IMPLICATIONS OF HABITAT RESTORATION FOR BUMBLE BEE POPULATION DYNAMICS, FORAGING ECOLOGY, AND EPIDEMIOLOGY

By

Knute Baldwin Gundersen

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ABSTRACT

IMPLICATIONS OF HABITAT RESTORATION FOR BUMBLE BEE POPULATION DYNAMICS, FORAGING ECOLOGY, AND EPIDEMIOLOGY

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Many insects provide valuable ecosystem services, including those that support our food supply. Beneficial insects such as pollinators fulfill part of this role by contributing to approximately one third of the global food crop production. Over the past few decades, pollinators have faced declining populations due to a variety of factors such as agricultural intensification, lack of floral and nesting resources, and disease. One method used in agricultural settings to help sustain pollinator populations is designating unfarmed habitat such as ditches and field margins for habitat enhancement in the form of hedgerows and wildflower strips. These floristically rich areas can be tailored to bloom both before and after crop bloom to help sustain pollinators during the time when crops are not in bloom. In turn, bee populations can benefit from the consistent availability of resources in these areas of habitat enhancement. This dissertation explores how habitat enhancement affects nesting density of a common wild pollinator, *Bombus impatiens*. Further, this research also aims to determine how foraging preferences change and how bumble bee disease transmission and prevalence respond to habitat enhancement. Research was conducted at 15 commercial highbush blueberry (*Vaccinium corymbosum*) fields in southwest Michigan containing either no restoration, a newly planted restoration, or a mature (5-8 year old) restoration in the field margin from 2015 to 2017. I found that sites with new and mature restorations significantly boosted *B. impatiens* population abundance in the surrounding landscape. Sites with new restorations also had significant increases in *B. impatiens* colonies within one year of establishment and a tripling in density over

the course of the three-year study. In my second study, at sites without restorations bumble bees foraged on a variety of flowers, whereas in sites with new and mature restorations, greater numbers of highly attractive flowers allowed bumble bees to retain floral constancy. Further, this study reveals the sensitivity of plant-pollinator networks to variation in habitat management in similar environments, highlighting the need for greater replication in studies that monitor changes in plant-pollinator networks over time. Finally, I found that the prevalence of *Crithidia bombi*, a common pathogen of bumble bees, in bumble bee gut tissue and on flower surfaces is significantly increased at sites with habitat enhancement. Flowers that were considered highly attractive were more likely to screen positive for a pathogen, highlighting the important role that flowers play in the horizontal transmission of pathogens. The results of this work shed new light on the underlying complexity of bee restoration in agricultural settings and will help inform best management practices for bee conservation in the future.

This dissertation is dedicated to Kateri,
who has supported my work in countless ways.

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CHAPTER 1

INFLUENCE OF HABITAT ENHANCEMENT ON BUMBLE BEE ECOLOGY AND PATHOGEN INTERACTIONS

INTRODUCTION

Many insect species provide valuable ecosystem services, including those that support our food supply (Gallai et al. 2009, Calderone 2012, IPBES 2016). Beneficial insects such as pollinators fulfill part of this role by contributing to approximately 35% of global food crop production (Kearns et al. 1998, Klein et al. 2007, Kremen et al. 2007). The specific value of pollination services attributed to pollinator groups (or even pollinators in general) is difficult to discern but there have been estimates of total global value at 235-577 billion due non-standardized sampling of yield data and extrapolation methods across studies (Hanley et al. 2015, IPBES 2016). The majority of crop pollination is done by honey bees. However, honey bees themselves are not always the most efficient pollinators for every crop system (Javorek et al. 2002, Winfree et al. 2007, Isaacs et al. 2016). Thus, alternative pollinator species like alfalfa leaf cutter bees (*Megachile rotundata*), *Osmia* spp., and bumble bees (*Bombus* spp.) are managed to supplement pollination in various crop systems where honey bees may not be as efficient (James and Pitts-Singer 2008, Winfree et al. 2008, Pitts-Singer and Cane 2011). Considering the declining honey bee populations in the United States (Natural Research Council 2006, vanEngelsdorp et al. 2010) and Europe (Potts et al. 2010), alternative managed pollinators and wild bees from the surrounding landscape are economically important because they can provide insurance to crop pollination when honey bees are not present or are in limited supply (Winfree et al. 2007). The declines in honey bees may also mean that losses in wild bee species have a proportionally larger negative effect on pollination services to plants (Winfree et al. 2007).

There is increasing evidence for declining populations of wild bees (Biesmeijer et al. 2006), including species that provide pollination services to crops (Allen-Wardell et al. 1998). Species richness from the genus *Bombus* was shown to have declined over the previous 140

years based on long-term data collection, although this was not the case for the other genera studied (Bartomeus et al. 2013). However, the population size response varied based on body size, diet breadth, and latitudinal range (Bartomeus et al. 2013). There is no one clear driver of bee species decline, due to factors affecting each bee species differently, but the most common possible reasons for declines in bee species include agricultural intensification (Kremen et al. 2002, Isaacs and Kirk 2010, Koh et al. 2016), habitat fragmentation (Kearns and Inouye 1997, Grixti et al. 2009), disease (Colla et al. 2006, Cameron et al. 2011, Graystock et al. 2013), climate change (Kerr et al. 2015), pesticide usage (Kevan 1975, Johansen 1977, Whitehorn et al. 2012, Arena and Sgolastra 2014, Feltham et al. 2014, Rundlof et al. 2015, Woodcock et al. 2017), and decreased genetic variability (Cameron et al. 2011, Lozier et al. 2011). Combinations of the above stressors such as pesticide pressure, habitat degradation, and lack of food resources can also have synergistic effects that could lead to compromised immune systems in bees, putting them at greater risk of pathogen infection (Goulson et al. 2015). Concern over declines in pollinators and the implications for agriculture and natural systems have led to a Presidential Memorandum calling for a Pollinator Health Task Force to promote the health of bees in the United States (Obama 2014, 2015).

One method of supporting pollinators, especially in agricultural settings, is by providing floral resources throughout the landscape (Kovacs-Hostyanszki et al. 2017). This can range from woody shrubs and trees to perennial or annual flowers planted in field margins or around ditches. However, the type and level of restoration practice employed is largely dependent on the grower’s willingness to spend the time and money to provide these resources, which can be costly. Specific floral provisioning methods can be achieved through the implementation of un-mown ditches alongside roads and crop fields (Grixty et al. 2009, Hopwood 2010), flowering

hedgerows (Hannon and Sisk 2009, M'Gonigle et al. 2015), cover crops (Rundlof et al. 2014, Eberle et al. 2017), and wildflower plantings (enhancements) (Carvell et al. 2007, Blaauw and Isaacs 2014, Williams et al. 2015, M'Gonigle et al. 2017). These enhancements contain a mix of perennial flowers that bloom throughout the growing season, providing bees with resources (pollen and nectar) needed to sustain nest growth and reproduction of bees in resource deficient landscapes. Within 1000 m of bumble bee nests, maintained spring and summer floral provisions from restoration based agri-environment schemes were shown to improve bumble bee persistence in the landscape across bumble bee generations Carvell et al. 2017). Wildflower plantings were also shown to be a source of bumble bee queens, indicating that targeted agri-environment schemes aimed to support pollinators also provide adequate nesting habitat for bumble bees (Carvell et al. 2017). Furthermore, Carvell et al. (2017) found that not all of the spring or summer flowering resources were positive indicators of bumble bee persistence from generation to generation. Thus, determining which flowers provide the best provisions to both bumble bee workers and queens is essential to providing a truly targeted agri-environment scheme to support bumble bees. Although bumble bees were found to increase in abundance (Blaauw and Isaacs 2014) and persistence from generation to generation (Carvell et al. 2017), we do not know how bumble bee carrying capacity in intensively agricultural systems responds to increases in floral floral resources provided by wildflower enhancements. Because bumble bee workers and queens are using managed wildflower plantings as both nesting and foraging resources, my research aims to determine how population density of bumble bees changes in response to the implementation of wildflower plantings. In this research, I used microsatellites as a tool to differentiate individual bumble bee nests.

Although wildflower plantings are generally regarded as beneficial to pollinator populations and the pollination of adjacent crops (Carvalheiro et al. 2012, Blaauw and Isaacs 2014, Williams et al. 2015), individual flowers can act as platforms for pathogen transmission between bees visiting those flowers (Durrer and Schmid-Hempel 1994). Previous studies have shown that pathogens can be spread through deposition on flower surfaces, through the nectar, and pollen (Durrer and Schmid-Hempel 1994, Singh et al. 2010, Salathe and Schmid-Hempel 2011, Meeus et al. 2011). Further, the effects of flower longevity, phenology, morphology, chemistry, and rewards on pathogen transmission has been explored (Shykoff et al. 1996, Shykoff et al. 1997, Biere and Honders 2006, Sasu et al. 2010), but there is little known regarding how large floral provisions affect pathogen transmission among bees in the landscape. For example, recent research has determined that many bee pathogens can be transferred through deposition or fecal-orally within a nest or at flowers (Singh et al. 2010, Cisarovsky and Schmid-Hempel 2014, Graystock et al. 2015) and multiple bee species can act as vectors for pathogens that are virulent for a different bee species (Ravoet et al. 2014). Another growing concern is that managed bees can transmit pathogens to wild bee colonies in a phenomenon known as pathogen spillover (Colla et al. 2006, Graystock et al. 2013, Graystock et al. 2014, 2015, 2016). In farms where habitat plantings are implemented, these concentrated areas of nutritional resources may also provide locations where the rates of pathogen transfer are elevated. This potentially negative effect of diseases would limit the potential of habitat plantings to enhance bee populations, but there is limited information on whether bees in these plantings are exposed to higher pathogen loads. My research also aims to elucidate whether wildflower plantings influence exposure of bumble bees to pathogens, and the role of different plant species in contributing to this pathogen exposure.

BUMBLE BEES

Bumble bees (Hymenoptera: Apidae, *Bombus* spp.) are one of the most well studied taxa of bee due to their large size, coloration allowing for easy identification of most species, and because of an interest from the general public (Kearns and Thompson 2001). Bumble bees are haplo-diploid organisms that have an annual life cycle, where a single mated queen will emerge from hibernation in the early spring or summer to begin provisioning a nest. Over the course of the summer, the queen will lay eggs, producing workers that can help bring in provisions from flowers in the surrounding landscape. Towards the end of the colony life cycle, the bumble bee queen will begin to produce reproductive (gynes and drones). The newly emerged gynes will then make a foraging trip, during which she will mate with one or several drones from other colonies. Afterwards, the new gyne will search out a location for her hibernaculum, which will last until the next spring or summer (Kearns and Thompson 2001).

They are also significant pollinators of wild and cultivated plants because they are commonly abundant, efficient at collecting and transferring pollen between flowers, and are generalist pollinators (Waser et al. 1996, Kearns and Thompson 2001). Many of the specialty crops grown in the U.S., such as watermelon (Kremen et al. 2002, Winfree et al. 2007), pumpkin (Artz and Nault 2011), and blueberry (Dogterom et al. 2000, Javorek et al. 2002) are pollinated more efficiently by bumble bees than by honey bees due to their ability to buzz pollinate (Buchmann and Hurley 1978). In some cases, wild bee populations, including bumble bees, are able to provide similar levels of pollination services as honey bees that are stocked in certain crop fields, such as watermelon (Kremen et al. 2002). Native pollinators such as bumble bees were also found to adequately pollinate blueberry in small and isolated fields (Isaacs and Kirk

2010) likely due to their ability to pollinate blueberry in cool conditions and with higher crop fidelity (Tuell and Isaacs 2010).

Due to the efficiency of bumble bees as pollinators and the recent declines in honey bee populations, several companies, such as Koppert Biological Systems Inc. (Howell, MI) and Biobest (Leamington, Ontario) have started to sell *Bombus impatiens* as an alternative pollinator for use in open field settings in eastern North America. Commercially produced bumble bees are well known for their effectiveness in greenhouse crops, such as tomatoes and peppers, which has made them a popular option for greenhouse pollination (Dogterom et al. 1998, Palma et al. 2008). Commercially produced *Bombus impatiens* is also used in open agricultural settings such as blueberry in Michigan. However, commercially produced bumble bees can have higher pathogen prevalence than wild bees in the surrounding landscape (Colla et al. 2006) including pathogens specific to honey bees (Graystock et al. 2013, Graystock et al. 2015), which could be spread to other bee species in the environment. Pathogen spillover from commercial bumble bees to wild bumble bees can infect up to 35% of wild bumble bees in a given landscape, and the pathogens are able to spread outwards from a point source at rates up to two kilometers per week (Otterstatter and Thomson 2008). While commercially produced bumble bees can be an effective alternative to honey bees in many pollination-dependent crops, growers run the risk of facilitating the spread of pathogens to native bumble bees and potentially also to honey bees (Graystock et al. 2013).

The spread of bee pathogens is an important factor in the decline of some bumble bee species (Colla et al. 2006, Cameron et al. 2011), but they also face habitat loss. This has reduced the availability of forage for bees, especially in agricultural settings (Grixti et al. 2009). For example, Koh et al. (2016) found that modeled native bee abundance declined in 23% of the U.S.

land area from 2008-2013, which was likely due to the conversion of natural or semi-natural land cover to agriculture. The declining abundance of bumble bees is demonstrated most starkly by the disappearance of several species of bumble bees from their native ranges, such as *B. affinis* (Hatfield et al. 2015), *B. fervidus*, *B. pensylvanicus* (Colla and Packer 2008), *B. franklini* (Thorp 2005), *B. occidentalis* (Thorp 2005, Colla and Ratti 2010), and *B. terricola* (Grixty et al. 2009). The U.S. Fish and Wildlife Service has even listed *B. affinis* as an endangered species due to its recent disappearance from most of its native range (U.S. Fish and Wildlife Service, 2017).

WILDFLOWER PLANTINGS

Agricultural intensification results in a decline of invertebrate abundance, diversity, and richness (Robinson and Sutherland 2002, Andersson et al. 2013), limiting the potential for natural pest control and pollination. Wild pollinators are negatively affected by intensification through a lack of nesting and surrounding foraging habitat (Steffan-Dewenter and Tscharntke 1999, Isaacs and Kirk 2010). One specific technique to conserve and enhance native pollinator populations is to implement native wildflower plantings in or alongside crop field margins (Marshall and Moonen 2002, M'Gonigle et al. 2017). Management of non-cropped habitat in agricultural settings can also be a cost-effective means of maximizing crop yield through increased pollination (Nicholls and Altieri 2013, Blaauw and Isaacs 2014).

Kremen et al. (2002) found that when honey bees are not present, at least 20 native bee species are needed for proper pollination of watermelon, a crop that blooms over long periods and is dependent on bee pollination. This highlights that management of wild pollinators can be important for providing crops with suitable and sustainable levels of pollination (Kremen et al. 2002). In an effort to reverse the trends of declining pollinator populations, U.S. growers and

landowners have access to economic incentives to boost native bee populations through USDA agencies such as the Farm Service Agency (FSA) and Natural Resources Conservation Service (NRCS). Through these agencies, growers can receive payments for maintaining pollinator friendly habitats in marginal farmland or even obtain financial aid to plant pollinator friendly seed mixtures on farms, covering up to 90% of seeding cost (Dicks et al. 2016).

In Michigan, a combination of native perennial flowering plants and grasses can be established as wildflower plantings, which bloom from early spring through fall (Figure 1.1) (Carvell et al. 2007, Tuell et al. 2008, Blaauw and Isaacs 2012, 2014). By overlapping the flowering periods of several plant species, resources are consistently provided to native pollinators and other beneficial arthropods throughout the growing season (Tuell et al. 2008). This local increase in floral diversity and abundance throughout the season is expected to increase the diversity and abundance of bees as well (Potts et al. 2003, Williams et al. 2015). The overall benefit to crop yield may be substantial due to an increase in abundance of natural enemies and pollinators in crop fields (Haaland et al. 2011, Wratten et al. 2012, Blaauw and Isaacs 2014). Furthermore, Blaauw and Isaacs (2014) found that improved crop yields due to ecosystem services of wild pollinators can offset the costs of a wildflower planting after 3-4 years.

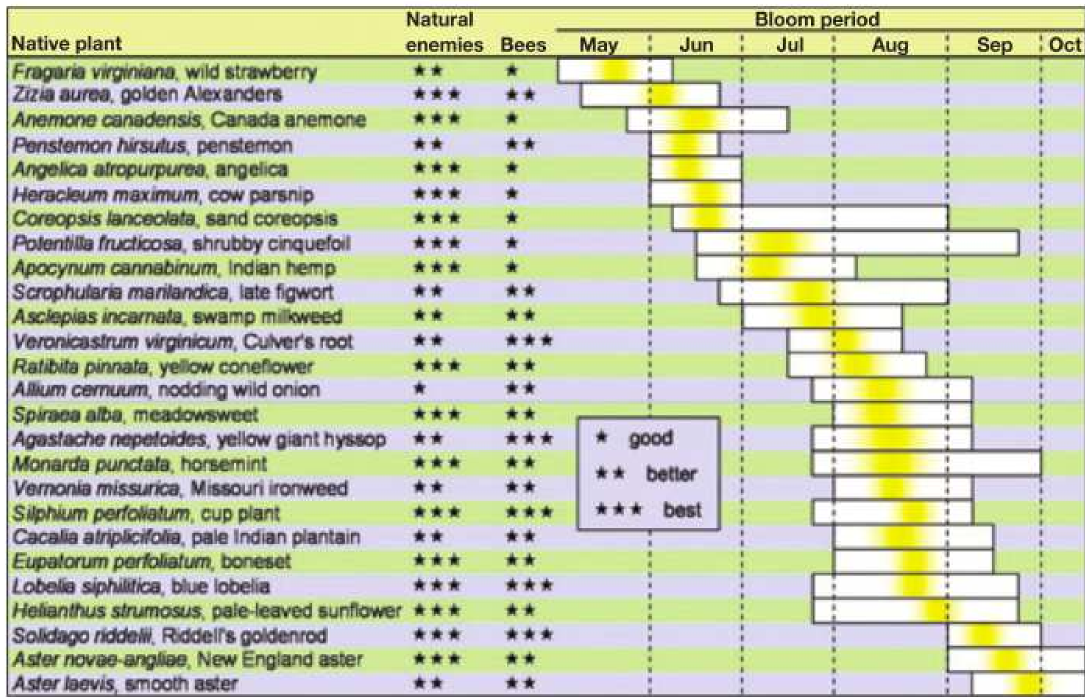

Figure 1.1. Michigan native wildflower species, with their relative attractiveness to natural enemies and pollinators, as well as their bloom periods during the summer (Isaacs et al. 2008).

Although wildflower plantings seem to have positive local effects on pollinator diversity and abundance, the underlying mechanisms are not fully understood. Plantings may increase local nest density or simply concentrate pollinators from the surrounding area onto the available floral resources (Jha and Kremen 2013, Morandin and Kremen 2013). In addition, little is known regarding the potential effect of wildflower plantings on pathogen spread among native pollinators in agricultural landscapes.

BUMBLE BEE NEST DENSITY

Targeted agri-environment schemes such as wildflower plantings have been shown to increase the chance of bumble bee survival and success into the next generation and they are significant sources of bumble bee queens in the spring (Carvell et al. 2017). Because wildflower plantings act as nesting sites for bumble bee queens, understanding how bumble bee populations change

over time in response to habitat enhancement is essential to determine how impactful these types of targeted agri-environment schemes are for bumble bees. Bumblebees are social insects, with haplodiploid reproductive systems in which sister bees from the same nest live together. Using population genetics techniques, nest density can be determined based on the relatedness of workers and drones in a hive through the use of microsatellite markers (Darvill et al. 2004, Knight et al. 2005, Sidhu 2013). Microsatellites are a common tool used to determine relatedness between sister bees (Estoup et al. 1993). Each microsatellite marker amplifies at a particular locus within the bee genome. When multiple different microsatellite markers are used in tandem, differences in the alleles amplified can be used to differentiate bees from different colonies through the use of maximum likelihood methods (Jones and Wang 2010).

Depending on the species of bee, different numbers of microsatellite markers have been developed in order to determine nest differentiation. For example, Knight et al. (2005) used 8 microsatellite markers for *B. pascourum* and *B. pratorum*, and 9 for *B. terrestris* and *B. lapidarius.* Microsatellite markers are unique to different species, and for the North American species, *B. impatiens*, there are 9-12 microsatellite markers used to differentiate colonies (Sidhu 2013, Lopez-Uribe et al. 2017, Suni et al. 2017).

Several studies have utilized microsatellites to determine nest density of bumble bees in farmland, by collecting bees at several sites with radii of 50m along transects from 1.5km (Knight et al. 2005) to 10km (Darvill et al. 2004). A recent study captured bees at 18 separate sites, each with an 800m sample radius, and recorded the coordinates for each bumble bee captured (Wood et al. 2015). Darvill et al. (2004) found that bumble bee nest density varied substantially from site to site and between species (13 – 193/km^2) along their transects. However, their sampling ran through several different landscape types, including mixed farmland, gardens,

and wooded areas. The difference in nest density may be due to the differences in the amount of forage resources available at each site along the transect (Darvill et al. 2004). Carvell et al. (2011) suggest that there is a positive relationship between the amount of forage resources available and the overall nest density of bees in the area, meaning that sites with high floral abundance could support higher nest densities. Wood et al. (2015) also found that in UK farmland, those sites with more than 2% of the land dedicated to flower rich resources for bees for more than three years had positive impacts on bumble bee nesting density in four species of bumble bees, compared to sites without pollinator-focused schemes. While the benefits of habitat plantings for pollinators are increasingly well documented, the mechanisms underlying these effects remain unclear, and should be understood to know how to develop conservation strategies.

PLANT-POLLINATOR NETWORKS

Networks are a commonly used tool to help visualize and analyze data for characterizing biological interactions ranging from two tiered interactions such as plant-pollinator networks (Memmott et al. 1999) to large-scale, community wide interactions (Pocock et al. 2012). Plant-pollinator networks or webs can be developed for ecological communities of pollinator species visiting single or multiple plant species for nectar and pollen. These interactions depict the abundance of the plants and pollinators as well as the frequency of interactions between the two (Memmott 1999). Plant-pollinator webs are capable of showing the entire community structure of pollination systems including plant and pollinator phenology (Figure 1.2)(Russo et al. 2013). They can also be used to determine the most commonly visited plant species and foraging

preferences of bee species within networks, potentially enabling adaptive selection of flowering plants that support bees but have lower potential for pathogen spread.

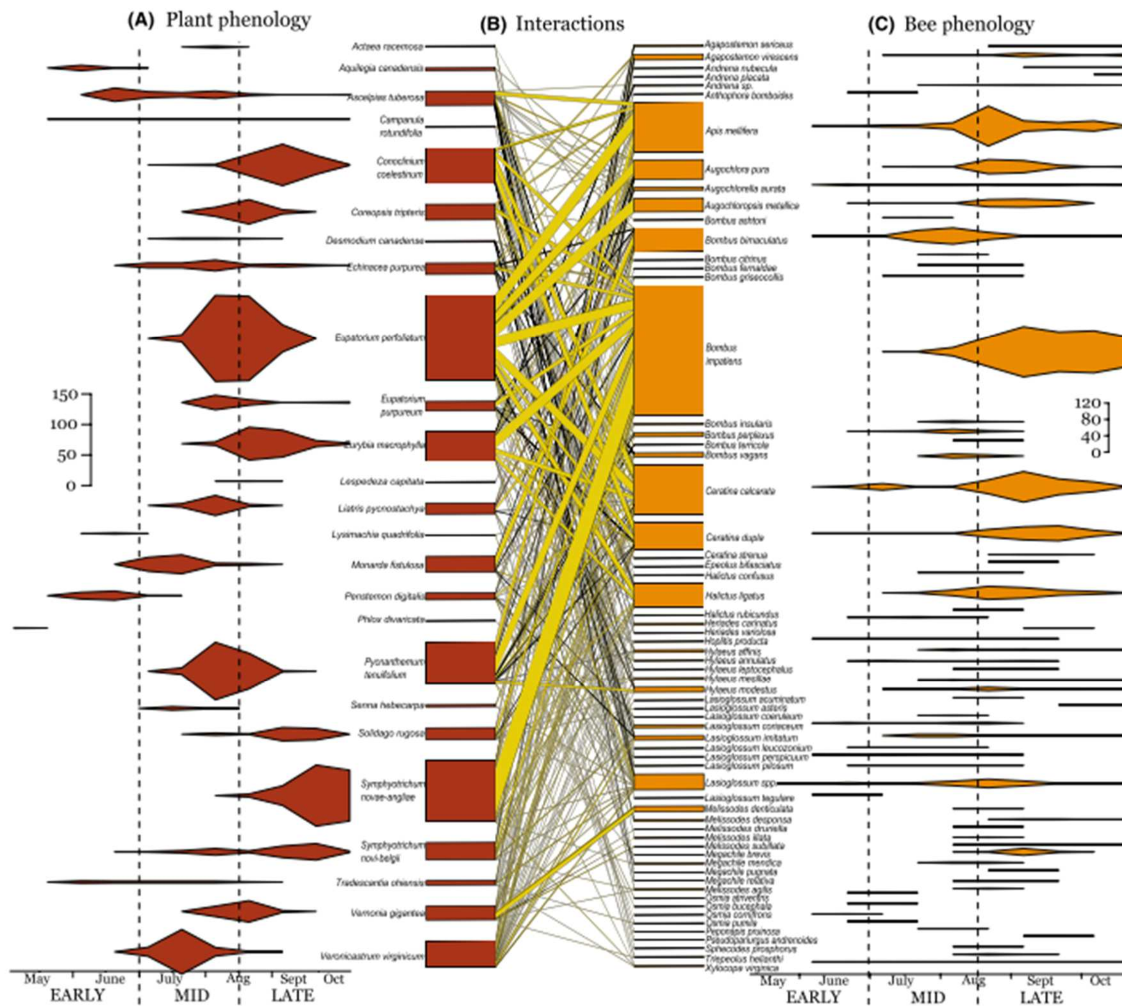

Figure 1.2. Example of a plant-pollinator network showing plant and bee phenologies (violin plots). The bars next to the yellow interactions represent the relative abundance of each of the plant or bee species, while the yellow lines linking plants to bees represent their interactions, with wider lines representing interactions that occurred more frequently (Russo et al. 2013).

Plant-pollinator webs can also be analyzed at the network level rather than just the species level as mentioned above. This allows scientists to explore important network level plant-pollinator hypotheses such as how generalist pollinator species interact with specialist plant species; whether pollinators within a network display complementarity; and whether plant-pollinator

networks are more robust to extinction than random networks (Dormann et al. 2009). These hypotheses can be tested by analyzing metrics such as connectance, nestedness, C-score, extinction slope, and robustness of the entire network (Dormann et al. 2009). The level of connectance and nestedness in a network for example can reflect how many pollinators and plants are interacting with one another out of all the possible interactions available in the network. These metrics provide insight into the proportion of plant and pollinator generalists or specialists within a network and are robust to variations in network size and collection methods such as sampling intensity (Gotelli and Graves 1996, Bascompte et al. 2003, Bluthgen et al. 2006, Dormann et al. 2009). Network level analyses also have practical applications and can give restoration ecologists insight into the development of wildflower plantings through the restoration process, which plant or pollinator species should be given priority in the context of the specific restoration project (Devoto et al. 2012), or how changes in land-use can affect declines in pollinators (Weiner et al. 2014). From network level analyses, we can also determine foraging strategies employed by specific groups of pollinators (Stone and Roberts 1992, Atmar and Patterson 1993, Gotelli and Graves 1996, Bersier et al. 2002), which can be used to explore pollinator response to habitat enhancement.

The sampling that goes into creating a plant-pollinator network is highly time consuming, usually resulting in low numbers of study sites being used for replication (Pocock et al. 2012, Russo et al. 2013, Tucker and Rehan 2016, Parra-Tabla et al. 2017). This is not universal, however, as highlighted by sampling of similar sites in multiple environments (Ramirez-Flores et al. 2015) and studies of multiple sites within a single environment (Santamaria et al. 2017). Although studies that focus intensely on capturing the minutia of a plant-pollinator community at a single site are valuable, they lack replication and may not accurately reflect the community

found at similar sites. This has implications for the extrapolation of these data to a broader context. My research aims to explore the variation in networks in different habitat enhancement treatments within similar local landscape contexts.

Plant-pollinator networks have typically been developed using only plants and insects. However, there are some cases where three or more components have been examined, including work by Henson et al. (2009), which linked plants to pollinators and pollinators to parasites/pathogens in a three-tier network (Figure 1.3). In a similar structure, Pocock et al. (2012) examined many ecological interactions on a farm including those between plants and pollinators.

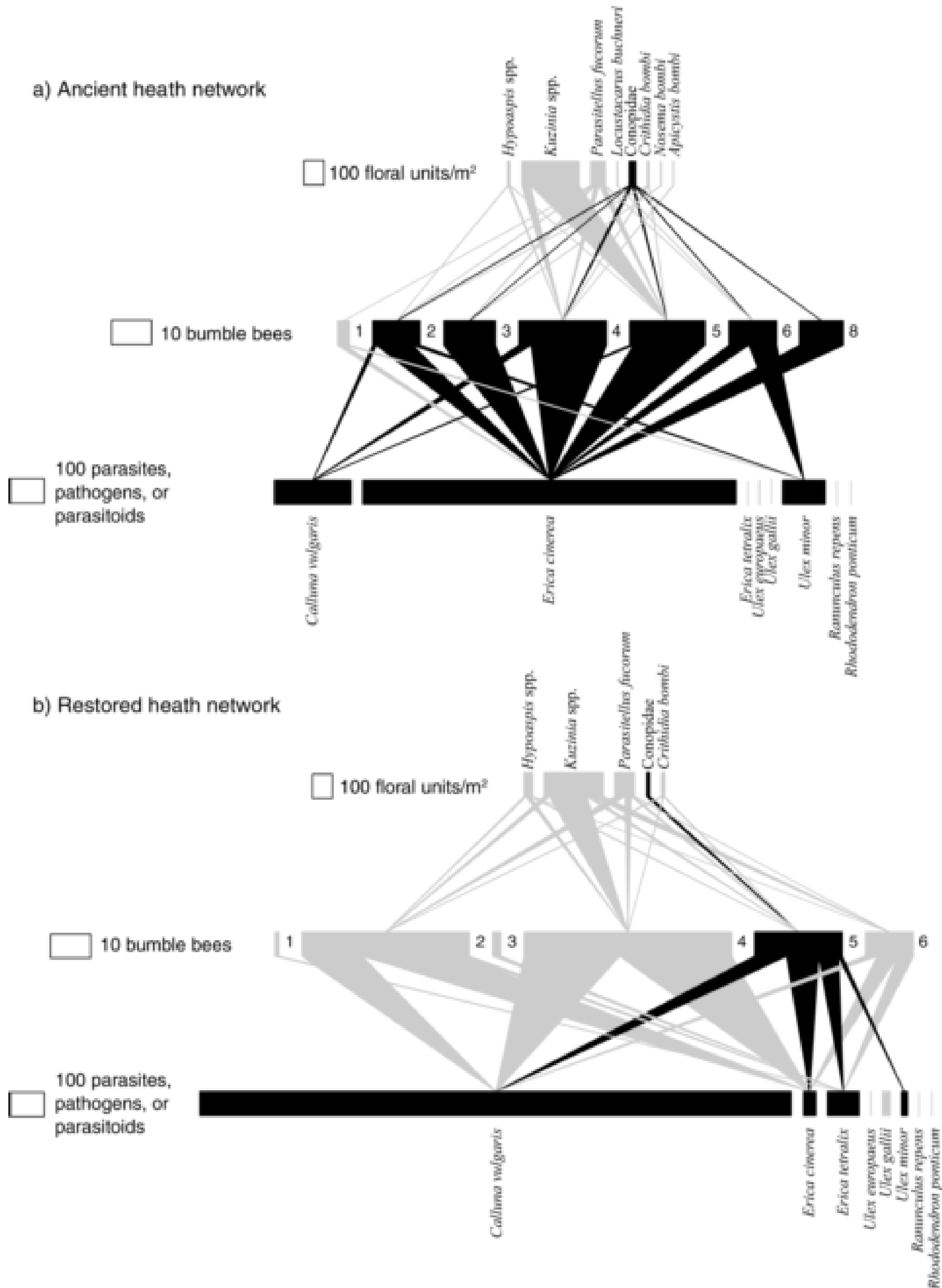

Figure 1.3. A comparison of plant-pollinator and pollinator-pathogen networks in ancient and restored heathland (from Henson et al. 2009). The top interaction at each treatment reflects a plant-pollinator interaction web with horizontal bars representing relative abundance of plant and pollinator observations, while the triangles connecting them represent the relative frequencies of interactions between the plants and pollinators. The bottom level represents the pollinator-pathogen interactions, meaning that pollinators "interacting" with specific pathogens are infected or carrying those specific pathogens.

Community wide approaches should be used to elucidate the effects of wildflower plantings on the plant-pollinator interactions (Allen-Wardell et al. 1998). The implementation of native wildflower plantings in field margins is expected to increase plant pollinator interactions over time due to increased abundance and richness of flowers and bees in the plantings due to increased flower and resource diversity (Potts et al. 2003). But, little is known regarding how wildflower plantings influence pathogen abundance and transfer amongst bees foraging in these areas. My project will use this framework to examine the role of pathogens in an ecological web of plants and bees and some of the key diseases that affect bumble bees.

THE ROLE OF PATHOGENS IN BUMBLE BEE DECLINE

Pathogen infection is one of the major contributors to pollinator decline, particularly in bumble bees (Colla et al. 2006, Cameron et al. 2011, Graystock et al. 2013, Koch et al. 2017). Certain species of bumble bees may be more impacted than others due to variation in range, size, abundance, resistance to infection, and level of exposure to pathogens (Price et al. 1986, Williams and Osborne 2009, Gillespie 2010). This includes commercially reared species such as *B. impatiens* Cresson, among which pathogens are common (Otterstatter et al. 2007, Graystock et al. 2013) yet populations of commercial bumble bees are relatively stable. Several important pathogens found in bumble bee colonies affecting their performance are the neogregarine *Apicystis bombi*, the trypanosome *Crithidia bombi*, and the microsporidia *Nosema bombi* (Durrer and Schmid-Hempel 1994, McIvor and Malone 1995, Graystock et al. 2013, Plischuk et al. 2017). Many bumblebee species can also vector diseases to other economically important pollinators such as honey bees (*Apis mellifera*) (Graystock et al. 2013, Manley et al. 2015).

Pathogens can be spread either vertically or horizontally. In vertical transmission, the pathogens are spread from generation to generation by the overwintering bumble bee queens. In

horizontal transmission, pathogens can be spread in multiple ways including sexual transmission (Roberts et al. 2015), plant-bee foraging interactions (McArt et al. 2014, Graystock et al. 2015, Koch et al. 2017), contact between bees and feeding brood within the colony (Folly et al. 2017). Durrer and Schmid-Hempel (1994) originally showed this by allowing bumble bees infected with *C. bombi* to forage on pathogen-free flower inflorescences for three hours before provisioning pathogen-free *B. lucorum* and *B. terrestris* workers with the same flowers. Several days later *C. bombi* was detected in 39.1% and 21.4% of the *B. lucorum* and *B. terrestris* workers respectively (Durrer and Schmid-Hempel 1994). Since then, much of the focus has shifted to understanding the driving mechanisms behind foraging and bee epidemiology such as the influence of floral traits (McArt et al 2014), secondary metabolites in nectar (Richardson et al. 2015, Palmer-Young et al. 2017a) and pollen, and diet breadth (Koch et al. 2017). Although horizontal transmission of diseases at flowers is receiving a significant amount of attention, a better understanding of the underlying mechanisms of how floral traits influence bee epidemiology is still needed and is a critical component to understanding how to improve pollinator health (Koch et al. 2017).

Apicystis bombi is a protozoan (Neogregarinorida: Lipotrophidae) found in bumble bee fat, intestinal tissues, and the spermathecae of queens (Liu et al. 1974). *Apicystis bombi* is widespread, occurring in North America, South America, Europe, and Asia (Lipa and Triggiani 1992, Colla et al. 2006, Plischuck and Lange 2009, Morimoto et al. 2013, Plischuk et al. 2017). It can be transmitted both fecal-orally and during reproduction (Liu et al. 1974). When *A. bombi* is ingested by a host bumble bee, sporozoites will pass through the midgut wall into the body cavity and will multiply in the fat, turning the tissue white (Lipa and Triggiani 1996). Infections can lead to complete loss of fat tissue (Schmid-Hempel 2001), which could result in decreased hibernation success in queens. Bumble bees infected with *A. bombi* were also more likely to be

infected with more than one pathogen (Rutrecht and Brown 2008) or virus (Graystock et al. 2015).

Crithidia bombi (Trypanosomatida: Trypanosomatidae) is widespread in North America, South America, and Europe, and frequently infects *Bombus* spp. (Shykoff and Schmid-Hempel 1991, Cordes et al. 2012, Schmid-Hempel et al. 2014, Plischuk et al. 2017). These single-celled flagellates are found in the gut tissue and malpighian tubules (Shykoff and Schmid-Hempel 1991). *Crithidia bombi* often does not kill overwintering queens, so there is a possibility for vertical transmission (Durrer and Schmid-Hempel 1994). It is also able to spread horizontally from bee to bee through fecal-oral transmission (Durrer and Schmid-Hempel 1994, Schmid-Hempel 1998, Colla et al. 2006), which can result in up to 30% infection rates in an area. Despite being so prevalent in nature, *C. bombi* is a relatively mild pathogen that reduces the size of ovaries, causing slower growth of bumble bee colonies earlier in their life cycles (Shykoff and Schmid-Hempel 1992).

Nosema bombi (Dissociodihaplophasida: Nosematidae) is a microsporidian found in North America, South America, Asia, and Europe (Cordes et al. 2012, Huth-Schwarz et al. 2012, Schmid-Hempel et al. 2014, Plischuk et al. 2017). It can be transferred vertically, from generation to generation, and horizontally between nests and other bumble bees through pollination interactions and within nest interactions (Fisher and Pomeroy 1989, Colla et al. 2006). *Nosema bombi* are typically found in the gut tissue and Malpighian tubules of bumble bees (McIvor and Malone 1995). When *N. bombi* are found in high concentrations, the intestinal tract and Malpighian tubules can be completely destroyed. However *N. bombi* is not found in the ovaries, so this does not affect the ability of queens to lay new eggs (McIvor and Malone 1995). *Nosema bombi* can also drastically lower the amount of sperm in male bumble bees, induce

mortality and diarrhea in workers, and it can cause disorientation in workers, which can prevent the bees from fulfilling their roles in the nest effectively and efficiently (Otti and Schmid-Hempel 2007). Although *N. bombi* can have severe negative impacts on bumble bees, when *B. terrestris* were treated with *N. bombi* from different sources, they varied in the severity of infection, suggesting that the genotype of a particular colony may play a role in how intense an infection can become (Schmid-Hempel and Loosli 1998).

The pathogens above have great potential to damage bumble bee populations over large geographic areas. For example, *Bombus occidentalis* has virtually disappeared from the Pacific Northwest, and this is considered to be the result of increased importation of commercially reared bumble bees with high pathogen loads (Colla and Ratti 2010). Although the use of commercially reared bumble bees is helpful for pollination services in crops, the negative effects were not discovered until too late for this species.

Pathogen transfer at flowers was first documented by Singh et al. (2010) during a greenhouse experiment in which infected honey bees transmitted Israeli Acute Paralysis virus (IAPV) to bumble bees while foraging. Further, Graystock et al. (2015) found that flowers can act as pathogen dispersal platforms when both infected honey bees and bumblebees were able to deposit pathogens on flowers, which were subsequently picked up by other honey bees and bumble bees. This could be due to spore-cuticle adhesion, where pathogenic spores adhere to the cuticle of the bee and then rub off onto the flower. Also, specific flowering species, such as those with bell shaped flowers, had increased bee pathogen prevalence, which could have been due to the increased handling time by bees (Graystock et al. 2015). Overall, this research suggests that many parasites can be spread by multiple pollinator species visiting the same flowers (Graystock et al. 2015). Bees drawn to resource rich wildflower plantings, especially in floristically limited

areas such as intensively managed agro-ecosystems, may have a higher chance of becoming infected or spreading disease through fecal-oral transmission or spore-cuticle adhesion at mutually visited flowers. However, negative effects of pathogen transmission may be offset partially by certain floral phytochemicals found in nectar that could help bumble bees resist infection by parasites (Palmer-Young et al. 2017a). However, parasite resistance may also prevent this from occurring in the long term through evolutionary resistance to phytochemicals in nectar (Palmer-Young 2017b).

Given the potential for pathogen transfer among bees at flowers, an unintended consequence of wildflower plantings being added to farm landscapes is that they may be facilitating pathogen spread amongst bees. This could negatively affect pollinator populations over time, and if this is happening there may be ways to adapt conservation strategies to reduce pathogen spread. But first there needs to be a better understanding of pathogen levels in wildflower plantings and the dynamics of these organisms on bumble bees that have access to floral resources.

SUMMARY

This thesis was developed to explore the implications of wildflower plantings for the community of bumble bees visiting landscapes around blueberry fields, the density of bumble bee nests, the flower-plant interactions, and the abundance of pathogens that bees may be exposed to. This research was conducted in west Michigan at a series of sites with varying levels of restoration for pollinators. My first objective assesses the role of habitat enhancement on bumble bee population density. My second objective explores how bumble bee foraging ecology changes in response to habitat enhancement. Lastly, my third objective investigates the role of habitat enhancement on disease transmission in bumble bees. The results of my research will directly impact bee

conservation efforts in agricultural systems, identifying new insights to management techniques to support pollinator populations. Further, my research will shed new light on an increasingly important topic in bee conservation: the role of habitat enhancement on disease transmission in bumble bee communities.

CHAPTER 2:

HABITAT ENHANCEMENT FOR POLLINATORS INCREASES LOCAL POPULATIONS OF BUMBLE BEES

INTRODUCTION

Wild bees provide the valuable ecosystem service of pollination to many economically important crops (Free 1970, Klein et al 2007, Aizen et al. 2008, Garibaldi et al. 2013), and bumble bees are key crop pollinators in temperate regions (Winfree et al. 2007). However, the populations of some bumble bee species have been declining in Europe and North America for a variety of reasons that may include disease (Colla et al. 2006, Cameron et al. 2011, Graystock et al. 2013), habitat fragmentation (Kearns and Inouye 1997, Grixti et al. 2009), agricultural intensification (Kremen et al. 2002, Isaacs and Kirk 2010, Koh et al. 2016), and increased pesticide use (Potts et al. 2010, Arena and Sgolastra 2014, Feltham et al. 2014). In agricultural landscapes in particular, wild bees face a combination of these stressors, which can negatively affect their populations over time (Goulson 2015). The conversion of natural habitat to agricultural land is expected to negatively affect wild bee populations in approximately 23% of the United States land area including regions that encompass 39% of the US's pollinator-dependent agricultural land (Koh et al. 2016).

Declines in bee abundance and diversity can have negative effects on crop production and quality, with insufficient pollination resulting in sub-optimal fruit set of small or misshapen fruit (Tromp 1990, Isaacs and Kirk 2010, Klatt et al. 2013). Pollination effectiveness can vary widely among species of pollinator visiting flowers, with *Bombus* species being able to transfer and deposit more pollen than other bee species on a per visit basis (Dogterom et al. 2000, Winfree et al. 2007). They are therefore a target for conservation practices in farmland, to increase populations while also supporting the level and consistency of crop pollination.

Bumble bees are of particular interest for measuring the response of animals to conservation practices, due to the relative ease of studying their populations compared to smaller bee species

or much larger animals (Woodard et al. 2015, Woodard and Jha 2017). Although pollinator supportive land management practices have been shown to increase bumble bee forager and nest abundance, little is known about how they influence bumble bee nesting density over time. Hedgerows or wildflower plantings alongside crop fields can provide resources for bees throughout the growing season (Carvell et al. 2007, Carvell et al. 2011, Pywell et al. 2011, Blaauw and Isaacs 2014, M'Gonigle et al. 2015, Williams et al. 2015), providing continuous nectar and pollen resources for bumble bees before and after bloom in habitats that are typically devoid of foraging resources outside of crop bloom. The continuity of floral resources can increase bumble bee abundance, density, and diversity at sites with pollinator friendly habitat sown in the field margin (Carvell et al. 2007, Wood et al. 2015a). This can also support reproduction of bumble bee colonies that are within foraging distance of habitat enhancements (Carvell et al. 2011) and can thus increase between-season persistence of bees (Pywell et al. 2011). This increase in reproduction can then lead to spillover of workers and subsequent queen bumble bees into the surrounding landscape, which could improve crop pollination at the landscape scale (Carvell et al. 2011). Another benefit of wildflower plantings is that they can also act as attractive nesting resources for ground nesting bees (Lye et al. 2009). Together, these studies suggest that bumble bee populations benefit from the additional nectar and pollen forage in agricultural settings when crops are not in bloom. However, the higher populations of bees at restorations could also be caused by a temporary concentration response to the local resources, without an increase in overall population size. Using a combination of microsatellite genetic analysis methods (Estoup et al. 1995) and foraging range estimates for bumble bees provide a method to test the hypothesis that habitat enhancement near crop fields increases bumble bee populations.

Previous research using microsatellites to estimate the number of bumble bee nests has primarily been in Europe, with more recent developments in North America. Darvill et al. (2004) provided the first analysis of the foraging range and nest density of British bumble bees using microsatellites, estimating 13 *B. terrestris* colonies/km^2 compared with 193 for *B. pascorum*. A similar approach was taken by Knight (2005) who compared four bumble bee species, finding 26-117 nests/km^2. Later, Knight et al. (2009) reported a positive correlation between forage availability and nest density in *B. pascuorum.* Wood et al. (2015b) then used microsatellite analysis to determine that farms implementing higher level conservation practices that include more floral resources had a 2-3 times higher nest density of two bumble bee species than farms without this investment in more habitat for bees. Most recently, Carvell et al. (2017) went one step further and used non-lethal microsatellite techniques to measure the rate of survival of *B. terrestris*, *B. lapidarius*, and *B. pascuorum* lineages from year to year across a farm with varying levels of habitat enhancement. Semi-natural vegetation cover, spring flower cover preferred by queens, and spring and summer flower cover preferred by workers increased the probability that a queen would successfully overwinter and start a new colony the following spring.

In the United States, Rao and Strange (2012) used microsatellites to better understand foraging distance and nest density of *B. vosnesenskii* in Oregon. They found that maximum foraging range is at least 11.57 km and that nesting density ranged from 0.76/km^2 to 22.16/km^2 in agricultural settings. Using microsatellites, *B. impatiens* nests have been estimated in cucurbit fields in Pennsylvania (Sidhu 2013), with up to 213 colonies/km^2 supplying bees to these small crop fields. Sidhu (2013) also determined that the proportion of land cover at 250 m was positively related to the number of *B. impatiens* colonies providing workers to visit pumpkin flowers, suggesting that *B. impatiens* may have a smaller foraging range and higher nesting

density in small diversified farms. Microsatellites have also been used to infer the foraging range and nesting density of *B. vosnesenskii* as a function of habitat type and habitat composition (Jha and Kremen 2013). These authors found that floral richness rather than floral cover had the greatest positive impact on bee foraging range within a landscape and that *B. vosnesenskii* nesting density was negatively correlated with the amount of paved habitat in the landscape.

Microsatellites can also be used to determine how conservation practices affect bumble bee populations over multiple years. *Bombus impatiens* is a key pollinator of wild plants and crops across eastern North America (Winfree et al. 2007, Williams et al. 2014), and recent studies show that its abundance can be increased by addition of pollinator habitat enhancement plantings in fruit farms (Blaauw & Isaacs 2014). However, it remains unclear whether the observed response is due to attraction of foraging bees to the plantings at the expense of other unenhanced areas or if it reflects population growth in response to the local increase in resources.

This study was conducted to test the hypothesis that habitat enhancement in the form of sown wildflower plantings for pollinators causes a local increase in bee populations. This was done using microsatellite markers to estimate the population size of the common eastern bumble bee (*B. impatiens*) at farms without plantings for pollinators, compared with newly restored plantings that matured during the study, and plantings that were mature at the start of this study. I postulated that *B. impatiens* nest density would be greatest at sites with mature habitat enhancement and that bumble bee nest density would increase over time at sites with newly restored habitat.

METHODS

Site selection

Bombus impatiens were sampled at fourteen blueberry farm sites in southwestern Michigan during 2014-16. The sites consisted of five with no habitat enhancement, four with a recently restored habitat for pollinators (0-2 years old during the study), and five sites with a mature habitat enhancement for pollinators (5-8 years old) (Fig. 2.1). Sites with new and mature habitat enhancements differed primarily in that sites with new habitat enhancements typically did not see high establishment of planted species until the third year of the study, whereas sites with mature enhancements had full establishment of planted species for all three years. The area of restoration at each site ranged from 0.1-3.06 ha in size (Table 2.1), and the restorations were all established using a mix of 15-20 species of native Michigan wildflower species that are attractive to the local bee fauna (Tuell et al. 2008). The sites with no restoration and the newly restored habitats were in field margins adjacent to crop fields of highbush blueberry (*Vaccinium corymbosum*). The other five sites contained mature habitat enhancement for pollinators, planted with similar mixtures of native wildflower species, but more than 3 years ago. Four of these mature sites were adjacent to highbush blueberry and one was adjacent to an apple and pear orchard. All sites were greater than two kilometers apart to ensure that the same bumble bee colonies were not sampled from multiple sites.

Figure 2.1. Map of southwest Michigan including sites selected for this study (yellow dots).

Habitat establishment

Sites with new and mature habitat enhancements were prepared in a similar manner, despite being seeded in different years. Site preparation involved a glyphosate treatment to the area in which the planting was to be before seeding. Next a seed mixture containing a mix of native

Michigan wildflowers and grasses were planted at sites with a new (Table A1) and mature (Table A2) habitat enhancement in the fall or early spring. Sites were maintained during the first and second year of establishment by mowing in the spring followed by spot herbicide treatments to control weedy species from taking over enhancements. Sites without habitat enhancement were left to standard farm practice, which entailed a consistently mown grassy margin.

Floral surveys

In 2015 and 2016, the plant community at each site was characterized using two 50m transects that were placed along the field margin and along the edge of the field margin. Each transect was randomly placed in their respective area using a random number generator to determine the starting coordinates of each transect. Five 1m^2 quadrats were placed 10m apart along each transect. Within each quadrat, flowering flower species were identified and counted. Flowers were counted by counting each individual flower head if on separate stalks or by counting each spike or raceme with flowers on it for flowers with inflorescences. Flowers were only counted when in bloom during the surveys. Flowering plants were identified on site and in the laboratory using Newcomb's Wildflower Guide (Newcomb 1987) and Weeds of the Northeast (Uva et al. 1997). Flower species abundance, density, richness, and diversity were calculated based on flower counts obtained from transects.

Sampling and observations

During July-October in 2014-16, up to 200 *B. impatiens* were collected from each of the 14 sites. The sites were sampled for a maximum of six hours in 2014, but because that yielded too few bees particularly at the control sites without plantings, in 2015 and 2016 each site was sampled

up to 14 hours or until 200 bees were collected, whichever came first. The total number of *B. impatiens* and the total amount of time spent sampling were recorded for each sampling event (Table 2.1). Worker bumble bees were collected, but often males were collected as well. These were not analyzed in this study (see details below).

Each collected *B. impatiens* was aerial netted and placed into a 22ml plastic scintillation vial. Vials were kept in 1-gallon plastic bags marked with the location and date of collection, placed in a cooler with ice packs for up to eight hours, and then transferred to a temporary storage freezer at -20°C for up to five days, after which they were all stored at -80°C. Once frozen, each bee was identified to species and caste (Williams et al. 2014). One hind leg of each bumble bee was clipped off and cut into 4-6 pieces before being placed in a single well of a 96-well polymerase chain reaction (PCR) plate. Forceps and micro scissors were sterilized between samples by dipping them into a 10% bleach solution for at least 5 seconds, followed by two separate rinses to remove residual bleach. Plates were then stored in a -80°C freezer until further processing. Twenty voucher specimens from each year were pinned, labeled, and stored in the Albert J. Cook Arthropod Research Collection at Michigan State University (See Appendix D).

Microsatellite analysis

DNA was extracted from each bee by adding 5 μL Proteinase K (10mg/mL) (Sigma Aldrich, St. Louis, MO) and 150 μL 5% Chelex solution (Bio-Rad, Hercules, CA) to each well containing a bumble bee leg in a 96-well PCR plate. The plates were then placed in a thermocycler at 95°C for 3.5 minutes, then 30 cycles of 95°C for 30 seconds, 55°C for 1.25 minutes, and 72°C for 45 seconds, followed by 72°C for 15 minutes before placing on infinite hold at 15°C (Sidhu 2013).

DNA amplification was performed using PCR with an optimized master mix composed of PCR reagents including: 317 µL H_20, 220 µL GoTaq Flexi© 5X buffer solution (Promega, Madison, WI), 61.6 µL $MgCl_2$, 22 µL bovine serum albumin (Promega, Madison, WI), 66 µL of 10 µM dNTP (Promega, Madison, WI), 8.8 µL GoTaq Flexi©(Promega, Madison, WI). To this solution, I added the following primer pairs (Life Technologies, Carlsbad, CA): BTMS0062 (16.5 µL), B124 (22.0 µL), BL15 (23.0 µL), B96 (24.2 µL), BTMS0081 (15.4 µL), BL11 (12.0 µL), BT30 (10.0 µL), Btern01 (7.7 µL)(Sidhu 2015), BT28 (11.0 µL), and BT10 (5.5 µL) (Integrated DNA Technologies, Coralville, IA) (Table 1). All microsatellite markers were multiplexed together in the same reactinon. Microsatellite selection was based on methods outlined by Lozier and Cameron (2009) and improved by Sidhu (2013). Primer concentrations were further optimized in October 2014 at the USDA ARS Pollinating Insects Research Unit in Logan, Utah. In total, 10 primer pairs were used to determine relatedness of each bumble bee. Finally, PCR products were run on an Applied Biosystems 3730xl automatic sequencer for DNA size fragment analysis, which provided electropherograms depicting the length of each allele from selected microsatellite loci. The size standard, LIZ 500 (ThermoFisher Scientific, Waltham, MA) was used to determine the length of the alleles. Each allele was hand scored using Geneious vR8 (Biomatters Limited, Newark, NJ).

Data analysis

Genotypes from the microsatellite loci analysis were analyzed using Geneious v.R8. Bumble bee genotype tables were analyzed with the PopGenReport package (R Studio v.1.0.136) to determine if null alleles were present. To determine whether there were null alleles at a given site and year, a Brookfield test were performed (Brookflield 1996). Relatedness of each *B. impatiens* was determined using COLONY 2.0.4.5 (J. Wang 2008, Zoological Institute of London,

England) v1.2 (Lozier and Cameron 2009), which uses a maximum likelihood method to determine sibling-sibling relationships. Requirements for relatedness estimation of *B. impatiens* in this software were: female monogamy, without inbreeding, haplo-diploid, no sibship prior, long run, and full-likelihood analysis method. Males were excluded from COLONY analysis due to inconsistencies in assigning them to a specific family with high probability.

The number of bumble bees collected per minute of sampling and the number of unique colonies found to be represented in the bees collected at each site were compared among the three treatments using a repeated measures analysis of variance, and by analysis within each year using linear mixed effects model to account for changes over the 3 years of the study and error arising from variation among sites. A subsequent Tukey's Honest Significant Difference (HSD) test was performed if the regression was significant. To test whether flower species richness or flowering plant abundance differed between treatments, years, and sampling rounds, a repeated measures ANOVA was used for each. Further, based on the number of colonies detected per site, an estimated foraging range of 500, 1000, and 2000 m were used to estimate the nest density of *B. impatiens* at each site.

RESULTS

Bombus impatiens collections

The number of *B. impatiens* collected per minute was calculated to determine whether habitat enhancement affected the apparent abundance of *B. impatiens* at each site (Table 2.1). A linear mixed effects model was determined that treatment had a significant impact on the number of *B. impatiens* colonies collected per minute across all years (χ^2 = 15.86, d.f. = 2, 11, p = 0.0004). In the same model, year had no effect on number of *B. impatiens* collected per minute (χ^2 = 2.54,

d.f. = 2, 11, p = 0.28). Further, there was no interaction between treatment and year (χ^2 = 3.93, d.f. = 4, 11, p = 0.42). Thus, over the three-year period, *B. impatiens* collections did not vary within treatment from year to year.

Table 2.1. Sites selected for *Bombus impatiens* collections. The number of workers collected and time spent collecting are averaged across 2014-2016. ** These sites had very poor establishment of wildflowers, and thus are considered to be sites with no restoration.

Treatment (enhancement type)	Site	Area of planting (ha)	Average ± S.E. number of workers collected	Average ± S.E. time spent collecting (min)	Average ± S.E. number of workers collected per minute
None	REE	-	34.7 ± 4.8	555.0 ± 97.6	0.077 ± 0.017
	FPP	-	59.7 ± 31.2	544.0 ± 142.7	0.144 ± 0.065
	LCC	-	60.7 ± 30.1	430.0 ± 85.4	0.173 ± 0.086
	HSAF	-	18.3 ± 7.2	445.3 ± 116.6	0.078 ± 0.052
	HDK	-	82.0 ± 27.7	502.3 ± 71.5	0.156 ± 0.037
	HBT**	-	4.0 ± 1.2	510.0 ± 77.0	0.020 ± 0.014
	BSA**	-	25.7 ± 19.0	490.0 ± 163.6	0.061 ± 0.030
			40.7 ± 10.4	**496.7 ± 45.5**	**0.101 ± 0.020**
New	FSL	0.10	115.3 ± 35.7	430.0 ± 75.1	0.236 ± 0.033
	LPL	0.17	130.0 ± 40.4	433.3 ± 37.1	0.290 ± 0.071
	BJO	0.15	105.7 ± 49.3	530.3 ± 84.8	0.182 ± 0.077
		0.14 ± 0.02	**117 ± 7.1**	**464.6 ± 57.0**	**0.236 ± 0.035**
Mature	ROOD	3.06	73.7 ± 58.0	453.7 ± 81.0	0.225 ± 0.136
	GAL	0.96	129.7 ± 29.6	488.7 ± 51.9	0.259 ± 0.097
	GET	1.30	156.3 ± 73.3	301.7 ± 38.1	0.579 ± 0.264
	UED	1.23	204.0 ± 12.1	318.7 ± 71.2	0.749 ± 0.242
		1.64 ± 0.48	**140.9 ± 27.2**	**390.7 ± 94.3**	**0.453 ± 0.107**

Bombus impatiens colony estimation

A total of 3875 *B. impatiens* workers and males were genotyped at 10 loci. An average of 112.9 alleles were present at each site across all 10 loci (Table A3). I detected null alleles across all loci at several sites (Figure A1-41). Upon revisiting these sites, there was an excess of homozygotes present, which is likely due to inbreeding depression. Further, I detected few scattered occurrences of null alleles at several other sites, typically at locus BL15 and BT28 (Figure A1-41). Again, this was due to an excess of homozygotes at those loci. Because the presence of null alleles was not universal across all sites and loci, all loci were kept for subsequent colony differentiation analysis.

The number of *B. impatiens* bumble bee colonies estimated per site during each year of sampling was tested for normality using a Shapiro-Wilk normality test, indicating that the data were normal among years and within each treatment ($p > 0.05$). A mixed effects model determined that both treatment and year had a significant influence on the number of colonies detected at each site (Treatment: $\chi^2 = 16.48$, d.f. = 2, 11, $p = 0.00043$; Year: $\chi^2 = 14.19$, d.f. = 2, 11, $p = 0.0008$). The treatment:year interaction term was not significant ($\chi^2 = 3.72$, d.f. = 4, 11, $p = 0.444$) (Figure 2.2).

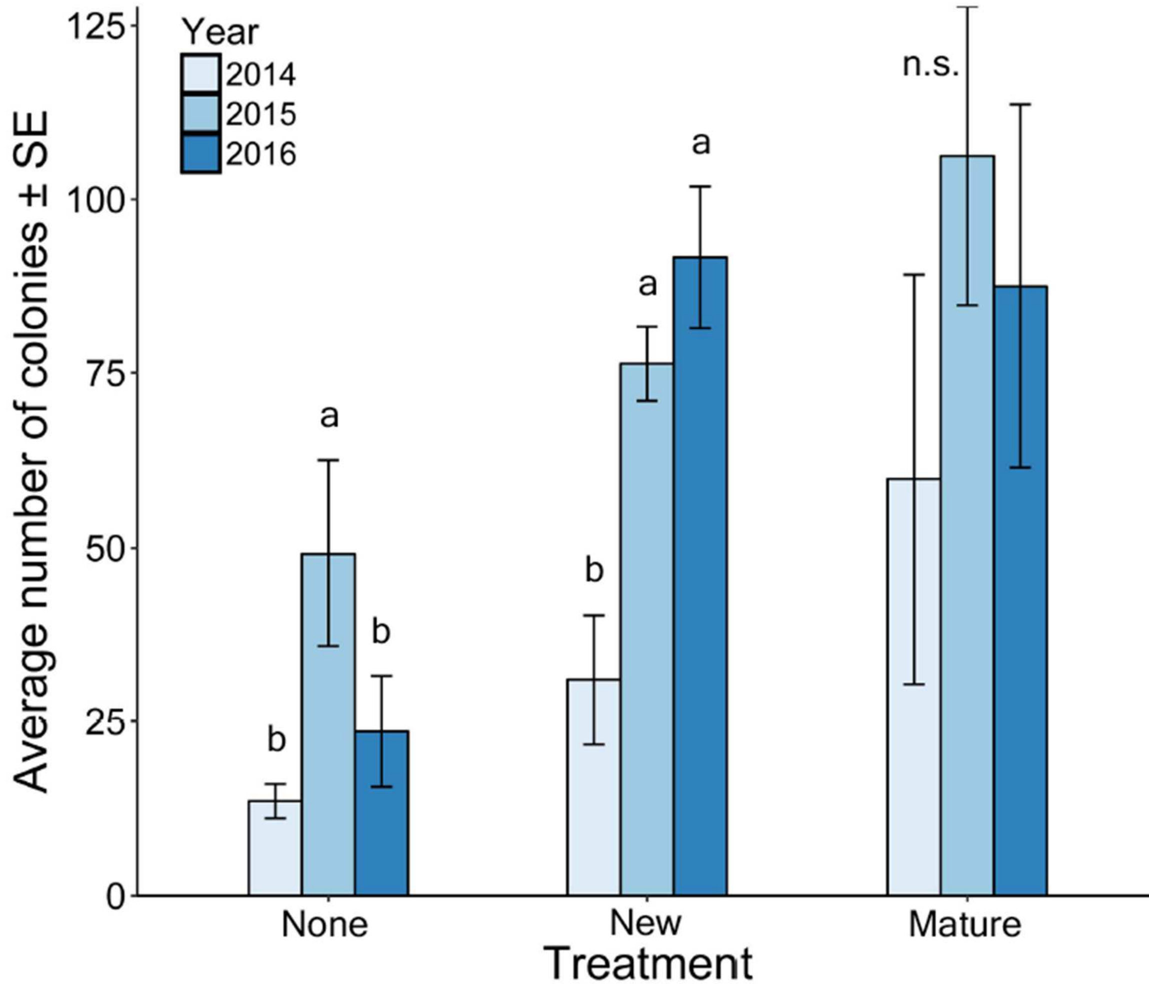

Figure 2.2. The average number of *Bombus impatiens* colonies found in each treatment by year. Letters above columns represent results from a one-way ANOVA and post-hoc Tukey's honest significance comparison among treatments in each year.

To determine whether the number of *B. impatiens* colonies found in each of the three treatments changed over the course of the study, a one-way ANOVA was conducted for each treatment, using year as an independent variable. The number of *B. impatiens* colonies found at sites without restoration was not stable among years (F = 4.12, d.f. = 2, 18, p = 0.034) (Figure 2.2), with a significant increase from 2014 (13.6± 2.5) to 2015 (49.1 ± 13.3) (Tukey's HSD: p = 0.031). However, there was no overall change at the unrestored sites, with no significant difference in the number of colonies between 2014 and 2016 (Tukey's HSD: p = 0.718). Sites

with a new habitat enhancement increased significantly over the years (F = 13.62, d.f. = 2, 6, p = 0.01). There was more than a doubling of the number of *B. impatiens* colonies from 2014 (31.0 ± 9.3) to 2015 (76.3 ± 5.4), with another increase in 2016 (91.7 ± 10.2). The number of colonies did not differ significantly between 2015 and 2016 in these newly restored sites. In sites with a mature habitat enhancement, the number of *B. impatiens* colonies found was consistently high from the start of the study, and did not differ from year to year using this test (F = 0.82, d.f. = 2, 9, p = 0.47) (Figure 2.2).

Depending on foraging range, *B. impatiens* nest density ranged from 17.3-135.3 colonies/km^2 (500 m), to 4.3-33.8 colonies/km^2 (1000 m), and 1.1-6.9 colonies/km^2 (2000 m) (Table 2.2).

Table 2.2. Average nesting density of *Bombus impatiens* colonies per km based on a 500, 1000, and 2000 m foraging radius.

		Colonies/km^2 ± SE for a given foraging radius		
Treatment	Year	500 m	1000 m	2000 m
None	2014	17.3 ± 3.1	4.3 ± 0.8	1.1 ± 0.2
	2015	62.6 ± 16.9	15.6 ± 4.2	3.91 ± 1.1
	2016	30.0 ± 10.1	7.5 ± 2.5	1.9 ± 0.6
New	2014	39.5 ± 11.8	9.9 ± 3.0	2.5 ± 0.7
	2015	97.12 ± 6.8	24.3 ± 1.7	6.1 ± 0.4
	2016	116.7 ± 13.0	29.2 ± 3.3	7.3 ± 0.8
Mature	2014	76.1 ± 37.5	19.0 ± 9.4	4.8 ± 2.3
	2015	135.3 ± 27.3	33.8 ± 6.8	8.5 ± 1.7
	2016	111.4 ± 33.3	27.9 ± 8.3	6.9 ± 2.1

The proportion of semi-natural habitat in the landscape surrounding the bumble bee collection sites at 300, 500, 1000, and 1500 m radius was analyzed to determine whether this influenced the number of *B. impatiens* colonies found at the sites. The proportion of semi-natural habitat had no significant effect on the number of colonies represented in the collections (p>0.05) (Table A4).

In order to determine whether floral abundance or floral species richness influenced the number of colonies represented by the sampled bees, both variables were compared between treatment, sampling round, and year using a repeated measures ANOVA. Neither treatment nor year had a significant effect on plant species richness (Treatment: $F = 2.28$, d.f. = 2, 9, $p = 0.158$; Year: $F = 0.204$, d.f. = 2, 9, p = 0.0.663) (Table 2.3). Further, treatment and year had no significant effect on flowering species abundance (Treatment: $F = 0.40$, d.f. = 2, 9, $p = 0.682$; Year: $F = 2.91$, d.f. = 2, 9, $p = 0.122$). Similar results occurred when sampling round was added to the repeated measures ANOVA ($Treatment_{richness}$: $F = 4.29$, d.f. = 2, 8, $p = 0.054$; $Round_{richness}$: $F = 2.98$, d.f. = 2, 8, $p = 0.108$; $Treatment_{abundance}$: $F = 0.021$, d.f. = 2, 8, $p = 0.979$; $Round_{abundance}$: $F = 0.137$, d.f. = 2, 8, $p = 0.874$).

Table 2.3. Average floral abundance, species richness, and standard error (S.E.) measured at the center point of each site in the field margin. A one-way ANOVA and post-hoc Tukey's honest significant difference test were used for comparison between treatments for each round.

Year	Sampling round	Treatment	Average ± S.E. floral abundance	Average ± S.E. floral species richness
2015	1	none	17.8 ± 4.9	5.7 ± 1.0
		new	16.0 ± 3.5	11.0 ± 1.5
		mature	16.3 ± 2.7	10.5 ± 0.9
	2	none	20.9 ± 10.3	7.7 ± 1.8
		new	22.8 ± 9.8	14.3 ± 3.5
		mature	30.7 ± 7.7	7.8 ± 1.7
	3	none	23.2 ± 7.8	8.6 ± 1.3
		new	25.7 ± 2.5	15.3 ± 2.8
		mature	17.6 ± 3.0	11.3 ± 2.7
	4	none	32.8 ± 13.3	8.5 ± 1.6
		new	56.3 ± 23.1	12.7 ± 1.2
		mature	51.2 ± 13.9	8.0 ± 1.1
2016	1	none	46.7 ± 17.3	9.2 ± 1.8
		new	13.5 ± 4.0	12.3 ± 0.3
		mature	26.1 ± 1.9	7.0 ± 0.9
	2	none	5.9 ± 2.0	5.5 ± 0.9
		new	6.4 ± 1.2	11.0 ± 1.0
		mature	15.8 ± 5.3	6.3 ± 1.7
	3	none	3.0 ± 1.1	4.3 ± 0.7
		new	15.5 ± 13.7	6.5 ± 2.5
		mature	7.3 ± 1.6	6.3 ± 1.3
	4	none	4.7 ± 1.5	4.8 ± 1.1
		new	13.1 ± 4.4	5.7 ± 1.3
		mature	9.1 ± 3.8	5.0 ± 1.4

DISCUSSION

My results show that habitat enhancement for pollinators in a commercially managed farm can positively affect populations of a bumble bee species, significantly increasing their populations after only one year of restoration. Furthermore, this is the first study to empirically demonstrate that populations of *Bombus impatiens* can be directly affected by a commonly used habitat enhancement practice in agricultural systems.

Bombus impatiens colony estimation

Habitat enhancement may support local bee populations through increased pollen and nectar resources as well as bare, undisturbed ground for nesting (Williams et al. 2010). Social bees in particular need consistent sources of pollen and nectar throughout the summer and fall to support their large colonies and to produce gynes that can successfully overwinter and found a new colony the following year (Pelletier and McNeil 2003). To ensure consistency of floral resources, flowers with slightly overlapping bloom times can be planted so that bees have access to forage throughout their colony lifecycle (Tuell et al. 2008). Further, ground-nesting resources may be in short supply on farms due to common practices such as tilling, which can destroy underground nests or disturb overwintering gynes (Williams et al. 2010). The complex vegetative structure and forage available in field margins from rural stewardship programs in the UK were also found to be highly attractive to bumble bee queens searching for nests and provisions for their first batch of offspring (Lye et al. 2009).

In sites with no habitat enhancement, there was fluctuation in the estimated number of colonies from year to year, suggesting that the increase in the estimated number of colonies from 2014 to 2015 could be due to increased sampling effort at sites without restoration in 2015 and

2016. At these sites, the weedy flowers commonly visited by *B. impatiens* may fluctuate in abundance and location due to herbicide usage or mowing (Doucet et al. 1999, Marshall et al. 2003). Thus, *B. impatiens* foraging on flowers found at these sites may have population fluctuations due to the low abundance and accessibility of flowers. Although there was no difference in floral abundance between treatments, sampling occurred within a 300 m radius of the field perimeter, so the floral abundance and richness measures likely did not capture all of the floral abundance and richness that bumble bees could have accessed at each site. Furthermore, floral abundance may not be the best measure of whether a site with restoration is working or not. Rather, the species of flowers planted and how attractive they are to target pollinators is likely playing a major role in the increase in *B. impatiens* populations (Warzecha et al. 2017).

Sites with newly restored habitat showed increases in *B. impatiens* colony abundance after the first year of restoration, demonstrating that habitat enhancement can support a greater number of *B. impatiens* colonies relatively quickly after restoration activities are started. This supports recent studies demonstrating that targeted pollinator habitat in and around agricultural systems is positively related to bee abundance, reproduction, and probability of survival (Goulson et al. 2010, Carvell et al. 2015, Carvell et al. 2017). The speed of response is surprising, however, as many of the sown species of wildflowers had not yet bloomed in this first year. While these bees require nectar and pollen floral resources, they also need nesting sites (Kells and Goulson 2003, Williams et al. 2010), and it is possible that the clearing of vegetation provided additional opportunities for the gynes to nest locally, allowing a greater proportion to remain in or near the restoration sites through the winter.

My finding that sites with mature restorations had consistently larger populations than sites without habitat enhancement over the course of this study (Figure 2.2) suggests that the

constant availability of preferred bumble bee forage may prevent large swings in population size due to variability in the location and abundance of flowering resources. This result supports Pywell et al. (2011) who showed that sown perennial flowers can support more pollinator species over time. Although floral abundance and richness had no effect on the number of *B. impatiens* colonies found at each site (Table 2.3), this could be due to a variety of weedy species found in abundance at sites without restoration that were not attractive to bumble bees. In contrast, sites with new and mature restorations had plants such as *Monarda fistulosa* and *Symphyotrichum novae-angliae* that were shown to be attractive to bumble bees and other native bees (Tuell et al. 2008). Improving the stability of native bee populations by having an area dedicated to habitat enhancement, where bee attractive plants can establish, may also help maintain consistent pollination services for commercial agriculture.

Assuming a 500 m foraging radius, *B. impatiens* were found to nest within the landscapes in this study at densities ranging from 1.3 colonies/km^2 (site with no restoration) to 178.3 colonies/km^2 (site with a mature restoration) over the course of this three year study (Sidhu 2013). Sites with newly restored habitats on average increased nesting density from 39.5 ± 11.8 colonies/km^2 in the first year to 97.2 ± 6.8 colonies/km^2 in the second year to 116.7 ± 13.0 colonies/km^2 by the third year. Further, sites with mature restorations maintained nesting density from 76.1 ± 37.5 colonies/km^2 in the first year to 135.3 ± 27.3 colonies/km^2 in the second year and 111.4 ± 33.3 colonies/km^2 in the third year (Table 2.2). Overall, these data indicate that habitat enhancement in highly managed agricultural landscapes increases bumble bee abundance and that nesting density increases in the landscape surrounding these restorations. Interestingly, in Michigan highbush blueberry, sites with new and mature restorations still had lower nesting density than Pennsylvanian pumpkin farms (up to 213 colonies/km^2), where no habitat

enhancement took place (Sidhu 2013). This may be due to the diverse landscape found around pumpkin farms in Pennsylvania, which may contain a combination of more nesting habitat and flowering resources when being compared to the highly fragmented landscape around Michigan blueberry farms. However, we still need to better understand bumble bee movement and foraging dynamics at a species level in order to more accurately determine nesting density. While some authors have used 500 m as an average foraging radius (Sidhu 2013), other studies have shown that some bumble bee species can forage at up to 11.57 km (Rao and Strange 2012). Further, average foraging distance is highly variable between individuals and also between different species of bumble bees (Knight et al. 2005) and in different landscapes, making it imperative to improve our understanding of foraging distances in bumblebees.

Field margins with restored habitat for pollinators may also be providing shelter for bees from direct pesticide exposure by attracting bees away from foraging on flowers within the crop when pesticides are being applied to the crop. Alternatively, Botias et al. (2015) found that bees can be exposed to pesticides such as neonicotinoids over the entire growing season, not just during crop bloom, from residues found in the pollen and nectar collected from wildflowers in arable field margins along cropping systems. This is of particular concern due to the persistence of neonicotinoids in the environment and can have adverse negative effects over time, such as reduced gyne production and colony growth rate (Whitehorn et al. 2012).

Pathogens and parasites may also be of concern for bees foraging in areas with habitat enhancement. Due to the increased bee populations visiting areas with restored floral habitat, there is likely a greater chance for horizontal pathogen transmission (Singh et al. 2010, Graystock et al. 2015). Also, the increased population sizes of bees will likely attract parasitoids that use bee species as their hosts. Despite the possible increase in pathogen transmission, there

is also evidence that bee immune systems are better equipped to fight pathogens and parasites due to phytochemicals found in nectar (Palmer-Young et al. 2017). In particular, generalists, such as bumble bees are likely to ingest combinations of phytochemicals from a variety of flowers which can synergistically interact to reduce infection or even inhibit certain parasites entirely (Palmer-Young et al. 2017). Despite evidence of pathogen resistance in the presence of rich floral diets, increased rates of pathogen and parasitoid exposure could have negative effects on bee populations, but the magnitudes of these effects on bees remain unknown.

Although potential negative effects from pesticides and pathogens may be of concern, there is evidence from this study that the positive effects of habitat enhancement in field margins offset the negative effects from pathogen and pesticide exposure. My results suggest that bottom up effects such as food and nesting resource availability may have a disproportionately large impact on bumble bee populations when compared top down effects such as increased exposure to pathogens and pesticides. From a bee conservation standpoint, targeted habitat enhancement can be a valuable tool to promote bee populations in agricultural systems.

CHAPTER 3

HOW DOES HABITAT ENHANCEMENT IN FARMS AFFECT BUMBLE BEE-FLOWER INTERACTIONS?

INTRODUCTION

Several bumble bee species such as *Bombus impatiens* and *B. griseocollis* make up the most commonly observed wild bee species in natural environments and in agricultural landscapes (Javorek et al. 2002, Winfree et al. 2007, Artz et al. 2011). Because of their abundance, in systems requiring animal mediated pollination, wild bumble bees can provide substantial pollination services to crops (Kremen et al. 2002, Winfree et al. 2007, Artz et al. 2011, Garratt et al. 2016). Bumble bees are also generalist pollinators that visit a variety of wild plant species, with a high degree of flexibility in their foraging behavior (Laverty and Plowright 1988, Vaudo et al. 2015). They are also well known for their ability to buzz pollinate flowers (Buchmann 1978), causing anthers to release pollen, which can then be transferred to other flowers of the same species. This combination of efficiency, abundance, and breadth in diet makes bumble bees valuable pollinators of both cultivated crops and natural plant communities (Winfree et al. 2007, Vaudo et al. 2015).

While they may be locally abundant in some settings, populations of some bumble bee species have also experienced declines across their distribution ranges in Europe and North America (Potts et al. 2010, Cameron et al. 2011, Bartomeus et al. 2013). These bees require nectar and pollen resources (Goulson et al. 2002, 2004, Vaudo et al. 2015) as well as relatively undisturbed nesting areas such as forest boundaries or habitat with tussock-type vegetation for nesting habitat (Svensson et al. 2000, Kells and Goulson 2003), both of which can be limited in intensively managed landscapes. Conservation programs are being developed to support these bees by providing greater floral resource abundance in farmland (M'Gonigle et al. 2015, Williams et al. 2015, Dicks et al. 2016), but there is an urgent need to understand how best to implement these programs so they have the desired outcomes. Restoration practices such as

planting hedgerows and wildflower strips have been shown to support pollinators due to the constancy of flowers throughout the growing season and because of the deficiency of floral resources in surrounding landscapes (Tuell et al. 2008, Blaauw and Isaacs 2014, M'Gonigle et al. 2015, Williams et al. 2015, Chapter 2). These types of habitat enhancement are generally designed to sustain bee populations throughout the rest of the growing season when the focal crop is not in bloom (Tuell et al. 2008) and increasingly are being considered to increase wild bee abundance in adjacent crop fields to increase crop yield (Kremen et al. 2002, Blaauw and Isaacs 2014, Venturini et al. 2017). To do this, wildflower strips in field margins can be tailored specifically to the crop system that they are planted in by creating wildflower mixes with plant species that bloom before and after the bloom time of the crop, and that are highly attractive and rewarding to bees (Tuell et al. 2008, Isaacs et al. 2017). Bees are then attracted to sites with habitat enhancement due to greater flowering resources (Spaethe et al. 2001, Glettii and Barrett 2008), floral display (Ishii 2006, Tuell et al. 2008), plant species richness (Jha and Kremen 2013), pollen and nectar rewards (Brunet et al. 2015), or a combination of floral traits (Leonard and Masek 2014).

Queen bumble bees need pollen and nectar when they emerge in the spring to successfully establish a colony and the colonies continue to need these resources through the summer during their annual life cycle (Kearns and Thomson 2001, Heinrich 2004). These resources are often gathered from woody plants such as blooming trees and shrubs, and from ground covers such as clovers, with greater reliance on annual forbs as the season progresses (Macior 1968, Park et al. 2015). The growing number of workers in each nest forage further from the nest to collect resources in the summer and into the fall. In simplified landscapes, bees forage on plant species that can survive in intensive land management systems, such as hardy

weedy species that are persistent in the environment. Plant diversity is also greatly decreased in these settings due to herbicide usage (Tscharntke et al. 2005, Egan et al. 2014), resulting in fewer foraging resources available for bees.

Jha and Kremen (2013) found that *B. vosnesenskii* would forage further to find more species rich wildflower patches and to find areas with less patch-to-patch variation in the availability of floral resources to suit their nectar and pollen needs. When resources are scarce within their foraging range, bumble bees will visit a variety of plant species, changing species often as flowers from the same species may not be common enough in the landscape to support floral constancy (Chittka et al. 1997, Goulson 1999). Thus, in simplified agricultural landscapes with few flowering resources, bumble bees will either have to forage on the first flowers they encounter or risk longer foraging trips to find resource rich flower patches.

Insights into the foraging ecology of bumble bees can be gained through plant-pollinator network analysis, which reveals the plant-pollinator interactions (Memmott et al. 1999). Network analysis can be an informative tool for analyzing complex community-level patterns such as the nestedness of the network, competition for resources, and degree of generality or specialization of species, which are not immediately obvious from the individual interactions themselves (Dormann et al. 2009). Network analysis also provides an opportunity to measure changes over time or to explore differences among treatments. In particular, network parameters such as C-score (competitiveness between pollinator species) (Stone and Roberts 1992), nestedness (Atmar and Patterson 1993), and generality (mean number of prey species for each predator) (Bersier et al. 2002) can provide insights into changes in foraging ecology in response to variation in the environment, such as habitat enhancement.

The connections between plants and bees have been studied using interaction networks (Memmott et al. 1999, Russo et al. 2013, Tucker and Rehan 2016), but many pollination network studies have focused on sampling a single site over a given time period (Pocock et al. 2012, Russo et al. 2013, Tucker and Rehan 2016, Parra-Tabla et al. 2017); however, studies including multiple sites such as Ramirez-Florez et al. (2015) and Santamaria et al. (2017) are becoming more prevalent to reflect the variation among field sites. I expect that bumble bee foraging strategies will differ in areas that have large patches of flowers such as restored habitat compared to locations with few floral resources that are scattered through the local landscape, and in this study I will use analysis of networks developed at replicated sites that vary in their level of habitat enhancement for pollinators.

This chapter explores how foraging ecology of bumble bees changes in sites with and without habitat enhancement in the form of wildflower plantings in field margins of commercially produced blueberries. The objectives of this study were to (1) determine whether the number of bumble bee-flower visits vary between sites with a mature habitat enhancement, a new one, or no habitat enhancement and (2) to determine which flower species are preferred by bumble bees in these three treatments. The relevant hypotheses were that (1) there will be more bumble bee-flower visits in sites with mature and new habitat enhancements than those without enhancement and (2) flower species with greater abundance will be visited more commonly than those flower species that are less common.

METHODS

Site selection

I selected 15 commercial blueberry fields in southwestern Michigan that either had no habitat enhancement (standard-practice mown field margin), a new habitat enhancement in the field margin (2-3 years since seeding), or a mature habitat enhancement in the field margin (5-8 years). The fifteen fields were located on different farms, spaced at least 2 km apart. One of the new restoration sites was sprayed with glyphosate during the first year to manage problematic weeds, and was therefore converted into a site without habitat enhancement. This resulted in 6 sites without, 4 sites with a new, and 5 sites with a mature habitat enhancement (see Figure 2.1 for site locations).

Habitat establishment

Sites with new and mature habitat enhancements were prepared in a similar manner, despite being seeded in different years. Site preparation involved one or more glyphosate treatments prior to seeding. Next, a seed mixture containing a mix of native Michigan wildflowers and grasses were planted at sites with a new (Table A1) and mature (Table A2) habitat enhancement in the fall or early spring. Sites were maintained during the first and second year of establishment by high (6 inch) mowings to prevent seed production by weeds and by spot herbicide treatments to control problematic weedy species. Sites without habitat enhancement were left to standard farm practice, which entailed consistently mown grass-dominated margins.

Plant and pollinator collections

In 2015 and 2016, bumble bees and the flowers they visited were collected during four 30-minute sampling periods (rounds) conducted from the beginning of July through mid-September. Each bumble bee-flower pair collected was considered an interaction. Each of the four sampling rounds occurred 3-4 weeks apart, depending on weather. Sampling occurred between 9:00-17:00 hours and only in conditions that were above 16.0 °C and not raining. Bumble bees and flowers were collected in separate but corresponding 22 mL plastic scintillation vials and were frozen at -80 °C for further identification in the laboratory. To ensure standardization of sampling across all sites and collectors, the timer was stopped after each sample was collected. Bumble bees were identified using "Bumble Bees of North America" by Richardson et al. (2014). Voucher specimens are held at the A.J. Cook Arthropod Research Collection at Michigan State University (See Appendix D).

Separately, the plant community at each site was characterized using two 50m transects placed along the field margin and along the edge of the field margin. Each transect was randomly placed in their respective area using a random number generator to determine the starting coordinates of each transect. Five $1m^2$ quadrats were placed 10m apart along each transect. Within each quadrat, flowering plants were identified to species and counted. If growing on separate stems each individual flower head was counted, whereas for flowers with compound inflorescences each spike or raceme with flowers on it was counted. Flowers were only counted when in bloom during the surveys. Flowering plants were identified on site and in the laboratory using Newcomb's Wildflower Guide (Newcomb 1987) and Weeds of the Northeast (Uva et al. 1997). Voucher specimens were deposited into the Michigan State University Herbarium. Flower

species abundance, density, richness, and diversity were calculated based on flower counts obtained from these transects.

Bumble bee abundance and interaction analysis

Bumble bee species abundance and richness were compared among treatments throughout the entire season as well as during each sampling round using a linear mixed effects model with treatment and year as fixed effects and site as a random effect. To determine differences among rounds, a separate mixed effects model was developed using treatment and round as fixed effects and site as a random effect.

The number of bumble bee-flower interactions (ie: the sum of the number of bumble bees visiting flowers that were collected during each sampling round) was assessed for normality using a Shapiro-Wilk test. Differences in the number of interactions between treatments over the course of the season were compared using a linear mixed effects model using treatment and year as fixed effects and site as a random effect. To determine if there were differences in the number of interactions between treatments across each round of sampling, a linear mixed effects model was developed using treatment and sampling round as fixed effects and site as a random effect to control for potential variation in sites.

To determine what floral factors influenced the number of interactions at each site, a generalized linear model was developed using Akaike's Information Criterion (AICc) and Schwarz's Bayesian criterion (BIC). The full model included the explanatory variables: year, proportion of highly attractive plant species present, plant species richness, and plant abundance. The best-fit model was selected based on low AIC/BIC score and whether both criteria agreed on the same model.

Floral preference analysis

Counts of bees on specific flowering plants from bumble bee-flower collections and floral survey data were compiled for each site over the four sampling rounds during both years. Using these data, I determined floral preference using the number of times each plant species was visited over the course of a year divided by the average density (plant m^{-2}) at which those flowers were found from the flower surveys. This bumble bee ranking preference was determined for each plant species at each site, to provide the preference by bumble bees for each flower species relative to its density in the area sampled. Flower species that were visited by bumble bees but were not found in the floral surveys were assigned densities equivalent to the lowest average density of flowers observed in the surveys at a rate of 0.1 m^{-2}. Once bumble bee ranking preference was calculated, a linear regression was used to determine which flower species were visited preferentially to other flower species, by identifying species that placed above the best fit lines.

Network analysis

Bumble bee and flower collection data were used to create a two-tier plant-pollinator interaction web using the “bipartite” package in R (Memmott 1999, Dormann et al. 2009). The connectance, nestedness, generality, extinction slope, C-score, linkage density, and diversity were calculated from each individual network. The bumble bee species richness and the proportion of flower species present that were visited were also calculated at each site. Network parameters were compared among the three treatments using a linear mixed effects model with treatment and year as fixed effects and site as a random effect in order to account for possible variation between sites.

RESULTS

Bumble bee community

I found six bumble bee species during this study, with the following overall abundance per 30 minute sample: *Bombus impatiens* (5.2 ± 0.6), *B. griseocollis* (2.0 ± 0.4), *B. vagans* (0.6 ± 0.2), *B. bimaculatus* (0.5 ± 0.1), *B. fervidus* (0.3 ± 0.1), and *B. citrinus* (0.1 ± 0.1). The overall abundance of this community differed among treatments throughout the study. Of the six species present only the most abundant *B. griseocollis* (F = 12.58, d.f. = 2,12, p = 0.001) and *B. impatiens* (F = 6.41, d.f. = 2,12, p = 0.013) were significantly different in their abundance among the treatments (Figure 3.1). *Bombus griseocollis* was more abundant in new and mature plantings compared with areas that were not enhanced, whereas *B. impatiens* was significantly more abundant in the mature plantings than the other two treatments.

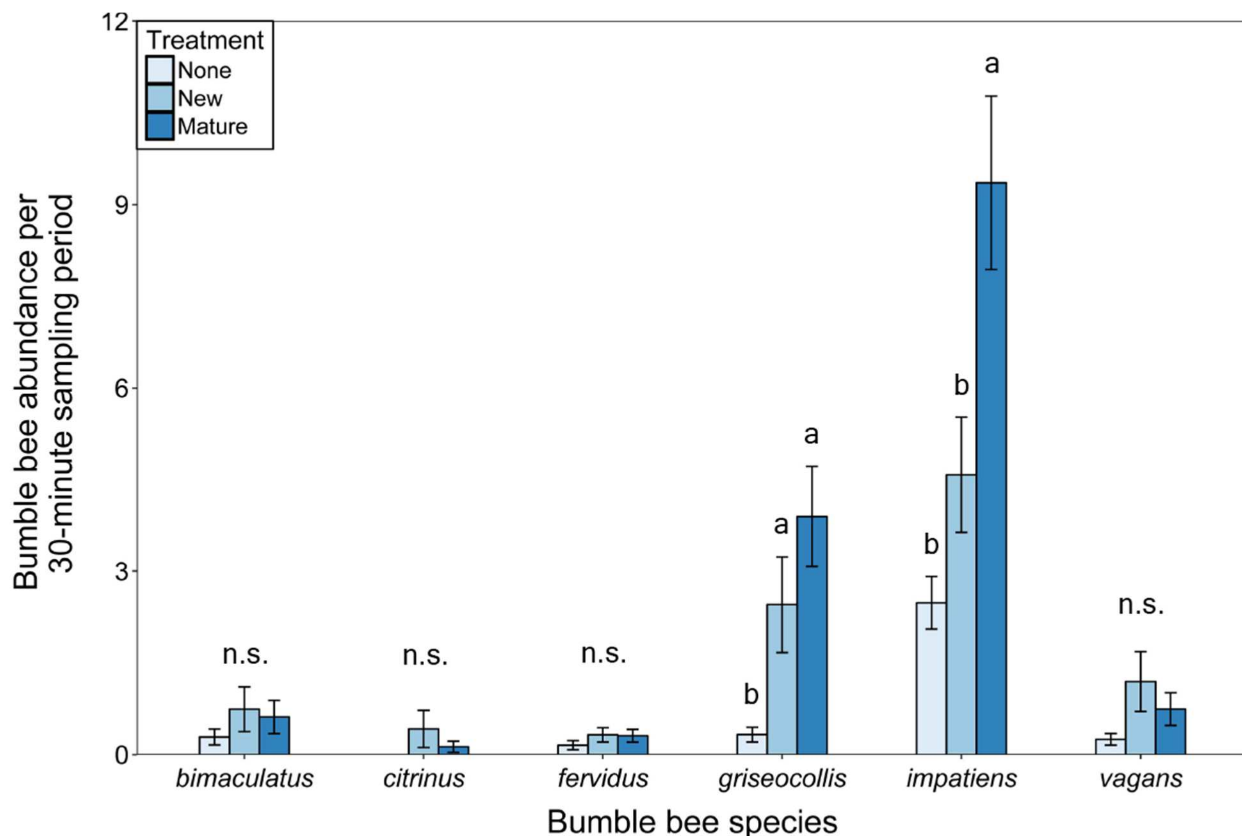

Figure 3.1. Average abundance of each bumble bee species collected per 30-minute sampling period between treatments over the course of 2015-2016.

Flower density

Flower density data were not normally distributed (Shapiro-Wilk test, $p < 0.01$), thus data were fitted to a Poisson distribution in subsequent analyses. Across both years, flower density was not significantly different among treatments ($F = 0.078$, d.f. = 2,12, $p = 0.926$) (Table 3.1).

Table 3.1. Average flower density ± standard error (SE) across enhancement treatments in 2015 and 2016.

Treatment	Average flower density (per m^2) ± SE
None	20.99 ± 4.51
New	35.58 ± 7.34
Mature	23.27 ± 3.41

Flower density was also compared among treatments for each sampling round. In both years, flower density did not differ significantly between treatments or among rounds (Treatment: $F = 0.112$, d.f. = 2,9, $p = 0.895$; Round: $F = 0.42$, d.f. = 2,9, $p = 0.990$) (Table 3.2).

Table 3.2. Average flower density ± standard error (SE) by sampling round and treatment during two years in three pollinator restoration treatments.

Sampling Round	Treatment	Average flower density (per m^2) ± SE
1	None	28.88 ± 9.66
	New	21.19 ± 3.49
	Mature	25.87 ± 4.27
2	None	19.25 ± 10.40
	New	29.76 ± 9.26
	Mature	24.43 ± 6.31
3	None	17.66 ± 5.98
	New	40.8 ± 12.10
	Mature	15.3 ± 3.35
4	None	17.13 ± 8.78
	New	50.06 ± 23.34
	Mature	27.75 ± 10.78

Bee-flower interactions

The bee-flower interaction data were not normally distributed (Shapiro-Wilk, $P_{2015} < 0.001$, $P_{2016} < 0.001$) and were subsequently fit to a Poisson distribution. There was a significant difference among treatments in the number of bumble-bee flower interactions per 30 minute sample across both years ($F = 10.77$, d.f. = 2, 12, $p = 0.002$) (Table 3.3), with the most interactions observed in the mature enhancements. A post hoc Tukey's HSD test revealed that sites with mature enhancements had significantly more interactions per 30-minute sampling period than sites with new enhancements ($p = 0.025$) or those without enhancements ($p = 0.001$). Sites with new enhancements and without enhancements did not differ significantly ($p = 0.108$). There was no year effect, indicating that the year of collection did not impact the number of interactions between treatments ($F = 0.095$, d.f. = 1,12, $p = 0.76$).

Table 3.3. The average number of bumble bee-flower interactions observed in sites with three levels of habitat enhancement

Year	Restoration type	# sites	Bumble bee-flower interactions ± SE
2015	None	6	3.8 ± 0.7
	New	4	9.4 ± 1.5
	Mature	5	16.6 ± 2.2
2016	None	6	3.6 ± 0.7
	New	4	10.0 ± 2.0
	Mature	5	13.6 ± 2.5

Across each season, there was a treatment, round, and treatment-round interaction effect on the average number of interactions observed at each site (Treatment: $F = 13.18$, d.f. = 2,13, $p < 0.001$; Round: $F = 16.10$, d.f. = 3,97, $p < 0.001$; Treatment-Round: $F = 12.61$, d.f. = 6,97, $p < 0.001$). A post-hoc multiple comparisons test showed that during rounds 1 and 2, mature enhancement sites had significantly more interactions than sites without enhancement (Round 1: estimate = 13.06 ± 2.16, $z = 6.05$, $p < 0.001$; Round 2: estimate = 11.94 ± 2.11, $z = 5.66$, $p < 0.001$). Sites with mature enhancements during round 4 also had fewer interactions (estimate = -8.26 ± 2.11, $z = -3.92$, $p = 0.001$) than sites with a new enhancement. Lastly, sites with without enhancements also had fewer interactions than sites with new and mature enhancements during round 2 (estimate = -12.48 ± 2.77, $z = -4.50$, $p < 0.001$) (Figure 3.2A,B).

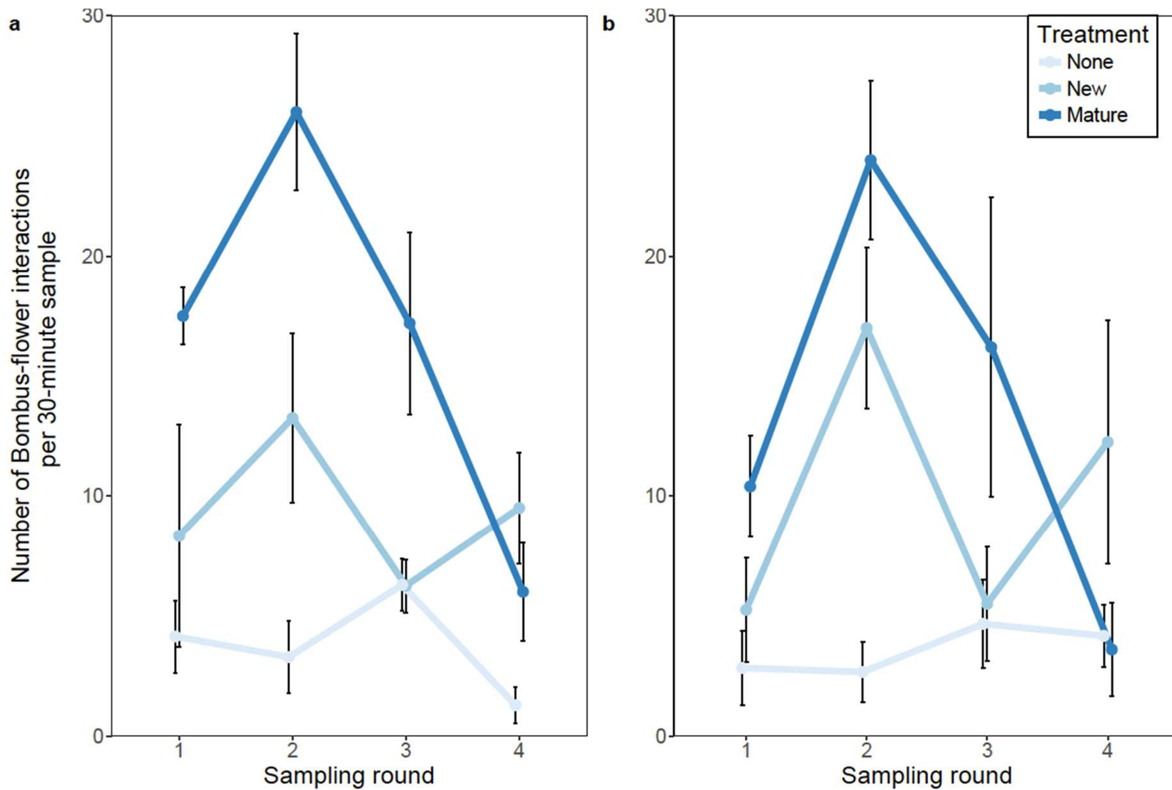

Figure 3.2. The average number of bumble bee-flower interactions observed (± S.E.) during each round of sampling of three different wildflower enhancement treatments during (a) 2015 and (b) 2016.

Stepwise AIC and BIC analyses were used to select the model that best predicted the number of interactions throughout the season at each site. The original model included the year, proportion of highly attractive species present at a site, plant species richness at each site, and total flower abundance at each site. The best-fit model selected by AIC included the proportion of highly attractive plant species ($p < 0.001$) and plant species richness ($p < 0.001$) at each site (Table 3.4).

Table 3.4. Stepwise AIC and BIC to determine best-fit model for determining the number of bumble bee-flower interactions at a given site.

Initial model: # interactions ~ year + proportion highly attractive plants + plant species richness + plant abundance

Step	d.f.	Residual deviance	AIC	ΔAIC	BIC	ΔBIC
Initial model	24	269.94	430.44	2.73	437.45	5.54
(-) year	25	270.08	428.58	0.78	434.18	2.27
(-) year, (-) plant abundance	26	271.21	427.71	0	431.91	0

Best-fit model: # interactions ~ proportion highly attractive plants + plant species richness

Floral preference

Although flower density did not affect the overall number of interactions with bumble bees, I was interested in which flowers were preferred by bumble bees in each of the restoration treatments. In sites without habitat enhancement, a total of 29 species were visited out of the 96 flowering plant species present. In sites with new enhancements, bumble bees visited 40 species of the 95 flowering species present. In the sites with mature enhancements, bumble bees visited a total of 30 flowering plant species of the 80 species present. In order to determine which plant species were highly attractive, bumble bee rank preference was calculated. Based on this, a linear regression was fitted to the dataset for each treatment. Based on the bumble bee rank preference, sites without enhancements had 22 plant species that were highly attractive to bumble bees (Figure 3.3). Sites with new enhancements had 14 plant species that were highly attractive (Figure 3.4) and sites with mature enhancements had 13 plant species that were considered highly attractive to bumble bees (Figure 3.5).

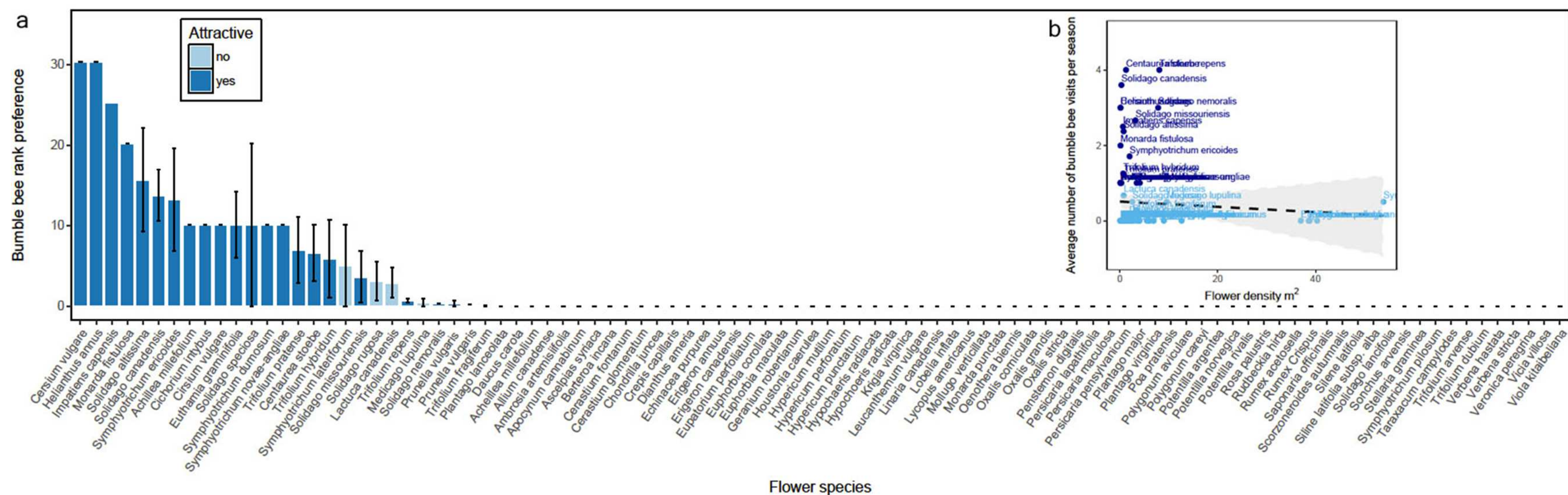

Figure 3.3 (a) Bumble bee rank preference of flower species found at sites without habitat enhancements; **(b)** average number of interactions by floral density for each plant species with a linear regression (dashed line) and 95% confidence intervals (gray shaded area). Plant species were considered attractive if they were above the shaded area (confidence interval) of the regression analysis.

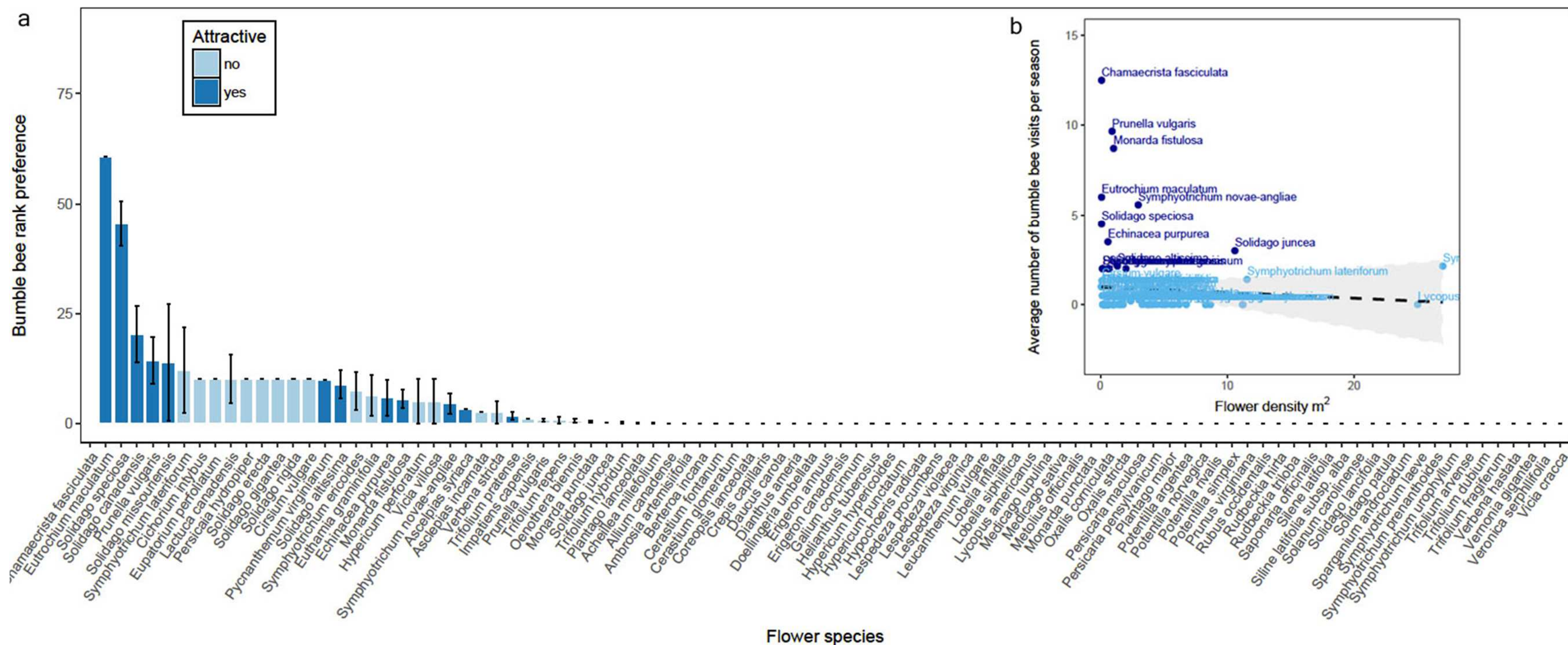

Figure 3.4 (a) Bumble bee rank preference of flower species found at sites with new habitat enhancements; **(b)** average number of interactions by floral density for each plant species with a linear regression (dashed line) and 95% confidence intervals (gray shaded area). Plant species were considered attractive if they were above the shaded area (confidence interval) of the regression analysis.

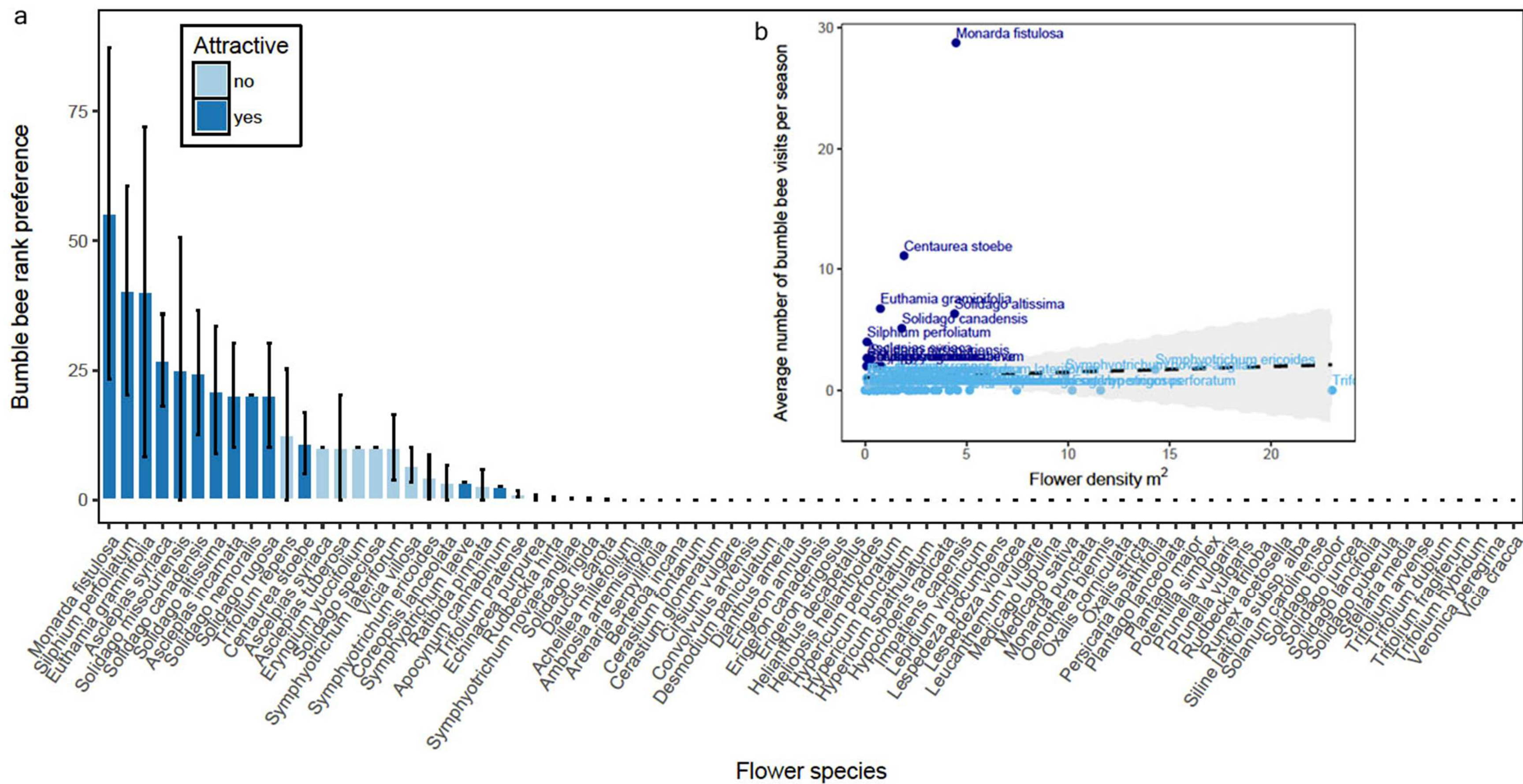

Figure 3.5 (a) Bumble bee rank preference of flower species found at sites with mature habitat enhancements; **(b)** average number of interactions by floral density for each plant species with a linear regression (dashed line) and 95% confidence intervals (gray shaded area). Plant species were considered attractive if they were above the shaded area (confidence interval) of the regression analysis.

Network analysis

A mixed effects ANOVA revealed that the richness of plant species visited by bumble bees differed significantly among treatments (F = 6.44, d.f. = 2,13, p = 0.011). This was twice as high in sites with new restorations (10.62 ± 1.13 species) than sites without restoration (5.27 ± 0.81) (Tukey's HSD, p = 0.015). Sites with a mature restoration (9.30 ± 1.00) also had higher richness but did not differ significantly in the number of plant species visited by bumble bees when compared to sites without restoration (Tukey's HSD, p = 0.15), and there was no differencebetween new and mature sites (Tukey's HSD, p = 0.58). Bumble bee species richness was significantly different among treatments (Mixed effects ANOVA, F = 3.92, d.f. = 2,13, p = 0.047). However, when comparing between each treatment on their own, bumble bee species richness was not significantly greater at sites with a mature (4.10 ± 0.36 species) (Tukey's HSD, p = 0.254) and new restoration (4.25 ± 0.45) (Tukey's HSD, p = 0.509) compared to sites without restoration (2.91 ± 0.21). Sites with mature and new restorations also did not differ in bumble bee species richness (Tukey's HSD, p = 0.932).

Networks were created from the data on bumble bees visiting flowers for each site during each year (Figures B1-16). Network parameters were calculated from each site during each year and were compared among the treatments using a mixed effects ANOVA to account for variation among sites and between years (Table 3.5). While the newly enhanced sites typically had the higher values for the network parameters, these differences were small and there was no significant difference in generality, linkage density, nestedness, robustness, Shannon-Weiner diversity, or vulnerability between treatments (p > 0.05) for either of the sample years (Table 3.5).

Table 3.5. Plant-pollinator network parameters for bumble bees compared among restoration treatments in 2015-2016 using a mixed effects model with year and treatment as fixed effects and site as a random effect.

Network parameter	Restoration type	Average ± SE	d.f.	F-value	Significance (p-value)
Generality	None	3.28 ± 0.60			
	New	4.01 ± 0.38	10, 13	0.90	0.43
	Mature	3.11 ± 0.38			
Linkage density	None	2.25 ± 0.37			
	New	2.85 ± 0.22	10, 13	0.52	0.61
	Mature	2.53 ± 0.21			
Nestedness	None	16.82 ± 3.83			
	New	29.35 ± 2.86	10, 13	3.14	0.08
	Mature	19.97 ± 3.36			
Robustness	None	0.67 ± 0.03			
	New	0.72 ± 0.02	10, 13	1.81	0.20
	Mature	0.72 ± 0.02			
Shannon-Weiner diversity	None	1.71 ± 0.12			
	New	2.25 ± 0.13	10, 13	2.50	0.12
	Mature	1.90 ± 0.18			
Vulnerability	None	1.69 ± 0.18			
	New	1.61 ± 0.20	10, 13	1.14	0.35
	Mature	1.96 ± 0.12			

DISCUSSION

In this study I found that as wildflower plantings for pollinators establish over time, the abundance of common bumble bees and the frequency of interactions between bumble bees and flowers in those plantings increases (Figure 3.1, Table 3.3). Flower density did not always follow the same pattern, however, and this is unexpected because flower density and abundance are typically correlated with the abundance of bees (Carvell et al. 2007, Wood et al. 2016). My results also indicate that bumble bees were highly attracted to specific flowering plant species. This difference in the number of interactions was most pronounced during the second and third rounds of sampling, which took place between late July and mid-late August in both years, suggesting that this is a critical time period for bumble bee foraging and a period in the season when providing attractive resources will be important for colony success. For example, colonies of the most abundant bumble bee species, *B. impatiens*, have typically grown close to their maximum size at this time of the year (Williams et al. 2014), so they are sending out more foragers to support and maintain colony growth at this time. Also, at sites with new or mature restorations, this time period corresponds with the peak bloom of *Monarda fistulosa*, a highly attractive plant to some bumble bee species, which was sown at sites with new and mature restorations (Tuell et al. 2008, Rowe 2017). This is further supported by the proportion of highly attractive plant species having a significant positive effect on the number of bumble bee-flower interactions at a site (Table 3.4).

Bumble bees were observed visiting a total of 62 plant species over the course of this study. Many were considered to be highly attractive to them (Figure 3.3-3.5) reflecting the generality of bumble bee foraging behavior (Williams et al. 2014, Vaudo et al. 2015) and also supporting the idea that when targeting restoration for specific pollinator species, focusing on a

few attractive species may be sufficient for providing the resources they need. From an agricultural production perspective, habitat enhancement could be tailored to support pollinators that are highly abundant or efficient pollinators of the specific crop to help increase yield (Kleijn et al. 2015). In this case, *B. impatiens* is a primary wild pollinator of blueberry in this region (Gibbs et al. 2016), and results from this study may inform how best to provide food resources in farm settings.

At sites without restoration, bumble bees foraged on a variety of weedy flower species, including 22 species that were highly attractive to them (Figure 3.3). Combined with the comparatively low numbers of interactions, the lack of preference may indicate that bumble bees do not show floral constancy in this setting, sampling many rewarding plants (Chittka et al. 1997, Goulson 1999). Another plausible explanation for the lack of floral constancy is that bumble bees may be foraging on the nearest flowers they can find in a relatively sparse landscape. This highlights the challenging nutritional situation for bumble bees in many farms, where they have limited resources for feeding during the summer months once the crop bloom is complete. This is expected to have significant implications for the carrying capacity of such sites, due to the important link between nutrition and population size (Hines and Hendrix 2005, Roulston and Goodell 2011, Vaudo et al. 2015). In contrast, at sites with new and mature habitat enhancements, bumble bees foraged on a combination of sown and weedy plant species, exhibiting preference for a few of the most dominant seeded species such as *M. fistulosa* and *S. altissima*. Although bumble bees had more interactions per 30-minute sampling period at sites with habitat enhancement compared to no restoration, they still foraged on the same number of plant species. At sites with new and mature restorations, bumble bees preferentially foraged on 14 and 13 plant species respectively, compared to 22 at sites without a restoration. This is

another indicator that bumble bees had more floral constancy at sites with an abundance of flowering plant species and highlights the value of directed restoration for supporting bees.

Mature restoration sites had high visit rates to spotted knapweed, *C. stoebe*, and to some species of *Solidago* that were not part of the sown wildflower mix (Figure 3.5). The increased number of interactions with these species reflects their abundance in the sites due to sub-optimal establishment after the sites were seeded. For example, at one of the mature restoration sites, bumble bee visits to *C. maculosa* accounted for 42 of the 52 interactions (80.7%) in 2015 and 29 of the 34 interactions (85.3%) in 2016. This particular site was dominated by *C. stoebe*, but bumble bees still visited it at other restored sites, showing that bumble bees are highly attracted to this species even though it is considered a highly invasive weed in Michigan (State of Michigan 2017). This also shows that site selection is critical for pollinator habitat enhancements, because if invasive plants are present and site establishment is not highly effective, they can be overtaken by weedy species that could threaten the goals of the restoration (Emery and Gross 2005, Rowe 2010, Williams et al. 2015, Trowbridge 2017).

Plant pollinator networks from each treatment (Figures B1-16) suggest some differences among the treatments, but the high variation among sites within each of the treatments resulted in no statistical differences in the network parameters. This finding is interesting because many studies survey a single site extensively and expect that the values obtained from their network analyses to be representative of their system (Pocock et al. 2012, Russo et al. 2013, Tucker and Rehan 2016, Parra-Tabla et al. 2017, Chacoff et al. 2018). I found that even within a single treatment, the variation in network parameters among sites was quite high (Table 3.5), indicating that sampling a single site may not be representative of an entire system, and also highlighting that higher levels of within-treatment replication than used in this study are needed (for example,

see Chacoff et al. 2017). Because plant-pollinator networks require intensive sampling, studies of these networks could substitute higher replication for sampling intensity (a major factor constricting the sampling of more than one site) to accurately characterize a particular system. Alternatively, my experimental design only captured bumble bee species visiting flower species for two hours over the course of each summer. This could also potentially increase variation in network parameters due to lack of resolution in the networks.

Although there were no statistical differences among treatments for each of the network parameters, some of these results could be explained by the generalist-type foraging behavior exhibited by bumble bees. Most bumble bees are generalist to some degree and can be found foraging on a variety of different plant species (Figures B1-16) (Williams et al. 2014). Thus, certain network measurements like links per species, robustness, generality, and vulnerability, which are particularly sensitive to specialist species being present, would not show differences between treatments when all the species being recorded are generalists. However other parameters like generality (mean number of flower species per bee species) should still be different between treatments in this study because of the lack of forage resources available to bees in sites with no restoration (Table 3.5). This again highlights the need for greater replication when characterizing plant-pollinator networks.

Analysis of network parameters showed that both bumble bee and plant species richness were greater at sites with mature and new restorations when compared to sites without restoration. Although there was higher bee and plant species richness at new and mature restorations, bumble bees still showed floral constancy among the most visited plant species (Goulson 1999, Jha and Kremen 2013). The increase in the number of plant species visited may be because bumble bees do not have a directed recruiting mechanism to floral resources

(Goulson 1999, Dornhaus and Chittka 2001). Thus, bumble bees must find forage resources without previous knowledge of what is present in the landscape. Another explanation is that bumble bees are more willing to forage on new flower resources in wildflower plantings because the cost of visiting a different plant species in these flower-dense an area with many resources is relatively low (Hodges 1985, Townsend-Mehler et al. 2011).

Overall, results of the floral preference analyses conducted here provide another line of evidence that when bumble bees are exposed to habitats with a low density of highly attractive plant species, they will forage on a greater variety of species because it may be risky to fly long distances to find plants that provide better resources. Habitat enhancement for pollinators typically creates patches with many weedy or sown flower species that are highly attractive to bumble bees, which support floral constancy as reflected in my floral preference analysis. Further, this study illustrates how plant-pollinator networks are sensitive to habitat management as well as to the variation in plant establishment between sites in similar environmental settings. This highlights the need for replication in studies that explore how plant-pollinator networks vary in response to habitat enhancement.

Implementing habitat enhancements in farmscapes can attract pollinators to a variety of food resources that can help sustain their colonies over the growing season when crops are out of bloom (Isaacs et al. 2017) Sites that had mature habitat enhancements had significantly more interactions than sites with new and without enhancements, indicating that over time, the full establishment of these plantings can greatly benefit bumble bees in florally sparse landscapes. This further supports the line of evidence showing that enhancements can support and boost pollinator populations over time (Chapter 2).

CHAPTER 4

FATAL ATTRACTION: THE ROLE OF HABITAT ENHANCEMENT IN BUMBLE BEE EPIDEMIOLOGY

INTRODUCTION

Bumble bees are important pollinators in a variety of pollination-dependent cropping systems around the world (Winfree et al. 2007, Kennedy et al. 2013). However, some populations of bumble bees have been experiencing declines in their populations and distribution ranges over the past few decades (Williams and Osborne 2009; Potts et al. 2010; Cameron et al. 2011; Bartomeus et al. 2013) due to a variety of factors that include agricultural intensification (Koh et al. 2016), habitat fragmentation (Grixty et al. 2009), pesticide usage (Rundlof et al. 2015; Woodcock et al. 2017) and pathogen infection (Graystock et al. 2013; 2015; McArt et al. 2017). Further, there is a strong line of evidence pointing towards multiple stressors acting synergistically to negatively affect bumble bee populations (Brunner et al. 2014; Goulson et al. 2015; Sanchez-Bayo et al. 2016; McArt et al. 2017).

Bumble bees require nectar and pollen food sources for colony survival and growth (Goulson et al. 2002; Vaudo et al. 2013). They also need nesting resources, which can be relatively uncommon in intensively managed agricultural systems (Kells and Goulson 2003). Integrating these limiting resources back into managed landscapes is considered one approach for stopping and potentially reversing the declines (Kovacs-Hostyanszki et al. 2017). Conservation efforts have led to extensive research on sown wildflower plantings in crop field margins to boost bee populations (M'Gonigle et al. 2015; Williams et al. 2015; M'Gonigle et al. 2017, Chapter 2). These plantings effectively increase the amount of highly attractive foraging resources available for bees to use and can support their colonies before and after crop bloom (Tuell et al. 2008, Blaauw and Isaacs 2014, Rowe 2017, Chapter 3) and increase nesting density over time (Carvell et al. 2017; Chapter 2). Bees in turn are attracted to pollinator-targeted restorations due to increased flowering resources (Gletti and Barrett 2008), plant species richness

(Jha and Kremen 2013; Chapter 3), pollen and nectar rewards (Brunet et al. 2015), or a combination of the above (Leonard and Masek 2014). I found that the number of bumble bee-flower interactions at sites in which pollinator restoration has taken place is significantly greater than at sites without restoration (Chapter 3).

Various pathogens including *Crithidia bombi*, *Apicystis bombi,* and *Nosema bombi* have been studied extensively in bumble bee systems, especially in relation to how they are spread from bee to bee (Durrer and Schmid-Hempel 1994; Colla et al. 2006; Otterstatter and Thomson 2007; Graystock et al. 2013, 2015). Durrer and Schmid-Hempel (1994) first showed that *C. bombi* could be transferred successfully from bumble bee to bumble bee after allowing uninfected bees to forage on flowers that had been foraged on by infected bees. More recently, Graystock et al. (2015) demonstrated that bumble bees were able to transmit bumble bee and honey bee specific pathogens at flowers. However both of these studies were lab experiments, which may not reflect the rates of transmission in a natural environment. Colla et al. (2006) proposed that pathogen transmission occurred at flowers, which is how *C. bombi* and *N. bombi* were transmitted from commercially reared bumble bees to wild bumble bees in the surrounding landscape. This line of evidence was bolstered by Graystock et al. (2013), when they found that bumble bees could become infected by eating honey bee pollen contaminated with a variety of pathogens both specific and unspecific to bumble bees. However, they were unable to rule out pathogen transmission from infected surfaces within the hive that the bumble bees had touched. These pathogens in particular have received a lot of attention for their potential connection to declines in bumble bee populations (Cameron et al. 2011, McArt et al. 2017). Although there has been a lot of research to address pathogen transmission between bees, there is little real world

evidence showing how pathogen transmission rates vary in response to changes in habitat such as restoration, flower species richness, and flower density.

Few studies have investigated the role of bee-plant-pathogen interaction networks for bee disease epidemiology. The study of these networks is critical to understanding the spread of disease among organisms and can provide insights into the key mechanisms or contributors to pathogen transmission and disease dynamics (Keeling and Eames 2005; Kamo and Boots 2006). Network analysis can also provide new insights into management techniques for restoration practices based on bee disease epidemiology. For example, Henson et al. (2009) aimed to discern the effect of plant-pollinator interactions on bumble bee disease interactions in restored heathlands in southern England; however, there was no direct link found between pathogens and flowers. The lack of a complete network prevented these authors from further explorations of the role of plant species in the prevalence of bumble bee pathogens in this system. In another study, Otterstatter et al. (2007) tracked the movement of experimentally inoculated bumble bees within their hives to determine how *C. bombi* is transmitted within the colony. They found that differences in the rates of infection in each colony were due almost entirely to the density of interactions among the bees in the colony (i.e., the number of interactions in the network), with more contact between bees leading to faster rates of infection. This indicates that contact between bumble bees within the hive drives pathogen transmission. However, they were unable to also rule out pathogens being spread on commonly encountered surfaces within the hives like brood clumps (Otterstatter et al. 2007).

Other surfaces where bees can contact pathogens also have the potential to effect pathogen transmission, and flowers are one important resource for bumble bees where they contact surfaces and may be at risk of pathogen infection. This was first demonstrated by Durrer

and Schmid-Hempel (1994) and then later by Graystock et al. (2015) who found that in a laboratory experiment, infected bumble bees could deposit both bumble bee-specific and honey bee-specific pathogens on flowers. These pathogens were subsequently transmitted to uninfected honey bees when foraging on those same flowers after bumble bees had been removed. This suggests that bee pathogens can be transferred at flowers but also that non-target bee species can also be a dispersal agent for pathogens. Although this study demonstrated that bee pathogens could be spread via shared use of flowers, they did not investigate the role of frequency of interactions amongst bees and flowers on pathogen spread, which is a vital piece to bee disease epidemiology. Additionally the research was conducted in the laboratory, and pathogen dynamics are expected to be quite different under outdoor field conditions.

Restoration of habitat for pollinators in farm landscapes has been shown to enhance wild pollinator abundance and diversity (Carvalheiro et al. 2011; Carvalheiro et al. 2012; Blaauw and Isaacs 2014), with benefit for crop pollination in some systems (studies mentioned above) but not in some others (Klein et al. 2012). While the benefits of this approach have been the main focus of much of the research, the potential for enhanced pathogen transfer is a potential negative aspect of these plantings that also requires investigation. If they serve to increase pathogen spread, or if particular plant species are more likely to be a hub for pathogen transfer, then this would need to be balanced against the benefits when considering flower-based conservation programs to enhance habitat for pollinators.

This study was conducted to test the hypothesis that habitat enhancement in the form of sown wildflower plantings for pollinators increases pathogen prevalence of three main bumble bee pathogens, *C. bombi, A. bombi,* and *N. bombi*. Furthermore, I aimed to determine the role of flowers in pathogen transmission amongst bumble bee species in the wild. This was done by

collecting and screening bumble bees and the flowers they were visiting for the three pathogens mentioned above and an interaction network between bumble bees, plants, and pathogens. I hypothesized that the increase in flower availability at sites with restoration plantings would have increased pathogen prevalence in bees and that the more commonly visited flower species would be more likely to act as pathogen dispersal platforms for bumble bees than other flowering plants.

METHODS

Site selection

I selected 15 field sites within a matrix of agricultural and natural land use in southwestern Michigan, each with a commercial blueberry field managed using standard crop management methods. Of the 15 sites, five had a mature (more than 5 years since establishment) habitat enhancement planting for pollinators in the field margin, four had a new (0-3 years) habitat enhancement planting for pollinators in the field margin, and six had the standard practice of mown grass as the field margin (see Methods Chapter 3).

Plant and pollinator collections

In 2015 and 2016, bumble bees and the flowers they were visiting were collected four times throughout the summer at each site during 30-minute sampling periods. Bumble bee-flower sampling began in early to mid-July and each collection round occurred 3-4 weeks apart depending on weather conditions. Sampling occurred between 9:00-17:00 hours and only in weather that was above 16.0 °C and not raining. Bumble bees and the flowers they were visiting were collected in separate but corresponding 22 mL scintillation vials and frozen at -80 °C until

they could be identified and dissected in the laboratory. Bumble bees were identified using "Bumble Bees of North America" as a guide (Richardson et al. 2014). Plants were identified both in the field and in the laboratory using Newcombs Wildflower Guide (Newcomb 1987) and Weeds of the Northeast (Uva et al. 1997).

Pathogen DNA extraction

After identification, bumble bees and flower samples were dissected under a microscope. For bumble bees, the abdomen was carefully opened using sterilized forceps (dipped in a 10% bleach solution followed by two water baths). Then, all of the gut tissue was removed from the bumble bee abdomen, including Malpighian tubules, digestive tract, and fat bodies, and placed in a 1.5 mL plastic microcentrifuge tube.

Flower heads were dissected with a pair of sterile forceps by carefully pulling off up to 1000 mg of the flower petals, stigmas, anthers, and nectaries and placing them in a sterile microcentrifuge tube. Flowers were handled very carefully so as not to damage any of the tissue, which can emit compounds that can disrupt subsequent polymerase chain reactions (PCR). Bumble bee and flower specimens were extracted using a modified protocol with the Qiagen DNeasy kit (Table C1). Extracted samples were labeled and placed in a -20 °C freezer until further PCR processing.

Bumble bee and flower PCR and gel electrophoresis

Bumble bees and flowers were screened for *A. bombi, C. bombi, and N. bombi* using primer pairs specific to each pathogen species (Table C2). Each pathogen screen required a separate PCR master mix and thermocycler regime (Table C2). The resulting amplified DNA was run through a 1.5% agarose gel (3 g UltraPure™ Agarose, Thermo Fisher Scientific, Waltham, MA, in 200

mL 1X TBE buffer) containing 7 µL GelRed™ (Biotium, Fremont, CA) for visualization of DNA in the gel. Before gel electrophoresis, 1 µL of DNA Gel Loading Dye (6X) (ThermoFisher Scientific) was added to the PCR product for each sample. PCR product and loading dye were centrifuged for 1-2 seconds to ensure mixing of PCR product and loading dye. Gels were loaded by pipetting 6.5 µL of PCR product/loading dye mixture into each well in an OWL D3-14 Wide Gel Electrophoresis System (Thermo Fisher Scientific). Ten µL of Ready-to-Use 100 BP DNA Ladder (Biotium) was added to the first and last well in each gel. Gels for *A. bombi* and *C. bombi* were run at 90 V and 250 mA for 90 minutes, whereas *N. bombi* samples were run at 120 V and 250 mA for 45 minutes. A photo of each gel was taken while on a Benchtop 2UV Transilluminator (UVP, Jena, Germany). Gels were then scored either by hand or in Adobe Photoshop Elements Editor v.11.0 (Adobe Systems, San Jose, CA) due to the closeness of each well. A sample was scored as being positive if the band produced from the given sample matched with the positive control.

Data analysis

In each site in each round (or over the course of the year), the proportion of bumble bees or flowers that screened positive for a given pathogen was calculated. The proportion of bumble bees and flower heads that screened positive in each site or round were arcsine transformed before statistical comparisons were performed.

Data were tested for normality using a Shapiro-Wilk normality test. All pathogen prevalence comparisons were completed using a mixed effects linear model with year, treatment, and round as fixed variables and site as a random effect followed by Tukey's honest significant difference test. However, as some statistical differences were likely obscured due to high

variability between years, the above analysis was followed by either a one-way analysis of variance followed by a or using a Kruskal-Wallis rank sum test followed by a Dunn's test for comparisons among treatments and rounds for each year individually. All data were analyzed using R statistical software (R Core Team 2013).

Comparisons of pathogen prevalence between bumble bee and flower species was determined with a generalized linear model fitted with a beta-inflated distribution using the gamlss package in R (Rstudio, "gamlss") because of the proportion-type data that included both zeros and ones (Ospina and Ferrari 2010). The only test that required a different distribution was for how often flowers were screened positive for *Nosema bombi* because there was only a single positive. Thus, beta-zero-inflated distribution was used in this case. If a significant difference was found between species, a Tukey-Kramer multiple comparisons test was performed to determine statistical groupings among species. This type of analysis was also done specifically to determine whether the two most common species of bumble bees, *B. impatiens* and *B. griseocollis*, had different levels of pathogen prevalence among treatments.

I also determined whether the presence of a pathogen being found in a bumble bee was correlated to the flower that the bee visited screening positive for that pathogen. Thus, I used a logistic regression to determine this relationship by using a generalized linear model fit to a binomial distribution.

Data from pathogens being present or absent on flowers or in bees was tied to each individual bumble bee-flower interaction (see Chapter 2) to create an interaction network. The three tier (bumble bee species-flower species-pathogen species) was created in R using the igraph, network, sna, and ndtv packages. Links to flower species that were highly attractive (see

Chapter 2) were highlighted to depict how pathogen transmission may occur at highly attractive plants.

RESULTS

All three pathogens that were screened for in this study (*C. bombi, A. bombi,* and *N. bombi*) were detected in bees and on flowers, with higher overall detections in the bees than on the flowers. Treatment had a significant effect on both the proportion of bumble bees and flower heads that were detected with at least one of the three pathogens over both years (bumble bees: Chisq = 7.14, d.f. = 2, 12, $p = 0.028$; flowers: Chisq = 6.07, d.f. = 2, 12, $p = 0.048$). However, a post-hoc Tukey-Kramer revealed that there were no significant pairwise differences between treatments for each of these analyses ($p > 0.05$ for each pairwise test). When the data were separated by year, there were significant differences between treatments in bumble bees in 2016 but not in 2015 and there was no difference among treatments in the flowers (Figure 4.1).

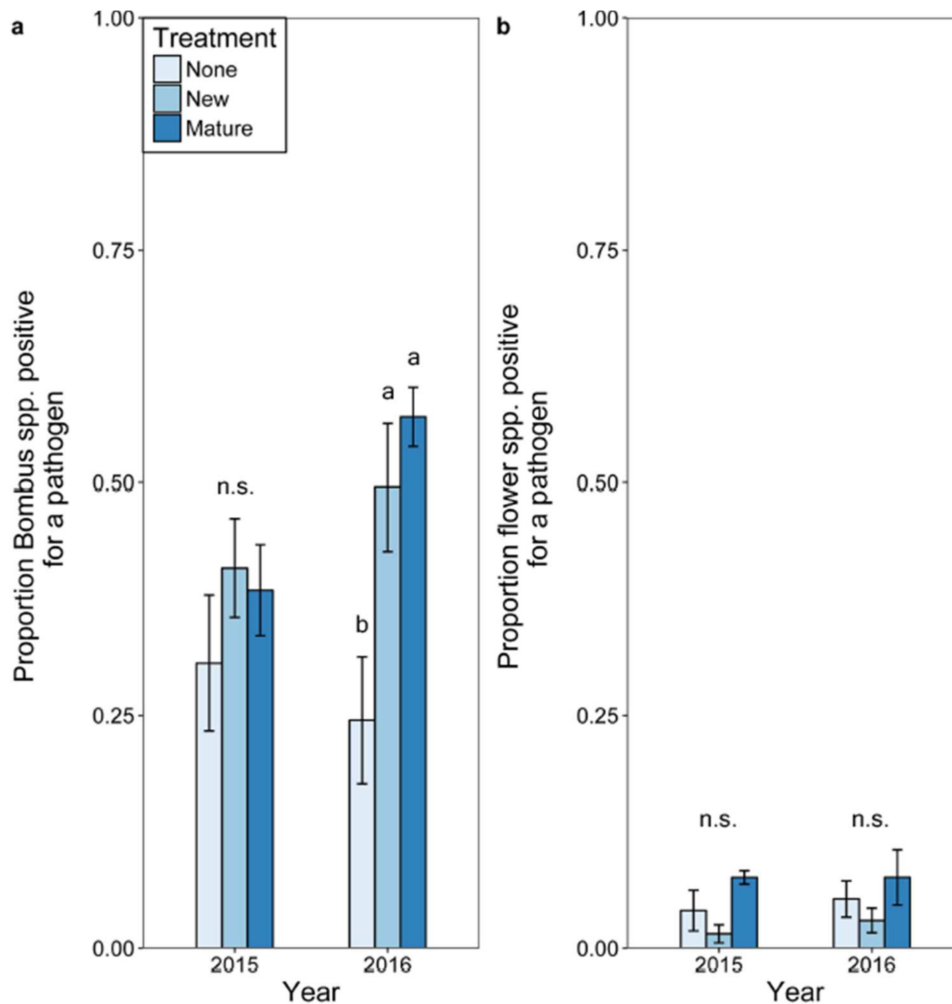

Figure 4.1. (a) Overall proportion of bumble bee and (b) flower samples that were screened positive for one of three pathogens (*Crithidia bombi, Apicystis bombi,* or *Nosema bombi*). Tukey's HSD (for bumble bees) and a Dunn Test (for flowers) were used to assign groupings among treatments.

In 2016, sites with new (0.53 ± 0.06) and mature restorations (0.51 ± 0.04) had significantly higher pathogen prevalence than sites without restoration (0.31 ± 0.06) (One-way ANOVA: F = 5.02, d.f. = 2, 28, p = 0.01).

Over the course of both years, a mixed effects model indicated that treatment had a significant effect on the proportion of bumble bees that screened positive with *C. bombi* (Chisq = 8.61, d.f. = 2, 13, p = 0.013) (Figure 4.2). However, there were no significant differences between treatments in a Tukey-Kramer multiple comparisons test (p > 0.05 for each pairwise

test). When years the data were analysed separately by year, in 2015, there were no significant differences among the restoration treatments in the proportion of bumble bees infected with *C. bombi* (one-way ANOVA: F = 0.95, d.f. = 2, 13, p = 0.412). However, in 2016, there was a significant difference among the wildflower planting treatments, with significantly higher rates of infection with *C. bombi* in bees collected at new (0.45 ± 0.06) and mature restorations (0.48 ± 0.04) compared to sites without restoration (0.17 ± 0.07) (one-way ANOVA: F = 7.90, d.f. = 2, 12, p = 0.01) (Figure 4.2).

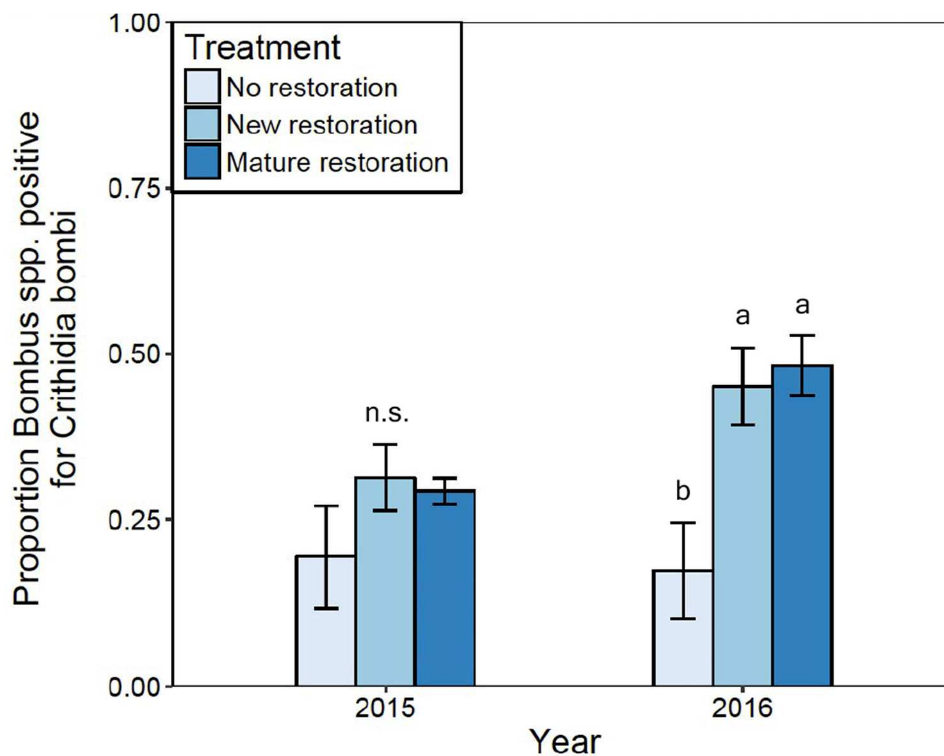

Figure 4.2. Proportion of *Bombus* spp. infected with *Crithidia bombi* between treatments in 2015-2016. Tukey's HSD was used to assign groupings between treatments.

There was no treatment effect on the proportion of bumble bees that screened positive for both *A. bombi* (Chisq = 0.06, d.f. = 2, 12, p = 0.725) (Figure 4.3) or *N. bombi* (Chisq = 2.33, d.f. = 2, 12, p = 0.312) (Figure 4.4).

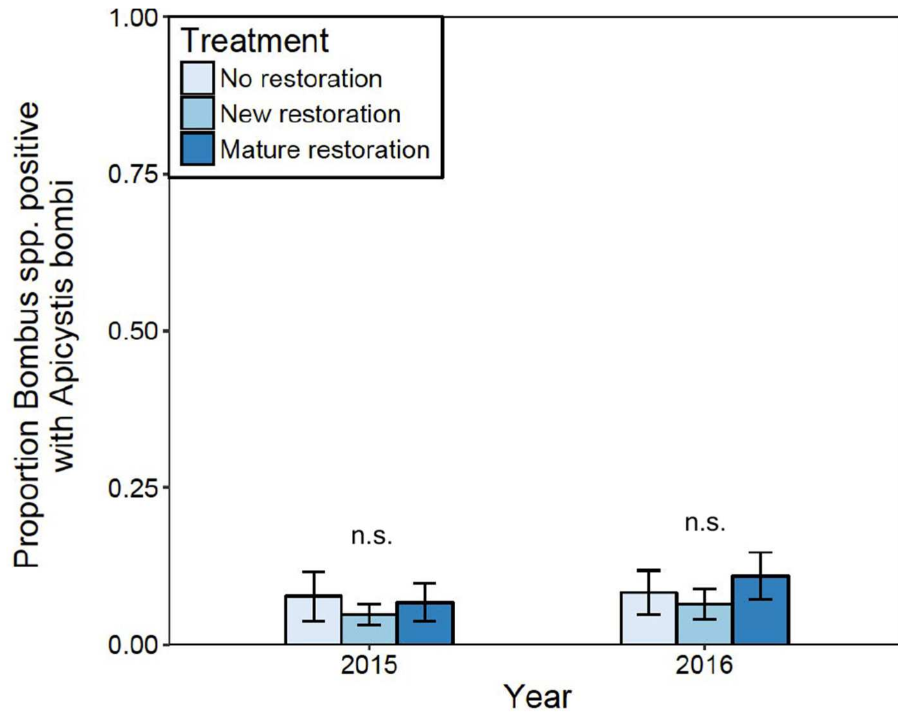

Figure 4.3. Proportion of *Bombus* spp. infected with *Apicystis bombi* between treatments in 2015-2016. A Dunn's test was used to assign groupings between treatments.

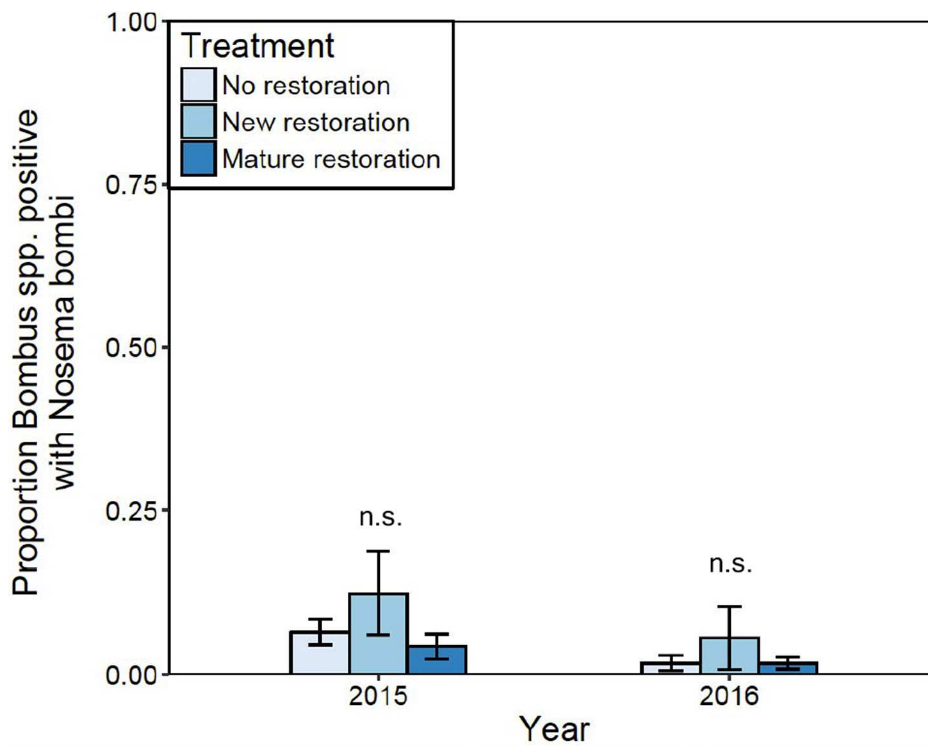

Figure 4.4. Proportion of *Bombus* spp. infected with *Nosema bombi* between treatments in 2015-2016. A Dunn's test was used to assign groupings between treatments.

The proportions of bumble bees infected with *C. bombi*, *A. bombi,* and *N. bombi* were compared among sampling rounds in both years using a mixed effects model, which indicated a significant difference between rounds for *C. bombi* (Chisq = 23.10, d.f. = 3, 11, $p < 0.001$), but was not significant for *A. bombi* (Chisq = 4.37, d.f. = 3, 11, $p = 0.224$) or *N. bombi* (Chisq = 4.10, d.f. = 3, 11, $p = 0.251$). A Tukey-Kramer multiple comparisons test revealed that the first round of sampling had a greater proportion of bumble bees that screened positive for *C. bombi* than sampling rounds 2-4 (round 2-1: $z = -2.81$, $p = 0.026$; round 3-1: $z = -3.142$, $p = 0.009$; round 4-1: $z = -3.91$, $p < 0.001$). Sampling rounds 2-4 did not differ from one another ($p > 0.05$). There was no difference among sampling rounds on the proportion of bumble bees that screened positive for both *A. bombi* and *N. bombi* (*A. bombi*: Chisq = 4.37, d.f. = 3, 11, $p = 0.224$; *N. bombi*: Chisq = 4.10, d.f. = 3, 11, $p = 0.251$).

Comparisons among the bumble bee species revealed significant variation in the proportion of bees with *C. bombi* (gamlss: $\sigma < 0.01$, $\nu = 0.01$, $\tau < 0.01$). A post-hoc Tukey-Kramer multiple comparison test found that *B. bimaculatus* (0.53 ± 0.09), *B. citrinus* (0.60 ± 0.19), *B. griseoccolis* (0.45 ± 0.07), and *B. vagans* (0.61 ± 0.08) had higher rates of *C. bombi* prevalence than *B. fervidus* (0.05 ± 0.03). Only *B. bimaculatus* and *B. vagans* had significantly higher *C. bombi* prevalence than *B. impatiens* (0.22 ± 0.03) (Figure 4.5).

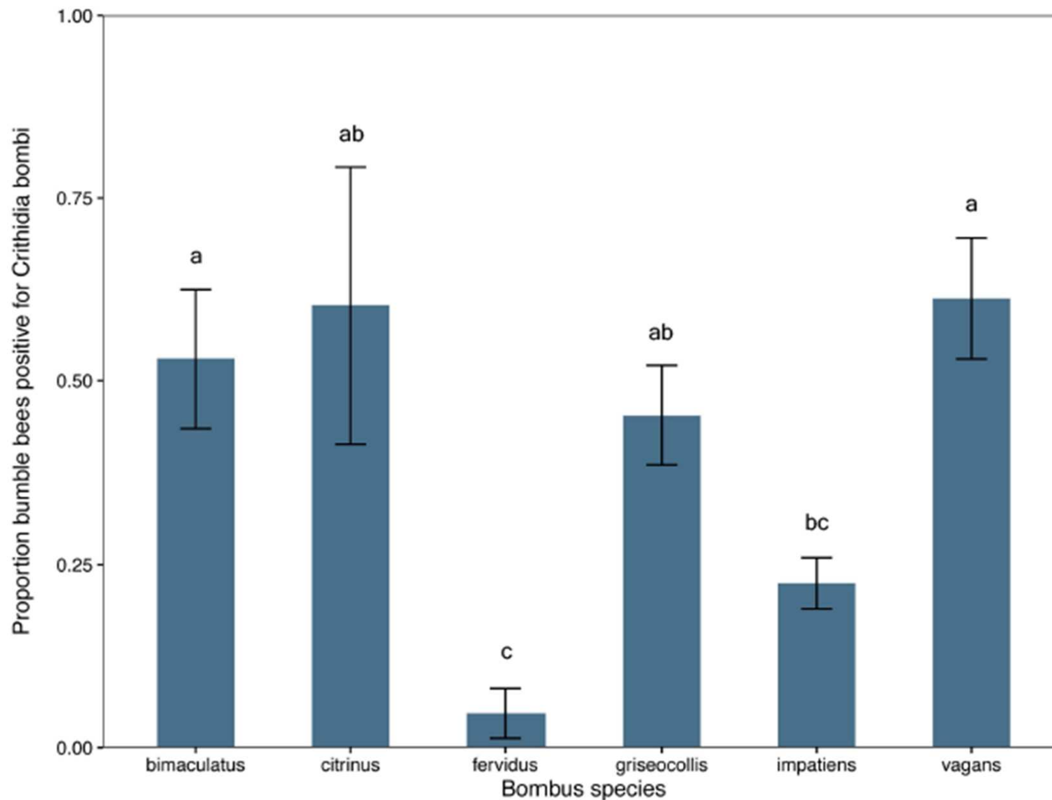

Figure 4.5. Proportion of bumble bee species that tested positive for *Crithidia bombi* in 2015 and 2016 across all treatments. Tukey's HSD was used to assign groupings between treatments.

Bumble bee species varied significantly in their levels of *A. bombi* pathogen prevalence (gamlss: $\sigma < 0.01$, $\nu < 0.01$, $\tau < 0.01$) (Figure 4.6). However, due to the variability within some species, there was no significant difference in *A. bombi* prevalence between species.

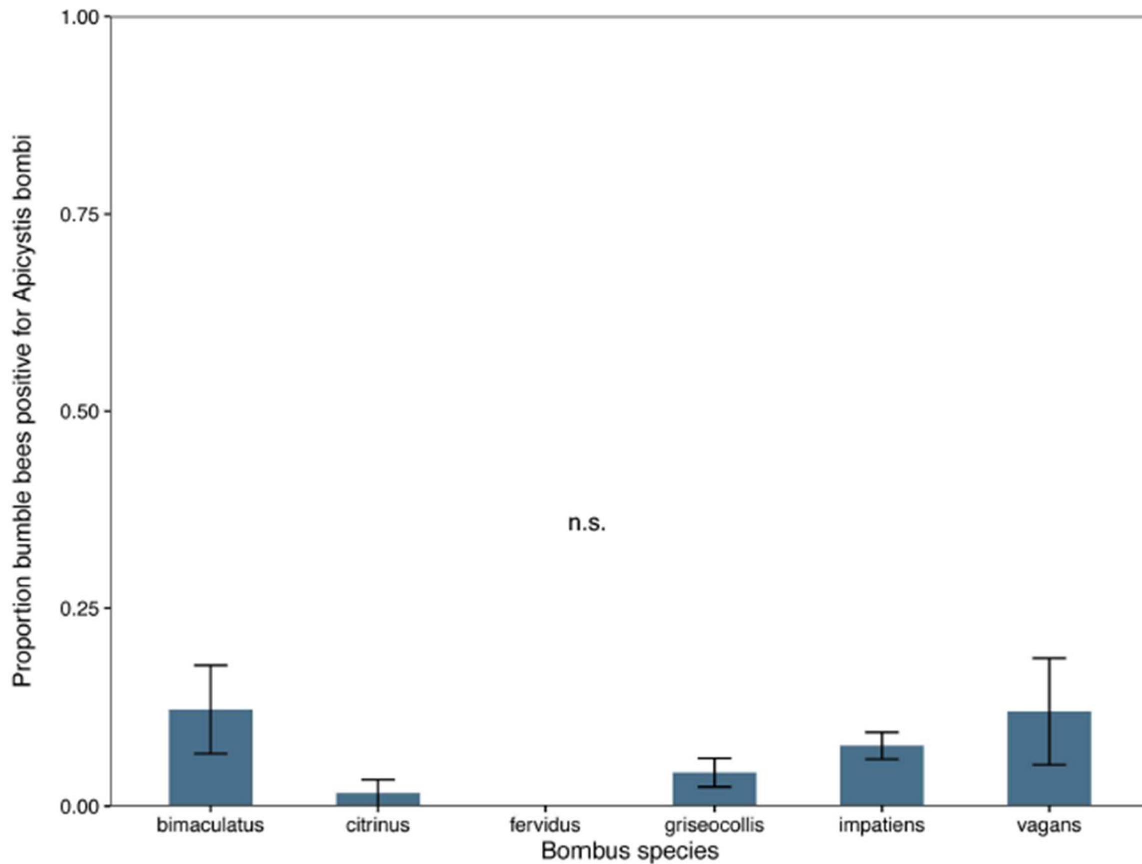

Figure 4.6. Proportion of bumble bee species that tested positive for *Apicystis bombi* in 2015 and 2016 across all treatments. Tukey's HSD was used to assign groupings between treatments.

Bumble bee species differed significantly in the prevalence of *N. bombi* (GAMLSS: $\sigma < 0.01$, $\nu < 0.01$, $\tau < 0.01$). A post-hoc Tukey-Kramer multiple comparison test found that *B. fervidus* (0.41 ± 0.11) had significantly higher rates of *N. bombi* prevalence than *B. bimaculatus* (0.02 ± 0.02), *B. griseocollis* (0.11 ± 0.05), and *B. impatiens* (0.01 ± 0.01). *Bombus citrinus* (0.36 ± 0.19) also had significantly higher rates of *N. bombi* prevalence than *B. impatiens*. *Bombus bimaculatus, B. griseocollis, B. impatiens, and B. vagans* (0.17 ± 0.07) did not differ significantly (Figure 4.7).

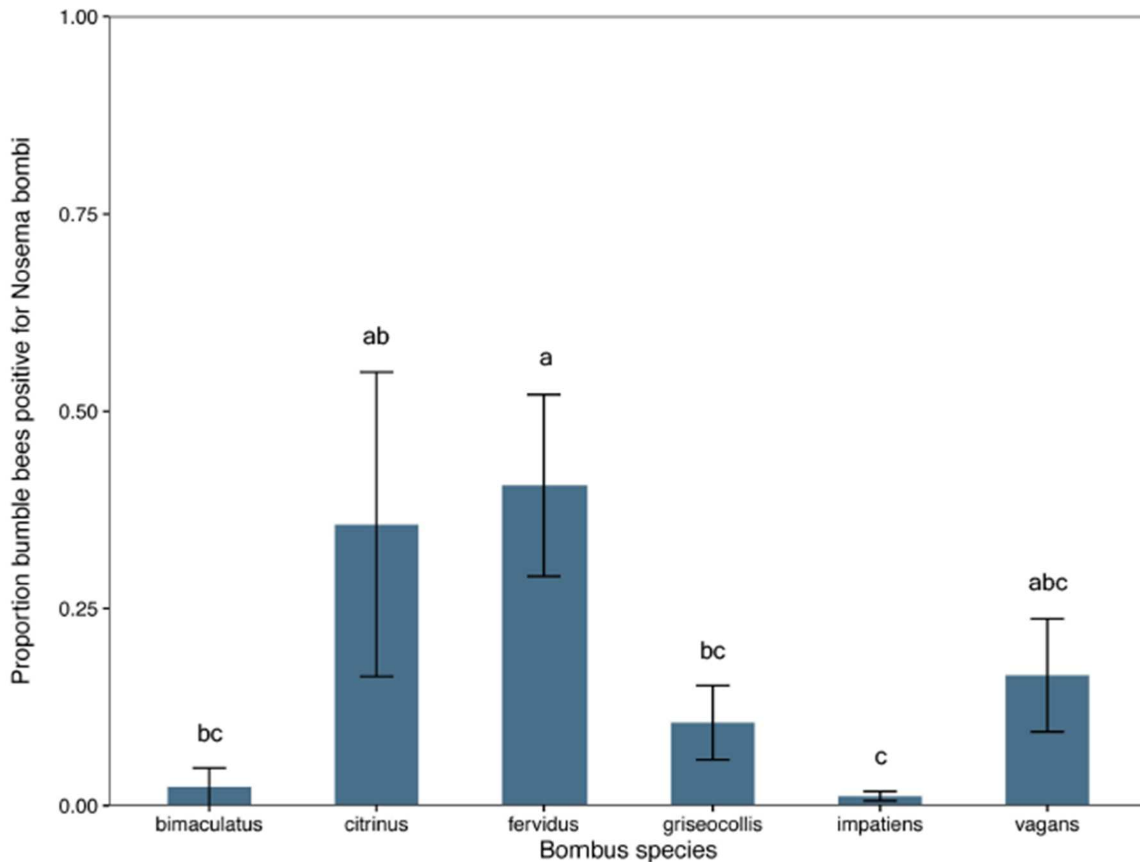

Figure 4.7. Proportion of bumble bee species that tested positive for *Nosema bombi* in 2015 and 2016 across all treatments. Tukey's HSD was used to assign groupings between treatments.

Pathogens were detected on flowers sampled at the three types of habitat, but at much lower levels than on the bumblebees. A mixed effects model revealed that there was a significant treatment effect on the proportion of plants that screened positive for a pathogen (Figure 4.1). However, a Tukey-Kramer multiple comparisons test revealed there was no significant difference between treatments ($p > 0.05$)

I also found that plant species varied significantly in the presence of *C. bombi* (gamlss, beta inflated: $\sigma < 0.01$, $\nu < 0.01$, $\tau < 0.01$), *A. bombi* (gamlss, beta inflated: $\sigma < 0.01$, $\nu < 0.01$, $\tau < 0.01$), and *N. bombi* (gamlss, beta zero-inflated: $\sigma < 0.01$, $\nu < 0.01$). The plant species most likely to be found with the presence of *C. bombi* were *Apocynum cannabinum* ($p = 0.01$) and *Monarda fistulosa* ($p = 0.03$) (Table 4.1). More plant species were connected to detection of *A.*

bombi, and for this pathogen the plant species with significant positive effect on its presence were *Centaurea maculosa* ($p < 0.01$), *Cirsium vulgare* ($p = 0.04$), *Mondarda fistulosa* ($p < 0.01$), *Solidago altissima* ($p = 0.01$), and *S. graminifolia* ($p = 0.02$) (Table 4.2). There were no plant species with significant effect on the presence of *N. bombi*. A post hoc Tukey-Kramer multiple comparison test was used to determine the differences in likelihood of being screened positive for *C. bombi, A. bombi,* and *N. bombi*, however, there were no differences between species for any of the pathogens (*C. bombi*: $F = 0.72$, d.f. = 60, 235, $p = 0.93$, *A. bombi*: $F = 0.98$, d.f. = 60, 235, $p = 0.53$, *N. bombi*: $F = 0.18$, d.f. = 60, 235, $p = 1.0$).

Table 4.1. Plant species with significant impacts on the odds of screening positive for *Crithidia bombi*. Non-significant plant species were not included in this table. Multiple comparisons revealed that plant species did not harbor *Crithidia bombi* more often than others. Estimates are given as log odds values.

Model: Proportion flowers with *Crithidia bombi* ~ plant species, **distribution:** Beta-inflated			
Plant species	**Estimate ± SE**	**t-score**	**Significance (p-value)**
Apocynum cannabinum	1.70 ± 0.60	2.82	0.01
Asclepias syriaca	1.03 ± 0.62	1.68	0.10
Cirsium vulgare	1.03 ± 0.62	1.68	0.10
Lotus corniculatus	1.03 ± 0.62	1.68	0.10
Monarda fistulosa	-1.24 ± 0.58	-2.13	0.03
Solidago altissima	0.91 ± 0.52	1.75	0.08
Symphyotrichum ericoides	1.03 ± 0.55	1.86	0.06

Table 4.2. Plant species with significant impacts on the odds of screening positive for Apicystis bombi. Non-significant plant species were not included in this table. Multiple comparisons revealed that plant species did not harbor *Apicystis bombi* more often than others. Estimates are given as log odds values.

Model: Proportion flowers with Apicystis bombi ~ plant species, **distribution:** Beta-inflated			
Plant species	**Estimate ± SE**	**t-score**	**Significance (p-value)**
Centaurea stoebe	-1.63 ± 0.43	-3.82	0.0002
Cirsium vulgare	0.87 ± 0.42	2.04	0.04
Euthamia graminifolia	-1.46 ± 0.61	-2.39	0.02
Monarda fistulosa	-1.69 ± 0.40	-4.26	<0.0001
Solidago altissima	-1.65 ± 0.645	-2.56	0.01

The likelihood of both a bumble bee and a flower head screening positive for *C. bombi, A. bombi,* and *N. bombi*, did not increase if the bumble bee had that pathogen (*C. bombi*: F = 0.06, d.f. = 1070, p = 0.95, *A. bombi*: F = 1.85, d.f. = 1070, p = 0.06, *N. bombi*: F = -0.004, d.f. = 1070, p = 1.0). Furthermore, there was no relationship between the proportion of flowers that screened positive for a pathogen and the proportion of bumble bees that were also infected for any given pathogen (All pathogens combined: T = 0.53, d.f. = 1, 30, p = 0.60; *C. bombi*: T = -1.42, d.f. = 1, 30, p = 0.17; *A. bombi*: T = 1.24, d.f. = 1, 30, p = 0.23; *N. bombi*: T = -0.39, d.f. = 1, 30, p = 0.70).

Bumble bee-flower-pathogen interaction networks were created for sites without an enhancement (Figure 4.8), with a new enhancement (Figure 4.9), and with a mature enhancement (Figure 4.10). Links between flower species deemed to be highly attractive to bumble bees (see Chapter 2) were highlighted to help visualize how highly attractive plants relate to possible pathogen transmission.

No enhancement

- Plant spp.
- Bombus spp.
- Pathogen spp.

#	Species
1	*Apocynum cannabinum*
2	*Asclepias syriaca*
3	*Asclepias incarnata*
4	*Asclepias tuberosa*
5	*Symphyotrichum laeve*
6	*Symphyotrichum lateriforum*
7	*Centaurea stoebe*
8	*Coreopsis lanceolata*
9	*Daucus carota*
10	*Echinacea purpurea*
11	*Eryngium yuccifolium*
12	*Euthamia graminifolia*
13	*Monarda fistulosa*
14	*Ratibita pinnata*
15	*Rudbeckia hirta*
16	*Silphium perfoliatum*
17	*Solidago altissima*
18	*Solidago canadensis*
19	*Solidago missouriensis*
20	*Solidago nemoralis*
21	*Solidago rigada*
22	*Solidago rugosa*
23	*Solidago speciosa*
24	*Symphyotrichum ericoides*
25	*Symphyotrichum novae-angliae*
26	*Trifolium pratense*
27	*Trifolium repens*
28	*Vicia villosa*
29	*Bombus bimaculatus*
30	*Bombus citrinus*
31	*Bombus fervidus*
32	*Bombus griseocollis*
33	*Bombus impatiens*
34	*Bombus vagans*
35	*Crithidia bombi*
36	*Apicystis bombi*
37	*Nosema bombi*

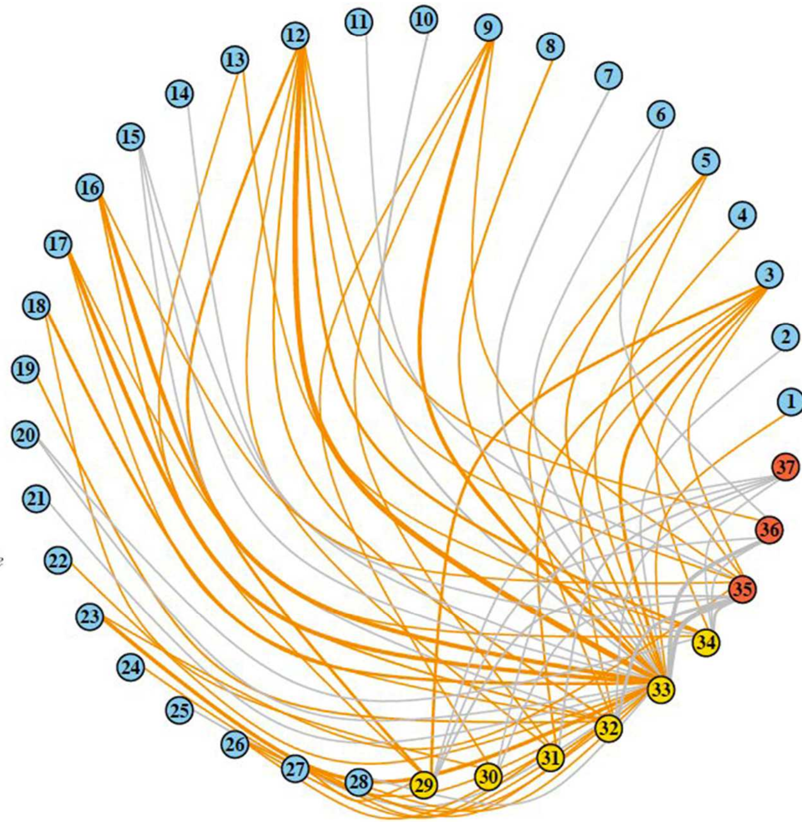

Figure 4.8. A combined bumble bee-flower-pathogen network from all sites without an enhancement, where each node represents a bumble bee, plant, or pathogen species. The thickness of each line linking any two nodes is proportional to the relative frequency of the interaction. Orange links highlight interactions with highly attractive plants.

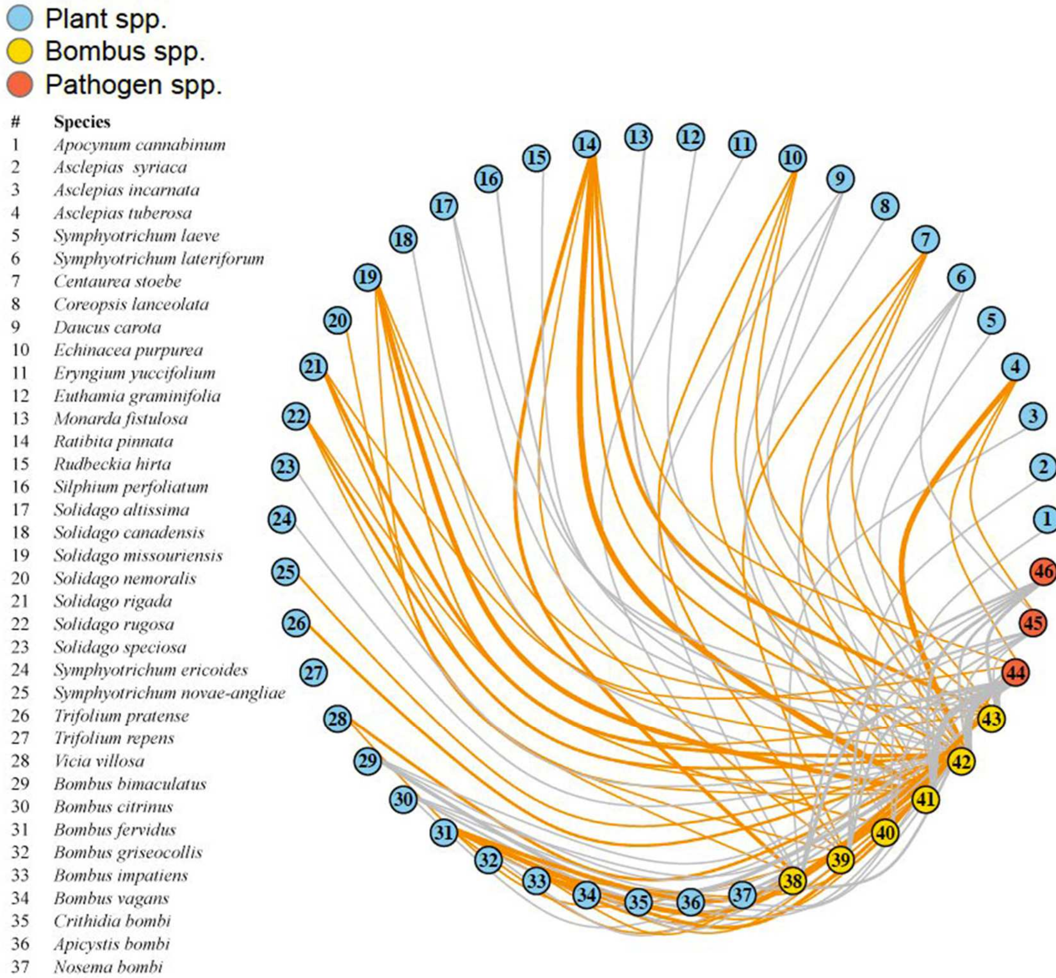

Figure 4.9. A combined bumble bee-flower-pathogen network from all sites with a new enhancement, where each node represents a bumble bee, plant, or pathogen species. The thickness of each line linking any two nodes is proportional to the relative frequency of the interaction. Orange links highlight interactions with highly attractive plants.

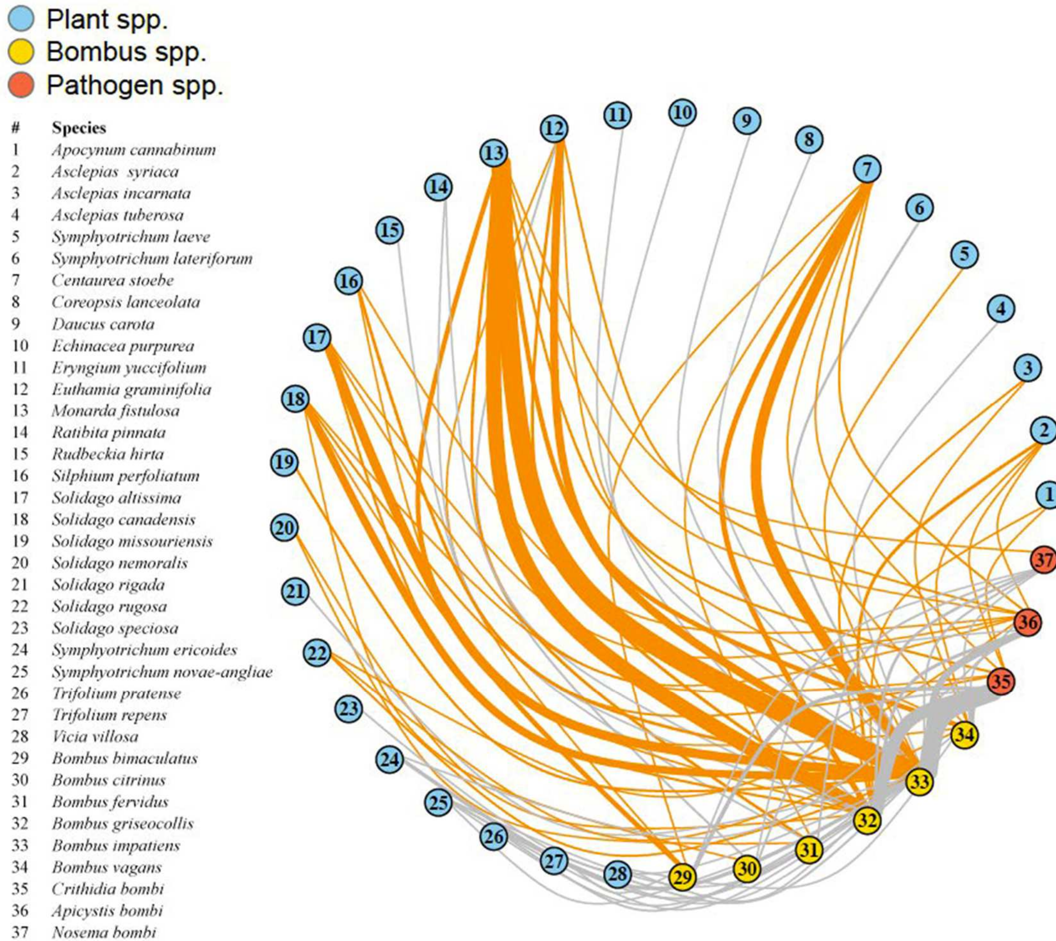

Figure 4.10. A combined bumble bee-flower-pathogen network from all sites with a mature enhancement, where each node represents a bumble bee, plant, or pathogen species. The thickness of each line linking any two nodes is proportional to the relative frequency of the interaction. Orange links highlight interactions with highly attractive plants.

DISCUSSION

In this study, I found that there was a significant effect of pollinator habitat enhancements on the proportion of bumble bees and flowers that screened positive for bee pathogens. When a Tukey-Kramer multiple comparisons test was performed, the year-to-year variation in the proportion of bees and flowers obscured statistical differences between individual treatments. When each year

was analyzed separately, the proportion of flowers that screened positive for a pathogen did not vary between treatments in either year (Figure 4.1b), but in 2016 the proportion of bumble bees that screened positive for a pathogen was greater in sites with new and mature enhancements compared to sites without an enhancement (Figure 4.1a). This has significant implications for the approaches being developed for bee conservation in agricultural landscapes because sown wildflower strips are thought of as being beneficial to bee populations. However, habitat enhancements may also be facilitating pathogen transfer between bees. The results of this study indicate that areas where many attractive flowers are clustered together may act as disease transmission hotspots, similar to how high traffic public locations, shared office space, and drinking water have higher rates of disease transfer among humans (Jaakkola and Heinonen 1995; Fayer et al. 2000; Weber et al. 2010; Qi and Du 2013).

I suggest two possible mechanisms for this result: first, the local populations of bees are increasing with respect to added floral resources from habitat enhancements (see Chapter 2) and thus more local bees are drawn to the habitat enhancement where pathogens can be transferred. Second, in landscapes that are devoid of flowering resources for bees such as highly managed commercial farms, bees from beyond the local landscape with long foraging ranges (Beekman and Ratneiks 2000, Rao and Strange 2012) may be visiting the resource rich habitat enhancement and the sheer number of bees from different colonies may increase the likelihood that pathogens will be present. Both of these mechanisms would be supported due to the increase in bumble bee visitation rates in sites with new or mature restorations compared to sites without restoration (see Chapter 3). It remains to be seen whether the negative effects of the pathogens outweigh the positive benefits of access to a greater amount and diversity of pollen and nectar from the greater plant community provided by the restorations (Chapter 3). However, my results in Chapter 2 in

which the colony density of *Bombus impatiens* was found to be greater in farms with mature restorations, indicates that at least for this species the balance is positive for adding wildflower plantings despite an increased risk of *C. bombi* infection. Further investigation of different bee species may reveal species-specific differences in the relative importance of nutrition and pathogen exposure.

These results are also consistent with the idea that pollinator restoration practices for farms as currently designed, generally adding one large planting in a field margin, may not be the optimal strategy for creating foraging habitat for bees when bee disease dynamics are added to the equation. New habitat enhancement strategies should be explored such as planting several small habitat enhancements throughout farms to determine whether this minimizes pathogen transfer due to fewer bees visiting each of the smaller more spread out wildflower enhancements. This could result in lower pathogen prevalence amongst bees, while still providing food resources to bees within the landscape.

This is the first study that has successfully screened flowers for bee pathogens in outdoor settings. Several studies have screened for bee pathogens on flowers in a lab (Durrer and Schmid-Hempel 1994, Graystock et al. 2015), but others have attempted this in natural settings and were unsuccessful (Schmid-Hempel et al. 2001). In total, roughly 8% of flowers at sites with mature restorations we screened were positive for bumble bee pathogens. Considering how many flowers bumble bees can visit in a day, bumble bees must be encountering pathogens frequently in sites with high pathogen prevalence on the flowers. Because pathogen loads are particularly high in both bumble bees and flowers in mature restorations, this provides further evidence that bumble bees are transferring pathogens at flowers, and I therefore expect this will lead to greater pathogen infection of colonies after bees visit the plantings

Interestingly, the flowers that were most likely to have *C. bombi, A. bombi,* and *N. bombi* were also a similar set of plant species that were most preferred by bumble bees (see Chapter 3 for highly preferred flower species) (Figures 4.8-4.10). This provides another line of evidence showing that flowers commonly visited by bees are more likely to act as bee pathogen dispersal platforms. Because all parts of the flower were used in the pathogen DNA extraction, the dispersal mechanism of pathogens by bees to flowers is still unknown. However, based on symptoms and locations in the of each parasite in the bumble bee body, *C. bombi* and *N. bombi,* are likely to be deposited on the flower heads through defecation and would subsequently be picked up via spore-cuticle adhesion when a new bumble bee visits the flower (Graystock et al. 2015).

Although evidence points towards flowers being dispersal platforms for bee pathogens, there was no correlation between bumble bee pathogen infection and the flower they were visiting having the same pathogen. This is likely due to the small number of flowers that screened positive for any given pathogen. Interestingly though, the data are consistent with bees not spreading pathogens to every flower they visit. One possible explanation for this is that there may be a specific behavior that increases the chance of deposition of a pathogen on a flower head, such as defecation onto the flower, but is not performed during every flower visit. Additionally, the half-life of pathogen viability under hot sunny conditions may be relatively short, as they some pathogens such as *C. bombi* can be killed with UV light, preventing further infection (Naughton et al. 2017). Thus, there would likely be a patchwork of viable and non-viable pathogens across a planting.

Bumble bees differed among species in the prevalence of each pathogen. Interestingly, both *Bombus griseocollis* and *B. impatiens*, the most common bumble bee species in my study

sites, had significantly lower levels of pathogen prevalence for both *C. bombi* and *N. bombi*. This is contrary to previous findings, where common species typically had higher parasite loads (Durrer and Schmid-Hempel 1995). These results indicate that bumble bee species differ in their susceptibility to different pathogens in the environment. There are several explanations for these results: (1) bumble bees may vary in immune function across species (Schmid-Hempel 2003); (2) bumble bee species may exhibit certain behaviors that increase their chance of becoming infected, such as preferentially foraging on flower species that are more likely to have pathogens; (3) synergistic effects from pesticide exposure may affect bumble bees immune function differently, rendering certain species to be more susceptible to infection than others (Pettis et al. 2013, Goulson 2015); and (4) certain bumble bee species may be less prone to infection when highly nutritious nectar and pollen are available (Vaudo et al. 2015) or because certain plant species may contain secondary metabolites in their nectar and pollen, which can prevent infection (Palmer-Young et al. 2017).

Although bumble bees show variation in susceptibility, the rates of pathogen prevalence are very high across all species in comparison to other studies of bumble bees in the wild (Colla et al. 2006, Otterstatter and Thomson 2008, Kissinger et al. 2011). Bumble bees in agricultural settings face a variety of stressors such as pesticides (Woodcock et al. 2017), habitat fragmentation (Grixti et al. 2009), and lack of foraging resources which can lower immune function in bees, making them more likely to suffer from pathogen infection too (Goulson 2015). Commercial highbush blueberry fields in particular are highly managed for a variety of pests, including spotted wing drosophila, which has seen a significant increase in pesticide usage in recent years (Diepenbrock et al. 2016). This in combination with the general lack of flowering resources may be reducing bumble bees ability to fend off pathogens in this environment.

Overall, this study provides further evidence that flowers can act as platforms for bee pathogen dispersal in open field conditions. Further, this study provides critical knowledge regarding bee epidemiology in response to a common conservation method in agricultural systems, which has implications for future management of “pollinator-friendly” habitats. Understanding the mechanisms behind drivers of bee decline such as pathogen spread can help us maximize the efficiency of conservation efforts. This in turn will not only restore bee populations in agricultural systems, but will also help to ensure stable pollination services to crops.

CHAPTER 5

FUTURE DIRECTIONS IN BEE CONSERVATION AND EPIDEMIOLOGY

The research shown in my dissertation shows that habitat enhancement can successfully increase *Bombus impatiens* population density and that the increases in density are driven by both floral richness and several highly attractive plant species that are found in these restorations. However, I found that one consequence of habitat enhancement is that these habitat enhancements become a hub for pathogen transmission thus resulting in increased levels of pathogen prevalence among bees that forage at restorations. Combined, these findings help inform the development of strategies that can be used to conserve bumble bee populations in agricultural systems.

Bee conservation is a rapidly expanding field with much of the research focused on how best to restore or boost bee populations in agricultural systems, as recent analyses show that many wild bee populations are declining in agriculturally intensified landscapes (Potts et al. 2010; Kennedy et al. 2013; Koh et al. 2016; McArt et al. 2017). The goal of bee restoration practices, and perhaps restoration practices in general, is not addressed in most bee conservation research (i.e.. restoring pollinator communities to what they once were historically or boosting their abundance solely for pollination services) (Suding 2011). Kleijn et al. (2015) show evidence that conservation of all pollinator species may not be needed if the end goal of restoration is to support crop pollination, as a small proportion of the overall bee community typically provides increases in crop pollination. If this is the case, restoration and conservation practices should be developed that can support specific pollinator species for the benefit of the extra crop pollination. This is not to say that only a few pollinators should be conserved; rather, one must distinguish what the goals of restoration are and how those goals may impact pollinator communities over time. If the goal of restoration is to to restore bee communities as a whole in an agricultural setting, one must consider the possible consequences of external factors on bee populations such as pathogen and pesticide exposure alongside potential for geographic isolation

and long term genetic stability which could occur in fragmented landscapes. However, if restorations are planted with the goal of increasing crop yield, it may be best to target populations of a few key pollinator species that are known to perform well in agricultural settings.

Targeted habitat enhancement for the benefit of pollinators is a technique used to boost bee populations in agricultural systems (Marshall and Moonen 2002; Blaauw and Isaacs 2014; M'Gonigle et al. 2017). This is because food (nectar and pollen) and nesting (Kells and Goulson 2003) resources are relatively scarce for bees in agriculturally intensified landscapes. One of the goals of targeted habitat enhancement for bees in agricultural systems is to provide an area of land where floral resources can be available before and after crop bloom so the wild bee community can persist throughout the summer after crop bloom ends. The response of bees to habitat enhancement has been documented many times; however, these studies measure only changes in bee abundance (Potts et al. 2003; Blaauw and Isaacs 2014; Wood et al. 2015). In each of these cases, we do not know whether the increase in abundance is attributed to an increase in population density or a concentration effect from bees travelling to a highly rewarding resource patch from farther out in the surrounding landscape. My study attempted to determine which of the two scenarios is taking place by estimating the number of colonies of bumble bees (*B. impatiens*) foraging near wildflower plantings. I found that there are greater numbers of colonies found foraging at sites with habitat enhancement than sites without (Chapter 2); however, the increase in colony density is difficult to measure because the foraging range of *B. impatiens* is likely plastic depending on how colony foraging dynamics change in response to highly rewarding forage resources being present in the landscape. Currently, studies including my own use estimations of foraging range, but these are sometimes based on other bumble bee species,

which may or may not share similar foraging ranges to *B. impatiens.* Determining the foraging range of common bee species is an important next step towards learning how many bee colonies persist in the landscape, and how they respond to resource availability. Further, there is a growing need to determine how flexible bee foraging ranges are, in order to best inform targeted bee restoration projects for maximum effectiveness.

The next step in my research was to determine how habitat enhancement affects bumble bee foraging preference (Chapter 3) and consequently how pathogen prevalence in bumble bees also responded (Chapter 4). One of the main findings of these two studies was that flowers that were visited most often and were thus considered most attractive to bumble bees were more likely to screen positive for a pathogen. Previous research has established that bumble bees are able to spread pathogens from one bee to another through the shared use of flowers under laboratory conditions (Durrer and Schmid-Hempel 1994; Graystock et al. 2015). My research shows that the horizontal transmission of pathogens at flowers is likely a mechanism for pathogen transmission; however, there is a need for future research to determine how important this type of transmission is relative to transmission within a colony or vertical pathogen transmission from one generation to the next. Based on my findings, I would argue that pathogen transmission through the shared use of flowers plays a large role in the spread of pathogens to bees because sites that had more bumble bee-flower interactions (sites with restorations) also had a higher incidence of pathogen detection in bees and on flowers. Pathogens can be spread in different ways depending on their life history traits, so this may not be the case for all bee pathogens. Still, the mechanism by which pathogens are transferred at flowers, which can cause infection in bees, requires deeper investigation. Further, a enhanced understanding of how

pathogen dynamics in areas with restoration affects bees over longer time scales would be of great interest.

Although habitat enhancement in agricultural settings can promote bumble bee populations locally within the landscape, one potential negative aspect of habitat enhancement is that large clusters of floral resources can draw in bees and can act as a hub for horizontal pathogen transmission. Thus, the tradeoff between the increase in floral resources and the possibility for an increase in pathogen prevalence in bee populations must be considered when restoring habitat in agricultural settings. Although the boost in populations is inherently a benefit for bees, as seen in my Chapter 2, if that results in almost a doubling in pathogen prevalence, restorations may not be as effective as they were originally intended to be. With growing concern regarding the effects of multiple stressors acting synergistically and further harming bee populations, the increase in pathogen exposure at flowers provided in farms where bees may also be exposed to pesticides may be a tipping point for some species of bees that are unable to fight off infection.

This all leads back to the ultimate goal of targeted restoration. Bee populations can increase, but some species may be negatively affected by a potential increase in pathogen prevalence in a significant way. For the sake of restoring pollinator species richness, habitat enhancement in florally scarce landscapes may not be the best option due to the negative effects associated with pathogen exposure. However, some bee species such as *B. impatiens* do not seem to be as negatively affected by higher pathogen prevalence for two reasons: (1) *B. impatiens* population density tripled over a three year period in response to restoration and; (2) *B. impatiens* had significantly lower pathogen prevalence than most other bumble bee species that were also studied. Thus, if the goal of restoration is to increase the proportion of wild pollinator

species that contribute significantly to crop production, targeted restoration for certain bee species like *B. impatiens* is a viable option.

There are two main areas of study that warrant future investigation based on the research presented in this dissertation. The first pertains to how flower species composition and abundance of highly attractive flowers influence pathogen spread in bees. I found that flowers that were considered highly attractive to bees were also significantly more likely to screen positive for a pathogen. Thus, new research should focus on whether plant species composition can be manipulated to decrease pathogen exposure to bees. The second area of research concerns how pollinator populations respond to different sizes and shapes of targeted habitat enhancement, how that interacts with the surrounding landscape matrix and further, how pathogen transmission is affected. For example, bee populations may gain the same nutritional benefit from multiple smaller restorations scattered throughout the local landscape as they do when a single large restoration is present. However, pathogen transmission may also be lowered when there are multiple restorations that bees can visit because bees that are doorstep foragers will likely forage at the closest restoration to them, thus reducing the likelihood of pathogen transfer at each restoration.

During this research, I overcame several significant obstacles in the analytical aspects of my studies, which should be noted for future studies so these issues can be avoided. In order to perform microsatellite analysis to obtain colony abundance estimations, it is essential to be able to sequence amplified DNA from a PCR reaction. In my study, I had issues getting my PCR reactions to work due to a slight error in reagents that were being used. Instead of mixing dNTPs on my own and adding them to my master mix, I purchased dNTPs from the same company, but they were pre-mixed. During an elongated troubleshooting process, I found out that the pre-

mixed dNTPs were suspended in a salt solution that inhibited my subsequent PCR reactions, whereas the unmixed dNTPs were suspended in molecular grade water. This experience highlights the need to follow protocols exactly as they are provided (see Chapter 2 methods).

Additionally, as pathogens were being analyzed for Chapter 4, I encountered issues getting certain primers to amplify for the pathogen, *Nosema bombi*. Originally I used the primer pair BOMBICAR, which has been used in some studies; however, I learned that the extreme sensitivity of the primer pair could sometimes result in many false positives (ie.. all of my samples screened positive for *N. bombi* while using this primer pair). Working with a colleague, Dr. Tripodi, from the USDA-ARS lab in Utah, I modified my screening method to use a primer pair that reliably amplified *N. bombi* DNA. Thus, I would caution future studies to be wary of extremely sensitive primer pairs because under some conditions, they can lead to false positives.

My last major hurdle in Chapter 4 , and my most challenging hurdle by far, was to develop a method to extract and screen bumble bee pathogens off of flowers because these methods are not well developed in the literature. After many rounds of troubleshooting and conversations with Dr. Graystock, from Cornell University, I found that soaking flower heads in proteinase K and buffer ATL for 2 hours in a heat block at 56 °C was the best way to extract pathogen DNA without also contaminating the DNA with secondary compounds commonly found in plant tissue that can disrupt DNA amplification in the PCR step. We plan to publish our results in a separate paper this upcoming year.

Overall, the research contained in my dissertation provides a picture of the complexity underlying bumble bee restoration in agricultural systems. Bumble bees require floral resources to sustain their colonies; however, they are also exposed to pathogen infection, which can negatively affect their populations. Further research exploring the costs and benefits of habitat

enhancement for bees must be considered to realize the full potential of restoration practices to support pollinator populations in agricultural settings.

APPENDICES

APPENDIX A:

CHAPTER 2 SUPPLEMENTAL TABLES AND FIGURES

Table A1. Plant species composition for seed mix used in new habitat enhancements.

New habitat enhancement seed mix	
Common name	**Scientific name**
Flowers	
Yarrow	*Achillea millefolium*
Windflower	*Anemone canadensis*
False indigo	*Baptisia alba*
Sand coreopsis	*Coreopsis lanceolata*
Purple coneflower	*Echinacea purpuria*
Rattlesnake master	*Eryngium yuccifolium*
Grass-leaved goldenrod	*Euthamia graminifolia*
Bee balm	*Monarda fistulosa*
Horse mint	*Monarda punctata*
Evening primrose	*Oenothera biennis*
Foxglove beardtongue	*Penstemon digitalis*
Hairy beardtongue	*Penstemon hirsutus*
Mountain mint	*Pycnanthemum virginianum*
Gray headed coneflower	*Ratibida pinnata*
Black-eyed Susan	*Rudbeckia hirta*
Figwort	*Scrophularia marilandica*
Gray goldenrod	*Solidago nemoralis*
Stiff goldenrod	*Solidago rigida*
Showy goldenrod	*Solidago speciosa*
Smooth aster	*Symphyotrichum laevis*
New England aster	*Symphyotrichum novae-angliae*
Arrow-leaved aster	*Symphyotrichum urophyllum*
Ohio spiderwort	*Tadescantia oheinsis*
Hoary vervain	*Verbena stricta*
Golden alexanders	*Zizia aurea*

Table A2. Plant species composition for seed mix used in mature habitat enhancements.

Mature habitat enhancement seed mix	
Common name	**Scientific name**
Flowers	
Golden Alexanders	*Zizia aurea*
Foxglove beard-tongue	*Penstemon digitalis*
Sand coreopsis	*Coreopsis lanceolata*
Black-eyed Susan	*Rudbeckia hirta*
Swamp milkweed	*Asclepias incarnata*
Butterfly milkweed	*Asclepias tuberosa*
Bee balm	*Monarda fistulosa*
Joe-pye weed	*Eupatorium maculatum*
Boneset	*Eupatorium perfoliatum*
Blue lobelia	*Lobelia siphilitica*
Yellow coneflower	*Ratibida pinnata*
Cup plant	*Silphium perfoliatum*
Stiff goldenrod	*Solidago rigida*
New England aster	*Symphyotrichum novae-angliae*
Smooth aster	*Symphyotrichum laevis*
Grasses	
Canada wild-rye	*Elymus canadensis*
Indiangrass	*Sorghastrum nutans*
Big bluestem	*Andropogon gerardii*

Table A3. Number of samples and allele counts at each locus, site, and year.

			Number of alleles detected at each locus										
Year	**Site**	**# Samples**	**BL15**	**B124**	**Btern01**	**BT28**	**BTMS 0062**	**BT10**	**BL11**	**BT30**	**B96**	**BTMS 0081**	**Total Alleles**
2014	BJO	12	8	12	6	1	15	13	9	4	6	3	**77**
	BSA	12	7	10	5	2	17	10	10	4	7	3	**75**
	FPP	13	9	11	10	1	18	13	12	3	10	2	**89**
	FSL	36	10	18	11	2	30	19	16	3	11	4	**124**
	GAL	106	15	21	14	4	38	23	17	7	17	4	**160**
	GET	13	8	9	5	1	16	13	6	4	8	2	**72**
	HBT	4	4	6	5	1	8	8	6	4	6	3	**51**
	HDK	23	7	12	8	2	23	16	12	3	10	2	**95**
	HSAF	12	7	12	9	1	15	15	12	4	6	2	**83**
	LCC	10	6	11	7	1	17	11	9	3	7	2	**74**
	LPL	44	10	17	12	3	29	16	15	4	12	3	**121**
	REE	21	7	14	8	2	23	18	13	2	8	2	**97**
	ROOD	5	6	6	5	3	8	6	5	2	5	2	**48**
	UED	118	16	27	17	3	34	22	16	8	16	4	**163**
2015	BJO	67	11	20	11	1	32	19	14	5	12	4	**129**
	BSA	52	11	17	11	3	30	19	16	6	14	3	**130**
	FPP	69	11	18	13	1	27	19	15	5	12	4	**125**
	FSL	79	10	24	17	4	32	20	16	7	14	3	**147**
	GAL	44	16	19	14	3	27	19	17	8	14	4	**141**
	GET	106	12	22	18	4	38	23	19	5	17	4	**162**
	HBT	4	4	5	3	1	5	5	6	2	3	2	**36**
	HDK	90	12	24	13	3	36	23	17	6	14	5	**153**
	HSAF	16	8	13	8	1	18	13	10	3	7	2	**83**
	LCC	82	13	22	11	2	36	20	15	8	15	3	**145**
	LPL	82	16	23	16	3	39	24	16	6	14	5	**162**
	REE	18	7	13	6	1	21	16	12	2	10	2	**90**
	ROOD	132	18	28	21	6	38	24	21	7	15	6	**184**
	UED	137	13	24	13	2	38	21	18	8	14	4	**155**
2016	BJO	82	13	20	14	3	34	21	16	6	15	3	**145**
	BSA	1	2	2	2	1	2	1	2	1	1	1	**15**
	FPP	33	12	13	11	1	28	20	14	3	12	3	**117**
	FSL	82	14	21	12	3	31	21	16	4	13	4	**139**
	GAL	73	13	23	10	1	34	20	13	5	12	3	**134**

Table A3 (cont'd)

GET	114	10	21	13	4	35	24	17	6	17	7	**154**
HBT	2	3	2	3	1	2	3	2	2	1	1	**20**
HDK	51	11	17	12	3	33	20	15	5	12	4	**132**
HSAF	4	6	6	5	2	7	7	7	3	6	3	**52**
LCC	43	10	17	12	1	32	17	14	6	15	3	**127**
LPL	112	16	25	16	3	41	21	17	5	16	7	**167**
REE	28	8	13	9	2	24	16	14	5	9	2	**102**
ROOD	20	9	10	10	1	22	14	11	5	10	4	**96**
UED	141	15	23	17	5	38	21	18	8	18	7	**170**
Average	**54.2**	**10.1**	**16.0**	**10.5**	**2.2**	**25.5**	**16.5**	**13.0**	**4.7**	**11.0**	**3.4**	**112.9**

Table A4. Output from one-way ANOVA to test whether the proportion of semi natural habitat at different radii from the site of collection influenced the number of *Bombus impatiens* colonies found.

Radius	Year	ANOVA output
300 m	2014	p = 0.619
	2015	p = 0.279
	2016	p = 0.640
500 m	2014	p = 0.768
	2015	p = 0.762
	2016	p = 0.851
1000 m	2014	p = 0.891
	2015	p = 0.504
	2016	p = 0.708
1500 m	2014	p = 0.857
	2015	p = 0.237
	2016	p = 0.638

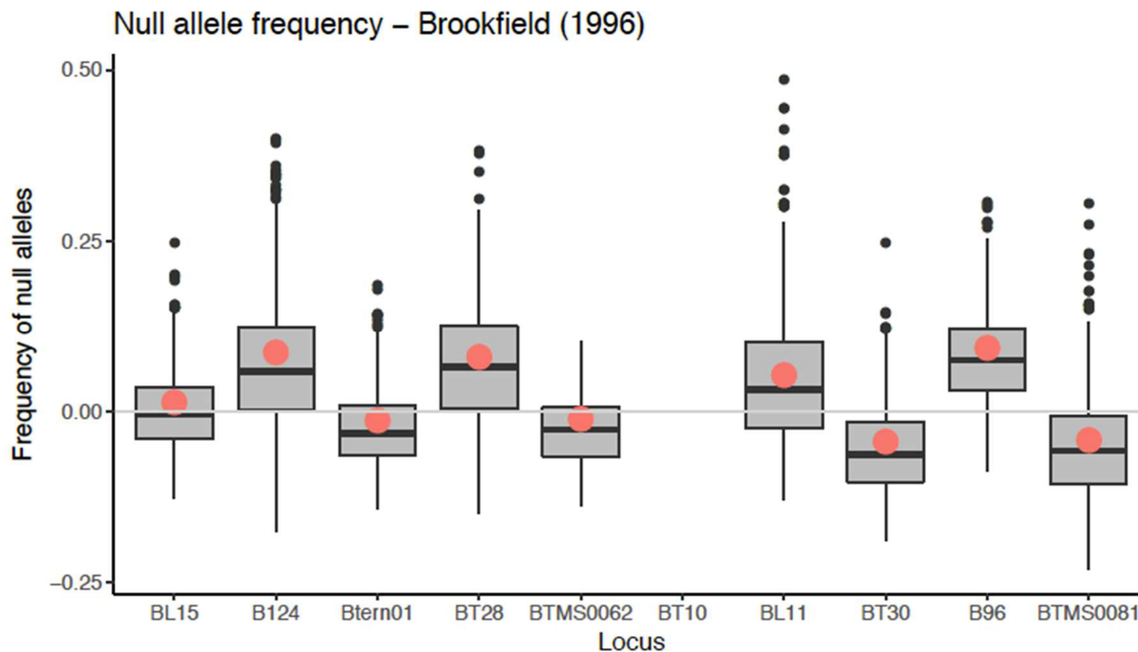

Figure A1. Presence of null alleles at site BJO in 2014. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

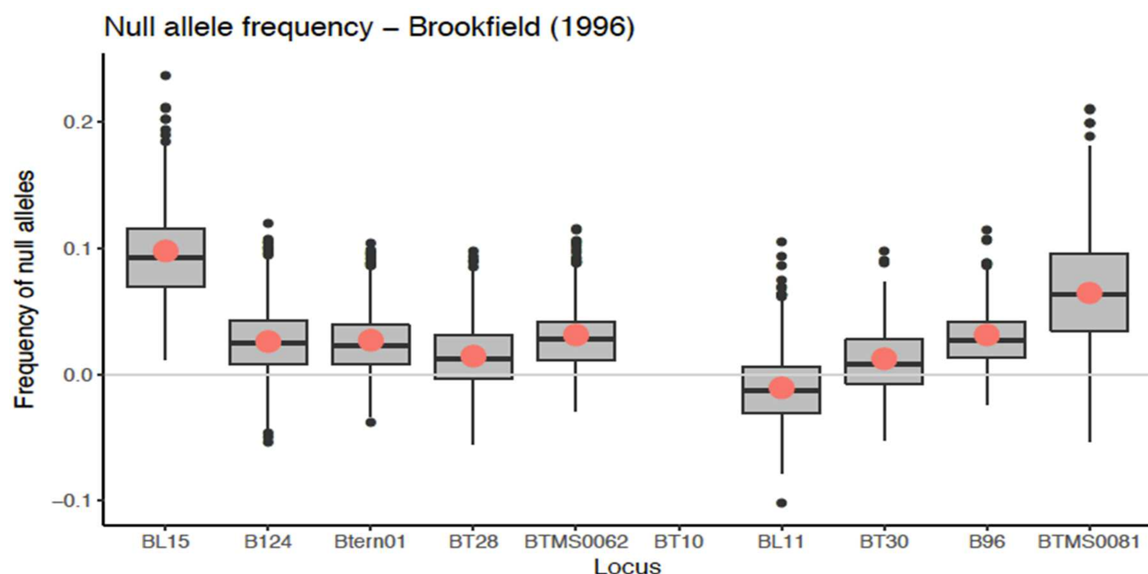

Figure A2. Presence of null alleles at site BJO in 2015. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

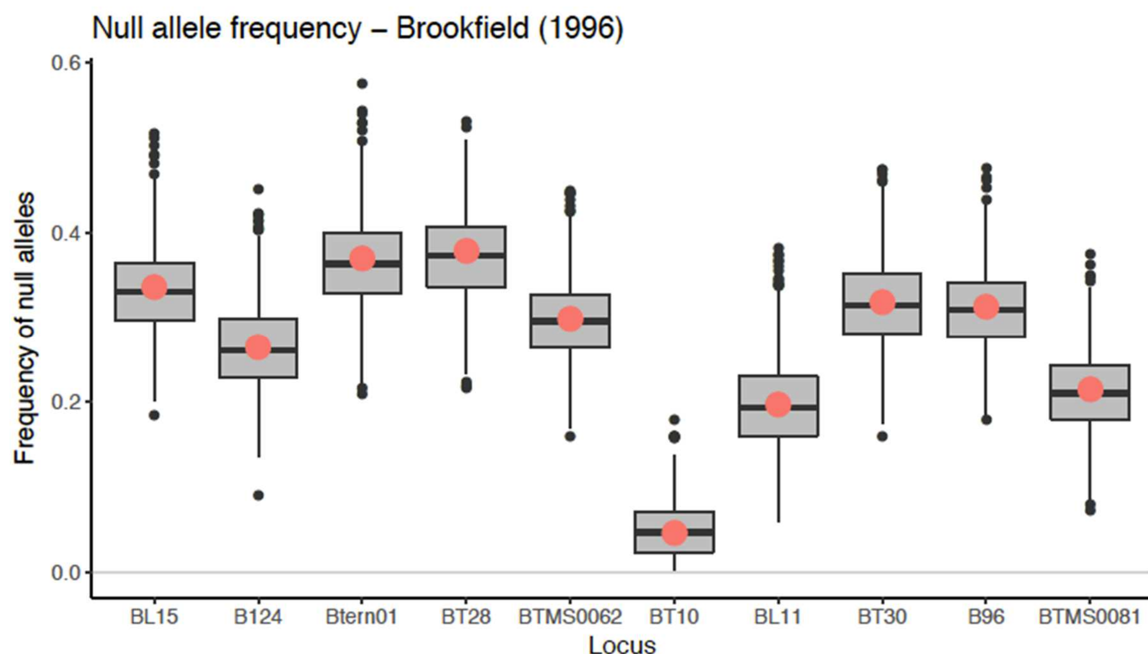

Figure A3. Presence of null alleles at site BJO in 2016. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

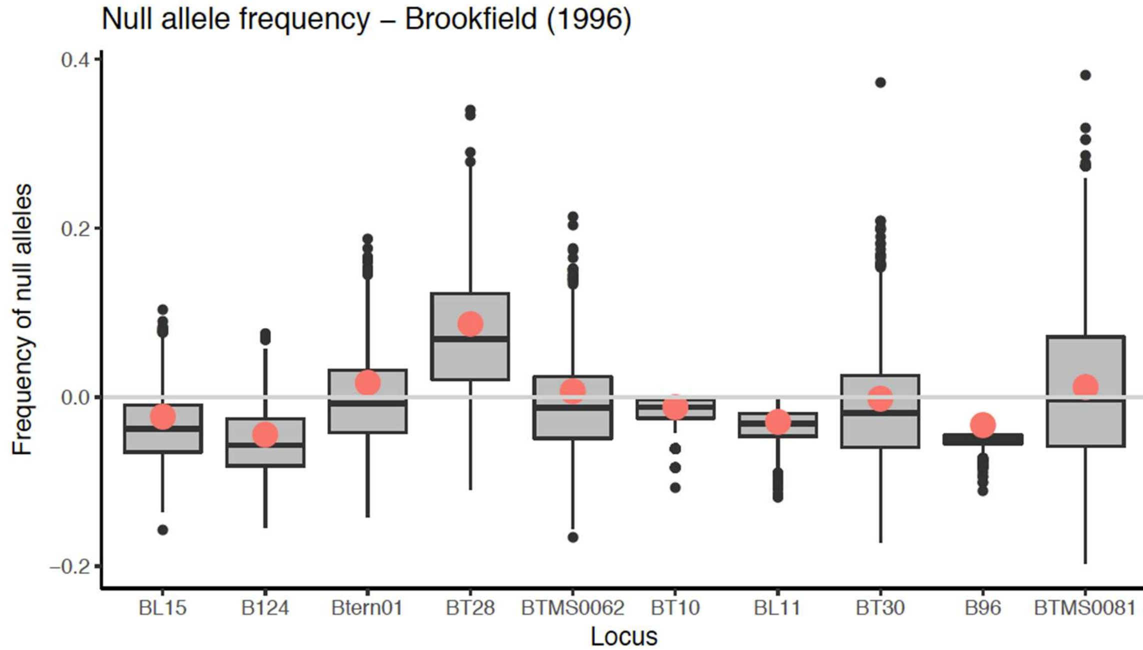

Figure A4. Presence of null alleles at site BSA in 2014. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

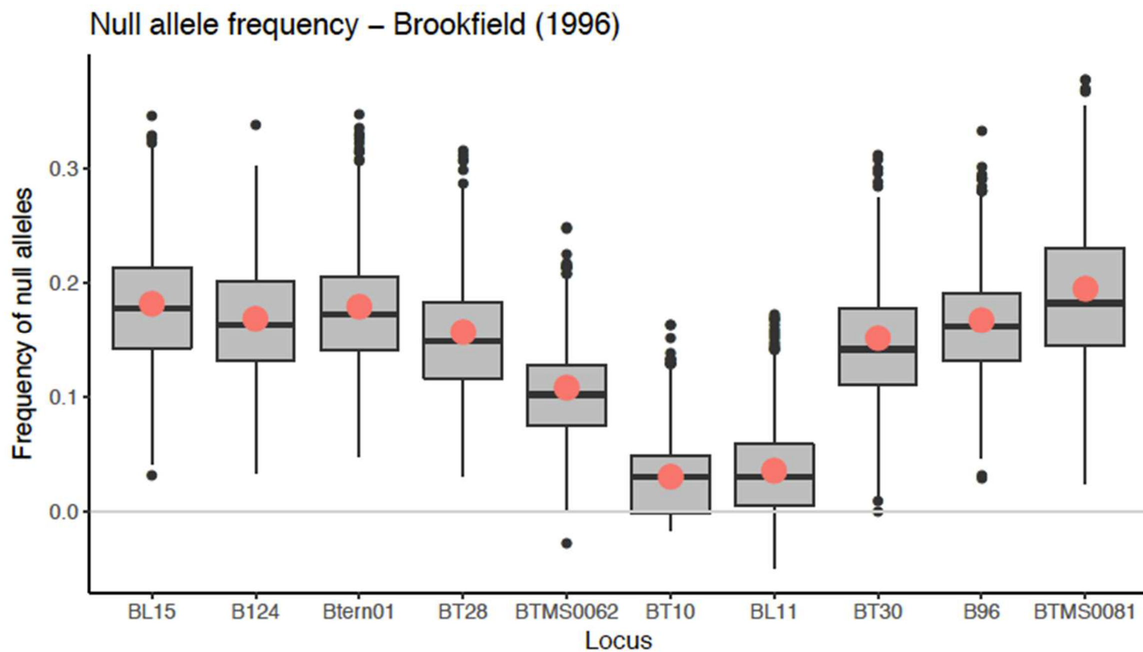

Figure A5. Presence of null alleles at site BSA in 2015. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

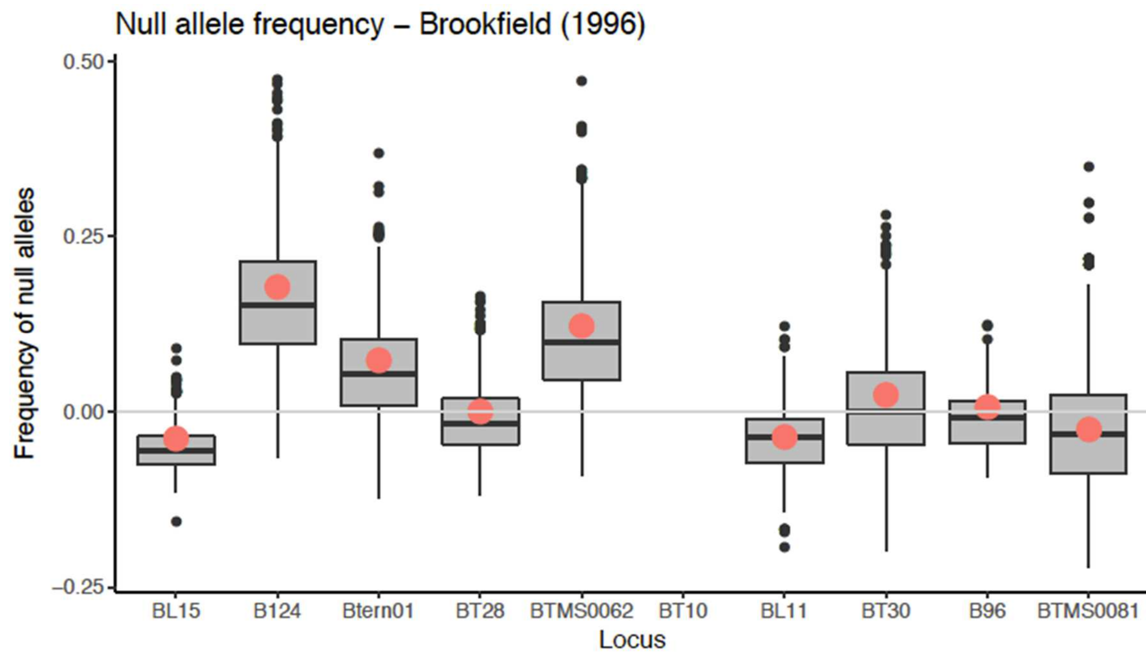

Figure A6. Presence of null alleles at site FPP in 2014. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

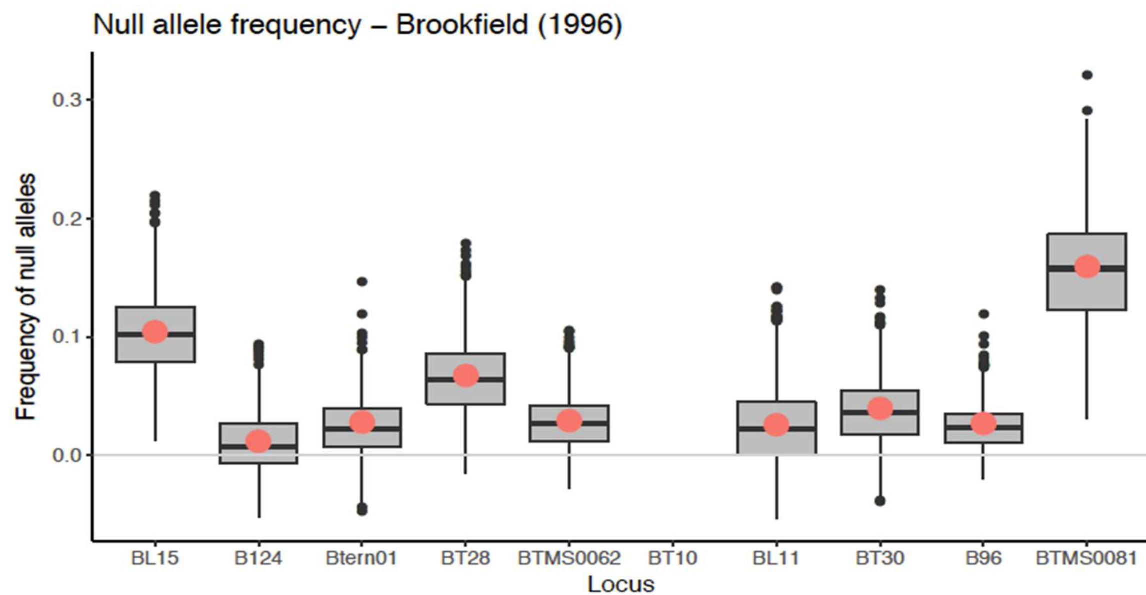

Figure A7. Presence of null alleles at site FPP in 2015. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

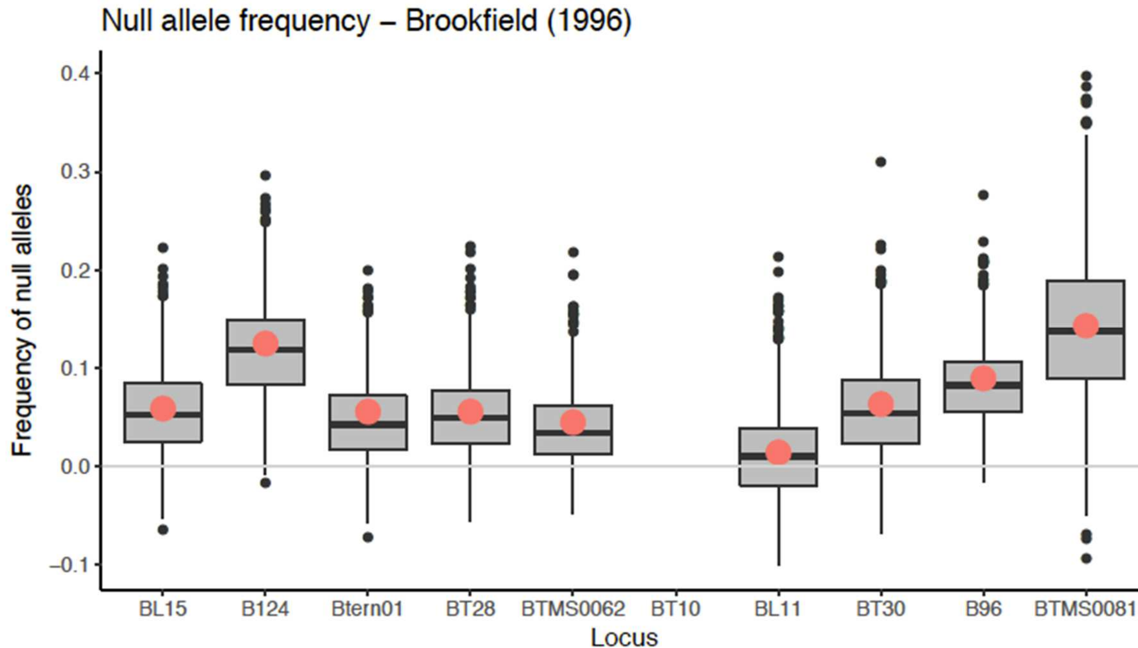

Figure A8. Presence of null alleles at site FPP in 2016. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

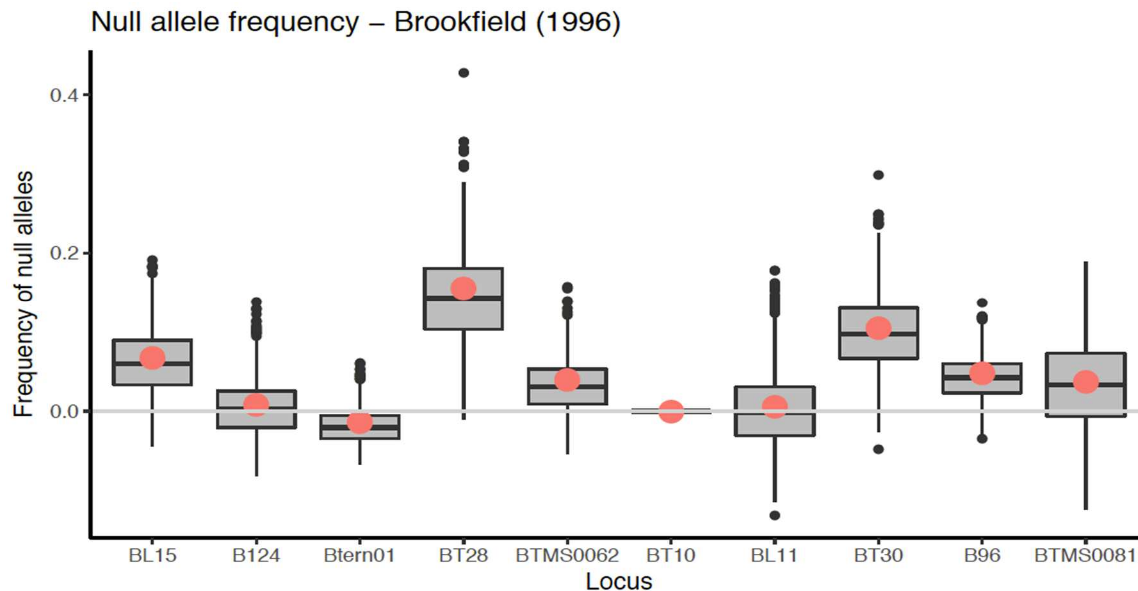

Figure A9. Presence of null alleles at site FSL in 2014. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

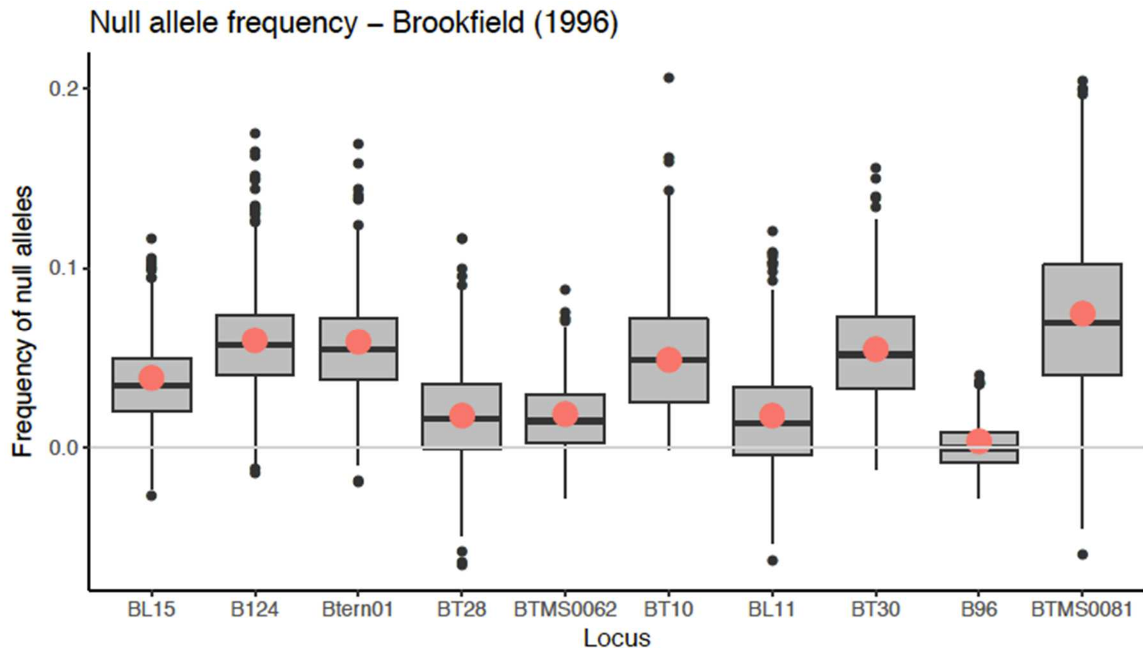

Figure A10. Presence of null alleles at site FSL in 2015. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

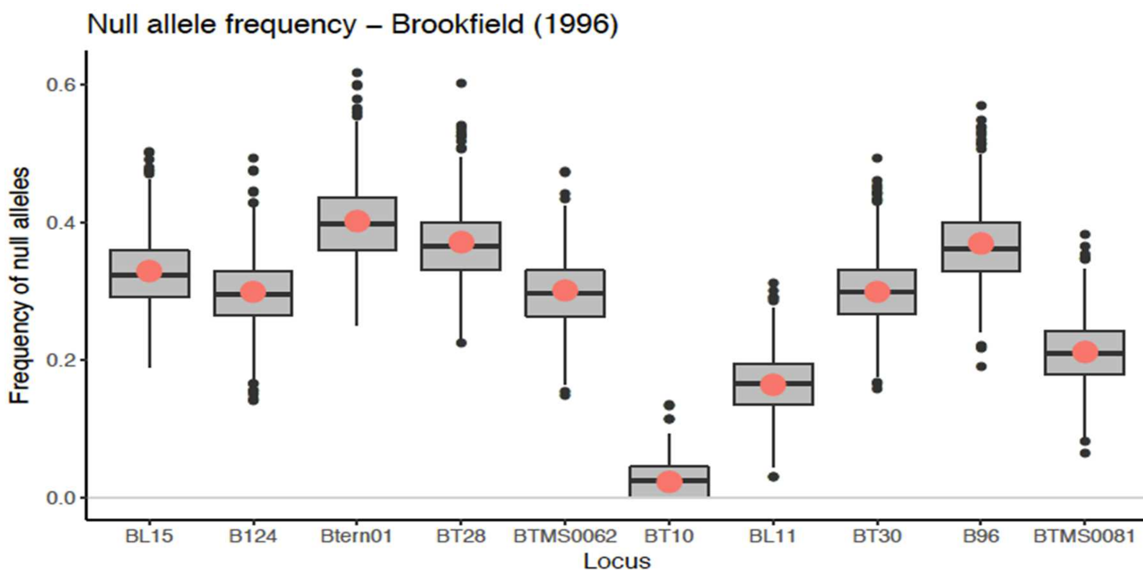

Figure A11. Presence of null alleles at site FSL in 2016. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

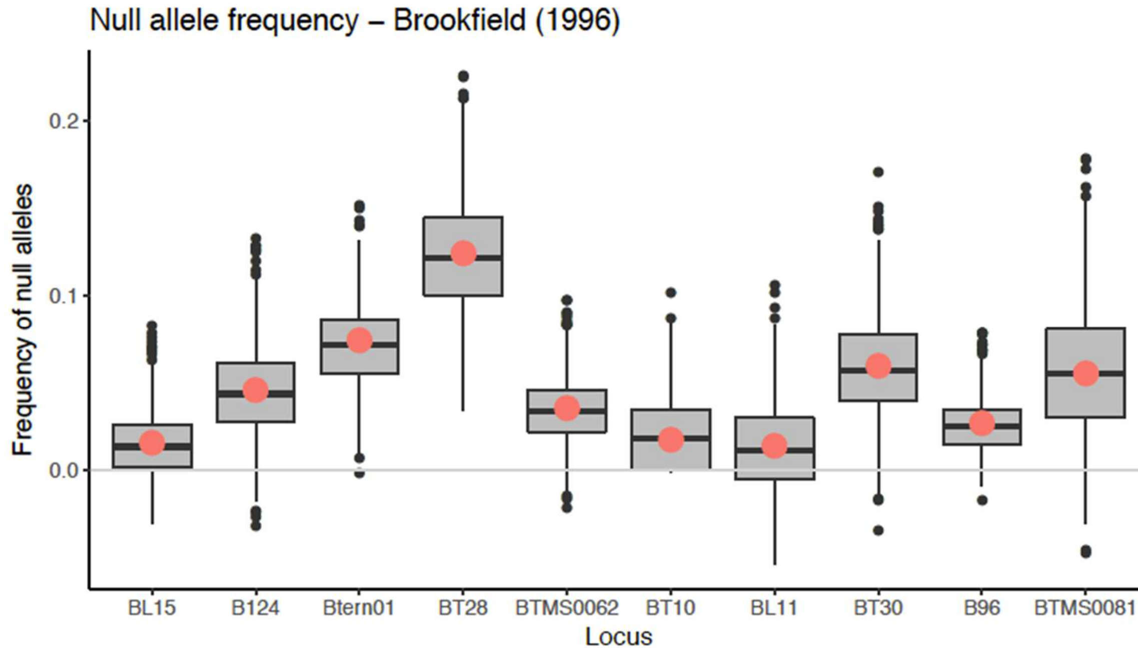

Figure A12. Presence of null alleles at site GAL in 2014. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

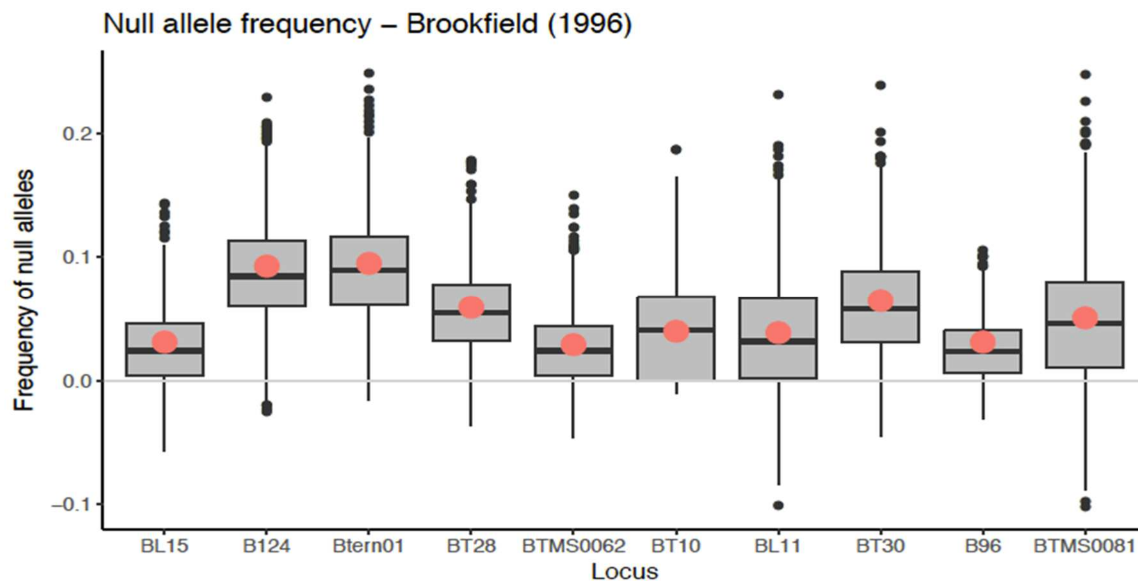

Figure A13. Presence of null alleles at site GAL in 2015. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

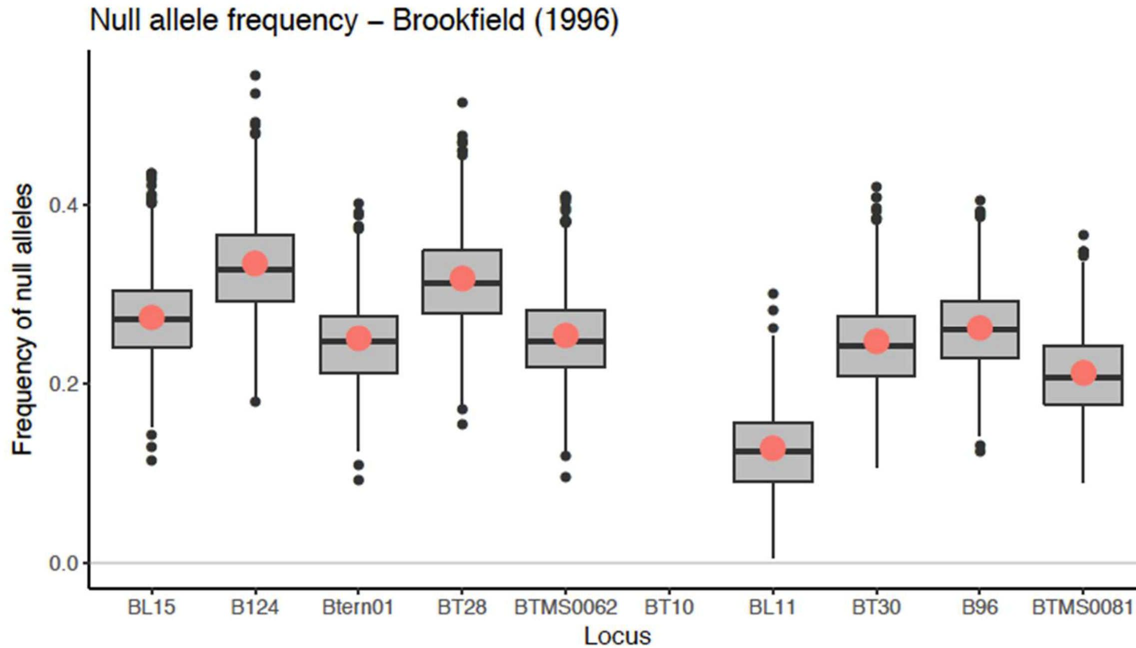

Figure A14. Presence of null alleles at site GAL in 2016. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

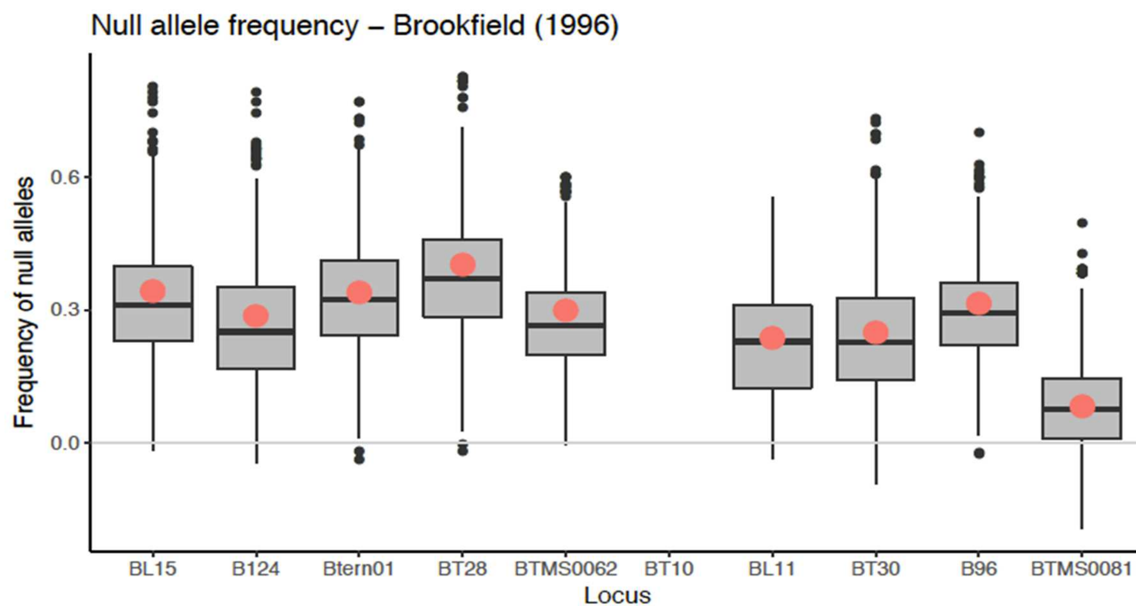

Figure A15. Presence of null alleles at site GET in 2014. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

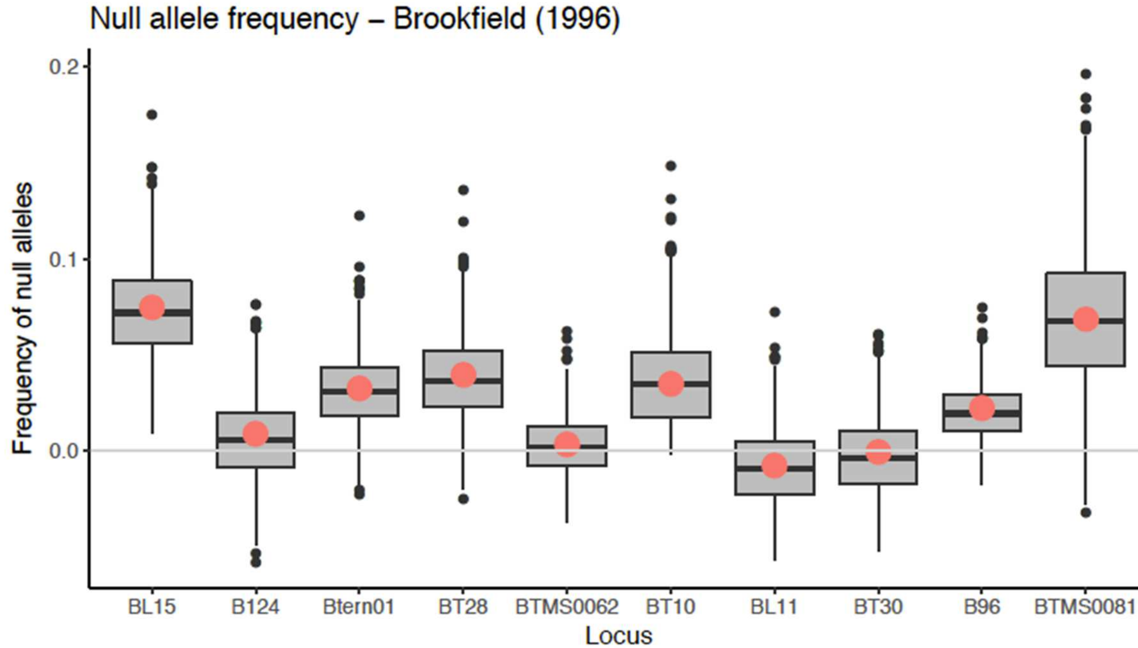

Figure A16. Presence of null alleles at site GET in 2015. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

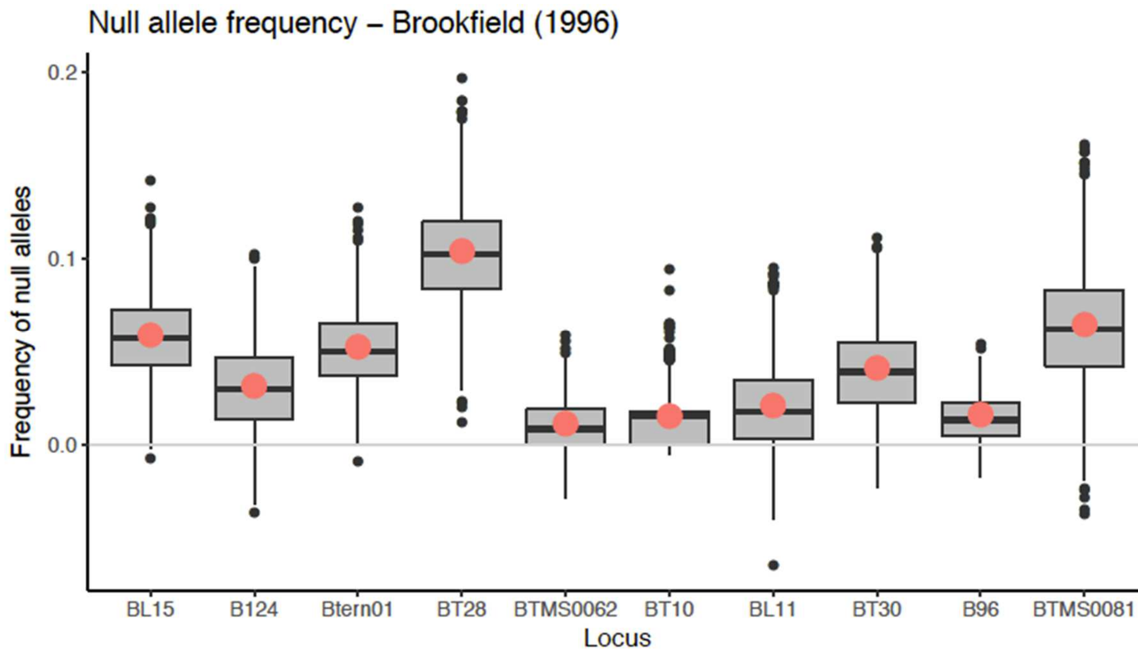

Figure A17. Presence of null alleles at site GET in 2016. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

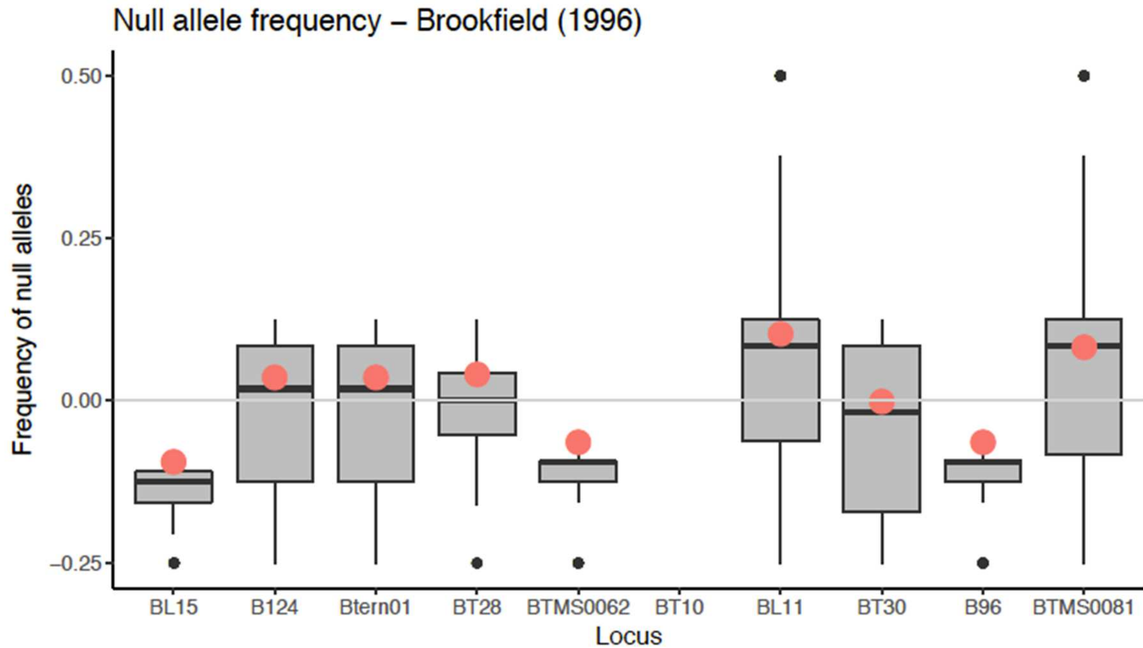

Figure A18. Presence of null alleles at site HBT in 2014. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

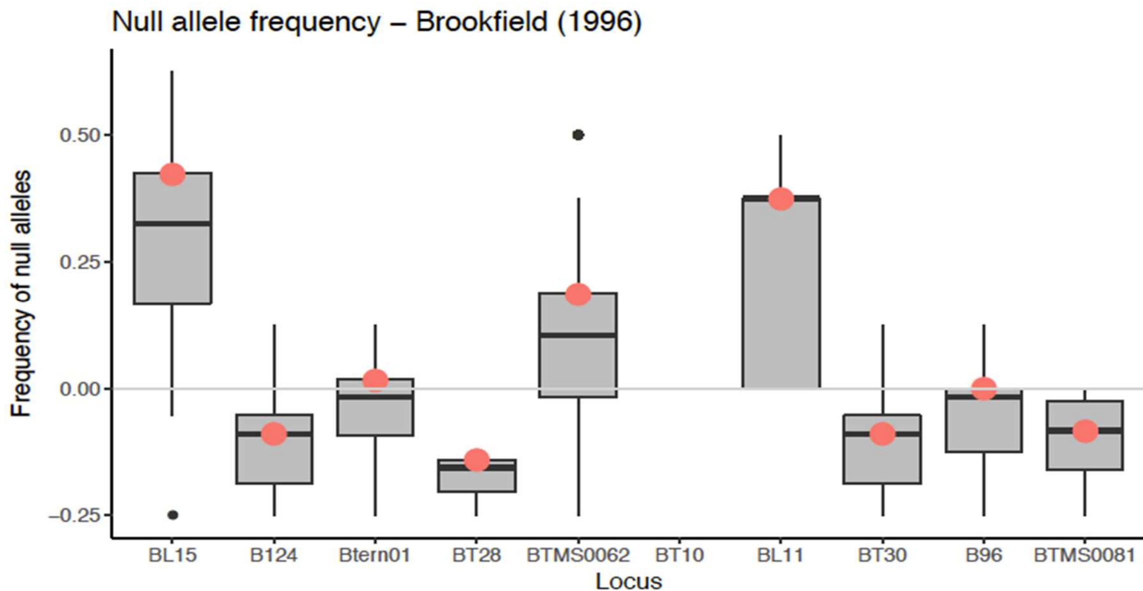

Figure A19. Presence of null alleles at site HBT in 2015. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

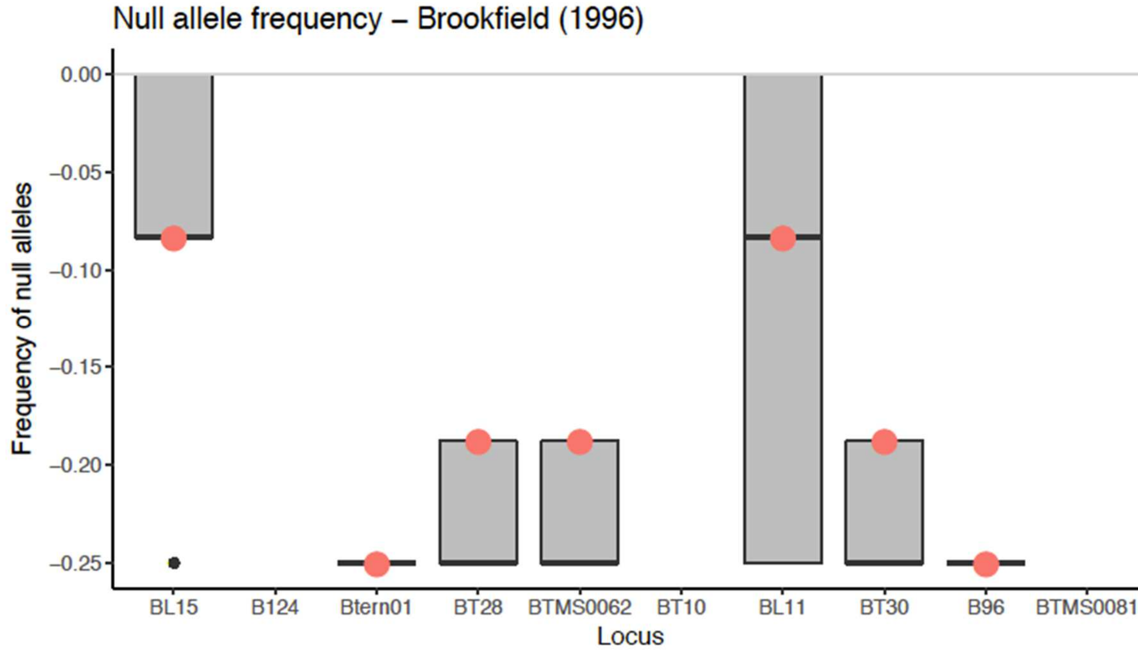

Figure A20. Presence of null alleles at site HBT in 2016. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

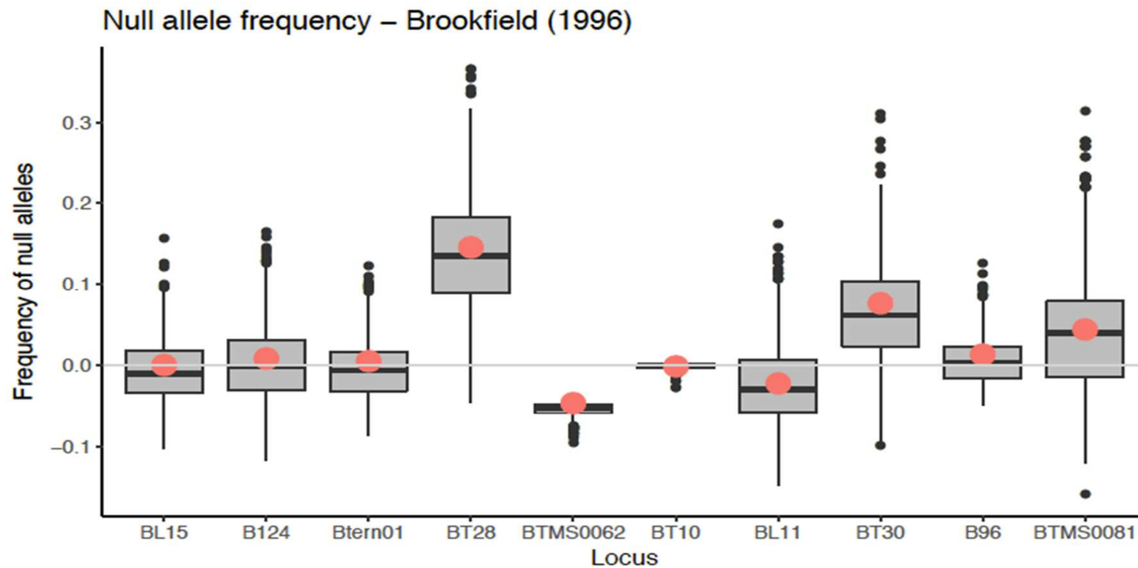

Figure A21. Presence of null alleles at site HDK in 2014. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

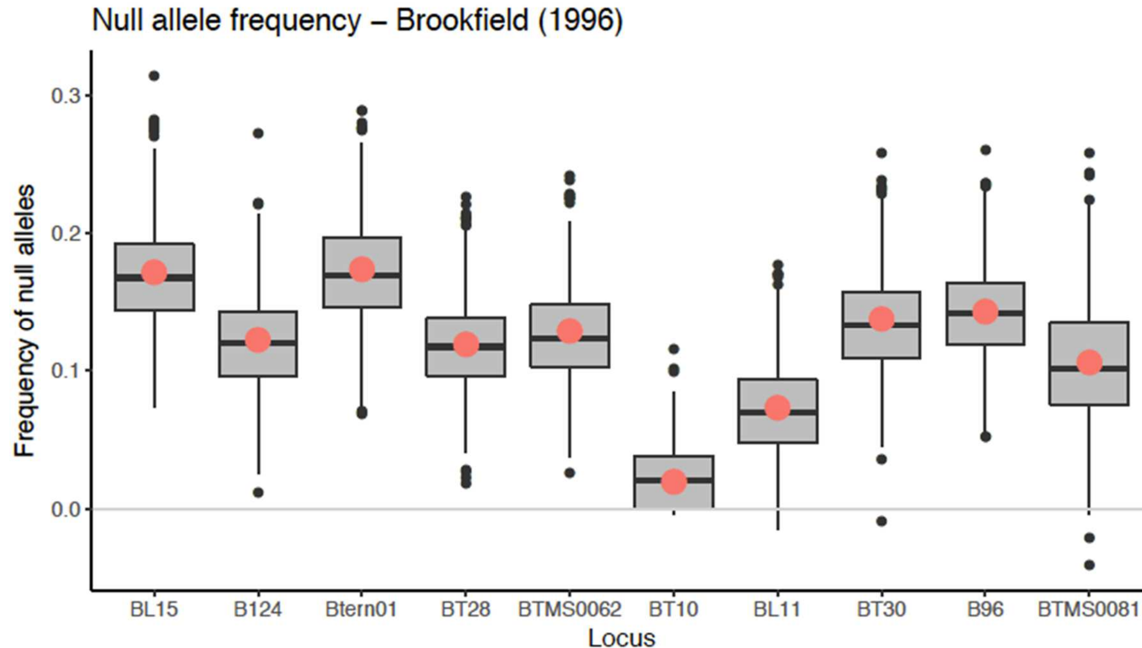

Figure A22. Presence of null alleles at site HDK in 2015. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

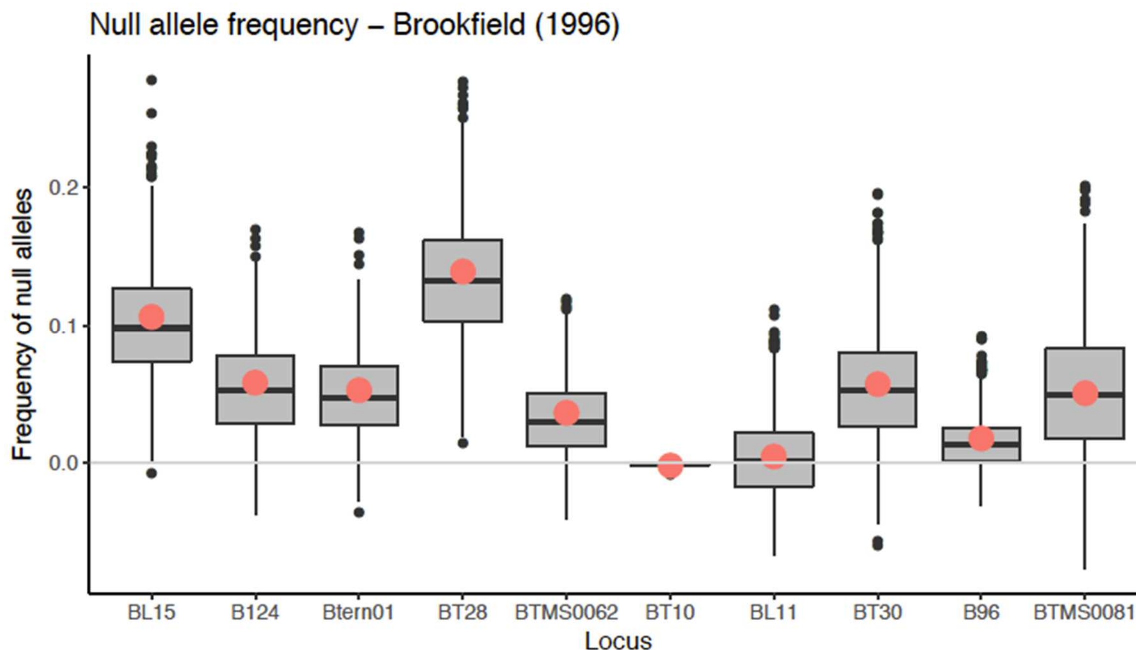

Figure A23. Presence of null alleles at site HDK in 2016. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

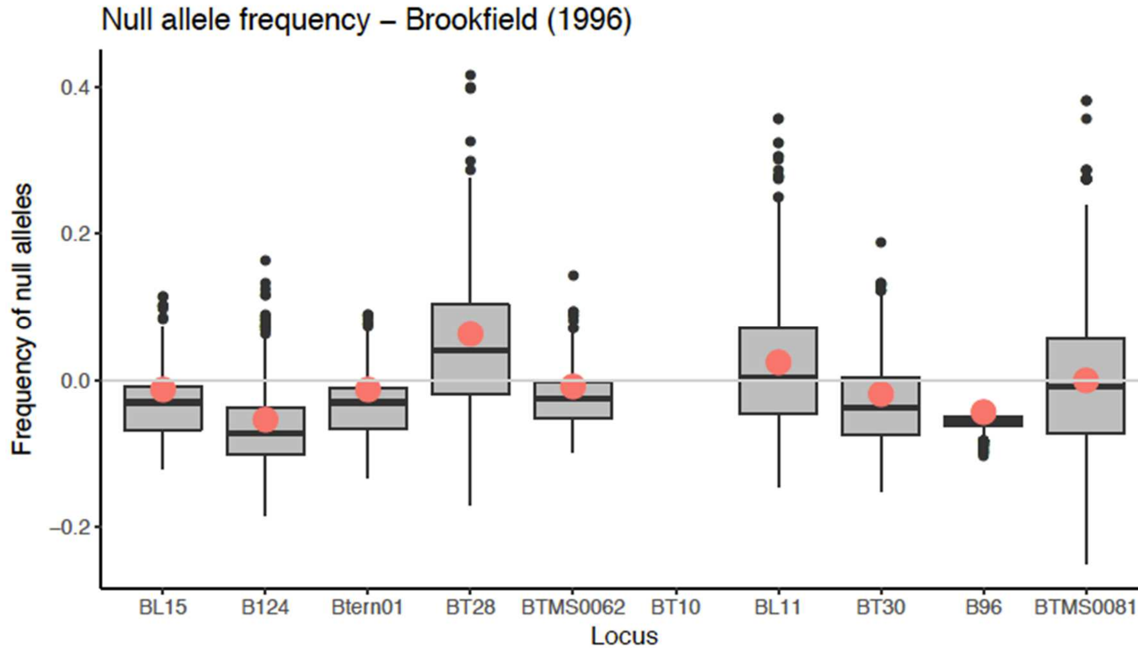

Figure A24. Presence of null alleles at site HSAF in 2014. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

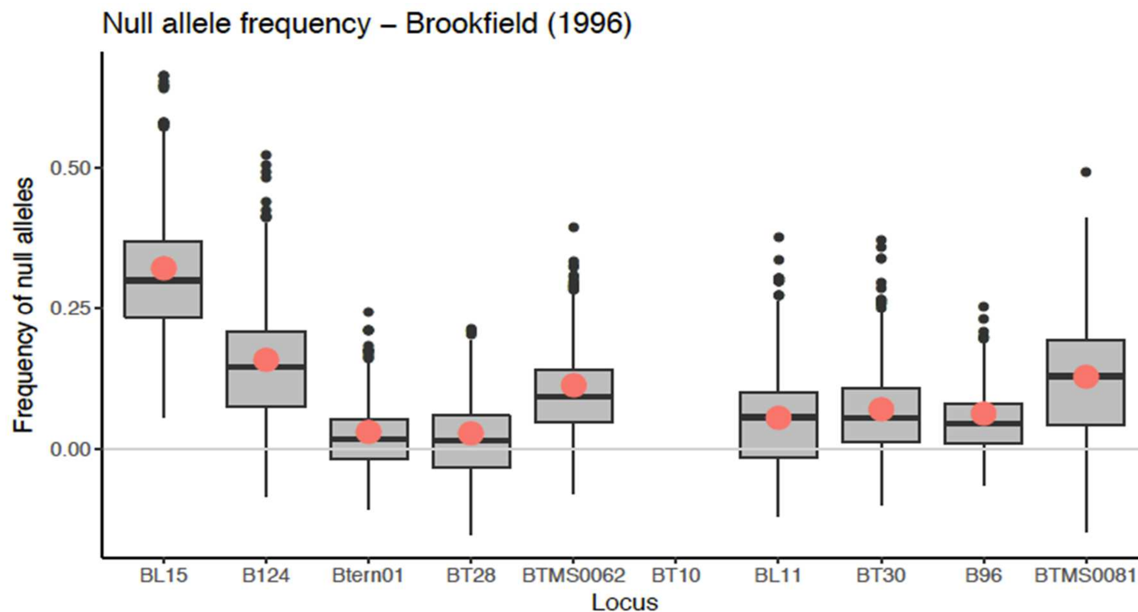

Figure A25. Presence of null alleles at site HSAF in 2015. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

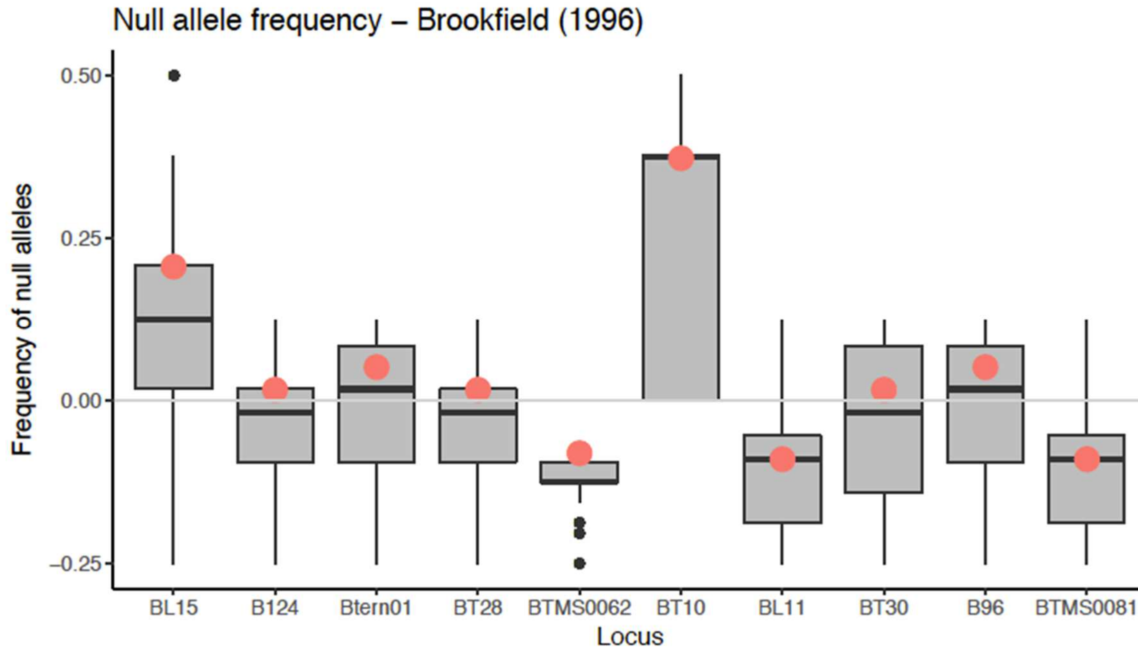

Figure A26. Presence of null alleles at site HSAF in 2016. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

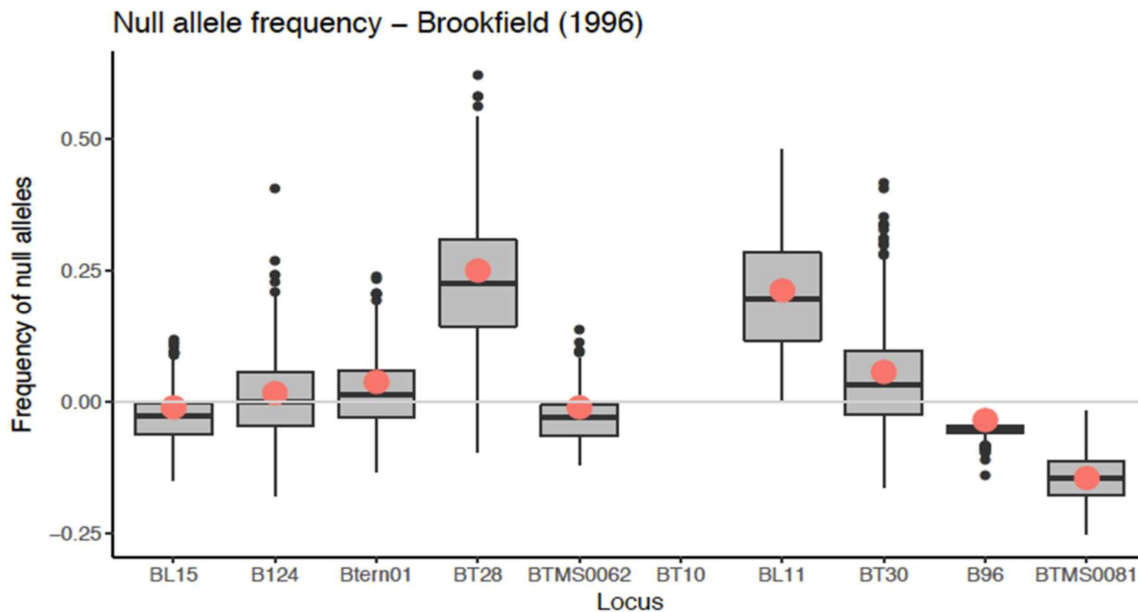

Figure A27. Presence of null alleles at site LCC in 2014. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

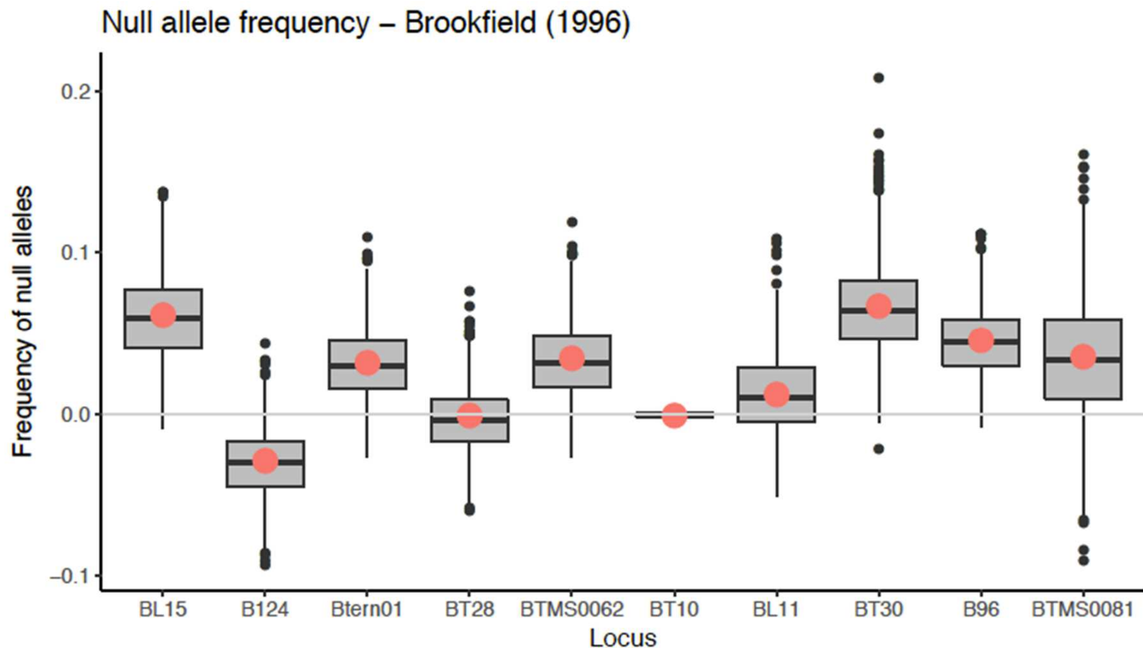

Figure A28. Presence of null alleles at site LCC in 2015. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

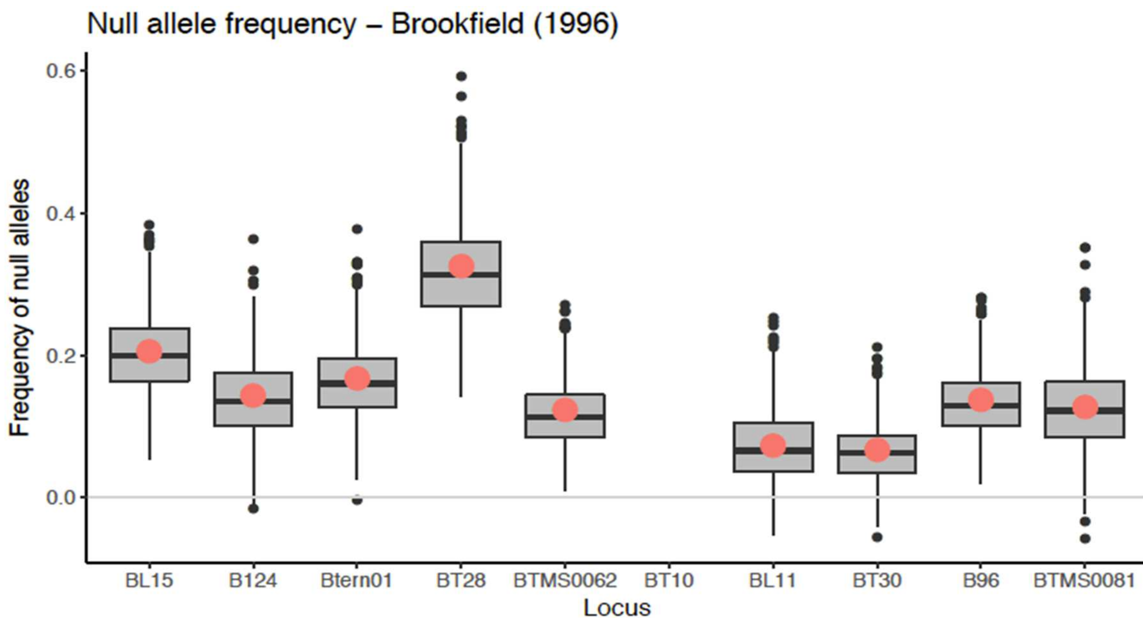

Figure A29. Presence of null alleles at site LCC in 2016. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

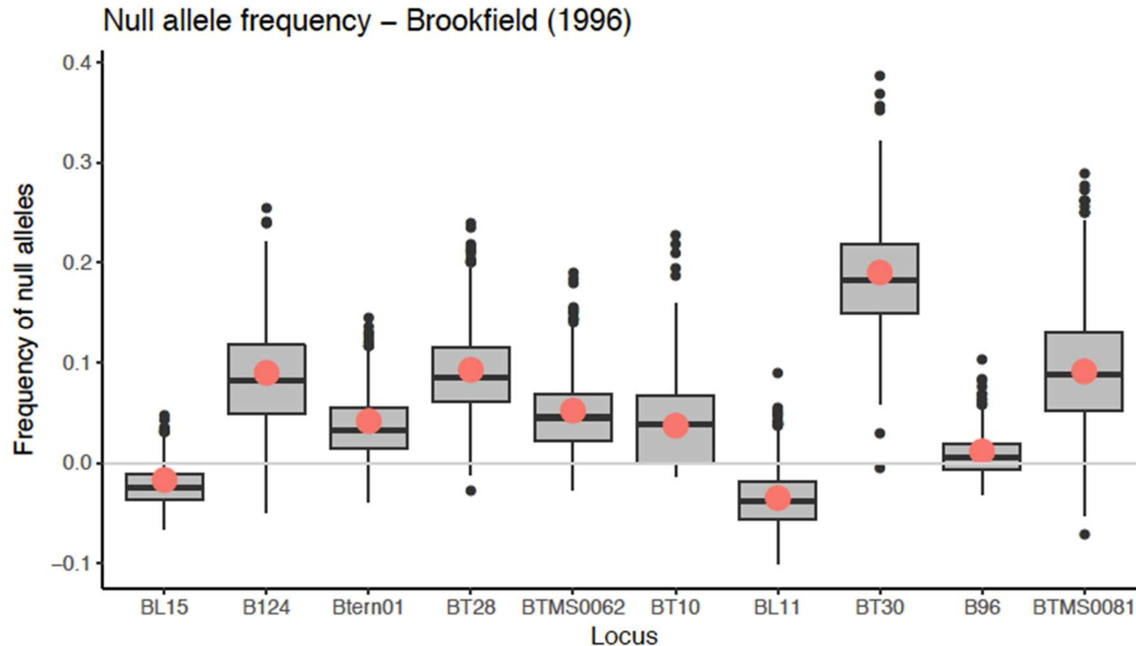

Figure A30. Presence of null alleles at site LPL in 2014. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

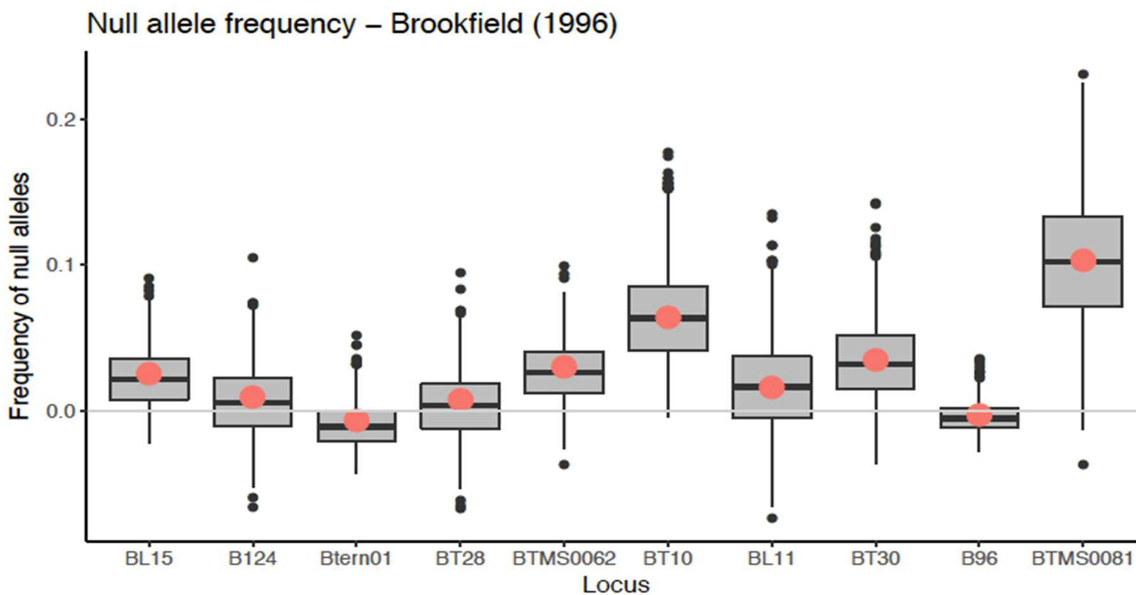

Figure A31. Presence of null alleles at site LPL in 2015. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

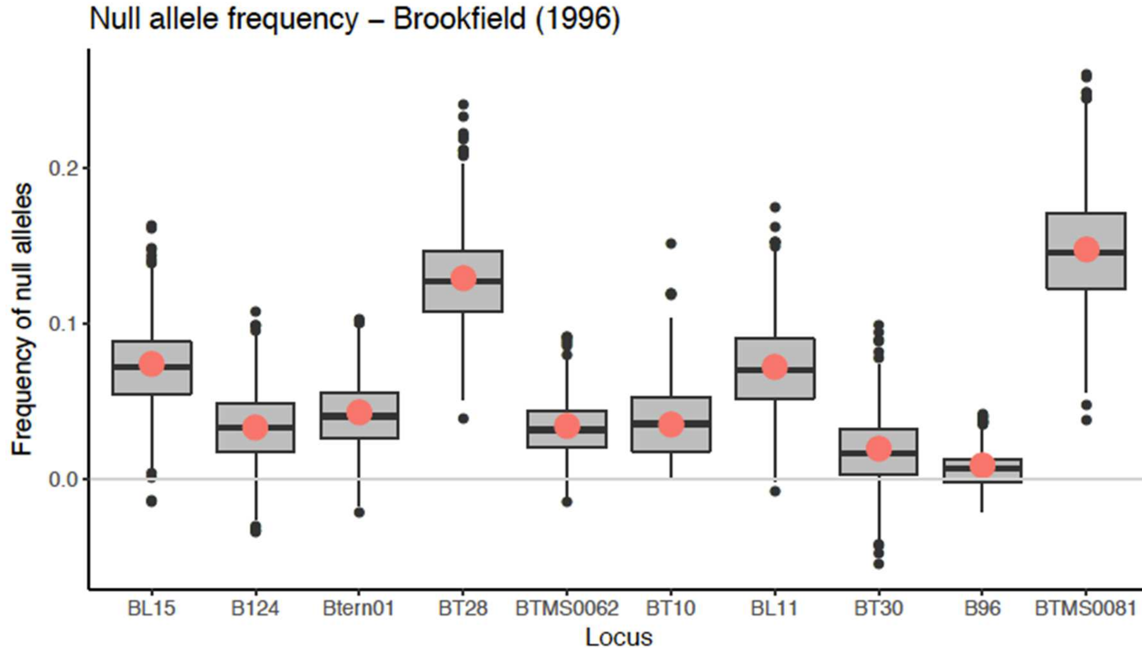

Figure A32. Presence of null alleles at site LPL in 2016. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

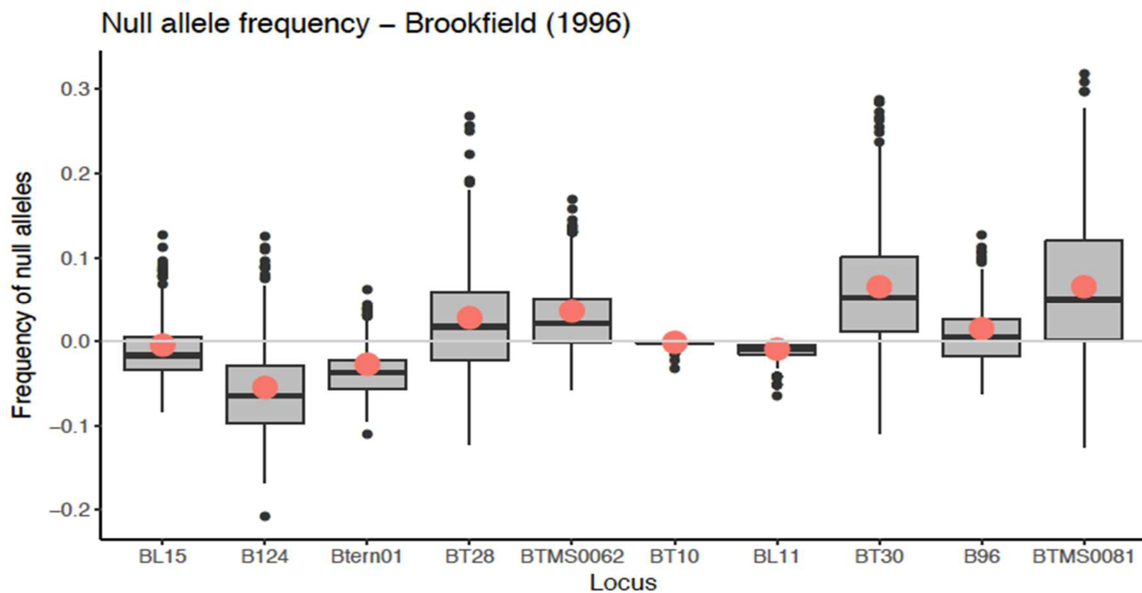

Figure A33. Presence of null alleles at site REE in 2014. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

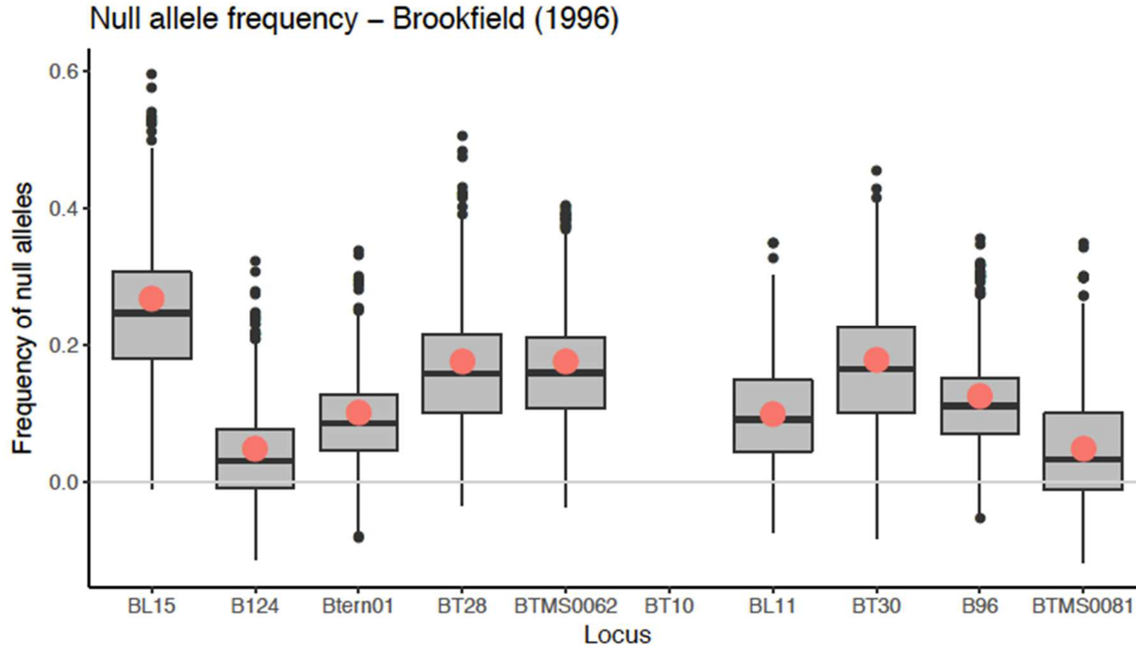

Figure A34. Presence of null alleles at site REE in 2015. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

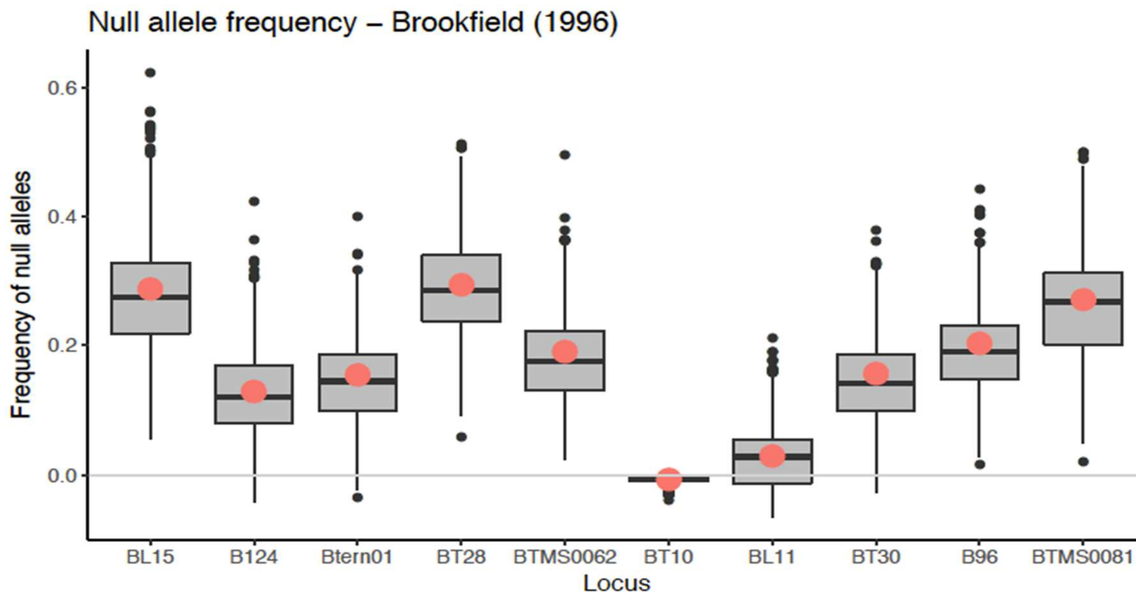

Figure A35. Presence of null alleles at site REE in 2016. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

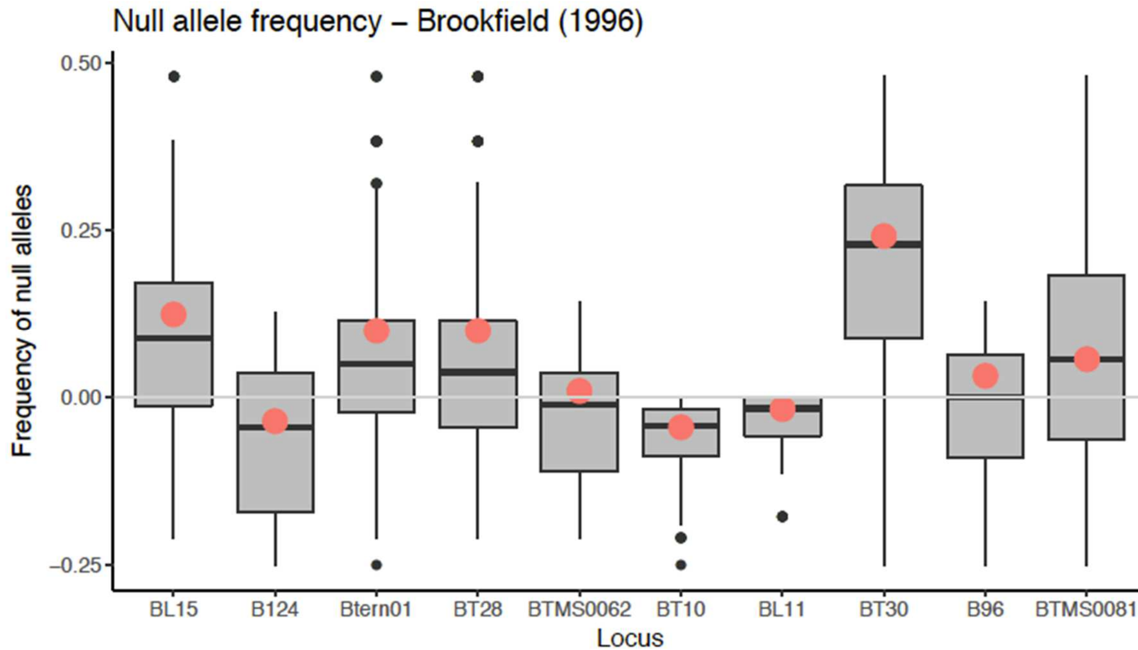

Figure A36. Presence of null alleles at site ROOD in 2014. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

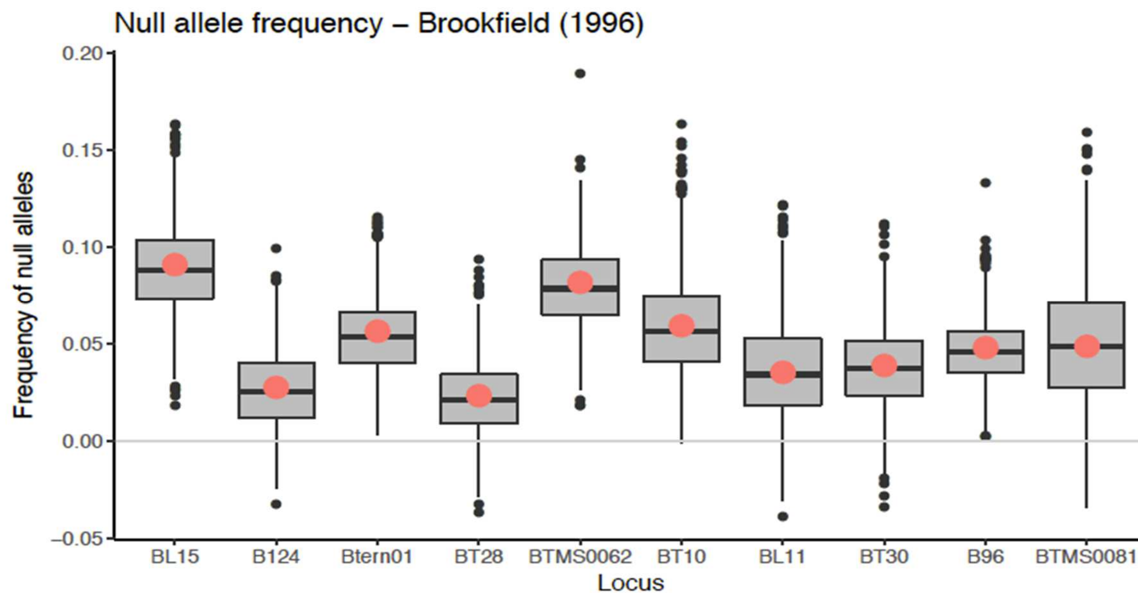

Figure A37. Presence of null alleles at site ROOD in 2015. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

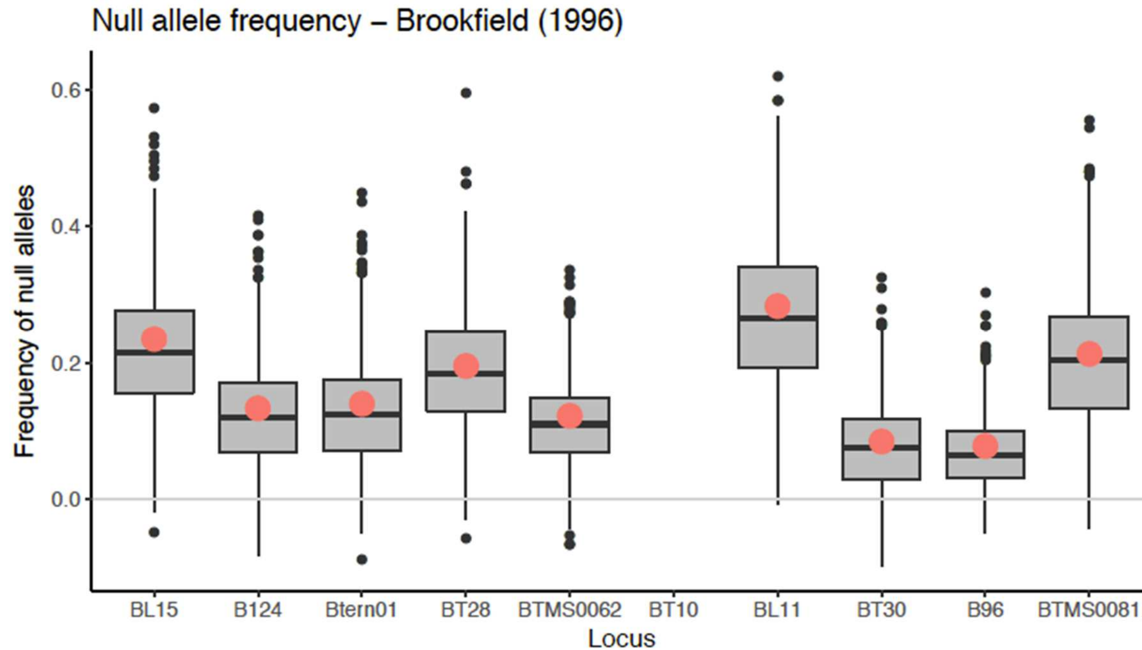

Figure A38. Presence of null alleles at site ROOD in 2016. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

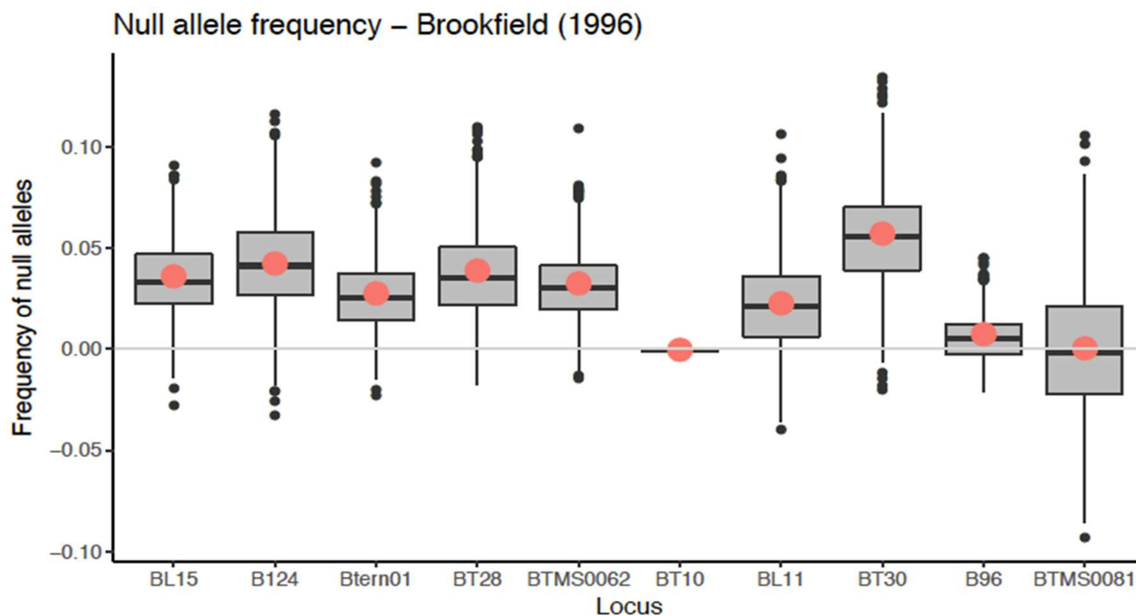

Figure A39. Presence of null alleles at site UED in 2014. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

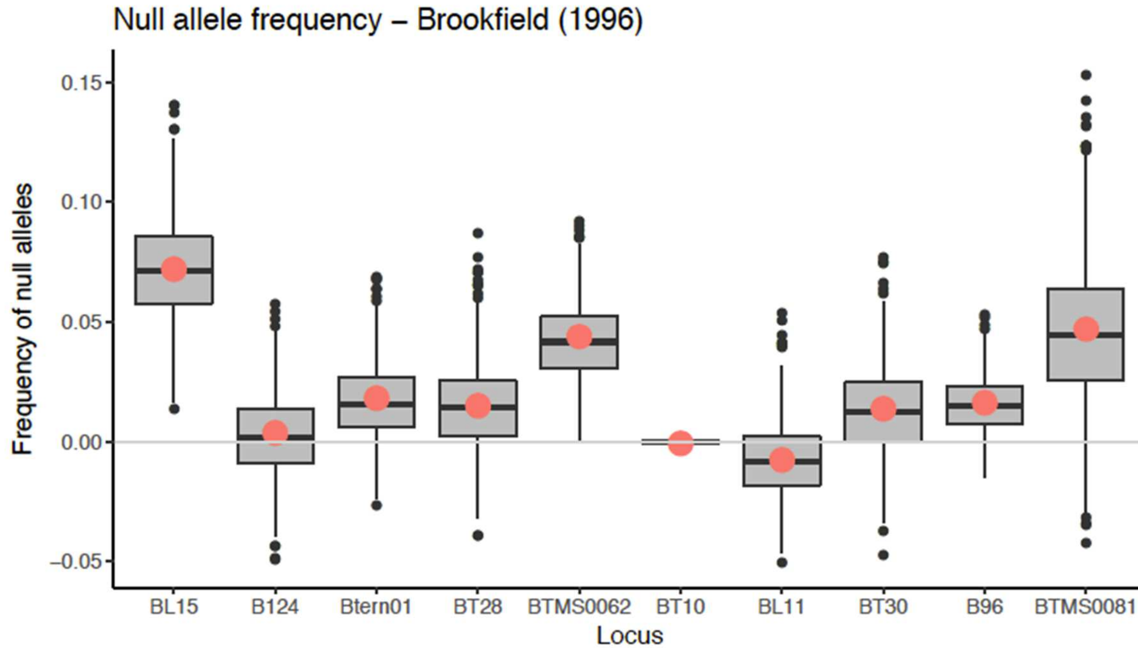

Figure A40. Presence of null alleles at site UED in 2015. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

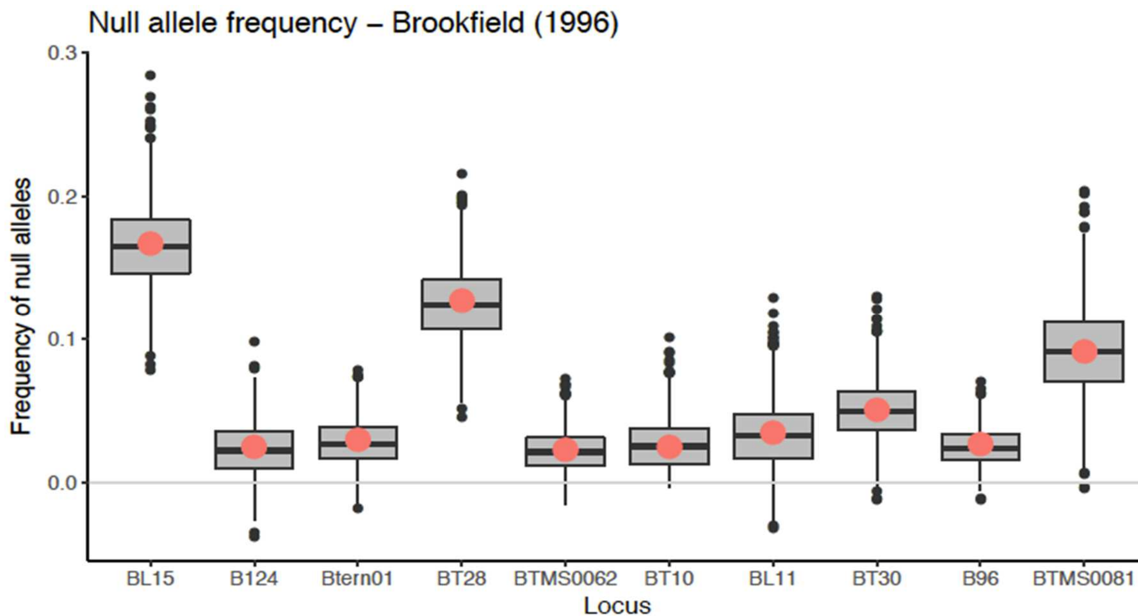

Figure A41. Presence of null alleles at site UED in 2016. Box whicker plots that cross 0 are not considered to have null alleles at that locus. Boxes show the 25th (lower edge), 50th (solid line), and 75th (upper edge) percentiles. Whiskers represent 1.5x the interquartile range. Solid black dots indicate outlier alleles at that locus and red dots represent the average frequency of null alleles.

APPENDIX B:

CHAPTER 3 SUPPLEMENTAL TABLES AND FIGURES

Table B1. Average number of bumble bee-flower interactions by sampling round and treatment each year. Parentheses following average interactions ± SE represent statistical groupings between treatments.

Year	**Round**	**Restoration type**	**n**	**Average number of bumble bee-flower interactions ± SE**		**Kruskal-Wallis χ^2**	**Significance**
2015	1	None	7	4.1 ± 1.5	b		
		New	3	8.3 ± 4.6	b	**7.34**	**p = 0.03**
		Mature	4	17.5 ± 1.2	a		
	2	None	7	3.3 ± 1.5	b		
		New	4	13.3 ± 3.5	a	**11.04**	**p < 0.01**
		Mature	5	26 ± 3.3	a		
	3	None	7	6.3 ± 1.1			
		New	4	6.3 ± 1.1		5.16	p = 0.08
		Mature	5	17.2 ± 3.8			
	4	None	7	1.3 ± 0.7	b		
		New	4	9.5 ± 2.3	a	**9.18**	**p = 0.01**
		Mature	5	6.0 ± 2.0	a		
2016	1	None	6	2.8 ± 1.6			
		New	4	5.3 ± 2.2		5.51	p = 0.06
		Mature	5	10.4 ± 2.1			
	2	None	6	2.7 ± 1.3	b		
		New	4	17.0 ± 3.4	a	**10.28**	**p = 0.01**
		Mature	5	24.0 ± 3.3	a		
	3	None	6	4.7 ± 1.8			
		New	4	5.5 ± 2.4		0.81	p = 0.67
		Mature	5	16.2 ± 6.2			
	4	None	6	4.2 ± 1.3			
		New	4	12.3 ± 5.1		3.74	p = 0.15
		Mature	5	3.6 ± 1.9			

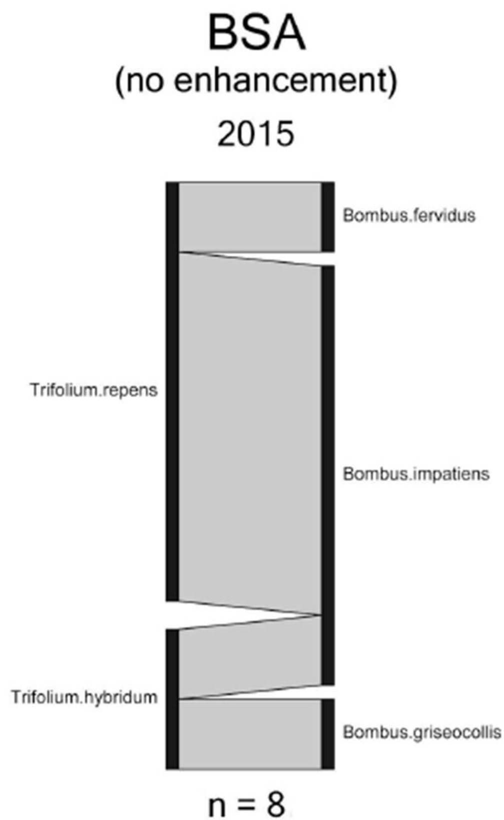

Figure B1. A plant pollinator network in 2015 at site BSA (no enhancement) with plant species on the left side and pollinator species on the right side with n referring to the number of interactions comprising the network. A plant-pollinator network could not be constructed at this site in 2016 due to lack of observations.

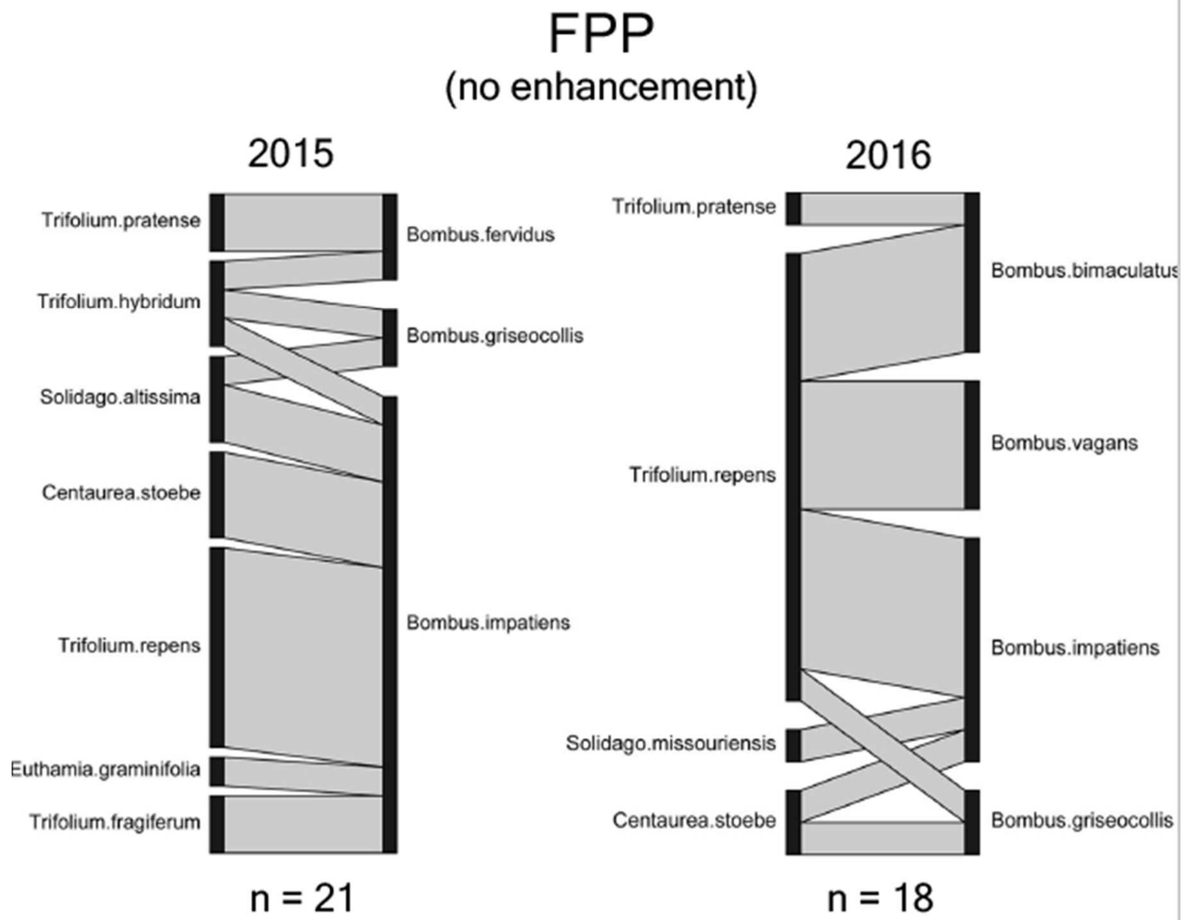

Figure B2. Plant pollinator networks in 2015-16 at site FPP (no enhancement) with plant species on the left side and pollinator species on the right side with n referring to the number of interactions comprising the network.

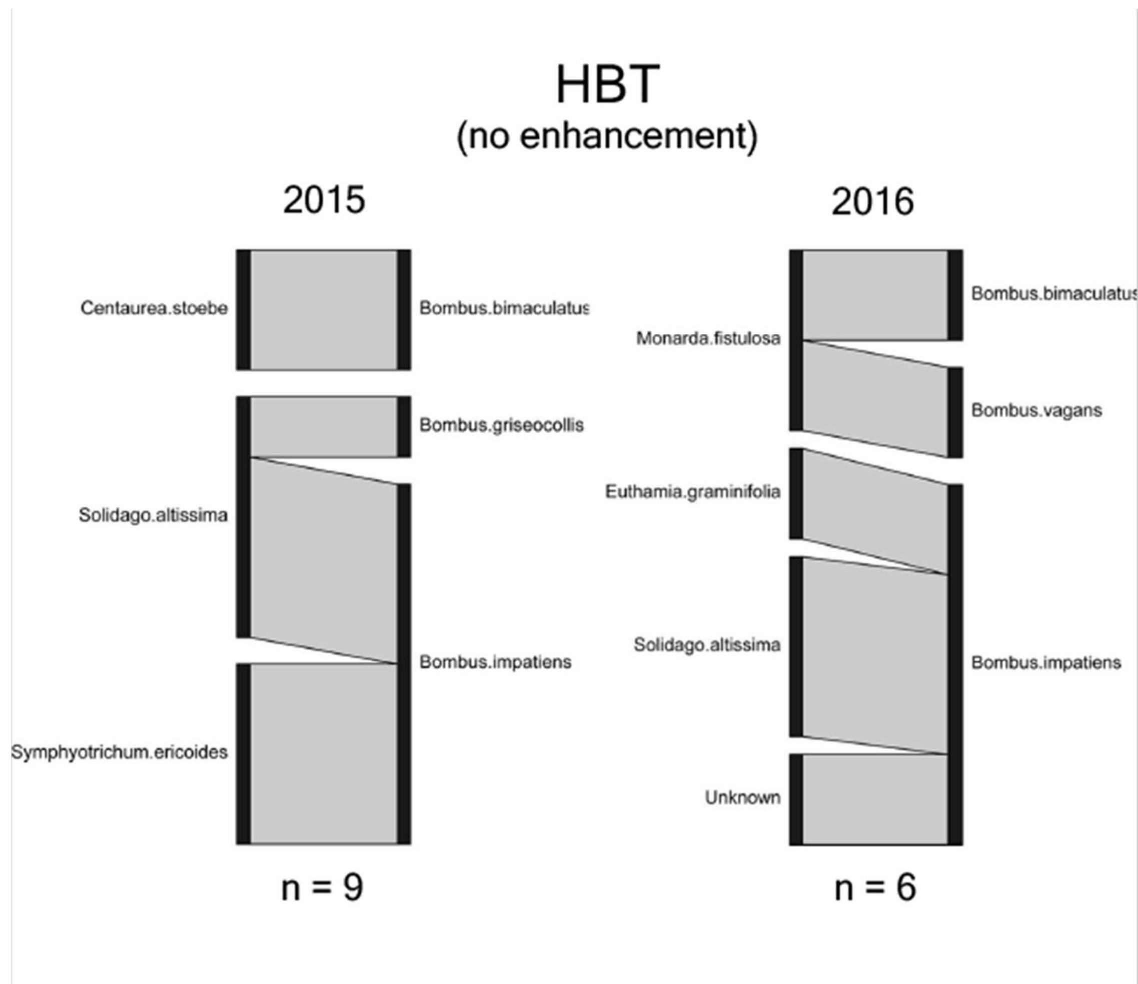

Figure B3. Plant pollinator networks in 2015-16 at site HBT (no enhancement) with plant species on the left side and pollinator species on the right side with n referring to the number of interactions comprising the network.

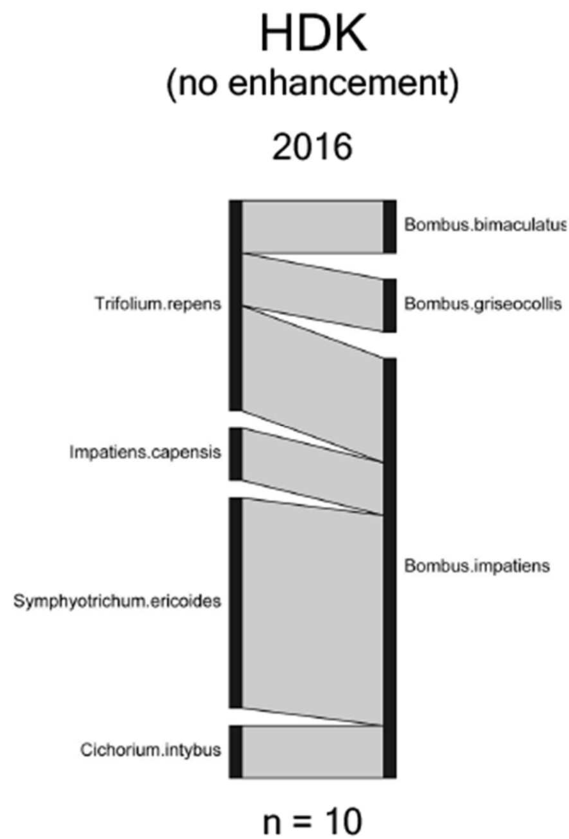

Figure B4. A plant pollinator network in 2016 at site HDK (no enhancement) with plant species on the left side and pollinator species on the right side with n referring to the number of interactions comprising the network. A plant-pollinator network could not be constructed at this site in 2015 due to lack of observations.

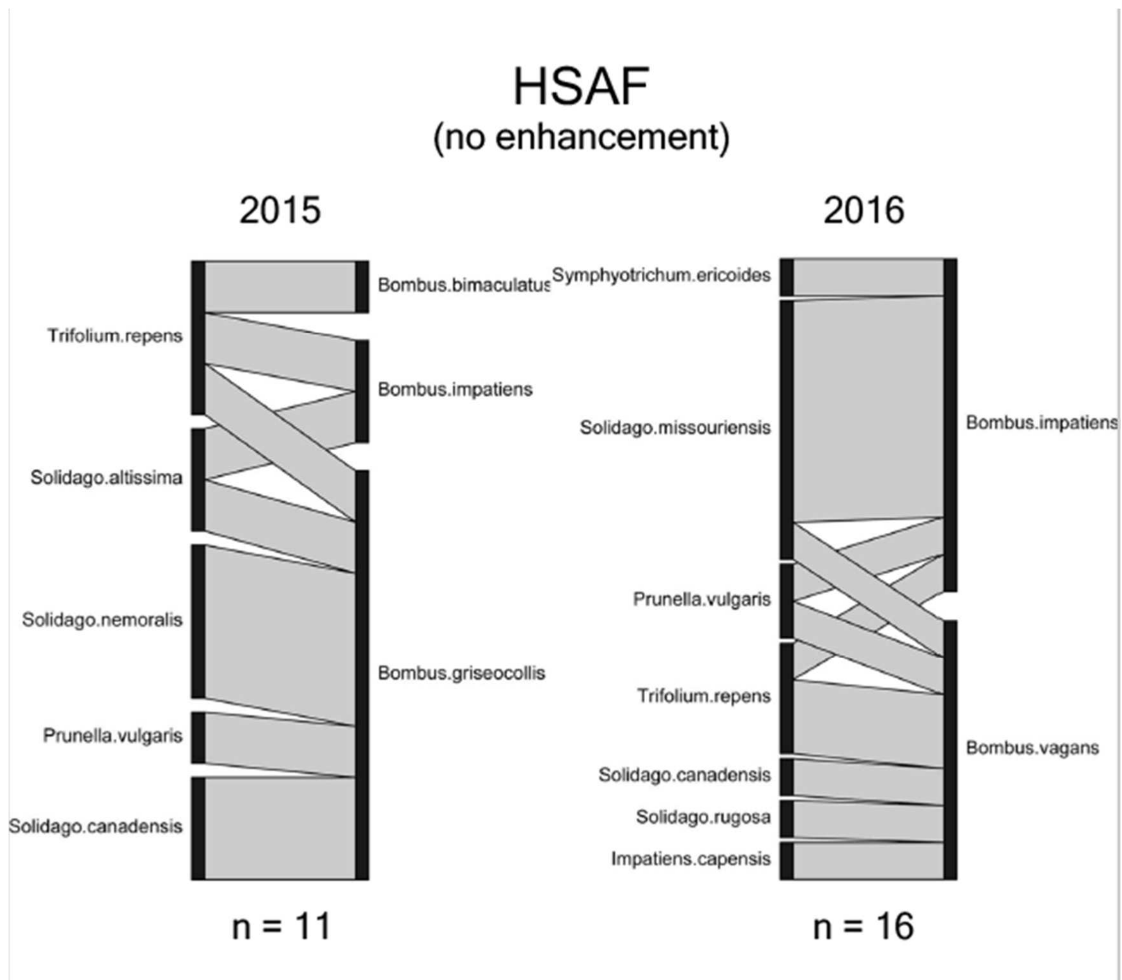

Figure B5. Plant pollinator networks in 2015-16 at site HSAF (no enhancement) with plant species on the left side and pollinator species on the right side with n referring to the number of interactions comprising the network.

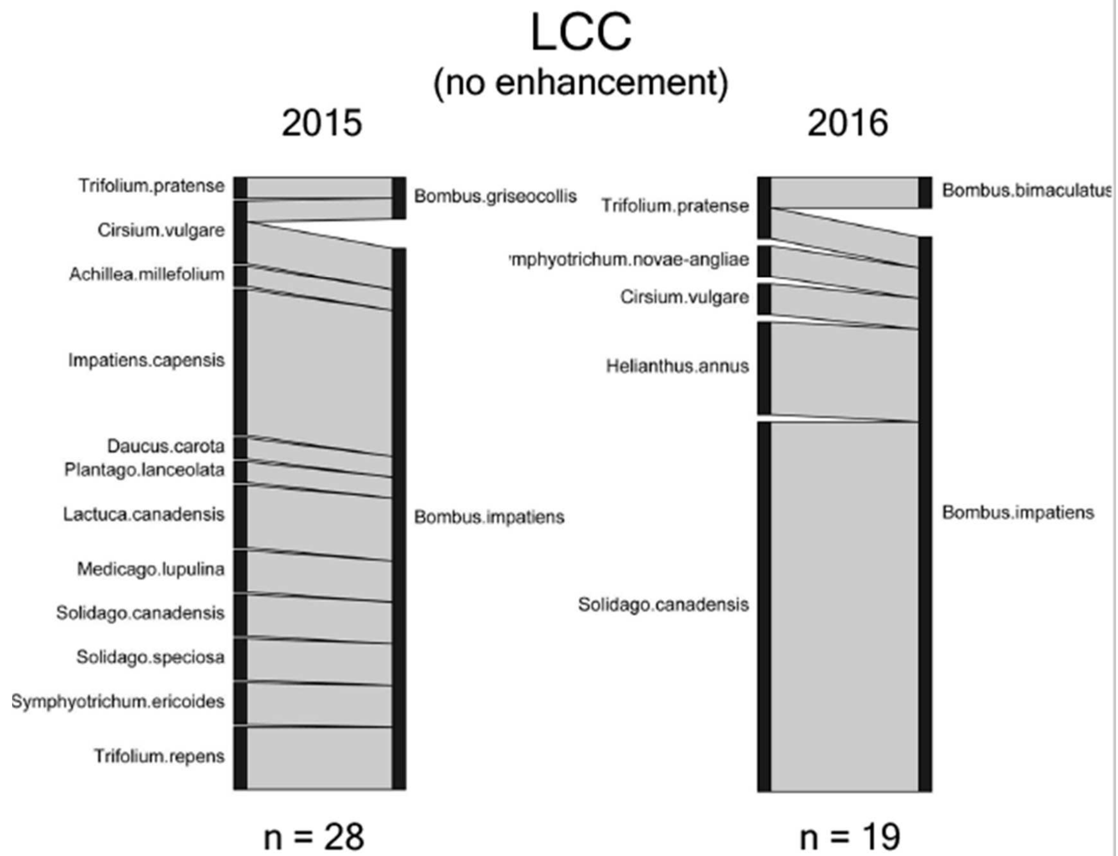

Figure B6. Plant pollinator networks in 2015-16 at site LCC (no enhancement) with plant species on the left side and pollinator species on the right side with n referring to the number of interactions comprising the network.

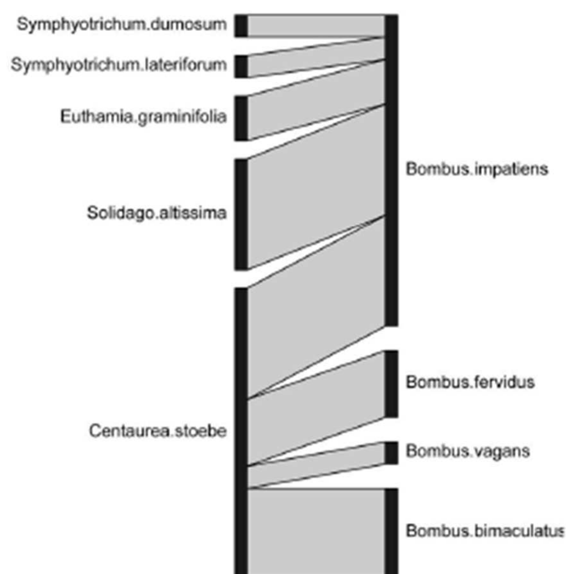

Figure B7. A plant pollinator network in 2015 at site REE (no enhancement) with plant species on the left side and pollinator species on the right side with n referring to the number of interactions comprising the network. A plant-pollinator network could not be constructed at this site in 2016 due to lack of observations.

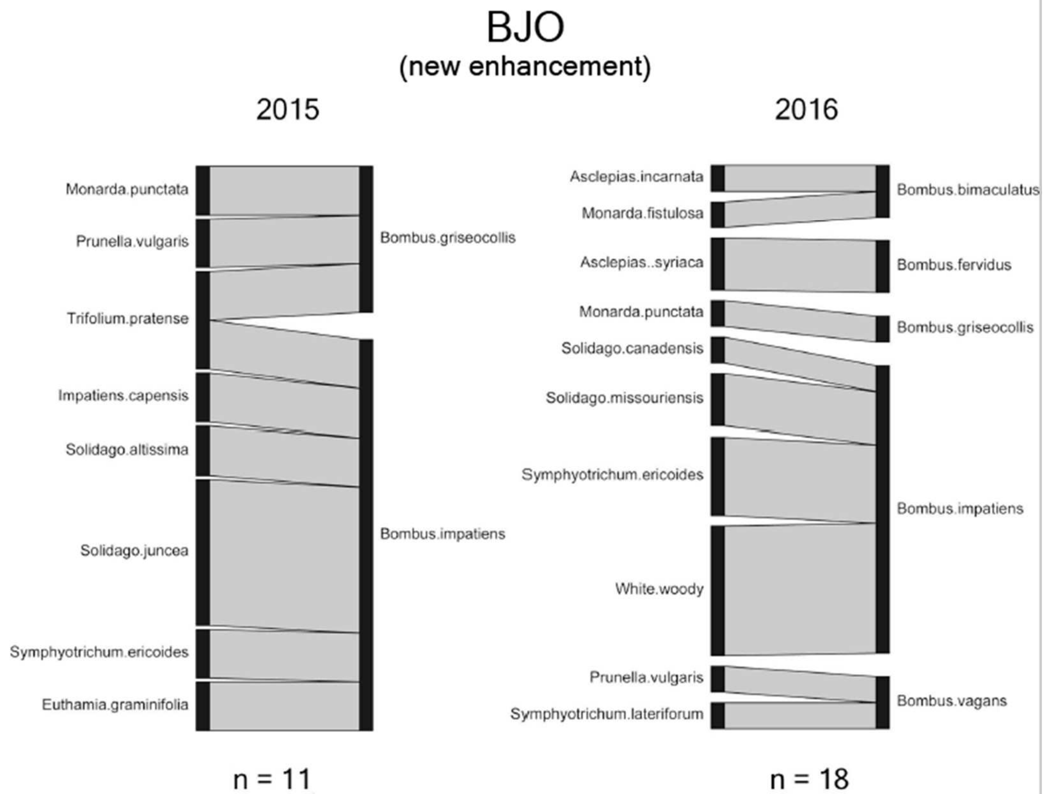

Figure B8. Plant pollinator networks in 2015-16 at site BJO (new enhancement) with plant species on the left side and pollinator species on the right side with n referring to the number of interactions comprising the network.

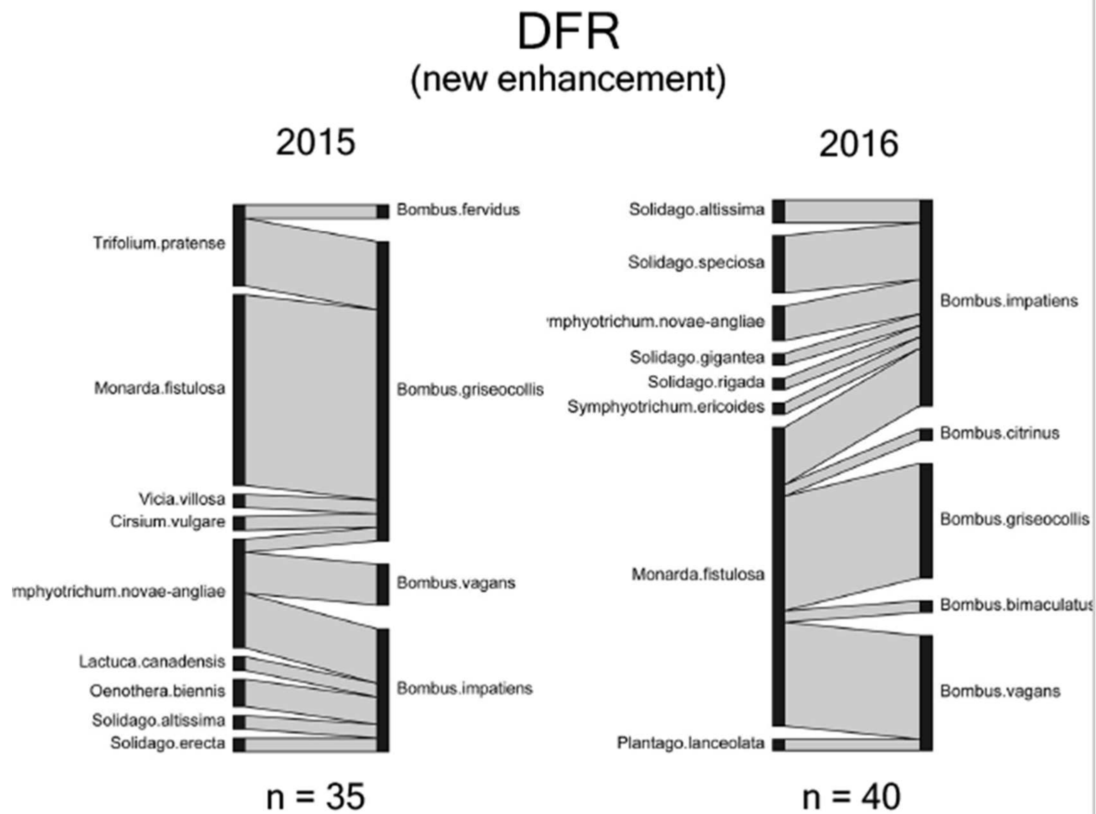

Figure B9. Plant pollinator networks in 2015-16 at site DFR (new enhancement) with plant species on the left side and pollinator species on the right side with n referring to the number of interactions comprising the network.

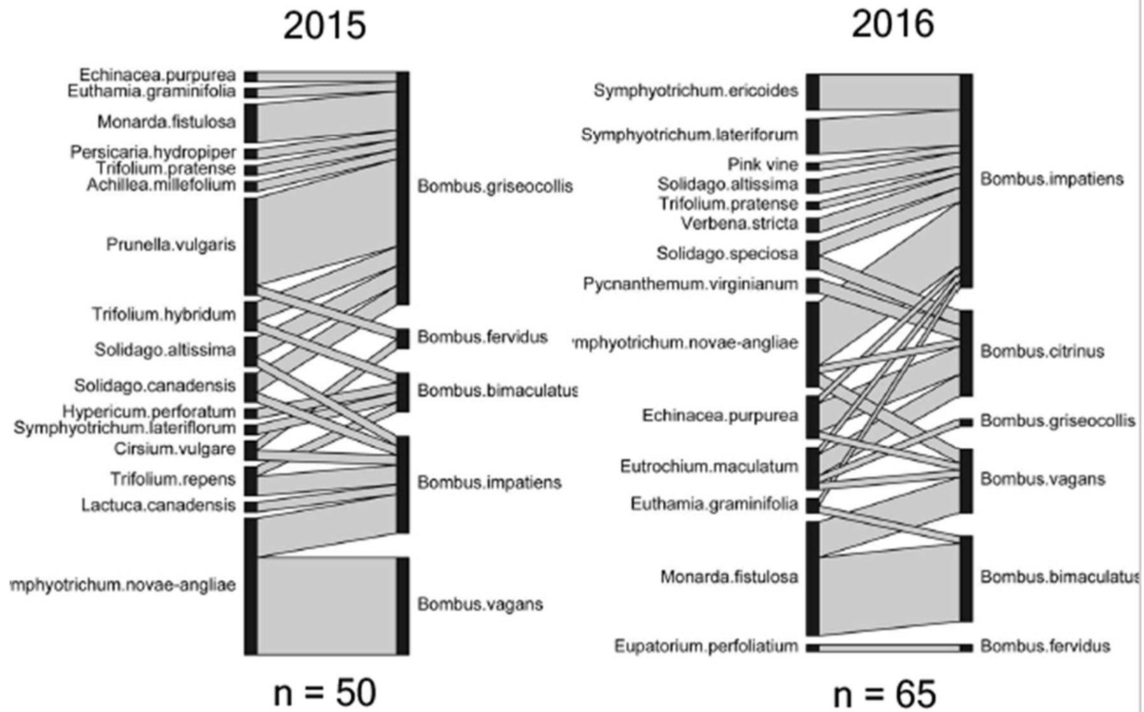

Figure B10. Plant pollinator networks in 2015-16 at site FSL (new enhancement) with plant species on the left side and pollinator species on the right side with n referring to the number of interactions comprising the network.

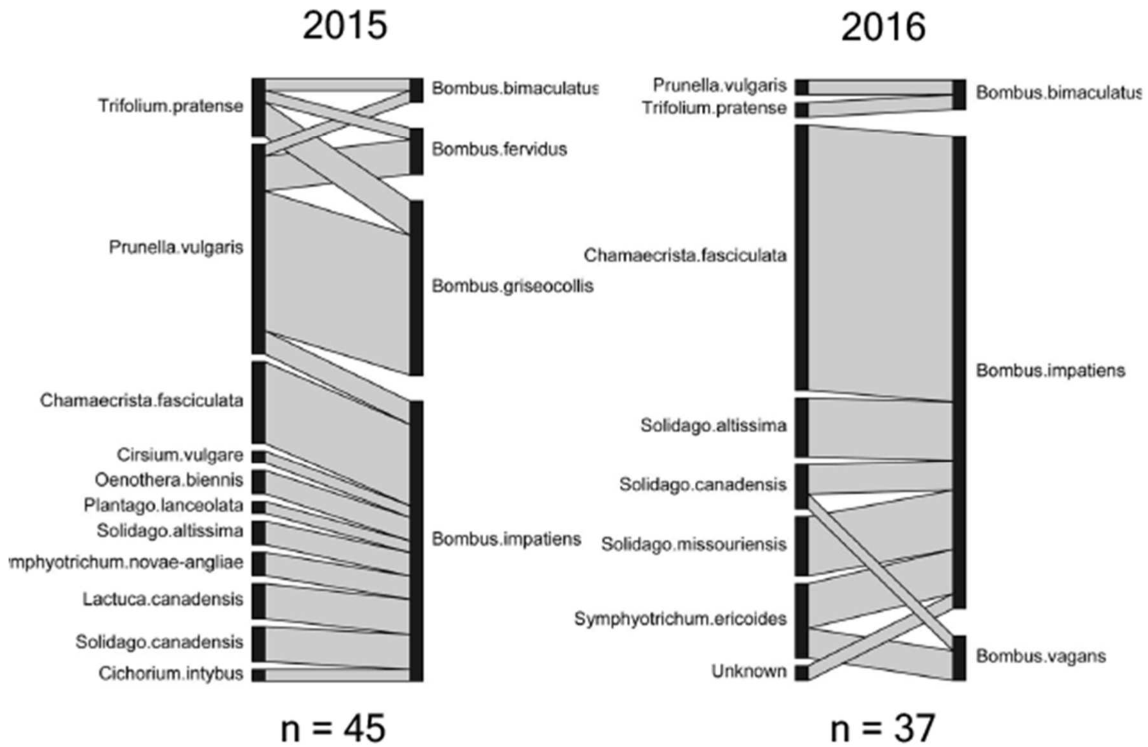

Figure B11. Plant pollinator networks in 2015-16 at site LPL (new enhancement) with plant species on the left side and pollinator species on the right side with n referring to the number of interactions comprising the network.

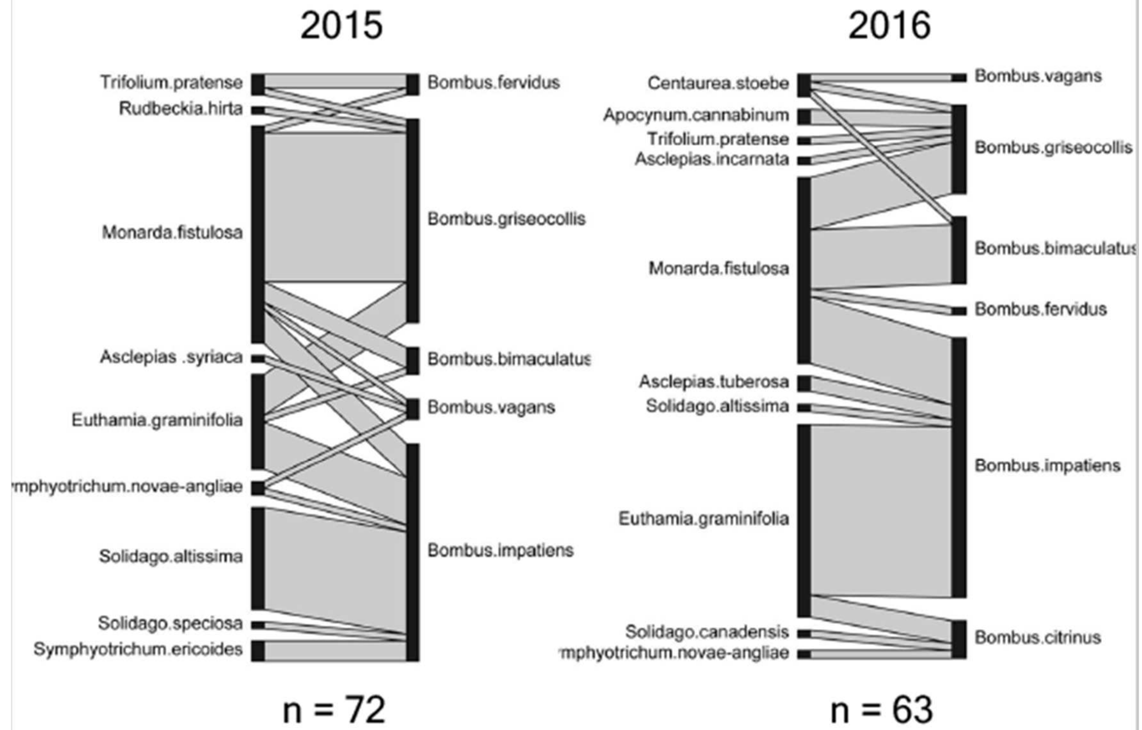

Figure B12. Plant pollinator networks in 2015-16 at site GAL (mature enhancement) with plant species on the left side and pollinator species on the right side with n referring to the number of interactions comprising the network.

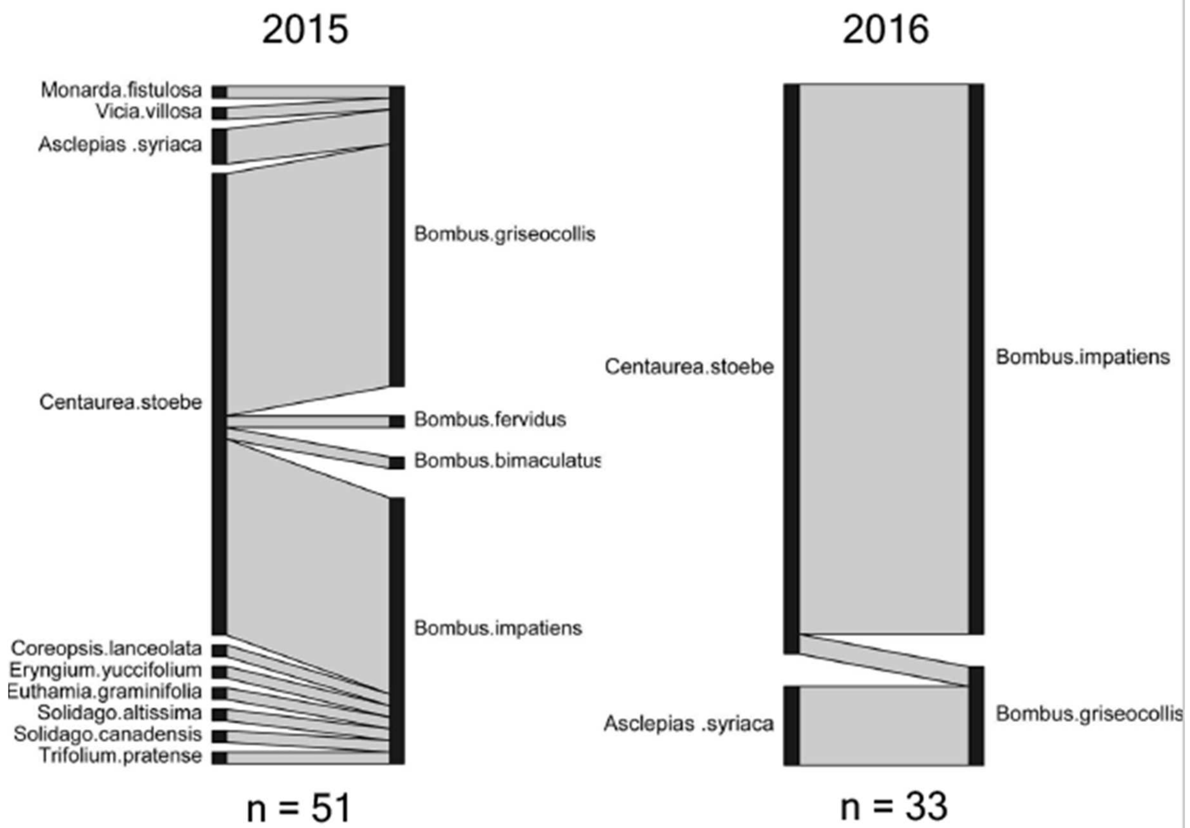

Figure B13. Plant pollinator networks in 2015-16 at site GET (mature enhancement) with plant species on the left side and pollinator species on the right side with n referring to the number of interactions comprising the network.

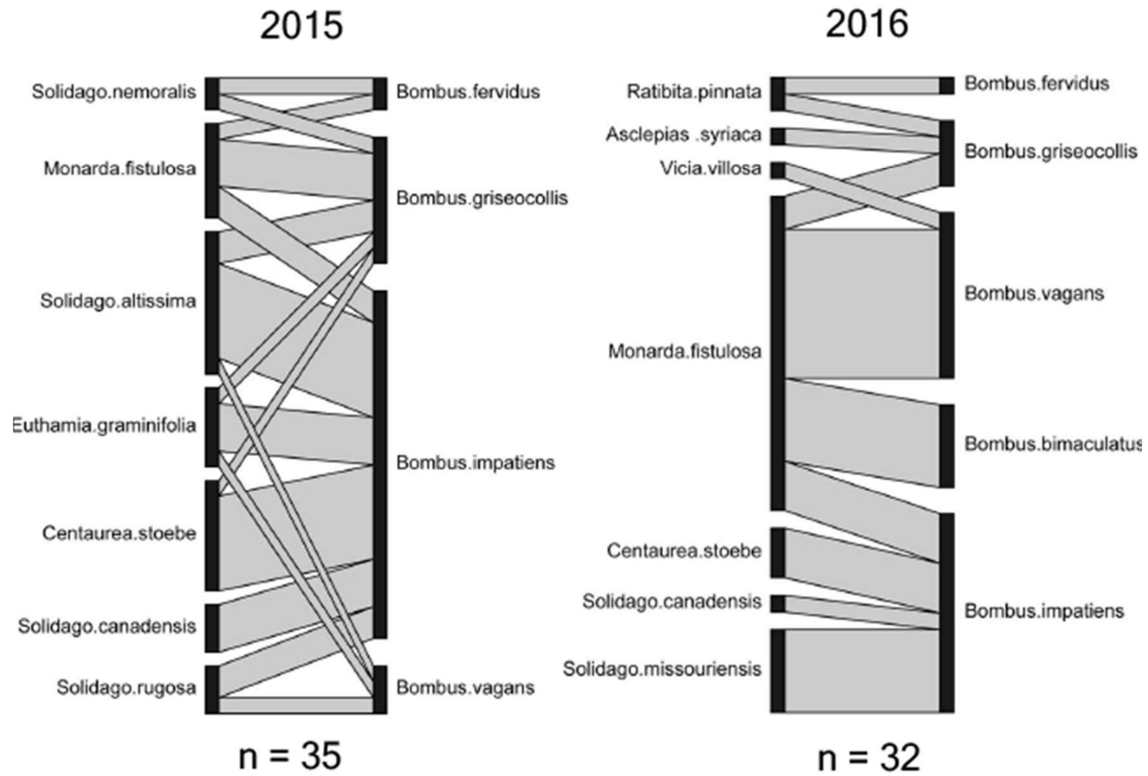

Figure B14. Plant pollinator networks in 2015-16 at site OTTO (mature enhancement) with plant species on the left side and pollinator species on the right side with n referring to the number of interactions comprising the network.

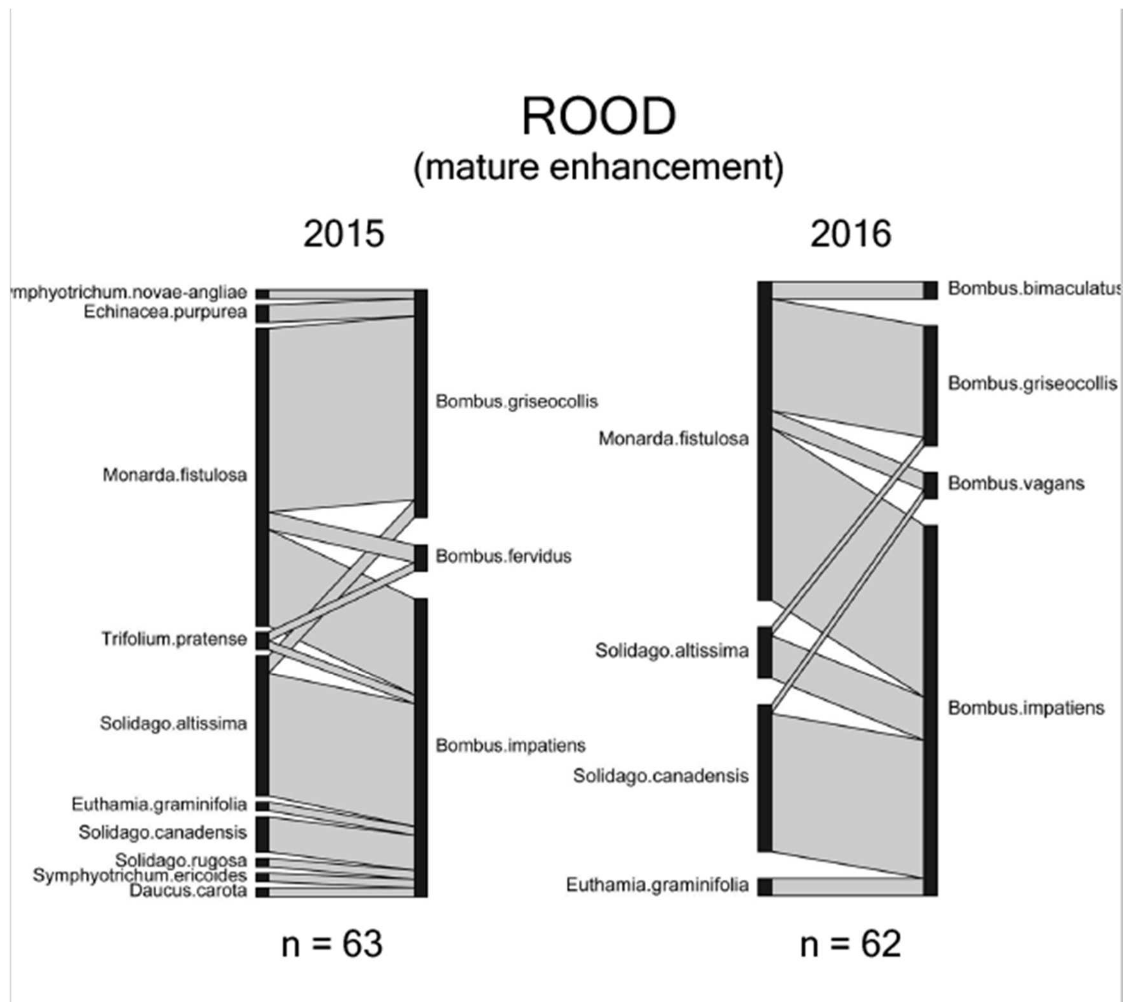

Figure B15. Plant pollinator networks in 2015-16 at site ROOD (mature enhancement) with plant species on the left side and pollinator species on the right side with n referring to the number of interactions comprising the network.

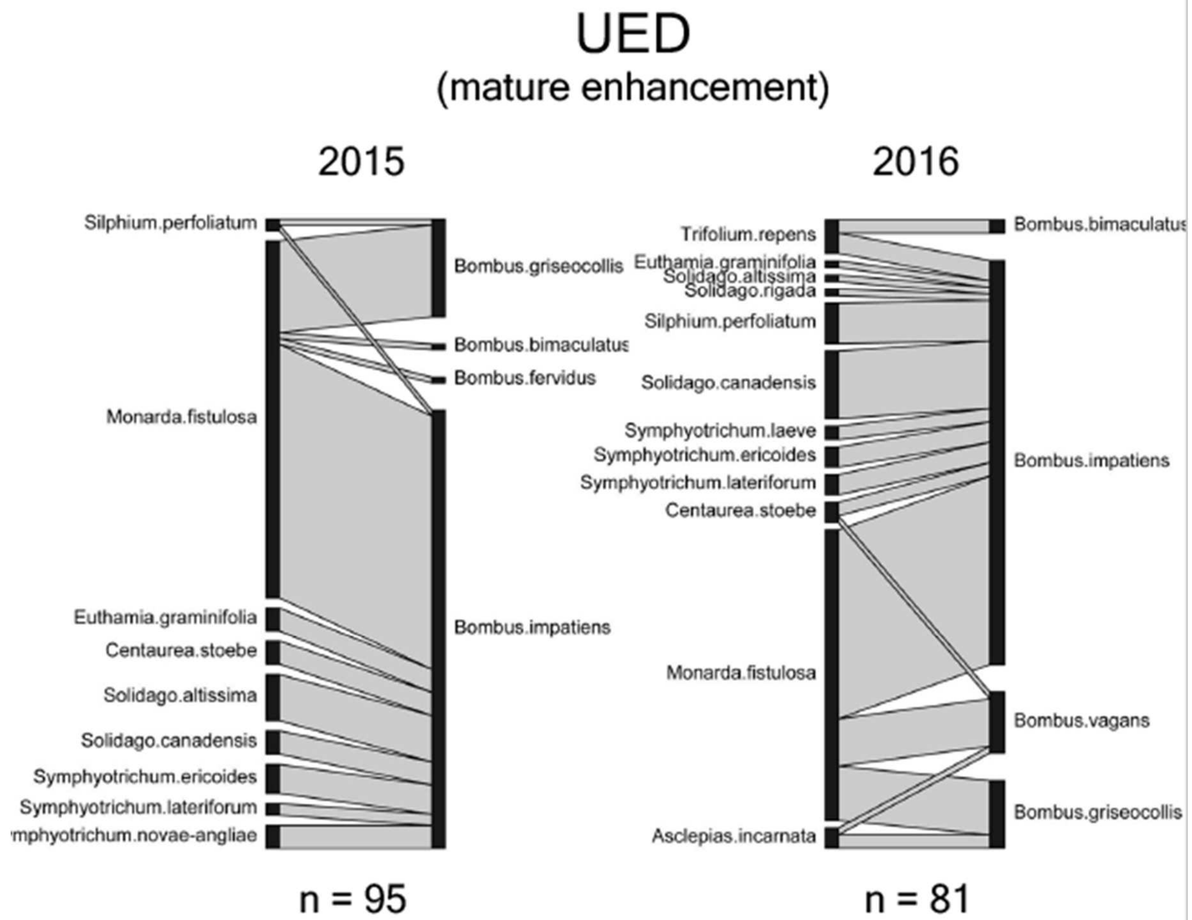

Figure B16. Plant pollinator networks in 2015-16 at site UED (mature enhancement) with plant species on the left side and pollinator species on the right side with n referring to the number of interactions comprising the network.

APPENDIX C:

CHAPTER 4 SUPPLEMENTAL TABLES

Table C1. Modified Qiagen Dneasy Extraction for *Bombus* and flower specimens used in this study.

Step	Procedure/Reagent	Amount
1	Add Proteinase K to sample	5 µL
2	Add Buffer ATL to sample	45 µL
3	Homogenize sample with sterile micropestle (skip this step for flower samples)	
4	Vortex sample + short centrifuge to mix reagents and sample	
5	Incubate for 1 hour at 56 °C (2 hours for flowers)	
6	Add Buffer AL to sample	50 µL
7	Add 95% ethanol to sample	50 µL
8	Pipette mixture into mini spin column (pipette only the fluid in the tube for flower samples)	>100 µL
9	Centrifuge at 15,000g for 1 minute	
10	Add Buffer AW1 to mini spin column	125 µL
11	Centrifuge at 15,000g for 1 minute	
12	Add Buffer AW2 to mini spin column	125 µL
13	Centrifuge at max speed for 3 minutes	
14	Discard flow collection tube	
15	Add mini spin column to sterile 1.5 mL microcentrifuge tube	
16	Add Buffer AE to mini spin column	50 µL
17	Incubate at room temperature for 1 minute	
18	Centrifuge at 12,500g for 1 minute	
19	Discard mini spin column	
20	Save resulting extracted DNA left over in the 1.5 mL microcentrifuge tube and freeze at -20 °C	

Table C2. Master mix and thermocycler regime for *Apicystis bombi, Crithidia bombi,* and *Nosema bombi.*

			Master mix							Thermocycling protocol			
Pathogen	Primers	Citation	dNTP (mM)	MgCl2 (µL)	Buffer (µL)	Taq (µL)	Primer (µL)	DNA Template (µL)	Total Volume (µL)	Step 1: Denaturing Sec, °C	Step 2: Replication Sec, °C	Step 3: Elongation Sec, °C	Amplicon size (bp)
Apicystis bombi	Neo F (5'-3'): CCAGCATGGAAT AACATGTAAGG Neo R (5'-3'): GACAGCTTCCAA TCTCTAGTCG	Meeus et al. 2010	Promega 0.4	(25 mM) 1.5	5x Buffer 2	GoTaq Felxi 0.25	0.5 of each	1.0	10	120, 94	30x 30, 94 30, 60 45, 72	180, 72	260
Crithidia bombi	SEF (5'-3'): CTTTTGGTCGGTG GAGTGAT SER (5'-3'): GGACGTAATCGG CACAGTTT	Meeus et al. 2010	-	-	-	2x PCRBIO HS Taq Mix Red 6.25	0.5 of each	1.0	12.5	120, 94	35x 30, 94 30, 57 45, 72	180, 72	420
Nosema bombi	NB185F (5'-3'): ACTAAGCCAATG TTCCACGTT NB185R (5'-3'): CCAGTAAACCCA CTTTCACAGAT	Tripodi (unpublished)	Apex 1	-	Apex 3.3	GoTaq Flexi 0.2	1.0 of each	1.0	25.2	120, 94	10x 30, 94 30, 60 45, 72 30x 30, 94 30, 57 45, 72	300, 72	185

APPENDIX D:

INSECT VOUCHER SPECIMEN RECORDS

RECORD OF DEPOSITION OF VOUCHER SPECIMENS

The specimens listed below have been deposited in the named museum as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the voucher number have been attached or included in fluid preserved specimens.

Voucher Number: 2017-14

Author and Title of thesis:
Knute Gundersen
"Implications of habitat restoration for bumble bee population dynamics, foraging ecology, and epidemiology"

Museum(s) where deposited:
Albert J. Cook Arthropod Research Collection, Michigan State University (MSU)

Specimens:
Voucher specimens are stored in the Michigan State University Albert J. Cook Arthropod Research Collection satellite bee collection that resides in Dr. Rufus Isaacs' laboratory.

Specimens:

Family	Genus-Species	Life Stage	Quantity	Preservation
Apidae	*Bombus bimaculatus*	adult	10	alcohol
Apidae	*Bombus citrinus*	adult	10	alcohol
Apidae	*Bombus fervidus*	adult	10	alcohol
Apidae	*Bombus griseocollis*	adult	10	alcohol
Apidae	*Bombus impatiens*	adult	10	alcohol
Apidae	*Bombus vagans*	adult	10	alcohol

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