SUPPORTING *BOMBUS* AND OTHER BEES IN *CUCURBITA* AGROECOSYSTEMS

A Thesis in

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by

Erin Delaney Treanore

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The thesis of Erin Delaney Treanore was reviewed and approved* by the following:

Shelby Fleischer
Professor of Entomology
Thesis Advisor

Christina Grozinger
Distinguished Professor of Entomology

Heather Hines
Assistant Professor of Entomology and Biology

Gary Felton
Professor of Entomology
Head of the Department of Department of Entomology

*Signatures are on file in the Graduate School
ABSTRACT

The decline of pollinators, in particular bees, has been an extensively researched and discussed topic in the last few decades. This decline was noted in both honey bees and native bee species, with concern increasing for some bumble bee species. A myriad of stressors have been implicated in this decline, but pathogens, pesticides, parasites and loss of habitat resulting in poor nutrition, canvas a majority of suspected and interacting factors. Agricultural land intensification houses a number of these stressors, often resulting in both increased likelihood to pesticide exposure and a loss of access to diverse assemblages of floral resources. Paradoxically, agriculture can also function to provide a large pulse of the floral resources that bees depend on. In this thesis I target this issue by focusing on a commonly found agroecosystem within the Mid-Atlantic region, pumpkin, or *Cucurbita pepo*, and examine (1) How cover-cropping can be used as a form of floral provisioning to provide additional resources for generalist pollinators (2) The quality of the floral resources provided within these floral provisionings, and (3) Effects of insecticide usage on both *Cucurbita pepo* and its generalist pollinators.

*Cucurbita pepo* presents a challenging system to work with due to its rotational nature, which creates limitations on the type of floral provisioning that can be used. I examined how a dual-flowering planting (fall-flowering and spring-flowering) using mixtures of common cover-crop species could fit into this system while supporting relevant pollinators. I also observed how various seeding rates affected the floral density of the planting, and how that effect persisted into the behavior of the pollinators. I found that seeding rate did not significantly affect either the floral density, or the behavior of the pollinators, suggesting that growers have flexibility in the seeding rate they use.

I then focused on the quality of the floral resources provided in these plantings, *e.g.*, the pollen, to observe how *Bombus impatiens* fed on this pollen would respond. I approached
this by using no-choice bioassays using queenless *B. impatiens* microcolonies and pollen from pumpkin, two common cover crop species from two plant families commonly visited by *Bombus*, a multifloral diet, and an artificial honey bee diet. After seven days, I examined the size of their ovaries, their change in weight, and their waxing behavior to gauge their response. I confirmed that adults feed on pollen, and found that pollen quality had a significant impact on the size of their ovaries, as well as ability to produce wax.

Lastly I examined the *Cucurbita pepo* system itself by looking at systemic insecticide usage and possible risks of exposure for *Bombus* and *Apis mellifera*. I examined how different methods of neonicotinoid treatment effects the floral display within *Cucurbita pepo*, and how this persisted into the foraging behavior of the observed pollinators. Looking at plant response, I found that the amount of insecticide applied had a significant effect on the number of flowers produced by the plant. *Apis mellifera* and *Bombus* demonstrated alternative preference to treatments, with treatment having a significant effect on foraging behavior of *Apis mellifera* but not *Bombus*.

By approaching pollinator conservation in an agroecosystem using an integrative approach, I aimed to emphasize the importance of considering all components of an agroecosystem. Although this research was conducted within the Mid-Atlantic, the ideas and concepts can be considered within the context of other systems and regions. These studies and findings in this thesis were meant to improve our understanding and ability to support pollinators through threat mitigation and a more targeted approach to floral provisioning.
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Chapter 1 Introduction to Pollinators in Agroecosystems and Bee Declines

Introduction

In the last decade, the decline of pollinators has been documented as a serious threat to both agricultural and natural ecosystems (Goulson 2010). In addition to loss of biodiversity, pollinators are often critical to ecosystem function, in particular for the movement of pollen from flower to flower and thus successful plant reproduction. Although some plants have evolved to reproduce asexually, in numerous plant groups reproduction can only occur when male gametes, i.e., pollen, come into contact with the female reproductive structure of the flower, or the stigma. This mechanism allows for fertilization of the ova and the ensuing development of the seed and fruit. The transfer of this genetic material in pollen can occur through autogamy (self-pollination) or allogamy (cross-pollination) yet for cross-pollination to occur an additional vector for pollen is often necessary. Pollen can be transferred from the reproductive parts of different flowers through wind, water, or animals, but insects have evolved to be highly specialized for this role (O’Toole and Raw 1991). Plants that have come to rely on these animal and insects groups as a pollen vector are known as zoophilous plants. Among the insects, bees (Hymenoptera: Apoidea) fulfill this niche in numerous ecosystems, making our understanding of their health and biology especially critical.
Pollinators

Bees have been recognized for their value to humans since the Antiquity, when they were first managed for honey production (VanEngelsdorp and Meixner 2010). The European honey bee, or *Apis mellifera*, has been the primary managed pollinator for both honey production and for pollination services. The large colony size, highly organized social nature, generalist foraging behavior, and perennial life cycle make them ideal for crop pollination and portability. Beyond honey bees, bumble bees, or *Bombus* have also recently been of interest for management purposes. Depending on the species, bumble bees often exist in a colony numbering a few hundred individuals. Considered primitively social in nature, generalists in their foraging behavior, and with an annual colony life cycle (in a majority of species), bumble bees can also meet the pollination needs of numerous crop species. Bumble bees are unique in their ability to vibrate their wings at a frequency that releases pollen from the anthers of flowers. This technique, known as buzz pollination, is the most effective method of gathering pollen from numerous plant species, making them an incredibly valuable pollinator.

In addition to honey bees and bumble bees, at many as 20,000 of other bees species exist across North America and the world (Michener 2007). These bees range in their life cycle, phenology, sociality, color, size, and dietary preferences. Many of these native bees are solitary but in comparison to honey bees, little is known about their ecology and role in both managed and unmanaged ecosystems. Recently their importance in agriculture, and thus for humans, come to light, increasing the interest in our understanding of them (Garibaldi et al. 2013). Recent studies have demonstrated that for some crops native bee species may actually be more effective pollinators than honey bees (Winfree et al. 2007, Garibaldi et al. 2013). Within blueberry it’s been shown that *Bombus* and *Andrena* are such efficient pollinators that the can pollinate multiple flowers in the same amount of time a single flower is pollinated by a honey bee (Javorek et al.
2002). Likewise, the blue orchard bee, or *Osmia lignaria*, is such an effective pollinator of tree fruits that growers are being provided with the information necessary to manage their own (Bosch and Kemp 2001). Furthermore, the presence of both honey bees and native bees within an agricultural landscape may actually create an antagonistic relationship that increases the movement of bees from flower to flower, and thus, the rate of pollination (Greenleaf and Kremen 2006a, Brittain *et al.* 2013). Yet despite these recent studies, the need exists to continue improving our understanding of native bee populations, ranges, and their overall ecology.

**Pollinator declines**

Pollinators have been extensively studied and probed within the scientific community for over a century; Darwin probed the role of moths in orchid pollination within *The Origin of Species*. Yet despite this scientific interest and abundance of research, the growing interest in pollinator ecology and health from policymakers and the public is relatively recent. This can be partially attributed to the declining numbers of honey bees that caught the media’s attention in the early 2000’s. Honey bees acted as the canary in the coal mine, when many beekeepers noted high numbers of their honey bees were disappearing. As honey bees are the primary pollinators in agroecosystems, their decline increased awareness of this problem in the public and corporate spheres. Unique to this problem is the absence or low number of adult workers in the hive without any distinct signs of affliction (Oldroyd 2007). This combination of symptoms was coined Colony Collapse Disorder, or CCD. Studies looking at factors leading to this condition implicated a synergistic relationship between pathogens, pesticides, parasites, and other stressors, such as habitat loss (Oldroyd 2007, VanEngelsdorp *et al.* 2009, VanEngelsdorp and Meixner 2010). Furthermore, additional studies have shown that native bees also experience adverse effects from
the previously mentioned pathogens, pesticides, and parasites (Vanbergen 2013, Brown et al. 2016).

The perils associated with the decline of pollinators also persist into the economic value associated with their role in agricultural systems. Studies have shown that crop values associated with insect pollination may be as high as $153 billion worldwide (Gallai et al. 2009). Approximately 87 of 124 main crops have been recognized as dependent on pollinators (Klein et al. 2007). Even more worrisome, some crop species such as Cucurbita pepo are classified as essentially dependent on animal pollination for successful reproduction and fruiting (Klein et al. 2007). Significant efforts are being made across the United States and world to address these declines in agricultural systems, with much attention being given to habitat enhancement in agricultural systems.

Habitat loss

As previously mentioned, habitat loss and fragmentation, in addition to land intensification in agricultural landscapes, all have been implicated as a key factor in the decline of pollinators (Senepathi et al. 2016). The loss of nutritional resources, e.g., pollen and nectar, combined with the loss of nesting resources are central to this issue. Land intensification, often for agricultural purposes, reduces the diversity of floral resources available and often the seasonality of floral bloom as well. In agricultural settings where tillage is employed, some findings have suggested that improper tillage practices can adversely affect larval survival of the ground-nesting squash bee, Peponapis pruinoisa (Ullman et al. 2016). Yet in the face of these declines, some bee species are actually benefitting from human activity, with domestication of Cucurbita pepo being cited as critical for facilitating the spread of Peponapis pruinoasa, across North America (Lopez-Uribe et al. 2016). More often though, generalists, e.g., bumble bees,
rather than specialist bee species, have been shown to benefit from mass-flowering resources (Westphal *et al.* 2009). In addition to land alteration for agriculture, some urbanized landscapes have been shown to have greater bee richness than agricultural land (Baldock *et al.* 2015). Despite these examples, the general consensus of studies finds habitat loss due to agriculture and the declining available forage to be detrimental to bee populations, while also calling for retention and augmentation of pollinator habitat to address this issue.

**Pesticides**

In addition to habitat loss, pesticide usage can significantly negatively impact pollinators in agricultural landscapes, particularly when acting in synergy with other stressors. A particular class of pesticides, neonicotinoids, have recently gained enormous attention for their hypothesized negative effects on pollinators. One of the primary reasons for this alarm is that all classes of neonicotinoids are systemic, meaning that various degrees of these chemicals are incorporated into the plant tissue (*e.g.*, phloem, xylem, leaf tissue) depending on the application method, formulation, and type of neonicotinoid used. Unfortunately, studies working with various crops and application methods have also found that these chemicals can translocate into the pollen and nectar of numerous flowering plant species, including zoophilous *Cucurbita* species (Dively and Kamel 2012, Stoner and Fitzer 2012).

Neonicotinoid insecticides have been implicated in numerous studies as having adverse health effects on pollinators. For example, honey bees larva exposed to imidacloprid had reduced olfactory associative behavior as adults, which could adversely affect their foraging behavior. Decreased learning behavior was also exhibited by adult honey bees that had ingested imadacloprid (Tan *et al.* 2014). Studies working with native bees have found similar adverse health effects. *Bombus terrestris* chronically exposed to field relevant doses of imidacloprid
exhibited impaired foraging performance (Gill and Raine 2014). As of 2016, only seven studies had been published on neonicotinoids and solitary bee species, with more than half of these studies being done with imidacloprid, calling for a comprehensive and thorough effort by researchers to address this knowledge gap (Hopwood et al. 2016).

**Parasites and Pathogens**

Like most living organisms, bees are also afflicted with a number of viruses and parasites which can be detrimental to their health and survival. Accordingly, they have also been cited as potential causes of their decline. Black queen cell virus (BQCV), Israeli paralysis virus (IAPV), deformed wing virus (DWV) and sacbrood virus (SBV) have been shown to cause significant colony loss in honey bees, but they have also been found in bumble bees, solitary bees, and other insect orders, suggesting a concerning possible transmission between species (Manley et al. 2016). In addition to these viruses, endoparasites including the microsporidians Nosema ceranae, N. apis, and N. bombii, have been found in both Apis and Bombus (Cameron et al. 2011, Chen et al. 2008) with N. bombii suggested to having played a significant role in the widespread decline of bumble bees in North America (Cameron et al. 2011). Ectoparasites, specifically mites, also are a significant contributor to bee decline, with Varroa destructor cited as the most destructive ectoparasite of Apis mellifera. Although less data exist on native bees, recent research suggests that honey bees may be a potential source of pathogens for solitary bees (Singh et al. 2010, Furst et al. 2014, Ravoet et al. 2014).
Thesis objectives

All of the above stressors both alone and acting synergistically have been noted as playing some role in the decline of pollinators. To consider both the importance of pollinators in agroecosystems, and the paradoxically detrimental effects of agricultural intensification, I will study pollinators within this context. I will be using *Cucurbita pepo* and the agricultural relevant and necessary group *Bombus* as a model system, while focusing on the threats posed by poor nutrition and pesticides.

I approach the cause of poor nutrition, or loss of flowering resources in **Chapter 2**, where I will examine how floral provisioning can be targeted to a specific pollinator and agricultural system. This is a component of Project Integrated Crop Pollination (ICP), a nationwide project approaching crop pollination in a way similar to that of Integrated Pest Management (IPM). There are six objectives identified by Project ICP, and I focus on the second: “developing habitat management practices to improve crop pollination”. By utilizing a dual-flowering planting (fall-flowering and spring-flowering) and also utilizing annual species, I plan to integrate the needs of specific agroecosystems with needs of relevant pollinators. Furthermore, I also examine how various seeding rates affect the floral density of the planting, and how this can persist into the behavior of the pollinators. As seeding rates can affect the level of competition between species and the resources available, I hypothesize that growth will differ between plots, with this being reflected in the behavior of the pollinators as well.

In **Chapter 3** I examine the nutritional quality of the pollen provided in these floral provisioning plantings as compared to pumpkin pollen, and how it affects the health and fitness of *Bombus impatiens*. I approach this by using no-choice bioassays using queenless *B. impatiens* microcolonies and pollen from pumpkin, two common cover crop species from two plant families commonly visited by *Bombus*, a multifloral diet, and an artificial honey bee diet. I hypothesize
that *Bombus impatiens* fed pollen from cover crop species will respond positively as compared to pumpkin pollen and a no-pollen control. By examining what the nutritional quality of resources available in a Mid-Atlantic agroecosystem, I will explore how floral provisioning plantings can be improved and targeted to specific pollinators.

Lastly in **Chapter 4**, I will examine the *Cucurbita pepo* system itself by looking at pesticide usage and possible risks of exposure for *Bombus* and *Apis mellifera*. Specifically, I examine how different methods of neonicotinoid treatment effects the floral display within *Cucurbita pepo*, and if these effects influence the foraging behavior of the observed pollinators. As a majority of studies examining how sublethal exposure affects *Bombus* are lab-based and lacking realistic conditions, I hypothesize that under field conditions *Bombus* will not alter its foraging behavior as a result of neonicotinoids. In doing so, I hope to improve our understanding of how pollinator pesticide exposure can occur and what can be done to mitigate it.

By using an integrative approach to pollinator conservation in an agroecosystem I emphasize the importance of considering all components of an agroecosystem. These suggestions and approaches used are meant to improve our understanding and ability to support pollinators within Mid-Atlantic agroecosystems, through threat mitigation and a more targeted approach to floral provisioning.
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Chapter 2

Targeted Floral Provisioning in Mid-Atlantic Agroecosystems: Adapting Cover Crop Mixtures to Support Generalist Pollinators

Introduction

In the last hundred years, humans have been rapidly reducing biodiversity across the globe. Various human activities have been recognized in causing this decline, with one of the key drivers being agricultural activity and intensification (Tscharntke et al. 2005, Potts et al. 2010, Dupont et al. 2011). Within this trend, the decline of pollinators is recognized as a serious threat to successful plant pollination and thus reproduction, in both agricultural and natural ecosystems (Goulson 2010, Biesmeijer et al. 2006). For pollinators, agricultural intensification can result in the loss of valuable floral resources and nesting habitat, resulting in major stress inflicted upon their populations (Goulson 2010). To combat these stressors and this decline, the augmentation of pollinator habitat and flowering resources has been demonstrated as a useful and successful strategy to support pollinators in agricultural systems (Goulson et al. 2008). Numerous studies have demonstrated the effectiveness of this strategy in supporting pollinators, especially when done strategically and in a targeted manner that considers the surrounding landscape (Klein et al. 2007, Pywell et al. 2011, Scheber et al. 2013).

In perennial agricultural systems floral provisioning is being employed with the addition of flowering strips providing diverse pollen and nectar resources, in what could otherwise be planted as a monoculture. These plantings have been shown to increase native pollinator
populations and the pollination services they provide within adjacent row crops. (Blauuw and Isaacs 2014 (a)). In turn, the retention and subsequent increase in the populations of these native pollinators can actually increase crop yield overtime (Blauuw and Isaacs 2014 (a), Blauuw and Isaacs 2014(b)) which can in turn aid in paying for the associated costs of floral provisioning. Although beneficial for pollinators, the addition of a perennial flowering strip can present challenges to growers utilizing a rotational cropping system, or if land-sparing is not a feasible option within their operation. Initial establishment costs and labor investment can prove to be considerably exhaustive in systems where crop placement is largely unknown for upcoming years. In these systems, the use of annuals versus perennials can provide the same benefits, while increasing the flexibility regarding location and timing of the planting within a system. By integrating these flowering species into current management practices, such as cover cropping, dual-function plantings can actually be created. Cover cropping is advantageous as it can manage weeds through competition (which has proven to be problematic for floral provisioning with herbaceous perennials in agricultural systems) but it also allows for the grower to benefit from pollination services in addition to the more traditional ecosystem services associated with cover crops e.g., nitrogen fixation or weed management.

**Cover Cropping**

By traditional definition, cover cropping is the establishment of a crop, traditionally a grass or legume, to a field that would normally be fallow, resulting in the overall improvement of the agroecosystem through ecological and economic benefits. The traditional planting timeline in the northeast United States consists of seeding of the cover crop in the fall, overwintering, and termination prior to the cash crop. The use of cover crops in an agricultural system is one way in which ecosystem services can be augmented to better meet the needs of the grower. An
ecosystem service is one of the numerous benefits that humans can attain from an ecosystem, examples being; provisioned services in the form of timber acquisition, or supporting services, such as the formation of soil (Millennium Ecosystem Assessment 2005). By doing so, cover crops then can be used for weed management, improving soil health, erosion control, retaining water, reduction of pathogens, retention of nutrients, and indirect management of pest populations through the support of natural enemies. Different benefits can be attained through the use of various management strategies, with factors such as seeding rate, species mixture, and planting date all impacting which ecosystem services are most available to the grower and the functionality of the cover crop planting as a whole (Murrell et al. 2017).

Research on cover crops has broadened their usages and benefits, including the integration of “intercropping” or intermixing cover crops within cash crop plantings. Preliminary research suggests that the use of intercropping and cover crop species may actually increase the availability of nitrogen for the cash crop species, highlighting the need for more studies on the role of intercropping in soil nutrient cycling (Lowry and Brainard 2016). Another research trajectory involves the development of cover crop ‘cocktails’, which are species mixtures with increased functionality due to their diversity. The use of mixtures, as opposed to a single species stand has also been shown to further increase the diversity of benefits and ecosystem services for the grower (Finney et al. 2016). When examining below ground interactions, such as the effects of cover crops to soil nitrogen retention in a system, it has been shown that the functional traits of the cover crops species in addition to the biomass of the cover crops should be considered (Finney et al. 2016). Considering above ground interactions, functional traits of cover crops should also be considered when used to support pollinators, especially in regards to cover cropping as a form of floral provisioning.

Numerous cover crop practices currently support pollinators, assuming they are managed in a way that allows them to reach flowering. Legumes, particularly clovers, have been
commonly used as cover crop species for their nitrogen fixing capabilities. Moreover, the use of clover can be valuable resources for late-season colony growth for bumble bees in particular (Rundlof et al. 2014). Cover cropping can also produce mass flowerings, which through massive resource provisioning can support early-season colony growth in bumble bees (Westphal et al. 2009). For social species, such as bumble bees, considering the phenology of the cover crop bloom in accordance to the phenology of colony needs can better support these pollinators. By marrying the practices of cover cropping with floral provisioning, a more flexible and targeted agri-environmental enhancement scheme can be developed.

Targeted Floral Provisioning

As previously discussed, the provisioning of additional floral resources in the form of flowering strips or cover crops is thought to be beneficial (Goulson et al. 2008) but our understanding of which pollinators are actually supported by these plantings is still growing. Within the United Kingdom different bumble bee species can be differentially attracted to a planting depending on the flowering mixture; long-tongued bee species were more attracted to flowering agricultural legumes whereas short-tongued species were more attracted to a diverse native wildflower mixture (Carvell et al. 2007). Limited to a specific region and set of bumble bee species, these results could vary among geographic regions and landscapes. Accordingly, more comprehensive studies on the degree to which individual plant species are attractive to various pollinators need to be conducted.

To contribute to this field, we examined how a dual-flowering mixture supported pollinators within an agroecosystem. We also examined how different seeding rates affected plant species establishment and growth, and how this could persist into the behavior of the pollinator community. Varying seeding rates were used, as it has been shown that seeding rates can affect
how individual cover crop species perform within a planting (Murrell et al. 2017) and lower seeding rates can also reduce associated costs, which has been shown to be of the major consideration of growers when adopting sustainable agricultural practices (Rodriquez et al. 2009). To focus on an agriculturally relevant pollinator, we targeted our planting phenology to meet the needs of *Bombus*. Research in our lab and from the surrounding region, including Virginia, West Virginia, Maryland, and New York (Schuler et al. 2005, Artz et al. 2011) has repeatedly found that within the pumpkin cropping system, two wild species of primary pollinators are of importance: *Bombus impatiens* and *Peponapis pruinosa*. Because *P. pruinoisa* is a specialist on *Cucurbita* spp., the generalist *Bombus impatiens* is the primary native crop-pollinating species intended to be supported by our floral provisioning. As *Apis mellifera* is an agriculturally relevant pollinator, albeit not native, we also examined their interactions with species within our planting. Lastly, we examined the diversity of native bees present within our planting and which plant species were of most necessity to them. By studying how pollinators are utilizing specific cover crop species through field studies, and what factors may be driving their visitation, we can critically assess the usefulness of our plantings. In our study we will examine the floral density, nutritional value of the pollen, and the structure and size of the flower itself, i.e., the floral area. In doing so we can better predict what factors are important to consider when creating a cover crop mixture and future floral and cover crop plantings can be better targeted to meet the needs of relevant pollinators.
Materials and Methods

Planting Establishment

Plantings were established at Penn State University Russell E. Larson Research and Education Center (RELREC) near Rock Springs, Pennsylvania and at the Southeast Agricultural Research and Extension Center (SEAREC) in Landisville, Pennsylvania. Two planting types were established at both locations, a fall-seeded spring-flowering mixture, and spring-seeded fall-flowering mixture that was designed to fully encompass the majority of time outside of pumpkin bloom, with oats used as a nurse crop intended to winterkill. Plant data were collected on the species establishment, density of growth, and size of floral display. Bee data were collected on visitation per plant within the plot by *Bombus*, plant preference of *Apis mellifera* and the general diversity of native pollinators within the planting. These measures were taken from April until June, and from August until October, representing the two time-spans of bloom during the season. These measurements began in the fall of 2015 and continued until the spring of 2017 at both locations, resulting in two replicates of the spring-flowering and the fall-flowering trials.

Extension agents and regional seed retailers were consulted to provide guidance on the seed mixtures. Commonly used cover crop species within the region that were both cost-effective and known to successfully flower while providing additional ecosystems services were selected to be used in the plantings. The spring-flowering species mixture consisted of: canola (*Brassica napus* ‘Wichita’), Crimson Clover (*Trifolium incarnatum* ‘Dixie’), Hairy Vetch (*Vicia villosa* ‘Purple Bounty’) and Oats (*Avena sativa*). The fall-flowering species mixture consisted of: Buckwheat (*Fagopyrum esculentum*), White Mustard (*Sinapis alba* ‘Braco’), Sunn Hemp (*Crotalaria juncea*), and Sunflower (*Helianthus annuus* ‘Perodovik’). Planting dates were approximately September 10<sup>th</sup> and July 10<sup>th</sup>, respectively. Some variation occurred from year to
year, but plantings were seeded within two weeks of those dates across all years and locations. Specific planting dates for all years can be found in Table 2.1. Prior to planting, sites were burned down with the herbicide Gramoxone (if weed pressure was present) and after the mixture was seeded in the ground, glyphosate was used to kill any weeds that persisted. Three different seeding rates were used to compare how flower density could affect plant growth, bee visitation, and bee diversity at the Rock Springs location. Information on the seeding rates can be found in Tables 2.2 and 2.3. An example of the floral-provisioning and control plots can be found in Figure 2.1.

Control sites

To understand what floral resources existed in our agroecosystem under normal management conditions, a control site was also selected in the nearby vicinity (within ca. 500 m) of our planting. This site was of similar size to the planting itself and was part of a normal management scheme for that farm. On every date that floral sampling occurred within our planting, measures were also conducted in the control site. The same sampling protocol was also used for bee sampling and observations within the control site. In the event that no activity was recorded after ten 1m² quadrats were observed, observations were stopped.

Seeding rates and drill information

The spring-flowering planting at Rock Springs was installed using a no-till Esch drill in strips allowing for the varying seeding rates. Strips were eight feet wide by 12.2 meters long with alley ways ranging between 8 and 10 feet allowing for separation between the strips. It was setup
as a random complete block design, with 3 treatments and 3 reps, and this planting design can be seen in Figure 2-2. Seeding rates ranged from what was considered approximately 100% of the total recommended seed drop in a mixture, to 50% of the recommended inclusion, creating a seeding gradient of approximately 100%, 75%, and 50%.

In the fall of 2015 the fall-flowering mix at our Landisville location was planted with the same 3 seeding rates as can be seen in the Rock Springs location (Table 2.2), but space limitation meant the remainder of the plantings were mass-flowering plantings with no differing seeding rates. The standard seeding rate used in the mass-flowering within the Landisville planting can be found in Table 2.1. The mix was planted using a 17 on a John Deere NoTill Drill. The spring-flowering cover crop planting at Landisville was put in with two different installments. The oats and hairy vetch were put in the grass box of the drill and planted at 2.5 centimeters of depth. Canola and clover were then placed in the large box of a JD 1590 grain drill and planted at a depth of 1.9 centimeters.

**Establishment Measures**

Establishment measures included the germination and establishment of individual species within the planting early in their respective growing seasons. Area cover measures for the spring flowering planting were taken on a single date in April upon development of true leaves for all species, meaning that all plants were 6 inches or shorter in height. For the fall-flowering planting, measures were taken under the same guidelines on a single date in August across all years and locations. Exact dates can be found in Table 2.1. Establishment measures were taken using 0.25 m² quadrat placed randomly within in the planting, using 20 samples per plot, resulting in a total of 80 samples per bloom period e.g., fall or spring, across the two years combined. In the case of Rock Springs where a randomized complete block was used, random placement of a quadrat
Floral Measures

After initial establishment measures were taken, data were collected on the dominant floral cover across the season of bloom. Sampling occurred every 7-10 days, weather permitting. Twenty samples per sampling date, with the exception of the planting located at the SEAREC in fall 2015, when thirty quadrats were taken, per sampling date. Flowers were only counted if they were open, non-wilting, and possessing reproductive parts. For the spring flowering planting, this began in mid-April and lasted until mid-June. For the fall-flowering planting this period began in mid-August and lasted until early October. Exact dates of sampling can be found in Table 2.1.

For both establishment and dominant floral cover measurements, floral cover was quantified by placing 0.25 m² quadrats randomly within the plantings. Data were collected on the total floral cover of each of the target plant species. Total area was calculated by counting the total number of flowering units of a single species within a quadrat for representative flowers, and multiplying that by the average size of floral display for that species. Following each sampling of a planting, five units, and five flowers of each species were measured and averaged for that date. Depending on floral morphology, either the diameter, or the length and width of the flower were taken. Exact method used for each species can be found in Table 2-5. In the case of floral clusters, e.g., buckwheat, measures were taken on the length and width of the flowering cluster in addition to the flower.

In addition to floral cover, data were also collected on the total weed cover and bare ground within a quadrat. In the case that volunteer weed species also occurred within the quadrat, samples were taken and efforts were made to identify plant to species
Bee Observations and Active Sampling

To understand and quantify which pollinators were being supported within the plantings, bee data were collected within 24 hours of a floral sampling date. Measurements were only conducted on dates with the following conditions: >16 °Celsius (61 °F), <3.5 m/s (8 miles/hr) wind, and sunny, partly cloudy or bright overcast. Because of this, starting time ranged from 8:00 until 12:00. Weather measurements were collected before and after each observation period, and data on air temperature, average wind speed, and sky condition were collected using a Kestrel 2000™ thermo-anemometer 28 (www.kestrelmeters.com).

Bee observations were performed for a total of 40 minutes split into two-minute windows (20 total) with the exception of the fall measures performed at SEAREC in 2015, which used 90 second windows. During each of these timed windows, a 1m² quadrat was observed. In the case that the planting was a randomized complete block, quadrats were randomly placed within each block allowing each seeding rate to be appropriately sampled. During each observation period, visitation data were collected on Bombus, e.g., how many visits to a single flower occurred within the given time period. Sight identification to species was done if possible, and differentiation between castes was also noted, i.e., queen or worker. The number of honey bees per quadrat and the plant of visit were also noted. For the remainder of bee species, active sample collection was performed with nets. All successfully collected netted samples were immediately put on ice and plant species collected from was documented. Any missed visitors were identified to a phenotypically descriptive morphospecies category, with plant of visit also being noted. All collected bees were identified to species as part of Project Integrated Crop Pollination Project (icpbees.org), pinned, barcoded, and added to the bee database. Bee species were identified primarily by Jason Gibbs, Assistant Professor and Curator at the University of Manitoba. Vouchers are housed in the Fleischer lab, Department of Entomology, Penn State.
Pollen collection and nutritional analysis

Pollen was collected by hand from six of the floral provisioning species at plantings located at Penn State University Russell E. Larson Research and Education Center (RELREC) and the Southeast Agricultural Research and Extension Center (SEAREC). For plant species where pollen collection was challenging due to small floral parts, e.g., Crimson clover (Trifolium incarnatum), cut flowers were brought back to the lab where they were placed in fresh water. Pollen collection was then done over the following two days with paintbrushes. With some species, pollen production was so minimal they were unable to be analyzed (e.g. buckwheat and white mustard)

To calculate the concentrations of protein, lipid, and carbohydrates within the pollen, two different analyses were performed. The Bradford assay was used to calculate the protein concentration and a modified assay from Van Handel and Day 1988 was used to calculate carbohydrate and lipid concentrations, with more detailed information being included in the Appendix. Approximately three mg of each species were needed for the combination of the lipid, carbohydrate, and protein assays.

Statistical Analyses

All analyses were performed in JMP Pro 13.0 software. A multivariate repeated measure was performed to examine the effect of seeding rate on the dominant floral cover across the season at Rock Springs. Data were pooled from both years of the study. Species and seeding rate were both included as fixed effects within our analysis. A two-way ANOVA was used to examine average floral cover produced across the three seeding rates. Average number of bees within each grouping category per quadrat, per week was calculated. A multivariate repeated measure was
then used to examine the average number of *Bombus* and *Apis mellifera* present during each observation period across the season. Data were pooled from across both years of the study. Because seeding rate was included in the first fall (2015) at Landisville, we could also include site as a factor within the fall analysis (but it was not included in the spring). Plant species, site, and time were included as fixed effects within this analyses. The seeding rates were broken down into three subgroups, with additional information being provided in Table 2.7. Patterns of plant species preferences across the bee groups were examined using principal component analyses. Total number of bees associated with each plant were summarized across all years and all locations for the PCA analyses. To compare the average number of visits a flower received (across the study) with the average cost of seed, and the nutritional value of the pollen, linear regressions were performed. Because all pollen nutritional data was averaged across the season, the other variables were handled similarly e.g., flower visitation.

**Results**

**Establishment and Floral Measures**

Establishment data of the plots across both years and location were plotted to visualize the early growth of the spring-flowering planting in Figure 2.2 and the fall-flowering planting in Figure 2.3. There were no instances when a species did not successfully establish, with establishment relatively similar across all species. Inability to compete by species such as cowpea likely occurred therefore after establishment as the plot developed. The relative contribution of each species to the total floral display at both locations, and the temporal dynamic of this floral display, was examined for both the spring-flowering (Figure 2.4a, b) and fall-flowering plantings (Figure 2.5 a, b). At both spring-flowering plantings, canola was dominant earliest in the season,
followed by crimson clover, and lastly by hairy vetch. Our fall-flowering species bloom order held similar consistency between locations, beginning with buckwheat and ending with a sunflower bloom. White Mustard appeared to bloom later at our Rock Springs location, and more sunn hemp floral display was also observed as compared to Landisville. In addition to the sown species, data were collected on weed presence, represented by the “volunteer” category, which never exceeded 1% of the total cover on any date for either of the spring-flowering or fall-flowering plantings.

Considering seeding rate, there was no effect of seeding rate on total floral area within the planting at the Rock Springs location for both the spring-flowering (Figure 2.6a, $F_{2,490} = 0.06$, $p=0.94$) planting. Plant species ($F_{3,490}=26.68$, $p=<0.001$) was also (expectedly) significant in its effect on total floral cover across the observation period. We performed the same analysis for the Rock Springs fall-flowering planting (Figure 2.7 and found there to be no significant difference across our 3 seeding rates ($F_{2,351}=0.74$, $p=0.47$). Both time ($F_{8,344}=7.74$, $p=<0.0001$) and plant species ($F_{4,351}=30.52$, $p=<0.0001$) were (expectedly) significant in their effect on total floral cover across the observation period. Summarizing the data from across the season and across seeding rates, we found significant differences in total floral area between plant species for both the spring-flowering (Figure 2.6 b) and the fall-flowering (Figure 2.7 b) planting. Performing a two-way ANOVA showed that while seeding rate did not significantly influence the average fall-flowering total floral display across the season, plant species did have an effect ($F_{4,35}=30.52$ $p=<0.0001$). Within the spring-flowering planting, we found that seeding rate did not significantly influence the spring-flowering total floral display ($F_{2,86}=2.48$ $p=<0.08$), whereas plant species did ($F_{2,86}=9.55$ $p=<0.001$).

**Pollen Nutrition**
After analyzing the macronutrient concentrations of the pollen, we ranked which pollen types contained the most protein, lipids, and carbohydrates, and the ratios in which they existed to one another, which is included in Table 2-4. We found the crimson clover to contain the highest amount of protein, followed by the hairy vetch, both legumes within our planting. The sunflower contained the lowest amount of protein, and also had the lowest protein to lipid ratio within our planting. Considering the carbohydrate concentration within the pollen, we found the sunflower had the highest concentration of carbohydrates, followed by the crimson clover.

Floral Area and Bee Composition

Data on flower measurements were synthesized and can be found in Table 2.5, in addition to the method that was used to determine the size e.g., length and width, or diameter. Average size in this table was pooled across all years and locations. Sunflower produced the largest floral area on average per flower (143 cm$^2$), and crimson clover produced the smallest (0.52 cm$^2$), with all other species ranging between) falling between 0.7-7 cm$^2$.

The total bee composition that had either been collected or identified, or sight identified in the field, was used to create a comprehensive list which can be found in Table 2.8. A total of 43 different bee species were identified, with 34 found at Rock Springs and 26 found at Landisville. Within those 43 bees species, four of these species were *Bombus* with two additional morphospecies of *Bombus* observed, but not positively identified. Assessing the biodiversity of the region, or within our planting, was a not the primary goal of this project, so no further analyses were performed, due to both sample size and collecting methods. Spring 2017 samples were not included within this table due to the timing of the study, but will be housed in the same location after identification.
**Bee Behavior within and between Sites**

Our multivariate repeated measure looking at the total number of *Apis mellifera* and *Bombus* counted during a 2-minute observation period each week showed seeding rate did not affect either of these bee taxa. Within *Apis mellifera*, seeding rate did not have a significant effect on the number observed in the fall-flowering ($F_{2,211}=0.76, p=0.48$) or the spring-flowering planting ($F_{2,131}=0.34, p=0.71$). This was also the case for *Bombus* observed within the fall-flowering ($F_{1,211}=11.59, p=0.23$) and spring-flowering planting ($F_{1,131}=0.715, p=0.49$).

We did observe that there was consistently a significant effect of time and plant species on the number of bees observed (Table 2.8). Site was also significant within our fall-flowering planting for *Bombus* ($F_{1,211}=11.59, p=<0.0008$) and *Apis mellifera* ($F_{1,211}=28.11, p=<0.0001$). Although we performed observations under a standard set of weather conditions, differences in seasonal temperature variations could have resulted in earlier emergence of *Bombus* queens between the locations, and thus altered colony sizes at the same point in the seasons. As honey bees are often an artifact of human activity, alteration in colony number in the surrounding region may have manifested within our data.

To explore the cost of the seed, quality of the resource produced, e.g., pollen, and the behavior of the pollinators within our planting, we performed a linear regression that compared the relationships between these variables. Because the nutritional data was the pollen collected from across the season, we reduced the visitation data to the average number of visitors a plant received regardless of seeding rate, or site. We found there to be weak negative relationship between the average number of visitors a flower received and the cost of the seed ($R^2=0.072$). Contrastingly, we found there to be a positive and stronger relationship between the quality of the pollen (as quantified by the P:L ratio) and the cost of the seed. Because of a limitation on the amount of pollen we were able to collect, we were unable to include white mustard or buckwheat.
in our analysis. Although the legumes we included in our mix were more expensive, e.g., hairy vetch, as compared to sunflower, the quality of the pollen could be used in the justification of the cost. Although the higher cost of seed did not always equate to increased rates of visitation. Depending on the pollinator of interest, and the other resources available in area, some higher resource quality plant species may be more beneficial to invest in.

The principal component analyses we performed provided support for which plants were best supporting each bee taxa. In Figure 2.16, or the spring-flowering planting, Components 1 and 2 explained 46.5% and 38.7% of the variation, respectively. Here we observed Axis 1 separated *Apis mellifera* out from the other two bee categories, primarily from their use of crimson clover. *Bombus* were also closely correlated with hairy vetch, which was one of the reasons hairy vetch was selected for this seeding mixture. In Figure 2.17, Components 1 and 2 explained 40.2% and 38.6% of the variation, respectively. Axis 2 separated out *Bombus* from the other two bee categories, on the basis of their use of sunflower.

**Effect of Surrounding Landscape and Control Sites**

To examine differences between location the total number of bees observed at each site across *Bombus*, *Apis mellifera* and other bees, observations within the planting and within the control at each location were summarized; this data can be found in Table 2.9. There was a noticeable higher number of *Bombus* at Rock Springs, as compared to Landisville (Table 2.9) whereas Landisville had much higher numbers of honey bees in the fall. This could be largely attributed to the landscape composition and its usage with in the immediate region surrounding our sites.

The most dominant plant species at all of our control sites were dandelion and white clover. There was a dearth of flowering resources at our control sites because of strict
management and consequently total bee counts were very low. Data for the total number of each bee taxa collected at control locations can be found in Table 2.9. The control sites had >20 bees across all seasons and locations, whereas our floral provisionings had >100 bees across all seasons and locations.

**Discussion**

The main objective of this study was to examine if, and how, floral provisioning could be targeted to fit a specific agroecosystem within the mid-Atlantic region, and whether varying attributes could be manipulated to better meet the needs of both relevant pollinators and the grower. By altering seeding rate within our study, we explored techniques for reducing costs associated with floral provisioning, while still supporting pollinator populations. Assessing which plant species were attractive to various bee species allowed us to ascertain which plants may be the most necessary to include in plantings, with our nutritional analysis of the pollen providing us a more comprehensive understanding of the resources provided. Examining the associated costs and how these could be reduced through alteration of seeding rates, we explored options to improve adoption of this practice. By utilizing this integrative approach to designing floral provisioning plantings, we were able to collect data that will aid in supporting pollinators in agroecosystems.

**Plant behavior**

Looking at how each of the plant species fared within our planting, provides us with the information to optimize floral provisioning plantings using cover crops. Our spring-flowering
mixture initially included Austrian winter pea, and our fall-flowering mixture initially included cowpea, but neither of these species were able to successfully compete within our mixtures, causing us to drop them from the planting (data not shown). Despite the benefits they have been noted to provide, e.g., extrafloral nectaries within cowpea, a different planting technique should be used if they are to be included in mixtures to support pollinators. We found that sunn hemp struggled within our mixture at times, either as a result of being outcompeted or due to the climatic zone. Although it did survive in our mixture, it would be most advantageous to include within warmer growing zones. Despite the fact we were on the outskirts of the growing region of crimson clover as winter annual, we found that it produced quite full stands and was a well-utilized species within our planting in both years.

**Seeding Rate**

As expected, plant species had a significant effect on total floral cover across the observation period. Our findings, however, demonstrate that altering seeding rates did not significantly impact the total floral area of the planting. Our three seeding rates did not result in a significantly different amount of floral cover at the Rock Springs spring-flowering or fall-flowering planting. We also saw minimal weed presence in all of our plantings, which was represented by all “volunteer” categories within our plantings, regardless of the seeding rate, which could suggest that the lowest seeding rate we selected was enough to both suppress weeds. The fall-flowering mix, which costs an average of $1.40 per pound would cost the grower approximately $70 if employed at the 50 lb/acre seeding rate. Dropping the seeding rate down to 30 lb/acre would reduce the cost by almost half while maintaining efficacy, which could be critical when working with larger pieces of land or over a period of time.

Because the average floral display of the species we used varied dramatically, e.g.,
sunflower vs. buckwheat, it was not surprising that the total floral area was significantly affected by the plant species. Our planting was designed to provide a sequential bloom across the season, meaning that at certain time points total floral area was primarily dominated by a single species. As each plant species used within our planting varied with its floral display, the ensuing significant effect of time was a natural progression of the planting. More importantly, seeding rate was not significant on the total floral area of the planting, suggesting that seeding rate is less critical in maintaining a floral cover within a planting.

**Seeding Rate Influence on Bee Behavior**

Our findings showed that within both the fall and spring-flowering plantings, seeding rate did not have a significant effect on the average number of *Apis mellifera* or *Bombus* counted. As we found there to be no effect of seeding rate on the average floral area across the season in both our fall-flowering (Figure 2.8) and spring-flowering (Figure 2.10) planting, the lack of an effect within the bee behavior reinforces this. With this knowledge, growers are allowed flexibility to select a seeding rate that will best meet the horticultural needs of their plantings, without impacting the pollinator community or the ecosystem services they provide.

**Influence of Time and Location on Bee Behavior**

Although seeding rate did not influence the average number of *Bombus* or *Apis mellifera* observed in our plantings, we found that the time and plant species did have a significant effect on the number of bees observed (Table 2.8). The natural phenology of a bumble bee colony results in altered numbers of bees out foraging for resources, which could explain the effect of
time. Secondarily, although we did not track honey bee colony placement (as there were none in the immediate vicinity), it would be assumed that unseen colonies could have been moved at any point in the season, resulting in a variation in the number out foraging. There was also a significant interaction between the plant species and time, which would also explain this result. One of the more interesting findings was the discrepancy in the total number of bumble bees and honey bees observed between our locations (Table 2.9). In particular, this speaks to the effect of the surrounding landscape on the pool of pollinators that will be supported by a floral provisioning planting and are actively providing a crop with the service of pollination. A multi-scale study would be beneficial in understanding how landscape habitat composition affects the total species pool of the region, and how agri-environmental management schemes could be altered to better support these bee species.

**Plant Species Attractiveness between Pollinator Groups**

Instrumental in our study were the data that correlated different plant species with the bees that were visiting them. Floral morphology, phenology, resources, color, scent, and a multitude of both known and unknown factors can all influence what will and can visit a specific species. Looking at our principal component analyses, our data suggested that different plant species had different levels of importance across bee taxa. For example bumble bees tended to visit hairy vetch, and we also observed *Bombus* queens visiting hairy vetch in the spring, which was one of the critical goals of our planting. Crimson clover was more frequently utilized by honey bees than hairy vetch. Hairy vetch also had perhaps one of the more complex morphologies, which may have affected handling time of pollinators depending on both their age and experience (Laverty 1994) and affected the total number of bees utilizing it. Canola demonstrated higher attractiveness to honey bees, with crimson clover utilized at some points as
well. Overall, our spring-flowering planting supported the generalist pollinators we intended, e.g., *Bombus* and *Apis mellifera*, with also a surprisingly diverse assemblage of native bees collected (Table 2.6).

Our fall-flowering planting, which consisted of sunflower, sunn hemp, buckwheat, and white mustard, demonstrated much more selectivity to individual plant species across the different bee taxa. Sunflower was often utilized bumble bees, and buckwheat often visited by honey bees and other bees (Figure 2.17). Despite sunn hemp providing some of the higher quality of pollen (when considering protein content), it proved to be rarely visited, which could be a combination of the flower morphology and the overall density within the planting. Although sunflower could be considered the lowest quality pollen within our planting in terms of protein content and P:L ratio (Table 2.5), the large floral display as compared to that of our other species could explain the higher number of visitors, in particular by bumble bees, as larger floral displays, regardless of floral rewards, have been shown to attract higher numbers of inexperienced bees (Makino and Sakai 2007). Furthermore, the structure of buckwheat lends itself to that of smaller visitors, e.g., honey bees and other bees, with anecdotal field observations (EDT) suggesting that larger bumble bees and carpenter bees are often too heavy to be supported by an inflorescence of buckwheat. Overall, we found that our fall-flowering planting, with the exception of sunn hemp, was well utilized by the generalist pollinators we intended, as well as a diverse assemblage of native pollinators.

**Conclusions**

While it has been shown by numerous studies that floral-provisioning within agroecosystems can be beneficial in supporting pollinators (Klein *et al.* 2007, Pywell *et al.* 2011, Goulson *et al.* 2008), there exists a gap in research uniting applied practices, e.g., cover cropping,
with basic questions concerning plant species attractiveness and floral resource quality. Our study attempts to address this question, by examining the seeding rate, the floral area, the pollinator community, and the quality of the floral resources provided. We demonstrate that floral provisioning can be adapted to fit into a rotational agroecosystem through use of cover crop species while also remaining cost effective for the grower through manipulation of seeding rate. Future studies should also examine how and if, yield of crops within the agroecosystem are affected, which would further play into the cost-benefit analysis for the grower. Furthermore, we demonstrate that alteration of seeding rates does not affect the total floral cover of the planting, and also, the number of bees utilizing this planting. To further disentangle the effects of seeding rates on pollinators, future work should also examine if floral attractants, e.g., pollen or nectar quality, or even scent, are affected through variation of seeding rate. With these findings in mind, a tangible goal of a planting should be decided by the grower prior to its planting e.g., enhancing bee richness and diversity, conserving agriculturally relevant bee species, provisioning of high quality resources, soil nutrient retention with bee conservation as a peripheral benefit, etc. To aid in accomplishing these goals, more rigorous studies conducted across a variety of climatic zones and agroecosystems need to be at the forefront of this research area.
References


Table 2-1 Dates of Seeding and Measures

<table>
<thead>
<tr>
<th>Location</th>
<th>Planting Date</th>
<th>Date of Establishment Measures</th>
<th>Dates of Floral Density and Bee Measures</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fall-Flowering</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>July 7th, 2016</td>
<td>August 2nd, 2016</td>
<td>August 9th 2016 - September 29th, 2016</td>
</tr>
<tr>
<td></td>
<td>July 18th, 2016</td>
<td>August 2nd, 2016</td>
<td>August 10th - 2016 September 9th, 2016</td>
</tr>
<tr>
<td><strong>Spring-Flowering</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rock Springs</td>
<td>September 11th, 2015</td>
<td>April 10th, 2016</td>
<td>April 23th, 2016 - June 14th, 2016</td>
</tr>
<tr>
<td></td>
<td>September 9th, 2016</td>
<td>February 24th, 2017</td>
<td>April 26th, 2017 - June 15th, 2017</td>
</tr>
<tr>
<td></td>
<td>September 19th, 2016</td>
<td>March 6th, 2017</td>
<td>April 26th, 2017 - June 15th, 2017</td>
</tr>
</tbody>
</table>

Table 2-2 Rock Springs Seeding Rates

<table>
<thead>
<tr>
<th>Fall-flowering</th>
<th>Cover Crop</th>
<th>Variety</th>
<th>Treatment 1 100%</th>
<th>Treatment 2 75%</th>
<th>Treatment 3 50%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(lbs/ac)</td>
<td>(kg/ha)</td>
<td>(lbs/ac)</td>
</tr>
<tr>
<td>Sunn Hemp</td>
<td>n/a</td>
<td></td>
<td>8</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Sunflower</td>
<td>Perodovik</td>
<td></td>
<td>10</td>
<td>11.2</td>
<td>7.5</td>
</tr>
<tr>
<td>White Mustard</td>
<td>Braco</td>
<td></td>
<td>5</td>
<td>5.6</td>
<td>3.75</td>
</tr>
<tr>
<td>Buckwheat</td>
<td>n/a</td>
<td></td>
<td>18</td>
<td>20.1</td>
<td>13.5</td>
</tr>
<tr>
<td>Cowpeas</td>
<td>Iron &amp; Clay</td>
<td></td>
<td>15</td>
<td>16.8</td>
<td>11.25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Spring-flowering</th>
<th></th>
<th></th>
<th>(lbs/ac)</th>
<th>(kg/ha)</th>
<th>(lbs/ac)</th>
<th>(kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oats</td>
<td>Armor</td>
<td>30</td>
<td>33.6</td>
<td>30</td>
<td>33.6</td>
<td>30</td>
</tr>
<tr>
<td>Canola</td>
<td>Wichita</td>
<td>5</td>
<td>5.6</td>
<td>3.75</td>
<td>4.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Crimson Clover</td>
<td>Dixie</td>
<td>20</td>
<td>22.4</td>
<td>15</td>
<td>16.8</td>
<td>10</td>
</tr>
<tr>
<td>Hairy Vetch</td>
<td>Purple</td>
<td>15</td>
<td>16.8</td>
<td>11.25</td>
<td>12.6</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Buckwheat: n/a
Table 2-3  Landisville Seeding Rates

<table>
<thead>
<tr>
<th>Cover Crop</th>
<th>Variety</th>
<th>Species</th>
<th>Rate (lbs/A)</th>
<th>Rate (kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunn Hemp</td>
<td>n/a</td>
<td>Crotalaria juncea</td>
<td>8</td>
<td>9.0</td>
</tr>
<tr>
<td>Sunflower</td>
<td>cv. Perodovik.</td>
<td>Helianthus annuus</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>White Mustard</td>
<td>Braco</td>
<td>Sinapis alba</td>
<td>5</td>
<td>5.6</td>
</tr>
<tr>
<td>Buckwheat</td>
<td>n/a</td>
<td>Fagopyrum esculentum</td>
<td>20</td>
<td>22.4</td>
</tr>
<tr>
<td>Cowpeas</td>
<td>Iron &amp; Clay</td>
<td>Vigna unguiculata</td>
<td>15</td>
<td>16.8</td>
</tr>
</tbody>
</table>

* only included for one year and then dropped from mix

Table 2-4 Pollen Macronutrient Concentrations

<table>
<thead>
<tr>
<th></th>
<th>Protein</th>
<th>Lipid</th>
<th>P:L</th>
<th>Carbohydrate</th>
<th>P:C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crimson Clover</td>
<td>147.61</td>
<td>33.11</td>
<td>4.46</td>
<td>258.00</td>
<td>0.57</td>
</tr>
<tr>
<td>Hairy Vetch</td>
<td>36.51</td>
<td>12.93</td>
<td>2.82</td>
<td>203.85</td>
<td>0.18</td>
</tr>
<tr>
<td>Canola</td>
<td>136.25</td>
<td>70.54</td>
<td>1.93</td>
<td>124.31</td>
<td>1.10</td>
</tr>
<tr>
<td>Sunnhemp</td>
<td>123.54</td>
<td>71.95</td>
<td>1.72</td>
<td>123.10</td>
<td>1.00</td>
</tr>
<tr>
<td>Pumpkin</td>
<td>140.73</td>
<td>96.78</td>
<td>1.45</td>
<td>43.28</td>
<td>3.25</td>
</tr>
<tr>
<td>Sunflower</td>
<td>116.23</td>
<td>116.09</td>
<td>1.00</td>
<td>302.08</td>
<td>0.38</td>
</tr>
</tbody>
</table>
Table 2-5 Method used to Measure Flower and Average Size of a Flower

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>Method</th>
<th>Average Flower Area cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canola</td>
<td>Diameter</td>
<td>2.83</td>
</tr>
<tr>
<td>Crimson Clover</td>
<td>Length*Width</td>
<td>0.52</td>
</tr>
<tr>
<td>Hairy Vetch</td>
<td>Length*Width</td>
<td>0.84</td>
</tr>
<tr>
<td>Buckwheat</td>
<td>Diameter</td>
<td>1.33</td>
</tr>
<tr>
<td>Sunflower</td>
<td>Diameter</td>
<td>143.14</td>
</tr>
<tr>
<td>White Mustard</td>
<td>Diameter</td>
<td>1.32</td>
</tr>
<tr>
<td>Sunn Hemp</td>
<td>Length*Width</td>
<td>6.44</td>
</tr>
</tbody>
</table>

Table 2-6 Landisville and Rock Springs species level identifications

<table>
<thead>
<tr>
<th>Genus</th>
<th>species</th>
<th>Fall-Flowering</th>
<th>Spring-flowering</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L</td>
<td>RS</td>
</tr>
<tr>
<td>Agapostemon</td>
<td>virescens</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Andrena</td>
<td>carlini</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Andrena</td>
<td>dunningi</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Andrena</td>
<td>forbesii</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Andrena</td>
<td>imitatrix</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Andrena</td>
<td>milwaukeeensis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Andrena</td>
<td>miserabilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Andrena</td>
<td>nasonii</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Andrena</td>
<td>perplexus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Andrena</td>
<td>rugosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Andrena</td>
<td>virginiana</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Andrena</td>
<td>wilkella</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apis</td>
<td>mellifera</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Augochlora</td>
<td>aurata</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Bombus</td>
<td>bimaculatus*</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Bombus</td>
<td>fervidus*</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Bombus</td>
<td>griseocollis*</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Bombus</td>
<td>impatiens</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Calliopsis</td>
<td>andreniformis</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>
**Ceratina mikmaqi** X  
**Colletes inaequalis** X  
**Halictus confusus** X X  
**Halictus ligatus** X X  
**Halictus rubicundus** X X  
**Lasioglossum callidum** X X  
**Lasioglossum cinctipes** X  
**Lasioglossum ephialtum** X  
**Lasioglossum hitchensi** X X  
**Lasioglossum imitatum** X X  
**Lasioglossum laevissimum** X  
**Lasioglossum lineatulum** X X  
**Lasioglossum nymphaearum** X  
**Lasioglossum paradigmirandum** X X  
**Lasioglossum pilosum** X X X  
**Lasioglossum spp.** X X X  
**Lasioglossum truncatum** X X  
**Lasioglossum versatum** X  
**Megachile brevis** X  
**Melissodes trinodis** X  
**Osmia cornifrons** X  
**Peponapis pruinosa** X X  
**Syrphidae spp.** X X X X  
**Xylocopa virginica** X X X X

*individual was not collected and identified, but rather identified by the observer through sight.*

**Table 2-7 Seeding rate symbols broken down**

<table>
<thead>
<tr>
<th>Seeding Rates</th>
</tr>
</thead>
</table>
| A  | 15-30 kg/ha  
| B  | 31-50 kg/ha  
| C  | 60-80 kg/ha  

Table 2-8 Effect of Site, Time, Plant Species, and the interaction between Time and Plant Species on the Average Number of Bees Observed in a Given Week

<table>
<thead>
<tr>
<th>Site</th>
<th>Time</th>
<th>Plant Species</th>
<th>Time*Plant Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spring</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>flowering</td>
<td><strong>Bombus</strong></td>
<td>Not applicable</td>
<td><em>(F&lt;sub&gt;7&lt;/sub&gt;,&lt;sub&gt;125&lt;/sub&gt;=0.76, p=0.62)</em> (F&lt;sub&gt;3&lt;/sub&gt;,&lt;sub&gt;131&lt;/sub&gt;=44.21, p=0.0001) (F&lt;sub&gt;21&lt;/sub&gt;,&lt;sub&gt;359.50&lt;/sub&gt;=7.31, p=&lt;0.0001)</td>
</tr>
<tr>
<td></td>
<td><strong>Apis mellifera</strong></td>
<td>Not applicable</td>
<td>*(F&lt;sub&gt;8&lt;/sub&gt;,&lt;sub&gt;124&lt;/sub&gt;=1.09, p=0.38) (F&lt;sub&gt;3&lt;/sub&gt;,&lt;sub&gt;131&lt;/sub&gt;=1.72, p=0.17) (F&lt;sub&gt;24&lt;/sub&gt;,&lt;sub&gt;360.24&lt;/sub&gt;=11.35, p=&lt;0.0001)</td>
</tr>
<tr>
<td><strong>Fall</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>flowering</td>
<td><strong>Bombus</strong></td>
<td>F&lt;sub&gt;1&lt;/sub&gt;,&lt;sub&gt;211&lt;/sub&gt;=11.59, p=&lt;0.0008 F&lt;sub&gt;8&lt;/sub&gt;,&lt;sub&gt;204&lt;/sub&gt;=2.33, p=0.0203 F&lt;sub&gt;8&lt;/sub&gt;,&lt;sub&gt;204&lt;/sub&gt;=2.33, p=0.0141 F&lt;sub&gt;24&lt;/sub&gt;,&lt;sub&gt;592.3&lt;/sub&gt;=4.84, p=&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Apis mellifera</strong></td>
<td>F&lt;sub&gt;1&lt;/sub&gt;,&lt;sub&gt;211&lt;/sub&gt;=28.11, p=&lt;0.0001 F&lt;sub&gt;8&lt;/sub&gt;,&lt;sub&gt;204&lt;/sub&gt;=3.69, p=0.0005 F&lt;sub&gt;3&lt;/sub&gt;,&lt;sub&gt;211&lt;/sub&gt;=19.85, p=&lt;0.0001 F&lt;sub&gt;24&lt;/sub&gt;,&lt;sub&gt;592.26&lt;/sub&gt;=9.70, p=&lt;0.0001*</td>
<td></td>
</tr>
</tbody>
</table>

*Bolded boxes are significant

Table 2-9 Number of bees across different taxa counted at both locations and their respective controls

<table>
<thead>
<tr>
<th></th>
<th>Rock Springs (Fall)</th>
<th>Rock Springs (Fall Control)</th>
<th>Rock Springs (Spring)</th>
<th>Rock Springs (Spring Control)</th>
<th>Landisville (Fall)</th>
<th>Landisville (Fall Control)</th>
<th>Landisville (Spring)</th>
<th>Landisville (Spring Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bombus</strong></td>
<td>396</td>
<td>10</td>
<td>260</td>
<td>3</td>
<td>190</td>
<td>1</td>
<td>128</td>
<td>0</td>
</tr>
<tr>
<td><strong>Apis mellifera</strong></td>
<td>339</td>
<td>6</td>
<td>472</td>
<td>6</td>
<td>811</td>
<td>4</td>
<td>342</td>
<td>13</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td>218</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>152</td>
<td>0</td>
<td>136</td>
<td>6</td>
</tr>
</tbody>
</table>
Figure 2.1 Examples of a (a) spring-flowering floral provisioning plot at our Rock Springs site, versus our (b) control site.
Figure 2.2 Example of plot setup with the various seeding rates with each letter representing a different seeding rate e.g., 50%, 75%, 100%.

Figure 2.3 Frequency distribution of % cover (cm² cover / m²) of spring-flowering species measured at the time of initial establishment (Data pooled across locations and years, N=80)
Figure 2.4 Frequency distribution of % cover (cm\(^2\) cover / m\(^2\)) of fall-flowering species measured at the time of initial establishment (Data pooled across locations and years, N=80)

a)
Figure 2.5 Spring-flowering (a) Landisville and (b) Rock Springs average floral cover within a 0.25 m² quadrat. Data were averaged across seeding rate and year.
Figure 2.6 Fall-flowering (a) Landisville and (b) Rock Springs dominant floral cover averaged across both seeding rate and year. Measures were taken with a 0.25 m² quadrat.

Figure 2.7 Rock Springs spring-flowering planting total floral cover averaged across seeding rates and year (F<sub>2,490</sub>=0.0592, p=0.94)
Figure 2.8 Rock Springs spring-flowering planting seasonal total of floral cover between plant species and seeding rates. (2-way ANOVA seeding rate ($F_{2,868}=2.48$) $p=<0.08$), plant species ($F_{2,868}=9.55$) $p=<0.001$)

Figure 2.9 Rock Springs fall-flowering planting total floral cover averaged across seeding rates and year ($F_{2,351}=0.7416$ $p=0.47$)
Figure 2.10 Rock Springs fall-flowering planting total floral cover between plant species (2-way ANOVA Seeding Rate ($F_{2,35}=0.74$) $p<0.47$), Plant species ($F_{4,35}=30.52$) $p<0.0001$).

Figure 2.11 Repeated measure of the average number of *Apis mellifera* observed within a 1m$^2$ quadrat during each individual week within the spring-flowering planting ($F_{2,131}=0.34$, $p=0.71$).
Figure 2.12 Repeated measure of the average number of *Bombus* observed within a 1m² quadrat observation period during each individual week within the spring-flowering planting ($F_{1,131}=0.715, p=0.49$)

Figure 2.13 Repeated measure of the average number of *Apis mellifera* observed within a 1m² quadrat during each individual week within the fall-flowering planting ($F_{2,211}=0.76, p=0.48$)
Figure 2.14 Repeated measure of the average number of Bombus observed within a 1m$^2$ quadrat during each individual week within the fall-flowering planting ($F_{1,211}=11.59, p=0.23$)

Figure 2.15 Linear regression of the average number of visitors a flower received across all bee taxa, ($Y=8.114+0.4926*\text{Cost}, R^2=0.071$), and the quality of the resources provided, as related to the approximate cost of the seed per lb ($) ($Y=0.6878+0.3538*\text{cost}, R^2=0.262$)
Figure 2.16 Principal component analysis based on the number of bees that were noted visiting different spring-flowering plant species

Figure 2.17 Principal component analysis based on the number of bees that were noted visiting different fall-flowering plant species
Chapter 3

Exploring the Effects of Pollen Nutrition on the Health and Fitness of Bombus impatiens Through the Use of Microcolonies

Introduction

Floral provisioning plantings are designed to broadly support managed and native pollinators in systems where floral resources may otherwise be missing, or limited to a single species. By providing diverse assemblages of plantings, bees can gather pollen, providing them with their primary sources of lipids and proteins, and nectar, providing them with their primary source of carbohydrates. The proteins and lipids contained in the pollen are crucial for larval development, and the carbohydrates in nectar provide adults with the necessary energy for flight and foraging (Brodschneider et al. 2010, Heinrich 1975). Compared to managed bees, less is known about the necessity of pollen in the diet of adult native bees, but recent studies have found that pollen ingestion is critical for egg development for adult solitary bee species (Michener 2007, Cane 2016). Because of the necessity of these resources, nectar and pollen are important not only on the individual level in solitary species, but also for colony growth and establishment in social species (Goulson 2010). The necessity of these rewards, in combination with floral characteristics, e.g., color, shape, size, and scent, drive this complex relationship between pollinators and flowers.

Pollen

For a plant, it has been assumed that the primary purpose of pollen architecture is to protect the internal genetic material and allow for successful reproduction. This is achieved
primarily with a thick protective layer, known as the exine, which is comprised of complex carbohydrates that can be difficult for animals to digest (Roulston and Cane 2000). External to the exine is the pollenkitt, a layer consisting of fatty hydrocarbons that in addition to a plethora of other potential functions, may actually provide pollinators with cues that could dictate pollen selection (Pacini and Hesse 2005). The combined nutrition of these layers provide pollen-feeding organisms some of the essential nutrients necessary for their survival. If, and to what extent, pollinators are driving selection of pollen macronutrient levels is largely unknown, but studies suggest that pollen macronutrient levels may be conserved within plant genera and families rather than driven by pollinator visitation (Roulston et al. 2000).

The variation in pollen nutrition and the implications on bee health and development had not been as seriously considered as nectar had until recently. Although pollen nutritional profiles remain relatively static within species, some abiotic conditions can alter them, which can also be observed in pollinator foraging behavior. A study recently found that Cucumis sativus grown in vermicomposted amended soil produced pollen with higher protein content as compared to chemically fertilized potted soil (Cardoza et al. 2012). Furthermore, Bombus impatiens workers fed on pollen from these C. sativus grown in vermicomposted soils increased the amount of time spent on each flower and had an increased ovary size. In addition to adapting foraging behavior to variation within a single plant species, bees also adapt their foraging strategies across plant species in response to variation in pollen resources. Honey bees have been demonstrated to prioritize pollen quantity over quality, visiting larger resource patches, whereas bumble bees prioritized “high quality” pollen over larger resource patches (Leonhardt and Blüthgen 2012). Additionally, bumble bees may actually preferentially forage for pollen that falls within a specific range of protein to lipid ratios (Vaudo et al. 2016). In addition to seeking higher quality pollen, it has been demonstrated that bumble bees may actually increase foraging distances for more
diverse patches of resources (Jha and Kremen 2013). The previous findings suggest that both the quality of the resource and the diversity of resources available drive foraging behavior.

As *Bombus* foraging behavior appears to be highly driven by pollen quality, this begs the question of what physiological need drives the compulsion for this “high quality” pollen. Pollen quality has been shown to affect the reproductive capacity and development of the individual within *Apinae*, but additionally, the quality of the pollen must also meet the colony needs as a whole (Génissel *et al.* 2002). How exactly bees adapt their pollen foraging behavior to meet the needs of the colony is still unclear, but studies show it to be tightly regulated within *Apinae* (Kitaoka and Nieh 2009). In the previous study, it was also shown that bumble bees increase their rate of foraging depending on the amount of pollen stores, and also, when exposed to pollen odors if stores of pollen were low. An improved understanding of how pollen quality can affect the various caste levels within a colony will be necessary to understanding the drivers of foraging behavior.

**Nectar**

Nectar has been extensively studied for its function as a pollinator attractant and as the primary reward for pollinators (Baker and Baker 1983). As a floral reward, nectar volume, sugar composition, and sugar concentration all drive pollinator visitation (Cnaani *et al.* 2006). Most plant nectar is composed of three different sugars, glucose, sucrose, and fructose, although some minor sugars are also present in various plant species (Nicholson and Thornburg 2007). Compared to pollen, nectar demonstrates much more nutritional plasticity both within single species and across species (Nicholson and Thornburg 2007, Herrera *et al.* 2006, Nicolson and Van Wyk 1998). Sugar concentrations, and relatedly viscosity, within nectar, are particularly vulnerable to a number of abiotic conditions, including: humidity, soil conditions, water content,
and even the presence of yeasts and bacteria (Herrara et al. 2006). In addition to these factors, recent studies have found that historical, environmental, and evolutionary processes may also shape the nectar chemistry (Chalcoff et al. 2006).

Because of the variability of nectar and the numerous factors that can alter nectar chemistry, it can be difficult to generalize pollinator-host preference. Although honey bees, which are considered long-tongue bees, are seemingly more attracted to sugar concentrations between 30%-50% and short-tongued bees are more attracted to nectar between 45%-60% (Roubik and Buchman 1984), sugar concentration, and thus pollinator attraction, should be considered in accordance with environmental, historical, and any other source of variation. Furthermore, nectar chemistry has evolved to be quite complex in some species, containing proteinaceous and non-proteinaceous amino acids, secondary compounds, and even antimicrobial compounds (Nepi et al. 2012). Although these components have been less studied, amino acids may actually be critical to our understanding of pollinator behavior, as some such as proline, are phagostimulatory and also important for flight in some pollinators (Nepi et al. 2012). Despite the extensive consideration of nectar for its role as a pollinator attractant and manipulant, less has been given to its role in maintaining pollinator health and fitness. The complexity of nectar chemistry reveals that numerous unanswered questions exist in our understanding of the effects nectar may have on bee health and development.

Foraging behavior

Pollinator foraging behavior is thought to be driven by floral traits involved in a complex co-evolutionary relationship over millions of years. These suites of floral traits are been broadly described as pollination syndromes, creating a general, albeit, incomplete framework for classifying plant-pollinator relationships. Although pollen and nectar quality are not traditionally
considered in pollination syndromes, how, and to what extent they influence pollinator foraging behavior must be considered. Examining the implications of floral rewards on pollinator health must be examined in order progress this field. We aimed to contribute to this field by examining the pollen quality of *Cucurbita pepo*, a species that has been extensively studied for its unique nectar attributes, but less so for its pollen. Field observations show that *C. pepo* is a less attractive resource for a range of pollinators (Sidhu 2013), including *Bombus*, when *Solanum spp.* were present (Fleischer unpubl.). Additionally, in approximately 21 hours of observed bumble bee and honey bee foraging within *C. pepo*, no instances were recorded of active pollen collection (personal observations, EDT). The previous information led us to the hypothesis that attributes of pumpkin pollen, whether macronutrient ratios or secondary compounds, make it an undesirable resource for *Bombus*.

Because of the hypothesized unattractive nature of pumpkin pollen, providing *Bombus* within this system with additional floral resources could be increasingly important, particularly in regards to health and fitness. To test this hypothesis, we conducted no-choice bioassays using queenless *B. impatiens* microcolonies and pollen from pumpkin, two common cover crop species from two plant families commonly visited by *Bombus*, a multifloral diet, and an artificial honey bee diet. We examined how pollen from these cover crops compared to the pollen provided in mass amounts by *Cucurbita pepo*. In doing so, we were able to remove floral signals, nectar, foraging distance and any other factors that could affect pollinator health and fitness. Expanding on the hypothesis that pollen macronutrient ratios drive foraging behavior in bumble bees (Vaudo et al. 2016), we aim to understand if pollen macronutrient ratios may also be affecting their health and fitness. We examined health and fitness by looking at each individual bee and their average ovary size and the average weight change. Furthermore, for each microcolony, we examined whether or not wax was produced, which can be seen as a critical aspect of colony maintenance for workers.
Materials and Methods

Microcolonies

Three *Bombus impatiens* colonies per replicate were purchased from Koppert Biological Systems (Howell, MI). These parental colonies were fed commercial sugar water and held at approximately 30% relative humidity and 29 °Celsius for 48 hours between colony arrival and the beginning of the experiment. During the experiment, conditions were monitored using HOBO (Onset Corporation™) data loggers. This experiment was repeated twice: in January 2016 and December 2016, in a complete randomized block design, with 5 treatments in the January block, 6 treatments in the December block, and 5 replicates (microcolonies) per treatment and block. Measurements were taken from queen-less microcolonies, an effective method of examining the effects of diet quality on bumble bees (Regali and Rasmont 1995). To create these microcolonies, three adult *B. impatiens* workers similar in size were removed from the queenright parental colonies. Each individual was chilled at -20 °Celsius until movement slowed, weighed (ML54 Analytical Balance, Mettler Toledo, Switzerland) and marked (red, blue, or no color, TESTORS® enamel marker [http://www.testors.com/](http://www.testors.com/)) before being added to a 6.6 × 8.3 × 9.5 cm vented plexiglas chamber. Microcentrifuge tubes with a hole (2.0 μl) were placed thru the top of the microcolonies and filled with a 50% sucrose solution, which was changed out daily.

Diet Treatments

In the January 2016 replicate, pollen from sunn hemp (*Crotalaria juncea*) and sunflower (*Helianthus annuus*) was collected by hand from flowers within floral provisioning plantings at the Russell E. Larson Research and Education Center (RELREC) near Rock Springs, PA, and the
Southeast Agricultural Research and Extension Center (SEAREC) in Landisville, PA. Pollen for this January 2016 replicate was collected from September-October of 2015 and kept at -20° Celsius until the start of experiment. Pumpkin (*Cucurbita pepo*) pollen for the January 2016 replicate was collected in Columbia County in 2014 by Anthony Vaudo of Pennsylvania State University and kept at -20 ° Celsius until the start of the experiment. For the December 2016 replicate, sunn hemp, sunflower, and pumpkin pollen was collected from September-October of 2016 following the same protocol at the Russell E. Larson Research and Education Center from September-October of 2015.

**Diet Treatments and Ambient Conditions**

Treatments consisted of six different diets, each given to five microcolonies: (i) Sunflower (*Helianthus annuus*), (ii) Sunn hemp (*Crotalaria juncea*), (iii) Pumpkin (*Cucurbita pepo*), (iv) Multifloral (*Gaillardia pulchella, Rudbeckia hirta, Coreopsis tinctoria, Cucurbita pepo* and *Chamaecrista fasciculate*), (v) MegaBee, and (vi) sucrose syrup only. The January 2016 replicate included sunflower, sunn hemp, pumpkin, MegaBee, and exclusively 50% sucrose. The December 2016 replicate added the multifloral treatment. The 50% sucrose syrup was also provided to all microcolonies. In the January 2016 replicate, plastic royal jelly thimbles were placed at the bottom of the microcolony and filled with the various pollen species, using 1 thimble per pollen treatment. These thimbles were replaced with 7 mL square weigh boats for the December 2016 replicate. Microcolonies were provided with both 50% sucrose syrup and pollen *ad libitum*. Fresh pollen was added daily and microcolonies were inspected for wax production. Two drops of sucrose syrup were added to the pollen within their respective microcolony containers to form a paste-like substance. Total weight of all pollen and the 50% sucrose syrup was unable to be calculated due to the bees removing pollen without consumption from the
feeding dishes.

Relative humidity was kept at approximately 30% and ambient temperature was approximately 30 °Celsius. The experiment ran for seven days and any bees that died during the course of the experiment were removed and date of removal was noted. At the end of the seven days all bees were once again weighed and stored at -20 °Celsius for dissection. For the seven days that the experiment ran in January 2016, conditions were kept at 11:13 L:D. For the December 2016 replicate, microcolonies were kept in darkness for the entirety of the experiment.

**Pollen Nutritional Analyses**

To calculate the concentrations of protein, lipid, and carbohydrates within the pollen, two different analyses were performed. Macronutrient concentrations can be found in Table 3.1. The Bradford assay was used to calculate the protein concentration and a modified assay from Van Handel and Day was used to calculate carbohydrate and lipid concentrations, as described in the Appendix (Vaudo et al. 2016.b.)

**Measurements of Bee Health and Fitness**

All bees that survived the entire experiment were examined for weight change and ovary development. Immediately prior to being placed in the microcolony, each individual was marked and weighed on a (ML54 Analytical Balance, Mettler Toledo, Switzerland), and weighed immediately upon end of the experiment. Weight change was then calculated. Ovary development was examined per the protocol used in Amsalam et al. 2015. A Leica MZ6 (200X) microscope with an ocular reticle was used to measure the length of the three largest terminal oocytes in ocular units, which were then converted to mm. For each bee, the three largest oocytes for each
individual bee were averaged, and an average ovary size calculated for each bee. During the second replicate of dissections, we also noted whether or not there was pollen present in the midgut the bumble bees. Photos were taken with a Leica MZ6 (200X) Microscope as well in a subset of the midgut samples which can be observed in Figure 3.1.

Microcolonies were examined for whether or not wax production occurred daily, with photos taken from each individual microcolony at the experiment end. Type of wax production was also noted whenever possible e.g., honeypot.

Data analyses

Data analyses were conducted with JMP 13.0 Pro Software. To look at the effect of the treatment on survival and on wax production, we used a Fisher’s Exact Test. We performed a simple logistic regression to test if initial starting weight influenced survival. A simple linear regression was performed to examine the relationship between weight change and ovary size, and survival as a function of the initial weight. We performed a 1-way ANOVA of treatment on the average weight change as well as ovary size, followed by an analysis of covariance (ANCOVA) to compare the effect of treatment on average ovary size per bee in the presence of variation due to weight change. Block (January versus December), and parental colony were included as random variables. Mean ovary size and average weight change was compared between microcolonies with pollen versus those with only sucrose syrup with post hoc test to determine the necessity of pollen in the adult diet had a direct effect.

Results

We found no significant difference among treatments for the survival of the workers
(Fisher’s Exact Test p=0.95 Figure 3.2). In all of our treatments, 90% or more of the bees survived (Table 3.1), resulting in a sample size of at least 27 in all treatments (with the exception of the multifloral diet which was only included in the second replicate, and thus had a final sample size of 15). In total, we took final weight measurements and dissections to determine ovary size from 152 bees across all treatments. Initial starting weight also had no significant influence on survival ($F_{1,165}=0.67$, p=0.42, Fig. 3.3)

Ovary size as measured by the oocyte size can is demonstrated in Figure 3.4, which shows the variation among ovaries in the sucrose syrup diet treatment versus the multifloral diet treatment. Because the relationship we wanted to most clearly understand was that of diet treatment on ovary development, we factored out other possible variables that could be affecting ovary size. As bees that gained weight could also be assumed to increase the size of their ovaries, we wanted to elucidate whether weight change alone was exclusively driving ovary development. We performed a linear regression (Figure 3.5) to test whether ovary size was influenced by weight change and found there to be a statistically significant relationship but one that explained only 5% of the variation in ovary size ($Y=1.601+6.673*X$, $R^2=0.052$), suggesting that change in weight was not the primary contributor to the variation in ovary sizes that were observed.

By looking at diet treatment and its effects on both ovary development (Figure 3.6) and weight change (Figure 3.7), a general idea of pollen nutritional quality can be observed. The multifloral and sunn hemp fed bees resulted in bees with larger ovaries, and gained the most weight. To better elucidate the relationship between dietary treatment and ovary size, we created a model that included weight change as a covariate, and block, parental colony as random effects (Fig. 3.8). From our analysis of covariance, we found that diet treatment had a significant effect ($F_{5,131.9}=4.32$ p=0.0064, p=0.001) to change ($F_{5,131.6}=2.13$ p=0.065) on the average ovary size and there was also a strong interaction between treatment and weight change ($F_{5,152}=3.67$ p=0.0064). Between the treatments we examined, the sunn hemp and multifloral diet fed bees produced bees
with the largest ovaries. Bees fed on sucrose syrup alone had noticeably smaller ovaries, suggesting that pollen is a critical component in the diet of adult bees.

(Table 3.1) provides an overview of all the pollen nutrient values, as well as the ovary size and weight change of bees within that treatment group. We grouped the treatment categories into either pollen plus sucrose syrup, versus sucrose syrup alone, to further explore the necessity of pollen in the diet of adult bumble bees. In Figure 3.4 the difference in ovary size between microcolonies supplemented only with sucrose syrup versus those supplemented with sucrose syrup and a pollen diet or pollen diet substitute can be observed. By reducing our analyses down to these categories, e.g., w/pollen or without, we found that inclusion of pollen in the diet had a significant effect on ovary development ($F_{1,152}=26.56 \ p<0.0001$). We also performed the same analysis while looking weight change (Figure 3.10) but found no significant effect ($F_{1,152}=0.21 \ p=0.65$).

Another effect of dietary treatment examined was the production of wax using the microcolony as the individual unit. We performed a Fisher’s Exact Test to look at whether dietary treatment also affected the microcolonies’ ability to produce wax (Figure 3.11) and found a significant effect of diet treatment (Fisher’s Exact test $p=0.002$). Microcolonies fed diets that were higher in fat e.g., sunflower, produced the most wax, suggesting that consideration of all macronutrients is critical within the evaluation of pollen quality.

**Discussion**

The main objective of this study was to explore the role of pollen nutritional quality from a physiological standpoint within *Bombus impatiens*, specifically by examining the role it may play in reproductive development and worker survival, and thus may influence worker health and colony maintenance. By examining pumpkin and common cover crop species, we were able to
gauge resource quality of what would be commonly found in a fall agroecosystem within the Mid-Atlantic region. We asked if there were physiological implications of specific macronutrient concentrations, as it has been demonstrated that *Bombus impatiens* foraging behavior specializes on a specific range of P: L ratios (Vaudo et al. 2016), and how different ratios may affect them physiologically. By creating a controlled lab study with the use of bioassays, we were able to collect data that supports the idea that pollen consumption by *Bombus impatiens* adults is critical in maximizing their ovary development, with the nutritional quality and diversity of the pollen available playing a key role.

**Evidence of Adult Pollen Consumption across Varying Pollen Quality Differentially Affecting *Bombus impatiens***

Our findings further demonstrate that pollen with varying nutritional quality will differentially affect individual ovary development in a social bee species, *Bombus impatiens* (Table 3.1, Fig. 3.4, Fig 3.6). Although there is a positive interaction between weight change and treatment that also affects ovary development, treatment alone significantly impacts the development of ovaries. Similar studies looking at *Bombus terrestris* support our findings that pollen quality may influence the reproductive output of the individual (Genissel et al. 2002). Although we did not carry this study out on a colony level basis, microcolonies accurately reflect the nutritive value of pollen on the colony level as well (Tasei and Aupinel 2008).

**Pollen Quality, Diversity, and Macronutrients***

Our results support the idea that both pollen diversity and pollen quality affect ovary development in bumble bees. Because we were limited to a subset of species that can be used in
cover cropping within this region, our indicators of pollen quality, e.g., protein, or P:L ratio, do not necessarily canvas the spectrum of ranges that exist. Sunn hemp, the only legume we studied, had the highest P:L ratio that we worked with, a 1.72 P:L ratio, which is still relatively low. Despite this, workers from the microcolonies fed with pollen from sunn hemp had ovaries that were on average $2.12 \pm .73$ mm in size, which were the largest we observed. Conversely, the second largest ovaries we observed ($1.97 \pm .70$ mm) were from the multifloral diet treatment, which had the lowest P:L ratio (0.95) of all the pollen types we worked with. Other lab based studies have also demonstrated the benefits of having a diverse pollen diet while also considering nutrient composition (Moerman et al. 2017), which only further demonstrates the importance of bumble bees having access to flowering patches that will provide both a diverse and high quality set of floral resources.

**Insights into Foraging Behavior**

Considering these findings from a field context, bumble bees have been shown to forage farther for more diverse patches of resources, with foraging behavior in general found to be highly plastic (Jha and Kremen 2013). Also demonstrated is that bumble bees will selectively forage for higher quality pollen, specifically pollen with a higher protein content (Leonhardt and Blüthgen, 2012). Recent research has demonstrated through choice experiments that bumble bees will preferentially consume pollen falling into a specific ratio of proteins to lipids (~5:1) (Vaudo et al. 2016.a., Vaudo et al. 2016.b.). Looking at our results through the lens of these findings, it could be suggested that the fitness benefits attained from higher quality resources outweigh the increased energy expenditure in their selective foraging to seek out these resources. With habitat fragmentation and loss of floral resource becoming increasingly problematic, increased foraging distance, particularly for sensitive life stages such as newly emerged queens could be detrimental
to their populations as a whole. By reducing the distance between high quality resource and diverse flowering patches, conservation efforts would be instrumental in reducing this stressor.

**Comparison of Diet Treatments and Field Behavior**

One of the more interesting results of our study was the contrast between what we observed in the field and what was observed during these bioassays. From our dissections there was clear evidence that *Bombus impatiens* was ingesting pumpkin pollen. In pumpkin field observations, amounting to 21 hours of total observation time, not a single *Bombus* was observed consuming pumpkin pollen, perhaps highlighting the need for future studies on the pollinators of *Cucurbita* and their ecology. Anecdotal field observations and videos actually demonstrate such an aversion to pumpkin pollen they will remove it from their body (personal observation, EDT). These results demonstrate that *Bombus* will consume pumpkin pollen if it is the only resource available, but this behavior may actually have adverse health effects. This also highlights the need and important for a diversity of additional resources within this agroecosystem.

Although the benefit of some mass-flowering crops for pollinators has been shown (Westphal *et al.* 2009), understanding the differences between mass-flowering plant species and their effects on pollinators is critical. In the case of *Cucurbita*, many are known to produce cucurbatacins, or bitter tasting biochemical compounds thought to inhibit herbivory. These secondary compounds could be detrimentally affecting generalist pollinators, with previous studies demonstrating that pollen containing chemical defenses inhibiting bees, in particular generalists, from digesting them (Sedivy *et al.* 2011). Individuals who consumed pumpkin pollen increased their by weight an average of 0.002 ± 0.02 mg, but in numerous individuals (11/28) weight was actually lost. The only other diet that caused individuals to lose weight was the artificial diet, which showed individuals lost on average 0.005 ± 0.021 mg. Despite these adverse
health effects per individual, ovary size was maintained in both of these pollen diets as compared to the solely sucrose syrup diet, which produced individuals with significantly smaller ovaries. We hypothesize that even in the presence of a pollen-like substance, individuals will allocate resources to their ovaries, prioritizing fitness over their own health. In the case that no pollen or pollen-like substance was present, e.g., only sucrose syrup, workers were observed to gain weight but at the cost of ovary size. In this case, it could suggest that nutrients were instead allocated to the individual’s own survival and reproductive function was not prioritized.

Conclusions

Within Bombus, our study and others show that pollen quality may dictate the reproductive output of the individual (Moerman et al. 2017). In Apinae, the quality of the pollen will affect the colony dynamic and larval growth, and is also critical for the reproductive potential of adult worker bees. Although nectar is the primary substance that sustains adults within Apinae, our study emphasizes that pollen cannot be discounted as a necessary food source. Previous studies of pollen in the diet of adult worker honey bees show that it could play a critical role in the development of hypophrängeal glands in younger workers (Crailsheim et al. 1991). Even within adult solitary bees, specifically Osmia californica, it has been recently shown that pollen feeding by adult females is critical to maximizing reproductive output (Cane 2017). The degree to which pollen quality regulates this is still being explored, but established by this study and others, is that pollen quality does affect adult bee development and health. With a dearth of floral resources being implicated in pollinator decline (Biesmeijer et al. 2006) more attention is being given to support pollinator conservation through targeted habitat enhancement strategies. Yet proper emphasis must be placed on providing both high-quality and a diverse assemblage of resources to pollinators. This highlights the need for more studies exploring the nutritional needs
across bee species and across life stages. Marrying high quality floral resources with diverse and targeted floral enhancements will be critical to pollinator conservation efforts moving forward.
References


Table 3-1. Macronutrient concentrations and average oocyte size and weight change

<table>
<thead>
<tr>
<th>Diet Treatment</th>
<th>Protein</th>
<th>Lipid</th>
<th>P:L</th>
<th>Carbohydrates</th>
<th>P:C</th>
<th>Ovary size (mm)</th>
<th>Weight change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunn hemp</td>
<td>123.54</td>
<td>71.95</td>
<td>1.72</td>
<td>123.10</td>
<td>1.00</td>
<td>2.12 ± .73</td>
<td>.016 ± .019</td>
</tr>
<tr>
<td>Pumpkin</td>
<td>140.73</td>
<td>96.78</td>
<td>1.45</td>
<td>43.28</td>
<td>3.25</td>
<td>1.69 ± .79</td>
<td>.002 ± .021</td>
</tr>
<tr>
<td>Sunflower</td>
<td>116.23</td>
<td>116.09</td>
<td>1.00</td>
<td>302.08</td>
<td>0.38</td>
<td>1.76 ± .72</td>
<td>.011 ± .022</td>
</tr>
<tr>
<td>Multifloral diet</td>
<td>60.49</td>
<td>63.48</td>
<td>0.95</td>
<td>63.85</td>
<td>0.95</td>
<td>1.97 ± .70</td>
<td>.019 ± .019</td>
</tr>
<tr>
<td>Artificial diet*</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>&gt;10.0</td>
<td>1.59 ± .77</td>
</tr>
<tr>
<td>Sucrose syrup (only)</td>
<td>0</td>
<td>0</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>1.02 ± .48</td>
<td>.009 ± .041</td>
</tr>
</tbody>
</table>

*A more detailed description of the macronutrients within this diet can be found in Table 3-3.

Table 3-0. Number of bees from each treatment group (Replicate two of the experiment) containing pollen within their gut (Fisher’s exact, p<0.0001)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P:L ratio</th>
<th>P:C ratio</th>
<th>Proportion of bees with pollen in gut</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose syrup</td>
<td>n/a</td>
<td>n/a</td>
<td>0/15</td>
</tr>
<tr>
<td>MegaBee</td>
<td>&gt;10</td>
<td>n/a</td>
<td>1/15</td>
</tr>
<tr>
<td>Sunn Hemp</td>
<td>1.72</td>
<td>1.00</td>
<td>5/15</td>
</tr>
<tr>
<td>Pumpkin</td>
<td>1.45</td>
<td>3.25</td>
<td>12/15</td>
</tr>
<tr>
<td>Sunflower</td>
<td>1.00</td>
<td>0.38</td>
<td>10/15</td>
</tr>
<tr>
<td>Multi</td>
<td>0.95</td>
<td>0.95</td>
<td>7/15</td>
</tr>
</tbody>
</table>
Table 3-3 MegaBee Diet Patent (https://www.google.com/patents/US20120308686 (June 12, 2017)

<table>
<thead>
<tr>
<th>From Patent Description:</th>
</tr>
</thead>
<tbody>
<tr>
<td>40-60% protein</td>
</tr>
<tr>
<td>2-4% lipid</td>
</tr>
<tr>
<td>40-60% carbohydrate</td>
</tr>
<tr>
<td>Particles less than 35 µm</td>
</tr>
</tbody>
</table>
Figure 3.1 Midgut of a worker bee from the 2016 December replicate that was fed the pumpkin diet (Leica MZ6 Microscope at 200X)

Figure 3.2. Distribution of *Bombus impatiens* worker survival across the six diet treatments (Fisher’s Exact Test p=0.95). There were 30 bees in each treatment group with the exception of the multifloral diet, which only had 15.
Figure 3.3 Logistic regression of Survival vs. Initial Weight (mg) of *Bombus impatiens* workers ($F_{1,165} = 0.67, p = 0.42$)

Figure 3.4 Photos of the ovaries taken using a Leica MZ6 microscope at 200X of a *Bombus impatiens* worker fed off of the multifloral diet versus the sucrose syrup diet, respectively.
Figure 3.5 Regression of average oocyte size per bee and average weight change (\(Y=1.601+6.673\times X, R^2=0.052, p=0.0046\))

Figure 3.6 Average ovary size across the different diet treatments as a 1-way ANOVA (\(F_{5,152}=7.48, p=0.001\))
Figure 3.7 Average weight change across the different diet treatments as a 1-way ANOVA ($F_{1,152}=2.93, p=0.0151$)

Figure 3.8 Weight change and oocyte size, with the various diet treatments represented. Diet ($F_{5,131.9}=4.32, p=0.0001$) was significant and there was a strong interaction between diet*weight change ($F_{5,131.6}=2.13, p=0.065$)
Figure 3.9 Average size of ovaries in *Bombus impatiens* between pollen supplemented with sucrose syrup and sucrose syrup alone ($F_{1,152}=26.56$, $p < .0001$).

Figure 3.10 Average change in weight of each individual worker between a diet of pollen supplemented with sucrose syrup and a sucrose syrup diet alone treatments. Individuals were measured immediately before and after the experiment ($F_{1,152}=0.21$ $p=0.65$).
Figure 3.11 Number of microcolonies that produced wax with $n=30$ for each treatment, with the exception of the multifloral diet where $n=15$ (Fisher’s Exact test $p=0.002$).
Chapter 4

Assessing Effects of Neonicotinoid Use on *Cucurbita pepo* and its Pollinators: A Field Based Approach

Introduction

The plant genus *Cucurbita* contains a number of economically and dietary significant crops, including pumpkin, squash, and zucchini. For example, in the state of Pennsylvania alone growers produced 79,400,000 pounds of pumpkin worth >$14.6 million, a figure that can be largely attributed to the growing demand for pumpkins (USDA NASS 2015 data). In addition to the economic importance of the field pumpkin, or *Cucurbita pepo*, this species also has enormous ecological importance, with its domestication being cited as critical for facilitating the spread of the spread of the specialist squash bee, *Peponapis pruinosa*, across North America (Lopez-Ulribe et al. 2016). Besides this specialist pollinator, whose larvae feed exclusively on the pollen and nectar from *Cucurbita pepo*, studies from the northeastern United States have also found bumble bees and honey bees to be important pollinators for *Cucurbita pepo* (Artz et al. 2011, Schuler et al. 2001). Unlike some crop species that can rely on other methods for pollination, e.g., wind, *Cucurbita pepo* is essentially dependent on animal pollination for successful reproduction and fruiting (Klein et al. 2007). Troublesome though to this intimate and necessary relationship is the high prevalence of pesticide use within *Cucurbita*. This plant genus is susceptible to a number of fungal, bacterial and viral plant pathogens, including numerous plant viruses and two bacterial pathogens, which are transmitted by insects. Alone, bacterial wilt, the disease caused by *Erwinia tracheiphila*, which is vectored by beetles, has been shown to cause yield losses up to 80% (Latin 1993), which can result in significant economic losses for growers. Pathogens that are transmitted by insect vectors, including cucumber beetles (*Diabrotica* spp. and *Acalymma vittatum*), squash
bugs (*Anasa* spp.) and aphids (*Aphis* spp.) call for an effective and rapid management strategy, which has been found within a particular class of pesticides, or neonicotinoids (Fleischer *et al.* 1998).

**Brief History of Neonicotinoid Use**

Since their introduction to the agricultural community in the early 1990’s, neonicotinoids have been identified as an effective mechanism for controlling common pests within agricultural systems (Elbert *et al.* 2008). Even more attractive to their use, neonicotinoids are considered to be safer than some other pesticide classes, in part due to their decreased toxicity to vertebrates while maintaining effectiveness within invertebrates. All classes of neonicotinoids are systemic, which means that various degrees of these chemicals are incorporated into the plant tissue (*e.g.* phloem, xylem, and leaf tissue, *etc.*) depending on the application method, formulation, and type of neonicotinoids used. Unfortunately, studies working with various crops and application methods have also found that these chemicals can translocate into the pollen and nectar of numerous flowering plant species. Treated seeds of sunflowers resulted in trace amounts being incorporated into their pollen and nectar, although the amounts were quite minute (Schmuck *et al.* 2001). Studies working with *Cucurbit pepo* and neonicotinoids also found traces in the nectar varying by orders of magnitude, dependent on application method as well as environmental conditions and plant stress (Dively and Kamel 2012, Stoner and Fitzer 2012). The finding that plants treated with neonicotinoids are retaining these chemicals in their pollen and nectar is particularly concerning for the pollinators relying fully, or in part on these substances for their survival.
Why are Neonicotinoids a concern for pollinators?

Neonicotinoid insecticides have been implicated in numerous studies as having adverse health effects on pollinators. Exposure can occur through a number of mechanisms, and across life stages. For example, studies have found up to 30 different types of pesticides within honey bee colonies (Mullin et al. 2010) suggesting exposure occurring at all life stages. In honey bees, larva exposed to imidacloprid had reduced olfactory associative behavior as adults, which could adversely affect their foraging behavior (Tan et al. 2014). Decreased learning behavior was also exhibited by adult honey bees that had ingested imadacloprid (Tan et al. 2014). Studies working with native bees have found similar adverse health effects. In terms of foraging behavior, *Bombus terrestris* chronically exposed to field relevant doses of imidacloprid exhibited impaired foraging performance (Gill and Raine 2014). In addition to foraging behavior, neonicotinoid exposure can also manifest within colony development. Managed *Bombus terrestris* colonies exposed to field relevant dosages of imidacloprid showed queen production to be the most severely affected colony variable, which could result in reduced bumble bee populations (Baron et al. 2017). Furthermore, wild caught *Bombus terrestris*, *B. lucorum*, *B. pratorum* and *B. pascuorum* queens had reduced ovary development after ingestion of thiamethoxam (Baron et al. 2017). Although these sublethal effects are highly concerning, neonicotinoids are also lethal under some conditions, with the LD$_{50}$ being highly dependent on the active ingredient, mechanism of exposure, and life stage (Iwasa et al. 2004, Blacquière et al. 2012). As of 2016, only seven studies had been published on neonicotinoids and solitary bee species, with more than half of these studies being done with imidacloprid, calling for a comprehensive and thorough effort by researchers to address this knowledge gap (Hopwood et al. 2016). Those that have been conducted have shown neonicotinoids to be detrimental to their health and survival. Acute contact with imidacloprid was shown to negatively affect the health of alkali bees, alfalfa leafcutter bees,

Perhaps one of the most concerning results from the abundance of research on neonicotinoids and pollinators is the finding that under lab-controlled conditions, bumble bees will preferentially consume nectar like substances laced with thiamethoxam or imidacloprid, even though their life span will be shortened as a result (Kessler *et al*. 2015). If bumble bees do prefer neonicotinoid laced resources, more studies are needed to examine if this trend holds true in field realistic settings. The lack of field studies has been cited as one of biggest shortcomings within this area of research (Lundin *et al*. 2015). In our study we aimed to contribute to addressing this knowledge gap by testing whether pollinators increased or decreased their visitation to neonicotinoid treated plants in a field setting. We selected *Cucurbita pepo* as our model system, in part due to the dearth of studies working with insect pollinated fruit and vegetable crops and pesticide exposure (Lundin *et al*. 2015). Moreover, *Cucurbita pepo* makes an ideal model system due to the high demand for systemic insecticides to manage the insect-vectored pathogens, and with the finding that higher concentrations of imidacloprid may translocate into *Cucurbita pepo* pollen and nectar, as compared to other crops (Stoner and Eitzer, 2012). We used treated vs. untreated pumpkin (*Cucurbita pepo*) seeds, as well as a field dosage treatment of neonicotinoids applied via the soil, to generate realistic doses. Soil drenching is performed by directly applying labeled rates to the base of the plant, which also allowed us to increase the amount of pesticide that we hypothesized would be taken up into the pollen and nectar (Dively and Kamel 2012). We also wanted look at the response of *Cucurbita pepo* to the neonicotinoid treatment, specifically by examining the rate of flowers produced, as well their size of their floral display. As floral display and signaling is a critical foraging cue for bees, understanding if this variable is altered by neonicotinoids is necessary.
By looking at a number of variables limited specifically to plant attributes, we wanted to disentangle the effect of the neonicotinoids on the plant traits from the bee behavior. In doing so, we intended to examine if *Bombus* and *Apis mellifera* increased their rate of visitation to floral resources that could contain neonicotinoids, which would provide additional support to the lab finding that bees will preferentially consume substances containing neonicotinoids (Kessler *et al.* 2015). By also looking at the plants response to the various treatments, we wanted to examine whether bee behavior was being affected by the neonicotinoids directly or indirectly through the plant response to the various treatments. We hypothesized that *Bombus* and *Apis mellifera* would be unable to differentiate between treatments containing neonicotinoids and those that do not. With field conditions and degradation of the active ingredients over time, we hypothesized that in a multi-week field study common pollinators of *Bombus* and *Apis mellifera* would not increase their visitation rate or length of visit to flowers from plants exposed to neonicotinoids.

**Materials and Methods**

**Planting Establishment and Soil Drenching**

To better understand the effects of thiamethoxam use on *Cucurbita pepo* and its pollinators, we applied four different dosages as a replicated complete block with four blocks (*Appendix 4.1*) within a 0.4 hectare (one-acre) pumpkin field. Both treated (FarMore FI400, [www.syngenta-us.com/seeds/vegetables/farmore/farmore.aspx](http://www.syngenta-us.com/seeds/vegetables/farmore/farmore.aspx)) and untreated *Cucurbit pepo* ‘Gladiator’ F1 seeds were ordered from Harris Seeds (Rochester, NY). Treated and untreated seeds were soil drenched with Platinum® ([http://www.syngenta-us.com](http://www.syngenta-us.com)) insecticide, with each plant being exposed to 0.094 grams of active ingredient using a split application of soil...
drenching. In total, we had four treatments: untreated seeds, untreated seeds with soil drenching, treated seeds, and treated seeds with soil drenching.

**Transplanting**

Seeds were grown in 72-cell transplant trays until the first true leaves had emerged, at which point they were transplanted to the field (July 25th). In the initial planting, 10 plants were placed per row with 1.2 m (4 feet) separating plants. We used two 12-meter rows per plot. Immediately after transplant, drip irrigation was run for 5-7 hours until the soil was sufficiently moist. Irrigation was done across the season, on average twice a week. Two days after transplanting, any plants that did not survive the initial planting were replaced.

**Pesticide application**

To create a gradient of pesticide doses, Platinum® was diluted to allow for a field relevant dosage per plant to be added. A homogenous solution for drenching the soil was produced by adding 0.94 grams of Platinum® insecticide to 15 L of water. This solution was applied to 20 plants, with 705 mL of solution applied to each plant, exposing each plant to 0.047 grams of active ingredient. Immediately after application, the drip irrigation system was turned on for 4 hours. This soil drench application was done twice, resulting in a split application, the first application being ten days after transplanting (August 3rd, 2016) and the second being three weeks after the first soil drenching (August 23rd, 2016). In total plants that received the soil drench were exposed to 0.094 grams of thiamethoxam, which was done as per label recommendations for growers.
Plant Measures

To understand whether systemic insecticide use was impacting the floral display of plants, data were collected on total number of flowers per plant, flower sex, and flower diameter every week beginning six weeks after transplant date. Flower diameter was measured on one randomly selected flower per treatment row, with sex being noted. As 97% of the flowers produced ended up being male, all measurements were taken on male flowers. These measurements were performed weekly across the bloom period in the month of September beginning on September 7th and ending on September 27th, 2016. As there were four treatments per block and four blocks in the experiment, a total of 160 plants were measured each week. During each observation and measurement period, data were collected on floral display per plant.

Bee Observations

On the same day that data were collected on floral number and size, bee observations were also conducted. Bee observations were only conducted on dates with the following conditions: >16 °Celsius (61 °Fahrenheit), <3.5 m/s (8 miles/hr) wind and sunny, partly cloudy or bright overcast. Because of the phenology of pumpkin flowers and weather conditions, observation starting time ranged from 7:00 am until 9:00 am, and lasting until flowers closed. In the event that pumpkin flowers were already beginning to close, observations were not conducted. To conduct bee observations, a flower (of either sex) from each plant in the row was observed for a total of 90 seconds. Flowers were chosen at random, with the exception of flowers that had ants within the nectaries which were excluded. Sex of the flower was noted and the observed flower was one that was always used for the plant measures.
During the observation period, any bee visitors that came into contact with the reproductive parts of the flower were recorded. Honey bees and bumble bees were the only observed visitors. Whenever possible, activity upon the flower was also recorded (e.g., collecting pollen or nectar). The duration of the visit for each visitor was recorded with a Sportline 220 stopwatch (www.sportline.com) and any situation where the visitor left and then returned was counted as a separate visit.

Analyses

All data analyses were conducted with JMP Pro 13.0 Software. When examining the response of plants to the different treatments, a univariate repeated measure with a mixed model approach to allow for random effects was used. Weeks after planting, treatment, and average number of flowers were included as fixed effects, as was the interaction between time and average number of flowers. The block was nested within treatment and included as a random effect in the model. When examining the average number of flowers produced across the season, we performed a Kruskal-Wallace followed by a post-hoc Dunn Test with joint ranking. Within all analyses concerning bee behavior, a repeated measure was performed as a univariate repeated measure, using treatment, weeks after planting, average number of flowers per plant, the interaction between average numbers of flowers per plant* weeks after planting, and treatment*weeks after planting as a fixed effect. Block, was nested within treatment and included as a random effect in the model. Visit duration data was computed as the average across the season to improve normality and an analysis of variance using a standard least squares approach was used with block, average number of flowers per plant, and average flower diameter as covariates. In all cases where models were selected, the model with the lowest corrected Akaike Informational Criterion (AICc) was chosen.
Results

Plant Response to Neonicotinoids

Examining first the response of plants to the different treatments, the average number of flowers per plant across the season, regardless of treatment were grouped, allowing us to examine the total floral resources available in a given week (Figure 4.2). The effect of treatment was isolated using a repeated measure examining the average number of flowers produced per plant (of both sexes) across all treatments and during all four weeks of the observation period (Figure 4.3). Both treatment ($F_{3,160}=9.53$ $p<0.001$) and time ($F_{3,160}=194.36$ $p<0.001$) had a significant effect on the number of flowers produced per plant. Week eight had the highest number of flowers produced across all treatments, with the drenched treated and the treated groups producing the most flowers at this time point. For seasonal averages of total number of flowers (both sexes) produced by the plant, as well as the average size of the male flowers, the untreated seeds produced significantly fewer flowers ($\chi^2 (3, 613=17.28) p=0.0006$). There was no effect of treatment on flower diameter ($F_{3,59}=1.17, p=0.33$) (Table 4.1).

Bee Response to Neonicotinoids

While understanding the response of the plant to the treatment was critical, understanding the behavior of pollinators and their foraging behavior was the focus of this study. Due to timing, the two pollinators that were present at the time of observations were *Apis mellifera* and *Bombus*. Examining the number of visits a flower received for each week across the observation period by *Bombus* per 90-seconds (visitation rate), treatment was not statistically significant ($F_{3,40.4}=2.37$, $p=0.09$), nor was the interaction between treatment and weeks after planting ($F_{9,109.8}=1.53$, $p=0.1441$) whereas weeks after planting (time) ($F_{3,130.7}=7.03$ $p=0.0002$) and the average number
of flowers per plant (F_{1,131.3} = 8.82, p=0.0035) had a significant effect (Figure 4.3). The interaction between time and average number of flowers was also significant (F_{3,138.7} = 2.87, p=0.04).

To further understand how neonicotinoid usage could be affecting the foraging behavior of the bees, the average length of a single visit from was pooled across the season. Block, average number of flowers per plant, and average flower diameter were all included as covariates in our analysis. There was no effect of treatment or any other variables on the average visit duration (Figure 4.4 (F_{8,17} = 1.79, p=0.38)).

A univariate repeated measure looking at visitation rate, and an analysis of variance of visitation duration, within *Apis mellifera* was used to examine their foraging behavior and the possible effect of treatment. Both treatment (F_{3,41.6} = 3.43, p=0.025) and time (F_{3,131.6} = 40.21, p<0.0001) had a significant effect on the average number of visits a flower received, as did the interaction between these two variables (F_{9,110.9} = 3.61, p=0.0006). We found that the treated group of seeds received the highest number of visits as compared to the rest of the groups. As this could be attributed to flower number, this was also a variable we examined, but found that the average number of flowers per plant (F_{1,129.4} = 0.01, p=0.91) had no significant effect. There was also no significant interaction between average number of flowers and time (F_{3,139} = 0.36, p<0.78) (Figure 4.6). Examining the visitation duration within only *Apis mellifera* visits and again found there to be no significant effect on the length of the visit (Figure 4.7 (F_{8,12} = 1.79, p=0.274)).

The last factor examined was the general foraging behavior and phenology of both taxa across the observation period to understand seasonal dynamics. The average number of visits per 90 second period for both taxa in Figure 4.8, shows the transition from primarily *Bombus* early in the season to *Apis mellifera* in the latter half of the season. In all of the hours of observations (~21 hours) there was not a single instance of either *Bombus* or *Apis mellifera* collecting pollen from any *Cucurbita pepo* flower, but rather there were documented instances of them actively removing it from their body.
Discussion

The main objective of this study was to examine how the common social bee species visiting *Cucurbita pepo*, e.g., *Apis mellifera* and *Bombus* interacted with floral resources that had been exposed to neonicotinoids, specifically thiamethoxam, in a field realistic experiment. We hypothesized that within a field setting, additional factors, e.g., weather, plant growth, temperature, pest damage, and time after application, would alter how these taxa interacted with floral resources that had been exposed to these chemicals. As a result of these external factors, we hypothesized that bees would be unable to differentiate between plants treated with neonicotinoids and those that did not, and thus rates of visitation and visitation duration would remain the same across all treatments. By creating a complete randomized block with four different treatments; untreated seeds, untreated seeds with a soil drench, treated seeds, and treated seeds with a soil drench, that we hypothesized creating an increasing level of neonicotinoids concentrations. In doing so, we were able to collect seasonal data on (1) the response of *Cucurbita pepo*, in terms of flower production and size, and (2) the foraging behavior of common social bee species within *Cucurbita pepo*, looking at both visitation rate and visit duration, to these various treatments.

Evidence for Altered Plant Floral Display and Foraging Behavior by Various Dosages of Neonicotinoids

Our findings demonstrate that when various amounts or application methods of neonicotinoids are applied, a *Cucurbita pepo* plant will significantly alter the number of flowers produced (Table 4.1). Plants that were in the drenched treated group produced $4.16 \pm 0.25$ (Mean $\pm$SE) flowers per plant whereas untreated seeds produced on average $2.50 \pm 0.22$ (Mean $\pm$SE) flowers per plant. Most (97%) of the flowers were males, and we did not allow the female flowers
to develop to the point of fruit, therefore we cannot make any conclusions about how the yield would be affected. Looking at the seasonal dynamics of flower production (Figure 4.2), we can also observe that plants exposed to neonicotinoids, either through seed treatment or soil drenching, produced flowers at a faster rate during weeks 6 and 7, than those not exposed to neonicotinoids. The diameters of all flowers, however, were not significantly affected by the treatment (Table 4.1). In addition to the difference in plant floral display, we also found that the observed bee genera responded differentially to floral display, seasonal phenology, and in some cases, to treatment. This did not support our hypothesis that we would see no preference towards a plant containing neonicotinoids versus one without. Although we observed significant differences between treatments within both taxa, the cause of these differences in foraging behavior was not clear.

**Local Apis mellifera Foraging Behavior**

First looking at *Apis mellifera* and the factors that affected their foraging behavior, we find different variables to be significant and to different degrees. As mentioned, treatment had a significant effect on the average number of flowers a visit received. Although we were unable to test the exact amount of neonicotinoids present within the pollen and nectar, it could be that the concentrations in the pollen and nectar fell into the range that was demonstrated to be preferential (10-100 nM) in the study conducted Kessler *et al.* 2015. However, the variation in visitation rate by *Apis* among treatments did not follow what we would expect to be a dose response, if we assume the drenched seed treatment would also have the highest amount of neonicotinoid residue. In the third week there was the clearest preference by *Apis mellifera* to the treated group, and not the drenched treated group, which was producing the highest number of flowers per plant at this
time point. This dynamic suggests that sheer flower number per plant was not the key factor driving this relationship.

Weeks after planting, or time, was also a significant variables in terms of explaining differences within foraging behavior. Considering *Bombus* behavior (Figure 4.8) or the decrease in *Bombus* numbers could explain the increased foraging by *Apis mellifera*. A 2011 study looking at visitation rate within pumpkin fields by *Apis mellifera* found there to be an antagonistic relationship between *Peponapis pruinoisa* (but not *Bombus*.) and visitation rate (Artz et al. 2011). Because the only other taxa present at the time of our study were *Bombus*, their presence may have filled this niche, creating an antagonistic relationship. We also observed a significant interaction (p=0.0006) between treatment, and weeks after planting within *Apis mellifera*, but not within *Bombus* spp., which could be an interesting relationship to examine in future studies.

**Local *Bombus* Foraging Behavior**

Examining *Bombus* we found interesting dynamics within the effect of our treatments as well. Treatment alone was not statistically significant (p=0.12) while looking at the average visitation rate across the season, while both time (p<0.0001) and average number of flowers (p=0.0003) were. There was also a significant interaction between these two variables (p=0.0087), which make senses as the plant will continue producing more flowers across the season, and eventually produced the maximum number of flowers in week three of our study (Figure 4.2). As week number eight also had the highest floral density and one of the lowest visitation rates, this result would lend support to the idea that within *Cucurbita pepo* the number of flowers within a field will negatively affect the visitation of *Bombus* per flower (Artz et al. 2011). Assuming our *Bombus* population was fixed within the region (if not decreasing due to time of season) whereas number of flowers was increasing, it could be that the visitation rate was
saturated by this dichotomous relationship. Looking at which factors could explain the significance of time, the third week may have coincided with the change of internal colony dynamics as this was when the first gyne was observed out foraging. This would mean colony-level factors that were not accounted for influenced visitation, in addition to factors within our experiment.

Our findings demonstrate that there is a difference in foraging behavior exhibited by *Apis mellifera* and *Bombus* in regards to their rate of visitation and across the observation period (September 7th-September 27th). Looking at foraging behavior between treatments within *Bombus*, treatments containing neonicotinoids were preferentially visited, which although not statistically significant (p=0.09) is apparent in week seven in Figure 4.3. *Apis mellifera* also preferentially visited treated seeds treatments, with Figure 4.5 clearly elucidating this relationship during week eight. When we consider additional reasons as to why we saw increased visitation to these treatments, a number of factors come to mind. We did not measure the pest damage (although pest pressure was minimal), it is possible that reduced pest pressure to these treatments resulted in a more appealing scent to *Bombus*, as it has been shown in *Cucurbita pepo* that leaf damage can alter the floral scent in male flowers (Theis *et al*. 2009, Shapiro *et al*. 2012). Considering the floral scents emitted, it could also be the case that the neonicotinoid treatment affected the scent of the flower itself, without improving the quality of the pollen or nectar, which would explain why we saw increased visitation to these flowers without a significant effect on the length of time spent on the flowers.

**Conclusions**

In our study we observed that neonicotinoids significantly impacted floral traits within *Cucurbita pepo*, which, in turn, affected the dynamic foraging behavior of *Bombus* and *Apis*
*Apis mellifera.* Because we were unable to analyze the concentration of neonicotinoids within our pollen and nectar, we are limited in our ability to state the extent to which they directly influenced the behavior of the bees. From our comparison of treatments with and without neonicotinoids, we can definitively state that the presence of neonicotinoids influences *Apis mellifera* foraging behavior (Figure 4.6), but not in the ranking that would be expected if it directly followed a dose response. Although the influence on *Bombus* foraging behavior (Figure 4.3) was not significant (p=0.09) when all variables were considered, the earlier time points (weeks 6 and 7) also suggest increased visitation to treatments that have been exposed to neonicotinoids. *Cucurbita pepo* flowers are only open for one day, but the plant produces copious numbers of flowers over an extended time frame. Future work should focus on the dynamics of neonicotinoid residues in pollen and nectar and the degree to which they remain in the tissue across the season. Comparing our study to studies that have analyzed pollen and nectar neonicotinoids concentrations and working with similar application methods (Dively and Kamel 2012, Stoner and Eitzer 2012), we would expect bee visitors’ exposure to be within sublethal levels, or lower. Although we did not explore the physiological implications of our findings, numerous studies have demonstrated adverse sublethal affects upon both of the above taxa (Tan *et al.* 2014, Gill and Raine 2014), which raise concern regarding our finding that these bees will increase their visitation to plants which have more flowers after exposure to neonicotinoids under field settings. As previous studies demonstrate that neonicotinoids differentially affect the health of different bee taxa (Mayer and Lunden 1997), and our findings show that neonicotinoids differentially affect their behavior, we highlight the need for a broad approach to this study area. This study provides a springboard off of which future studies looking at how pollinator behavior interacting with systemic insecticides in field settings can be launched, where it clearly becomes important to consider dynamic effects on flowering behavior. A collaborative and united effort by researchers
to investigate how, and the extent to which neonicotinoids are influencing field behavior across all bee taxa, will be necessary to advancing this field.

Table 4-1 Number of flowers (♂ and ♀) per plant and diameter of flower across the September 7 to 27 observation period.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Flowers per Plant (♂ =1888, ♀=61)</th>
<th>♂ Flower Diameter (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drenched Treated</td>
<td>4.16 ± 0.25 (A)</td>
<td>16.15± 0.50 (A)</td>
</tr>
<tr>
<td>Drenched Untreated</td>
<td>3.21 ± 0.20 (A)</td>
<td>17.10 ± 0.58 (A)</td>
</tr>
<tr>
<td>Treated</td>
<td>2.95 ± 0.27 (A)</td>
<td>16.18 ± 0.53 (A)</td>
</tr>
<tr>
<td>Untreated</td>
<td>2.50 ± 0.22 (B)</td>
<td>15.58 ± 0.84 (A)</td>
</tr>
</tbody>
</table>

*Treatment means with the same letter were not significantly different.*
Figure 4-1. Pumpkin Plot Plan Layout
Figure 4-2. Average number of flowers produced per plant (all treatments) across all four weeks of the observation period (September 7th - September 27th)

Figure 4-3 Average number of flowers produced per plant within each treatment across the observation period (September 7th - September 27th). Both treatment ($F_{3,160}=9.53$ $p<0.001$) and time ($F_{3,160}=194.36$ $p<0.001$) had a significant effect on the number of flowers produced per plant.
Figure 4-4 Average number of visits a flower received per 90 second window by *Bombus* across the month of observation ($F_{3,40.4}=2.37$, $p=0.09$)

Figure 4-5. Seasonal average visit duration to a single flower by *Bombus* ($F_{8,17}=1.79$, $p=0.38$)
Figure 4-6 Average number of visits a flower received per 90 second window by *Apis mellifera* across the month of observation ($F_{3,41.6}=3.43$, $p=0.025$)

Figure 4-7 Seasonal average visit duration by *Apis mellifera* ($F_{8,12}=1.79$, $p=0.274$)
Figure 4-8 Foraging behavior of *Bombus* and *Apis mellifera* (September 7th-September 27th). Black line indicates the week in which *Bombus* gynes observed foraging.
References


Chapter 5 Conclusions and Future Directions

Thesis Objectives

Within this thesis, I examined how generalist bee species were affected by various agroecosystem attributes, specifically within *Cucurbita pepo*, and how these attributes could be altered to better support them. Pursuing the idea that agricultural intensification is a causal agent in exacerbating bee decline, I approached this topic from various different angles that considered both systemic insecticide usage, as well as the associated loss of flowering resources and subsequent effects of poor nutrition. In Chapter 2, I examined how floral provisioning could be adapted to fit a rotational agroecosystem through the use of flowering cover crop mixtures, various seeding rates, and a dual-season planting. I further examined the plant species within this floral provisioning mixture in Chapter 3 by examining the nutritional value of their pollen in a lab-based microcolony study while using the agriculturally relevant pollinator *Bombus impatiens* as a model organism. In Chapter 4 I examined the *Cucurbita pepo* system and how neonicotinoid use could be affecting both the foraging behavior, flowering behavior of the plant, and methods of exposure for generalist foraging bee species. By identifying key threats to pollinators within a Mid-Atlantic agroecosystem, e.g., pesticide exposure and a lack of floral resources, our pollinator conservation efforts can be more efficiently targeted.

Observing the agroecosystem as a whole, numerous studies have demonstrated the positive effects of implementing floral provisioning in supporting bee populations (Klein *et al*. 2007, Goulson *et al*. 2008, Pywell *et al*. 2011). There also exists an abundance of data on cover cropping and the ecosystem services they provide to growers, with very few considering the
implications for pollinators. Here we show the ease of integrating floral provisioning to support pollinators into a common cover crop mixture, but also highlight the need for more studies examining both applied practices, e.g., cover cropping, with basic questions concerning plant species attractiveness and floral resource quality. Taking this research a step further, the question of floral resource quality and nutrition comes into play. It’s been established that both *Apis mellifera* and *Bombus* will preferentially forage for higher quality and diverse resources (Leonhardt and Blüthgen, 2012, Jha and Kremen 2013, Vaudo *et al.* 2016) suggesting these variables should also be incorporated into future conservation efforts, particularly when considering our findings from Chapter 3. Lastly, as we consider the efforts to conserve and support pollinators within agroecosystems, we also must consider the potential risks associated with this practice e.g., increased pesticide exposure, which we explore in Chapter 4. Moving forward in this vein of research, it will be important to conduct future studies within the context of an agroecosystem, rather than within a single feature of the system.

In Chapter 2, I examined how floral provisioning can be targeted to a specific pollinator and agricultural system. By utilizing a dual-flowering planting (fall-flowering and spring-flowering) and also utilizing annual species, I was successfully fit floral provisioning into a rotational agricultural ecosystem, such that it was logistically feasible, phenologically relevant, matching plant flowering and bee activity, and was heavily utilized by bee species highly relevant to providing pollination services. By further examining how various seeding rates affect the floral density of the planting and the behavior of the pollinators, I showed that seeding rate did not significantly reduce either of these attributes. With this data, recommendations to growers can be made that may result in more cost effective options, and in turn, potentially result in a higher number of growers adopting these practices.

In Chapter 3 I demonstrated that the nutritional quality of the pollen in floral provisioning plantings commonly found within the Mid-Atlantic region differentially affected the
fitness of *Bombus impatiens*, as quantified by ovary development. Beyond ovary development, I was also able to show that diet significantly affected waxing behavior, shedding light on the complexity of resource quality and allocation within *Bombus impatiens* as a function of resource quality. This study also documented the necessity of adult pollen consumption by *B. impatiens*. Considering the significant efforts being made to augment pollinator habitat, an issue I approached in Chapter 2, serious consideration should be given to the quality of the floral resources, as well as the intended pollinator to be supported, when designing these flowering strips. In order to sufficiently address this issue, more comprehensive studies need to be conducted examining the nutritional needs of various pollinator species in addition to the nutritional quality of the pollen.

Lastly in Chapter 4, I examined the *Cucurbita pepo* system itself by looking at insecticide usage and possible risks of exposure for *Bombus* and *Apis mellifera*. I examined how different methods of neonicotinoid treatment affected the floral display within *Cucurbita pepo*, and how this persisted into the foraging behavior of the observed pollinators. The data collected from this study showed that generalist pollinators within *Cucurbita pepo* preferentially visit plants treated with neonicotinoids, although the cause of this dynamic is not completely clear. Regardless of whether flower number, or floral resource quality as a result of neonicotinoid treatment is causing this interaction, under choice conditions exposure to neonicotinoids is increased as a result. As a majority of studies examining how sublethal exposure to neonicotinoids are lab-based, I attempted to begin filling this massive knowledge gap with this study. In doing so, I unearthed a complex dynamic between neonicotinoids and generalist pollinators in *Cucurbita pepo* and laid the groundwork for more comprehensive future studies.

In addition to concrete findings, this thesis also cast light on the myriad of unanswered questions that beg to be addressed in future studies. To address some of the questions posed in Chapter 2, seeding rates within floral provisioning strips should be further examined with their
subsequent effects on additional characteristics also considered. For example, if and how, yield of crops within the agroecosystem are affected by variation in seeding rate (as a result of pollinator retention within the system), and to expand on the effects of seeding rates on plant-pollinator dynamics, floral attractants, e.g., pollen, nectar, and scent, should also be examined. Along with seeding rate, optimization of cover crop mixtures for provisioning pollinators need to consider classic horticultural management factors, such as the influence of soil, water, and plant nutrients, all of which could influence floral display. Considering floral attractant and resources, our results from Chapter 3 lead to a number of questions concerning pollen quality, nutrient allocation, and the use of microcolonies for nutritional studies. As we observed certain pollen types to be better for ovary development and waxing behavior, taking a step back from this finding and considering the results from Chapter 2, we see that high-quality pollen resources do not always translate to increased visitor number in the field (as was observed with sunn hemp). Moreover, future studies should also examine if nutrient allocation within an organism e.g., bumble bee, will differ as a result of pollen quality, and if so, what internal processes may be dictating this.

To lastly consider Chapter 4, a number of directions can and should be taken to examine if Bombus and Apis mellifera do preferentially visit neonicotinoid treated plants within the context of a field experiment. Because there was a complex relationship between plant behavior, neonicotinoids, and bee behavior, future studies should examine this dynamic more closely. With the question of floral number begging attention, the use of artificial flowers could be an easily manipulated variable to disentangle this relationship. Moving forward as whole, this topic should be approached systematically, with an establishment of neonicotinoid concentrations in the pollen and nectar being the most informative and necessary to establish. Secondarily, examining bee visitation across the length of a day, length of a season and across numerous bee and crop species, will be necessary to build a solid foundation for this research area. Lastly, examining if and the
extent to which health and physiology is affected within visitors exposed to neonicotinoids will provide a more comprehensive picture of this issue.

By approaching pollinator conservation in an agroecosystem using an integrative approach, I hope to emphasize the importance of considering all components of an agroecosystem. These suggestions and approaches used are meant to improve our understanding and ability to support pollinators within Mid-Atlantic agroecosystems, through threat mitigation and a more targeted approach to floral provisioning.
References


Appendix

Chapter 2: Supplementary information

See Chapter 3 Methodology for information on nutritional analyses

Chapter 3: Supplementary information

Methodology used for the nutritional analyses (Vaudo et al. 2016)

We analyzed the protein concentration of pollen using the Bradford assay. To prepare the samples for analysis, we divided the pollen into three 1mg replications for each individual plant species in 1.5mL Eppendorf microcentrifuge tubes (Eppendorf North America, Hauppauge, NY). To facilitate breaking of the pollen wall, three drops of 0.1M NaOH were added to each sample and then ground with a microcentrifuge pestle. After grinding, the sample was filled to 1.5mL of 0.1M NaOH and vortexed. All samples were allowed to sit for 24 hours. We conducted the Bradford assay with the Bio-Rad Protein Assay Kit microassay 300 µL microplate protocol using bovine γ-globulin as the protein standard (Bio-Rad Laboratories, Inc., Hercules, CA). Due to the high protein concentration of the pollen, we diluted 50 µL of each replicate into 100 µL 0.1M NaOH in each well of a BD Falcon 300 µL sterile non-tissue culture treated 96 well plate (BD, Franklin Lakes, NJ). Absorbance readings at 595nm were measured using a SpectraMax 190 spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA) and protein concentrations calculated using simple linear regression analysis from the protein standards using SoftMax Pro v.4.0 software (Molecular Devices, LLC 2001)
Pollen lipid and carbohydrate concentrations were determined using a modified protocol from Van Handel 1988 (65). To prepare the samples for analysis, we divided the pollen into three 1mg replications for each individual plant species in 1.5mL Eppendorf microcentrifuge tubes. We added 0.2mL 2% sodium sulfate into each tube and homogenized the samples with a microcentrifuge pestle. We washed each sample into a glass tube with 1.6mL chloroform/methanol and centrifuged the samples at 3000 rpm for 5 min. We transferred the supernatant to a clean glass tube, added 600 µL DI water, and centrifuged the sample at 3000 rpm for 5 min. We separated the top carbohydrate/water/methanol fraction for sugar analysis and remaining chloroform fraction was used for lipid analysis. For carbohydrate analysis, we heated each sample at 100°C to evaporate the solvent to ~100µL. We added anthrone/sulfuric acid reagent to equal 5mL and heated at 100°C for 17 min. Each sample was removed from the heat and allowed to cool. We used two technical replications for each biological replication and measured absorbance at 625nm using a SpectraMax 190 spectrophotometer. Carbohydrate concentrations were calculated using simple linear regression analysis from anhydrous glucose standards using SoftMax Pro v.4.0 software.

The lipid/chloroform fraction was heated at 100°C to evaporate the solvent. We added 0.2mL sulfuric acid to the sample and heated at 100°C for 10min and then added vanillin/phosphoric acid reagent to equal 5mL, removed from heat, and allowed to cool. We used two technical replications for each biological replication and measured absorbance at 525nm using a SpectraMax 190 spectrophotometer. Lipid concentrations were calculated using simple linear regression analysis from vegetable oil standards using SoftMax Pro v.4.0 software. Pollen concentrations of protein, carbohydrate, or lipids is reported as µg nutrient/mg pollen and subsequent protein:carbohydrate (P:C) and protein:lipid ratios (P:L) were determined for each plant species.