Honey Bee Colony Resource Acquisition, Population Growth, and Pollen Foraging in Diversified Native Grass-Wildflower Grazing Systems

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Abstract

Compounding evidence suggests a current or impending sixth mass extinction event and pollinator crisis. While several factors contribute to pollinator declines, the most notable driver is habitat loss and degradation. Agricultural grasslands provide crucial habitat for wild and domesticated fauna, regulate water and nutrient cycles, store carbon, and maintain soil stabilization. However, conventional tall fescue pastures, which dominate the southeastern United States, limit pollinator habitat, reduce ecosystem services, and diminish cattle productivity if infected with toxic endophytes. Establishing wildflowers (WFs) and native warm season grasses (NWSGs) into tall fescue pastures has the potential to boost both pollinator ecosystem services and cattle productivity. This study monitored the differences in honey bee colony health, productivity, and pollen foraging behavior between diversified and conventional grazing pastures in south west Virginia. Chapter 1 sought to evaluate the health and productivity of hives within NWSGW+ diversified and conventional grazing systems. Chapter 2 sought to 1) determine whether honey bees used sown wildflower species in diversified pastures as significant sources of pollen, 2) compare species composition and nutritive value of pollen collected from hives within diversified and conventional pasture systems, and 3) evaluate temporal trends in pollen collection. Floral surveys revealed diversified pastures had almost 4x greater mean bloom density than conventional pastures, with over half of all blooms recorded in diversified pastures belonging to unsown species. Results from this study suggest that colonies in diversified pasture systems may have a slight advantage in resource acquisition, population

growth, and winter survival following the first year of establishment, though further research is needed. Pollen DNA metabarcoding revealed that honey bees in both diversified and conventional pasture systems have similar diets, and that sown species were foraged upon primarily in the fall. Samples collected from diversified pasture systems yielded greater pollen weight, species richness, and protein content. In conjunction with previous research, these results indicate that diversified pastures could ultimately provide a more complex nutritional support system for pollinators in grazing agroecosystems. However, individual pasture renovation may not be large enough to yield statistically significant differences in honey bee colony success.

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General Audience Abstract

Many studies have suggested that we are currently experiencing or entering a sixth mass extinction event and pollinator crisis. While several factors contribute to pollinator declines, the most notable driver is habitat loss and degradation. Agricultural grasslands provide crucial habitat, regulate water and nutrient cycles, store carbon, and maintain soil stabilization. However, conventional tall fescue pastures, which dominate the southeastern United States, limit pollinator habitat, reduce ecosystem services, and can diminish cattle productivity. Establishing wildflowers (WFs) and native warm season grasses (NWSGs) into tall fescue pastures has the potential to boost both pollinator and cattle health and productivity. This study monitored the differences in honey bee colony health, productivity, and pollen foraging behavior between pastures diversified with WFs and NWSGs and conventional grazing pastures in south west Virginia. Chapter 1 evaluated the health and productivity of hives within diversified and conventional grazing systems. Chapter 2 sought to 1) determine whether honey bees used sown wildflower species in diversified pastures as significant sources of pollen, 2) compare species types and value of pollen collected from hives within diversified and conventional pasture systems, and 3) evaluate temporal trends in pollen collection. Floral surveys revealed diversified pastures had almost 4x greater mean bloom density than conventional pastures, with over half of all blooms recorded in diversified pastures belonging to species we didn't purposefully plant. Results from this study suggest that honey bee colonies in diversified pasture systems may have a slight advantage in resource acquisition, population growth, and winter survival following the

first year of establishment, though further research is needed. A Pollen DNA analysis revealed that honey bees in both diversified and conventional pasture systems have similar diets, and that they collected pollen and nectar from the flowers we planted primarily in the fall. Honey bee colonies in diversified pastures collected more protein-rich pollen from a wider variety of flowers. These results indicate that diversified pastures could ultimately provide a more complex nutritional support system for pollinators in grazing agroecosystems. However, individual pasture renovation may not be large enough to yield large differences in honey bee colony success.

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Literature Review

Abstract

Global pollinator populations are rapidly declining primarily due to climate change, habitat fragmentation, reduced floral resource diversity, pests, parasites, pathogens, management practices, and reduced genetic diversity. This poses many detrimental ecological and economic consequences. Agricultural grasslands provide many important ecological services such as providing crucial habitat for wild and domesticated fauna, regulating water and nutrient cycles, storing carbon, and maintaining soil stabilization mechanisms. However, the traditional nature of fescue pastures, such as in the eastern US, can limit pollinator habitat and resulting ecosystem services, as well as limit cattle productivity. Implementing wildflowers and native warm season grasses (NWSGs) into fescue grazing land has the potential to simultaneously boost pollinator ecosystem services and cattle productivity. As a super generalist pollinator that consistently interacts with nearly every aspect of their ecosystem, the European honey bee (*Apis mellifera*) is an excellent bioindicator of floral resource abundance and the general "health" of an ecosystem. Additionally, recent advancements in palynology, the study of pollen, now allow for an efficient and accurate understanding of pollinator resource preferences that can better inform future pollinator management decisions and conservation efforts. This review focuses on the declines of bees (Order: Hymenoptera), the current research associated with monitoring and supporting their conservation, and the "Bee-Friendly Beef" concept of integrating wildflowers and native warm-season grasses into grazing pastures.

Global Pollinator Declines

There is compounding evidence of a current or impending sixth mass extinction event and substantial evidence suggesting that pollinators, such as birds, small mammals, and insects are in crisis (Thomas et al. 2004; Wake and Vredenburg 2008; Hallmann et al. 2017; Kopec and Burd 2017; Marshman et al. 2019). Specifically, reports of sharp declines in pollinating insect taxa, such as Lepidopterans, Dipterans, Coleopterans, and Hymenopterans have become increasingly common (Marshman et al. 2019). Acting independently and in unison, climate change, habitat fragmentation, reduced floral resource diversity, pests, parasites, pathogens, management practices, and reduced genetic diversity are among the most notable drivers of pollinating insect declines (Watanabe 1994; Potts et al. 2010; Genersch 2010; Kremen and Miles 2012; Goulson et al. 2015; Jarvis 2018). The interactive, non-additive effects of these drivers may increase the speed and severity of lethal and sublethal factors (Potts et al. 2010).

Climate Change

Mismatches in temporal, spatial, morphological and physiological interdependencies may obstruct vital interactions between pollinators and their floral hosts (Memmott et al. 2007; Schweiger 2008; Hegland et al. 2009). These events occur at all organizational levels, often altering the temporal activity, population genetics, phenology, and community composition and functionality, sometimes leading to extinction events (Stone and Willmer 1989; Parmesan et al. 1999; Thomas et al 2001; Williams et al. 2007; Memmott et al. 2007; Hegland et al. 2009; Potts et al. 2010).

Rare and declining pollinators often have more specialized climatic niches and thus may experience heightened climatic effects (Williams et al. 2007; Schweiger et al. 2010). Wild and

managed bees are among the most abundant and efficient floral visitors (Simpson and Neff 1981). Yet, generalist pollinators, such as the European honey bee (*Apis mellifera*), may offer enough pollination redundancy to prevent local floral extinction, which could in turn partially support wild bee populations (Memmott et al. 2004).

Habitat Fragmentation and Pollinator Nutrition

Abundant and well-connected habitat is crucial to maintaining biodiversity (Hanski 2007, Blignaut and Aronson 2008, McGuire et al. 2016, Neokosmidis et al. 2016, Pavageau et al. 2018, Thompson et al. 2019; Raven and Wackernagel 2020; Larkin and Stanley 2021). However, the rate of widespread habitat fragmentation and destruction has drastically increased over the past hundred years as a direct result of urbanization and agricultural intensification (Steinfeld 2006; Satterthwaite 2010; Larkin and Stanley 2021). These anthropogenic land use changes negatively affect bee species richness and diversity, and are particularly detrimental to specialist pollinators (Biesmeijer et al. 2006; Senapathi et al. 2016; Kopec and Burd 2017; Vray et al. 2019; Powney et al. 2019). Poor nutrition can lead to starvation and can also increase susceptibility to viral and fungal diseases in wild and managed pollinator species (Woodard and Jha 2017; Dolezal and Toth 2018). Bee population dynamics and energetic processes can be mechanistically linked via the nutritive value of the surrounding ecosystem (Woodard and Jha 2017). Unsurprisingly, the global decline of wildflowers has been strongly correlated with the decline in wild bee abundance and diversity as well as a reduction in honey bee colony success (Biesmeijer et al. 2006; Bretagnolle and Gaba 2015; Brummitt et al. 2008).

Pests, Parasites, and Pathogens

There is substantial evidence of viral and fungal transmission between wild and managed bees, yet pollinator disease ecology is almost exclusively studied in European honey bees (James and Zengzhi 2012; Goulson et al. 2015; Murray 2019). Some viruses, such as deformed wing virus (DWV) and acute bee paralysis virus (ABPV), have been reported to infect both bumble bees (*Bombus* spp.) and honey bees (*Apis* spp.), and are carried by *Varroa destructor* mites, which may affect more than just honey bees (McMenamin et al. 2018; Rhodes 2018). Other infectious agents are generally host-specific, however, globalization often places unexpecting pollinators at risk from unfamiliar pathogens (Graystock et al. 2013; Genersch et al. 2006; Genersch 2010). In conjunction with unfavorable environmental conditions, the presence of pests, parasites, and pathogens may help to explain otherwise inexplicable colony losses (Cox-Foster 2007; Neumann and Carreck 2010). It should be noted that these conditions have functionally eliminated wild honey bee populations in Europe rendering them reliant on anthropogenic management practices (Moritz et al. 2007; De la Rúa 2009). As a robust, non-native species, wild honey bees can theoretically survive, and even thrive, in some areas of the US. However, the required management practices to ensure colony survival, especially pest and parasite control, make survival unlikely (Caron 2013).

Environmental Pollution and Pesticides

Exposure to toxic materials, such as heavy metals, pesticides, acaricides, fungicides, herbicides and other pollutants, adversely affects wild and managed pollinators (Celli and Maccagnani 2003; Potts et al. 2010; Johnson 2013; Barganska et al. 2015; Rhodes 2018). While the application of such chemicals is often purposeful and regulated in agriculture, there is

compounding evidence of anthropogenic byproducts appearing in floral tissues without direct application, particularly in more urban environments (Leita et al. 1996; Zwolak 2019). Heavy metals are known to be particularly persistent and have been observed in and on live and dead honey bee bodies, as well as within hive attributes such as royal jelly, a nutritive secretion used for raising queens (Leita et al. 1996). Similarly, when seeds are treated with certain insecticides, the chemicals enter the vascular system of the plants and emerge in their pollen, nectar, and guttation droplets thus exposing foraging pollinators to persistent sublethal and lethal effects (van de Sluijs et al. 2013; Pisa et al. 2014; Sanchez-Bayo 2014; Tosi et al. 2018).

Genetic Diversity & Management Practices

Bees are particularly sensitive to the effects of small populations as their single-locus sex determination can lead to the production of sterile diploid males, making them more prone to local and regional extinction events (Kluser 2007; Meixner et al. 2010; Caron 2013). Selective breeding practices in managed pollinating species, most notably the European honey bee, often directly reduce genetic diversity, hindering natural resistance to diseases, foraging success, and other important behaviors (Tarpy 2003; Jones et al. 2004; Graham et al. 2006; Mattila and Seeley 2007; Meixner et al. 2010).

Ecological Consequences

The loss of pollinating species may not be easily reversible (Allen-Wardell et al. 1998). Diverse pollinator communities are positively associated with diverse plant communities and diverse plant communities are the foundation for diverse ecosystems (Fontaine et al. 2005). Pollinating specialist and generalist species provide high-value pollination services for both crop

production and native plants (James and Pitt-Singer 2008; Hung et al. 2018; Potts et al. 2016, Winfree et al. 2018). Several non-agricultural, ecologically significant plants depend upon pollination (Breeze et al. 2011). As pollinator populations decline, floral resources are also reduced, placing more stress on ecosystems and reducing local and global biodiversity (Potts et al. 2010). For example, hawthorns (*Crataegus)*, a pollinator-dependent shrub, provides crucial winter forage for birds and other wildlife (Breeze et al. 2011). Ultimately, the magnitude to which ecosystems directly or indirectly depend on pollinators is inexplicable. A rapid collapse of pollinating populations, similar to the current global trend, will yield the loss of many important species.

Economic Consequences

Approximately 70% of the 124 primary crops used for human consumption depend on pollinators, most notably bees (Klein et al. 2007). The value of these crops is approximately $57 billion for the United States and $172 billion globally per annum (Losey & Vaughan 2006; Gallai et al. 2009; Breeze et al. 2011). As the global agricultural industry continues to rapidly expand, the demand for natural pollinators is becoming further amplified (Ghazoul 2005). Effective pollinator conservation efforts are now more necessary than ever before.

Opportunities for Pollinator Conservation

Predominantly urban and monoculture agroecosystems provide insufficient nourishment and habitat for bees (Lebuhn et al. 2012; Plascencia and Philpott 2017;). Establishing natural areas and managed buffer strips has been shown to increase pollinator diversity and abundance and may even increase crop success (Klein et al. 2004, Greenleaf and Kremen 2006, Kim et al.

2006, Holzschuh et al. 2007; Krewenka et al. 2011; Smart et al. 2016; Plascencia and Philpott 2017; Evans et al. 2018; Wagner et al. 2020; McCullough et al. 2021; Wagner et al. 2020). Honey bee performance and health increased with greater proximity to natural land (Ricigliano 2019) and wild bee community success was positively correlated with honey bee success (Evans et al. 2018). As pollination ecosystem services are regulated by floral resource availability, integrating native forbs into grazing systems will have a cascading effect on the surrounding ecosystem (Woodard and Jha 2017).

Agricultural Grasslands

Grasslands and rangelands provide crucial habitat for permanent and migratory birds, wild and domesticated mammals, soil fauna, arthropods, and interspersed flora (White et al. 2000). Additionally, they regulate water and nutrient cycles, store carbon, and maintain soil stabilization mechanisms (White et al. 2000; Powell et al. 2001; Wilson et al. 2014; Sirimarco et al. 2018; Bengtsson et al. 2019; Balasubramanian et al. 2020). Grasslands and rangelands account for approximately 265,000 hectares of land used primarily for grazing in the United States (USDA-ERS 2017). Cool season grasses such as tall fescue (*Schedonorus arundinaceus* Schreb.), orchardgrass (*Dactylis glomerata* L.), and Kentucky bluegrass (*Poa pratensis* L.), as well as other forages such as white clover (*Trifolium repens*), and weedy vegetation dominate grazing land in the eastern US (Ball et al. 1991; Hoveland 2000; Maresh Nelson et al. 2019).

Tall Fescue

Between 1940 and 1960, tall fescue (*Schedonorus arundinaceus* Schreb.) became the primary perennial cool-season grass in the temperate-subtropical transition zone of the eastern

United States (Sleper and West 1996). Under grazing pressure, it is the only perennial cool-season grass that can compete with native warm-season grasses as it is typically infected with a fungal endophyte (E*pichloë coenophiala)* that is correlated with drought and grazing tolerance (Bacon et al. 1977; Hoveland 1993; Kallenbach 2015; Ball et al. 2019). Tall fescue, like most cool-season grasses, expresses a bimodal growth pattern, in which they are dormant during the hotter mid-summer months (Moser and Hoveland 1996). Consequently, when pastures are primarily composed of tall fescue, there is a dearth in forage availability for cattle and other grazing animals (Moser and Hoveland 1996).

E*pichloë coenophiala* also produces an ergot alkaloid that is toxic to cattle when consumed in large quantities (Hoveland et al. 1980; Roberts and Andrae 2004). Offering access to alternative forages has demonstrated weight gain and heightened reproductive success as cattle will limit their grazing of endophyte-infected tall fescue (Maresh Nelson et al. 2019; Drewnoski et al. 2009). Native warm-season grasses (NWSGs) such as big bluestem (*Andropogon gerardii* Vitman), little bluestem (*Schizachyrium scoparium* Nash), and indiangrass (*Sorghastrum nutans* L.) are viable non-toxic alternative forage for cattle that peak in biomass production when cool-season grass productivity is low (Brown 1999, Tracy et al. 2010; Burns and Fisher 2013; Keyser et al. 2016; Backus et al. 2017).

Developing a Seed Mix

Implementing native forbs into grazing systems can serve as a vital pollinator resource, cover crop, and in some cases increase cattle health and productivity (Klaus et al. 2021a; Klaus et al. 2021b; Tracy et al. 2010). While reconstructing endemic flora is unrealistic, provisioning and regulating ecosystem services through pasture renovation demonstrates great potential for

pollinator conservation. Habitats with a higher plant diversity attract and support a greater abundance and diversity of bees, creating a positive feedback loop between the pollinating and pollinated species (Fontaine et al. 2005; Rundlöf et al. 2018; Bendel et al. 2019; Wagner et al. 2020).

For bees, native perennial wildflowers are more attractive and typically provide adequate nutritive and nonnutritive value via greater diversity in morphology and bloom phenology than non-native annual species (Frankie et al. 2005; Tuell et al. 2008). In the case of pasture renovation, commercial availability, and non-toxicity to cattle must also be considered.

Honey Bees as Bioindicators

As a "super-generalist" pollinator, honey bees are among the most interconnected organisms in any ecosystem (Valido et al. 2019). Honey bees can be useful bioindicators at both spatial and temporal scales in agricultural landscapes as they strongly interact with the air, soil, water, vegetation and other environmental aspects within 7 km (the mean foraging radius) of their hive (Caron 2013; Ruiz et al. 2013; Quigley et al. 2019). Research has focused primarily on using honey bees as bioindicators of pollution but they can also be used to approximate the available floral resources of an ecosystem (Abou-Shaara 2014; Couvillion et al. 2015).

Indicators of Environmental Pollution

As a bioindicator, honey bees can signal numerous environmental qualities such as the presence and quantity of persistent organic pollutants, radioactive elements, heavy metals, and other environmental pollutants through the residues found in honey, pollen, and larvae and the overall health of a colony (Celli and Maccagnani 2003; Barganska et al. 2015). Honey bees

purposefully interact with the flora, water, and soil of their surrounding environments and incidentally interact with undesirable particles within these aspects, often bringing them back to their hives.

The highly viscous nature of honey can easily attract and trap particles from the abrasion of rock, anthropogenic structures and processes, oceanic aerosols, and organic debris (Sager and Maleviti 2014; Sager 2017). However, the foraging strategy of bees discourages contact with local highways and roads (Leita et al. 1996). Anthropogenic chemicals can also enter the vascular systems of plants and emerge in their pollen, nectar, and guttation droplets thus exposing foraging pollinators as interfloral particles (van der Sluijs et al. 2013; McGrath and Mainguy 2014; Pisa et al. 2014; Sanchez-Bayo 2014; Tosi et al. 2018). Honey bees have even been used to detect the presence of pathogenic microorganisms such as (*Erwinia amylovora*), a bacteria that causes fire blight, a harmful disease affecting the Rosaceae family (Martin et al. 2004).

Indicators of Resource Availability & Ecosystem Dynamics

As a honey bee colony grows, foragers must collect sufficient nutritional resources. Therefore, the ultimate success of a honey bee colony is directly correlated with floral abundance and diversity. Honey bees heavily recruit foragers to highly rewarding flower patches, often strongly competing with and sometimes overshadowing wild pollinator species (Aizen et al. 2008; Giannini et al. 2015; Torne-Noguera et al. 2016). However, the nestedness, modularity, and redundancy of the heightened interconnectedness of honey bees may increase the overall 'cohesiveness' and subsequent success of mutualistic pollination networks (Aizen et al 2008; Martín González 2010; Bascompte and Jordano 2014; Russo et al. 2014; Valido et al. 2019).

A single honey bee is capable of visiting 1500 blooms per day (Caron 2013). However, in forests, where floral resources may be reduced, honey bees can collect honeydew produced by aphids, treating it as they would nectar (Sager 2017). By measuring hive attributes such as colony growth, health, and the origins of acquired floral resources, one can infer the attractiveness and support an ecosystem can offer pollinators.

Approximating Floral Resource Composition with Pollen (Chapter 2)

As angiosperms and gymnosperms account for approximately 86% of the global floral profile, palynology, the study of pollen, can offer important insights into a wide variety of ecosystems. Palynology is particularly useful for understanding temporal and spatial plant-pollinator relationships, ancient plant communities, food quality and provenance monitoring, airborne allergen monitoring, and forensic investigations (Bell et al. 2016). Historically, pollen samples have been identified through time-consuming microscopy methodology but recent advancements in DNA barcode technology have allowed for the use of chloroplast and nuclear markers (Richardson et al. 2015a; Bell et al. 2016; Ivleva et al. 2005)

Pollen grains, the male gametes of angiosperm and gymnosperm plants, are typically between 2 and 250 μm in diameter (Weber 1998). The outer layer (exine) of pollen is made of sporopollenin, a highly cross-linked biopolymer that is extremely resistant to decay and is considered to be one of the strongest natural biopolymers in existence (Mackenzie et al. 2015; Ivleva et al. 2005; Kesseler and Harley 2006; Stanley and Linskens 1974). The inner layer (intine) is made of cellulose, a vital structural carbohydrate, and the internal pollen cavity (cytoplasm) contains the organelles and genetic material necessary for fertilization (Stanley and Linskens 1974; Ivleva et al. 2005; Kesseler and Harley 2006; Mackenzie et al. 2015).

Floral Rewards

Pollen-producing plants can be zoophilous (animal-pollinated, typically insects (entomophilous)), anemophilous (wind-pollinated), or ambophilous (pollinated by both animals and wind) (Weber 1998; USDA). Zoophilous host plant species provide both nutritive and nonnutritive rewards for their pollinators (Simpson and Neff 1981).

Nutritive rewards primarily include nectar and pollen. Nectar can contain common sugars, such as glucose (G), sucrose (S), and fructose (F), more complex sugars (oligosaccharides), enzymes, amino acids, vitamins, and lipids (Simpson and Neff 1981; Percival 1961). Nectar composition varies among genus and species, as well as flower age and environmental conditions (Percival 1961). Pollen can contain a variety of simple and complex proteins, enzymes, and secondary metabolites in various combinations. Additionally, pollinators may obtain consumable fatty oils (lipids), stigmatic secretions, or other plant tissues upon floral visitation (Simpson and Neff 1981). Plant domestication may alter the composition of nutritive rewards, particularly in the case of vital secondary metabolites, and can consequently reduce the related health benefits for bees (Egan et al. 2018). Nonnutritive rewards include nest-building materials, shelter, nuptial gifts, mating sites, and heat sources (Rands and Whitney 2008; Simpson and Neff 1981).

Honey Bee Foraging and Nutrition

As super-generalist and eusocial pollinators, honey bees collect and store nectar and pollen from a wide variety of entomophilous (Memmott et al. 2004; Valido et al. 2019) and anemophilous sources (Sabugosa-Madeira et al. 2008; Quinlan et al. 2022). Colony growth and

survival are dependent on the quantity and quality of available floral resources (Caron 2013; Cook et al. 2003)

Nectar, containing necessary carbohydrates and secondary metabolites, is typically consumed directly by adult bees or is regurgitated and dehydrated within the hive to yield honey, which is then consumed by both adult and larval bees (Caron 2013). Historically, honey bees have been documented to exhibit a strong preference for nectar sources that have nearly equivalent proportions of glucose (G), sucrose (S), and fructose (F), such as white clover (*Trifolium repens*) (Percival 1961). However, more recent research has attributed nectar preference to the physical properties, such as viscosity, of nectar more so than the chemical composition (Nicolson et al. 2013). Additionally, differences in genotype and learned behaviors may affect a foragers propensity to collect nectar and pollen from certain sources (Latshaw and Smith 2005).

Pollen primarily provides bees with proteins, minerals, fats, and other essential nutrients not found in nectar (Villanueva et al., 2002; Markowicz Bastos et al., 2004; Almeida-Muradian et al., 2005; Human and Nicolson, 2006; Caron 2013). To collect pollen, honey bees first touch the stamen and are subsequently covered in pollen dust. They then moisten the pollen with mouth secretions and use their hind legs to compact the pollen into corbicular pellets for transportation (Krell 1996; Campos 1997; Caron 2013). Corbicular pollen composition and quantity varies according to location, weather, season, time of day, and other environmental factors (Campos et al. 2008; Barth 2010; Lau et al. 2019).

Pollen protein content can range from approximately 2.5% to 61% independent of pollination phenology (Roulston et al. 2000; Keller et al. 2014). Bees typically avoid collecting pollen that is unusually poor or rich in protein (Roulston et al. 2000). They do, however,

demonstrate learned floral preference takes precedence over floral scent or color (Cook et al. 2003). Intraspecific pollen protein concentrations can vary according to environmental conditions, including rising atmospheric carbon dioxide levels which have been shown to systematically reduce pollen protein content in non-leguminous species (Ziska et al. 2016). As honey bees are notoriously adaptable generalist pollinators, they may alter their foraging behaviors to account for reduced nutritional value (Latshaw and Smith 2005; Nicolson et al. 2013). However, in the case of vital fall forage species, such as goldenrod (*Solidago spp.*), a replacement may not be possible (Ziska et al. 2016).

Physical and Chemical Palynology

Pollen identification is a diverse and challenging field. A multitude of physical (microscopy) and chemical (spectroscopy) methods have been used, separately and in conjunction, to yield identification results. Each has its own advantages and disadvantages. Recent advancements in DNA barcode technology have allowed for the use of chloroplast and nuclear markers to identify pollen to family, genus, and species (Ivleva et al. 2005; Richardson et al. 2015a; Richardson et al. 2015b; Bell et al. 2016).

Palynological microscopy relies upon the physical attributes of pollen granules, whereas spectroscopy relies upon exinal spectral light dictated by chemical composition. Common physical pollen identification methods include optical (OM) and scanning electron microscopy (SEM) (Lynch and Webster 1975; Bahadur et al. 2018; Rahl 2008). However, sporopollenin, the primary component of the exine, is autofluorescent and is therefore not able to be marked by fluorophores or nano quantum dots for identification (Traverse 2007). The characteristics used in identification include ornamentation, polarity, size, shape, aperture structure, symmetry, texture,

and sculpturing (Bahadur et al. 2018). Angiosperm pollen varies enormously, whereas gymnosperm pollen lacks sculpture (Traverse 2007). Using exclusively physical identification techniques, the pollen of extant plants can typically only be identified to genus, not species (Traverse 2007). Additionally, there is currently not a single physical palynological identification technique that can satisfactorily capture pollen shape and texture simultaneously, but rather a combination of techniques is necessary (Sivaguru et al. 2012).

Common chemical pollen identification methods include fluorescence (Boyain-Goitia et al. 2003), laser-induced breakdown (LIBS) (Laucks et al. 2000), infrared (Zimmermann and Kohler 2014), fourier transform (FT) Raman, FT-infrared raman (FTIR) (Kendel and Zimmermann 2020), FT-mid-infrared raman (FTMIR), FT-near-infared raman (FTNIR) (Bleha et al. 2021), and other types of spectroscopy (Schulte et al. 2008). Flow cytometry, a method somewhere between physical and chemical analysis that is typically used in biomedical research and diagnostics, has recently been applied to palynology as it is capable of identifying cell size, granularity, total DNA, surface receptors, and intracellular proteins (Dunker et al. 2020).

In both physical and chemical analyses, previously sampled pollen grains are compared to those in question (Laucks et al. 2000). However, low taxonomic resolution of pollen records, primarily due to homoplasy and limited sampling, have limited analyses (Mander et al. 2014). Physical palynology is also strongly limited by time and skill as to make accurate morphological comparisons requires extensive training (Mander et al. 2014). In conjunction with physical and chemical palynology or on its own, new DNA metabarcoding techniques can accurately and efficiently parse pollen families, genera, and species from honey and corbicular pellets and successfully extrapolate desirable floral resources.

Environmental DNA (eDNA) & Metabarcoding

Traditional methods of DNA sequencing were only capable of identifying a single specimen (Sanger et al. 1977). However, the more recent invention of next-generation sequencing (NGS) techniques, particularly in the case of environmental DNA (eDNA) metabarcoding, has allowed for the inference of biodiversity in a wide variety of disciplines (Hajibabaei 2011; Shokralla et al. 2012; Galimberti 2014). DNA metabarcoding uses small regions of DNA with high interspecific variation and low intraspecific variation to identify the species composition of a sample (Keller et al. 2015; Bell et al. 2016). In animals, the COI region of the mitochondrial genome is used as a barcode, and in plants the rbcL, matK, trnH-psbA regions of the chloroplast genome or the nuclear ribosomal ITS region are most commonly used, either in combination or individually, to determine the species composition of a sample (Coleman 2003; Han et al. 2013).

For identifying pollen species, DNA metabarcoding is typically faster, more comprehensive, and offers a higher taxonomic resolution (70-90% of plant taxa typically identified to species) than physical and chemical identification methods, and only requires basic molecular biology laboratory equipment (Bell et al. 2017; Richardson et al 2015b). Additionally, it does not require the aforementioned highly-specialized knowledge necessary to identify pollen visually (Chen et al. 2010; Bell et al. 2017; Bell et al. 2016; CBOL Plant Working Group). In applying metabarcoding techniques to honey bee research, corbicular pollen analysis has revealed a wide variety of foraging preferences among diverse environments (Potter et al. 2019; Richardson et al 2015b; Richardson et al. 2015a) as well as provided efficient phytopathogen surveillance (Tremblay et al. 2019).

In urban environments, sown wildflowers comprised a majority (69%) of honey and wild bee forage but other, unsown floral species were also utilized within the matrix (Potter et al. 2019). In mixed agricultural landscapes, trees (such as Sapindaceae, Oleaceae, and Rosaceae), dandelion (*Taraxacum officinale*) and mustard (Brassicaceae) pollen were among the most frequently observed honey bee forage species (Richardson et al 2015b). In the presence of grazing systems, white clover (*Trifolium repens*) is commonly reported (Milla et al. 2021). It is to be expected that, in Virginia and in the absence of sown wildflowers, there will be similar tree and clover compositions. In grazing systems diversified with wildflower and native warm-season grasses, such as those involved in the Bee-Friendly Beef Initiative, both sown and wild species should appear in corbicular metabarcoding pollen analyses.

Summary

As both wild and managed pollinators continue to decline, conservation efforts are imperative. Previous research has suggested that establishing wildflowers within numerous ecosystem types can help to promote and conserve pollinator diversity. More specifically, Wagner et al. 2020 has demonstrated that implementing wildflowers and native warm-season grasses (NWSGs) into tall fescue grazing systems can increase both bee abundance and diversity. Despite an absence of known apiaries within a 0.5 km radius of research sites, Wagner et al. 2020 also found several honey bees amidst wild pollinators visiting the diversified pastures, suggesting that these pastures are attractive to important pollinators.

As a super generalist pollinator that consistently interacts with nearly every aspect of their ecosystem, the European honey bee (*Apis mellifera*) is an excellent bioindicator of mutualist pollinator networks, floral resource availability, and the general “health” of an

ecosystem. However, there are few studies that use honey bees as a bioindicator of habitat revitalization and no previous studies that use honey bees to measure the effectiveness of wildflower implementation within grazing pasture systems. The study described in this master's thesis sought to evaluate the efficacy of habitat rehabilitation as it applies to pasture composition and pollination ecosystem services.

Chapter 1

Honey Bee Colony Resource Acquisition and Population Growth in Native Grass-Wildflower Grazing Systems

Abstract

Agricultural grasslands provide crucial habitat for wild and domesticated fauna, regulate water and nutrient cycles, store carbon, and maintain soil stabilization mechanisms. However, conventional tall fescue pastures, which dominate the southeastern United States, limit pollinator habitat, reduce ecosystem services, and diminish cattle productivity if infected with toxic endophytes. Establishing wildflowers (WFs) and native warm season grasses (NWSGs) into tall fescue pastures has the potential to boost pollinator ecosystem services and cattle productivity. As a super generalist pollinator that consistently interacts with nearly every aspect of their ecosystem, the European honey bee (*Apis mellifera*) is an excellent bioindicator of mutualist pollinator networks and the general "health" of an ecosystem. This study monitored differences in weight, area allocated population growth, adult population, area allocated to resource acquisition, and brood pattern observed in honey bee colonies between diversified and conventional grazing pastures in south west Virginia. Floral surveys revealed diversified pastures had almost 4x greater mean bloom density than conventional pastures, with over half of all blooms recorded in diversified pastures belonging to unsown species. Results from this study suggest that individual pasture renovation may not be enough to cause statistically significant differences in honey bee colony metrics. Colonies in diversified pasture systems demonstrated a slight advantage in long-term colony success but further research is needed to identify driving factors in honey bee colony success in grazing systems.

Introduction

Pollinator Crisis

Compounding evidence suggests a current or impending sixth mass extinction event and pollinator crisis (Thomas et al. 2004; Wake and Vredenburg 2008; Hallmann et al. 2017; Kopec and Burd 2017; Marshman et al. 2019). Climate change, habitat fragmentation, pesticides, pollution, reduced floral resource diversity and abundance, pests, parasites, pathogens, poor management practices, and reduced genetic diversity are among the most notable lethal and sublethal drivers of pollinating insect declines (Watanabe 1994; Potts et al. 2010; Genersch 2010; Kremen and Miles 2012; Goulson et al. 2015; Jarvis 2018). The magnitude to which ecosystems directly or indirectly depend on pollinators is inexplicable. Yet it is known that a rapid collapse of pollinating populations, comparable to the current global trend, will yield the loss of crucial biota and staggering economic consequences.

Mismatches in temporal, spatial, morphological, and physiological interdependencies may obstruct vital interactions between pollinators and their floral hosts (Memmott et al. 2007; Schweiger 2008; Hegland et al. 2009). The rate of widespread habitat fragmentation and destruction has drastically increased over the past hundred years as a direct result of urbanization and agricultural intensification (Steinfeld 2006; Satterthwaite 2010). Unsurprisingly, the global decline of wildflowers has been strongly correlated with both a decline in wild bee abundance and diversity as well as a reduction in honey bee colony success (Biesmeijer et al. 2006; Brummitt et al. 2008; Bretagnolle and Gaba 2015).

Anthropogenic land use changes negatively affect bee species richness and diversity, and are particularly detrimental to specialist pollinators (Biesmeijer et al. 2006; Senapathi et al. 2016; Kopec and Burd 2017; Powney et al. 2019; Vray et al. 2019). Generalist pollinators, such as the

European honey bee (*Apis mellifera*), may offer enough pollination redundancy to prevent local floral extinction, which could, in turn, partially support wild bee populations (Memmott et al. 2004). Yet, some studies note heightened competition and pathogen transmission among wild pollinator populations within resource-sparse ecosystems with managed honey bee populations (James and Zengzhi 2012; Goulson et al. 2015; Woodard and Jha 2017; Dolezal and Toth 2018; Murray 2019).

Honey Bees as Bioindicators

The European honey bee (*Apis mellifera*) is responsible for directly pollinating over two million acres of crops in the United States (Morse and Calderone 2000). However, traditional apicultural practices have been substantially modified over the last 25 years to account for drastic colony losses and reduced honey production (Odoux et al. 2014). Consequently, sustainable beekeeping is becoming increasingly more difficult. Supplementing hives with more diverse, wild forage has become standard for over 30% of the U.S. commercial crop-pollinating industry (USDR 2021). Optimizing the U.S. pollination industry with a thorough understanding of honey bee and wild bee ecology is a pressing issue (Odoux et al. 2014).

As a “super-generalist” pollinator, honey bees are among the most interconnected organisms in any ecosystem (Valido et al. 2019). A single honey bee is capable of visiting upwards of 1500 blooms per day (Caron 2013). Honey bees can be useful bioindicators at both spatial and temporal scales in agricultural landscapes as they strongly interact with the air, soil, water, vegetation and other environmental aspects within 7 km (the mean foraging radius) of their hive. (Caron 2013; Ruiz et al. 2013; Quigley et al. 2019). These factors determine the functionality of a pheromone feedback system that maintains physical and behavioral

equilibrium within the hive (Hatjina et al. 2014). Recent research has focused primarily on using honey bees as bioindicators of various pollutants but they can also be used to approximate the available floral resources, and pollinator-suitability of an ecosystem (Abou-Shaara 2014; Couvillion et al. 2015).

Grasslands

Predominantly urban and monoculture agroecosystems provide insufficient nourishment and habitat for bees (Lebuhn et al. 2012; Plascencia and Philpott 2017). Establishing natural areas and managed buffer strips has been shown to increase pollinator diversity and abundance and may even increase crop success (Klein et al. 2004, Greenleaf and Kremen 2006, Kim et al. 2006, Holzschuh et al. 2007; Krewenka et al. 2011; Smart et al. 2016; Plascencia and Philpott 2017; Evans et al. 2018; Wagner et al. 2020; McCullough et al. 2021). Honey bee performance and health increases with proximity to natural land (Ricigliano et al. 2019) and wild bee community success is positively correlated with honey bee success (Evans et al. 2018). As pollination ecosystem services are regulated by floral resource availability, integrating native forbs into grazing systems will have a cascading positive effect on the surrounding ecosystem (Woodard and Jha 2017).

Grasslands and rangelands account for approximately 265,000 hectares (55%) of land used primarily for grazing in the United States (USDA-ERS 2017). Native and managed grasslands can provide food and nesting resources for bees, the quality and quantity of which largely depends on floral species composition and grazing practices (Pavlu et al. 2003; Pavlu et al. 2007; Scohier et al. 2013; Enri et al. 2017; Lazaro et al. 2016; Smith et al. 2016; Shapira et al. 2019). Intentional establishment of native forb and legume species in grazing pastures has

demonstrated great potential for pollinator conservation (Woodcock et al. 2014; Orford et al. 2016

In the eastern US, grazing land is dominated by cool-season, non-native grasses such as tall fescue (*Schedonorus arundinaceus* Schreb.), orchardgrass (*Dactylis glomerata* L.), and Kentucky bluegrass (*Poa pratensis* L.), as well as other forages such as white clover (*Trifolium repens*), and weedy vegetation (Ball et al. 1991; Maresh Nelson et al. 2017; Hoveland 2000). Implementing native forbs and legumes into grazing systems can serve as a vital pollinator resource, cover crop, and in some cases increase cattle health and productivity (Woodcock et al. 2014; Orford et al. 2016; Ekroos et al. 2016; Kovacs-Hostyanszki et al. 2017; Klaus et al. 2021a; Klaus et al. 2021b; Tracy et al. 2010). While reconstructing endemic flora is unrealistic, provisioning and regulating ecosystem services through pasture renovation demonstrates great potential for pollinator conservation.

The purpose of this study was to evaluate honey bee colony health and productivity in pastures diversified with native grasses and wildflowers (NWSGW+). To achieve this objective, honey bee hive performance was measured across multiple sites that were diversified with native grasses and wildflowers and then compared with hive performance on conventional tall fescue pastures in southwest Virginia. The following hive-related metrics were measured: 1) colony weight, 2) area allocated to pre-eclosion population metrics (eggs, larvae, capped brood), 3) adult population metrics, 4) the area allocated to stored resources (honey, nectar, pollen), and 5) brood pattern. In addition to hive-related metrics, descriptive information on plant species composition and floral abundance also was collected at the study sites. The following hypotheses were tested: 1) hives within NWSGW+ diversified grazing systems will be more productive, based on

metrics, than those in conventional grazing systems, and 2) diversified sites would have greater plant diversity and floral abundance that could be attributed to the sown wildflowers.

Methodology

Study Sites

This study was conducted in Patrick, Carroll, Grayson, Craig, Giles, and Montgomery counties in southwestern Virginia, US (Table 1; Figure 1). The region is primarily characterized by managed agricultural and grazing land, fragmented deciduous forests, and semi-natural grassland habitats among ridges and valleys. Major agriculture commodities include livestock (chickens, cattle, turkeys, etc.), soybeans, corn, hay, and tobacco (VFBAF). Grazing land is primarily composed of cool-season non-native pasture grasses, such as tall fescue (*Schedonorus arundinaceus* Schreb.), orchardgrass (*Dactylis glomerata* L.), and Kentucky bluegrass (*Poa pratensis* L.), legumes, such as white clover (*Trifolium repens*), and weedy native and non-native vegetation. Surrounding forests are dominantly composed of oak, hickory, and pine but have some patches of maple, birch, beech, sourwood and tulip poplar. Forbs and legumes found in grazing land, grasslands, and forest edges are major food resources for honey bees. Some trees, such as maple, sourwood, and tulip poplar offer major spring forage.

Peak summer temperatures register above 35°C (95°F) whereas winters have average low temperatures as low as -3.3°C (26°F). The average temperature typically decreases 6.4° C (11.5° F) with every 1000-m (3280-ft) increase in elevation (Woodard and Hoffman 1991). The average annual precipitation varies from approximately 850 mm (33.5 in) to 1270 mm (50 in) (Fleming 2016).

Table 1. Site characteristics. Site ID: D and C refer to diversified and conventional, respectively.

Site ID	Location	Elevation (m)	Hectare	Dominant Soil	Seed Mix	Date Sown
D1	36°33'44.2"N, 80°10'40.0"W	310	1.8	Minnieville Loam	A	June 5th, 2020
D2	36°45'29.4"N, 80°32'04.8"W	827	1.9	Chester-Glenelg Loam	A	June 6th, 2020
D3	36°41'59.5"N, 80°44'57.4"W	810	2	Chester-Glenelg Loam	A	June 7th, 2020
D4	36°47'08.7"N, 80°12'11.4"W	426	2.6	Braddock Fine Sandy Loam	A	June 8th, 2020
D5	36°42'29.3"N, 81°17'11.5"W	1,174	4.3	Tate Loam	B	May 20th, 2020
C1	36°39'40.8"N, 81°01'21.2"W	772	6.7	Pigeonroost Loam	-	-
C2	36°42'52.3"N, 81°03'09.0"W	765	4.3	Tate Loam	-	-
C3	37°25'44.2"N 80°23'03.2"W	555	4	Nicelytown Tilt Loam	-	-
C4	37°16'43.2"N, 80°31'25.9"W	682	4	Frederick Gravelly Silt Loam	-	-
C5	37°13'30.5"N, 80°26'59.1"W	626	1.6	Groseclose and Poplimento Soils	-	-

* See table 3 and 4 for seed mix descriptions.

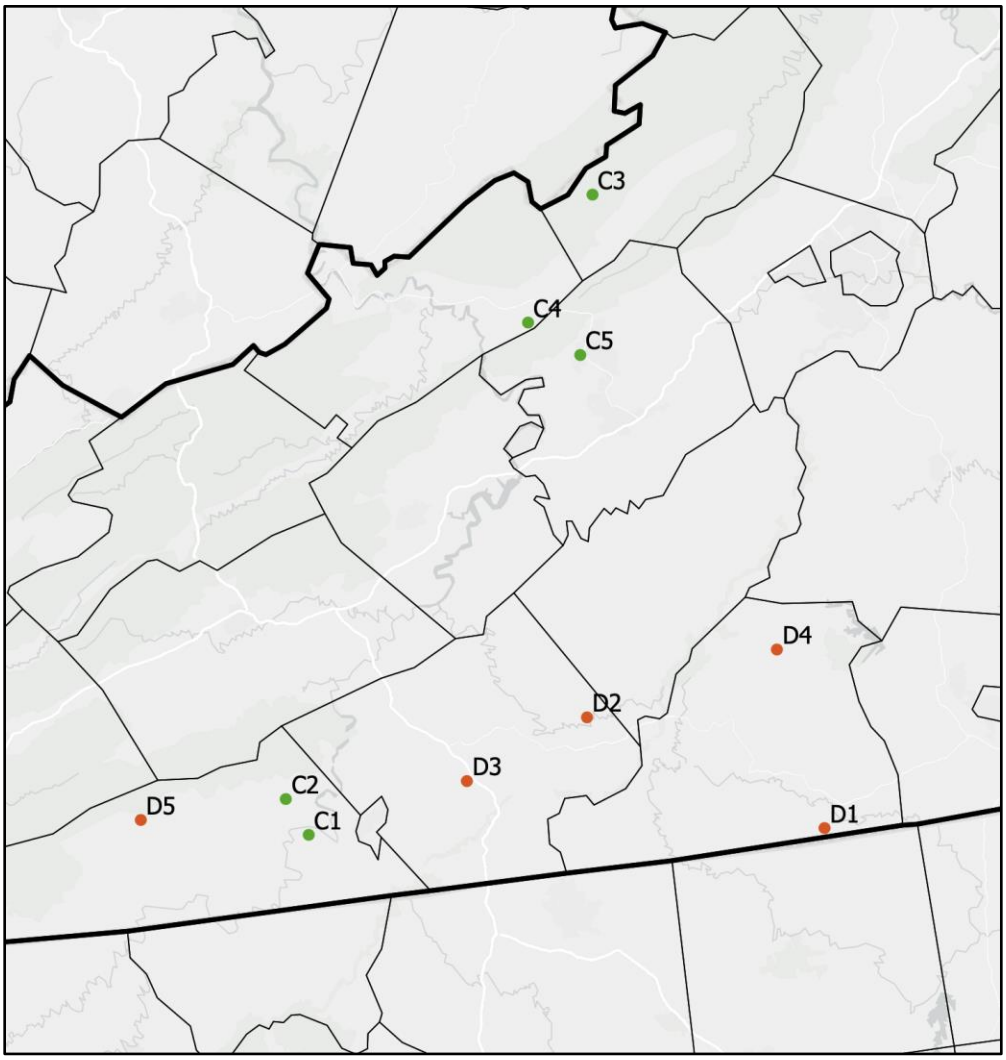


Figure 1. Map of diversified (orange) and conventional (green) sites.

GIS Landscape Analysis

The landscape composition within a 3 km and 5 km radius around each apiary was extracted and tabulated from the USGS National Land Cover Database (2019). The average percent land cover was recorded for each site treatment type (NWSGW+ diversified; conventional) (Figure 2).

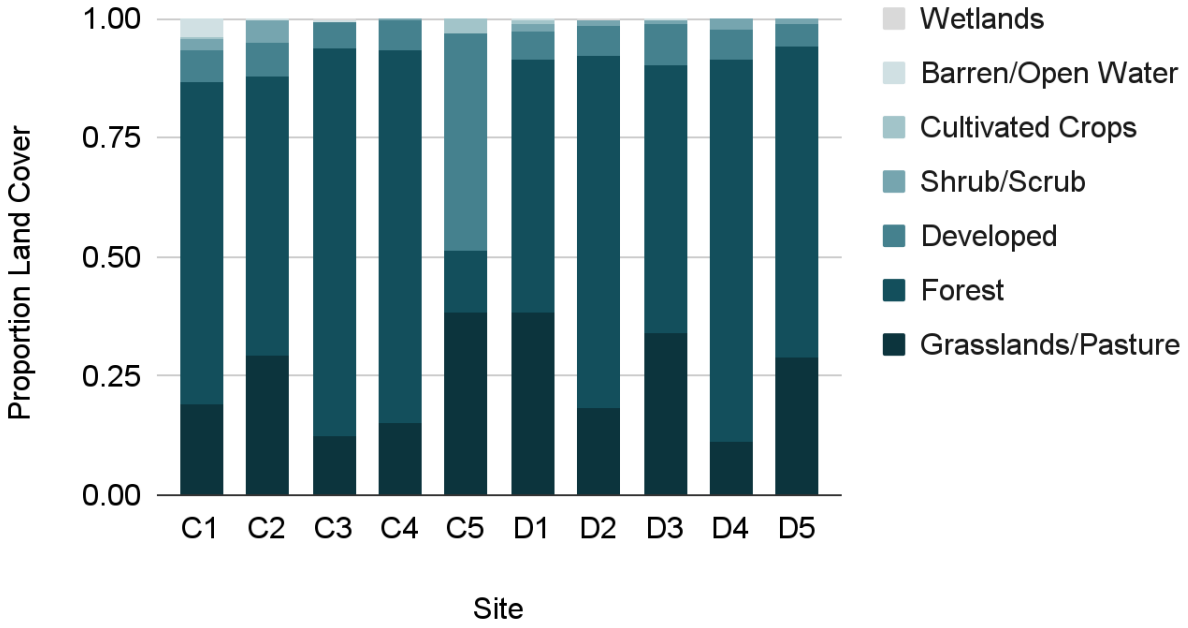


Figure 2. Proportional land use surrounding each apiary (3 km radius) as tabulated from the USGS NLCD (2019).

Site Establishment and Seed Mixes

Ten beef cattle farms in southwestern Virginia were selected for this study (Table 1; Figure 1). All sites were unimproved, permanent pasture land (average slope 5-10%) dominated by cool-season pasture grasses such as tall fescue (*Schedonorus arundinaceus* Schreb.), orchardgrass (*Dactylis glomerata* L.), and Kentucky bluegrass (*Poa pratensis* L.), white clover (*Trifolium repens*), and weedy vegetation. Five sites were selected for native warm season grass and wildflower (NWSG/WF) diversification (D sites, Table 1; Figure 1) and five sites served as controls with no diversification (C sites, Table 1; Figure 1) Prior to planting, all sites were sprayed with Roundup (glyphosate, 2 qt/ac) (Bayer USA, Parsippany, NJ) in fall 2019 and in spring 2020 to suppress existing vegetation. No soil amendments were applied to sites to adjust fertility.

Sites were no-till planted between May 20th and June 8, 2020. Three sites (D1, D2, D3) were planted with Truax FLEX-II no-till drill - (Truax Company, New Hope, MN) and the remainder (D4, D5) with a Dew Drop Drill (Little Sioux Prairie Co, Spencer, IA). Four sites (D1, D2, D3, D4) were sown with a 13 species mixture (10 WF, 3 NWSG) (Table 2; Table 4) and one site (D5) was sown with a 16 species mixture (14 WF, 3 NWSG) (Table 3; Table 5).

Wildflower species were selected for bloom phenology, non-toxicity to cattle, morphological diversity, and commercial availability. Native warm season grasses were included to provide summer forage for cattle. Seeds were obtained from Roundstone Native Seed LLC (Upton, KY). The seed mixtures were a 4:1 ratio of NWSG to WF proportioned on an area basis such that each NWSG species would be sown at 13 seeds/sq ft and each WF at 1 seed/sq ft.. This seed density translated into approximately 8 lbs/ac planting rate for NWSG mix and 2 lbs/ac for WF mix. Planting rate was increased for Dew Drop drill (10 lbs NWSG, 2.5 lbs WF) as it is broadcast seeder.

Table 2. Seed Mix A: Sown species at sites D1, D2, D3, and D4 in 2020. Recommended seeding rates and seeds per pound were based on information in the Virginia Plant Establishment Guide (Hall et al. 2011) and information from Roundstone Native Seed LLC (Upton, KY).

Scientific Name	Common Name	Mix Proportion	kg/ha seeding rate
Andropogon gerardii	Big Bluestem	0.32	3.59
Schizachyrium scoparium	Little Bluestem	0.20	2.24
Sorghastrum nutans	Indian Grass	0.30	3.36
Coreopsis lanceolata	Lance Leaved Coreopsis	0.02	0.22
Linum perenne	Blue Flax	0.02	0.22
Tradescantia ohiensis	Ohio Spiderwort	0.01	0.11
Rudbeckia hirta	Blackeyed Susan	0.01	0.11
Echinacea purpurea	Purple Coneflower	0.04	0.45

Agastache foeniculum	Lavender Hyssop	0.003	0.03
Ratibida pinnata	Gray-headed Coneflower	0.01	0.11
Helianthus maximiliani	Maximillian Sunflower	0.03	0.34
Solidago rigida	Rigid Goldenrod	0.01	0.11
Gaillardia pulchella	Indian Blanket	0.02	0.22

Table 3. Seed Mix B: NWSGs and wildflowers seeded at site D5 in 2020.

Scientific Name	**Common Name**	**Mix Proportion**	**kg/ha seeding rate**
Andropogon gerardii	Big Bluestem	0.15	1.31
Tridens flavus	Purple Top	0.05	0.47
Desmanthus illinoensis	Illinois Bundleflower	0.14	1.29
Desmodium canadense	Showy Tick Trefoil	0.16	1.46
Cassia fasciculata	Partridge Pea	0.19	1.69
Aster laevis	Smooth Aster	0.01	0.11
Coreopsis lanceolata	Lance Leaved Coreopsis	0.07	0.66
Coreopsis tinctoria	Plains Coreopsis	0.01	0.05
Solidago juncea	Early Goldenrod	0.01	0.04
Pycnanthemum tenuifolium	Slender Mountain Mint	0.01	0.03
Oenothera biennis	Evening Primrose	0.01	0.11
Heliopsis helianthoides	False Sunflower	0.10	0.87
Helianthus angustifolius	Narrow-Leaved Sunflower	0.03	0.29
Monarda fistulosis	Bergamot	0.01	0.057
Ratibida columnifera	Upright Coneflower	0.01	0.13
Helianthus maximiliani	Maximillian Sunflower	0.06	0.50

Table 4. Seed Mix A (D1, D2, D3, D4): Bloom Phenology.

	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Coreopsis lanceolata												
Linum perenne												
Tradescantia ohiensis												
Rudbeckia hirta												
Echinacea purpurea												
Agastache foeniculum												
Ratibida pinnata												
Helianthus maximiliani												
Solidago rigida												
Gaillardia pulchella												

Table 5. Seed Mix B (D5): Bloom Phenology.

	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Desmanthus illinoensis												
Desmodium canadense												
Cassia fasciculata												
Aster laevis												
Coreopsis lanceolata												
Coreopsis tinctoria												
Solidago juncea												
Pycnanthemum tenuifolium												
Oenothera biennis												
Heliopsis helianthoides												
Helianthus angustifolius												
Monarda fistulosis												
Ratibida columnifera												
Helianthus maximiliani												

In-Pasture Plant Composition

Percent cover of sown wildflower and NWSG species, weedy species, and bare ground was visually estimated in 1-m^2 square quadrats in pastures adjacent to the apiaries in June, July, and August of 2022. Percent cover of plant species was then estimated using a modified Daubenmire method (Daubenmire 1959). Within these quadrats, blooming species were

identified and counted to determine the species richness, evenness, and diversity of active floral resources in each pasture. A Sørensen-Dice index was applied to determine the similarity of blooming resources among site treatments.

Honey Bee Management

All honey bee colonies were established via nucleus hives from a local professional apiculturist in mid-April, 2021. At the time of establishment, all queens were less than one year old and colonies were inspected to ensure disease-free status. The hives were standard pinewood Langstroth hives (10-frame) with plasticell foundation. A total of twenty hives, across ten apiaries. Five of the apiaries were established within conventional grazing systems, five were established within diversified NWSGW+ grazing systems. Within each apiary, partner hives were placed 3 feet apart on 1.5 tall cinder block foundations (Figure 3A). Each hive box was painted with Behr exterior latex paint in shade P100-4^{M}, P450-3^{U}, or MQ4-46^{M}. The first boxes of partner hives were ensured to be different shades to help bees differentiate the hives and mitigate pest, parasite, or pathogen transfer within the apiary. Each colony received two standard deep (D) boxes before receiving medium honey supers (M) as they grew (Figure 3B).

Pre-swarm queen cells were destroyed to manage swarming. A second box (deep, ~10 inches tall) and honey supers (medium, ~7 inches tall) were added as reserves grew. Honey supers were not harvested. Hives were treated with Formic Pro in late summer twice, one sheet for two weeks each, to mitigate *Varroa destructor* mite populations. Each hive was fed a 1:1 sugar water solution ad libitum for 6 weeks in spring, and a 2:1 sugar water solution ad libitum for 6 weeks in the fall to compensate for known resource shortages. Site D5 was excluded due to catastrophic environment-caused hive destruction. Site C5 was excluded due to its close

proximity to many *Prunus* trees, which provided bees with rich flower resources that were not present at other sites. Colonies that did not overwinter successfully were replaced with nucleus hives from Bedillion Bees in Pennsylvania in early May, 2022.

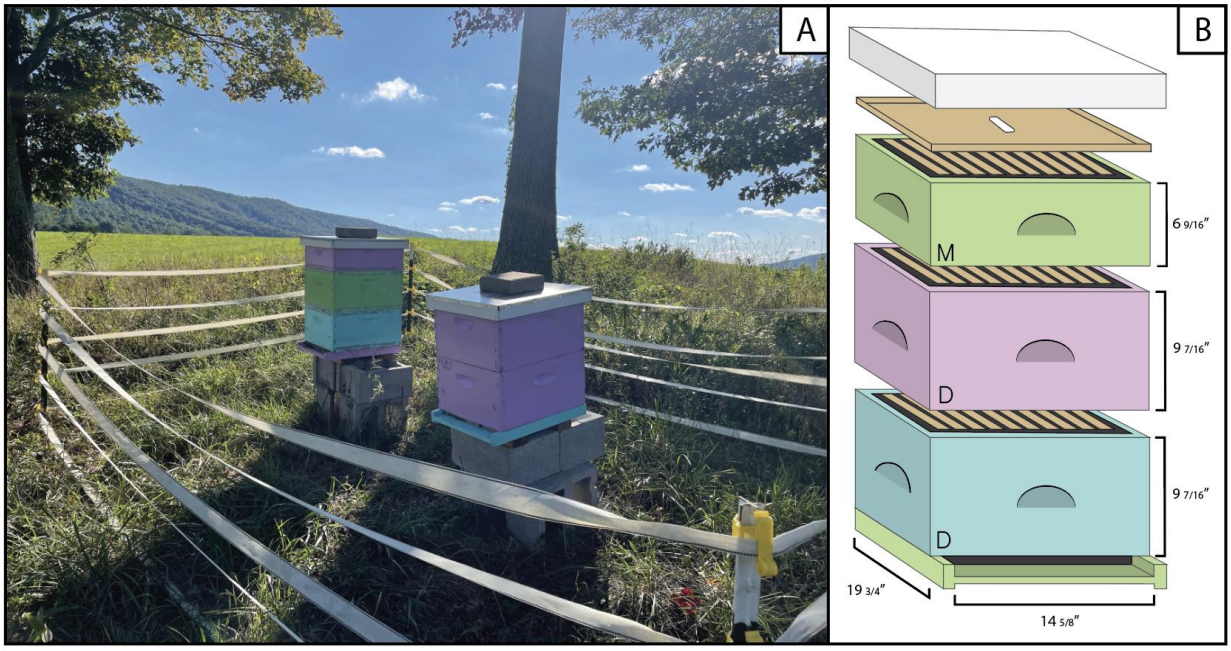


Figure 3. A: Apiary system (C4). B: Dimensions of a 3-box hive (two deep boxes (D) and one medium (M)).

Data Collection

The following colony attributes were monitored in 2021 and 2022: 1) hive weight, 2) pre-eclosion population metrics (eggs, larvae, capped brood), 3) stored resource metrics (nectar, honey, pollen), 4) adult population and overwintering, and 5) brood pattern. Demographic events, such as swarming and requeening, and management events such as artificial feeding, box additions, and varroa treatments were recorded. Additionally, the presence of ailments (parasites, pests, and diseases) observed during inspections were recorded.

Hive attributes were measured 10 times in 2021, and 8 times in 2022 throughout the sampling season (April-September). Sampling occurred biweekly April-July, triweekly July-

August, and biweekly August-September. This schedule reduced redundancy during the observed slow growth period in mid-late summer, and was conducive to swarm management and feeding schedules. Bees were kept on the frames during observations to reduce disturbance.

Hive Weight

In 2021, raw hive weight was recorded via SolutionBee© scales (Solutionbee LLC, Raleigh, NC). In 2022, all hives were weighed with a tripod-scale system following regular hive inspections. For both years, colony weight was calculated by subtracting the weight of empty hive gear (ie. bottom board, frames, boxes, lids, etc.) from the raw weight to account for the differences in required space.

Pre-eclosion Population & Stored Resource Metrics

The area allocated to pre-eclosion population metrics (eggs, larvae, capped brood), stored resource metrics (nectar, honey, pollen), empty cells, and foundation was estimated to the nearest 5% for each frame (Figure 4) (Delaplane et al. 2013). Proportional allocated area was calculated according to the usable frame surface area of deep and medium Langstroth frames (Table 5).

Table 5. Usable frame area according to standard deep and medium frame measurements used to calculate allocated area.

Box Type	Frame Width (cm^2)	Frame Height (cm^2)
Deep	48	23
Medium	48	16

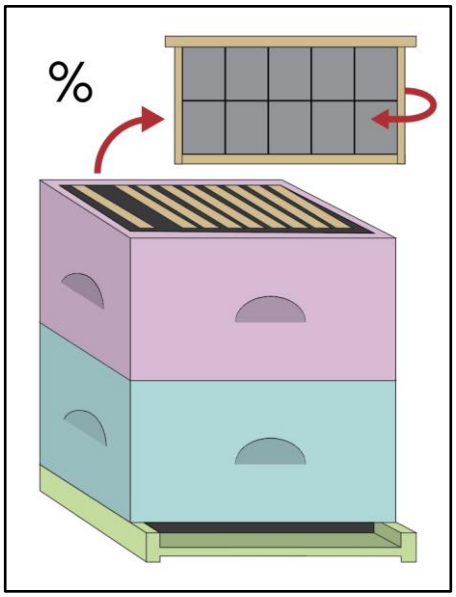


Figure 4. Frame metrics with 5% grid.

Adult Population & Overwintering

The number of full frames of bees (FOB) was recorded in spring and fall for both 2021 and 2022 as an estimate of the adult bee population (Nasr et al. 1990; Delaplane et al. 2013). Colonies were considered deceased when the total number of FOB was equal to 0. This metric is particularly useful in quantifying relative overwintering success.

Brood Pattern

Brood pattern was rated on a scale of 0-3, from poor to excellent, respectively. These ratings are defined as followed: 0 indicated an absence of brood, 1 indicated a "poor" pattern in which there is some capped brood but large gaps in laying are present, 2 an "average" amount of capped brood with some linked gaps, and 3 indicated an "excellent" pattern in which there are few gaps (3) (Lee et al. 2019; Guzman-Novoa and Page 1999) (Figure 5). A Two-way ANOVA was used to identify significant relationships between site treatment, sampling period, and brood pattern.

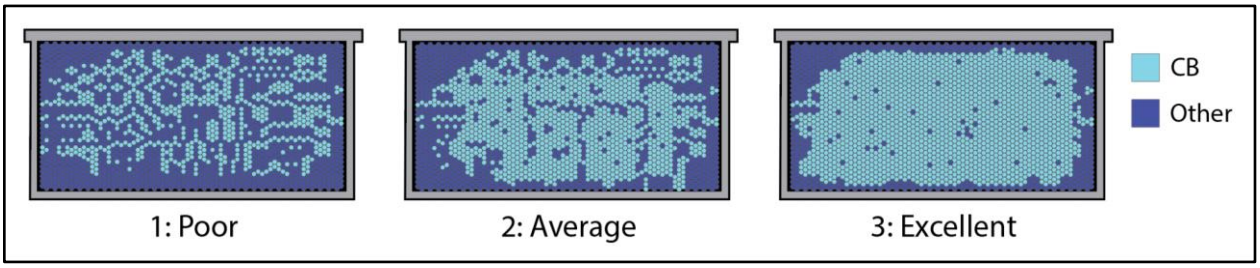


Figure 5. Brood pattern ranking system (0-3).

Parasite, Pest, and Disease Pressure

Ailments (parasites, pests, and diseases) were recorded as observations during inspections and categorized as small hive beetles (SHB), wax moths (WM), yellow jackets (YJ), and ants (A), European foulbrood (EFB), American foulbrood (AFB), parasitic mite syndrome (PMS), chalkbrood/stonebrood (CB/SB), nosema (NS), or an otherwise unidentifiable high viral load (HVL). Standard alcohol mite washes were conducted three times throughout the sampling seasons of each year (Gregorc and Sampson 2019). Ailments were only reported as a factor contributing to brood pattern.

Statistical Analysis

All statistical analyses were performed in R 2022.07.0. The Shannon Diversity Index was calculated for in-pasture plant composition. Linear mixed-effect models were used to evaluate the relative and interactive effects of site treatment, and sample period in regard to colony weight, pre-eclosion population metrics, stored resource metrics, adult population (FOB) and overwintering success, brood pattern, and in-pasture plant composition. Hive metrics were analyzed by year to account for the reestablishment of lost hives in 2022 and were considered

statistically significant if $P < 0.10$. Hives that survived the winter of 2021-2022 were included with re-established (2022) hives in the 2022 analyses.

Results

Hive Weight

Hive weight ranged from 9.8 kg to 77.8 kg with a mean weight of 37 kg. In 2021, hive weight ranged from 13.5 kg to 77.8 kg with a mean weight of 40 kg. Overall, hive weight was not significantly associated with pasture treatment type ($P > 0.10$), sample period ($P > 0.10$), or the interactive term ($P > 0.10$). In 2022, hive weight ranged from 10 kg to 72.6 kg with a mean weight of 34.3 kg. Overall, hive weight was not significantly associated with pasture treatment type ($P > 0.10$), sample period ($P > 0.10$), or the interactive term ($P > 0.10$). Hives in diversified pastures tended to be 25% heavier on average than those in conventional pastures (Figure 7).

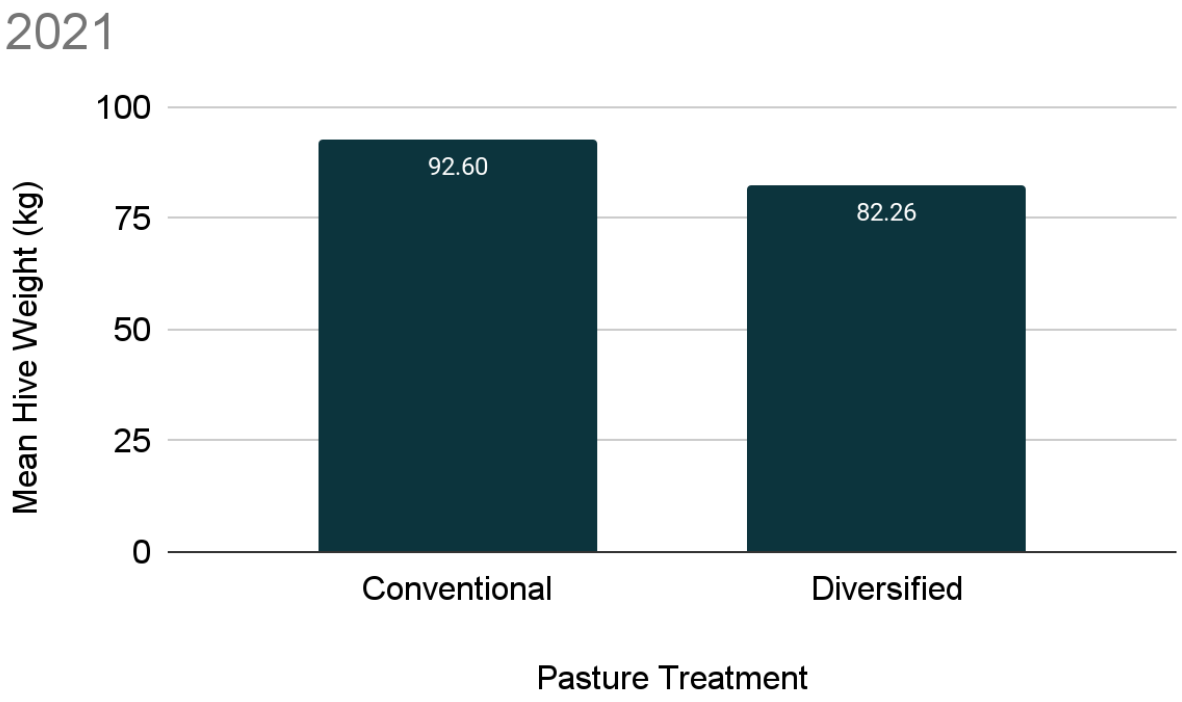


Figure 6. Mean hive weight (kg) for hives in conventional and diversified pasture systems in 2021.

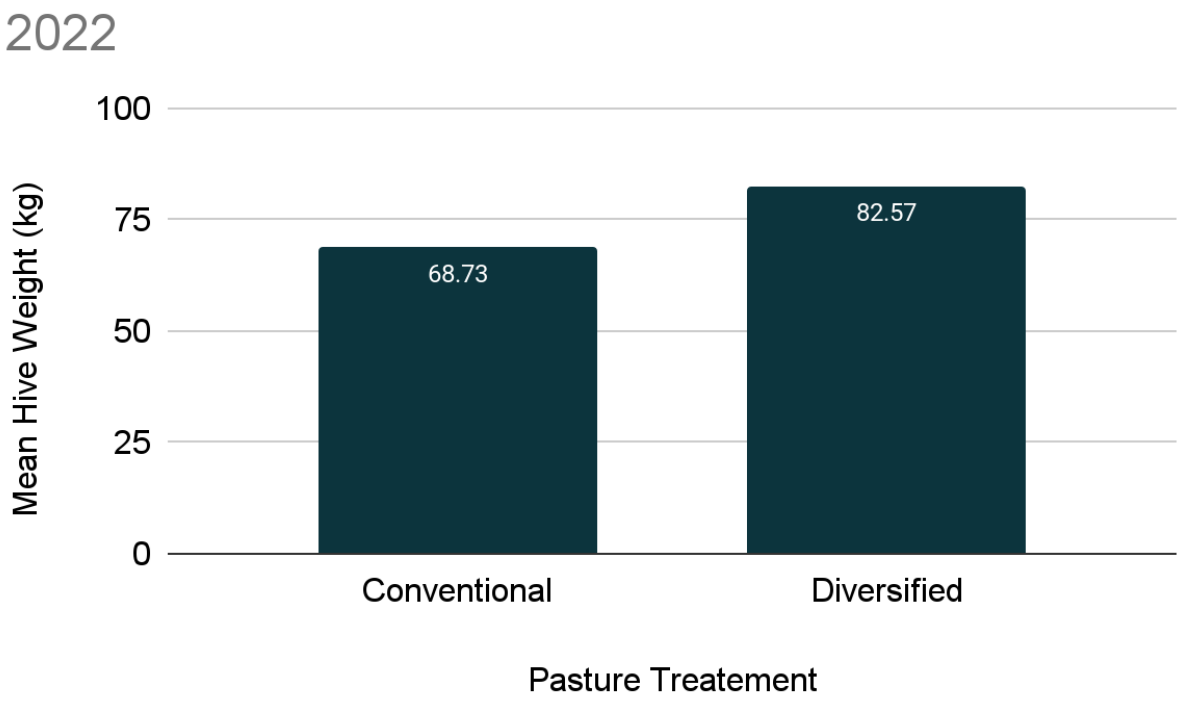


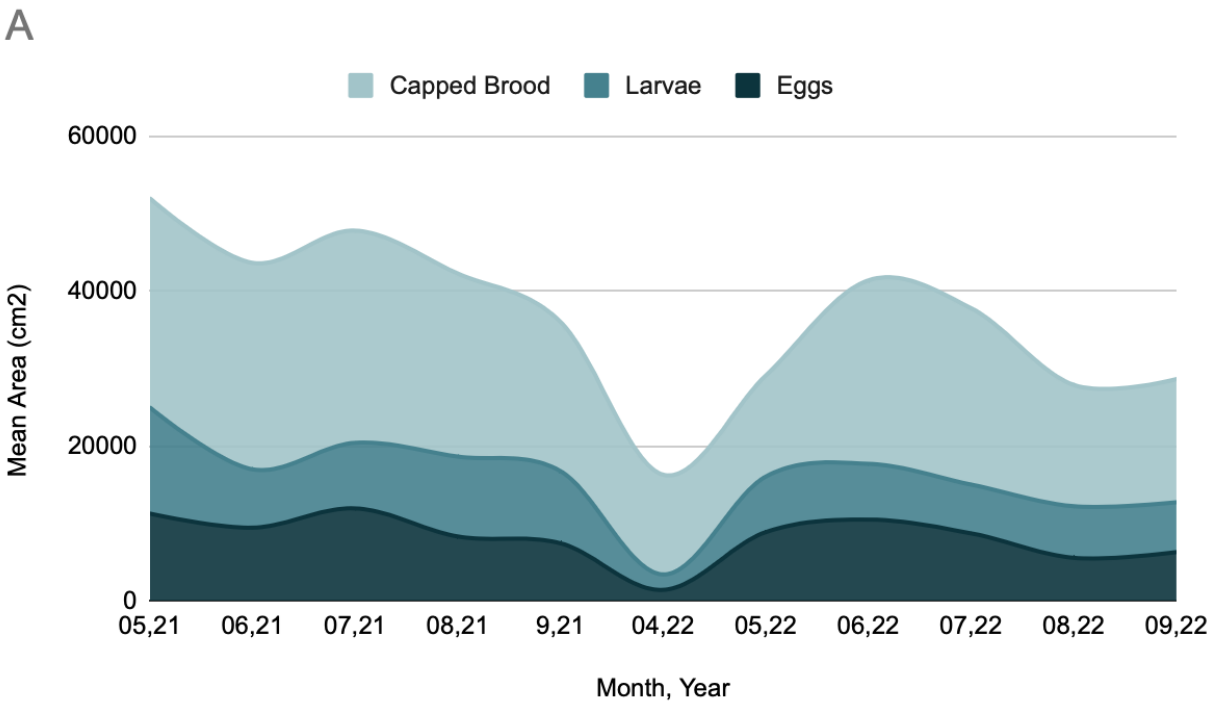
Figure 7. Mean hive weight (kg) for hives in conventional and diversified pasture systems in 2022.

Pre-Eclosion Population Metrics

In both 2021 and 2022, the area allocated to eggs, larvae, and capped brood was not significantly associated with pasture treatment type ($P > 0.10$) (Table 6; Figure 8). In 2021, the area allocated to eggs and capped brood was significantly associated with the sample period ($P < 0.05$), but not the interactive term. The area allocated to larvae was not significantly associated with the sample period or the interactive term. In 2022, none of the pre-eclosion population metrics were significantly associated with sample period, treatment, or the interactive term (Table 6).

Table 6. Mean area (cm^2) allocated to pre-eclosion metrics (eggs, larvae, capped brood) and p-value of pasture treatment type.

	2021			2022		
	Divers.	Conv.	P-value	Divers.	Conv.	P-Value
Eggs (cm^2)	9,497	8,545	0.81	7,224	8,299	0.57
Larvae (cm^2)	9,733	9,566	0.85	6,168	8,464	0.76
Capped Brood (cm^2)	24,381	23,437	0.46	17,884	19,541	0.16



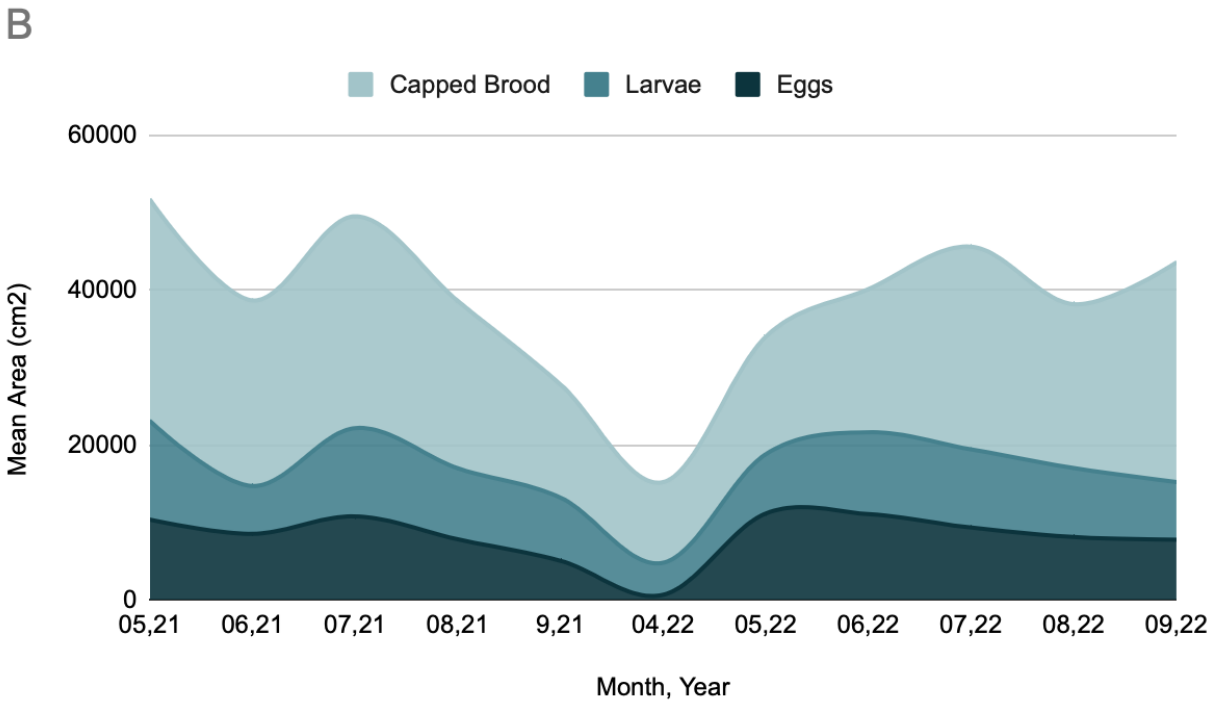


Figure 8. Mean area allocated to eggs, larvae, and capped brood of hives in (A) NWSGW+ diversified and (B) conventional pasture systems.

Adult Population & Overwintering Success

Adult population estimates ranged from 0 to 24 frames of bees (FOB) with a mean of 8.3 FOB (Figure 9). On average, the adult population was higher for hives within diversified systems compared to conventional systems. Overall, the adult population was not significantly associated with pasture treatment type ($P > 0.10$), sample period ($P > 0.10$), or the interactive term ($P > 0.10$).

In 2021, upon hive establishment, the adult population was equivalent across all hives and treatments. Prior to the fall FOB count, 2 hives were lost, 1 from a diversified system and 1 from a conventional system. In the fall of 2021, the adult population ranged from 0 to 24 with a

mean of 14.25 FOB (Figure 9). Numerically, hives within diversified pasture systems had 1.37 FOB greater on average than hives in conventional pasture systems.

In the spring of 2022, 43% of hives in diversified pasture systems had survived the winter compared with 29% of hives in conventional pasture systems survived the winter. The adult population ranged from 0 to 16 with a mean of 3.6 FOB. Numerically, hives within diversified pasture systems had 3.5 FOB more than hives in conventional pasture systems (Figure 10). In the fall of 2022, the adult population ranged from 0 to 20 with a mean of 7.9 FOB (Figure 9). However, the adult population was not significantly associated with pasture treatment type ($P > 0.10$).

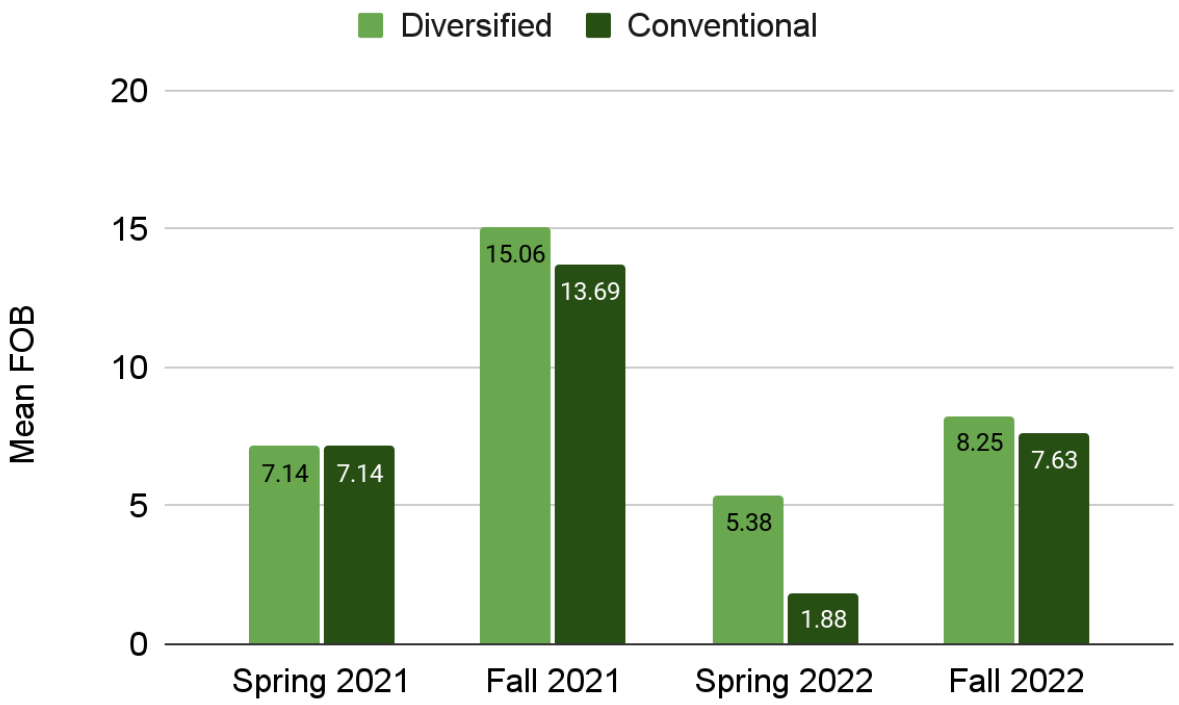


Figure 9. Mean number of frames of bees (FOB) in colonies in diversified and conventional pasture systems throughout 2021 and 2022 sampling seasons.

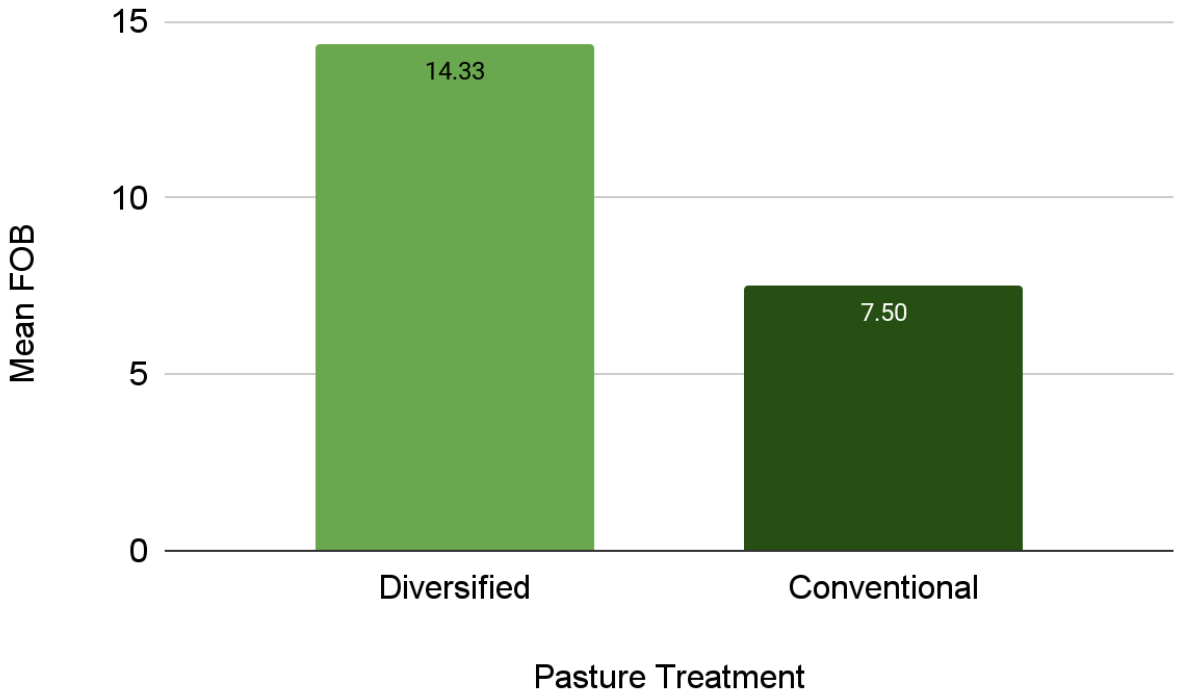


Figure 10. Mean number of frames of bees (FOB) of colonies in diversified and conventional pasture systems that survived the 2021-2022 winter.

Stored Resources

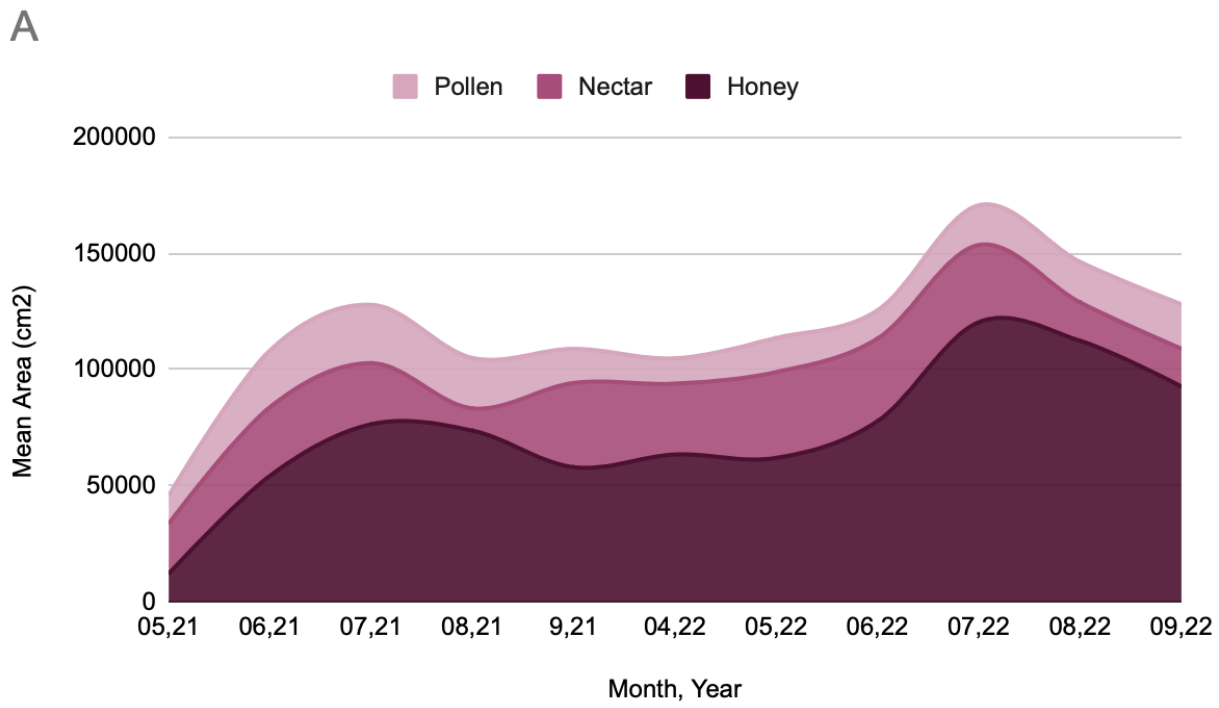
In 2021, the mean area allocated to honey, nectar, and pollen was not significantly associated with pasture treatment type ($P > 0.10$) (Table 7; Figure 11). The mean area allocated to honey was significantly associated with the sample period ($P < 0.05$) and the interactive term ($P < 0.10$). However, the mean area allocated to nectar and pollen were not significantly associated with the sample period or the interactive term ($P > 0.10$).

In 2022, the mean area allocated to nectar was significantly greater in diversified pasture systems compared to conventional grazing systems ($P < 0.10$), but the mean area allocated to pollen was significantly greater in conventional systems compared to diversified pasture systems ($P < 0.05$) (Table 7). The mean area allocated to honey was not significantly associated with pasture treatment ($P > 0.10$) (Table 7). On average, colonies in diversified systems increased the

mean area allocated to stored resources by 35% whereas those in conventional systems experienced a 3% decrease from 2021 to 2022, however this was not statistically significant.

Table 7. Mean area (cm^2) allocated to stored resource metrics (honey, nectar, and pollen) and p-value of pasture treatment type.

	2021			2022		
	Divers.	Conv.	P-value	Divers.	Conv.	P-Value
Honey (cm^2)	53,606	69,692	0.79	87,281	74,962	0.16
Nectar (cm^2)	23,828	20,648	0.66	28,762	19,297	0.08*
Pollen (cm^2)	19,210	23,302	0.93	14,841	15,930	0.02*



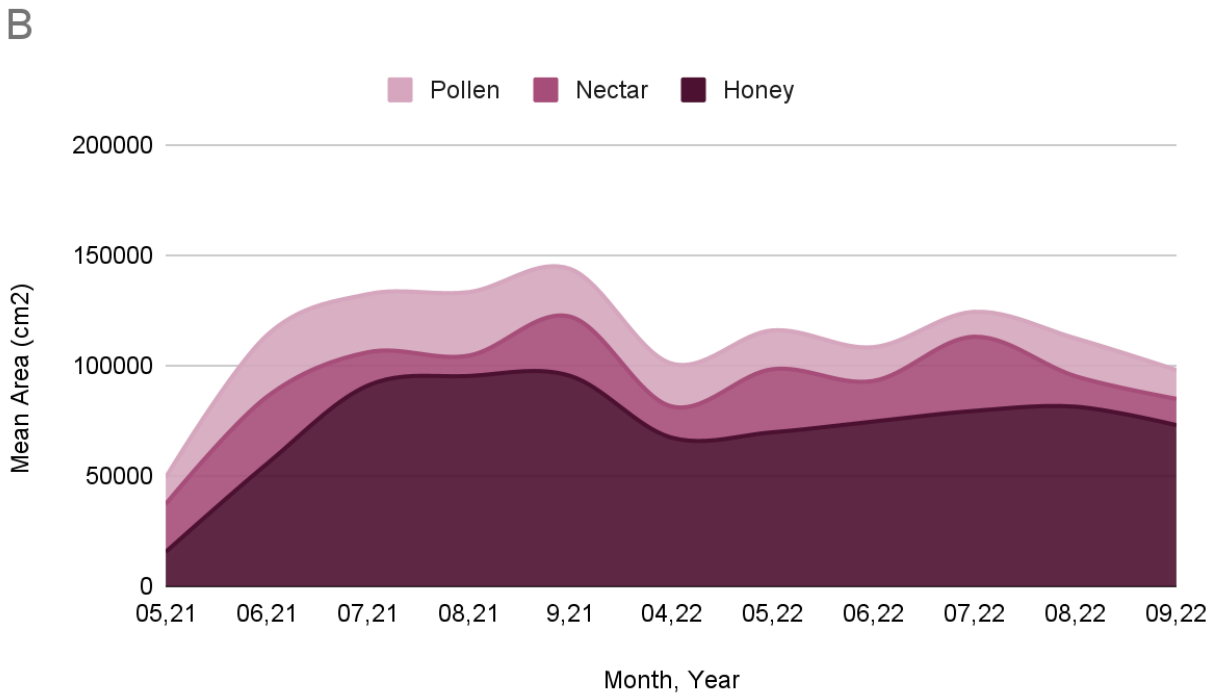


Figure 11. Mean area allocated to pollen, nectar, and honey of hives in (A) NWSGW+ diversified and (B) conventional pasture systems.

Brood Pattern

In 2021, brood pattern ranged from 0 to 3 with a mean of 2.06. Overall, brood pattern was not significantly associated with sample period, pasture treatment type, or the interactive term ($P > 0.10$). Numerically, hives in diversified pastures tended to be 0.17 points higher than those in conventional pastures (Figure 12). In 2022, brood patterns from all sites and sampling periods ranged from 0 to 3 with a mean of 2.16. Overall, brood pattern was significantly associated with pasture treatment type ($P < 0.10$), sample period ($P < 0.10$), but not the interactive term ($P > 0.10$). Numerically, hives in diversified pastures tended to be 0.26 points higher on average than those in conventional pastures (Figure 12).

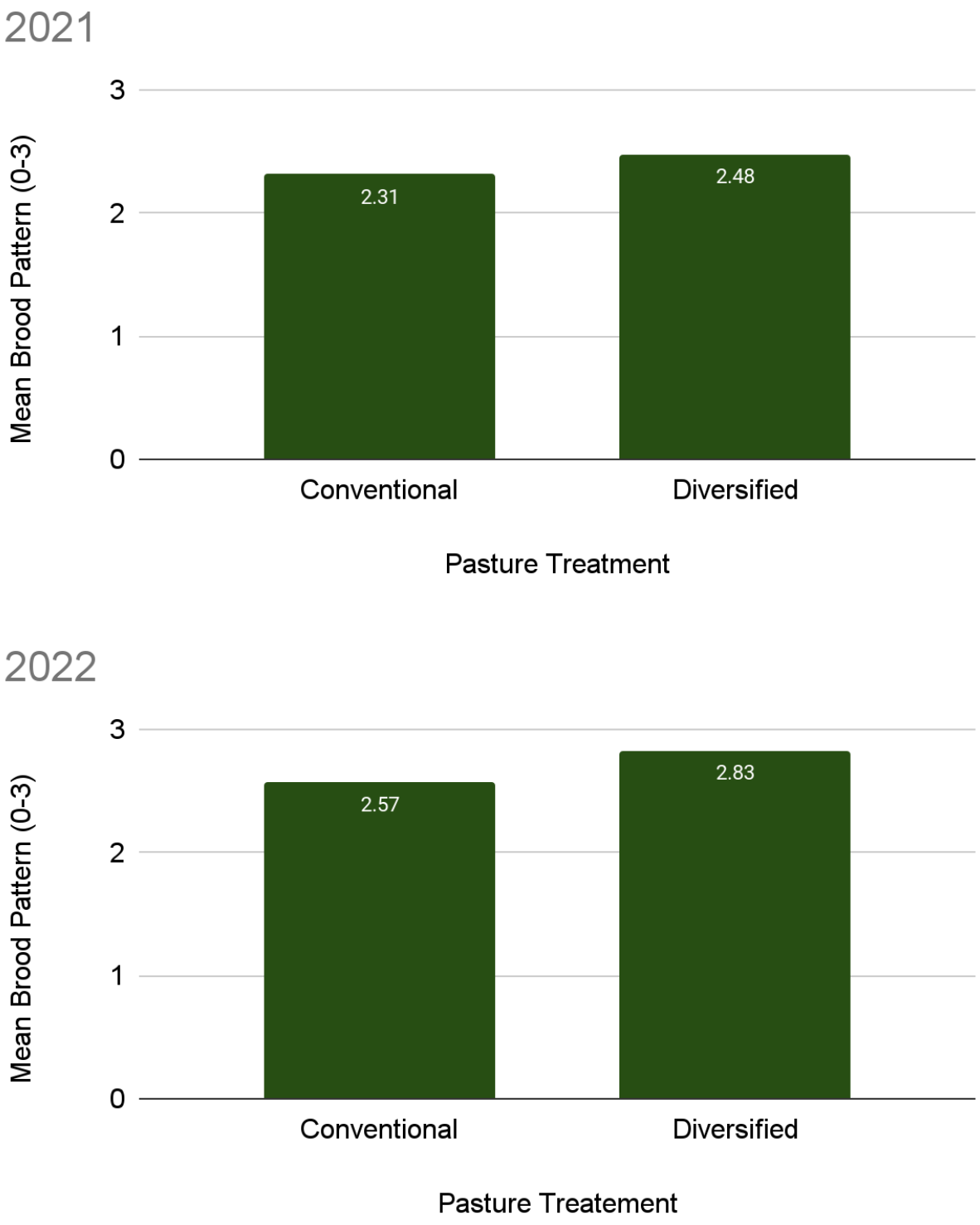


Figure 12. Mean brood pattern in NWSGW+ diversified and conventional pasture systems in 2021 and 2022.

Plant Species Composition of Pastures

In 2020, percent cover averaged 27%, 5%, and 14% for sown, weed, and bare ground, respectively in diversified pastures (Figure 13). Average sown wildflower density was 3.4 stems per m^2. *Echinacea purpurea* was the dominant sown wildflower species, representing an average of 14 % of total stems, followed by *Helianthus maximiliani* (11%) and *Helianthus angustifolius* (11%) (Figure 14; Figure 15). *Sorghastrum nutans* accounted for 70% of total sown grass, followed by *Schizachyrium scoparium* (15%), and *Andropogon gerardii* (13%) (Figure 14; Figure 15). Average sown grass density was 3.4 stems per m^2.

In 2021, sown, weedy, and bare ground accounted for 34%, 46%, and 19% respectively of ground cover (Figure 13). Average sown wildflower density was 13 stems per m^2. *Rudbeckia hirta* was the dominant sown wildflower species, representing an average of 34% of total stems counted, followed by *Coreopsis lanceolata* (30%) and *Helianthus maximiliani* (11%) (Figure 14; Figure 15). *Sorghastrum nutans* accounted for 49% of total sown grass, followed by A*ndropogon gerardii* (49%), *Schizachyrium scoparium* (2%) (Figure 14; Figure 15). Average sown grass density was 4.5 stems per m^2. Of the 26 sown species, 21 were observed in 2020, decreasing to 19 in 2021.

In 2022, sown species accounted for 32%, unsown species 58%, and bare ground 8% of diversified pasture coverage on average (Figure 13). In conventional pastures, unsown species accounted for an average of 92.3%, and bare ground accounted for an average of 7.7% of pasture coverage. An average of 40.21% of all blooms in diversified pastures were sown species and 59.79% were unsown species (Figure 15). *Rudbeckia hirta* was the dominant sown wildflower species, representing an average of 16.54% of total blooms counted (31.05% of sown species), followed by *Coreopsis lanceolata* (11.86% of total; 22.27% of sown) and *Ratibida pinnata* (7.4% of total; 13.88% of sown). *Trifolium repens* was the dominant unsown wildflower species,

representing an average of 15.71% of total blooms counted, followed by *Trifolium pratense* (13.87%) and *Erigeron sp.* (9.01%) (Figure 14).

Between 2020 and 2022, mean sown species coverage increased by 5.73%, unsown species coverage decreased by 0.7%, and bare coverage decreased by 5.02% in diversified pastures (Figure 13). No species included in NWSGW+ seed mixes were observed in conventional pastures.

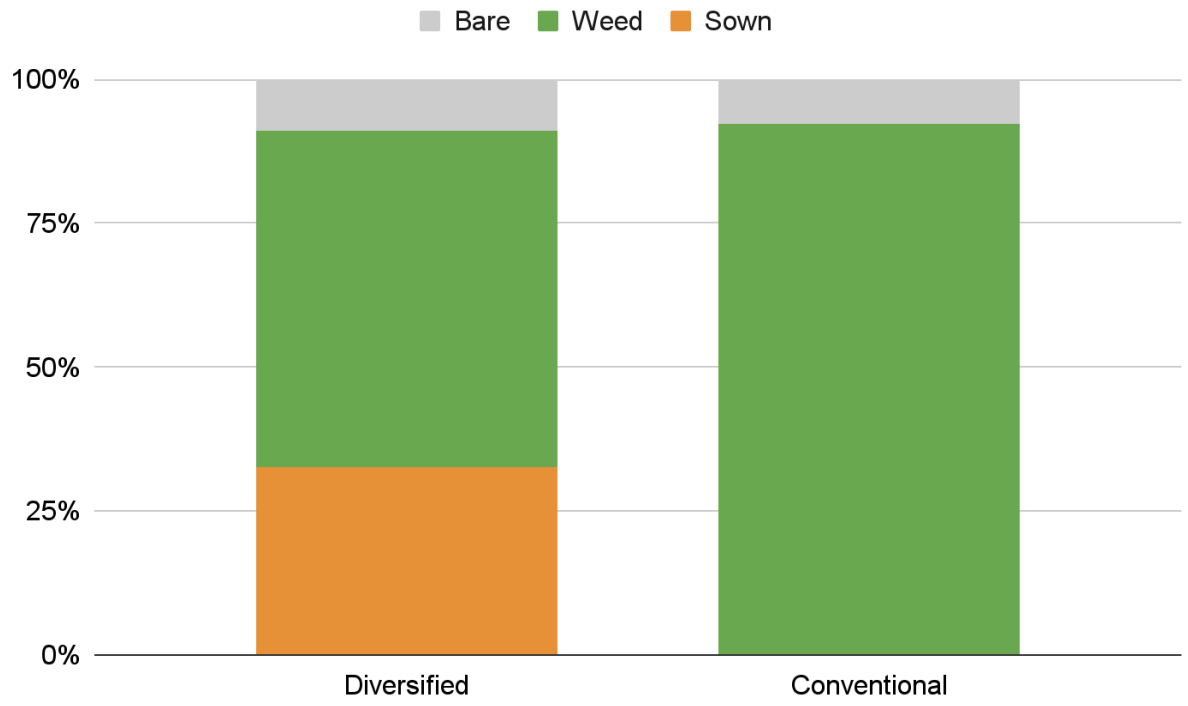


Figure 13. Mean percent cover type for NWSGW+ diversified and conventional pastures (2022).

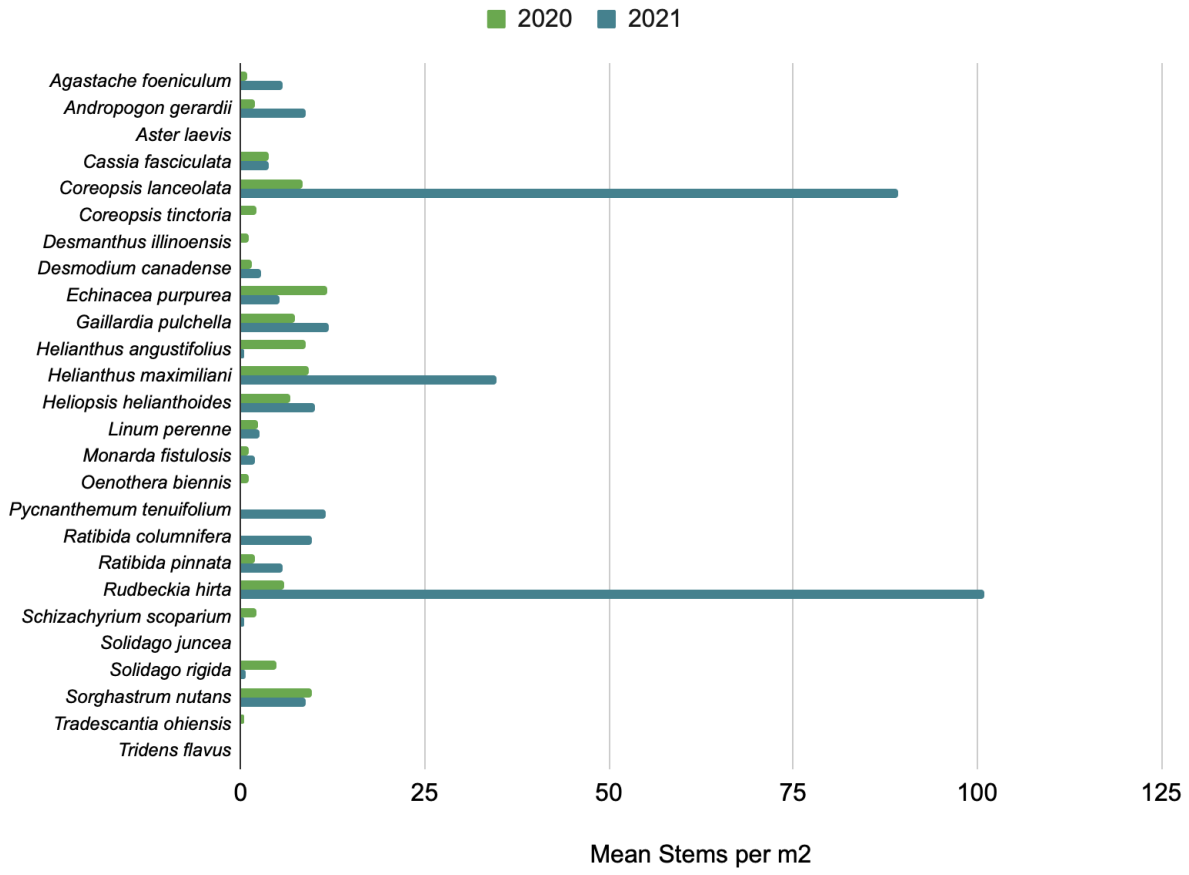


Figure 14. Mean sown species stem density of NWSGW+ diversified pastures (2020, 2021).

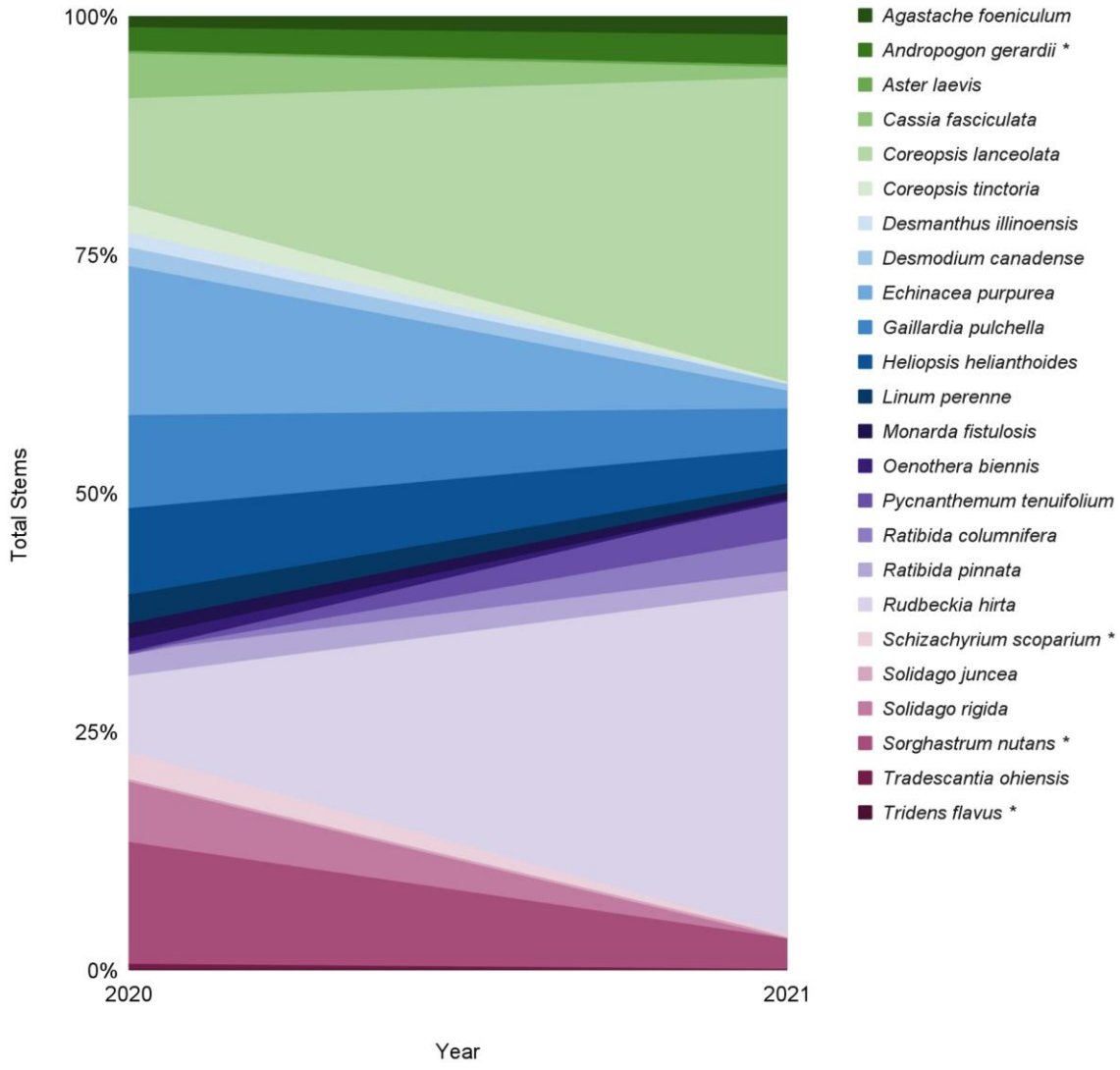


Figure 15. Relative abundance of sown species in NWSGW+ diversified pastures (2021-2022).

In-Pasture Floral Resources

A total of 4,472 blooms were recorded in 2022. Both unsown native and non-native species were significantly related to NWSGW+ pasture diversification ($P < 0.10$). In diversified pastures, mean bloom density by species ranged from 0 to 0.8 blooms per m^2 with a mean density of 0.076 and was highest in June and lowest in August (Figure 16). Sown species

accounted for 53.28%, unsown native species for 13.76%, and unsown non-native species for 32.95% of total blooms observed (Figure 20).

On average, the most abundant in-bloom sown wildflower species in diversified pastures was *Coreopsis lanceolata* (18.21%) in June, and *Monarda fistulosis* in July and August (6.35%; 9.11%). The most abundant in-bloom unsown wildflower species in diversified pastures was *Bellis perennis* in June (15.27%), *Erigeron sp.* in July (53.96%), and *Verbena urticifolia* in August (20.25%) (Figure 18). Unsown native species accounted for 13.04%, and unsown non-native species accounted for 86.96% of total blooms (Figure 20). No sown species were observed in conventional pastures. In conventional pastures, the most abundant in-bloom species was *Cichorium intybus* in June (25.46%), *Sisymmbrium officianale* in July (42.32%), and *Polygonum sp*. in August (28.6%) (Figure 17; Figure 19). In early summer, a majority of the total blooms observed in diversified pastures belonged to unsown species (Figure 20). However, a majority of blooms belonged to sown species in late summer (Figure 20).

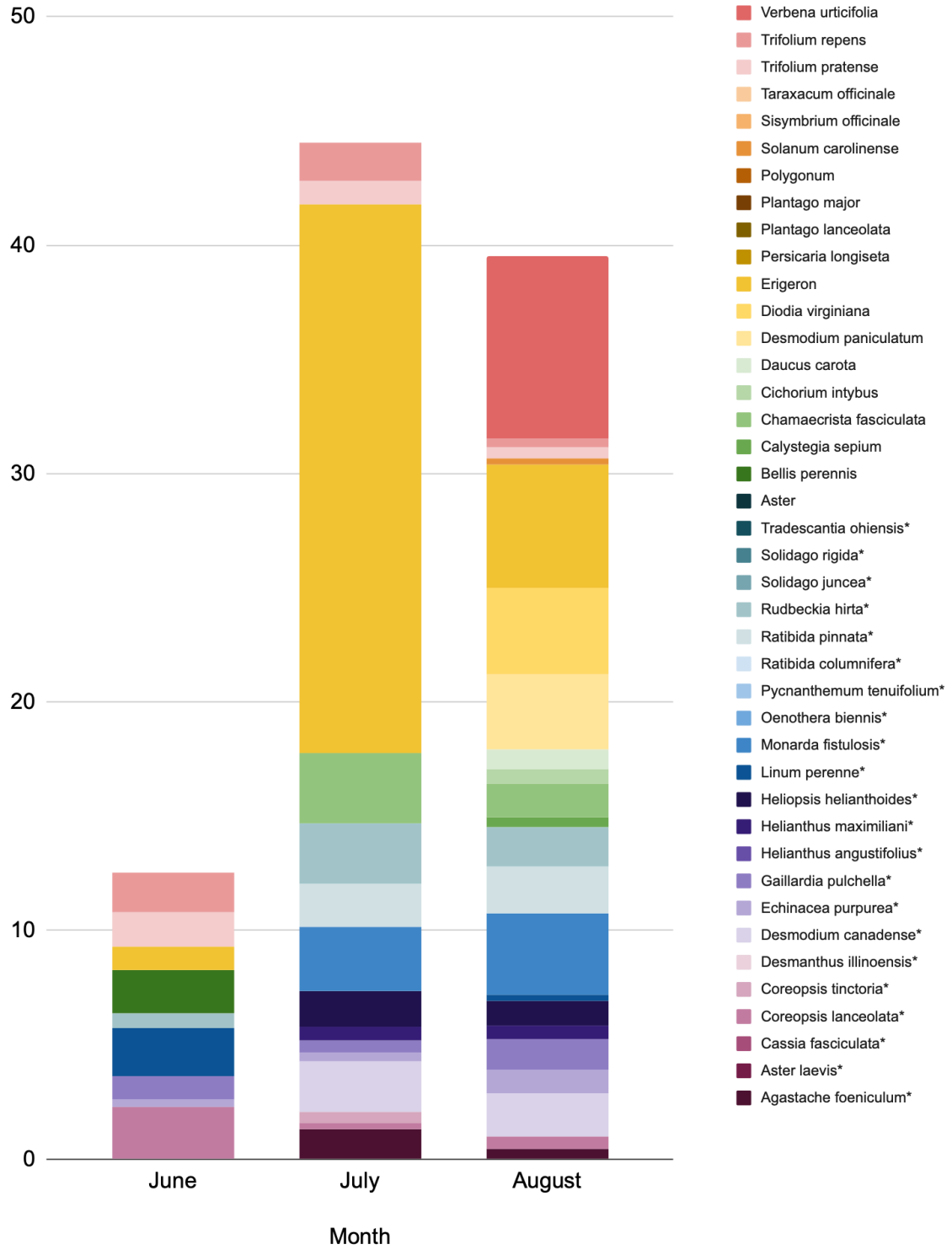


Figure 16. Mean bloom density of sown (*) and unsown species in NWSGW+ diversified pastures (2022).

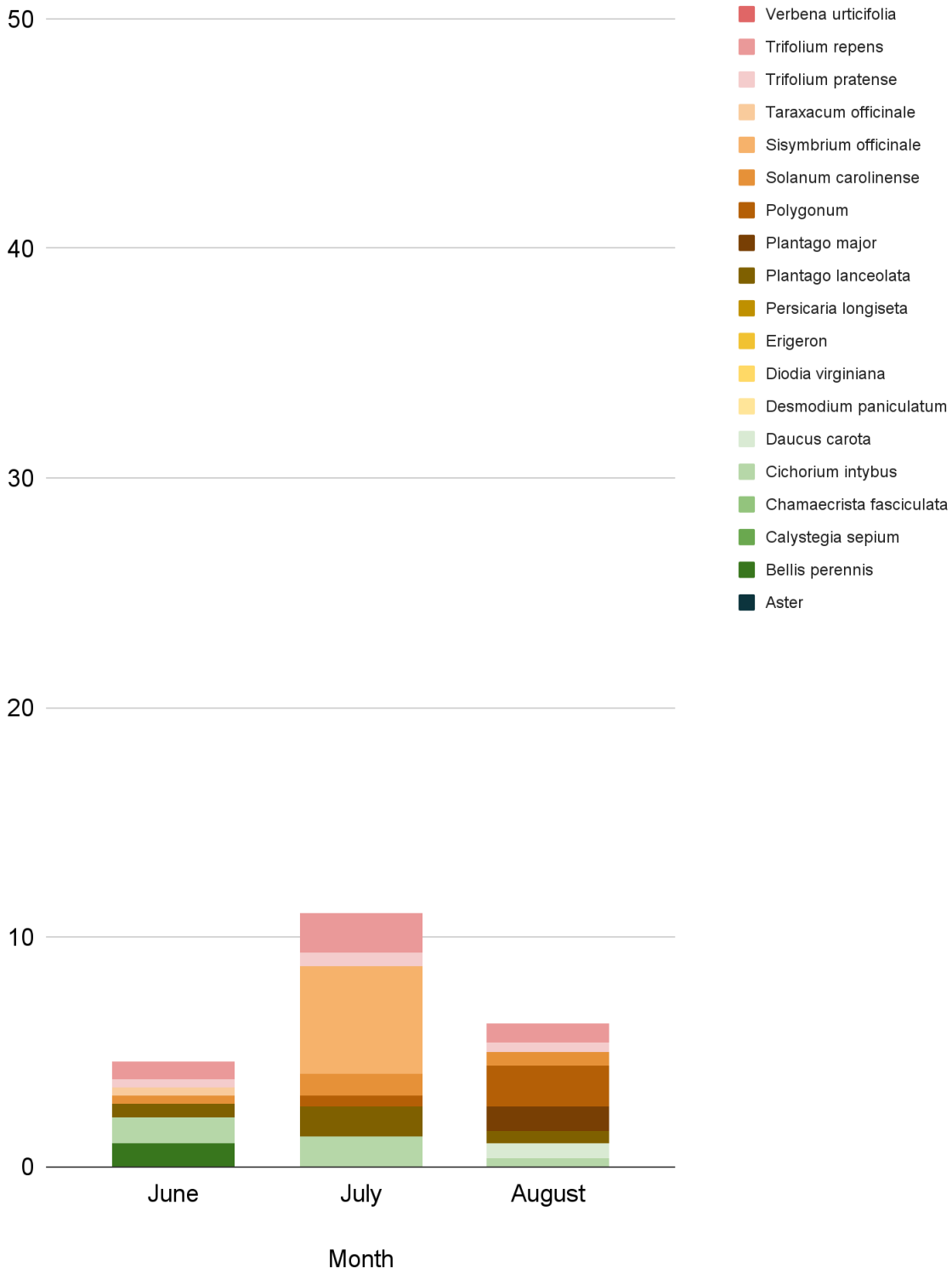


Figure 17. Mean bloom density of sown (*) and unsown species in conventional pastures (2022).

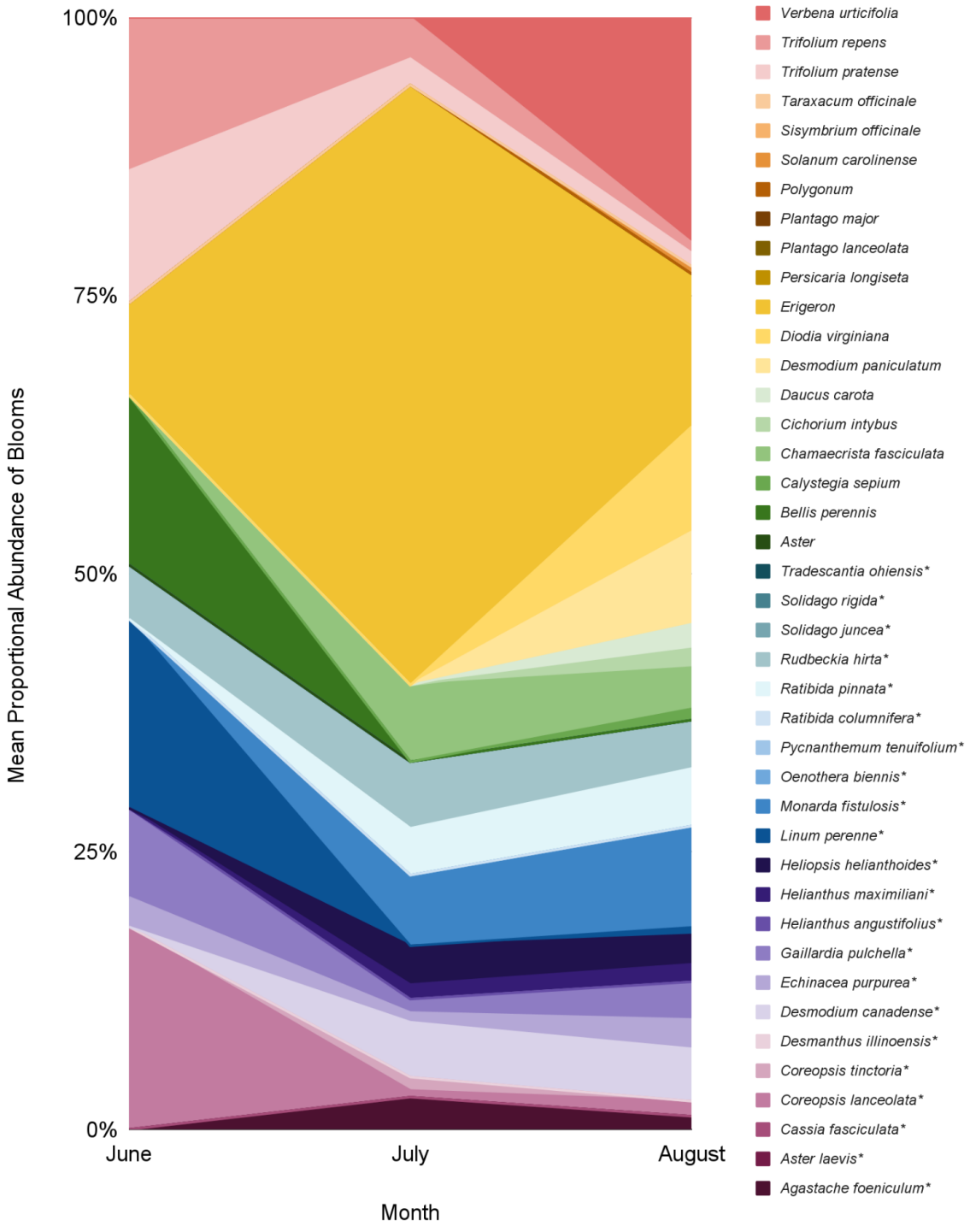


Figure 18. Proportional abundance of blooms by sown (*) and unsown species in NWSGW+ diversified pastures (2022).

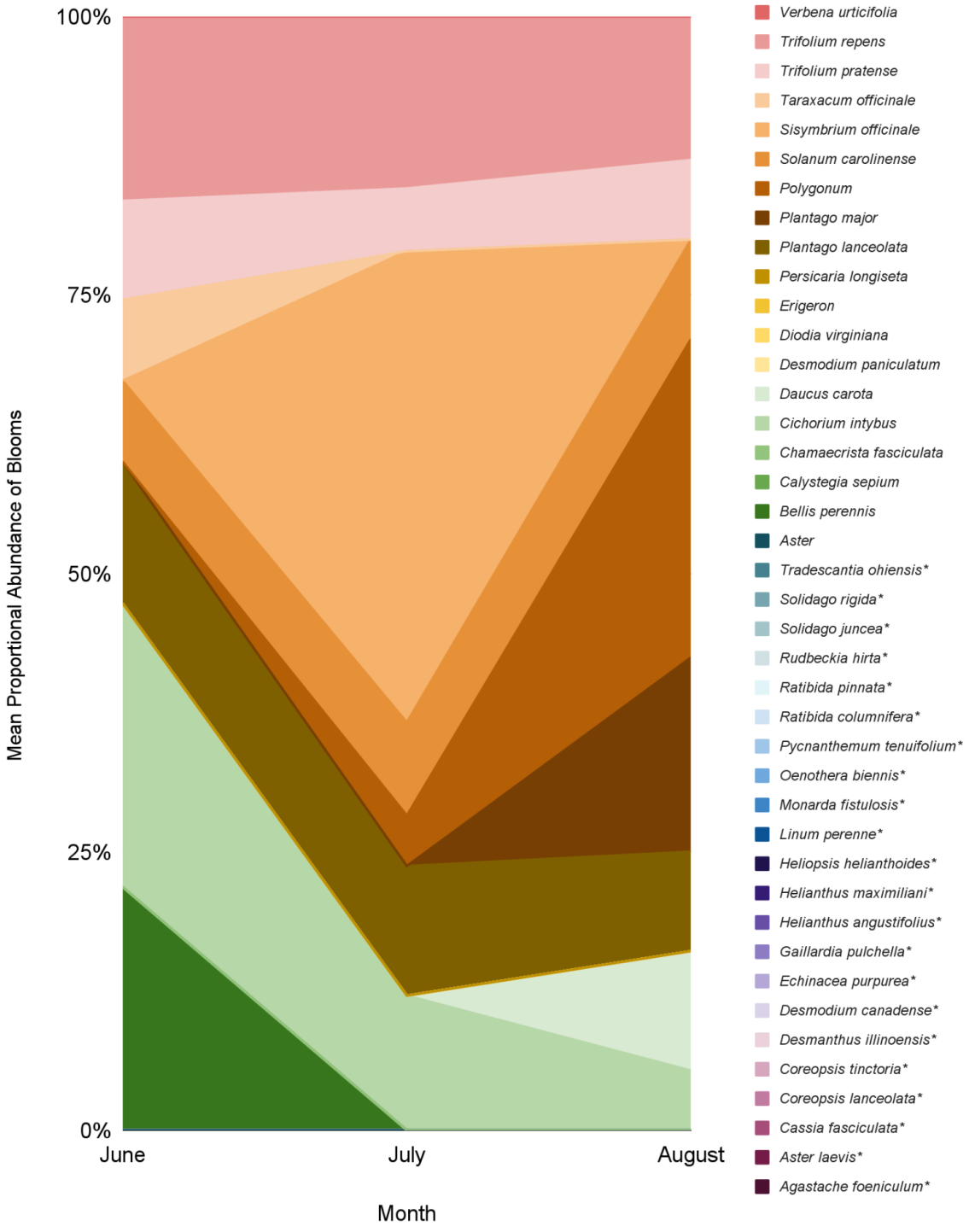


Figure 19. Proportional abundance of sown (*) and unsown blooms in conventional pastures (2022).

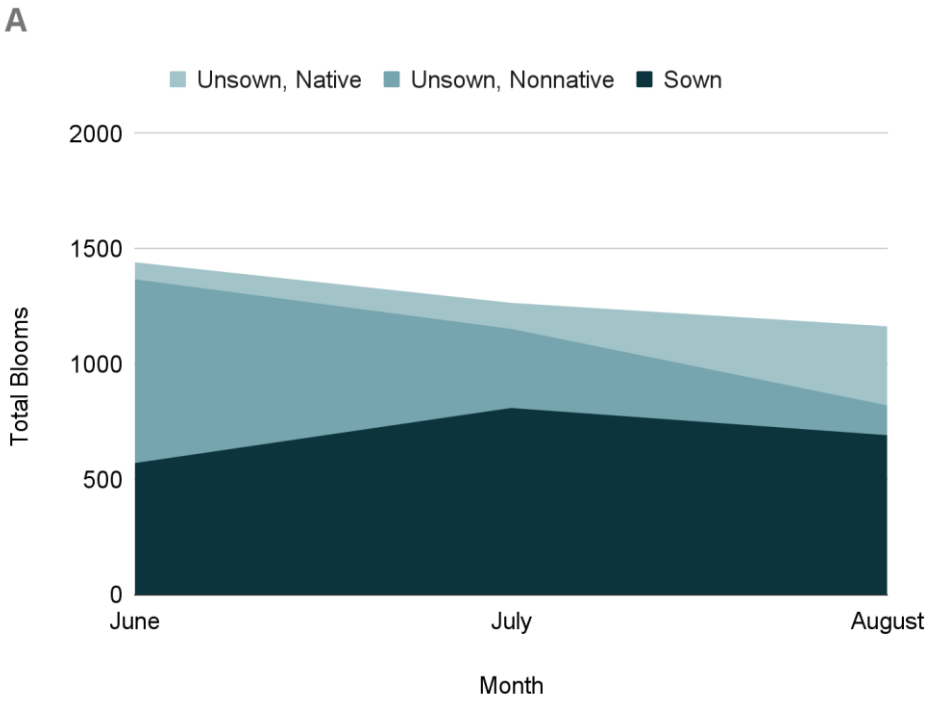


Figure 20. Total sown, unsown nonnative, and unsown native blooms in NWSGW+ diversified pastures (2022).

Discussion

Hive Weight

Mean colony weight decreased in conventional pasture treatments from 2021 to 2022, whereas colonies in diversified treatments showed a slight increase. The positive correlation between floral resource quality and honey bee colony success is well known (Biesmeijer et al. 2006; Bretagnolle and Gaba 2015; Brummitt et al. 2008). Yet the true effect of the surrounding landscape on honey bee colony weight remains unclear. Previous research has shown that colony weight will not vary significantly due slight differences in surrounding land types (St. Clair et al. 2020; McMinn et al. 2022). However, cropland has been shown to be more favorable than more florally deprived landscapes (such as urban environments) (Sponsler and Johnson 2015).

The differences observed in mean colony weights between 2021 and 2022 suggest that colonies in NWSGW+ diversified pasture systems may be slightly more robust and may have greater long-term success compared to those in conventional pasture systems. However, it is also possible that the NWSGW+ diversified pastures may not be large enough to cause a significant difference in accumulated colony weights. A longer sample period is needed to determine the long-term effects of NWSGW+ diversification on accumulated colony weight.

Pre-Eclosion Population Metrics

In 2021, colonies in both pasture treatments exhibited a bimodal pattern with peaks in early June (SP5) and early September (SP8). In mid-June, several colonies swarmed, leading to a reduced area allocated to population growth as the new queens mated and began to lay eggs. The bees established in 2021 exhibited a strong genetic predisposition to swarming behaviors (i.e. constantly raising a surplus of queen cells). Swarming was consistent across the pasture treatments and was not found to be statistically significant in colony metrics.

Many hives did not successfully overwinter from 2021 to 2022 leading to a reduced area allocated to population growth in the spring of 2022 (SP11). The area allocated population growth peaked in late June (SP14) and early August (SP16) for colonies in diversified pasture systems. For colonies in conventional pasture systems, the area allocated population growth peaked in late June (SP14) and mid-September (SP18). This suggests that there may be a slight lag in population growth ability in conventional systems.

Adult Population & Overwintering

On average, honey bee colonies in NWSGW+ diversified pasture systems had larger adult populations in the fall of 2021, spring of 2022, and fall of 2022 though these differences were not found to be statistically significant. The largest difference in pasture treatment was observed in the spring of 2022 predominantly due to the differences in overwintering survival. Of the three colonies that did survive the winter in diversified pasture systems, the mean FOB was 14.3. Of the two colonies that survived the winter in conventional pasture systems, the mean FOB was 7.5. Not only did colonies in diversified pasture systems have a greater winter survival rate as a whole, but had a 52% larger population size in colonies that did survive.

In temperate climates, honey bees enter a distinct physiological state to survive the winter. Successful winter survival is one of the most important aspects of beekeeping and has been strongly correlated with a multitude of factors including population metrics, quantity and quality of stored nutritive resources, queen health, genotype, exposure to pesticides, and *Varroa destructor* mite populations (Dooremalen et al. 2012; Doke et al. 2015; Brosi et al. 2016). Larger colonies use stored resources more efficiently and have greater potential for growth and further resource acquisition following winter survival (Doke et al. 2015).

Stored Resources

Overall, colonies in both NWSGW+ diversified and conventional pasture systems showed a positive trend in the mean area allocated to stored nutritional resources from establishment to the end of the 2022 sampling season.

In 2021, colonies within both pasture types had the greatest mean area allocated to stored resources in late July (SP6) and late September (SP10). The peak observed in late September can

partially be attributed to the storage of 2:1 sugar water fed to the bees starting in mid-September (SP9). At the end of the 2021 sampling season, colonies within diversified pasture systems had an average of 133,443 cm2 allocated to stored nutritional resources whereas colonies in conventional systems had 145,182 cm2.

In 2022, colonies within both pasture types had the greatest mean area allocated to stored resources in mid-July (SP15). The colonies were not fed in the fall of 2022. At the end of the 2022 sampling season (SP18), colonies within diversified pasture systems had an average of 128,028 cm2 allocated to stored nutritional resources whereas colonies in conventional systems had 97,948 cm2.

On average, colonies in diversified systems demonstrated a 35% increase in the mean area allocated to stored resources whereas those in conventional systems experienced a 3% decrease from 2021 to 2022. This, in conjunction with other hive attributes, will affect the overwintering success rates going into the spring of 2023. According to these results, colonies in NWSGW+ diversified pasture systems may have greater long-term success compared to those in conventional pasture systems.

Brood Pattern

Throughout 2021 and 2022 colonies in diversified pasture systems had slightly higher rated brood patterns than colonies in conventional systems. However, these differences were not found to be statistically significant. As an indicator of queen health, colony strength, pest and disease pressure, and environmental suitability, slight differences in brood pattern could become biologically significant in determining overwintering success (Lee et al. 2019; Guzman-Novoa and Page 1999). Ultimately, it is unclear whether the slight differences observed in brood

patterns were a result of greater bloom availability in diversified pasture systems or a result of random genetics, pest or disease pressure, or unrecorded environmental factors.

Establishment of NWSGW+ Pastures

Species richness of NWSG+ diversified pastures changed significantly each year. Previous research has demonstrated that both sown wildflower and NWSG species may exhibit annual and biennial habits, in which productivity may peak or wane three to four years post-establishment (Bonin and Tracy 2012, Tracy and Bonin 2013, Bonin et al. 2014). Annuals are often eventually replaced by perennial species (De Cauwer et al. 2005).

A few dominant species were more successful while many sown species struggled to fully establish. *Aster laevis*, *Solidago juncea*, and *Tridens flavus* were sown but not observed in pastures. Diversified pastures were primarily dominated by *Rudbeckia hirta* (39% total blooms, 2022) and *Coreopsis lanceolata* (25.54% total blooms, 2022). Competition, habitat suitability, complex germination behaviors, and re-seeding capability are likely to have driven the ultimate establishment outcomes of these species (Aldrich and Norcini 2006). *Rudbeckia hirta* is known to be an aggressive floral competitor, and *Coreopsis lanceolata* is well equipped to re-seed in excess.

For goldenrod species, such as *Solidago juncea*, and other wetland-preferring wildflowers, dryer pasture habitat type, similar to those in this study, may not be suitable for their success. However, leguminous native forbs such as *Desmanthus illinoensis* and *Chamaecrista fasciculata*, and some non-native forbs observed in NWSGW+ diversified pastures, such as *Trifolium repens* and *Trifolium pratense,* are well suited to pasture habitat and

intraspecific interactions (USDA- NRCS 2022). A greater focus on seed mix design will prove to be useful in future NWSGW+ pasture renovation efforts.

In-Pasture Floral Resources

Honey bee colony success is directly related to the abundance of high-quality, diverse, and consistent floral resources (Mattila and Otis 2006; Jevtić et al. 2009; Gallant et al. 2014; Requier et al. 2015; Brosi et al. 2016; Smart et al. 2016). Despite some establishment failures, NWSGW+ diversified pastures had significantly higher bloom densities and species richness throughout the growing season. To prepare pastures for NWSGW+ seed mix establishment, existing vegetation was first suppressed, allowing unsown dormant and successive forb species to establish. Mean bloom density was 4.96x greater in diversified pastures than in conventional pastures. When evaluating the functionality of pollinator habitat restoration efforts, it is important to recognize unsown species as potentially advantageous forage.

Over half of all blooms recorded in diversified pastures were unsown species (59.79%). The most abundant unsown species in 2022 were *Trifolium repens, Trifolium pratense, and Erigeron* species. Both *Trifolium repens* and *Trifolium pratense* are non-native, pasture-dwelling leguminous forbs that are known to be valuable forages for honey bees (Goodwin et al. 2011). Native *Erigeron* species are considered to be durable pioneers that primarily exist in dry, mountainous areas and grasslands. Other frequently observed unsown non-native species included *Bellis perennis*, *Cichorium intybus*, and *Sisymbrium officinale*. Native species included *Verbena urticifolia* and *Polygonum*.

In both diversified and conventional pastures, a large majority of unsown species were non-native. As honey bees are a super-generalist non-native species, this is less likely to

negatively affect their foraging behavior than it is for wild pollinators. However, countless studies have documented both honey bee and wild pollinator use of non-native floral species (Nicolson et al. 2013; Richardson et al. 2015b; Seitz et al. 2020).

Conclusion

Despite having nearly 4x more blooms in diversified pasture systems, honey bee health and productivity did not differ substantially between diversified and conventional pasture systems overall. In the first year, colonies in conventional systems typically outperformed those in diversified pasture systems. However, in 2022, the opposite occurred. This could be attributed to the differences observed in over-wintering success in the spring of 2022. Having a more robust adult population in early spring, as seen in colonies in diversified systems, allows for vital hive responsibilities to occur at a much faster rate leading to greater population growth and area allocated to stored nutritive resources. This suggests that colonies in diversified pasture systems may have an advantage in population growth, winter survival, and overall health in subsequent years.

These observed differences could potentially become more prevalent and statistically significant as time continues. Additionally, a larger colony sample size may reveal more consistent trends. Previous research has shown that a greater abundance of diverse floral resources will provide more robust nutritional resources (i.e. honey, nectar, and pollen) for honey bee colonies (Bonoan et al. 2018; Woodard and Jha 2017; Dolezal and Toth 2018) and that land cover does significantly influence honey bee colony success (Kuchling et al. 2018). Considering the similar land cover tabulated within a 3km for this study, it is possible that the pastures

subjected to floral diversification could have been too small to support detectable differences between colonies in different pasture treatments.

It is important to also consider the other factors such as pollution, pest and disease pressure, resource competition, genetic predispositions, and environmental stressors that play an important role in determining overall honey bee colony success and health. Larger sample sizes, larger pasture renovations, and longer study periods could reveal more consistent trends. Still, NWSGW+ diversified pastures demonstrate potential for providing a more complex nutritional support system for honey bees and other insect pollinators in grazing agroecosystems.

Chapter 2:

Honey Bee Pollen Foraging in Native Grass-Wildflower Grazing Systems

Abstract

Compounding evidence suggests a current or impending sixth mass extinction event and pollinator crisis. While several factors contribute to pollinator declines, the most notable driver is habitat loss and degradation. Agricultural grasslands provide crucial habitat for wild and domesticated fauna, regulate water and nutrient cycles, store carbon, and maintain soil stabilization mechanisms. However, conventional tall fescue pastures, which dominate the southeastern United States, limit pollinator habitat, reduce ecosystem services, and diminish cattle productivity if infected with toxic endophytes. Establishing wildflowers (WFs) and native warm season grasses (NWSGs) into tall fescue pastures has the potential to boost pollinator ecosystem services and cattle productivity. As a part of a larger project (Chapter 1) where tall fescue pastures were diversified with NWSGs and WFs, the objectives of this study sought to: 1) determine whether honey bees used sown wildflower species in diversified pastures as significant sources of pollen, 2) compare species composition and nutritive value of pollen collected from hives within diversified and conventional pasture systems, and 3) evaluate temporal trends in pollen collection. Floral surveys revealed diversified pastures had almost 4x greater mean bloom density than conventional pastures, with over half of all blooms recorded in diversified pastures belonging to unsown species. Pollen DNA metabarcoding revealed that honey bees in both diversified and conventional pasture systems have similar diets, and that sown species were foraged upon primarily in the fall. Samples collected from diversified pasture systems yielded greater pollen weight, species richness, and protein content. In conjunction with

previous research, these results indicate that diversified pastures could ultimately provide a more complex nutritional support system for pollinators in grazing agroecosystems.

Introduction

Globally, pollinators are rapidly declining. Climate change, habitat fragmentation, reduced floral resource diversity, pests, parasites, pathogens, management practices, and reduced genetic diversity are among the most notable drivers of these declines (Watanabe 1994; Potts et al. 2010; Genersch 2010; Kremen and Miles 2012; Goulson et al. 2015; Jarvis 2018). The interactive, non-additive effects of these drivers may also increase the speed and severity of lethal and sublethal factors (Potts et al. 2010). Habitat fragmentation and reduced floral resource diversity directly contribute to nutritional deficits that can lead to starvation and increased susceptibility to viral and fungal diseases in wild and managed pollinator species (Woodard and Jha 2017; Dolezal and Toth 2018). Understanding the nutritional requirements and foraging preferences of pollinators is vital to effective conservation efforts.

As super-generalist pollinators, honey bees collect and store nutritive materials from a wide variety of entomophilous (Memmott et al. 2004; Valido et al. 2019) and anemophilous sources (Sabugosa-Madeira et al. 2008; Quinlan et al. 2022). Nectar provides carbohydrates, enzymes, amino acids, vitamins, and lipids (Simpson and Neff 1981; Percival 1961), whereas pollen primarily provides proteins, minerals, lipids, sterols, and other essential micronutrients not found in nectar (Villanueva et al. 2002; Markowicz Bastos et al. 2004; Almeida-Muradian et al. 2005; Human and Nicolson 2006; Caron 2013; Requier et al., 2015; Filipiak et al., 2017; Bonoan et al., 2018; Chakrabarti et al., 2019; Crone and Grozinger, 2021).

Pollen protein content can range from approximately 2.5% to 61% independent of pollination phenology (Roulston et al. 2000; Keller et al. 2005), yet bees typically avoid collecting pollen that is unusually poor or rich in protein (Roulston et al. 2000) especially after initial foraging experiences (Cook et al. 2003). As notoriously adaptable pollinators, honey bees may alter their foraging behaviors to account for reduced nutritional value (Latshaw and Smith 2005; Nicolson et al. 2013). The protein content of honey bee foraged pollen has been demonstrated to decrease in late summer to early fall and differ annually (Quinlan et al. 2022)

Seasonal weather conditions can affect forage availability and honey bee thermoregulation expenses (Schweiger et al. 2010). In temperate climates, honey bee pollen foraging habits vary temporally. In spring, colonies require large quantities of pollen to rebuild food stores and support substantial brood production. In summer, colonies continue brood production and food storage. Brood production stops in late fall and resumes in late winter, requiring the consumption of pre-existing pollen stores. An abundance of high-quality, diverse, and consistent floral resources is the principal factor determining honey bee colony growth, productivity, resilience, and overwintering success (Mattila and Otis 2006; Jevtić et al. 2009; Dooremalen et al. 2012; Gallant et al. 2014; Doke et al. 2015; Requier et al. 2015; Brosi et al. 2016; Smart et al. 2016; Alaux et al. 2017).

Grasslands and rangelands account for approximately 265,000 hectares of land used primarily for grazing in the United States (USDA-ERS 2017). In the eastern US, this land is dominated by cool-season grasses such as tall fescue (*Schedonorus arundinaceus* Schreb.), orchardgrass (*Dactylis glomerata* L.), and Kentucky bluegrass (*Poa pratensis* L.), as well as other forages such as white clover (*Trifolium repens*), and weedy vegetation (Ball et al. 1991; Maresh Nelson et al. 2019; Hoveland 2000). Agroecosystems that lack substantial forb diversity,

such as conventional tall fescue pastures, provide insufficient nourishment and habitat for bees (Lebuhn et al. 2012; Plascencia and Philpott 2017;). Implementing native forbs into grazing systems can serve as a vital pollinator resource, cover crop, and in some cases increase cattle health and productivity (Klaus et al. 2021a; Klaus et al. 2021b; Tracy et al. 2010; Woodcock et al. 2014). While reconstructing endemic flora is unrealistic, provisioning and regulating ecosystem services through pasture renovation demonstrates great potential for pollinator conservation.

Typically, native perennial wildflowers are more attractive and provide more adequate nutritive and nonnutritive value via greater diversity in morphology and bloom phenology than non-native annual species for both wild and managed bees (Frankie et al. 2005; Tuell et al. 2008). However, in agricultural landscapes, honey bees have been observed predominantly relying on anemophilous trees (*Quercus, Acer*, *Fraxinus*), the rose family (Rosaceae), the honeysuckle family (Caprifoliaceae), crucifers (Brassicaceae), and herbaceous plants (*Solidago, Trifolium, Plantago, Taraxacum*) for pollen (Sabugosa-Madeira et al. 2008; Richardson et al. 2015a; Quinlan et al. 2022). Honey bees are known to demonstrate strong temporal-taxonomic transitions in foraging patterns among different floral habitats (Richardson et al. 2015a). This behavior could encourage honey bees to seek out sown species in florally-diversified systems.

This study aims to identify the differences in honey bee pollen foraging in pasture systems in southwestern Virginia, a landscape located within the Fescue Belt that is dominated by deciduous forests, mixed forests, and pastures. As a part of a larger study (Chapter 1), tall fescue pastures at five locations in southwest Virginia were diversified with wildflowers and paired with conventional tall fescue pastures. Honey bee hives were established at each paired location. The objectives of this study sought to 1) determine whether honey bees used sown

wildflower species as significant sources of pollen, 2) compare species composition and nutritive value of pollen collected from hives within diversified and conventional pasture systems, and 3) evaluate temporal trends in pollen collection. Pollen DNA metabarcoding was used to determine floral origin and a series of standard Bradford Assays were used to determine pollen protein content. Insights into the role of sown native wildflowers in honey bee foraging behaviors could help to inform future habitat restoration programs regarding honey bee nutrition outcomes. Honey bees exhibited similar patterns of floral utilization in both NWSGW+ diversified and conventional pasture systems, and sown floral species are utilized primarily in the fall.

Methodology

Refer to Chapter 1 for information regarding site location, surrounding land cover, floral establishment, and honey bee management.

Pollen Collection and Storage

Entrance pollen traps were placed on each hive for a 24-hour period three times (05/25, 7/27, and 10/01) in 2021. Entrance traps were used in favor of bottom-mounted traps to mitigate sample contamination by bees incidentally regurgitating pollen from their honey stomachs, (Vasquez and Olofsson, 2009) and contact between pollen foragers and stored pollen. A total of 57 samples were collected across the 20 hives; 3 hives did not collect any pollen for the third sample period. The pollen was frozen at -30 °C until weighed and processed for metabarcoding and protein content analysis.

Pollen Weight

Pollen samples were weighed prior to metabarcoding and protein content analysis. Pollen traps retain 0% ± 5% of all the collected pollen brought back to the hive. By assuming the percent of retained pollen is stable, the weight serves as a proxy of daily collected pollen mass per colony.

Pollen Amplification and Sequencing

As outlined in McMinn et al. 2022, Pollen samples were homogenized by combining 5 g of pollen, or the total pollen sample if <5 g, with distilled water to achieve a final concentration of 0.25 g/mL pollen. The homogenized samples were then agitated with a Burrell Wrist-Action laboratory shaker (Burrell Scientific, LLC, Pittsburgh, PA, USA) for 2 minutes at 1200 spm. An 0.8 mL aliquot of the blended solution was combined with 1.0 mL of 0.7 mm zirconia beads (Fisher Scientific, Hampton, NH, US) in a 2.0 mL bead beater microcentrifuge tube (Fisherbrand Free-Standing Microcentrifuge Tubes; Fisher Scientific, Hampton, NH, US). Tubes were then placed in a bead-beater (Mini-BeadBeater-16; BioSpec Products, Bartlesville, OK, USA) for 3 minutes to disrupt the pollen coat and release the DNA.

DNA was extracted using the QIAGEN DNeasy Plant Mini Kit (Qiagen USA, Valencia, CA). The ITS2 barcoding locus was amplified using a 3-step PCR amplification process in which the product of each step was used as the primer for the next (Richardson et al., 2015, 2019; McMinn et al. 2022). Primers in the 3rd PCR step were modified to append sample indexing and lane hybridization oligos to the PRC2 amplicons. Gel electrophoresis confirmed library quality. Libraries were then purified using a SequalPrep Normalization Plate kit (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced on a 15 million reads, 300 base-pair

standard Illumina MiSeq Flow Cell at the Molecular and Cellular Imaging Center in Wooster, Ohio.

Bioinformatics

Using the NCBI plant reference database for the ITS2 locus, sequences were identified to the genus and species levels using the MetaClassifier (Sponsler et al., 2020) pipeline via the Owens cluster at the Ohio Supercomputer Center. Paired-end reads were first merged using PEAR (Zhang et al. 2014) and converted to FASTA format using Seqtk (Shen et al. 2016). Paired-end reads were then aligned to NCBI plant reference database sequences (https://plants.sc.egov.usda.gov/) using VSEARCH (Rognes et al. 2016) with a minimum query coverage of 80% and a minimum identity of 92.5%. The median proportions were then calculated for each marker.

Following sequence alignment, all taxa detected at 0.1% proportional abundance or greater were included in analyses to avoid low-abundance false positives. Relative to other detected taxa, proportional abundance reflects the quantity of a given taxa present within a sample. A total of 53 samples were adequately sequenced. The 4 (7%) of under sequenced, and subsequently dropped, libraries included two May samples for sites C2 and C3 and July samples for E1 and E2. Pollen samples were assessed at both genus and species levels.

Trait-Based Analysis

Detected genera were classified by native status (native or exotic to contiguous United States), and sown status (presence in sown seed mix). Trait data were obtained from the USA Plants Database (USDA, NRCS 2022).

Pollen Protein Content

Pollen protein content was determined using a Bradford ProteinAssay with the Bio-Rad QuickStart Bradford Assay Kit (Catalog #5000201, Bio-Rad, Hercules, CA). Collected pollen samples that were >5 g of pollen first were homogenized using a mortar and pestle. Then 5 mg of each homogenized sample was transferred to a new tube along with 1.5 ml of 0.1 M NaOH buffer solution. Each sample was vortexed for 20 seconds and left at ambient room temperature for 24 hours. After the incubation period, samples were centrifuged at 2,000 x g for 30 seconds to precipitate solids. In a cuvette, 35 µl of substrate and 1.5 ml Bradford dye were combined and incubated in the dark for 25 minutes to promote protein binding. Absorbance readings at 595 nm were measured using a spectrophotometer. A QuickStart Bovine Serum Albumin (Bio-Rad, Hercules, CA) dilution series was used to initialize a standard curve. The quadratic equation of the standard curve was then applied to pollen spectrophotometer readings to determine the protein concentration.

Statistical Analysis

All statistical analyses were performed in R 2022.07.0. Pollen samples were assessed at both genus and species levels. Richness, evenness, and diversity indices were calculated. Linear mixed-effect models were used to evaluate the relative and interactive effects of site treatment, sample period, in regard to pollen weight, diversity, richness, evenness, and protein concentration. Two-way ANOVA models were used to evaluate the relative and interactive effects of site treatment (NWSGW+ diversified; conventional) and month in regard to in-pasture floral composition, pollen weight, indices, and protein content.

Results

Refer to chapter 1 for information regarding the establishment of NWSGW+ pastures and in-pasture floral resources.

Pollen Weight

Pollen weight from all sites and sampling periods ranged from 0 g to 58 g with a mean weight of 5.6 g. Pollen weight tended to be highest in samples collected in May and lowest in September (Figure 21). Among diversified sites, the weight of pollen samples ranged from 0 g to 58 g with a mean weight of 6.6 g (Figure 21). Among conventional sites, the weight of pollen samples ranged from 0 g to 18 g with a mean of 4.6 g (Figure 21). Pollen weight data showed an interaction between month and pasture treatment ($F = 4.0$, $df = 2$, $P = 0.0244$). Pollen weight tended to decline from May to September in diversified pastures but remained consistent across sample periods in the conventional sites (Figure 21).

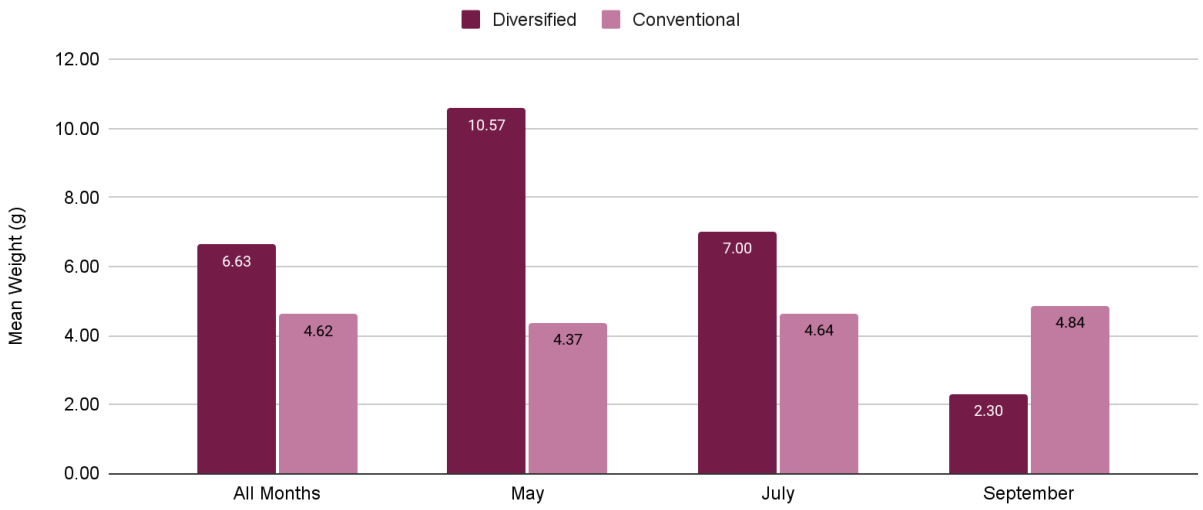


Figure 21. Mean pollen sample weight from hives at NWSGW+ diversified and conventional sites in May, July, and September.

Genus-Level eDNA Indices

A total of 113 genera were detected in pollen samples collected in May, July, and September (Figure 22). Among NWSG+ diversified sites, genus richness ranged from 38 to 69, with a mean of 52.6, and was highest in September (Figure 23). Genus evenness ranged from 0.36 to 0.06, with a mean of 0.10, and was highest in July. Genus diversity ranged from 0.988 to 2.1, with a mean of 1.6, and was highest in May (Figure 23). Among conventional sites, genus-level richness ranged from 31 to 70 with a mean of 52.4, and was highest in July. Genus evenness ranged from 0.07 to 0.32, with a mean of 0.12, and was highest in September. Genus diversity ranged from 0.5 to 2.4, with a mean of 1.5, and was highest in May.

Genus richness was not significantly associated with site treatment alone ($F = 0.013$; $df = 1$; $P = 0.9095$), but was significantly associated with month ($F = 3.447$; $df = 2$; $P = 0.04$) and the interaction between site treatment and month ($F = 15.06$; $df = 2$; $P < 0.0001$). Genus evenness was significantly associated with month ($F = 8.16$; $df = 2$; $P < 0.0001$) but was not significantly associated with site treatment ($F = 2.17$; $df = 1$; $P > 0.10$) or the interaction between site treatment and month ($F = 1.12$; $df = 2$; $P > 0.10$). Pollen genus diversity was not significantly associated with site treatment ($F = 0.637$; $df = 1$; $P > 0.10$), month ($F = 2.226$; $df = 2$; $P > 0.10$), or the interaction between site treatment and month ($F = 2.442$; $df = 2$; $P > 0.10$).

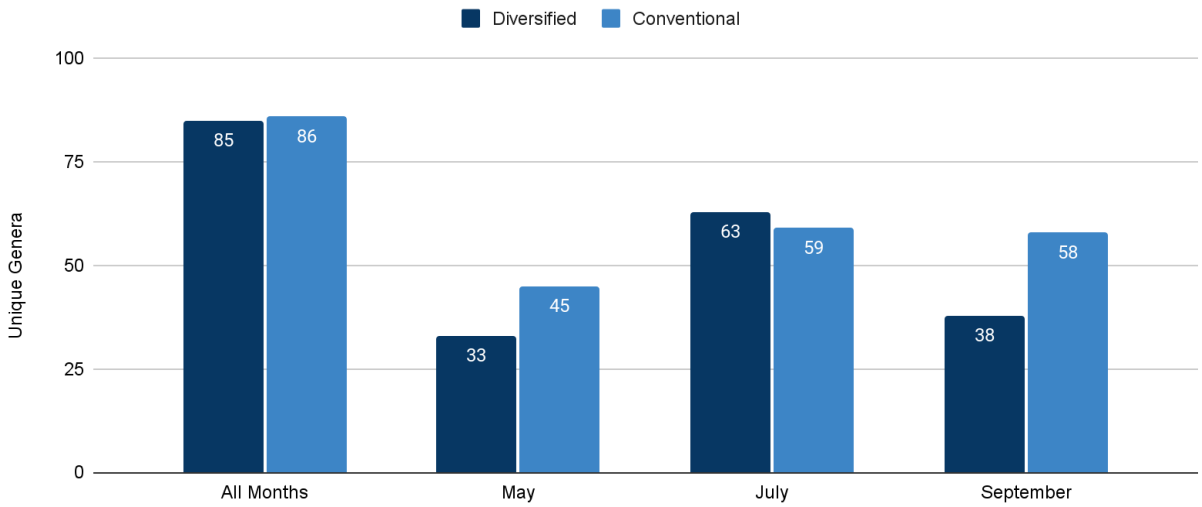


Figure 22. Total unique genera detected in pollen samples from hives at NWSGW+ diversified and conventional sites in May, July, and September.

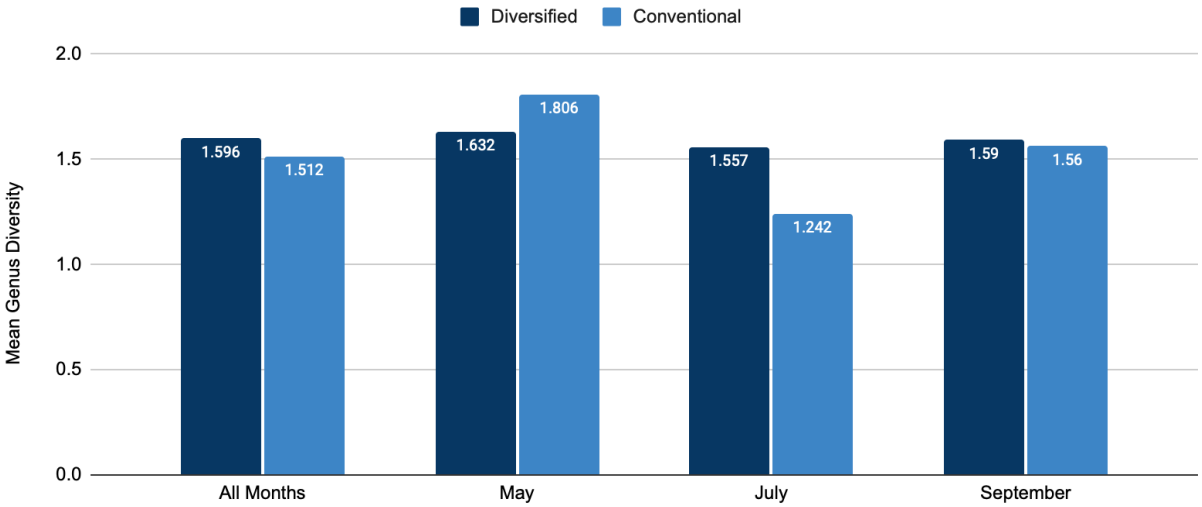


Figure 23. Mean genus diversity of pollen samples from hives at NWSGW+ diversified and conventional sites in May, July, and September.

Genus-Level eDNA Composition

Overall, *Trifolium* (clover), *Plantago* (plantain), and *Toxicodendron* (poison ivy) were the most abundant genera detected in pollen samples collected from hives in NWSGW+

diversified pasture systems (Figure 24). Within conventional pasture systems, the most abundant genera detected were *Trifolium*, *Symphyotrichum* (aster), and *Solidago* (goldenrod) (Figure 24).

In May, *Trifolium, Toxicodendron*, and *Rosa* (rose) were the most proportionally abundant genera detected in pollen samples collected from hives in NWSGW+ diversified pasture systems (Figure 25). Whereas pollen samples collected from conventional sites were primarily composed of *Trifolium*, *Rubus* (bramble), and *Rosa*.

In July, *Trifolium*, *Plantago*, and *Verbascum* (mullein), were the most proportionally abundant genera detected in pollen samples collected from hives at NWSGW+ diversified sites (Figure 25). Whereas pollen samples collected from conventional sites were primarily composed of *Trifolium*, *Verbascum*, and *Plantago* (Figure 25). Proportional species abundance was not significantly ($P > 0.05$) associated with site treatment.

In September, *Trifolium, Solidago*, and *Symphyotrichum* were the most proportionally abundant genera detected in pollen samples from hives at NWSGW+ diversified sites (Figure 25). Whereas pollen samples collected from conventional sites were primarily composed of *Symphyotrichum*, *Solidago*, and *Brassica* (crucifer) (Figure 25). The abundance of pollen genera did not differ between diversified and conventional sites ($P > 0.05$) in any month.

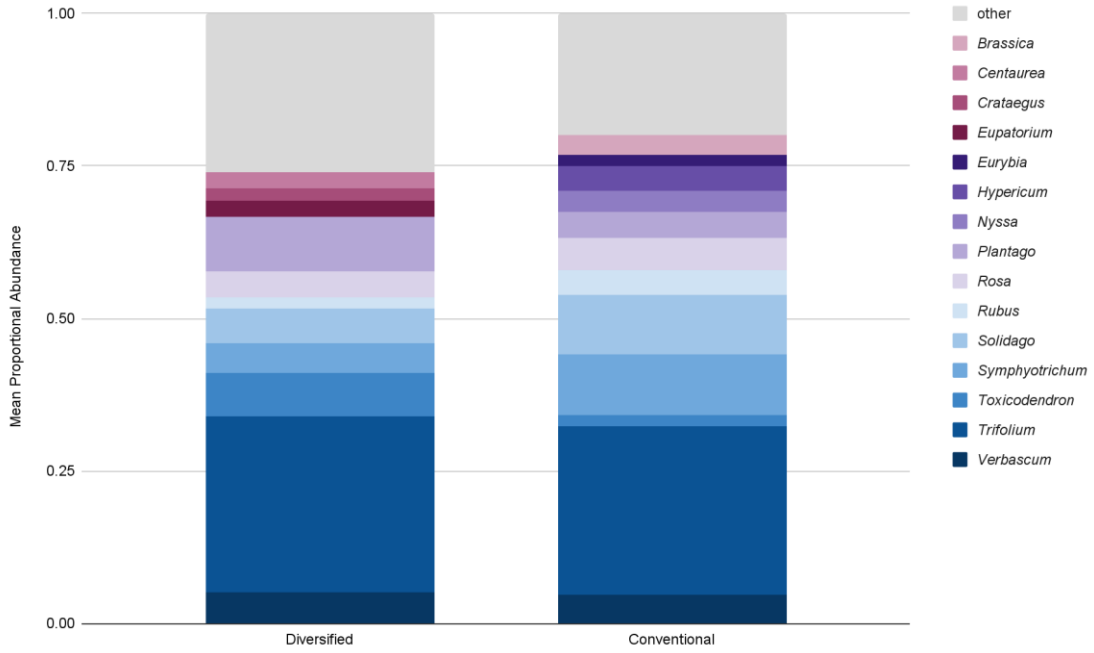


Figure 24. Mean proportional abundances of genera detected (>5%) in pollen samples from hives at NWSG+ diversified sites and conventional sites for all sampling months.

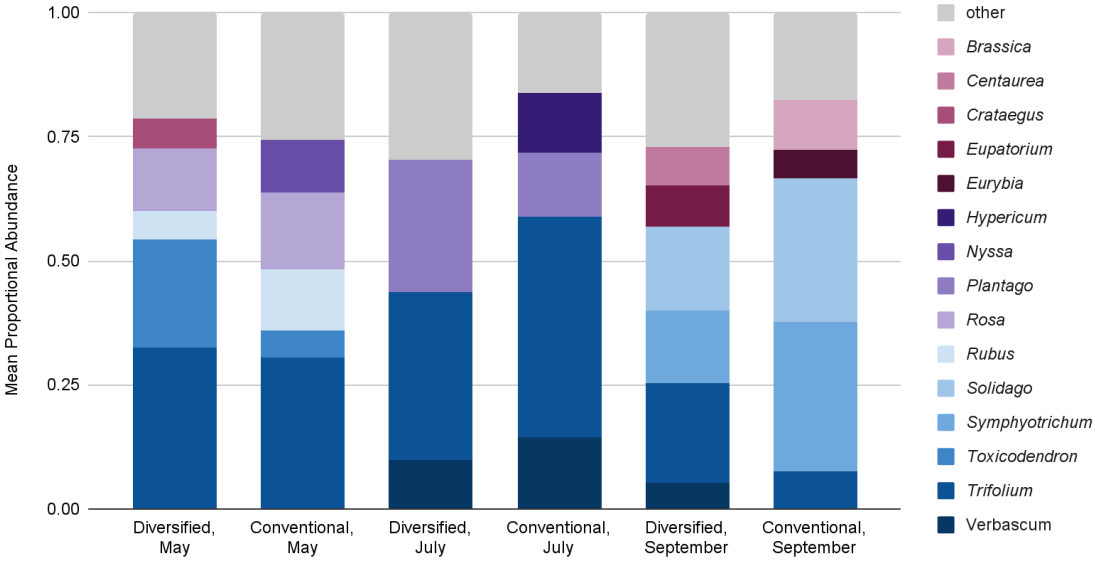


Figure 25. Mean proportional abundances of genera detected (>5%) in pollen samples from hives at NWSG+ diversified sites and conventional sites in May, July, and September.

Species-Level eDNA Indices

A total of 275 species were detected in pollen samples collected in May, July, and September (Figure 26). Among NWSGW+ diversified sites, species richness ranged from 34 to 73 with a mean of 52, and was highest in May (Figure 27). Species evenness ranged from 0.08 to 0.48, with a mean of 0.13, and was highest in July. Species diversity ranged from 1.4 to 2.9, with a mean of 2.2, and was highest in May. Among conventional sites, species-level richness ranged from 24 to 78 with a mean of 50, and was highest in May. Species evenness ranged from 0.08 to 0.41, with a mean of 0.15, and was highest September. Species diversity ranged from 1.2 to 2.86, with a mean of 1.98, and was highest in May.

Pollen species richness was not significantly associated with site treatment alone ($F = 0.5$; $df = 1$; $P = 0.48$), but was significantly associated with month ($F=13.2$; $df=2$; $P = 0.00003$) and the interaction between site treatment and month ($F = 9.4$; $df = 2$; $P = 0.0004$). Pollen species evenness was significantly associated with month ($F = 3.8$; $df = 1$; $P = 0.03$) but was not significantly associated with site treatment ($F = 2.4$; $df = 1$; $P = 0.13$) or the interaction between site treatment and month ($F = 2.7$; $df = 2$; $P = 0.08$). Species diversity was significantly associated with month ($F = 3.8$; $df = 2$; $P = 0.03$), but was not significantly associated with site treatment ($F = 3.2$; $df = 1$; $P =0.08$) or the interaction between site treatment and month ($F = 1.584$; $df = 2$; $P = 0.2$).

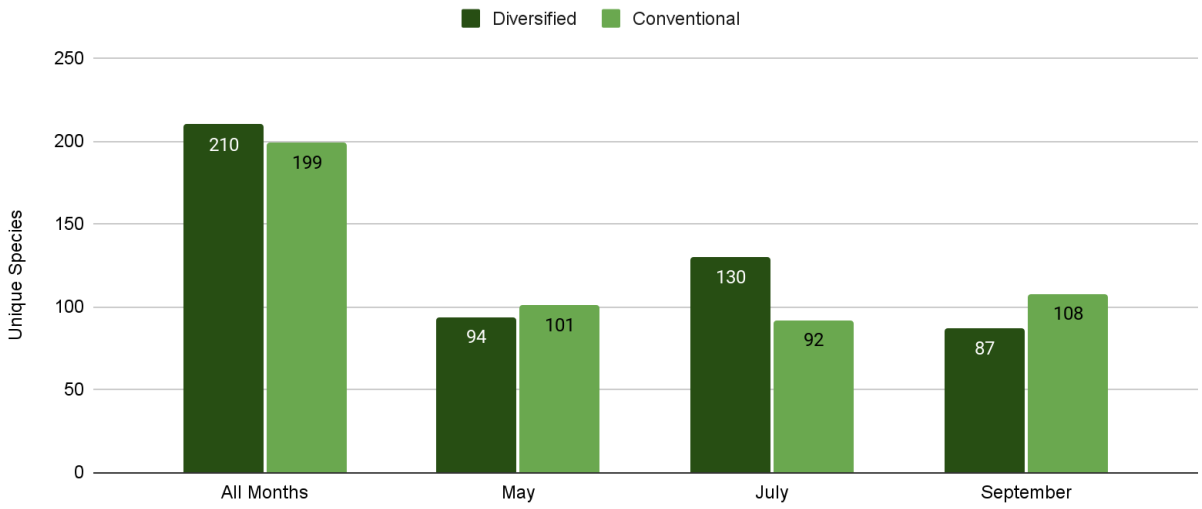


Figure 26. Total unique species detected in pollen samples from hives at NWSGW+ diversified and conventional sites in May, July, and September.

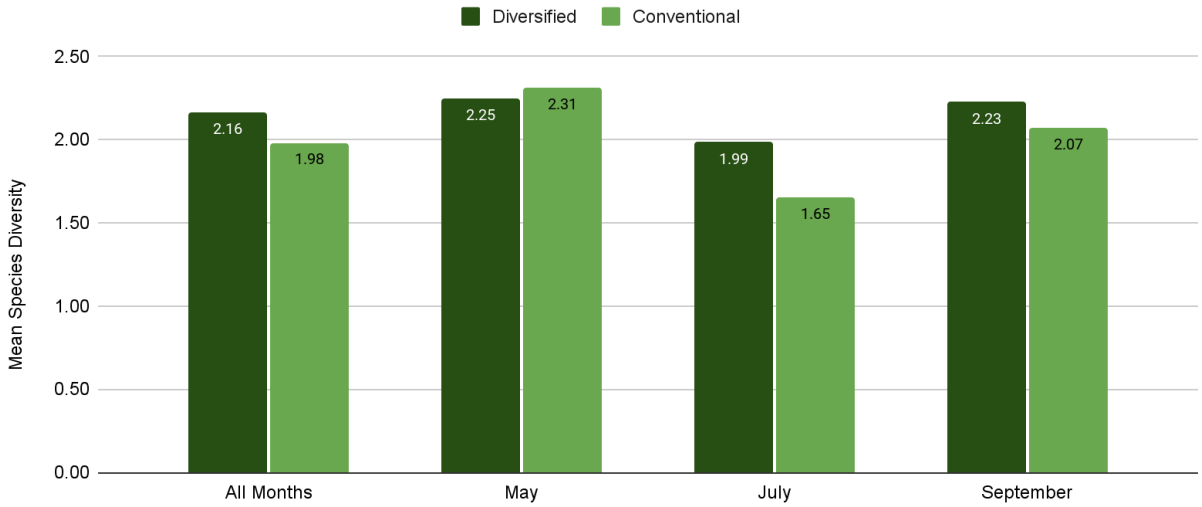


Figure 27. Mean species diversity of pollen samples from hives at NWSGW+ diversified and conventional sites in May, July, and September.

Species-Level eDNA Composition

Overall, *Trifolium repens* (white clover), *Plantago lanceolata* (ribwort plantain), and *Toxicodendron diversilobum* (pacific poison oak) were the most proportionally abundant genera detected in pollen samples collected from hives in NWSGW+ diversified pasture systems (Figure 28). Within conventional pasture systems, the most proportionally abundant genera detected

were *Trifolium repens*, *Symphyotrichum boreale* (northern bog aster), and *Solidago juncea* (early goldenrod) (Figure 28).

In May, *Toxicodendron diversilobum*, *Trifolium repens*, and *Trifolium nigrescens* (small white clover) were the most proportionally abundant species detected in pollen samples collected from hives at NWSGW+ diversified sites (Figure 29). Pollen samples collected from conventional sites were primarily composed of *Trifolium repens*, *Rosa rubus* (bramble rose), and *Nyssa sylvatica* (black gum) (Figure 29). Proportional species abundance was not significantly ($P < 0.1$) associated with site treatment.

In July, *Trifolium repens*, *Plantago lanceolata*, and *Verbascum thapsus* (great mullein), were the most proportionally abundant genera detected in pollen samples collected from hives at NWSGW+ diversified sites (Figure 29). Pollen samples collected from conventional sites were primarily composed of *Trifolium repens*, *Verbascum thapsus*, and *Plantago lanceolata* (Figure 29). Proportional species abundance was not significantly ($P > 0.05$) associated with site treatment.

In September, *Trifolium repens*, *Symphotrichum boreale*, and *Solidago juncea* were the most proportionally abundant species detected in pollen samples from hives at NWSGW+ diversified sites (Figure 29). Pollen samples collected from conventional sites were primarily composed of *Solidago juncea*, *Symphotrichum boreale*, and *Symphyotrichum novae* (New England aster) (Figure 29). Proportional species abundance was not significantly ($P > 0.05$) associated with site treatment.

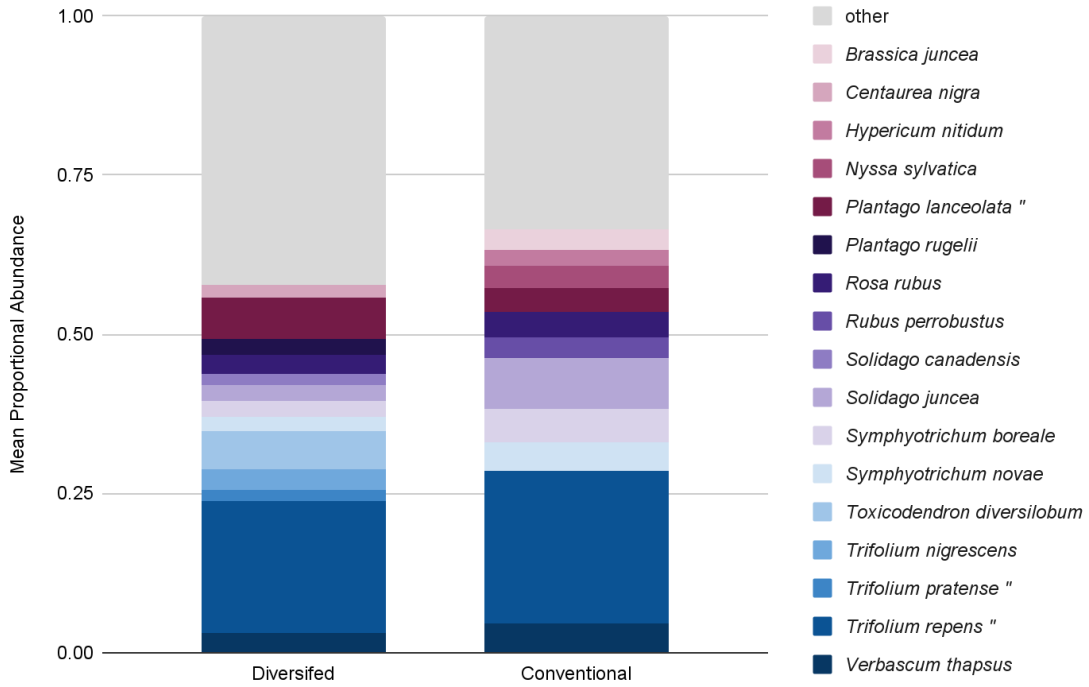


Figure 28. Mean proportional abundances of species detected (>5%) in pollen samples from hives at NWSG+ diversified sites and conventional sites for all sampling months. (” : species observed-in-pasture).

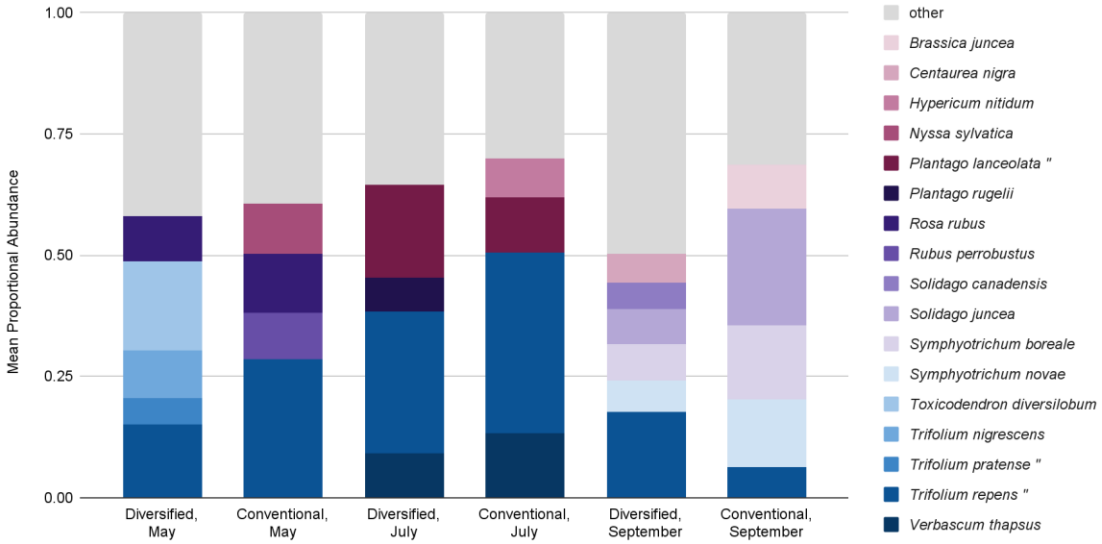


Figure 29. Mean proportional abundances of species detected (>5%) in pollen samples from hives at NWSG+ diversified sites and conventional sites in May, July, and September (” : observed in pasture).

Sown & In-Pasture Species

Among diversified sites, three sown wildflower species were detected in pollen samples (Figure 30). *Solidago juncea* was omitted because it was not observed in pastures. Mean abundance of sown species differed by month and was highest in September ($F = 2.8$, $df = 2$, $P = 0.07$). *Gaillardia pulchella* accounted for 4% of all pollen species in September samples with *Coreopsis lanceolata* and *Rudbeckia hirta* accounting for less than 1%.

Among both conventional and diversified pasture systems, 12 observed-in-pasture (OIP) wildflower species were detected in pollen samples (Figure 30). Abundance of pollen from OIP species ranged from 0 to 0.9 with a mean of 0.36. Of the OIP species, pollen from *Trifolium repens*, *Plantago lanceolata*, and *Solanum carolinense* were most abundant (Figure 30). Abundance of sown and OIP species differed according to site treatment and month but was not significantly associated with site treatment alone.

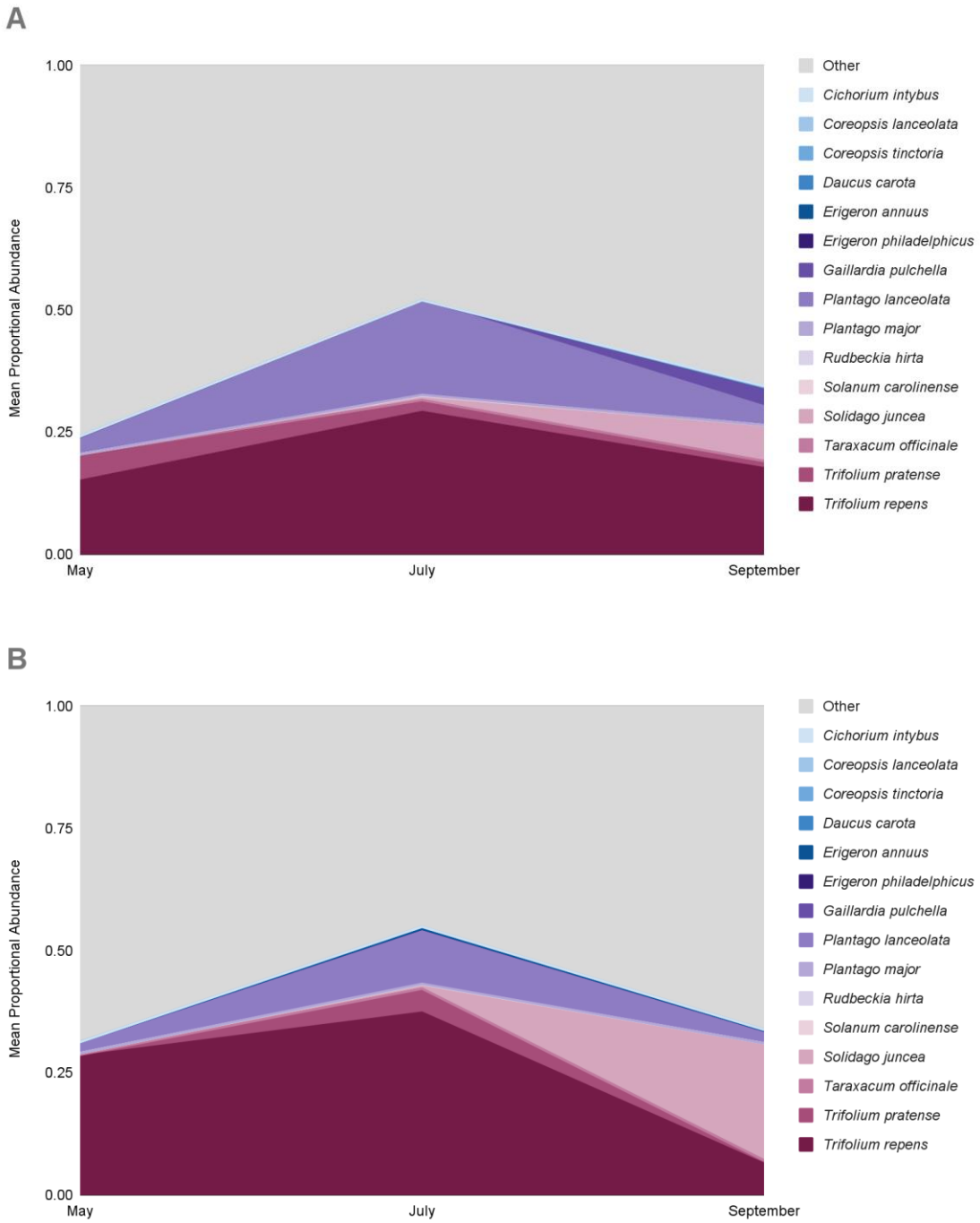


Figure 30. Mean proportional abundance of OIP wildflower species detected in pollen samples in May, July, and September in (A) NWSGW+ diversified and (B) conventional pasture systems.

Protein Content

Protein concentration of pollen samples from all sites and sampling periods ranged from 188 ug/ml to 826 ug/ml with a mean concentration of 430 ug/ml. Pollen protein concentration was highest in samples collected in May and lowest in September. Pollen protein concentration varied by sample period ($F = 11.2$; $df = 2$; $P < 0.001$) and pasture treatment ($F = 4.2$; $df = 1$; $P = 0.049$), but no interaction was detected ($P = 0.570$). Pollen from diversified sites had higher protein concentrations compared with conventional sites - especially in July and September (Figure 31).

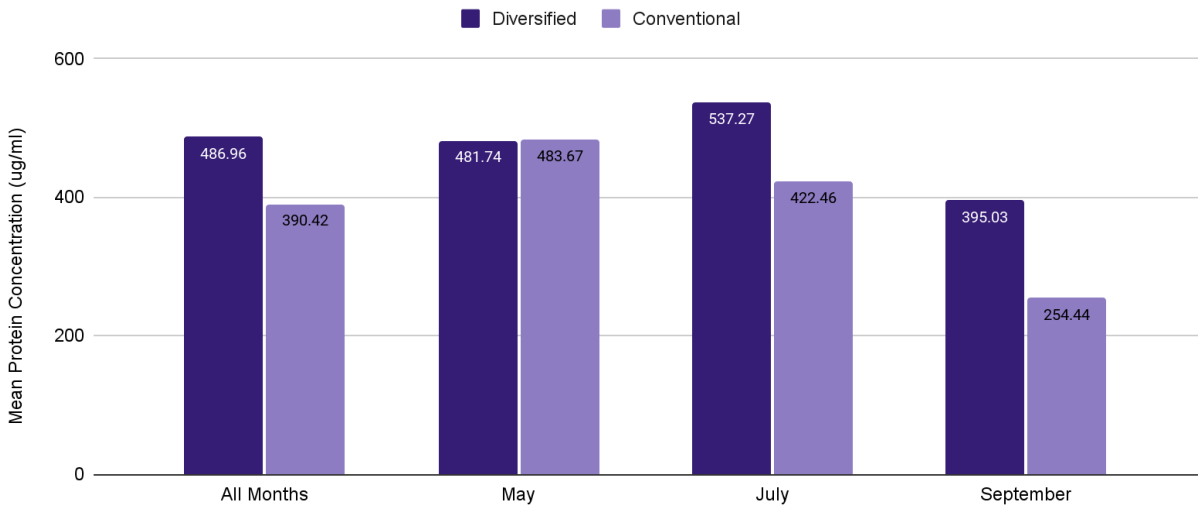


Figure 31. Mean pollen protein concentration (ug/ml) from hives at NWSGW+ diversified and conventional sites in May, July, and September.

Discussion

Refer to chapter 1 for information regarding the establishment of NWSGW+ pastures and in-pasture floral resources.

Pollen

Pollen provides protein, lipids, amino acids, sterols, and other micronutrients necessary to sustain honey bee colonies. Foragers are known to be capable of assessing the amino acid and fatty acid content of pollen, but cannot distinguish sterol composition (Ruedenauer et al. 2021). Pollen foraging behavior is dependent on a multitude of factors including, but not limited to, floral resource availability, environmental conditions, season, brood requirements, population, and overall colony health. As a super-generalist species, honey bees will actively collect nutritionally diverse pollen to balance colony micronutrient content (Bonoan et al. 2018). Understanding this behavior can be especially useful in determining ecosystem-wide floral resources.

Pollen Weight

Colonies in diversified pasture systems collected a total 38 g of pollen more than those in conventional pasture systems. On average, pollen samples from diversified sites were 2 g heavier than samples collected from conventional sites. The largest difference in mean pollen weight was observed in May in which colonies in diversified systems collected an average of 6.2 g more pollen than colonies in conventional systems. The magnitude of these differences changed significantly throughout the season.

Colonies in diversified pasture systems collected less pollen throughout the growing season. This trend is consistent with previous research regarding seasonal variation in pollen

foraging behavior (Schweiger et al. 2010). Colonies in conventional pasture systems collected less, but more consistent amounts of pollen throughout the year. Assuming the percent of retained pollen is stable, weight serves as a proxy of daily pollen mass per colony (10 ± 5% total) (Lavie and Fresnaye 1963; Require et al. 2015). Over time, daily differences in collected pollen weight are important to the brood-rearing capabilities of a colony.

The differences observed in pollen weight across pasture treatments could reflect larger energetic costs required to travel further for pollen foraging in conventional sites as NWSG+ diversified pastures had 9.95x greater bloom density in June, 3.32x greater in July, and 9.18x in August compared to conventional pastures. With substantially lower in-pasture bloom densities, colonies in conventional systems may have been more limited in spring and summer forage, requiring pollen foraging efforts to continue well into the fall to account for smaller pollen stores.

eDNA Sequencing

Pollen metabarcoding revealed a strong relationship between biodiversity indices and season. Pollen species richness demonstrated a temporal relationship with pasture treatment, whereas species evenness and diversity were independently associated with month. This suggests temporally variable bloom availability and forage quality in grazing agroecosystems. While biodiversity indices are important tools, it is also important to consider the unique floral phenology and nutritional value of utilized species. Pollen samples were composed primarily of small herbaceous species including *Trifolium* (clover) and *Plantago* (plantain), taller herbaceous species including *Verbascum* (mullein), *Solidago* (goldenrod), and *Symphyotrichum* (aster) and woody flowering species including *Toxicodendron* (poison ivy), *Rosa* (rose), and *Nyssa* (gum).

Trifolium repens was the most consistent and proportionally abundant species detected across both pasture types throughout all sampling periods. This suggests both a great local abundance and strong foraging preference for *T. repens* pollen. Globally, *T. repens* is a substantial forage for honey bees (Goodwin et al. 2011) and has been identified as a very rich source of protein (Ghosh et al. 2020). As a low-growing legume with smaller blooms, nearby flowers, such as those sown in diversified pastures, may increase *T. repens* visitation rates (Krimmer et al. 2019). A similar effect may influence *Plantago* foraging behavior, especially considering its anemophilous habit.

Woody species (T*oxicodendron, Rosa, Nyssa*) were more dominant in spring pollen samples collected from conventional pasture systems. Flowering woody plants are a valuable springtime resource for bees in many habitat types and often boast greater bloom density than grasslands (Somme et al. 2016; Mach & Potter 2018; McMinn-Sauder 2022). Taller herbaceous species (*Verbascum, Solidago, Symphyotrichum*) were detected more in mid-summer and fall as woody species ceased blooming.

Only three of the 25 sown wildflower species were detected in pollen samples from diversified pasture systems (*Coreopsis lanceolata, Gaillardia pulchella, Rudbeckia hirta*), despite a consistent abundance of available sown blooms. Sown species accounted for less than 1% of species by proportional abundance in May and July, and less than 5% in September. It is possible that *Gaillardia pulchella* offers substantial fall forage for honey bees when many other species have ceased blooming.

As a non-native species, honey bees are more likely to forage upon species within their historical environments such as European *Trifolium, Erigeron,* and *Plantago* species (Ghosh et al. 2020). Additionally, honey bees exhibit strong resource loyalty in which they will

functionally deplete a single source nectar or pollen source before moving on. This is a primary characteristic of their forager recruitment behavior. Unlike honey bees, many native bee species have obligatory host-specific foraging behaviors. It is possible that the sown wildflower species are evolutionarily and nutritionally less desirable for honey bees. The wildflower species planted in diversified pasture systems are more likely to better support native pollinators.

Protein Content

Pollen protein content can range from 2.5 to 61%. However, honeybees will typically forage on zoophilous and anemophilous species that contain at least 12% protein (Roulston et al. 2000). Mean protein concentration was 96 ug/ml higher in pollen samples collected in diversified pasture systems compared to those collected in conventional systems. In May, pollen protein concentrations were similar across treatments, but in July and September pollen collected from diversified sites had higher protein concentrations. Due to the low proportional abundance of sown species detected in the pollen sample DNA metabarcoding, it is likely that this difference is a result of protein-rich unsown species becoming more established in diversified systems such as *Trifolium* spp. (Ghosh et al. 2020). However, as pollen samples were previously homogenized for metabarcoding analysis, it was impossible to distinguish protein concentrations among species. The differences observed in protein could also be attributed to protein-rich woody pollen sources in the surrounding environment such as *Acer spp., Quercus spp., and Salix spp.* (Roulston et al. 2020).

Conclusion

Results from this study suggest that honey bees in both diversified and conventional pasture systems have similar diets. In south western Virginia, weedy pastures, field-edges, forests, and roadsides provide valuable pollen forage for honey bees in agroecosystems, such as conventional tall fescue pastures. Future land conversion, deforestation, and weed control practices could threaten the nutritive value of grazing agroecosystems for honey bees.

Floral surveys revealed diversified pastures had almost 4x greater mean bloom density than conventional pastures, but over half of all blooms recorded in diversified pastures were unsown species. Pollen weight, species richness, and protein content were positively correlated with diversified pasture systems. This suggests that successful pasture diversification may not need to be entirely reliant on the establishment of sown species, but could partially rely on seed banks and nearby species to colonize disturbed pastures. NWSGW+ diversified pastures demonstrate great potential for providing a more complex nutritional support system for pollinators in grazing agroecosystems.

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