Understanding the links between soil, plants, and pollinators.

Submitted by

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Declaration

I certify that all material in this thesis is my own work, unless clearly stated, and that no material has been previously submitted and approved for the award of a degree by this or any other University.

I certify that the world length of this thesis, not including bibliography, is within the guidelines of 80,000 words, as suggested by the University of Lancaster. The word length is 52,344

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Chapter 1 has been edited from an article published in an internationally peer-reviewed journal. The published article was entirely written by Thomas David, with guidance from Jonathan Storkey and Carly Stevens.

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Chapter 2 contains statistical analyses of data previously collected by others and used with permission. The data from UK acid grassland sites (results shown in Table 2.1, section 2.4.1) was collected by Stevens et al. (2004). The Park Grass Long-term Experiment data was procured from the electronic Rothamsted Archive (e-RA).

Abstract

Global nitrogen (N) deposition has risen steeply since the mid-19th Century and is forecasted to rise further. This increased flux of N to ecosystems is increasingly considered as a worldwide driver of environmental change. Impacts to botanical communities have been shown across a range of ecosystems and regions, but evidence on consequential impacts to further trophic levels is currently limited. Our understanding of how N affects plant-pollinator interactions is relatively poor. Declining trends in pollinating insects have been widely reported during recent decades and a key factor in this is losses of floral resources.

The research chapters presented in this thesis seek to address the wide knowledge gap of how N affects pollinators by investigating how soil N affects 1) the composition of floral functional traits in a plant community, 2) intra-specific variation in key floral functional traits: phenology and nectar, and 3) the potential impacts to plant-pollinator interaction networks. This research was undertaken using the Park Grass Long-term Experiment (PGE) at Rothamsted Research, UK, a long-term nutrient-enrichment experiment. The study found extensive impacts of N on the provision of floral resources. Floral resources were lost with N-enrichment, with morphologically specialised floral units particularly threatened. Flowering phenology of plant species was influenced by N-enrichment with a notable contrast between early-season species, which had an extended flowering duration, and peak-season species that had a reduced flowering duration. Responses of nectar traits varied across species but were shown to respond to N applications. The observed pollinator visitation networks were impacted by the soil treatments; N additions typically led to Diptera-dominant communities and networks that were potentially less robust to stochastic events. The research project provided evidence that soil N can act as an underlying driver of plant-pollinator networks and should be considered as a factor in pollinator ecology and trends.

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Thanks to my family; my parents, two sisters, brother, dogs and cats. I didn't visit you as much as I would have liked during the last four years, but I've thought of you often.

Finally, thank you to Susana, who has made me smile every day.

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Abbreviations

AIC – Akaike information criterion

ANOVA – analysis of variance

BIC – Bayesian information criterion

CWM – community weighted means

FAMD – factor analysis of mixed data

FFD – first-flowering date

fl - flower

FYM – farmyard manure

GABA – gamma-Aminobutyric acid

GDD5 – growing-day degrees, with 5° C threshold

ha – hectare

HPLC – high performance liquid chromatography

K – potassium

kg – kilogram

kt – kiloton

LFD – last-flowering date

LMER – linear mixed-effects model

M – mole

MANOVA – multivariate analysis of variance

Mg – magnesium

ml - millilitre

N – nitrogen

Na – sodium

NaCl – sodium chloride

NH_4 – ammonia

NH_x – reduced N

NODF – nestedness metric based on overlap and decreasing fill

NO_y – oxidised N

NO_3 – nitrate

P – Phosphorus

PAD – pulsed amperometric detection

PGE – the Park Grass Long-term Experiment

REML – restricted maximum likelihood

sp. – species

tg - teragram

yr – year

µg - microgram

µl - microlitre

µM - micromole

<u>Species codes</u>

Ar – *Ajuga reptans*

As – *Anthriscus sylvestris*

Cf – *Cerastium fontanum*

Cm – *Conopodium majus*

Cn – Centaurea nigra

Fm – *Fritillaria meleagris*

Hr – *Hypochaeris radicata*

Hs – Heracleum sphondylium

Lc – *Lotus corniculatus*

Lh – *Leontodon hispidus*

Lp – *Lathyrus pratensis*

Ka – Knautia arvensis

Pl – *Plantago lanceolate*

Ra – *Ranunculus acris*

Rb – *Ranunculus bulbosus*

Rf – *Ranunculus ficaria*

Sm – *Sanguisorba minor*

To – *Taraxacum officinale agg.*

Tp – *Trifolium pratense*

Trag – *Tragopogon pratensis*

Vc – *Veronica chamaedrys*

CHAPTER 1

UNDERSTANDING HOW CHANGING SOIL NITROGEN AFFECTS PLANT-POLLINATOR INTERACTIONS

This chapter has been edited from a review published in:

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1.1 Abstract

Many pollinating insects, across taxa and regions, have declined during the 20th century. Amongst the drivers of these trends, soil eutrophication and acidification caused by nitrogen (N) have not been broadly researched. Anthropogenic influences have greatly increased the global deposition of N to soils during the past century; this is increasingly recognised as a threat to global biodiversity. The fundamental role of soil in plant growth and health means that alterations to soil conditions will likely have consequences for plant-pollinator interactions. Soil-N can impact on botanical communities, often reducing species-richness due to quick growth of competitive grasses and altering the nutritional qualities of vegetation. Research shows this can affect other trophic levels, such as herbaceous insects, but little is known about the impacts to pollinators. There is evidence that relevant floral traits for pollinators, such as phenology and nectar traits, can be affected by soil-N. However, the extent of these impacts, across species, are poorly understood. Importantly, we currently lack sufficient research to determine if and how pollinators will be impacted by botanical responses to changing soil fertility. This review collates the research and evidence of how soil-N affects botanical species composition and relevant floral traits and discusses how pollinating insects and plant-pollinator interactions might be impacted. We conclude by identifying the key knowledge gaps in this subject; the lack of research that includes pollinators into studies of how N additions affect botanical traits, poor understanding of inter-specific variation in botanical responses to N, synthesis of botanical traits to form a comprehensive understanding, and the inclusion of other abiotic and biotic drivers into studies.

1.2 Introduction

The life cycles of pollinating insects are sustained through flowering plants, which provide nutrition for adults and their progeny. Environmental impacts on plants can therefore have consequences for pollinators, and vice versa; if pollinators are affected it can have implications for the quality of pollination services delivered (Fontaine *et al.* 2006; Fründ *et al.* 2013). Plant-pollinator interactions are vital in supporting terrestrial biodiversity and the functional stability of ecosystems; it is estimated that 87.5% of global plant species rely on biotic pollination (Ollerton, Winfree and Tarrant, 2011). Pollinators are also important for the yields of many agricultural crops (Gallai *et al.* 2008; Garibaldi *et al.* 2011, 2014; Rader *et al.* 2016); 87 of the 115 leading global food crops, accounting for 35% of our agricultural produce, rely on biotic pollination (Klein *et al.* 2007). Insects are the key pollinators in most systems (Gallai *et al.* 2009; Patiny, Rasmont and Michez, 2009; Potts et al. 2010; Wardhaugh, 2015; Ollerton, 2017). However, it is well-documented that insect pollinators are in decline across a range of taxa and ecoregions, due to a combination of factors that include habitat degradation, land-use change, parasites, pesticides, and the transport of non-native commercial pollinators (e.g. Williams and Osborne 2009; Potts *et al.* 2010; Winfree *et al.* 2011; Vanbergen *et al.* 2013; Rundlöf *et al.* 2015). Soil eutrophication, caused by atmospheric nitrogen deposition and increased use of inorganic and organic fertilisers, is known to contribute to declining habitat quality and is increasingly recognised as a substantial threat to global biodiversity (Phoenix *et al.* 2006; Bobbink *et al.* 2010; Bleeker *et al.* 2011; Erisman *et al.* 2014; Schoukens 2017). However, the effect of elevated biologically-available nitrogen (N) on plant-pollinator interactions has received very little research attention (Harrison and Winfree, 2015).

Excessive soil enrichment with N can occur through fertiliser application, agricultural run-off, and through atmospheric N deposition. We have an increasingly well-developed understanding of the multiple ways through which N deposition can impact plant communities (Bobbink *et al.* 2010). The eutrophication of soil by N, often a limiting nutrient, typically boosts above-ground primary productivity, which favours fast-growing, nitrophilic plants. This leads to taller vegetation and creates stronger competition for light, with smaller and slower growing species often suffering as a result. (Mountford, Lakhani and Kirkham, 1993; Crawley *et al.*, 2005). Excessive fluxes of N to soil can lead to soil acidification, particularly in poorly buffered soils (Dise *et al.* 2011; Stevens *et al.* 2010), which can increase the availability of toxic compounds and heavy metals such as aluminium in the soil for plant take-up. This further drives changes to botanical communities through filtering the species pool according to plant tolerances to soil pH and heavy metal loadings (Dise *et al.* 2011; Stevens *et al.* 2010). When in the ammonia form, N can be directly toxic (Pearson and Stewart, 1993), although this is species-specific and associated with point source contamination (Bobbink *et al.* 2010). Soil-N enrichment poses varied further impacts through interactions with secondary stressors, such as climate change and other environmental factors (e.g. Caporn *et al.* 2000; Tylianakis *et al.* 2008; Porter *et al.* 2013), altering soil microbial communities (e.g. Farrer and Suding 2016), and increasing susceptibility to pests and pathogens (e.g. Brunsting and Heil 1985; Strengbom *et al.* 2003, 2006).

This introductory review will address the most widespread impacts: chronic eutrophication and, in poorly-buffered soils, acidification (Bobbink, Hornung and Roelofs, 1998). The review will begin by introducing the recent history of anthropogenic alterations of the N cycle and how this is shown to impact on invertebrates. The second part will discuss how pollinator-relevant botanical traits are impacted by N additions, and how pollinators might respond. The review will continue into a discussion of some key knowledge gaps. Finally, the review will give an overview of the research questions specifically addressed by thesis and how this will contribute to our understanding.

1.3 Historic nitrogen production and deposition

Humans have dramatically altered the N cycle, mostly through agricultural and energy industries (Vitousek *et al.*, 1997; Cornell *et al.*, 2003; Galloway *et al.*, 2004; Fowler *et al.*, 2013). Global anthropogenic N production increased from 15 Tg N yr^{-1} in

1860 to 187 Tg N yr^{-1} in 2005 (Galloway *et al.* 2008) and is now the dominant supply of biologically available N to soils. Global atmospheric N deposition consequentially increased from 34 Tg N yr^{-1} in 1860 to 100 Tg N yr^{-1} in 1995, and is estimated to double between 1995-2050 (Galloway *et al.* 2004). Inorganic fertiliser application increased in Europe during the last century, although some countries have decreased application since the 1980's (Stoate *et al.*, 2001).

Atmospheric N deposition is currently highest in the developed temperate regions of the Northern Hemisphere (Dentener *et al.* 2006; Bleeker *et al.* 2011). Since the 1980's, the deposition rate in Europe and North America has begun to level off and decrease in some areas, due to more efficient technologies and the plateauing of N fertiliser application (Cornell *et al.* 2003; Goulding *et al.* 2015); at current levels it is typically 10-25 kg N ha^{-1} yr^{-1} (Dentener *et al.* 2006; Bleeker *et al.* 2011). In many ecosystems this still falls above the estimated critical threshold for sensitive ecosystems of 15 kg N ha^{-1} yr^{-1} (Phoenix *et al.* 2006). In China, the rate can exceed 50 kg N ha^{-1} yr^{-1} and is increasing (Liu *et al.* 2013). Atmospheric N deposition is typically lower in remote undeveloped areas of the Southern Hemisphere, although the rate is expected to increase dramatically in the future (Dentener *et al.* 2006; Galloway *et al.* 2008). This is concerning, as many of the world's biodiversity hotspots are found in the Southern Hemisphere and will be in danger of rising levels of N deposition in excess of the critical thresholds of sensitive ecosystems (Phoenix *et al.* 2006; Bleeker *et al.* 2011).

In the UK, N emissions rose from 312 kt N yr^{-1} in 1900 towards a peak of 787 kt N yr^{-1} between 1980-1990, and have since declined substantially, to 460 kt N yr^{-1}, in 2000 (Fowler *et al.* 2004). This fall in emissions is in part due to focussed policy programs (such as Clean Air Act legislation in 1993), more efficient technologies, and reducing emissions associated with transport and utilities (ROTAP, 2012). Despite this reduction in emissions, the flux of N deposition to terrestrial ecosystems changed little between 1980-2007, remaining close to 400 Gg N yr^{-1} (ROTAP, 2012). As such, 58% of N-sensitive habitats experienced deposition levels above critical levels in 2009, only decreasing from 62% in 1980. A noted priority is minimising ammonia (NH_4) emissions resulting from agricultural practices; the UK has had more success in lessening oxidised nitrogen (NO_y) emissions than reduced nitrogen (NH_x) emissions and so ambient atmospheric NH_x concentrations remain high (Erisman *et al.* 2007; ROTAP, 2012). UK atmospheric levels and deposition fluxes have therefore become increasingly proportionally dominated by NH_x. Acidification of habitats, associated with sulphur (S) or N deposition, is an ongoing but gradually alleviating problem in the UK. Particularly threatened are acid-sensitive habitats; 54% of acid-sensitive habitats in 2006-2008 were strongly acidified above critical threshold levels (ROTAP, 2012).

1.4 Soil N impacts on pollinators and other trophic interactions

Our knowledge of N-driven impacts to higher trophic levels is lacking (Stevens, David and Storkey, 2018). Research on plant-herbivore and plant-herbivore-predator interactions has demonstrated that plant-mediated impacts of soil-N enrichment can affect invertebrate development and populations (e.g. Jefferies and Maron, 1997; Haddad *et al.* 2000; Throop and Lerdau, 2004; Chen *et al.* 2010; Pöyry *et al.* 2017). It is acknowledged that the impacts to invertebrate herbivores can occur through various pathways, typically: alterations to the quantity of foliar N, alterations to dietary qualities of vegetation, microclimatic cooling, change in botanical species composition, and phenology (Nijssen *et al.* 2017; Poyry *et al.* 2017; Stevens, David and Storkey, 2018).

Soil-N enrichment, and increased take-up by plants, typically leads to higher concentrations of foliar N and therefore increased N availability for herbivorous insects (Awmack and Leather, 2002). This often positively correlates with increased invertebrate growth rate and body size (Throop and Lerdau, 2004). A study of over 1,000 Scandinavian lepidopterans found the larvae of butterflies and moths that use host plants with higher foliar N concentrations were larger (Poyry *et al.* 2017). These responses are understandable, given that larger animals typically have higher N content (Fagan *et al.* 2002). A study of *Lycaena tityrus*, however, found that, although larval size increased with high foliar N, adult size decreased (Fischer and Fiedler, 2000). This phenomenon could have been caused by the observed faster pupal development times. The faster development rates of insects feeding from high-N plant material can also lead to increased voltinism (Fischer and Fiedler, 2000; Awmack and Leather, 2002; Poyry *et al.* 2017). Although voltinism typically indicates a positive response of population, Fischer and Fiedler (2000) found increased adult mortality in their study species, suggesting that faster development time could compromise adult success. Experimental tri-trophic interaction studies found increased aphid body size in individuals feeding from N-fertilised plants (Aqueel and Leather, 2012; Banfield-Zanin *et al.* 2012). There were implications for the coccinellid predators of aphids in these studies; the *Coccinellidae* ate fewer aphids, likely due to increased aphid body mass, (Aqueel and Leather, 2012) and Banfield-Zanin *et al.* (2012) found higher predator mortality when feeding on aphids from plants receiving high synthetic fertiliser applications. This shows the potential detrimental consequences of N deposition cascading through a food chain.

Increased foliar N can lead to the distortion of stoichiometric balances in vegetation, thus affecting the quality of diet for insects (Throop and Lerdau, 2004; Carnicer *et al*. 2015; Filipiak and Weiner, 2017). Shifted ratios of N with other key elements and nutrients such as carbon (C), phosphorous (P), calcium (Ca), and magnesium (Mg) can impact on the stoichiometric balances of animals through higher trophic levels (Audusseau *et al.* 2015). Herbivorous insects feeding on plant matter with proportionally higher N could encounter nutrient deficiency. Evidence shows that lepidopteran larvae feeding from high N:P vegetation have lower body mass and decreased chance of survival (Apple *et al.* 2009). Lepidopterans are typically P-limited organisms, with a low N:P ratio (Fagan *et al.* 2002), it’s possible that similarly nutrient-limited herbivores could encounter developmental or growth problems when feeding from highly N-enriched vegetation. On the other hand, increased N proportionally to other nutrients and elements could favour larger insects and those not limited by other nutrients (Fagan *et al.* 2002). In addition to stoichiometric imbalances, dietary quality can be compromised through increased toxicity of plant defensive compounds (Tao *et al.* 2015).

Cooler microclimates amongst tall grass or near the ground can be created through N enrichment; as nitrophilic grasses grow, increasing the vegetative sward, less light reaches the ground. Although causal research is somewhat lacking, correlative studies show that cooler microclimates could have a strong impact on invertebrate development and populations (Nijssen *et al.* 2017). This will most likely affect species that are reliant on warm springs and those undergoing important developmental stages during this time, such as egg-larvae hibernating lepidopterans (Wallisdevries and van Swaay, 2006). In a study of three separate N-driven mechanisms, Klop *et al.* (2015) found microclimatic cooling the most likely cause of *Lasiommata megera* declines in the Netherlands. This species hibernates as larvae, lepidopteran species that hibernate as pupae or adults are not severely impacted (Wallisdevries and van Swaay, 2006).

N-driven alterations to plant communities can lead to the loss of plant species with important ecological roles such as host plants for specialised insects. This can impose significant pressure on closely linked species. For example, Swedish butterfly species that are dependent on nutrient-poor habitats have declined substantially, concomitantly many species associated with nutrient-rich habitats benefitted from the changing botanical communities (Ockinger *et al.* 2005). This suggests a significant role of N-driven botanical species turnover in invertebrate communities. The availability of dietary resources is also dependent on phenological synchronicity. Soil-N enrichment can affect flowering phenology (e.g. Cleland *et al.* 2006), which may have implications for flower-visiting insects. Phenological changes in the emergence of new leaves could constrain the provision of suitable dietary vegetation for herbivores, as suggested by Throop and Lerdau (2004). Research into the effect of N deposition on this pathway is lacking and further work needs to be done to identify if synchronicity is affected.

Research into the impact of drivers of global change often considers species on a spectrum of generalists to specialists, with specialist species typically more vulnerable to environmental changes (e.g. Warren *et al.* 2001; Clavel *et al.* 2011; Potts *et al.* 2010; Carvalheiro *et al*. 2013). However, not all specialist and oligolectic invertebrate species respond in the same way to N-deposition. Responses of specialist insects to N deposition are often strong, but the direction of response may differ according to their lifestyle and host plants (Poyry *et al.* 2017). Those with an N-rich diet or utilising nitrophilic host plants could benefit, whereas those associated with N-poor habitats could decline, as observed in a study of the range expansions of 282 Swedish butterfly species between 1973 and 2010 (Betzholtz *et al.* 2013). The increased concentrations of foliar N and shifts in botanical species composition led Poyry *et al.* (2017) to predict that large, dispersive, multivoltine insects and those with generalised habits or specialised to nitrophilic plants will dominate insect communities with increasing N deposition. This could lead to homogenisation of biodiversity and overall species loss with higher fluxes of N to terrestrial ecosystems; indeed, Haddad *et al.* (2000) found decreased insect diversity and species richness, across herbivores and predators, with N additions.

Although research into herbivorous invertebrates shows N deposition acts through higher trophic levels than plants, we still have a poor understanding of how insect pollinators are affected by N deposition. Bees, key insect pollinators, are obligate flower visitors and can technically be considered herbivores as they feed on plant produce: nectar and pollen. Given the recorded declines in many pollinating insects, including bees, identifying whether and how N acts as a driver is imperative. Consequential effects on pollination services and biodiversity could be severe if N acts as a substantial driver on insect pollinators.

Pollinating and flower-visiting insects could be strongly impacted by environmental drivers that act on plants, due to their intrinsic interactions with floral units. Therefore, N-driven botanical species turnover and loss of species richness could have strong implications for flower visiting insects by altering the landscape of floral dietary resources. The detrimental effect of botanical species loss on pollinator populations and diversity has been extensively shown (e.g. Potts *et al.* 2003; Carvel *et al.* 2006; Fründ *et al.* 2010; Senapathi *et al.* 2015). Specialist, oligolectic, pollinators are more threatened by losses or changes in floral resources (Weiner *et al.* 2011), as they lack the flexibility to use alternative resources, unlike generalists. As such, evidence shows a close correlation in the population trends of specialised pollinators and specialised entomophilous plants in Britain and the Netherlands (Biesmeijer *et al.* 2006). The historic decline of key floral resources is shown to be a strong factor in historic declines of oligolectic pollinators in the Netherlands (Scheper *et al.* 2014).

Bees are also shown to respond to the quality of floral resources such as sugar (Vandelook *et al.* 2019) and amino acid content (Cook *et al.* 2003). The quality and botanical origin of nectar and pollen can lead to stoichiometric shifts in key nutrients in the bee (Abbas *et al.* 2014), illustrating how stoichiometric changes caused by N deposition act through further trophic levels. An experimental study by Hoover *et al*. (2012) showed that bumblebees were more attracted to, and fed more from, synthetic nectar representative of N-enriched plants than synthetic control nectar. However, this nectar also induced a higher mortality rate in the foraging bumblebees. This emphasises the need to better understand the impacts of N deposition on pollinating insects.

Baude *et al.* (2016) postulated that trends in UK pollinator diversity may be related to UK atmospheric N deposition during the past century. From the early 20th century until approximately the 1980s, atmospheric N deposition increased (Fowler *et al.* 2004; RoTAP 2012; Storkey *et al.* 2015) while total nectar provision, nectar diversity and pollinator diversity all decreased (Carvalheiro *et al.* 2013; Baude *et al.* 2016). Since the 1990's, when N deposition in the UK began to plateau and decline, nectar diversity and total nectar provision increased alongside an alleviation in the decline of pollinator diversity (Carvalheiro *et al.* 2013; Baude *et al.* 2016). Research into the relationship between N deposition and plant-pollinator interactions and pollinator assemblages is required to determine if these correlative trends need to be considered more meaningfully in pollinator debates. With more data and evidence, we can begin to develop a mechanistic understanding of how pollinators are affected by changes in soil N across taxa and ecoregions in the context of the multitude of other factors impacting pollinator populations (Vanbergen *et al.* 2013). Long-term ecological experiments such as the Park Grass Experiment at Rothamsted Research (Hertfordshire, UK) have an important role to play in elucidating these mechanisms (Storkey *et al.* 2016).

1.5 Pathways of how N impacts on pollinators

When pollinating insects forage in the natural world, they choose from a selection of floral resources. Botanical species pool and flowering phenology determine the availability and selection of these floral resources. The most common reward that pollinating insects seek is nectar. The increased availability of N, commonly a limiting nutrient, can potentially alter relevant botanical traits. Therefore, N addition could disrupt or, conversely, strengthen individual plant-pollinator interactions; possibly affecting the structure of networks, and pollinator communities (Fig. 1.1). Botanical species composition and two important floral traits, phenology and nectar, set out potential pathways through which N could impact plant-pollinator interactions and pollinator communities. These pathways will be the focus of the thesis, and the following sections of this chapter will review the scientific evidence for the effects of N on these pathways.

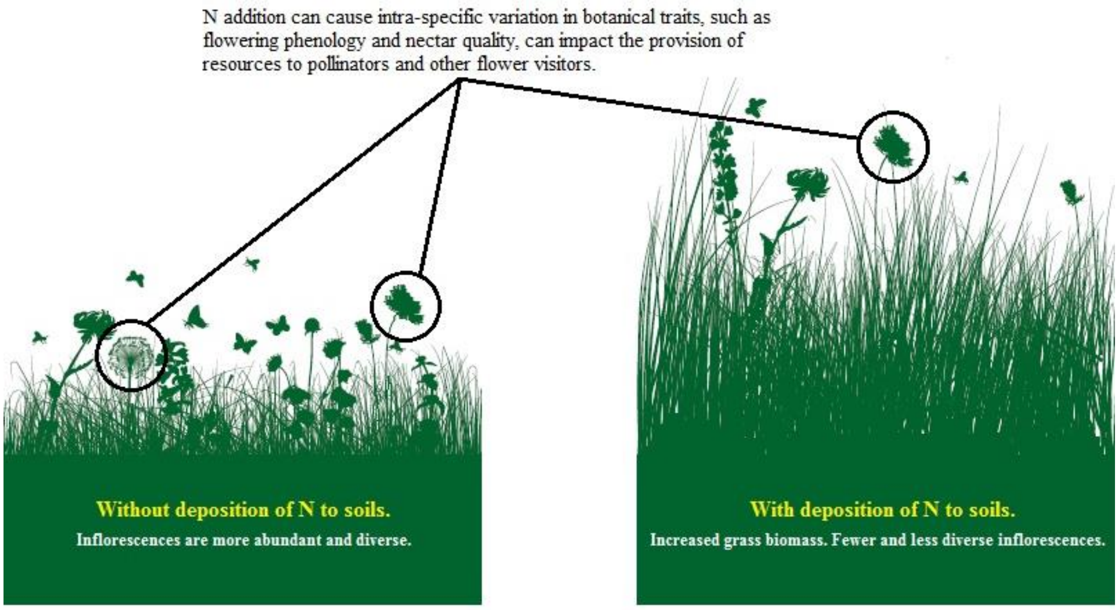


Figure 1.1 Increased fluxes of N to soils can alter the landscape and affect available food resources for pollinators.

The image depicts the loss of flowering species and dominance of grass typical of elevated N deposition in temperate grasslands. Our understanding of the impacts to floral traits, such as phenology and nectar, is lacking.

1.5.1 Species composition

Soil eutrophication lessens the limitation of a key resource nutrient; consequentially altering plant competition dynamics, typically causing increased biomass of fast-growing competitive grasses at the expense of flowering forbs and legumes (e.g. Suding *et al.* 2005; Helsen *et al.* 2014; Goulding *et al.* 2015; Storkey *et al.* 2015). Soil acidification reduces pH, frees potentially toxic heavy metals such as aluminium, and decreases the cation-exchange capacity and availability of beneficial minerals (Stevens *et al.* 2010b; Phoenix *et al.* 2012). This causes further selectivity, with plants sensitive to low pH and heavy metals, such as aluminium, declining (Stevens *et al.* 2010b), especially in poorly-buffered soils such as acidic grasslands, heaths, and forests (Clark *et al.* 2007; Diekmann *et al.* 2014; Field *et al.* 2014). Therefore, soil eutrophication and acidification are important drivers of reduced botanical species richness (e.g. Stevens *et al.* 2010a, b; Duprè *et al.* 2010; Wesche *et al.* 2012; Field *et al.* 2014). However, even when species richness does not decrease there can be shift in botanical community structure and a decline in flowering forbs, reducing the richness of useful species for pollinators and other flower visiting insects (Phoenix *et al.* 2012).

The dominance of grasses tolerant to N and low pH translates into reduced diversity and abundance of plants that provide nectar and pollen for pollinators (Wesche *et al.* 2012; Helsen *et al.* 2014). Habitat degradation and the loss of entomophilous plants is known to be a key factor in insect declines (e.g. González-Varo *et al.* 2013). The loss of nectar and pollen resources are a detriment for current and future generations of pollinator communities.

Evidence from temperate and montane grasslands suggests that the rate of botanical species loss increases with higher levels of N deposition (Stevens *et al.* 2010a; Humbert *et al.* 2016). Therefore, the impact to pollinators may be more severe at greater deposition levels. In the Northern Hemisphere, where N deposition has been high, this may have contributed to the negative trends of many insect pollinators during the 20th century (Wallisdevries, Van Swaay and Plate, 2012; Carvalheiro *et al.*, 2013), as suggested by Baude *et al.* (2016).

Studies from Mediterranean and tropical ecosystems show N deposition can encourage the spread of exotic nitrophilic plant species at the expense of native forbs (Bobbink *et al.* 2010). Encroachment of exotic grasses could greatly harm the native pollinator community by decreasing native nectar and pollen resources. The invasion of exotic entomophilous plants can have more complex dynamics for pollinator communities and the consequences will likely vary with botanical species and invaded community (Bartomeus, Vilà and Santamaría, 2008). The additional nectar and pollen resources can be beneficial for flower-visitors and attract many generalist pollinating insects. However, invasive plant species can compete with native species and potentially dominate pollen transport networks, which threatens the reproduction of native plant species (Lopezaraiza-Mikel *et al.* 2007). While generalist insect pollinators may benefit, there is a concern that specialised native pollinators will suffer if their associated food plants decline (Weiss 2009).

There is a large diversity of plant-pollinator interactions, with plants and pollinators adapting specialised traits to improve the efficiency of their interaction. Insect pollinators occupy a range of niches and can be categorised into functional groups that reflect size, feeding apparatus, and foraging behaviours. Some functional groups are linked with certain functional groups of floral traits, such as long-tongued bees and the deep corollas of *Trifolium pratense*. To understand more accurately how soil-driven shifts in plant community composition affect pollinating insects, we need to know how specific plant functional groups, species, and traits within a community respond. The loss of certain botanical species and traits threatens specialist interactions and is a cause of historic pollinator loss (Kleijn and Raemakers 2008; Scheper *et al.* 2014). For example, in the Netherlands, declines in N-sensitive host plants has led to the local extinctions of their associated butterfly species (Öckinger *et al.* 2006). Meanwhile, butterflies that utilise nitrophilic host plants are increasing in abundance (Wallisdevries, Van Swaay and Plate, 2012; WallisDeVries and van Swaay, 2017). The high levels of N deposition in Harpenden, UK during the 1970's-1980's dramatically reduced the ability of *Fabaceae*, such as *Trifolium pratense*, to grow naturally (Storkey *et al.* 2015). Since the 1990's, the plateauing and decline of N deposition allowed Fabaceae to recover. This may have contributed to the pattern of declines in long-tongued bumblebees, which were most severe during 1970-1989 and have become less accentuated since the 1990s (Carvalheiro *et al.* 2013). When closely associated food plants decline, the degree of impact to pollinators will depend on how severely the botanical trait is affected, whether the pollinator has a polylectic or oligolectic diet and if there are alternative food sources available, and whether oligolectic pollinators are able to show plasticity and adapt their foraging behaviour (Kleijn and Raemakers 2008).

Functional trait analysis of the botanical community can identify whether specific floral traits will increase or decrease in a community with N deposition. This, in turn, can be used to inform us which pollinator functional groups will be more affected by N deposition. Reduced overall functional diversity of the plant communities, and a decrease in entomophilous plants, have been found in European grassland communities and in floodplains in North Germany (Wesche *et al.* 2012; Helsen *et al.* 2014). Reduced floral diversity equates to reduced diversity of functional nectar and pollen traits, which can lead to impoverished species richness of bee assemblages (Potts *et al.* 2003, 2010; Fründ, Linsenmair and Blüthgen 2010; Weiner *et al.* 2014). Stevens, David and Storkey (2018) performed a trait-analysis on a dataset of acidic grasslands across the UK to give an insight into how the botanical degradation caused by N deposition and soil acidification might affect upland pollinator communities. The authors found that plants with floral structures suited to larger bees (e.g. bumblebees) and long-tongued pollinators, such as zygomorphic flowers or deep corollas, were more absent in areas that experienced higher levels of N deposition and with more acidic topsoil. The reduction of suitable forage plants within these habitats, caused by high levels of N deposition, can impose stress upon and threaten the associated functional groups of pollinators, such as long-tongued bumblebees. Generalist floral resources typically visited by smaller bee species were not negatively affected by N deposition or soil acidification. The results suggest that, in acidic grasslands, bees that visit generalist open flowers will not be as heavily impacted as specialist, long-tongued pollinators and large-bodied bees. The analysis also found that plants with lower nectar production are more prevalent in areas with higher levels of N deposition (Stevens, David and Storkey 2018), indicating that N deposition may reduce the overall quantity of nectar produced in upland acidic grasslands.

Following the decline or termination of N application, botanical communities can remain in a state of low diversity that reflects historic soil enrichment. Experiments have shown these conditions can persist for over 20 years following the cessation of N addition (Isbell *et al.* 2013). Although the overall community structure may be slow to recover, in some communities the occurrence of N-sensitive species can increase within a couple of decades of declining N fluxes (Clark and Tilman 2008; Storkey *et al.* 2015). Evidence from the Park Grass Long-Term Experiment has shown that the decreasing levels of atmospheric N deposition since the 1990’s has allowed a recent resurgence of *Fabaceae* and other important nectar and pollen resources (Storkey *et al.* 2015).

1.5.2 Phenology

Soil N content and additions can potentially alter the phenology of plants, but our understanding of how the flowering phenology of entomophilous plants responds across taxa is poor. The mechanisms through which flowering phenology responds to increased fluxes of N to soil are unclear. There are typically three mechanisms that cause a plant to move resource allocation from growth to reproduction; autonomous regulation, environmental cues, and stress (Simpson *et al.* 1999; Song *et al*. 2013; Takeno, 2016). It is possible that N could act on all three of these, dependent on local factors and the plant species. Autonomously, it’s been shown that flowering date clearly correlates with plant maximum height and the time at which the plant reaches a certain height (Vile *et al.* 2006; Sun and Frelich, 2011). Therefore, N could potentially delay flowering in dominant nitrophilic plants and those able to exploit the extra nutritional resources that grow taller with soil eutrophication. Alternatively, the increased vegetative sward leading to cooler microclimates amongst tall plants may limit the photoperiod accessible to smaller plants. This could theoretically delay flowering for small plants that are able to maintain populations amongst the sward. Finally, excessive soil N can induce stress in plants particularly if the N form, NH_x or NO_y, is not what the plant is adapted to, such as NH_x inducing plan cell acidification in calcareous grassland species (Bobbink *et al*. 1998). Stress often accelerates flowering, as the plant rushes to ensure reproductive success at the expense of immediate growth (Wada and Takeno, 2010; Takeno, 2016).

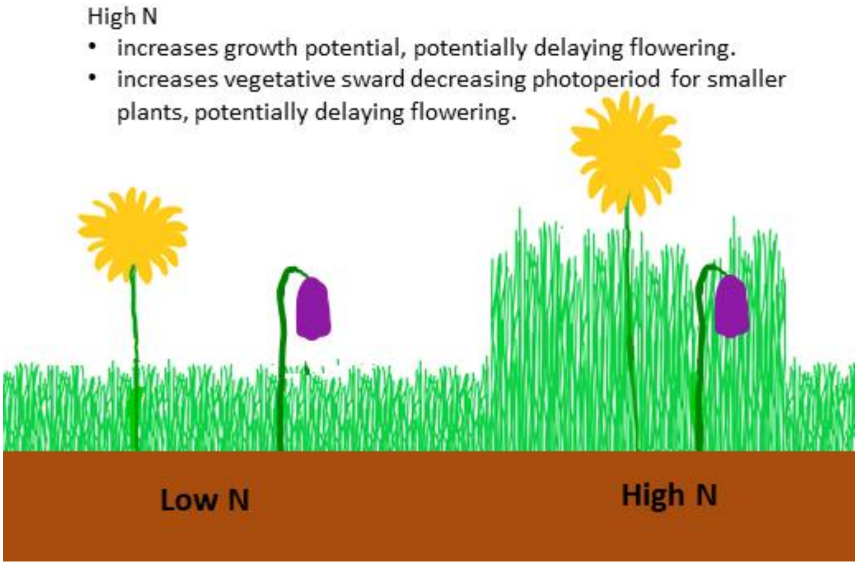


Figure 1.2. A simple illustration depicting how N additions could affect the autonomous and environmental cues for flowering phenology.
By increasing the potential for growth, nitrophilic flowering plants could spend more time in the growth phase before switching to reproductive phase. The increased vegetative sward limits the solar energy reaching smaller flowering plants, reducing their photoperiod and potentially delaying flowering.

Evidence from a field trial of experimental plots in coastal Central California found that addition of N to soils delayed flowering in grasses and slightly accelerated flowering of annual forbs (Cleland *et al.* 2006). This may be due to annual forbs increasing the partitioning of newly acquired nutrition to reproduction, whereas perennial grasses that can spread vegetatively might not partition so much energy towards floral units. Alternatively, advanced stress-induced flowering could have occurred amongst forbs whereas the N-rich soil may have delayed the switch from growth to reproduction in nitrophilic grasses. In long-term experiments in UK, the ericaceous dwarf shrub *Vaccinium myrtillus* produced flower buds earlier with N additions (20 kg N ha^{-1} yr^{-1} in acidic grasslands, 60 kg N ha^{-1} yr^{-1} in heathlands). Hoover *et al.* (2012), also found that N enrichment accelerated the flowering of *Cucurbita maxima* in a potted experiment. Other studies show that forbs can vary in their phenological response to increased soil N, and do not always flower earlier. In a field experiment in a temperate steppe of North China, the flowering phenology of most forbs did not respond to the experimental addition of 100 kg N ha^{-1} yr^{-1}. However, *Heteropappus altaicus* had a delayed date of first flowering and *Allium bidentatum* had a shortened bloom duration (Xia and Wan 2013). In a Tibetan alpine meadow, the flowering phenology of three *Ranunculaceae* were monitored in response to N addition. *Anemone trullifolia* and *Caltha scaposa* responded; dates of first and last flowering were delayed. *Trollius farreri* showed no response (Liu *et al.* 2017). This is an example of inter-species variation of response within taxonomic families.

The Park Grass Long-Term Experiment at Rothamsted Research, has shown that chronic eutrophication can lead to local adaptation in flowering phenology (Silvertown *et al.* 2006). The field plots have received consistent N applications for over 150 years and populations of a grass species, *Anthoxanthum odoratum*, flower at alternative times according to soil treatment, preventing pollen exchange between some populations (Snaydon and Davies 1982). This asynchrony between populations can theoretically lead to speciation. There has not yet been a similar study published that investigates the divergence of populations of entomophilous plants in Park Grass.

It is largely unknown how shifts in flowering phenology will influence pollinator communities (Miller-Rushing *et al.*, 2010). Models predict that shifts in flowering phenology can disrupt interactions, potentially leading to network instability, reduced pollinator abundances, and potentially local extinctions (Memmott *et al.*, 2007; Fabina, Abbott and Gilman, 2010). However, realistically, if species are able to adapt and find and utilise alternative food resources the impact should not be so severe (Benadi *et al.*, 2014). Therefore, high plant species richness could buffer detrimental effects of phenological shifts, by providing alternative forage. The impact to pollinators will likely vary across ecosystems, depending on the plant and pollinator species present and on the extent of N enrichment. Unlike the effect of climatic warming, which can also accelerate insect emergence dates (Bartomeus *et al.*, 2011; Ovaskainen *et al.*, 2013), soil N enrichment will likely only affect plants, not their pollinators. Therefore, there is a potential for phenological asynchrony and of uncoupled specialised interactions, which can have negative implications for both pollinator communities and plant reproduction (Burkle, Marlin and Knight, 2013; Kudo and Ida, 2013).

1.5.3 Floral production and morphology

Nitrogen addition has been shown to affect floral production, however, the responses are species-specific and often dependent on the amount of N. Several studies found that low additions of N to soils can enhance floral production, meaning more floral units per plant (Muñoz *et al.* 2005; Burkle and Irwin 2009a, b; Hoover *et al.* 2012). Increased floral production, can drive an increase in pollinator visitation per plant (Muñoz *et al.,* 2005; Burkle and Irwin, 2010), and has been linked with improved insect pollinator diversity (Potts *et al.* 2010). In long-term experiments in UK heathlands, with N applications ranging between 7.7-60 kg N ha^{-1} yr^{-1}, flowering of the dominant shrub *Calluna vulgaris* increased with N, with flowering at a maximum at the highest levels of N addition (Phoenix *et al.* 2012). In a subalpine meadow field experiment, Burkle and Irwin (2010) found that total floral production per plot, when including all species, was highest with low levels of N additions (10 kg N ha^{-1} yr^{-1}) compared to control or high N (200 kg N ha^{-1} yr^{-1}) plots. These two studies indicate dose-dependent responses, but in contrasting ways. Dominant species may be more likely to maintain enhanced floral production with high N additions, as shown by Phoenix *et al.* (2012). On the other hand, for other species there is a narrow window of N addition in which enhanced floral production can benefit pollinators, but at higher levels floral production declines and positive effects diminish (Burkle and Irwin, 2010). In the aforementioned subalpine study, the floral production of *Potentilla pulcherrima* decreased with high N addition (200 kg N ha^{-1} yr^{-1}) (Burkle and Irwin, 2010). Declines in forb floral production were also observed in long-term UK grassland experiments (Phoenix *et al.* 2012). The authors noted how forb flowers declined more noticeably than forb cover, and may be a more sensitive indicator of responses to N deposition. As floral production is a more important metric than vegetation cover for pollinators, this is important to bear in mind when considering data on species composition. Gijbels *et al.* (2015) and Burkle and Irwin (2010) did not find any effect of N on floral production per plant in *Gymnadenia conopsea* and *Ipomopsis aggregata*, respectively.

The range of responses of floral production to N addition shows that impacts to pollinators could vary across ecosystems, soil types, and botanical communities, as well as with levels of N addition.

Burkle and Irwin (2010) found that low N addition increased the length and width of corollas of *Ipomopsis aggregata* flowers. In many flowering plants with elongated corollas, the floral morphology has evolved to restrict access to unwanted visitors and selectively favour certain insects (Nilsson 1988). Significant changes to corolla morphology caused by N addition occur on much faster timescales than the co-evolved adaptations, and without the likewise adaptations of insect proboscises. Therefore, the elongation of corollas may exclude some of the preferred pollinators. Furthermore, if increased soil N causes corollas to widen, opportunistic and unwanted flower-visitors may have better access to the valuable nectar supply. However, there is no evidence to determine whether alterations of floral dimensions or morphology will impact upon specialised pollinators or pollination services in the real world.

1.5.4 Floral rewards

Pollinating insects visit flowers to obtain the nutritional rewards that sustain them; nectar and pollen. Although pollen is digested by some adult insects, and is required for the development of bee larvae, nectar is the most commonly sought reward and reason for visiting inflorescences. For this reason, the thesis will focus on nectar, specifically nectar sugars, as floral rewards. Nectar is sugar-rich and contains other compounds, including amino acids, lipids, and defensive compounds, and forms and integral part of the diet of many flower-visiting insects. Nectar secretion and sugar content varies greatly between plants (e.g. Petanidou *et al.* 2006; Heil *et al.* 2011; Hicks *et al*. 2016) and is affected by abiotic and biotic factors (Zimmerman, 1988). However, we lack a clear and in-depth mechanistic understanding of how various factors affect nectar traits (Heil *et al.* 2011). It has been demonstrated that below-ground conditions and plant-plant competitive dynamics affect nectar production and sugar content (Baude *et al.* 2011; Barber and Soper Gorden 2014). Therefore, soil N enrichment is likely to generate indirect impacts on nectar traits through alteration of plant competitive dynamics. However, there are few studies exploring how nectar traits are affected by soil N. Furthermore evidence shows the responses of nectar traits to soil eutrophication are not uniform; our ability to forecast impacts of N additions on nectar traits of plants is confounded by the evidence of inter-specific response variation.

In a study on the effects of nutrient additions to nectar traits, N addition did not increase the nectar secretion of *Trifolium pratense*, an N-fixing *Fabaceae*, but it did increase secretion rate in *Antirrhinum majus* (Shuel 1956). In another study, nectar secretion increased in *Ipomopsis aggregata* but not in *Linum lewisii* or *Potentilla pulcherrima* (Burkle and Irwin 2009a; Burkle and Irwin 2010). However, the increase in nectar secretion of *A. majus* and *I. aggregata* was only observed at low levels of N addition (10 kg N ha^{-1} yr^{-1} for *I. aggregata*). At high levels of N addition (200 kg N ha^{-1} yr^{-1} for *I. aggregata*), nectar secretion decreased for both species (Shuel 1956; Burkle and Irwin 2010). This suggests that while low N deposition might bring benefits for nectar-feeding insects, excessively high deposition will not.

The composition of sugars within nectar is an important determinant of insect visitation and niche division. A more diverse selection of nectar sugar compositions can improve the richness of pollinator assemblages (Potts *et al.* 2003), due to the preferences and requirements of different pollinator guilds. For instance, Hymenoptera pollinators prefer high sucrose nectars, Diptera visit lower sucrose nectars, and Lepidopteran flower-visitors are intermediate (Petanidou 2005). Some species show sex-specific nectar niches; Rusterholtz and Erhardt (2000) found that females of *Lysandra bellargus*, a threatened European Lepidoptera, preferred high glucose nectars whereas males preferred high sucrose. Ceulemans *et al.* (2017), studying *Succisa pratensis*, demonstrated that the composition of sugars can be affected by soil nutrient enrichment with N and phosphorous (P); glucose proportionally decreased. Whether this response is uniform across more species is unknown; *S. pratensis* is an N-sensitive species (Hill 1999) and there may be inter-specific response variation. It is possible that alterations to the composition of sugars in nectar can impact on the diets of pollinator guilds and disrupt the interaction niches. Whether the alteration of nectar sugar composition is enough to influence plant-pollinator networks is unknown and requires further research.

Floral nectars contain many other compounds, in addition to sugars, that can directly impact the nutritional qualities and attractiveness of nectar (Alm *et al.* 1990; Carter and Thornburg 2004; González-Teuber and Heil 2009; Nepi *et al.* 2012; Nepi 2014). Amino acids are found ubiquitously in nectar (Baker 1977), and are a necessary component of insect diets. The amino acid content of nectar can be a driver of bee health and mortality; Paoli *et al.* (2014) found high amino acid diets increased the lifespan of queen-right *Apis mellifera* workers, but decreased the lifespan of queenless workers. Excessively high levels of amino acids may be problematic or even dangerous for pollinating insects, for example naturally high concentrations of the non-protein amino acid β-alanine in the nectar of *Gentiana lutea* have been observed to stun and reduce motor skills in foraging bees (Nepi 2014).

High soil N content, and N additions, have been shown to increase total amino acid content in most studies (Gardener and Gillman, 2001; Gijbels, Van den Ende and Honnay, 2014; Gijbels *et al.*, 2015), with Gardener and Gillman (2001) observing a linear relationship between N addition and nectar amino acid content. Of the individual amino acids, asparagine and glutamine are commonly observed to increase with N addition (Gardener and Gillman 2001; Gijbels *et al.* 2015; Ceulemans *et al.* 2017). Where levels of soil N are excessive, glutamine can be excreted from some plant species to avoid cellular damage, which could explain the increased proportion of glutamine in the nectar of fertilised plants (Gardener and Gillman 2001; Gijbels *et al.* 2015). Other amino acids observed to increase with soil N addition in individual studies are serine in *Gymnadenia conopsea*, a Lepidoptera-pollinated *Orchidaceae* (Gijbels, Van den Ende and Honnay, 2014), and proline in *Agrostemma githago* (Gardener and Gillman 2001). There are no common trends in the amino acids that decrease in production; rather the responses are specific to individual experiments and study species. Gijbels *et al.* (2015) observed a decrease in arginine, aspartic acid, and glutamic acid in the nectar of *Gymnadenia conopsea*. These three amino acids inhibit the chemosensory cells of Diptera (Shiraishi and Kuwabara 1970). Although *G. conopsea* is Lepidoptera-pollinated, if this response is found in other plants it could affect the taste perception and foraging choices of Dipteran pollinators, such as *Syrphideae* (Gijbels *et al.* 2015). Gamma-aminobutyric acid (GABA), proline, and glutamine share a common biosynthetic pathway from glutamine alpha-ketoglutarate. In a study on the effects of N addition to the nectar amino acid profile of *Agrostemma githago*, Gardener and Gillman (2001) observed a decrease in GABA alongside an increase in proline and glutamine. This suggests a potential response mechanism whereby proline and glutamine are present at higher concentrations at the expense of GABA (Gardener and Gillman 2001). GABA, and its close association with NaCl salt, has phagostimulatory effects on insects but can be dangerous for larvae at high concentrations (Nepi 2014). Therefore, it is not clear whether a decrease in GABA would affect pollinators positively or negatively. The increase in proline concentration could be useful for pollinators; it is rapidly metabolised and is useful for the conversion of energy in initialising flight in invertebrates (Carter *et al.* 2006). It also serves as an attractant; Bertazzini *et al.* (2010) found honeybees were more attracted to proline than serine, alanine, or control solutions. However, its attractiveness is highest at moderate levels, with bees becoming less interested at higher concentrations (Carter *et al.* 2006).

As with sugars, amino acids in nectar are shown to be correlated with certain functional groups of pollinators. Petanidou *et al.* (2006), studying a Mediterranean plant community of 73 species, found pollinator preference was the strongest determinant of nectar amino acid composition, more so than life-history or taxonomic group. This is highly suggestive of an ecological role of nectar amino acids that influence plant-pollinator networks. For example, phenylalanine and γ-aminobutyric acid (GABA) are found in high concentrations in plants pollinated by bumblebees and long-tongued solitary bees (Petanidou *et al.* 2006; Nepi 2014). Petanidou *et al.* (2006) also observed that total amino acid content was correlated with the number of long-tongued visitors. Sex-specific niches also exist with regards to amino acids; females of the threatened *Lysandra bellargus* butterfly prefer a diet high in amino acids (Rusterholtz and Erhardt 2000), potentially to enhance fecundity (Mevi-Schutz and Erhardt 2005). The variation in nectar preferences and niches across insect pollinator taxa suggests an important role for nectar chemistry in the diets of insects. However, it is not known if the changes to nectar brought about by N deposition will impact on plant-pollinator interactions.

We are lacking evidence of how soil N affects the production of defensive secondary compounds in floral nectar (González-Teuber and Heil 2009). Alkaloids, glycosides, and phenolics play a role in selectively limiting flower visitors (Carter and Thornburg 2004; Nepi 2014). At low levels these substances can be important for the health of pollinating bees, by reducing parasite loads (Richardson *et al.* 2015; Richardson, Bowers and Irwin 2016). However, in excessively high concentrations alkaloids are repellent and can reduce mobility and motor function of bees (Manson *et al.* 2013). The addition of N can increase the production of natural defensive compounds in vegetation (Chen, Olson and Ruberson, 2010); if a similar response is found in floral nectar there may be consequences for pollinating insects. There has also been no research into how floral volatiles, which are used by bees during early foraging trips to identify sources of nectar and pollen (Dötterl and Vereecken 2010), are affected by soil N. Without a broad understanding that encompasses the less prevalent components of nectar, it is difficult to forecast how nectar traits affected by soil N will impact pollinators.

Although there are few studies into how soil N affects nectar traits, research that includes the impact on pollinators is even sparser. Ceulemans *et al.* (2017) housed colonies of *Bombus terrestris* with *Succisa pratensis*, to investigate the effects of nutrient enrichment on floral reward chemistry and colony development. The study found that colonies housed with enriched *Succisa pratensis* had more dead and ejected larvae from *Bombus terrestris* colonies, leading to a smaller colony with fewer workers during the colony's mid-life. The study did not identify whether a specific component of nectar or pollen was the cause, but it is evidence that high soil enrichment may be detrimental to colony health. Hoover *et al.* (2012) artificially made *Cucurbita maxima* nectar from a 'recipe' of sugars and amino acids that represented the concentrations found in control and N-enriched plants. In this experiment, *Bombus terrestris* workers preferentially visited and consumed nectar of enriched plants, but their lifespan decreased. These two studies reveal negative impacts of soil N enrichment on the development and longevity of a key pollinating species, which could potentially lead to impoverished pollinator populations and pollination services. However, it should be noted that the artificial nectar used by Hoover *et al.* (2012) only contained sugars and amino acids, without the less prevalent secondary compounds, so is not necessarily applicable to real-world responses. Evidence on other key pollinating species, such as wild solitary bees, would be useful to ascertain the occurrence of this response.

Many flower-visitors feed on or collect pollen, including agricultural pests (such as the pollen beetle) and important pollinators. Bees collect pollen to rear their young, and the botanical origin, quantity, and chemistry of pollen can affect the health and mortality of future generations of these key pollinators (Roulston, Cane and Buchmann, 2000; Potts *et al.*, 2003; Sedivy, Müller and Dorn, 2011; Vanderplanck *et al.*, 2014). Foraging bees are able to recognise and preferentially forage pollen that has a higher content of essential amino acids (Cook *et al.* 2003). Pollen with higher protein and amino acid content lead to more and larger larvae (Génissel *et al.* 2002; Vanderplanck *et al.* 2014) and without sufficient amounts of key amino acids, larvae are unable to develop and survive (Roulston and Cane 2000). Therefore, the nutritional qualities of pollen are important for ensuring sustainable pollinator communities.

Ceulemans *et al.* (2017) found soil enrichment with N and P altered the sugar composition of *Succisa pratensis* pollen; glucose increased, and fructose decreased. The authors also recorded a shift in the amino acid profile, with asparagine and ornithine increasing alongside a decrease in arginine, glycine, and threonine. However, as the content of proteins, amino acids, and other compounds in pollen varies across botanical species (Szczesna 2006), there is likely inter-species variation in the response to increasing soil N. As noted previously, Ceulemans *et al.* (2017) found that floral rewards from nutrient-enriched plants caused more dead and ejected larvae from *Bombus terrestris* colonies, although the study did not establish if this response was due to changes in nectar or pollen qualities. Burkle and Irwin (2010) did not find any change to the production of pollen per individual flower with N addition. However, the increase in floral production of *Ipomopsis aggregata* with low N addition (10 kg N ha^{-1} yr^{-1}) resulted in greater overall pollen production per plant. On the other hand, with high N addition (200 kg N ha^{-1} yr^{-1}), *Potentilla pulcherrima* floral production decreased, causing an overall decrease in pollen production per plant. Lau and Stephenson (1993) found that *Cucurbita pepo* pollen grains from high N soils were 5% larger, however, whether this translates to higher overall nutritional content was not explored. Bees develop dietary niches with pollen specialisms more often than nectar specialisms, due to the importance of pollen in rearing offspring. These are driven by secondary metabolites found in low concentrations in pollen, and closely-related bee species can differ in their tolerances of some pollen compounds (Sedivy, Müller and Dorn 2011). There is not yet any research into how soil N affects the defensive compounds of pollen; if so, this could create stress and mortality for the larvae of some pollen specialists.

1.6 Knowledge gaps

1.6.1 The impact to pollinators

While there is some research that investigates how N additions affect the discussed botanical traits, we have very few studies concerning the actual impacts to pollinators. This is a key knowledge gap; the lack of causative research into how botanical traits affected by soil N consequentially affect pollinators. With N deposition set to rise in many parts of the globe in the future, it is important to know how pollinators will respond to background additions of N. Without research that incorporates pollinators into the experimental set-up it is impossible to know if, and how strongly, plant-pollinator interactions will be affected. It is important to know how the developmental and reproductive fitness of pollinators are affected by atmospheric N deposition or the addition of fertilisers. We also need to ascertain if responses are consistent across botanical and insect taxa, or if some interactions and functional groups are more threatened than others. Finally, there is also evidence that low additions of N can lead to changes in floral traits that may benefit pollinators, whereas the effects at higher rates of addition can be adverse (Muñoz *et al.* 2005; Burkle and Irwin 2009a, b; Burkle and Irwin 2010; Hoover *et al.* 2012). Clarity is required as to the thresholds beyond which N addition becomes detrimental for pollinators in various habitats.

Many studies use generalist pollinators, such as *Apis mellifera*, as a study-species. It is important to consider the wider pollinator community, as some specialist pollinator functional groups or species may be more threatened by N deposition. WallisDeVries and van Swaay (2017) developed an N index for butterflies based on hostplants, which illustrates the species that have been most impacted by N deposition and soil eutrophication. It may be possible to develop similar indices to help forecast the effect of atmospheric N deposition on other pollinators. There are oligolectic bee species with narrow diet breadths, such as *Chelostoma sp.* (Sedivy *et al.* 2008; Denisow and Wrzesień 2015), and long-tongued bumblebees with identifiable key forage plants. However, most bee species forage relatively opportunistically, which complicates the development of an N-sensitivity index encompassing all bee species. Given the inconsistency of responses to increasing soil N between plant species across the range of processes discussed above, it is likely that whether N additions lead to a net positive or negative response of pollinators will be dependent on specific plant / invertebrate assemblages. Developing a predictive framework will, therefore, require network analyses of plant-pollinator interactions along a soil fertility gradient as opposed to correlative models of total pollinator abundance or diversity.

1.6.2 Inter-specific variation and the synthesis of botanical traits

Research into how soil N affects relevant botanical traits, including flowering phenology and, especially, nectar and pollen chemistry are lacking (Nijssen, Wallis De Vries and Siepel, 2017), making it difficult to understand the impacts of N additions to pollinators. This task is further confounded by the inter-species variation found for these traits, evidenced through the studies to date. Many experiments so far have used individual study-species, and therefore we lack enough data to be able to understand the mechanistic reasons for these species-specific responses. Further field studies incorporating a range of botanical taxa can help to address this knowledge gap. Life-history traits of plants, such as annuality and perenniality, or N-preferences as denoted by Ellenberg N values (Hill 1999), may offer a predictive tool for the responses of botanical traits (Burkle and Irwin 2009a). Developing our understanding of inter-specific response variation will enhance our ability to forecast how ecosystems will respond to N addition.

To understand the overall impact of soil N on pollinator communities, we need to synthesise our understanding of the various botanical traits. This will improve the real-world applicability of research. For example, increased floral production and nectar secretion are beneficial responses for pollinators; but this may be completely negated, and the overall response detrimental, if the botanical species declines, if flowering phenology is dramatically shifted, or if production of toxic defensive compounds becomes excessive. When possible, future research should focus on a suite of botanical traits to more accurately determine the overall impact to insect pollinators.

If we combine this with an improved understanding of inter-specific variation, we can synthesise knowledge of various botanical traits and botanical taxa. We can then more accurately forecast how N addition alters plant communities for prospective pollinating insects. It would be useful to incorporate knowledge of nectar and pollen chemistry into our understanding of the compositional changes in botanical species, to reflect the actual changes of nutritional resources in an affected plant community. For example, *Taraxacum officinale agg.* is N-tolerant and is popularly visited by insects for nectar and pollen. However, an increase in *T. officinale* at the expense of other flowering plants is not necessarily beneficial; its pollen is lacking in key amino acids and bee larvae cannot survive on it alone (Roulston and Cane 2000; Génissel *et al.* 2002). By understanding the responses of plants to N addition across taxa and traits, and synthesising this knowledge, we can more accurately understand the impact to pollinators.

1.6.3 Biotic interactions

A key impact of atmospheric N deposition or the addition of fertilisers is the changing dynamics of botanical competition for increased nutritional resources. The interactions of plants as they compete for resources can also be a factor that affects floral and nectar traits (Baude *et al.* 2011). Therefore, field studies, or at least potted experiments that are representative of realistic botanical communities, will more reliably depict how plants respond to N addition. Studies of individual species might help to address general knowledge gaps in the subject, but we cannot be certain of the real-world applicability of these results without incorporating the effect of plant competition dynamics.

Soil biota and herbivores are affected by N deposition and impacts to these groups may have effects on pollinators. Below-ground biota and processes affect floral traits, and can alter pollinator visitation in a variety of ways (Scheu 2001; Barber and Soper Gorden 2014). Again, the responses of nectar and pollen chemistry are understudied; Barber and Soper Gordon (2014) note that little research has investigated the effect of soil biota on the nutritional value of floral rewards. Herbivory, which can be influenced by soil N (Phoenix *et al.* 2012), can also affect pollination, generally by reducing the attractiveness of plants (A'Bear, Johnson and Jones, 2014). The effects of atmospheric N deposition on ecosystems are complex, involving indirect interactions amongst trophic and functional groups of organisms. Therefore, synthesis of research into how N affects rhizosphere, plants, and herbivory would better inform our understanding of how N deposition affects pollinators.

1.6.4 Abiotic interactions

Future environmental scenarios will involve a complex system of multiple drivers. Studies have shown that aspects of climate change can act interactively with fertiliser use strategies, with the strength and direction of the interaction varying between studies (de Chazal and Rounsevell, 2009). Atmospheric N deposition is only a single driver of global environmental change and will not necessarily act alone (Tylianakis *et al.*, 2008; Burkle and Alarcon, 2011; González-Varo *et al.*, 2013). Realistically, an ecosystem will experience N deposition alongside increased atmospheric CO_2 and a changing climate. Evidence shows these drivers can act interactively to alter botanical traits differently than when acting individually (Cleland *et al.* 2006; Tylianakis *et al.* 2008; Hoover *et al.* 2012). For example, although climate warming is known to accelerate flowering phenology of many plant species (Fitter et al., 1995; Thórhallsdóttir, 1998; Miller-Rushing and Primack, 2008), the response can also be affected by soil N (Cleland et *al.* 2006, Hoover *et al.* 2012). Excessive soil N enrichment can exacerbate the stress plants experience from climatic events such as draughts and severe frost (Phoenix *et al.*, 2012). If these events increase in frequency with future climate change, plants may suffer further.

Our knowledge of the impacts of N deposition on pollinators is patchy. While studies that consider the effects of N are important and required, we should consider that botanical responses can be intensified or dampened by other drivers of environmental change. Research that addresses the combined effect of these drivers of environmental change are necessary to more clearly understand how pollinator communities will be affected in the future.

1.7 Aims of the thesis; research approaches to address knowledge gaps in the impact of soil nitrogen on plant-pollinator interactions

With this thesis, I have set out to study the impacts of soil N-enrichment on plant-pollinator interactions. To do this, I have conducted research on the Park Grass Long-term Experiment (PGE), based at Rothamsted Research, Harpenden, UK. This is a long-term chronic nutrient enrichment experiment based on a mesotrophic grassland (full description is given in Chapter 2, section 2.3.1). From the suggested pathways of potential N-driven impacts on plant-pollinator interactions, I sought to identify the impacts to the composition of floral resources in botanical communities, to two key floral traits, phenology and nectar, and to the pollinator communities observed interacting with the experimental plots (Fig. 1.2). As this area of research is still relatively unexplored, the proposed hypotheses are broad and aim to improve our understanding of this driver.

Research has been conducted that shows loss of species richness and losses in overall forb cover (e.g. Stevens *et al.*, 2004; Field *et al.*, 2014), but this is not informative enough if we want to know the impacts to higher trophic levels such as pollinators. Depending on the species that are lost, loss of species richness and forb cover can mean different things for pollinators. The functional roles of floral resources for pollinating insects vary, for example, from open morphology to restrictive specialist morphology, or from early-flowering to late-flowering plants. Functional traits analyses are a more accurate way of investigating the impacts of abiotic drivers to ecosystem characteristics, function, and stability (Díaz and Cabido, 2001; Garnier *et al.*, 2007). By studying the functional trait level impacts, we can gain a more informative insight into the effects of N deposition or applications on the provision of floral resources in a plant community. However, few have used these analyses to study the floral traits of plant communities, and fewer still have taken an in-depth look at the impacts of N on the composition of floral traits within botanical communities. I explored these relationships in chapter 2.

Previous research from Snaydon and Davies (1982) found evidence of local adaptation in the flowering phenology of a common grass, *Anthoxanthum odoratum*, in the PGE. This adaptation in phenology was recorded on the plot-scale in response to N treatments. However, similar studies have not been conducted on localised plot-scale phenological adaptations of flowering forbs and legumes. Such effects could be impactful to pollinating insects by altering the abundance and provision of available resources. Given that the PGE has a history in showing evidence of phenological local adaptation, it is a suitable study site to investigate N-driven phenological shifts in a range of flowering forbs and legumes (chapter 3).

The second floral trait I have studied, using the plant species from the PGE, is nectar (chapter 4). Nectar is more widely foraged from flowers than pollen, as it is the primary reason for many insects to visit flowers. Pollen is of course vital for bees for feeding their progeny, but other insects do not digest it so easily (Roulston and Cane, 2000). To investigate the impacts to the broad pollinator community, nectar is potentially a more useful trait to study because of its wide-ranging usage. The nutritional values of nectar vary amongst plant species and the specific sugar compositional signatures can influence pollinator visitors according to traditionally-thought pollinator syndromes (Petanidou, 2005; Abrahamczyk *et al.*, 2017; Vandelook *et al.*, 2019). Therefore, shifts in nectar traits could have implications for insect foraging behaviour.

Finally, in chapter 5 I assessed how pollinating insects respond to these alternative plant communities by studying the representative pollinator communities observed to visit separate PGE plots. All plots are subject to the same latent pollinator community, which may influence results by causing some overlap in species assemblages. The study investigated how N-driven impacts to the provision of floral resources impacts on pollinator visitation and foraging choices. Furthermore, the study showed whether certain functional groups of pollinators are favoured over others by N-enrichment. By recording the plant-pollinator interactions, I determined whether N-enrichment distorts plant-pollinator interaction networks and if this occurs in ways that could affect the ecosystem stability.

RESEARCH QUESTIONS AND HYPOTHESES

- PROVISION OF FLORAL RESOURCES, ON FUNCTIONAL TRAIT LEVEL.
    - N addition will lead to fewer insect-pollinated plants.
    - N addition will lead to the loss of morphologically specialist floral units, such as long corollas.
    - Floral trait composition will recover from eutrophication after 20 years but will still differ from historic nil plots.
- FLOWERING PHENOLOGY OF FORBS AND LEGUMES.
    - N additions will lead to changes in flowering phenology, but with inter-specific variation, due to different tolerances to N amongst species.
    - The patterns of community-scale in-flower species richness throughout the season will be affected by soil N.
- NECTAR TRAITS
    - N addition will lead to changes in sugar composition and provision.
    - Inter-specific variation in responses will be recorded.
- POLLINATOR COMMUNITIES AND INTERACTION NETWORKS
    - With N addition, the flower-visiting insect community will be less species-rich, with fewer specialised pollinators such as bumblebees.
    - With changes to the amount of specialist floral resources and pollinating insects, the nestedness of plant-pollinator networks will

Figure 1.2. The structure of the PhD thesis. This figure broadly presents research questions and hypotheses of the four results chapters.

CHAPTER 2

IMPACTS OF NITROGEN ADDITIONS TO FLORAL FUNCTIONAL TRAIT COMPOSITION OF GRASSLANDS, AND THE RECOVERY OF COMMUNITIES FROM NITROGEN.

Part of this chapter is based on an analysis conducted by the author, as part of a joint review, published in:

Functional Ecology (2018); 32(7): 1757–1769

2.1 Abstract

Declines in insect pollinators have been widely recorded. It has been established that these are due to many contributing factors, of which loss of floral resources and habitat degradation are key. However, there has been insufficient research conducted on how nitrogen (N)-driven changes to botanical communities affect pollinating insects. There is evidence that N-enrichment of soils leads to loss of botanical species richness in temperate grasslands, but not enough research on the effects to the structure of the botanical community, for example the functional traits. In this study, I used functional traits analyses, using community weighted means of relevant floral traits, to determine how N deposition and applications affect botanical communities in ways that are impactful for pollinators and other flower-visiting insects. I studied two different temperate grassland types, acid grasslands and mesotrophic grasslands, to identify if there are similarities or contrasts across community types. The analysis showed that morphologically specialist flowers are more likely to be impacted by N additions. Importantly, nectar-rich plants were observed to decline, leading to reduced nectar-provision. The study found differences between the two forms of N applied to mesotrophic grassland communities, and further differences between the acidic and mesotrophic grasslands. Therefore, to forecast impacts of N enrichment to pollinator communities, it will be important to take the ecosystem type and the form of N additions, the ratio of oxidised and reduced N, into account. Furthermore, soil pH was shown to mitigate against severe impacts to the floral community. The most detrimental impacts were observed in plots with poorly buffered soil, in which soil acidification occurred alongside N additions.

2.2 Introduction

2.2.1 Nitrogen deposition and applications

The industrialisation of energy and agriculture during the 19th century led to severely escalated trends in nitrogen (N) production (Galloway, 1998; Galloway *et al.*, 2004; Fowler *et al.*, 2013). This consequentially increased the fluxes of N addition to ecosystems, whether through atmospheric deposition or via fertiliser application. From 1860–1995, global atmospheric N deposition tripled, with deposition projected to rise further in the future, potentially doubling between 1995–2050 (Galloway *et al.*, 2004). Deposition levels are not uniform across the world; higher deposition occurs around more heavily industrialised regions. For example, China, India, North-West Europe, and Eastern USA experience high levels of N deposition relative to the Southern Hemisphere (Dentener *et al.*, 2006; Galloway *et al.*, 2008; Kanakidou *et al.*, 2016). In China and India, these levels are expected to rise further, whereas in North-West Europe deposition rates have plateaued and begun to decline since the 1990s (Sutton *et al.* 2011). Many regions in the Southern Hemisphere experience lower levels of N deposition, although fluxes are predicted to rise in the near-future above potentially critical threshold levels for sensitive ecosystems (Phoenix *et al.*, 2006; Bobbink *et al.*, 2010; Bleeker *et al.*, 2011).

Research from a range of bioregions shows N deposition affects botanical communities (Bobbink *et al.*, 1998; Bobbink *et al.*, 2010). Recent studies have shown that N-driven impacts on plant individuals and communities can consequentially affect higher trophic levels (Aqueel and Leather, 2012; Banfield-Zanin *et al.*, 2012; Ceulemans *et al.*, 2017; Nijssen *et al.*, 2017; Pöyry *et al.*, 2017). However, our overall understanding of the impacts to consumers remains littered with knowledge gaps that need to be addressed (Stevens *et al.*, 2018). The impact of N-driven changes to botanical communities on insect pollinators is a specific knowledge gap that needs further study (Harrison and Winfree, 2015; David *et al.*, 2019).

The two most common forms of nitrogen that enter ecosystems via the soil are reduced, NH_x, and oxidised, NO_y. The primary sources of NH_x and NO_y, respectively, are agriculture and fossil fuel combustion (Galloway, 1998; Stevens *et al.*, 2011). Although, globally, NH_x is the dominant form of N entering terrestrial ecosystems (Dentener *et al.*, 2006; Stevens *et al.*, 2011), deposition of these two forms varies temporally and spatially, leading to wide-ranging NH_x:NO_y deposition levels and soil contents. For example, since 1860 the NH_x:NO_y ratio has decreased in North America and Europe, but has increased in South and Central America, East Africa, and China (Dentener *et al.*, 2006). The ratio is expected to increase in these three regions in the near future (Dentener *et al.*, 2006). However, it is not entirely clear whether there is a difference in how deposition of the two separate forms of N affect botanical communities and traits. Historic total N deposition is well correlated with historic NH_x deposition, so separating their effects can be challenging (Stevens *et al.*, 2011). Furthermore, the form of N in soils is highly changeable, depending on soil factors, meaning that deposition of NH_x can be nitrified into NO_y by nitrifying bacteria (Galloway, 1998; Van Den Berg *et al.*, 2016). However, in poorly buffered grassland soils, it is likely that NH_x could be more severe. The nitrification of NH_x into NO_y species leads to increased soil acidification and base cation leaching, freeing up potentially toxic aluminium in the process (Stevens *et al.*, 2011). Faster-growing annual plants found in grassland communities are typically more adapted to high nitrification soils and so preferably take up NO_y, as assimilation of NH_x can cause cell acidification in these plants (Britto and Kronzucker, 2002; Lucassen *et al.*, 2003).

2.2.2 Pollinator declines

Insect pollinators fulfil an invaluable ecosystem service; carrying out or improving the reproduction and yield, globally, of an estimated 87.5% of plant species (Ollerton *et al.*, 2011), accounting for 35% of our total agricultural produce (Klein *et al.*, 2007). Nevertheless, during the last century many pollinating insects have declined, in species richness, abundances, and ranges (Biesmeijer *et al.*, 2006; Potts *et al.*, 2010; Burkle and Alarcon, 2011; Vanbergen *et al.*, 2013; Ollerton, 2017). It is now established that this is due to a plethora of factors, acting independently and interactively, although a key reason in many systems is the degradation or loss of floral landscapes and resulting lack of nectar and pollen foraging resources (Carvell *et al.*, 2006; Rader *et al.*, 2014; Senapathi *et al.*, 2015). Enrichment of soils by N is known to affect primary productivity and alter botanical communities, so could contribute to the state of floral communities available to pollinating insects. Improving our understanding of how N deposition impacts botanical communities can inform our understanding of historic pollinator trends and give us improved insight into how pollinator communities will respond to the deposition or application of N in the future and the potential for mitigation or habitat restoration.

2.2.3 Impacts of N to botanical communities

In temperate grasslands, N additions are shown to lead to a decline in botanical species richness, through a filtering of the species pool (Suding *et al.*, 2005; Clark and Tilman, 2008; Maskell *et al.*, 2010; Southon *et al.*, 2013). The most common driver of these declines is the excessively increased fertility of soils (Bobbink *et al.*, 1998; Crawley *et al.*, 2005). Increasing N, a limiting nutrient for plant growth in many ecosystems, typically leads to a boom in primary productivity with competitive and fast-growing plants exploiting the enhanced resources. This comes at the expense of smaller and slower-growing plant species, which are generally outcompeted and thus decline. Typically, this shift in botanical community composition is represented by an increase in grasses, and a decrease in legumes and flowering forbs. High plant diversity improves ecosystem functioning and stability (Weisser *et al.*, 2017), and has specifically been linked to increased diversity of pollinating insects (Potts *et al.*, 2003; Fründ *et al.*, 2010; Weisser *et al.*, 2017).

To improve our understanding of how assemblages of pollinating insects are impacted by shifts in botanical communities, we need a more informative metric than species richness or diversity. The use of functional traits analyses has recently gained momentum as a useful method that gives a more informative view of how shifts in biodiversity impact ecosystem functioning (Tilman *et al.*, 1997; Díaz and Cabido, 2001; Heemsbergen *et al.*, 2006; Garnier *et al.*, 2007). Recent research focussing on the traits that respond to environmental change ('response traits' *sensu* Lavorel and Garnier, 2002) revealed that N deposition impacts the functional trait composition of a plant community, independent of species loss (Helsen *et al.*, 2014). This study showed that insect-pollinated plants declined with N deposition whilst wind-pollinated plants flourished. However, the study did not consider more specific traits that take into account the wide diversity of plant-pollinator interactions (so called ‘effect traits’ *sensu* Lavorel and Garnier, 2002).

The interactions between plants and pollinators are varied, from accidental and opportunistic insect visits on simple open flower heads to more specialised interactions based on morphological adaptations or the metabolisation of secondary metabolites (Fenster *et al.*, 2004; Whittall and Hodges, 2007; Krömer *et al.*, 2008; Sedivy *et al.*, 2011; Vanderplanck *et al.*, 2014). The relevancy, extent, and applicability of pollinator syndromes is still debated (Johnson and Steiner, 2000; Fenster *et al.*, 2004; Ollerton *et al.*, 2009). However, plants do play different roles within a pollinator network, some have more central roles, linking many visitors as generalists, while others have more selective interactions (Bascompte *et al.*, 2003; Vázquez and Aizen, 2004; Dupont and Olesen, 2009). Floral morphological structures are shown to have evolved alongside pollinator feeding apparatus (Borrell, 2005; Rodríguez-Gironés and Santamaría, 2006; Johnson and Anderson, 2010). Furthermore, plants with more specialist pollination or nectar presentation mechanisms, such as vibratile pollination or the long-corollas of *Fabaceae*, often produce highly nutritional floral rewards (Roulston *et al.*, 2000; Hanley *et al.*, 2008). The effect, therefore, of losing two different floral resources could have contrasting impacts on a pollinator community. For instance, losing more specialised floral resources that provide nutritious resources for oligolectic insects could impact a narrow group of pollinators, potentially severely. On the other hand, losing plants with functionally simplistic open flowers visited by polylectic insects will have a different effect; if the plant produces plentiful high-quality nectar and is visited by a diverse array of insects, the consequences of losing a widely-utilised, well-linked, resource could be significant for the insect community, although detrimental impacts could be buffered if alternative functionally similar floral resources are available.

2.2.4 Aims of the study

With this study, I aimed to determine how N-enrichment of soils affects the composition of floral resources in botanical communities. With declines in forbs and legumes commonly observed, it is expected that there are fewer insect-pollinated plants with greater soil-N additions. Using functional trait analyses, with a community weighted mean (CWM) approach, the study will investigate which specific groups of floral traits are most affected by N-driven shifts in botanical composition. This will give insight into which functional groups of insect pollinators are most or least threatened by future levels of N deposition and application. This analysis can also reveal the likely impacts of historical N deposition on pollinator communities, thereby supporting our understanding of pollinator trends in recent decades.

The responses of different ecosystems and ecoregions to N-enrichment can vary (Bobbink *et al.*, 2010; Maskell *et al.*, 2010; Field *et al.*, 2014), so I considered two different types of temperate grasslands; acidic grasslands and a mesotrophic grassland meadow. Both grassland types, in a natural state, are potentially nectar-rich and able to support pollinator communities (Baude *et al.*, 2016). To study acid grassland sites, I utilised nationwide data previously collected by Stevens *et al.* (2004). For a representative mesotrophic grassland meadow community, I studied the Park Grass Long-term Experiment (PGE), based at Rothamsted Research, which has had nutrient treatments applied consistently since its founding in 1856. The treatment structure of the PGE allows the effects of two common forms of reactive N entering soils, ammonia (NH_4) and nitrate (NO_3), to be separated from each other and from the effect of changes in soil pH. This is useful, as it can be challenging in studies of atmospheric N deposition to distinguish between the effects of total N, NH_4, and soil acidification (Bobbink *et al.*, 1998; Stevens *et al.*, 2011). Applications and deposition of N can acidify soils with poor buffering capacity, through the release of H^+ protons from NH_4 nitrification or through leaching of NO_3 (Barak *et al.*, 1997; Schroder *et al.*, 2011; Lu *et al.*, 2014). Soil acidification represents an additional selection pressure on the plant community, often reducing species richness further (Clark and Tilman, 2008; Stevens *et al.*, 2010; Diekmann *et al.*, 2014; Field *et al.*, 2014). With declines in forb richness recorded in temperate grasslands with N deposition and soil acidification (Duprè *et al.*, 2010; Stevens, Thompson, *et al.*, 2010; Field *et al.*, 2014; Helsen *et al.*, 2014) I hypothesised that 1) there will be fewer insect-pollinated plants, specifically 2) fewer plants with morphologically specialised floral units.

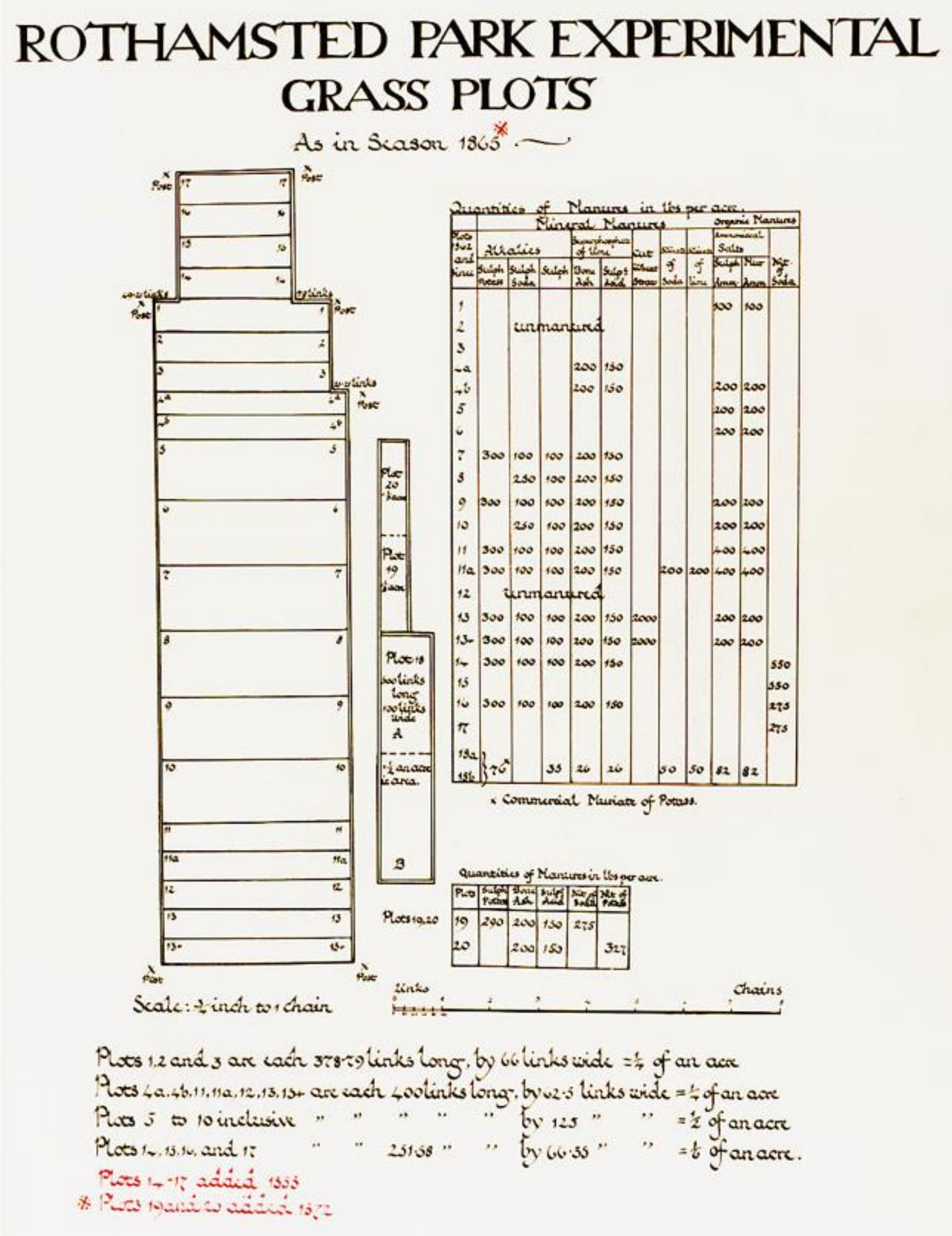
Finally, the decline of N deposition rates in some regions, such as North-West Europe, has led to a keen interest in the response and recovery of botanical communities to declining rates of N deposition. Although there are indications that some N-sensitive species can recover in abundance following the cessation of N addition (Clark and Tilman, 2008; Storkey *et al.*, 2015), the plant community can retain relics of historic N-enrichment for many years, such as low-diversity (Isbell *et al.*, 2013). Therefore, in addition to studying the effects of N-enrichment on functional traits, I also determined how temperate grassland communities recover from historic N additions over time using time series of data from PGE plots that have had N fertiliser withheld for the last three decades. Considering prior research, I further hypothesised that 3) the floral trait composition of N-enriched plant communities will slowly recover from eutrophication, but not to the extent that they are perfectly comparable with historic nil plots.

2.3 Materials and methods

2.3.1 The Park Grass Long-term Experiment

The Park Grass Experiment (PGE), based at Rothamsted Research, Hertfordshire, UK, is a field-based nutrient-addition long-term experiment. The experiment was founded in 1856 to study the effects of fertilisers on hay yield. It is now a unique and useful resource for studying the long-term effects of chronic N addition, mineral addition, and soil acidification, on temperate mesotrophic grasslands. When the experiment began, the 2.8 ha field had been a permanent grassland for at least 100 years, was a uniform site on silty clay loam soil, near-level, moderately well-drained, with pH 5.5 topsoil, and would have been classified as an MG5 type mesotrophic grassland according to the National Vegetation Classification (Rodwell 1992; Dodd *et al.,* 1994; Silvertown *et al.*, 2006). The PGE was grazed by sheep from 1856-1875, but since 1875 a second cut has been taken and immediately removed in October each year. Therefore, grazing is not a variable that needs to be considered for modern experiments using the PGE. The original fertiliser treatments continue to be applied to the PGE, with minor alterations to treatment structure in some plots.

There are 19 main plots (numbered 1–4, 6–20) (Fig. 2.1). Plot 3 and 12 receive no fertiliser or minerals and are effectively control plots. In total, 15 of the main plots receive consistent N and mineral treatments in various combinations and levels. N is applied as one of two separate forms, ammonium sulphate (delivering NH_4) or sodium nitrate (delivering NO_3), and generally at three different levels (48 kg ha^{-1} yr^{-1}, 96 kg ha^{-1} yr^{-1}, 144 kg ha^{-1} yr^{-1}). Recently, plot 15 had a 144 kg NO_3 ha^{-1} yr^{-1} treatment incorporated, so now there are plots receiving 144 kg N of both forms of N (although the changes to plot 15 are not shown in Fig. 2.2). A 30 kg ha^{-1} yr^{-1} treatment is also used on plot 20 in conjunction with organic farmyard manure. Organic manure treatments are applied uniquely to two of the plots, plot 19 and 13. Plot 19 receives farmyard manure once every four years, plot 13 also receives farmyard manure once every four years but also pelleted poultry manure once every four years in the intermediate years. The provision of nutrients by organic manure treatments is shown in Fig 2.2. The minerals applied to the PGE are phosphorous (P), potassium (K), sodium (Na), and magnesium (Mg). Mineral treatments are applied as either P, K, Na, Mg; P, Na, Mg; P, K; P; K, using the following quantities: P 35 kg ha^{-1} yr^{-1}, K 225 kg ha^{-1} yr^{-1}, Na 15 kg ha^{-1} yr^{-1}, Mg 10 kg ha^{-1} yr^{-1}. However, from 2017 onwards P application was scaled back to 17 kg ha^{-1} yr^{-1}. There is one plot that also receives 135 kg silicone (Si) ha^{-1} yr^{-1}. From 1903, liming treatments began to be incorporated, to prevent soil acidification that was occurring in some plots. Between 1903–1964, plots were divided into two; limed and unlimed. From 1965, the liming treatment was expanded to divide plots 1–18 into four subplots; a (limed to pH 7), b (limed to pH 6), c (limed to pH 5), and d (unlimed). Plots 19, 20, and part of plot 18 have had liming treatments withheld since 1968. From 1990, plots receiving 96 kg N ha^{-1} yr^{-1} of either form (NH_4 on plot 9, NO_3 on plot 14) were divided into two, the N treatments were continued in one half and withheld from the other half. From 1995 the same system was applied to the farmyard manure treatments in plot 13. From 2015 plot 7, receiving P K Na Mg, was also divided, with P withheld from one half. These plots are now 9/1, 9/2, 14/1, 14/2, 13/1, 13/2, 7/1, 7/2, with treatments withheld from all x/1 plots – now called ‘transition plots’. Today, 101 subplots exist, a full plan can be found in Fig. 2.2. The PGE was designed and created before modern statistical theory was developed by Sir Ronald Fischer (in fact Fischer developed his statistical theories partly from his experience of working on the Rothamsted Research classical experiments, such as the PGE). Therefore, it lacks spatial replication of plots, and researchers must find ways to account for this, such as using spatial pseudo-replication across subplots or from separate individuals within larger plots, or by taking replicates across time. The PGE is cut in mid-June to sample and record biomass. The herbage is left to dry in-situ for up to a week, which allows seeds to return to the soil. Following sampling, the plots are completely mown and cleared, and are left to regrow for a second, summer-autumn, season. The field site is then cut a second time in mid-October.



ROTHAMSTED PARK EXPERIMENTAL GRASS PLOTS

As in Season 1865*

Quantities of Manures in lbs per acre.

Plots 1862 and since	Mineral Manures								Organic Manures		
	Alkalies			Superphosphate of Lime		Cut Wheat Straw	Silicate of Soda	Silicate of Lime	Ammoniacal Salts		Nit. of Soda
	Sulph Potass	Sulph Soda	Sulph	Bone Ash	Sulph Acid				Sulph Amm	Mur Amm	
1									100	100	
2	unmanured										
3											
4a				200	150						
4b				200	150				200	200	
5									200	200	
6									200	200	
7	300	100	100	200	150						
8		250	100	200	150						
9	300	100	100	200	150				200	200	
10		250	100	200	150				200	200	
11	300	100	100	200	150				400	400	
11a	300	100	100	200	150		200	200	400	400	
12	unmanured										
13	300	100	100	200	150	2000			200	200	
13-	300	100	100	200	150	2000			200	200	
14	300	100	100	200	150						550
15											550
16	300	100	100	200	150						275
17											275
18a 18b	76×		35	26	26		50	50	82	82	

× Commercial Muriate of Potass.

Quantities of Manures in lbs per acre.

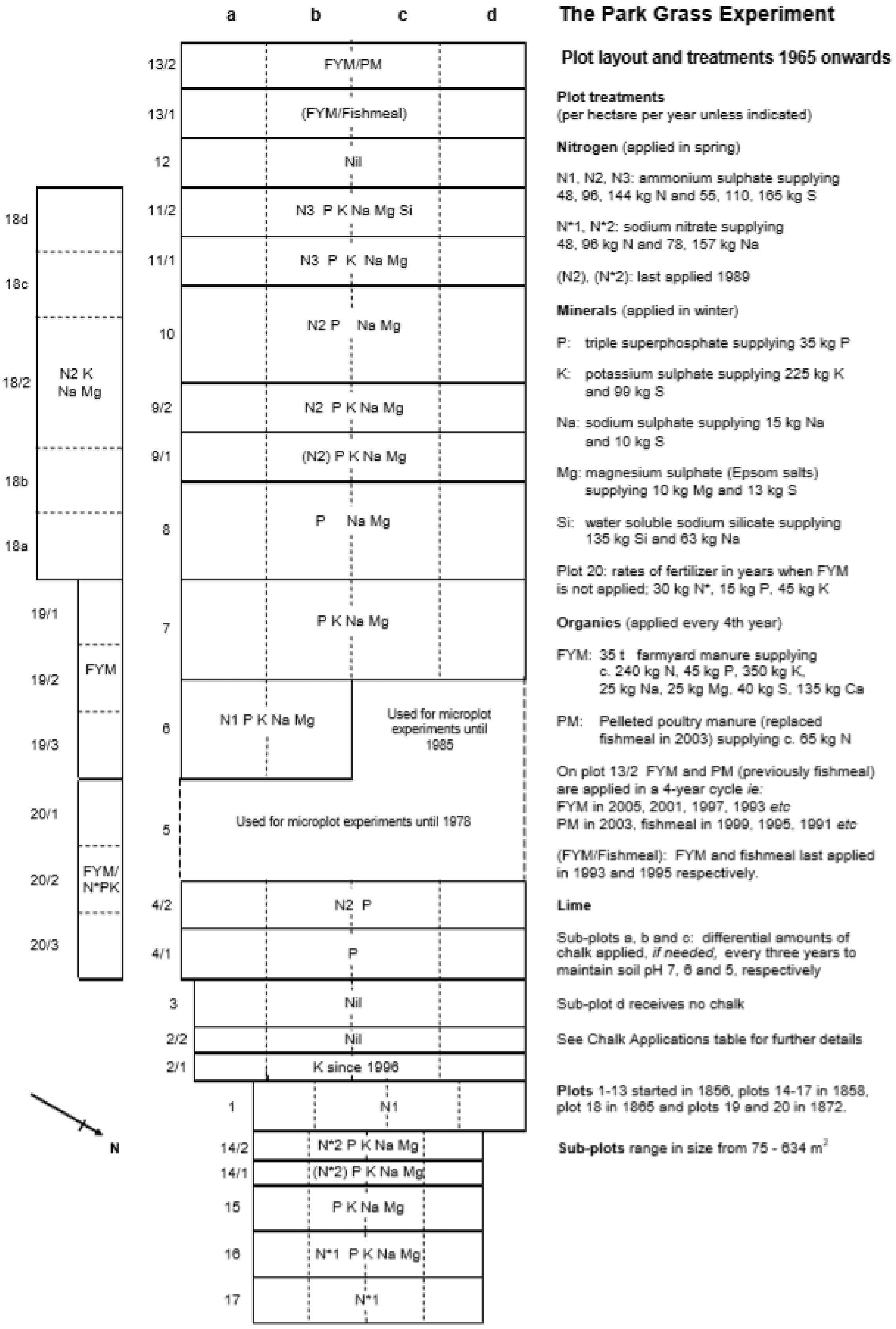
Plots 19, 20

Plots	Sulph Potass	Bone Ash	Sulph Acid	Nit of Soda	Nit of Potash
19	290	200	150	275	
20		200	150		327

Scale: ½ inch to 1 chain

Plots 1, 2 and 3 are each 378.79 links long, by 66 links wide = ¼ of an acre
Plots 4a. 4b. 11. 11a. 12. 13. 13- are each 400 links long, by 62.5 links wide = ¼ of an acre
Plots 5 to 10 inclusive " " " " " by 125 " " = ½ of an acre
Plots 14. 15. 16. and 17 " " 251.58 " " by 66.35 " " = ⅙ of an acre.
Plots 14-17 added 1858
* Plots 19 and 20 added 1872

Figure 2.1. Original plan of the Park Grass Long-term Experiment from 1865, its founding year, showing placement of plots and treatment application table (Rothamsted Research e-RA, 2019).



The Park Grass Experiment

Plot layout and treatments 1965 onwards

Plot treatments
(per hectare per year unless indicated)

Nitrogen (applied in spring)

N1, N2, N3: ammonium sulphate supplying 48, 96, 144 kg N and 55, 110, 165 kg S

N*1, N*2: sodium nitrate supplying 48, 96 kg N and 78, 157 kg Na

(N2), (N*2): last applied 1989

Minerals (applied in winter)

P: triple superphosphate supplying 35 kg P

K: potassium sulphate supplying 225 kg K and 99 kg S

Na: sodium sulphate supplying 15 kg Na and 10 kg S

Mg: magnesium sulphate (Epsom salts) supplying 10 kg Mg and 13 kg S

Si: water soluble sodium silicate supplying 135 kg Si and 63 kg Na

Plot 20: rates of fertilizer in years when FYM is not applied; 30 kg N*, 15 kg P, 45 kg K

Organics (applied every 4th year)

FYM: 35 t farmyard manure supplying c. 240 kg N, 45 kg P, 350 kg K, 25 kg Na, 25 kg Mg, 40 kg S, 135 kg Ca

PM: Pelleted poultry manure (replaced fishmeal in 2003) supplying c. 65 kg N

On plot 13/2 FYM and PM (previously fishmeal) are applied in a 4-year cycle *ie*:
FYM in 2005, 2001, 1997, 1993 *etc*
PM in 2003, fishmeal in 1999, 1995, 1991 *etc*

(FYM/Fishmeal): FYM and fishmeal last applied in 1993 and 1995 respectively.

Lime

Sub-plots a, b and c: differential amounts of chalk applied, *if needed*, every three years to maintain soil pH 7, 6 and 5, respectively

Sub-plot d receives no chalk

See Chalk Applications table for further details

Plots 1-13 started in 1856, plots 14-17 in 1858, plot 18 in 1865 and plots 19 and 20 in 1872.

Sub-plots range in size from 75 - 634 m^2

Figure 2.2. Plan of the Park Grass Experiment from 1965 onwards, showing plots and treatments (Rothamsted Research e-RA, 2019).

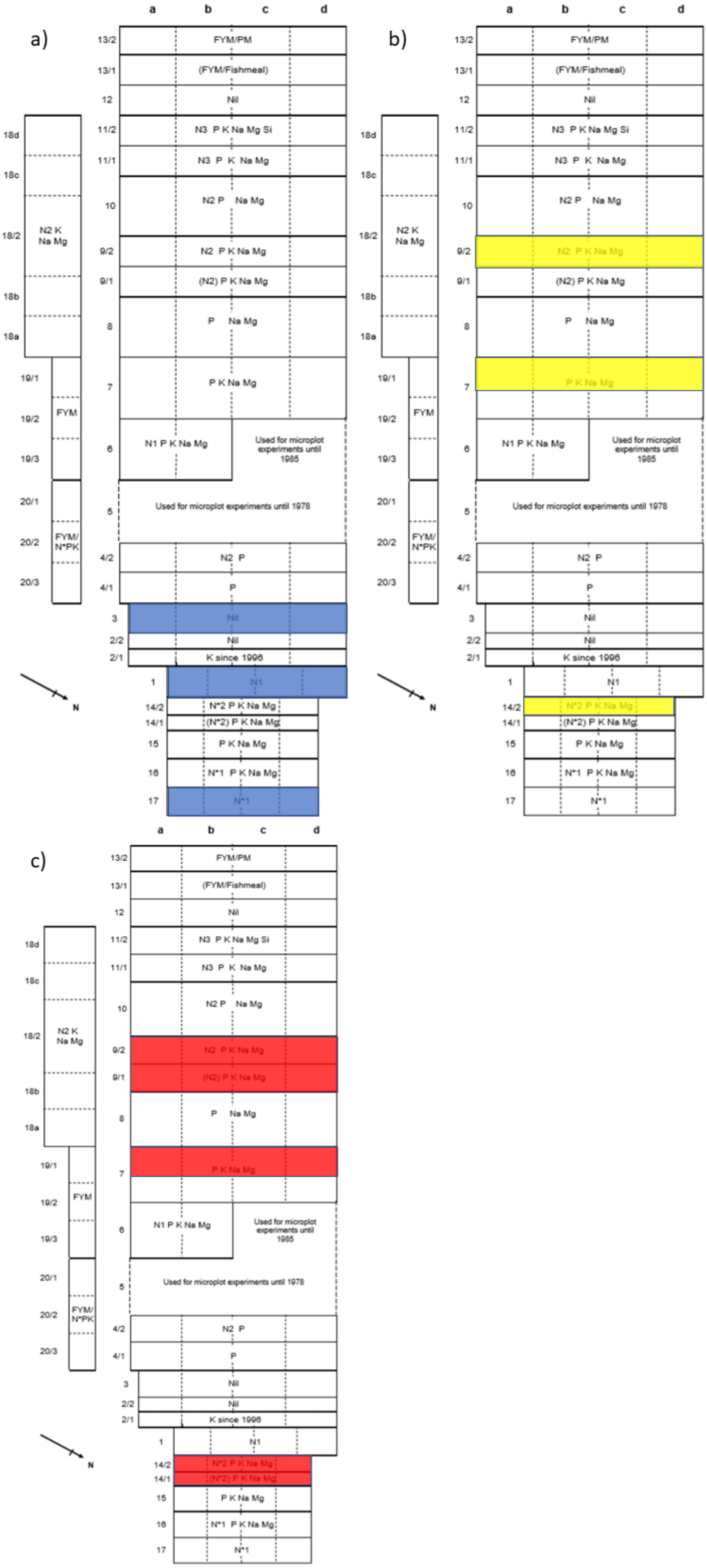


Figure 2.3. The PGE study plots used for the Chapter 2 analyses.
Blue plots in a) are plots 1, 3, and 17; studying 48 kg ha^{-1} yr^{-1} N additions. Yellow plots in b) are plots 7/2, 9/2, and 14/2; studying 96 kg ha^{-1} yr^{-1} N additions. Red plots in c) are plots 7/2, 9/1, 9/2, 14/1, 14/2; studying recovery from 96 kg ha^{-1} yr^{-1} N additions.

2.3.2 Park Grass Experiment study-plots and acid grassland sites

For the study of CWM of floral traits, I selected study plots with a comparable treatment structure for the analyses (Fig. 2.3). Plots 3 (control; no N addition), 1 (48 kg ha^{-1} yr^{-1} NH_4), and 17 (48 kg ha^{-1} yr^{-1} NO_3) were studied to investigate the effects of N addition on functional botanical trait composition. 48 kg ha^{-1} yr^{-1} N, alongside background N deposition calculated as 6.84 kg N ha^{-1} yr^{-1} in 2013 (Storkey *et al.*, 2015), is comparable with the highest levels of N deposition found in regions of China, India, North Europe, and Eastern USA, and will reflect more widespread potential near-future deposition levels if global fluxes continue to rise. I also used plots 7/2 (control; no N addition), 9/2 (96 kg ha^{-1} yr^{-1} NH_4), and 14/2 (96 kg ha^{-1} yr^{-1} NO_3), all of which receive the P, K, Na, Mg mineral treatment in addition to N. The subplots used for these analyses were ‘b’ (pH 6) and ‘d’ (unlimed). I acquired species abundance data from previous field studies conducted on the PGE (Crawley *et al.*, 2005; Storkey *et al.*, 2015). Data from 1991-2000 and from 2010-2012 was used. Data were relative biomass of each species per plot and year calculated on dry weights.

To study the recovery of botanical communities when N is withheld, we used the plots that had been split in 1990 to test the recovery of communities from a state of high N application; plots 9/2, 9/1, 14/2, and 14/1. Plots 9/2 (96 kg ha^{-1} yr^{-1} NH_4) and 14/2 (96 kg ha^{-1} yr^{-1} NO_3) continue to have the N treatments applied in addition to P, K, Na, Mg mineral applications. Since 1990, plots 9/1 and 14/1 have not had any N additions, and only receive the mineral applications. The comparable control plot to the applied N and withheld N plots is plot 7/2, which has always received the mineral applications and without N applications.

Data from the acid grassland sites was collected by Stevens *et al.* (2004). All sites were grazed and protected for conservation. In total, the authors surveyed 68 acidic grasslands in the UK on a gradient of N deposition ranging from 6.2–36.3 kg ha^{-1} yr^{-1} during the years 2002–2003. The sites ranged in altitude between 15–692 m altitude, 50.5–58.2° latitude, 6.6–10.6° C mean annual temperature, 594–3038 mm annual precipitation, and 3.7–5.7 pH (Stevens *et al.*, 2004). The sites were classified as U4 according to the National Vegetation Classification (Rodwell, 1992). The authors sampled the grassland communities with 2 m by 2 m quadrats identifying all higher plants and bryophytes to a species level and estimating percentage cover, taking a total of 5 replicates per site.

2.3.3 Functional traits

Botanical trait data was assembled using the following sources: flowering phenology, floral colour, typical insect-visitors, and pollinator syndrome morphology from the BIOFLOR database (Klotz *et al.* 2002), provision of nectar volume and nectar-sugars from the Agriland database (Baude *et al.*, 2016), flowering phenology and floral dimensions from the "The wildflower key" (Rose and O'Reilly, 2006), and Ellenberg values were taken from the ECOFACT report (Hill, 1999). Further information can be found in Appendix 1.

CWM scores were calculated from the species abundance data for each botanical trait response. For continuous variables (nectar traits, floral sizes), the CWM denotes a mean score of that trait in the community. For traits with ordinal levels (month of flowering phenology, Ellenberg value), the CWM is the mean level across the community. For categorical variables (taxonomic family, floral colour, pollinator syndrome, and typical insect-visitors), the CWM is the proportion that each level of the variable is represented within the botanical community. When analysing the effect to phenology as a response trait, grasses were omitted from the study, to focus on the flowering times of useful inflorescences. When studying all other response traits, all botanical species, including grasses, were included, to reveal the impact to the overall community. Meteorological data was obtained from direct measurements taken by the Rothamsted meteorological enclosure. Mean daily rainfall and temperature values from 1st March until the sampling date were used in the analyses. These dates cover the typical growing season of the PGE and using data from 1st March has been previously shown to be significantly related to biodiversity and yield mass (Silvertown *et al.*, 1994).

2.3.4 Statistical analysis

All data analysis was conducted using RStudio (R Core Team, 2018) with the lme4 package (Bates *et al.*, 2015), graphs were produced using the ggplot2 package (Wickham, 2016). Mixed-effects models, with REML, were fitted to analyse the data (Garnier *et al.*, 2007). The random effects used for all models using the PGE data were sample date (continuous; 167–190 Julian date), wet N deposition (continuous; 4.62–9.88 kg N ha^{-1} yr^{-1}), mean daily temperature (continuous; 8.21–11.07° C), mean daily rainfall (continuous; 0.81–3.55 mm), and year (categorical; 1991–2000, 2010–2012). All continuous random effects were nested within year for the analyses. Wet N deposition was included as a random effect here to account for background atmospheric N deposition which can alter the PGE plant communities independently of the experimental treatments (Storkey *et al.*, 2015). The functional traits were analysed as independent response variables throughout. To determine the effect of N addition on PGE communities, the following fixed effects were used: N form (categorical; nil, NH_4, NO_3), pH (continuous; 3.4–7.2), and the interaction between N form and pH. To account for the possibility that the two forms of N additions would affect the assemblages differently, thus creating different baselines from which to study recovery, we analysed the recovery from NH_4 and NO_3 separately. The fixed-effects used for the recovery analysis were: N status (categorical; applied, withheld) and years of recovery (continuous; 1–22 counting the years since cessation in 1990). For the recovery analysis, pH was incorporated as a random effect to focus on the effects of withholding N. To determine the extent of recovery after 20 years, the N-recovery communities were compared directly with historic control plots, using data from 2010–2012. For these mixed-effects models, the fixed effects were: N status (categorical; nil, withheld NH_4, withheld NO_3), pH (continuous; 4.3–7.1), and the interaction between N status and pH.

The mixed-effects models for analysing the acidic grassland communities incorporated N deposition (continuous; 7.7–40.86 kg N ha^{-1} yr^{-1}), topsoil pH (continuous; 3.69–5.37), and the interactive term as fixed effects. The random effects were altitude (continuous; 15–500 m), mean annual precipitation (continuous; 486.6–719.1 mm), and latitude (continuous; 50.54–59.23° N). Previous analysis of the data by Stevens *et al.* (2004) showed that altitude and mean annual precipitation were the environmental variables that best explained changes in species richness, whilst latitude accounted for factors that can influence the geographic ranges of plant species. These variables were utilised as random effects for a more elegant and succinct analysis. Other environmental and management variables, such as grazing, that were not so impactful to species richness (Stevens *et al*. 2004), were not included in the analysis.

All models were simplified with stepwise regression, by removing the least significant term beginning with the interactive variables. At each step the models were assessed for goodness of fit of the data by checking the standardised residuals, the Akaike information criterion (AIC), and the Bayesian information criterion (BIC) scores, and the Hessian convergence matrix.

2.4 Results

2.4.1 UK-wide acid grassland sites

Stevens *et al.* (2004) found that species richness declined with N deposition across the surveyed UK acidic grassland sites. In agreement with Stevens *et al.* (2006), the analysis also shows that the proportion of forbs decreased at sites with higher N deposition (Table 2.1). The proportion of legumes, however, was unaffected. Of the dominant forb families, only *Ericaceae,* and *Ranunculaceae* showed a significant response to the fixed-effect variables. *Ericaceae* declined with N deposition, *Ranunculaceae* declined with more acidic soils.

Overall, there were proportionally fewer insect-pollinated plants with higher levels of N deposition (Table 2.1). The only morphologically adapted flower types observed to significantly decline were those with bell-style flowers. Higher N deposition led to fewer nectar-rich plants, meaning an overall reduction in potential nectar production with higher N deposition. Plants with smaller inflorescences were more common in acidic soils. Plants with blue-purple or yellow flowers, and those with

UV-patterned flowers, were less common in acidic soils. The average date of flowering cessation was also earlier at sites with more acidic pH.

Table 2.1. Output of LMER models studying the impact of N deposition, topsoil pH, and the interactive term on the floral functional trait composition of UK acid grasslands.
The rows show all response variables that were impacted significantly by the explanatory variables. All values are statistically significant (t > 2) effect sizes given by the final simplified ("+" denotes increases, "-" decreases).

	N deposition (kg N ha^{-1} yr^{-1})	**Topsoil pH**	**N deposition * Topsoil pH**
Lifeform & Family (change in proportion composition)			
Forb	-0.00335		
• *Ericaceae*	-0.00209		
• *Ranunculaceae*		+0.00337	
Insect-pollinated	-0.00421		
Morphological pollinator syndrome (change in proportion composition)			
Bell	-0.00226		
Nectar traits (change in provision; kg^{-1} ha^{-1} yr^{-1} production, ug^{-1} fl^{-1} day^{-1} sugar)			
Nectar production	-1.366		
Floral size (change in size, mm)			
Landing area		+2.87	
Floral colour (change in proportion composition)			
Blue-Purple		+0.0753	-0.00365
Yellow		+0.0392	
White	+0.00261		
UV pattern		+0.0364	
Flowering phenology (change in month of phenological event)			
End flower		+0.0597	

2.4.2 Park Grass Experiment – 48 kg N ha^{-1} yr^{-1} treatments, without minerals

Ammonia, NH_4, application to the experimental grassland sites led to botanical communities with proportionally more grasses, fewer legumes, and fewer forbs (Table 2.2). Of the dominant forb families, *Lamiaceae* and *Ranunculaceae* uniformly decreased in proportion, whereas the decline in *Apiaceae* was buffered by high pH. In soils with pH exceeding 6, *Apiaceae* was more common with NH_4 addition.

Table 2.2. Output of LMER models studying the impact of 48 kg N ha^{-1} *yr*$^{-1}$ *N applications and topsoil pH on the CWM of floral functional traits of the PGE field site.*
The rows show all response variables that were impacted significantly by the explanatory variables. All values are statistically significant (t > 2) effect sizes given by the final simplified ("+" denotes increases, "-" decreases).

	NH_4	**NO_3**	**pH**	**NH_4*pH**	**NO_3*pH**
Lifeform & Family (change in proportion composition)					
Grass	+0.0906		-0.187		
Legume	-0.0359	-0.0355			
Forb	-0.0619		+0.177		
• *Apiaceae*	-0.0761	-0.181		+0.0152	+0.0316
• *Asteraceae*		+0.0602	+0.111		
• *Lamiaceae*	-0.00373	-0.00223			
• *Ranunculaceae*	-0.00936				
Insect-pollinated	-0.0554		+0.124		
Morphological pollinator syndrome (change in proportion composition)					
Simple flower	-0.258		-0.0282	+0.0413	
Bee-form	-0.0327	-0.0406	+0.00933		
Lip	-0.00392	-0.00242			
Nectar traits (change in provision; kg^{-1} ha^{-1} yr^{-1} production, ug^{-1} fl^{-1} day^{-1} sugar)					
Nectar production	-688.15			+125.17	
Nectar sugars			+10.153		
Floral size (change in size; mm)					
Landing area			+3.56		
Corolla depth	-1.285	-1.119	+0.62		
Floral colour (change in proportion composition)					
Blue-Purple			+0.0342		
Yellow	-0.0466	+0.0519	+0.0736		
White	-0.166	-0.0302		+0.0264	+0.048
UV pattern		+0.077	+0.0622		
Flowering phenology (change in month of phenological event)					
Start flower	+0.13				
End flower		+0.294	+0.188		
Duration		+0.792			
Season	+0.3801				

There were overall fewer insect-pollinated plants, with both generalist and specialist floral-units declining. Nectar-rich plants sharply declined with NH_4 additions in acidic soils, but in soils with pH above 6, there was no such decline. NH_4 enrichment favoured plants with shallower corollas. Plants bearing yellow flowers declined with NH_4 addition, those bearing white flowers, such as *Apiaceae* declined at low pH, but increased at high pH. The typical flowering phenology of NH_4-enriched plant communities was found to be delayed slightly, with a later peak flowering date.

NO_3-enrichment caused a decrease in legumes, but no significant effects to the proportion of total forbs or grasses (Table 2.2). Of the dominant forb families, *Apiaceae* declined with NO_3 addition in low pH soils, but increased in soils above pH 6. *Lamiaceae* declined in proportion and *Asteraceae* increased.

With 48 kg NO_3 ha^{-1} yr^{-1} applications, in the absence of other minerals, there was no significant impact found in the proportion of total insect-pollinated plants (Table 2.2). As with NH_4-enrichment, plants with shallower corollas were favoured. Plants with flowers morphologically adapted for bee visitors, such as flag and lip blossoms, declined, but those with simple open flowers were not impacted by NO_3. The typical nectar provision was unaffected by NO_3-enrichment. Plants bearing yellow flowers and those bearing flowers with UV reflectivity patterns both increased with NO_3 addition. Plants bearing white flowers declined in low pH but were more stable in high pH soils. The average start of flowering was unaffected, as was the peak flowering season. The average end date of community flowering was delayed by NO_3-enrichment and there was an increased prevalence of plants with a longer flowering duration.

Soil pH, which ranged between pH 4–7.1, was an important factor, with more acidic soils having an overall detrimental effect on plant communities (Table 2.2). Soil acidification led to higher proportions of grasses and fewer forbs, although legume proportion was unaffected. With more acidic soils, *Asteraceae* also declined in both treatments and control plots, *Apiaceae* declined in N-enriched plots. Insect-pollinated plants overall decline with more acidic soils, especially those with specialised morphological adaptations, although there were more plants with simple open flowers. Acidic soils favoured plants with smaller flowers, shallower corollas, and less rewarding nectars.

2.4.3 Park Grass Experiment – 96 kg N ha^{-1} yr^{-1} treatments, with minerals

The higher addition of NH_4, when applied to mineral-enriched soils, caused more severe effects to the plant community. Grass proportions increased, and forb and legume proportions decreased, by larger values than the 48 kg N ha^{-1} yr^{-1} additions (Table 2.3). *Asteraceae* and *Ranunculaceae* declined with NH_4 addition, *Asteraceae* declined with NH_4 addition in plots with low pH, no forb family increased in proportion.

Overall, there were fewer insect-pollinated and morphologically specialised plants (Table 2.3). Only the proportion of plants bearing simplistic open flowers was unaffected by NH_4 addition. These all declined more steeply under 96 kg N ha^{-1} yr^{-1} additions than 48 kg N ha^{-1} yr^{-1} additions. The plant community became less rich in overall nectar provision and nectar sugars. There was a decline in plants bearing blue-purple, yellow, and UV patterned flowers, although the proportion of plants bearing white flowers was unaffected. Overall, NH_4 enrichment also led to plants with larger floral units or with shallower corolla depth becoming more prevalent.

NO_3 additions of 96 kg N ha^{-1} yr^{-1} caused a proportional decline in forbs and legumes, alongside an increase in grasses (Table 2.3). However, the decline of forbs was not uniform across taxonomic families. *Apiaceae* increased by a small amount, while *Asteraceae* was dramatically reduced in soils with lower pH.

Overall, the proportion of insect-pollinated plants was reduced by NO_3 enrichment (Table 2.3). Plants with simple open floral units thrived with high NO_3 addition. The average nectar production of plants was unaffected, but we found plants producing nectar with lower sugar content became more prevalent. As with NH_4 enrichment, we observed declines in plants bearing blue-purple, white, and UV patterned flowers, but an increase in the proportion of plants bearing white flowers. This is potentially driven by the increased *Apiaceae* composition. Overall, NO_3-enrichment favoured plants with an earlier flowering date and an earlier peak bloom window.

Table 2.3. Output of LMER models studying the impact of 96 kg N ha^{-1} yr^{-1} N applications and topsoil pH on the CWM of floral functional traits of the PGE field site.

The rows show all response variables that were impacted significantly by the explanatory variables. All values are statistically significant (t > 2) effect sizes given by the final simplified ("+" denotes increases, "-" decreases).

	NH$_4$	**NO$_3$**	**pH**	**NH$_4$*pH**	**NO$_3$*pH**
Lifeform & Family (change in proportion composition)					
Grass	+0.329	+0.282	-0.0767		
Legume	-0.162	-0.211	+0.0225		
Forb	-0.166	-0.0714	+0.0544		
• *Apiaceae*		+0.0701	+0.035		
• *Asteraceae*	-0.626	-0.546	-0.0873	+0.0999	+0.0852
• *Ranunculaceae*	-0.0222				
Insect-pollinated	-0.263	-0.216	+0.0572		
Morphological pollinator syndrome (change in proportion composition)					
Simple flower		+0.0745	+0.0424		
Bee-form	-0.109		+0.0337		
Nectar traits (change in provision; kg^{-1} ha^{-1} yr^{-1} production, ug^{-1} fl^{-1} day^{-1} sugar)					
Nectar production	-1144.21		-113.08	+164.89	
Nectar sugars	-70.385	-88.686	+12.068		
Floral size (change in size; mm)					
Landing area	+31.601	+18.892			
Corolla depth	-2.153		+1.202		
Floral colour (change in proportion composition)					
Blue-Purple	-0.176	-0.186			
Yellow	-0.0694	-0.0769	+0.0279		
White		+0.0495	+0.0288		
UV pattern	-0.0644	-0.0713	+0.0283		
Flowering phenology (change in month of phenological event)					
Start flower		-0.261	-0.529		
End flower			-0.165		
Duration			+0.482		
Season		-0.451	-0.743		

Soil pH was an important factor with applications of 96 kg N ha^{-1} yr^{-1}. At more acidic pH, there were overall less insect-pollinated plants; fewer forbs and legumes, and more grasses (Table 2.3). There were fewer *Apiaceae* and, in nil soils without N addition, more *Asteraceae*. More acidic soils led to proportional decreases in both plants producing open simplistic flowers and flowers morphologically specialised for bees. With NH_4-enrihcment, plants with higher nectar production became more prevalent with higher pH. In plots without NH_4 addition, more acidic soils led to increased dominance of plants with lower nectar production, but with more sugar-rich nectars. Soils with higher pH were favourable for plants with deeper corollas, and for plants bearing yellow, white, and UV-patterned flowers. We also found plants with an earlier flowering season, but a prolonged flowering window were associated with higher soil pH.

2.4.4 Park Grass Experiment – Effect of withholding N from plots

Withholding the application of N to the experimental plots caused a shift in the pollinator-relevant functional traits of the botanical communities. There are effects caused by both the cessation of NH_4 and NO_3 application (Table 2.4). Most of the studied botanical traits respond instantly when NO_3 is applied and then withheld, indicating a quicker recovery rate. On the other hand, we see fewer immediate reactions to NH_4 cessation. Recovery from NH_4 application is often seen over the long-term, indicating that communities will take longer to recover from NH_4 additions.

When N is withheld, grasses decreased in abundance and both forbs and legumes increase. With NH_4 cessation, this effect is gradual, occurring over years, whereas we see more immediate effects when NO_3 is withheld (Table 2.4). With regards to the dominant forb families, *Apiaceae* typically decrease in abundance following N cessation, over time with NH_4 cessation and more immediately with NO_3 cessation. *Asteraceae* and *Ranunculaceae* increase in abundance with NH_4 recovery. In the case of NO_3 recovery, *Asteraceae* and *Ranunculaceae* initially increase, but in the long-term decrease in overall composition, possibly due to the proportional increase in *Fabaceae*. Overall insect-pollinated plants increase in plant communities recovering from N application, immediately in the case of NO_3 cessation, and in the long-term in the case NH_4 cessation.

Plants with floral units morphologically adapted to bee pollination increase with the retention of both N treatments, more gradually with NH_4 (Table 2.4). Plants bearing simple flowers only increase in proportion in communities recovering from NO_3, not those recovering from NH_4. When plant communities recover from N applications, flowers with more rewarding nectar increase in proportion, immediately in the case of NO_3 recovery, and more gradually in the case of NH_4 recovery. With long-term recovery from NH_4 additions, we find more plants with smaller inflorescences, but there are no other significant effects to floral size.

There is an increase in plants producing blue flowers when either form of N is withheld (Table 2.4). Communities recovering from NH_4 addition have a long-term increase in plants with yellow inflorescences, and an immediate increase in plants with UV pattern. On the other hand, communities recovering from NO_3 addition have an immediate increase in plants with yellow and UV signalling blossoms, which plateaus over the long-term. Plants with white inflorescences, however, immediately decline following N cessation, but begin to increase over the long-term.

The average flowering phenology of the plant communities are affected by NO_3 withdrawal (Table 2.4). The average peak flowering season is delayed, as are the average first and last flowering dates. Over time, plants with shorter flowering duration increased in proportion in NO_3 recovery plots. Withholding NH_4 led to early-flowering plants becoming more dominant. Over time, this response declined.

Table 2.4. Output of LMER models studying the change in the CWM of the functional floral traits when N is withheld.

The rows show all response variables that were impacted significantly by the explanatory variables. All values are statistically significant (t > 2) effect sizes given by the final simplified ("+" denotes increases, "-" decreases).

	NH_4 withheld	**yrs. recovery**	**NO_3 withheld**	**yrs. recovery**
Stress tolerance (average value of community)				
Ellenberg N	-0.286	-0.0189	-0.872	
Ellenberg R	-0.365	+0.0161	-0.089	
Lifeform & Family (change in proportion composition)				
Grass		-0.015	-0.303	
Legume		+0.0109	+0.112	+0.011
Forb		+0.0045	+0.162	-0.009
• Apiaceae		-0.00151	-0.0312	-0.00215
• Asteraceae	+0.0443		+0.0364	-0.00151
• Ranunculaceae		+0.000385	+0.0815	-0.00516
Insect-pollinated		+0.0118	+0.245	
Morphological pollinator syndrome (change in proportion composition)				
Simple flower			+0.0467	-0.00492
Bee-form		+0.0109	+0.113	+0.0113
Nectar traits (change in provision; kg^{-1} ha^{-1} yr^{-1} production, ug^{-1} fl^{-1} day^{-1} sugar)				
Nectar production		+9.626	+141.335	+5.552
Nectar sugars		+1.322	+27.829	
Floral size (change in size; mm)				
landing area		-0.813		
Floral colour (change in proportion composition)				
Blue		+0.00967	+0.102	+0.00494
Yellow		+0.0609	+0.159	-0.00585
White			-0.053	+0.00449
UV	+0.0523		+0.15	-0.00572
Flowering phenology (change in month of phenological event)				
Start flower	-0.68	+0.0495	+0.321	+0.0147
End flower			+0.347	-0.0172
Duration				-0.0285
Season	-0.579	+0.0522	+0.315	+0.0293

2.4.5 Park Grass Experiment – 20 years of recovery from N additions

After 22 years of recovery from N additions, there are fewer significant differences between the nil plots and recovery plots (7, 9/1, 14/1) than between the nil plots and the N addition plots (7, 9/2, 14/2) (Table 2.5). Communities recovering from NH_4 addition still have higher compositions of grasses, although this is alleviated slightly in high-pH soils. The proportion of legumes in plant communities recovering from N addition is not significantly different from historical nil plots. However, the proportion of forbs remains lower in the recovery plots than the historic nil plots. The proportion of *Apiaceae* and *Asteraceae* is relatively unchanged by withholding N additions for 22 years, as they show the same responses found in N-addition plots. However, withholding N has allowed *Ranunculaceae* to increase. The proportion of *Ranunculaceae* in communities recovering from NH_4 is similar to historic nil plots, and it is greater in NO_3-recovery plots.

When comparing historic nil plots with N-recovery plots, there is no significant difference in the proportion of insect-pollinated plants (Table 2.5). This shows recovery from the impoverished entomophilous proportion found in N-application plots. The proportion of plants bearing simple flowers, after 22 years of recovery, is now greater in communities recovering from NO_3 than in historic nil plots, whilst communities recovering from NH_4 addition remain poor with regards to foraging resources for generalist Diptera species. The shift towards poor nectar production caused by NH_4 additions has recovered after 20 years of withheld N. However, plots recovering from N addition still have typically lower nectar sugar provision. Plant communities recovering from NO_3 addition still have more plants with larger inflorescences, but there was no difference in average corolla length found between historic nil plots and recovery plots

The proportion of blue, purple, and yellow flowers, which declined with N addition, has recovered after 22 years (Table 2.5). There is now no discernible difference between historic nil plots and recovery plots. This is also the case for plants that bear flowers with UV patterns. However, the proportion of plants with white flowers remains low in low-pH NO_3-enriched plots.

After 22 years of withholding N, there is little difference in the average flowering phenologies of the plant communities (Table 2.5). Plots recovering from NO_3 addition maintain a slightly higher proportion of early-flowering plants, but this no longer translates into an early peak flowering season.

In the recovery of plant communities, pH played an important role for the compactions of forbs, particularly *Asteraceae,* increasing these at higher pH in more buffered soils (Table 2.5). Grasses were less dominant in plant communities recovering from NH_4 addition in high pH soils. Well-buffered soils helped the recovery of plants producing white flowers in plant communities recovering from NO_3 additions. Overall, considering all plots soil pH had wide-reaching impacts on the floral community. The detrimental effect of soil acidification was reasonably uniform across functional traits. Plants producing simple flowers and morphologically specialises flowers both declined with soil acidification, although bee-specialised flowers declined at a faster rate. There was a decline in plants producing yellow and UV patterned flowers with soil acidification. The shift in botanical community caused by soil acidification typically led to increased dominance of plants with an early flowering season, although this did not affect overall flowering duration.

Table 2.5. Output of LMER models studying the recovery of CWM floral functional traits following 20 years of withheld N, in comparison to historic control plots.

The rows show all response variables that were impacted significantly by the explanatory variables. All values are statistically significant (t > 2) effect sizes given by the final simplified ("+" denotes increases, "-" decreases).

	NH_4 recovery	**NO_3 recovery**	**pH**	**NH_4 recovery*pH**	**NO_3 recovery*pH**
Lifeform & Family (change in proportion composition)					
Grass	+1.725			-0.29	
Legume			+0.104		
Forb	-1.327	-1.096	-0.134	+0.219	+0.184
• Apiaceae		+0.0552	+0.0322		
• Asteraceae	-0.817	-0.792	-0.129	+0.14	+0.129
• Ranunculaceae		+0.0294			
Insect-pollinated			+0.138		
Morphological pollinator syndrome (change in proportion composition)					
Simple flower		+0.0808	+0.0405		
Bee-form			+0.104		
Nectar traits (change in provision; kg^{-1} ha^{-1} yr^{-1} production, ug^{-1} fl^{-1} day^{-1} sugar)					
Nectar production			+85.53		
Nectar sugars	-71.039	-71.175	+12.375		
Floral size (change in size; mm)					
Landing area		+16.741	+12.609		
Corolla depth					
Floral colour (change in proportion composition)					
Blue-purple					
Yellow			+0.0431		
White		-1.087			+0.202
UV pattern			+0.0427		
Flowering phenology (change in month of phenological event)					
Start flowering		-0.191	-0.166		
End flowering			-0.105		
Duration flowering					
Season			-0.383		

2.5 Discussion

The findings are in agreement with previous analyses of these datasets that showed increased grass proportions and decreased forbs and legumes with higher levels of N additions to soils (Stevens *et al.*, 2004; Storkey *et al.*, 2015). It is also in agreement with other studies from grasslands (e.g. Duprè *et al.*, 2010; Stevens, Duprè, *et al.*, 2010) and other habitats (e.g. Maskell *et al.*, 2010; Field *et al.*, 2014; Humbert *et al.*, 2016). Furthermore, the analyses confirmed that N-driven impacts to the botanical community alters the array of functional floral resources in ways that could be impactful for pollinator assemblages. This suggests that, in regions with higher deposition, N may have acted as a driver of negative pollinator trends during the recent century. Increasing deposition levels in the Southern Hemisphere could impact on the composition of floral resources available to pollinators in the future. The impact to floral communities was not uniform; responses varied between functional floral groups and taxonomic families. Furthermore, responses varied according to the level of N addition and between the two grassland types, acidic and mesotrophic. Therefore, the precise responses of grassland communities to escalating N deposition or additions, and subsequent consequences for pollinators, will be dependent on the local habitat context.

2.5.1 The response of taxonomic families

The response of taxonomic families was non-uniform, with families differing in their response to N additions. However, there were some consistencies in the responses of individual botanical families across study systems and N application levels. For example, *Ranunculaceae* were consistently sensitive to NH_4, in the PGE they declined at both levels of NH_4 addition whilst they declined in acid grasslands with increasing soil acidification, a common consequence of NH_4 deposition or applications in poorly buffered soils (Barak *et al.*, 1997; Duprè *et al.*, 2010; Stevens *et al.*, 2011; Van Den Berg *et al.*, 2016). On the other hand, *Ranunculaceae* did not notably decline with NO_3 additions. It may be that this taxonomic family is, in general, less able to tolerate the potential cell acidification and toxicity resulting from high levels of NH_4 uptake (Britto and Kronzucker, 2002; Lucassen *et al.*, 2003; Stevens *et al.*, 2011). Many *Ranunculaceae* species are used by wide groups of insect pollinators, including dipterans and hymenopterans (Appendix 5), although there is also evidence of specialisation through toxicity of secondary compounds to the larvae of some bee species (Sedivy, Müller and Dorn, 2011). The proportion of *Apiaceae* and *Asteraceae*, when compared across N applications levels, showed an interesting inverse in their responses. With 48 kg N ha^{-1} yr^{-1}, *Apiaceae* declined in unbuffered N-enriched plots but increased with higher levels of NO_3 applied with minerals. On the other hand, *Asteraceae* increased with 48 kg N ha^{-1} yr^{-1} applications of NO_3 but declined in unbuffered plots with high levels of N applied with minerals. There was no response in *Apiaceae* or *Asteraceae* abundance in acid grassland sites to N deposition or topsoil pH, so the responses could be dependent on the context of the ecosystem; these two taxonomic families were more prevalent in the PGE and had a low baseline in the acid grassland sites. *Ericaceae* did not grow in the mesotrophic PGE site as it is typically found on more acidic grasslands (Rodwell, 1992). Legumes, on the other hand, did not decline in acid grassland sites with N deposition or soil acidification, but declined consistently with N application in the PGE site, with greater losses observed at higher applications of N.

Leguminous flowers tend to produce nutritious nectar, high in protein and essential amino acids (Cook *et al.*, 2003; Hanley *et al.*, 2008). The decline in legumes represents a loss of significant floral resources that are rewarding, and relatively specialised. Previous studies have also found linear losses of overall botanical species richness with N addition (Stevens *et al.*, 2010; Humbert *et al.*, 2016), so clearly the level of N input is an important determinant of the extent of impact. The primary mechanism of species loss in temperate grasslands is the altered competitive dynamics, due to increased soil fertility, and resulting increase in grass biomass (Bobbink *et al.*, 1998). Legumes, which fix N through their rhizobium, are likely to lose a competitive advantage with excessive N additions (Suding *et al.*, 2005; Skogen *et al.* 2011). Interestingly, legumes did not significantly decline with N deposition in the UK acid grassland sites. This is likely because the proportion of legumes in these communities was already considerably low (mean = 0.0268, standard deviation = 0.063), even in sites with the lowest levels of deposition. Deposition levels in Europe were typically higher during the late 20th Century, which had impacts on plant communities (Storkey *et al.*, 2015). This could have shifted the baseline of legume proportions in acid grassland sites and obscured results.

The loss of forbs was not uniform across families. In the PGE, *Ranunculaceae* consistently declined with NH_4 enrichments. *Apiaceae* and *Asteraceae* also frequently declined in poorly buffered soils with N additions. However, in well-buffered soils with pH 6 or greater, *Apiaceae* and *Asteraceae* plants proportionally increase. The most common *Ranunculaceae* found in the PGE are *Ranunculus acris* and *Ranunculus bulbosus*, both of which have relatively low Ellenberg N values (Hill, 1999). Therefore, although the more N-tolerant, but infrequent, *Ranunculus ficaria* and *Ranunculus auricomus* can maintain populations despite N additions, the more abundant *Ranunculaceae* species are threatened by N additions. Conversely, the most common *Apiaceae*, *Anthriscus sylvestris* and *Heracleum sphondylium* have high Ellenberg N values (Hill, 1999), which allow N-enriched plots to maintain high proportions of *Apiaceae*. In the acid grassland sites, the decline of *Ericaceae* plants is a concerning result for bee species of acid grasslands. *Ericaceae* are incredibly rewarding nectar and pollen resources (Baude *et al.*, 2016) that are visited by a wide suite of bees. Furthermore, *Ericaceae* were one of the more prevalent and dominant taxonomic families across the acid grassland sites, so the loss of these plants with N deposition reflects a loss of a significant floral resource for pollinating insects.

2.5.2 Impacts to functional trait composition

Morphological floral structure

Higher N additions, whether as applications to the PGE or deposition to the acid grassland sites, consistently led to declines in plants that bear more specialised flowers, that are typically favoured by bees. For example, the loss of the bell flowers of *Ericaceae* and *Liliaceae* in acid grassland sites depletes important resources utilised by a range of bee species (Cane *et al.*, 1985; Scott *et al.*, 2016). In the PGE, 48 kg N ha^{-}

^{1} yr^{-1} applications in the absence of other minerals led to declines in the lip-form flowers of *Lamiaceae*. Labiate flowers are often highly specialised for pollination by bees, rather than generalist insects (Claßen-Bockhoff, 2007; Wester and Claßen-Bockhoff, 2007; Westerkamp and Claßen-Bockhoff, 2007). The labiate *Lamiaceae* flowers found in the PGE, such as *Ajuga reptans* and *Prunella vulgaris*, suit visitation by bees. In fact, in the PGE, N additions of 48 kg N ha^{-1} yr^{-1} caused declines in overall 'bee-form' flowers. These include the flag-blossoms of *Fabaceae*, labiate blossoms of *Lamiaceae*, and the bell flowers of *Liliaceae*. This finding also helps to explain the decline in average corolla size found with 48 kg N ha^{-1} yr^{-1} additions, of either N form. Longer corollas have typically co-evolved with specialist pollinators with long tongues (Nilsson 1988; Johnson and Anderson, 2010), such as some bumblebees. Research has also shown that plants that selectively favour specialist pollinators produce more rewarding pollen (Hanley *et al.*, 2008) and nectar (Petanidou *et al.*, 2006; Vandelook *et al.*, 2019). Therefore, more oligolectic bee species that have strongly linked interactions with these plants may be more at risk from the impacts of N deposition, regardless of the form of N. Furthermore, the loss of these floral types implies a deterioration of the quality of floral rewards provided by a floral community.

Historic N deposition levels from the 20th Century have been high in Europe, North America, China, and India. The loss of specialist floral resources, such as *Fabaceae*, could have contributed to the declines of more specialised pollinating insects, such as long-tongued bees (Rasmont *et al.*, 2005; Biesmeijer *et al.*, 2006; Patiny *et al.*, 2009; Baude *et al.*, 2016). With global N deposition levels set to increase in most regions of the world, with the possible exception of Europe (Dentener *et al.*, 2006; Galloway *et al.*, 2008), this poses an ongoing threat to many insect pollinators that are already impacted by a myriad of detrimental factors (Potts *et al.*, 2010; Vanbergen *et al.*, 2013). Many regions in the Southern Hemisphere currently experience low, but rising N deposition (Galloway, 1998; Dentener *et al.*, 2006; Fowler *et al.*, 2013). We lack research regarding the impact of N deposition to many biologically diverse regions in the Southern Hemisphere (Bobbink *et al.*, 2010). However, it's likely that these regions have not yet experienced the same consequences of N deposition that the Northern Hemisphere has during recent decades. If deposition levels continue to rise in the future in the Southern Hemisphere, there could be detrimental impacts to oligolectic pollinators and wider pollinator communities.

However, this is not the full picture; many pollinators that exhibit specialised feeding apparatus still forage opportunistically from alternative floral resources. Therefore, those pollinators that do show plasticity and flexibility in their foraging choices will not be so impacted if plant communities remain biodiverse enough to ensure alternative floral resources. In this respect, there is a difference between the two forms of N. In the PGE, applications of NH_4 drove a decline in simple flowers as well as specialised flowers, whereas NO_3 additions led to increases in simple flowers. Furthermore, high levels of NO_3 addition favoured plants that produce larger open flowers. The detrimental impact of NH_4 to all floral resources in the mesotrophic grassland site could be due to the inherent adaptations and tolerances of many plant species commonly found in such grasslands. These plant favourably take up NO_3, not NH_4, as they are unable to tolerate the cell acidification caused by NH_4 uptake (Britto and Kronzucker, 2002; Lucassen *et al.*, 2003; Stevens *et al.*, 2011). On the other hand, NO_3 additions did not completely deplete floral resources, but altered the structure of the floral community, towards communities dominated by the larger simplistic flowers of *Apiaceae* and more generalist *Asteraceae* such *Taraxacum officinale agg.* and *Leontodon hispidus*. These plants produce copious amounts of sugar-rich nectar (Baude *et al.*, 2016; Hicks *et al.*, 2016), so benefit a wide range of flower-visiting insects. Furthermore, their large blossoms can cater to a larger abundance of insects, with many feeding simultaneously from the same floral unit. Mesotrophic grasslands in Europe and Eastern USA, that currently have a low NH_x:NO_y ratio (Dentener *et al.*, 2006; Stevens *et al.*, 2011), could see a shift towards such floral communities. However, many regions across the world, including India, China, and the majority of the Southern Hemisphere currently experience high NH_x:NO_y rations. In these regions, the loss of floral resources across floral groups should be a real concern if N deposition continues to increase.

Floral colour

Although it is not clear to what extent floral colour influences pollinator foraging choices, there is evidence of insect flower-visitors and pollinators showing strong preferences for certain colours or for UV reflectivity (Dyer *et al.*, 2006; Forrest and Thomson, 2009; Reverté *et al.*, 2016). For instance, Reverté *et al.* (2016) found bees were commonly associated with purple flowers, lepidopterans with lilac flowers, coleopterans with white flowers, and dipterans and wasps with UV-yellow flowers. The decline in blue-purple flowers at high levels, 96 kg N ha^{-1} yr^{-1} in the PGE (in the presence of other minerals) therefore suggests a decline in flowers that are visually more attractive to bees. With a lack of these floral units, naïve bees might be less likely to be attracted to and forage in these locations, possibly moving on to more appealing habitats. The increase in yellow and UV patterned blooms with 48 kg NO_3 ha^{-1} yr^{-1} additions is possibly driven by the increase in *Asteraceae* such as *T. officinale* and *L. hispidus*. This emphasises the positive impact NO_3 addition can have on these generalist resources, which are commonly favoured by dipterans such as *Syrphidae* (Reverté *et al.*, 2016). This shift in the floral community, along with the increase in simplistic open flowers, could promote the visitation of dipterans and wasps to plant communities undergoing high NO_3 additions. Of course, if plants bearing flowers of the preferred colour decline, flower visitors will in all likeliness opportunistically forage alternative blossoms if able.

Floral nectar

Although the applicability of morphological pollinator syndromes in plant-pollinator interactions is not fully clear, the necessity of nectar for pollinating insects is incontrovertible. Nectar is the vital reason for why most insects visit flowers, and so N-driven changes to the provision of floral nectar by a botanical community will likely have consequences for the broad pollinator community. As previously noted, certain nectar-rich plants were observed to decline with N additions, such as *Ericaceae* in acid grasslands. Nectar-rich plants declined with N deposition in acid grasslands and with low levels of NH_4 and high levels of both N forms in the PGE. The nectar production of plant communities with low levels of NO_3 applied was not affected, possibly due to the increased growth of *Asteraceae* and *Apiaceae*, many species of which are relatively nectar-rich plants (Baude *et al.*, 2011; Hicks *et al.*, 2016). Although the overall volume of nectar provided by plant communities decreased, the average nectar-sugar provision only declined under the highest levels of N additions in the PGE. The high levels, 96 kg N ha^{-1} yr^{-1}, are not necessarily field-realistic and are far higher than typical background deposition in the Northern Hemisphere. However, this does serve as a warning of escalating N deposition.

Flowering phenology

The N-driven alterations to plant communities could lead to shifts in the phenology of floral resources, for example, if earlier blooming or later blooming plants are favoured. With 48 kg NH_4 ha^{-1} yr^{-1} application in the PGE, the plant community moved towards later flowering plants, which could lead to a delayed onset of flowering and peak flowering date. Furthermore, as there was no change in the typical cessation of flowering, this suggests a curtailed flowering season caused by NH_4 additions. A shorter flowering season limits the floral resources available to pollinators and can have impacts on the pollinator community (Memmott *et al.*, 2007). However, there were contrasting impacts caused by NO_3 applications. With 48 kg NO_3 ha^{-1} yr^{-1}, there was no impact to early flowering plants, but NO_3 additions seemed to favour species with a later flowering date. In contrast, 96 kg NO_3 ha^{-1} yr^{-1} applications with minerals favoured early flowering plant species, but not late flowering species. The lack of a positive effect on late flowering species here could be caused by the strong increase in soil fertility and competitive dynamics from N and mineral applications, that does not allow late-flowering species to thrive in the same way that early species can. Nevertheless, the results suggest that NO_3 additions can lead to plant communities with extended flowering seasons, while NH_4 additions lead to shorter flowering seasons. This is an interesting contrast between the two N forms. When we consider real-world N deposition, clearly the ratio of NH_x:NO_Y will be an important determinant of the impact to pollinator communities. In both acidic grasslands and mesotrophic grasslands, we found that more acidic pH led to a decline in late-flowering plants. Therefore, the buffering capacity of soils undergoing N deposition or applications will help to determine the effect to pollinators active late in the season. This trend in acidic soils could be problematic, for example, for newly produced gynes of bumblebee colonies that require plentiful nectar and pollen resources to prepare for winter hibernation.

2.5.3 Soil acidification

In studies of atmospheric N deposition, it can be difficult to separate the effects of N and pH, especially with NH_x which are highly correlated with soil acidification and total historic N deposition (Bobbink *et al.*, 1998; Stevens *et al.*, 2011). A benefit of using the PGE, is the treatment structure that distinguishes between N applications and soil pH. The findings presented in this chapter reveal the importance of well-buffered soil in mesotrophic grasslands for floral resources and therefore for pollinator communities. Soil acidification caused plant communities to become more grass-dominant, at the expense of forbs, with an overall decline in insect-pollinated plants. This is in agreement with other studies, that have shown the detrimental impact of N-driven soil acidification on plant communities (Stevens *et al.*, 2010; Diekmann *et al.*, 2014; Field *et al.*, 2014). Notably, *Asteraceae* and *Apiaceae* declined with more acidic pH. As previously discussed, plants from these families are important nectar and pollen resources in communities with NO_3-enrichment. The decline of these plants with soil acidification shows how much more threatened plant communities on poorly buffered soils are by N deposition. The detrimental impact to pollinators with soil acidification is further emphasised by the decline in sugar-rich nectars, thereby limiting not only the availability of floral resources, but the nutritional quality of the floristic communities.

Many plants found in acid grassland sites will be more tolerant of, and naturally adapted to, the soil acidification and plant cell acidification caused by NH_x deposition than mesotrophic grassland sites (Britto and Kronzucker, 2002; Stevens *et al.*, 2011). Nevertheless, we observed impacts of soil acidification on the floral community, in agreement with Stevens *et al.* (2004). Furthermore, some of the observed responses were similar to the responses found in the PGE, suggesting that there may be common trends in the responses of plant communities to N-driven soil acidification. This is a useful finding, as variation in responses to N deposition and soil acidification is a factor that tends to complicate the accuracy of forecasting consequences. In both ecosystem types, there was a shift towards plants with smaller inflorescences, and losses in plants that bear blue-purple, yellow, or UV patterned flowers. These are flowers preferentially foraged by bees, wasps, and dipterans (Reverté *et al.*, 2016).

2.5.4 Recovery from high N additions

Studies of plant communities recovering from high levels of N deposition and addition have shown conflicting signs of recovery; while N-sensitive plant species can increase in abundance, improving overall species richness (Storkey *et al.*, 2015), in other experiments, the overall abundance of flowering units seems to remain stagnated, with a community structure that remains representative of N-enrichment: the dominance of grasses (Isbell *et al.*, 2013). CWM analysis with the mass ratio hypothesis can give insight into the state of recovering plant communities (Grime, 1998; Diaz *et al.*, 2007; Kimball *et al.*, 2016). Unlike previous studies which found increased forb presence in plant communities recovering from eutrophication, this study gave insights into the recovery of specific floral functional traits therefore showing the recovery of plant communities in the context of plant-pollinator interactions.

The results show that withholding N application allowed plant communities to recover, and there are promising signs for plant communities and interacting pollinators in regions where N deposition sharply drops in the future. However, whilst these signs are positive, the extent and speed of recovery depended on the form of N that the plant communities were recovering from. Recovery from NH_4 occurred over a longer time frame than recovery from NO_3 enrichment, which was more immediate. After 22 years of recovery, from 1990–2012, the plant communities still retained slight relics of historic N-enrichment. Communities recovering from NH_4 addition retained high grass proportions representative of historic N pollution. Although proportions of forbs in communities recovering from N pollution remained low, proportions of legumes had recovered. Legumes seem to be more able to capitalise on the decline of grasses and the new opportunities for growth freed up by a decline in grasses. This could be due to their N-fixing rhizobium, with which they can take advantage of low-nutrient soils. The finding is in agreement with Storkey *et al.* (2015), although the authors also found numerous recovering forb species in addition to legumes. The recovery of legumes is promising for many bee species, particularly bumblebees that commonly visit legumes such as *T. pratense* and *L. corniculatus*. If this response has also occurred with decreasing N deposition in Northwest Europe and Britain, it may be having positive effects for these important pollinator species. An analysis of bumblebee trends during the recent decades revealed that bumblebee declines were strongest pre-1990, after which they were somewhat alleviated (Carvalheiro *et al.*, 2013). This reflects the trends of N deposition in the UK, which escalated towards a peak in the late 1980's before beginning to plateau and decline from the 1990's onwards (Fowler *et al.*, 2004; Storkey *et al.*, 2015). With N deposition rates forecasted to continue dropping in Northwest Europe, this is a promising sign, but only if the associated plant and pollinator species can recolonise areas from which they have been lost. The forb community showed signs of recovery, but not to the same extent as legumes. The recovery dynamics of forbs differed between communities recovering from NH_4 and NO_3. While communities recovering from NH_4 have shown a slow increase in forb proportion, communities recovering from NO_3 had an immediate boost in forb proportion, which has since plateaued, potentially due to legumes becoming more dominant. That the forb community recovered to higher levels in higher soil pH shows the importance of soil pH not only in preventing species loss from N additions, but also in the recovery of plant communities and recolonization of N- and pH-sensitive species.

The immediate recovery of plants exhibiting many pollinator-relevant traits from NO_3 addition is encouraging, as is the albeit slower recovery rate from NH_4 addition. After 22 years of recovery there was no statistical difference between nil plots and recovery plots in the proportions of plant species that bear flowers morphologically suited to bee visitation, such as labiate flowers, bell flowers, and flag flowers. In addition, the continued high presence of simplistic open flowers with NO_3 recovery shows that the recovery of morphologically specialised plants was not at the expense of simplistic floral structures, which are important resources for a wider suite of pollinating insects. The proportions of blue-purple, yellow, and UV patterned flowers in communities recovering from N additions were not statistically different from nil plots, which also suggests that with recovery from N attractive floral resources for bees and dipterans is available. The flowering phenology, too, was relatively similar to that found in nil plots, although plant communities recovering from historic NO_3 enrichment had a greater density of early-flowering plants. This did not come at the expense of late-flowering species or flowering duration. The tendency towards early-blooming plants is potentially a positive consequence for early-emerging bee species that will be foraging for reinvigorating nectar and pollen to start nests and colonies. However, despite the many positive signs of recovery, there is one important way in which plant communities did not recover, the provision of sugar-rich nectars. Although many nectar-rich plants recover, these were not necessarily rich in nectar-sugars, which is an important factor to support an abundant and diverse pollinator assemblage (Potts *et al.*, 2003; Fründ, Linsenmair and Blüthgen, 2010; Weiner *et al.*, 2014).

2.5.5 Caveats to consider and limitations of the study.

The response variables used in the analyses of this chapter data refer to vegetative biomass. The data used are from previous years (1990-2000, 2010-2012 for PGE; 2002-2003 for UK acid grasslands), collected by other researchers, and does not contain information on flowering units. I inferred floral resource provision from these datasets. This is an important caveat to consider, as true floral production within species will vary across sites and communities. Abiotic drivers, including N, can affect floral production (e.g. Burkle and Irwin, 2010). Furthermore, trait values used in the analysis were standardised within species, with values obtained from trait databases such as BIOFLOR (Klotz *et al.* 2002). Realistically, floral traits such as phenology, floral dimensions, and nectar secretion are relatively plastic and vary with abiotic conditions, including N. The full range of expression of these traits is not taken into consideration. In consideration of the two caveats described above, the results should be regarded with care, as the veracity of the results relies on biological simplifications. Realistically, the response of plant communities will vary according to locally mediated conditions.

The study does, however, offer a novel view with useful insights of how N and pH act as drivers on the supply of floral resources to pollinator communities.

The PGE plots recovering from eutrophication had high levels of N applied historically; 96 kg N ha^{-1} yr^{-1}. This is incredibly high in the context of current N deposition levels but could be applicable to future deposition levels, if fluxes continue to increase in regions such as China that currently experience high N deposition (Galloway *et al.* 2004). Furthermore, the PGE N-recovery plots also receive mineral fertilisation (P, K, Na, Mg). Therefore, the results of recovery reported in this chapter could be symptomatic of high-fertility soils and associated plant communities. Recovery responses of plant communities could vary across sites due to localised soil conditions. Importantly, however, the study shows that plant communities can recover from severe soil eutrophication, an important finding.

The consideration of phylogenetic constraints in studies of functional traits has been heavily debated (e.g. Ackerly and Donoghue, 1995; Westoby *et al.* 1995; Freckleton *et al.* 2002). The independence of functional traits from phylogeny varies with traits, for example quantitative traits such as floral dimensions or nectar production are often less constrained by phylogenetic nonindependence (Westoby *et al.* 1995). In this analysis, traits that were more heavily influenced by phylogeny were yellow flowers with *Asteraceae* (8 of 24 acid grassland species; 7 of 17 PGE species), and white flowers with *Apiaceae* in the PGE (4 of 10 species). Other functional trait groups were well represented across phylogenetic group. For example, ‘bee-form’ flowers included species of *Asteraceae* (e.g. *C. nigra*), *Lamiaceae* (e.g. *A. reptans*), *Fabaceae* (e.g. *T. pratense*), and other taxonomical families.

Phylogenetic and ecology are not mutually exclusive, and phylogenetic correction can under-represent variation derived from ecology or environmental drivers (Westoby *et al.* 1995). The importance of phylogenetic correction of datasets for comparative analysis is dependent on the research question (Ackerly and Donoghue, 1995). The aim of this study was to determine how soil N and pH affect the floral landscape and resources for prospective foraging pollinators. For this research question, the phylogenetic correction of trait analysis was not deemed necessary.

2.6 Conclusion

The study applied an informative analysis towards determining the potential impact of N deposition and addition on the floral landscape. Degradation of the floral landscapes, particularly in grasslands, has been noted as a key driving factor of shrinking pollinator insect diversity and abundances in recent decades (Goulson *et al.*, 2008; Potts *et al.*, 2010; Vanbergen *et al.*, 2013; Senapathi *et al.*, 2015). The role of N-enrichment, a driving factor of shifts in botanical communities, warrants research efforts. As the study showed, responses of botanical communities can differ across different types of ecosystems; this study considered two types of grasslands, but responses could vary even more between further unlike ecosystems such as temperate grasslands and tropical forests. Furthermore, the impacts of N deposition are further confounded by the different impacts caused by NH_x and NO_Y, meaning that responses of botanical communities could vary depending on the localised NH_x:NO_Y deposition ratios. Ambitious research undertaken across a range of ecoregions could help inform world-wide ecosystems. With deposition levels already high and forecast to increase further in China and India may already be experiencing impacts that will intensify further. The southern Hemisphere, where current deposition levels are relatively low, houses many biologically rich hotspots, and is at risk of the impacts of rising N deposition levels surpassing critical loads (Phoenix *et al.*, 2006; Bleeker *et al.*, 2011).

This study showed, with functional trait analysis, that recording loss in species richness does not necessarily give enough insight into the shift in functional traits of the botanical communities. Certain floral traits remain stable in the face of N deposition, whereas others are more susceptible to declines. However, the different responses caused by NH_4 and NO_3 illustrate the varied responses that could be caused by atmospheric N deposition and the complexity in forecasting these. Impacts to plant-pollinator interactions will likely be influenced by the ratio of NH_x:NO_Y. The study also showed the importance of soil buffering capacity; many of the traits studied, particularly key pollinator traits, declined further with soil acidification. Throughout the study, the results suggested that more specialist floral units will likely decline in the face of increasing N deposition and application rates. Losses of these key resources could have had impacts on declines of insect pollinators during the 20th Century (Rasmont *et al.*, 2005; Carvalheiro *et al.*, 2013; Baude *et al.*, 2016). The evidence of recovery of floral communities, with N cessation, shows that there is a ray of hope. Efforts have been made to decrease N deposition, with levels in Europe plateauing and declining (Fowler *et al.*, 2004; Storkey *et al.*, 2015). If deposition rates in Europe continue to drop, and if such trends are observed across the world, this will help pollinator communities which are already under stress of numerous other factors (Goulson *et al.*, 2008; Potts *et al.*, 2010; Vanbergen *et al.*, 2013).

CHAPTER 3

THE IMPACTS OF NITROGEN ON THE FLOWERING PHENOLOGY OF INDIVIDUAL SPECIES AND A GRASSLAND COMMUNITY

3.1 Abstract

It is recognised that soil eutrophication and acidification from nitrogen (N) addition impacts botanical communities, and that this may have understudied implications for further trophic levels. The health of many pollinators is tied to plants; for example, bees, key pollinators in many systems, must forage nectar and pollen to develop their colonies. Flowering phenology also determines the abundance and diversity of floral resources available to pollinators. This botanical trait can be affected by abiotic drivers, although research is lacking on the impact of soil N. In light of insect pollinator declines, we need to improve our understanding of this. Using the Park Grass Experiment (PGE), I set out to determine how different forms and levels of N applications, as well as soil acidification, affect flowering phenology. By collecting data over an 8-year period, I was able to study the many treatments of the PGE with good statistical power. I found impacts to flowering phenology caused by N, and contrasting effects of the two forms, ammonia (NH_4) and nitrate (NO_3). NH_4 delayed flowering onset and shortened the overall flowering duration for many species. NO_3 had a more varied effect. Overall, there was a contrast in the responses of early-flowering species and late-flowering species. The onset of flowering was advanced, and flowering duration increased for the earliest species, while flowering was curtailed with a shorter duration observed for later-flowering plants. Inter-specific variation is a common problem in forecasting responses to soil-N additions, but the contrast between these groups is suggestive of a pattern. The boost in early-flowering species is positive for newly emerging insect pollinators, while the early curtailment of later-flowering species is detrimental during the peak season for many pollinating insects.

3.2 Introduction

3.2.1 Nitrogen deposition and impacts of nitrogen to flowering phenology

Globally, atmospheric nitrogen deposition has risen sharply since the industrialisation of the mid-19th century and is forecast to continue increasing in the near-future (Galloway *et al.*, 2004, 2008; Dentener *et al.*, 2006; Fowler *et al.*, 2013). From 1860 to 1990 global atmospheric deposition tripled, from 31.6 to 103 Tg N yr^{-1}, and is expected to approximately double between 1990-2050 (Galloway *et al.*, 2004). These rises are not uniform worldwide, however. The deposition rate is highest in the northern hemisphere and, despite plateauing in Europe and North America, is continuing to rise in other regions, such as China (Liu *et al.*, 2013). In the southern hemisphere, the deposition rate is lower, but is predicted to increase in the near-future, with potential consequences to biologically rich ecosystems (Phoenix *et al.*, 2006; Bleeker *et al.*, 2011).

Increases in nitrogen deposition affect plant community composition; in grasslands, this is primarily through heightened competition due to increased soil fertility (Bobbink, Hornung and Roelofs, 1998; Crawley *et al.*, 2005; Bobbink *et al.*, 2010; Field *et al.*, 2014; Helsen *et al.*, 2014; Storkey *et al.*, 2015). However, the fundamental role of N in plant growth means that changes in soil-N could have further effects on botanical traits. These impacts are largely understudied and represent a knowledge gap (Nijssen, Wallis De Vries and Siepel, 2017; Stevens, David and Storkey, 2018). There has been little research conducted into how nitrogen additions affect flowering phenology; furthermore, the evidence we do have illustrates variation across studies, species, and systems. In the field, overall forb communities have been observed to flower earlier (Cleland *et al.*, 2006) and later (Smith *et al.*, 2012) in response to nitrogen additions. Recent research into the effects of nitrogen fertilisation on flowering phenology has not differentiated between reduced (NH_x) oxidised (NO_y) nitrogen and has only considered specific forms, either NH_x (Smith *et al.*, 2012; Xi *et al.*, 2015; Liu *et al.*, 2017) or NO_y (Cleland *et al.*, 2006), or an NH_xNO_y mixture (Xia and Wan, 2013). As the two forms have varying effects to botanical communities, across ecosystems and soil types (Southon *et al.*, 2013; Van Den Berg *et al.*, 2016), distinguishing between them could improve our understanding of potential responses across regions and NH_x:NO_y ratios. Furthermore, studies have shown that flowering phenology responses varies amongst species (Xia and Wan, 2013; Xi *et al.*, 2015; Liu *et al.*, 2017). This inter-specific variation is a common theme that confounds the development of a clear overview of how plant communities and ecosystems might respond to increased soil nitrogen. Within a plant community, the functional traits and the key interactions plants share with other trophic groups varies across species. To improve our understanding of the inter-specific variation in botanical responses to nitrogen, and thus improve the accuracy of our forecasting ability, it is useful to study a wide range of species across taxonomic group, season, and ecoregion. A collection of evidence across a wider range of species will give us more insight into the varied responses of ecosystems to nitrogen additions and help to show under-lying patterns in how responses are linked between species.

3.2.2 Importance of flowering phenology for pollinating insects

Flowering phenology is an important botanical trait for pollinating insects that can affect the provision of floral nectar and pollen resources. Flowering phenology is a relatively plastic trait that can shift in response to abiotic conditions, such as climate warming (Fitter and Fitter, 2002; Parmesan and Yohe, 2003; Cleland *et al.*, 2007; Hegland *et al.*, 2009), with the potential to decrease the food supply for pollinating insects (Memmott *et al.*, 2007). Whereas climate warming can directly lead to earlier pollinator emergence as well as flowering (Gordo and Sanz, 2006), soil-N additions will likely only impact plant phenology, not pollinator phenology directly. Shifts in plant phenology without likewise shifts in pollinator emergence and activity dates could potentially lead to phenological mismatches. Considering recent declining trends of pollinator insects (Biesmeijer *et al.*, 2006; Patiny, Rasmont and Michez, 2009; Potts *et al.*, 2010; Powney *et al.*, 2019), this is an area that requires further research. It is now acknowledged that declining trends are due to many interacting factors, of which the loss of floral resources and foraging opportunities is a key driver (Potts *et al.*, 2003; Brown and Paxton, 2009; Scheper *et al.*, 2014). Therefore, drivers that limit the abundance of floral nectar and pollen resources, such as shifting flowering phenology, need to be strongly considered as a factor. Bees, obligate flower visitors, are key pollinators in many systems. The mistiming of key floral resources for oligolectic bee species, such as *Chelostoma* sp. (Sedivy, Müller and Dorn, 2011) or long-tongued bumblebees, could exert stresses upon individuals and colonies. For polylectic bees and other generalist insect pollinators, the detrimental impacts of shifting floral phenology could be buffered and somewhat negated by high botanical biodiversity (Bartomeus *et al.*, 2013). However, with insufficient alternative floral resources, shifts in flowering phenology could impose limitations on the broad insect-pollinator community. Therefore, it is useful to know how the flowering phenology of a botanical community is affected, not only individual species. By furthering our understanding of how soil N affects the flowering phenology of individuals and communities we can more broadly forecast the impacts to pollinator communities.

3.2.3 Aims of the study

Using the Park Grass Long-Term Experiment (PGE), at Rothamsted Research (Hertfordshire, UK), I investigated the impacts of the form of N, the amount of N, and topsoil pH on the flowering phenology of common calcareous grassland plant species that are visited by pollinators. With the treatment structure of the PGE, it is possible separate these effects to make hypotheses of N independent of pH, something that can be challenging in other studies (Stevens *et al.*, 2011). The mechanisms that cause a plant to begin flowering are typically autonomous, environmental, or stress-induced (Takeno, 2016), therefore I hypothesised 1) species-specific responses to the treatments. This would be in accordance with other studies that have found contrasting responses of flowering phenology to N additions (e.g. Cleland *et al.* 2006; Xia and Wan, 2013, Liu *et al* 2017). Furthermore that 2) the form of N will be an important factor, as plant species vary in their tolerances to reduced or oxidised N forms. I further hypothesised that 3) onset of flowering will be related to the species' tolerance to N, with nitrophilic species flowering later. Also, that smaller plants will flower later with N additions, due to reduced photoperiod caused by larger vegetative sward. In this study I also measured the effects of the N treatments on the in-flower species-richness of plant communities throughout the season. Alongside the lower species richness that is typically caused by N addition, I also hypothesised that 4) the patterns of community-scale in-flower species richness throughout the season would be affected by the increased soil resources. This study is informative in two ways; it separates the effects of nitrogen form, amount, and topsoil pH in a study into flowering phenology, and it relates changes in community-scale flowering phenology caused by nitrogen addition to the species richness that is useful for flower-visitors and pollinators.

3.3 Materials and Methods

3.3.1 Field site; Park Grass Long-Term Experiment

The full description of the PGE can be found in Chapter 2 (section 2.3.1). The study presented in this chapter uses data collected from 78 of the 101 subplots, encompassing the full range of treatments applied to the PGE. Plots 7/1, 9/1, 13/1, 14/1, and 15 were excluded from the analysis (Fig. 3.1), as these have had treatment alterations in recent years, as explained in section 2.3.1. Our study is interested in the effects of long-term, chronic, nitrogen addition and these 5 plots are not comparable to the other PGE plots in this sense. Plot 20 was also excluded, as it contains a unique treatment combination that incorporates an alternative level of N (30 kg ha^{-1} yr^{-1}) in addition to organic farmyard manure.

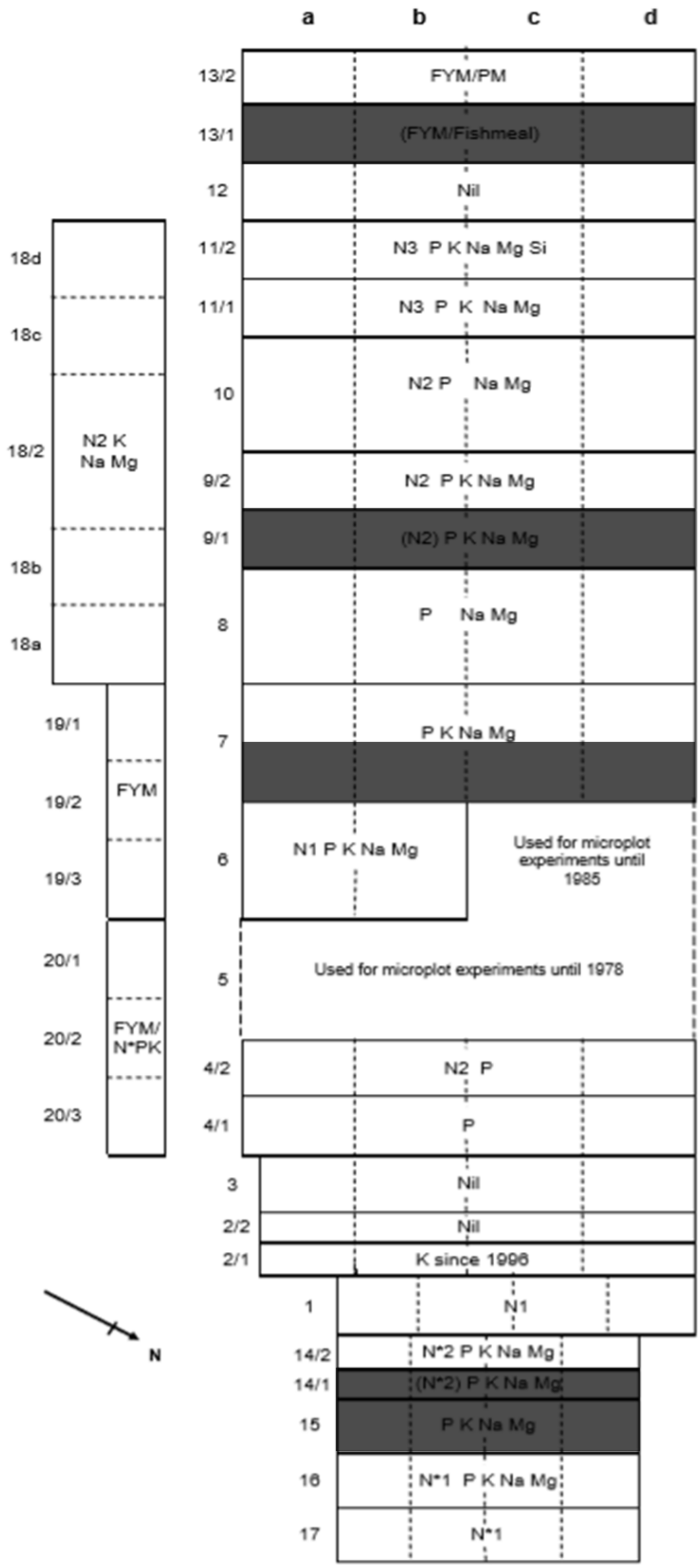


Figure 3.1 The PGE plan of plots used for Chapter 3.
Shaded plots were not used in the analysis as the treatments have been altered within the last 20 years, all other plots were used.

3.3.2 Phenology monitoring and data collection

Flowering phenology was monitored across all PGE plots between 2011-2018, during the first cut season from the first flowering until mid-June. The second cut season was not monitored, as the June cut date varies between years and would influence the date of regrowth. To develop the dataset, I walked around each plot approximately twice a week from the time the first flower was observed until the first cut in June, recording all botanical species in flower, including grasses. This gives community-scale data on the first-flowering dates (FFD) for all plant species found on PGE. The dataset also shows the last-flowering date (LFD) and duration of flowering for all species that cease flowering prior to the June cut. Furthermore, the dataset shows the species richness of in-flower plants for each plot community throughout the season.

In total, 61 species of plants were recorded; 15 grasses, nine legumes, and 37 forbs. Of the forbs and legumes, 15 forbs and three legumes, flowered consistently in the first season, in enough plots that it was viable to assess the impact of the soil treatments on FFD. The June cut interrupts the flowering season of 8 of these 18 species, and so I studied the LFD and overall flowering duration of the 10 species for which it was possible to do so, all of which were forbs (Table 3.1).

Table 3.1 Chapter 3 study species, representative codes used in the study, taxonomic family, response variables tested, and PGE flowering season.

'Code' shows the species code, which is used to refer to the species throughout further figures and tables. 'Response var.' denotes the response variables that were studied for each species; first-flowering date (FFD), last-flowering date (LFD), and total flowering duration (duration). 'Season' denotes the months during which the plant is in flower in the PGE, cut means the species is still in flower at the time of the cut in late-June

Code	Species	Family	Response var.	Season
To	*Taraxacum officinale*	*Asteraceae*	FFD, LFD, duration	Feb–May
Ra	*Ranunculus acris*	*Ranunculaceae*	FFD	Feb–cut
Rf	*Ranunculus ficaria*	*Ranunculaceae*	FFD, LFD, duration	Feb–April
Fm	*Fritillaria meleagris*	*Liliaceae*	FFD, LFD, duration	March–April
As	*Anthriscus sylvestris*	*Apiaceae*	FFD, LFD, duration	April–May
Rb	*Ranunculus bulbosus*	*Ranunculaceae*	FFD, LFD, duration	April–May
Pl	*Plantago lanceolata*	*Plantaginaceae*	FFD	April–cut
Ar	*Ajuga reptans*	*Lamiaceae*	FFD, LFD, duration	April–June
Cf	*Cerastium fontanum*	*Caryophyllaceae*	FFD, LFD, duration	April–June
Tp	*Trifolium pratense*	*Fabaceae*	FFD	April–cut
Vc	*Veronica chamaedrys*	*Plantaginaceae*	FFD, LFD, duration	April–June
Cm	*Conopodium majus*	*Apiaceae*	FFD, LFD, duration	April–June
Sm	*Sanguisorba minor*	*Rosaceae*	FFD, LFD, duration	April–June
Trag	*Tragopogon pratensis*	*Asteraceae*	FFD	April–cut
Hr	*Hypochaeris radicata*	*Asteraceae*	FFD	May–cut
Lh	*Leontodon hispidus*	*Asteraceae*	FFD	May–cut
Lc	*Lotus corniculatus*	*Fabaceae*	FFD	May–cut
Lp	*Lathyrus pratensis*	*Fabaceae*	FFD	May–cut

3.3.3 Statistical analysis

Flowering phenology of individual species

The impacts of the treatments on the FFD, LFD and the overall flowering duration of species within the communities were analysed using mixed-effects models with the lme4 package (Bates *et al*., 2015) in RStudio (R Core Team, 2018). Mixed-effects, fitted with REML, incorporated relevant treatments as fixed-effects and other treatments, plots, and years as random-effects. The fixed-effects were nitrogen type (categorical; nil, NH_4, NO_3), nitrogen amount (continuous; 0–144 kg N ha^{-1} yr^{-1}), pH (continuous; 3.6–7.2), and the two-way and three-way interactions between these variables. To focus on the research question, the effect of N, I incorporated the following random-effects: P (categorical; applied, not applied), K (categorical; applied, not applied), Na and Mg (categorical; applied, not applied), organic farmyard manure (categorical; applied, not applied), year (categorical; 2011–2018) and plot (categorical). Initially, I used Julian day and growing-day degrees, with thresholds of 0° C and 5° C, as response variables. Growing-day degrees with a threshold of 5° C (henceforth referred to as GDD5) fit the data best and helped to account for inter-annual variations in temperature, so the final statistical analyses were run with GDD5 as the response variable. For *Ranunculus acris*, the data was not normal and required a log-transformation to determine the best model. The GDD5 response variable for other species was normally distributed and did not require transformation. The initial models were simplified by removing the least significant variables, one-by-one and beginning with the interaction terms, checking the residuals and model assumptions at each step.

Community in-flower species richness and seasonal phenology

From the dataset, I calculated the total species richness of blooming plants in each plot for each day in which phenology was monitored over the 8 years. Using the ggplot2 package (Wickham, 2016) in RStudio, I plotted the data of species richness against GDD5 to investigate how N addition affected the overall species richness of in-flower plants throughout the season. GDD5 was more accurate in fitting the data than both Julian day and growing-day degrees with a threshold of 0° C, as it helped account for inter-annual temperature variation. The data was plotted with 95% confidence intervals to show statistically significant differences between the treatments throughout the season. For the remainder of this chapter, the term ‘species-richness’ will refer to the in-flower species-richness, counting only species in flower and not those in a purely vegetative state.

During the 2016-2018 seasons, I conducted pollinator transects of the PGE plots, recording insect flower-visitors (data is analysed and presented in full in chapter

5). Using this dataset, I identified simple phenological markers of important seasonal times for the bee communities, in GDD5: the date of first recorded activity in a season to represent first emergence, and the median dates of bumblebee, honeybee, and solitary bee activity to represent the dates of peak activity for these groups. These seasonal markers were used in this chapter to compare how N addition form, amount, or topsoil pH affect the species-richness of floral resources at important times during the season for insect pollinators.

3.4 Results

3.4.1 First flowering date (FFD)

Of the 18 study-species analysed for FFD response, 17 responded in some way to the treatments; only *Tragopogon pratensis* did not show a response to any explanatory variables (Table 3.2). Sixteen of the study species responded to the quantitative amount of N applied (whether independently or interactively); all but *T. pratensis* and *Hypochaeris radicata*. Responses to the two forms of N treatments, NH_4 and NO_3, varied. Applications of NO_3 caused a varied range of responses amongst the species; significantly delaying FFD for two species, accelerating five species, and not being significant for the other 10. The responses to the reduced form, NH_4, were more consistent; delaying the FFD of five species and not being a significant factor for the other 12. In fact, a simple plot of the mean FFD of the study species from control plots, NH_4 plots, and NO_3 plots shows that no species had an earlier FFD under NH_4-enrichment (Fig. 3.2). NH_4 did not delay the flowering of *Leontodon hispidus* independently, but the interactive with pH shows that *L. hispidus* FFD will be delayed in communities with high pH and NH_4 addition. The FFD response of six species was further affected by the amount of N applied; two advanced and four delayed with higher N applications.

We often find a significant effect by an N variable accompanied by a significant interaction between the N variable and pH (Table 3.2). In nine of the 18 occasions in which an N variable acts as a significant independent variable, there were non-additive significant interactions between the N and pH. Where there is a significant interactive term between an N variable and pH, the response caused by N form or addition will vary depending on the pH of the soil. Acting independently, soil pH affected the FFD of six species in contrasting ways; at higher pH, FFD was accelerated for three species and delayed for three species.

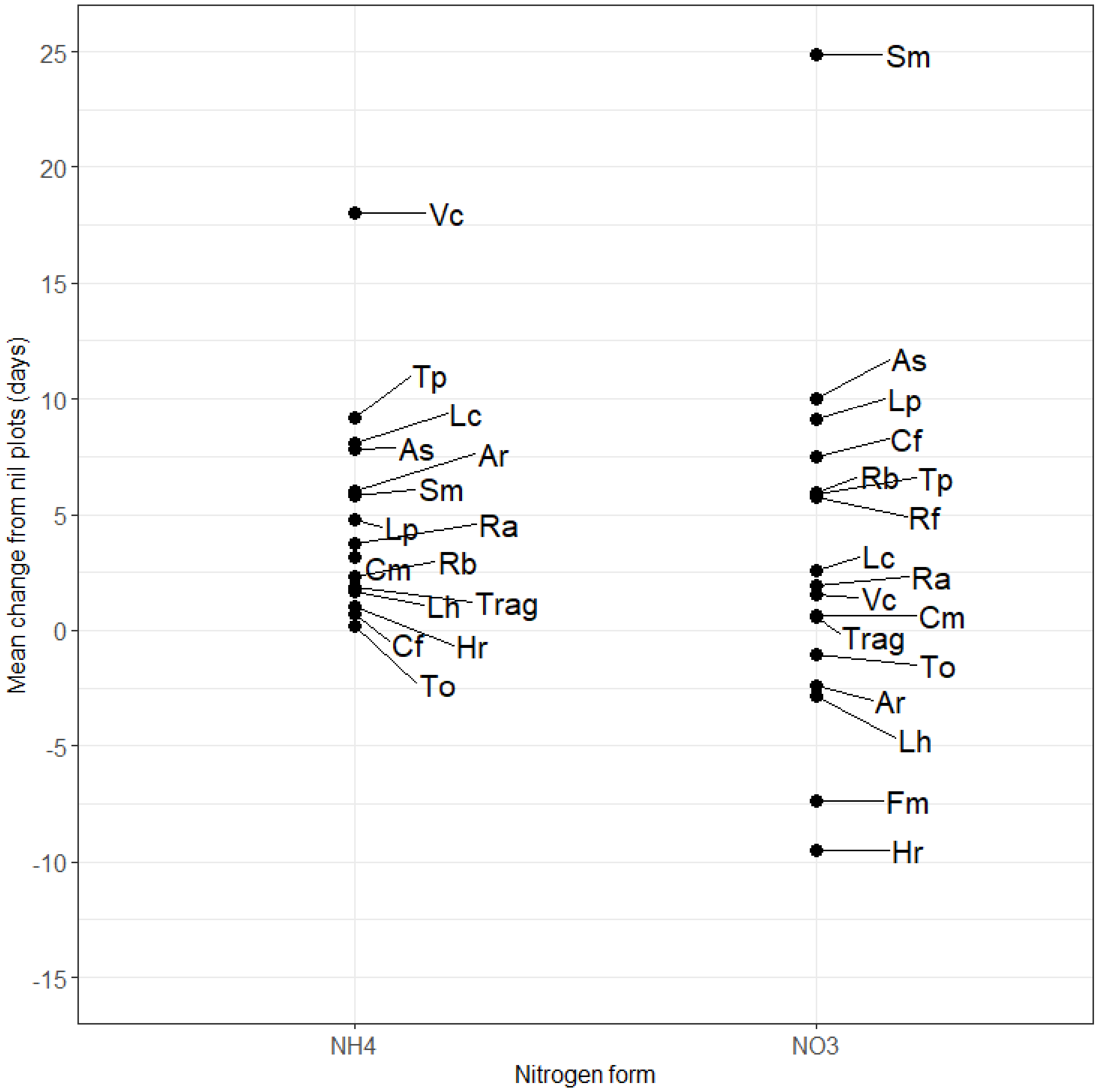


Figure 3.2. Summary figure showing the mean effect of NH_4 and NO_3 treatments on the first-flowering date (FFD) of all 18 study species.

Table 3.2. Output of LMER models studying the impact of treatments on first-flowering date (FFD) of the study-species.
All presented values are statistically significant (t > 2) effect sizes given by the final simplified models ("+" denotes a delayed GDD5, "-" denotes an earlier GDD5).

Species	**NH_4**	**NO_3**	**N amount**	**pH**	**NH_4:N amount**	**NH_4:pH**	**NO_3:pH**	**N amount:pH**
To	+60.0	-83.4		+14.0		-10.0	+13.1	
Ra			-1.4	-6.3	+0.9			+0.2
Rf			-0.3					
Fm		-498.9					+68.6	
As				-12.9				+0.1
Rb		+210.2					-28.9	
Pl		-59.1						
Ar	+31.9							
Cf		+67.38						
Tp	+146.2		+0.7			-22.8		
Vc			+2.08					
Cm	+66.2	-130.9	+0.4	+20.9		-14.3	+15.3	
Sm				-20.9				
Trag								
Hr				+84.3				
Lh		-195.8				+18.3		
Lc	+84.5							
Lp			+0.06					

3.4.2 Last flowering date (LFD)

Mixed-effects models of LFD for the 10 study-species also reveal inter-specific variation (Table 3.3), although *Ranunculus ficaria* and *Ranunculus bulbosus*, from the same taxonomic family, share similar responses to the treatments. The LFD of both is earlier with higher additions of nitrogen. The earlier LFD caused by high additions of N is not as severe in higher pH soils, as shown by the significant interaction between N amount and pH.

Table 3.3. Output of LMER models studying the impacts of the treatments on the last-flowering date (LFD) of the study-species.

All presented values are statistically significant (t > 2) effect sizes given by the final simplified models ("+" denotes a delayed GDD5, "-" denotes an earlier GDD5). The years column indicates the number of years for which it was possible to analyse the flowering duration (The full flowering season of some species was sometimes interrupted by the PGE cut).

Species	**Years**	**NH_4**	**NO_3**	**N amount**	**pH**	**NH_4: pH**	**NO_3:pH**	**N amount:pH**
To	8			+0.3	+14.5			
Rf	8			-2.9				+0.5
Fm	8				-17.6			
As	3				+19.8			
Rb	4			-2.1				+0.3
Ar	5				+12.4			
Cf	2		+66.4	-1.2				
Vc	5				+33.2			
Cm	3			-1.2	-16.0	+16.2		
Sm	2			-10.4	+43.2			+1.4

The amount of N applied, regardless of form, was an important factor affecting the LFD for many species. Higher applications of N prolonged the LFD for *Taraxacum officinale agg.* but curtailed the LFD for five species. We found significant interactions between nitrogen amount and pH for *R. ficaria*, *R. bulbosus*, and *Sanguisorba minor*. With high N addition, the LFD of these three species is later in high-pH soils. Regarding the form of N, NH_4 was not an independent significant factor for the LFD response of any species, but interactively with higher pH delays the LFD of *Conopodium majus*. NO_3 only caused a significant effect to the LFD of *Cerastium fontanum*, delaying the cessation of flowering. Soil pH acts as a significant independent factor for several species, but with a varied effect between species. In higher pH soils, the LFD is earlier for two species, delayed for five species, and unaffected for three species.

3.4.3 Flowering duration

There was often overlap in the flowering seasons of many of the studied species (Fig. 3.3). However, there was a slight divide between the flowering season of the three earliest species, *T. officinale*, *R. ficaria*, and *Fritillaria Meleagris*, and the season of other species. The three earliest species began flowering before any grasses in the PGE, whereas other study species flowered amongst grass species.

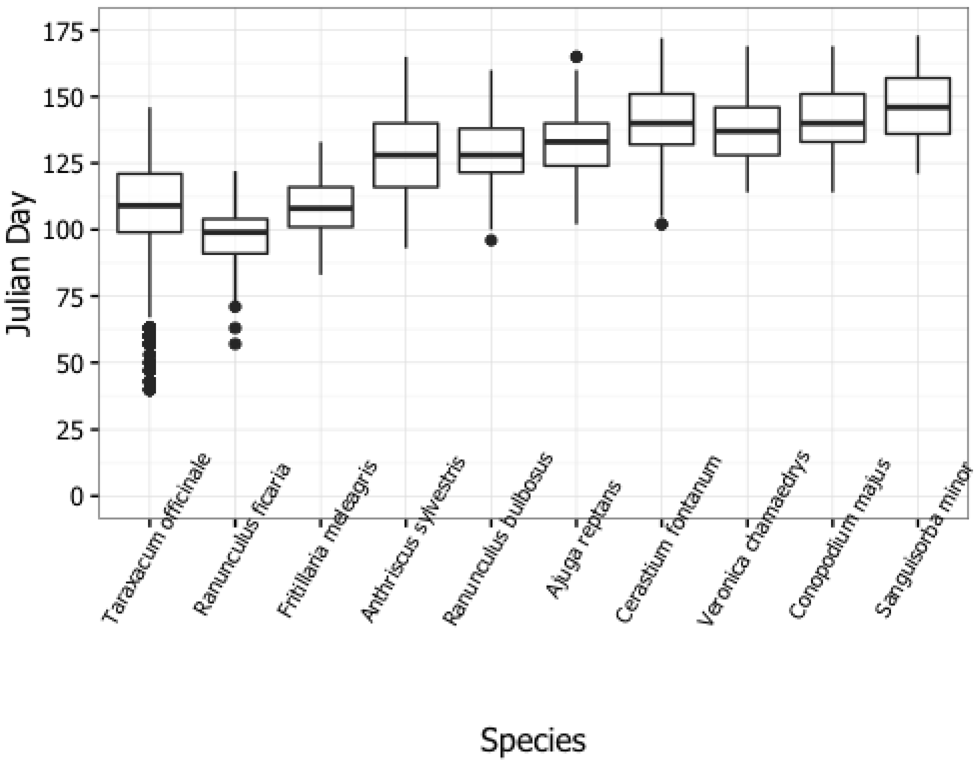


Figure 3.3. The recorded flowering times for the 10 study species for flowering duration.
All data points, each observation from every survey bout, is shown here. Therefore, the figure shows each day in which the 10 species were in flower across the PGE between 2011-2019 (1st cut only; January – June).

The amount of N applied is a significant factor for the overall community-scale flowering duration of eight of the 10 study-species (Table 3.4). Higher amounts of N addition increased the flowering duration of the two of the three earliest species: *R. ficaria*, and *F. meleagris*. Two of the early species also had extended flowering duration caused by the form of N; NO_3 application increased duration of *F. meleagris* flowering, the interaction of high pH and NH_4 application increased duration of *T. officinale* flowering. The flowering duration of *Anthriscus sylvestris* and *Ajuga reptans* was not affected by the amount of N applied, but all other species had shortened flowering duration with higher amounts of N application. The durations of *A. reptans* and *C. majus* were also reduced further when N was in the NH_4 form. The contrast between the flowering duration of early and later species in response to N is also clearly visible when mean values across all experimental plots were taken (Fig. 3.4).

Table 3.4. Output of LMER models studying the impact of the treatments on flowering duration of the study-species.

All presented values are statistically significant (t > 2) effect sizes given by the final simplified models ("+" denotes a delayed GDD5, "-" denotes an earlier GDD5. The years column indicates the number of years for which it was possible to analyse the flowering duration (The full flowering season of some species was sometimes interrupted by the PGE cut).

Species	Years	NH_4	NO_3	N amount	pH	NH_4: pH	N amount: pH
To	8					+12.6	
Rf	8			+0.5			
Fm	8		+50.7	+0.8	-34.1		
As	3				+26.206		
Rb	4			-2.378			+0.29
Ar	5	-72.2					
Cf	2			-0.9			
Vc	5			-1.98	+46.64		
Cm	3	-180.1		-1.5	-38.2	+35	
Sm	2			-3	+77.7		

Independently, higher soil pH increased the overall flowering duration of three species, decreased the duration of two species, and did not affect the flowering duration of the other five species. There were interactive terms between nitrogen amount and pH, for *R. bulbosus*, and between NH_4 and pH, for *T. officinale* and *C. majus*. There were no significant interactive effects between NO_3 and pH. For all three species, the flowering duration is longer when nitrogen is added to high-pH soils rather than low-pH soils.

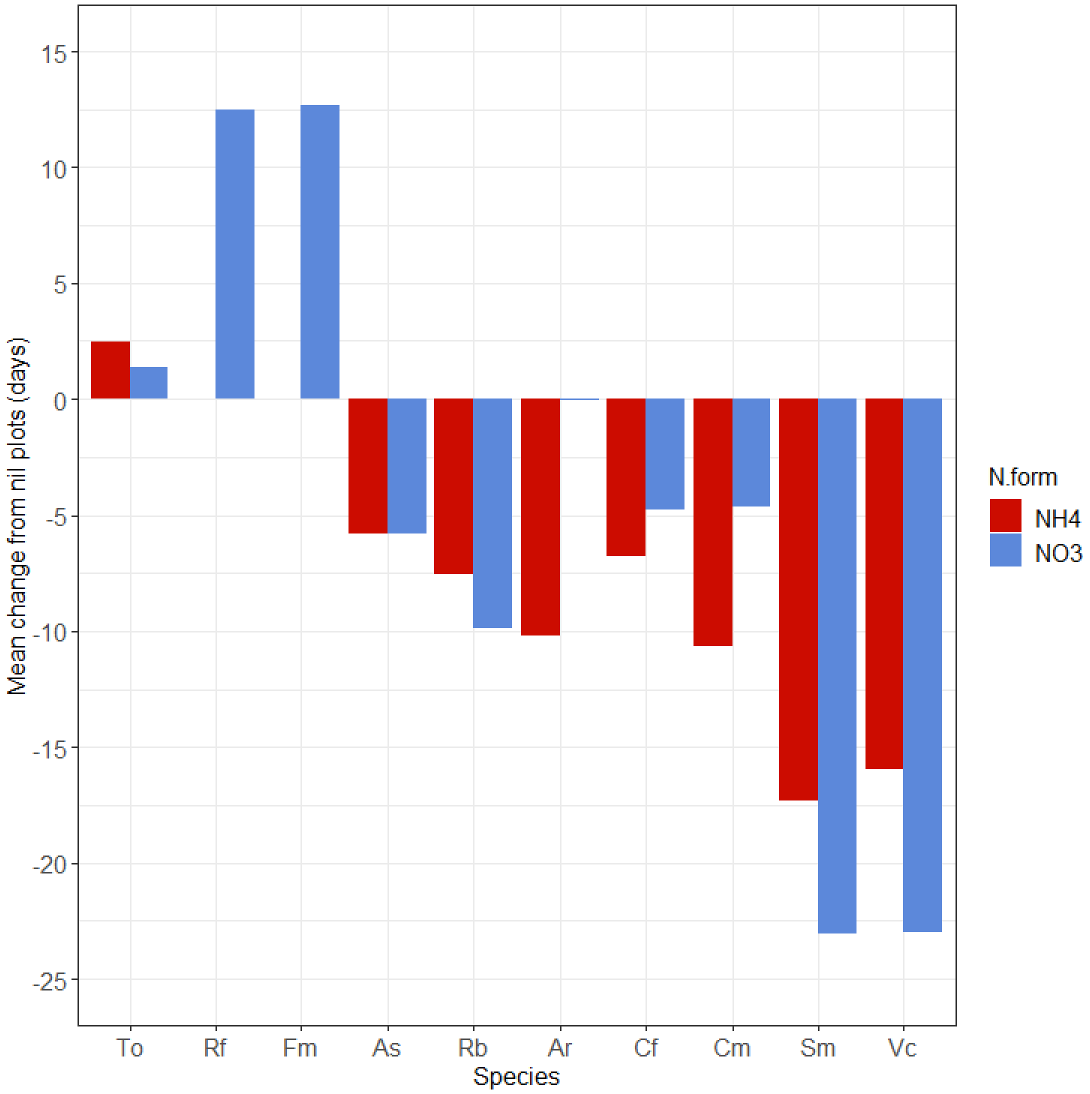


Figure 3.4. Summary figure showing the mean impact of the N treatments on the flowering duration of the 10 study-species.

3.4.4 Community phenology

In total, 1117 bee visitations to flowers were observed, 613 by bumblebees, 335 by solitary bees, and 169 by honeybees (Table 3.5). The median earliest observations of solitary bees and bumblebees visiting a flower was 156.5 GDD5, for honeybees this was 168.8 GDD5. The median observation, across all observations of all years, in GDD5 was 491.2 for all bees, 565.3 for bumblebees, 294.9 for solitary bees, and 301.8 for honeybees. For the comparisons of community phenology and species richness, I used the data for all bees, solitary bees, and bumblebees. Honeybees were excluded as their median date closely resembled that of solitary bees, and relatively few honeybees were encountered in the transects.

Table 3.5. Phenological markers used for the emergence and peak activity of bees, obtained from pollinator transect surveys. '1st Emergence' and 'median' values are given in GDD5.

Pollinators	**No. observations**	**1st emergence**	**Median**
All bees	1117	156.5	491.2
Bumblebee sp.	613	156.5	565.3
Solitary sp.	335	156.5	294.9
Honeybee	169	168.8	301.8

Nitrogen additions of both forms decreased species-richness of flowering plants in the mid-season (Fig. 3.5). NH_4 additions decreased in-flower species richness more than NO_3 additions. Plots receiving NH_4 additions had the lowest species richness of flowering plants throughout the entire season. Plots with NO_3 addition had an intermediary species-richness during the mid-season.

The overall shape of the pattern in species richness throughout the season was similar between control and NH_4 plots, despite the significantly more diverse assemblage of flowering plants in nil plots (Fig. 3.5). The application of NO_3 led to a shifted pattern in community-scale flowering phenology, with a much sharper initial increase in the richness of flowering plants in the early season. NO_3-enriched plots were, on average, earlier to have a species in flower than control plots or NH_4-enriched plots. This early flourish led to a small early peak at the time of bee emergence during which NO_3-enriched plots were the most florally species-rich. This was followed by a far more gradual rise in species-richness, resulting in control plots surpassing NO_3 plots in floral species-richness at approximately 275 GDD5, shortly before the median time of peak solitary bee and honeybee activity. The slowing of the NO_3 increase occurs from 200 GDD5, approximately the same time as the first peak of flowering grasses (Fig. 3.6). For the remainder of the season, throughout the times of peak bee activity, control plots had the highest species richness. At the very end of the season, there was no significant difference between plots receiving either form of N, both of which were lower than control plots (Fig. 3.5).

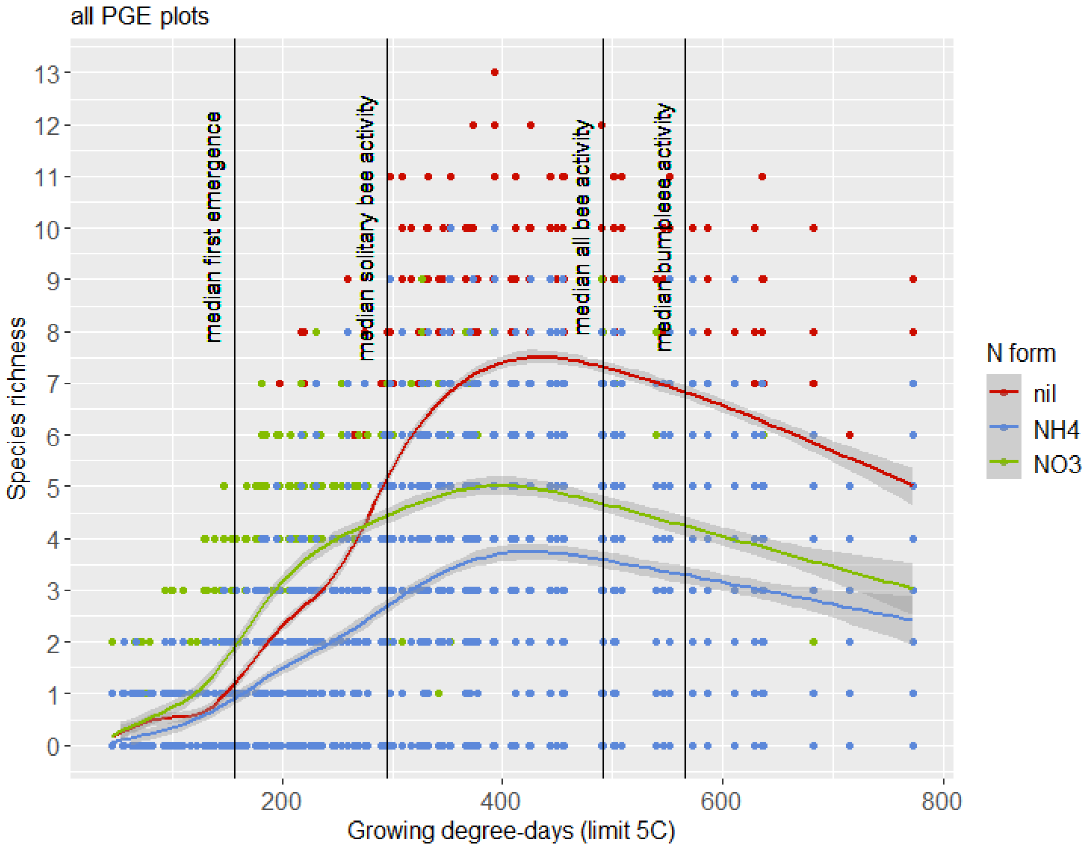


Figure 3.5. Species-richness of in-flower plants throughout the season, for all the PGE plots. 95% confidence interval is shown by the shaded area of each treatment line. The phenological markers of bee activity are shown by the vertical lines; median dates of first emergence, solitary bee activity, all bee activity, and bumblebee activity. Red data points show nil N plots, blue data points NO3-enriched plots, and green data points NH4-enriched plots.

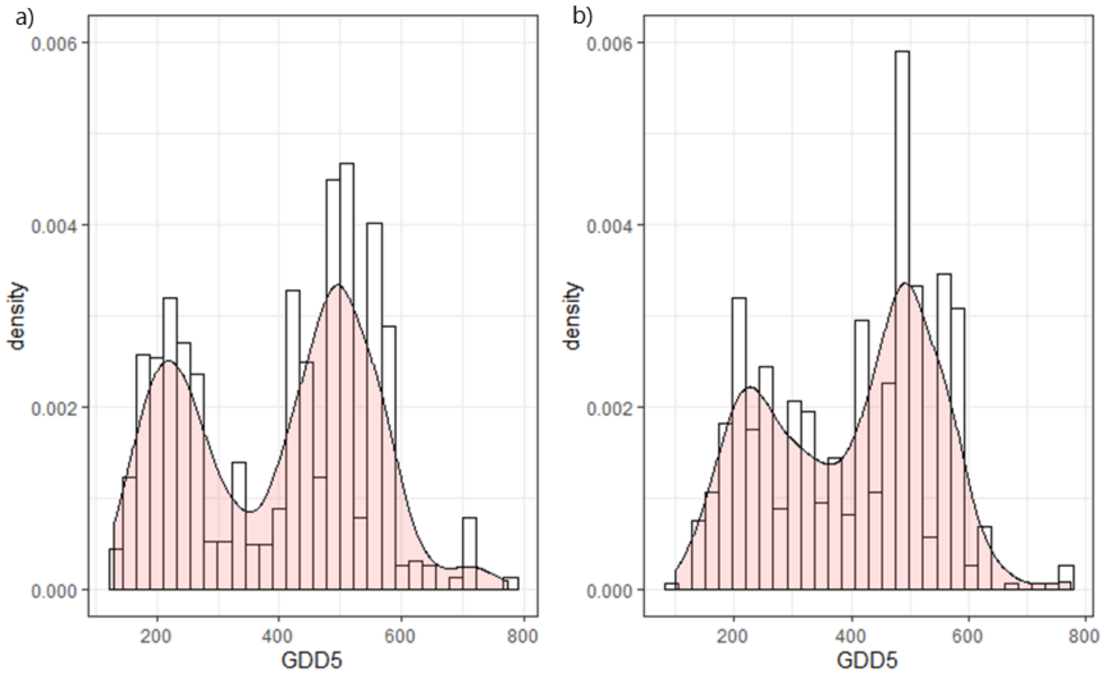


Figure 3.6. Density graphs showing flowering activity throughout the season for all grass species in a) NH_4-enriched plots and b) NO_3-enriched plots.

The magnitude of decline in flowering species-richness caused by N applications was affected by the amount of N added (Fig. 3.7). At lower additions (48 kg N ha^{-1} yr^{-1}), the decline was not so severe, particularly for plots receiving NH_4 additions. At the time of median solitary bee activity, there was no difference between NO_3-enriched plots and control plots. During the seasons of median bumblebee and overall bee activity, there was no significant difference in the species-richness between plots receiving the two forms of N, although both treatment types were more species-poor than control plots. At high levels of N addition (96 kg N ha^{-1} yr^{-1}), the plots receiving N treatments were far more species poor, with reduced species-richness throughout the median seasons of bee activity.

With liming treatment, which buffers against soil acidification, seasonal species-richness trends reflect the overall trends shown across all plots. In plots without liming treatment, in which soil acidification can occur, there are strong detrimental effects of NH_4 addition (Fig. 3.8). On average, there are no flowering plants in plant communities receiving NH_4 additions in unbuffered soils throughout the entire growing season. On the other hand, unlimed plots with NO_3 addition have the characteristic high early-season peak of NO_3-enriched plots.

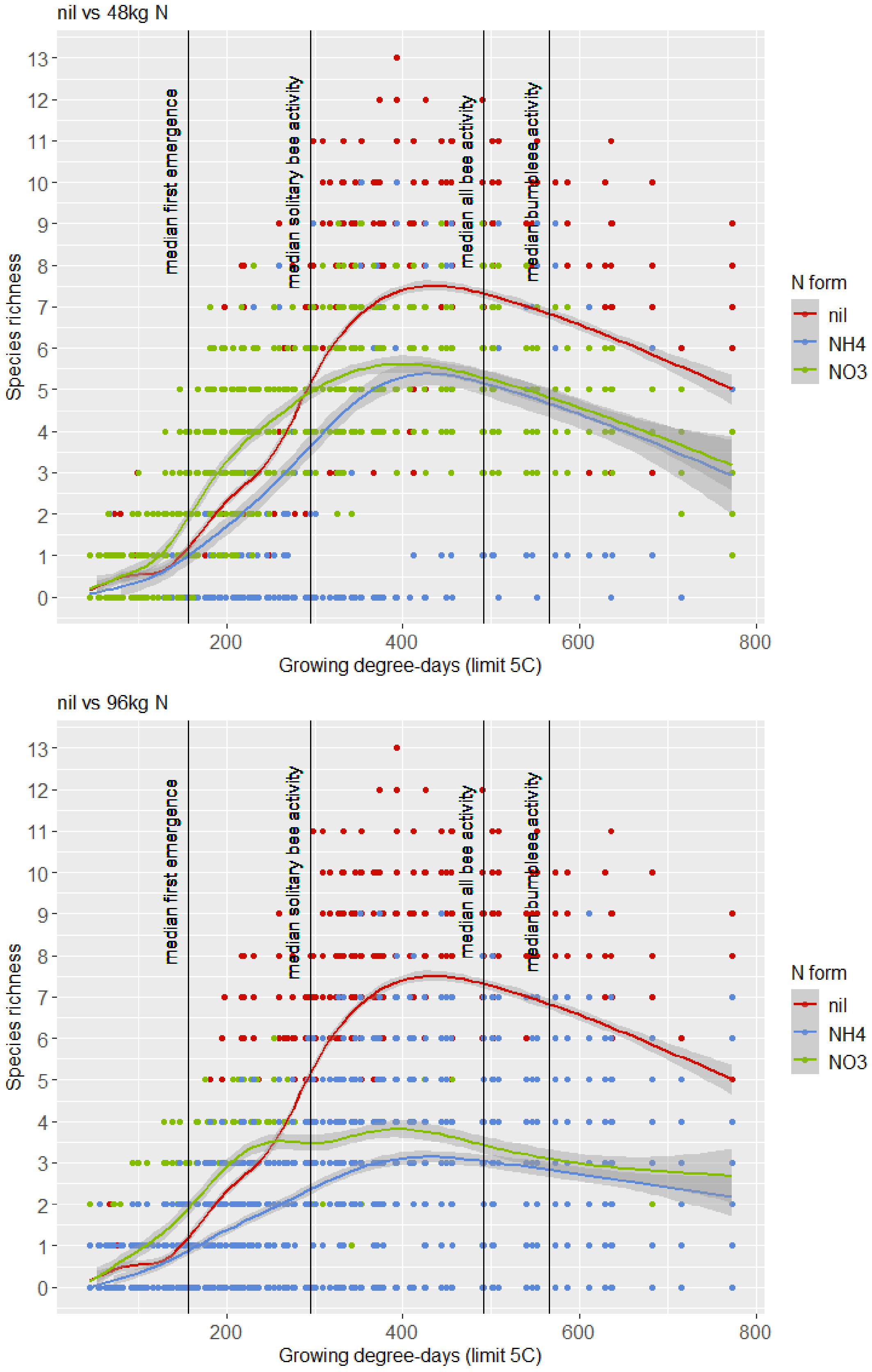
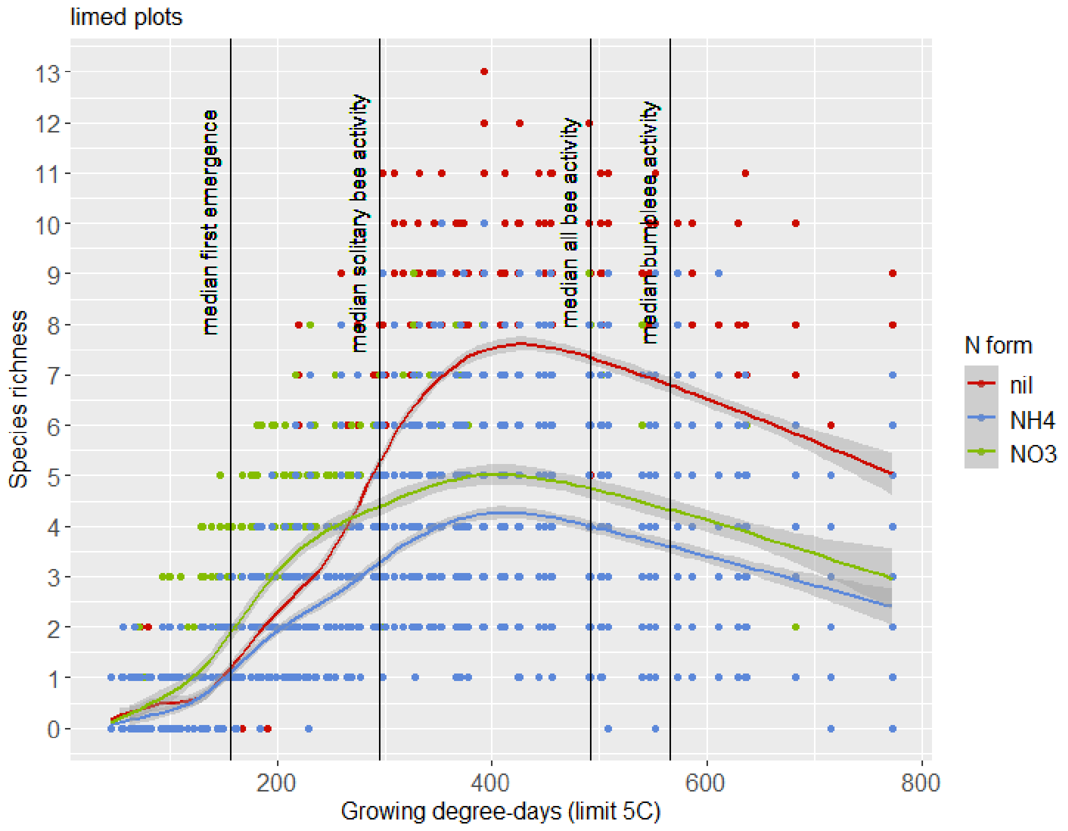


Figure 3.7. Species-richness of in-flower plants throughout the season, for a) plots receiving 48 kg N ha^{-1} yr^{-1}, b) plots receiving 96 kg N ha^{-1} yr^{-1}.

95% confidence interval is shown by the shaded area of each treatment line. The phenological markers of bee activity are shown by the vertical lines; median dates of first emergence, solitary bee activity, all bee activity, and bumblebee activity.



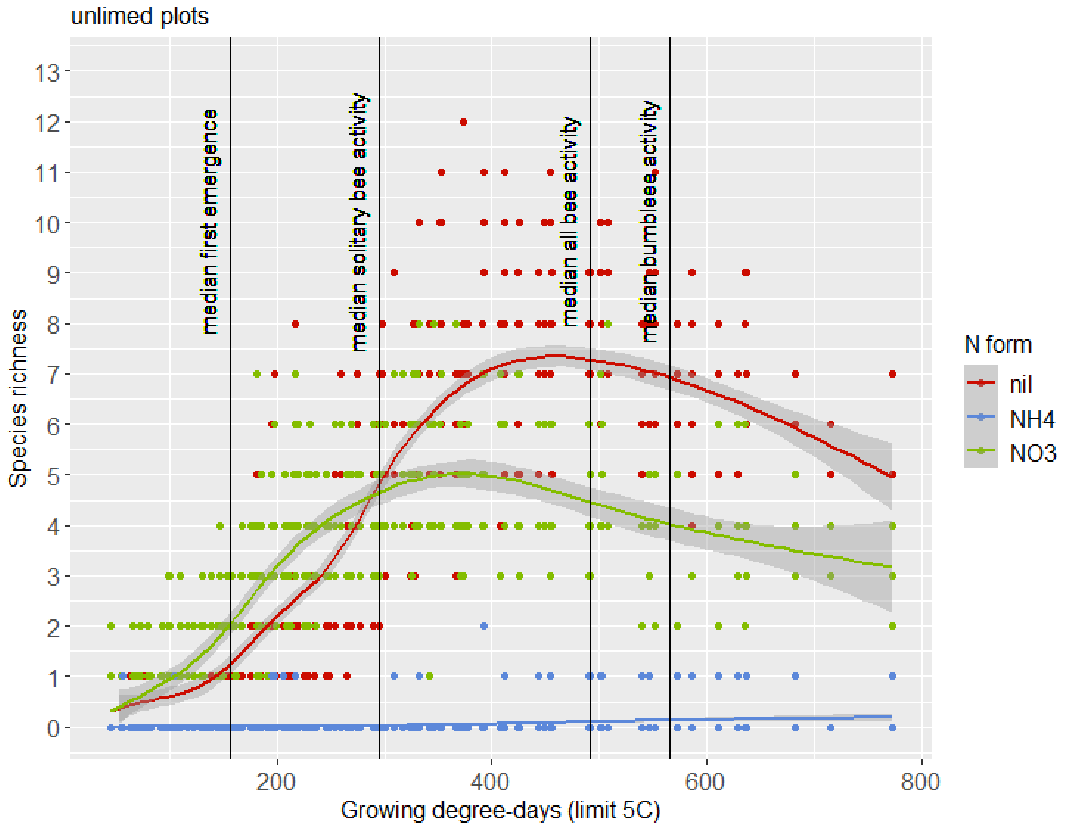


Figure 3.8. Species-richness of in-flower plants throughout the season, for a) limed plots, b) unlimed plots.

95% confidence interval is shown by the shaded area of each treatment line. The phenological markers of bee activity are shown by the vertical lines; median dates of first emergence, solitary bee activity, all bee activity, and bumblebee activity.

3.5 Discussion

3.5.1 General impact of N additions and N form

The results of the mixed-effects models show us that N additions to soil clearly influence the flowering phenology of plants in-situ. For almost all study species, N-driven changes to the dates of first-flowering, last flowering, and overall flowering duration were observed. The study also found that NO_3 addition shifted the typical seasonal species-richness curve. Some research has shown evidence of linear responses of species-richness to N additions, with more severe losses occurring with higher N inputs (Stevens, Duprè, *et al.*, 2010; Humbert *et al.*, 2016). The presented results agree with these studies; while N additions deplete flowering species richness in the peak season, the greatest loss of species was found with higher inputs of N (Fig. 3.7). A species-poor plant community that is dominated by grasses will likely reduce the range of nutritional values available to pollinating insects, with detrimental consequences for pollinator communities (Potts *et al.*, 2003, 2010; Fründ, Linsenmair and Blüthgen, 2010; Weiner *et al.*, 2011) and the development of bee larvae and colonies (Génissel *et al.*, 2002).

The findings of this study also show that the level of N additions can be important for flowering phenology, with more exacerbated changes to phenology occurring with higher inputs of N. Therefore, high levels of addition and deposition to soils could be impactful to ecosystems in multiple ways. The response of pollinators to soil N will be determined by N-driven impacts to floral traits, such as phenology, not only by changes in species composition. Even if flowering forbs and legumes rich in nectar and pollen maintain populations under N-enrichment, shifts in flowering phenology will affect the provision of floral resources. The flowering cessation and flowering duration were more frequently explained by the amount of N applied, rather than the form of N. Therefore, changes in soil fertility could determine the flowering duration of many botanical species within plant communities rather than the different soil processes and mechanisms of nutrient uptake associated with alternate N forms. The prevalence of N amount as a significant factor suggests that impacts to flowering phenology could occur across the globe regardless of spatial variation in NH_x:NO_y ratios.

Although the impacts to flowering cessation and duration were more commonly driven by the amount of N applied, the form of N was revealed as an important determinant of the onset of flowering for many species. Previous studies into the impacts of N on flowering phenology have focused on individual forms of N or on an NH_x:NO_y mix. None have researched both NH_x and NO_y and considered them as separate entities with potentially different effects or response mechanisms. Nonetheless, the collection of previous research has suggested contrasting effects between the two forms of N, with NO_y more commonly advanced forb flowering (Cleland *et al.*, 2006; Smith *et al.*, 2012; Xia and Wan, 2013; Liu *et al.*, 2017). In the PGE, when NH_4 was applied to experimental plots, all 18 study-species had a mean date of first flowering that was either the same or later than control plots; no species had an advanced FFD mean (Fig. 3.2), although the mixed-effects models only show statistically significant effects for five of the species. This finding is consistent with other studies that also found delayed effects to flowering caused by NH_x additions (Smith *et al.*, 2012; Xia and Wan, 2013; Liu *et al.*, 2017). On the other hand, FFD responses to NO_3 additions were far more varied (Fig. 3.2), mixed-effects models showing statistically significant responses that were both delayed and advanced across the various study-species. The clear contrast in responses between the two forms suggests mechanistic differences in how the separate forms of N affect plants, whether through changes in competitive dynamics, soil processes, or uptake by plants.

Evidence of the impacts of soil N on flowering phenology could be of use alongside research into the effects of climatic warming on phenology. Insect emergence and flowering phenology can both be advanced by climatic warming (Fitter and Fitter, 2002; Hovenden *et al.*, 2008; Khanduri, Sharma and Singh, 2008; Hegland *et al.*, 2009; Ibáñez *et al.*, 2010; Thackeray *et al.*, 2010). In some cases, insect emergence is more sensitive to warming and is accelerated further than flowering phenology (Gordo and Sanz, 2006; Parmesan, 2007; Forrest, 2016), although this is not necessarily an ubiquitous phenomenon. As N-enrichment likely will only impact directly on flowering phenology, not on insect emergence, there is the potential for phenological mismatches where NH_4 causes FFD delays whilst pollinators emerge earlier. However, global drivers of environmental change, such as temperature, atmospheric carbon, and nitrogen deposition, acts together and can have interactive effects on flowering phenology (Cleland *et al.*, 2006; Hoover *et al.*, 2012). A greater research effort to synergise this research would be helpful in understanding the impacts of these drivers across the globe.

3.5.2 Inter-specific variation; taxonomic families

There were no clear links between the species-specific responses and N-driven impacts on the common triggers of flowering (autonomous, environmental, or stress-induced). I hypothesised that nitrophilic plants may flower considerably later, due to the increased potential for vegetative growth, also that the smallest plants would flower considerably later due to reducing photoperiod from shading. However, there was no discernible pattern in flowering onset amongst the inter-species variation. Of the three most nitrophilic plant species, *A. sylvestris*, *R. ficaria*, and *T. officinale*, (respective Ellenberg N scores of 7, 6, 6 (Hill *et al.* 1999)), *A. sylvestris* was one of the more delayed plants by N. However, flowering of *R. ficaria* and *T. officinale* was not strongly delayed by N additions. The smaller peak-season species prone to being overshadowed were *C. fontanum*, *C. majus*, and *V. chamaedrys*. With NH_4 addition, *V. chamaedrys* flowering was especially delayed, however there were negligible delays with NO_3 additions, or for the flowering of *C. majus* and *C. fontanum* with either N additions.

Inter-specific variation in responses to N additions can complicate predictability; addressing this variation by determining factors that group responses can improve our ability to forecast consequences which will improve conservation or mitigation attempts. Burkle and Irwin (2009) posited that life-history traits, namely annuality and perenniality, were important in explaining inter-specific variation of responses to N additions. All species used in the study of the PGE were perennial, so it was not possible to agree or disagree with this suggestion. However, it was possible to gain some insight into the variation of responses within taxonomic families. The study species cover a range of taxonomic families, although the numbers of species within taxonomic families is low. All 3 *Fabaceae* species, *L. corniculatus, L. pratensis* and *T. pratense*, had delayed FFD in response to N additions. Another study, on the legume *Cicer arietinum,* also showed delayed onset of flowering in response to N additions (Namvar and Sharifi, 2011). *Fabaceae* possess rhizobacteria in their roots that fix N from the air; therefore, they do not exploit increased soil fertility in the same way as other plants, which could lead to differences in the responses of floral traits of legumes and forbs. For *Fabaceae* that maintain populations in N-enriched soils, the shift in competition dynamics could limiting flowering opportunities, thus delaying the onset of flowering or leading to shortened duration. It was not possible to study the duration of flowering for the *Fabaceae* species in the PGE, as the flowering seasons of these plants are interrupted by the mid-Summer cut. *Fabaceae* produce protein-rich floral rewards (Hanley *et al.*, 2008) that are favoured by many bees (Carvell, 2002), and so the delayed flowering could have consequences for bumblebee species. However, the relevance of taxonomic family in grouping phenological responses to N additions was inconsistent and therefore is not reliable. The two early *Ranunculaceae, R. acris* and *R. ficaria*, had a slightly advanced date of flowering onset with higher levels of N addition but *R. bulbosus* did not fit this pattern. However, there were interesting similarities in the flowering cessation of *Ranunculaceae*, with earlier flowering cessation in both *R. bulbosus* and *R. ficaria* in response to N additions. Furthermore, the magnitude of the response of both species was exacerbated in acidic soils. Although similarities were recorded in the phenological responses of *Ranunculaceae,* further similarities throughout the family are not supported by the literature; a study of the flowering phenology of three montane *Ranunculaceae* species found contrasting responses to N additions (Liu *et al.*, 2017). Furthermore, the lack of similar responses within *Asteraceae* and *Apiaceae* suggests that taxonomic family is an unreliable indicative factor and should not be used widely for predicting inter-specific variation of flowering phenology responses.

3.5.3 Seasonality as an explanatory factor of inter-species variation patterns

Throughout the results there is a clear contrast in how early-flowering and late-flowering species responded to N additions. Four species in the PGE commonly flower before any grass species flowers, *T. officinale, R. ficaria, R. acris,* and *F. meleagris*. After this, the earliest grasses begin thriving and flowering, e.g. *Anthoxanthum odoratum, Alopecurus pratensis, Carex flacca,* and *Luzula pratensis*. During and following this, the remaining forbs and legumes flower. Results from the mixed-effects models consistently showed a contrast between species flowering before and after the first group of grasses. The primary impact of N on species richness is through changes in competitive dynamics, which causes grasses to thrive at the expense of forbs and legumes (Bobbink, Hornung and Roelofs, 1998; Crawley *et al.*, 2005). The alteration of competitive relations could be the mechanism that explains the contrast between these two response groups. Before the grasses thrive, the earliest flowering plant species can take advantage of the extra soil nutrition and exploit the available space and light. Vigorous grass growth during the Spring then leads to a loss in flowering opportunities for forbs and legumes, causing curtailed flowering for many species (Table 3.3; Table 3.4). Alternatively, the accelerated flowering of early plants could be explained by increased stress caused by soil nitrogen applications. Stress, whether through temperature, water availability, salinity or pathogens can lead to earlier flowering of plants as they look to ensure the successful continuation of future generations (Kazan and Lyons, 2016). However, *R. ficaria* and *T. officinale* are relatively tolerant of N, although *F. meleagris* is less so (Hill, 1999). Furthermore, this mechanism does not explain the clear contrast between early species and mid-season species.

When considering the N applications as separate forms, NH_4 and NO_3, we see that the positive effects of N application on the flowering duration of early plant species are more commonly associated with NO_3 application, with less benefit from NH_4 application. For example, the flowering durations of *R. ficaria* and *F. meleagris* were prolonged by NO_3 additions, whereas they were not able to tolerate or grow in NH_4 plots. These plant species are more typical of neutral or calcareous grasslands than acidic grasslands (Rodwell 1992). Therefore they are likely not so well adapted to the cell acidification that can occur with NH_4 uptake (Britto and Kronzucker, 2002; Lucassen *et al.*, 2003; Stevens *et al.*, 2011).

The boost to early flowering provided by NO_3 is well-illustrated by the seasonal patterns in community flowering species-richness (Fig. 3.5). The NO_3-enriched plant communities were first to flower and had higher in-flower species-richness during the early season, when the first bees were observed foraging. Plant communities experiencing NO_3 deposition with relatively little NH_4 will likely have a more diverse array of floral resources for newly emerging bee species, such as *Bombus terrestris*, *Bombus pratorum*, and many *Andrena sp.*. The increased variety of resources can be important for queens emerging from hibernation, in need of nectar to reenergise and pollen to establish new colonies and rear initial workers and offspring. For example, plant communities featuring only *T. officinale* in the early season may pose problems for developing bee colonies (Génissel *et al.*, 2002); this plant species, although producing abundant sugar-rich nectar (Hicks *et al.*, 2016), lacks several important amino acids (Roulston and Cane, 2000), causing problems in larval development when foraged on its own (Génissel *et al.*, 2002). However, most regions across the world experience deposition ratios that are NH_4-dominant or relatively even (Dentener *et al.*, 2006). Therefore, in real-world plant communities, these positive effects to early plants might be distorted by the detrimental impacts caused by NH_4.

In the mid-Spring season, the effect of limited flowering opportunities is revealed. The flowering species richness of NO_3 plant communities slowing, whilst that of control plots continues rising and eventually surpasses NO_3-enriched plots between 250–300 GDD5. Depending on the year, this is generally between mid-March – mid-April. Therefore, from approximately mid-April through the remainder of Spring and Summer, control plots were clearly the most species rich. It is at this time that colonies of bumblebees are at their largest and require a large amount of nectar and pollen to feed adult bees and newly produced workers, gynes, and males. A more diverse selection of floral resources benefits the diversity and density of bee communities (Potts *et al.*, 2003; Fründ, Linsenmair and Blüthgen, 2010; Weiner *et al.*, 2011; Vaudo *et al.*, 2015) and can aid bees against other detrimental factors such as parasitism (Di Pasquale *et al.*, 2013). The stagnation of species richness in NO_3-enriched plots could cause colonies, which may be well established due to rich early resources, to become stressed as floral resources dwindle during the peak season. The time frame for the restriction of flowering species richness in NO_3 plots overlays the typical period of early grass flowering (Fig. 3.6), which further emphasises the possibility that grasses are limiting flowering opportunities for N-tolerant forbs.

3.5.4 Soil acidification

The structure of the PGE allows us to separate the effects of N addition and pH and shows that topsoil pH can be an important interactive factor that influences flowering phenology and species richness. As an independent variable, the effect of pH on phenology varied, but when incorporated as an interactive term, cautionary impacts of soil acidification on flowering phenology were revealed. Often, a significant effect of N was accompanied by a significant interaction between N and pH that signified the ability of well-buffered soils to mitigate severe impacts of N additions. In soils where the pH was maintained at 6 or 7, there was often less impact caused by N to many species. Plant-pollinator networks in poorly buffered soils undergoing high N deposition could therefore be more prone to phenological asynchrony. With regards to the species richness of plants in flower, the impacts of NH_4 in poorly buffered soils is severe, with a near total loss of flowering plants throughout the season.

3.5.5 Limitations of the study and caveats to consider.

The treatment structure of the PGE has useful attributes, such as the separation of N and pH as drivers. However, the separation of NO_3 and NH_4 leads to difficulty in the inference of realistic responses. Atmospheric N deposition occurs as a mixture of oxidised and reduced N, albeit on a gradient of NH_x dominance to NO_y dominance. It would be useful to study the responses of flowering phenology to a mix of N forms. The study was conducted using the PGE, a single field site that simulates various nutrient enrichment scenarios and the subsequent plant communities. N deposition is a driver of global change, affecting ecosystems across the world with varying effects and magnitudes (Bobbink *et al.* 2010). The findings of this chapter are informative, but only pertain to temperate grasslands. The impact of soil eutrophication and acidification on flowering phenology could vary across communities according to locally mediated biotic and abiotic factors. Further research to ascertain, first if the results of the PGE study are applicable to other temperate grasslands, and second to identify responses in other ecoregions and ecosystem types would be useful. This may help us to gauge the magnitude of soil eutrophication and acidification as driving factors of pollinator populations and pollination services.

3.6 Conclusion

Applications of N to the mesotrophic soils of the PGE affected the flowering phenology of a wide suite of plants. The prevalence of impacts of N additions, on FFD, LFD, and duration, of all study species, suggests that chronic N additions and deposition could have wide-reaching effects on plant-pollinator interactions through floral traits. Real-world predictability of the impacts of N-enrichment can be complicated by the varied responses amongst plant species, but in this study a clear contrast between early flowering plants and those in the peak season was shown. A possible mechanism for this was suggested to be the increased grass biomass associated with N-enriched soils, with the enhanced competitive relations limiting flowering opportunities. While there were potentially positive impacts to the flowering duration of early flowering-plants, plants in the important peak season of bee activity had reduced flowering durations, thereby limiting the total provision of floral resources during the season of highest insect-pollinator abundance.

A contrast was also found between the two forms of N applied in the PGE, reflecting the alternate preferences and tolerances of plants to uptake certain forms of N. This will cause plant communities to respond differently depending on the ratio of NH_x:NO_Y they are receiving. Understanding the difference in responses to the different forms of N will help to improve the accuracy with which we can forecast impacts of N. This can then improve our ability to plan mitigation or conservation approaches to tackle negative consequences of N deposition.

The flowering durations of most study species responded to the amount of N applied, regardless of the form. This suggests that with higher levels of N deposition or application, limitations to flowering duration could occur which could impact on the provision of floral resources of nectar and pollen to wild pollinators. The consequences of the reported impacts to flowering phenology to pollinators will likely be species- and interaction-dependent. For example, strongly specialist pollinators with a narrow diet breadth could be more severely impacted than generalists if their primary floral resources have a reduced flowering duration. The impact to pollinators could be buffered by high biodiversity of flowering plants (Bartomeus *et al.*, 2013), although N applications were also shown to decrease the species richness of in-flower forbs and legumes during the peak season.

CHAPTER 4

THE IMPACT OF NITROGEN ADDITIONS ON THE NECTAR TRAITS OF ENTOMOPHILOUS GRASSLAND PLANTS

4.1 Abstract

Many insects visit flowering plants primarily for the floral nectars produced by nectary glands. Visiting insects gain energy through feeding on the carbohydrate-rich viscous liquid. Nectar traits, such as secretion, sugar composition, and sugar concentration, vary across plant species and can influence insect visitation. Nectar traits are also relatively plastic and can vary within plant species in response to abiotic drivers. The impact of soil nitrogen (N), a pervasive driver of global change through N deposition, on variation of nectar traits remains poorly understood. This is one of many knowledge gaps in the question of how N-enrichment of soils affects plant-pollinator interactions. In this chapter, I study the impact of N applications, topsoil pH, and other mineral applications on six entomophilous plant species that grow in mesotrophic grasslands and supply rich nectar and pollen resources to a range of insects. The study found nectar trait responses to N in most of the study species. Strong responses of *Ajuga reptans* to NH_4 additions were recorded, with reduced nectar volume and lower sugar concentration of nectar in plots where the treatment was applied. The nectar volume of *Centaurea nigra* also responded to both forms of N treatment, NH_4 and NO_3, resulting in lower provision of sugars per flower. The concentration of sugars in *C. nigra* nectar were strongly affected by the application of other minerals. The two *Apiaceae* species, *Anthriscus sylvestris* and *Heracleum sphondylium*, both had lower proportions of sucrose with increasing levels of N addition. On the other hand, *Trifolium pratense* and *Knautia arvensis* did not respond to N treatments. This shows that, although inter-specific variation is apparent, deposition and applications of N to soils can have detrimental impacts on the nectar traits of a wide range of plants, such as reduced provision of sucrose and total sugars. The richness and reward of nectar traits throughout a plant community can have implications for the richness of pollinator assemblages. Therefore, when studying the impacts of N on pollinators, it is important to look beyond species richness, and consider floral traits.

4.2 Introduction

4.2.1 The importance of floral nectars for insect pollinators and flower-visitors

Floral nectars are an important source of nutrition for insect pollinators and other flower visitors. Nectar is the primary reason for insects to visit flowers. To feed their progeny, bees also forage for pollen, but many other adult insects have difficulties consuming pollen (Roulston and Cane, 2000). There are other instances of bees foraging flowers for fatty oils (Steiner and Whitehead, 1991), but nectar is the key reward that is sought after by insects across taxa including hymenopterans, dipterans, coleopterans and lepidopterans. Insects feed on this viscous sugar-rich liquid, produced by the nectary gland, to gain the energy they require to function. The predominant components of nectar are water and sugar; the most common sugars are disaccharide sucrose and the hexose monosaccharides fructose and glucose, which are found in floral nectars from nearly all plants species (Wykes, 1952; Percival, 1961; Southwick, Loper and Sadwick, 1981). Other sugars can be present, such as maltose, melibiose, and raffinose, but in far lower concentrations (Wykes, 1952; Percival, 1961). Floral nectars also contain other components, including amino acids, alkaloids, and lipids, that may have important roles in insect dietary nutrition, bacterial immunity, and potentially for protecting nectar from unwanted visitors (Baker, 1977; Adler, 2000; Carter and Thornburg, 2004; González-Teuber and Heil, 2009; Nepi *et al.*, 2012). Sugars, as the predominant components of nectar, and the primary draw of insect visitors due to their calorific reward, are the focus of this study.

The concentration of total sugars in floral nectars range widely, but median and mean values tend to fall around 40% (Pamminger *et al.*, 2019). As such, insect pollinators forage nectars with a wide range of sugar concentrations (Roubik and Buchmann, 1984; Forcone, Galetto and Bernardello, 1997; Kajobe, 2007), although evidence suggests that certain groups of insects preferentially forage nectar with specific sugar-richness. When a flower-visitor is feeding from nectar, the optimal sugar-concentration is a trade-off between the calorific gain given by the sugars and the energy required to access and drink the viscous liquid (Kim, Gilet and Bush, 2011). Following this logic, deeper corollas, which typically require more energy to forage than open simplistic flowers, should be more rewarding (Harder, 1986). Furthermore, different insect groups have varying energy demands; for example, a large bee will require more energy than a dipteran to initiate and maintain flight muscles. As such, long-tongued bees that forage longer corollas prefer more concentrate nectar than, for example, generalist dipterans that forage open flowers (Krömer *et al.*, 2008; Kim, Gilet and Bush, 2011; Vandelook *et al.*, 2019). The optimum range of sugar concentrations for bees therefore, to give them net energy gain in order to power flight muscles, is 55-60% (Kim, Gilet and Bush, 2011). For hymenopterans foraging for nectar, the aim is to maximise efficiency; overly long corollas can cost energy to forage, whereas corollas that are too short often contain nectar that is not rewarding enough (Klumpers, Stang and Klinkhamer, 2019). Other insect groups typically forage more dilute nectar, 30-40% for lepidopterans, and lower still for dipterans (Kim, Gilet and Bush, 2011; Vandelook *et al.*, 2019). Pyke and Waser (1981) found that bird-pollinated flowers were typically more dilute than bee-pollinated flowers, also relating concentration to pollinator syndrome, potentially because of the larger appetite and feeding apparatus of birds compared to bees.

An important characteristic of floral nectar is the composition of the main sugars. Studies of sucrose:glucose:fructose, or more commonly sucrose:hexose, ratios have shown sugar compositions vary across species. The compositions are relatively stable within species (Wykes, 1953), but can be affected by abiotic conditions such as relative humidity (Corbet, Unwin and Prys-Jones, 1979). Interestingly, the majority of studies suggest that nectar sugar composition, specifically the proportion of sucrose, is strongly linked with proposed pollinator syndromes, floral morphologies, and typical visitors (Percival, 1961; Southwick, Loper and Sadwick, 1981; Krömer *et al.*, 2008; Witt, Jürgens and Gottsberger, 2013; Abrahamczyk *et al.*, 2017; Vandelook *et al.*, 2019), although not all studies have found this relationship (Chalcoff, Aizen and Galetto, 2006). For example, bee-pollinated flowers with longer corollas, such as various *Fabaceae* and *Lamiaceae*, tend to have sucrose-dominant nectars (Wykes, 1953; Southwick, Loper and Sadwick, 1981; Petanidou, 2005). In turn, specific functional groups of pollinators, when foraging, have developed corresponding preferences for certain nectar profiles. Long-tongued bees preferentially forage from plants producing high sucrose nectars (Elisens and Freeman, 1988; Petanidou, 2005; Petanidou *et al.*, 2006). Furthermore, long-tongued bees are able to detect increases in sucrose and adapt their foraging behaviour accordingly (Cnaani, Thomson and Papaj, 2006), suggesting an adaptive preference for such nectars. Short-tongued bees and lepidopterans prefer more balanced nectars and the most generalist insect flower visitors, such as many dipterans and coleopterans, preferentially feed from hexose-dominant nectars (Elisens and Freeman, 1988; Petanidou *et al.*, 2006). Whilst plants with deep corollas produce sucrose-dominant nectar, the open floral structures of *Apiaceae* and *Rosaceae* have hexose-dominant nectars and *Asteraceae* and *Dipsacaceae* more evenly balanced sugar profiles (Southwick, Loper and Sadwick, 1981; Petanidou, 2005). These species are visited by a wider suite of insects, from generalist bees to dipterans. The range of sugar compositions found in floral nectars and corresponding insect preferences suggests that nectar traits have a role in the adaptive evolution of pollinator foraging habits and plant-pollinator interactions (Abrahamczyk *et al.*, 2017; Vandelook *et al.*, 2019). Specialist nectar-feeding bird species can digest and assimilate polysaccharide sucrose sugars whereas opportunistic nectivorous birds are only able to assimilate monosaccharide hexose sugars such as glucose and fructose (Martinez del Rio, 1990; Martinez del Rio, Baker and Baker, 1992; Dupont *et al.* 2004). There is insufficient evidence to ascertain if this contrast is also true between specialist insect pollinators, such as bees, and opportunistic nectar feeders, such as many dipterans. In addition to the nectar niches according to pollinator functional groups are possible gender-specific preferences for certain nectar types. Rusterholtz and Erhardt (2000) found that females of the threatened lepidopteran *Lysandra bellargus* preferred to forage glucose-rich nectars whereas the males preferred high-sucrose nectars.

4.2.2 How might nitrogen impact on floral nectars, and the consequences for pollinators?

Although patterns have been found in nectar traits, the secretion and chemical composition of floral nectars is not necessarily stable within species across plants and flowers, or even within flowers across time (Corbet *et al.*, 1979; Roubik and Buchmann, 1984; Herrera, Pérez and Alonso, 2006; Kajobe, 2007). Abiotic and biotic factors affect nectar traits, and this is therefore a relatively plastic trait that can vary according to environmental change. The chemical composition of nectar can change throughout the day, as temperatures increase and relative humidity falls, typically leading to higher sugar richness (Southwick, Loper and Sadwick, 1981; Roubik and Buchmann, 1984; Kajobe, 2007) and sucrose proportions (Corbet *et al.*, 1979; Corbet, Unwin and Prys-Jones, 1979) being found around midday. There is evidence that aboveground herbivory and belowground microbiota can also affect nectar traits although this is an area that needs further research to reveal the extent (Barber and Soper Gorden, 2014). Abiotic drivers of global change, for example increased atmospheric carbon dioxide (Osborne *et al.*, 1997; Rusterholz and Erhardt, 1998; Lake and Hughes, 1999; Erhardt, Rusterholz and Stöcklin, 2005) and climatic warming (Hoover *et al.*, 2012; Scaven and Rafferty, 2013; Mu *et al.*, 2015; Takkis *et al.*, 2015), can affect nectar production and composition.

Nitrogen (N) deposition, a driver of global change, and direct applications of N to soil also have impacts on nectar traits. This could consequentially have effects on plant-pollinator interactions, pollinator net energy gain, and therefore pollinator populations. However, research into the subject is limited and we lack a thorough understanding. Nectar trait responses can vary according to the rates of addition. Burkle and Irwin (2009, 2010) found increased nectar production in *Ipomopsis aggregata* at low levels of N additions, but not at high levels. Furthermore, species-specific responses are apparent across studies (e.g. Shuel 1956; Petanidou *et al.* 1999; Burkle and Irwin 2009, 2010; Baude *et al.* 2011). The functional lifecycles, N take-up strategies, and taxonomic differences between plants could explain some of this inter-specific variation in nectar trait response. For example, Shuel (1956) found *Antirrhinum majus* had increased nectar production with N and nutrient additions while *Trifolium pratense*, a *Fabaceae*, showed no response. A study using litter amendments also did not record any response from a leguminous plant *Medicago sativa* (Baude *et al.* 2011). Leguminous plants take up N through N-fixing rhizobacterium, so do not exploit increases in soil N like grasses and flowering forbs. This could explain why the responses of legumes can differ from those of forbs. Annual plants and perennial plants are also shown to differ in their nectar trait responses. *Ipomopsis aggregata*, an annual plant, increased nectar production with N additions, whereas the perennial *Linum lewisii* did not (Burkle and Irwin, 2009). In a separate study, the addition of plant litter amendments to soil caused the nectar sugar content of *Lamium amplexicaule*, an annual herb, to increase (Baude *et al*. 2011). The nectar traits of a semi-perennial herb, *Mimulus guttatus*, did not respond to litter amendments. It should be noted that increasing soil N can also lead to decreased nectar production, *Gymnadenia conopsea* growing in higher N soil produced less floral nectar (Gijbels *et al.* 2014). Petanidou *et al.* (1999) found the nectar trait responses to nutrient additions (N, potassium (K), and phosphorous (P)) of an herbaceous species differed from that of woody species. Glucose proportionally increased in the nectar of woody species, *Thymus capitatus* and *Satureja thymbra*, whilst in the herbaceous *Stachys cretica* sucrose increased. Other studies have similarly found changes to the sugar profiles of floral nectar with N or nutrient additions. Glucose proportionally decreased in *Succisa pratensis* nectar with nutrient additions (Ceulemans *et al.* 2017). In *Cucurbita pepo*, glucose decreased relative to fructose with NH_4NO_3 additions, while a proportional decrease in sucrose was also recorded (Hoover *et al.* 2012).

Increased soil N has often been shown to increase amino acid content of floral nectars (Gardener and Gillman, 2001; Gijbels *et al*. 2014, 2015), although not in all studies, Petanidou *et al.* (1999) did not find any change to amino acid content in the nectars of phrygana plants. Changes to amino acid profiles caused by N and nutrient additions (Gardener and Gillman, 2001; Ceulemans *et al.* 2017) could also affect pollinator feeding habits, as certain guilds of pollinators are shown to prefer specific amino acids in high concentrations, such as *Megachilidae* with gamma-aminobutyric acid (GABA).

The most prevalent way that N affects plant communities is through a change in competitive dynamics, which can also affect nectar traits (Baude *et al.*, 2011). The presence of strongly competitive species can negatively impact the nectar traits of adjacent annual herbaceous species, with a study showing decreased nectar production and sugar provision in *L. amplexicaule* when grown with *M.* guttatus (Baude *et al.* 2011). There could be other indirect biotic effects from herbivory.

Herbivory can increase with N addition, and this can have implications for nectar production and quality (Adler *et al.* 2006). Therefore, whether through increased stress, nutrition, or competitive dynamics, it's possible that N can act as a factor on plant-pollinator interactions through nectar traits. The altered floral rewards and interactions could have consequential impacts on the development and stability of colonies of important pollinators (Ceulemans *et al.*, 2017).

Global N deposition has risen sharply during the last 150 or so years, tripling from 34 Tg N yr^{-1} in 1860 to 100 Tg N yr^{-1} in 1995, and with further increases predicted (Galloway *et al.*, 2004). Direct applications of N to soils as agricultural practice further adds to the flux of N to soils and ecosystems. Historically, higher N additions are spatially related to regions of more intensive industry, such as Europe, North America, India, and China, with lower fluxes in the Southern Hemisphere. While global deposition rates are expected to continue rising, deposition in Europe has recently begun to plateau and decline (Erisman, Grennfelt and Sutton, 2003; Fowler *et al.*, 2004). The impact of this is gaining consideration as a driver of global change, and researchers are increasingly concerned about how ecosystems might be affected (Tylianakis *et al.*, 2008; Nijssen, Wallis De Vries and Siepel, 2017; Stevens, David and Storkey, 2018). Our understanding of the effect to botanical species compositions is relatively good, despite knowledge gaps regarding some ecoregions, such as the tropics (Bobbink *et al.*, 2010). However, our knowledge of the impacts to higher trophic levels is patchy (Stevens, David and Storkey, 2018), with a particular lack of knowledge regarding plant-pollinator interactions (David, Storkey and Stevens, 2019). Habitat degradation is considered a major driver of pollinator declines (Vanbergen and Iniative, 2013; Rader *et al.*, 2014). Excessive N-enrichment could well contribute towards this driver by reducing botanical species richness. However, N additions could also lead to changes in the quality of resources. The interactions between plants and pollinators are strongly influenced not only by botanical species composition, but also floral traits, such as nectar quantity, composition, and quality. With insect pollinators declining across taxa and ecoregion to a plethora of factors, the role of N-driven alterations to botanical communities demands further research.

4.2.3 Aims of the study

With this study, I aimed to determine how chronic N additions and soil acidification affect the nectar traits of some key floral resources, with a view of the impact to nectar provision for foraging insect pollinators. Due to the varied responses observed through the literature, it is necessary to study several species. Using the Park Grass Experiment (PGE), at Rothamsted Research, species have been selected that span a range of pollinator syndromes, tolerances, and life history traits as well as occurring on a range of fertiliser treatment plots. The impact of nitrogen applications, soil acidification, and mineral additions on nectar quantity, composition, and quality were studied, with a view to how plant-pollinator interactions might be impacted. The study will address the hypotheses that 1) N additions will lead to changes in floral nectar traits and that 2) species will differ in their responses, with forbs showing more response than the legume species. Finally, the study addresses the hypothesis that 3) soil eutrophication will lead to a degradation in nectar sugar provision by plant communities, due to changes to nectar traits and floral abundance.

4.3 Materials and methods

4.3.1 Park Grass Long-term Experiment

Nectar samples were taken from the PGE (See Chapter 2 for full summary of the PGE). Samples were taken from plots 3b, 3d, 1b, 1c, 17b, 17d, 7/2b, 7/2d, 16b, 16d, 14/2b, 14/2d, 13/2b, 13/2d (Fig. 4.1). These plots incorporate a range of N treatment forms (nil, NH_4, NO_3, organic farmyard manure), N amounts (0, 48 kg ha^{-1} yr^{-1}, 96 kg ha^{-1} yr^{-1}), pH (5–6.3), and mineral applications (with or without P, K, Na, Mg), as shown in Table 4.1. The plots were chosen to give comparisons across forms of N, amounts of N, and soil pH. Incorporating plots receiving minerals into the study gives an extra dimension to study the application of N. As the Park Grass Experiment lacks pure plot replication, it is important to look for hidden replication that can improve the statistical power of analyses. The plots also encompass a range of botanical species richness.

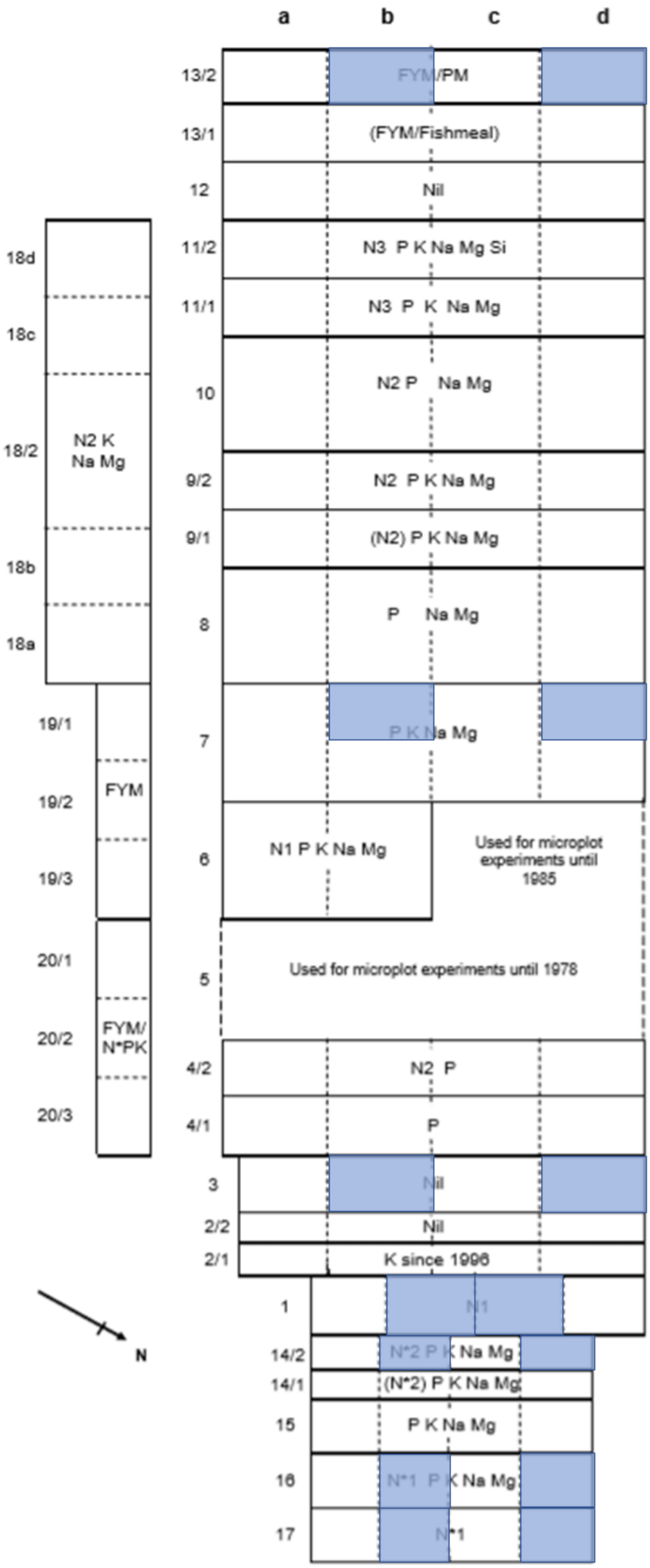


Figure 4.1 The Park Grass Experiment plan, showing all study plots used for nectar sampling. Shaded plots show the PGE plots from which nectar samples were taken.

Table 4.1 The PGE study plots used to sample nectar; the N and mineral treatments, and soil pH.

'Org' denotes organic farmyard manure applications, the "*" symbol in mineral column denotes minerals in the form of farmyard manure applications. Organic manure is a rotation of farmyard manure (supplying c. 240 kg N ha^{-1} yr^{-1}, 45 kg P ha^{-1} yr^{-1}, 350 kg K ha^{-1} yr^{-1}, 25 kg Na ha^{-1} yr^{-1}, 25 kg Mg ha^{-1} yr^{-1}, 40 kg S ha^{-1} yr^{-1}, 135 kg Ca ha^{-1} yr^{-1}) and poultry manure (supplying c. 65 kg N ha^{-1} yr^{-1}), applied every 2 years on rotation. For mineral application, "+" is applied and "-" is not applied. Mineral application is 225 kg K ha^{-1} yr^{-1}, 15 kg Na ha^{-1} yr^{-1}, 10 kg Mg ha^{-1} yr^{-1}, 122 kg S ha^{-1} yr^{-1}, 17 kg P ha^{-1} yr^{-1} (in 2016)/35 kg P ha^{-1} yr^{-1} (in 2017 and 2018, to account for P accumulation).

Plot	N form	N amount	pH	Minerals
3b	Nil	0	6.1	-
3d	Nil	0	5.1	-
1b	NH_4	48	6.0	-
1c	NH_4	48	5.1	-
17b	NO_3	48	6.2	-
17d	NO_3	48	5.8	-
7/2b	Nil	0	6.1	+
7/2d	Nil	0	5.1	+
16b	NO_3	48	6.0	+
16d	NO_3	48	5.6	+
14/2b	NO_3	96	6.3	+
14/2d	NO_3	96	6.1	+
13/2b	Org	72.5	6.0	*
13/2d	Org	72.5	5.2	*

4.3.2 Nectar collection

Nectar was collected from a range of plant species across the Park Grass experiment over three years, 2016, 2017, 2018. Three factors were important in selecting species to sample; 1) that they were important nectar sources for pollinating insects, 2) that they grew in enough experimental plots to make investigating the impacts of the N treatments possible, and 3) that it was possible to obtain raw nectar from the plant, without the need for rinsing or diluting methods. Rinsing the nectary gland with water dilutes the nectar and some of the solution can be lost through evaporation or by falling from the nectary gland, which makes it difficult to accurately determine actual concentrations. Also, when rinsing the nectary gland with water, it can take up to 30 minutes for the amount of sugar taken up into the solution to plateau (Corbet *et al.*, 1979), which is a significant time constraint. Therefore, I focused on plants from which raw floral nectar could be obtained. The plants that matched this criterion were *Ajuga reptans, Anthriscus sylvestris, Centaurea nigra, Heracleum sphondylium, Knautia arvensis*, and *Trifolium pratense* (Table 4.2).

A. reptans, C. nigra, and *T. pratense* were the focus of the study, and were sampled more (Table 4.3), for the following reasons. Firstly, they have large populations in several plots across treatments. Secondly, they are important nectar resources visited by bees (Carvell *et al.*, 2006) for their rich nectar and pollen rewards (Hanley *et al.*, 2008; Hicks *et al.*, 2016). Thirdly, they have contrasting life history traits: *T. pratense* is a *Fabaceae* with N-fixing rhizobacteria, *C. nigra* is a competitive plant species that grows in all of the studied PGE plots including the competitive high N plots, *A. reptans* is smaller plant that has been crowded out and lost from the more competitive plots – those with high N or mineral applications.

Table 4.2. The study species from which nectar samples were obtained.
Code denotes the two-lettered code used to refer to the species for the remainder of the chapter. The PGE plots are the study-plots in which the species grow, with applied treatments shown by the N treatments column (Org is organic farmyard manure).

Species	Code	Family	PG plots	N treatments	Typical pollinators
Ajuga reptans	Ar	Lamiaceae	3b, 3d, 17b, 17d, 1b, 1c, 13b, 13d	Nil, NO_3, NH_4, Org	Bumblebees
Anthriscus sylvestris	As	Apiaceae	7b, 14b, 14d, 16b, 16d, 13b, 13d	Nil, NO_3, Org	Diptera Hymenoptera Coleoptera
Centaurea nigra	Cn	Asteraceae	All plots	Nil, NO_3, NH_4, Org	Bumblebees Solitary bees
Heracleum sphondylium	Hs	Apiaceae	7b, 14b, 14d, 16b, 16d, 13b, 13d	Nil, NO_3, Org	Diptera Hymenoptera Coleoptera
Knautia arvensis	Ka	Caprifoliaceae	3b, 3d, 1b, 1c	Nil, NH_4	Bumblebees Solitary bees
Trifolium pratense	Tp	Asteraceae	3b, 3d, 17b, 17d, 7b, 7d, 16b, 16d, 14b, 14d, 13b, 13d	Nil, NO_3, NH_4, Org	Bumblebees

Table 4.3. The number of nectar samples taken from the study species from each plot.

Sp.	**Park Grass Experimental Plots**													
	3b	3d	1b	1c	17b	17d	7b	7d	16b	16d	14b	14d	13b	13d
Standing crop														
Ar	21	20	6	8	34	22							10	16
As							3		3	3	3	3	3	3
Cn	23	17	6	6	10	13	18	22	4	16			9	22
Hs							3		3	3	3	3		
Ka	9		4	4										
Tp	23	6			7	7	17	6	14	6	8	6	6	13
24-hour nectar secretion														
Ar	12	11	4	4	14	10							6	8
Cn	6	8	6	6		4				10				12

To sample the standing crop of nectar, floral units were bagged with finely meshed bags of organza fabric for 24 hours prior to nectar collection (as per Corbet, 2003). Mesh organza/bridal veil fabric bags have the smallest influence on nectar secretion and chemistry, so are the preferred material for plant bagging (Wyatt *et al.,* 1992). The entire umbel of *Apiaceae*, the entire capitulum of *Asteraceae, Caprifoliaceae*, and *Fabaceae*, and the entire flowering spike of *Lamiaceae* were bagged (Fig. 4.2). This restricts visitation by insects and allows nectar stores to build up so that nectar can be collected. The following day, 24 hours after a floral unit was bagged, microcapillary tubes (Drummond; 1µl) were used to collect the nectar from the nectary glands of the flowers. The volume of the nectar gathered by the microcapillary tubes was measured, using digital callipers, and the microcapillary tubes were then placed in a labelled Eppendorf and frozen at -20 °C prior to laboratory analysis. Several flowers were sampled per plant (*Ajuga reptans* 5–28 flowers; *Anthriscus sylvestris* 10–20; *Centaurea nigra* 4–32; *Heracleum sphondylium* 8–27; *Knautia arvensis* 7–20; *Trifolium pratense* 5–12. Means and standard errors shown in Table 4.4), for two reasons. Firstly, HPLC analysis required 20 µl of diluted sample to run. Secondly, nectar production commonly varies across flowers of an individual plant, and with flower age (Petanidou, Van Laere and Smets, 1996; Herrera, Pérez and Alonso, 2006); sampling several flowers per plant replicate would give us a mean value per flower per plant replicate. As nectar traits can vary according to the time of day and relative humidity (Corbet *et al.*, 1979; Corbet, Unwin and Prys-Jones, 1979; Southwick, Loper and Sadwick, 1981), nectar sampling was conducted with a blocked design structure; morning, midday, and afternoon. Nectar sampling of each plant species from specific plots were distributed evenly across these blocks. To account for the lack of plot replication in the Park Grass Experiment, replicates were taken from the same plot but always from separate groups of plants, on the opposite sides of a plot.

Figure 4.2. Examples of 4 bagged flowers. (clockwise from top left) Centaurea nigra, Heracleum sphondylium, Trifolium pratensis, Knautia arvensis.

To sample the 24-hour nectar production, floral units that had been sampled for the nectar standing crop were bagged again for another 24 hours. Nectar was sampled from the same flowers 24 hours later. Only *A. reptans* and *C. nigra* were effectively sampled for 24-hour nectar secretion on a large scale, as the plants have larger flowers with open corollas which could be sampled on subsequent days without damaging the floral tissue with microcapillary tubes. Again, the samples were measured and stored at -20 °C prior to laboratory analysis.

4.3.3 Laboratory analysis

The nectar samples were analysed for sugar content using high performance liquid chromatography (HPLC). Samples were thawed, re-measured, and expelled into filtered HPLC-grade H_2O, at first to a 1:30 dilution. I then determined the optimum dilution for each sample with pilot HPLC runs. A Dionex ICS-3000 (Thermo Scientific) system was used to analyse the samples. The flow rate was 0.25 ml min^{-1}. Elution buffers were HPLC-grade H_2O (A), 1 M sodium acetate (B), 0.5 M sodium hydroxide (C). The running conditions began with 80% A:20% C at 0 minutes, changing on a gradient towards 100% C at 5 minutes. 100% C was maintained between 5–8 minutes. Between 8–8.5 minutes the gradient changed to 80% B:20% C, which was maintained between 8.5–11 minutes. At 11.5 minutes the gradient changed to 80% A:20% C, which was maintained until the run ended at 17 minutes. Each run injected 20 µl of a sample onto a CarboPack PA1 column (column 2 mm x 250 mm), at a temperature of 25° C. A pulsed amperometric detection (PAD) detector, with gold electrode, was used to detect the analytes. A mix of glucose, fructose, and sucrose standards were used, at 1 µM, 2 µM, 5 µM, 10 µM, 15 µM, 20 µM, and 25 µM concentrations. Standards were run before and after the sample runs, and a blank H_2O sample was run every 10 samples to check for contamination. Calibration curves were calculated for glucose, fructose, and sucrose from the external standards. This data was used to transform the results of the HPLC sample runs into µM and µg µl^{-1} concentrations.

4.3.4 Surveys of floral abundance to determine nectar sugar provision per area

The overall floral abundance of study species in the plant communities was surveyed throughout the seasons by using quadrats. Quadrats, sized 0.25m^2, were thrown 20 times systematically around each study plot. The total number of floral units for each plant species was counted for every quadrat. Quadrat surveys were conducted approximately every two weeks, around the same time as nectar sampling.

4.3.5 Statistical analysis

All statistical analyses were performed using R Studio (R Core Team, 2018). Graphs were produced in R Studio, using the ggplot2 package (Wickham, 2016). The analysis of nectar production and sugar concentration was split into two broad groups, standing crop and 24-hour production, to differentiate between the latent nectar that has been resting in the corolla and freshly produced nectar. The impacts of the treatments were analysed with linear mixed-effects models, using the lme4 package for R Studio (Bates *et al.,* 2015). N treatment (categorical; nil, NH_4, NO_3, organic farmyard manure), N amount (continuous; 0 kg N yr^1, 48 kg N yr^1, 96 kg N yr^1), pH (continuous; 5.0–6.3), and P, K, Na, Mg Minerals (categorical; applied, not applied, organic farmyard manure) were fixed effects. Biomass (continuous; 0.822–6.300 kg dry weight samples), nested within year (categorical; 2016, 2017, 2018), were random effects. These models were implemented to find the effects of the treatments on the response variables: nectar volume and production, sucrose:hexose ratio, concentration of sugars per volume and per flower. To analyse the effect on sugar concentration per volume, nectar volume per flower was incorporated (continuous; 0.004–0.783) nested within year as a random effect, to account for variation caused by volume.

The total counts of floral abundance from quadrats (0.25m^2 quadrats thrown 20 times) were divided by four for each plot, giving mean floral abundance m^{-2}. For each nectar sample, the total concentration of sugars was multiplied by the contemporaneous information on floral abundance to give the provision of sugars (µg m^{-2}) for each species in the studied plant communities. Mixed-effects models were used to ascertain the effect of the treatments on sugar provision. The response variable was total quantity of sugars (continuous; 0-115748 µg m^{-2}), the fixed effects were N treatment (categorical; nil, NH_4, NO_3, organic farmyard manure), N amount (continuous; 0 kg N yr^1, 48 kg N yr^1, 96 kg N yr^1), pH (continuous; 5.0–6.3), and P, K, Na, Mg Minerals (categorical; applied, not applied, organic farmyard manure). Year (categorical; 2016, 2017, 2018) was the random effect.

All models were simplified step-wise by removing the least significant variable, checking residuals and model assumptions at each step, until we obtained the final model. Post hoc Tukey HSD tests were conducted on the final models to ascertain the significance of between-level comparisons.

4.4 Results

In total, 596 nectar samples were taken across the three years for the study species. The observed mean values of the nectar traits varied amongst the study species (Table 4.4; Fig 4.3). Across all plots, regardless of treatments, the flowers with the most voluminous nectar rewards were understandably those of *A. reptans*, with the open flowers of the *Apiaceae* species *A. sylvestris* and *H. sphondylium* having the lowest volume nectar standing crops. In agreement with other studies, the sucrose:hexose ratio is high in flowers requiring long-tongued insect visitors and lower in the open flowers of the *Apiaceae* species. Due to the larger volume of nectar found in the flowers, *A. reptans* have the most sugar-rich nectar per flower, but other species were more rewarding in terms of the sugar-richness per volume. Nectars from *A. sylvestris, C. nigra, H. sphondylium,* and *T. pratense* were all more concentrate than *A. reptans* nectar, whilst nectar from *K. arvensis* was the most dilute.

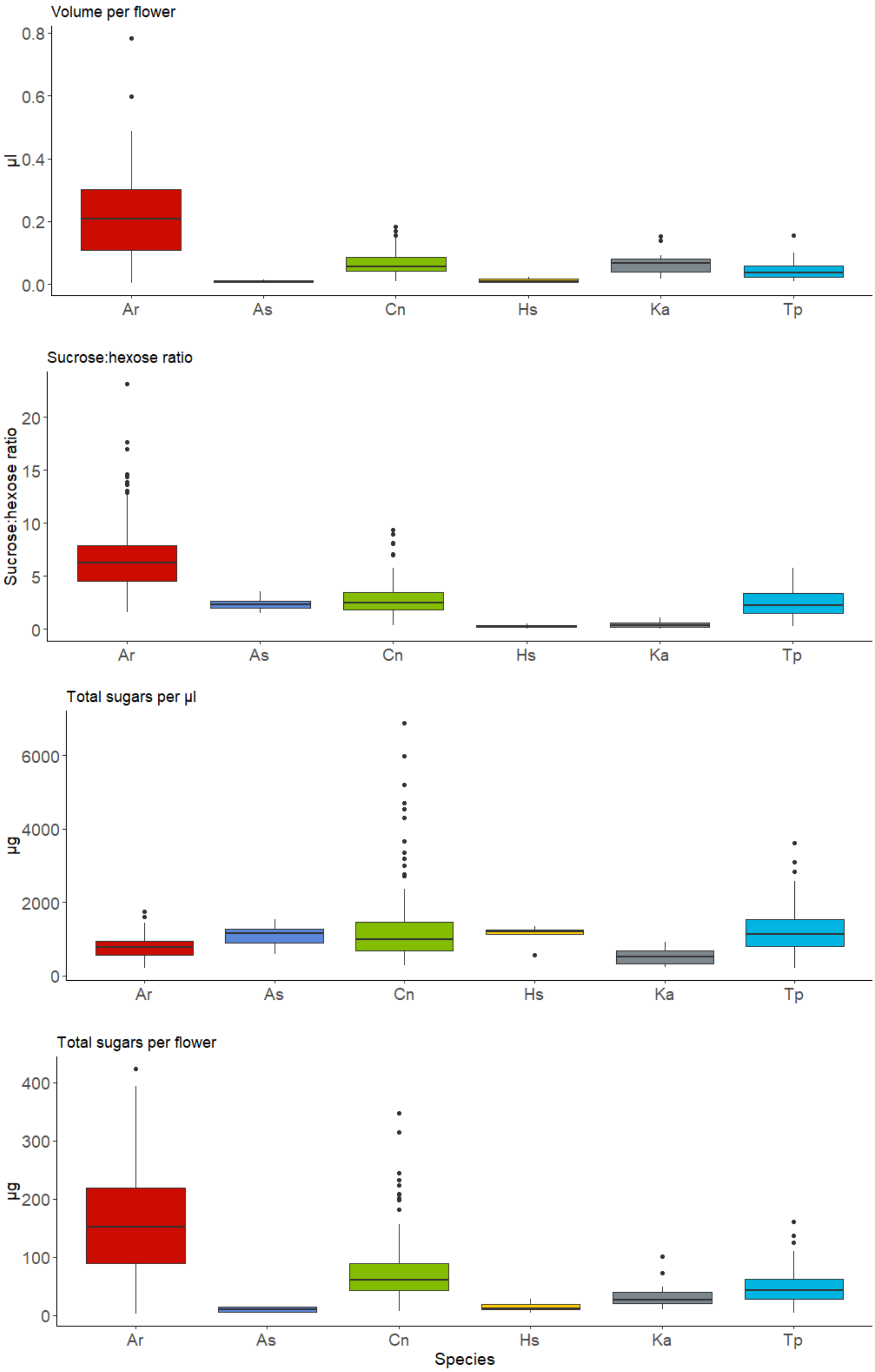


Figure 4.3. Summary of key nectar traits from the six study-species; volume standing crop per flower, sucrose:hexose ratio, total sugars per µl, and total sugars per flower.

Table 4.4. Summary statistics of the nectar samples from the study species.
The table shows the mean value, and standard error of the means, for the number of flowers sampled per replicate, the volume of nectar per flower, the ratio of sucrose:hexose, the concentration µg sugars per µl, and total the µg sugars per flower.

Species	**Fl. rep^{-1}**	**Vol. fl^{-1} (µl)**	**S:H ratio**	**Sugars (µg µl^{-1})**	**Sugars (µg fl^{-1})**
Standing crop					
Ar	14.63 ± 0.50	0.19 ± 0.011	7.32 ± 0.30	888.70 ± 22.39	157.85 ± 8.48
As	15.17 ± 1.04	0.0091 ± 0.0010	2.364 ± 0.15	1097.95 ± 78.42	10.08 ± 1.27
Cn	24.55 ± 0.60	0.076 ± 0.0031	2.62 ± 0.10	1026.18 ± 52.98	70.77 ± 3.87
Hs	19.62 ± 1.47	0.013 ± 0.0017	0.270 ± 0.040	1152.51 ± 53.90	15.16 ± 2.14
Ka	12.87 ± 0.99	0.070 ± 0.0098	0.445 ± 0.085	532.16 ± 58.89	35.15 ± 6.30
Tp	9.58 ± 0.14	0.044 ± 0.0025	2.449 ± 0.12	1211.91 ± 55.19	48.08 ± 2.65
24-hour nectar					
Ar	14.37 ± 0.50	0.28 ± 0.016	5.59 ± 0.29	598.56 ± 18.80	167.46 ± 9.68
Cn	13.46 ± 0.49	0.045 ± 0.002	3.29 ± 0.25	1799.14 ± 152.86	80.16 ± 6.65

4.4.1 Nectar production

The application of NH_4 caused a detrimental effect on the 24-hour nectar secretion rate of *A. reptans* and *C. nigra*. The mean 24-hour nectar volume of *A. reptans* in NH_4 plots was 44% lower than plants in control plots (0.167 µl compared to 0.291 µl), while mean 24-hour nectar volume of *C. nigra* was 40% lower than plants from control plots (0.0308 µl compared to 0.0514 µl). Furthermore, that standing crop in flowers of *A. reptans* and *C. nigra* was also lower when in NH_4-enriched communities (Table 4.5, Fig. 4.4). The standing crop of *C. nigra* was negatively affected by both forms of N, *A. reptans* by NH_4 and not NO_3. Conversely, the standing crop of *A. reptans* nectar was higher in soils with organic farmyard manure applied, and in experimental plots with higher topsoil pH. For the leguminous *T. pratense*, only mineral applications affected nectar standing crop, increasing the volume.

Table 4.5. Output of LMER models studying the impact of treatments on the volume of nectar standing crop and 24-hour nectar secretion (µl).

All presented values are statistically significant (t > 2) effect sizes given by the final simplified models ('+' denotes positive effects, '-' denotes negative effects). Shaded cells show that where a species could not be sampled from a treatment in sufficient quantities.

Species	**NH_4**	**NO_3**	**Org**	**N amount**	**pH**	**Minerals**
Standing crop						
Ar	-0.119		+0.198		+0.103	■
As	■					
Cn	-0.0372	-0.015				
Hs	■					
Ka		■	■			■
Tp	■					+0.0145
24-hour nectar						
Ar	-0.125					■
Cn	-0.028					

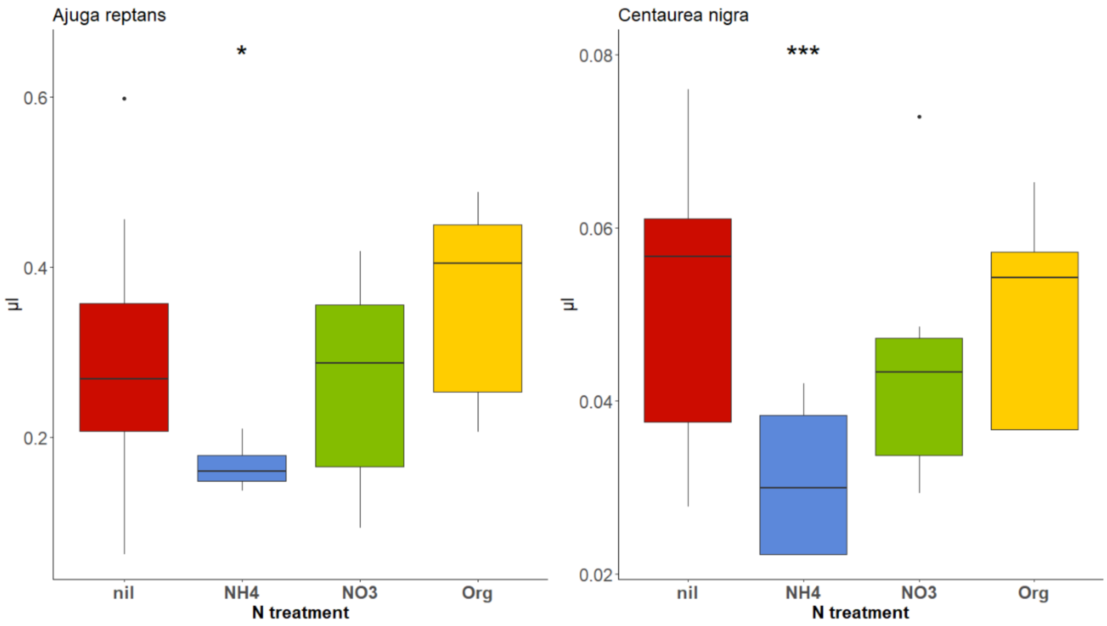


Figure 4.4. The 24 hour-nectar secretion of A. reptans and C. nigra, shown across nil and N treatment plots.

Significant difference between N treatments and nil plots, from post hoc Tukey tests, are shown. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

4.4.2 Sugar composition

The experimental treatments caused variation in the composition of nectar-sugars within the species (Table 4.6). NH_4 applications decreased the relative sucrose:hexose (S:H) ratio in *A. reptans* nectar (Fig. 4.5), while NO_3 application reduced the ratio in *A. sylvestris* and *H. sphondylium*, with more substantial drops in sucrose proportion at higher levels of NO_3 application (Fig. 4.6). Organic farmyard manure application increased the S:H ratio in *A. reptans*. In *A. reptans*, the impact of NH_4 to the S:H ratio was driven by the strong increase in fructose production (Fig. 4.7). The S:H ratios of *C. nigra, T. pratense,* and *K. arvensis* were not affect by the N treatments.

Table 4.6. Output of LMER models studying the impact of the treatments on the sucrose:hexose ratio of nectar.

All presented values are statistically significant (t > 2) effect sizes given by the final simplified models ('+' denotes positive effects, '-' denotes negative effects). Shaded cells show that where a species could not be sampled from a treatment in sufficient quantities.

Species	NH4	NO3	Org	N amount	pH	Minerals
Ar	-2.597		+2.716			■
As	■			-0.00103		
Cn						
Hs	■			-0.00111		
Ka		■	■			■
Tp	■					

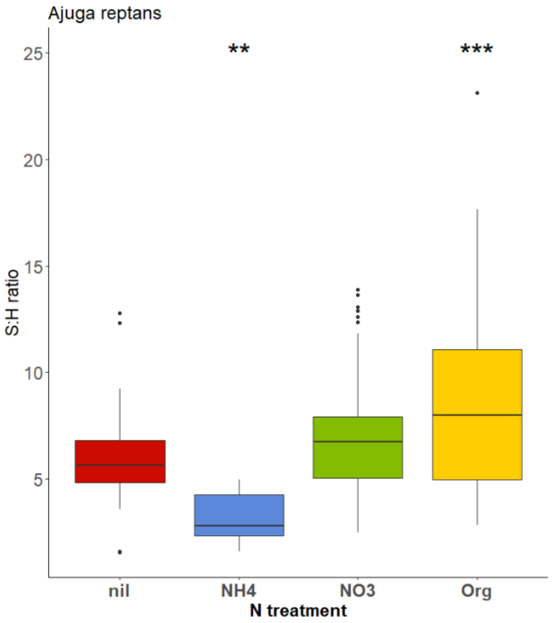


Figure 4.5. The S:H ratio of A. reptans nectar for plants from nil plots and all N treatment plots. Significant difference between N treatments and nil plots, from post hoc Tukey tests, are shown. * p<0.05; ** p<0.01; *** p<0.001.

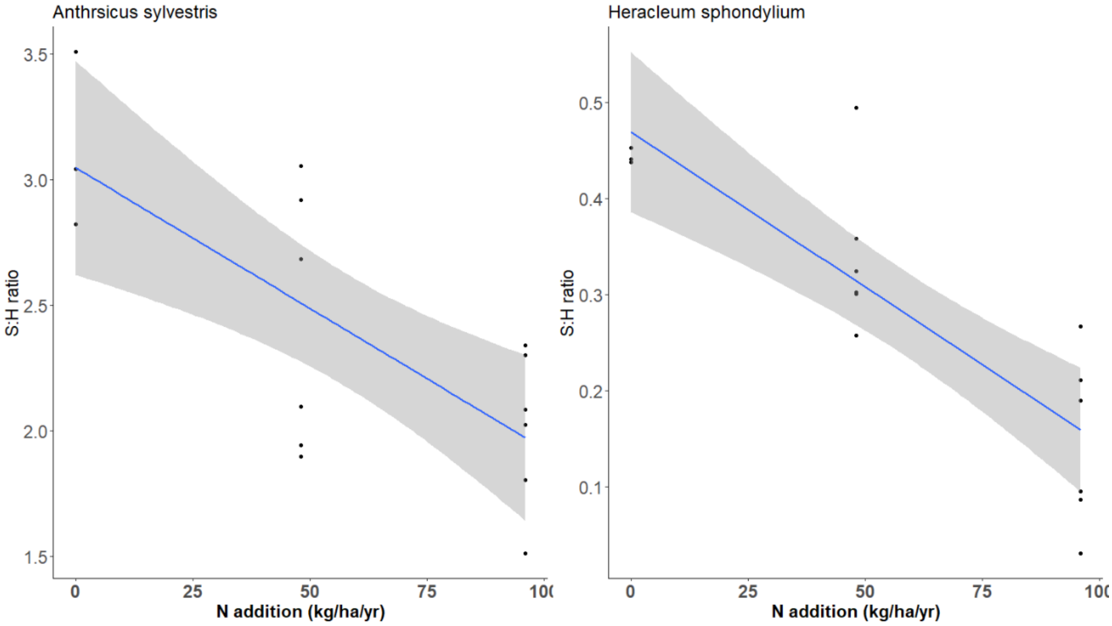


Figure 4.6. The impact of N addition levels on the S:H ratio of Apiaceae nectar.
The shaded region shows the 95% confidence interval.

4.4.3 Nectar quality; sugar concentration

In the latent nectar standing crop of *A. reptans*, organic fertilisers led to an overall decrease in sugars per volume (Table 4.7; Fig. 4.7). In *A. reptans* in NO_3-enriched plots, glucose concentration was lower, while fructose increased in concentration with NH_4-enrichment (Fig. 4.7). The concentration of sucrose in *H. sphondylium* nectar declined with higher levels of N additions. The total sugar concentration per volume of *A. reptans* was lower at higher soil pH. Mineral (P, K, Na, Mg) additions increased the total sugar concentration of *C. nigra* nectar, driven by increases of sucrose. Non-significant Tukey post hoc testing showed that I was not able to confidently ascertain a significant effect of N treatments on the nectar sugar concentrations of freshly secreted (24hr) *A. reptans* nectar. Only soil pH had a significant effect here, with lower glucose concentrations found at higher soil pH. Mineral additions increased the concentration of all three sugars, and total sugar concentration, in freshly secreted nectar of *C. nigra* (nectar secreted within 24 hr) (Table 4.7).

When studying total sugars produced per flower (Table 4.8), we see significant impacts of NH_4 to both *A. reptans* and *C. nigra* standing crops. There was lower glucose and sucrose provision, and lower total sugar concentration, per flower in *A. reptans* plants undergoing NH_4 enrichment, this was true for both nectar standing crop and for freshly secreted nectar over 24 hours (Fig. 4.8). There were fewer sugars found in the standing crop of *C. nigra* nectar in NH_4-enriched plots (Fig. 4.9). However, the N treatments did not affect the provision of sugars secreted in 24 hours by *C. nigra*; the sugar secretion was instead positively driven by mineral additions (Fig. 4.10). There were no other significant impacts on total sugar production, although other treatments had effects on individual major sugars. Higher soil pH led to an increase in the provision of fructose in *A. reptans* standing crop, and of glucose and fructose in *T. pratense* standing crop. Organic fertiliser led to increased provision of fructose and sucrose in *A. reptans flowers*. Higher levels of N application led to reduced sucrose provision in the standing crop of *H. sphondylium* flowers.

Table 4.7. Output of LMER models studying the treatments on the concentration of individual and total sugars per volume (μg μl^{-1}).

All presented values are statistically significant (t > 2) effect sizes given by the final simplified models ('+' denotes positive effects, '-' denotes negative effects). Shaded cells show that where a species could not be sampled from a treatment in sufficient quantities. For each species, the effect to total sugars (Total), glucose (Glu.), fructose (Fru.), and sucrose (Suc.) is given.

Species	**Sugar**	**NH$_4$**	**NO$_3$**	**Org**	**N amount**	**pH**	**Minerals**
Standing crop							
Ar	Total			-235.20		-150.79	■
	Glu.		-9.953	-17.394			■
	Fru.	+93.853		-32.475			■
	Suc.			-183.20		-141.18	■
As	Total	■					
	Glu.	■					
	Fru.	■					
	Suc.	■					
Cn	Total						+349.61
	Glu.						
	Fru.						
	Suc.						+217.83
Hs	Total	■					
	Glu.	■					
	Fru.	■					
	Suc.	■			-2.163		
Ka	Total		■	■			■
	Glu.		■	■			■
	Fru.		■	■			■
	Suc.		■	■			■
Tp	Total	■					
	Glu.	■					
	Fru.	■					
	Suc.	■					
24-hour nectar secretion							
Ar	Total						■
	Glu.					-10.068	■
	Fru.						■
	Suc.						■
Cn	Total						+1557.8
	Glu.						+280.62
	Fru.						+301.49
	Suc.						+909.3

Table 4.8. Output of LMER models studying the impact of the treatments on the concentration of total and individual sugars per flower (μg flower^{-1})

All presented values are statistically significant (t > 2) effect sizes given by the final simplified models ('+' denotes positive effects, '-' denotes negative effects). Shaded cells show that where a species could not be sampled from a treatment in sufficient quantities. For each species, the effect to total sugars (Total), glucose (Glu.), fructose (Fru.), and sucrose (Suc.) is given.

Species	**Sugar**	**NH4**	**NO3**	**Org**	**N amount**	**pH**	**Minerals**
Standing crop							
Ar	Total	-133.444		+39.78			■
	Glu.	-8.9					■
	Fru.					+9.55	■
	Suc.	-94.514					■
As	Total	■					
	Glu.	■					
	Fru.	■					
	Suc.	■					
Cn	Total	-40.28					
	Glu.	-10.769					
	Fru.	-16.491					
	Suc.	-47.627					
Hs	Total	■					
	Glu.	■					
	Fru.	■					
	Suc.	■			-0.046		
Ka	Total		■	■			■
	Glu.		■	■			■
	Fru.		■	■			■
	Suc.		■	■			■
Tp	Total	■					
	Glu.	■				+2.946	
	Fru.	■				+6.353	
	Suc.	■					
24-hour nectar secretion							
Ar	Total	-89.33					■
	Glu.	-5.814					■
	Fru.						■
	Suc.	-67.542					■
Cn	Total						+58.41
	Glu.						+10.617
	Fru.						+11.528
	Suc.						

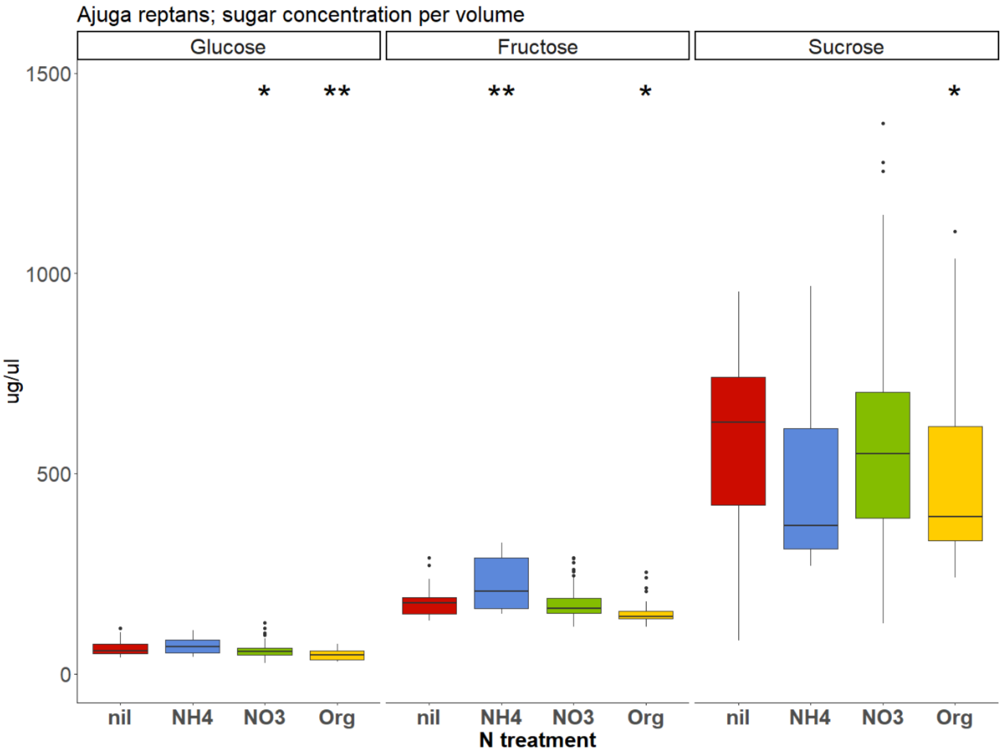


Figure 4.7. Concentration of individual sugars per volume (µg µl^{-1}) in A. reptans nectar, comparing amongst nil and N treatments.

The three separate sugar types are shown across facets. Significant difference between N treatments and nil plots, from post hoc Tukey tests, are shown. * p<0.05; ** p<0.01; *** p<0.001.

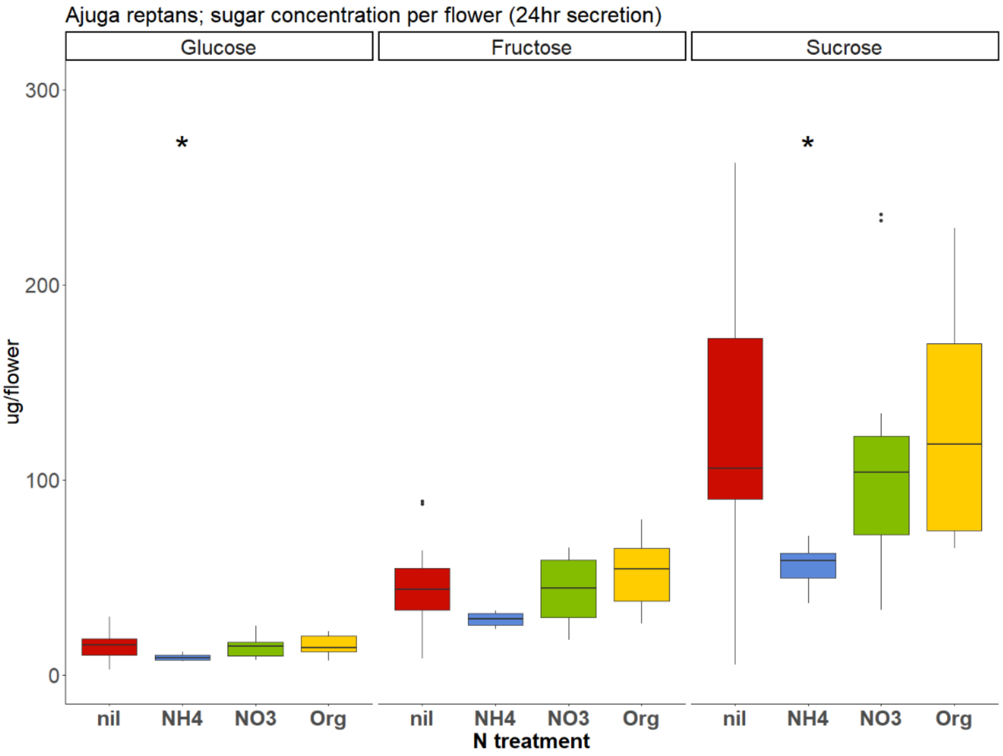


Figure 4.8. Concentration of individual sugars per flower (µg flower^{-1}) in A. reptans nectar, comparing amongst nil and N treatments.

The three separate sugar types are shown across facets. Significant difference between N treatments and nil plots, from post hoc Tukey tests, are shown. * p<0.05; ** p<0.01; *** p<0.001.

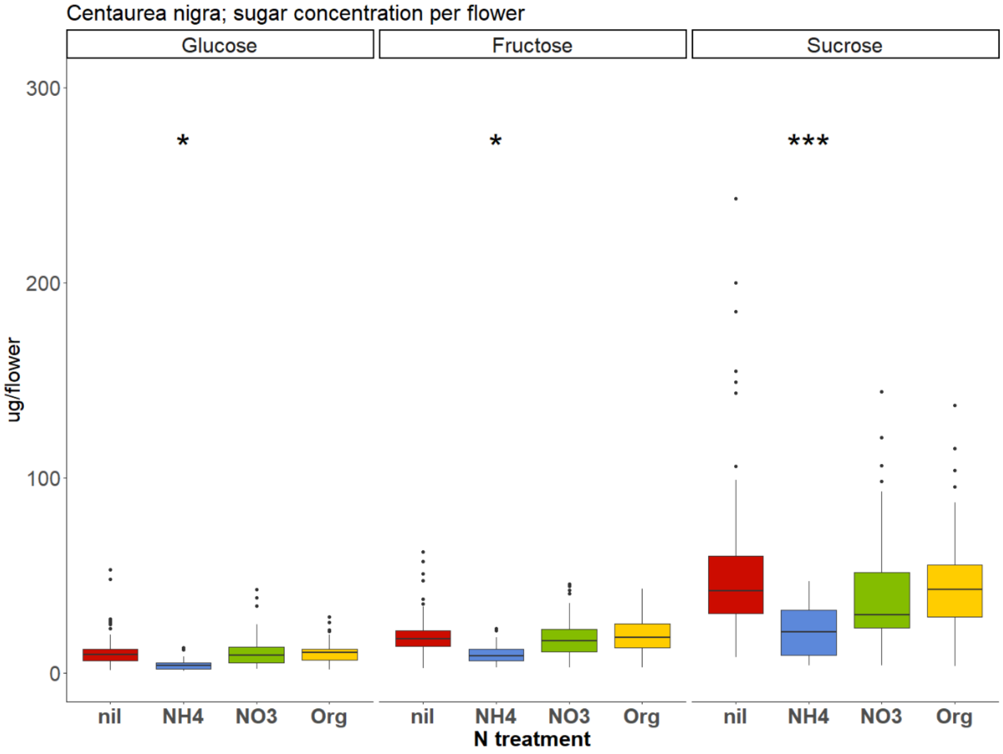


Figure 4.9. Concentration of individual sugars per flower (µg flower^{-1}) in C. nigra nectar, comparing amongst nil and N treatments.

The three separate sugar types are shown across facets. Significant difference between N treatments and nil plots, from post hoc Tukey tests, are shown. * p<0.05; ** p<0.01; *** p<0.001.

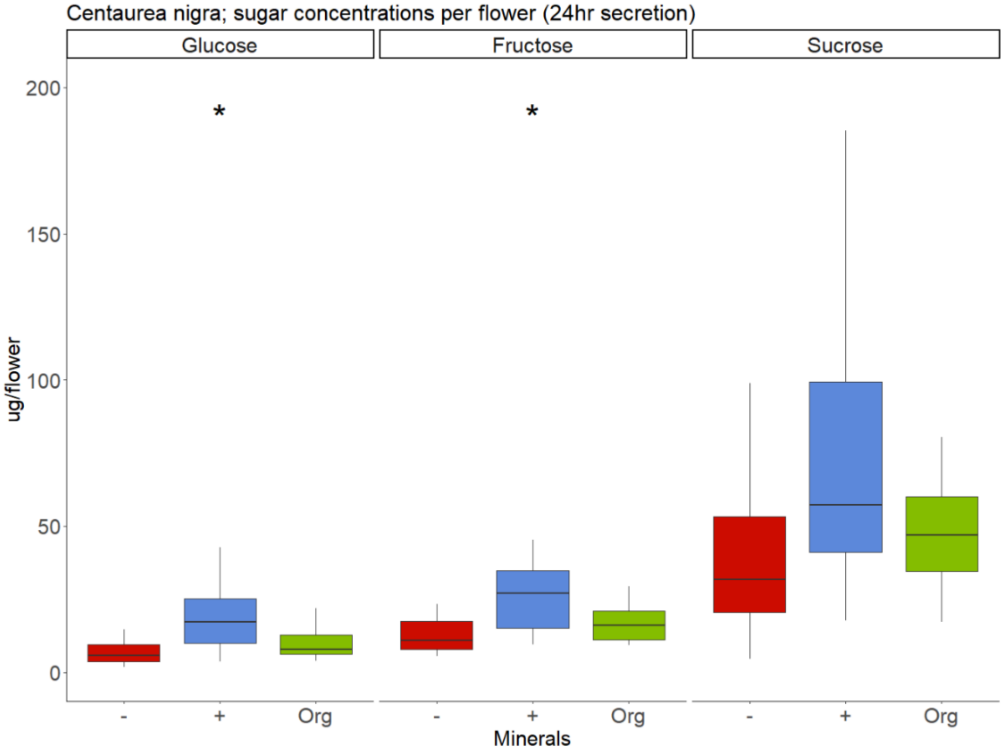


Figure 4.10. Concentration of individual sugars per flower (µg flower^{-1}) in C. nigra nectar, comparing amongst mineral treatments.

The three separate sugar types are shown across facets. Significant difference between N treatments and nil plots, from post hoc Tukey tests, are shown. * p<0.05; ** p<0.01; *** p<0.001.

4.4.4 Provision of sugars per unit area

The treatments had numerous effects on the quantity of nectar sugar provision per unit area (Table 4.9). The total sugar provision per area was lower in NH_4-enriched plots for all three species sampled from these plots, *A. reptans, C. nigra,* and *K. arvensis* (Fig. 4.11). Nectar sugar provision by *C. nigra* and *T. pratense* was lower in plant communities with NO_3 added. Only *C. nigra* provided fewer nectar sugars in plant communities enriched with organic farmyard manure. Soil pH had a significant effect on the sugar provision per area of four species. Acidification caused lower nectar sugar provision from *A. reptans* and the two *Apiaceae*, *A. Sylvestris* and *H. sphondylium,* but led to greater sugar provision by *C. nigra* (Fig. 4.12). Additions of minerals (P, K, Na, Mg) to plant communities increased *T. pratense* nectar sugar provision. The impacts to floral unit abundance closely reflect impacts to sugar provision (Table 4.10), with a single exception. Mineral additions caused greater *C. nigra* floral unit abundance but was not a significant driver of nectar sugar quantities.

Table 4.9. Output of LMER models studying the *mean provision of total sugars per area ($\mu g\ m^{-2}$) for each species and the effect of treatment on sugar provision.*
All presented values are statistically significant (t > 2) effect sizes given by the final simplified models ('+' denotes positive effects, '-' denotes negative effects). Shaded cells show that where a species could not be sampled from a treatment in sufficient quantities.

Species	**Mean**	**NH_4**	**NO_3**	**Organic**	**pH**	**Minerals**
Ar	484.88	-428.78			+290.64	■
As	9396.28	■			+13204	
Cn	412.93	-492.96	-235.66	-220.07	-471.76	
Hs	1591.77	■			+3936	
Ka	438.19	-437.8	■	■		■
Tp	9493	■	-9701.297			+14732.437

Table 4.10. Output of LMER models studying the mean floral abundance m^{-2} for each species and the effect of treatment on floral abundance.

All presented values are statistically significant (t > 2) effect sizes given by the final simplified models ('+' denotes positive effects, '-' denotes negative effects). Shaded cells show that where a species could not be sampled from a treatment in sufficient quantities.

Species	Mean	NH_4	NO_3	Organic	pH	Minerals
Ar	3.17	-2.6197			+1.28	■
As	858.18	■			+1287.9	
Cn	5.34	-3.0206	-3.1585	-1.8157	-4.9874	+1.7005
Hs	112.73	■			+384.29	
Ka	13	-13.611	■	■		■
Tp	170.61	■	-176.752			+265.483

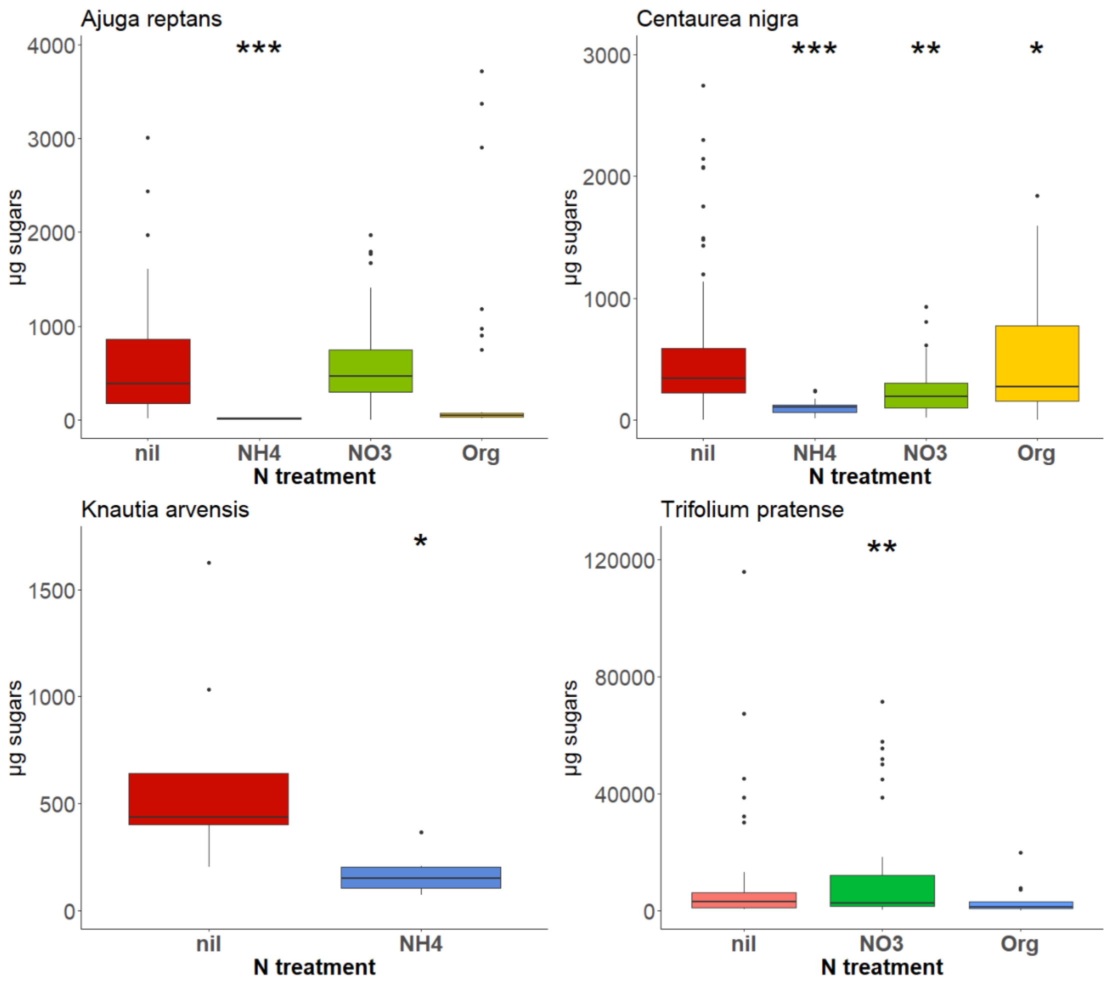


Figure 4.11. Comparative plots showing the total provision of sugars by a species per unit area (µg m^{-2}), across nil plots and N treatment plots.

Treatments that were significantly different from nil plots, from post hoc Tukey tests, are shown with * p<0.05; ** p<0.01; *** p<0.001.

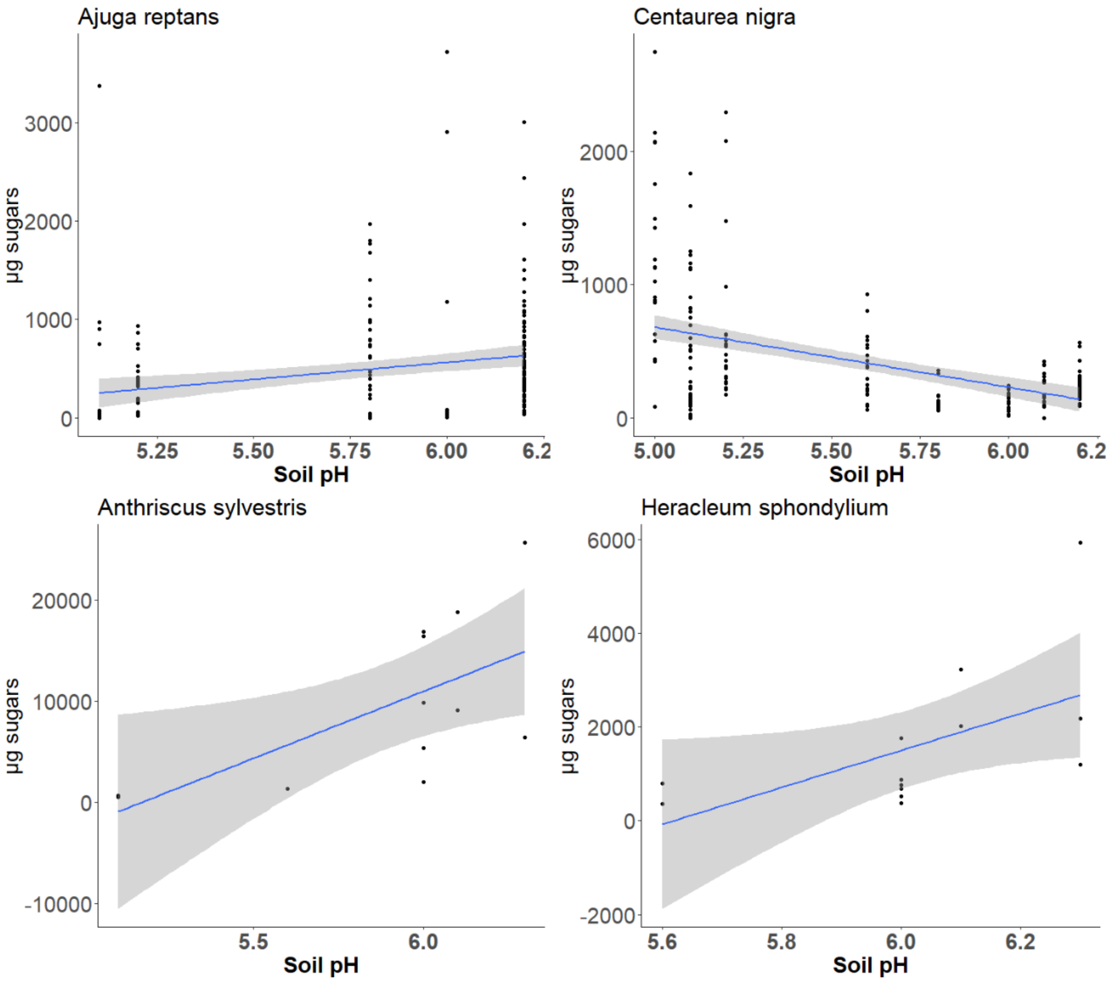


Figure 4.12. Comparative plots showing the total provision of sugars by a species per unit area (µg m^{-2}), across a pH gradient.

The shaded region of graphs indicates the 95% confidence interval.

4.5 Discussion

The results showed that soil N-enrichment led to varied changes in the nectar traits of most study species. The nectar traits of the forb *A. reptans* responded widely to the treatments, with many detrimental responses recorded with NH_4, mixed responses to organic farmyard manure treatments, but relatively few responses to NO_3 applications. The nectar secretion rate over 24 hours of the forb *C. nigra* responded negatively to NH_4-enrichment, which led to decreases in sugar content per flower. The legume *T. pratense* was less sensitive to N treatments, with no observed significant impacts to nectar traits. This could be due to the fundamental difference in how forbs and legumes take up N, forbs through the soil, and legumes through N-fixing rhizobium drawing it in from the air. However, sugar concentration of *T. pratense* nectar did decrease with soil acidification, so N deposition could indirectly affect the nectar traits of this species. Sucrose concentration was observed to decrease with higher additions of N in the two *Apiaceae* species, *A. sylvestris* and *H. sphondylium*, suggesting a taxonomically shared response trait. Soil nitrogen, pH, and fertility were widely impactful to the provision of nectar sugars by plant communities, with these impacts stemming from changes to nectar traits and the abundance of floral units. The following sections will discuss the results thematically according to their responses.

4.5.1 Impacts of N treatments; focus on *Ajuga reptans* and *Centaurea nigra*

NH_4 additions had negative effects on the nectar secretion rate over 24 hours of *A. reptans* and *C. nigra*. Furthermore, this seems to have had an impact on the standing crop of their nectar, as flowers of both species showed a reduced volume of standing crop when treated with NH_4. The standing crop of *C. nigra* was also depleted in plots where NO_3 had been applied, although NO_3 additions did not have a discernible effect on the 24-hour nectar production of *C. nigra*. The different responses to the two forms of N could be due to the natural tolerances and adaptations of the two plant species. Both are typically found in mesotrophic grasslands, and therefore are not so adapted to acidification caused by the nitrification of NH_4 and subsequent release of H^+ ions or through the inability to exchange H^+ ions with the rhizosphere during the process of uptake and metabolisation (Lucassen *et al.*, 2003; Van Den Berg *et al.*, 2005; Stevens *et al.*, 2011). This could cause stress to the plant. Nectar production is an energetically costly mechanism and, if under stress, the plant could focus less energy into nectar secretion.

The lower volumes of nectar provided in *C. nigra* flowers enriched with NH_4 or NO_3 were no more concentrated than nectar from *C. nigra* flowers in control plots. Therefore, the lower nectar volume simply equates to a reduction in the provision of sugars for prospective insect pollinators. All sugars declined in concentration in *C. nigra* flowers with NH_4 additions, with a steeper decline in sucrose than glucose or fructose. The disaccharide sucrose is thought to be an important nectar sugar for long-tongued bees, which respond positively to experimental increases in sucrose (Cnaani, Thomson and Papaj, 2006) and are frequently observed to forage sucrose-rich plants (Elisens and Freeman, 1988; Petanidou, 2005; Petanidou *et al.*, 2006). As *C. nigra* is an important late-season resources for many bee species, the lower provision of sucrose and total sugars is a potentially negative effect for the prospects of long-tongued bees foraging in the late-season, such as gynes and males of *Bombus hortorum* or the nationally scarce *Bombus ruderatus* (Falk, 1991).

The nectar produced by *A. reptans* in NH_4-enriched plant communities was not only less voluminous, but less sugar-rich (when expressed on a volumetric basis) and with a reduced sucrose:hexose ratio. Over a 24-hour period, *A. reptans* flowers produced less glucose and sucrose in NH_4-enriched soils. Therefore, NH_4 additions to soil or deposition of NH_4 will lead less rewarding flowers, especially for long-tongued bees that preferentially forage sucrose-rich nectar (Elisens and Freeman, 1988; Petanidou, 2005; Cnaani, Thomson and Papaj, 2006; Petanidou *et al.*, 2006). As *A. reptans* flowers require handling skills and an expenditure of energy to forage, as bees activate flight muscles to fly between flowers, the lessened reward makes these flowers less able to support intensive foraging bouts. There was, however, no negative impact of NO_3 addition on the nectar traits of *A. reptans*, perhaps due to the lack of plant cell acidification during NO_3 uptake (Stevens *et al.*, 2011). Therefore, if *A. reptans* experiences atmospheric N deposition that is more heavily composed of NO_3, the effect to nectar traits may not be so impactful. On the other hand, NH_4-dominant deposition, as occurs in most regions across the world (Stevens *et al.*, 2011), could be more impactful to the nectar of *A. reptans, C. nigra*, and other forbs that respond similarly.

Although *A. reptans* nectar standing crop in organic farmyard manure-treated plots was far higher than nil plots, there was no increase in 24hr nectar production associated with organic plots. As organic manure applications did not increase the production rate, it stands to reason that a build-up of nectar was occurring in plots with organic manure applications, possibly due to high biomass obscuring flowers or selective visiting of bees to alternative plants. The analysis of overall sugar concentrations per volume showed that the *A. reptans* nectar from organic plots was far less concentrated, with fewer total sugars, than nil and NO_3 plots. *A. reptans* flowers in organic manure-treated plots had similar levels of sugars to flowers from control plots, but in a more dilute and voluminous form. The mechanism for this is not clear, it could be that the high biomass of organic plots shades the plants and create a cooler microclimate that lessens evaporation of water from flowers. The creation of cooler microclimates amongst taller vegetation has been proposed as a pathway for impacts of N on fauna (WallisDeVries and Van Swaay, 2006; Nijssen, Wallis De Vries and Siepel, 2017), it's possible that this pathway can indirectly affect fauna through changes on food resources such as nectar for pollinators. When foraging more dilute nectar, the lack of calorific reward per sampling effort might have put bee visitors off and encouraged them to preferentially forage more rewarding plants. Often bees will forage for the most calorific rewarding nectar (Southwick, Loper and Sadwick, 1981), and the act of foraging for nectar is an important trade-off between the reward and the effort and energy expended whilst foraging (Kim, Gilet and Bush, 2011). In morphically specialised plants, the energy expenditure in foraging is greater and so the nectar needs to be more rewarding in order to provide a net energy gain (Harder, 1986). With an insufficient reward that does not give a net gain in energy, bees might simply look elsewhere. Due to the increased volume of the nectar standing crop, the sugar provision per *A. reptans* flower is actually not so different from nil plots. However, larger volumes of nectar require more energy to forage and a bee can only take up so much nectar (Kim, Gilet and Bush, 2011), furthermore bees typically prefer sugar-rich nectars (Krömer *et al.*, 2008; Klumpers, Stang and Klinkhamer, 2019; Vandelook *et al.*, 2019). Therefore, despite providing the same amount of total sugars, the dilute nectar is likely not as attractive or rewarding.

A. reptans nectar standing crop was lower in more acidified soil. The analysis of sugar concentrations per volume showed that, although *A. reptans* nectar in acidified soil was less voluminous, it was more concentrated. This suggests that there was no limit imposed by soil acidification on sugar production, only on the volume of water expelled through the nectary gland. Therefore, although soil acidification led to less nectar in *A. reptans* flowers, which perhaps could limit the abundance of long-tongued pollinators able to forage, the nectar was more rewarding which should render it more attractive to insects that will need to expend less energy to forage it.

A. reptans has an Ellenberg R value of 5, on a scale of 1-9, indicating that the plant is able to grow in slightly acidic grasslands (Hill, 1999). It was more acid tolerant than the other study species, which had Ellenberg R values ranging from 6-8. The fact that soil pH did not affect the output of sugars from *A. reptans* and did not affect the nectar traits of other species, suggests that it is not a prominent factor that will affect how N impacts of the attractiveness and nutritional qualities of nectar.

4.5.2 *Trifolium pratense* and the effect of mineral additions on nectar traits.

The volume of nectar standing crop of *Trifolium pratense* was not affected by the N treatments, or by pH, only by mineral additions. Furthermore, the other nectar traits of *T. pratense*, composition or quality, were not affected by N additions either. Legumes, due to the way they take up N, by fixing it from the atmosphere, do not exploit applications of N to soil in the same way other competitive plants do. This can cause them to decline in biomass due to competitive exclusion (Skogen, Holsinger and Cardon, 2011; Storkey *et al.*, 2015). However, the inability to exploit N applications could render them less susceptible to N-driven variation in floral traits. Shuel (1956) also found that *T. pratense* did not have altered nectar secretion in response to N, whereas a forb, *Antirrhinum majus*, did. The inter-specific variation in responses to N additions is something that complicates generating an overall view of how N affects ecosystems. Therefore, predictive patterns in responses, such as the contrast between legumes and forbs, is useful. Soil pH did have a small effect on the concentration of fructose and glucose available in the standing crop of *T. pratense* nectar. This suggests that, although N might not directly alter leguminous nectar traits, it is important to be wary of indirect impacts. Soil acidification is a locally mediated effect of N deposition, the severity depends on soil conditions and buffering capacity. This facet of N deposition could lead to changes in the provision of calorific floral resources for pollinators in communities that are under stress of acidification.

Previous research has shown the importance of phosphorous (P) for the N-fixing rhizobacterium of legumes and that, while N additions disadvantage legumes, additions of P can be beneficial (Ae *et al.*, 1990; Kirkham, Mountford and Wilkins, 1996; Vance, Uhde-Stone and Allan, 2003; Hasan *et al.*, 2016; van de Wiel, van der

Linden and Scholten, 2016). The growth of legumes, such as *T. pratense*, therefore responds directly to P additions. It is logical that the nectar traits of *T. pratense* showed more response to mineral additions than N additions. Mineral applications were also revealed to be a significant positive factor in the production of nectar-sugars by *C. nigra*. The volume of nectar production and the composition of sugars produced were not affected by the addition of minerals, but this treatment did drive an increase in sugar production, per volume and per flower. The increased soil nutrition from minerals, including P and potassium (K), could therefore greatly benefit pollinating insects through this increased calorific provision.

4.5.3 The nectar trait responses of the two *Apiaceae* species to N addition.

A limitation of the study is the small sample size obtained for some species. Nectar traits can vary between plants and flowers of the same species (Corbet, Unwin and Prys-Jones, 1979; Herrera, Pérez and Alonso, 2006) and, whilst taking cumulative samples across many flowers can help to account for inter-floral variation (Corbet, 2003), ideally more samples were needed from *A. sylvestris*, *H. sphondylium*, and *K. arvensis* to identify impacts from N additions. However, we did find consistent effects of N addition on the sucrose:hexose ratios of nectars from *A. sylvestris* and *H. sphondylium*. The two species are both from the *Apiaceae* family, and this response was not recorded in any other species. This could be an indication that nectar trait responses can be linked to taxonomic family, but we should be tentative with this conclusion. These are only two species and investigations of other *Apiaceae* species should be undertaken to confirm this. The response of sucrose:hexose ratios in *Apiaceae* nectar to N additions was linear, with the proportion of sucrose declining further with higher levels of N addition (Fig. 4.6). The flowers of *A. sylvestris* and *H. sphondylium* are large generalist umbellifers, visited by a wide suite of insects including dipterans, coleopterans, lepidopterans, and hymenopterans. Most bee species observed foraging these floral units in the Park Grass Experiment (Appendix 5) were short-tongued generalist species, that tend to prefer mixed or hexose-dominant nectars (Petanidou *et al.*, 2006), with few long-tongued bumblebees that prefer sucrose-rich nectars. Dipterans and coleopterans also, typically, prefer hexose-dominant nectars (Elisens and Freeman, 1988; Petanidou *et al.*, 2006). The N-driven shift in sugar ratios hexose will likely improve the attractiveness of *A. sylvestris* and *H. sphondylium*, and potentially other *Apiaceae*, to dipterans, coleopterans, and generalist short-tongued bees, whilst decreasing the attractiveness to more specialist bee species. The concentration of total sugars was not affected in either species, or in *K. arvensis*, in response to N. Given forecasted increases in global N deposition, this is perhaps a good sign, as the interaction between these important nectar resource and a wide assemblage of pollinators should not be too impacted by changes to the floral rewards.

4.5.4 Impacts to community-scale nectar provision

The detrimental impact of NH_4 on the nectar traits of calcareous grassland species is apparent throughout this chapter. All three study species of NH_4-enriched plots provided far less sugar to flower visitors. NH_4 is known to negatively affect the botanical communities of temperate grasslands (e.g. Pearson and Stewart, 1993; Stevens *et al.* 2011; Southon *et al.* 2013), including by reducing floral abundance. Furthermore, the analyses showed how NH_4 caused reduced nectar secretion (Table 4.5) and concurrently lower sugar concentrations per flower (Table 4.8). Through combining nectar traits data with quadrat-derived floral abundance data, the strong detrimental impact of NH_4-enrichment on sugar provision became clearly apparent. Although N deposition in the UK has plateaued and showed signs of decreasing in recent decades (ROTAP, 2012), NH_4-enrichment of terrestrial ecosystems remains a clear issue. The declines of insect pollinators are through a myriad of reasons, here I show that declines in the provision of calorific dietary resources due to soil eutrophication could be a contributing factor to pollinator declines. Given the continued high NH_4 deposition levels in the UK, this detrimental impact to floral resources could continue to stress pollinator insect communities in the future.

Soil acidification is also shown to contribute to reduced nectar sugar provision by plant communities. The two *Apiaceae* species studied, *A. sylvestris* and *H. sphondylium* supplied fewer sugars to prospective pollinators in soils with lower pH. Soil acidification did not alter the nectar traits of these plants, so this result is likely driven by the sharp fall in floral abundance with soil acidification (Table 4.10). The results show that it is important to consider multiple facets of N-enrichment. The nectar sugar provision of the *Apiaceae* species was not affected by N treatments, but in acid-sensitive soils with low buffering capacity, N deposition could indirectly impact on resource provision for flower-visiting insects. These two species are nectar-rich and serve the resource acquisition needs of a wide range of pollinators. The significant hit to sugar provision suggests N-driven soil acidification could greatly impact pollinator communities broadly.

Negative effects of soil acidification were not ubiquitous across study species, reflecting the common theme of species-specific responses to N. The sugar provision of *C. nigra* was in fact higher in more acid soils. In the UK, *C. nigra* has an Ellenberg R value of 6, implying adaptability to weakly acid or basic soils, however in Central Europe the Ellenberg R value is 3, suggesting it grows primarily in acid soils (Hill *et al*, 1999). The nectar traits of *C. nigra* did not respond to soil acidity in this study, so the likely driver of sugar provision was floral abundance, which increased in more acidic soils. The studied topsoil pH was consistently greater than pH 5; we cannot infer if the positive trend of sugar provision with soil acidification would continue in strongly acidic soils, but it is unlikely to do so where soil pH diverges from what the species can tolerate. Although weakly acid soil was beneficial for *C. nigra* sugar provision overall, N-driven soil acidification could cause a net decrease in nectar sugar provision, as all N treatments decreased nectar sugars per unit area.

A major problem of N deposition is the negative impacts it can have on leguminous plants (Storkey *et al*, 2015). This could be a substantial issue for bees, key pollinating insects, as leguminous plants are key floral resources. The nectar and pollen of legumes is often highly nutritious (Carvell *et al.* 2006) and can form an integral part of the diet of some long-tongued oligolectic and bees (Goulson and Darvill, 2004). This study emphasised the strong negative potential of N deposition for bees. Although N did not affect nectar traits of *T. pratense*, the reduction in floral abundance with N additions led to plant communities providing significantly less *T. pratense* floral sugar resources. Leguminous plants take up N through atmospheric fixation by rhizobacteria, thus when N is added to soils *T. pratense* loses a competitive edge due to the increased resources available to nitrophilic plants. Many long-tongued bees have declined in the UK in recent decades (Biesmeijer *et al*. 2006), particularly pre-1990 (Carvalheiro *et al.* 2013), when N deposition was high. It has been suggested by other authors (Carvalheiro *et al.* 2013; Baude *et al.* 2016) that N deposition could have contributed to a decline in the provision of floral resources for pollinators with detrimental consequences for associated pollinator species. The findings presented here, namely the decline of nectar sugar provision with N additions, emphasise this. Conversely, *T. pratense* floral sugar provision increased with mineral additions (P, K, Na, Mg), likely due to increased floral abundance. Studies have shown the positive impact P and K can have on the growth and N-fixing capabilities of *Fabaceae* (Premaratne and Oetrli, 1994; Carsky *et al.* 2001; Mmbaga *et al.* 2014). The mineral additions in the PGE allowed *T. pratense* to maintain high populations, which contributed to increased floral abundance and the resultant high sugar provision of mineral-enriched plant communities.

4.5.5 Limitations and caveats of the study, suggested future research

A caveat of the study is that only a select group of plant species were studied; those from which raw nectar sampling was possible without dilution. As mentioned in the methods section, dilution of nectar can lead to uncertainties in calculating true concentrations of sugars, and therefore in the inference of results. If all plant species in the community were sampled, it would be possible to determine the impact of N and soil pH on the total provision of sugars by a plant community. This would be incredibly useful and informative for addressing our lack of understanding of how these drivers effect pollinator populations.

Nectar production and composition is known to vary across flowers and individuals within a species, particularly due local abiotic drivers such as relative humidity and temperature (e.g. Corbet *et al.* 1979) and so large sample sizes are required to be sure of conclusions. Relatively few samples were taken from *A. Sylvestris, H. sphondylium,* and *K. arvensis*. This reduced the power of analysis and made the reporting and inference of significant results difficult for these species.

The experiments reported in this chapter were conducted in a field site. With this I was able to determine the impacts to representative plant communities under realistic conditions (i.e. with plant competitive dynamics and climate). It would be useful to support this study with controlled experiments and spatially distributed field studies. Laboratory or greenhouse experiments in controlled environments can be useful to verify the impacts to individual study species. Furthermore, it could be useful to test for heritable nectar traits by using seeds from PGE populations. Alternatively, representative communities with multiple species could be constructed in controlled studies. This would incorporate alterations to plant competitive dynamics, a key aspect of soil eutrophication, into the study. The study of plant communities in spatially separated field sites across the UK or temperate European grasslands would also support this research. The PGE contains distinct plant communities that are representative of communities undergoing various soil additions. However, they are not spatially replicated or independent. Studying plant communities across a gradient of N deposition and soil acidification would help to show real impacts to the provision of floral resources.

4.6 Conclusion

In this chapter it was shown that changes in soil N content can impact on important floral traits; nectar production, composition, and quality. This is an important finding, as it shows the consequences of N deposition on plant-pollinator communities could be affected by nuanced responses in floral traits. Nectar-sugar traits have been shown to influence pollinator visitation (Southwick, Loper and Sadwick, 1981; Petanidou, 2005), the health of individual pollinators, and the diversity of wider pollinator communities (Potts *et al.*, 2003). Therefore, when considering the role of N deposition on plant-pollinator interactions and currently declining trends in pollinators, it is important to look beyond species composition and consider the quality of resources, not just the quantity, as previously suggested by Nijssen, Wallis De Vries and Siepel (2017). There were detrimental effects caused by NH_4 additions, whilst NO_3 additions led to few changes in floral nectar traits across the species.

Therefore, impacts to plant-pollinator interactions could vary spatially according to NH_x:NO_y deposition ratios. To improve our understand further, we should consider other nectar traits, for example the provision of amino acids which also influence pollinator visitation (Petanidou *et al.*, 2006) and health (Nepi *et al.*, 2012), or secondary metabolites that can acts as deterrents (Adler, 2000) or help fight parasitic infections (Richardson *et al.*, 2015).

Chapter 5

THE IMPACT OF NITROGEN ADDITIONS ON GRASSLAND POLLINATOR COMMUNTIES AND PLANT-POLLINATOR INTERACTION NETWORKS

5.1 Abstract

With pollinating insects declining around the world across taxa and ecoregion, it is imperative to understand these trends to improve our ability to forecast, mitigate, and prevent further declines and extinctions. The ecosystem service provided by pollinating insects, for wild plants and crops, is hugely important and valuable. The role of nitrogen (N) deposition and direct applications of N to plant-pollinator interactions and pollinator communities is relatively unknown and requires further consideration as a potential factor in pollinator ecology. In this study, the impact of N enrichment on pollinator communities and plant-pollination interaction networks was studied, using the Park Grass Long-term Experiment (PGE) at Rothamsted Research, UK. Some plots on the PGE have received chronic N additions, either as inorganic fertilisers or organic manures, for over 160 years which has led to the establishment of varied plant communities due to the selective pressures of eutrophication, and acidification. The pollinator communities and network were assessed by conducting transects through the growing seasons of 3 years, in addition to surveying the provision of floral units by the experimental plots. Nitrogen enrichment did impact on pollinator visitation; fewer pollinators were observed foraging in nitrogen enriched plots. Although overall flower abundance was unaffected by N addition, functional diversity of the floral community decreased regardless of whether N was added as inorganic fertiliser or manure. N addition also led to a decreased proportion of *Fabaceae*. Bees were particularly affected, the loss of floral functional diversity led to reduced bee abundances and more Diptera-dominant pollinator assemblages. The relatively high connectance found in all plots suggests a latent robustness of mesotrophic grassland plant-pollinator communities in the face of N pollution. The changes to floral and pollinator communities led to altered interaction networks that were less nested, an indication of lower resilience to species loss or stochastic events.

5.2 Introduction

5.2.1 Pollinating insects

It is estimated that 87% of global plant species rely on biotic pollination from flower-visiting animals (Ollerton, Winfree and Tarrant, 2011). In wild ecosystems, this service can help to maintain and support biodiversity and wider ecosystem function. Biotic pollination is also an important ecosystem service in agriculture; approximately 35% of agricultural produce, from 87 food crops, requires biotic pollination to some extent (Klein *et al.*, 2007). Overall, insects are the most prevalent and common pollinators (Ollerton, Winfree and Tarrant, 2011). Species of birds (Stiles, 1978), bats (Fleming, Geiselman and Kress, 2009), mammals (Goldingay, Carthew and Whelan, 1991), and reptiles (Valencia-Aguilar, Cortés-Gómez and Ruiz-Agudelo, 2013) also act as pollinators of some plants around the world, whether through adapted specialised interactions or through accidental pollination through movement. However next to insect-pollination, these are comparatively rare cases. The behaviours of many insects are closely tied with flowers; whether for territory or looking for mates, predators searching for insect prey, or, commonly, to feed on the rewarding sugar-rich nectar that is produced by the nectary glands of flowers. Bees, especially, are key pollinators for many wild plants and crops. Due to the need to forage pollen for their progeny, visiting flowers is a necessity for bees. Because of this close bond, they have adaptations that make them highly effective pollinators, such as hairy bodies with corbicula for collecting pollen. In turn, certain flowers have adapted floral morphologies and mechanisms to encourage the efficient pollination service that bees can supply. However, bees are not the only insect pollinators (Jauker and Wolters, 2008; Orford, Vaughan and Memmott, 2015; Hahn and Bruhl, 2016; Rader *et al.*, 2016). Many studies of pollinator communities focus only on bees, despite evidence that other insects can acts as pollinators, which can lead to a narrow and obscured view of actual pollinator communities. It is important to consider the broader picture when we study pollinator communities.

In recent decades, broad declines in insect pollinators have been recorded, although these trends are not uniform across taxa (Rader *et al.*, 2014; Powney *et al.*, 2019). The declines in pollinating insects are due to many, often interacting, factors (Goulson, Lye and Darvill, 2008; Potts *et al.*, 2010; Vanbergen and Iniative, 2013). Habitat loss, in particular, is a key driver of insect pollinator declines (Winfree, Bartomeus and Cariveau, 2011; Rader *et al.*, 2014; Senapathi *et al.*, 2015). The loss of floral resources constitutes a decline in food resources for pollinators, meaning that insects are not able to gain the nutrition they need to function and to reproduce. Studies show that insect pollinators that have a more narrow diet breadth typically are most vulnerable to habitat degradation and the loss of floral resources (Winfree, Bartomeus and Cariveau, 2011; Rader *et al.*, 2014; Powney *et al.*, 2019). Generalist insects that are flexible in their foraging behaviour and visit more generalist plants to support their diet are typically less threatened.

5.2.2 Structure of plant-pollinator networks

The interactions of plants and pollinators can be viewed as networks, specifically as mutualistic networks; as both plant and pollinator benefit from the interactions. It is in effect a food web, whereby the flower-visiting insects are feeding from plant material (Ings *et al.*, 2009). Therefore, plants can affect the pollinator community, through the provision of resources. More abundant and diverse floral communities, with a variety of floral niches, enhances pollinator abundance and diversity (Potts *et al.*, 2003; Bartomeus *et al.*, 2013; Vaudo *et al.*, 2015). Similarly, higher levels of pollinator diversity improves pollination services and can positively drive increases in plant diversity (Fontaine *et al.*, 2006; Woodcock *et al.*, 2019) and can improve the seed set and reproduction of plants in the community (Hoehn *et al.*, 2008; Albrecht *et al.*, 2012). Enhanced biodiversity, functional richness, and abundance of plants and pollinators generally increases the number of interactions in a network (Bascompte *et al.*, 2003; Vázquez and Aizen, 2004). This complexity promotes a more stable community, and so a more biologically-rich network is typically more resilient to losses, extinctions, or stochastic events (Memmott, Waser and Price, 2004). This is important, as research from agricultural landscapes has shown that plant-pollinator networks have the potential to be fragile without a stable structure (Pocock, Evans and Memmott, 2012).

The study of interaction networks has become more sophisticated, with the development of topographical metrics that describe the characteristics and structure of networks (Dormann *et al.*, 2009; Vázquez *et al.*, 2009; Blüthgen, 2010). The concept of specialisation versus generalisation is much debated, and recently doubted, in pollinator theory (Waser *et al.*, 1996; Fenster *et al.*, 2004; Ollerton *et al.*, 2009), although there is evidence to show that floral morphologies and traits can influence the identity of insect foragers (Fenster *et al.*, 2004; Rosas-Guerrero *et al.*, 2014). Plant-pollinator networks are typically far more specialised than seed disperser networks, for example (Blüthgen *et al.*, 2007; Stang, Klinkhamer and Van Der Meijden, 2007). Although there are cases of close-tied specialist interactions (Vázquez and Aizen, 2003), there are indications that true, strong specialisation is likely overestimated in plant-pollinator networks (Vázquez and Aizen, 2004; Petanidou *et al.*, 2008). Instead, plant-pollinator networks tend to exhibit asymmetric specialisation, i.e. specialists frequently interact with a subset of generalists in addition to more specialised individuals (Vázquez and Aizen, 2004). If the populations of food resources are stable across years, being a specialist pollinator can be beneficial (Waser *et al.*, 1996). Likewise, if populations of closely associated specialist pollinators are stable, plants can benefit from more selective pollinator visitation. However, if abundances vary across years and are liable to external pressures, in general it is safer to exhibit generalist behaviours or reproductive morphology (Waser *et al.*, 1996). In this way, flexible feeding behaviour and high plant diversity can buffer against impacts of global change, such as altered flowering phenology (Bartomeus *et al.*, 2013).

The structure of specialist insects interacting with both specialist and generalist plants, leads to high nestedness, another common feature of plant-pollinator networks (Bascompte *et al.*, 2003). Highly nested networks are typically more robust and resilient to species loss, due to the varied interactions and resources visited by specialist species (Burgos *et al.*, 2007; Almeida-Neto *et al.*, 2008). However, the extent of potential damage caused by lost species is dependent on that species connectance. While nested networks are robust against the loss of poorly-linked species, losses of species with many links in the network can be potentially catastrophic (Memmott, Waser and Price, 2004; Vázquez *et al.*, 2009). Higher levels of overall connectance throughout the network typically denotes better network stability in the face of stochastic events and loss of species richness, as more alternative feeding resources are utilised by the upper trophic level (Gilbert, 2009). Although network metrics, such as these, are useful and descriptive, they are liable to manipulation or errors through rarely occurring species or lack of sampling effort (Jordano, 1987; Blüthgen, Menzel and Blüthgen, 2006; Blüthgen *et al.*, 2008; Petanidou *et al.*, 2008). An observation of a single unique interaction can be mistakenly identified as a specialist interaction, even if the interaction occurs between two generalists (Jordano, 1987). This can distort the estimation of specialisation of networks and as a result, true specialisation is often overestimated (Petanidou *et al.*, 2008). Furthermore, each appearance of a new species causes connectance metrics to decrease, as the proportion of all possible links is now fewer (Jordano, 1987). On the other hand, nestedness tends to increase with species richness (Bascompte *et al.*, 2003; Vázquez and Aizen, 2004).

Interaction networks are a useful tool for identifying the impacts of global environmental change (Tylianakis *et al.*, 2008; Burkle and Alarcon, 2011). Interactions helps support biodiversity, and typically proffer a better insight into ecosystem function, stability, and services than species richness. The functioning and stability of real-world ecosystems is built around interacting organisms, not species that are isolated from each other. By studying interaction networks, we can see the impacts to species richness, but also to the function of ecosystems ,how species interact with each other, whether losses of food resources are occurring, and we can also identify if networks become hinged on interactions that if lost will severely impact the assemblage (Tylianakis, Tscharntke and Lewis, 2007). Given current pressures of drivers of global environmental change (Tylianakis *et al.*, 2008), the use of interaction networks offer a more insightful view of how drivers can impact our ecosystems in the future (Burkle and Alarcon, 2011).

5.2.3 Nitrogen as a driver of environmental change

Nitrogen (N) deposition, which globally has escalated steeply since the industrialisation of the mid-19th Century (Galloway *et al.*, 2004; Dentener *et al.*, 2006; Fowler *et al.*, 2013), and direct applications of N to soils can impact on botanical communities (Stevens *et al.*, 2004; Bobbink *et al.*, 2010; Field *et al.*, 2014; Storkey *et al.*, 2015). The most prevalent mechanism is though soil eutrophication and altered competition dynamics (Bobbink, Hornung and Roelofs, 1998; Bobbink *et al.*, 2010). Typically, competitive grasses take advantage of the increased soil nutrition and exclude slower-growing and smaller species, in effect causing declines in many flowering forbs and legumes (Crawley *et al.*, 2005). This is, in effect, a floral deterioration of the landscape, with foraging resources for pollinating insects being lost. Habitat loss is regarded as a key driver of pollinator declines (Vanbergen and Iniative, 2013; Rader *et al.*, 2014; Harrison and Winfree, 2015), but the contribution of plant community responses to eutrophication is poorly understood. Plants with more specialised floral structures were observed to decline more with N additions (Chapter 2). Floral morphology can be a significant predictor of pollinator identity for many plants (Lázaro, Hegland and Totland, 2008), with many deeper flowers often providing more rewarding nectars (Klumpers, Stang and Klinkhamer, 2019). Therefore, it’s likely that specialist insects are most threatened by N-deposition, as is also the case with habitat loss (Winfree, Bartomeus and Cariveau, 2011; Rader *et al.*, 2014). As such, N-pollution should be considered a contributor to the degradation of pollinator habitats.

Beyond the impacts to botanical composition, there is evidence of soil N causing variation in the expression of floral traits that can be key in attracting insects and supplying them with nutritional benefits (David, Storkey and Stevens, 2019), such as phenology (e.g. Cleland *et al.*, 2007), nectar quantity and quality (e.g. Gijbels, Van den Ende and Honnay, 2014), and morphology (e.g. Hoover *et al.*, 2012). Despite the numerous knowledge gaps in our understanding of how N pollution impacts on plant-pollinator interactions and pollinator communities, this subject is gaining consideration as a potential driver (Ings *et al.*, 2009; Harrison and Winfree, 2015; David, Storkey and Stevens, 2019). Incorporating our understanding of N-pollution impacts to botanical communities into pollinator research can help to improve our knowledge of pollinator trends and give insight to targeted conservation means or preventative strategies.

There are very few studies that have incorporated pollinators into studies of N-driven impacts on floral traits, and these have contrasting results. Burkle and Irwin

(2010) found that low additions (10 kg N ha^{-1} yr^{-1}) of N to soil in experimental plots in the Rocky Mountains actually promoted forb growth and flower production, which enhanced pollinator visitation. Positive effects, however, were not observed with 20 kg N ha^{-1} yr^{-1} additions. The field site used by the authors received low background N deposition, just over 4 kg N ha^{-1} yr^{-1} (Burkle and Irwin, 2010). The results suggest that low N deposition levels currently being experienced by some regions in the Southern Hemisphere could in fact benefit floral production of temperate and montane grasslands. However, these positive effects will diminish with rising N deposition in the near future, and with further escalating levels impacts could become detrimental (Phoenix *et al.*, 2006; Bleeker *et al.*, 2011). The N deposition levels experienced by many regions in the Northern Hemisphere are currently far in excess of the low, 10 kg N ha^{-1} yr^{-1}, N treatment level used by Burkle and Irwin (2010), so the positive effects reported by the authors likely do not apply to grasslands in these regions. Although low N additions caused some potentially beneficial responses, other studies found N addition detrimentally impacted the potential for nectar resources to support bumblebee colonies (Hoover *et al.*, 2012; Ceulemans *et al.*, 2017). Artificially-made nectar that was representative of *Cucurbita pepo* nectar produced under N-enrichment was more attractive to bumblebees than nectar representative of no N additions, but it led to increased mortality of workers (Hoover *et al.*, 2012). Furthermore, another study of nutrient enrichment on *Succisa pratensis* and bumblebees found that colonies feeding on nutrient-enriched plants had more dead larvae during the colonies' early-mid lifespan (Ceulemans *et al.*, 2017).

Research into the effects of N on plant-pollinator interaction networks are limited and findings have thus far been inconclusive. Burkle and Irwin (2009; 2010) found that, although floral abundance and pollinator visitation were linked and affected by N additions, network structure and the core plants and pollinators were not. However, the experimental N-addition was only applied during the three years of sampling. Therefore, the study could only monitor immediate changes to a heterogenous pollinator community caused by immediate responses to N applications and was not able to explore the impact of chronic N increases. Studies of long-term N-applications can give a better insight of the effects of N deposition and chronic N pollution (Ings *et al.*, 2009). .

5.2.4 Aims of the study

For this study, I used the Park Grass Long-Term Experiment (PGE) at Rothamsted Research, UK, to investigate the effects of chronic N additions on pollinator communities and plant-pollinator networks. The PGE has received consistent N treatment applications for over 160 years, so reveals the impacts of long-term N-enrichment on plant communities. Although the experimental plots are located within the same field site, so have the same local pollinator community, the botanical communities and floral resources differ strongly between plots due to the long-term treatments. Therefore, we can predict how high levels of N deposition could selectively narrow down pollinator assemblages in real-world scenarios when scaled up to the landscape scale. As N-enriched plots typically have lower species richness and functional richness of important floral resources (Crawley *et al.*, 2005; Silvertown *et al.*, 2006), I hypothesised that 1) the flower-visiting insect community will be less species-rich and with fewer specialised pollinators such as bumblebees. Furthermore, that due to the loss of specialist floral resources, 2) the nestedness and complexity of plant-pollinator networks will decline with N addition. Investigating how pollinator communities and plant-pollinator interaction networks are affected by N additions can help inform management and conservation efforts in the future, with global N deposition rising and pollinators facing numerous stress-inducing factors.

5.3 Materials and methods

5.3.1 Park Grass Long-Term Experiment

The study used the Park Grass Long-Term Experiment (PGE), of which a full description is given in Chapter 2 (section 2.3.1). Sampling all 101 plots of PGE is not feasible, so a subset of study-plots was selected. In Europe, atmospheric N deposition is becoming more NO_3-dominant (Dentener *et al.*, 2006), and the impacts of NO_3 to floral traits are more mixed than the impacts of NH_4, which makes predicting the impacts of NO_3 addition to pollinators more difficult. Therefore, this chapter focused on the experimental plots receiving NO_3 application to identify if this form of N-enrichment affected plant-pollinator interactions and pollinator communities. A problem with conducting experiments on the PGE is the lack of plot replication, the experiment was designed prior to modern statistical theory, and so there is no replication of treatment. To account for this, researchers must use temporal replication and search for hidden replicates by using treatments as blocking factors. For this study, I used subplots with different topsoil pH and plots receiving mineral applications to create more replicates with which to study the impacts of N addition (Table 5.1; Fig. 5.1).

Table 5.1. Study plots used for pollinator transect sampling and the studied treatments for each plot.

Organic manure is a rotation of farmyard manure (supplying c. 240 kg N ha^{-1} yr^{-1}, 45 kg P ha^{-1} yr^{-1}, 350 kg K ha^{-1} yr^{-1}, 25 kg Na ha^{-1} yr^{-1}, 25 kg Mg ha^{-1} yr^{-1}, 40 kg S ha^{-1} yr^{-1}, 135 kg Ca ha^{-1} yr^{-1}) and poultry manure (supplying c. 65 kg N ha^{-1} yr^{-1}), applied every 2 years on rotation. For mineral application, "+" is applied and "-" is not applied. Mineral application is 225 kg K ha^{-1} yr^{-1}, 15 kg Na ha^{-1} yr^{-1}, 10 kg Mg ha^{-1} yr^{-1}, 122 kg S ha^{-1} yr^{-1}, 17 kg P ha^{-1} yr^{-1} (in 2016)/35 kg P ha^{-1} yr^{-1} (in 2017 and 2018, to account for P accumulation).

Plot	N application	pH	Mineral application
3b	Nil	6.1	-
3d	Nil	5.1	-
17b	NO_3 48 kg N ha^{-1} yr^{-1}	6.2	-
17d	NO_3 48 kg N ha^{-1} yr^{-1}	5.8	-
7/2b	Nil	6.1	+
7/2d	Nil	5	+
16b	NO_3 48 kg N ha^{-1} yr^{-1}	6	+
16d	NO_3 48 kg N ha^{-1} yr^{-1}	5.6	+
14/2b	NO_3 96 kg N ha^{-1} yr^{-1}	6.3	+
14/2d	NO_3 96 kg N ha^{-1} yr^{-1}	6.1	+
13/2b	Organic manure	6	-
13/2d	Organic manure	5.2	-

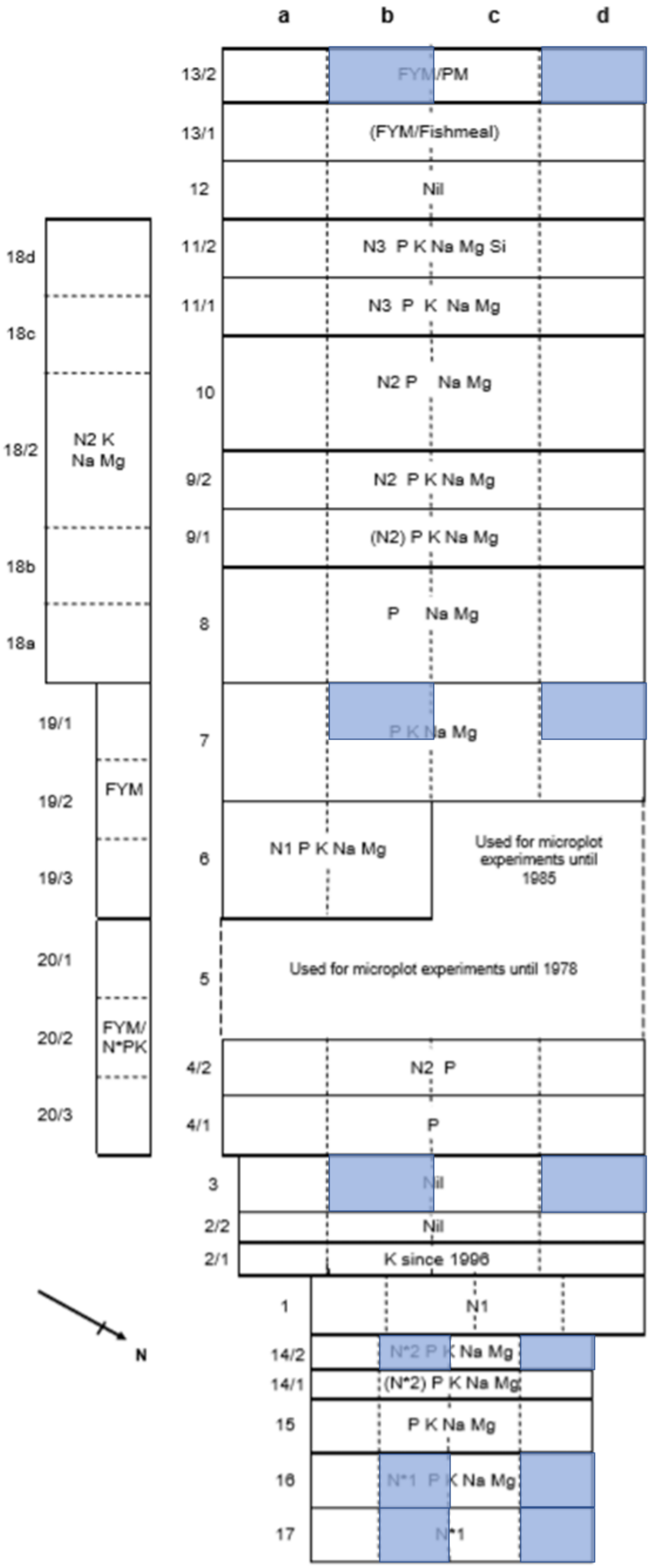


Figure 5.1. The Park Grass Experiment, with chapter 5 study plots clearly shaded.
Shaded plots show the PGE plots where plant-pollinator interactions were recorded.

5.3.2 Pollinator transect sampling

Pollinator transects were conducted in the spring, summer, and autumn of 2016, 2017, and 2018. Transects were chosen as the sampling method as they more accurately show the true network structure, are time efficient, and are the optimum method for grassland meadow habitats (Gibson *et al.*, 2011). I conducted the pollinator transects by walking the perimeter of a plot, recording each interaction between an insect and flower within two metres of the plot's edge, as recommended by Gibson *et al.* (2011). Only insects feeding from the nectar or contacting the sexual parts of the flower were recorded. The sizes of the PGE study plots are not equal. The smallest study plots, 14/2d and 14/2b, have a perimeter of 34.64 m, to compensate I only surveyed 34.64 m of all the PGE study plots per transect. Insects were caught and frozen for identification under microscope. Bees were identified to species level; other insects were identified to family level. The plant species visited was also recorded.

Each year's sampling commenced when all the study plots were in flower, with replicate transects taken during the growing season until the PGE cut date at the end of June, when the vegetation on all plots was cut. Pollinator transect sampling then continued when the regrowth brought each study plot back into flower, typically early August, until the final cut in October. Each sampling day was split into three blocks: morning (08:00–11:00), midday (12:00–15:00), afternoon (16:00–18:00), to account for the effect of time of day on the active pollinator community. All plots were studied within a single day, with four plots in each block. Sampling bouts were therefore grouped into three full sampling days, within which each plot was sampled three times, once in each block. The three sampling days were conducted within a week when the climatic conditions were similar across all three days. I aimed to conduct a group of three sampling days approximately every two weeks throughout the growing period. Overall, I made eight of these bouts in 2016 and 2017, and nine bouts in 2018 (Table 5.2). This amounted to 24 transects per plot in 2016 and 2017, and 27 transects per plot in 2018; a total of 75 transects per plot over the three years.

Pollinator transects were carried out in suitable weather, using the insect pollinator sampling protocol of the national pollinator and pollination monitoring pilot as a guide (Carvell *et al.* 2016). Transects were conducted in a minimum of 13° C, and in temperatures between 13 – 17° C, cloud cover was to be <50%. When possible, sampling days took place when wind speed was below Beaufort Scale 5 (mean wind speed 10 m s^{-1}). However, this was not possible in extended windy periods, the maximum mean wind speed during a sampling day was 15 m s^{-1}. The blocking structure of conducting transects across three days took this into account, and so wind speed was similar across plots within a sampling bout.

Table 5.2. The sampling dates for pollinator transect sampling.
The dates show when pollinator transects were conducted. Within each period, the blocked structure of three transects per plot were conducted.

Month	2016	2017	2018
April	28/04 – 06/05	10/04 – 12/04 29/04 – 02/05	18/04 – 20/04
May	19/05 – 23/05	13/05 – 16/05 22/05 – 26/05	07/05 – 08/05 08/05 – 09/05
June	07/06 – 09/06 30/06 – 03/07	07/06 – 11/06	06/06 – 08/06 13/06 – 15/06
August	12/08 – 17/08 26/08 – 31/08	06/08 – 10/08 14/08 – 16/08	05/08 – 07/08 30/08 – 02/09
September	13/09 – 14/09	22/09 – 25/09	07/09 – 10/09
October	14/10 – 17/10		10/10 – 10/10

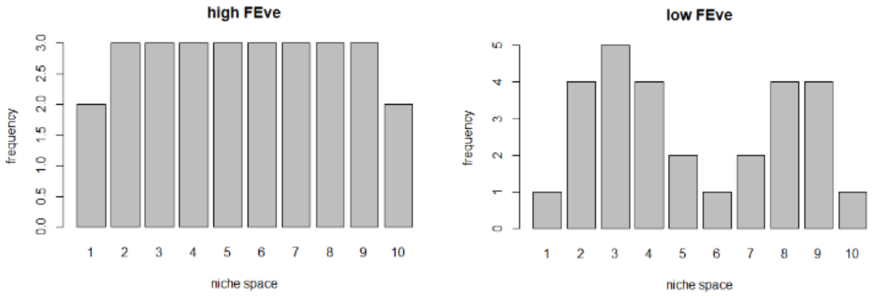
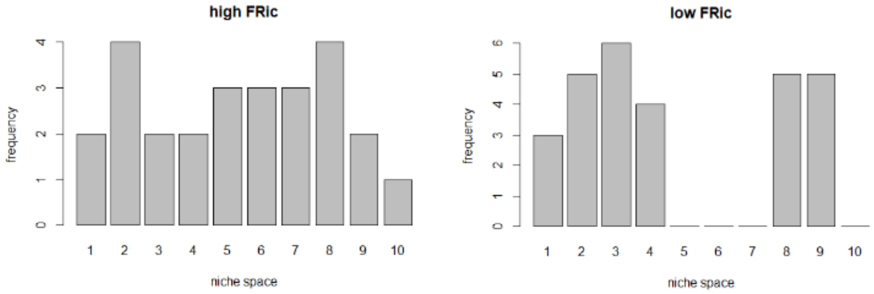
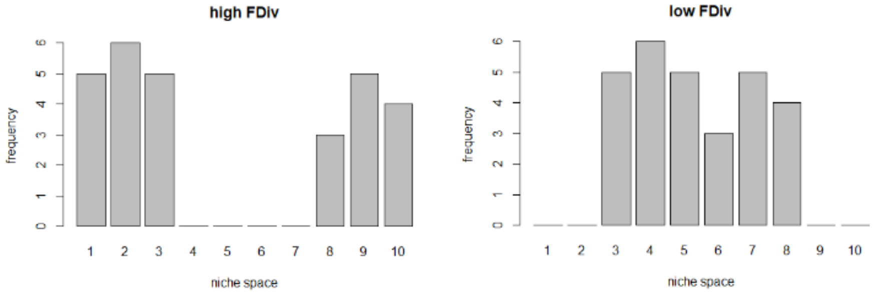
5.3.3 Floral quadrat survey

For each bout of three pollinator transect surveys, the floral community of the PGE study plots was also assessed. This was done with 0.25 m^2 quadrats, 20 quadrats were thrown along the surveyed transect route in each study plot. For each quadrat I counted the number of floral units of each plant species. Trait data was used to compute metrics that described the floral community of each plot, Rao's quadratic entropy, functional divergence, functional evenness, and functional richness. Trait data was acquired from the following sources: floral colour, typical insect visitors, and pollinator syndrome morphology from the BIOFLOR database (Klotz *et al.* 2002), nectar traits from the Agriland database (Baude *et al.*, 2016), flowering phenology and floral size measurements trait data was collected from direct measurements in PGE.

5.3.4 Statistical analysis

All statistical analyses was conducted using R Studio (R Core Team, 2018), with graphics produced using the ggplot2 (Wickham, 2016) and bipartite (Dormann, Gruber and Fründ, 2008) packages. The transects resulted in a large dataset of observations. To study the impact to the pollinator community, this was compiled into summary statistics with each data point representing a study plot within a year. With the floral quadrat data and traits information, the following indices of functional diversity were calculated: Rao's quadratic entropy (Rao, 1982; Botta-Dukát, 2005), functional divergence (Fdiv), functional evenness (FEve), and functional richness (FRic) (Mason *et al.*, 2005) (Table 5.3); these metrics integrate the contribution of multiple traits and are weighted by species abundance. The functional diversity indices were calculated using the 'FD' package in R Studio (Laliberté *et al.* 2010, 2014). The mean abundance of floral unit per quadrat and the proportion of *Fabaceae, Apiaceae, Asteraceae, Ranunculaceae,* and *Lamiaceae* floral units was also calculated for each plot and year. These five plant families were the most dominant and common forb and legume families throughout the PGE plots. The impacts of treatments on the floral community were tested with ANOVA, the explanatory variables were N treatment (categorical; nil, NO_3, organic farmyard manure), N amount (continuous; 0-96 kg N ha^{-1} yr^{-1}), mineral application (categorical; not applied, applied, applied as organic farmyard manure), and pH (continuous; 5-6.3).

Table 5.3. The functional diversity indices used for the analyses.
The four indices are defined, and illustrative theoretical examples are given for functional evenness, richness, and divergence.

Index	Definition
Functional evenness (FEve)	The evenness of trait expression within niche space. High FEve occurs where a functional trait is evenly distributed across values through niche space. Low FEve occurs where specific trait expressions are proportionally more common. 
Functional richness (FRic)	The richness of expressions of a functional trait. High FRic occurs when niche space is broadly occupied, low FRic when fewer values of niche space are filled. 
Functional divergence (Fdiv)	The divergence between functional trait expressions in niche space of a community. High FDiv occurs when traits are distantly separated across niche space, low FDiv when traits occupy a closely grouped area of niche space. 
Rao's quadratic entropy	Rao's index combines an index of species diversity with measurements of species distinctiveness (effectively the mean Euclidian distance between the functional traits of species). It is a measure of dissimilarity between species and is weighted by abundance and diversity.

All explanatory variables (N treatment, N amount, pH, mineral addition, and all floral indices) and response variables of the insect pollinator community were used to form a factor analysis of mixed data (FAMD) plot, to identify explanatory and response variables that could be grouped. The FAMD plot was constructed and analysed using the 'FactoMineR' package (Le *et al.* 2008). FAMD is a principal component analysis that can analyse data of quantitative and qualitative variables simultaneously. This is unlike principal component analyses (PCA) or multiple correspondence analyses (MCA) which study only quantitative and qualitative variables, respectively. Variables are scaled and normalised to allow balanced analysis of the variables. This method of analysis is useful for studying datasets composed of mixed variables and for graphically portraying the relationships and grouped associations between the variables. A MANOVA was also used to give further evidence of linked groups and identify drivers of the response variables. Linear mixed-effects models, using the lme4 package (Bates *et al.* 2015), were used to determine the direction and magnitude of the identified explanatory variables for each response variable. Year was incorporated as a random effect to account for the effect of year on insect abundances. These models were reduced with stepwise simplification, removing the least significant terms and checking model assumptions at each step to achieve the final simplified model.

To study the structure of the plant-pollinator interaction networks the raw dataset was converted to quantitative interaction matrixes, one for each plot within a year. The bipartite package in R Studio (Dormann, Gruber and Fründ, 2008; Dormann *et al.*, 2009) was used to calculate the following networks metrics for each interaction network: "nestedness metric based on overlap and decreasing fill" (NODF) (Almeida-Neto *et al.*, 2008; Almeida-Neto and Ulrich, 2011), weighted NODF, connectance, weighted connectance, links per species, Alatalo interaction evenness, the number of compartments, and H2' (Table 5.4). Null models of each interaction network were generated and the network metrics of these were compared to the true observed network. The null model approach can help to correct for network distortions caused by network size or infrequent interactions (Vázquez and Aizen, 2003; Dormann, 2011; Gibson *et al.*, 2011). The 'r2d' null model method holds the observed values for rows and columns but allows the number of links to vary with null model creation. I used r2d null models for connectance, weighted connectance, links per species, and the number of compartments, as these metrics describe the quantity and strength of interactions. The ‘swap’ null model method is more conservative, holding the row and column values in addition to the number of interactions. This method was used to create null models for NODF, weighted NODF, Alatalo interaction evenness, and H2’. For each null model, 1,000 iterations were generated, and these were used to determine if the real networks differed from null models in a way that was statistically significant. The difference between the null models and true observed networks for each network metric was calculated – this difference is known as the D-Value (Vázquez and Aizen, 2003). The bottom-up robustness of the pollinator community for each observed network, of a plot within a year, was also calculated using the bipartite package (Dormann, Gruber and Fründ, 2008) in RStudio. The robustness function models extinction cascades following the loss of species; with 1,000 iterations, plant species were removed systematically beginning with the one with least abundance, and the impact on pollinator species richness was calculated. The robustness score was also used as a response variable to test for the impacts of the nutrient and pH treatments. To analyse how the treatments impacted network metrics, I used linear mixed effects models, incorporating N (categorical; NO_3 and Organic), N amount (continuous; 0–96), and pH (continuous; 5.1–6.3) as fixed effects, and year (categorical; 2016, 2017, 2018) and minerals (categorical; applied or not) as a random effect. Linear models were used to investigate the interaction between N treatment and year. The network metrics were response variables, explanatory variables were N treatment, year, and the interactive term. For all linear mixed effects models and linear models described, I simplified the models using stepwise simplification, dropping the least significant term and checking the model assumptions at each step. Post hoc Tukey tests were utilised to check the accuracy of significant comparisons.

Table 5.4. Network metrics used for plant-pollinator network analysis. Metrics are defined and the suitable null model method stated.

Network metric	Definition	Null model method
Connectance	The proportion of all possible interactions that are observed (Dunne, Williams and Martinez, 2002).	R2d
Weighted connectance	Useful for quantitative webs. The metric is weighted by the strength of interactions (Dunne, Williams and Martinez, 2002).	R2d
Links per species	The mean amount of links per species node.	R2d
Number of compartments	Compartments are isolated subsets within the network, not connected to another compartment.	R2d
NODF	A more consistent index for nestedness (Almeida-Neto *et al.* (2008, 2011).	Swap
Weighted NODF	A quantitative NODF metric that takes network strengths into account (Almeida-Neto *et al.*, 2008; Almeida-Neto and Ulrich, 2011).	Swap
Alatalo interaction evenness	An index of evenness that is suitable for bipartite networks and handles outliers well (Alatalo, 1981; Muller *et al.*, 1999).	Swap
H2'	A measure of specialisation ranging from 0 (no specialisation) to 1 (complete specialisation) .	Swap

5.4 Results

5.4.1 Summary statistics of the insect community

Across the 3 years of pollinator transects, encompassing 75 transects for each of the 12 plots, 5076 interactions were observed between plants and flower-visiting insects (Appendix 2; Appendix 3). Overall, Diptera were the most numerous taxonomic order at 3145 observations (61.9%), followed by Hymenoptera at 1569 (30.9%); 1115 of which were bees (21.9%). The composition and abundance of insects in the insect communities was not uniform across plots (Table 5.6). Neither was the data uniform across years (Table 5.5). The year had a significant effect on the abundances of insects observed, with far fewer insects observed visiting flowers in 2018; notably the reduced abundance led to lower taxonomic richness. However, the composition of the observed flower-visiting community was more stable across years, as there was no significant effect of year on the proportions of Diptera and bees (Fig. 5.2). The sampling season of 2018 was notably hotter and drier than 2016 and 2017 (mean temperature, with standard errors, from 1st March–31st September was 14.54 ± 0.31° C in 2016, 14.35 ± 0.28 ° C in 2017, and 15.4 ± 0.3 ° C in 2018. Mean rainfall, with standard errors, from 1st March–31st September was 1.71 ± 0.28 mm in 2016, 1.9 ± 0.3 mm in 2017, and 1.39 ± 0.27 mm in 2018).

Table 5.5. Output of ANOVA of the impact of sampling year on insect pollinator observations and community.

Response variables for which study year was a significant factor are indicated by * p<0.05; ** p<0.01; *** p<0.001.

	df	F-value	P-value
Total abundance	2	5.82	0.0069 **
Insect family richness	2	5.68	0.0076 **
Total bees	2	6.23	0.0051 **
Total Bombus	2	3.38	0.046 *
Total Apis	2	7.56	0.0020 **
Total solitary bees	2	7.59	0.0019 **
Total Diptera	2	3.40	0.045 *
Total Syrphidae	2	6.41	0.0044 **
Total wasps	2	8.94	0.00079 ***
Total sawflies	2	11.88	0.00013 ***
Total Coleoptera	2	2.42	0.10
Total Lepidoptera	2	2.43	0.10
Proportion bees	2	0.16	0.86
Proportion Diptera	2	0.24	0.79
Bee species richness	2	4.80	0.015 *
Unique interactions (all-family)	2	7.48	0.0021 **
Unique interactions (bees-species)	2	5.90	0.0064 **

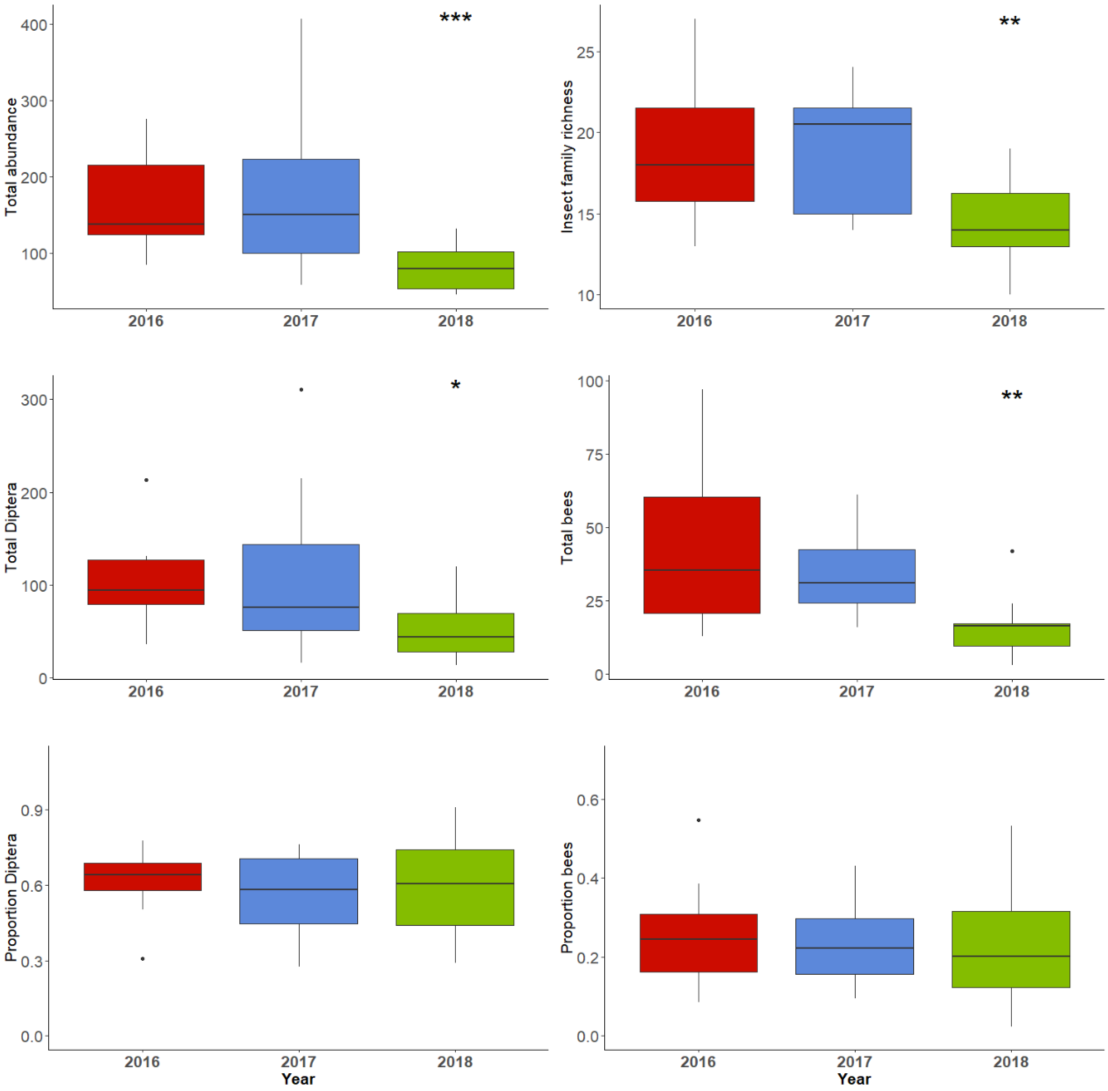


Figure 5.2. Aspects of the observed insect community across the 3 study years. Significantly different years are indicated by * p<0.05; ** p<0.01; *** p<0.001.

Table 5.6. Summary statistics of the insect communities of each plot, with mean values across three study years.

	3b	**3d**	**17b**	**17d**	**7b**	**7d**	**16b**	**16d**	**14b**	**14d**	**13b**	**13d**
Overall abundance	214.00	139.00	137.67	115.00	398.67	96.00	332.33	110.67	418.67	171.33	118.00	183.00
Insect family richness	22.33	20.33	19.67	14.00	16.33	12.67	18.00	14.33	18.67	17.33	17.33	18.33
Bee species richness	13.33	9.33	8.67	9.00	9.00	8.33	8.33	8.33	9.00	6.67	7.67	10.00
Unique interactions (family)	45.67	40.00	37.00	26.33	35.00	21.67	32.00	27.33	32.33	31.00	32.67	37.33
Unique bee interactions (species)	22.00	14.67	9.67	10.33	15.33	13.00	11.00	10.67	11.00	10.50	10.33	12.67
Bee abundance	65.00	20.67	16.00	18.67	50.67	37.67	38.33	25.67	30.00	17.00	28.00	24.67
Bombus abundance	42.67	10.67	4.67	4.67	35.67	27.33	22.00	14.00	14.33	3.67	12.67	12.00
Apis abundance	13.67	5.00	3.00	3.67	6.67	5.33	5.00	3.67	4.00	2.00	2.67	1.33
Solitary bee abundance	8.67	5.00	8.33	10.33	8.33	4.33	11.33	8.00	11.67	11.33	12.67	11.33
Diptera abundance	113.33	92.67	94.33	86.33	317.00	42.00	268.00	81.67	369.33	138.00	72.33	125.00
Syrphidae abundance	25.33	20.00	23.33	18.67	17.00	4.33	13.33	9.67	15.33	15.67	15.33	17.00
Wasps abundance	2.67	2.67	0.67	0.33	2.00	0.00	3.33	1.00	4.33	3.00	0.33	1.67
Sawflies abundance	22.33	11.00	6.33	2.00	18.00	4.00	12.00	3.00	9.00	5.00	6.00	11.67
Coleoptera abundance	5.33	7.67	16.67	6.67	11.33	4.67	6.33	6.00	4.33	6.33	7.67	14.00
Lepidoptera abundance	4.00	5.67	3.67	1.00	0.33	7.67	1.33	0.00	0.33	2.00	3.33	3.00
Proportion bees	0.34	0.21	0.14	0.23	0.14	0.39	0.10	0.22	0.07	0.09	0.24	0.14
Proportion Diptera	0.47	0.57	0.64	0.65	0.79	0.42	0.83	0.72	0.88	0.81	0.62	0.66

5.4.2 Floral assay of botanical communities

The N treatments impacted the floral composition of the plant communities (Table 5.7). NO_3-enrichment led to reduced species richness, but not organic farmyard manure additions did not. Both forms of N addition, NO_3 and organic farmyard manure decreased the functional divergence and richness of the floral communities (Fig. 5.3). Plots receiving NO_3 or organic manure treatments also had smaller proportions of *Fabaceae* but larger proportions of *Ranunculaceae*. The N treatments did not significantly affect other aspects of the floral community. The relative proportions of *Apiaceae* and *Asteraceae* were also similar between control and N- or manure-treated plots. Mineral treatments also affected floral communities (Table 5.7), leading to floral communities that were less functionally rich, less taxonomically rich, and with higher proportions of *Fabaceae* and *Ranunculaceae* flowers alongside lower proportions of *Asteraceae* and *Lamiaceae* flowers (Fig. 5.4). Other aspects of floral community were unaffected by mineral treatments.

Table 5.7. Output of ANOVA of the effect of N treatments and mineral treatments on the floral community of the study plots.

Significant factors are indicated by . p<0.1; * p<0.05; ** p<0.01; *** p<0.001.

	N treatment			**Mineral treatment**		
	df	F-value	P-value	*df*	F-value	P-value
FDiv	2	12.62	0.000085 ***	2	3.11	0.058 .
FEve	2	1.18	0.32	2	0.43	0.96
FRic	2	5.62	0.0079 **	2	17.65	0.000061 ***
Rao's QE	2	1.41	0.26	2	1.83	0.18
species richness	2	6.20	0.0052 **	2	10.4	0.00031 ***
floral abundance	2	0.06	0.94	2	2.60	0.090 .
% Fabaceae	2	10.76	0.00025 ***	2	3.61	0.038 *
% Asteraceae	2	1.55	0.23	2	15.10	0.000022 ***
% Apiaceae	2	0.20	0.82	2	0.86	0.43
% Ranunculaceae	2	14.97	0.000024 ***	2	3.43	0.0044 *
% Lamiaceae	2	1.28	0.29	2	6.11	0.0055 **

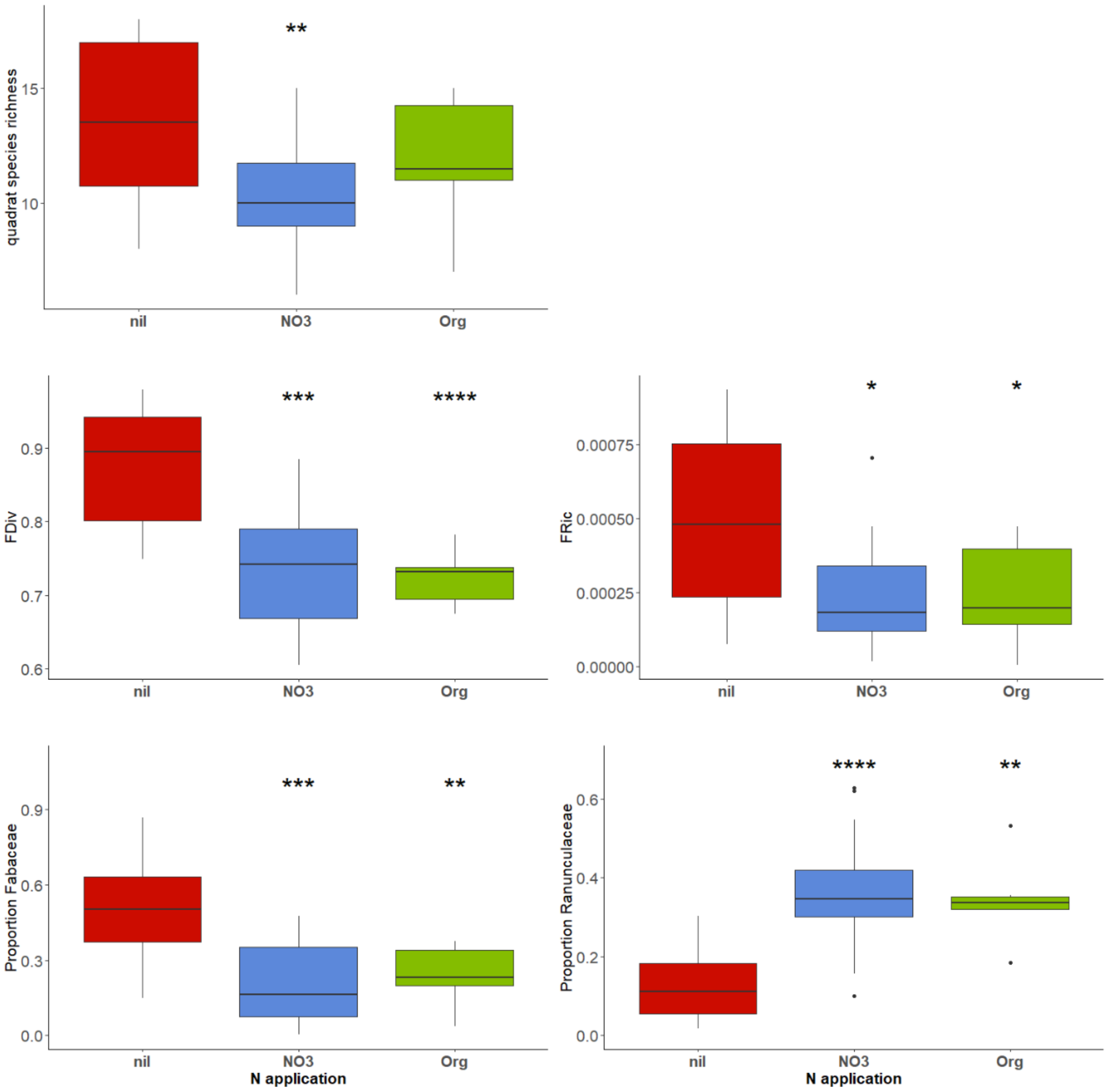


Figure 5.3. The significant effects of NO_3 and organic (Org) treatments on the floral community of the study plots, contrasted with nil N.

Treatments that are significant different from control are indicated by * p<0.05; ** p<0.01; *** p<0.001.

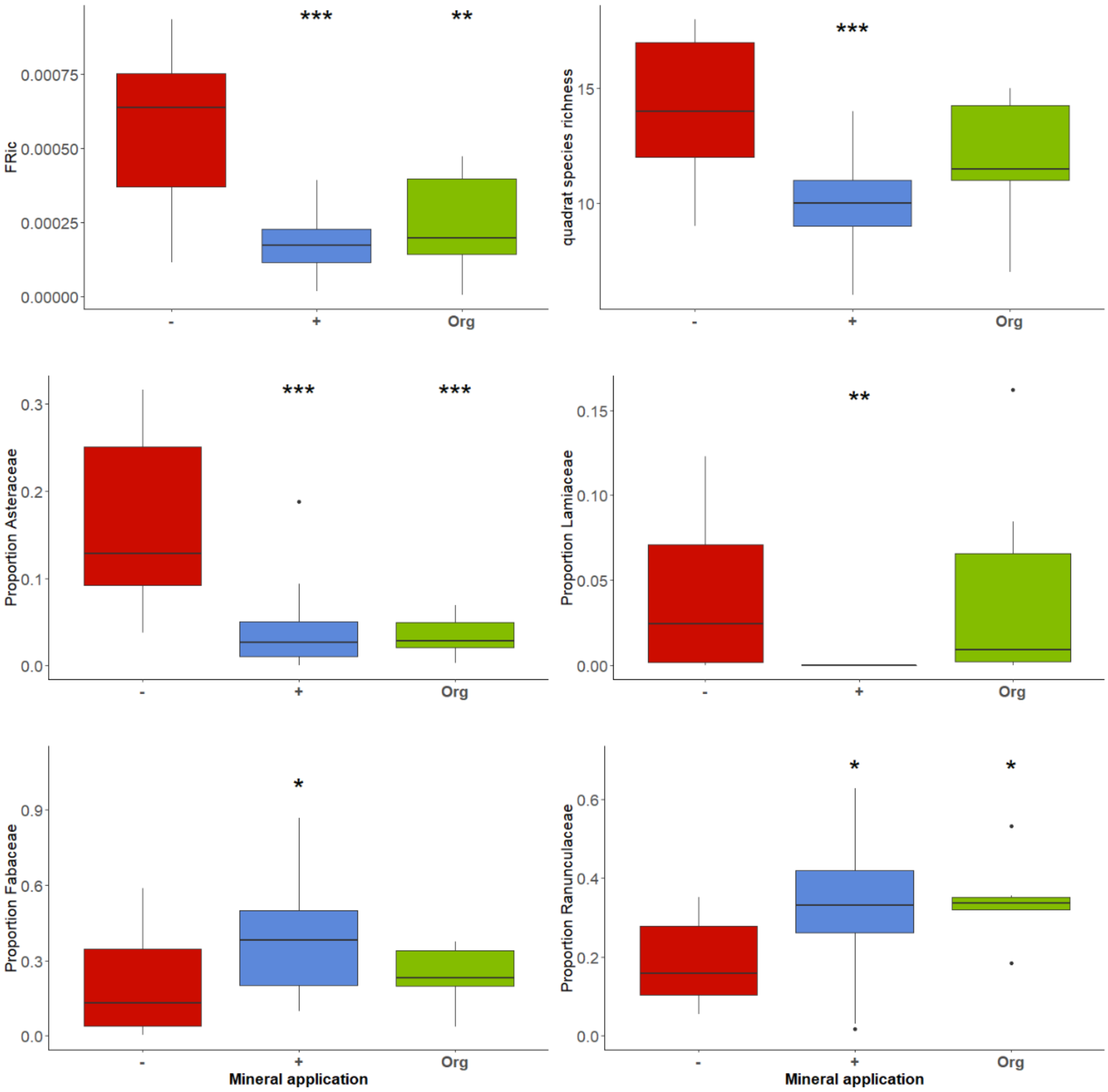


Figure 5.4. The significant effects of mineral and organic (Org) treatments on the floral community of the study plots, contrasted with nil minerals.

Treatments that are significant different from control are indicated by * p<0.05; ** p<0.01; *** p<0.001.

5.4.3 Multivariate analysis of insect community

The first two dimensions of the FAMD explained 42.8% of the variation of the data and revealed a clear separation in the floral and pollinator communities of the study plots across years, with nil plots separated from NO_3, which overlapped slightly with the manure-treated plots (Fig. 5.5). When studying the grouped response and explanatory variables, certain aspects of the floral and pollinator communities are revealed to be grouped (Fig. 5.6). N amount, the proportion of *Ranunculaceae* flowers, and the proportion of Diptera were well grouped, with the proportion of *Apiaceae* acting in the same direction but contributing less. In opposition is the strong grouping of the proportion of *Fabaceae*, the proportion of bees, and two indices of floral functional diversity; Rao's quadratic entropy and functional divergence, with functional evenness contributing in the same direction but with less magnitude. Close to this group is the total abundance of Lepidoptera, the floral functional richness, plant species richness, and the proportion of *Lamiaceae* which contributes less but in the same direction. The total number of bees, bumblebees, honeybees, bee species richness and unique bee interactions are also tightly-grouped. Other variables, such as the abundance of Diptera, wasps, sawflies, solitary bees, and insect family richness are loosely grouped around the overall total abundance of insects in a plot. Many of the described groups were confirmed by the MANOVA analysis (Appendix 4).

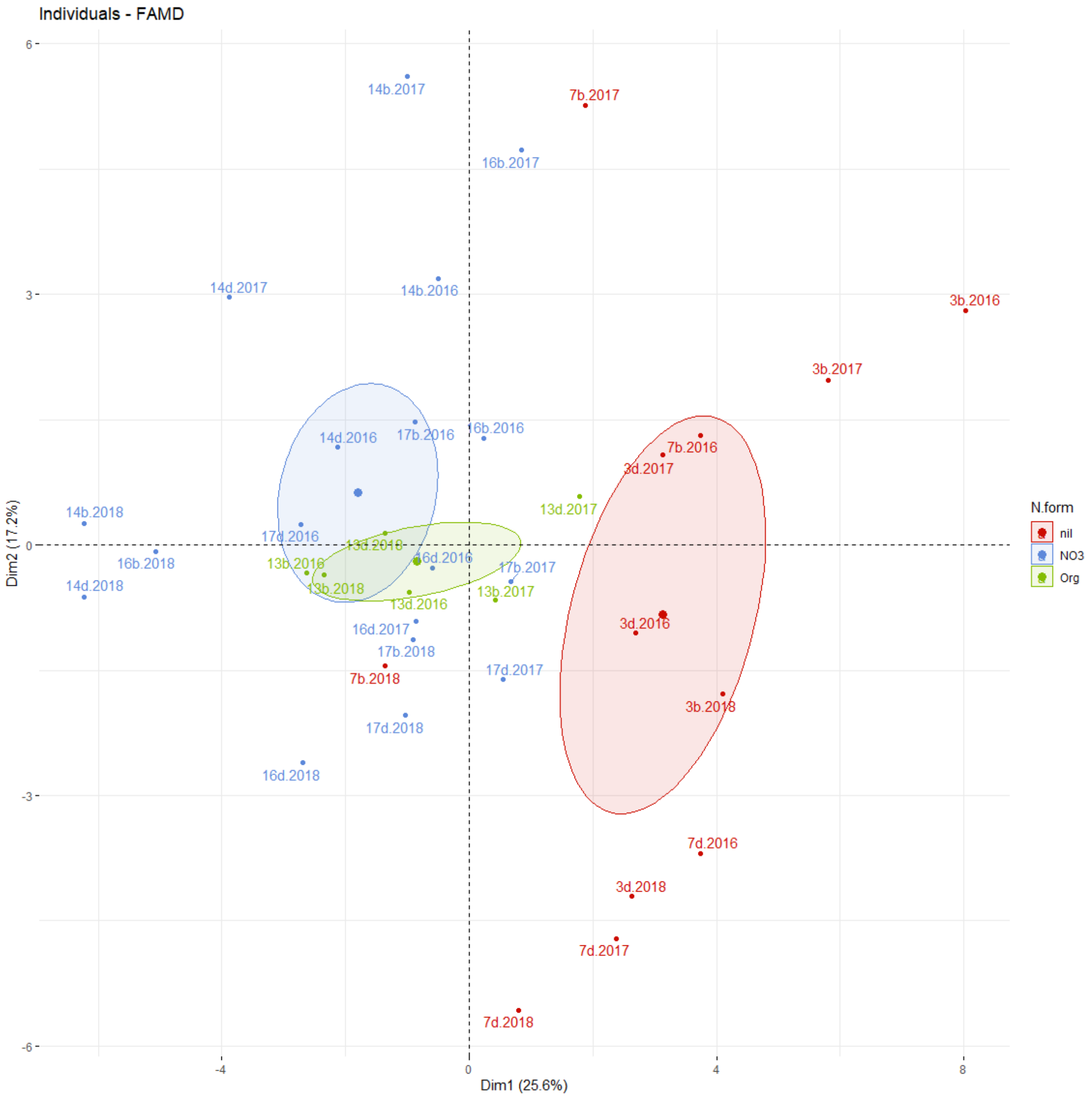


Figure 5.5. FAMD correspondence analysis plot of individual plant-pollinator networks of plots for each year, grouped by N treatment.

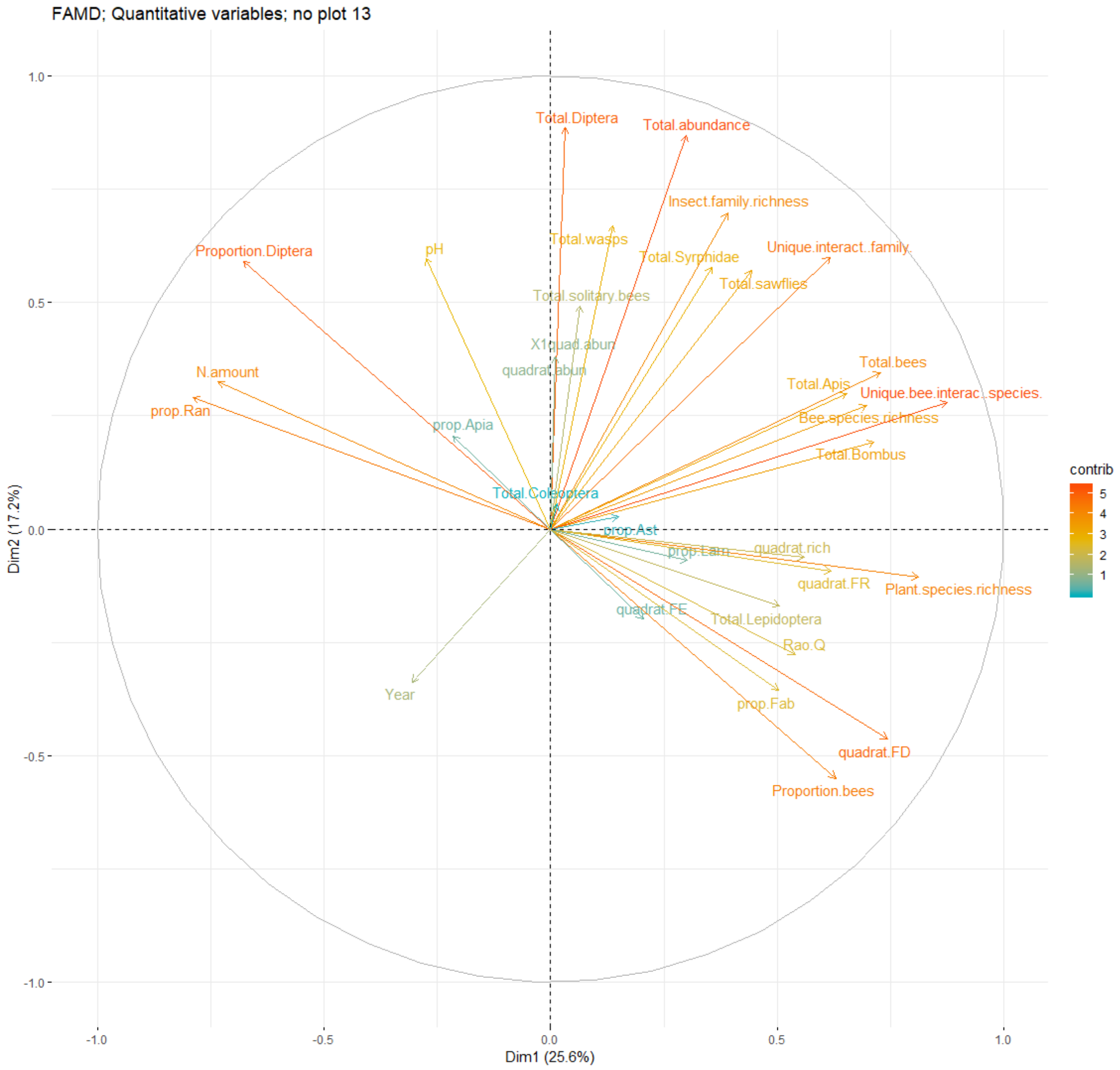


Figure 5.6. FAMD correspondence analysis plot of the quantitative variables, showing direction and contribution towards the data's variation.

The results of the univariate mixed-effects models, with year as a random factor, showed the effect sizes and significant factors influencing the insect pollinator community (Table 5.8). N additions, in the NO_3 form, decreased the overall abundance of flower-visiting insects and the number of taxonomically unique interactions observed. In particular, NO_3-enrichment led to declines in lepidopterans and bees, of which bumblebees and honeybees noticeably declined, and also declines in the number of unique interactions involving bees. With greater levels of NO_3 addition, the pollinator community shifted further towards being Diptera-dominant. Organic manure applications also led to fewer honeybees and fewer unique interactions involving bees. Despite this finding, the abundance of solitary bees increased in plots receiving either NO_3 or organic manure. Sawfly abundance decreased with NO_3 and with organic manure applications. With higher pH soils, several aspects of the insect community increased; overall insect abundance, overall bee, bumblebee, Diptera, and *Syrphidae* abundances, and the number of unique interactions. Several significant effects were caused by the floral community, independent of N additions. Higher functional divergence of the floral resources increased bee species richness and the amount of unique bee interactions. It also caused a shift in the community towards bees becoming proportionally more prevalent and Diptera less so. Functional evenness, functional richness, and Rao's quadratic entropy did not register significant effects on the pollinator community. Plant communities producing more floral units had higher overall bee visitation, with more solitary bees and also more wasps and sawflies. Higher proportions of *Fabaceae* led to higher proportions of bees, potentially driven by increased bumblebee abundance, in addition to fewer sawflies. The proportion of *Apiaceae* was positively linked with the proportion of Diptera, while the proportions of *Asteraceae, Lamiaceae* and *Ranunculaceae* were not significant factors.

Table 5.8. Output of LMER models studying the impacts of treatments and botanical explanatory factors on response variables of the pollinator communities.

All presented values are statistically significant (t > 2) effect sizes given by the final simplified models ('+' denotes positive effects, '-' denotes negative effects). FDiv is the Functional divergence of floral community, fl abun is floral abundance. %Fab, %Apia are the proportion of *Fabaceae, and Apiaceae.*

	NO$_3$	Org	N amount	pH	Minerals	FDiv	fl abun	%Fab	%Apia
Overall abundance	-55.85			+72.9					
Insect family richness					-2.86				
Bee species richness						+10.91			
Unique interactions (family)	-6.92			+6.98	-7.92				
Unique bee interactions (sp.)	-5.22	-3.75		+3.71		+17.7			
Bee abundance	-18.68			+23.62			+76.58		
Bombus abundance	-11.7			+15.11				+44.23	
Apis abundance	-4.11	-5.67							
Solitary bee abundance	+3.76	+5.73					+1.78		
Diptera abundance				+102.71	+69.33				+246.56
Syrphidae abundance				+6.03	-9.58				
wasp abundance							+1.08		
sawfly abundance	-13.3	-9.47					+5.98	-20.98	
Coleoptera abundance									
Lepidoptera abundance	-2.06								
Proportion bees			-0.001			+0.59		+0.27	
Proportion Diptera			+0.002			-1.21			

Table 5.9. D-values of network metrics: NODF, weighted NODF, connectance, weighted connectance, links per sp., Alatalo interaction evenness, number of compartments, and H2'. Statistical significance between null and real models is shown by * p<0.05; ** p<0.01; *** p<0.001.

Plot	**NODF**		**W. NODF**		**Connectance**		**W. Connectance**	
	D-value	Sig	D-value	Sig	D-value	Sig	D-value	Sig
3b.16	4.753		9.466	***	0.063	***	0.065	***
3b.17	3.815		5.272		0.094	***	0.053	***
3b.18	7.899		10.648	**	0.038	***	0.061	***
3d.16	-4.539		0.282		0.032	***	0.033	**
3d.17	-0.014		11.856	**	0.039	***	0.026	***
3d.18	1.050		0.659		0.041	***	0.041	***
17b.16	3.183		6.354		0.041	***	0.054	***
17b.17	1.385		1.495		0.083	***	0.077	***
17b.18	2.650		1.857		0.094	***	0.071	***
17d.16	-0.047		2.549		0.127	***	0.085	***
17d.17	2.033		4.774		0.090	***	0.067	***
17d.18	4.485		1.630		0.057	***	0.046	**
7b.16	9.838		17.885	***	0.124	***	0.103	***
7b.17	2.350		12.384	**	0.103	***	0.069	***
7b.18	-0.653		10.340	*	0.133	***	0.110	***
7d.16	11.242	*	15.696	**	0.108	***	0.100	***
7d.17	-0.989		12.380	***	0.062	***	0.027	
7d.18	9.013	***	6.647		0.098	***	0.096	***
16b.16	0.023		7.189		0.092	***	0.073	***
16b.17	-3.586		5.657		0.187	***	0.082	***
16b.18	11.344	*	17.496	**	0.009		0.045	***
16d.16	-11.377	*	4.863		0.122	***	0.063	***
16d.17	4.582		7.823	*	0.178	***	0.114	***
16d.18	-6.032		1.536		0.083	***	0.072	***
14b.16	0.075		5.283		0.070	***	0.055	***
14b.17	-4.504		-3.208		0.171	***	0.063	***
14b.18	-0.330		12.915		0.080	**	0.058	**
14d.16	-12.866	***	2.248		0.093	***	0.037	***
14d.17	8.736	*	9.904	*	0.073	***	0.055	***
14d.18	12.341	**	14.871	*	0.059	*	0.072	*
13b.16	-3.634		-3.446		0.084	***	0.070	***
13b.17	-2.809		7.247	*	0.125	***	0.096	***
13b.18	-6.073		11.252	*	0.108	***	0.093	***
13d.16	-8.864	**	2.099		0.110	***	0.062	***
13d.17	-0.652		6.003	*	0.122	***	1.500	***
13d.18	6.474		13.284	**	0.114	***	1.259	***

Table 5.9. (continued).

Plot	**Links per sp.**		**Alatalo**		**No. compart**		**H2’**	
	D-value	Sig	D-value	Sig	D-value	Sig	D-value	Sig
3b.16	0.489	***	0.133	***	0.039		-0.263	***
3b.17	0.691	***	-0.019		-0.869	*	-0.053	
3b.18	0.240	***	0.236	***	0.111		-0.232	***
3d.16	0.249	***	0.069		0.542		-0.041	
3d.17	0.331	***	0.031		0.187		-0.109	*
3d.18	0.267	***	0.011		-0.740		-0.013	
17b.16	0.263	***	0.196	***	0.208		-0.235	***
17b.17	0.480	***	0.100	*	0.078		-0.084	*
17b.18	0.425	***	0.020		0.079		-0.032	
17d.16	0.476	***	0.224	***	0.038		-0.321	***
17d.17	0.526	***	0.039		0.089		-0.042	
17d.18	0.305	***	0.009		0.172		0.007	
7b.16	0.762	***	0.212	***	-0.984	***	-0.366	***
7b.17	0.650	***	0.072		0.039		-0.286	***
7b.18	0.634	***	0.224	**	-0.969	***	-0.212	***
7d.16	0.572	***	0.156	*	0.046		-0.388	***
7d.17	0.371	***	-0.029		0.276		-0.014	
7d.18	0.487	***	0.083		-2.760	***	-0.109	*
16b.16	0.489	***	0.189	***	0.013		-0.442	***
16b.17	1.081	***	0.008		0.023		-0.085	
16b.18	0.034		0.099	***	-0.887	**	-0.507	***
16d.16	0.664	***	0.133	**	0.000		-0.166	***
16d.17	0.832	***	0.045		0.000		-0.088	*
16d.18	0.366	***	0.093		0.145		-0.082	
14b.16	0.441	***	0.112	***	0.001		-0.425	***
14b.17	0.796	***	-0.003		0.000		-0.071	
14b.18	0.248	**	0.092	*	-0.996	***	-0.635	***
14d.16	0.425	***	0.186	***	0.000		-0.279	***
14d.17	0.335	***	0.041		0.025		-0.141	
14d.18	0.182	*	0.144	**	-0.914	***	-0.240	**
13b.16	0.463	***	0.029		0.200		-0.028	
13b.17	0.722	***	0.130	***	0.047		-0.090	**
13b.18	0.494	***	0.074		0.096		-0.164	***
13d.16	0.718	***	0.151	**	0.039		-0.149	***
13d.17	0.653	***	0.222	***	0.564		-0.142	***
13d.18	0.612	***	0.182		0.581	*	-0.057	*

5.4.4 Network structure

Barring a few exceptions, the connectance, weighted connectance, and links per species of the observed plant-pollinator networks differed from the null models (Table 5.9). Fewer plant-pollinator networks differed from the null models with regards to the other metrics. The D-values as response variables reveal the impacts that the N treatments and pH had upon the network structure. The NO_3 treatment decreased weighted NODF, with fewer links per species at higher N application levels (Table 5.10). Organic farmyard manure treatments decreased the unweighted NODF. There was a greater number of unlinked compartments in networks of NO_3-enriched plots. Bee-plant interaction networks illustrate the increased compartmentalisation vividly, as shown by visually comparing networks from 17b (48 kg N ha^{-1} yr^{-1}, pH 6) and 3b (nil N, pH 6) (Fig. 5.7). Higher soil pH increased the evenness of networks and led to less specialised networks. Although the connectance of most networks was significantly different from null models, there was no difference across plots caused by the N treatments. Although nestedness was influenced by N treatments, there was no statistically significant impact to the robustness of the pollinator community across all years.

Table 5.10. The output of LMER models studying the impacts of the treatments on the D-value of network metrics.

All presented values are statistically significant ($t > 2$) effect sizes given by the final simplified models ('+' denotes positive effects, '-' denotes negative effects).

	NO_3	**Organic**	**N amount**	**pH**
Connectance				
Weighted connectance				
NODF		-6.367		
Weighted NODF	-4.362			
Links per species			-0.001	
Alatalo interaction evenness				+0.047
Number of compartments	+0.375			
H2'				-0.129
Robustness				

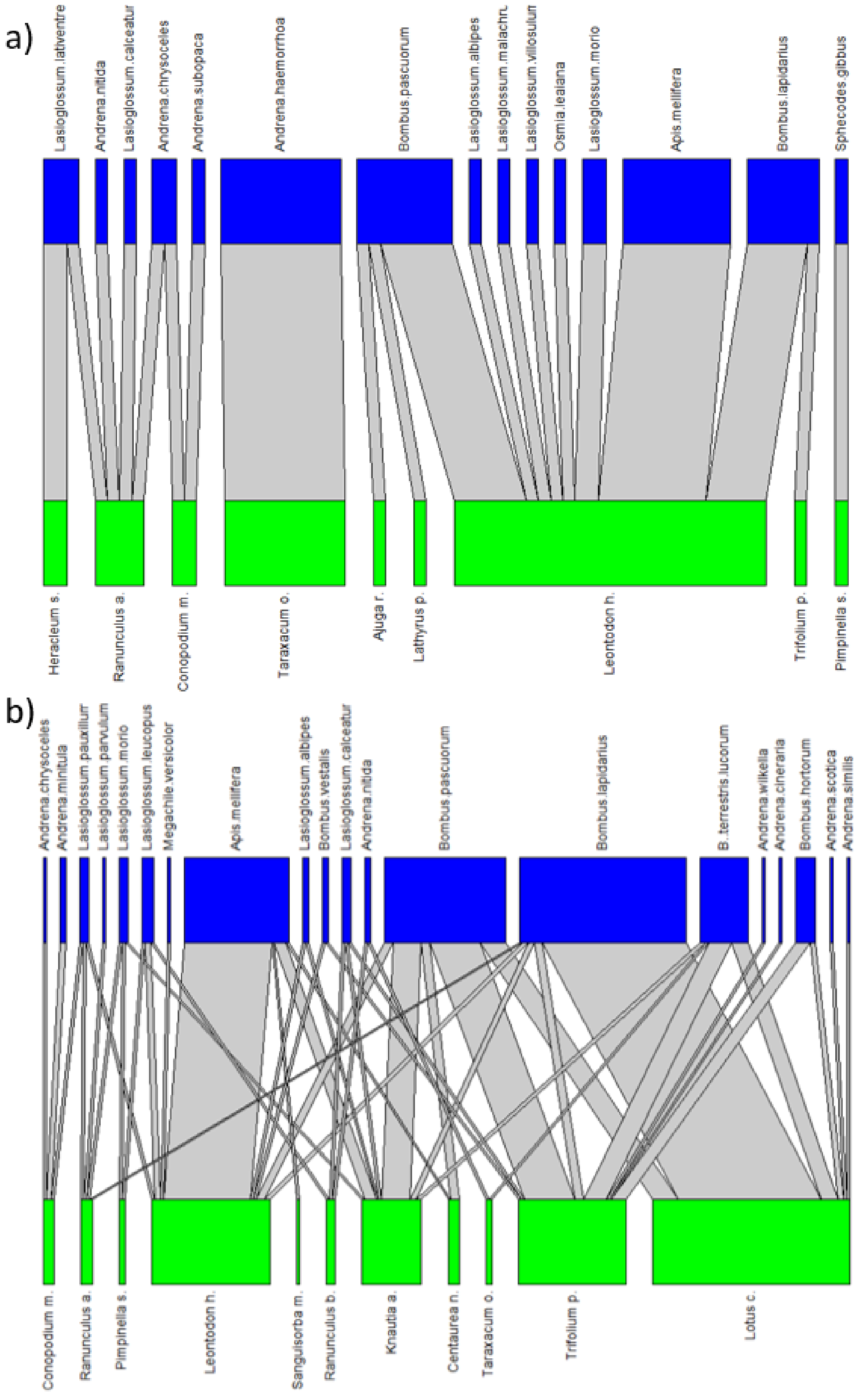


Figure 5.7. Bee-plant interaction networks of a) plot 17b (48 kg N ha^{-1} yr^{-1}) and b) plot 3b (nil N), across all years.

Bee species, identified to species, presented on the top, plant species on the bottom. Links portray the weighted interactions between insects and plants. 17b network obs. = 48, 3b network obs. = 195.

Year was a significant factor for the links per species, the Alatalo evenness, the robustness of pollinator communities, and the H2 metrics, N treatment alone was not a significant factor. There were significant interactions between Year and N treatment for Alatalo evenness and the robustness of pollinator communities, and a near-significant interaction on the weighted connectance of networks (Table 5.11). Post hoc Tukey tests showed that the robustness of NO_3- and organic-associated pollinator communities was significantly lower in the hot, dry summer of 2018 (Fig. 5.8). The structure of plant-pollinator networks of NO_3 plots were more susceptible to annual variation; there were significant differences between years for Alatalo interaction evenness (Fig. 5.9), and links per species (Fig. 5.10). Post hoc Tukey tests did not reveal significant differences for any network metrics between years for nil plots.

Table 5.11. The ANOVA output showing the impact of N treatment, year, and the interactive term on network metrics.

Significant factors are indicated by . p<0.1; * p<0.05; ** p<0.01; *** p<0.001.

	N treatment			**Year**			**N treatment*Year**		
	df	F-value	P-value	df	F-value	P-value	df	F-value	P-value
NODF	2	2.36	0.11	2	1.8	0.19	4	0.96	0.45
weighted NODF	2	1.91	0.17	2	0.87	0.43	4	2.03	0.12
connectance	2	1.16	0.33	2	2.75	0.082 .	4	1.09	0.38
weighted connectance	2	1.75	0.19	2	0.1	0.9	4	2.64	0.055 .
links per sp	2	1.4	0.26	2	6.58	0.0047 **	4	0.95	0.45
Alatalo evenness	2	0.01	0.99	2	9.62	0.0007 ***	4	3.13	0.031 *
Cluster coefficient	2	1.98	0.16	2	3.01	0.066 .	4	1.78	0.16
H2	2	0.98	0.39	2	3.58	0.042 *	4	0.77	0.55
Pollinator robustness	2	0.59	0.56	2	15.4	0.000034 ***	4	3.6	0.018 *
Network robustness	2	1.88	0.17	2	2.54	0.09 .	4	0.93	0.46

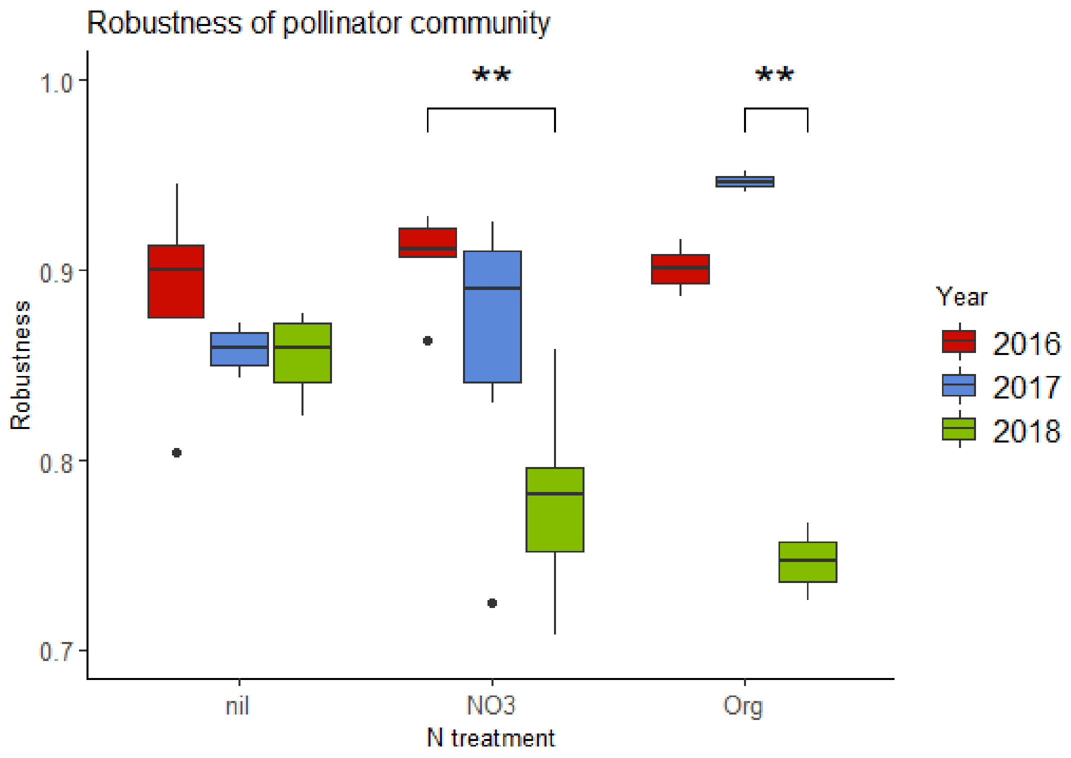


Figure 5.8. The robustness of pollinator communities, shown across years within treatment groups; nil, NO_3 treatment, organic treatment.

Statistically significant comparative differences, from post hoc Tukey tests, are indicated by * p<0.05; ** p<0.01; *** p<0.001.

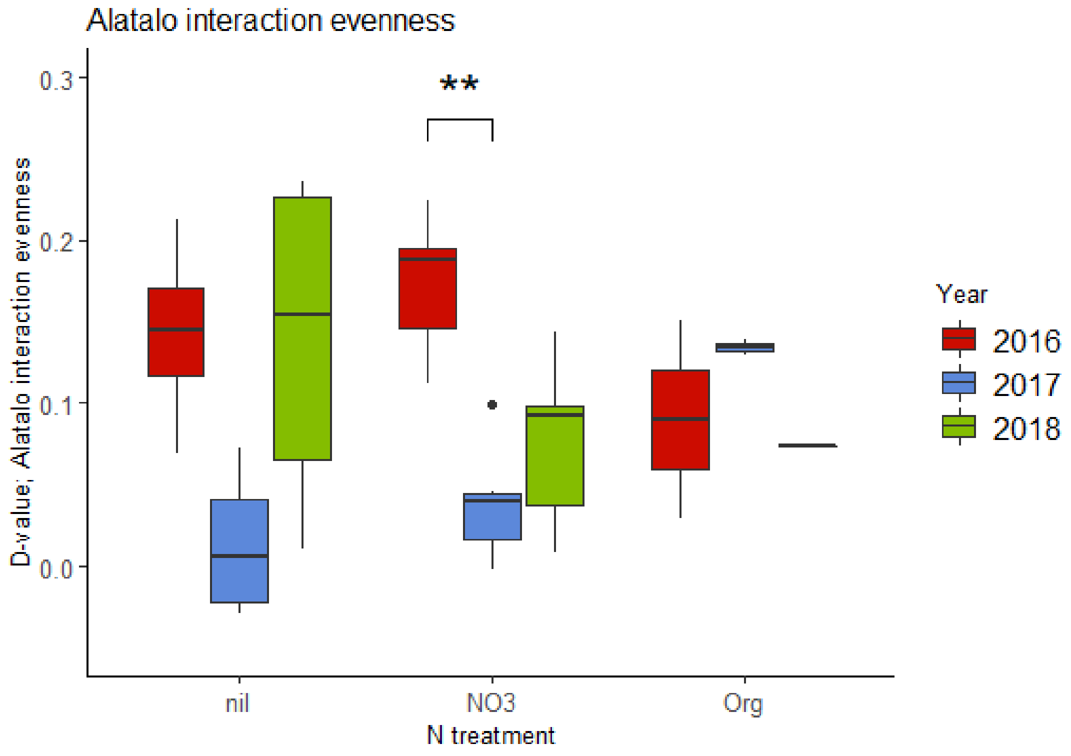


Figure 5.9. The Alatalo interaction evenness of plant-pollinator networks, shown across years within treatment groups; nil, NO_3 treatment, organic treatment.

Statistically significant comparative differences, from post hoc Tukey tests, are indicated by * p<0.05; ** p<0.01; *** p<0.001.

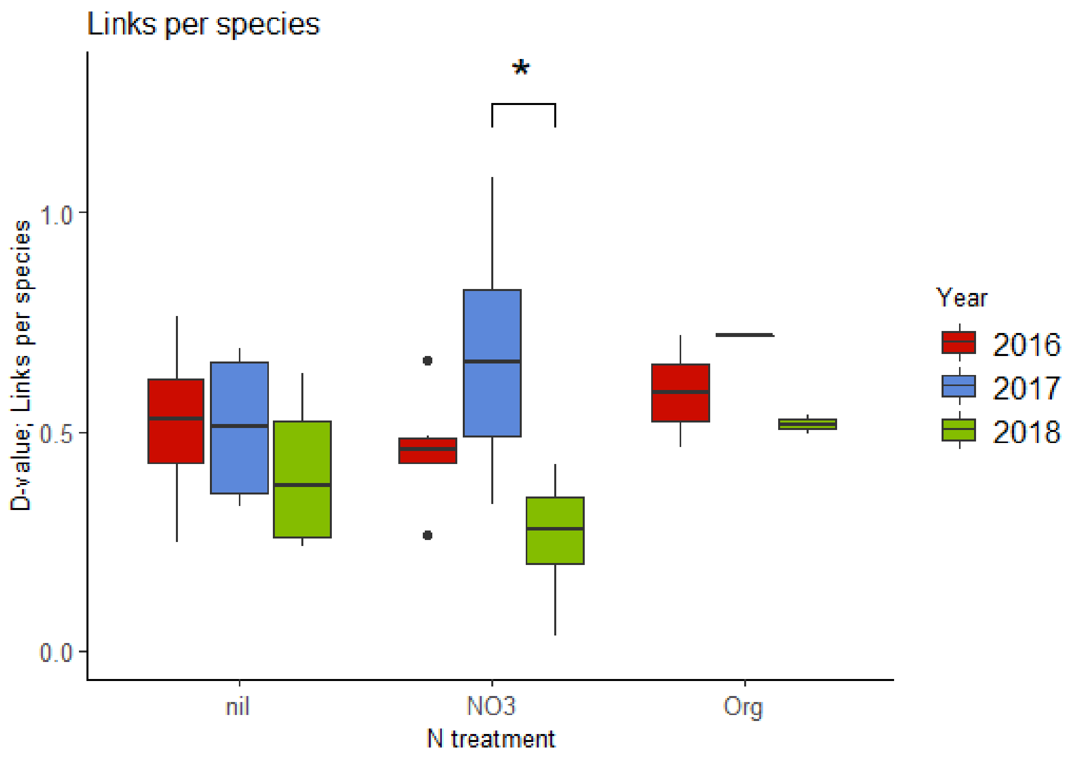


Figure 5.10 The links per species of plant-pollinator networks, shown across years within treatment groups; nil, NO_3 treatment, organic treatment.
Statistically significant comparative differences, from post hoc Tukey tests, are indicated by * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

5.5 Discussion

5.5.1 Pollinator abundance

The results of the mixed-effects models revealed that chronic addition of N to the PGE mesotrophic grassland experimental plots has created plant communities which are, overall, less attractive to the pollinator community. These experimental plots are representative of temperate grassland communities undergoing very high N deposition, such as some regions in Europe, Eastern US, China and India, (Galloway *et al.*, 2004; Dentener *et al.*, 2006) and so these findings indicate that N deposition and applications might have a role in the declines of insect pollinators and other flower-visiting insects. Plant communities in PGE receiving NO_3 applications had fewer insect flower-visitors overall. Increased pollinator visitation can occur with higher floral production (Burkle and Irwin, 2010; Viik *et al.*, 2012), but in PGE, N additions did not lead to an increase in overall floral abundance, so there is likely an alternative mechanism drawing in pollinators. Chronic NO_3 applications did alter the functional diversity of the floral community in PGE, decreasing functional divergence and richness, while also decreasing species richness and the proportion of *Fabaceae*, which could explain the decreased pollinator visitation. The relationship between floral diversity and insect abundance has been recorded in other studies of grassland sites (Potts *et al.*, 2003). With higher levels of functional divergence and richness, more floral niches are available which means a wider potential assemblage of insects could be attracted to foraging from the floral community. Furthermore, flowers with more specialised floral structures have frequently been shown to have more sucrose-rich nectar (Wykes, 1952; Southwick, Loper and Sadwick, 1981; Petanidou, 2005). The loss of these rewarding floral niches and their potential for specialist interactions likely played a role in decreasing the abundance of insects visiting N-enriched plots. Alongside the N-driven loss in floral functional diversity and abundance of insects was a reduction in the number of unique interactions observed. The loss of unique interactions is likely very closely linked with these two factors. With greater diversity of organisms and interactions comes enhanced community resilience (Okuyama and Holland, 2008), so the decline in interaction-richness associated with N applications could increase the sensitivity of these networks to stochastic events. Indeed, the robustness of NO_3 plant-pollinator networks decreased greatly during the warmer, drier 2018 season. Unique interactions were also lower with mineral application, as was insect family richness. As with N additions, applications of multiple limiting nutrients can cause competitive plants to thrive and outcompete other plants, leading to loss of botanical diversity (Harpole *et al.*, 2016). In PGE, mineral application did not affect the functional divergence or functional evenness of the floral communities but did significantly reduce the functional richness (Fig. 5.4). Therefore, there are likely fewer functional niches of floral structures and pollinator syndromes for the insect community to visit. Clearly the impact of high additions of nutrients to mesotrophic grasslands that are naturally adapted to a reasonable level of nutrient-limitation can have negative impacts on the pollinator community. The abundance of insect foragers, of bees and Diptera, and the number of unique interactions both declined with soil acidification, too. Given the propensity of N to acidify soil (Stevens *et al.*, 2011; Fowler *et al.*, 2013; Goulding *et al.*, 2015), this adds another dimension to the potential impact of unchecked N deposition and applications to soils with poor buffering capacity. Given the decline in abundances of insect pollinators (Goulson, Lye and Darvill, 2008; Vanbergen and Iniative, 2013; Powney *et al.*, 2019), alongside increasing N deposition (Galloway *et al.*, 2004; Dentener *et al.*, 2006), further studies into this correlation are perhaps important to determine the impact of N to better inform conservation efforts.

5.5.2 Pollinator community composition.

The foraging of bees in experimental plots was closely grouped with various aspects of the floral community, according to the factorial analysis (Fig. 5.6). Increases in floral niches with higher functional divergence and richness of flowers will likely favour bee richness, due to their possessing more specialised feeding apparatus than dipterans. Previous studies have shown strong links between the diversity of floral resources and more species-rich and abundant bee communities (Potts *et al.*, 2003), and the results from the PGE support this. Furthermore, bumblebees are typically more attracted to florally rich areas, and can fly preferentially towards more floristically diverse and abundant patches (Jha and Kremen, 2013). Therefore, if wild pollinators selectively forage from patches with enhanced floral diversity plant communities that suffer more with N deposition, such as those in poorly-buffered soils prone to acidification, could see reduced pollination services and impoverished local pollinator community. The proportion of *Fabaceae*, in particular, was closely linked to the proportion of bees and the abundance of bumblebees. *Fabaceae*, and other flowers with deep corollas, are typically sucrose-rich (Wykes, 1952; Southwick, Loper and Sadwick, 1981; Petanidou, 2005). Bumblebees have been shown to favour this nectar trait (Cnaani, Thomson and Papaj, 2006) and the protein-rich pollen (Carvell, 2002). With declines in *Fabaceae*, it is unsurprising that N-enrichment decreased the abundances of bumblebee visitation.

The results of the mixed-effects models revealed that with NO_3-enrichment, although there were fewer bumblebees and honeybees, and fewer bees overall, there were in fact more solitary bees. This was also true for experimental plots receiving organic manure; solitary bee visitation was higher. Solitary bees are wild pollinators that can contribute greatly towards pollination services of a wide range wild plants and crops (Breeze *et al.*, 2011; Ollerton *et al.*, 2012; Garibaldi *et al.*, 2014). The increased visitation from these important pollinators with NO_3 and organic manure enrichment may seem positive, but a closer look at the bee communities by species reveals that the increase in solitary bee activity is not uniform across the season and was in fact heavily compartmentalised (Fig. 5.7). There is a significant boost to a single species, the early-season *Andrena haemorrhoa* (orange-tailed mining bee), visiting *Taraxacum officinale agg.* (Fig. 5.11). This heavily-weighted interaction dominated the solitary bee assemblages in the N- and manure-enriched plots such as 17b (Fig. 5.7). Throughout the remainder of the season the abundance of solitary bees in the 17b network is no greater than that found in control plots. The weight of this interaction between a common early *Andrena* species and competitive early-flowering plant could be caused by vigorous growth of *T. officinale* in the early season, able to take advantage of increased soil fertility prior to the increased grass vigour that occurs from mid-Spring. Other studies have found that the flowers can bloom earlier with N-enrichment (Cleland *et al.*, 2006; Hoover *et al.*, 2012; Xi *et al.*, 2015). The phenological study of PGE also found that NO_3-enrichment led to a florally improved early season with increased *T. officinale* flowering duration (Chapter 3). This is a clear potential benefit to early-emerging pollinators, such as *A. haemorrhoa*, that require nectar and pollen to restore energy after hibernation. However, it's noticeable that *A. haemorrhoa* only interacted with *T. officinale*, leading to a compartmentalised and poorly connected network, which is typically less robust to species loss. Furthermore, *T. officinale* is lacking in several essential amino acids, meaning that if used alone it causes poor development and high mortality of bee larvae (Roulston and Cane, 2000; Génissel *et al.*, 2002). If the foraging bees are not able to find alternative forage plants to supplement this, it could be problematic. However, if supplementary and nutritious forage is available, early bee species could be benefitted by this and go on to develop nest sites and colonies prior to the flowering of crops such as oilseed rape, and apple and orchard fruits.

Figure 5.11. Andrena haemorrhoa foraging on Taraxacum officinale, March 2016. Photograph taken from plot 17b of the PGE.

On the other hand, the bee interaction network of control, nil, plots are more species-rich and far better connected with many more unique interactions (e.g. Fig. 5.7), which can help resilience and robustness in the face of species loss or stochastic events. This is, in part, due to the more varied resources that bring in higher abundance and richness of bee species. In NO_3-enriched networks, pollinator visitation typically focuses around the generalist *Asteraceae* plants, *Leontodon hispidus*, with a few visits to *Ranunculaceae* and other plants. On the other hand, networks in control plots see high levels of visits to two *Fabaceae* species *Lotus corniculatus* and *Trifolium pratense*, and *Knautia arvensis*, in addition to *L. hispidus* and a suite of other plants.

At higher levels of N addition, the functional diversity of the floral community decreased as did the proportion of *Fabaceae*, and with this shift in floral composition came a movement towards more Diptera-dominated pollinator communities. Diptera can be efficient vectors of pollen transport, particularly the more bristly, in part due to their sheer abundance (Ssymank *et al.*, 2008; Breeze *et al.*, 2011; Orford, Vaughan and Memmott, 2015). However, in most cases they are less effective and efficient pollinators than bees. Bees are also often more specialised pollinators than dipterans, providing pollination services for many flowers with deeper corollas or specially adapted pollination mechanisms in addition to more simplistic open flowers, including many crops (Klein *et al.*, 2007; Garibaldi *et al.*, 2014). Therefore, unchecked increasing levels of N deposition or local N application could lead to poor resources for foraging bee species and subsequent shift towards more dipteran-dominated pollinator assemblages.

Regarding other flower-visiting insects, the abundances of other hymenopterans, sawflies and wasps, were affected by floral abundance. These insects frequently visit flowers to feed on nectar or to predate on and parasitise other insects, and in doing so can act as pollination vectors. The abundance of flowers, with the greater feeding opportunities they bring is likely a draw for these insects. Lepidopteran visitation was also negatively associated with NO_3 application. In the PGE, relatively few lepidopteran visits were recorded overall, but they were typically to *Asteraceae* and *Fabaceae* (Appendix 5). It's possible that fewer lepidopterans were observed visiting NO_3-enriched plots because the floral community was less functionally-rich and with fewer *Fabaceae*.

5.5.3 Plant-pollinator network structure.

Most plant-pollinator networks across plots and years were significantly more connected, with more links per species, than the generated null models. Although the robustness of the pollinator community to species loss was not significantly affected when considering the networks across all years, better connected networks are theoretically more robust and resilient than randomly generated networks of interaction links (Dunne, Williams and Martinez, 2002; Gilbert, 2009). The sensitivity of the less-nested NO_3 networks to potential stochastic events was shown by the sharp fall in the robustness of pollinator communities during a hot and dry summer. The effect of inter-annual variation on plant-pollinator networks is known (Burkle and Irwin, 2009), but this study reveals that N-enrichment can harm network stability in summers with extreme weather events. In the hot and dry summer of 2018, the NO_3 plots became significant less robust, with fewer links per species, and with an uneven network. On the other hand, control plots were not significantly affected by this stochastic event and remained stable throughout the three study-years. This finding is evidence that drivers of global change should not be considered independently. The responses of plant-pollinator interactions will be determined by many factors. In this study, the potential for N deposition to interactively shape plant-pollinator interaction networks with changing climate is shown.

All networks were highly connected, more so than null models, with relatively stable connectance metrics through the years under various N treatments. This indication that the connectance of plant-pollinator networks in mesotrophic grasslands remains stable when undergoing N-pollution is good news. The connectance metric of networks can be distorted by observations of new species, which drive connectance down when they make their first interaction (Jordano, 1987). The fact that connectance was statistically similar between control and N-enriched plots shows that although control pots were more species rich, there were ample resources for more rare species to form many interactions.

Just as new species typically drive connectance down, they can also increase nestedness (Bascompte *et al.*, 2003; Vázquez and Aizen, 2004). Plant communities receiving applications of NO_3 and organic manure harboured plant-pollinator networks that were less nested, possibly due to the lower species richness and number of unique interactions in these networks. Both treatments resulted in plant communities that had a less functional diverse floral composition, which is important for attracting and providing resources for a wider diversity of inset pollinators (Potts *et al.*, 2003; Fontaine *et al.*, 2006; Burkle and Alarcon, 2011). Furthermore, the control plots contained more morphically specialised flowers, such as *Fabaceae*. These flowers tend to produce more sucrose-rich nectar (Wykes, 1952; Petanidou, 2005; Petanidou *et al.*, 2006; Klumpers, Stang and Klinkhamer, 2019), which is generally the preferred nectar type of long-tongued bumblebees (Elisens and Freeman, 1988; Petanidou, 2005; Cnaani, Thomson and Papaj, 2006; Petanidou *et al.*, 2006). With these floral units, the control plots have a more rewarding assemblage of resources for specialist pollinators. Furthermore, the control plots have enough abundance of generalist floral units of *Asteraceae, Apiaceae,* and *Ranunculaceae* to supply alternative foraging opportunities to specialist pollinators, thus leading to the more nested structure (Appendix 5). This network structure typically conveys resilience (Memmott, Waser and Price, 2004; Burgos *et al.*, 2007; Almeida-Neto *et al.*, 2008; Vázquez *et al.*, 2009), as impacts of plant species loss can be buffered by high biodiversity and alternative foraging choices (Stang, Klinkhamer and Van Der Meijden, 2007).

Many of the observed networks were less specialised than null models, often significantly so. Although plant-pollinator networks are traditionally thought of as specialist networks, recent research suggests that this may not be the case (Waser *et al.*, 1996; Vázquez and Aizen, 2004; Petanidou *et al.*, 2008). Plant-pollinator networks are still typically more specialist than many other webs, such as seed dispersal webs (Blüthgen *et al.*, 2007; Stang, Klinkhamer and Van Der Meijden, 2007), and can feature strongly-connected reciprocal interactions (Vázquez and Aizen, 2003), but most networks are characterised by opportunistic insects with relatively plastic foraging behaviour visiting a wide suite of plants to obtain floral rewards (Petanidou, Van Laere and Smets, 1996; Waser *et al.*, 1996; Ollerton *et al.*, 2009). In this study of the PGE, N applications did not statistically affect the specialisation index H2' of the networks. Although NO_3-enrihcment led to the loss of key resources for bumblebees, *T. pratense* and *L. corniculatus*, bumblebees in enriched plots still focussed their foraging efforts on a single pervasive and rewarding species, in this case *L. hispidus* (Fig. 5.7). Many pollinators, including specialist bumblebee species, can show remarkable flower constancy when foraging (Waser, 1986; Goulson, 2000), and thus the degree of specialism within a network might not be so impacted by the loss of a key interactor species if an alternative abundant and nutritious species is available. The H2' specialism metric was impacted by soil pH, however, as was the Alatalo interaction evenness metric. More acidified soils led to networks that were less even and more specialised. The specialisation metric can be obscured by strongly linked partners that are not necessarily specialised (Vázquez and Aizen, 2004; Petanidou *et al.*, 2008), for example by pollinating insects visiting abundant plants. Soil acidification creates another selective pressure on the botanical community (Stevens *et al.*, 2010), typically leading to acid-tolerant plants dominating these communities. If the acid-tolerant newly-dominant plant provides nutritious and plentiful nectar and pollen resources, insect pollinators can be drawn to it out of necessity. This leads to an uneven networks metric that is dominated by one or a few species (Tylianakis, Tscharntke and Lewis, 2007). The indication of specialisation, however, is potentially misleading as it is not a true specialisation in the sense of co-adaptations, but purely out of necessity. In the PGE, this difference in structure occurred between plots of different pH, for example between plots 3b (nil N, pH 6.1) with an even network and 3d (nil N, pH 5.1) which was dominated by visits of wide range of insects, of both dipterans and hymenopterans, to *Pimpinella saxifraga* (Appendix 5).

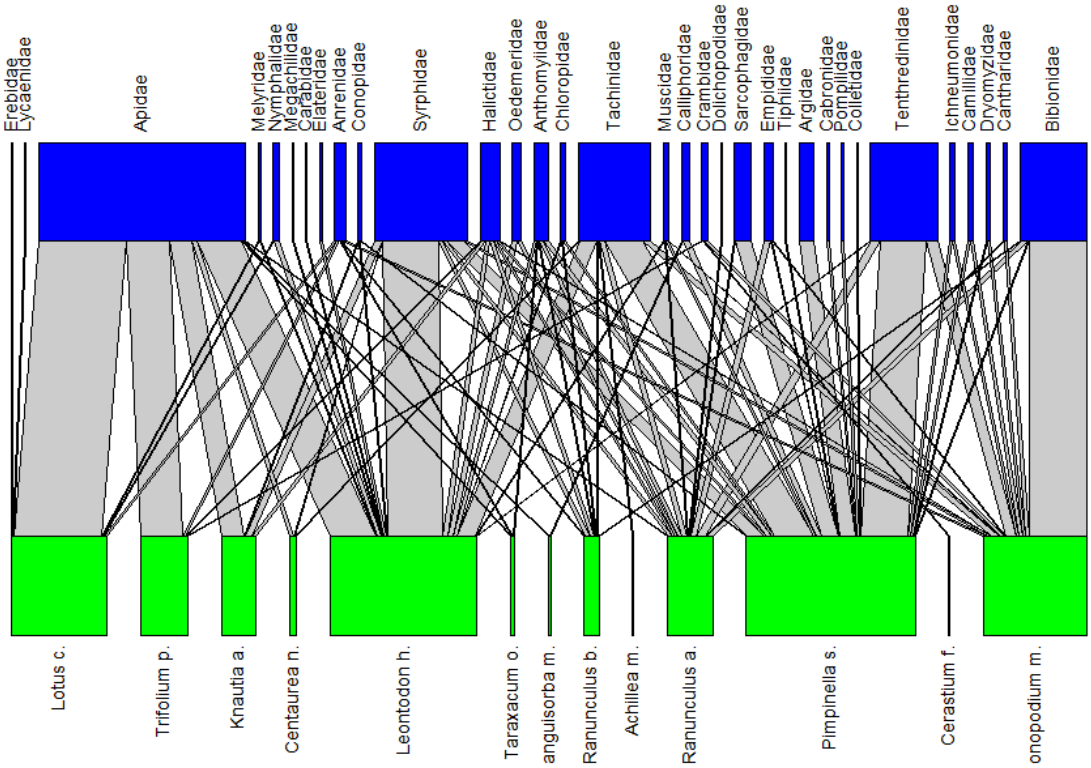


Figure 5.12. Plant-pollinator interaction network of plot 3b (nil N, pH 6.1), across all years.
All insect flower visitors, identified to family, observed in plot 3b at the top. Plant species on the bottom. Links portray the weighted interactions between insects and plants. Total network observations = 549.

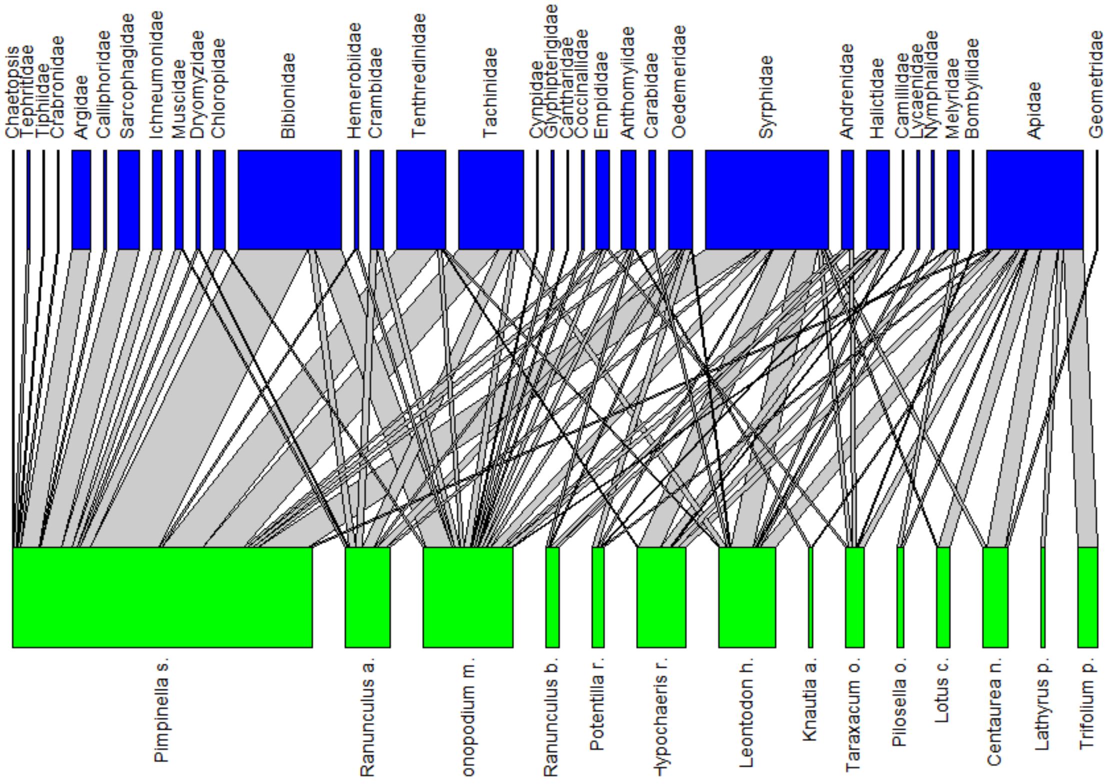


Figure 5.13. Plant-pollinator interaction network of plot 3d (nil N, pH 5.1), across all years. All insect flower visitors, identified to family, observed in plot 3b at the top. Plant species on the bottom. Links portray the weighted interactions between insects and plants. Total network observations = 325.

5.5.4 Caveats of the study, limitations, and suggestions of further studies

The PGE is a single field site, plots are not spatially independent. Therefore, the pollinator communities of plots are in fact subsections of a single pollinator community. The ambient pollinator community of the PGE is not necessarily representative of pollinator communities found in spatially separated sites experiencing N deposition. The major implications of this caveat are two-fold. On one hand, some interactions and specific pollinator species observed in this study might not be found in similar real-world plant communities that are spatially independent. For instance, there could be fewer long-tongued bumblebee species in a region with widespread high N deposition that has caused declines in legumes. In such a scenario, interactions involving bumblebees would be rarer. In the PGE however, the ambient pollinator community is not limited by floral resources in this way. The second implication of this caveat is that the observed interactions in the PGE were subject to choice and preference. In a true plant-pollinator network, foraging insects could visit flowers out of necessity, whereas in the PGE the same interaction might not be observed due to the presence of a preferred floral resource. To account for this caveat, it would be useful to conduct pollinator surveys of spatially independent sites across an N gradient. This would help to reinforce our understanding of how soil eutrophication and acidification impact on plant-pollinator interaction networks and pollinator communities.

5.6 Conclusion

Chronic applications of NO_3 in the PGE had significant effects on the pollinator communities associated with experimental plots, and on the plant-pollinator interaction networks of these plots. Nitrogen addition led to reduced overall visitation, a reduced diversity of species and interactions, and a shift in pollinator community towards more heavily Diptera-dominant assemblages. The loss of bee abundance is potentially of significance, considering these insects are especially effective pollinators of a wide range of wild plants and crops (Klein *et al.*, 2007; Kremen *et al.*, 2007; Garibaldi *et al.*, 2014) and are noted to have declined during recent decades (Ollerton *et al.*, 2014; Powney *et al.*, 2019). The findings of this study suggest that N deposition could indeed have contributed to these declines. Many of these impacts to the pollinator community could be linked to changes in the floral community in the plots, but changes in the floral traits could also have played a factor. For example, earlier flowering phenology of *T. officinale* in NO_3-enriched plots led to strongly compartmentalised interactions developing between *T. officinale* and an early *Andrenidae* bee species. The lack of connection between this compartment and others suggested that, although the early provision of resources generally benefits early solitary bees, the interaction is not robust; loss of these early floral resources or phenological mismatch could have consequences for the early bees. The structure of networks was also altered by N addition, typically becoming less nested and less even, and therefore potentially less resilient to change or extreme weather events (Memmott, Waser and Price, 2004; Burgos *et al.*, 2007; Vázquez *et al.*, 2009).

However, the significantly high connectance of all experimental plots suggests that there remains good latent stability in these mesotrophic grassland plant-pollinator networks, despite heavy N additions (Gilbert, 2009).

With regards to global atmospheric N deposition, future rising levels could indeed impact on pollinator communities, by depriving insects of key floral resources and reducing the functional divergence, evenness, and richness of floral communities. This study only considered the impacts of NO_3 addition, which findings presented in the previous research chapters had shown to be less detrimentally impactful to plant community composition and floral traits than NH_4 additions (Chapter 2; Chapter 3; Chapter 4). Considering that the form of N that was less impactful to floral traits and composition still altered pollinator communities, reduced bee visitations, and altered network structure, effects of NH_4 could potentially be more severe. Given that most of the world experiences N deposition that is moving more heavily towards NH_4 dominance over NO_3, this is a research area than warrants further research.

NO_3-enrichment was found to weaken the robustness of plant-pollinator communities in a particularly hot and dry year. With climate change widely predicted to lead to increased frequency of extreme weather and drier temperate summers (Semenov and Barrow, 1997; Rosenzweig *et al.*, 2001), the revealed interaction between inter-annual variation and N-enrichment is a worrying indicator of weakened plant-pollinator networks under the two pervasive drivers of global change, climate change and N-deposition. With a more concerted research effort, we can better understand how N-deposition acts as a driver of global change and affects pollinator communities, which can help us to implement mitigation or conservation strategies.

Chapter 6

GENERAL DISCUSSION

6.1 Summary of research questions

With global N deposition forecasted to increase in the near future (Galloway *et al.*, 2004; Dentener *et al.*, 2006; Fowler *et al.*, 2013), improving our understanding of its impacts to biodiversity and ecosystem functioning should be imperative. A wide range of studies have shown that soil N-enrichment affects plant species richness (Stevens *et al.*, 2004; Maskell *et al.*, 2010; Field *et al.*, 2014), but the knowledge of impacts to functional traits and consequences to higher trophic levels is patchy (Nijssen, Wallis De Vries and Siepel, 2017; Stevens, David and Storkey, 2018). In particular, the impacts of N on plant-pollinator interactions is a knowledge gap (David, Storkey and Stevens, 2019). This considerable knowledge gap has been discussed in the thesis, and the research chapters provide insight into how pollinator communities and their foraging habits and interactions with plants might be altered by soil N. Overall there were 3 overarching ideas behind the structure of the thesis.

1. How do chronic additions of N to soil affect the community of plants in ways that are relevant to pollinators?
2. How do important floral traits, phenology and nectar, respond within species to chronic additions of N to soil?
3. Do chronic additions of N to soils alter pollinator visitation responses, pollinator communities, and interaction networks?

The research chapters presented a wide array of results that showed the impacts of soil N on floral communities, floral traits, and pollinator communities. In this chapter, I will thematically discuss the findings.

6.2 Loss of specialist floral resources and the implication for pollinators

The loss of floral resources, through changing land use or habitat degradation is considered one of the main drivers of pollinator declines (Goulson, Lye and Darvill, 2008; Rader *et al.*, 2014; Senapathi *et al.*, 2015). Whilst these declining trends are widespread across pollinating taxa, declines caused by deterioration of floral resources are not uniform across taxa; more specialist pollinators, such as those with more elaborate feeding apparatus, are more at risk (Winfree, Bartomeus and Cariveau, 2011). The loss of specialised floral resources has been strongly connected in these declines. For example, in the UK and Netherlands declines in specialist floral resources and specialised insect pollinators are closely correlated (Biesmeijer *et al.*, 2006), while Rasmont *et al.* (2005) suggest the loss of deep corolla forage plants as a driving factor of long-tongue bumblebee declines.

Chapter 2 presents a novel study that identifies shifts in floral traits, within forbs and legumes, that add to our understanding of effects of N on species richness and proportion of different functional groups. The evidence presented in Chapter 2 shows a loss of specialist floral resources with N applications or deposition, which can have impacts to the pollinating assemblages foraging from the experimental plots, as shown in Chapter 5. This is an important finding, and suggests that heavy N deposition could have contributed towards habitat degradation and the loss of floral resources which is a key factor in specialised pollinator declines in Europe (Goulson, Lye and Darvill, 2008; Winfree, Bartomeus and Cariveau, 2011; Rader *et al.*, 2014). The increase and subsequent plateau and decline in N deposition in some regions of the UK during the late 20th Century closely reflects trends in nectar provision across the UK (Baude *et al.*, 2016), which declined at times of high N deposition and rebounded when deposition rates started to fall. This close pattern of trends across N deposition, nectar provision, and insect pollinators suggests that N could be a contributing driver to pollinator trends, alongside other drivers such as land use change, pathogens, parasites, and pesticides. This could have contributed to a slowing down of the declining trends in long-tongued bumblebees during recent decades (Carvalheiro *et al.*, 2013). In contrast, with deposition rates in many regions around the world set to increase in the near future, there could be widespread impacts to pollinator communities due to the impacts on floral assemblages. The southern hemisphere, where deposition levels are widely expected to surpass critical threshold levels (Phoenix *et al.*, 2006; Bleeker *et al.*, 2011), is at risk of N-driven impacts to ecosystems that could have severe consequences for specialist pollinators. However, it is important to note that the study presented in these chapters is of a mesotrophic grassland, with an additional analysis of acidic grasslands. While these two grassland types both showed a decline in specialist floral units with increasing N additions, this does not mean similar responses will be found throughout other ecosystems. Many other temperate ecosystems respond to N deposition with a loss of botanical species richness (Bobbink *et al.*, 2010; Nijssen, Wallis De Vries and Siepel, 2017). There are currently knowledge gaps regarding the impact of N deposition to other ecoregions, including tropics, and more research should be conducted to address this (Bobbink *et al.*, 2010). More research is required to determine if the reported impacts are widespread across other ecosystems.

As shown in Chapter 4, the flowers with longer corollas, *Ajuga reptans, Centaurea nigra,* and *Trifolium pratense*, had more sucrose-dominant nectar. This is in agreement with other studies that have found this link between sucrose-richness and pollinator syndromes and floral morphology (Wykes, 1953; Southwick, Loper and Sadwick, 1981; Petanidou, 2005; Vandelook *et al.*, 2019). Therefore, the loss of specialised floral resources with N deposition may also constitute a decrease in the quality of nectar provided by a plant community. Furthermore, N-enrichment not only decreased the prevalence of these floral units in a plant community, it also altered nectar traits such as the lower provision of sugars by NH_4-enrihced *A. reptans*. The effect of high N deposition could constrain pollinator communities further if, in addition to a loss of foraging resources, the available floral resources are less nutritionally rewarding. The provision of nectar, or lack thereof, has been widely suggested as a factor that limits the abundance and diversity of pollinator communities (Potts *et al.*, 2003; Wallisdevries, Van Swaay and Plate, 2012).

6.3 The varied impacts of NH_4 and NO_3

In studying the impacts of N on floral trait composition and expression of floral functional traits, marked contrasts between the responses to reduced and oxidised N, were observed. This is an important finding as real-world N deposition typically occurs on an NH_x:NO_y ratio gradient. The difference in impacts between the two forms of N has been discussed by previous researchers (Stevens *et al.*, 2011; Van Den Berg *et al.*, 2016). However, relatively few experimental studies have considered the two forms as separate entities with potentially contrasting impacts on floral communities and traits, or on plant-pollinator interactions. Most focus on a single form or a mix of the two. With the PGE, it is possible to separate the effects of NH_4 and NO_3, to provide a better insight into how responses of plant and pollinator communities might vary across the world depending on the dominant form of N deposition.

This study has shown that NH_4 applications, widely speaking, were more detrimentally impactful than NO_3 applications, with broader declines in floral units (Chapter 2), delayed dates of first-flowering (Chapter 3), and reduced sucrose and total sugar production (Chapter 4). With regards to the floral community that was available for pollinator visitation, NH_4-enrichment led to declines in both specialist and generalist floral units. The loss of generalist floral units alongside specialist floral units could be particularly impactful, as a wide suite of pollinating insects will be affected and there will be fewer alternative foraging resources to buffer against losses of specialised resources for long-tongued insects. The loss of well-connected resources can be far more severe for the pollinator community (Memmott, Waser and Price, 2004), and lead to the eroding of nested network structures which offer more resilience as they can buffer against species loss (Vázquez and Aizen, 2004; Vázquez *et al.*, 2009). NO_3 application, on the other hand, allowed species of *Asteraceae* and *Apiaceae* to maintain stable populations. These species produce floral units that were widely used by the pollinator community, connecting many species (Appendix 5). However, although these generalist resources prevailed under NO_3 additions, the pollinator community may have become overly reliant on these species, such that, if they were lost the consequences could be wide-reaching. Chapter 3 showed how flowering cessation was earlier and overall flowering duration shorter for all species which flowered after the first peak of grasses, e.g. *Anthoxanthum odoratum* and *Alopecurus pratensis*. This occurred regardless of N form, but the flowering onset of all species were either delayed or unaffected by NH_4 addition, whilst NO_3-enrichment had varied effects on flowering duration. NH_4 additions also led to reduced nectar production and lower sugar and sucrose concentration in *A. reptans* and lower volumes were recorded in *C. nigra,* but it is not known if NH_4 deposition will affect the nectar traits of a wider suite of plant species.

Although NH_4 additions were more impactful to floral traits and the floral community, and from this there are likely severe consequences for pollinator communities in environments experiencing high rates of NH_4 deposition, there were still impacts to pollinator communities caused by NO_3. Therefore, although NH_4 has potentially more severe consequences, both forms of N additions were shown to have consequences for pollinator communities.

Besides the contrasting impacts of NH_4 and NO_3 applications, mineral applications caused variation in floral community composition and floral traits, which also had an effect on pollinator visitation networks. This research project has focused on the impacts of N, with a view to improve our understanding of a pervasive global driver of environmental change. However, the impact of other soil mineral applications warrants further research.

6.4 Addressing inter-specific response variation

As well as community level responses, there was inter-specific variation in responses to soil N-enrichment. Other authors have also reported this issue (e.g. Burkle and Irwin, 2009). The life history traits of plants are potentially an important factor in how they respond to the enhanced nutrition in N-enriched soils, as shown by Burkle and Irwin (2009) who postulated that annuality and perenniality could be used to split response groups. It was not possible to identify effects caused by plant lifespan, as all studied species were perennial, but seasonality was revealed as an important factor, particularly for the flowering duration of forbs. This is an important floral trait for pollinating insects that determines the provision of floral resources. For closely-linked interactions, this is perhaps an even more critical factor, as asynchrony with a dependent food resource could cause stress through malnutrition potentially that can cause problems for function, reproduction, and the stability of populations (Memmott *et al.*, 2007). The sharp contrast observed between the earliest flowering plant species in the PGE, *Taraxacum officinale agg.*, *Ranunculus ficaria*, and *Fritillaria meleagris*, and those that flowered from the mid-spring onwards was clear. In fact, the effect of N addition on flowering duration was negatively linked with the first flowering of the plant species, with later species having a shorter flowering period in plots where N was added. This could be due to competitive exclusion of flowering opportunities due to strong growth of grasses – N addition was therefore having an indirect effect on flowering time. The implication of the findings is that, with N addition, flowering plants that grow with less local competition from highly competitive plants could have a longer flowering duration. This applies to early spring or winter flowering plants in temperate grasslands, but also to mid-season plants in some higher latitude ecosystems. Flowering plants in far north tundra ecosystems can increase in biomass with N deposition, due to the sparsity of natural competition (Bobbink *et al.*, 2010). In such situations, the extra soil nutrition could translate to longer flowering periods which will benefit pollinator communities through increased provision of nectar and pollen resources.

The literature is too limited to make assumptions on the relevance of taxonomic family for floral trait responses. Variable results have been shown within taxonomic families, such as three alpine species of *Ranunculaceae* (Liu *et al.*, 2017) and three steppe species of *Rosaceae* (Xia and Wan, 2013). In the study of PGE, there were also inconsistent responses within family. All three *Ranunculaceae* species, (*R. acris, R. ficaria,* and *R. bulbosus*), had delayed dates of first-flowering with N additions, whilst the two species for which flowering cessation was studied, *R. ficaria* and *R. bulbosus*, responded similarly to the N treatments with delayed last-flowering dates. The three studied *Fabaceae* species, *Trifolium pratense*, *Lathyrus pratensis,* and *Lotus corniculatus*, also had similar responses in first-flowering to N addition, a delayed onset of flowering. On the other hand, the plants within the *Asteraceae* family (*Taraxacum officinale agg., Leontodon hispidus, Hypochaeris radicata,* and *Tragopogon pratensis)* and *Apiaceae* family (*Anthriscus sylvestris* and *Conopodium majus*), showed no similarities in their responses. However, the sucrose:hexose ration of floral nectar from two *Apiaceae* species, *A. sylvestris* and *H. sphondylium*, had similar responses to N additions, decreasing with higher additions of N. While this is an interesting result that is suggestive of a grouped response, we cannot make assumptions from only two species of a taxonomic family. Further studies could be undertaken to determine in what ways taxonomic family can link responses to drivers of global change such as N deposition.

The delayed flowering observed for *Ranunculaceae* species and *Fabaceae* species should be considered in the context of pollinator emergence and activity dates. Insect pollinators emergence is shown to accelerate in response to climate warming, potentially even more so than plant flowering acceleration under climate warming (Parmesan and Yohe, 2003; Gordo and Sanz, 2006; Parmesan, 2007). It is doubtful that N deposition will directly impact on the emergence date of insect pollinators, and therefore the shift in flowering dates could lead to phenological asynchrony and potentially cause problems for strongly-specialised interactions. The *Fabaceae* family, for example, includes many species that are of importance to effective and efficient pollinating insects, with morphologically specialised floral structures and rewarding floral rewards. Delays in these resources, if not matched by a change in the phenology of long-tongued pollinators, could lead to limited provision of suitable foraging resources for pollinators that are already at risk of declines (Rasmont, Pauly and Terzo, 2005; Goulson, Lye and Darvill, 2008; Winfree, Bartomeus and Cariveau, 2011).

6.5 Impact of soil acidification and importance of soil buffering capacity

Soil acidification is an major consequence of N-deposition, alongside soil eutrophication (Bobbink *et al.*, 2010), often leading to species loss (Stevens *et al.*, 2004; Stevens, Thompson, *et al.*, 2010). In the PGE, only NH_4 has led to soil acidification of topsoil, with unlimed NH_4-enriched plots between pH 3.7–4, whereas unlimed NO_3-enriched plots range between pH 5.6–6.1. In comparison, unlimed nil plots range between pH 5.0–5.1. In the PGE, pH was often an important factor, high pH often mitigating some of the more severe consequences of N-enrichment, such as limiting the declines in nectar-rich or morphologically-specialised flowers (Chapter 2) or reducing the magnitude of impact to flowering phenology (Chapter 3). The propensity of topsoil to become acidified by N deposition or applications is dependent on the buffering capacity of the soil and is variable between ecosystem types. The PGE field site was founded on MG5 mesotrophic grassland; this grassland type can encompass a range of pH levels and is typically prone to acidification and resulting impacts to floral composition (Critchley *et al.*, 2002). In general, unimproved grasslands, including calcareous and acidic grasslands which harbour botanical communities with high value to further trophic levels are more at risk of soil acidification than agriculturally managed grasslands (Critchley *et al.*, 2002). Acidic grasslands can contain high botanical species richness and provide nutritiously rewarding floral resources for pollinating insects (Baude *et al.*, 2016), but the weak buffering capacity of the topsoil means that acidification is one of the main consequences of N deposition that leads to declines in species richness in these systems (Bobbink *et al.*, 2010; Duprè *et al.*, 2010). Temperate forests that have a history of agricultural management also typically have higher pH and are less prone to acidification than those with an unmanaged history (Blondeel *et al.*, 2018). Globally, there is potential for N deposition to cause topsoil acidification to a wide array of ecoregions. For example, high N additions have been shown to acidify tropical soils, causing a shortage in topsoil base cations calcium (Ca) and magnesium (Mg) (Lu *et al.*, 2014). The study of tropical soils, however, did not find increased mobilisation of aluminium ions ($Al3^+$), which is a known detrimental consequence of N-driven soil acidification in temperate grassland systems (Duprè *et al.*, 2010; Stevens, Thompson, *et al.*, 2010; Stevens *et al.*, 2011). Considering the above, N-driven soil acidification should be regarded as a potentially strong factor influencing plant-pollinator interactions and wild pollinator assemblages across a range of ecoregions globally.

6.6 Limitations of the study

An issue of the experimental set-up was that all experimental plots were contained within the same field site, the PGE, and so were subject to the same background pollinator community. Therefore, the observed N-driven impacts to pollinator communities might not necessarily reflect field-realistic impacts. The different networks across plots are influenced by insect preferences, not visitations driven by necessity. In reality, long-tongued bumblebee species may still maintain populations in N-enriched plant communities if there are suitable and rewarding floral resources throughout the season. On the other hand, without an array of specialist flowers providing rewarding nectar and pollen, long-tongued bumblebee species might not be accounted for in N-polluted ecosystems, either being lost due to the lack of sucrose-rich resources or because they choose to forage elsewhere in more rewarding habitats (Jha and Kremen, 2013). It would be useful to find spatially separated sites that resemble PGE plots and plant communities, to determine if the observed plant-pollinator networks of the PGE plots are similar to field-realistic ones.

The lack of replication in the PGE is also a major limitation for studies conducted in the field site. The experiment was designed prior to modern statistical theory, and so researchers must work to find alternative methods of replication in the PGE. One can use temporal replication, by conducting long-term studies over several seasons, as was undertaken with the chapters presented in this thesis where sampling was conducted throughout the seasons over at least three years. When sampling plants from the individual plots, pseudo-replication is unavoidable as there is no replication of treatments. At best, one can obtain samples from clearly separated individuals from opposite sides of the plot. When comparing the treatments of plots, blocking factors can be used to create hidden replication, for instance studying N additions across plots that differ in topsoil pH or mineral applications. A limitation of Chapter 5 is that only NO_3-enriched plots were studied. In the PGE, due to previous manipulative studies, there is no longer an unlimed plot that incorporates 48 kg NH_4 ha^{-1} yr^{-1} with mineral additions. Therefore, the decision was taken to focus on NO_3-enrichment, for which there was a full suite of comparable plots. Regardless, previous research chapters had suggested the impacts of NO_3 addition were more varied and less severe than those of NH_4 plots, so it was useful to investigate how NO_3 plots affect pollinator visitation and networks.

The unique long-term status of the PGE and the value of its contribution towards ecological research (Silvertown *et al.*, 2006) means that it is difficult to conduct manipulative studies without permanently altering or damaging the integrity of the experimental plots.

6.7 Conclusions and future questions

Understanding and halting pollinator declines is an important undertaking. The loss of pollinators constitutes the loss of an important ecosystem service that can support ecosystem stability, biodiversity, and, importantly for human society, agricultural yields. For the range of benefits, it is difficult to put an exact value on the ecosystem service of biotic pollination (Hanley et al., 2015; Melathopoulos, Cutler and Tyedmers, 2015), but it 2009 the value to agriculture was estimated at $153 billion (Gallai et al., 2009). In this study, deposition or applications of N to soils has been revealed to impact floral communities and important floral traits, leading to distorted pollinator communities. Alongside other research into the effects of N to higher trophic levels, this is a good start in understanding a pervasive global driver of environmental change. However, knowledge gaps remain that should be considered in order to take this subject forward (Fig. 6.1). N deposition does not act alone in our changing world. It is one of many potential drivers on ecosystems. Globally, N deposition will act alongside aspects of climate change, such as increased temperatures and more frequent extreme weather events (Fig. 6.1). On local scales, there will be interactive biotic factors, such as soil microbiota, and abiotic factors, such as soil buffering capacity. Deposition rates vary worldwide and, importantly, the ratio of NH_x:NO_y also varies. Improving our understanding of the impacts of distinct forms, across a range of ecoregions and ecosystem types will do much to enhance our understanding of this driver (Fig. 6.1). As deposition rates vary across the globe, current studies in regions that have experienced high historic N deposition will have a distorted baseline and so will not necessarily encapsulate the full story of N deposition from lower levels (Bobbink *et al.*, 2010). Many regions that currently experience a low but rising level of N deposition are understudied, such as tropical regions and Mediterranean ecosystem types (Bobbink *et al.*, 2010). These ecosystems house high levels of biodiversity and so it is imperative to improve our understanding of the risks these ecosystems face from future rising N deposition to formulate mitigation and conservation measures (Fig. 6.1). Whilst it has been shown that N deposition impacts plant communities, improved understanding of the effects to pollinator communities could be conducted by undertaking regional studies that incorporate spatially separated plant and pollinator communities undergoing different levels of N-enrichment. Alternatively, studies of plant-pollinator interaction networks through time, encompassing changing levels of N deposition, will also provide insight to N-driven impacts to pollinator communities. However, the danger of this approach is that it takes many years of study, by which time the effects of N-pollution could be well-established, thus harming our conservation or mitigation efforts.

OPEN QUESTIONS AND IDEAS FOR FUTURE RESEARCH

- INTERACTION WITH ABIOTIC DRIVERS OF GLOBAL CHANGE, I.E. ASPECTS OF CLIMATE CHANGE.
- INTERACTION WITH LOCAL ABIOTIC AND BIOTIC FACTORS.
- CONSIDERATION OF GLOBAL N DEPOSITON AS RATIO OF $NH_X:NO_Y$.
- UNDERSTANDING BASELINES.
- MORE RESEARCH EFFORTS IN LESSER-STUDIED ECOREGIONS, SUCH AS TROPICS.
- SYNTHESIS OF N DEPOSITON RESEARCH AND POLLINATOR STUDIES

Figure 6.1. Research considerations to better understand the impacts of N on plant-pollinator interactions and pollinator communities.

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APPENDICES

Appendix 1. The floral functional traits used in Chapter 2. The flower forms are scored individually, e.g. Ajuga reptans scores 0-simple flower, 1-bee form, 0-bell form, 1-lip form. PGE obs denote median values taken across PGE observations.

The flower forms are scored individually, e.g. Ajuga reptans scores 0-simple flower, 1-bee flower, 0-bell form, 1-lip form. 'PGE obs' denote median values taken from across PGE observations.

Trait	Data type	Levels/range	Unit	Reference
Simple flower	Binary	1; 0	1 if morphology type true, 0 if not	Klotz *et al.* 2002
Bee form	Binary	1; 0	1 if morphology type true, 0 if not	Klotz *et al.* 2002
Bell form	Binary	1; 0	1 if morphology type true, 0 if not	Klotz *et al.* 2002
Lip form	Binary	1; 0	1 if morphology type true, 0 if not. E.g.	Klotz *et al.* 2002
Nectar production	Quantitative	0.01 - 4733.31	kg ha-1 yr-1	Baude *et al.* 2016
Nectar sugars	Quantitative	0.11 - 1892.83	µg fl-1 day-1	Baude *et al.* 2016
Landing area	Quantitative	2.5 - 70	mm	Rose & O'Reilly, 2006; Klotz *et al*. 2002; PGE obs
Corolla depth	Quantitative	2 - 22.5	mm	Rose & O'Reilly, 2006; Klotz *et al*. 2002; PGE obs
Colour	Qualitative	Blue-purple; Yellow; White		Klotz *et al.* 2002
UV patternation	Binary	1; 0	UV patternation present or absent	Klotz *et al.* 2002
Start flower	Ordinal	1; 2; 3; 4; 5; 6; 7	corresponds to month	Rose & O'Reilly, 2006; Klotz *et al*. 2002
End flower	Ordinal	5; 6; 7; 8; 9; 10; 11; 12	corresponds to month	Rose & O'Reilly, 2006; Klotz *et al*. 2002
Duration	Quantitative	2 - 12	number of months	Rose & O'Reilly, 2006; Klotz *et al*. 2002
Season	Ordinal	1; 2; 3; 4; 5; 6; 7; 8	values indicate season, from 1 "pre-spring" to 8 "midsummer"	Rose & O'Reilly, 2006; Klotz *et al*. 2002
Ellenberg N	Ordinal	1; 2; 3; 4; 5; 6; 7	N tolerance. 1 - low soil fertility, 9 - high soil fertility	Hill, 1999
Ellenberg R	Ordinal	2; 3; 4; 5; 6; 7; 8	reaction (pH) tolerance. 1 - acid tolerant, 9 - acid sensitive	Hill, 1999

Appendix 2. Pollinator families observed across all transects bouts. Total quantities, and quantities from each plot.

	Total	**3b**	**3d**	**17b**	**17d**	**7b**	**7d**	**16b**	**16d**	**14b**	**14d**	**13b**	**13d**
Hymenoptera													
Andrenidae	201	9	6	14	17	16	9	25	14	22	25	29	15
Apidae	786	169	47	23	24	127	100	81	53	55	19	47	41
Argidae	69	12	9	5		13	1	9	3	7	4	1	5
Cephidae	3						2			1			
Chrysididae	3							1		1	1		
Colletidae	1	1											
Crabronidae	15	2	1	1	1	1		3		3	2	1	
Cynipidae	1		1										
Figitidae	5								1	3	1		
Halictidae	121	16	11	10	14	9	4	7	6	13	7	6	18
Ichneumonidae	39	4	5	1		5		7	2	6	5		4
Megachilidae	6	1		1				2	1				1
Pompilidae	5	2								2			1
Tenthredinidae	310	55	24	62	6	41	9	36	7	21	10	17	22
Tiphiidae	4	1	1	2									
Diptera													
Anthomyiidae	99	12	7	10	10	9	1	10	6	12	7	10	5
Bibionidae	1380	54	50	66	54	274	48	217	63	320	90	37	107
Bombyllidae	1		1										
Calliphoridae	51	6	1	1	2	2	1	15	2	8	10	1	2
Camillidae	14	5	1	1								6	1
Chaetopsis	2		1									1	
Chloropidae	47	4	6	7	4	1		3		16	2	1	3
Conopidae	4	3		1									
Dolichopodidae	6	1			1				1	1			2
Dryomyzidae	8	3	2					1				2	
Empididae	201	7	6	11	9	82	3	23	8	19	7	14	12
Muscidae	38	5	4	6		4		7		5	4	3	
Pallopteridae	1			1									
Sarcophagidae	203	14	10	4	5	30	1	45	18	24	40	7	5
Scathophagidae	3				1				1	1			
Syrphidae	574	76	60	57	56	51	13	41	29	47	48	47	49
Tachinidae	496	59	32	35	27	60	7	66	22	69	59	36	24
Tephritidae	8		1	3						2			2
Tipulidae	9								1			6	2
Lepidoptera													
Crambidae	33	5	6	2	1		7	3		1	3	1	4
Erebidae	1	1											

Geometridae	2		1										1
Glyphipterigidae	3		1				2						
Hesperiidae	1											1	
Lycaenidae	5	1	1				2						1
Noctuidae	4			2		1		1					
Nymphalidae	15	5	1	7	2								
Scythrididae	1											1	
Coleoptera													
Cantharidae	45	4	1	4	2	7	4	9	4	6	2	2	
Carabidae	23	1	3	2		4	1		3	1	3	1	4
Cerambycidae	1							1					
Coccinellidae	10		1	1		4		1	1		2		
Elateridae	43	2		4	2	12	3	3	2		3	7	5
Melyridae	39	2	6	9	3	1	1		1	1	6	1	8
Oedemeridae	129	7	12	28	14	6	4	5	7	5	3	12	26
Scarabaeidae	4			2			1						1
Neuroptera													
Chrysopidae	1											1	
Hemerobiidae	2		2										

Appendix 3. Bee species observed across all transects bouts. Total quantities, and quantities from each plot.

	Total	**3b**	**3d**	**17b**	**17d**	**7b**	**7d**	**16b**	**16d**	**14b**	**14d**	**13b**	**13d**
Andrena chrysoceles	12	1		2	3			1	1		1	3	
Andrena cineraria	8	1			1	1		2	1	1	1		
Andrena dorsata	1					1							
Andrena fulva	1		1										
Andrena haemorrhoa	143			10	11	11	5	21	11	17	22	23	12
Andrena minutula	3	2	1										
Andrena nitida	15	2	1	1	1	1		1	1	4			3
Andrena scotica	4	1	1			2							
Andrena similis	1	1											
Andrena subopaca	5			1	1		2					1	
Andrena wilkella	2	1									1		
Apis mellifera	172	38	16	10	12	21	17	16	12	13	7	5	5
Bombus hortorum	64	7	1		1	11	6	12	6	5	1	1	13
Bombus hypnorum	4		1		2		1						
Bombus lapidarius	109	59	9		1	12	14	5	1	3		3	2
Bombus pascuorum	354	44	18	9	8	66	44	46	32	26	8	36	17
Bombus pratorum	4						1	1		2			
Bombus terrestris/lucorum	98	19	5		3	18	21	7	4	8	3	4	6
Bombus vestalis	3	2								1			

Halictus tumulorum	1												1
Hoplitis claviventris	1												1
Lasioglossum albipes	13	2	2	1	2	1			1	3		1	
Lasioglossum calceatum	12	3		1	2	1	1		1			3	
Lasioglossum lativentre	11		1	3	4		1		1				1
Lasioglossum leucopus	8	4	1			1	1						1
Lasioglossum malachrum	6			1		2				1	2		
Lasioglossum morio	24	3	2	2	6	1	2	1			3		4
Lasioglossum parvulum	2	1	1										
Lasioglossum pauxillum	6	3	2			1							
Lasioglossum villosulum	5		2	1							1		1
Megachile versicolor	2	1							1				
Nomada goodeniana	6								2		1	3	
Nomada ruficornis	1								1				
Nomada striata	2										1		1
Osmia leaiana	1			1									

Sphecodes ephippius	6	1	1	4	
Sphecodes gibbus	3			2	1
Sphecodes hyalinatus	2			2	

Appendix 4. Summary of MANOVA output showing significantly linked response and explanatory variables, which informed LMER analysis of pollinator communities.

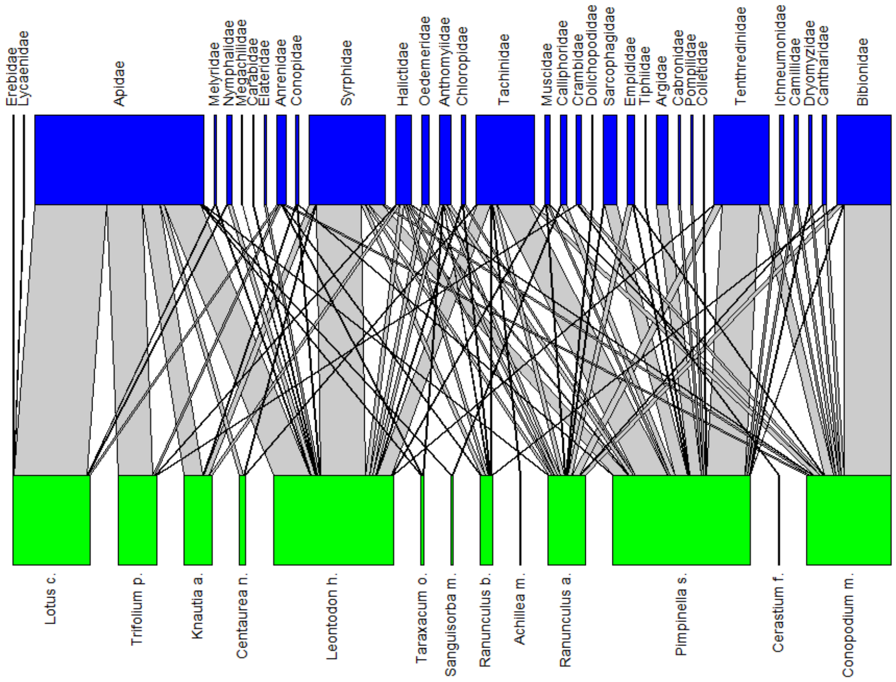
Significance levels indicated by . p<0.1; * p<0.05; ** p<0.01; *** p<0.001. FDiv, FEve, FRic, Rao Q are the Functional divergence, functional evenness, functional richness, and Rao Quadratic Entropy of the floral community. Fl abun is floral abundance. %Fab, %Ast, %Apia, %Ran, %Lam are the proportion of *Fabaceae, Asteraceae, Apiaceae, Ranunculaceae, and Lamiaceae.*

	N form	**N amount**	**pH**	**Minerals**	**FDiv**	**FEve**	**FRic**	**Rao Q**	**fl abun**	**%Fab**	**%Ast**	**%Apia**	**%Ran**	**%Lam**
Overall abundance	.		**								*			
Insect family richness			*	*				.			*			*
Bee species richness	.				**				.		*			
Unique interactions (family)	.		*	.										
Unique bee interactions (sp.)	**		.		*	.								
Bee abundance	*		.		.									
Bombus abundance	**		.	.						.				
Apis abundance	*					.								*
Solitary bee abundance	.								.					
Diptera abundance		*	**	*						*	**	*		
Syrphidae abundance			*	*				.						
wasp abundance		.				.			*		.			

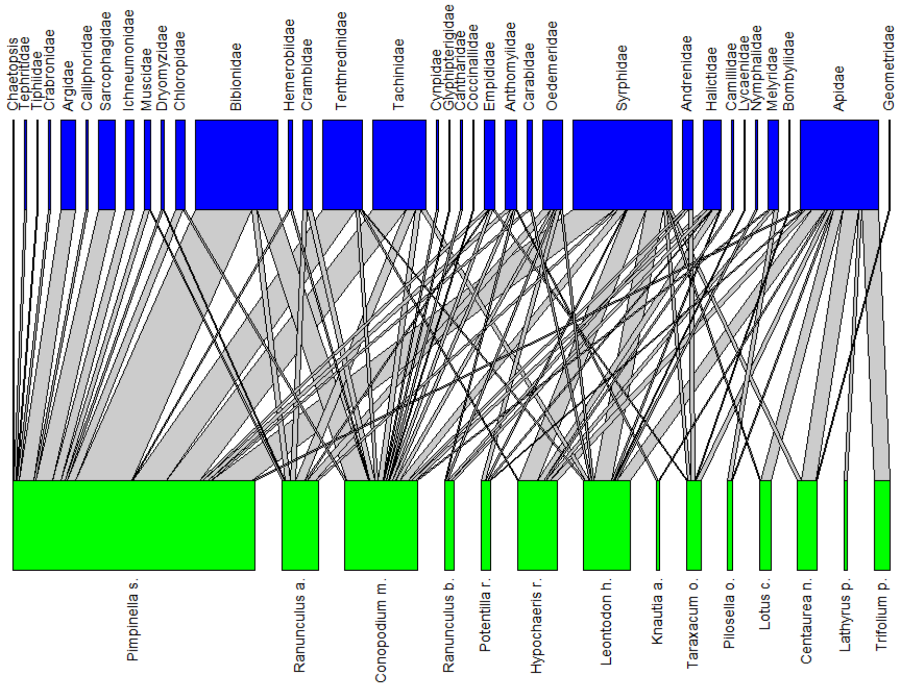
sawfly abundance	*		*				**	*	
Coleoptera abundance				.		.			*
Lepidoptera abundance	*			.				*	
Proportion bees	***	**	**		***			***	*
Proportion Diptera	***	**	**		***			***	

Appendix 5. Plant-pollinator interaction networks from the 12 study plots.

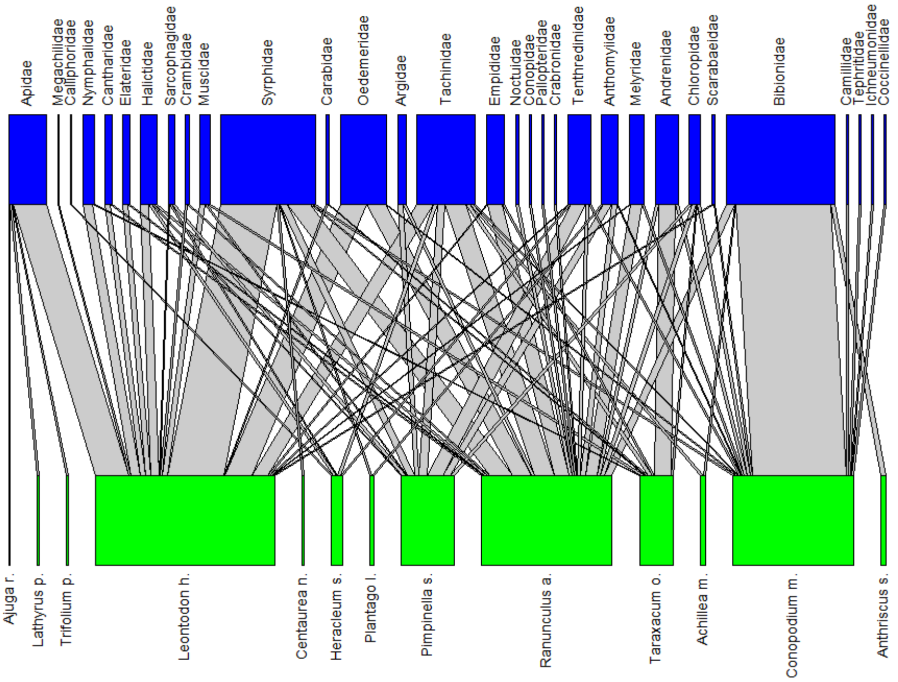
Plot 3b (nil N, pH 6)



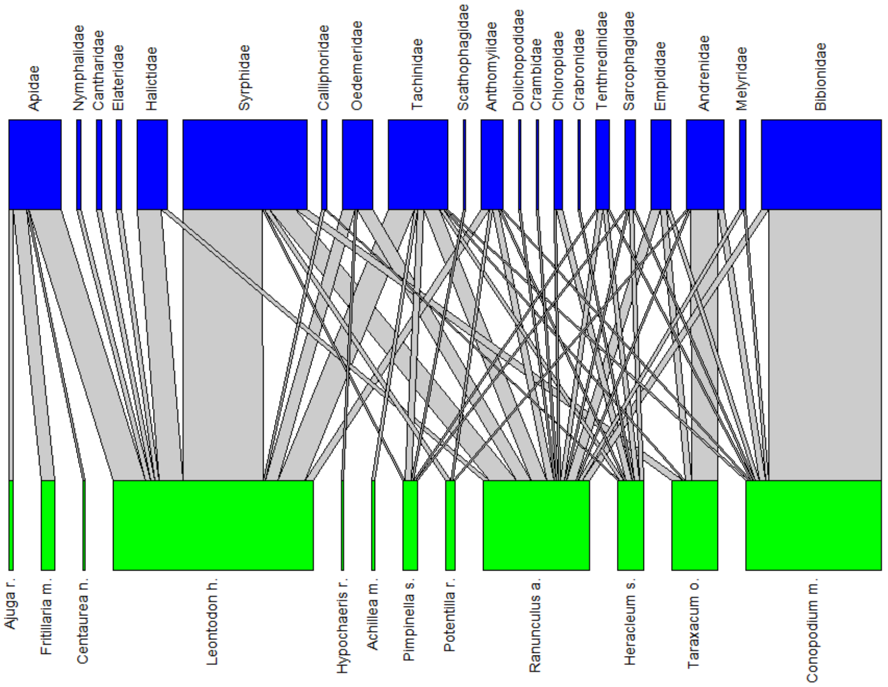
Plot 3d (nil N, unlimed)



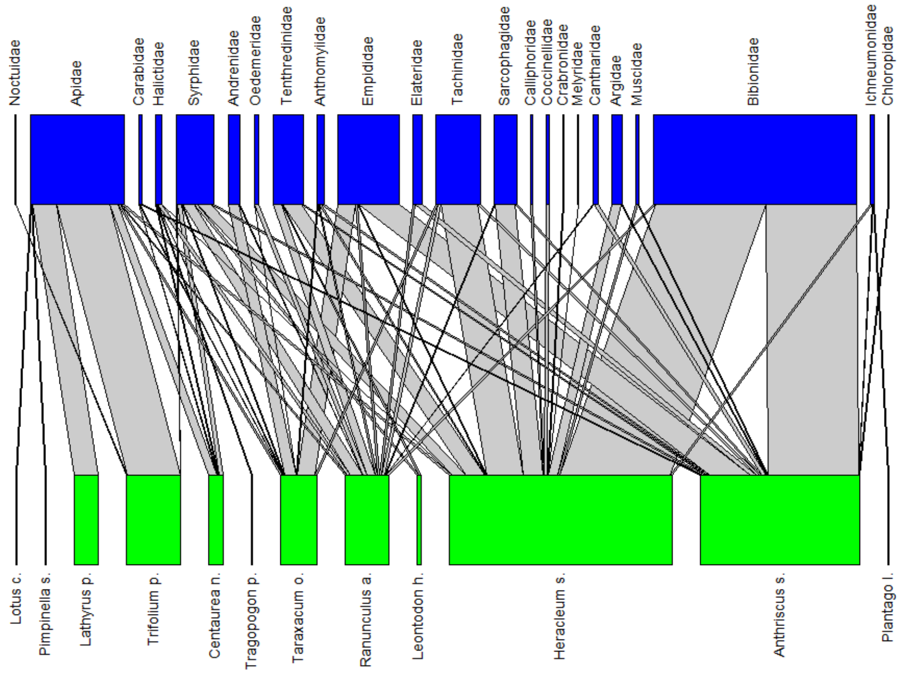
Plot 17b (48 kg NO_3 ha^{-1} yr^{-1}, pH 6)



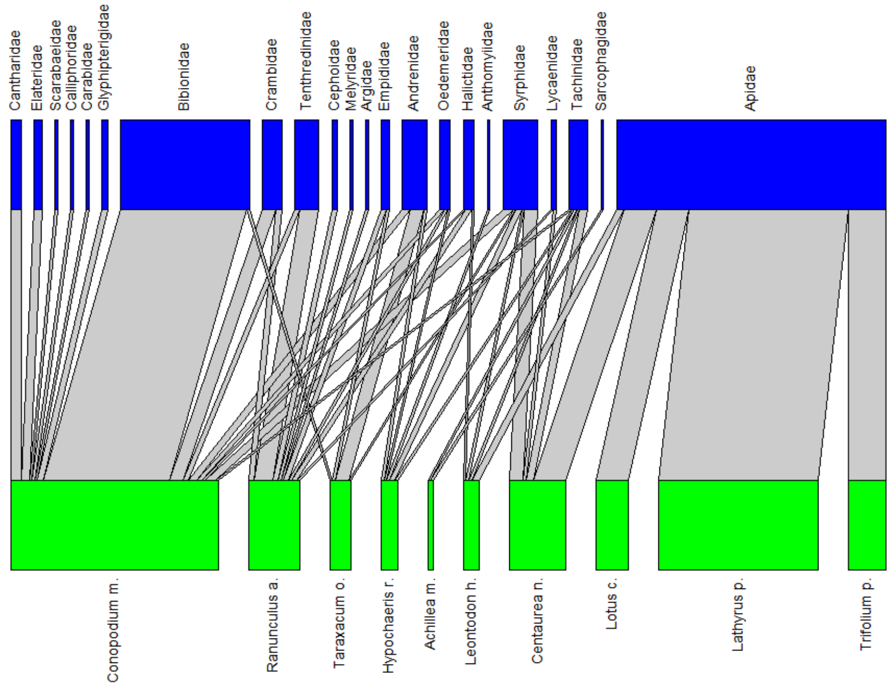
Plot 17d (48 kg NO_3 ha^{-1} yr^{-1}, unlimed)



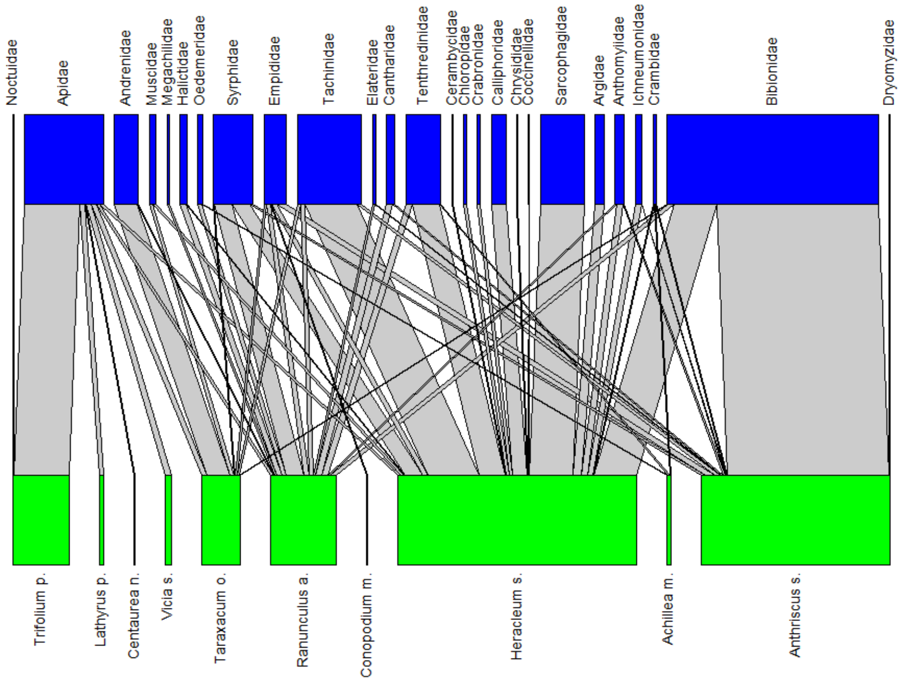
Plot 7/2b (nil N, minerals, pH 6)



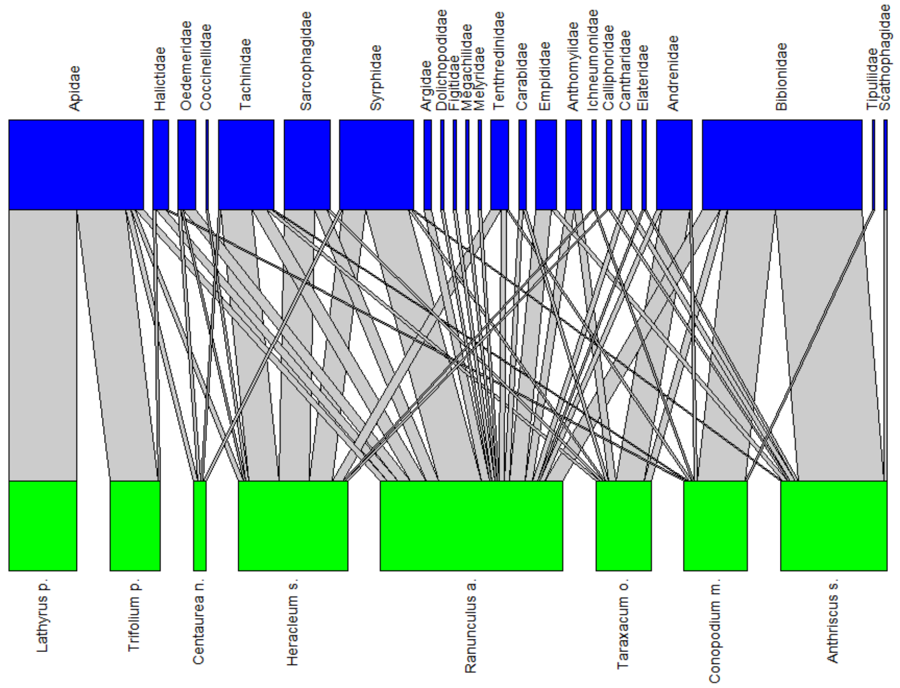
Plot 7/2d (nil N, minerals, unlimed)



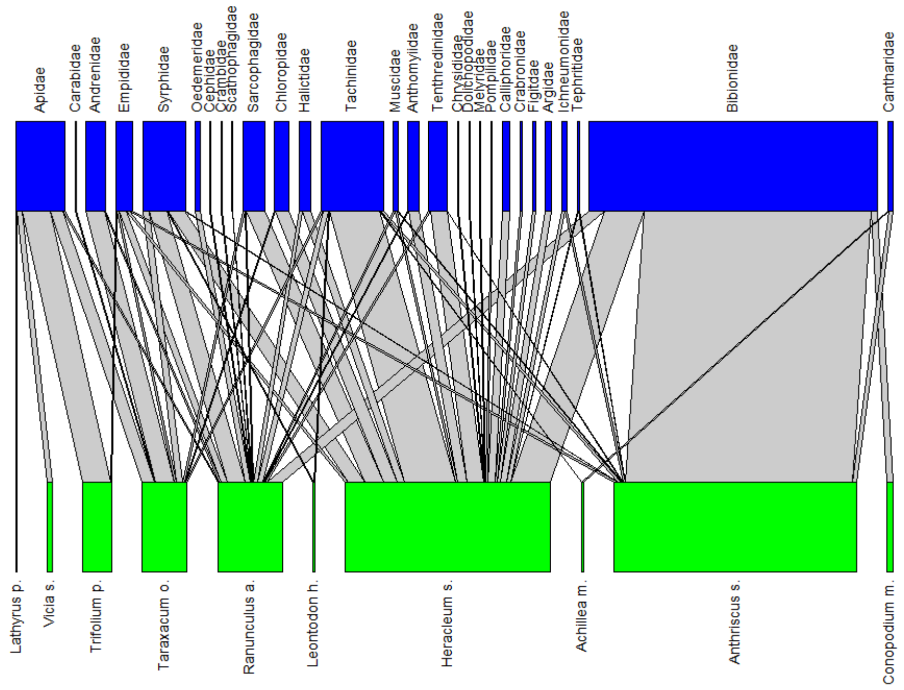
Plot 16b (48 kg NO_3 ha^{-1} yr^{-1}, minerals, pH 6)



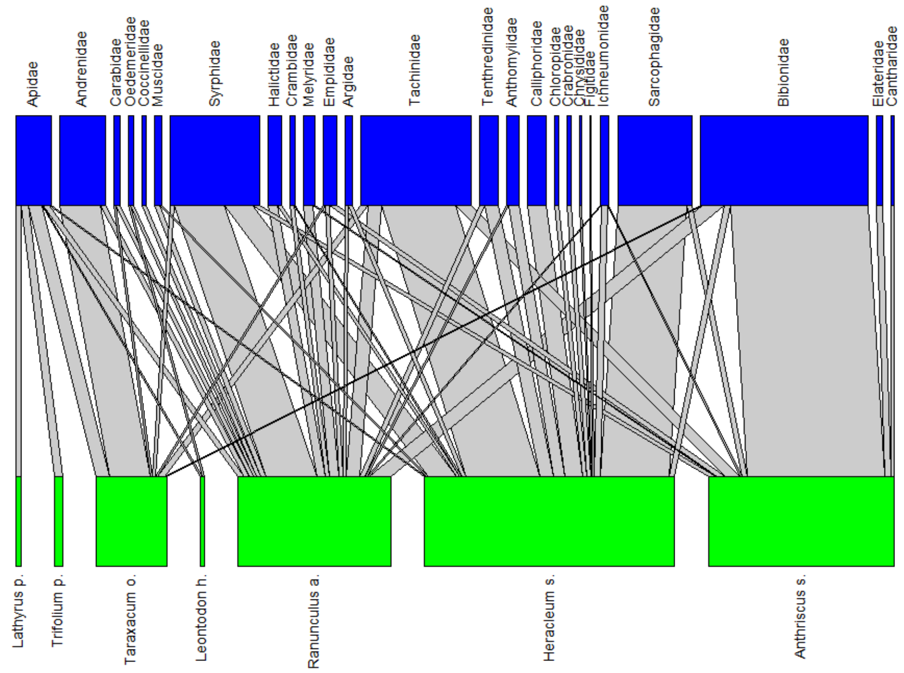
Plot 16d (48 kg NO_3 ha^{-1} yr^{-1}, minerals, unlimed)



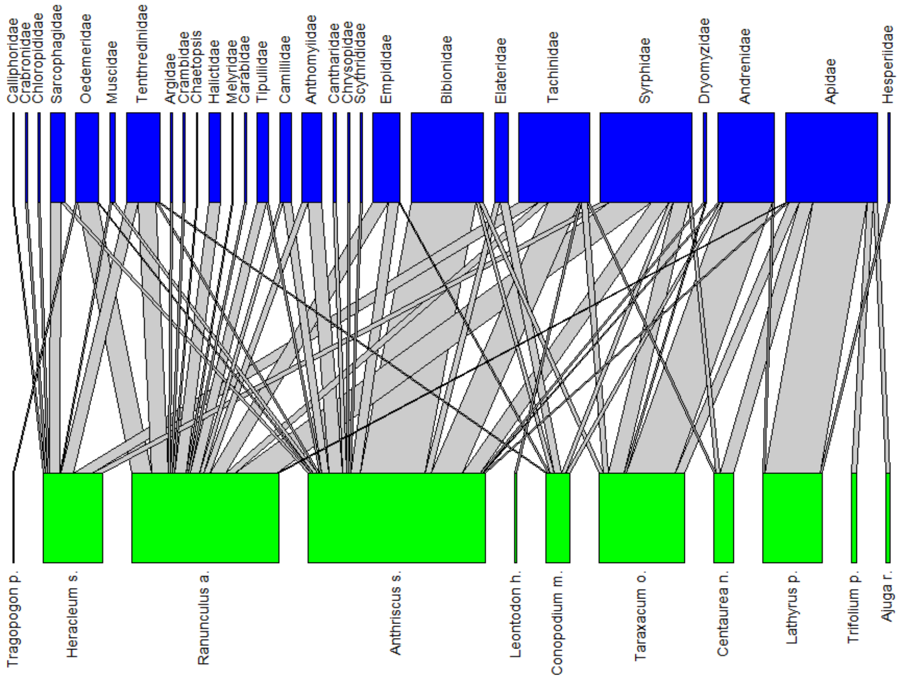
Plot 14/2b (96 kg NO_3 ha^{-1} yr^{-1}, minerals, pH 6)



Plot 14/2d (96 kg NO_3 ha^{-1} yr^{-1}, minerals, unlimed)



Plot 13/2b (FYM, pH 6)



Plot 13/2d (FYM, unlimed)