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The effect of nutrient pollution on food quality of the pollinator *Bombus terrestris*

Eva HULSMANS

Supervisor: Prof. Ir. O. Honnay
Laboratory of Plant Conservation and Population Biology

Co-supervisor: Dr. T. Ceulemans
Laboratory of Plant Conservation and Population Biology

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Summary

Pollinators are declining worldwide and one possible underlying cause is large scale land use change leading to habitat loss and degradation. This mainly involves a decline of food resources and an increased inflow of pollutants, e.g. nutrients, in the remaining habitats. This nutrient pollution may affect pollinators directly through the loss of diverse food resources. However, it may also affect pollinators indirectly by altering nectar and pollen composition of plants, possibly influencing the quality of food sources for pollinators. Here, we investigate the effect of changed food composition following nutrient enrichment on colonies of the bumblebee *Bombus terrestris* in a mesocosm experiment. We found that bumblebees fed with nectar and pollen of plants of *S. pratensis* that underwent nutrient addition showed more dead larvae and less live workers in their hives. When analyzing the composition of the nectar and pollen, we found lower proportions of the important amino acids glycine and arginine in the pollen and less glucose, which is an important energy source for *B. terrestris*, in the nectar. These findings provide one of the first indications that pollinators can be negatively affected by changes in food quality induced by ecosystem nutrient pollution. This may have far reaching implications for the conservation of pollinators as nutrient pollution continues to rise worldwide. Additionally, our results may affect plant populations as well, as less available pollinators and changed pollination behavior could lead to impaired cross-fertilization and fruit set of plants, leading to higher levels of genetic erosion.

Samenvatting

Bestuivers gaan wereldwijd achteruit en een van de mogelijke oorzaken hiervan is grootschalige landgebruiksverandering. Dit leidt tot habitatverlies en –degradatie, wat o.a. resulteert in het verlies van voedselbronnen en een verhoogde toevoer aan vervuilende stoffen, bijvoorbeeld nutriënten, in de resterende habitat patches. Nutriëntenvervuiling kan bestuivers direct beïnvloeden via het verlies aan diversiteit van voedselbronnen. Daarnaast kan het ook een indirect effect uitoefenen op bestuivers door het veranderen van de chemische samenstelling van nectar en pollen van planten, wat mogelijk de voedselkwaliteit voor bestuivers kan beïnvloeden. In deze studie onderzochten we het effect van een veranderde voedselsamenstelling na toevoeging van nutriënten aan *S. pratensis* planten op kolonies van de hommel *Bombus terrestris* in een mesocosmos experiment. We vonden dat hommels die zich voedden met nectar en pollen van planten waaraan nutriënten werden toegevoegd meer dode larven en minder levende werksters in hun kolonies hadden. Na analyse van nectar- en pollencompositie bleek dat pollen van deze planten minder van de belangrijke aminozuren glycine en arginine, en hun nectar minder glucose, een belangrijke energiebron van *B. terrestris*, bevatte. Deze resultaten zijn een van de eerste aanwijzingen dat bestuivers negatief beïnvloed kunnen worden door veranderingen in voedselkwaliteit als gevolg van nutriëntenvervuiling. Dit kan belangrijke implicaties hebben voor het behoud van bestuivers aangezien nutriëntenvervuiling blijft toenemen wereldwijd. Daarnaast kunnen onze bevindingen ook effect hebben op plantenpopulaties, aangezien een verdere afname van bestuivers en een verandering in hun gedrag kan leiden tot verminderde kruisbestuiving en vruchtzet bij planten, verder leidend naar meer genetische erosie.

1. Introduction

1.1 Pollination

During sexual reproduction, the genetic material of two individuals is combined to produce genetically unique individuals. This genetic diversity is advantageous in an unpredictable or changing environment, as it allows for quick adaptation. To sexually reproduce and allow fertilization to occur, one male and one female gamete need to be combined. However, plants are sessile and cannot actively move towards each other to combine gametes. Therefore, plants require vectors in order to bring male gametes (pollen) together with female gametes (ovules).

1.1.1 Process

Pollination is the transfer of pollen to the stigma of a plant's gynoecium. It is usually followed by fertilization and is crucial for sexual reproduction in flowering plants. When the pollen of an individual gets transferred to a stigma of the same individual, it is referred to as self-fertilization or autogamy. This is in contrast to cross-fertilization where pollen from a different individual transfers to a stigma. Cross-fertilization can happen by chance through the production of large quantities of pollen that are dispersed by wind or water. However, a more directed way to achieve cross-fertilization is animal-mediated pollination. Pollination by animals is stimulated by rewards, in the form of food such as pollen and nectar. These pollinators involuntarily act as vectors that transport pollen from one flower to another when collecting these rewards (Abrol, 2011). Animal-mediated pollination assures a more directed movement of pollen to other plants, in contrast to wind pollination, making pollination by animals more efficient. This is important, as pollen is the most energetically costly plant part per gram of organic tissue when compared to other plant parts (Petanidou and Vokou, 1990). However, producing attractants and rewards for animal vectors occurs at a very high energetic cost as well. This leads to a tradeoff between two states: i) a lot of pollen with little rewards and attractants and ii) less pollen with more rewards and attractants (Culley *et al.*, 2002).

1.1.2 Vectors of pollen transfer

A wide variety of about 300,000 animal species, including insects, birds, bats and mammals are known to act as plant pollinators (Proctor, 1978; Faegri and van der Pijl, 1979). Insects are the most important group as more than 70% of angiosperms are insect-pollinated (Kearns and Inouye, 1997). Among insects, bees provide most pollination in natural and agricultural ecosystems (Delaplane and Mayer, 2000; Free, 1993; Klein *et al.*, 2007). Bees belong to the superfamily Apoidae in the order of Hymenoptera and include groups like honeybees, solitary bees, and bumblebees. It is estimated that 25,000 to 30,000 bee species worldwide are obligate flower visitors, relying on flower rewards as their

sole source of nutrition (Buchmann and Nabhan, 1996). Worldwide, the honeybee (*Apis melifera* L.) is by far the most important and best studied pollinator for crops and plants in natural ecosystems (Klein *et al.*, 2007; Free, 1993). Honeybees pollinate plants of over 100 families (Danforth, 2007) and for this reason they are intensely managed by beekeepers all over the world.

Whereas honeybees are vital, the potential value of bumblebees (*Bombus sp.*) in agriculture has long been recognized as well. Their recent domestication has boosted their economic importance, making them important pollinators, too (Delaplane and Mayer, 2000). Bumblebees are valuable because most species have long tongues, which are longer than those of honeybees, meaning they are better at pollinating flowers with deep corollas (Hobbs *et al.*, 1961; Holm, 1966). Additionally, bumblebees are known to exhibit buzz-pollination behavior. This is important for around 15,000-20,000 plant species, among which important crops like tomato and potato, which have anthers that only release pollen through small openings in their tips. Visiting animals then extract this pollen from the anthers through vibrations or 'buzzes'. Buzz-pollination is found among many other bee species as well, but do not include the honeybees (De Luca and Vallejo-Marin, 2013). Furthermore, bumblebees are native pollinators throughout wild plant communities of temperate ecosystems (Memmott *et al.*, 2004; Fontaine *et al.*, 2006; Hegland and Totland, 2008). Unlike other bees, bumblebees are usually concentrated in the north and (sub)alpine regions. They thrive in cool and strongly seasonal climates because of two reasons. Firstly, their queens, which are the only individuals to survive the winter, are cold resistant. Secondly, the physiology and temperature control mechanisms of bumblebees are well suited for low temperatures. Therefore, bumblebees have a lower threshold temperature for activity, compared to honeybees (Corbet *et al.*, 1991).

Although managed pollinators deliver extremely valuable pollination services, it has been shown that wild pollinators also provide important pollination to crops around the world. On a global scale, the economic value of these services is even comparable to pollination by honeybees (Kleijn *et al.*, 2015). However, only a small portion of common bee species provides most crop pollination, with only 2% of bee species providing 80% of pollination services (Kleijn *et al.*, 2015). At smaller spatial scales, however, there is little doubt that a more diverse pollinator community generally provides better pollination services (Garibaldi *et al.*, 2016). Overall, wild species pollinate more effectively than honeybees. For instance, fruit set increased with wild insect visitation in all studied crop systems by Garibaldi *et al.* (2013), compared to only 14% of the systems with honeybee visitation. Additionally, increasing wild insect visitation enhanced fruit set twice as much as increased honeybee visitation.

1.1.3 Attractants and rewards

Since many plants strongly depend on animal pollination (Section 1.1.4), they have developed several floral traits to attract and reward pollinators (Darwin, 1862; Galen and Newport, 1987). In the first step of pollination, pollinators use attractive floral traits to assess the quality of reward in the flower. These floral attractants include inflorescence size, the number of flowers, flower size, shape, color, and floral

scent (Haratym and Weryszko-Chmielewska, 2012; Woodcock *et al.*, 2014; Schiestl, 2015). Pollinators can learn if a flower is rewarding very fast, after which they learn to avoid it. Therefore, most plants produce honest attracting signals, leading to rewards.

Rewards can include floral oils and resins. However, these are fairly unusual rewards that are limited to relatively few genera (Reis *et al.*, 2000). Floral oils are collected by highly specialized bees (Michener, 2007). These bees use floral lipids instead of, or together with, nectar to be mixed with pollen for larvae feeding. Additionally, some bees use these oils for water-resistant cell linings (Cane *et al.*, 1983; Melo and Gaglianone, 2005). On the other hand, floral resins are mainly used for nest construction (Armbruster, 1984).

a) Nectar and pollen composition and function

The most common rewards, however, are nectar and pollen. Nectar is thought to be a major energy source for pollinators, as its high carbohydrate content provides energy for flight, colony maintenance, and general daily activities (Ellis *et al.*, 2013). On the other hand, pollen is of paramount importance to bee nutrition as well as it is their sole protein source, unlike in the case of nectar feeding Lepidoptera and Diptera (Goulson, 2003; Smeets and Duchateau, 2003). This protein content is essential for brood production and the development of young bees (Ellis *et al.*, 2013).

Floral nectar is a chemically complex solution, containing high concentrations of the sugars glucose, fructose, and sucrose in varying concentrations. The second most important components are amino acids (AAs) and proteins, including enzymes and preservatives (Carter and Thornburg, 2004). The AAs may influence insect taste preference and nutrition (Gardener and Gilmann, 2002; Gonzalez-Teuber and Heil, 2009). For instance, it has been shown that butterflies that were fed artificial nectar without amino acids had a lower fecundity (Mevi-Schütz and Erhardt, 2005). Other components include ions, antioxidants, lipids, terpenoids, and various secondary compounds, as well as cytoplasmic remnants (Nicolson and Thornberg, 2007). The energetic value of nectar depends on its volume and total sugar concentration (Nicolson, 2011).

Pollen contains proteins, lipids (including phytosterols), carbohydrates, starch, vitamins, and minerals (Day *et al.*, 1990; Vanderplanck *et al.*, 2014). However, protein is nutritionally the most important component. Plants that must be pollinated by insects have been shown to produce pollen with higher protein levels than plants that do not depend on this service (Hanley *et al.*, 2008). Nevertheless, if high-protein pollen is lacking sufficient amounts of essential AAs required for growth, it is reduced in nutritional value (Loper and Cohen, 1987). This means that nutritional quality might be better expressed through AA composition rather than protein levels. For bees, pollen is the main amino acid source, mainly for some essential amino acids (e.g. leucine, valine, and isoleucine; De Groot, 1953). Proline, aspartic, and glutamic acids are important as energy and nitrogen sources, although they are not essential (Chapman, 2012). Furthermore, phytosterols are important components of pollen, as they are precursors of molting hormones and bees cannot synthesize them themselves (Cohen, 2004).

All of these essential components of nectar and pollen and their concentrations vary greatly between plant species (Roulston *et al.*, 2000; Vanderplanck *et al.*, 2014).

1.1.4 Tradeoffs in producing attractants

Attracting pollinators is not endlessly beneficial to plants. Producing attractants and rewards comes at a great energetic cost, e.g. nectar production can take up to 37% of the daily amount of photosynthates in long-lived flowers (Harder and Barrett, 1992). At a certain point, these costs may outweigh its reproductive benefits. For instance, Pyke (1991) found indications for a tradeoff between an increase in number of seeds through animal-mediated pollination and a decrease due to the costs of producing nectar. Furthermore, plants that produce excessive rewards can change pollination behavior and thus increase the chance of self-pollination and inbreeding (Irwin and Adler 2008; Iwata *et al.* 2012; Mamut *et al.* 2014). For instance, overly-rewarding plants might cause individual pollinators to consecutively visit the flowers of the same plant, increasing the chance at pollination with pollen from different flowers of the same plant (geitonogamy; de Jong *et al.*, 1993). Alternatively, if a flower contains high rewards, pollinators might spend more time on it, increasing the chance that pollen from the same flower is deposited on the stigma (Dudash, 1991; Harder and Barrett, 1995). Next to a higher risk of inbreeding, this can also lead to clogging of stigma surfaces, preventing the germination and tube growth of other pollen grains, an increased risk in fruit abortion, and a reduction of the amount of pollen available for export to other individuals (Barrett, 2002; Keller, 2002). Because overly-rewarding plants have to deal with these mechanisms that reduce their fitness, there is a trade-off between a selection of sufficiently attracting floral traits and getting pollinators to forage quickly between different plants (de Jong *et al.*, 1993).

1.1.5 Importance of pollination as an ecosystem service

Ecosystem services are defined as the direct and indirect ways humans benefit from the functioning of ecosystems. These services are many and diverse, but overall can be grouped into four broad categories. First of all, products that are directly obtained from ecosystems are called ‘provisioning services’. These products include provisioning of food, water, timber, etc. The next group of services are referred to as ‘regulating services’. These benefit humans via the regulation of important processes such as the purification of water and air. The next group, called ‘cultural services’, constitute non-material benefits that people obtain through spiritual enrichment, cognitive development, reflection, recreation, and aesthetic experiences. The fourth and last group of services is referred to as ‘supporting services’. They are deemed necessary for the production of all other ecosystem services and their functioning. As briefly mentioned in section 1.1.2, pollination supports plant communities in both natural and agricultural settings and thus belongs to the latter group (MEA, 2005). We will elaborate on its functioning here.

a) In natural ecosystems

Almost 90% of all wild plant species rely on animals for pollination (Ollerton *et al.*, 2011). This means that without pollinators, many plants would not be able to set seed and reproduce (Kearns *et al.*, 1998). Indeed, a reduction of pollination quantity and quality can lead to a lower seed set and higher inbreeding for many plant species (Kearns and Inouye, 1997; Kwak *et al.,* 1998; Tomimatsu and Ohara, 2002). Plant species in small and fragmented populations may be particularly vulnerable to pollination changes (Oostermeijer *et al.*, 2000; Luijten *et al.* 2000). That is, if the isolation between fragments exceeds the foraging range of pollinators, if the local pollinator population becomes too small, or if pollinators avoid plant populations that are too small, reduced pollination services may be the outcome (Kearns *et al.*, 1998). As a result, pollinator limitation in fragmented landscapes can lead to a reduction in seed output by 50-60%, thus further reducing the fitness of plant populations (Jennersten, 1988; Pavlik *et al.*, 1993). The loss of plant populations by fragmentation, and consequently the reduction in gene flow, will further be accelerated by inbreeding, genetic drift, and demographic stochasticity (Rathcke and Jules, 1993; Ellstrand and Elam, 1993).

At the basis of effective pollination services are diverse pollinator communities. For instance, a high insect species richness can increase the chance that a certain plant species is visited by its appropriate pollinator (Saville *et al.*, 1997). This relationship is thought to be caused by niche complementarity, i.e. the tendency for coexisting species to differ in at least one ecological niche dimension, as well as the presence of more specific taxa in a diverse pollinator community (Albrecht *et al.*, 2012).

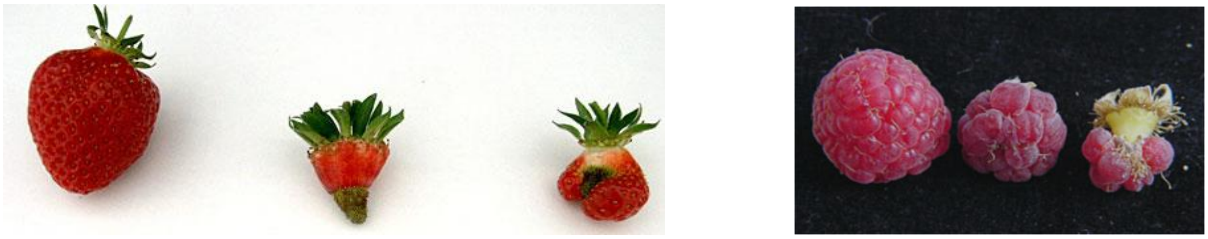

Fig. 1: Fruit set under differing pollination regimes. Strawberry (Fragaria x annanasa Duch.) after open pollination (left), passive self-pollination (middle), and wind pollination (right) (Photo by Kristine Krewenka, Agroecology, Göttingen, Germany). Raspberry (Rubus ideaus L.) after open pollination (left) and passive self-pollination (middle and right) (Photo by Jim Cane, Bee Research Institute, Longan, USA)

b) In agricultural ecosystems

The importance of pollination as a key ecosystem service in agriculture cannot be underestimated, as more than two thirds of crops worldwide depend on animal pollination (Klein *et al.*, 2007). Pollinators in agricultural settings help to produce more, heavier, and more symmetrical fruits and seeds. For instance, bee pollinated strawberries, in contrast to wind or self-pollinated ones, are heavier, show less malformations, are more red, show lower sugar-acid ratios, and are firmer, thus increasing their economically important shelf life (Klatt *et al.*, 2014). Similar results have been found in raspberries as

well (Fig. 1). Secondly, even crops that do not directly require pollination for harvest, like plants that produce timber and fibers, still need pollination for sexual reproduction. Additionally, crops that do not rely on animal pollination to produce seeds still benefit from the availability of pollinators in their surroundings. For instance, in the case of cotton, which is usually wind-pollinated, effective pollination by bees was able to enhance the yield by 20-30% (Allen-Wardell *et al.,* 1998). Surprisingly, the production of meat and dairy products is also dependent on pollination as the cattle feeds on plants such as clover (*Trifolium* sp.) and alfalfa (*Medicago sativa* L.) that need pollination to reproduce (Dias *et al.*, 1999). All of these pollination services in agriculture add up so that the global economic value of animal pollination is estimated at approximately €153 billion. This represents 9.5% of the agricultural value in the world for human food production in 2005 (Gallai *et al.*, 2009).

1.2 Pollinator decline

1.2.1 Observations

Throughout the world, vertebrate and invertebrate pollinators are rapidly declining and are even on the verge of extinction in some areas (Nabhan, 1996; Matheson *et al.*, 1996). It can sometimes prove tricky to show the decline of wild pollinators because of the rarity of some species, the lack of baseline information, and the large spatial and temporal variability of their populations. Nevertheless, evidence of this decline is usually found directly from isolated case studies in a specific taxon, in a specific place, and at a specific time. Additionally, declines can also be found indirectly through studying abundance of pollinators over gradients of anthropogenic disturbance. That is, if pollinator populations are reduced in areas of human disturbance, and the amount of disturbed area increases over time, then it can be expected that pollinators will also decline over time (MEA, 2005).

Direct proof of diminishing pollinator populations has been shown in at least one region of every continent, excluding Antarctica. Direct declines of solitary bees have been found for the UK and Germany (MEA, 2005) and decreasing numbers of honeybees have been shown in the USA by the USDA (2012) and in Europe by EPILOBEE (2016). Furthermore, bumblebees have suffered declines in recent decades, in Europe as well as in North America (Buchmann and Nabhan, 1996; Westrich *et al.*, 1998). For instance, in a study of bumblebees and cuckoo bees in western and central Europe, 80% of taxa were threatened in at least one country and 30% of taxa were threatened throughout their range. Additionally, four taxa went extinct during the period from 1951-2000 (Kosior *et al.*, 2007). Likewise, declines of bumblebees have been shown in North America by Cameron *et al.* (2011). They found that the relative abundance of the four studied species (out of a total of five bumblebee species in the region) declined by up to 96%. In addition, their surveyed geographic ranges contracted by 23 up to 87% as recently as during the last twenty years.

Although these documented declines are significant and important, the extent and pattern of the decline remains elusive. Therefore, there is an urgent need in pollinator research for long-term monitoring on an international scale (Goulson *et al.*, 2015). Nevertheless, because of the strong functional importance of pollinators, these declines have raised concerns worldwide (Biesmeijer *et al.*, 2006; Ghazoul, 2005). In response to these concerns, policymakers and organizations throughout the world have initiated so called 'Pollinator initiatives' to protect this vulnerable group. For instance, the government of the UK has set out to fund research aimed at stopping pollinator declines with a budget of £10 million (Ollerton *et al.*, 2011).

1.2.2 Possible causes of worldwide decline

Possible causes of these worldwide declines are plentiful and diverse. Successful animal-mediated pollination requires both plant and animal, making the diversity of both parties important for pollination. Although different drivers threaten biodiversity directly (Sala *et al.*, 2000), disruptions to crucial interactions, like pollination, are likely to precede actual biodiversity loss (Tylianakis *et al.*, 2008). Overall, the decline of pollinating species and the disturbance of their mutualistic relationship with plants is a global threat and elucidating its different causes is of great importance (Kearns *et al.*, 1998). In the following section we provide a comprehensive outline of the different causes under study so far.

1.2.2.1 Competition with invasive species

Insect pollinators in the Americas have had to compete with Africanized honeybees ('killer bees'), reducing their numbers (Efstathion *et al.*, 2015). African queen bees (*Apis mellifera scutellata*) were accidentally introduced in São Paulo, Brazil in 1957 and they have spread throughout Latin-America and the south of the US (Schneider *et al.*, 2004). These bees challenge endemic wildlife because they are highly defensive and reproduce faster than native bees (Winston, 1992). Furthermore, they are less selective about where they nest, outcompeting native animals (Schmidt and Hurley, 1995).

Other pollinator populations have declined as well when having to compete with non-native pollinators. For instance, introduced hives of *Bombus terrestris* in Japan were able to take over native bumblebee nests by killing their queens. Next to detrimental effects through direct contact, competition for food is a real threat as well. For instance, an up to 90% overlap in plant species use was reported between alien honeybees and native *Bombus* species in the USA (Thomson, 2006). Furthermore, not only invasive pollinators are a threat to native pollinators. Competition of invasive plant species with preferred host plants may also lead to declines in native pollinator populations. This might especially be the case for more specialized species (Traveset and Richardson, 2006).

1.2.2.2 Disease

Diminishing numbers of honeybees in the developed world have been linked to infection by two parasitic mites: *Varroa jacobsoni* (Roy *et al.*, 1988) and *Acarapsis woodi* (Sammataro *et al.*, 2000). Varroa is especially harmful as it is the primary vector of many viruses that are related to honeybee colony losses (Le Conte *et al.*, 2010). Furthermore, by feeding on bee hemolymph fluids, Varroa suppresses host immunity and increases host virus load (Highfield *et al.*, 2009). Pollinators are often co-infected by a diverse set of pathogens including bacteria, viruses (e.g. deformed wing virus; Cameron *et al.*, 2010), and microsporidians (e.g. *Nosema bombi;* Otti and Schmid-Hempel, 2008; Rutrecht and Brown, 2009). This co-infection may explain the difficulty of pinpointing single agents causing honeybee losses (Potts *et al.*, 2010). These parasites of managed pollinators have been known to spill over to wild pollinators as well. For instance, domesticated honeybees in the UK have been shown to be infectious agents with respect to sympatric wild honeybees (Fürst *et al.*, 2014).

1.2.2.3. Environmental change

Ongoing unprecedented environmental change is influencing ecosystems worldwide. The main cause of this change is a steep increase in the demand for food, timber, fiber and fuel (MEA, 2005). To meet this demand, agriculture has strongly intensified, deforestation has increased, and the use of fossil fuel sources has grown (UNEP, 2005). These factors lead to many different mechanisms that can threaten pollinators either directly, by decreasing nesting and habitat opportunity, or by affecting their food.

Particularly the drop in available food sources is well documented. Bees, being obligate pollinators, are dependent on flowering plant species for their survival in varying degrees of specificity. A global loss of 20,000 flowering plant species is predicted in the following decades, which will have strong effects on pollinators worldwide (Heywood, 1995). In this respect, species that are specialized on certain plant taxa are more likely to be at risk than more generalist groups, as the loss of abundancy of these taxa immediately reduces their food sources (Kearns and Inouye, 1997; Kearns *et al.,* 1998).

a) Climate change

Climate change may threaten pollination through phenological mismatches. Consequences of phenological shifts following a doubling in atmospheric CO_2 were simulated through an extensive empirical network of interactions between pollinators and plant species. Within this model, phenological shifts reduced floral resources for 17-50% of all pollinators (Memmott *et al.*, 2007). In addition to effects on the timing of species' life cycles, global warming can also cause shifts in the ranges of many species (Chen *et al.*, 2011). For instance, range compression has been observed among bumblebee species across continents (Kerr *et al.*, 2015).

b) Land use change

Next to changes in climate, global changes in land use have been observed as well. Initially, most ecosystems in Europe were subject of centuries of extensive farming practices. This resulted in semi-

natural and particularly nutrient-poor habitats that often contained very species rich communities (Bignal and McCracken, 1996). However, during the second half of the 20th century, population growth and technological advances led to land use changes and agricultural intensification. These changes have an effect on natural ecosystems through several mechanisms. Firstly, habitat loss and fragmentation, followed by the loss of resource diversity and habitat degradation, seem among the most important consequences of land use changes for pollinator declines (Potts *et al.*, 2010). For instance, it has been shown that species richness of bee communities in agricultural landscapes declines when the amount of semi-natural patches in the landscape decreases (Le Feon *et al.*, 2010; Garibaldi *et al.*, 2011).

Habitat loss is generally considered to be the most important driver of pollinator declines as land use changes have resulted in the disappearance of many semi-natural habitats (Spek, 2004; Brown and Paxton, 2009; Blomqvist, 2005). Because declines in pollinator communities are paralleled by the declines in their associated wild plants and crops, the loss of suitable habitat and its corresponding plant communities could result in declines of pollinator communities as well (Biesmeijer, 2006; Ricketts *et al.*, 2008; Winfree *et al.*, 2009)

Another important driver of pollinator declines is habitat fragmentation, since a declining fragment size has been shown to have a negative impact on bee species richness and abundance. However, this effect was not identical across different groups of bees, with detrimental effects being stronger in bees that are solitary, parasitic and/or collect specialized pollen resources (Steffan-Dewenter *et al.*, 2006). Additionally, fragmentation tends to increase spatial isolation and edge effects in habitats, also harming pollination.

Furthermore, resource diversity for pollinators is decreasing, as local plant diversity has been shown to decline in most habitats (Lavergne *et al.*, 2006). Since these declines seem to affect obligatory outcrossing animal-pollinated plants in particular, it suggests a decline of available food sources for pollinators (Biesmeijer, 2006). This has indeed been observed in the UK, where 76% of plants species used by bumblebees declined between 1978 and 1998 (Carvell *et al.*, 2006). Additionally, modern farming often involves monocultures over large areas, reducing the floral diversity pollinators rely on even further. These crops might give bursts of food availability during short periods of time; however, they are not sufficient to feed pollinators throughout the active season (Rands and Whitney, 2010). Even marginal lands are being increasingly cultivated, which results in a loss of nesting opportunities and food sources. For instance, the UK has lost 30% of its hedgerow habitat, which provides nesting habitat and floral resources for wild bees (Osborne *et al.*, 1991). In addition, the increasing amount of grazing cattle has led to a drop in flowers in grasslands and meadows as well as trampling of pollinator nests (Kearns and Innouye, 1997).

Finally, habitat degradation may affect pollinators by the loss of food and nesting resources and the use of agrochemicals with (sub)lethal effects. These agrochemicals include pesticides that can cause mortality to bees by direct intoxication and can ultimately result in pollinator diversity and abundance

shifts (Alston *et al.*, 2007; Brittain *et al.*, 2010). Systemic pesticides, like neonicotinoids, are widely used in the developed world. They spread throughout plant tissue and can accumulate in nectar and pollen, thus creating a harmful food source for pollinators. This produces sub-lethal effects on pollinator behavior and performance (Cresswell, 2011; Gill *et al.*, 2012). For instance, neonicotinoids exposure can impair brain functioning and learning abilities, and can reduce the foraging performance, growth rate, and queen production in bumblebees (Gill *et al.*, 2012; Henry *et al.*, 2012; Whitehorn *et al.*, 2012; Palmer *et al.*, 2013). Additionally, food storage behavior by social bees can lead to accumulation of these products in the hive, further aggravating the situation (Mullin *et al.*, 2010). On the other hand, the use of herbicides and fertilizers can also affect pollinators indirectly by decreasing floral resources. For instance, wheat fields that were managed organically had a significantly higher bee diversity and abundance compared to conventionally managed fields (Holzschuh *et al.*, 2008). Unfortunately, the impact of agrochemicals is not limited to cultivated lands alone, as these chemicals are known to drift into surrounding habitat. In this respect, increasing arable land in its surroundings has been shown to negatively affect insect-pollinated plants, independent of local habitat characteristics (Clough *et al.*, 2014).

1.3 Nutrient pollution

1.3.1 Nutrient cycles

Nutrients in the soil provide essential building blocks for plants that are necessary for plant growth and development. Nitrogen (N), primarily needed for protein, and phosphorus (P), mainly needed for DNA, RNA, and energy transfer, are key limiting nutrients in many terrestrial ecosystems (Elser *et al.*, 2007; Fay, 2015). These ecosystems are often adapted to conditions of low nutrient availability (Bobbink *et al.*, 1998). However, human activities since the industrial revolution have resulted in an approximate 100% and 400% increase of reactive N and P fluxes, respectively, in global nutrient cycles (Fig. 2; Fallowski *et al.*, 2000; Galloway *et al.*, 2008; Peñuelas *et al.*, 2011). Local concentrations of nitrogen have even been found to exceed background concentrations by 50 times (Penuelas *et al.*, 2011).

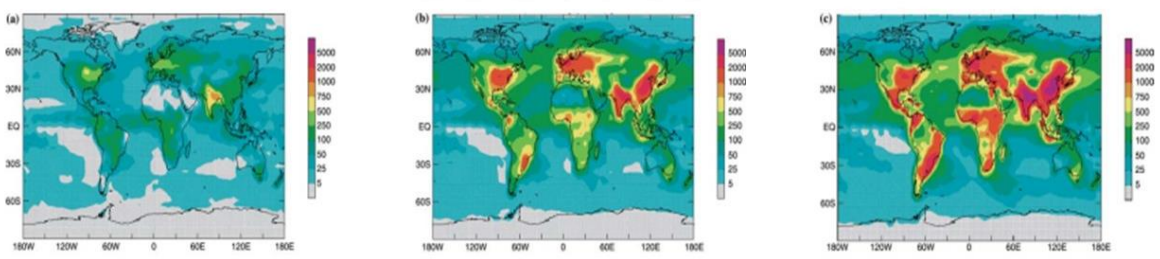

Fig. 2: Global spatial patterns of total inorganic N deposition in 1860 (a), early 1990s (b), and 2050 (c) in mg N m-2 y-1 (adapted from Galloway et al., 2004)

Without human intervention, reactive N is formed by natural processes, such as lightning and biological N formation by bacterial fixation. However, the development and large-scale implementation of the Haber-Bosch procedure in 1902, converting unreactive, atmospheric N_2 to reactive nitrogen, caused a steep increase of anthropogenic nitrogen in ecosystems. Anthropogenic sources of N include combustion of fossil fuels and biomass (NO_x), use of fertilizer and manure (NH_3), and dumping of industrial and household waste in the environment (organic N). Almost all atmospheric reactive N is deposited on the earth surface after being transported over ranges from tens to thousands of kilometers, thus introducing biologically active nitrogen in ecosystems (Bobbink *et al.*, 2010).

Phosphorus in terrestrial ecosystems occurs naturally through weathering of rock and periodic flooding, and anthropogenically by the application of manure and fertilizer (Newman, 1995; Turner and Haygarth, 2001). Additionally, P is deposited from the atmosphere, although it does not have a stable gaseous form, but instead is mainly transported through aerosols. Hence, the distances it travels from the source of pollution are far less than those of nitrogen (Graham and Duce, 1979). Though aerosols and inundation with P rich water are part of natural processes, their levels have recently increased dramatically. This is because the amount of aerosols has risen due to combustion processes and biomass burning (Mahowald *et al.*, 2008). In addition, the use of inorganic P fertilizers and concentrated live stock has greatly increased P values in surface waters (Anderson, 1997). In contrast to N, phosphorus is highly immobile in the environment and remains tightly bound to soils containing high clay fractions, calcium, and sesquioxides (Addiscot and Thomas, 2000; Hinsinger, 2001). This means that P will reside in the environment for a much longer time and its impact will thus last much longer.

1.3.2 Effects of nutrient pollution

Increased nutrients have had dramatic effects on ecosystem functioning and biodiversity. Hence, they have been identified as one of the most important threats to biodiversity (Smith *et al.*, 1999; Sala *et al.*, 2000; Galloway *et al.*, 2008). Negative effects in ecosystems of N addition include direct toxicity to individual species, long-term negative effects of ammonia and ammonium (as they are toxic to sensitive species), and lowered resistance to secondary stress and disturbance. Furthermore, resistance may be lowered by a lower vitality of the plants after N toxicity or disturbance can be higher because of increased herbivory after N content in the plant has increased (Pearson and Stewart, 1993; Kleijn *et al.*, 2008; Bobbink *et al.*, 2010).

However, one of the primary consequences of nutrient pollution is eutrophication, or the increase of above-ground biomass following nutrient enrichment. This can result in increasing light limitation, making it harder for some less-competitive species to recruit seedlings (Craine *et al.*, 2009; Hautier *et al.*, 2009). This means that high-nutrient soils favor good light competitors with a high allocation to aboveground leaves and stems. Additionally, the atmospheric deposition of acidifying N compounds can lead to acidification of soils. This can result in a decrease of soil quality, damage to plant roots,

increased stress sensitivity, and excessive concentrations of nitrate, Al^{3+}, and other metals in the groundwater (Horswill *et al.*, 2008). Furthermore, changed nutrient concentrations can also lead to disruptions in below-ground mutualisms with arbuscular mycorrhizal fungi (AMF). These fungi are known to provide access for plants to previously unusable P forms. Increased P may then then lead to a shift towards plant species that do not require these AMF for P uptake, resulting in a loss of species (Johnson *et al.*, 2008). All of these factors contribute to changes in species composition, resulting in an unprecedented loss of biodiversity (Tilman *et al.*, 2011).

1.4 Floral attractants and rewards in environments under global change

Changing environments, and more specifically nutrient pollution, have been shown to affect floral traits. In particular, the number of flowers per plant, mean corolla and petal width, and reproductive capabilities of the individual plants can be affected (Burkle and Irwin, 2009). These factors play an important role in pollinator attraction, yet do not explicitly affect pollinator food sources. Environmental conditions can however also affect the quantity and composition of the pollen and nectar produced by flowers. Changes in these rewards impact the food sources of pollinators directly.

1.4.1 Nectar secretion rate changes and pollinator response

Individual flowers in a species, and even within an individual, may vary in the rate at which they produce rewards. This variation can be attributed to variations in micro-environment, genotype, age of the plant, and age of the flower (Goulson, 1999). Generally, nectar secretion and pollen production is found to be higher when nutrients are added (Lau and Stephenson, 1994; Petanidou *et al.*, 1999; Gijbels *et al.*, 2014).

Interestingly, bumblebees and honeybees are able to make a distinction between rewarding and non-rewarding flowers without having to actually probe it. Distinguishing rewarding flowers can be achieved through multiple mechanisms. Honeybees checked nectar content by hovering in front of the flower and sometimes shortly making contact with the corolla. Flowers that were then rejected contained on average less nectar than actually visited flowers (Duffield *et al.*, 1993). Additionally, bumblebees can visually check pollen content in open flowers and it is possible that they assess nectar in the same way (Thorp *et al.*, 1975, 1976; Kevan, 1976; Zimmerman, 1982; Cresswell and Robertson, 1994). It is plausible that they can also predict nectar volume through its scent or the scent of yeast fermentation products present in it or from humidity gradients surrounding the flower (Crane, 1975; Corbet *et al.*, 1979; Heinrich, 1979; Williams *et al.*, 1981). Morphological floral traits, such as flower size, age, sex, and symmetry might also be used to spot more rewarding flowers (Shykoff *et al.*, 1997).

1.4.2 Nectar and pollen composition changes

Although it was first thought that nectar composition within a species remained constant, regardless of differences in habitat (e.g. Baker and Baker, 1976), more sophisticated techniques have shown otherwise. For instance, Lanza *et al.* (1995) demonstrated that nectar amino acid composition can vary between and even within populations. Varying CO_2 levels have been shown to affect this composition as well (Rusterholz and Erhardt, 1998). Furthermore, soil nutrient addition can affect the concentration and composition of AAs in floral rewards, as it can alter physiological activities and growth of plant tissue (Gardener and Gilman, 2001). For instance, Gijbels *et al.* (2015) showed that experimental nutrient addition changed nectar AA composition in the orchid *Gymnadea conopsea*, while other floral traits remained constant. Fertilized plants had more pollinia removed and a higher fruit set, but their fruit contained less and more selfed seeds. Another study added soil enhancing humus litter to investigate the effect of nitrogen addition. It was found that below-ground competition between plants following these additions can induce changes in plant development and affect nectar composition, particularly sugar concentration (Baude *et al.*, 2011). Similarly, P additions led to higher phosphorus contents in pollen produced by *Curcubito pepo* (Tau and Stephenson, 1994).

1.5 Effect of nectar and pollen composition following nutrient pollution on pollinators

Hoover *et al.* (2012) showed that nitrogen treated plants had an altered AA and sugar chemistry. Individual bumblebees feeding on nectar from these plants had a reduced survival of on average 8 days. In another study, bumblebees visited flowers of plants treated with organic fertilizer significantly longer and found the flowers faster compared with those treated with chemical fertilizer. The flowers of plants in these organic treatments seemed larger, which was however not significant, and their pollen had a higher protein content. The bumblebees feeding from the organic treatment also had bigger and more active ovaria, a trait associated with high quality food (Cardoza *et al.*, 2012). Since nectar and pollen are indispensable food sources for bee colonies, their survival and growth depend on its quantity and nutritional quality (Cook *et al.*, 2003). The quantity of these food sources is shown to be strongly affected by land use changes in ecosystems worldwide, to the extent that even nectar availability on the scale of a flower is decreasing following nutrient pollution (Section 1.4.1). Although the effects of reduced food quantity are being unraveled, little is known about the effects of changes in food composition yielding a possible reduction in food quality for pollinators.

1.6 Hypotheses

In this study, we aim to assess the stress and colony size of bumblebee colonies when fed with flowering plants that have been subject to soil nutrient addition in a greenhouse mesocosm experiment. Our expectation is that soil nutrient addition affects amino acid and sugar composition in nectar and pollen of the flowers, thus changing the food quality for bumblebees and, in turn, affecting bumblebee colony size and stress.

Our specific hypotheses were:

i) Nectar and pollen AA composition of flowers of plants will be affected by nutrient addition
ii) Bumblebee colonies will use this nectar and pollen as a food source
iii) Bumblebee colony size and stress levels will be negatively affected when their food sources have undergone nutrient addition, resulting in:
    a. Less workers with a lower dry weight
    b. Less reproduction
    c. Higher and faster mortality
iv) Plants are affected by nutrient addition and produce more above-ground biomass, but are impaired regarding effective reproduction due to altered pollination patterns
    a. More flowers
    b. More rosette leaves and flowering stems that are higher
    c. Less seeds with a lower germination rate

2. Methodology

2.1 Bombus terrestris

2.1.1 Description

Bombus terrestris L. (buff-tailed bumblebee) is a common bumblebee species in temperate areas across the globe. The queens of this species are 20 to 22 mm long, while workers range between 11 and 17 mm, and males 14 to 16 mm. *B. terrestris* is colored black, except for a yellow band on the thorax and second abdominal segment. Queens are colored a yellowish brown/buff from the fourth segment down, hence their name. Workers look similar to the queen, with their tails having a whiter appearance and a subtle buff colored line separating the tail from the abdomen. Males on the other hand have a buff-tinged tail (Fig. 1; Sladen, 1912).

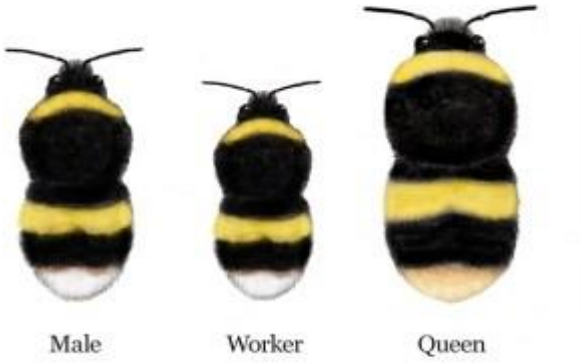

Fig. 3: *B. terrestris* morphology (Source: https://bumblebeeconservation.org/about-bees/identification/common-bumblebees/)

This short-tongued bumblebee is considered a generalist, as it is able to collect floral rewards from many plant species in a large variety of habitats. Characteristics facilitating this generalist behavior include ecological flexibility, a relatively early seasonal emergence (mainly of queens), long mean foraging distances, buzz-pollination behavior, and nectar robbing (Matsumura *et al.*, 2004).

B. terrestris is distributed almost globally. It is found as a native pollinator throughout continental Europa and adjacent areas. This includes Mediterranean islands, southern Scandinavia, England, most of Scotland and some Atlantic islands (Velthuis and Van Doorn, 2006). Furthermore, the agricultural value of this species has long been recognized and utilized. For instance, in 1885 and 1906 hundreds of queens were caught in the UK and released in New Zealand to improve seed set of red clover (*Trifolium pratense* L.; Hopkins, 1914). More systematically, the domestication and rearing of locally collected *B. terrestris* started in 1988 in Belgium and the Netherlands. Domesticated colonies have now been brought to Korea, Japan, China, Taiwan, Mexico, Chile, Argentina, Uruguay, South Africa, Morocco, and Tunisia (Dafni, 1998; Hingston *et al.*, 2002). For instance, the annual importation to Japan is 60,000 colonies (Goka and Japanese Bumblebee Companies Association, 2003). The species has spread throughout these regions as a result of commercialization and is known to exhibit invasive behavior there (Goulson, 2010).

2.1.2 Life cycle

As most *Bombus* species, *B. terrestris* has an annual life cycle. This cycle is described extensively by Goulson (2010), among others, and is summarily reported here. In February or March, queens emerge

from hibernation looking for suitable, underground nesting sites. They usually consist of pre-existing holes in the ground, often burrows abandoned by rodents. When the nest is chosen, the queen supplies it with pollen, which she molds into a lump. This is where she lays her first 8 to 16 eggs. The outside of this pollen lump is then covered in a layer of wax mixed with pollen. At this point, she also forms a honeypot by the entrance of the nest, where she stores nectar. After an incubation period of about four days, the eggs hatch. During this time, the queen broods the pollen lump by generating a lot of heat. The emerging larvae then consume part of the pollen and reside in it. The whole is now referred to as the brood clump. Other than incubating the brood, the queen also needs to forage for nectar and pollen. This period is thought of as the most delicate phase in the *Bombus* life cycle, as insufficient foraging and/or inclement weather can cause the queen and young colony to succumb. In a period of ten to fourteen days, the larvae go through four instar stages, after which they spin a strong silk cocoon and pupate. After another approximately 14 days the pupae hatch, making the total development time four to five weeks. However, this is strongly dependent on temperature and food supply. The different life stages in a commercially reared *B. terrestris* nest are shown in Fig. 4. The first batch of adults are almost all workers. Within a few days after their hatching, the queen stops her foraging behavior, as this activity is now taken over by some of the new workers. Others help the queen tend the brood, as she continues to lay eggs. The colony can grow rapidly from this point onwards, potentially increasing tenfold in weight in three to four weeks.

Fig. 4: Picture of 5 to 6 week-old *B. terrestris* nest, reared by Biobest Belgium NV

When the nest reaches a critical size, the colony switches to start producing males and new virgin queens, also called reproductives. In Hymenoptera, the haploid males are produced from unfertilized eggs, meaning that queens can choose the sex of their offspring when laying their eggs. Furthermore, any egg laid by a worker is bound to be male as they have never mated before. The amount of reproductives produced in a nest generally depends on the colony size. That is, small colonies will not produce any at all, medium-sized hives will only produce males, and bigger ones will produce both. However, the timing in which colonies switch to rearing reproductives can vary greatly. For instance, some nests will switch while the colony is still small, after approximately ten days after the emergence of the first workers. Other colonies make this switch later, on average 24 days after the first workers appear (Duchateau and Velthuis, 1988). Young queens leave the nest to forage, while still returning back to it at night and in between foraging bouts. However, they do not provide the nest with food, but rather build up their own fat reserves. On the other hand, males do not play a role in the nest at all, as they leave the hive permanently after only a few days. Outside the nest, they forage for food and look for a mate. After the reproductives leave the nest, it rapidly degenerates.

Bombus males look for potential mates by leaving pheromones in several places early in the morning. During the course of the day, they check these spots on a regular flight circuit. When they encounter a young queen during their flight, they try to mate with it (Williams, 1991). *B. terrestris*, as most other bumblebee species, only mates once. This means that all offspring of one queen are full siblings. After mating, the queen might continue to feed for a while, but she starts searching for a suitable hibernation site not long after. Once an appropriate spot is found, the queen digs a hole in the ground a few centimeters deep. Here, she forms a small oval room where she will go into diapause until spring. As some queens can go in diapause as early as June, they have to overcome a long period without food. They do this by relying on fat reserves that fill their abdominal cavity. The different stages of a generic *Bombus* nest are shown in Fig. 5.

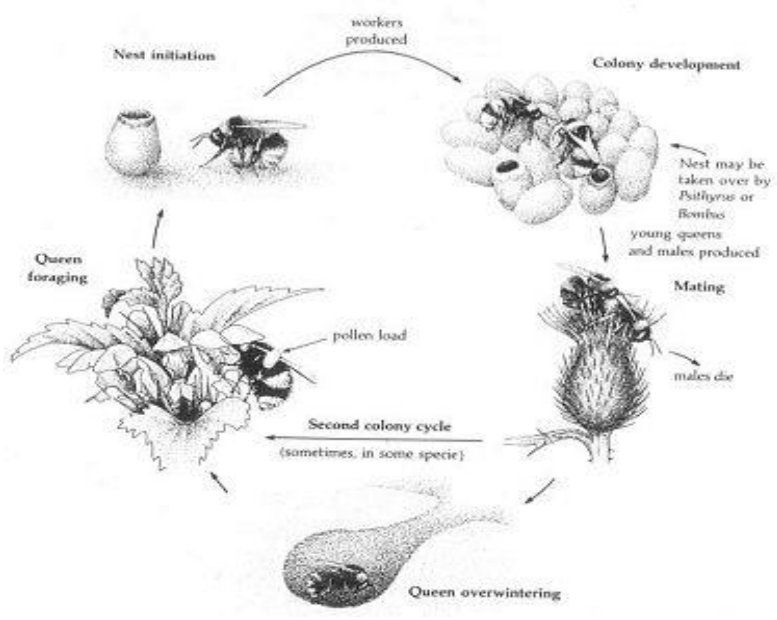

Fig. 5: Depiction of *Bombus* life cycle
(Source: http://www.bumblebee.org/lifecycle.htm)

2.2 *Succisa pratensis*

Succisa pratensis Moench (Devil's bit scabious; Fig. 6) is a perennial rosette herb with a short vertical rhizome (Adams, 1955). It flowers in August and September, and doing so, it produces one to 21 flower heads on up to ten different flowering stems. These stems are about 20 to 80cm tall, although this can vary greatly in different environments. Every flower head consists of 70-110 of violet four-lobed tube flowers (van der Meer *et al.*, 2014). Reproduction usually occurs sexually through the production of seeds, but vegetative propagation can happen sporadically by the formation of side rosettes (Adams, 1955; Jongejans and de Kroon, 2005). Genets can live up to 25 years and seed banks are transient to short-term persistent (McDonald *et al.*, 1996; Hooftman *et al.*, 2003; Wallin *et al.*, 2009).

This plant is found throughout temperate zones of Eurasia in nutrient poor grasslands, both on acidic and basic soils. Typical habitats include heathlands, unfertilized hay meadows, and calcareous fens (Adams, 1955). Although it is still a quite common species, it has been subjected to changes in land use, habitat fragmentation, and habitat degradation, which caused its area of distribution to decrease by 74% since 1935 (van der Meijden *et al.*, 2000). The remaining populations are usually small and isolated (Vergeer *et al.*, 2003).

Fig. 6: *S. pratensis* (Source: Carl Axel Magnus Lindman [Public domain or Public domain], via Wikimedia Commons)

Although *S. pratensis* is self-compatible, outcrossing is preferred as it promotes seed set. Fertilization is usually animal-mediated through insects including bees, bumblebees, tachinids, and hoverflies. As this plant flowers relatively late in the season, it is an important source of nectar and pollen for many insects right before winter (Vergeer *et al.*, 2003).

2.3 Mesocosm experiment and set-up

In this experiment, we created mesocosms in bugdorms (60x60x60cm; BugDorm Store, Taiwan) using colonies of *Bombus terrestris* L. as pollinators, and *Succissa pratensis* Moench plants as the main food source. We obtained commercially available, four-week-old hives of *B. terrestris* (Biobest Belgium NV). These hives were equipped with see-through tops so the colonies could be easily observed. They were kept in cardboard boxes as to not expose the nest to light. The hives were supplemented with sugar water containers. In the bugdorms, hives were provided with commercially available *S. pratensis* plants (Ecoflora, Belgium) so that the amount of flowers was similar across mesocosms. We added 700ml of nutrient or control solutions (Table 1) to these plants over the course of two weeks, starting three weeks before the start of the experiment. The nutrient solution consisted of 350ml of N and 350ml of P solution. Additionally, the plants received continuous treatment during the experiment to ensure all new bumblebee food sources were affected by the treatment. This was achieved by supplying the plants with a diluted solution throughout the experiment (Table 1).

The experiment was conducted in a greenhouse with average temperatures of 24.36 ± 5.12 °C, relative humidity of 61.10 ± 30.75% and light intensity averaged over daytime of 106.04 ± 53.95 W/m². We had two treatments: i) fertilized, with plants that got the nutrient solution and ii) non-fertilized, with plants that got the control solution. The fertilized and non-fertilized treatments each had 17 replicates and were randomized in the greenhouse. All mesocosms were provided with water in a petri dish ad libitum. Additionally, we supplied commercially available pollen (Weyn's Honing, Belgium) as an extra food source. This is because we wanted to test for a difference in food quality, not quantity. Therefore,

Table 1: Composition of solutions for fertilization of the *S. pratensis* plants

	Treatment	Solution	Dilution before use
Initial solution	N	28,3g NH_4NO_3 + 13,9g NaCl + 1l H_2O	
	P	16,4ml concentrated H_3PO_4 + 13ml 50% NaOH solution + 750ml H_2O	380ml in 2l H_2O
	Control	13,9 NaCl in 1l H_2O	
Diluted solution	NP	500g KNO_3 + 56g KH_2PO_4 in 10l H_2O	10l in 2000l H_2O
	Control	400g KCl in 10l H_2O	

we had to ensure that the bumblebees were supplied with enough food. However, considering they needed to use *S. pratensis* as their main food source, we gave limited amounts of pollen to the fertilized and non- fertilized treatments and refilled equally across treatments when deemed necessary. The weight of consumed pollen was recorded per mesocosm. Mesocosms were supplied with four plants in the first two weeks, after which the plants were taken out of the mesocosms for three days to harvest nectar and pollen. Afterwards, the mesocosms were supplied with three plants for the rest of the experiment. The set-up of the mesocosms is illustrated in Fig. 7

Throughout the duration of the experiment, we monitored several variables of either direct interest or necessary as supporting information. Firstly, we observed the colonies weekly by opening the cardboard boxes the hives were in. We did this after sunset to minimally disturb the colony and so that most of the workers had returned to the hive for the night. Doing so, we counted the number of the different life stages of the colonies to assess colony size and fecundity, as this is strongly correlated with colony fitness (Mattila and Seeley, 2007). Furthermore, we record the number of dead larvae in the hive, which has a delayed effect on colony size. Also the weight of workers and queen was

Fig. 7: Left: Components of the mesocosms used in the experiment with a *B. terrestris* colony (a), *S. pratensis* plants (b), pollen (c), and water (d). Right: Depiction of the actual experiment in the greenhouse

recorded, as a lower weight can be the result of stress and can further limit reproduction in individuals (Scofield and Mattila, 2015). We also documented the date of queen death, as this rapidly leads to colony degradation in wild colonies (Rutrecht and Brown, 2009). Additionally, a detailed photograph was taken for further analysis and counting of life stages and honeypots (Table 2). Furthermore, we periodically collected all the dead workers found outside of the nest. All weights were determined after drying the bumblebees at 50°C for 24 hours.

As a way of verifying whether the nutrient treatment of the *S. pratensis* plants had actually affected the composition of the provided pollen and nectar for the bumblebees, we analyzed the AA and sugar composition of nectar, pollen, and honey. This was achieved by taking bulked samples from five flowers in the most recently opened flower head of every plant. We did this by pipetting 10 µl of 50% azide water up and down in the flower five times. Azide is a biocide and stops bacterial growth immediately. By storing our nectar samples in this solution, we keep the composition of our samples relatively constant, as microbial communities have been shown to degrade floral nectar (Herrera *et al.*, 2008). Furthermore, we sampled pollen by cutting off anthers that had pollen on them. We collected everything in Eppendorf tubes and kept them in the freezer (-20°C) until further analysis.

To verify that the bumblebees were feeding off the provided *S. pratensis* plants, we observed foraging behavior. Doing so, we recorded if workers were foraging either on a flower head, on the available pollen, or on both during censuses of 5mins. The number of active workers and the number of open flowers was also recorded. Throughout the experiment, we made sure to obtain as many seeds as possible from every plant by harvesting them when they were ripe.

We ended the experiment after 9 weeks when most of the colonies had collapsed and most of the plants had stopped flowering, no longer fulfilling their function as food source. We put the hives in the freezer at -20°C for 24h to sacrifice remaining living life stages. Next, we collected the queen and workers separately, after which they were dried and weighed. To assess the food quantity taken up by the hives during the experiment, we counted the number of full and empty honeypots and these were weighed after being dried for 24h at 50°C. Finally, the total weight of the containers with extra sugar water was determined to assess how much was consumed during the experiment.

Then, we measured several plant above-ground biomass characteristics (Table 2) and counted the seeds for each plant. Next, we prepared two petri dishes per plant with moist paper and put out 30 seeds per dish. These were sealed with parafilm and kept in a germination room at room temperature with 16h of light per day. After five weeks, the germination rate was determined.

2.4 Analysis of nectar and pollen AA and sugar composition

We analyzed nectar and pollen composition with a HPAEC-PAD on an ICS3000 chromatography system (Dionex, Sunnyvale, CA, USA). Samples of the two treatments were analyzed randomly. The analysis and detection was carried out at 32 °C with a flow rate of 250 µL per min. Sugar analysis was performed by injecting 15 µL of diluted sample on a Guard CarboPac PA 100 column (2 x 50 mm; Dionex) in series with an analytical CarboPac PA 100 column (2 x 250 mm; Dionex). Sugars were eluted in 90 mM NaOH, with an increasing NaAc-gradient from min 0–6, the NaAc-concentration increased linearly from 0–10 mM from min 6–16. From min 16–26, the NaAc-concentration increased linearly from 10–100 mM; finally, the concentration increased linearly from 100–175 mM from min 16–26. The columns were then regenerated with 500 mM NaAc for 1 min and equilibrated with 90 mM NaOH for 9 min before the next run started. AA analyses started by injecting 15 µL of diluted sample on an AminoPac PA 10 column (2 x 50 mm; Dionex) in series with an analytical AminoPac PA 10 column (2 x 250 mm; Dionex). AAs were eluted in 50 mM NaOH for 13.8 min. From 13.8 to 17.8 min, the NaOH concentration increased with curve 8 (concave) from 50 to 80 mM. From 17.8 to 25.8 min, NaOH concentration decreased from 80 to 60 mM, while the sodium acetate concentration increased from 0 to 400 mM with curve 8. These concentrations were kept constant from 25.8 to 41.8 min. The columns were then regenerated with 125 mM NaOH and 500 mM sodium acetate for 1 min and equili- brated with 50 mM NaOH for 10 min before the next run started. Retention times of both sugars and AAs were calibrated every four samples by injecting a mixture with standard sugars or AAs with known concentrations. The concentrations of the different sugars and AAs in each analyzed sample were estimated by comparing the area under the chromatogram peaks with standards using Chromeleon software (Dionex, Sunnyvale, CA, USA). In general, individual sugar and AA composition is less variable than its concentration, meaning that their relative contribution may be of greater biological importance (Gardener and Gilman, 2001; Gijbels *et al.*, 2014). This is why, when statistically comparing the AA and sugar compositions between treatments, we use proportions of compounds, rather than their absolute amounts.

2.5 Statistical analysis

We analyzed the effect of treatment (fertilized *vs.* non-fertilized plants) and week in the experiment on the stress indicators, as well as the number of live workers, that were recorded weekly by means of repeated measures ANOVAs. Next, we compared the date of queen death between treatments through a right-censored, Weibull-distributed survival analysis. Additionally, bumblebee dry weights between treatments were compared through independent sample t-tests.

We analyzed the effect of treatment on AA and sugar composition of nectar and pollen between treatments using multivariate statistics. Specifically, we performed permutational multivariate

Table 2: Measured variables of plant and pollinator during the experiment. Variables in italics were measured weekly throughout the duration of the experiment.

Variables of interest	Colony size and fecundity	*Number of live workers* *Number of gynes and males* *Number of eggs* *Number of larvae (phase 1-2 and 3-4)* *Number of pupae*
	Indicators of stress	*Number of dead larvae* Date of queen death Mean weight of workers Weight of queen
Supporting variables	Efficacy of treatment	AA and sugar composition of nectar and pollen of plants - Shannon diversity index and Evenness of AA composition - Total amount of AA - Hexose sugars/sucrose ratio
	Plant above-ground biomass and reproductive traits	Max and mean height of rosette leaves Max and mean height of flowering stems Number of rosette leaves Number of flowering stems Number of flowers Number of seeds Number of seeds per flower Germination rate
	Foraging behavior of pollinator	Time of day Number of open flowers Foraging activity on flower Foraging activity on extra pollen Number of active bumblebees
	Food quantity	*Number of honeypots* *Weight of pollen consumed* Weight of honeypots Number of empty and full honeypots Weight of remaining sugar water

ANOVAs (PERMANOVA) through adonis after verifying the assumption of homogeneous multivariate dispersions (999 permutations; vegan package, R; Oksanen *et al.*, 2013). If a significant difference in composition between groups was found, we compared AA and sugars between treatments post-hoc through Wilcoxon signed-rank tests. Then, we adjusted the obtained p-values through Bonferroni corrections. Furthermore, we performed nonmetric multidimensional scaling (NMDS) on the AA and sugar composition matrices through Bray-Curtis distances (vegan package, R). Afterwards, we fitted treatment as an explaining variable on these ordinations. We then tested its significance permutationally using envfit (1000 permutations; vegan package, R). Next, we determined the total amount of AA, as well as the Shannon and Evenness diversity indices of AA composition in each sample. Additionally, we calculated the ratio of hexose sugars (fructose and glucose) over sucrose in all samples. As *B. terrestris* is a short-tongued bumblebee, this group is known to prefer hexose-rich nectar (fructose and glucose) over sucrose-rich nectar (Baker and Baker, 1990; Dupont *et al.*, 2004; Krömer *et al.*, 2008). As such, this ratio might provide insights in the nutritional sugar value of our food sources. All measures describing sugar and AA composition in pollen and nectar were analyzed with Wilcoxon signed-rank tests, as they all proved to be non-normal.

Prior to analyzing the collected plant variables, we standardized the number of seeds by dividing the total number of seeds produced by a plant by its number of flower heads and checked the plant traits in Table 1 for normality. Number of flowering stems, number of flowers and germination rate did not fulfill this assumption, so we compared their means between treatments through a Wilcoxon signed-rank test. Then, we analyzed the remaining plant variables with an independent sample t-test.

To assess whether the bumblebees used the plants as their main food source, we tested if there was a difference between the amount of foraging on *S. pratensis* flowers and commercial pollen through a binomial-distributed GLMM using glmer (package lme4, R). Food source was a fixed factor and colony a random factor. Analogously, we analyzed the difference between fertilized and non-fertilized treatments with treatment as a fixed factor.

The effect of treatment and time on the number of honeypots as well as the amount of pollen consumed throughout the experiment was analyzed with repeated measures ANOVAs. Ultimately, the number of empty and full honeypots, their weight, and the remaining weight of the sugar water was compared between treatments with independent t-tests.

3. Results

3.1 Variables of interest: indicators of stress

We found a significant negative effect of nutrient addition and week on the number of dead larvae in the colonies (Fig. 8), but not of their interaction. However, the assumption of sphericity was violated for week and this was, as for all following repeated measures ANOVAs, corrected for with Greenhouse-Geisser, Huynh-Felft, and Lower-bound corrections. Nevertheless, this did not affect the significance of the negative effect of week on the number of dead larvae (Table 3). On the other hand, we did not find a significant effect of treatment on the number of live workers in the colonies (Fig. 9), but the interaction between treatment and week was significant. However, sphericity was violated for the interaction term and the most conservative correction was not significant. As with the number of dead larvae, week had a negative significant effect, but sphericity was violated. When corrected, it remained a significant effect on the number of live workers in the colony (Table 4).

Table 3: Results of repeated measures ANOVA testing for the effect of condtion, week, and their interaction on the number of dead larvae in the colony. Where the assumption of sphericity was violated, we displayed corrected statistics as well in increasing order of conservativity. *** p < 0.001, ** p < 0.01, * p < 0.05

Effect	Correction used	F	p-value
Treatment	None	6.18	0.025 *
Week	None	15.92	< 0.001***
	Greenhouse-Geisser	15.92	< 0.001 ***
	Huynh-Feldt	15.92	< 0.001 ***

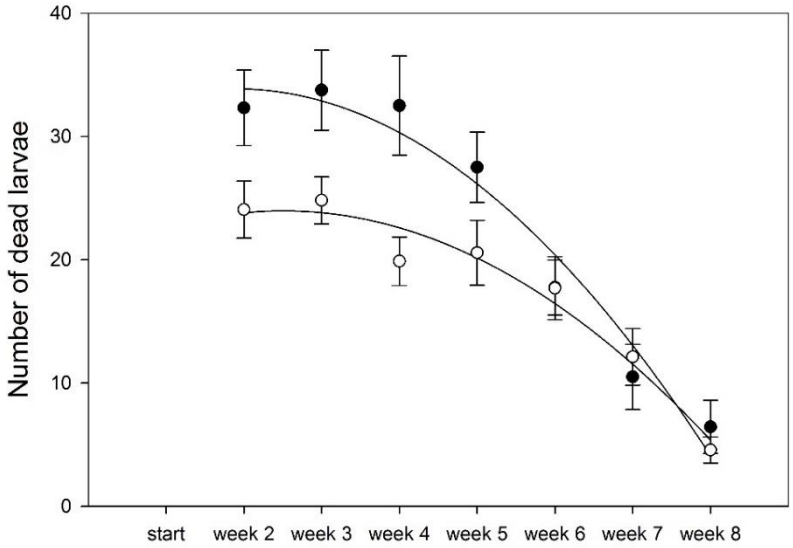

Fig. 8: The number of dead larvae through time in hives of fertilized (black) and unfertilized (white) treatments. We found a significant negative effect of nutrient addition (F=6.18, p=0.025) and week (F=15.92, p < 0.001)

	Lower-bound	15.92	< 0.001 **
Treatment * Week	None	1.76	0.13

Table 4: Results of repeated measures ANOVA testing for the effect of condtion, week, and their interaction on the number of live workers in the colony. Where the assumption of sphericity was violated, we displayed corrected statistics as well. *** p < 0.001, * p < 0.05, . p < 0.1

Effect	Correction used	F	p-value
Treatment	None	1.82	0.20
Week	None	238.83	< 0.001 ***
	Greenhouse-Geisser	238.83	< 0.001 ***
	Huynh-Feldt	238.83	< 0.001 ***
	Lower-bound	238.83	< 0.001 ***
Treatment * Week	None	2.50	0.021 *
	Greenhouse-Geisser	2. 50	0.091 .
	Huynh-Feldt	2. 50	0.079 .
	Lower-bound	2. 50	0.14

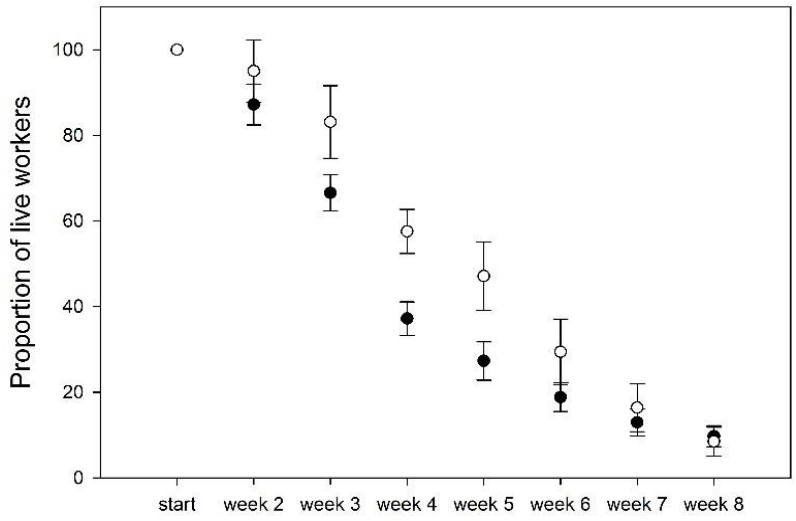

Fig. 9: The proportion of live workers through time in hives of fertilized (black) and unfertilized (white) treatments. Week 1 was choosen as a reference point. We found a significant effect of week (F=238.83, p < 0.001) a marginally negative effect of the interaction between nutrient addition and week (F=2.50, p=0.091). In the middle of the experiment, from week 3 to 5, the treatment has the biggest effect on the proportion of live workers. This effect diminishes at the start and end of the experiment.

The mean weight of workers did not significantly differ between the fertilized (0.058 ± 0.021g) and unfertilized treatments (0.057 ± 0.011g; t=0.19, df=24.81, p=0.85). The weight of queens did not significantly differ between the fertilized (0.19 ± 0.042g) and the non-fertilized treatments (0.22 ± 0.049g; t = -1.3745, df = 23.157, p = 0.1824) either. In our computed survival model, that fulfilled the assumption of non-systematic deviance, the chance of queen survival through time did not differ

between treatments either (LR χ^2=952.84, p=0.69; Fig. 10). The mean time of death in the fertilized treatment was modelled to be 7.04 ± 0.57 weeks and 6.74 ± 0.53 weeks in the non-fertilized treatment.

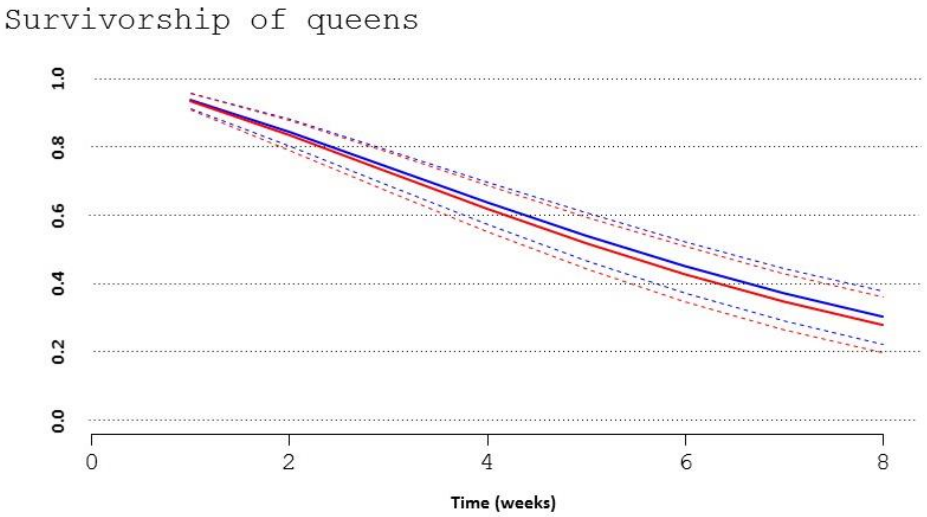

Fig. 10: The chance of queen survival in our computed survival models in fertilized (blue) and non-fertilized (red) treatments. We found no significant difference between treatments (LR χ^2=952.84, p=0.69)

3.2 Supporting variables

3.2.1 Efficacy of treatment

a) Nectar

The nectar produced by fertilized and unfertilized plants differed significantly in total AA amount, with unfertilized plants having a higher quantity of AA in their nectar (Table 5). However, unfertilized plants had lower indices of diversity describing their AA composition (Table 5). When analysing the AA composition of nectar sampled from unfertilized and fertilized treatments, the NMDS showed a significant difference between unfertilized and fertilized treatments (R^2=0.067, p= 0.021; Fig. 11). This was confirmed by PERMANOVA analysis (F=6.46, R^2=0.057, p=0.002). Specific AAs that differ between treatments were revealed by post-hoc testing (Fig. 12). Ordination by NMDS of the sugar composition in nectar revealed marginally significant differences between treatments (R^2=0.20, p=0.066). Similar results were obtained from the PERMANOVA (F=2.91, R^2=0.10, p=0.064). Post hoc analysis showed that these differences can be attributed to a marginal difference in relative glucose contributions between treatments (p=0.081; Fig. 13). We found no significant difference between the ratio of hexose sugars and sucrose in nectar of plants of the two treatments.

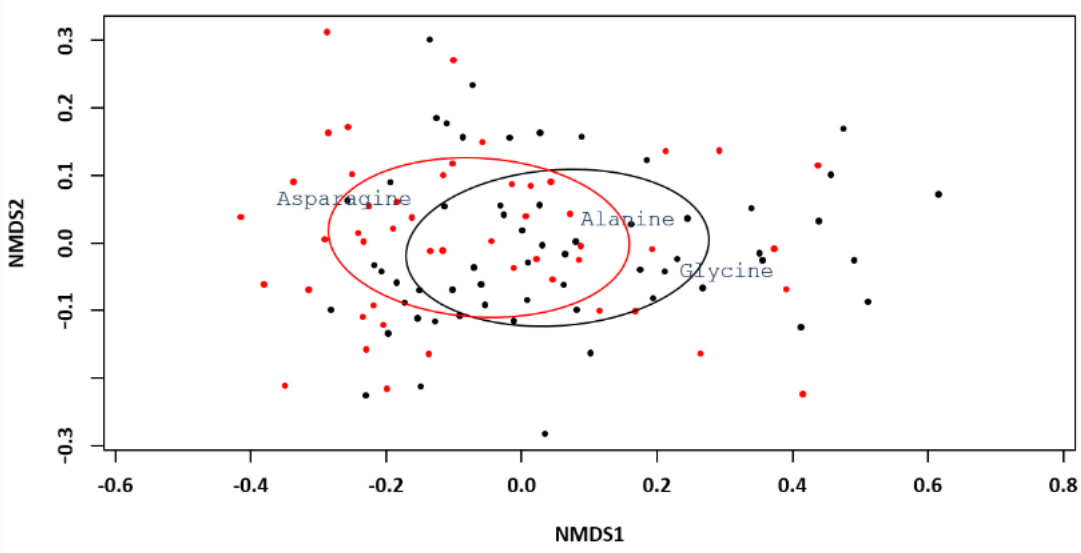

Fig. 11: NMDS plot of AA composition of nectar produced by fertilized (red) and unfertilized (black) plants. Ovals represent standard deviation for their respective treatments. We found a significant effect of treatment on this ordination (R^2=0.067, p= 0.021). Only significantly differing AAs after Bonferroni correction are shown.

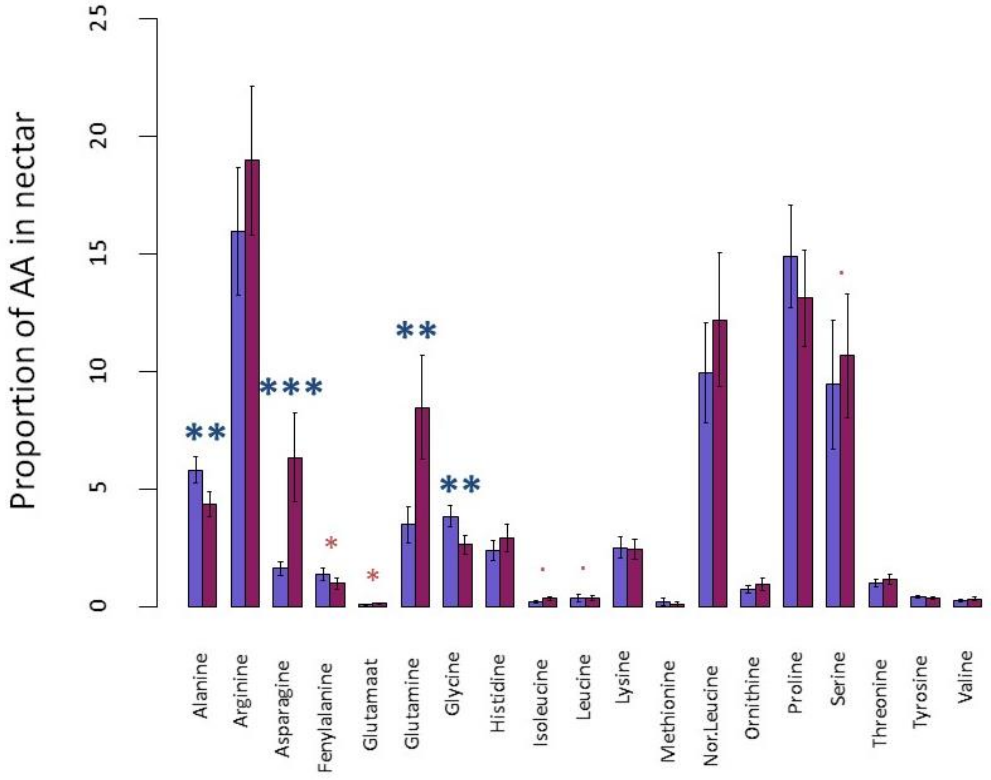

Fig. 12: Relative amounts of individual AAs in nectar between treatments (blue: unfertilized, red: fertilized). Significant differences between treatments are shown (red), with those significant after Bonferroni corrections in blue (*** P < 0.001; ** P < 0.01; * P < 0.05; . P < 0.1)

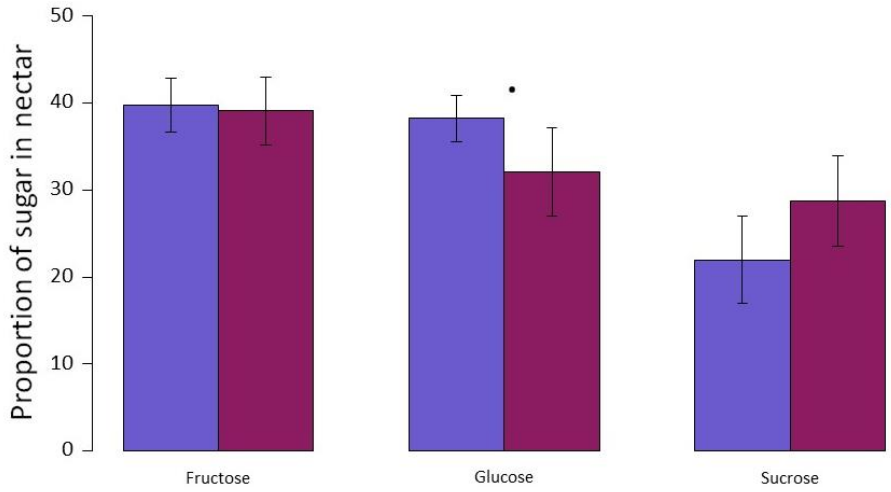

Fig. 13: Relative amount of sugars present in the nectar samples of unfertilized (blue) and fertilized (red) plants. There is marginally significantly more glucose in the nectar of unfertilized plants (p=0.081).

Table 5: Mean ± sd of describing measures of nectar and pollen AA and sugar composition. Differences between groups were tested with Wilcoxon signed-rank tests and significance of W-values is indicated (* $p < 0.05$, ** $p < 0.01$).

		Fertilized	Not fertilized	W
Nectar	Total AA (µmol/l)	482.27 ± 414.75	808.52 ± 886.40	1765 *
	Sugar ratio	249.35± 1111.84	207.51 ± 503.06	1578
	Shannon index AA composition	2.33 ± 0.28	2.16 ± 0.33	1017 **
	Eveness AA composition	0.70 ± 0.08	0.65 ± 0.10	1017 **
Pollen	Total AA (µmol/l)	1019.36 ± 808.47	572.70 ± 323.06	428 **
	Sugar ratio	128.03 ± 319.82	151.35 ± 556.13	726
	Shannon index AA composition	0.91 ± 0.52	0.88 ± 0.42	766
	Eveness AA composition	0.27 ± 0.16	0.27 ± 0.13	766

b) Pollen

We found a significant positive effect of nutrient addition on the total amount of AAs in pollen (Table 5). However, we found no difference between the two diversity indices (Table 5). When analysing the AA composition of pollen sampled from fertilized and unfertilized plants, the NMDS showed a significant difference between treatments (R^2=0.32, $p < 0.001$; Fig. 14). This was also the case for the PERMANOVA we performed (F=6.99, R^2=0.084, $p < 0.001$). Significantly differing AAs are shown in Fig. 15. Ordination by NMDS of the sugar composition in pollen revealed a significant difference between treatments as well (R^2=0.13, p=0.003). Similar results were found through PERMANOVA (F=6.33, R^2=0.076, $p < 0.001$). Post-hoc testing indicated that there is more fructose (p=0.002) and less glucose (p=0.012) in the pollen of unfertilized plants (Fig. 16). The sugar ratio of hexose sugars and sucrose did not significantly differ (Table 5).

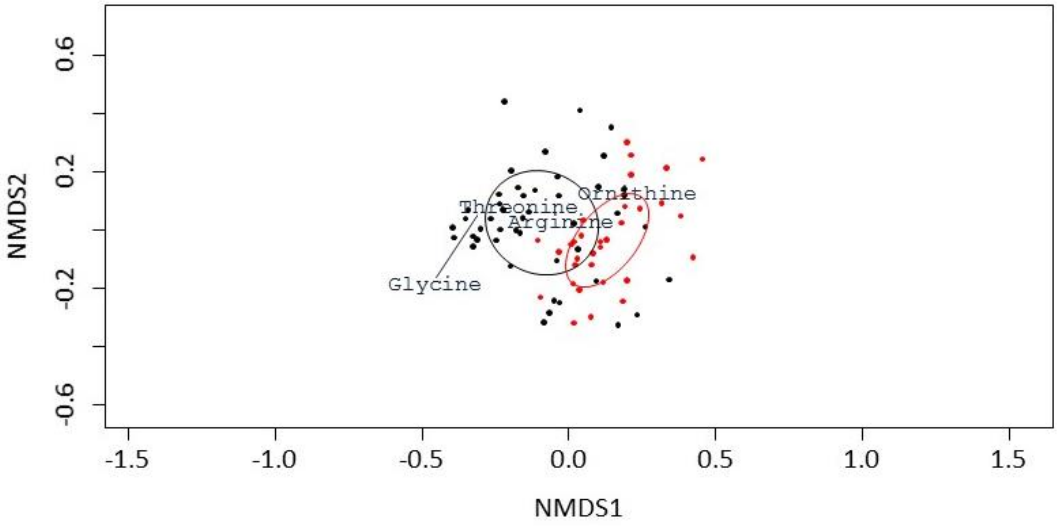

Fig. 14: NMDS plot of AA composition of pollen produced by fertilized (red) and unfertilized (black) plants. Ovals represent standard deviation for their respective treatments. We found a significant effect of treatment on this ordination (R^2=0.32, $p < 0.001$). Only significantly differing AAs after Bonferroni correction are shown.

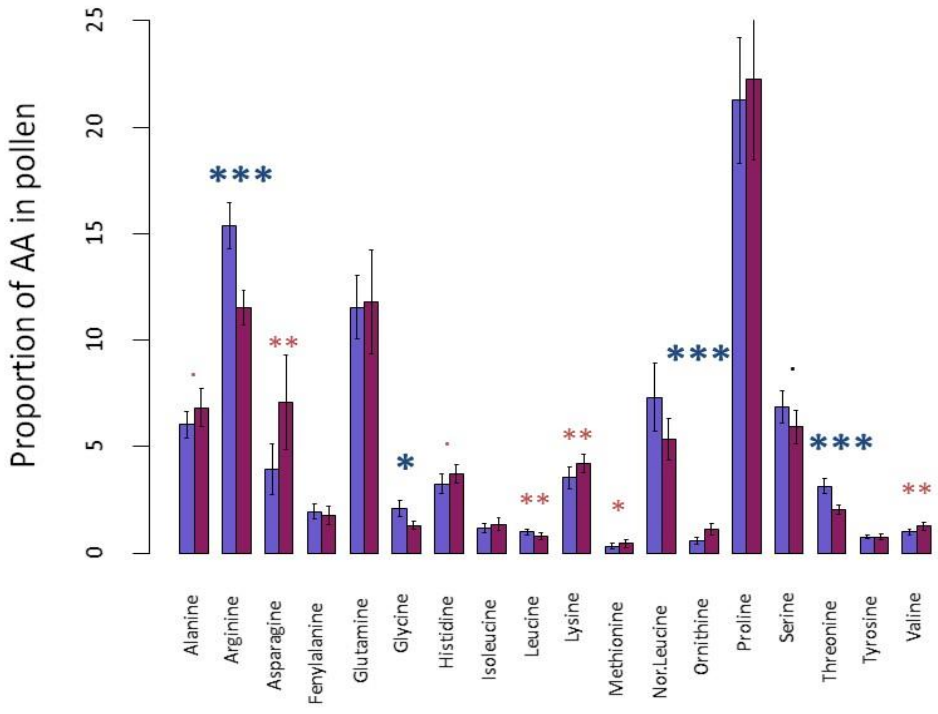

Fig. 15: Relative amounts of individual AAs in pollen between treatments (blue: unfertilized, red: fertilized). Significant differences between treatments are shown (red), with those significant after Bonferroni corrections in blue (*** $P < 0.001$; ** $P < 0.01$)

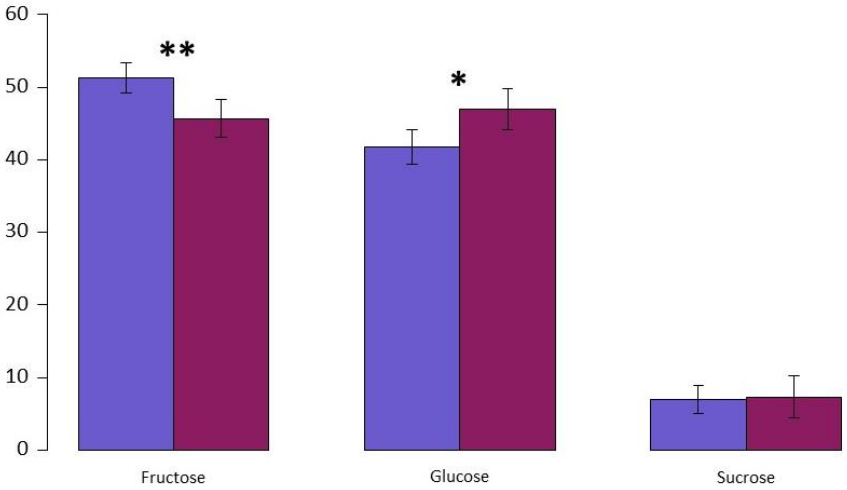

Fig. 16: Relative amount of sugars present in the pollen samples of unfertilized (blue) and fertilized (red) plants. There is significantly more fructose (p=0.012), but less glucose (p=0.002) in the pollen of unfertilized plants.

3.2.2 Plant traits

We found that some, although not all, variables of plant biomass production varied between treatments (Table 7). Specifically, we found significantly more biomass in the rosettes of fertilized plants, when compared to non-fertilized plants. Furthermore, fertilized plants produced significantly more flowers and marginally significantly more seeds per plant. However, there was no longer a difference in seed production when standardized per flower. Additionally, we found no difference in germination rate and flowering stem height between treatments.

Table 6: Mean ± sd for measured plant variables across treatments. *** p < 0.001, * p < 0.05, . p < 0.1

Variable	Fertilized	Non-fertilized	t/W-value
Max rosette height (cm)	11.17 ± 4.34	9.83 ± 3.72	1.96 .
Mean rosette height (cm)	6.76 ± 2.28	5.96 ± 2.36	2.04 *
Max flowering stem height (cm)	76.51 ± 29.53	77.28 ± 28.02	-0.16
Mean flowering stem height (cm)	68.83 ± 26.08	70.04 ± 26.73	-0.27
No. rosette leaves	43.07 ± 16.35	29.79 ± 13.98	5.17 ***
No. flowering stems (*)	3.64 ± 2.35	3.65 ± 1.87	2347
No. flowers (*)	14.37 ± 10.31	10.71 ± 6.09	2973 *
No. seeds	301.34 ± 186.47	243.72 ± 183.89	1.83 .
No. seeds per flower	24.58 ± 15.07	25.49 ± 15.80	-0.35
Germination rate (*)	0.024 ± 0.040	0.028 ± 0.041	2230.5

(*) Statistical test used is Wilcoxon signed-rank test. All other variables are tested with independent t-tests.

3.2.3 Foraging behaviour

We found no significant difference through the GLMM comparing overall foraging activity between bumblebees in fertilized and unfertilized treatments (χ^2=2.17, p=0.14). However, we did find a significant difference between foraging activity on flowers and on commercial pollen (χ^2=16.65, p < 0.001). An observation of a bumblebee harvesting pollen from *S. pratensis* is given in Fig. 17.

Fig. 17: Bumblebee worker caught after foraging on *S. pratensis*. The white pollen that is typical for *S. pratensis* on its left back leg is distinguishable by color from the

3.2.4 Food quantity

The repeated measures ANOVA of the number of honeypots in the colony through time showed no significant difference between treatments. Week had a significant positive effect on the number of honeypots, but the interaction term did not (Table 8; Fig. 18). Since the recordings for commercial pollen uptake were not done weekly, but rather when the colonies needed it, we examine the effect of time of recording, as opposed to week in the experiment. The time of recording had a significant positive effect on the amount of commercial pollen taken up by the colonies (Table9). Treatment and the interaction term, however, did not significantly affect commercial pollen uptake (Table 9). Similarly, we found no significant differences between treatments for various other measures of quantity of food taken up by the bumblebees (Table 10).

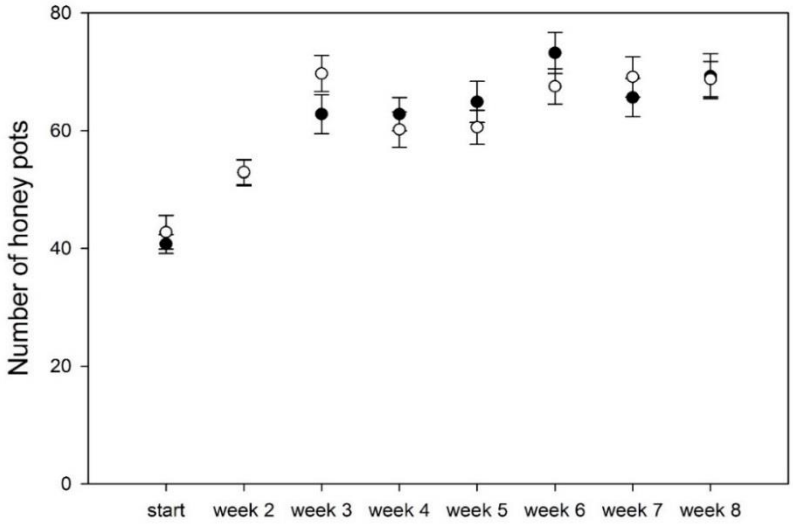

Fig. 18: The number of honeypots through time in hives of fertilized (black) and unfertilized (white) treatments did not significantly differ (F=0.23, p=0.64).

Table 7: Results of repeated measures ANOVA testing for the effect of condtion, week, and their interaction on the number of honeypots in the colony. Where the assumption of sphericity was violated, we displayed corrected statistics as well.

Effect	Correction used	F	p-value
Treatment	None	0.23	0.64
Week	None	37.75	< 0.001
Treatment * Week	None	1.32	0.245
	Greenhouse-Geisser	1.32	0.27
	Huynh-Feldt	1.32	0.26
	Lower-bound	1.32	0.27

Table 8: Results of repeated measures ANOVA testing for the effect of condtion, week, and their interaction on the amount of commercial pollen consumed by the colony. Where the assumption of sphericity was violated, we displayed corrected statistics as well.

Effect	Correction used	F	p-value
Treatment	None	0.37	0.55
Time of recording	None	9.26	< 0.001
	Greenhouse-Geisser	9.26	< 0.001
	Huynh-Feldt	9.26	< 0.001
	Lower-bound	9.26	0.008
Treatment * Time of recording	None	0.71	0.64

Table 9: Mean ± sd of measures representing quantity of food uptake

Variable	t-value	p-value	Fertilized	Non-fertilized
No. empty honeypots	0.13	0.89	32.12 ± 11.45	31.41 ± 18.57
No. full honeypots	0.39	0.70	25.12 ± 12.06	23.18 ± 16.42
Weight honeypots (g)	0.29	0.77	37.46 ± 9.62	36.47 ± 10.17
Weight sugar water (g)	-0.25	0.81	2059.53 ± 109.99	2069.65 ± 126.35

4. Discussion and conclusion

4.1 Effects of soil nutrient addition of food plants on bumblebee colony size and stress indicators

Colonies feeding from fertilized plants show more dead larvae as opposed to colonies that have been fed with unfertilized plants. As a higher number of dead larvae indicates higher stress levels of colonies, this result indicates that ecosystem nutrient pollution may have deleterious consequences for pollinators. In both treatments, the number of dead larvae drops over time. This is not surprising, as reproduction in our experiment was very limited and there were very few new larvae towards the end in both treatments. Similarly, we also found that that there are less workers in the fertilized treatment during weeks three to five of the experiment (although the interaction between treatment and week is no longer significant after correcting for sphericity). We suggest that treatment only affects the number of workers in the middle of the experiment, because there is a delay before the treatment influences the workers in the beginning. At the end of the experiment, colonies collapse in both treatments, meaning that the number of workers converges to low amounts across treatments. This confirms the results of Hoover et al. (2012) who also found a higher mortality of bumblebee workers in fertilized treatments. In concert with the higher number of dead larvae, these results further support the hypothesis that nutrient pollution negatively affects bumblebee colonies. At the end of the experiment, number of workers in the colonies collapses in both treatments. This is to be expected because the average life span of a *B. terrestris* worker was reached for the newest workers produced in the hives (7 weeks; Holland and Bourke, 2015) and there was little reproduction to replace them. When investigating queen survivorship weight there was no difference between treatments. This might be because the development of the queens took place long before the start of the experiment, meaning that the effect of the treatment is reduced for them.

In our experiment we only analyzed size and stress indicators of a colony as proxies for the effect of nutrient pollution on bumblebees. However, to assess more long term and potentially stronger effects of nutrient pollution, it would also be crucially informative to investigate the effects on the reproductive fitness of the bumblebees. Unfortunately, in this experiment there was little or no reproduction in both treatments as we found no production of new reproductive stages and of virgin queens or males despite monitoring for this. There are a number of reasons that might explain this lack of reproduction. Firstly, it is possible the hives might have been kept too warm in the greenhouse, as we observed that some of the bumblebees, independent of treatments, regurgitated fluid from their honey crops and applied it to their head. This could be a way of increasing evaporative water loss to bring down their body and hive temperature (Roberts and Harrison, 1998). When colonies are overheated, they stop reproduction immediately. Another stress factor might have been the weekly

observing of the hives; despite being done after sunset to ensure minimal disturbance. Lastly, bumblebees often have difficulties reproducing in greenhouse conditions, when compared to natural environments (Pers. Comm. Dr. M Pozo Romero)

4.2 Effect of fertilization treatment on nectar and pollen composition

a) Nectar

There is a higher total concentration of AAs in nectar of unfertilized plants when compared to plants that were fertilized, which might reflect a higher nutritional value. Then again, we found lower indices of AA diversity in nectar of unfertilized plants, meaning that its composition might provide a less complete food source than nectar of fertilized plants. The specific AA composition of nectar has been shown to affect its taste and scent. This means that the differences in AA composition we found between treatments could influence pollinator behaviour (Nicolson and Thornburg, 2007; Bertazzini *et al.,* 2010; Rodríguez-Peña *et al.,* 2013). Insect responses to different nectar AAs have been researched and discussed for the relevant AAs in our study below. However, these findings are sometimes contradictory and still very little is known about specific neurological or phago-stimulating effects of these different AAs on pollinators (Gijbels *et al.*, 2014). In addition, the contribution of specific AAs to overall nectar taste is largely unknown, as there are probably other compounds in nectar affecting its taste as well (Baker and Baker, 1982). As these contributions are also unknown, the analysis of nectar taste has great research potential (Gardener and Gillman, 2008). For clarity, two classifications of nutritional value and taste of AAs for insects are summarized in Table 11.

Asparagine, glutamine, alanine, and glycine are the AAs significantly differing in nectar samples between treatments. Firstly, the relative abundance of asparagine is higher in nectar of fertilized plants. This AA is shown to inhibit labellar chemosensory cells of flies (Table 11) and in the study by Petanidou (2005), it is avoided by all guilds and bee families. Also glutamine was more abundant in the nectar of fertilized plants. This AA is known to be important in the nitrogen metabolism and in gluconeogenesis, indicating its importance as an energy substrate for flight (Gardener and Gillman, 2001). Since it is a N-rich structure, it is possible that the higher amounts of glutamine in nectar of fertilized plants is the result of shunting excess N out of plant cells (Gardener and Gillman, 2001). On the other hand, alanine was less present in fertilized plants. The stingless bee *Melipona fuliginosa* has been shown to avoid this AA (Roubik *et al.*, 1995). However, it is proposed by Gijbels (2015) to be important for taste perception or metabolism in Lepidoptera. Next to alanine, there is also less glycine in nectar of fertilized plants. Kim and Smith (2000) showed that this AA is attractive to and elicits a feeding response in honeybees, as well as improves their learning performance. However, Inouye and Waller (1984) have shown that honeybees are repelled by unnaturally high concentrations of glycine,

while at natural concentrations it had little effect on feeding preference. Additionally, Petanidou (2005) found glycine to be avoided by most bee families.

Other AAs differed significantly between treatments before Bonferroni corrections. We discuss them here for completeness. Threonine, an intermediately essential AA, is found to be more abundant in unfertilized plants. However, this AA was avoided by most bee families studied by Petanidou (2005). Another intermediately essential AA, phenylalanine, was detected in higher amounts in the nectar of unfertilized plants as well. This AA is very common in nectar, albeit in variable amounts (Bose and Battaglini, 1978). It has been shown to be preferred by honeybees and to stimulate sugar cells in flies (Hendriksma *et al.*, 2014; Shiraishi and Kuwabra, 1970). Valine is present in higher amounts in fertilized plants. Despite it being one of the most essential AAs, it is shown by Petanidou (2005) to be avoided by most bee families studied. However, according to Shiraishi and Kuwabra (1970), valine is found to be sugar cell stimulatory. Proline, that was found more in nectar of unfertilized plants, is non-essential, but is considered an important source of nitrogen and energy (Chapman, 2012). It is mainly used to fuel the earliest or most energy-costly stages of insect flight and is the most abundant AA in honeybee hemolymph fluid (Micheu *et al.* 2000; Gade and Auerswald 2002). Proline is classified as salt cell stimulatory by Shiraishi and Kuwabra (1970). Glutamate, a non-essential AA, was found in very low relative amounts in the nectar, but was more abundant in nectar of fertilized plants. It was found to be avoided by most bee families studied by Petanidou (2005) and is shown to be general inhibitory to taste cells in flies (Shiraishi and Kuwabra, 1970).

B. terrestris is a short-tongued bumblebee. This group is known to prefer hexose-rich nectar (fructose and glucose) over sucrose-rich nectar (Baker and Baker, 1990; Dupont *et al.*, 2004; Krömer *et al.*, 2008). Although the sugar ratio in nectar did not differ between treatments, we did find significantly more glucose in nectar of unfertilized plants. While the proportional difference in concentration may be relatively small, bumblebees have been reported to discriminate between small differences in nectar concentration (Cnaani *et al.*, 2006). Since there is more glucose in the nectar of flowers of plants that were not fertilized, it might give the colonies feeding from it a benefit.

b) Pollen

Pollen quality is usually assessed through its total AA content, as bees need to gather amino acids from pollen, especially essential AAs. Although they are considered to be non-essential amino acids, proline, aspartate, and glutamate are important as energy and nitrogen sources (Chapman, 2012). This pollen quality is shown to be important for larval growth in bees (De Groot 1953; Roulston and Cane 2002). For social bee species with relatively short brood developmental times, like *B. terrestris*, high-quality pollen may be essential to guarantee the survivorship of the colony (Goulson *et al.*, 2005).

Honeybees prefer some pollen over others, regardless of other floral traits (Levin and Bodart, 1955). However, more attractive pollen may not always be better for the bees (Schmidt and Hanna, 2006). Keller *et al.* (2005) found that there was no difference in preference by honeybees for low or high quality pollen. Thus, they postulated that the bees might prefer enough pollen quantity, instead of

quality. This might also explain why they sometimes readily collect toxic pollen. However, Cook *et al.* (2013) found that honeybees prefer pollen with more essential AAs. This effect could not be unambiguously unraveled, as variation in other factors including pollen odor, other essential nutrients, phago-stimulants, defensive metabolites, and phago-deterrents might influence this preference as well. Comparing pollen within the same plant species, as in this study, might lose a lot of this variation. However, only synthetic pollen with known *a priori* AA compositions can elucidate its effect completely.

Table 10: Classification of AAs according to the degree in which the AA is considered essential in early studies by De Groot (1953) and taste classes for two species of fly (*Boettcherisca peregrina* and *Phormia regina*) as described by Shiraishi and Kuwabra (1970). Furthermore, we show which specific AAs were significantly more abundant in the nectar and pollen in each treatment. Text in bold indicates significance after Bonferroni corrections, text in roman letters indicates significance only before Bonferroni corrections, and text in italics indicates marginal significance before Bonferroni corrections.

Amino Acid	Degree of essentiality of AA (De Groot, 1953)	Taste classes (Shiraishi and Kuwabra,1970)	More abundant in nectar	More abundant in pollen
Leucine	Most essential	Sugar cell stimulatory	*unfertilized*	unfertilized
Isoleucine	Most essential	Sugar cell stimulatory	*fertilized*	
Valine	Most essential	Sugar cell stimulatory	fertilized	fertilized
Threonine	Intermediately essential	No effect		**unfertilized**
Phenylalanine	Intermediately essential	Sugar cell stimulatory	unfertilized	
Arginine	Intermediately essential	General inhibitory		**unfertilized**
Lysine	Intermediately essential	General inhibitory		fertilized
Tryptophan	Least essential	Sugar cell stimulatory		
Methionine	Least essential	Sugar cell stimulatory		fertilized
Histidine	Least essential	General inhibitory		*fertilized*
Tyrosine	Not essential	No effect		
Cystine	Not essential	No effect		
Serine	Not essential	No effect	*fertilized*	*unfertilized*
Hydroxyproline	Not essential	Salt cell stimulatory		
Alanine	Not essential	No effect	**unfertilized**	*fertilized*
Glycine	Not essential	No effect	**unfertilized**	**unfertilized**
Glutamate	Not essential	General inhibitory	fertilized	
Aspartate	Not essential	General inhibitory		
Proline	Not essential	Salt cell stimulatory		
Asparagine	Not discussed	No effect	**fertilized**	fertilized
Glutamine	Not discussed	No effect	**fertilized**	

As in nectar, there is less glycine present in pollen of fertilized plants as well. The preference of bees for this non-essential AA is uncertain, as contradictory evidence exists (see above). Although the intermediately essential threonine was insignificantly more abundant in nectar of unfertilized plants,

it is significantly more abundant in its pollen. In the study by Petanidou (2005), this AA was avoided by most bee families. Arginine, an intermediately essential AA that is richer in pollen of unfertilized plants, was found to be avoided by the stingless bee*, Melipona fuliginosa* (Roubik *et al.*, 1995). Furthermore, it has been shown to inhibit labellar chemosensory cells of flies (Shiraishi and Kuwabara, 1970). There was significantly more ornithine in pollen of fertilized plants. This is a non-proteinogenic AA present in the urea-cycle (Meijer *et al.*, 1990).

AAs that were significant before Bonferroni corrections are discussed here. Lysine, an intermediately essential AA, was found in higher proportions in pollen of fertilized plants. This AA was classified as generally inhibitory to chemosensory cells of flies (Table 11). Also valine, one of the most essential and salt cell stimulatory AAs, and asparagine, which have been found to be avoided by some insects (see above) were more abundant in pollen of fertilized plants. Leucine, a most essential AA that was more abundant in pollen of unfertilized plants, was avoided by most bee families studied by Petanidou (2005). On the other hand, methionine, which is one of the less essential AAs, was more abundant in pollen of unfertilized plants. Both of the latter AAs are sugar cell stimulatory in flies (Shiraishi and Kuwabara, 1970).

Similar to nectar, the sugar ratio in pollen did not differ between treatments. However, we did find a higher relative abundance of glucose in fertilized plant pollen, and more fructose in pollen from unfertilized plants. Thus, there was a different hexose sugar source, preferred by *B. terrestris*, dominant in each treatment. However, considering the main sugar source for bees is nectar, we expect the effect of sugar composition in nectar to be of greater importance than that of pollen.

In summary, the nectar of fertilized plants contained more glycine, which is found to be avoided by insects, and glutamine, which is an important AA in N metabolism and for flight. Conversely, more alanine, which is avoided by some insects but hypothesized to be important for taste perception and metabolism, and glycine, which is attractive and stimulates learning performance, is found in the nectar of unfertilized plants. Higher glycine content was also found in the nectar of unfertilized plants, as well as more threonine and arginine, which is intermediately essential for bees, yet avoided by some insects. As pollen is considered to be the main source of AAs for bees, we expect its AA composition to be more important for bee nutrition when compared to nectar AA composition.

4.3 Effect of fertilization treatment on plant traits

The higher rosette biomass and flower production found in the fertilized plants corresponds with earlier well-known observations of increased biomass production after nutrient addition (Bobbink *et al.*, 2010; Ceulemans *et al.*, 2012). These observations are thus considered a validation of our nutrient treatments. In this study, we found no difference in germination between treatments. However, earlier

nutrient addition experiments on *S. pratensis* showed a lower germination rate in fertilized plants (Cabuy et al., 2015) This was attributed to an increase of the duration of pollinator visits. Because this leads to a higher chance of transfer of pollen within one individual, it increases the risk of inbreeding. The lack of difference between treatments here might be explained because there was only a limited amount of plants in each bugdorm. This means pollen could only come from very few individuals within the same bugdorm. Furthermore, visitation duration and the number of visits to the same plant might have also increased in both treatments, due to the limited number of plants present. All of this may increase the chances of inbreeding in both treatments, leading to similar germination rates.

4.4 Effect of fertilization treatment on foraging behavior and food quantity

We found that bumblebees spent more time foraging on flowers than on commercial pollen. This is optimal, as we wanted the bees to use the flowers as their main food source. Additionally, there was no difference in foraging activity between treatments, implying that the quantity of food taken up by colonies may be similar in both treatments. This is further confirmed by the lack of difference between colonies for the number of honeypots in the hive and their weight, and the amount of commercial pollen and sugar water taken up by the colonies. All of these measures suggest that the food quantity between treatments was the same and that observed differences are thus probably the result of differences in food quality.

4.5 Conclusions and perspectives

In this study, we found that nutrient addition leads to a change in AA and sugar composition of nectar and pollen in flowers of *S. pratensis* plants. When bumblebees subsequently used these plants as food source, their colony size and stress was negatively affected, as colonies fed with fertilized plants showed more dead larvae and less live workers in their hives. It is possible that the negative effects we found may be attributed to lower concentrations of glycine and arginine in the pollen of fertilized plants, both important AAs for instance to improve learning performance in honeybees. Additionally, we found less glucose, which is an important energy source for short-tongued bees like *B. terrestris*, in the nectar of fertilized plants. However, contradictory to our hypotheses, we found a higher abundance of glutamine, an important AA for flight and N metabolism, in the nectar of fertilized plants. As the nectar AA composition may be of less importance to bee nutrition than pollen AA composition, its effect may also be limited. Based on these seemingly conflicting results, we recommend that elucidating the effect of pollen and nectar AA composition on bumblebee health and fitness should be subject to further research. Nevertheless, our results provide one of the first indications that pollinators could be negatively affected by changes in food quality induced by ecosystem nutrient pollution.

However, it should be noted that, although we found significant differences between treatments, the absolute effect of nutrient treatments found in this study was relatively small. As we only studied bumblebee colonies from a certain age (five to six weeks) onwards, the effect might be reduced. We expect that the negative effect may become more pronounced when this experiment is started with single queens immediately prior to founding a colony. Additionally, because we had very little reproduction in this study, we could not study how it is affected. Studies on the effect of changed food source composition on reproduction in bee colonies could reveal even stronger results.

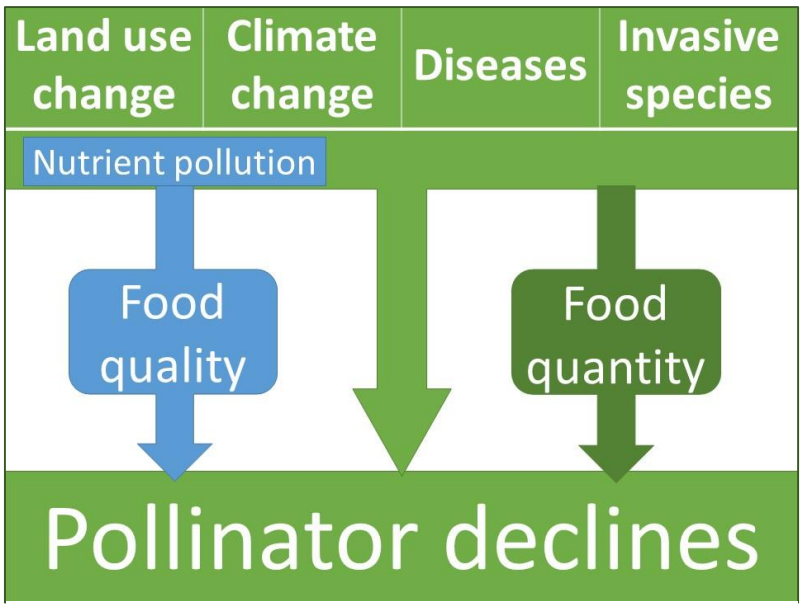

Fig. 19: Situation of our research question (blue) in other possible factors affecting pollinator declines, either directly or indirectly through food quality and quantity.

Lastly, there is relatively large variation in the counts recorded from the hives. This is probably due to strong natural variation between different colonies. However, a more precise way of assessing the hives and counting the different life stages would probably result in more powerful findings. Developing such a method, while minimally disturbing the colonies, seems of particular interest.

Despite its limitations, our results may have far-reaching implications for conservation of pollinators and maintenance of sufficient pollination ecosystem services in an era of ever-increasing nutrient pollution of natural and semi-natural habitats worldwide (Tilman *et al.,* 2001, Peñuelas *et al*., 2011). Indeed, current research seems biased towards investigating the effects of fewer food resources in a landscape under nutrient pollution due to the loss of plant species of nutrient poor habitats (food quantity). Our research however, indicates that the remaining food resources in these landscapes may also be of lower quality, possibly adding to negative environmental pressure on pollinator populations. These results also impinge on conservation strategies, as most aim at increasing pollinator food resource availability in agricultural landscapes, traditionally high in nutrients. This might undermine the ultimate goal of mitigating pollinator decline because of low nutritional quality of the added resources. In this respect, our results indicate that maintaining sufficient nutrient poor habitats may be crucial for the conservation of pollinators. Particularly in landscapes increasingly filled with detrimental pressures on pollinator fitness including pathogens, pesticides, and decreased floral resources, serious decline of food quality may be a crucial component to understand and mitigate the susceptibility of pollinators worldwide (Fig. 19).

Although we investigated size and stress of bumblebee colonies, our results may also have implications for plant populations themselves. Firstly, reduced bumblebee colony size could lead to less pollinators available to perform the valuable ecosystem service of pollination, possibly leading to impaired cross-fertilization of plants and hence higher levels of genetic erosion. Additionally, as changed food source composition has been shown to affect pollinator behavior (see earlier), it could increase selfing levels of plants as well (Gijbels et al. 2015). Elucidating the knock-on effects on plant population dynamics via both pathways merits attention in future research, as they may negatively affect plant fitness.

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Addendum

1. Risk analysis

During the experiment, we encounter chemical as well as biological types of risk.

Chemical

The nutrient solutions we use to fertilize the plants, contains chemicals that need to be handled with caution. These include:

NH_4NO_3: May cause fire or explosion; strong oxidizer.
Causes serious eye irritation.

H_3PO_4 and NaOH: Causes severe skin burns and eye damage.

KNO_3: May intensify fire; oxidizer

As general precautions, we always use gloves, a lab coat, and safety goggles when handling these products.

Biological

As we are working with bumblebees (Bombus terrestris), which is a stinging insect, there is a potential risk of getting stung. Most people experience local effects like pain, swelling, itching, and redness around the sting site. Some people will experience swelling in a larger area, not just immediately around the sting site. They may develop hives but no systemic effects. This is a mild allergic reaction and can last a few days. The area will be sore and uncomfortable but one should not give in to the temptation to scratch the stung area. Scratching may cause a break in the skin which could lead to an infection. In rare cases, a severe allergic reaction can occur. This situation is serious and can cause "anaphylaxis" or anaphylactic shock. Symptoms of anaphylaxis can appear immediately (within minutes) or up to 30 minutes later. Symptoms to watch for include: i) Hives, itching and swelling in areas other than the sting site, ii) swollen eyes and eyelids, iii) wheezing, iv) tightness in the chest and difficulty breathing, v) hoarse voice or swelling of the tongue, vi) dizziness or sharp drop in blood pressure, vii) shock, and eventually viii) unconsciousness or cardiac arrest (Canadian Centre for Occupational Health & Safety)

2. Honey

In this experiment, we analyzed the composition of honey produced by the bumblebee colonies as well. However, as these analyses were not directly relevant to our hypotheses, we did not discuss them in the main corpus of this thesis. All honey samples were collected from the hives at the end of the experiment, so the treatment affected its composition as much as possible. They were chemically and statistically analyzed exactly as discussed for the nectar and pollen sample (Section 2: Methodology).

Results

The honey produced by colonies feeding from fertilized and unfertilized plants did not differ in total AA amount, nor in diversity indices describing the AA composition (Table A1). When analyzing the AA composition of honey sampled from colonies in the two treatments, the NMDS showed no significant difference between them either (R^2=0.066, p=0.42). This was also the case for the PERMANOVA we performed (F=1.03, R^2=0.037, p=0.36; Fig. A1). Ordination by NMDS of the sugar composition in honey revealed a marginally significant difference between treatments (R^2=0.14, p=0.072). However, the PERMANOVA showed that the sugars in honey produced by colonies of the two treatments did differ significantly (F=3.75, R^2=0.12, p=0.033). Post-hoc testing indicated that there was more fructose in the honey produced from fertilized plants (p=0.048, Bonferroni-corrected; Fig. A2). The sugar ratio of hexose sugars and sucrose was significantly higher in honey produced from fertilized plants (p=0.038, Table 6).

Table A1: Mean ± sd of describing measures of honey AA and sugar composition. Differences between groups were tested with Wilcoxon signed-rank tests, however no significant differences were found.

	Fertilized	Not fertilized	W
Total AA (µmol/l)	670.57 ± 251.42	711.79 ± 299.67	116
Sugar ratio	18.46 ± 11.20	13.71 ± 11.36	57*
Shannon index AA composition	0.95 ± 0.39	0.85 ± 0.49	81
Eveness AA composition	0.28 ± 0.12	0.26 ± 0.15	81

Discussion

Overall, we found no significant difference in any of the measures or analyses of honey composition between treatments. We did, however, find higher fructose levels in the honey produced from fertilized plants. Interestingly, this means that the found differences in nectar composition did not reflect in the honey produced from it. In contrast to honeybees, bumblebees only store very limited amounts of honey at a time in their honeypots. This is only enough to feed the colony for a few days and they do not make winter stores as honeybees do, since only the queen survives and she hibernates throughout winter. The honey bumblebees produce is considered to be less transformed than that of honeybees. Nevertheless, because the composition of the honey is different than that of the nectar sources, we expect there to be some transformation anyway.

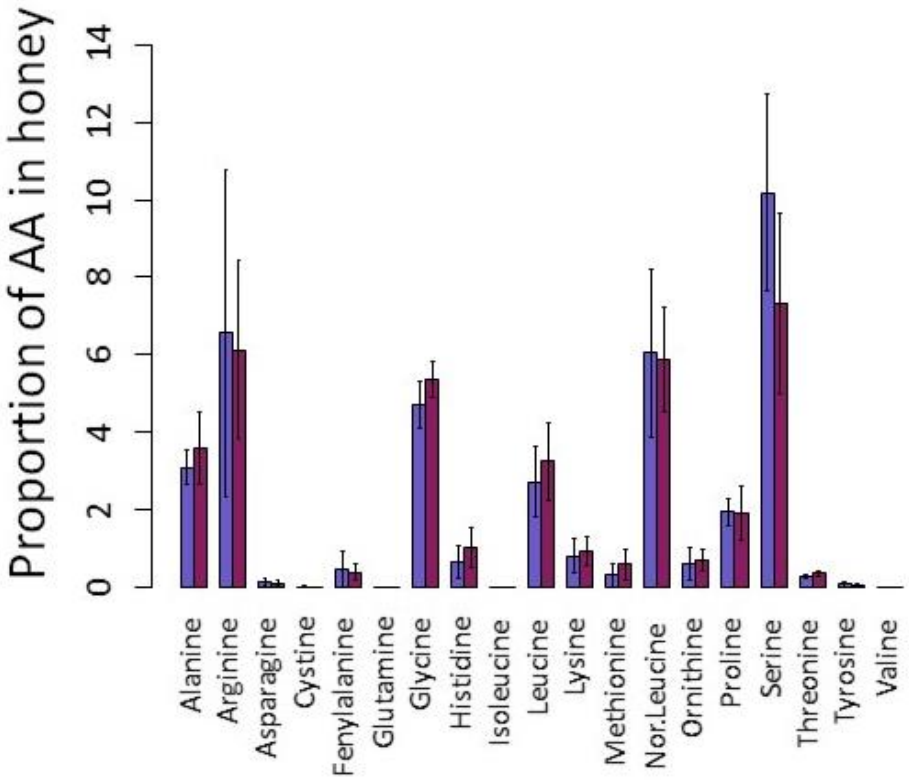

Fig. A1: Relative amounts of individual AAs in honey between treatments (blue: unfertilized, red: fertilized). We found no significant difference in AA composition of honey between these treatments.

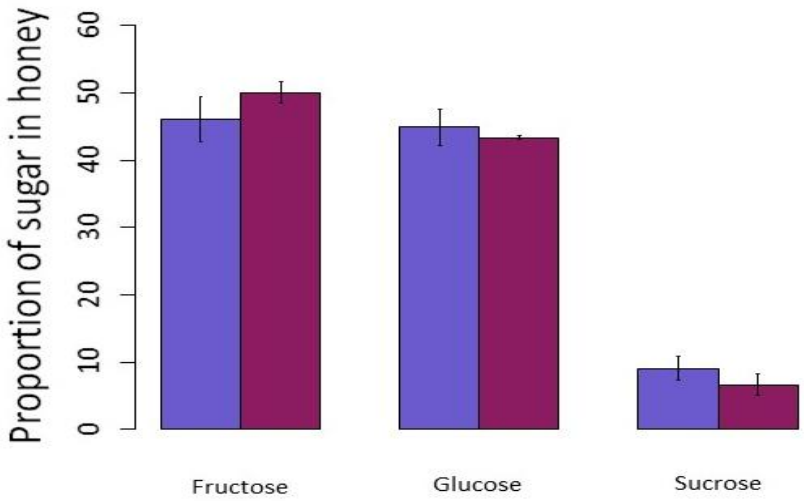

Fig. A2: Relative amounts of individual sugars in honey between treatments (blue: unfertilized, red: fertilized). We found no significant difference in sugar composition of honey between these treatments.

In honeybees, worker bees collect nectar during foraging and store it in their honey stomachs. During this time, the nectar is mixed with secretions of the salivary and hypopharyngeal glands. Enzymes from the secretions of the latter gland then begin to chemically alter the nectar, mainly breaking down larger saccharides, like sucrose, into monosaccharides. When in the hive, the forager bee transfers the nectar to a house bee. This house bee then drinks the nectar and may regurgitate and redrink it a few times more hereafter. Doing so, more secretions, and their enzymes, are being mixed with the nectar. After some time, the nectar is put in the honeycomb, where the nectar ripens into honey. This ripening consists of two processes: i) the conversion of sucrose into glucose or fructose and ii) the evaporation of excess water. Due to all of this intense contact with the bee, it is generally agreed that the AAs found in honey derives from the bee, instead of nectar or pollen (Ball, 2007). We suggest that this mechanism is similar in bumblebees, which would explain the lack of difference between honey AA composition. Since there are so many actors transforming the sugar composition as well, we see no direct link between nectar and honey sugar composition either.

3. Additional figures and tables

Table A2: Significance of difference of relative amounts of AAs present in the nectar samples in fertilized and unfertilized treatments. P-values are displayed before and after correcting for multiple comparisons by Bonferroni corrections

Amino acid	p-value	Bonferroni-corrected p-value
Alanine	< 0.001	0.005
Arginine	0.163	1.00
Asparagine	< 0.001	< 0.001
Phenylalanine	0.038	0.877
Glutamaat	0.036	0.838
Glutamine	< 0.001	0.003
Glycine	< 0.001	0.004
Histidine	0.28	1.00
Isoleucine	0.096	1.00
Leucine	0.066	1.00
Lysine	0.931	1.00
Methionine	0.521	1.00
Nor-Leucine	0.259	1.00
Ornithine	0.468	1.00
Proline	0.261	1.00
Serine	0.044	1.00
Threonine	0.541	1.00
Valine	0.145	1.00

Table A3: Significance of difference of relative amounts of AAs present in the pollen samples in fertilized and unfertilized treatments. P-values are displayed before and after correcting for multiple comparisons by Bonferroni corrections

Amino acid	p-value	Bonferroni-corrected p-value
Arginine	< 0.001	< 0.001
Ornithine	< 0.001	< 0.001
Lysine	0.009	0.25
Glutamine	0.83	1.00
Asparagine	0.009	0.25
Alanine	0.065	1.00
Threonine	< 0.001	< 0.001
Glycine	0.001	0.035
Valine	0.006	0.16
Serine	0.070	1.00
Proline	0.68	1.00
Isoleucine	0.19	1.00
Leucine	0.007	0.19
Methionine	0.048	1.00

Nor-Leucine	0.13	1.00
Histidine	0.093	1.00
Fenylalanine	0.34	1.00
Glutamaat	0.10	1.00
Aspartaat	0.78	1.00
Cystine	0.22	1.00
Tyrosine	0.69	1.00

Table A4: Significance of difference of relative amounts of sugars present in the nectar samples in fertilized and unfertilized treatments. P-values are displayed before and after correcting for multiple comparisons by Bonferroni corrections

Sugar	p-value	Bonferroni-corrected p-value
Glucose	0.027	0.081
Fructose	0.69	1.00
Sucrose	0.11	0.32

Table A5: Significance of difference of relative amounts of sugars present in the pollen samples in fertilized and unfertilized treatments. P-values are displayed before and after correcting for multiple comparisons by Bonferroni corrections

Sugar	p-value	Bonferroni-corrected p-value
Glucose	0.004	0.012
Fructose	0.001	0.003
Sucrose	0.80	1.00

Table A6: Significance of difference of relative amounts of sugars present in the honey samples in fertilized and unfertilized treatments. P-values are displayed before and after correcting for multiple comparisons by Bonferroni corrections

Sugar	p-value	Bonferroni-corrected p-value
Glucose	0.19	1.00
Fructose	0.016	0.048
Sucrose	0.037	0.11

ECOLOGY, EVOLUTION AND BIODIVERSITY CONSERVATION
Charles Deberiotstraat 32 - box 2439
3000 Leuven
tel. +32 16 3 23966
fax +32 16 3 24575
www.kuleuven.be