; Harder, 1990). Pollen carryover by pollinators during subsequent flower visits is thus restricted, because pollen is packed into scopae instead of being transported to the next flower. In concordance with this, bumblebees visiting 'Brightwell' flowers in 2004 carried lower proportions of cross-pollen relative to those collected in 'Climax'. The results from this study

suggest that blueberry cultivars that release more pollen per flower can actually be exposed to a higher degree of selfing.

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Table 5.1. Sampling dates and number of bumblebees collected from 'Brightwell' and 'Climax' blueberry plants in 2003 and 2004.

	Year 2003					
	21 Mar.	23 Mar.	29 Mar.	2 Apr.	14 Apr.	Total
Brightwell	0	0	7	10	11	28
Climax	1	8	7	5	2	23
	Year 2004					
	24 Mar.	27 Mar.	2 Apr.	5 Apr.	12 Apr.	Total
Brightwell	1	8	23	23	31	86
Climax	7	8	12	13	0	40

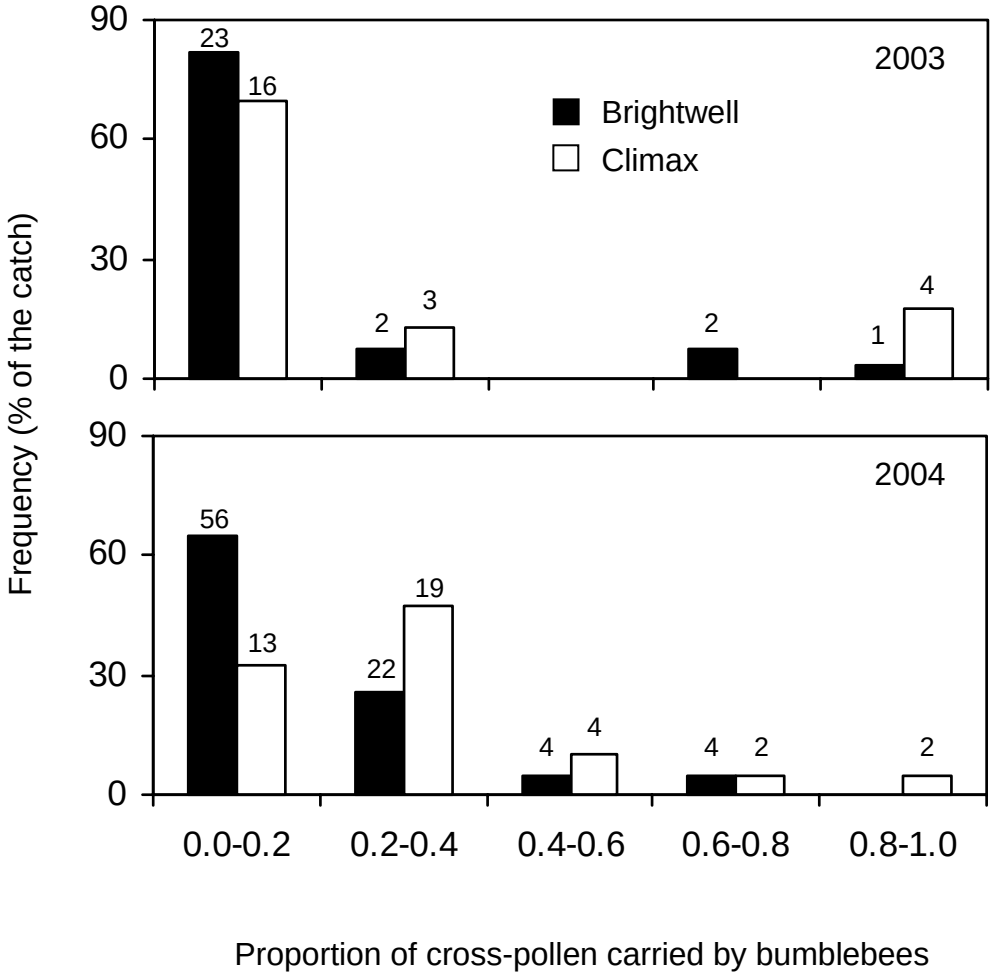

Fig. 5.1. Distributions of proportions of cross-pollen carried by bumblebees visiting 'Brightwell' and 'Climax' bushes in a mixed rabbiteye blueberry plot in 2003 and 2004. Sample sizes (number of bumblebees collected) appear above each bar.

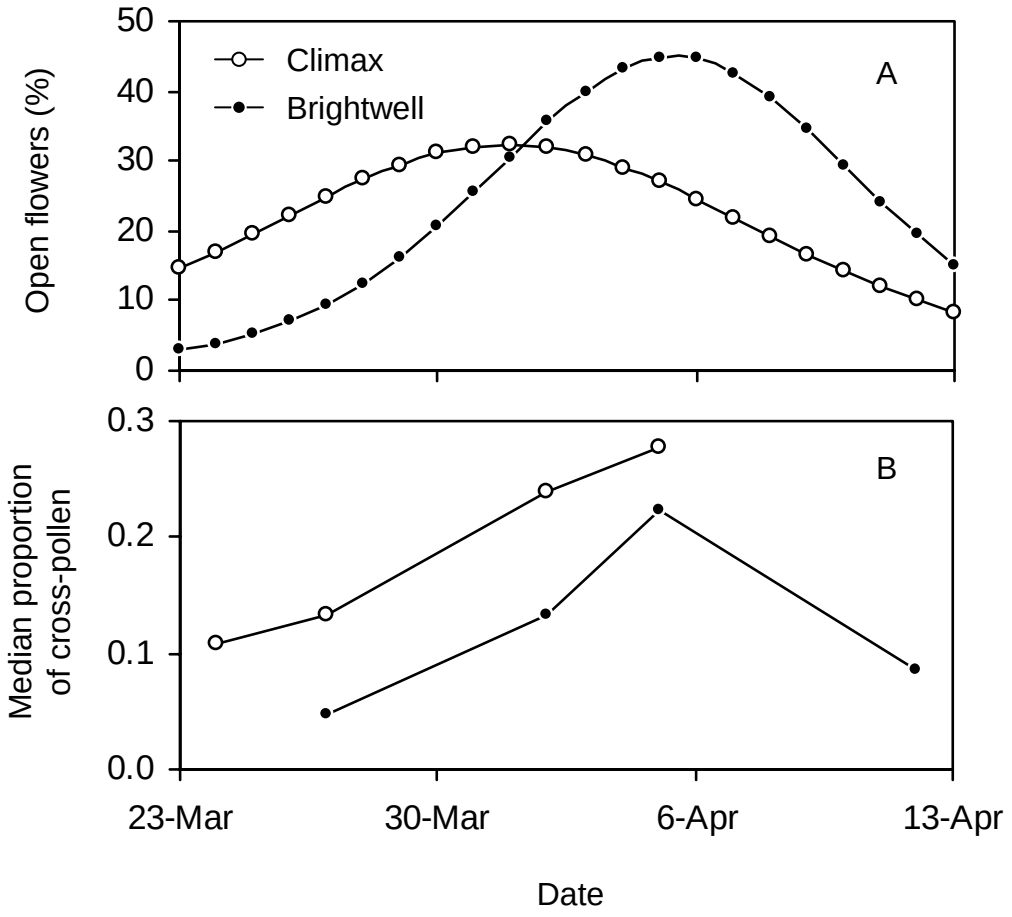

Fig. 5.2. A. Daily percentage of open flowers for each cultivar. B. Median proportion of cross-pollen carried by bumblebees visiting 'Brightwell' and 'Climax' flowers in a mixed blueberry plot. Both graphs show data from Spring 2004.

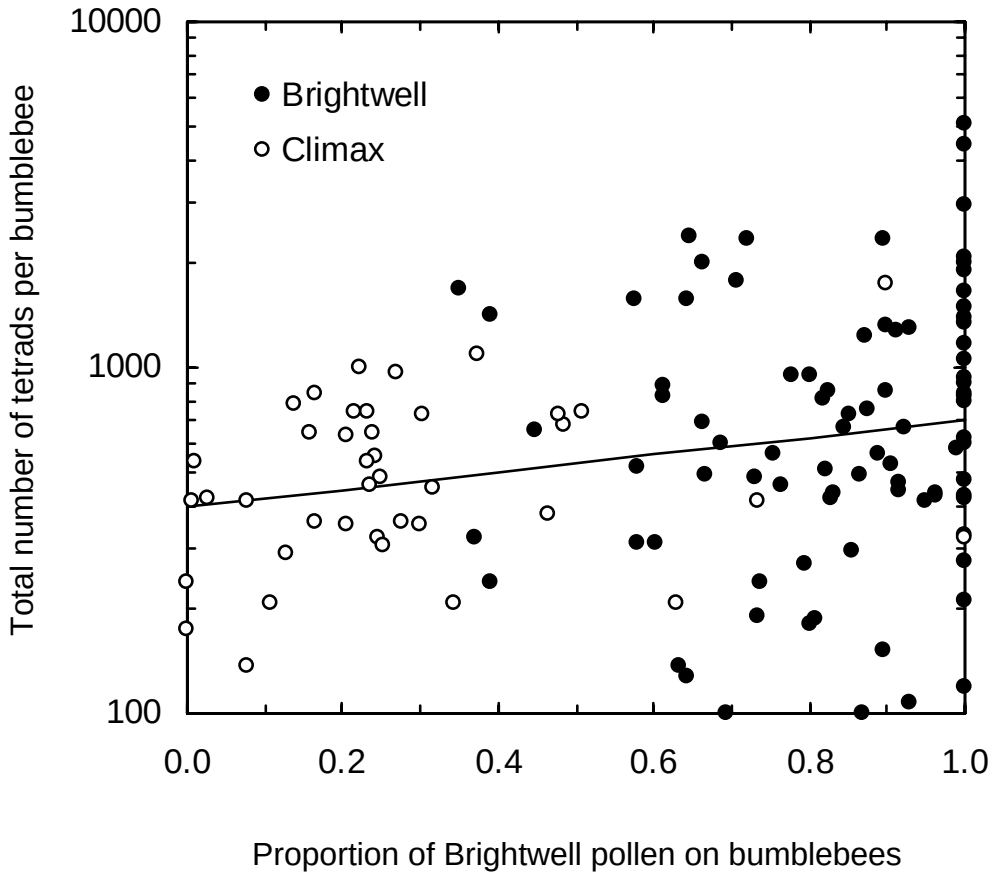

Fig. 5.3. Total number of tetrads and proportion of 'Brightwell' pollen carried by bumblebees visiting 'Brightwell' and 'Climax' flowers in a mixed blueberry plot during Spring 2004. The line was fitted across all data points (n = 126).

CHAPTER 6

EFFECTIVE POLLINATION PERIOD IN RABBITEYE BLUEBERRY[1]

[1]Brevis, P.A., D.S. NeSmith, and H.Y. Wetzstein. To be submitted to the *Journal of the American Society for Horticultural Science.*

Abstract

Effective pollination period (EPP) is the number of days during which pollination is effective to produce a fruit. The EPP is determined by ovule longevity, pollen tube growth rate, and length of stigmatic receptivity. This research was conducted to establish the EPP and to investigate the role of stigmatic receptivity in determining the EPP of rabbiteye blueberry (*Vaccinium ashei* Reade). The experiments were conducted in a growth chamber using blueberry plants of the cultivars Brightwell and Tifblue. Emasculated flowers were hand-pollinated at 0, 2, 4, 6, and 8 days after anthesis (DAA). Ripe fruit were harvested to record percent fruit set. Stigmatic receptivity was evaluated as the number of germinated tetrads on the stigma 24 h after pollination. For day/night temperatures of 23/10°C, fruit set reached maximum values when pollination was carried out 2 to 4 DAA depending on the cultivar. Flowers produced acceptable fruit set (>30%) during a period of 7 days (EPP = 7 days). This study provided the first quantitative evidence of late stigma maturation in blueberry, in that low stigmatic receptivity limited tetrad germination in flowers pollinated at 0 DAA; however, this variable and fruit set were not positively associated. Therefore, stigmatic receptivity was not the most limiting factor of the EPP. 'Tifblue', a cultivar with problems of low fruit set, exhibited low fruit setting capacity when pollination was carried out at 0 DAA. Data indicated that age-related variables other than stigmatic receptivity (i.e., ovule longevity and/or pollen tube growth rate) likely limit the EPP of rabbiteye blueberry and contribute to reproductive failure in 'Tifblue'.

The lifespan of an individual flower can be quite short; therefore, pollination must occur on the few days that the flower is receptive. Effective pollination period (EPP) is defined as the number of days during which pollination is effective to produce a fruit (Williams, 1965). Research has shown that the EPP plays a crucial role in controlling fruit set of various fruit crops (Sanzol and Herrero, 2001). The EPP is determined by the length of stigmatic receptivity, the rate of pollen tube growth, and the longevity of the ovules.

The EPP has been studied in a number of fruit crops, including almond [*Prunus dulcis* (Mill.) D.A. Webb], apricot (*P. armeniaca* L.), peach [*P. persica* (L.) Batsch.], plum (*P. domestica* L.), sour cherry (*P. cerasus* L.), sweet cherry (*P. avium* L.), apple [*Malus* ×*sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.], kiwifruit [*Actinidia deliciosa* (Chev.) Liang and Ferguson] and pear (*Pyrus communis* L.) (Burgos et al., 1991; Furukawa and Bukovac, 1989; Gonzalez et al., 1995; Guerrero-Prieto et al., 1985; Herrero, 1983; Keulemans and van Laer, 1989; Ortega et al., 2004; Toyama, 1980; Williams, 1965). Although blueberry (*Vaccinium* section *Cyanococcus*) often exhibits problems of low fruit set, the EPP of this small fruit crop has received little attention. Blueberry flowers remain receptive up to 8 days after anthesis (DAA) in highbush blueberry (*Vaccinium corymbosum* L.) (Moore, 1964), 6 DAA in lowbush blueberry (*V. angustifolium* Ait.) (Wood, 1962), and at least 5 DAA in rabbiteye blueberry (*V. ashei*) (Young and Sherman, 1978). According to these studies, cultivar differences within a species also occur. Young and Sherman (1978) evaluated pollen tube penetration two days after pollination in styles pollinated at different time intervals from anthesis. However, this variable is not a good estimate for either pollen tube growth rate or stigmatic receptivity. Therefore, there is no information available on the parameters determining the EPP in blueberry species.

Rabbiteye cultivars are grown extensively in the southeastern United States because they are generally well suited for the growing conditions of the region. However, poor fruit set is one of the most important horticultural problems of the rabbiteye blueberry industry (Scherm et al., 2001). Rabbiteye blueberries have a limited degree of self-fertility (El-Agamy et al., 1981); therefore, the standard practice is to interplant two or more cultivars that overlap in bloom to facilitate cross-pollination. While poor fruit set may be due to a lack of cross-pollination, there may also be problems with short EPPs. The objectives of this study were 1) to establish the EPP of rabbiteye blueberry and 2) to investigate the role of stigmatic receptivity in determining the EPP.

Materials and methods

Plant material and experimental conditions. Four- and five-year-old rabbiteye blueberry plants were used in 2003 and 2004, respectively. Bushes were grown in 19-L containers using pine bark as a growing medium. The cultivars Brightwell and Tifblue were chosen because of their commercial importance and their different performance with regards to fruit set. 'Brightwell' is usually very productive while 'Tifblue' often exhibits low fruit set (Lyrene, 2002; NeSmith and Krewer, 1999). Following chilling under natural conditions outdoors, dormant plants were stored at day/night temperatures of 10/5°C to slow bud development. To initiate the experiment, plants with floral buds at stage 1 to 2 (Spiers, 1978) were transferred into a growth chamber located at the University of Georgia Griffin Campus. Chamber conditions were 23/10°C day/night temperature, 40/85% day/night relative humidity with a 13-h photoperiod. In order to simulate more natural conditions, the temperature was increased to 28/15°C four weeks after

pollination and to 30/18°C two weeks later. Plants were fertilized with a 15N-9P-12K slow-release fertilizer (Osmocote) and watered daily.

Flower age at the time of pollination. Flowers were emasculated just prior to anthesis and hand-pollinated 0, 2, 4, 6, and 8 days after anthesis (DAA). Emasculation was performed at two-day intervals so that all flower-age treatments could be pollinated on the same day. Flowers of the same age were randomly located throughout the plant.

Pollen source. Fresh pollen was extracted on the day of pollination from newly opened flowers. Self- or cross-pollen was applied to the stigma of emasculated flowers using the surface of a steel rod. Although the number of tetrads applied was not controlled, the amount of pollen deposited on the stigma was below saturation (<100 tetrads) (Parrie and Lang, 1992). Previous in vitro assays indicated that pollen viability was high (>80% tetrad germination) for both cultivars (Brevis and NeSmith, 2003). One-hundred flowers were pollinated per plant, that is, 10 flowers per flower age × pollen source combination. Flowers were tagged individually with color-coded threads around the pedicel to identify each treatment combination. Three single-plant replications per cultivar were used in 2003 and 2004.

Fruit set and seed number per berry. Fruit were harvested when ripe (>90% blue in color). Fruit set was estimated as the proportion of pollinated flowers that produced ripe berries. Harvested fruit were stored in a freezer so that seed counts could be made at a later date. In 2003, all seeds were counted under a dissecting microscope regardless of their stage of development. In 2004, fully developed and aborted seeds were recorded separately, as described by Krebs and Hancock (1991).

Stigmatic receptivity. In the second year of the study, stigmatic receptivity also was evaluated on the same plants used to estimate fruit set. Thus, the total number of emasculated

flowers per plant was doubled to 200. Flowers for both fruit set and stigmatic receptivity evaluations were pollinated on the same days using similar pollen. Half of the flowers remained on the plant to estimate fruit set, as described earlier. The other half was detached from the plant one day after pollination. Styles were fixed in formaldehyde:acetic acid:alcohol (FAA) as described by Ngugi et al. (2002) and softened in 1 N NaOH overnight on a hot-plate (60°C). Cleared styles were mounted in squash preparations with 0.1% water-soluble aniline blue in 0.1 N K_3PO_4 (Martin, 1959). Tetrad germination was examined by fluorescence microscopy. Germinated tetrads on the stigma can be easily identified because those that do not germinate are washed off during the softening procedure. Stigmatic receptivity was assessed as the number of germinated tetrads on the stigmatic surface 24 h after pollination.

Statistical analysis. The experimental design was a split-plot with cultivar as the main-plot and a factorial arrangement of flower age × pollen source as the subplot, with 10 flowers per treatment combination. Averages per plant were calculated for the variables germinated tetrads on the stigma and seed number per berry. All response variables were transformed prior to analysis to reduce heterogeneity of variance. Fruit set was arcsine-square root transformed. Germinated tetrad and seed counts were square root transformed. Following analysis of variance, linear and quadratic effects were tested and curves were fitted across flower age values.

Results

Fruit set. The main effect of flower age at the time of pollination as well as the interaction term cultivar × flower age were significant for fruit set (Table 6.1). In cross-pollinated 'Brightwell', a slight increase in fruit set was observed from 0 to 2 DAA (Fig. 6.1A). In cross-pollinated 'Tifblue', fruit set recorded almost a two-fold increase from 0 to 4 DAA. Thereafter, fruit set for both cultivars declined with increasing flower age at pollination. Cross-pollinated rabbiteye blueberry flowers produced acceptable fruit set (>30%) during a period of 7 days. Although this threshold may be considered low, estimates of 30% fruit set have been recorded for rabbiteye blueberry under average commercial field conditions (NeSmith and Adair, 2004).

Fruit set was affected by cultivar. Overall, 'Tifblue' set a lower proportion of its flowers relative to 'Brightwell', although the extent of this difference depended upon flower age at the time of pollination. The greatest difference in fruit set between cultivars was observed during the first half of the flower lifespan. Fruit set of 'Tifblue' at 0 DAA was about one-third of that of 'Brightwell', following cross-pollination. The relative difference between cultivars at 0 DAA was even greater following self-pollination (Fig. 6.1B).

The main effect of pollen source was also significant ($P \leq 0.001$). Self-pollination reduced fruit set relative to cross-pollination. These results are consistent with previous studies (El-Agamy et al., 1981; Meader and Darrow, 1944). Pollen source did not interact with flower age at the time of pollination.

Seed number per berry. The main effects of flower age at the time of pollination, cultivar, and pollen source, as well as the interaction term cultivar × flower age were significant for total number of seeds per berry (Table 6.1). In general, seed count data followed a pattern

similar to that observed for fruit set (Fig. 6.1C-D). The effect of flower age at pollination on seed number per berry was not as strong as that on fruit set, however.

Seed counts in 2004 revealed that the number of fully developed seeds was correlated with the total number of seeds per berry ('Brightwell': r = 0.84, $P < 0.0001$, n = 112 berries; 'Tifblue': r = 0.79, $P < 0.0001$, n = 75 berries). Fully developed seeds represented a lower proportion of the total seed content in self- relative to cross-pollinated berries. Percentages of fully developed seeds following self- and cross-pollination were 8% and 10% in 'Brightwell', and 5% and 8% in 'Tifblue', respectively.

***Stigmatic receptivity*.** Flower age at the time of pollination was the only significant main effect for number of germinated tetrads on the stigma (Table 6.2). Stigmas pollinated on the day of anthesis were the least receptive, and stigmatic receptivity gradually increased with flower age at pollination (Fig. 6.2). Stigmas from older flowers supported germination of a higher number of tetrads, and pollen tubes grew further down the style in 24 h (Fig. 6.3). The high stigmatic receptivity on stigmas pollinated from 6 to 8 DAA, as evidenced by the number of germinated tetrads, contrasted with the rapidly decreasing percentage of fruit set observed among flowers of similar age. Stigmatic receptivity and fruit set were not positively associated; therefore, this variable apparently had little influence on the EPP.

Discussion

For day/night temperatures of 23/10°C, the EPP of rabbiteye blueberry was 7 days. Although it is difficult to compare results from this study with those from studies conducted in different environmental conditions, there seems to be little variation in the EPP among blueberry species. For the current study, the EPP in rabbiteye blueberry was similar to that found with

lowbush blueberry (7 days; Wood, 1962) and slightly shorter than that of highbush blueberry (9 days; Moore, 1964).

The EPP can be limited by short longevity of the ovules, slow pollen tube growth rate, or short length of stigmatic receptivity (Sanzol and Herrero, 2001). In this experiment with rabbiteye blueberry, stigmas on the day of anthesis were the least receptive. As flowers aged, stigmatic receptivity increased. Maturation of the stigma may cause an increase in exudate quantity or quality. If so, the higher number of germinated tetrads observed at 6 to 8 DAA would be a consequence of a greater capacity of the stigma to hydrate pollen and support germination. Late stigma maturation can play an important role in preventing autogamy (Bertin and Newman, 1993). Given that the anthers release pollen from the day of anthesis and the stigma is apparently not fully receptive until 6 DAA, it is unlikely that pollen could adhere to and germinate on the stigma of the same flower. Although Vander Kloet (1988) and Drummond and Groden (2000) indicated that blueberry flowers are generally protandrous, quantitative data to support this is lacking. To our knowledge, the results from this study are the first quantitative evidence of late stigma maturation in blueberry.

Under the conditions of this study, stigmatic receptivity did not account for the variation in fruit set. At 8 DAA, fruit set was low in both cultivars despite the high stigmatic receptivity. It is probable that the ovules of these flowers had started to degenerate before the pollen tubes reached the ovary. At 0 DAA, fruit set of cross-pollinated ‘Brightwell’ was about three times higher than ‘Tifblue’, despite the fact that there was no overall cultivar effect on stigmatic receptivity.

Fruit set estimates revealed a significant interaction between cultivar and flower age (Table 6.1). ‘Brightwell’ flowers pollinated at 0 and 2 DAA outperformed those of ‘Tifblue’.

This finding has practical implications. Given the same growing conditions, fruit set in 'Tifblue' is normally lower than in 'Brightwell' (Lyrene, 2002; NeSmith and Krewer, 1999). The current results indicate that age-related factors operating at the flower level, other than stigmatic receptivity, likely contribute to the failure to set adequate commercial fruit loads. The comparatively low performance of 'Tifblue' during the first half of the flower lifespan could be a consequence of factors such as delayed embryo sac maturation. Presence of immature ovules at anthesis is characteristic of some pear (Herrero, 1983) and almond (Pimienta and Polito, 1983) cultivars. In pollinated flowers of apricot, the proportion of ovules containing differentiated egg cells increased from only 20% at 2 DAA to 54% at 6 DAA (Burgos and Egea, 1993). It is hypothesized that delayed embryo sac development contributes to low fruit set in 'Tifblue', because pollination 0 to 2 DAA could result in pollen tubes reaching the ovary when most of the ovules are not receptive. Future research should examine ovule longevity and its role in the EPP of rabbiteye blueberry.

Self-pollination reduced fruit set and seed number per berry, as well as the proportion of seeds reaching full development. According to Hokanson and Hancock (2000), higher proportions of aborted seeds in self- relative to cross-pollination are indicative of early acting inbreeding depression. This mechanism is reported to be responsible for self-sterility in highbush and lowbush blueberries (Hokanson and Hancock, 2000; Krebs and Hanckock, 1988, 1990, 1991).

In summary, the EPP of rabbiteye blueberry was determined to be 7 days. Low stigmatic receptivity limited tetrad germination on immature stigmas, although this variable and fruit set were not positively associated. Age-related factors, other than the stigmatic receptivity (i.e.,

pollen tube growth and/or ovule longevity), likely limit the EPP in rabbiteye blueberry and contribute to the problems of low fruit set in the cultivar Tifblue.

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Table 6.1. Mean squares for fruit set and total number of seeds per berry for 'Brightwell' and 'Tifblue' rabbiteye blueberry pollinated from 0 to 8 days after anthesis with self- and cross-pollen under growth chamber conditions in 2003 and 2004[z].

Source	df	Mean squares	
		Fruit set[y]	Total seed number per berry[x]
Cultivar (C)	1	4.953**	76.18***
Main plot error	5	0.233	1.17
Flower age (A)	1	3.005***	19.12**
Pollen source (P)	1	1.175***	25.30**
C × A	1	3.277***	17.95**
A × P	1	0.005	3.65
C × P	1	0.002	0.73
Subplot error	102[w]	0.073	2.41

*, **, ***Significance at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively

[z]Data from both years were combined because year main effects and interactions were not significant ($P > 0.05$). Sample size was thus n = 6 plants.

[y]Analysis performed on arcsine-square root transformed data.

[x]Averages per plant were calculated prior to analysis. Analysis performed on square root transformed data.

[w]The df of the error term for seed number per berry was 80 due to missing values (seed count data was not available when fruit set was equal to zero).

Table 6.2. Mean squares for number of germinated tetrads on the stigma for 'Brightwell' and 'Tifblue' rabbiteye blueberry pollinated from 0 to 8 days after anthesis with self- and cross-pollen under growth chamber conditions in 2004.

Source	df	Mean squares[z]
Cultivar (C)	1	0.229
Main plot error	2	0.081
Flower age (A)	1	25.181***
Pollen source (P)	1	0.047
C × A	1	0.096
A × P	1	0.210
C × P	1	1.970*
Subplot error	48	0.393

*, **, ***Significance at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively

[z]Averages per plant (n = 3) were calculated prior to analysis. Analysis performed on square root transformed data.

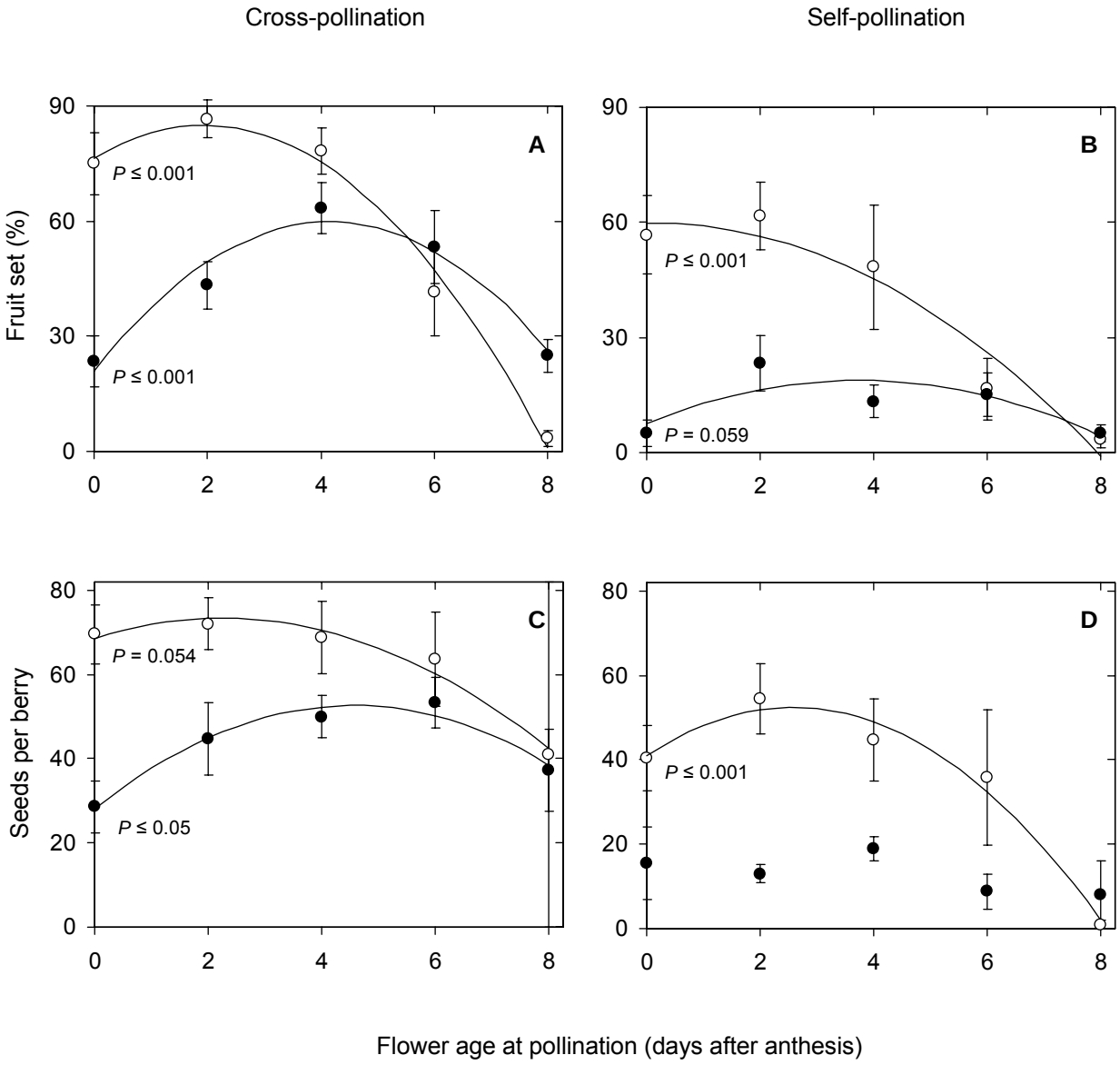

Fig. 6.1. Effect of flower age at the time of pollination on fruit set (A, B) and total number of seeds per berry (C, D) following cross-pollination (left panes) and self-pollination (right panes). The symbols refer to the rabbiteye cultivars Brightwell (○) and Tifblue (●). Values are arithmetic means (± SE) of untransformed data from two consecutive years (n = 6 plants, with 10 flowers assessed per plant). Curves shown were fitted across untransformed data. *P*-values indicate the significance of polynomial models fitted across transformed data.

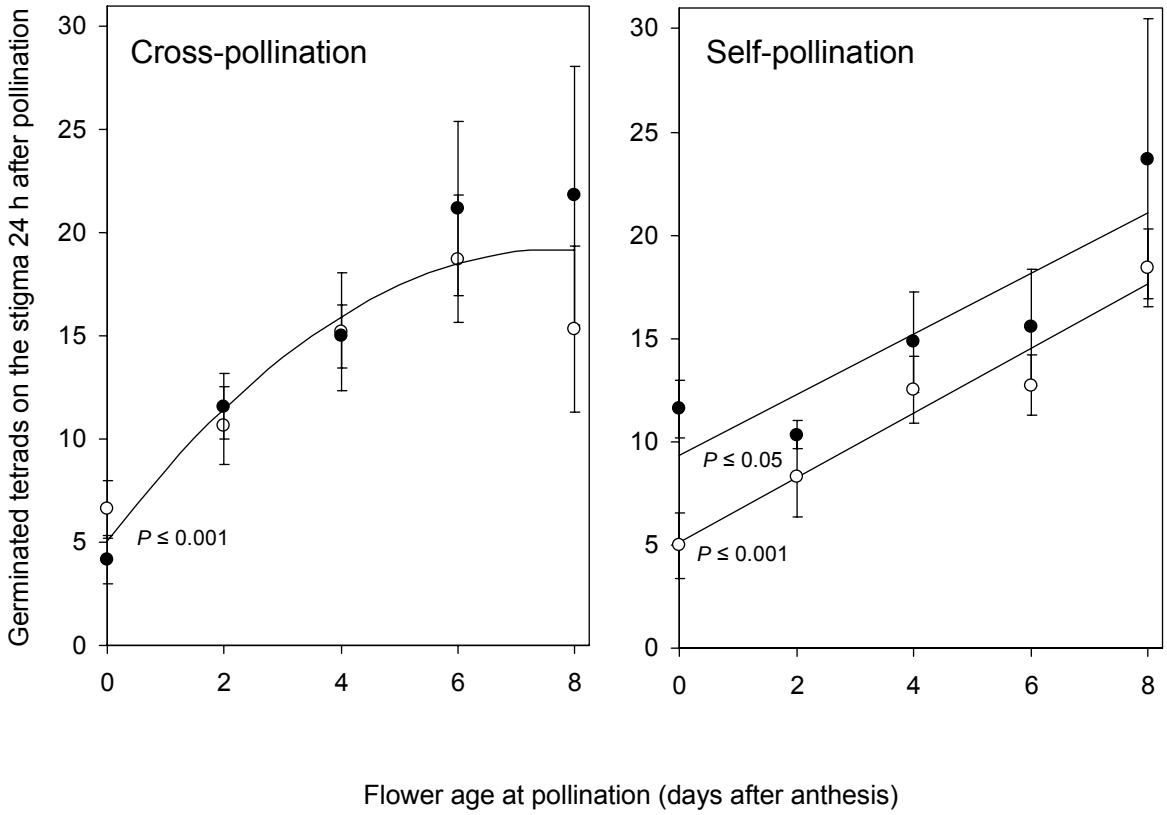

Fig. 6.2. Effect of flower age at the time of pollination on stigmatic receptivity following cross- and self-pollination. The symbols refer to the rabbiteye cultivars Brightwell (○) and Tifblue (●). Values are arithmetic means (± SE) of untransformed data from 2004 (n = 3 plants, with 10 stigmas assessed per plant). Curves shown were fitted across untransformed data. *P*-values indicate the significance of the models fitted across transformed data. A single curve is shown in cross-pollination because there was no cultivar effect ($P > 0.05$).

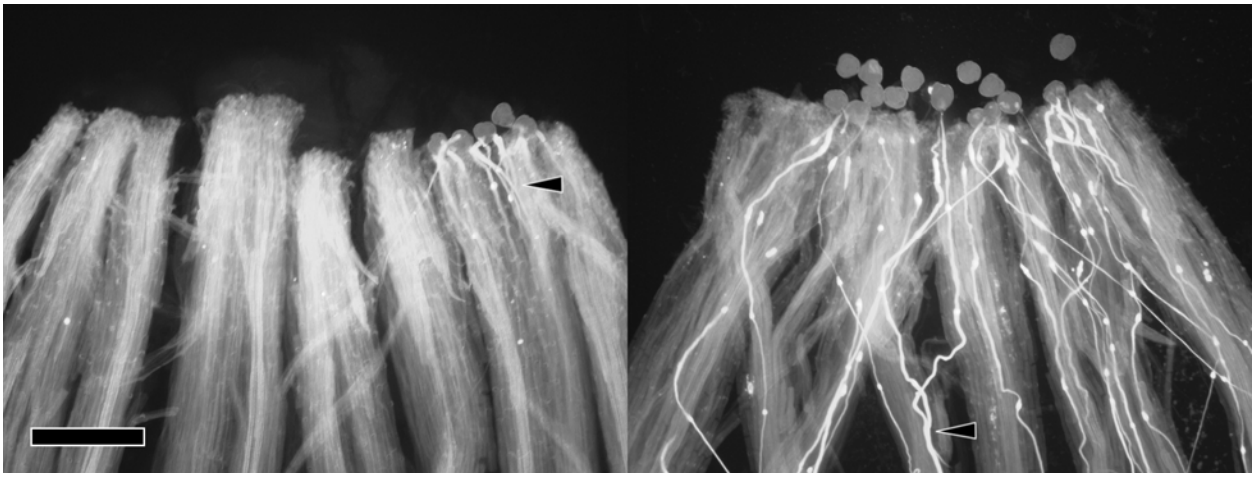

Fig. 6.3. Tetrad germination on cross-pollinated stigmas of ‘Brightwell’ 24 h after pollination. Flower age at the time of pollination was 0 (left) and 8 (right) days after anthesis. Pollen tubes are depicted with arrows. Scale bar: 250 μm.

CHAPTER 7

CONCLUSIONS

The research conducted as part of this dissertation addressed selected aspects of the pollination biology of rabbiteye blueberry (*Vaccinium ashei* Reade) in an effort to identify limiting factors for fruit set and yield. The studies presented here contribute to understand the role of pollen number and viability, pollen dispersion, and effective pollination period (EPP) in determining the pollen limitation of fruit set in rabbiteye blueberry.

On a per ovule basis, pollen production of the xenogamous *V. ashei* is very low relative to other species with similar breeding systems. The low pollen-ovule ratio of rabbiteye blueberry (402 ± 35) is apparently an indicator of the high efficiency of its pollen dispensing mechanism, characterized by poridically dehiscent anthers. Total pollen production and release vary among rabbiteye blueberry cultivars. High percentages of in vitro tetrad germination (>80%) suggest that pollen viability does not contribute to reproductive failure in rabbiteye blueberry.

Transport of cross-pollen by bumblebees was studied in a mixed 'Brightwell'-'Climax' blueberry planting using a novel technique based on frequency distributions of pollen diameter, measured with a particle counter. Predicted proportions of 'Brightwell' and 'Climax' pollen carried by bumblebees indicate that these pollinators visited flowers of each cultivar in a non-random fashion. The likelihood for cross-pollination was low and limited to the period of maximum bloom overlap. 'Climax' had a higher likelihood for cross-pollination than 'Brightwell', which may be due to a difference in pollen release per flower.

Flower longevity was studied in ‘Brightwell’ and ‘Tifblue’ rabbiteye blueberry under growth chamber conditions. For day/night temperatures of 23/10°C, the EPP was 7 days. This study provided the first quantitative evidence of late stigma maturation in blueberry, in that stigmatic receptivity limited tetrad germination in flowers pollinated on the day of anthesis; however, this variable was not the most limiting factor of the EPP. ‘Tifblue’, a cultivar with problems of low fruit set, exhibited low fruit setting capacity when pollination was carried out on the day of anthesis. Age-related factors, such as ovule longevity and pollen tube growth rate, likely limit the EPP and contribute to reproductive failure in the cultivar Tifblue.

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