Pollination ecology of lowbush blueberry (*Vaccinium angustifolium*) – The role of introduced pollinator communities, self- fertilization and somatic mutations on fruit set response

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Abstract

This thesis examines fruit yield variation and its causes in the lowbush blueberry (*Vaccinium angustifolium*). I found that yields exhibits significant variability in fruit within and between fields. An experiment involving controlled introductions of the three pollinator species commonly used in blueberry production—the honeybee (*Apis mellifera*), the bumblebee (*Bombus spp.*) and leafcutter bee (*Megachile rotundata*)—was also conducted. Increasing the abundance or diversity of the introduced pollinator community did not systematically increase fruit set across the experimental populations. There is weak evidence to suggest the combination of bumblebee and leafcutter bee may be more effective at increasing fruit yield than honeybee alone. The behaviour of introduced pollinator species differed between fields depending on the combination of pollinator species present, however, none of these changes was correlated with increased fruit set. As part of this work, self-pollinations and cross-pollinations were conducted in a large number of clones and variation in inbreeding depression of yield was detected among clones. To evaluate whether differences in accumulated deleterious mutations among clones were responsible for variation in inbreeding depression, a follow-up experiment manipulating access to self pollen was undertaken. In addition, differential genetic load was measured, using clone size as a proxy for somatic cell division. Neither clonal size nor self pollen access sufficiently explained the interclonal variation in self fruit set. Within the same fields, geitonogamously-pollinated fruit set was greater than autogamously-pollinated fruit set. These differences suggest the presence of somatic mutations, cell lineage selection, mitotic recombination, or epigenetic changes within lowbush blueberry clones, and they mirror results from studies of several perennial plant species that have revealed autogamy depression to be a significant factor in plant fertility. These results suggest that self-fertilization is an important element limiting fruit set that should be addressed in attempts to increase lowbush blueberry yield.

Résumé

Ce thèse explore la variance du rendement de fruit et les causes des différences dans le rendement vue dans l'espèce de bleuet *Vaccinium angustifolium*. J'ai trouvé qu'il existe une variabilité dans le rendement de fruit entre les champs et entre les individus du champs. Une expérience visant l'introduction des trois espèces pollinisateur utilisé le plus fréquement (*Apis mellifera*, *Bombus spp., Megachile rotundata*) au Nouveau-Brunswick a aussi eu lieu. Lors de l'augmentation de la diversité ou l'abondance il n'y avait aucun gain conséquent dans le rendement de fruit. Il y a un faible temoignage qui suggère que le rendement de fruit est plus élevé dans les champs qui inclut la combinaison pollinisateur de *Bombus* et *Megachile* comparé au champs seulement avec *Apis* introduit. Les mesures de comportement des pollinisateur introduit ont aussi varié entre les champs dépendant des espèces présent dans les champs, mais ces changements en comportement n'ont pas été lié au différences dans le rendement de fruit. Comme partie de cette recherche, des auto-pollinisations et des pollinisations croisée ont eu lieu dans plusieurs clones, avec une variabilité dans la dépression de consanguinité vue entre individus. Pour evaluer si des différences dans l'accumulation des mutations somatiques entre individus sont responsable pour la variabilité dans la dépression de consanguinité, une expérience qui a but de modifié accès de auto pollen a suivie. Pour mésurer la différence en charge génétique, la taille des clones a été utilisé pour représenter la division cellulaire somatique. Ni la taille des clones ou l'accès à l'auto pollen fut capable d'expliquer la variation de rendement de fruit entre les clones. Ces mêmes champs on aussi produit un taux de rendement de fruit plus élevé dans les fleurs fécondé avec le pollen geitonogamous comparé au fleurs fécondé avec le pollen autogame. Ces différences suggèrent la présence des mutations somatiques, la sélection lignée cellulaire, la recombinaison mitotique, ou des changement épigénétique dans les clones de bleuets, et ces résulats reflète d'autres études des espèces de plantes vivaces qui indique que la dépression autogame est une force significative dans la fertilié des plantes. Nos résultats suggèrent que l'auto pollinisation joue un rôle important dans la limitation du rendement de fruit et dois être considéré lorsqu'on essai d'augmenter le rendement de fruit chez le bleuet *V. angustifolium*.

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Introduction

Lowbush blueberry (*Vaccinium angustifolium*) is among the most important crops in the Maritime Provinces and Maine. Its success in the region lies mainly in its capacity to grow in poor soil conditions. Native stands of lowbush blueberry are often found in areas where no other crop would thrive: e.g., fire disturbed areas and soils characterized as being acidic, nutrient poor, thin and rocky (Kinsman, 1986). The relative ease of small scale production and adaptability to many soil types has allowed lowbush blueberry to become historically important to the settling of the region (Kinsman, 1986) and to become a productive part of agriculture in the region. In the last 20 years, much of the growth in the North American blueberry industry has occurred within Canada, with a 57% increase in area cultivated for lowbush blueberries occurring between 1992 and 2003 (Strik and Yarborough, 2005). In New Brunswick alone, since the early 1970s lowbush blueberry farm area has increased six-fold to over 12000 hectares with an accompanying ten-fold increase in production with over 15 million kg being harvested in 2008 (NB Blueberries, 2009). These increases in yield and scale in lowbush blueberry farming are in part derived from recent advancements in land improvement, soil fertility, pest management, harvesting and pollination management (Yarborough, 2004). The increases in yields have been a boon to New Brunswick farmers with the industry value at $25 million in 2008, leading to the ambitious goal of 4000lb per acre acre production being targeted for the upcoming 10 years (NB Blueberies, 2009). However, a key issue identified by the industry and researchers alike as a limiting factor is the variability in fruit set seen in fields (Bell et al. 2010, Hepler and Yarborough, 1991). In analyzing current

production practices and research on lowbush blueberry yield, two interrelated factors emerge as being possible elements in explaining the yield variability: pollination ecology and limitations due to genetic factors.

As a wild and long-lived perennial plant, lowbush blueberry is grown on a two-year cycle of field burning/pruning back of growth (year 1) followed by harvesting (year 2). All fields are derived from wild populations, requiring that natural vegetation be removed, creating fields of thousands of clones. The loss of both natural habitat and diversity in floral resources are suspected to affect native pollinator populations. Coupled with the increase in density of lowbush blueberry flowers as clone size increases undeterred, native pollination services rapidly become overwhelmed, requiring the import of managed pollinators into the field to achieve full fruit set. Advancements in bee rearing have led to three pollinators being used in different combinations in New Brunswick, *Apis mellifera*, *Bombus spp.*, and *Megachile rotundata*. The costs associated with using introduced pollinators are significant, and many farmers are unsure of the most effective way to deploy them within their fields It is well known that fruit set is reduced when flowers are self-pollinated. Yet given the size of clones, and the fact that many pollinators make only short flights between flowers and flower clusters, it is likely that much of the pollen transferred between flowers is self-pollen. Determining what combinations of pollinators and bee behaviours lead to greater yields will allow farmers to develop more efficient pollinator management strategies.

The perennial nature of *Vaccinium angustifolium* allows the plant to grow vegetatively throughout its lifetime, creating large areas of flowers and ramets from a single individual. Clone size can potentially play a large role in determining pollinator movements between. Increases in self-fertilization have been seen as the number of flowers or floral display size increase (De Jong 1992, Mitchell, et al, 2004). If clone size affects the level of self-pollination, any increases in introduced pollinator density may result in unnecessary increased costs for farmers. Manipulating the continuity of a floral display or increasing the availability of unrelated flowers/pollen may reduce the rate of self pollination and increase blueberry yields. Identifying the optimal size of a clone (in terms of low self-fertilization) allows farmers to manage blueberry clone size in their fields to maximize the benefits of introduced pollinators.

The results of self-pollinations may also be variable between clones and within a clone. As lowbush blueberry clones grow, deleterious mutations that occur during mitosis can accumulate within a plant and be passed onto developing gametes. The wild nature of source plants for lowbush blueberry fields introduces the possibility of differential genetic load between populations. Similarly, within a field individual clones may contain a different number of mutant alleles. The recessive nature of most mutations means they may not be expressed until the next generation, following self-pollination. This may result in seed or fruit abortion and other potential fitness effects. If we can correlate

inbreeding depression with some measure of growth or mitosis, we may be able to develop better management practices for lowbush blueberry clones, as well as models that allow us to understand the potential consequences of mutation and somatic growth to the long term fitness of perennial crops. These two themes, the effect of introduced pollinators on fruit yield along, and their interactions with clonal growth, along with the likely increase in self-pollination leading to declines in fruit yield, form the topic of this thesis.

Chapter 1. Implications of somatic mutations for perennial agriculture: A study of inbreeding depression in lowbush blueberry production

Modern agricultural production depends heavily upon fruit and seed production, especially by annual grain and oil seed crops such as wheat, rice, and maize, canola, and soybeans. While outputs from these crops have improved dramatically over the past few decades, there have been increasing proposals to shift agricultural production away from reliance solely on annual plants towards the increased use of perennials. Such a shift is expected to reduce both soil erosion and reliance on high input chemical fertilizers and herbicides (Glover and Reganold 2010) while promoting above and below soil biodiversity (Neher, 1994; Glover et al. 2010) and ecological processes (Glover et al. 2010). The obstacles to this shift if they can be overcome, could dramatically reduce the environmental impact of large scale agricultural.

While many aspects of plant growth influence the success of agricultural production, perhaps the most direct components are those related to reproduction (i.e. fruit and seed set. Fruit set within plants is a complex attribute determined by multiple factors. It is impacted by both the plant's ecology and genetic makeup. The perspective that I take in this chapter focuses on the genetic and pollination constraints associated with reproduction in perennials, constraints that may potentially limit fruit and seed set and thereby negatively impact the success of perennial agricultural. In particular, I consider how larger plant size and plant age, both inherent aspects of perennial plants, could lead to increased self-pollination and

consequent yield reduction due to inbreeding depression. Moreover, because perennial agriculture would rely on long-term propagation of individuals (essentially clonal reproduction), I consider whether and how somatic mutation accompanying the increased numbers of cell divisions in perennial plants could contribute to yield decline by exacerbating early-acting inbreeding depression (fruit and seed abortion) following self-fertilization.

These often overlooked aspects of plant reproduction in perennials are complex, and it is not possible to address them exhaustively. Moreover, while grain production is the major goal of perennial agriculture (Cox, 2006; Dehaan 2005; Glover, 2005), at this point there are few such perennial grain cropping systems available for direct study. On the other hand, perennial agricultural systems are well developed in the case of tree crops, such as citrus and stone fruit. Yet another perennial plant-based crop system (involving plants closer in size and stature to those envisioned as the basis of perennial grain production) are the small fruits (e.g. berries, grapes), which are typically shrubs or small vines. While training, pruning and propagation methods have been thoroughly examined, the impacts of clonality on long term fitness components has garnered less attention

A particular case in point is lowbush blueberry (*Vaccinum angustifolium*) production. Lowbush blueberry plants are derived from seed or from individuals already growing within recently cleared areas or that migrate from surrounding wild populations. This natural establishment from wild populations results in variation in age, size and phenotype. From the standpoint of agricultural production, the clonal growth of lowbush blueberry plants increases survivorship through providing

improved access to limiting soil nutrients. As well, clonality spreads the risk of mortality amongst ramets within the clone (Pan and Price, 2002). Such benefits allow lowbush blueberry to persist in harsher environments and in nutrient-poor soils that are inhospitable to many other crops. For example, lowbush blueberry establishment in Eastern Canada typically involves simply the clearing of marginal forests containing blueberry undergrowth, thus allowing for clonal spread of the blueberry field. With proper weed management, blueberry rhizomes can spread by up to 40 cm per season and take as little as two years to fully colonize a field (NSAC, 1997).

Increases in clone size can lead to greater reproductive capacity and net fitness benefits arising from the larger number of flowering shoots (Vallejo-Marin et al, 2012). However, as plants increase in size, they generally experience higher levels of self-pollination due to within-plant floral visits, also known as geitonogamy (De Jong, 1992; Mitchell, 2004; Feinsinger, 1978; Schemske, 1980b). At a certain size any benefits seen due to clonal growth may be counterbalanced by the detriments of increased self pollen capture in clones (Charpentier, 2002). Low self-fruit set of the lowbush blueberry is thought to arise in part from early-acting inbreeding depression (Krebs and Hancock, 1990). The resulting increase in self-pollination, due to higher self-pollen transfer within the clone as clone size increases is expected to reduce reproductive fitness and yield (De Jong, 1992). Thus by encouraging clonal growth we may increase the reproductive potential of individual plants, but these benefits may not be realized due to inbreeding depression.

The greater fitness resulting from outcrossed pollen has been an important factor in the evolution of sexual strategies to reduce self pollination (Reusch, 2001). Changes in floral morphology or phenology (i.e. dioecy, dichogamy) and the maintenance of self-incompatibility systems in plants are adaptations that have potentially developed and persisted in certain plant species due clonal growth (Vallejo-Marin, 2012). In comparison to annual crops, clonal and perennial species experience a greater number of somatic divisions along with an inevitable greater number of somatic mutations arising. Throughout their lifetime these mutations, which are thought to be primarily deleterious, are likely to accumulate in greater numbers in long-lived plants (Klekowski, 1989; Scofield and Schultz, 2006; Vallejo-Marin, 2012). Once mutations are establish in meristematic tissue, they can potentially become integrated into the gametic cells of plants. Upon fusion of gametes expressing similar mutations, inbreeding depression can result due to the increase in homozygosity of loci (Charlesworth, 1987). The two mechanisms by which inbreeding depression is thought to occur are: 1) overdominance, where heterozygotes display a fitness advantage over homozygotes in loci for the character under study, and 2) increased homozygosity for recessive or partially recessive deleterious mutations (Charlesworth, 1987). It is now generally accepted that it is primarily the homozygosity of deleterious mutations with recessive effects which is the main cause of inbreeding depression. (Charleswoth and Willis, 2009)

Levels of within-plant (self-) pollination and their consequences for fruit set can be studied by direct observation of pollinators, artificial self- and cross-pollination treatments, and manipulations of floral display size intended to alter the

level of geitonogamy. But it is more difficult to explore whether clonality leads to somatic mutation accumulation and yield reduction (i.e., above and beyond that which may be due to segregating genetic load in the population). Scofield and Schultz's Φ model (2006) attributes some of the variation in inbreeding depression to differential accumulation of deleterious mutations in clones. More recently, Schultz and Scofield (2009) introduced the so-called "autogamy depression test", a combination of controlled pollinations and fitness assays that allows one to determine whether there is a significant accumulation of somatic deleterious mutations in ramets. In essence, the test relies on the notion that each ramet within a clone develops mutations at independent loci and so it is expected that the likelihood of forming homozygotes for mutations (and observing consequent low reproductive output) will be highest when self-fertilization occurs within the same ramet (here defined as autogamous self-pollination) compared to instances in which self-pollination occurs pollinated between flowers on different ramets (here defined as geitonogamous self-pollination). This test can be applied to determine whether yield reduction due to somatic mutation is potentially a factor in clonal plant propagation.

In this study, we first assess overall levels of early-acting inbreeding depression, and then ask whether there is evidence that clonal growth (perenniality) increases deleterious mutation load due to somatic mutation accumulation, possibly leading to reproductive fitness decline in blueberry. As well, we examine this question for a number of wild perennial species for which appropriate data have been collected by other researchers, but have not been

analyzed before in this way. This was done by applying the autogamy depression test to pollination and yield data. We also examine how clonal spread itself influences the level of self-pollination and subsequent fruit abortion in blueberry. This was done by manipulating floral display size, and subsequently examining the effect of the manipulation on fruit set.

METHODS

Study site

The study area consisted of 24 commercial blueberry fields in Neguac, New Brunswick. Fields were chosen to take advantage of natural variation of blueberry clone sizes. All three commonly introduced pollinator species (*Apis mellifera, Bombus impatens Megachile rotundata*) were present within the field at typical stocking densities used by blueberry farmers.

Detection of inbreeding depression

In the summer of 2011 pollinations were carried out to determine the level of inbreeding depression in lowbush bluberry plants. Twenty-four blueberry fields were identified in the Neguac, New Brunswick region. Twelve putative clones of blueberry plants were delineated evenly along 60 meter transects in each field. Pollination treatments consisted of bagged self-pollinated inflorescences and inflorescences selected for supplemental pollination with outcross pollen.

Supplemental (outcrossed) pollinations were conducted by collecting pollen from clones located at least five meters away from the experimental transect. This pollen was then applied to stigmas of open flowers in the target clone. Self pollinations involved removal of the flower bag covering the labeled ramet, followed by sonication and collection of pollen from the target ramet. This pollen was then applied to stigmas of open flowers within the labeled ramet. Flower counts and supplemental self-pollination were conducted a minimum of three times in each of the 24 fields throughout the flowering period. Percent fruit set was calculated by counting the number of flowers pollinated, and then comparing that value to the number of berries on the selected branch once berries developed. Inbreeding depression was calculated using the common definition, 1- w_s / w_o (Agren and Shemske, 1993), where w_s represents the fitness of self fertilized progeny (= percent fruit set in self-pollinated inflorescences) and w_o the fitness of outcrossed progeny (= percent fruit set in outcross pollinated inflorescences).

Autogamy depression test

Initial clone identification

In 2012 a further 20 putative clones in a single field were identified. Initial clone determination was based on growth form, ramet color, constancy and timing of leaf development. Development of leaves was monitored with size adjusted in cases where leaf morphology differed from the suspected clone. Leaf tissue was then sampled from several edges in each putative clone to allow genotyping of ramets via microsatellites.

DNA extraction and microsatellite genotyping

From single leaves in each putative clone, genomic DNA was extracted using a QIAcube robot and spin column kits (DNAeasy Plant Mini prep) with the appropriate preloaded standard plant DNA extraction protocol (Qiagen, Valencia, California, USA). Microsatellite loci developed by Boches et al. (2005) from expressed sequence tag and genomic libraries were tested on plant material collected in 2010. Two well-resolved microsatellites loci (CA 344F and CA 794F) were chosen for clone identification. The primer sequences were 5'-CGGTTGTCCCACTTCATCTT-3' for CA 344F and 5'-TTACCAAAACGCCTCTCCAC-3' for CA794F. Each microsatellite was amplified from the four leaves collected from the different portions of the putative clones. PCR reactions were carried out in a final volume of 10 µL. The reaction mixture consisted of 2 µL of DNA solution, 5 U/µL of Taq DNA polymerase, 1 µL of 10X PCR buffer, 10 mM of dNTP mix, 2.5 mM $MgCl_2$, 2µM M13 Forward primer, 2µM M13 5'-IRDye labelled forward primer (@ 700nm). PCR-grade water was added to 10 µL. The cycling program consisted of 300 s of initial denaturation at 94 °C and 25 cycles of 95 °C for 40 s, 61 °C for 40 s and 72°C for 40s. This was followed by 15 cycles of 94 °C for 40s, 53°C for 40s, and 72 °C for 40 s, with a final single cycle of 71 °C for 600 s.

Microsatellite genotypes were determined using the LI-COR NEN Model 4300 DNA analyzer and the SAGA software package (LI-COR Biosciences, Lincoln, Nebraska, USA). Leaf samples showing non-identical base pair size in either

microsatellite locus, were treated as derived from unrelated clones, with the ramets of the unrelated portion of the clone removed from further analysis.

Autogamous and geitonogamous pollinations

The autogamy depression test (Schultz and Scofield, 2009) is designed as an experiment to determine the rate of deleterious somatic mutation. The test involves comparing the level of fruit set (and other fitness components) between two types of – pollinations: 1) autogamous (or within-flower); and 2) geitonogamous (within-plant, between-flower). Twenty of the largest clones ($8.7m^2$ – $34.2m^2$) were selected, and a total of 440 ramets were identified within these clones for pollinations (i.e., approximately 10 ramets per clone for autogamous pollinations and 10 for geitonogamous pollinations). These ramets were identified, labeled, and bagged prior to flowering (Figure 1).

Upon flowering, the ramets selected for autogamous pollination had their flowers sonicated, with the pollen being collected in a sterile tube and reapplied to flowers within that ramet. For geitonogamous pollinations, pollen was collected from donor ramets at the opposite edge of the clone in individual tubes and then applied to the ramet of study. Flower counts and pollinations were conducted every 3rd or 4th day until flower senescence. Bags were then removed to allow fruit maturation.

Clone manipulation tests to study the effect of self pollen access on fruit set

Clone identification

Within the field 120 clones were identified, delineated and measured. Clones were chosen based on having a clear separation from adjacent individuals, or through the possession of a distinct growth form different from neighboring clones. Clones were identified based on growth form, ramet color and leaf development. Multiple points along the clone were flagged, in order to allow for measuring of dimensions and clone area calculation. Once measured, the 120 clones were distributed evenly into three groups having representative individuals belonging to each size class. Five ramets were randomly selected and flagged for flower and berry counts. An additional ramet was flagged to determine outcross fruit set. Two different manipulations were conducted as described below.

Manipulation of access to self pollen (Treatment 1)

To determine whether reduced inflorescence number in a clone alters within pollen transfer, self pollination levels and subsequent fruit abortion, 20% of the clone area was netted with thin nylon mesh in a manner to restrict flowers within the clone from pollinators (Figure 2). Five ramets (non-covered) were randomly selected and flagged for flower and berry counts in open-pollination. An additional ramet was flagged to determine hand-outcrossed fruit set. Netting was removed from clones once flower senescence began .

Manipulation of genetic diversity (Treatment 2)

To determine whether increases in access to outcross pollen increases fruit set, 15% of the total area of each clone were replaced with material derived from transplanted unrelated clones in large plots (by placing pots with ramets from other clones within the confines of the target). Ramets from unrelated clones were selected based on their relative age and growth stage to ensure concurrent flowering (Figure 2). Five ramets were randomly selected and flagged for flower and berry counts. An additional ramet was flagged to determine outcross fruit set. Pots containing unrelated ramets were removed once flower senescence was observed.

Flower counts were conducted every 3rd day to 4th day. Fruit set was determined on flagged inflorescences of clones from all three groups of blueberry clones. Every ramet selected to be outcrossed had its flowers pollinated with non related pollen every 3rd to 4th day.

RESULTS

Inbreeding depression and its relationship with outcrossed fruit set

Upon self-pollination of 288 plants in 2010, the majority of individuals (Figure 4) showed low fruit set treatment (mean = 0.14 ± 0.17) compared to the outcrossing treatment (mean = 0.58 ± 0.31). A Wilcoxon signed rank test shows a significant difference between the means of self and outcross fruit set ($P < 0.0001$), suggesting the presence of inbreeding (Figure 6). Inbreeding depression ($1- w_s / w_o$, where w_s =self fruit set and w_o = supplemental fruit set) was calculated as 0.76.

Similarly calculation of lethal equivalents per zygote 2B= -12 ln (w_s /w_o) (Levin, 1984) were found to be significant, with some plants having levels as high as 46.8, while the entire population averaged 15.7 ± 11.5 lethal equivalents per zygote.

Autogamy depression test

Genetic fingerprinting of the 20 clones used in our study largely supported our initial identification of clones. Using the Ca 794F microsatellite loci, only a single leaf sample from one of the 20 clones was identified as being different from the targeted clone (Figure 3). The CA 344F microsatellite identified 2 clones with leaf samples shown not to be from a unique genetic individual. Inflorescences that border these regions of the clone were removed from further analysis.

Among the 20 clones analyzed in 2011, mean seed and fruit set were significantly lower in the autogamous pollination treatment than in ramets pollinated geitonogamously (Figure 7, Table 1). In 19 of 20 cases, geitonogamously pollinated flowers had higher fruit set, varying between 0.02-0.39 for autogamous fruit, and 0.12-0.83 for geitonogamous fruit. There also exists a great variation in fruit set between clones and between ramets within clones. These results show that autogamy depression exists in lowbush blueberry and potentially reduces the level of fruit set in naturally-pollinated plants.

Manipulations intended to modify self pollination

The clonal manipulation treatments did not lead to increased fruit set relative to the control group; i.e., neither the netting portions of clonal flowers to reduce the contribution of the self pollen (treatment 1), nor the interspersing of unrelated ramets within the target clone to increase the possibility of outcrossing (treatment 2). In fact, the control group (lacking any modifications to reduce self pollination) had the highest fruit set (Mean =0.63; SD = 0.28), compared with reducing self pollen through netting (Mean = 0.55; SD = 0.28) or adding unrelated pollen donors (Mean = 0.51; SD = 0.31) (Figure 8). The differences between groups are significant ($F=7.66$, $P < 0.02$), with a large proportion of ramets studied having highly variable fruit set (Figure 8).

Clone size as a predictor for fruit set

Clones size varied between 1.5 m^2 to 16.6 m^2 with arcsine fruit set levels being observed between 0.17 and 0.97. Contrary to our hypothesis, there was high variation in fruit set within clone size classes but no direct correlation between clone size and fruit set ($r^2=0.002$, $p=0.67$). Overall clone size (area) did not play a significant role in predicting fruit set in our selected individuals (Figure 9).

DISCUSSION

Inbreeding depression

The effect of inbreeding depression can manifest itself throughout the lifespan of an organism (Charlesworth 1987), and estimates of the overall inbreeding

depression in lowbush blueberry derived from fruit and seed set alone are likely to underestimate those based on fitness assays over the entire organism's life cycle. In wild species that exhibit high selfing, only mild inbreeding depression is typically observed, due to purging of deleterious mutation load (Charlesworth and Willis 2009). The low level of seedling establishment in blueberry fields (maintained primarily by vegetative propagation), may reduce the opportunity for purging of deleterious mutations in populations. Thus, inbreeding depression is expected to persist in these fields and remain as an important parameter in determining the yield. We observed a high level of self-sterility in low bush blueberry, with self fruit set near zero for more that 50% of the clones studied. Through comparison of outcross and self fruit set values obtained in this work, high levels of inbreeding depression and lethal equivalents were calculated. The degree of inbreeding depression (δ = 0.72) in our New Brunswick population is similar to inbreeding depression values previously identified in the related species, *Vaccinium myrtillus* (δ = 0.82) (Guillaume and Jacquenmart, 1999). The mean number of lethal equivalents found in the population (26.7) is slightly higher than previous studies conducted on lowbush blueberry (Bell et al., 2010). The number of lethal equivalents per zygote (2B) varied between 1.27 and 60.6 among the putative clones self-pollinated. This variation was present not only between fields but also with the individual plants within fields. These differences are likely important in fruit development, with self fertilization increasing the homozygosity in mutations that are deleterious, thereby causing fruit abortion. Many of the differences observed by both farmers and researchers in fruit set among and within field sites may be potentially explained by differences in genetic load.

The growth habit and longevity of lowbush blueberry may exacerbate these differences with somatic mutations accumulating differentially between clones and fields depending on local growth conditions and agricultural practices.

Autogamy depression: possible causes and consequences

The consequences of clonal growth for long term plant fitness have not been thoroughly examined in wild plant species, much less for those used in agriculture. A number of large long-lived clonal plants are known to researchers, many of these having existed for thousands (e.g., box huckleberry) to perhaps millions (e.g., quaking aspen) of years of years (Kemperman and Barnes, 1976; Wherry, 1976). The mechanisms by which plants propagate vegetatively are similar to branching, in that daughter cells from the parent meristematic tissue multiply and form a ramet, which often develops an independent root system (Cook 1983). This mechanism leads to the emergence of plant modules developing around the parent ramet, and that can go on to produce flowers and fruits. The underground nature of rhizomes, root buds, and other modes of asexual reproduction create difficulty in fully studying relationships between clone size and plant fitness. In our study, individuals of *Vaccinium angustifolium* were identified using distinctive phenotypic traits such as leaf color and growth form as well as with microsatellite markers. Any samples from a portion of a clone with microsatellite data disagreeing with that from the rest of the clone were removed from the autogamous-geitonogamous pollination analysis. The higher fruit set seen with geitonogamously-pollinated fruit suggests that autogamy depression is present in our lowbush blueberry population. This autogamy depression is

characterized by Scofield and Schultz (2009) as "a loss in viability in selfed progeny relative to outcrossed progeny" due to increases in the homozygosity of mutations relative to that in progeny produced from geitonogamy.

If deleterious mutations are responsible for autogamy depression, mutation accumulation within individuals and branches of clones must vary significantly. The few studies that have analyzed both types of self pollination have found similar results to ours with geitonogamy resulting in greater fruit set compared to autogamy across a number of plant species (Figure 10). The apparent prevalence of autogamy depression among a variety of taxa suggests that some assumptions about self pollination should be reconsidered. Autogamy is often thought of being less of harmful in fitness compared to geitonogamy, as it is a means to provide reproductive assurance and results in only low discounting of pollen (Eckert, 2000, Lloyd and Schoen, 1992). Geitonogamy on the other hand is thought to severely affect fitness and is a "negative consequence of cross fertilization" (Eckert 2000), as it requires the same elements of outcrossing but provides none of the benefit, causing seed discounting, pollen discounting while providing little reproductive assurance (Goodwillie at al. 2005). Our results suggest that further analysis should be conducted before qualifying the fitness effects of autogamy and geitonogamy.

Mutations occurring in distinct cell lineages within an individual are known to occur. For example distinct branches are sometimes seen as having different leaf colour, growth form and other characteristics such as pest resistance or chlorophyll deficiency (Gill et al. 1995; Klekowski, 1989). Gill (1995) proposed the genetic mosaicism hypothesis (GMH). It asserts that spontaneous mutations occur along

distinct branches of a perennial plant and are maintained due to meristematic growth and modularity. Fruit species in particular have hundreds of cultivars that have been selected from such somatic mutational events (Shamel, 1936). However, beneficial mutational events are generally thought to be rarer than those that are deleterious (Fisher, 1930). The somatic mutation rate must be significantly high enough in lowbush blueberry to cause autogamy depression and a differential mutational load amongst branches. Klekowski (1989) identified age as being an important factor in determining genetic load of a population, with longer reproductive lifespans (and accompanying higher somatic cell divisions) resulting in greater mutation frequency. In an extension of Klekowski's idea, Scofield and Schultz (2006) devised the Φ model (Φ representing mitotic divisions), assuming that deleterious mutations were a function of the number of mitotic divisions experienced by the plant during one generation. In their analysis of large and small angiosperms, Scofield and Schultz (2006) observed that larger statured plants displayed higher somatic mutation accumulation, increasing the frequency of recessive mutations, and leading to higher levels of inbreeding depression. Lowbush blueberry exhibits both longevity and increased mitotic divisions due to clonal growth with lowbush blueberry clones varying between 75 to 250 square feet, with clones as large as half a mile being observed (Yarborough, 1991). As lowbush blueberry clones grow and expand in the cleared forest sites, branches diverge from each other, and develop from rhizomes beneath the soil, creating cell lineages where subsequent cells (flower derived from these cells) will harbor their own unique population of mutations. Depending upon the age and length/size of the specific branch, gametes

that have developed from these meristematic cell lineages are likely to differ between ramets in number of deleterious mutations and the fitness effects of the mutations. Autogamous pollination results in lower fruit set due to higher proportion of homozygous mutations compared to pollinations due to geitonogamy.

Though autogamy appears to be present in our sampled clones, flowers pollinated geitongamously do not reach the levels of fruit set seen with outcrossing. A certain proportion of inbreeding depression in both the autogamously and geitonogamously pollinated fruit is likely due to the segregating mutations present in the population as a whole. There is also the possibility that the autogamy depression observed in our population is due solely to the segregating mutations alone. Under this hypothesis the differences seen between autogamously and geitonogamously pollinated fruit may be due to differences in the rate of propagation or retention of the mutant-bearing meristematic initials. The structure of the apical meristem (Figure 11) consists of two or three overlapping layers of cells that are capable of division in all planes and this may affect the maintenance and propagation of any initial mutant cells (Klekowski, 2003). The multiple layers of the apical meristem permits the maintenance of mutant cells in only certain positions within layers of the meristem, creating chimeras with mutant cells differing genetically and in cell fitness (Klekowski, 2003). This potential difference in cell fitness creates competition between the mutant and wild type cell, and leads to somatic selection where the healthiest cell becomes the ones that propagates/divides the fastest. Such somatic selection has been shown to reduce the mutational load of organisms, especially in the case of rare recessive mutations hidden from selection at the individual level through

natural selection (Otto and Hastings, 1998). Somatic selection within distinct plant cell lineages, formed either by clonal growth (ramets) or due simply to cell competition within the apical mersitem, may present an opportunity to reduce the deleterious effects of initial mutant cells or mutations arising during mitosis. A decline in ramet fitness is expected to occur when genetic drift overwhelms diplontic selection of deleterious somatic mutations (i.e., when selective removal of somatic cell lineages with higher mutational load is inefficient) (Klekowski, 2003). Using a stratified apical meristem model (Pineda-Krch and Lehtila, 2002) may further elucidate the role played by mutations and other genetic events in causing differential levels of fruit set, and potentially provide a better understing of elements that determine fruit set in clonal plants. Apical meristems of angiosperms are typically depicted as tunica corpus systems in which several layers of cells (tunica, layer 1 and 2) overlay the corpus (layer 3) from which all leaves, stems, and flowers originate (Figure 11). Estimates for somatic mutation rates in plants can vary widely between 10^{-4} to 10^{-7} per individual generation, while mitotic crossing-over occurs at rates of 10^{-5} to 10^{-4} per individual generation (Otto and Hastings, 1998). Large and long-lived plants benefit from having multiple units growing simultaneous. If somatic mutations confer fitness variation to specific cell lineages, then somatic selection may occur and contribute further to genetic mosaicism and within clone variability for fruit and seed set. Pineda-Krch and Lehtila (2002) assumed a three-dimensional structure for a stratified apical meristems consisting of seven cells in three layers. Through a stochastic model of meristem growth and cell mutation, somatic mutations were followed throughout thousands of cell generations, and probabilities of mutation

fixation were determined. The model shows how genetic heterogeneity (chimerism for mutations) can be maintained depending on the fitness of the mutant, and provides a mechanism for differential self fruit set in an individual. Identifying the underlying mechanism for the presence of autogamy depression in clonal plants should lead to a greater understanding of growth, reproduction and ageing among plants in the wild and among those used agriculturally.

Manipulation of self pollen environment to increase outcrossing

Agriculturalists are primarily concerned with pollinator visitation rates to ensure proper fruit set. Lowbush blueberry production requires that attention be paid to pollen flow within the field and clone. Excessive intraclonal pollination can potentially negate the benefits of higher visitation rates. With clonal crops, farmers may be seeing clone yields increase as clones gain greater coverage of open space in fields, without noticing that reproductive efficiency (berry/unit area) of their crop is declining (Ally et al., 2010). The clone manipulations conducted in this study were carried out in an attempt to encourage greater outcrossing between clones through reduction of access to ramets for self pollination or integrating outcross pollen within clones. These manipulations failed to increase yield and we must ask why, given the results of the inbreeding depression and autogamy depression studies. Indeed, the control clones, where no pollen environment modifications were performed exhibited slightly higher fruit set. One possibility is that we may have negatively affected cross-pollination or pollination as whole within the clone by introduction of the netting used and by disturbance.

Ongoing studies in these same fields have shown that pollinator introductions in blueberry fields do not result in significant overall increases in fruit yield. This may be related to the foraging behaviour of the introduced managed pollinators. In plants with high floral denisity we observed honeybees thoroughly visiting all the flowers on an inflorescence with little or no movement to or between adjacent plants. In our study, clone size was not a significant factor in determining overall fruit set. Individuals with high and low fruit set were present throughout the entire size range of clones. Similarly, the modification of clones to reduce self-pollen accessibility by pollinators was not successful. This may be due to the fact that even within a single ramet as many as 111 flowers were found, with flower counts over 50 not uncommon in our study site. This greatly exceeds the maximum flower counts of previous experiments (16 flowers), where it was found that 77.6% of all pollinator movement was within a single plant (Mitchell, 2004). Our manipulations of floral density may have been insufficient to affect self-pollination by pollinators due to the high number of flowers located within a small area (a single inflorescence). In the same vein, the introduction of unrelated flowering ramets may not have been sufficient to counter the extensive within-infloresence visits made by pollinators. Bumblebees have been observed visiting hundreds of flowers on a single shrub before moving, resulting in high levels of both autogamy and geitongamy (Hessing, 1988; Johnston, 1992). In a field devoid of other flowering plants, bees may be maximizing resource extraction per energy expenditure and thus making few long-distance (between clone or infloresence) flights. These results are reflected in observations from the previous season where many movements of pollinators were observed to occur within single

infloresences (unpublished data). Our manipulations of the pollen environment were conducted at a larger scale than this. The introduction of new clones or netting of the target clone would need to be performed at a smaller scale to determine whether scale was a factor in the failure to observe increases in fruit set.

Conclusion

Clonal agriculture

Approximately 80% of angiosperm species are capable of clonal growth or vegetative reproduction (Klime's at al. 1997). Many important agricultural species (potatoes, grapes, sugarcane) reproduce vegetatively. With these species the reproductive consequences of clonal growth is of less importance, as their yield and profitability to the farmer is not dependant upon pollination. Lowbush blueberry on the other hand, is similar to fruit orchards, where outcross pollination is an essential element for fruit production. High fruit set in apple orchards requires cross fertilization, and as such studies focused on pollen quality, pollen dispersal and orchard design have been undertaken to develop best practices for optimal fruit production (Kron and Husband, 2001). If we are to integrate the potential reduction in plant fitness due to mutations in our understanding of plant ageing, we need to plan our future agricultural developments accordingly. Projects such Natural Systems Agriculture (NSA) aim to mimic the native perennial prairies through perennializing major crops (wheat, sorghum and sunflower) and the domestication of several native perennial species (Jackson, 2002). The perennial nature of the crop will allow the

farm to forgo tilling thereby reducing soil erosion. The increase in topsoil stability allows for a more efficient use of nitrogen in the system, as nitrogen losses from annual crops are 30 to 50 times higher than in perennials (Cox, 2008). Early evidence also suggests that such a polyculture system will provide much of its own nitrogenous needs (Jackson, 2002). However, in the development of such a system, the accumulation of mutations has not yet been addressed. Perennial grasses are already purported to not be as productive due the amount of resources needed to maintain stubble and roots to ensure it survives indefinitely (Wilkins, 1991). Plant breeders also breed for uniformity in order to simplify harvest. In the aquatic species, *Decodon verticillatus*, a monomorphic ecotype of the species that reproduce asexually had higher levels of genetically based infertility compared to the trimorphic type (Dorken and Eckert, 2001). Accumulation of deleterious mutations may negate any selection for seed or fruit production if inbreeding depression is present. Somatic mutation accumulation as outlined by Klekowski (2003) may also create problems for perennial agriculture in a similar way that inbreeding depression affects reproduction. As crop plants grow and accumulate deleterious mutations, ramet fitness declines, which can result in competition between ramets throughout an individual clone. As less fit ramets are loss within the clone, genet size may decrease, increasing the possibility of genetic drift overwhelming within clone natural selection, resulting in the fitness of the entire organisms (genet) declining (Klekowski, 2003).

A greater understanding of the propagation and maintenance of mutants in plant cell lineages would be vital to ensuring long term production in perennial crops (Klekowski 2003). However, we must not only think of mutations as being negative.

At the beginning of the 20th century, 5000 of 8800 plant varieties known to European horticulture were developed from somatic mutations and then propagated by human (Whitham and Slobodchikiff, 1981). Careful selection and agricultural practices may indeed allow for the development of a perennial agriculture system, but overall success will likely be dependant on understanding the role of clonal growth and mutational events on long-term plant fitness.

Literature cited

Bell, D. J., Rowland, L. J., Stommel, J., and Drummond, F. A. (2010). Yield variation among clones of lowbush blueberry as a function of genetic similarity and self-compatibility. *Journal of the American Society for Horticultural Science, 135*, 259-270.

Charlesworth, D., and Charlesworth, B. (1987). Inbreeding depression and its evolutionary consequences. *Annual Review of Ecology and Systematics, 18*, 237-268.

Charlesworth, D., and Willis, J. H. (2009). The genetics of inbreeding depression. *Nature Reviews Geneicst, 10*, 783-796.

Charpentier, A. (2001). Consequences of clonal growth for plant mating. *Evolutionary Ecology, 15*, 521-530.

Cook, R. E. (1983). Clonal Plant Populations: A knowledge of clonal structure can affect the interpretation of data in a broad range of ecological and evolutionary studies. *American Scientist, 71*, 244-253.

Cox, T. S., Van Tassel, D. L., Cox, C. M., and DeHaan, L. R. (2010). Progress in breeding perennial grains. *Crop and Pasture Science, 61*, 513-521.

Cox, T. S., Glover, J. D., Van Tassel, D. L., Cox, C. M., and DeHaan, L. R. (2006). Prospects for developing Perennial Grain Crops. *BioScience, 56*, 649-659.

De Jong, T. J., Waser, N. M., Price, M. V., and Ring, R. M. (1992). Plant size, geitonogamy and seed set in *Ipomopsis aggregata*. *Oecologia, 89*, 310-315.

Dorken, M. E., and Eckert, C. G. (2001). Severely reduced sexual reproduction in northern populations of a clonal plant, *Decodon verticillatus* (*Lythraceae*). *Journal of Ecology, 89*, 339-350.

Eckert, C. G. (2000). Contributions of autogamy and geitonogamy to self-fertilization in a mass-flowering, clonal plant. *Ecology, 81*, 532-542.

Ellstrand, N. C., and Elam, D. R. (1993). Population genetic consequences of small population size: implications for plant conservation. *Annual Review of Ecology and Systematics, 24*, 217-242.

Feinsinger, P. (1987). Effects of plant species on each other's pollination: Is community structure influenced? *Trends in Ecology and Evolution, 2*, 123-126.

Fisher, R. A. (1930). The genetical theory of natural selection. Oxford, England: Clarendon Press.

Galetto L, Bernardello G, Isele IC, Vesprini J, Berduc A. (2000) Reproductive biology of *Erythrina crista-galli* (Fabaceae). *Annals Missouri Botany*, *87,* 127-145.

Gill, D. E., Chao, L., Perkins, S. L., and Jason, B. W. (1995). Genetic mosaicism in plants and clonal animals. *Annual Review of Ecology and Systematics, 26*, 423-444.

Glover, J. D. (2005). The necessity and possibility of perennial grain production systems. *Renewable Agriculture and Food Systems, 20*, 1-4.

Glover, J. D., Culman, S. W., DuPont, S. T., Broussard, W., Young, L., Mangan, M. E., and Wyse, D. L. (2010). Harvested perennial grasslands provide ecological benchmarks for agricultural sustainability. *Agriculture, Ecosystems and Environment, 137*, 3-12.

Gomez, J. M., and Zamora, R.. (1996). Wind pollination inhigh-mountain populations of *Hormathophylla spinosa* (*Cruciferae*). *American Journal of Botany*, *83*, 580–585.

Goodwillie, C., Kalisz, S., and Eckert, C. G. (2005). The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annual Review of Ecology, Evolution, and Systematics, 36*, 47-79.

Guillaume, P., and Jacquemart, A. L. (1999). Early-inbreeding depression *in Vaccinium myrtillus* and *V. vitis-idaea*. *Protoplasma, 208*, 107-114.

Guitian, P., J. Guitian, and Navarro, L.. (1993). Pollen transfer and diurnal versus nocturnal pollination in *Lonicera etrusca*. *Acta Oecologica*, 14, 219-227.

Hardin, J. W., Doerksen, G., Herndon, D., Hobson, M., and Thomas, F. (1972). Pollination Ecology and Floral Biology of Four Weedy Genera in Southern Oklahoma. *The Southwestern Naturalist, 16*, 403-412.

Hepler, P. R., and Yarborough, D. E. (1991). Natural variability in yield of lowbush blueberries. *HortScience, 26*, 245-246.

Jackson, W. (2002). Natural systems agriculture: a truly radical alternative. *Agriculture, Ecosystems and Environment, 88*, 111-117.

Jon, A., and Schemske, D. W. (1993). Outcrossing rate and inbreeding depression in two annual monoecious herbs, *Begonia hirsuta* and *B. semiovata*. *Evolution, 47*, 125-135.

Karron, J. D., Holmquist, K. G., Flanagan, R. J., and Mitchell, R. J. (2009). Pollinator visitation patterns strongly influence among-flower variation in selfing rate. *Annals of Botany, 103*, 1379-1383.

Klekowski, E. J., and Godfrey, P. J. (1989). Ageing and mutation in plants. *Nature, 340*, 389-391.

Klekowski, E. J. (2003). Plant clonality, mutation, diplontic selection and mutational meltdown. *Biological Journal of the Linnean Society, 79*, 61-67.

Krebs, S. L., and Hancock, J. F. (1990). Early-acting inbreeding depression and reproductive success in the highbush blueberry, *Vaccinium corymbosum* L. *TAG Theoretical and Applied Genetics, 79*, 825-832.

Kron, P., Husband, B. C., Kevan, P. G., and Belaoussoff, S. (2001). Factors Affecting Pollen Dispersal in High-density Apple Orchards. *HortScience, 36*, 1039-1046.

Lande, R., and Schemske, D. W. (1985). The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. *Evolution, 39*, 24-40.

Levin, D. A. (1984). Inbreeding depression and proximity-dependent crossing success in *Phlox drummondii*. *Evolution, 38*, 116-127.

Lloyd, D. G., and Schoen, D. J. (1992). Self- and cross-fertilization in plants. I. Functional dimensions. *International Journal of Plant Sciences, 153*, 358-369.

Mitchell, R. J., Karron, J. D., Holmquist, K. G., and Bell, J. M. (2004). The influence of *Mimulus ringens* floral display size on pollinator visitation patterns. *Functional Ecology, 18*, 116-124.

Navarro, L. (1999). Reproductive biology of *Anthyllis vulneraria subsp. vulgaris* (*Fabaceae*) in northwestern Iberian Peninsula. *Nordic Journal of Botany*, *19*, 281-287.

Navarro, L., and Guitián, J. (2002). The role of floral biology and breeding system on the reproductive success of the narrow endemic *Petrocoptis viscosa rothm*. (*Caryophyllaceae*). *Biological Conservation, 103*, 125-132.

NB Blueberries. (2009). New Brunswick Wild Blueberry Industry – Strategic plan. Bleut NB Blublerries, Fredericton, NB. http://www.nbwildblue.ca/en/growerinfo.shtml

Neher, D. A., and Lee Campbell, C. (1994). Nematode communities and microbial biomass in soils with annual and perennial crops. *Applied Soil Ecology, 1*, 17-28.

Otto, S., and Hastings, I. (1998). Mutation and selection within the individual. *Genetica, 102-103*, 507-524.

Pan, J. J., and Price, J. S. (2001). Fitness and evolution in clonal plants: the impact of clonal growth. *Evolutionary Ecology, 15*, 583-600.

Pellegrino, G., Gargano, D., Noce, M. E., and Musacchio, A. (2005). Reproductive biology and pollinator limitation in a deceptive orchid, *Serapias vomeracea* (*Orchidaceae*). *Plant Species Biology, 20*, 33-39.

Pineda-Krch, M., and Lehtila, K. (2002). Cell lineage dynamics in stratified shoot apical meristems. *Journal of Theoretical Biology, 219*, 495-505.

Rao, S. P., Atluri, J. B., and Reddi, C. S. (2001). Intermittent mass blooming, midnight anthesis and rockbee pollination in *Pterocarpus santalinus* (*Fabaceae*). *Nordic Journal of Botany, 21*, 271-276.

Reusch, T. B. H. (2001). Fitness-consequences of geitonogamous selfing in a clonal marine angiosperm (*Zostera marina*). *Journal of Evolutionary Biology, 14*, 129-138.

Rivera, G. L., Galetto, L., and Bernardello, L. (1996). Nectar secretion pattern, removal effects, and breeding system of *Ligaria cuneifolia* (*Loranthaceae*). *Canadian Journal of Botany, 74*, 1996-2001.

Santrandreu, M, and Lloret. F. (1999). Effect of flowering phenology and habitat on pollen limitation in *Erica multiflora*. *Canadian Journal of Botany, 77*, 734- 43

Saunders, N. E., and Sipes, S. D. (2006). Reproductive biology and pollination ecology of the rare Yellowstone Park endemic *Abronia ammophila* (*Nyctaginaceae*). *Plant Species Biology, 21*, 75-84.

Schemske, D. W. (1980). Evolution of floral display in the orchid brassavola nodosa. *Evolution, 34*, 489-493.

Schultz, S.T., and Scofield, D.G. (2009). Mutation accumulation in real branches: Fitness assays for genomic deleterious mutation rate and effect in large-statured plants. *The American Naturalist, 174*, 163-175.

Scofield, D. G., and Schultz, S. T. (2006). Mitosis, stature and evolution of plant mating systems: low-Φ and high-Φ plants. *Proceedings of the Royal Society B: Biological Sciences, 273*, 275-282.

Shamel, A. D., and Pomeroy, C. S. (1936). Bud Mutations in Horticultural crops. *Journal of Heredity, 27*, 487-494.

Strik, B. C., & Yarborough, D. (2005). Blueberry production trends in North America, 1992 to 2003, and predictions for growth. *HortTechnology, 15*, 391-398.

Tandon, R., Shivanna, K. R., and Ram, H. Y. M. (2001). Pollination biology and breeding system of *Acacia senegal*. *Botanical Journal of the Linnean Society, 135*, 251-262.

Tarasjev, A. (2005). Impact of genet size and flowering stage on fruit set in *Iris pumila* L. clones in wild. *Acta Oecologica, 27*, 93-98.

Traveset, A. (1995). Reproductive ecology of *Cneorum tricoccon* L. (*Cneoraceae*) in the Balearic Islands. *Botanical Journal of the Linnean Society, 117*, 221-232.

Vallejo-Marín, M., Dorken, M. E., and Barrett, S. C. H. (2010). The ecological and evolutionary consequences of clonality for plant mating. *Annual Review of Ecology, Evolution, and Systematics, 41*, 193-213.

Vaughton, G. (1988). Pollination and Seed Set of *Banksia spinulosa*: Evidence for Autogamy. *Australian Journal of Botany, 36*, 633-642.

Whitham, T. G., and Slobodchikoff, C. N. (1981). Evolution by individuals, plant-herbivore interactions, and mosaics of genetic variability: The adaptive significance of somatic mutations in plants. *Oecologia, 49*, 287-292.

Wilkins, P. W. (1991). Breeding perennial ryegrass for agriculture. *Euphytica, 52*, 201-214.

Tables and Figures

Table1. Mixed model ANOVA for fruit set with treatment (self-pollinated vs. outcross) being a fixed effect clone identity being a random effect.

Source	**S.S.**	**MS Num**	**DF**	**F ratio**	**Prob>F**
Treatment	2.91	2.91	1	12.82	0.002
Clone	27.76	1.46	19	6.34	0.0001
Clone*treatment	4.38	0.23	19	1.55	NS

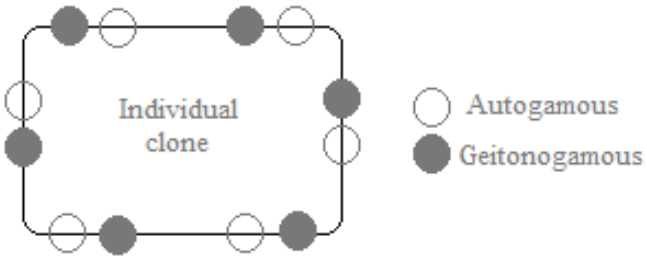

Figure 1. Experimental layout of autogamy depression test.

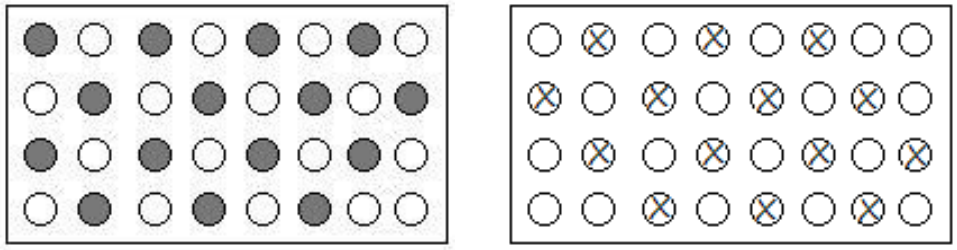

Figure 2. Diagram of experimental manipulations made for the attempted reduction self pollination. Shaded circles represent unrelated potted ramets introduced into to a target clone, while circles containing Xs represent ramets of a target clone that were netted.

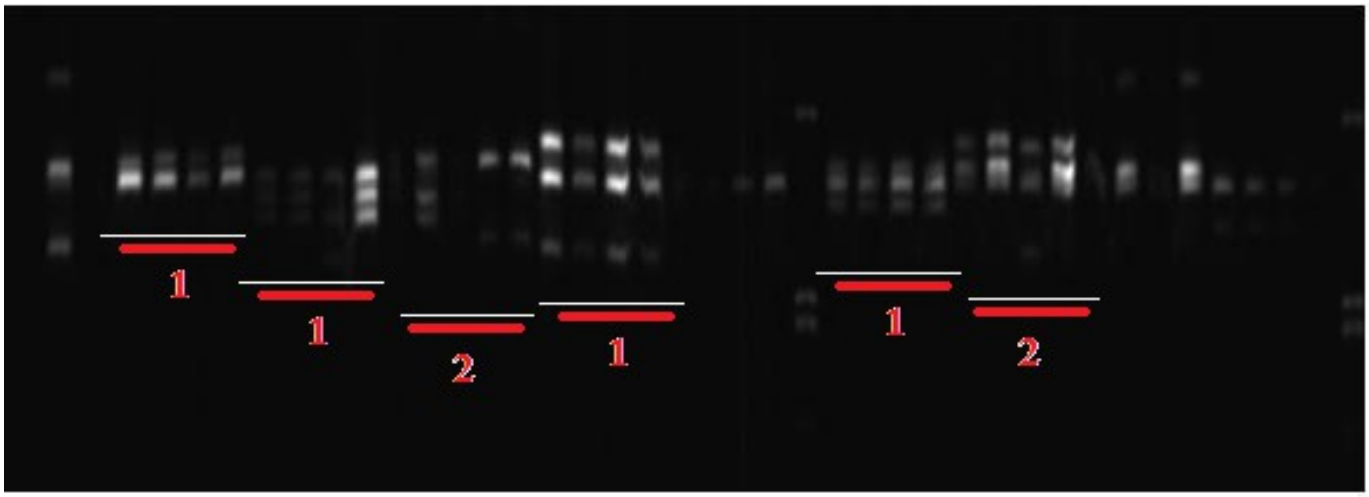

Figure 3. Image of a section of a 64-well LI-COR gel depicting a single microsatellite (CA344F) amplified from nine putative clones. Each clone is depicted with four unique leaf samples from the edges of the clone. 1- represents clones where all four of the leaf samples agree with our identification of a unique clone, 2- represents clones where one of the leaf samples from a clone were shown to be from a different genetic individual.

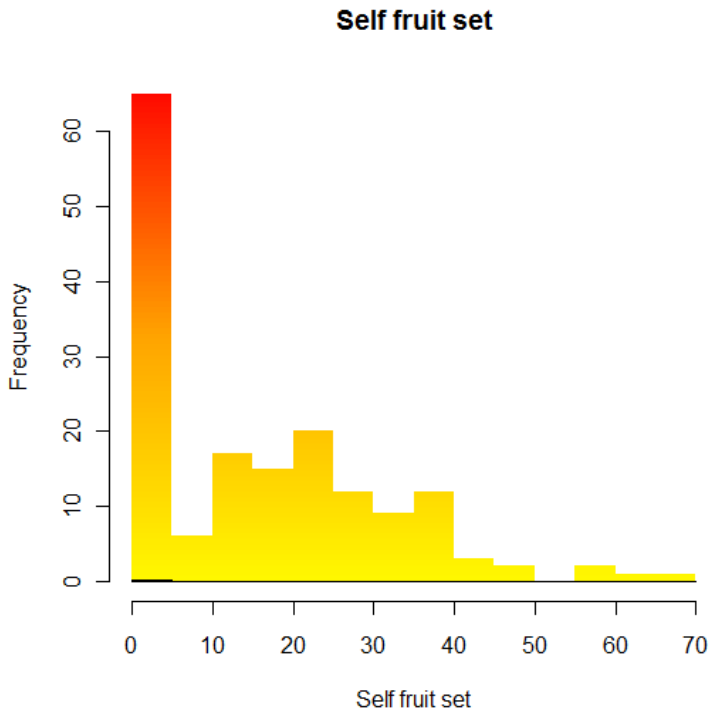

Figure 4. Frequency of self fruit set in 288 plants among the 24 fields in the study area.

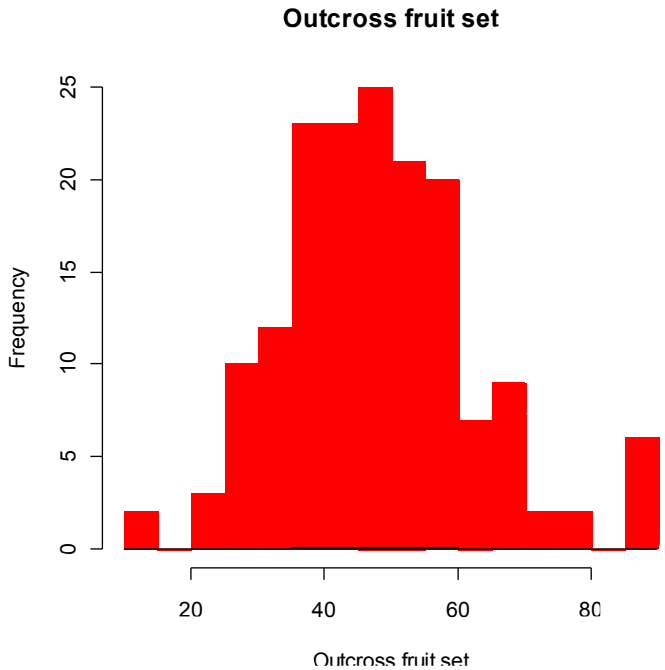

Figure 5. Frequency of outcross fruit set in 288 plants among the 24 fields in the study area.

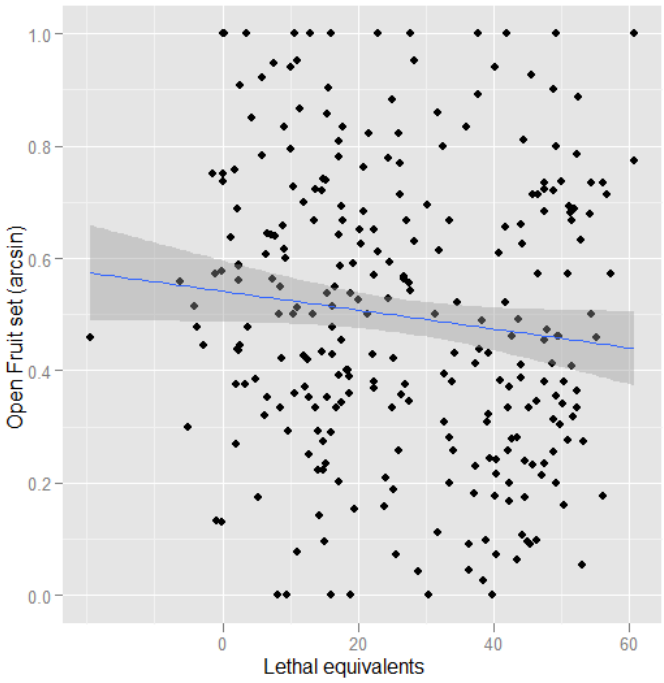

Figure 6. Open fruit set as a function of lethal equivalents [-12ln(*ws/wo*)], where *ws*=self fruit set *wo*=supplemental fruit set ($r^2 = 0.009$, P = 0.056).

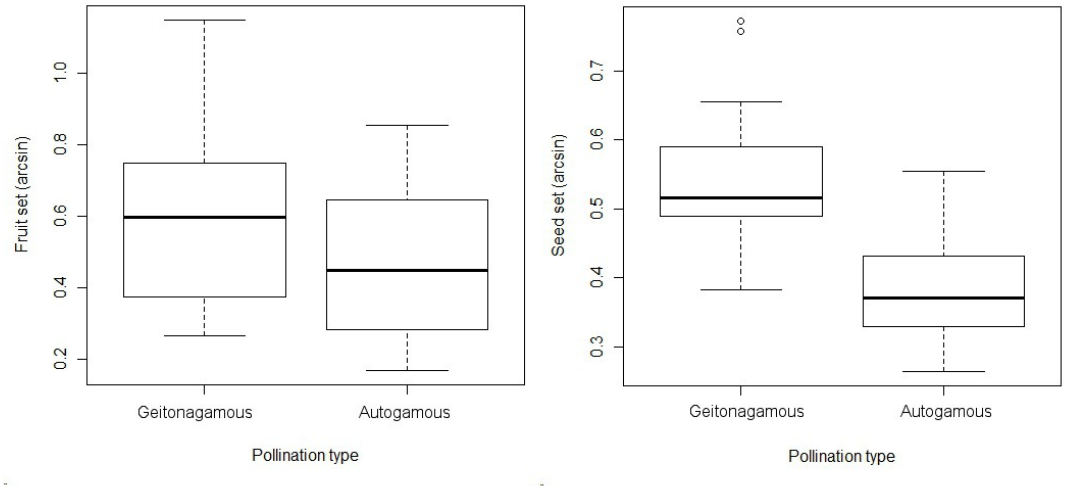

Figure 7. Fruit set (arcsine transformed) and seed set (arcsine transformed) from geitonogamous (mean= 0.6, n=40) and autogamous (mean=0.46, n=40) pollinations

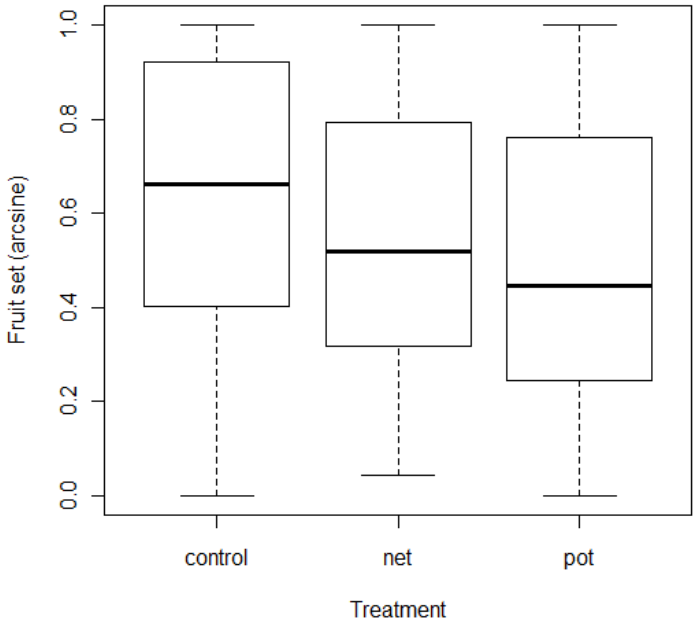

Figure 8. Mean fruit set (arcsine) in three treatments flowers.

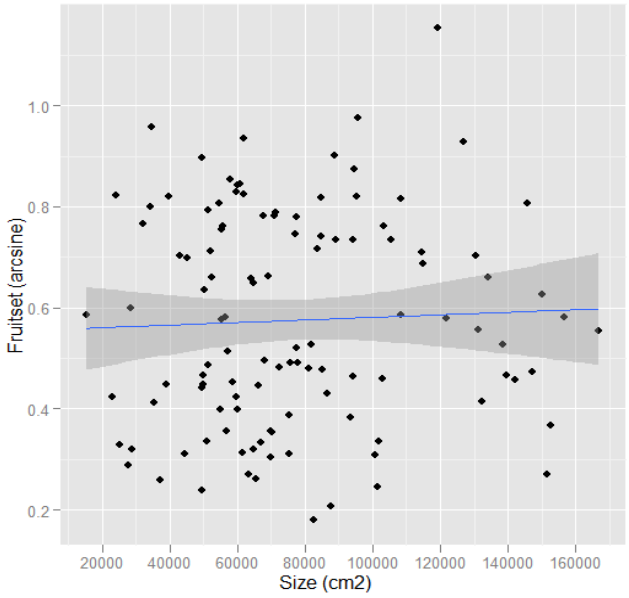

Figure 9. Fruit set as a function of clone size (cm^2), r^2= 0.0017 in 120 clones.

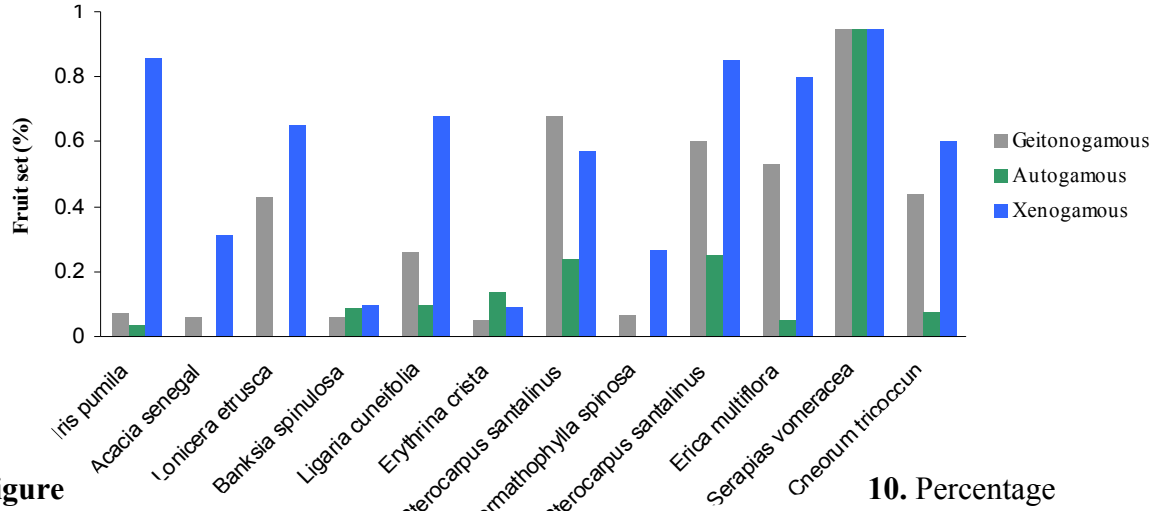

Figure 10. Percentage fruit set in autogamous, geitonogamous xenogamous (cross-pollinated) flowers in 12 perennial plant species (Galetto et al., 2000; Gomez and Zamora, 1996; Guitian et al., 1993; Hardin et al., 1972; Navarro, 1999; Navarro and Guitian, 2002; Pellegrino et al., Rao et al., 2001; Rivera et al., 1996; Santadreu and Lloret, 1999; Saunders and Sipes, 2006; Tandon et al., 2001; Tarasjev, 1995; Traveset, 1995).

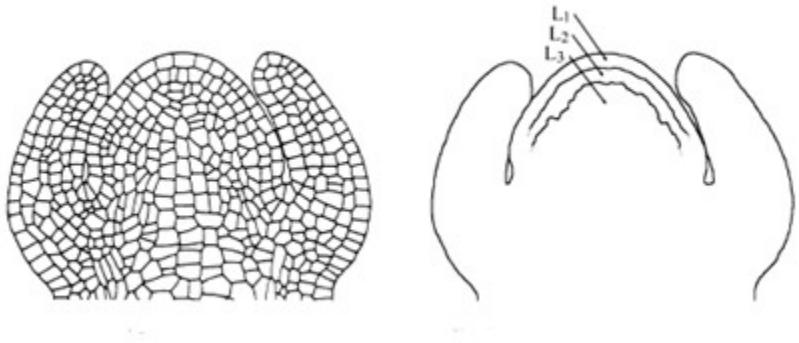

Figure 11. Typical depiction of a three layer stratified apical meristem. L1 and L2 represent tunica layers, and L3 the corpus. Adapted from Edward J. Klekowski (Klekowski, 2003)

Chapter 2. Fruit set response of lowbush blueberry (*Vaccinium* angustifolium) to introduced pollinators

Lowbush blueberry naturally grows as a clonal understory shrub and early successional species in both natural and manmade forest clearings. In eastern North America, lowbush blueberry cultivation has been ongoing for centuries, with the indigenous people and first settlers of North America maintaining patches of cleared area for harvest via forest burning (Eaton, 2004). However, it is only within the past 50 years that blueberry farming has began to adopt the principles and practices common in other agricultural crops. Modern commercial blueberry production requires conditions that are vastly different than those present in natural stands. Fields are established through the identification of areas with a high blueberry density, and these are then cleared of trees and shrubs, in effect mimicking the early successional forest stage. The wild blueberry clones are then encouraged to colonize the cleared areas via clonal growth, essentially creating a monoculture of lowbush blueberry. Improvements in cultural practices have led to the development of integrated pest management, irrigation, weed control and fertilization methods suited to the blueberry agroecosystem of Eastern North America. Through the introduction of higher intensity management in lowbush blueberry, significant yield increases have been seen, however, variability within and between fields in yield remains a common concern for blueberry farmers. For example, in the early 20th century blueberry yields averaged slightly less than 200kg per acre. By 1976 this figure doubled due to technological advances and increases in the intensity of management practices within

fields (Metzer and Ismail, 1976). More recently, Hepler and Yarborough (1991) analyzed one hundred blueberry clones from a population in Maine, and observed variation in yield ranging between 300 kg/ha to 17000 kg/ha.

In fruit crops that rely on insect pollinators, analysis of fruit set must incorporate the pollination ecology of a species. The presence of pollination deficits has been known for thousands of years in many different crop plants, with early Greek historians (300-500 b.c.) documenting lack of fruit and seed among dates and figs, along with techniques for hand pollinations to boost production (Galil, 1968; Kevan, 2001). Certain crops, such as apples, are documented as once having received sufficient pollination from native species prior to widespread introduction of pesticides to orchards (Batra, 1995). Similarly, Kremen (2002) found that in another species requiring high levels of pollination, watermelon (*Citrullus lanatu*), maintenance of the ecosystem and natural diversity of pollinators allowed production on par with fields where honeybees are introduced. Lowbush blueberry has the capacity to set fruit on all of flowers produced, however, in agricultural fields fruit set rarely surpasses 40% with native pollinators (Wood, 1969, Hepler and Yarborough, 1991).

To effectively exploit floral resources, pollinators have been required to adapt to the floral biology and phenology of plants within their ecosystem. Lowbush blueberry has co-evolved alongside a variety of native bee genera (*Andrena, Bombus, Halictus, Agapostomon, Augochlora, Augochlorella* and *Lasioglossum*) that are adapted to both inclement weather conditions sometimes present during the blueberry flowering season, as well as to the particular characteristics of the flower

(e.g., anthers that release pollen upon flower sonication) (Javorek, 2002; Sampson, 1993). The increases in blueberry densities seen in commercial fields relative to native stands following forest clearing, as well as the overall agricultural intensification of blueberry farming vastly increases the total number of flowers present, while at the same time reducing nesting and floral resources for native pollinators (Kremen, 2002; Drummond 2002).

Crop pollination by native bees has been shown to be a function of natural habitat availability in the ecosystem (Kremen 2004). The changes in lowbush blueberry agroecosystems provide a prime example of anthropogenic impacts upon the landscape that may affect pollination services. The effects of changes in land use on pollinators can vary in severity, depending on the sensitivity of the species of the species and diet specialization (Winfree, 2011). Wild bumblebees in Europe have been declining steadily with at least three species having gone extinct, with similar extirpations of species in North America occurring due to the ecological consequences of high intensity agriculture intensification (fragmentation, habitat loss, floral resource loss, pesticide use, and non native bee introductions) (Goulson et al. 2008). With the increasing trend in intensification of blueberry production and loss of native pollinators, farmers have become more reliant upon introduced managed pollinators. Honeybee use alone has increased approximately 120-fold in Maine during the past 40 years (Drummond, 2002). In certain years, the simple introduction of honeybees has resulted in the doubling of proportional fruit set in lowbush blueberry after the introduction of honeybees (Wood, 1960). These potential increases have led to the honeybee being the most widely used introduced pollinator

in agriculture (Potts et al. 2010). Our dependence on honeybees for the pollination of many crop species has now become a threat to the long term sustainability of agriculture. Between 1947 and 2005 59% of honeybee colonies were loss due to a confluence of factors including parasites (e.g., varroa mite), diseases, pesticides and other environmental factors (National Research Council, 2006; vanEngelsdorp et al. 2008). This reliance on a declining honeybee population for the majority of our pollination needs creates additional risk when we look at agricultural expansion. The proportion of agricultural crops requiring pollination is increasing at a much greater rate than the global stock of honeybees (Aizen and Harder, 2009). Due to the decreasing presence of native pollinators and low availability of honeybees (along with their associated high prices), lowbush blueberry farmers of Eastern North America are now employing the alfalfa leafcutter bee (*Megachile rotundata*) and bumblebees (*Bombus spp.*) in their fields to supplement honeybees as well as the dwindling population sizes of native pollinators.

While honeybees have been part of the agricultural landscape for thousands of years (Potts et al. 2010), the trend towards increasing use of alternative pollinators has only been under consideration for the past 50-100 years. During the early 1900's a number of researchers observed that pollination success differed between bee species, depending on the crop variety (Batra, 1995). This led to significant work on the domestication of formerly wild species that were shown to be effective and efficient pollinators, leading to the first attempts of domestic rearing. In the 1960's alfalfa leafcutter bees began to be produced commercially, but were only introduced to blueberry fields in the 1990's with over three hundred acres pollinated by leafcutters in

1995 (Argall, 1996). During this time, bumblebee mass production developed significantly (Roseler, 1985), providing blueberry farmers with access to three different managed pollinators. By using three different introduced pollinator species, New Brunswick blueberry farmers are attempting to recreate the diverse native pollinator community (or the level of pollination service) formerly present in blueberry heaths. The three species of introduced pollinators display foraging strategies and behaviours that are significantly different from each other, resulting in differential pollination efficiency and effectiveness (Javorek, 2002). This is particularly important in lowbush blueberry due to its life history and pollination adaptations. The lowbush blueberry flower is herkogamic with a physical separation between the male and female components of the flower, which upon dissections has been demonstrated to be effective in reducing self pollen deposition on the stigma (Bell, 2009). The pendulous flower and poricidal anthers contained within are adaptations selecting for buzz pollination by bees (Bell, 2009, Harder and Barclay, 1994). Upon self fertilization lowbush blueberry also exhibits a certain degree of self incompatibility, but varying levels of lower fruit set have been demonstrated (Bell et al., 2010; Aalders and Hall, 1961; Chapter 1 of this thesis), suggesting the presence of inbreeding depression. The higher fruit set observed with outcrossed pollen may be especially significant as the large size of blueberry clones, generally between 6 and 25 square meters (Yarborough,1991), create large patches of flowers derived from a single clone. Depending upon the adaptations and behaviour of the introduced pollinators, fruit set levels may vary with different species of pollinators, as some species may more effectively transfer outcross pollen between plants. In fields with multiple species of

introduced pollinators there may be antagonism or synergism in the level of outcrossing affecting fruit set. The implications for pollinator management and resulting fruit set are significant if the pollination efficiencies of species are altered in communities consisting of multiple introduced species.

Introduction of new pollinating species in any environment requires knowledge of their behaviour and interactions with the plants and the already present pollinators in the constructed agroecosytem. The differences between pollinators in their rate of pollination, foraging distances, sensitivity to environmental conditions will all play a role in determining the effectiveness and cost efficiency of managed pollination. Bumblebee queens are known to pollinate blueberry flowers at a rate of six to one compared to honeybees, while pollen harvesting leafcutter bees pollinate approximately three flowers for every single honeybee pollination (Javorek, 2002). Research on the use and effects of individual alternative pollinators in commercial blueberry fields has been ongoing since the early 1990's, with many commercial fields in New Brunswick now employing combinations of pollinator species in their fields. Recommended stocking densities for blueberry pollinators exist, but do not take into account the effects of interactions that may arise through the creation of introduced pollinator communities. Introductions of honeybees has been shown to affect the behaviour other bees in a community, with native short tongue bees avoiding areas frequented by honeybees while bumblebees altered the times of day which they foraged (Walther-Hellwig et al., 2006). Similarly, foraging rates of bumblebees are reduced in the presence of honey bees (Thomson, 2004). There may also be competition for floral resources, with honeybees depleting nectar and pollen,

reducing attractiveness of patches in a field (Goulson, 2003; Roubik, 1991). Moreover, the activity of honeybees may also be a further deterrent to other pollinators, with smaller bees being displaced from flowers by honeybees (Gross and Mackay, 1998). The behaviour (between plant movements, thoroughness of pollination) of pollinators is an important factor in determining the most efficient pollinators and potential combinations of pollinator species. Any changes that arise in mixed species communities due to interactions are likely to affect patterns of pollen movement within the field. To accurately assess pollinator efficacy in mixed pollinator species agroecosystems, information regarding changes in pollinator activity (number of flowers visited, pollination time, between plant movements, etc.) must be quantified and analyzed along with the resulting fertility response of our crop.

Differences in the microenvironment of lowbush blueberry clones may be associated with successful fruit set. Differences in temperature between regions may influence processes within the plant prior or during fruit set. Temperature is known to be an important factor in the growth rate and maturation of lowbush blueberry fruit (Hall and Aalders, 1968). Though photoperiod is known to be the primary initiator of bud development, temperature can potentially influence the onset, duration or level of flowering in blueberry species (Spann et al. 2004; Hall and Ludwig, 1961). Soil moisture is also an important factor affecting lowbush blueberry flowering (Benoit et al. 1984). In the relatively infertile, acidic, well drained soil common to lowbush blueberry (Hall et al., 1967), high temperature may lead to increase water stress in plants, potentially affecting flowering and fruit set. Pollinators also respond to

temperature, with different pollinator species having different climactic ranges within which they will forage (Corbet, et al., 1993; Stubbs, et al., 1994). Determining whether temperature is a characteristic unique to certain fields should allow us to better understand the flowering of blueberry as well as potential microclimatic influences on pollinator activity.

In the present chapter I present the results of work aimed at measuring fruit set responses of lowbush blueberry clones in eight different introduced pollinator combinations. Within these introduced pollinator communities I also present results on behaviour metrics for the whole introduced pollinator community, as well as changes in the behaviour of pollinator species in these combinations. The work was conducted to increase our understanding of the fruit set consequences of manipulating the diversity of pollinator community.

Methods

Pollinator environment manipulation

The study area consisted of blueberry fields in and around the region of Neguac, New-Brunswick (lat. 47”15° N, long. 65”04° W), encompassing an expanse of approximately 54 square kilometers. To evaluate fruit set changes due to differences in pollinator environments 24 blueberry fields were identified around the region. The 24 fields were divided into 8 pollinator treatment groups (each replicated in three fields) consisting of control (no introduced pollinators) fields, fields with a single introduced pollinator species (honeybee, bumblebee, or leafcutter bee), fields with

a combination of two introduced pollinators (honeybee and bumblebee, honeybee and leafcutter bee, or bumblebee and leafcutter bee) or fields with all three introduced pollinators. Pollinators were introduced to the fields a few days before bud opening (see Table 1 for numbers of pollinator units introduced per field).

Pollination treatments

A transect of approximately 60 meters was placed in each field, with the line originating near a field edge and progressing towards the middle. Twelve blueberry clones were delineated evenly along transects. For each clone three pollination treatments were compared. These consisted of: (1) an open-pollinated inflorescence (i.e., pollinated by introduced and native pollinators present in the field); (2) a bagged self-pollinated inflorescence; and (3) an inflorescence selected for supplemental pollination with pollen from a separate clone located at least five meters away from the experimental transect. Flower counts and supplemental pollination were conducted at a minimum of five times at all 24 sites throughout the flowering period. Once fruit set began, berries from each of the plants in the experimental transects were collected. Fruit set (proportion of flowers setting fruit) was derived from flower counts.

Pollinator behaviour

Throughout the flowering period pollinator foraging behaviour was observed by conducting "bee chases", in which pollinators were followed as they moved through the patch. To ensure that observations accounted for pollinator behaviour

within the putative clones studied, areas of pollinator observation were chosen based on the location of the transects. Single bees were followed as long as possible (until lost or ceasing activity), with data collection consisting of: (1) approximate number of clones visited; (2) number of flowers visited; (3) time spent foraging on flowers; and (4) total flight distance between inflorescences during the bee chase. To determine whether there existed differences between fields for these behaviours, ANOVAs were performed for each pollination behaviour and the eleven fields studied. ANOVAs were also performed between types of pollinators to investigate behaviour differences between species. Finally, ANOVAs were used to test for differences in pollination characteristics between fields for each of the pollinator species; i.e., to determine if pollinator species behave differently depending on the composition of the introduced pollinator community.

Site monitoring: Microclimate variability and pan trapping of insects

Nine of the fields distributed across the study area were equipped with HOBO pendant temperature data loggers set at 30 minute recording intervals. These fields were selected as they were deemed to representative of the various microclimate regions of Neguac, N.B. Pan traps were placed in each field to determine number and type of pollinators. Insects were collected every 24 hours, and preserved in order to be identified in the laboratory.

Results

Pollinator environment and fruit set analyzed without covariates

Mean proportional fruit set of control plants ranged from 0.431 for honeybee fields to 0.565 for the bumblebee and leafcutter field combinations (Figure 1). Pollinator treatment was non-significant—it did not systematically influence fruit set across the 24 experimental fields (ANOVA: $F = 1.06$, d.f. = 287, $P = 0.3895$).

Fruit set differences between pollination treatments analyzed with a covariate:

To account for potential differences in microenvironmental factors that different clones may be exposed to (e.g., variation in soil fertility, pathogens, water availability) supplemental fruit set was employed as a covariate in the analysis of the effect of pollinator species (or pollinator species mixture) on fruit set. A linear regression of control fruit set on supplemental fruit set (Figure 2) demonstrated that a positive relationship exists between these two separate fruit set measures within single clones, suggesting that indeed, clone-to-clone differences in microhabitat may account for some of the variation in fruit set. The level of supplemental pollination was shown to significantly influence fruit set ($P=3.6x10^{-8}$) in the different pollinator environments. When fruit set in specific pollinator combinations was analyzed using supplemental pollination as a covariate, we discovered that pollinator introduction treatments that consisted of honeybees introduced alone led to significantly lower fruit than other treatments. In contrast, fields with pollinator treatments that contained a combination of both leafcutters and bumblebees had higher fruit set

(Figure 3). Tukey's tests showed that these two treatments differ significantly (P=0.049).

Bee behaviour

There was no difference between fields in the average number of clones visited by pollinators (P=0.343). Between species a difference in number of clones existed (P=0.003), *Bombu*s on average visited more clones (1.8 ± 0.12) than honeybees 1.5 ± 0.20) and leafcutters (1.04 ± 0.22). In fields that contained bumblebees, significant variation ($P < 0.0001$) in mean number of clones visited by bumblebees existed depending on the pollinator combination in the field. In a field where only leafcutters were introduced, visiting bumblebees of unknown origin (feral or introduced) visited the greatest number of clones (6.7 ± 0.8), while a field with both *Apis* introduced and *Bombus* had bumblebees visiting 2.5 ± 0.4 clones on average. Two fields with only *Bombus* introduced had bumblebees visiting clones at a reduced rate (1.2 ± 0.6 and 1.0 ± 0.7), while a field all three pollinators resulted in *Bombus* visiting 1.46 ± 0.5 clones on average.

There was no significant difference between the number of flowers visited by pollinator communities between fields (P=0.08). Between pollinator species a significant difference in number of flowers visited per bee chase was found. *Bombus* visited significantly (7.26 ± 0.6) more flowers than honeybees (5.19 ± 1.05) and leafcutter bees (1.9 ± 1.19) ($P < 0.0001$). In fields where only bumblebees were introduced there was significant variation ($P < 0.0001$) in mean number of flowers

visited by bumblebees existed depending on the pollinator combinations in the field. In a field with only leafcutter bees introduced, bumblebees visited significantly more flowers (34.9 ± 3.3, $P < 0.0001$), while a field with bumblebee and honeybees introduced had bumblebees visiting 9.08 ± 1.9 flowers ($P < 0.0001$). A field with all three pollinators had a reduced rate of flower visitation by bumblebees with only 2.7 ± 2.4 flowers being visited ($P=0.008$), while a field stocked with only bumblebees resulted in bumble flower visitation rates of 3.3 ± 2.7 ($P=0.04$),

Fields differed significantly in the amount of time pollinators spend foraging on single flowers ($P < 0.0001$). In fields with honeybees and leafcutters, pollinators spent the most time foraging per flower (5.3 ± 0.81s), while in fields with only bumblebees, pollinators spent significantly less time foraging per flower, with respective means of 2.43 ± 1.4, 2.35 ± 0.96, 1.95 ± 1.6 and 1.5 ± 1.9 seconds. Between pollinator species, time spent foraging differed significantly ($P < 0.0001$). Time spent foraging on flowers by *Bombus* differed between field ($P=0.0016$), with the field containing both *Apis and Bombus* having the longest foraging time (4.1 ± 0.5 seconds). The three fields with only *Bombus* had the shortest time spent pollinating per flower with respective means of 1.55 ± 0.9, 2.53 ± 1.0, and 1.95 ± 0.88 seconds. A field with no introduce colonies of bumblebees had the shortest time spent foraging per flower, 1.02 ± 0.9 seconds. With honeybees, a significant difference existed for time spent foraging per flower in a field stocked with *Apis and Bombus*, having the highest 8.68 ± 1.6 seconds time spent foraging per flowers ($P=0.0001$).

Between fields no difference in distance travelled by pollinators was seen ($P=0.79$). Between pollinators species distance travelled between flowers differed

significantly (p=0.008). *Bombus* travelled the greatest distance 203 ± 25.1 cm, *Apis* 98.8 ± 43.3 cm and *Megachile* 51.2 ± 60.1 cm. No differences were detected in distance traveled by species in different fields.

Microclimate variability

There were no significant differences in temperature during the flowering period between the fields in our region of study (Table 2, Figure 5). Total number of days that met the minimum temperature (°C) required for each pollinator species was calculated (Table 3). Significant differences ($P < 0.0001$) were found between number of days that met the minimum temperature required by each pollinator species.

Fruit set variation by field

Mean proportional fruit set (arcsine) of control plants in the 24 fields ranged from 0.15 to 0.96 (Figures 6). The difference in fruit set among fields is significant (ANOVA: $F = 4.552$, d.f. = 287, $P < 0.0001$). Similarly, supplemental fruit set ranged from 0.42 to 0.85 (ANOVA: $F=1.776$, d.f. = 287, $P = 0.017$). Self fruit set among the 24 sites ranged from 0.055 - 0.227, but differences between fields were not significant (ANOVA: $F = 0.728$, d.f. = 287, $P = 0.82$).

Discussion

Fruit set in fields with different pollinator combinations

Vaccinium angustifolium can be considered to be a semi-domesticated crop that has been under some degree of cultivation but not intense artificial selection for hundreds of years. Lowbush blueberry as a monocultural crop is relatively new, however, and may not yet have responded to changes in cultural practices in the same fashion as other more heavily managed and bred crops. In our study there was no difference in fruit set between managed pollinator communities, despite that fact that the three introduced pollinator species are vastly different, exhibiting the entire gamut of sociality from the solitary *Megachile rotundata*, the primitive eusociality of *Bombus spp.,* to the well developed caste system of *Apis mellifera* (Michener, 1974). These species also exhibit significant differences in morphology, sensitivity to temperature, foraging strategies and nesting behaviours (Michener, 1974; Burril and Dietz, 1981; Hobbs,et al., 1967; Javorek, 2002). These behavioural and physiological differences may be important in allowing multiple species to exist in an environment. In an agroecosystem, functional diversity is generally associated with greater yields in crops such as cucurbits (Hoehn et al., 2008). Furthermore, it has previously been demonstrated that pollinator diversity, more so than pollinator abundance, is a good predictor of pollination and fruit set success (Klein et al., 2003). Our results partially support this latter idea as the lowest fruit set values were found in fields containing only honeybee colonies, with many thousand more individual foragers available compared to fields with the other two species. However, in our work, the diversity of introduced pollinators did not seem to influence levels of fruit set.

The nature of lowbush blueberry farming introduces a number of variables that cannot be controlled for in the field. Factors such as: soil conditions, water

status, disease pressure, may all influence the overall health of lowbush blueberry clones as well as their capacity to set fruit. The use of supplemental fruit set as a covariate in analyzing lowbush blueberry fruit set, was used to attempt to reduce the effect of these factors with respect to the fruit set response to different pollinator environments. Lowbush blueberry plants that have higher maximum supplemental fruit set also set fruit at a higher rate in open pollinated environments. This suggests that certain characteristics unique to the plant (health, nutrient availability) have a disproportionate effect on fruit set in blueberry fields. It also supports the idea (Bell et al. 2010; Hepler and Yarborough, 1991) that plant yield potential may be dependent on the genetic makeup of individual clones.

When supplemental pollination fruit set is included as a covariate we found that that the *Bombus – Megachile* pollinator combination leads to higher fruit set compared with other pollinator combinations. Experimental results have identified honeybees as being inferior to both leafcutter and bumblebees in lowbush blueberry pollen deposition (Javorek, 2002). Honeybees have also been shown to promote autogamy through a high rate of intraplant moments in a variety of plants that exhibit partial self compatibility similar to lowbush blueberry (Huryn 1997). Our results, though they remain difficult to interpret due to the variability in fruit set between treatments, fields and clones suggest that simply increasing pollinator abundance in a field does not result in increased fruit set, and may in fact reduce fruit set if pollinator behaviours lead to poor pollen deposition or increased self fertilization.

Bee behaviour

Pollination studies of most cropping systems are concerned mainly with visitation rates to ensure proper fruit set. Conversely blueberries require attention to also be paid to pollen flow dynamics within the field. Excessive intraclonal pollination can potentially negate any perceived benefits due to higher visitation rates (see Chapter 1). In our bee chases, bumblebees visited a greater number of clones during foraging bouts. Surprisingly, the field where bumblebees visited the greatest number of clones did not contain any introduced bumblebee colonies and only leafcutters. These bees may have travelled from adjacent fields or from feral nesting areas in adjacent forested area. The number of clones visited by *Bombus* was also significantly higher in a field containing both honeybees and bumblebees. Of these fields with the highest number of clones visited per bee chase, none of the blueberry clones monitored experienced increases in fruit set. The increase in potential outcrossing pollen was likely negated by the high number of flowers visited by bumblebees, as the two fields where bumblebees visited the greatest number of clones were also the same two fields where bumblebees pollinated significantly more flowers. This increase in potential self-fertilization may help explain why neither of these two fields were amongst the highest in fruit set. A study using fluorescent dye pollen analogues suggested that bumblebees collect a sufficient amount of pollen from a single flower to result in 59% self-pollination of the next flower (Rademaker et al., 1997). Pollen carryover is an important element to consider, in a study of *Mimulus ringens* Karron et al. (2009) found that selfing rates were 21% for the initial flower pollinated on a new clone, but increased to 78% by the time the fourth flower of that clone was visited by a self pollinating bumblebee. In lowbush blueberry, the

large floral display of individual clones potentially results in relatively few flowers receiving outcrossed pollen. As pollinators move between flowers on an inflorescence and adjacent ramets, the proportion of self pollen being transferred rapidly overwhelms the beneficial outcrossed pollen collected by pollinators from the previous clone.

When all three pollinators were present or when only *Bombus* were introduced in a field, *Bombus* visited significantly fewer flowers per bee chase compared to other pollinator combinations. With three pollinator species actively foraging, or only bumblebees foraging thoroughly within a field, floral resources may have been reduced, resulting in heterogeneity or patchiness of resources. Heinrich (1979) observed that bumblebees behaved differently in patches where floral resources were reduced, with flights between inflorescences being twice as long. This longer distance or more random flight patterns in a heterogenous depleted floral patch, may have resulted in floral visits being more difficult to track throughout the field over the course of a single bee chase. However such a scenario may lead to greater levels of outcrossing as bees actively pursue resources between a number of different patches, visiting relatively few flowers within each patch.

When both honeybees and bumblebees were in the same field, both species exhibited their highest values in time spent foraging per flower. Exploitative competition between honeybee and other pollinators have been observed (Dupont et al. 2004). The greater number of foragers found in a honeybee colony may deplete the nectar standing crop, potentially resulting in increases in foraging time per flower

in order to extract all the possible resources from every flower visited. In areas of high honeybee activity nectar scarcity results in bumblebee colonies shifting from foraging for pollen to nectar, further decreasing pollination effectiveness of *Bombus* (Dupont, 2004). Such phenomena and competition for resources potentially reduce pollen transfer and the rates of outcrossing, as nectar/pollen resources become scarce foraging must become more intense in relatively small regions

In fields with only bumblebees, the time spent foraging per flower was the lowest for *Bombus*. This efficiency in pollinating flowers may be due to scent marking by conspecifics allowing active foragers to only target flowers with abundant resources (Stout, 1998). This may be increased if floral resources are potentially limiting, allowing bumblebee pollinators to maximize resource extraction and minimize visits or time spent visiting depleted flowers. Floral complexity may also play a role, with reduced complexity leading to time spent foraging per flower decreasing, while also leading to an increase in self-fertilization (Ohashi, 2002).

We cannot accurately quantify the degree to which specific pollinators cause self-fertilization, however, the distance flown during foraging may correlate with the level of outcrossing. Total distance travelled was greatest in *Bombus*, but was similar across fields, and was not associated with greater fruit set in any of the fields. Displacement and shift in behaviours or pollination patterns have been previously observed in pollinator communities (Huryn, 1997), however, the changes observed in our study are not consistent with factors that are likely to increase outcrossing and

fruit set in our blueberry fields. The combination of pollinators that may potentially lead to the greatest fruit set, bumblebee-leafcutter, is associated with fields where bumblebees visit the greatest number of clones and flowers. Though increases in floral visitation may lead to higher levels self-fertilization (e.g., in the middle of a large clone) when plants are pollen limited any increase in pollination and flower visits may benefit fruit set response.

Temperature

There was no significant difference between the temperatures of different microregions. Upon closer examination of the number of days where the species-specific minimum temperature was met (Michener, 1974; Burril and Dietz, 1981; Hobbs et al., 1967; Javorek, 2002), we see that there were significantly fewer days where *Megachile* was capable of pollinating due to minimum temperatures required for pollination (Table 3). Having the lowest minimum temperature (8°C) allowed bumblebees to pollinate for the entire 19 days in all nine regions. Mean number of days that met minimum foraging temperatures was lower for honeybees (11 days) and leafcutters. This may lead to greater pollination throughout the region by bumblebees. Measurement of bee foraging activity may be more important than basic pollinator abundance in explaining plant responses to pollinator activity. In agriculture, recommendations for pollinator stocking rates generally focus on bee visitation rates and pollinator effectiveness (Vaissiere, 1991). If the weather conditions during the relatively short lowbush blueberry flowering peak (Figure 7)

are not conducive to pollination by certain species, fruit yield may not benefit from their inclusion in the pollinator community.

Fruit set variation between fields

The implications for pollinator management are significant if the pollination efficiencies of species are altered in communities consisting of multiple introduced species. The pollination effectiveness of the three species in our study is significantly different, but these differences cannot fully explain the variability in fruit set seen with our pollinator introductions. Fruit set variation between the field was significant between fields, suggesting an unidentified factor (or set of factors) present in each field. The differences in fruit set seen in the populations may be due to simple environmental conditions (soil conditions, water status) and/or intrinsic genetic factors of the clones themselves (clonal growth, varying degrees of self-sterility). In *Vaccinium corybosum* Krebs and Hancock (1991) found that self seed set differed between populations, with self sterility being as high 90% of a population, while only 40% of individuals exhibited self sterility in a nearby population. Differences seen in *V. angustifolium* yield tend to remain constant across multiple seasons (Hepler and Yarborough, 1991; Bell 2009), suggesting a constant element within the fields influences fruit set. The observed correlation of supplemental fruit set with the open pollinated fruit set supports the notion that certain blueberry clones are more productive than others. Hokanson and Hancock (2000) suggest that reduced fruit set

upon self pollination is indicative of inbreeding depression. If differences in genetic load exist between populations, this may account for differences between fields in fruit set. Fields with high genetic load, in a pollinator environment promoting significant self fertilization will result in a lower fruit set. Genetic relatedness has not been found to be a significant predictor of yield in outcrossing treatments (Bell, 2010), further supporting the notion that levels self fertilization rates may be the more limiting factor. Differences in observed open fruit set values may potentially be a result of the degree to which self pollination reduces fruit set through inbreeding depression (i.e. genetic load of populations) (Krebs and Hancock, 1991; Hokanson and Hancock, 2000) and the proportion of pollinations that result in self fertilizations (see Chapter 1).

Conclusions

Most studies conducted to date have focused on the effectiveness of pollinators in blueberry fields when there are introductions of singular pollinators (Javorek, 2002; Stubbs and Drummond, 2001). These studies have not considered resulting fruit set due to difference in pollinator behaviour in introduced pollinator communiries, nor has the quality of pollen (self- versus outcross-pollen) being transferred in such communities been considered. There may also be features of a blueberry field that are more likely to lead to increased self-pollination; e.g., the flowering phenology of clones within a field can vary to such an extent to create distinct patches of flowers at peak maturity. Floral resources in such a field may be

abundant over the entire flowering period, but may be limiting on a day-to-day basis. Pollination within these distinct patches may lead to increase pollen transfer within clones, or may result in a dominant though less effective pollinator (honeybees) excluding or limiting pollination by their more effective counterparts. The behaviour (between clone movements) of pollinators are an important element in determining the most efficient pollinators, any changes that arise in mixed species communities due to interactions are likely to affect pollen movement within a field. Our self-pollination trials demonstrated that self-incompatibility or high levels of inbreeding depression likely exists within our blueberry fields, with self-fruit set near zero for more that 50% of the clones studied (Chapter 1). Coupled with the potential variability in clone size, the degree of self pollination may account for some of the variability seen between pollinator treatments and fields. Excessive intraclonal pollination can potentially negate the perceived benefits of higher visitation rates, by increasing expression of mutant alleles in progeny resulting in seed or fruit abortion.

The cultural practices currently used in commercial blueberry fields likely promote self-fertilization by allowing individual clones to increase in size at will. If farmers emphasize cultural methods that allow clones to maximize their size, they may in fact be losing production (unit/area) due to inbreeding depression increases as a result of intraclonal pollinator movement. Pollinator stocking recommendations generally focuses on the size of the lowbush blueberry field (Drummond, 2002). However, in the increasingly complex introduced pollinator communities being created by blueberry farmers, many of these recommendations may need to be

reassessed. The structure of the blueberry agroecosystem will need to be accounted for in any pollination strategy. Dependant on the introduced species, as well as any native pollinator, placement and density of pollinator units may differ if behavioural alterations occur among pollinators. With pollinator rental/purchase prices steadily rising (Stubbs and Drummond, 1997b), a profitable farm pollination strategy must incorporate predictions of pollinator behaviour within specific fields. This also necessitates knowledge of the blueberry clones within your field. Differences in ramet density, floral density, size of blueberry clones and floral phenology will affect the effective outcrossing pollination rate. Combining agricultural field/crop profiles with expected pollinator behaviours may lead to better use of introduced pollinators, providing greater yields while minimizing yearly pollinator costs.

Literature Cited

Aalders L.E. and Hall, I.V. (1961). Pollen incompatibility and fruit set in lowbush blueberries.Canadian Journal Genetic Cytology, 3, 300-307.

Argall, J., K. E. MacKenzie, S. K. Javorek, and G. Chiasson. (1996). Alfalfa leafcutting bees for the pollination of wild blueberries. Wild blueberry fact sheet B.7.0., New Brunswick Department of Agriculture and Fisheries, Fredericton,New Brunswick.
http://www.gnb.ca/0171/10/0171100027-e.asp

Bell, D. J., Rowland, L. J., Polashock, J. J., and Drummond, F. A. (2008). Suitability of EST-PCR markers developed in highbush blueberry for genetic fingerprinting and relationship studies in lowbush blueberry and related species. *Journal of the American Society for Horticultural Science, 133*, 701-707.

Bell DJ. (2009). Spatial and genetic factors influencing yield in lowbush blueberry (*Vaccinium angustifolium* Ait.)) in Maine. PhD Dissertation. University of Maine, School of Biological Sciences.

Bell, D. J., Rowland, L. J., Stommel, J., and Drummond, F. A. (2010). Yield variation among clones of lowbush blueberry as a function of genetic similarity and self-compatibility. *Journal of the American Society for Horticultural Science, 135*, 259-270.

Benoit, G.R., Grant, W.J., Ismail, A.A., and Yarborough, D.E.. (1984). Effect of soil moisture on the potential and actual yield of lowbush blueberries. Canadian Journal of Plant Sciences. 64, 683-689.

Burril, R., M., and Dietz, A. (1981). The response of honey bees to variations in solar radiation and temperature. *Apidologie, 12*, 319-328.

Corbet, S. A., Fussell, M., Ake, R., Fraser, A., Gunson, C., Savage, A., and Smith, K. (1993). Temperature and the pollinating activity of social bees. *Ecological Entomology, 18*, 17-30.

Drummond, F. (2002). Honey bees and blueberry pollination fact sheet No. 629, Bees and Blueberry Pollination Cooperative Extension Bulletin 629, University of Maine Cooperative Extension and Department of Biological Sciences. UMaine Extension No. 2079

Dupont, Y. L., Hansen, D. M., Valido, A., and Olesen, J. M. (2004). Impact of introduced honey bees on native pollination interactions of the endemic *Echium wildpretii* (*Boraginaceae*) on Tenerife, Canary Islands. *Biological Conservation, 118*, 301-311.

Eck, P. (1988). Blueberry Science. Rutgers University Press, New Brunswick.

Galil, J. (1967). Sycomore wasps from ancient Egyptian tombs. Israel Journal of Entomology, 2, 1-9.

Gawleta, N., Zimmermann, Y., and Eltz, T. (2005). Repellent foraging scent recognition across bee families. *Apidologie, 36*, 325-330.

Goulson, D., Lye, G. C., and Darvill, B. (2008). Decline and conservation of bumble bees. *Annual Review of Entomology, 53*, 191-208.

Hall, I. V. and Ludwig, R. A. (1961). The effects of photoperiod, emperature and light intensity on the growth of the lowbush blueberry (*Vacinium angustifolium*.). *Canadian Journal of Botany, 39*, 1733-1739.

Hall, I. V., Aalders, L. E., Jackson, L. P., Wood, G. W. and Lockhart, C. L. (1967). Lowbush blueberry production in Canada. Canada Department of Agriculture, Ottawa, Ont. Pub. 1278.39, pp 67

I. V. Hall, L. E. Aalders. (1968). Fruit set and berry development of lowbush blueberry as affected by temperature. *Canadian Journal of Plant Science*, 48, 321-322

Hall, I.V., Aalders, L.E, Nickerson, N.L., and Vander Kloet, S.P. (1979). The biological flora of Canada. 1. Vaccinium angustifolium Ait., sweet lowbush blueberry. *Canadian Field-Naturalist*, 93, 415-430.

Harder LD, and Barclay R.M.R. 1994. The functional significance of poricidal anthers and buzz pollination: Controlled pollen removal from *Dodecatheon*. *Functional Ecology*, 8, 509-517.

Heinrich, B. (1979). Resource heterogeneity and patterns of movement in foraging bumblebees. *Oecologia*, 40, 235-245.

Hobbs, G.A., W. O. Nummi, J. F. Virostek. (1961). Food-gathering behaviour of honey, bumble, and leaf-cutter bees (Hymenoptera: Apoidea) in Alberta. *Canadian Entomologist*, 93, 409-419.

Hoehn, P., Tscharntke, T., Tylianakis, J. M., and Steffan-Dewenter, I. (2008). Functional group diversity of bee pollinators increases crop yield. *Proceedings of the Royal Society B: Biological Sciences, 275*, 2283-2291.

Hokanson, K., and Hancock, J. (2000). Early-acting inbreeding depression in three species of Vaccinium (Ericaceae). *Sexual Plant Reproduction,* 13, 145-150.

Huryn, V. M. B. (1997). Ecological Impacts of Introduced Honey Bees. *The Quarterly Review of Biology, 72*, 275-297.

Javorek, S. K., Mackenzie, K. E., and Vander Kloet, S. P. (2002). Comparative pollination effectiveness among bees (*Hymenoptera: Apoidea*) on lowbush blueberry (Ericaceae*: Vaccinium angustifolium*). *Annals of the Entomological Society of America, 95*, 345-351.

Karron, J. D., Holmquist, K. G., Flanagan, R. J., & Mitchell, R. J. (2009). Pollinator visitation patterns strongly influence among-flower variation in selfing rate. Annals of Botany, 103, 1379-1383.

Kevan, P.G, Clark, A, and Thomas, V.G. (1990). Insect pollinators and sustainable agriculture American Journal of Alternative Agriculture , 5, 13-22.

Klein, A. M., Steffan–Dewenter, I., and Tscharntke, T. (2003). Fruit set of highland coffee increases with the diversity of pollinating bees. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 270*, 955-961.

Krebs, S. L., and Hancock, J. F. (1991). Embryonic genetic load in the highbush blueberry, *Vaccinium corymbosum* (Ericaceae). *American Journal of Botany, 78* 1427-1437.

Kremen, C., Williams, N. M., Bugg, R. L., Fay, J. P., and Thorp, R. W. (2004). The area requirements of an ecosystem service: crop pollination by native bee communities in California. *Ecology Letters, 7*, 1109-1119.

Kremen, C., Williams, N. M., and Thorp, R. W. (2002). Crop pollination from native bees at risk from agricultural intensification. *Proceedings of the National Academy of Sciences, 99*, 16812-16816.

Michener, Charles D. (1974). The social behavior of the bees, a comparative study. Belknap. Cambridge, Massachusetts, pp. 404.

Natural Research Council (2006). Status of Pollinators in North America. *Committee on the Status of Pollinators in North America*. National Academic Press

Ohashi, K. (2002). Conseequences of floral complexity for bumblebee-mediated geitonogamous self-pollination in *Salvia Nipponica Miq*. *(Labiatae*). *Evolution, 56*, 2414-2423.

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., and Kunin, W. E. (2010). Global pollinator declines: trends, impacts and drivers. *Trends in Ecology and Evolution, 25*, 345-353.

Rademaker, M. C. J., De Jong, T. J., and Klinkhamer, P. G. L. (1997). Pollen dynamics of bumble-bee visitation on *Echium vulgare*. *Functional Ecology, 11*, 554-563.

Rands, S. A., and Whitney, H. M. (2010). Effects of pollinator density-dependent preferences on field margin visitations in the midst of agricultural monocultures: A modelling approach. *Ecological Modelling, 221*, 1310-1316.

Röseler, P.F. (1985). A technique for year-round rearing of *Bombus terrestris* (*Apidae, Bombini*) colonies in captivity. Apidologie 16, 165-170.

Spann, T. M., Williamson, J. G., and Darnell, R. L. (2004). Photoperiod and temperature effects on growth and carbohydrate Storage in southern highbush blueberry interspecific hybrid. *Journal of the American Society for Horticultural Science, 129*, 294-298.

Stout, J. C., Goulson, D., and Allen, J. A. (1998). Repellent scent-marking of flowers by a guild of foraging bumblebees (*Bombus spp*.). *Behavioral Ecology and Sociobiology, 43*, 317-326.

Stubbs, C. S., and Drummond, F. A. (1997a). Management of the alfalfa leafcutting bee, *Megachile rotundata* (*Hymenoptera: Megachilidae*), for pollination of wild lowbush blueberry. *Journal of the Kansas Entomological Society, 70*, 81-93.

Stubbs, C.S. and Drummond, F.A. (1997b). Blueberry and cranberry (Vacinium Spp.) Pollination: A comparison of managed and native bee foraging behaviour. *Acta Horticulturae*, 437, 341-344

Stubbs, C. S., Drummond, F. A., and Osgood, E. A. (1994). *Osmia ribifloris biedermannii* and *Megachile rotundata* (Hymenoptera: Megachilidae) Introduced into the lowbush blueberry agroecosystem in Maine. *Journal of the Kansas Entomological Society, 67*, 173-185.

Thomson DM. (2004). Detecting the effects of introduced species: a case study of competition between *Apis* and *Bombus*. Oikos, 114, 407–18

Thomson, D. (2004). Competitive interactions between the invasive european honey bee and native bumble bees. *Ecology, 85*, 458-470.

Vaissiere, B.E. (1991). Honey bee stocking rate, pollinator visitation, and pollination effectiveness in upland cotton grown for hybrid seed production. *Acta Horticulturae*, *288,* 359-363.

vanEngelsdorp, D., Underwood, R., Caron, D., Hayes, Jr., J (2007) An estimate of managed colony losses in the winter of 2006 - 2007: A report commissioned by the Apiary Inspectors of America. *American Bee Journal*, 147, 599-603.

vanEngelsdorp, D., and Meixner, M. D. (2010). A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *Journal of Invertebrate Pathology, 103, Supplement*, S80-S95.

Walther-Hellwig, K., and Frankl, R. (2000). Foraging habitats and foraging distances of bumblebees, *Bombus spp*. (*Hym., Apidae*), in an agricultural landscape. *Journal of Applied Entomology, 124*, 299-306.

Walther-Hellwig K, Fokul G, Frankl R, Buechler R, Ekschmitt K, Wolters V. (2006). Increased density of honeybee colonies affects foraging bumblebees. Apidologie 37, 517–532.

Winfree, R., Bartomeus, I., and Cariveau, D. P. (2011). Native pollinators in anthropogenic habitats. *Annual Review of Ecology, Evolution, and Systematics, 42*, 1-22.

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Tables and figures

Table 1. Mean standardized values observed during bee chases for: flowers visited and total time spent pollinating. Average movement size, number of clones visited and control fruit set also included. Pollinator units (number of honeybee hives, number of bumblebee boxes, and gallons of leafcutter) are included in parentheses.

site	Pollinator (units)	Flowers visited (per hour)	Time pollinating (per hour)	Distance (cm)	Clones visited	Control fruitset
DHBL	Bombus(10)	41.57	126.29	236.41	1.33	
	Apis(40)			169.33	1.67	0.600
	Megachile(6)	28	213.47	62.0	1	
DHB	Bombus(40)	68.42	230.6	174.11	2.63	0.622
	Apis(10)	79.69	544.32	102.54	1.58	
FL	Bombus	22.11	125.65	327.75	6.75	0.508
	Megachile(3)	7.13	43.43	33.5	1	
EHB	Bombus(1)	55.79	96.85	246.5	1.5	0.471
	Apis(4)	40.34	200.02	96	1.667	
RB	Bombus(4)	143.7	336.18	168.45	1.75	0.473
RH	Apis(8)	84.0	486.4	121.88	1.33	0.323
CH	Apis(60)	142.0	617.73	96.1	1.94	0.491
STYB	Bombus(2)	158	359.74	228.75	1	0.277
QB	Bombus(1)	146.81	334.89	167.64	1.21	0.676

Table 2. One way ANOVA analysis of temperature in 9 regions between May 26, 2010 and June 15, 2010.

Source of Variation	SS	df	MS	F	P-value
Between Groups	414.96	8	51.87	1.589	0.123
Within Groups	67865.2	2079	32.64		
Total	68280.1	2087			

Table 3. Number of days that met 3 distinct minimum foraging temperature (ºC) during the estimated flowering period.

Field	Number of days with Mean T(ºC) > 13[a]	Mean T(ºC) > 8[b]	Mean T(ºC) > 16[c]
Lavillette West	10	19	2
Lavillette East	13	19	6
Quarry A & B	8	19	1
Lawayqua'le	11	19	5
Fairisle	12	19	2
Drisdelle_small	12	19	2
Drisdelle_large	12	19	2
Stymiest south	14	19	5
Stymiest North	13	19	4
Mean (± S.D)	11.66 ± 1.8	19 ± 0.0	3.22 ± 1.78

a - Honey bee (*Apis mellifera*) minimum temperature (Hobbs et al., 1961; Corbet et al. 1993;)
b – Bumblebee (*Bombus spp.*) minimum temperature (Corbet et al., 1993)
c - Leafcutter bee (*Megachile rotundata*) minimum temperature (Corbet et al., 1993)

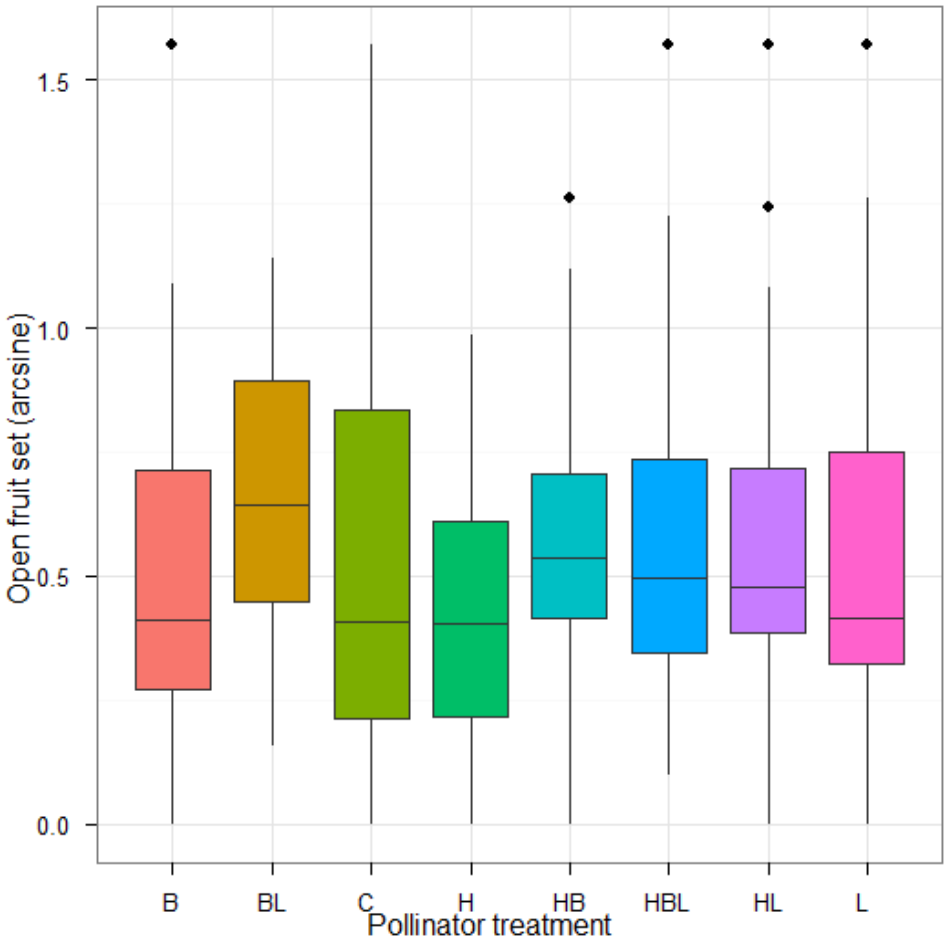

Figure 1. Open fruit (arcsin transformed) in 8 pollinator treatments**,** B=bumblebee, H=honeybee, L=leafcutter.

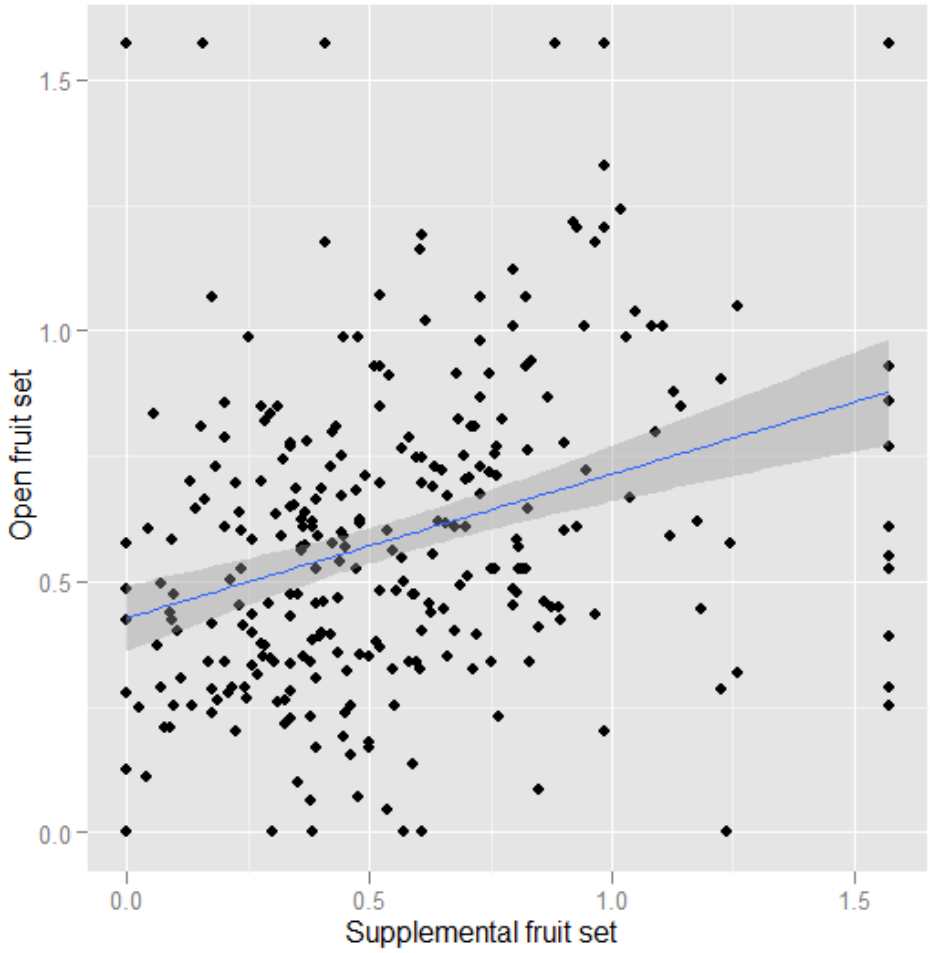

Figure 2. Linear regression between supplement fruit set and open fruit set (r^2=0.10, P<0.001).

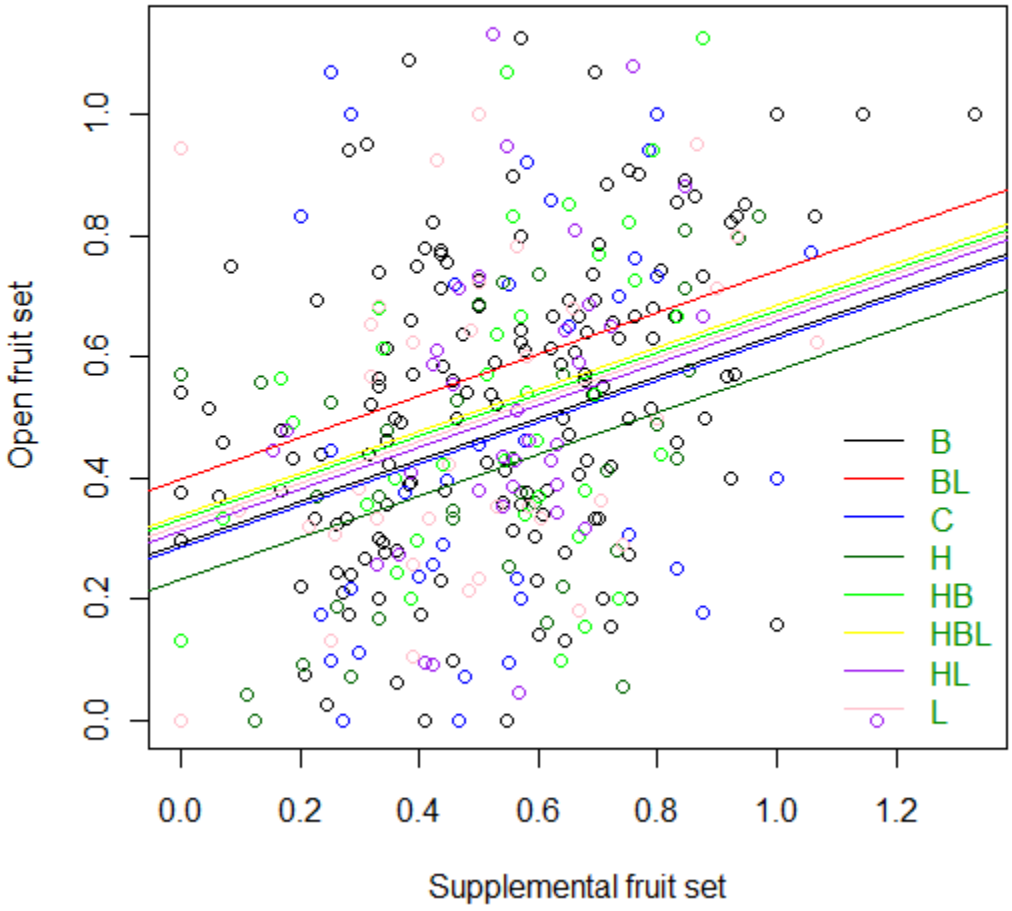

Figure 3. Open fruit set as a function of supplemental fruit in 8 pollinator treatments. Each line represents the ANCOVA regression for a particular pollinator treatment.

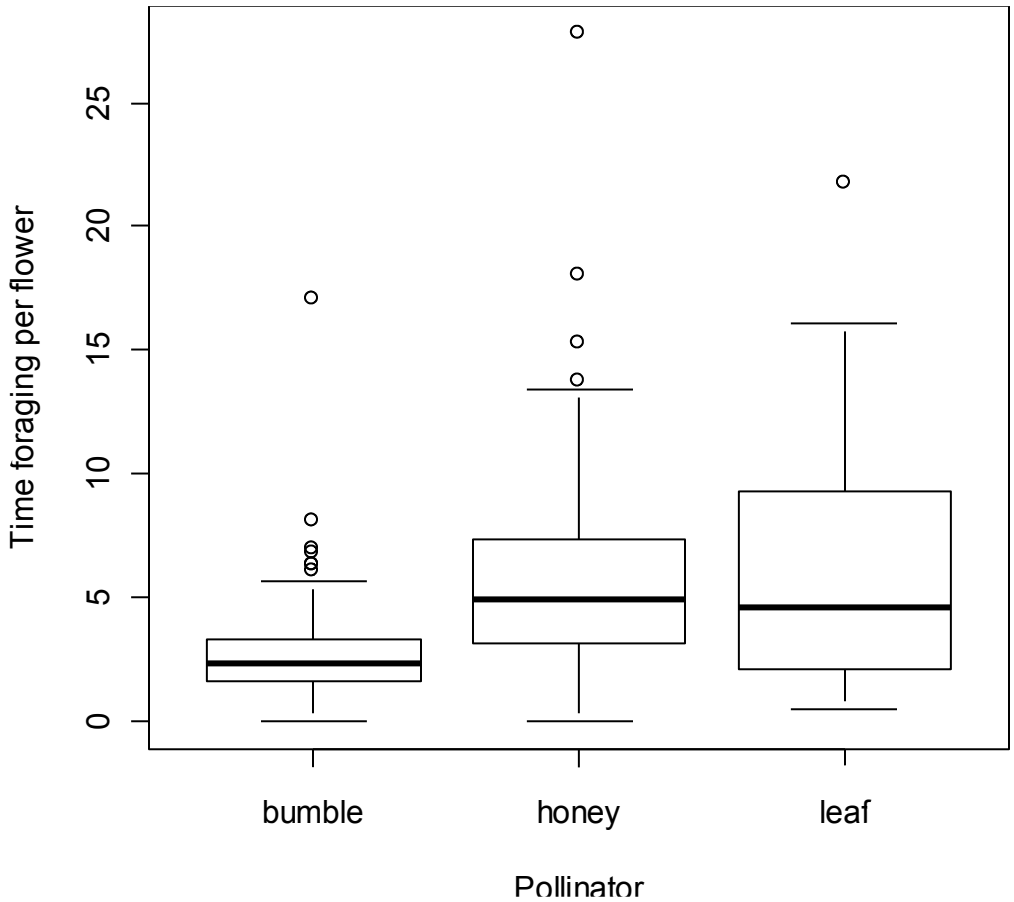

Figure 4. Time (seconds) spent foraging per flower in three pollinators (*Apis mellifera, Bombus sp., Megachile rotundata*).

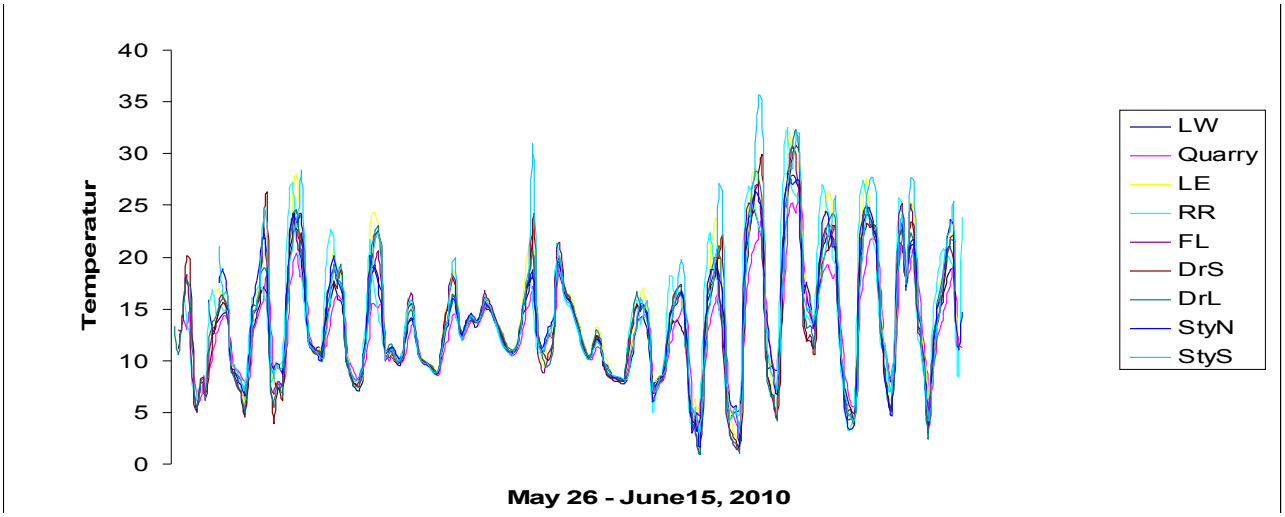

Figure 5. Temperature (ºC) at 9 different sites measured every two hours between May 26, 2010 and June 15, 2010

Key: LW (Lavillette West), Quarry (Quarry A & B), LE (Lavillette East), RR (La Way Qual'e), FL (Fairisle), DrS (Drisdelle small), DrL (Drisdelle large), StyN (Stymiest north), StyS (Stymiest south).

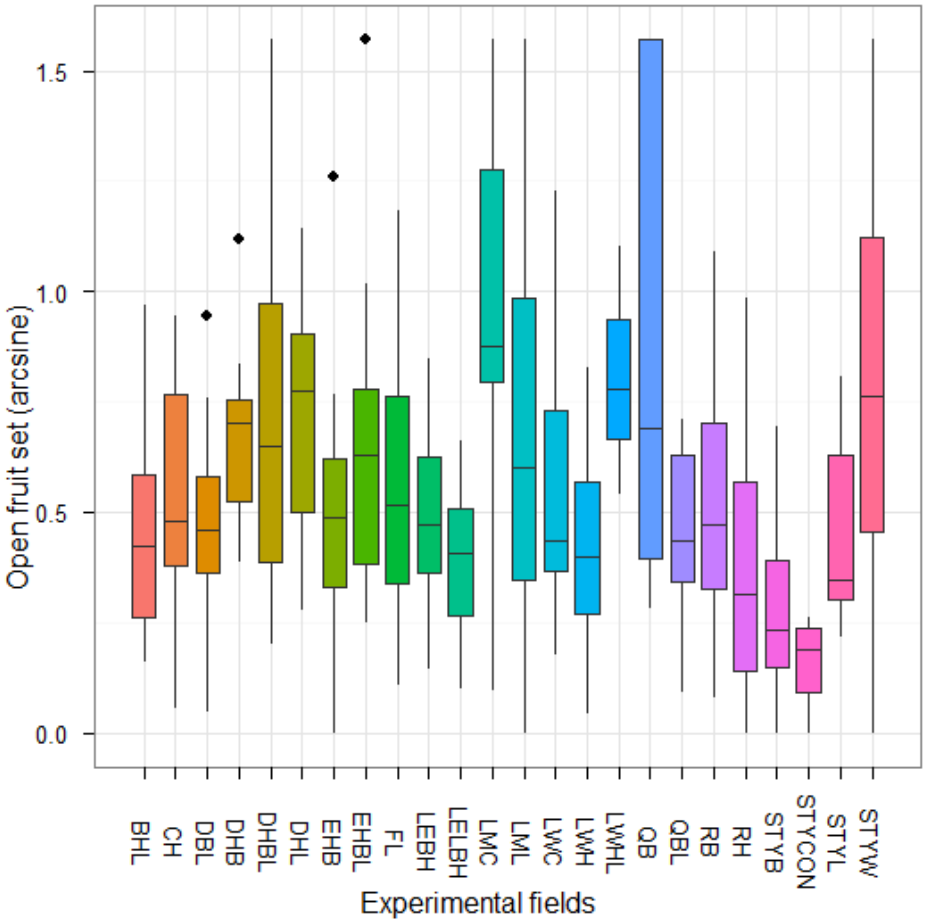

Figure 6. Open fruit set (arcsin transformed) in 24 experimental fields. Each colour represents a specific pollinator treatment (see key in figure)
B=bumblebee, H=honeybee, L=leafcutter.

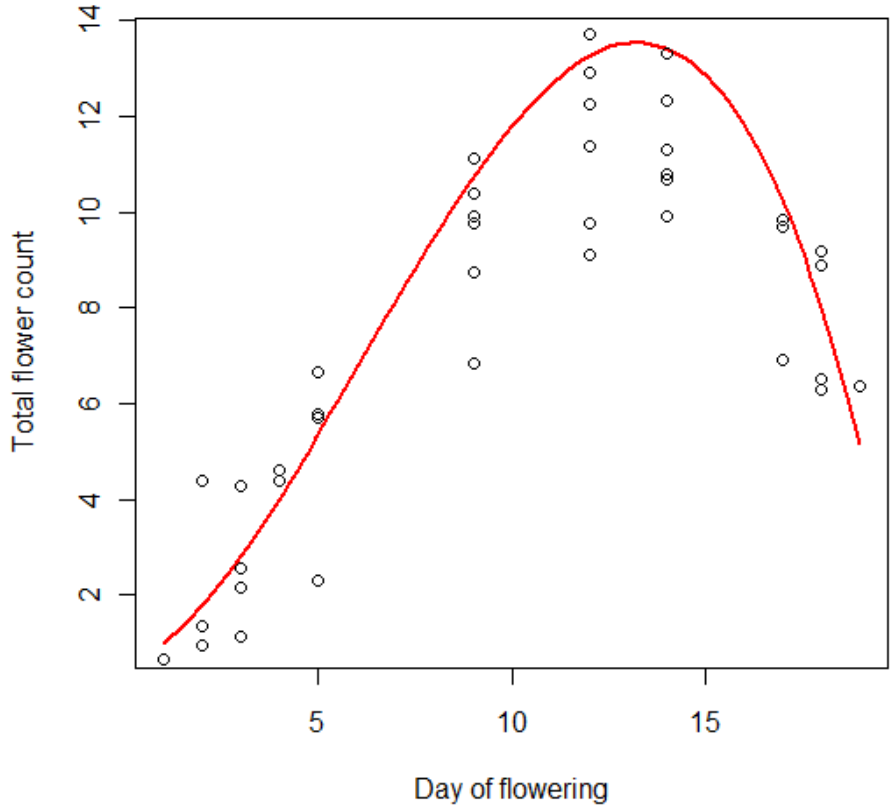

Figure 7. Mean number of flowers per inflorescences as the flowering season progresses. Flowers were monitored between May 26 till June 13, 2006. Regression line fitted using a 2nd degree polynomial ($r^2 = 0.67$, $P > 0.001$)

Summary

The clonal growth habit of *V. angustifolium* coupled with the self-incompatibility and inbreeding depression may account for much of the variability seen in fruit set. A clone's yield is an inherent function of its maternal genetic makeup, which can be influenced by the pollen environment in which it is found. The unfettered growth of lowbush blueberry clones, allowed by farmers, provides ample opportunity for the development of genetic differentiation within a clone. In this scenario we cannot consider all self pollen to be similar, as the resulting fruit set is likely to differ depending on the degree to which mutations are shared between the two cell lines being self fertilized. Pollinators are introduced to this environment by farmers attempting to maximize overall pollination levels in the field. However, increases in yield are not guaranteed through simple increases in pollinator visits to flowers. Ideally, we anticipate that a significant number of these visits result in cross fertilization, however, that is often not the case with the three most commonly used managed pollinators. The domestication process that lowbush blueberry is undergoing requires that blueberry growers adopt pollination management practices similar to other cropping systems while taking into account unique factors such as: buzz pollination, inbreeding depression and clonality of the plant. Blueberry farmers may benefit from future studying the effects of large clone size on intraclonal movement of pollinators and pollen at different scales within their fields. Determining the role of somatic mutations and the resulting different pollen environments in clones of various sizes is necessary to the pollination ecology of *V. angustifolium* and can further optimize fruit production.