Exploring strategies to enhance pollination and yield in the field bean, *Vicia faba* L.

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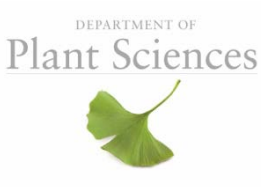

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Jake Moscrop

September 2022

Abstract

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Vicia faba is a legume crop valued for its high protein content and ability to fix nitrogen. The UK is a key producer of faba beans, which are likely to be increasingly important as a less environmentally harmful protein source. However, without insect pollination, *V. faba* yield declines by 32.9% on average. When faced with continuing bee declines, it is imperative that more is done to understand how floral traits of *V. faba* may be used to improve crop yield through pollinator attraction.

In this project, novel variation has been identified between commercial lines of *V. faba* in reward, attraction, and access traits. Using this variation, a hypothesis was formulated that *V. faba* lines possessing floral traits considered to be more attractive to bees would receive more bee visits. Data from two years of field trials supported this hypothesis, with bees showing preference for lines with more flowers and superior nectar sugar content. Field trials also supported the hypotheses that open pollination would have a positive effect on *V. faba* yield, and that lines with more attractive floral traits would receive a larger yield benefit with open pollination. These results have shown for the first time that *V. faba* floral trait variation affects bee attraction in the field and has significant effects on the pollination of the crop.

Following field trials, preferences experiments using *Bombus terrestris* foragers in controlled condition were used to identify *V. faba* floral traits likely to be most important for bee attraction. Together, field and controlled condition experiments suggested that floral colour, nectar concentration, display size, and scent are traits most likely to increase pollinator attraction to the crop. These experiments have also contributed to the wider understanding of pollinator attraction, adding to evidence that bees show preference for purple colours.

The evidence presented in this thesis suggests that by growing existing *Vicia faba* lines with floral traits including purple flowers with more concentrated nectar, farmers can ensure better crop yield in environments where pollinators are present. In addition, floral traits

should be considered as breeding targets by *V. faba* breeders, as they have great potential to increase crop pollination and support wild pollinator populations.

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Acronyms and abbreviations

A. mellifera	*Apis mellifera*
B. hortorum	*Bombus hortorum*
B. pascuorum	*Bombus pascuorum*
B. terrestris	*Bombus terrestris*
IQR	Interquartile range
NIAB	National Institute of Agricultural Botany
PGRO	Processors and Growers Research Organisation
V. faba	*Vicia faba*
VOC	Volatile Organic Compound
% v/v	Percent volume per volume
% w/w	Percent weight per weight

1 Introduction

1.1 Flowers and pollination

For humans, flowers are structures that we enjoy in nature and in our gardens. However, for angiosperms, flowers are a means of presenting reproductive organs to increase the chance of pollination. Pollination is the transfer of pollen grains from an anther to a stigma. Upon reaching a stigma, pollen grains germinate and the sperm they contain fertilise ovules contained in a flower's carpel. The first evidence of pollen-producing organs comes from the late Devonian (approximately 359 million years ago), with sperm packaged inside pollen grains for protection (Borg and Twell 2011). Later fossil evidence shows that approximately 280 million years ago insects were consuming pollen (Krassilov and Rasnitsyn 1996). It is thought that pollinivory eventually led to the evolution of insects as pollinators. When insect pollinators first evolved is still unclear. Evidence suggests that members of the now extinct order Bennettitales were pollinated by insects at least 100 million years ago (Peñalver et al. 2015).

After many millions of years of evolution, there now exists a diverse array of floral forms and animal pollinators, many of which form close interactions. As a result of interactions with animal pollinators, flowers now show incredible diversity in colour, form, size, symmetry, smell, patterning, structures, and reward. Some of these adaptations have become particularly specialised. Within the genus *Petunia,* some species have coevolved highly specific adaptations to different pollinators. *Petunia exserta* has intense red flowers with protruding stamens and pistils, adapted to attract hummingbirds and transfer pollen to and from a bird's body while it feeds. In contrast, the white flowers of *P. axillaris* have long corolla tubes and produce volatile benzenoids in a circadian rhythm matching the activity of its hawkmoth pollinator (Klahre et al. 2011; Yarahmadov et al. 2020). The interactions between pollinators and plants are vital to both the diversity of the natural world we see today and the human world as we know it. Through artificial selection humans have manipulated floral traits, mostly for our own enjoyment. However, we now have the ability to study and manipulate plant-pollinator interactions to both enhance plants for our enjoyment and to influence our food production.

1.2 Pollinators and humans

The close relationship between plants and insect pollinators now means that many plants intrinsic to the health of the planet rely on insects. This includes plants important to humanity, both directly as food crops, clothing, cosmetics and drugs, and indirectly, as essential parts of ecosystems.

It is difficult to properly quantify the contribution of specific crops to global food production due to the diversity of foods consumed across the world and the way of measuring crop production, whether it be the area grown, tonnes produced, or calories consumed. The much-cited review by (Klein et al. 2007) estimates that around 35% of global food production by mass depends on animal pollination. However, estimation by mass fails to recognise the unequal nutritional value of insect pollinated and wind pollinated foods. Klein also estimates that animal pollination benefits up to 75% of 115 globally important food crops. Many of these crops, although not making up the majority by volume, provide us with essential nutrients, unlike wind pollinated cereals which make up most of our calorific intake. These crops include many fruits, nuts and vegetables including kiwis, apples, tomatoes, squashes, strawberries, Brazil nuts, cocoa, coffee, and vanilla (Klein et al. 2007; Groeneveld et al. 2010; Garibaldi et al. 2011; Klatt et al. 2013b). It may be hard to properly estimate the "value" of pollination in units of mass, but it is almost certain that without animal pollinated foods our diets would be less healthy and less enjoyable.

Alongside the nutritional value of crops, services provided by insect pollination are essential for our economy, wellbeing, and our environment. In 2005, the value of the global pollination service for food production was estimated to be €153 billion (39% of the production value of human consumed crops) (Gallai et al. 2009). That estimate does not include the value of pollinators for seed production of crops to produce vegetative parts consumed by humans, crops used for animal feed, biofuels and ornamental crops. The true economic value of pollination will therefore be much higher. Clearly insect pollination is important for the global economy, but even for farmers, insect-pollinated crops provide higher revenue per unit area than wind pollinated crops (Ashworth et al. 2009). On average it has been estimated that insect pollinated crops including fruits e.g. strawberries, nuts e.g.

almonds, and stimulants e.g. coffee, averaged €761 per ton, whereas wind pollinated plants including cereals like rice, wheat, and corn, averaged €151 per ton (Gallai et al. 2009).

Of the insects which pollinate our crops, bees are by far the most important. Bees are responsible for pollination of 90% of the world's leading crops (Klein et al. 2007). Of the 20,000 plus species of bee found worldwide (Michener 2000), only honeybees and a minority of bumblebees are managed by humans. The honeybee pollination industry is particularly large in the USA with, for example, great interstate movement of honeybee hives needed to adequately pollinate almonds in California (Goodrich et al. 2019). However, for many crops grown outdoors, wild bees appear to provide a superior pollination service compared to honeybees (Garibaldi et al. 2013).

1.3 Pollinator declines

Since the 1950s there have been declines in both the abundance and diversity of pollinators (Vanbergen et al. 2014). Over the last two decades several studies have repeatedly reported declines in insect diversity and biomass, mostly in Europe and North America. A survey of flying insects reported a fall of 58.5% on average across the whole of the UK between 2004 and 2021 (Ball et al. 2022). Similarly, in Germany, a 76% decline in insect biomass was reported between 1989 and 2016 (Hallmann et al. 2017). Some global estimates predict the extinction of 40% of insect species within the next few decades (Sánchez-Bayo and Wyckhuys 2019). At this rate, a pollination catastrophe is unavoidable if no action is taken. Research increasingly suggests that exposure to agrochemicals, disease, environmental change, forage availability and nutrition are major drivers of decline. However, the extent to which each factor affects different pollinator species remains unclear (Whitehorn et al. 2012; Graystock et al. 2013; Stabler et al. 2015).

One of the most well reported drivers of global insect declines is exposure to agricultural insecticides (Potts et al. 2010). Perhaps the best studied and most harmful are neonicotinoids. At low levels, neonicotinoids disrupt learning, memory, navigation, and olfactory sensing in bees (Siviter et al. 2018; Muth and Leonard 2019; Muth et al. 2019). At higher doses they impair reproduction, reducing insect population sizes (Stuligross and Williams 2020). In light of this evidence all but one neonicotinoid insecticides have been

banned in the EU since 2020. However, in 2022 the UK government approved the emergency use of a neonicotinoid-containing product due to lack of alternative options (Bellis and Suchenia 2022). Current knowledge shows that the impacts of insecticides on insect health are complex and multifactorial, depending on routes of exposure, dose, biochemistry, sublethal effects and how effects at the organismal level impact bee colony health (Desneux et al. 2006; Krupke et al. 2012; Sponsler et al. 2019).

Emerging infectious diseases pose a risk to pollinator populations, both commercial and wild. Movement of pollinators beyond their normal range has accelerated movement of diseases into new areas. The US national honeybee disease survey detected a doubling of chronic bee paralysis virus (CBPV) between 2009 and 2014 and found significantly more Nosema in migratory honeybee hives (Traynor et al. 2016). A similar trend has been seen for CBPV in the UK, with exponential increase between 2007 and 2017 (Budge et al. 2020). As in other wild animals, many cases of emerging infectious diseases in wild insects are the result of disease spillover from manged species (Daszak et al. 2000). Multiple studies have found higher incidence of deformed wing virus, black queen cell virus and *Nosema bombi* in bumblebees neighbouring apiaries with high infection levels, and non-existence of deformed wing virus and *Crithidia bombi* in bumblebees when honeybee foragers are absent (Colla et al. 2006; Alger et al. 2019). Evidence suggests that the presence of managed bees is a significant source of disease to wild bees and that deployment of honeybees into landscapes to supplement pollination may be detrimental to native pollinator populations (Graystock et al. 2016).

Pollinators forage across a diverse range of landscapes for both pollen (a source of amino acids) and nectar (providing energy in the form of sugars). The diversity and abundance of floral resources available in a landscape directly affects the pollinator population size, activity, and species richness. Implementation of flower strips in intensively farmed agricultural landscapes increases abundance and diversity of bees and butterflies and the positive effects increase over time as wildflower strips become more established (Buhk et al. 2018). The positive effects of flower supplementation have been demonstrated in different agricultural environments, strongly suggesting that absence of pollinators in intensively farmed landscapes is in part due to lack of adequate forage (Campbell et al. 2017; Carvell et al. 2017). Some studies have shown that a period of 15 days without forage can have long

term negative impacts on honeybee colonies (Horn et al. 2016; Requier et al. 2017). In addition, maintaining a range of semi-natural habitats with complementary flowering phenologies is as important as total nectar production in farm landscapes with limited resources (Rundlöf et al. 2014; Timberlake et al. 2019).

Forage composition can be as important for bee health as forage abundance, particularly composition of pollen, a source of amino acids, lipids, vitamins, and minerals. Pollen composition and diversity affects longevity of individuals and colony size, with poor quality diets having negative impacts for pollinator health (Gregorc et al. 2019). Bees fed with higher "quality" pollen from a variety of species show greater tolerance to the parasite *Nosema ceranae* (di Pasquale et al. 2013; Tritschler et al. 2017). Similar effects are seen with tolerance to other infective agents including viruses and bacteria (Dolezal et al. 2019; Rutter et al. 2019). Quality of diet can increase resilience to field realistic concentrations of pesticides, with bees showing upregulation of detoxification enzymes, longevity-associated glycoproteins and faster metabolism when fed more diverse pollen as opposed to monofloral pollen (Barascou et al. 2021).

Like in all biological systems, drivers for bee declines cannot be considered in isolation. When bees are exposed to combinations of poor nutrition, disease, and insecticides, the harmful effects of each are exacerbated and are sometimes synergistic (Siviter et al. 2021). Habitat loss resulting in range restriction of insects aids disease transmission, which in turn can be exacerbated by climate stress (Rowland et al. 2021). When addressing the challenges facing bees, a multifaceted approach must be taken, which seeks to provide better habitat, more stable forage, and reduce exposure to stress.

One way in which we can improve forage for bees and ensure better food production for ourselves in through improvement of mass-flowering crops. The focus of this study has been to explore variation in floral traits of field beans and their influence on bee behaviour, both to enhance resources for bees and to improve crop yield for food production.

1.4 The study system: *Vicia faba*

Vicia faba, L. commonly known as the field bean, faba bean, fava bean, broad bean and tick bean, is an annual legume grown globally as a protein source for food and animal feed. Faba beans have been grown as a crop for thousands of years, and now exist in four main forms. *V. faba* var. *major* is the broad bean, with large broad seeds. *V. faba* var. *equina* is the horse bean, historically grown in Europe for feed. *V. faba* var. *minor* is the tick bean, with small round seeds. *V. faba* var. *paucijuga* again has small seeds and is historically grown in Asia (Duc 1997).

Faba beans are a multiuse crop, used as a human food source in Africa, the middle East and Asia and as animal feed and silage in Europe. Traditionally, faba beans are eaten as Medamis (stewed), Falafel, Bissara (cotyledon paste) and Nabet soup (using germinating seeds) in Egypt and the middle East (Bakr 1996). More recently, faba beans are being used as a protein-rich, gluten-free, and low-fat additive. Faba bean isolates have been used in gluten-free bread (Sozer et al. 2019), yoghurt, tofu (Jiang et al. 2020), pasta (Tazrart et al. 2016), as an egg replacement in reduced-cholesterol mayonnaise (Ouraji et al. 2020), and as a meat alternative (Sulaiman et al. 2018; do Carmo et al. 2021).

Faba beans also have ecological benefits. As a legume, the plants can fix atmospheric nitrogen due to a symbiosis with *Rhizobium* bacteria. Faba beans are particularly advantageous in rotational cropping as they can maintain high rates of biological nitrogen fixation in the presence of high amounts of available nitrogen in soil (Turpin et al. 2002). Faba beans can also provide a valuable break between intensive cereal crops, helping to diversify wild fauna and soil microbes beneficial to cropping systems (Köpke and Nemecek 2010). Faba beans are also valued by beekeepers as a beneficial forage crop for honeybees (Kirk 2004).

Currently most faba beans by mass are produced by China, Ethiopia, and the UK (**Figure 1.1**). The UK produces the highest yield per hectare in Europe and was the second largest exporter of faba beans in 2020 after Australia. Despite increases in production nationwide, yield continues to show great fluctuation between harvests (**Figure 1.2**). Recent research has suggested that the yield instability of faba beans results from susceptibility to environmental

stress and dependency on insect pollinators (Bishop et al. 2016b, c). The same is seen for many insect-pollinated crops, where yield instability increases with dependency on insect pollinators (Garibaldi et al. 2011). Recent declines in pollinator populations increase the risk of inadequate pollination and may be a contributing factor to yield instability which will only worsen with time. Yield instability remains a considerable deterrent to farmers growing the crop. However, breeders have not yet explored whether yield can be improved through manipulation of floral traits to improve pollinator attraction. Enhanced pollination may provide a novel solution to reduce yield instability, but first research is needed to better understand pollination of the crop, variation in floral characters and whether differences in floral traits affect bee attraction and ultimately yield.

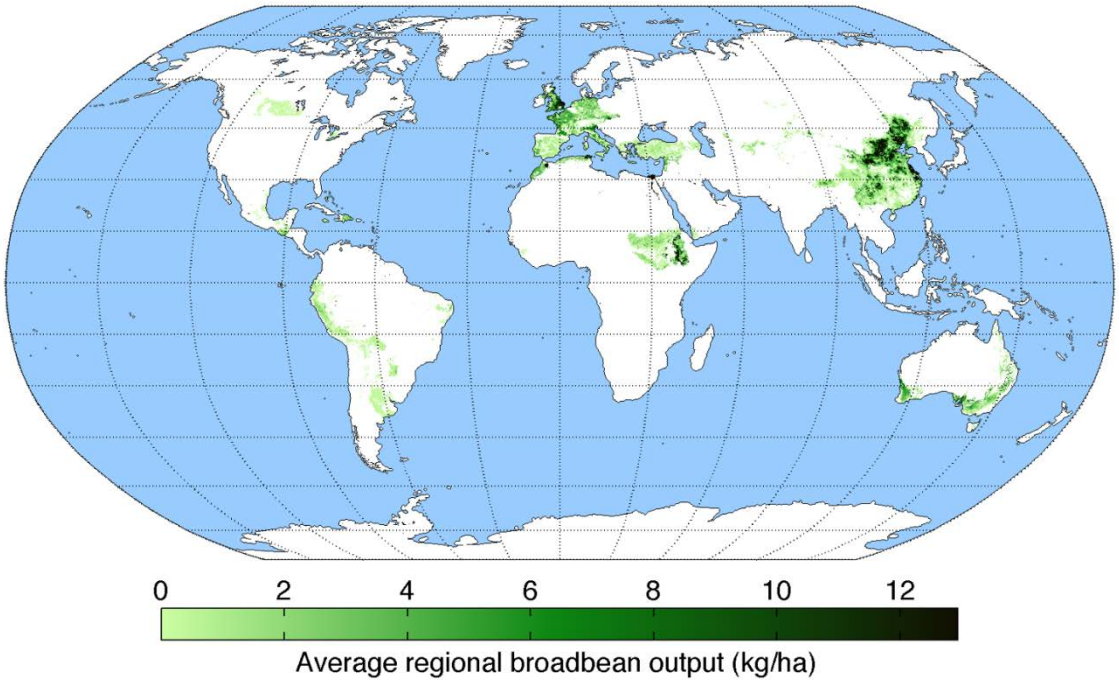


Figure 1.1 Global production of *Vicia faba*. Output is greatest in China, Ethopia and the UK. Figure from (Monfreda et al. 2008). White represents no *Vicia faba* production.

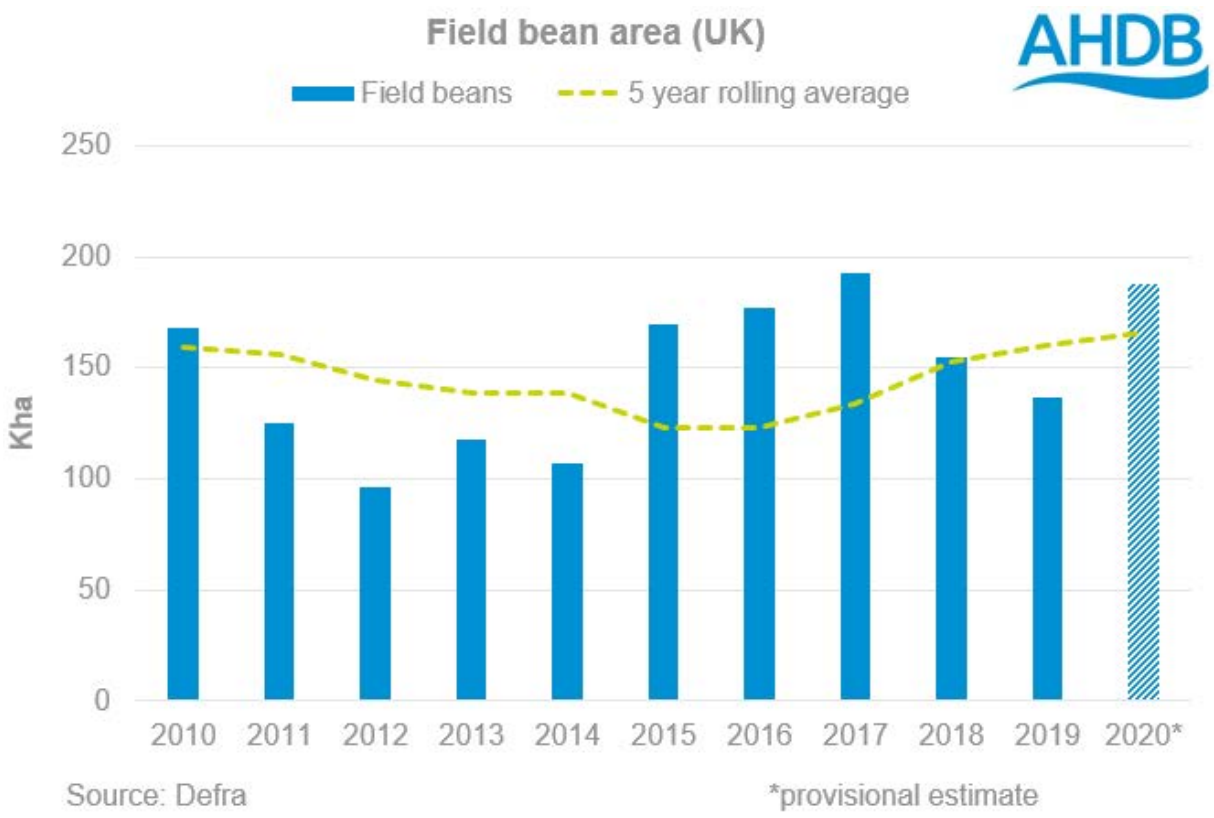


Figure 1.2 UK production of *Vicia faba*. *Vicia faba* production in the UK from 2010 to 2020 in Kilograms/ hectare. Figure from AHDB (https://ahdb.org.uk/news/analyst-insight-pulse-market-brief). Accessed 28 March 2023.

Vicia faba plants grow with an unbranched stem between 1 to 2 metres tall. The leaves are alternate and pinnate with 2-6 obtuse leaflets all ending in a small point (**Figure 1.3 A**). Flowers grow on short racemes in leaf axils and open successively, with younger flowers at the apex **(Figure 1.3 B**). Flowers are papilionaceous and when fully open have an upright standard petal (sometimes referred to as a flag petal) and a pair of wing petals enclosing a pair of fused keel petals **(Figure 1.3 C**). Flowers of most *V. faba* lines appear white to a human and often have darker coloured, vertical veins on the standard petal and a single dark spot on each wing petal. Pollination most commonly occurs when a flower is "tripped", ejecting the anthers and stigma out of the keel petal so that the stigma is covered with pollen, either from the same flower or from another flower, transported by a pollinator. In Europe, bumblebees are the most common and effective pollinator of *V. faba* (Stoddard 1986c). To trip a flower, a bee makes a "legitimate" visit, pushing its head into the flower, forcing the wing and keel petals downwards, rubbing the reproductive structures against the bee's underside while it drinks nectar from the back of the corolla (**Figure 1.4**). Alternatively,

bees can rob nectar from flowers, by chewing a hole in the corolla tube, avoiding the need to trip the flower (**Figure 1.4 D**). Bees can also obtain nectar from extrafloral nectaries, small secretory patches on the underside of stipules, often dark in colour **(Figure 1.4 E**). It is reasonable to assume that legitimate visits have the greatest probability of pollination and outcrossing (Kambal 1969).

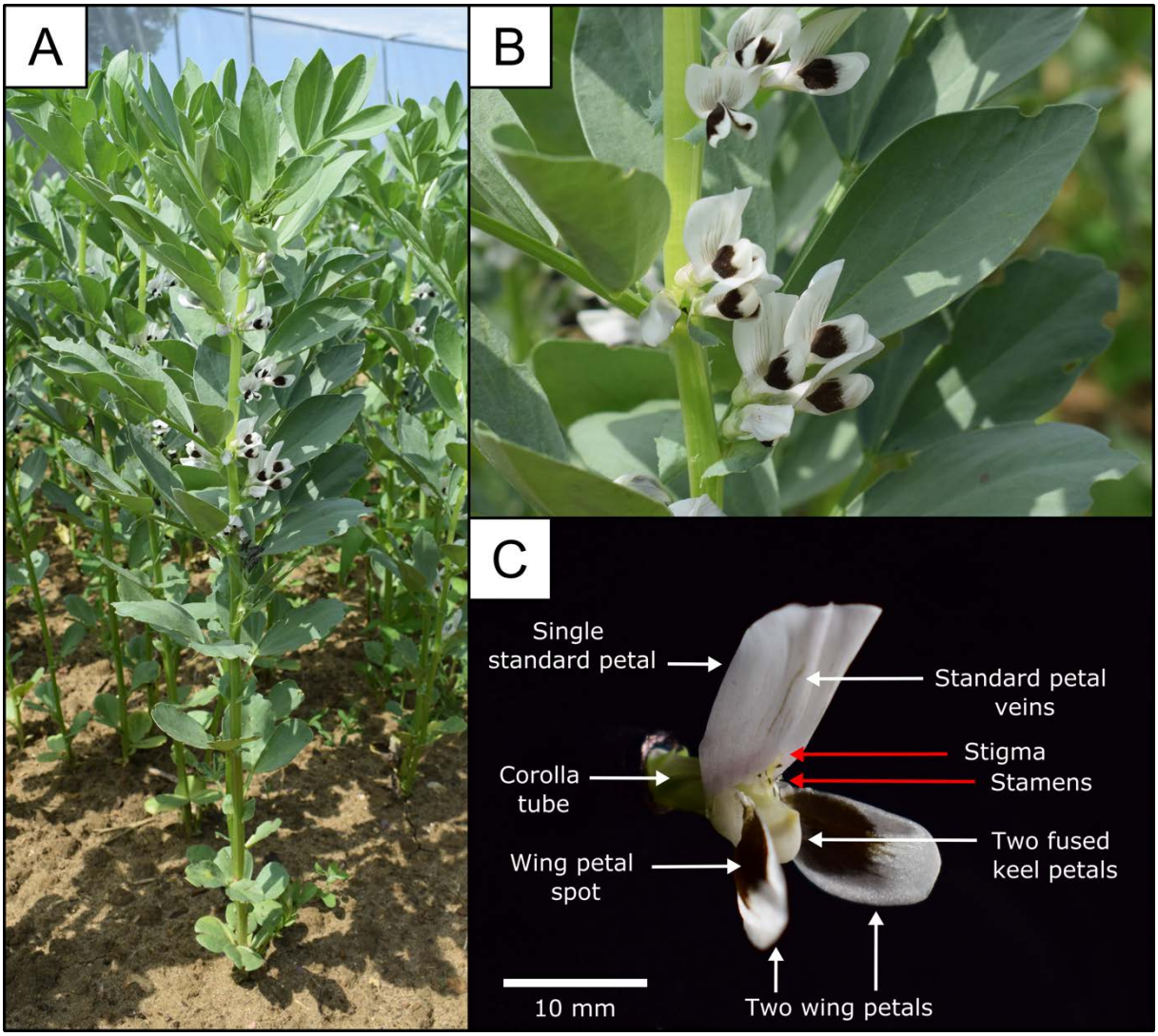


Figure 1.3 Morphology and pollination of *Vicia faba*. (A) A *V. faba* plant growing in a field showing alternating leaves. **(B)** Close up of a *V. faba* stem showing leaflets and flower racemes growing from leaf axils. **(C)** A tripped *V. faba* flower. Reproductive organs are highlighted with red arrows.

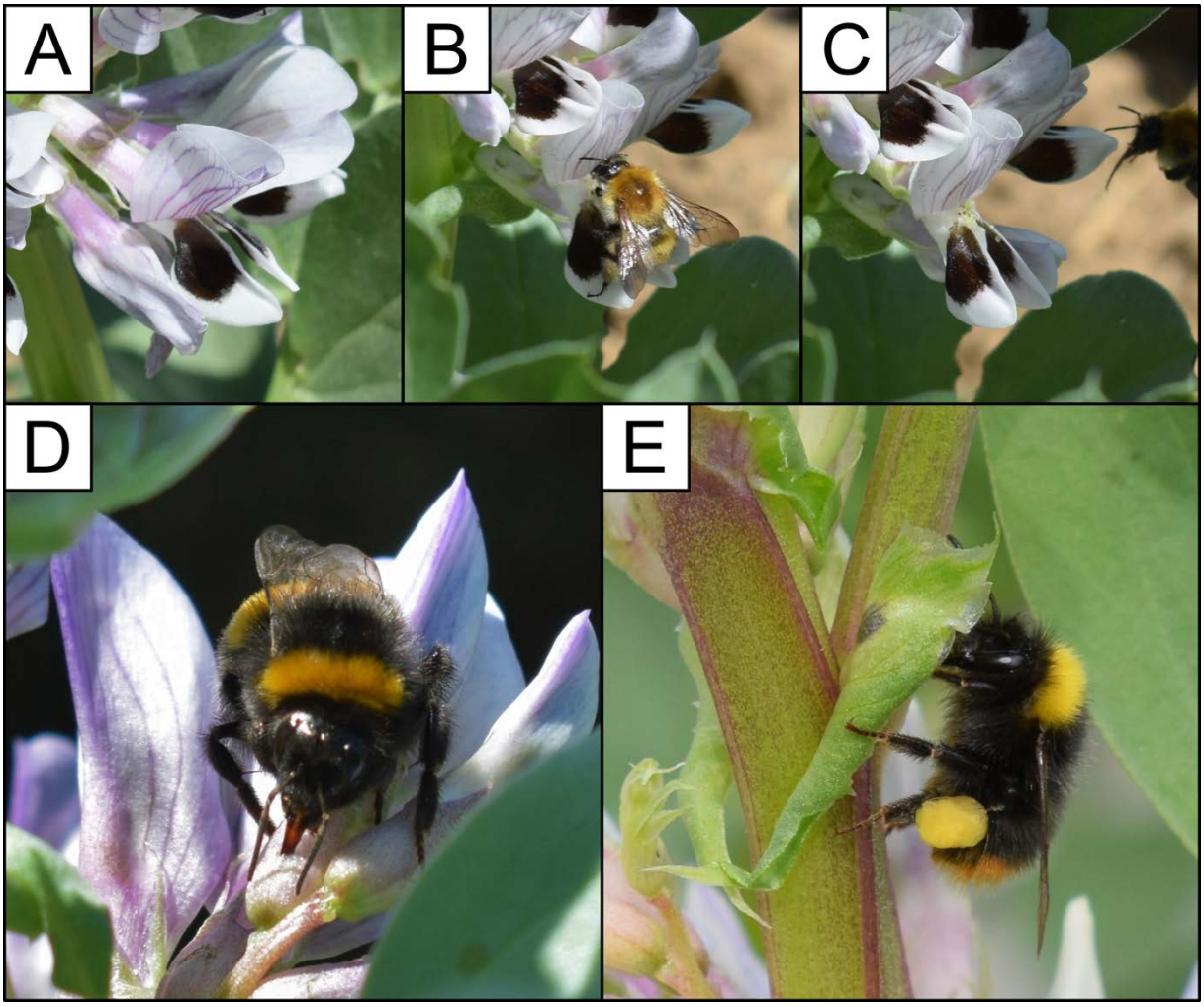


Figure 1.4 Tripping a *V. faba* flower. (A) An un-tripped flower. **(B)** A *Bombus pascuorum* forager lands on the flower forcing the wing and keel petals downwards, ejecting the stigma and anthers out of the fused keel petals. This is known as tripping. **(C)** Immediately after tripping, flowers temporarily remain open with stigma and anthers exposed. **(D)** A bumblebee (*Bombus terrestris*) nectar robbing a flower. **(E)** A bumblebee (*Bombus pratorum*) drinking nectar from an extrafloral nectary.

1.5 Floral traits as means of increasing pollination

Insufficient pollination may be a significant factor contributing to yield instability of *V. faba,* and supplementary pollination can both boost yield of *V. faba* under normal conditions, and attenuate yield loss during stress (Bishop et al. 2016a; Bishop et al. 2020). Enhancing pollinator attraction is a promising means of ensuring maximum yield and limiting negative effects of stress in *V. faba* . To be able to increase pollination of the crop we must first gather more information on the existing variation in floral traits between *V. faba* lines and assess the influence of floral traits on bee attraction. Key to this assessment is whether differences in floral traits affect legitimate flower visitation by bees in field conditions and ultimately crop yield.

A number of studies have presented results quantifying floral traits of *V. faba*, including nectar production (Osborne et al. 1996; Pierre et al. 1996; Aredewa et al. 2004), pollen production (Kambal et al. 1976; Stoddard 1986b; Carré et al. 1994), and volatile organic compounds (Sutton et al. 1992; Griffiths et al. 1999; Salerno et al. 2017). The objectives of each study differ, and each present data gathered from a maximum of two genotypes, apart from Kambal and colleagues (1976), who measured pollen production and style length in 12 genotypes. Although useful in their own right, these studies cannot easily be used to compare floral traits between genotypes. The most comprehensive assessment of floral trait variation between different genotypes was carried out by Bailes (2016), who examined multiple traits in up to 30 genotypes. Bailes (2016) reported substantial variation in flower and petal size, wing petal spot size, nectar concentration and volume, and the number of pollen grains produced by flowers. Bailes (2016) also observed differences in flower tripping force and floral volatiles between two lines: Fuego and Tattoo. The following sections summarise current published knowledge of floral trait variation and how this might influence pollination in *V. faba* and other plant species.

Floral scent

The influence of variation in specific traits on bee behaviour has been assessed by Bailes (2016), using data from *V. faba*, and by other studies examining bee behaviour in other systems. Floral traits may be broadly classified into attractive traits, which advertise the flower, and reward traits, which affect the payment obtained by a visitor. Floral scent is often one of the first attractive traits detected by bees and can aid bee orientation and landing (Arenas and Farina 2012). Bailes (2016) observed that, although bumblebees have no preference between the scent of the *V. faba* lines Fuego and NV676, they can distinguish between them. To date no other studies have reported pollinator preference between *V. faba* lines due to floral scent. Most studies examining insect responses to *V. faba* volatile compounds focus on pest responses to plant and leaf volatile compounds. *Aphis fabae* are attracted to volatiles of Sutton dwarf leaves, and egg parasitoid wasps (*Trissolcus basalis*) prefer to lay eggs in stink bugs (*Nezara viridula*) on water stressed *Aguadulce* plants due to their volatile profile (Nottingham et al. 1991; Webster et al. 2008; Salerno et al. 2017). The effect of floral volatiles on pollinator and pest behaviour has studied more widely in other systems including some crops. A study by Ceuppens et al. (2015) found that bumblebees prefer floral scent of the strawberry variety Sonata over the variety Elsanta. In contrast, a study by Mozūraitis et al. (2020) found no preference between scents of strawberry cultivars, by strawberry blossom weevil, *Anthonomus rubi*. In other systems, floral scent is known to function as a powerful long-range attractant, with Euglossine bees able to detect scent over 2 km away (Ackerman 1986). Floral scent can also facilitate flower detection, orientation and learning at closer range, and may provide directional cues to aid nectar detection (Effmert et al. 2006; Leonard et al. 2011).

Multiple attempts have been made to identify the Volatile Organic Compounds (VOCs) responsible for *V. faba* scent and their effect on bee behaviour in artificial experiments. A study by Sutton et al. (1992) identified (E)-β-ocimene as the main VOC emitted by flowers of Maris Bead alongside traces of α-pinene and limonene. Griffiths et al. (1999) reported a similar bouquet for Maris Bead, with (E)-β-ocimene making up the largest proportion of VOCs with (Z)-β-ocimene, α-pinene, linalool, and β-myrcene also identified. Bruce et al. (2011) identified (E)-Caryophyllene as the main VOC entrained from flowers of Sutton Dwarf, followed by linalool, limonene and α-humulene. Linalool is known to strongly attract

pollinators (Raguso and Pichersky 1999). However, some experiments using synthetic VOCs, have found that *Bombus impatiens* show no preference for linalool, but an innate preference for β-trans-bergamotene (Haber et al. 2021). Depending on chemistry, VOCs may diffuse differently providing unique concentration gradients which may affect attraction. It is important that future studies seek to properly examine bee behaviour in response to biologically relevant VOCs.

The genetic control of components of scent have been studied in different systems and in some cases can be manipulated by single genes. Work by Amrad et al. (2016) in *Petunia* found that R2R3-MYBs (*EOBII* and *ODO1*) regulate benzenoid biosynthesis in multiple species. Evidence also suggests that *ODO1* may be responsible for variation in benzenoid production, however, manipulation of the gene may have widespread effects, as R2R3-MYBs also affect flower colour (Sheehan et al. 2012). Further evidence has shown that introgression of a locus containing an *ODO1* polymorphism from unscented *P. exserta* into the normally scented *P. axillaris* abolishes scent production (Klahre et al. 2011). Klahre and colleagues (2011) also showed that hawkmoths prefer scented *P. axillaris* over unscented flowers. However, when presented with unscented white flowers and scented red flowers they struggled to make choices, suggesting that, at least for hawkmoths, a combination of scent and colour is important for flower selection. In *Prunus mume*, emission of specific benzenoids including cinnamyl alcohol and cinnamyl acetate is higher in pink flowers compared to white flowers, providing more evidence that it may be possible to manipulate floral scent and colour together to influence pollinator attraction (Zhang et al. 2019). However, it is important to note that manipulation of plant volatiles needs to be considered in the context of both agonists and antagonists. As shown by Knauer and Schiestl (2017), both *B. terrestris* pollinators and *Pieris brassicae* (that lay eggs on plants) are attracted to *Brassica rapa* plants because of the floral VOC phenylacetaldehyde.

Colour and patterning

Insects detect light using compound eyes made up of many small optical units termed ommatidia. Most insects are unable to detect red wavelengths of light, but unlike humans can perceive ultraviolet (Chittka et al. 1994). Measurement of spectral sensitivity of bee photoreceptors gives indications of what bees should be able to perceive. Colours of objects as perceived by bees have been modelled using a "colour hexagon" of bee visual space, based on electrophysiological characterisation of honeybee photoreceptors (Chittka 1992). This method allows us to calculate how easily a bee should be able to tell different colours apart and plot it in a graphical form. Previous work by Dyer et al. (2008) suggests that bumblebees may be less able to discriminate between colour distances of less than 0.07 hexagon units, but honeybees can discriminate between colours as little as 0.008 hexagon units apart (Dyer and Neumeyer 2005). The method also enables calculation of "green colour contrast", defined by Spaethe et al. (2001) as "the difference in signal provided by the green receptor between (a green) background and target for detection". In simple terms, it is hypothesised that the first visual signal used by a bee when detecting flowers is contrast against the background, which in nature is often green. Therefore, the "green contrast" of a colour may give an indication of how easily a bee can detect it. However, some argue that the influence of colour and contrast is far more complex, and green contrast does not have a significant effect on honeybee choices in controlled conditions (Leslie et al. 2018).

Estimations based on honeybee photoreceptors and resolution of ommatidia have also been used to produce "photographic" predictions of what flowers look like through a bee's eye (Hempel De Ibarra et al. 2015). Both this method and the bee hexagon method are useful tools to help us quantify colour variation as perceived by insects and the effect of colour on bee attraction. However, colour measurements need to be viewed in context. Studies have shown that perception of colour and resolution is likely to be influenced by flight angle, distance from an object and light levels (Spaethe et al. 2001; Dyer et al. 2008). However, more recent work has suggested that honeybees may be able to see at far better resolution that previously thought (Rigosi et al. 2017). Compared to honeybees, bumblebee photoreceptors may have less noise due to their size and may have different sensitivity depending on light conditions (Kapustjanskij et al. 2007; Dyer et al. 2008; Meyer-Rochow 2012). In controlled conditions, bumblebees show preferences between colours, with many

reports finding bias towards purple hues (Raine and Chittka 2007; Reverté et al. 2016). Similarly, flowers which are highly saturated may appear more attractive to bees, suggesting attractiveness of a colour is related to its detectability (Lunau et al. 1996).

Closely linked with floral colour is the distribution of colour across the flower surface, referred to as patterning. Flowers of most *V. faba* lines have veins which run vertically on the standard petal and in some cases can be highly saturated (visible in **Figure 1.4 A**). No previous work has been done exploring variation in standard petal veins, their genetic control or effect on bee behaviour in *V. faba*. Based on evidence from other plant species, it is reasonable to hypothesise that *V. faba* veins may function as a nectar guide, providing both an attractive and directional cue to bees. Experiments using artificial flowers show that pollinators identify models with "nectar guides" more quickly, and spend less time handling them, potentially increasing foraging efficiency (Waser and Price 1983; Dinkel and Lunau 2001). The genetic control of colour and patterning has been explored well in the genus *Antirrhinum*, which have zygomorphic flowers comparable to *V. faba*. Work by Schwinn et al. (2006) has shown that variation in anthocyanin pigmentation between *Antirrhinum* species can be attributed to differences in activity of the *Rosea* and *Venosa* loci which encode R2R3-MYB transcription factors. Work by Shang et al. (2011) has shown that venation of anthocyanin pigment in *Antirrhinum majus* is defined by the pattern of expression of the *Venosa* gene. Signal of *Venosa* also correlates with strength of venation between *Antirrhinum* species, suggesting that *MYB* genes determine intensity and distribution of veins in this genus. Crucially, Shang et al. (2011) also reported that plants with venation attract more bee visits than solid white flowers or solid pink flowers. Much work has also been done on the nectar guides of *Mimulus* spp., revealing that yellow nectar guides containing carotenoids are under control of an R2R3-MYB transcription factor named *GUIDELESS*, and mutation of this gene leads to lack of nectar guides and results in fewer visits by bumblebees (Owen and Bradshaw 2011; Yuan et al. 2013).

Another floral pattern present for most *V. faba* lines is wing petal spots, which often appear dark or black in colour to humans (visible in **Figure 1.3**). Sometimes called melanin spots by breeders, the spots have been linked with high tannin in seeds (Knott 1990). Work done by Bailes (2016) identified considerable variation in wing petal spot size between lines, from 20% coverage of the wing petal to 60% coverage. Like standard petal veins, the function of

wing petal spots of *V. faba* has not been explored greatly. The only bee preference experiments were completed by Bailes (2016), demonstrating that bumblebees prefer real flowers with wing spots over those without. There are no other papilionaceous flowers with wing petal spots as distinct as *Vicia faba*. A well-studied example of flower spots is those of *Gorteria diffusa*, which mimics flies in a sexual deception scenario to attract male fly pollinators (Johnson and Midgley 1997; Ellis et al. 2014). Given that foragers of *Bombus* species are female, a sexual deception mechanism is unlikely in *V. faba* flowers. The spots could still mimic bees, eliciting an aggregation response, as found in *Daucus carota* (Goulson et al. 2009). Considering the high contrast between the dark spots and white wing petals and their position, proximal to the reward, the spots may aid flower identification due to contrast or function as nectar guides, providing a directional cue. In an artificial system, the presence of black spots on a flat orange disc have been shown to reduce bumblebee search time when compared to plain orange discs, and bees can learn to recognise black spots in differential conditioning tests (de Jager et al. 2017).

A handful of studies have been published examining genetic control of yellow wing petal spots in *V. faba* due to the trait's association with low seed tannin content. Both Sjödin (1971) and Cabrera (1988) performed crosses between wild type (dark spotted) and yellow spotted varieties and found that the yellow phenotype was governed by a single inheritance factor, referenced in Hughes et al. (2020). Recently, Hughes et al. (2020) confirmed that inheritance of the yellow spot phenotype in the *V. faba* variety Gelber is due to a single recessive gene. Alleles responsible for a zero-tannin phenotype (named *zt1* and *zt2*) have been found to result in a complete absence of the wing petal spot and stem colour alongside reduction in flower phenolic compound emission (Zanotto et al. 2020). A recent study by Gutierrez and Torres (2019) has found that the *zt1* phenotype is the result of two mutations in the *VfTTG1* gene, which encodes a WD40-repeat protein. In multiple plant species, R2R3MYB and bHLH transcription factors interact with WD-repeat (WDR) proteins to activate genes in the anthocyanin biosynthetic pathway (Shang et al. 2011).

In other systems which have forms of petal spots, MYB transcription factors play a key role in patterning. In *Clarkia* species, an R2R3MYB transcription factor called *CgMyb1* activates petal spot formation and that changes in the promotor of *CgMyb1* can affect the position of spots (Martins et al. 2017). Different MYB transcription factors also affect anthocyanin

pigmentation in discrete regions of *Clarkia* flowers and the background flower colour (Lin and Rausher 2021). Variation in anthocyanin spots between populations of *Mimulus guttatus* is primarily due to variation in three R2R3MYB genes found at the same locus (*PLA1*) (Lowry et al. 2012). Manipulation of R2R3MYB activators and R2R3MYB repressors can be used to alter anthocyanin spot patterning, and *Bombus impatiens* foragers preferentially visit genotypes with a single larger spot than the wildtype with many small spots (Ding et al. 2020). Differences in expression level and promotor sequence of MYB transcription factors have also been found to affect floral anthocyanin patterning in *Lillium, Oncidium* and *Phalaenopsis* (Chiou and Yeh 2008; Hsu et al. 2015; Yamagishi 2021).

Floral morphology

Floral morphology can affect both the attractive surface available to pollinators and the ease of handling flowers. Substantial variation in standard petal height and wing petal area between *V. faba* lines has previously been identified by Bailes (2016). Standard petal height and wing area may be used as proxies for overall flower size and affect the size of the display visible to a pollinator on an individual flower scale. In multiple plant species, bees show preference for larger flowers (Inoue et al., 1995; Conner and Rush, 1996; Elle and Carney, 2003; Martin, 2004). In controlled conditions, larger artificial flowers can also be located more rapidly by bees (Spaethe et al. 2001), and in *V. faba*, links have been made between the size of the standard petal and outcrossing rate (Suso et al. 2005).

Alongside standard height and wing petal area, Bailes (2016) reported variation in corolla tube length. The corolla tube is located proximal to the stem of the plant, behind the petals as seen in **Figure 1.3 C**. In order to access the nectar reward, a bee must reach into the corolla tube and extend its proboscis. The length of the corolla tube can therefore affect the ease of access of nectar. Since tongue length can vary substantially between bee species (e.g the average length is 7 mm for *A. mellifera* and 13 mm for *Bombus hortorum*), corolla tube length has strong potential to affect pollinator visitation (Goulson et al. 2005).

On a similar accessibility point, the force required to open the papilionaceous flowers of *V. faba* may also exclude smaller pollinators. The force required to open flowers has been explored in alfalfa (*Medicago sativa*), and easier to open flowers set more seed in the field,

despite there being no significant difference in honeybee visitation (Knapp and Teuber 1990). Bailes (2016) examined the operative force of two *V. faba* lines which contrasted in flower size, finding large force variation between them, however no experiments have been done so far to explore whether operative force may affect bee visitation or yield in *V. faba*.

Floral display size

In general, larger flowers should be more visible to bees, and a positive relationship is seen between flower size and visitation rate (Conner and Rush 1996; Martin 2004). However, increased visitation to larger flowers could result from a larger available reward. On the plant level, the number of flowers produced by a plant will both affect the visibility of plants and potential reward available to pollinators. In *V. faba*, floral display size positively correlates with outcrossing (Suso et al. 2005). In other systems, the number of flowers available to pollinators also positively correlates with pollinator visitation (Grindeland et al. 2005; Makino et al. 2007; Parra-Tabla and Vargas 2007).

Energetic reward

Pollinators visit flowers to gain energetic reward, most often in the form of nectar, containing sugar, and pollen, containing protein. Whereas pollen has not evolved primarily to be consumed by pollinators, nectar is an adaptive secretion used to attract pollinators. A great number of studies have explored preferences of bees for nectar, generally finding that bees prefer flowers with greater quantities of nectar (Cnaani et al. 2006; Nayak et al. 2015; Mallinger and Prasifka 2017). Bees also show general preference for nectar with greater sugar concentration, as it provides more energy (Knopper et al. 2016; Bailes et al. 2018). Using *V. faba* relevant concentrations, Bailes et al. (2018) found that bumblebees prefer 55% w/w sugar solution over 40%. However, factors other than just nectar volume and sugar content have been shown to affect pollinator preference. Honeybees prefer nectar containing amino acids (Alm et al. 1990), and warmer less viscous nectar (Nicolson et al. 2013). A recent study found that bees may make trade-offs between sugar content and ease of drinking and offloading nectar when foraging, as nectar with more sugar is more viscous and takes longer to regurgitate (Pattrick et al. 2020). Enhancing nectar production and quality in *V. faba* appears a promising way of enhancing bee attraction, and may be a way to

better support bee populations. Bees also forage for pollen and show ability to discriminate between pollen based on quantity and quality (Robertson et al. 1999). Estimates of the number of pollen grains produced by *V. faba* flowers have been made by Suso et al. (2008), Carré et al. (1994), Kambal et al. (1976), and Bailes et al. (2018), considerable variation appears to exist between lines. Considering that bees can assess nectar quality while consuming it but collect pollen in corbiculae and therefore cannot assess quality while collecting it, *V. faba* lines with superior pollen quantity or quality would have to be bred to have recognisable cues for bees.

1.6 Objectives and hypotheses

Improving pollinator attraction to *Vicia faba* is a promising way of ensuring stable yield. To do this, several essential questions must first be answered, including how variable are floral traits among modern commercial *Vicia faba* lines, does the variation present between *Vicia faba* lines affect pollinator attraction, and do differences in *Vicia faba* floral traits affect yield? To advance knowledge in this area, this PhD has explored and tested the following objectives and hypotheses.

Objective 1: To identify novel floral trait variation in previously uncharacterised lines of *Vicia faba,* with emphasis on modern commercial lines.

Hypothesis 1: Modern commercial lines of *Vicia faba* show significant variation in previously unexplored floral traits.

The results of this objective are presented in Chapter 3.

Objective 2: To investigate the effect of *Vicia faba* floral trait variation on bee attraction in field conditions.

Hypothesis 2: In field conditions, *Vicia faba* lines with floral traits which are theorised to be more attractive to pollinators will attract more pollinators than lines with floral traits which are theorised to be less attractive to pollinators.

The results of this objective are presented in Chapter 4.

Objective 3: To evaluate the effect of pollinator exclusion on yield of multiple *Vicia faba* lines.

Hypothesis 3: In field conditions, *Vicia faba* plants have lower yield when pollinators are excluded, compared to when pollinators are not excluded.

The results of this objective are presented in Chapter 4.

Objective 4: To determine if floral trait variation between *Vicia faba* lines can influence yield.

Hypothesis 4: In field conditions, *Vicia faba* plants with floral traits which are theorised to be more attractive to pollinators will receive a greater yield benefit with open pollination than *Vicia faba* plants with floral traits which are theorised to be more attractive to pollinators.

The results of this objective are presented in Chapter 4.

Objective 5: To investigate the effect of extremes of variation in specific *Vicia faba* floral traits on bee behaviour in controlled conditions.

Hypothesis 5: *Bombus terrestris* foragers find the appearance of large wing petal spots more innately attractive than small wing petal spots.

Hypothesis 6: *Bombus terrestris* foragers find flower standard petal appearance, colour, and patterning of the *Vicia faba* line Maris Bead more innately attractive than that of the *Vicia faba* line NV129.

Hypothesis 7: *Bombus terrestris* foragers find the floral scent of the *Vicia faba* line Maris Bead more innately attractive than that of the *Vicia faba* line NV129.

The results of this objective are presented in Chapter 5.

2 Materials and Methods

2.1 Plant material

2.1.1 Genetic material used to study variation in floral traits of *Vicia faba*

To evaluate variation in floral traits between lines of *Vicia faba* L., seed was selected from the NIAB collection. Seed which had been produced by inbreeding for at least 5 generations was selected to minimise genetic variation within lines, except for Tiffany, Victus and Yukon, which were purchased from the supplier (LS Plant Breeding, Impington, Cambridge). Lines were selected based on personal observation of differences in the appearance of flowers and advice from experts at the National Institute of Agricultural Botany and the Processors and Growers Research Organisation. Emphasis was made to examine commercial *Vicia faba* lines which have not been characterised previously.

2.1.2 Growth conditions for floral trait variation studies at NIAB

For work exploring variation in floral traits, plants were grown in an insect-proof glasshouse at NIAB, Park Farm, Histon, Cambridge between November and May each year. Replicate plants from each line were grown in every month across the growing season to control for any environmental variation throughout the year. Plants were placed evenly across the growing space to minimize potential effects of light or humidity gradients. Seeds were sown directly into 1 litre pots (11 x 11 x 11 cm) of Levington Advance M2 Potting Compost. Glasshouse temperature was maintained at 18 – 25°C with 16 -18h daylight, depending on the month grown. When daylight levels fell below 20,000lux, 10,000lux High Pressure Sodium lamps were automatically activated. Humidity was maintained at approximately 45%. Anderline biological control agent (Bioline AgroSciences, Little Clacton, Essex) containing the predatory mite *Amblyseius andersoni* was used to control thrip (*Thysanoptera* spp.) levels.

2.1.3 Choice of flower stage used in experiments

As *V. faba* flowers mature from buds to open flowers, the standard petal changes from a state where the abaxial surfaces tightly enclose the bud to reflexing back away from the keel-wing complex until eventually the adaxial sides of the standard petal touch. The standard petal then collapses back to enclose the keel-wing complex containing the carpel. In studies exploring floral trait variation, fully open stage 5 flowers (**Figure 2.1**) were used, both for consistency and because fully open flowers are most likely to be visited by bees (Pierre et al. 1996). For experiments examining pollen production, stage 3 flowers were collected, to avoid pollen being lost from flowers.

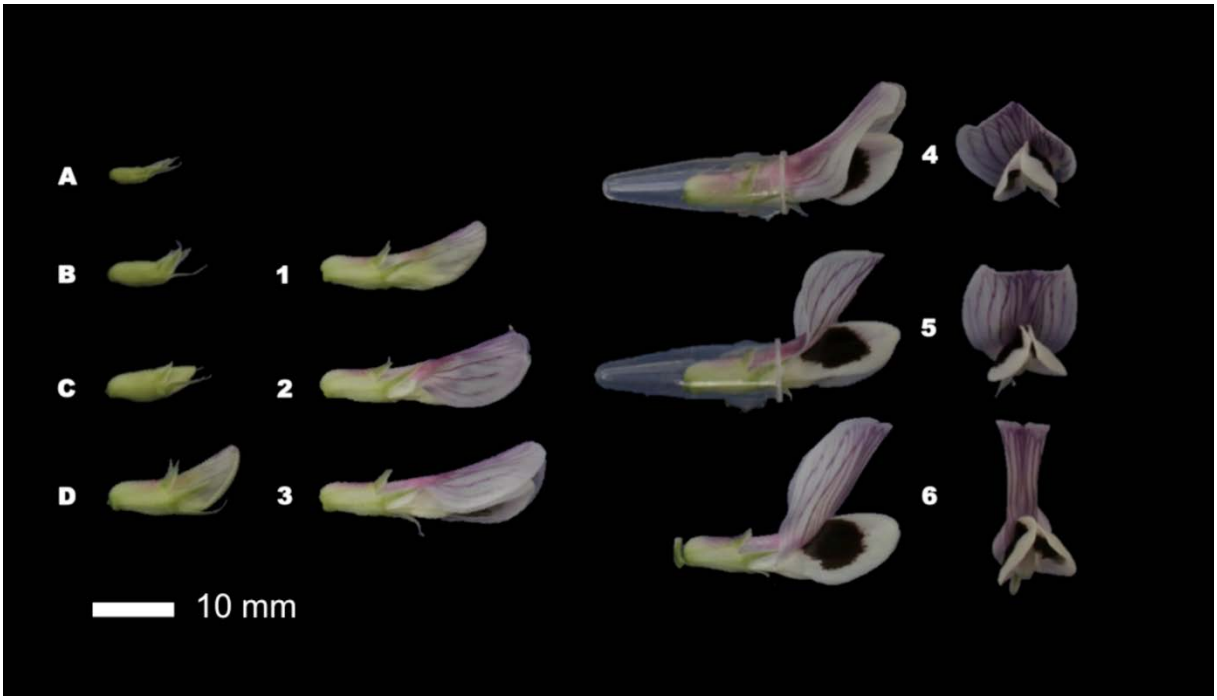


Figure 2.1 Growth stages of *V. faba* flowers. Growth stages of buds are classed as A, B, C and D. Later stages are classed as 1, 2, 3, 4, 5 and 6 according to Bailes (2016). Stage 5 flowers were used for floral trait variation experiments, except pollen production, for which stage 3 flowers were used.

2.2 Measurement of floral morphology

2.2.1 Imaging flowers

Stage 5 flowers were picked from a glasshouse at NIAB Park Farm and transported to the image analysis lab in NIAB Park Farm Barn 1 in 50 mL conical centrifuge tubes (hereafter referred to as 50 mL Falcon tubes) containing moist tissue paper to avoid desiccation.

Flowers were imaged against a "SpotCard" developed by Symington and Glover (2019). The SpotCard allowed metadata (line, plant, and flower number) to be stored in images, and extracted using FIJI (ImageJ) (https://fiji.sc). Coloured spots in each corner of the card provided a scale for data extraction. A red card background was selected to provide contrast against *V. faba* flowers (**Figure 2.2**). A minimum of five flowers were imaged from each plant, for a minimum of seven plants per line, for 11 *V. faba* lines.

Prior to imaging, flowers were pierced with a needle at the point where the standard petal starts to reflex. The needle was 25 mm in length, with a 10 mm middle segment coloured black. Flowers were pierced so that the upper surface was held 10 mm above the surface of the SpotCard (**Figure 2.3**). A disc of blue card was glued to the head of the needle to aid data extraction using FIJI. Once pierced, flowers were positioned in the top left segment of the SpotCard, backed by polystyrene, by sliding the needle tip into a pre-made hole (**Figure 2.2 A**). Lighting was provided by two LED lights set at 100% brightness. An image was taken of each flower using a Nikon D3300, positioned 55 cm above the card, with a 35 mm focal length, 1/50 shutter speed and f/10 aperture. Manual focus was set up before images were taken to ensure crisp focus. The shutter was released remotely using an Amazon Basics wireless remote control for Nikon.

After a photo had been taken of an intact flower, each flower was dissected to isolate the standard petal and two wing petals, which were placed in the other three sections of the SpotCard (**Figure 2.2 B**). The wing petal in the right segment was stuck down using double sided sticky tape, to ensure that it lay flat. The other wing petal was left to lie naturally. Another photo was taken of the dissected flower.

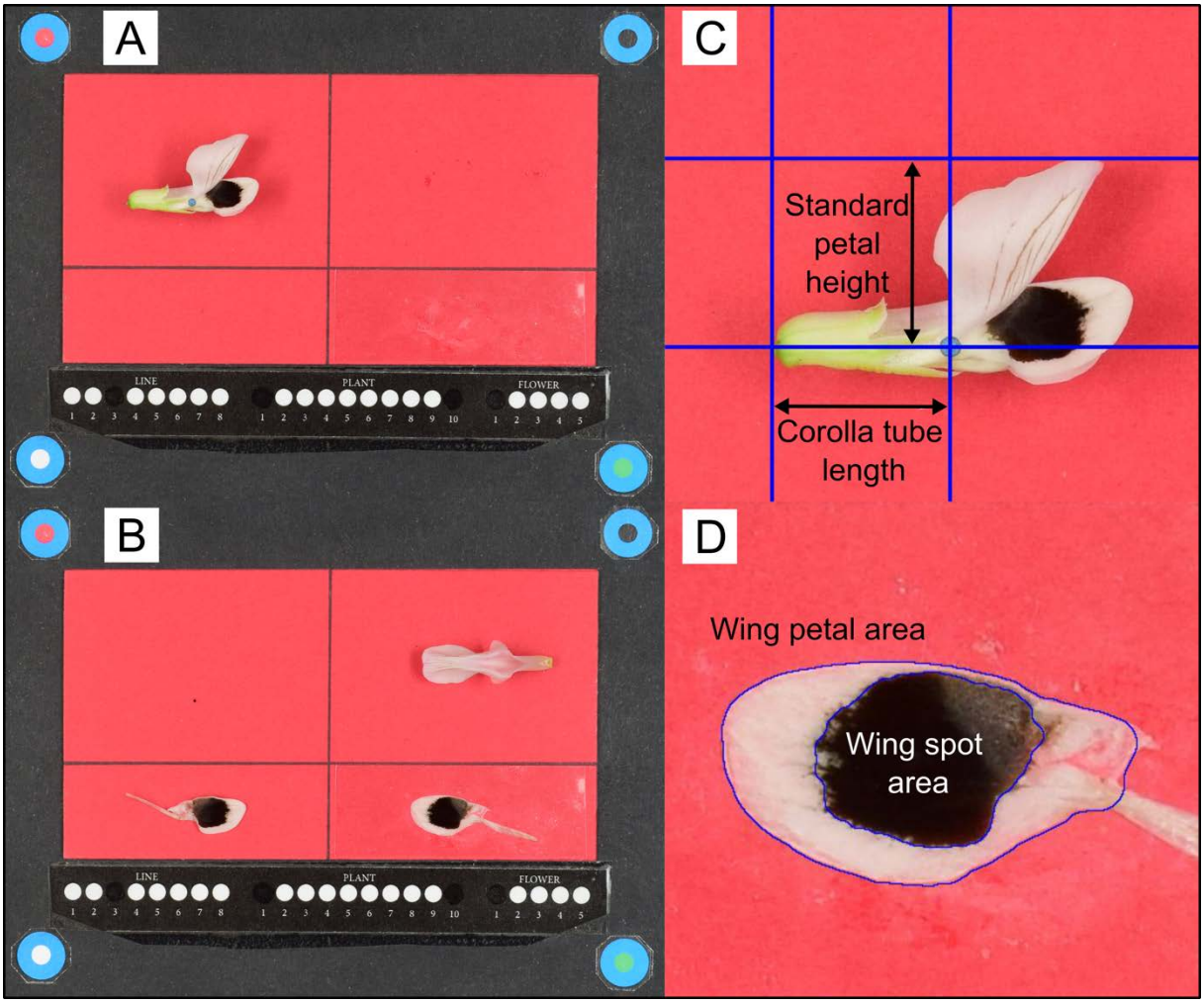


Figure 2.2 The "SpotCard" used for cataloguing floral measurements of *Vicia faba* flowers. Flowers were imaged against a SpotCard developed by Symington and Glover (2019). Plant metadata was recorded using the panel along the bottom of the SpotCard, visible in A and B. The corner circles provided a scale bar, being exactly 150 mm apart diagonally and 120 mm from left spots to right spots. **(A)** Whole flowers were imaged side on. **(B)** Flowers were then dissected to isolate wing and standard petals and imaged again. **(C)** Standard petal height was measured from the centre of the blue pin to the uppermost part of the standard petal. Corolla tube length was measured from the centre of the blue pin to the leftmost part of the corolla. **(D)** Wing petal area was measured using the perimeter of the main body of the wing petal area. Wing spot area was measured using the perimeter of the spot.

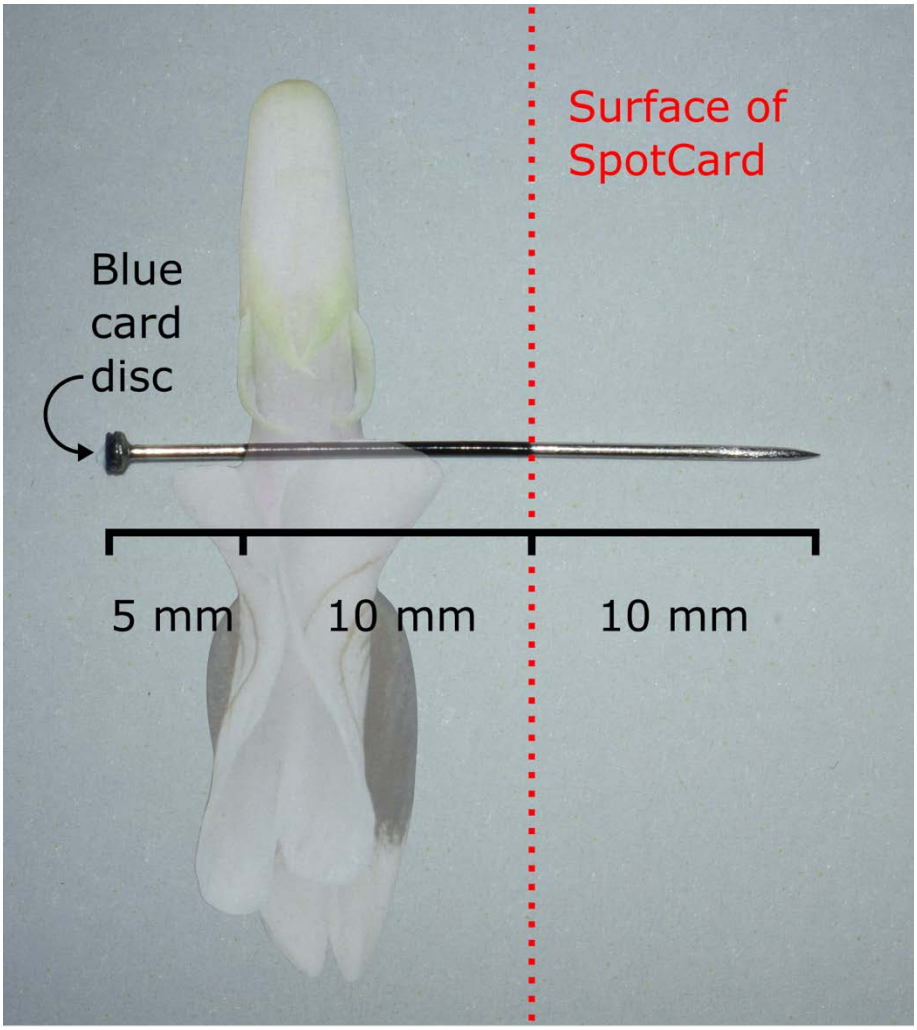


Figure 2.3 The needle used to mount *V. faba* flowers on the SpotCard. A needle was marked with a 10 mm wide black segment, 5 mm from the head of the needle. The upper boundary of the black segment marked the position to which the flower was pierced, and the lower margin indicated the depth to which the needle was inserted into the imaging card. This ensured that all flowers were positioned at the same height above the imaging card and lay at the same angle. A disc of blue card was glued to the head of the needle to aid in later image data extraction using FIJI.

2.2.2 Data extraction

Data from images was extracted using a version of the SpotCard macro, adapted for the *V. faba* SpotCard by Hamish Symington (Symington and Glover 2019, additional file 2). The macro used colour thresholding to detect the four corner spots. The image was cropped to the spots, which were used to set the scale in mm. The macro then cropped to specified areas of the image to produce cut-outs of the whole flower and the wing petal in the right-hand box. Cut outs of whole flowers were used to measure standard petal height, and corolla tube length. Standard petal height was measured from the centre of the blue pin to the uppermost part of the standard petal. Corolla tube length was measured from the centre of the blue pin to the leftmost part of the corolla. Cut outs of the wing petal were used to measure wing petal area and wing petal spot area. Wing petal area was measured using the perimeter of the main body of the wing petal area. Wing spot area was measured using the perimeter of the spot. Wing petal spot area was then converted to a percentage of the total wing petal area using Microsoft Excel.

2.3 Measurement of flower colour and patterning

2.3.1 Flower colour

Fresh stage 5 flowers were collected from the glasshouse at NIAB Park Farm and transported to the University of Cambridge Department of Plant Sciences in 50 mL Falcon tubes containing moist tissue paper to avoid desiccation. Reflectance spectra of three points of the flower were measured using a spectrophotometer (**Figure 2.4**).

(A) The adaxial face of the standard petal (avoiding veins)

(B) The abaxial face of the wing tip

(C) The abaxial wing-spot

These points were chosen as they represented the most visible parts of the flower and captured the majority of variation seen between the lines studied. It was not possible to accurately measure variation in standard petal vein colour using a spectrometer as veins were narrower than the spectrophotometer beam.

Samples were prepared by flattening the petal onto a matt black card using double-sided sticky tape to minimize experimental error from light scattering. The reflectance (%) of samples relative to a white standard was then recorded at wavelengths between 300 and 700nm using a spectrophotometer (Ocean Optics 2+) with a 10 ms integration time and the spectrum of the black background corrected for using a sealed dark box. Samples were illuminated with a Deuterium-Halogen light source (Ocean Optics DH 2000) and analysed with SpectraSuite software (version 1.0, Ocean Optics). The probe was held in a metal probe holder, positioned at 45° to the sample.

A minimum of three flowers were measured per plant, for a minimum of seven plants per line, for 11 *V. faba* lines. For each flower, three reflectance spectra were measured at each point. Reflectance spectra were averaged, smoothed and any negative values were corrected using the Pavo package in R (https://www.rdocumentation.org/packages/pavo/versions/2.8.0). Each average reflectance spectrum was converted into a co-ordinate in bee colour-space according to (Chittka 1992).

Coordinates were then exported from R and plotted using Microsoft Excel (Version 16.58) for expanded views of hexagon plots.

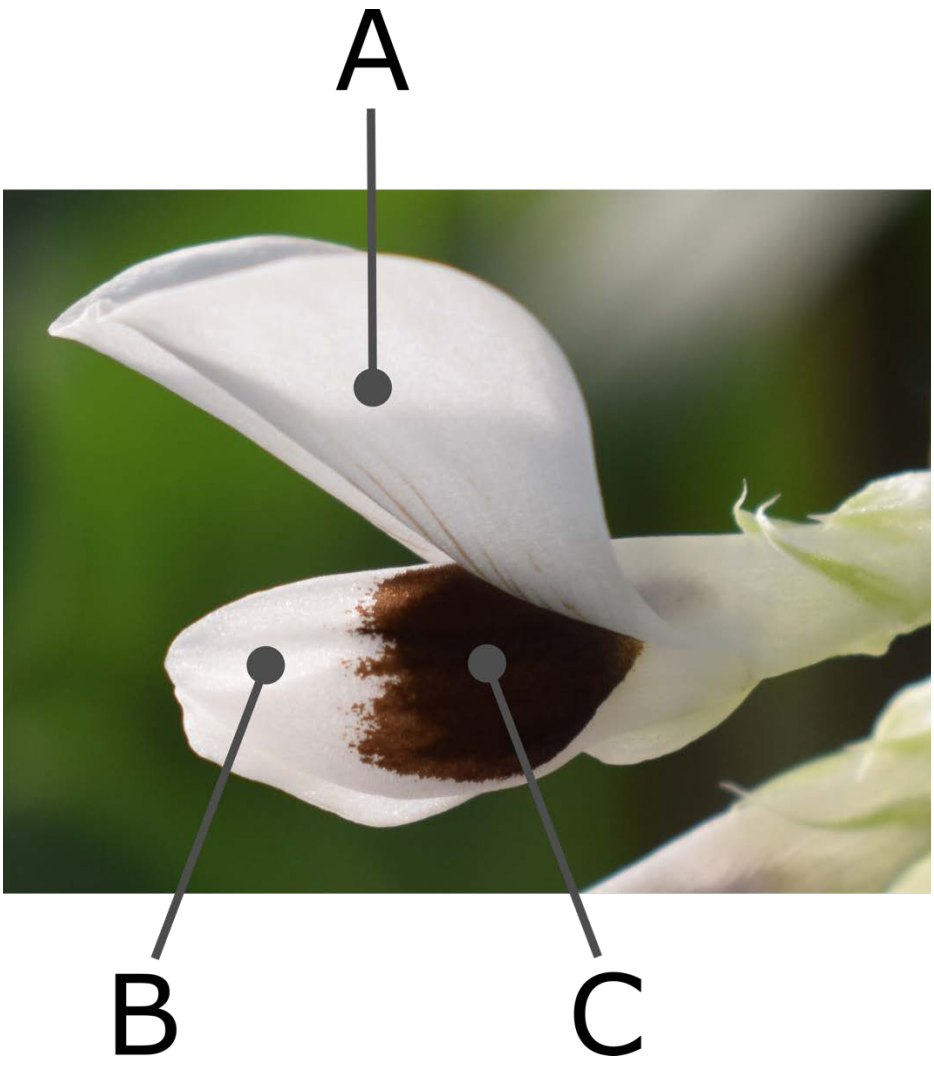


Figure 2.4 The points at which reflectance spectra were measured on a *V. faba* flower. Reflectance spectra of three points of the flower were measured during glasshouse floral trait variation experiments. **A** - the adaxial face of the standard petal (avoiding veins), **B** - the abaxial face of the wing tip, and **C** - the abaxial wing-spot.

2.3.2 Ultraviolet photography

Ultraviolet photographs of *Vicia faba* flowers were taken using a Sunscreenr™ UV camera for Android. Photographs were taken outdoors on a sunny day, out of direct sunlight. Visible spectrum photographs of the same flowers were taken for comparison using an iPhone 7. Both UV and visible spectrum photographs were taken in the same lighting.

2.4 Quantification of the number of flowers produced per node

The number of flowers was counted on each raceme of a node, with a node defined as the point where racemes are produced at the plant axil. A minimum of fifty nodes were sampled per line, for 31 lines. The minimum number of nodes sampled for any one plant was five. Flowers which had died or failed to develop were not counted. If a flower produced more than one raceme at any node the flowers of both racemes were counted.

2.5 Quantification of flower tripping force

The operative force required to trip a *V. faba* flower was measured for 30 lines. This was carried out using a method adapted from Córdoba and Cocucci (2011) and Bailes (2016). A minimum of 11 flowers were measured per line, for a minimum of two plants per line. For all but three lines, over 20 flowers were measured per line.

A dynamometer (model 20010, 10 g, PESOLA Präzisionwaagen, Switzerland) with a measurement range of 0.1 – 10 g was attached vertically on a fixed frame (**Figure 2.5**). Stage 5 flowers were suspended from the dynamometer using a metal clip, placed on the calyx. The dynamometer was tared to zero to account for the weight of the flower. A crocodile clip was attached to the flower's right wing petal in the centre. A motor was then activated via a PlayStation controller, which steadily lowered the clip downwards, exerting force on the flower. The clip was moved down until the flower tripped. Tripping was defined as the point at which the stigmatic surface was ejected from the fused keel petals and became visible. At this point, the force was read from the dynamometer to the nearest 0.1 g. Force was also recorded using an electronic balance, modified to fit the moving platform. However, this

method of measurement was abandoned as values displayed by the balance fluctuated excessively.

Flowers were collected from well-watered plants grown in a glasshouse at the NIAB Park Farm site and were measured in the glasshouse immediately after being picked. Flowers were measured between 10 am and 12 noon.

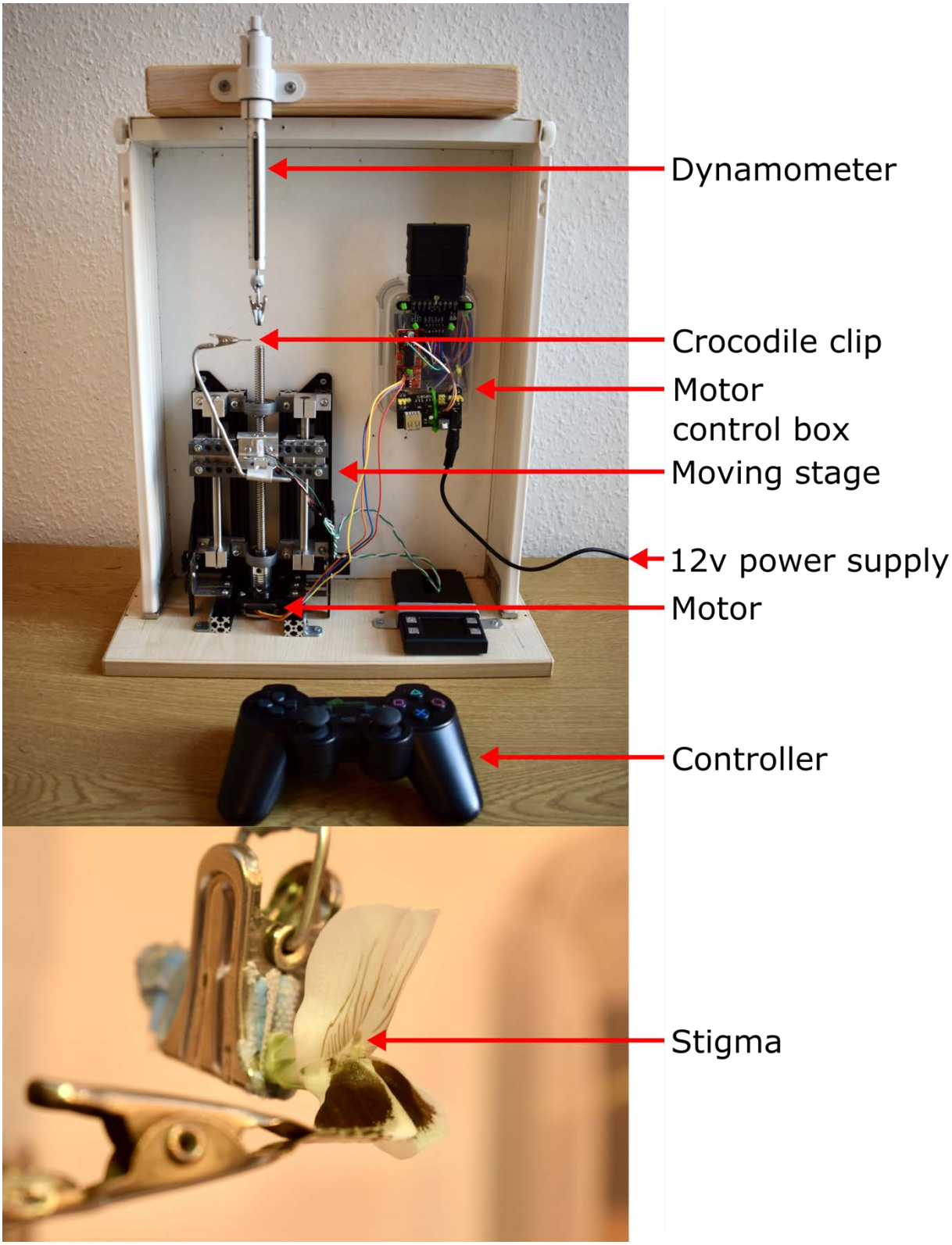


Figure 2.5 Apparatus used to measure flower tripping force. The force required to trip flowers was measured using a fixed dynamometer and stage, moved by an electronic motor, and operated via a PlayStation controller, made by Dr Carlos Lugo–Vélez. Stage 5 flowers were suspended from the dynamometer by a metal clip. A crocodile clip, fixed to the moving stage, was attached to the right-hand wing petal. The stage was moved downwards until the flower tripped, exposing the stigma, at which point the force was recorded.

2.6 Measurement of floral reward

2.6.1 Nectar volume

The volume of nectar produced by stage 5 flowers was measured in a randomised order between 10 am and 12 noon on plants which had been flowering for at least 1 week. Flowers were sampled from the middle nodes of the primary plant stem, avoiding the lowest three nodes and last flowering nodes if plants were nearing the end of flowering. The sampling time was decided based on previous work demonstrating that nectar production can vary over the course of a day and over the lifetime of a plant (Kakutani et al., 1989; Osborne et al., 1997). Nectar volume was measured for 11 lines in total.

Flowers were picked in the NIAB Park Farm glasshouse and transported to the image analysis lab in NIAB Park Farm Barn 1 in 50 mL Falcon tubes containing moist tissue paper to avoid desiccation. For each flower, the standard petal was removed by making a small incision along the calyx and then peeling back the standard, being careful not to disturb nectar which can collect at the base of the standard petal. The base of the standard petal was then removed using a scalpel and placed carefully in a 0.5 mL microcentrifuge tube with five holes pierced at the base and the lid removed (**Figure 2.6**). The reproductive complex, which contains the nectaries, was then isolated by removing the wing petals with tweezers. The reproductive complex was then placed in the 0.5 mL microcentrifuge tube with the nectary end pointing down. The 0.5 mL microcentrifuge tube was then placed inside a labelled and pre-weighed 1.5 mL microcentrifuge tube and the lid was closed. The sample was spun in a centrifuge at 13,000 rpm for 1 minute. The 0.5 mL microcentrifuge tube was then removed from the 1.5 mL tube, the contents disposed of, thoroughly washed using DI water, and dried ready for re-use. Nectar volume was estimated by re-weighing the 1.5 mL microcentrifuge tube, then using **Equation 1**.

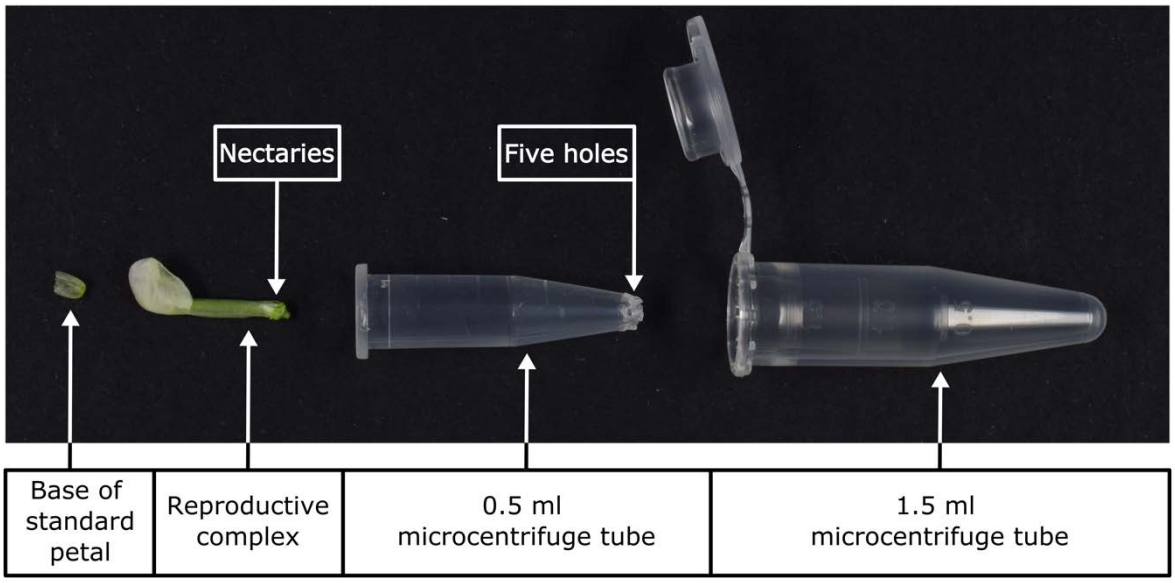


Figure 2.6 Apparatus used to extract nectar from *V. faba* flowers. The dissected base of the standard petal and the reproductive complex, containing nectaries, were placed inside a 0.5 mL microcentrifuge tube with five holes at the bottom and the lid removed. The 0.5 mL tube was placed inside a labelled and pre-weighed 1.5 mL microcentrifuge tube which was then closed and centrifuged at 13,000 rpm for 1 minute. The 0.5 mL tube was then removed from the 1.5 mL tube and the 1.5 mL tube was weighed again to estimate nectar volume per flower.

$$Volume\ (\mu l) = \frac{\boldsymbol{m}}{0.9988603 + 0.0037291\boldsymbol{C} + 0.000178\boldsymbol{C^2}}$$

Equation 1 Volume of nectar produced by *V. faba* flowers. The volume of nectar collected from a flower was estimated using the mass of nectar weighed in mg (***m***) and the concentration of its nectar in % sugar w/w (***C***) by converting the concentration into solution density following the data tables of Haynes (2015). All sugar in the nectar was assumed to be sucrose, the predominant sugar contained in *V. faba* nectar according to Pierre et al. (1996).

2.6.2 Nectar concentration

The sugar concentration of nectar collected by centrifugation was quantified as weight by volume (% w/w) to the nearest 0.5% using one of two handheld refractometers (Bellingham & Stanley, Tunbridge Wells; models Eclipse 45-03 and Eclipse 45-82) which measure in either 0.5 % divisions from 0 – 50 % or 0.2 % divisions from 45 – 80 %. A minimum of 80 flowers were sampled per line and a minimum of 10 plants per line for 11 *V. faba* lines.

2.6.3 Total sugar content of flowers

The total amount of sugar produced per flower was estimated using **Equation 2**, based on data tables from the CRC Chemistry and Physics Handbook (Haynes 2015). Sugar concentration (% w/w) was converted into molarity based on the assumption that the sugars within the nectar are all sucrose. The quantity of sugar (mg) was estimated based on the molecular weight of sucrose and the volume of nectar produced by the flower (µl). Estimation of nectar sugar content was based on the assumption that sucrose is the only sugar found in nectar. Sucrose is not the only sugar present in nectar, but it is the largest constituent. Other sugars recorded in *V. faba* nectar include glucose and fructose, which have similar molecular weights to sucrose and therefore the estimate made using sucrose should be very similar to the true value.

$$Total\ sugar\ (mg) = \frac{(0.028\boldsymbol{C} + 0.0002\boldsymbol{C^2})\ \times\ 342.3\ \times\ \boldsymbol{V}}{1000}$$

Equation 2 The mass of sugar (mg sucrose) produced by *V. faba* flowers. The total mass of sugar produced by a flower was calculated using the estimated volume of nectar produced (***V***) in µl and the sugar concentration measured as % w/w (***C***). The section within brackets represents the conversion of sugar from % w/w to molarity, where 342.3 is the mass (g) of 1 mole of sucrose.

2.6.4 Pollen production

Unopened stage 3 flowers were picked from plants in the NIAB Park Farm glasshouse and transported to the University of Cambridge Department of Plant Sciences in 50 mL Falcon tubes containing moist tissue paper to avoid desiccation. The standard petal and wing petals were carefully removed from each flower using a scalpel and forceps. The tip of the reproductive complex (made up of the stigma and anthers contained within two fused keel petals) was then isolated using scalpel and forceps. The two fused keel petals were then separated using forceps inside a 1.5 mL microcentrifuge tube, releasing the anthers and pollen into the tube. The anthers, stigma and keel petals were then laid inside the tube and 200 µl of a solution of modified Alexander stain, Agar and Tween (see Appendix A) was added to the tube before it was closed tightly. Alexander stain was used based on the work of Alexander (1987) and Peterson et al. (2010). Samples were then vortexed for 30 seconds at maximum speed to dislodge pollen from anthers and ensure distribution of stain. Samples were incubated at room temperature before being frozen at -20°C until pollen could be counted. Alexander stain coloured viable pollen grains red and non-viable grains blue (**Figure 2.1**).

For counting, samples were defrosted at room temperature for 15 minutes. Two counts of a 9 mm^2 grid were made for each sample using a haemocytometer slide and light microscope. Samples were inverted and flicked 10 times to homogenise samples before each count. The number of pollen grains stained red (viable) and blue (non-viable) were counted and the average pollen count for each sample was used to estimate the average number of pollen grains produced per flower and the proportion of viable and non-viable pollen grains. A minimum of 3 flowers were sampled per plant for a minimum of 9 plants per line for 11 lines.

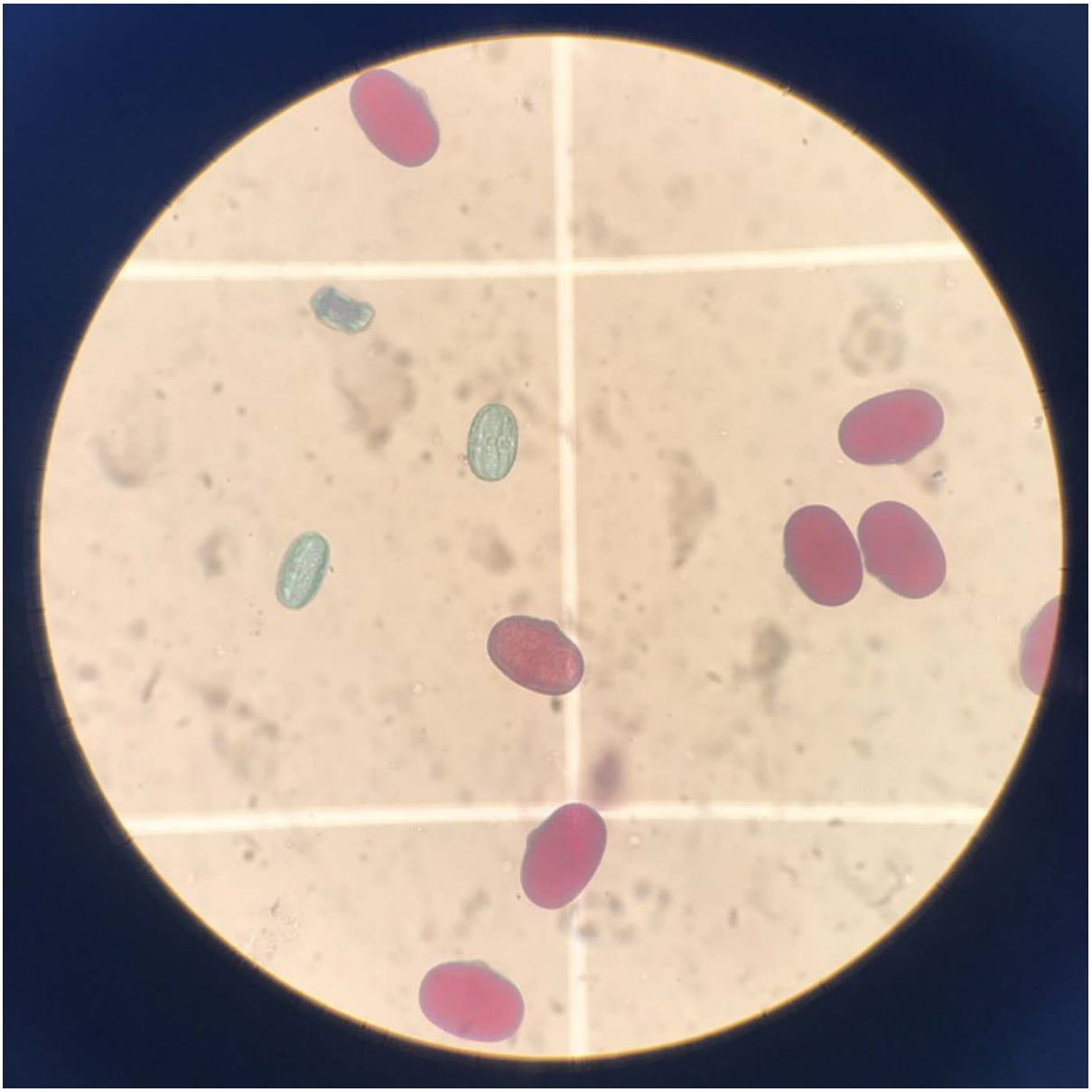


Figure 2.7 Pollen grains of *V. faba* stained using modified Alexander stain. Pollen samples were stained using modified Alexander stain (see Appendix A) as a means of estimating viability. Viable pollen grains with intact cytoplasm appeared red after staining and non-viable pollen grains with degraded cytoplasm appeared blue.

2.7 Bee experiments in controlled conditions

2.7.1 *Bombus terrestris* welfare and maintenance

The UK native subspecies of buff tailed bumblebee, *Bombus terrestris audax* (Harris, 1776), was used in bee behaviour experiments. *Bombus terrestris audax* was used as this species most commonly visits *Vicia faba* in the field (see chapter 4) and is commercially available for research. Colonies were supplied by Biobest, Belgium. Colonies were fed with 20% w/w sugar from a custom-made transparent feeder (**Figure 2.8**). This ensured bees were naïve to colours or scents used in experiments. Bee pollen (Sevenhills Wholefoods, organic raw polifloral bee pollen) was delivered directly into the colony box twice a week. Worker bees which fed on sugar solution and returned to the colony multiple times were marked with Queen bee marking numbers (E.H. Thorne, Market Rason, UK). Only workers marked as active foragers were used in behaviour experiments. Bees from at least two colonies were used for experiments to ensure that any observations were not colony specific. Colonies were connected to a 0.3 x 0.75 x 1.12 m plywood flight arena with a UV-transparent plexiglass lid via a gated plastic tube which allowed control over movement of bees between the colony box and arena. Sliding plywood doors on the sides of the arena allowed access to the arena. The floor of the flight arena was painted with Garden green water-based paint (Plasti-kote). A green background was used for all bee behaviour experiments involving visual cues, following the methods of (Lunau 1990; Dyer 2006; Bukovac et al. 2017). Lighting in the room was provided by 12 Sylvania 58W Professional Activa 172 tubes suspended from the ceiling. During experiments, a desk lamp with a Ecozone 25W daylight bulb positioned above the arena provided supplementary lighting.

Figure 2.8 Transparent feeder for *Bombus terrestris audax*. Bees were fed *ad libitum* using a transparent 200 mL custom-made feeder made using a petri dish and a 250 mL container. Bees accessed sugar solution using holes. Sugar solution was automatically replenished when the level dropped sufficiently. Using a transparent feeder ensured bees were naïve to any particular colour or scent used in experiments.

2.7.2 Spot size experiments

For spot size experiments, model flowers were made using epoxy resin. Dental silicone (Zhermack elite HD+ dental silicone, Zhermack, Badia Polesine, Italy) moulds were made of the adaxial surface of white rose petals to copy the conical cell surface. Conical cell structure of rose petals was confirmed using cryo- SEM prior to creating moulds. Casts were produced from moulds using Devcon epoxy resin coloured with 100 mg Zinc white Artist's pigment (Cornelissen & Son, London, UK) per 4,000 mg epoxy. Casts were assembled and mounted on wooden dowel using Velcro (**Figure 2.9 A and B**). Dowels were supported in 60 mL Sterilin Polystyrene containers (Thermo Scientific, Loughborough, UK), hereafter referred to as Hamilton jars, filled with polyurethane sponge and sealed using parafilm to prevent entry by bees. Two types of artificial flower were made, representing extremes of spot size, based on the work of Bailes (2016). One type of model had wing spots covering 20% of the petal area, and the other type of model had wing spots covering 60% of the petal area. Wing spots were drawn onto epoxy wings using a black Sharpie permanent marker pen and a template. Two templates were made for large and small wing spots, using the maximum and minimum percentage cover wing spot sizes observed by Bailes (2016). Prior to experiments, bees were fed using feeders made of four wells cut from a 96-well non-skirted PCR tube plate (Thermo Scientific, Loughborough, UK), mounted on green dowels, to train bees to forage at the same height as model flowers (**Figure 2.9 C**).

<u>Innate preference</u>

To test innate preference, an arena was cleared of bees and cleaned with soapy water, followed by 30% v/v ethanol. Four large and four small spotted flower models were placed in an equally spaced alternating array inside the arena. Immediately before a bee was released into the arena, 10 µl of 40% w/w sugar solution was loaded onto each model at the base of the standard petal (shown by the red arrow in **Figure 2.9 A**). The first 10 visits of a bee were recorded, and each flower model was reloaded with sugar solution after each visit. A visit was defined as a bee landing and drinking from a flower model.

<u>Differential conditioning</u>

For differential conditioning experiments, the arena was cleared and cleaned and four large and four small spotted flower models were placed in an equally spaced alternating array

inside the arena. One type of models (e.g. large spots) were loaded with 10 µl of 40% w/w sugar solution and the other (e.g. small spots) were loaded with quinine hemisulphate solution (0.12% w/w) as a punishment. Bees cannot distinguish between sugar and quinine solution using any cues other than taste (Whitney et al. 2008). The models visited by a bee were recorded and models visited by bees were reloaded after each visit. The first 200 visits made by a bee were recorded. When a bee was full and returned to the colony box the models were washed in distilled water, dried and placed back in the arena in a different position to the previous foraging bout to eliminate positional effects.

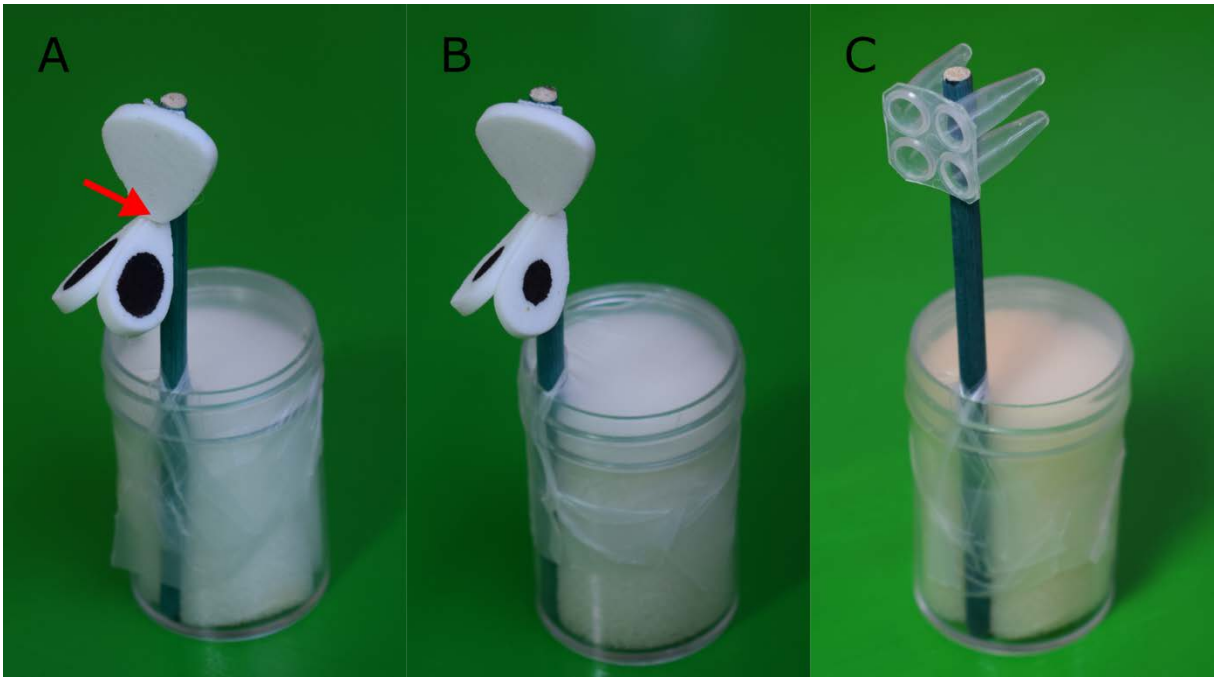


Figure 2.9 Model flowers used for spot size choice experiments. Two sets of epoxy flower models were made to test bee preference between extremes of wing petal spot size. **(A)** Large spotted models with spots covering 60% of the wing area. **(B)** Small spotted models with spots covering 20% of the wing area. **(C)** A 4 x 4 section of a PCR plate mounted on a dowel and Hamilton jar, used to train bees for the experiment. Red arrow indicates where flower models were loaded with 10 µl of 40% w/w sugar solution. The sugar solution droplet was held by the surface texture of the model.

2.7.3 Maris Bead and NV129 colour and patterning experiments

Four experiments to test bee preference between visual characteristics of Maris Bead and NV129 standard petals were carried out to explore factors which may contribute to differences in visitation rates observed in the 2021 field trial. The most striking visual differences between Maris Bead and NV129 were observed on the standard petals. The experiments used printed models of standard petals to exclude the influence of wing petal spots, nectar volume or concentration, or floral odour. Printed models were used as they reliably replicated the appearance of Maris Bead and NV129 standard petals and more closely matched the reflectance spectra of true flowers than pigmented epoxy models. Images of standard petals collected from the 2021 field in Stubton were taken using an imaging rig and standardised lighting at NIAB Park Farm. Images which were most representative of Maris Bead and NV129 were selected and cropped into 15 mm circles, to standardise petal size. Circles were printed on standard non-gloss business cards (VistaPrint) with a green background (**Figure 2.10**). The reflectance spectra of the printed petals were examined against spectra from the real petals to ensure that they were comparable in bee vision. A lid from a 0.2 mL PCR microcentrifuge tube (Thermo Scientific, Loughborough, UK) was attached onto the surface of the flower model using Pritt water-based glue to hold a sugar solution reward.

<u>Innate preference</u>

To test innate preference, printed flower models were held vertically 80 mm apart using BluTack (Bostik, Stafford, UK) in a 900 mm by 180 mm by 250 mm flight arena painted green with Painter's Touch Meadow Green spray paint (Rust-oleum, Illinois, USA)(**Figure 2.10 E)**. Each model was loaded with 10 µl of 40% w/v sugar solution. A bee was released into the arena and the first flower visited was recorded. A visit was defined as a bee landing and drinking. The bee was then captured in a black tube whilst drinking. The models were then replaced with new models to eliminate any scent marks and the orientation was swapped to eliminate positional effects. The bee was then released again to make a second choice. This process was repeated until 10 choices had been made.

The first experiment used images of complete standard petals to test bee preference for the colour and patterning of Maris Bead and NV129 in isolation from other floral traits (**Figure**

2.10 A). The subsequent experiments then focused on specific traits associated with the standard petals to evaluate their effect on bee innate preference in isolation. The second experiment tested the influence of average petal colour on bee preference, excluding the influence of more saturated petal veins. The average colour of Maris Bead and NV129 standard petals were generated using the average colour function in FIJI (ImageJ) (Image>Colour>Average Colour>CIELab averaging) and printed as 15 mm circles (**Figure 2.10 B**).

To test bee preference between the most intensely saturated part of the petals, vein colour was compared. The most saturated part of the veins was identified from the images of Maris Bead and NV129 standard petals using colour thresholding in FIJI (ImageJ), sampled, and printed as 15 mm circles (**Figure 2.10 C**). The reflectance of the vein colour printed models could not be compared to the colour of the true flower veins using a spectrophotometer due to true flower veins being narrower than the spectrophotometer beam, meaning that the colour of the veins could not be measured in isolation from the surrounding tissue.

Lastly, to test preference between vein patterning, images of Maris Bead and NV129 standard petals were desaturated using FIJI and veins were identified using thresholding. Veins were then traced in Inkscape using the Bezier curve tool to standardise thickness of the veins at 0.1 mm. The resulting models represented the vein pattern of Maris Bead, with 10.1 % of the disc area covered by veins, and NV129, with 5.9% of the disc area covered by veins. The models were printed as 15 mm diameter circles against a green background and an innate preference test was then performed as described in the above experiments. The choices of 20 bees were recorded in total. (**Figure 2.10 D**). An innate preference was tested as described above.

<u>Differential conditioning</u>

For differential conditioning experiments involving printed models, three of each model type were placed in an equally spaced alternating array inside the arena (**Figure 2.11**). One type of models (e.g. Maris Bead vein pattern) were loaded with 10 µl of 40% w/w sugar solution and the other (e.g. NV129 vein pattern) were loaded with quinine hemisulphate solution (0.12% w/w) as a punishment. Bees cannot distinguish between sugar and quinine solution using any cues other than taste (Whitney et al. 2008). The models visited by a bee were

recorded and models visited by bees were reloaded after each visit. A visit was defined as a bee landing and drinking. The first 100 visits made by a bee were recorded. When a bee was full and returned to the colony box the models were then replaced with new models to eliminate any scent marks and the orientation was swapped to eliminate positional effects. Responses of five bees were recorded when model type A was rewarded (e.g. Maris Bead vein pattern) and responses of five bees were recorded when model type B was rewarded (e.g. NV129 vein pattern).

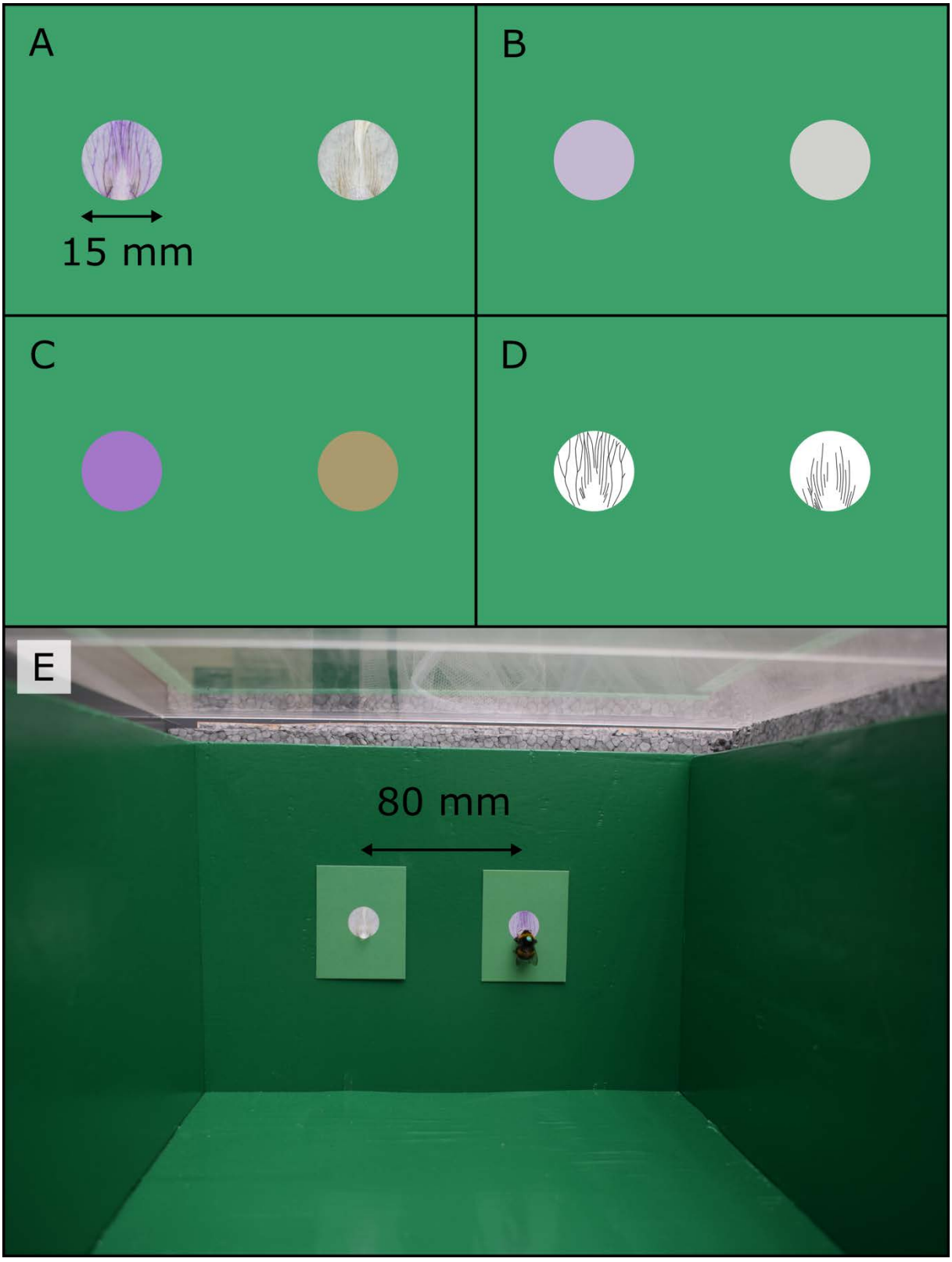


Figure 2.10 Setup for printed models in innate choice experiments. Four innate choice experiments were performed to assess bee preference for standard petal traits of the most visited line from the 2021 field trial (Maris Bead) and the least visited (NV129). **(A)** Maris Bead and NV129 standard petal full colour images. **(B)** Maris Bead and NV129 standard petal average colour. **(C)** Maris Bead and NV129 standard petal vein colour. **(D)** Maris Bead and

NV129 standard petal black and white images. **(E)** The 15 mm disks used for experiments were printed as standard non-gloss business cards (VistaPrint) with a green background. Experiments were performed in a 90 cm by 18 cm by 25 cm flight arena painted green with Rust-oleum Painter's Touch Meadow Green spray paint.

Figure 2.11 Setup for printed models in differential conditioning experiments. Three of each model type were placed in an equally spaced alternating array inside the arena. One type of model (e.g. Maris Bead vein pattern) were loaded with 10 µl of 40% w/w sugar solution and the other (e.g. NV129 vein pattern) were loaded with quinine hemisulphate solution (0.12% w/w) as a punishment. The first 100 drinking visits made by a bee were recorded. When a bee was full and returned to the colony box models were then replaced with new models to eliminate any scent marks and the orientation was swapped to eliminate positional effects. Responses of five bees were recorded when model type A was rewarded (e.g. Maris Bead vein pattern) and responses of five bees were recorded when model type B was rewarded (e.g. NV129 vein pattern).

2.7.4 Scent experiments

Innate preference

To test bee innate preference for floral scent the most visited and least visited lines from the 2021 field trial were compared (Maris Bead and NV129 respectively). Plants were grown at NIAB Park Farm, Histon until the start of flowering and were then transported to the Department of Plant Sciences, Cambridge. Bee choice experiments using flowers were performed between 10 am and 4 pm using stage 5 flowers picked from well-watered plants. Four flowers of each line were picked from separate plants and placed in tower feeders (**Figure 2.12**) designed to eliminate all other floral cues except for flower scent. Tower feeders were custom made to be washable and were based on towers used by Groen et al. (2016). Two towers, one containing Maris Bead flowers and one containing NV129 flowers, were placed 20 cm apart at the end of an arena that had been cleaned with 70% ethanol followed by distilled water to remove any scents. Towers were left for 10 minutes for scent to diffuse evenly. An active forager was then released into the arena and the first tower landed on was recorded. The bee was then captured in a black tube whilst drinking. The orientation of the two models was then swapped to eliminate positional effects and the towers were left for 10 minutes for scent to diffuse evenly. The bee was then released again to make a second choice. This process was repeated until 10 choices had been made. After all choices had been recorded the towers were washed in ethanol followed by distilled water to remove any lingering scents.

Differential conditioning

For differential conditioning experiments three towers containing four Maris Bead flowers and three towers containing four NV129 flowers were placed 20 cm apart in an alternating array inside the arena. One tower type (e.g. Maris Bead towers) were loaded with 10 µl of 40% w/w sugar solution and the other (e.g. NV129 towers) were loaded with quinine hemisulphate solution (0.12% w/w) as a punishment. Bees cannot distinguish between sugar and quinine solution using any cues other than taste (Whitney et al. 2008). Towers were left for 10 minutes for scent to diffuse evenly before a bee was released into the arena. The towers visited by a bee were recorded and towers visited by bees were reloaded after each

visit. A visit was defined as a bee landing and drinking. The first 100 visits made by a bee were recorded. When a bee was full and returned to the colony box the towers were replaced with towers that had been cleaned with ethanol to eliminate any scent marks and the orientation was swapped to eliminate positional effects. Towers were left for 10 minutes for scent to diffuse evenly before a bee was released into the arena again. Responses of five bees were recorded when Maris Bead towers were rewarded and responses of five bees were recorded when NV129 towers were rewarded.

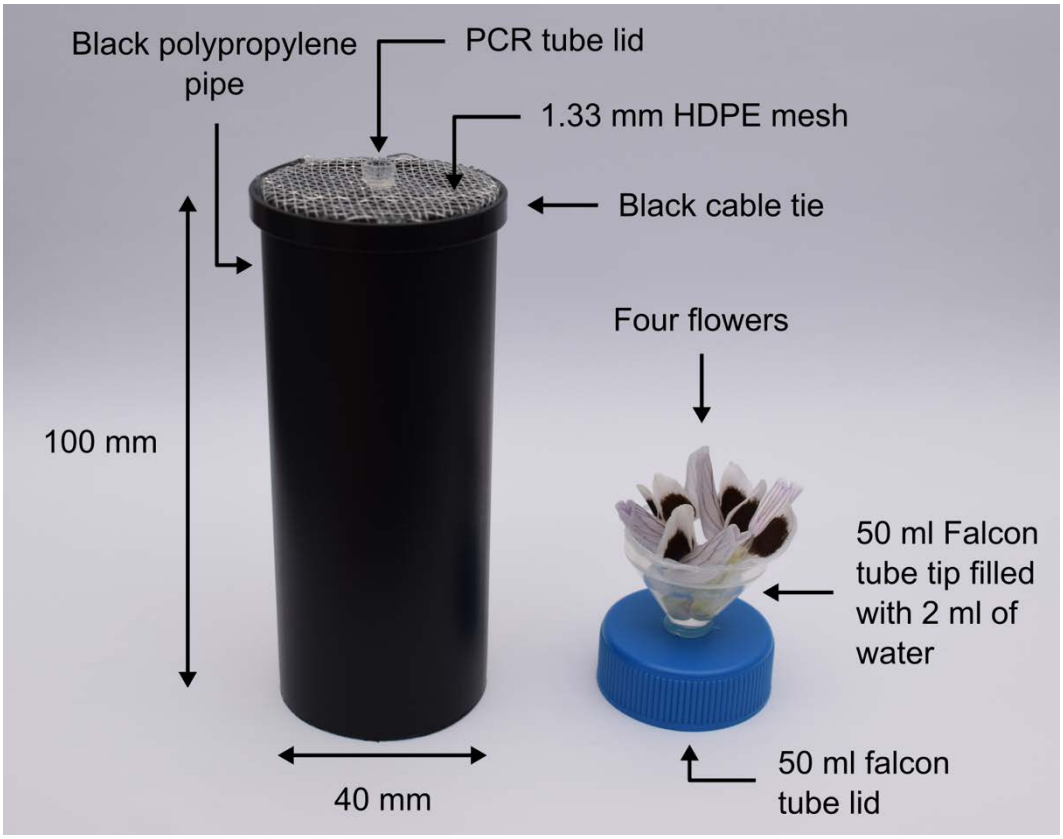


Figure 2.12 Tower feeders used in floral scent bee experiments. Towers were made of black polypropylene (from B&Q) topped with 1.33 mm HDPE mesh attached using hot glue (Ethylene-vinyl-acetate) and a section of black cable tie. A PCR tube lid was attached to the centre of the mesh using hot glue to hold a sugar reward. Four stage 4 flowers were placed underneath the tower in the base of a 50 mL Falcon tube hot glued onto a 50 mL Falcon tube lid. The base of each flower was submerged 2 mL of water.

2.8 Bee observations in the field

2.8.1 Field sites

In 2020, plots were planted at the NIAB trial ground, Histon Cambridge, What3Words: noon.chips.rift, grid reference: TL428627.

In 2021, plots were planted at the PGRO trial ground, Stubton, Lincolnshire, NG23 5DA, What3Words: those.consoles.holidays, grid reference: SK885490.

In 2022, plots were planted at the NIAB trial ground, Histon Cambridge, What3Words: known.went.snaps, grid reference, TL432623.

2.8.2 Line selection

V. faba lines were selected which showed extremes of floral trait variation as identified by work presented in Chapter 3, and data collected by Bailes (2016) (**Table 2.1**). In 2020, the *V. faba* lines Maris Bead and NV129 were compared due to their extremes of floral trait variation. In 2021, Fuego, Maris Bead, NV100, NV129, and Tiffany were compared. In 2022, Fuego, Lynx, Maris Bead, Tiffany, Vertigo and Yukon were compared.

Line	Fuego	Lynx	Maris Bead	NV100	NV129	Tiffany	Vertigo	Yukon
Flower apperance								
Nectar concentration (% w/w)	40.90	39.30	49.00*	32.00*	15.00*	32.64	42.03	28.71
Nectar volume per flower (ul)	2.94	4.27	1.58*	0.80*	0.71*	3.00	3.18	2.47
Total sugar per flower (mg)	4.36	6.22	2.54*	1.04*	0.78*	4.07	4.37	3.15
Number of flowers per node	7.42	6.21	7.46	4.98	4.31	7.47	6.27	7.09
Force required to trip a flower (mN)	21.21	23.54	24.28	19.81	36.07	21.55	26.17	16.89
Standard petal height (mm)	15.43	15.36	14.00*	17.00*	13.00*	15.38	17.07	17.15
Wing petal area (mm^2)	112.96	107.96	89.00*	86.00*	110.00*	111.61	120.31	105.03
Corolla tube length (mm)	12.34	12.42	13.00*	12.00*	14.00*	13.45	12.69	11.98
Wing petal spot size (%)	44.70	44.68	46.00*	42.00*	44.00*	45.46	48.69	-
Year grown	2021 2022	2022	2020 2021 2022	2021	2020 2021	2021 2022	2022	2022

Table 2.1 The V. faba lines compared in field trials in 2020, 2021 and 2022 . Selection of lines was based on floral trait variation data presented in Chapter 3, and data collected by (Bailes 2016). Lines varied in both appearance of flower, reward, number of flowers and the force required to access flower. Data were collected from plants under glasshouse conditions. Numbers marked with asterisks were obtained from Bailes (2016) from plants grown in the same conditions. Intensity of blue shading is from highest to lowest values for each trait.

2.8.3 2020 trial layout and drilling

Ten plots were drilled on the 12th of March 2020 in 1.5 meter by 9-meter plots planted at the NIAB trial ground, Histon, in a field containing other NIAB field bean trial plots of the same dimensions (**Figure 2.13**). Seeds were drilled at a density of 22 plants per square meter in four rows.

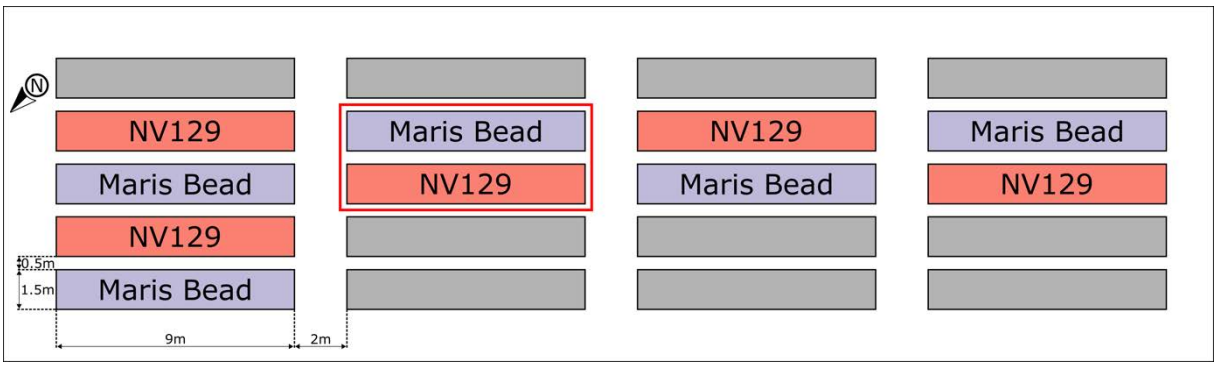


Figure 2.13 Field trial layout 2020. Ten plots were drilled on the 12th of March 2020 in 1.5 meter by 9-meter plots at the NIAB trial ground, Histon. Seeds were drilled at a density of 22 plants per square meter in four rows. Two lines of *V. faba* were drilled in an alternating pattern, these were Maris Bead (shown in purple) and NV129 (shown in red). Plots were surrounded by field beans from other NIAB trials (shown in grey). A red box indicates the two plots which were observed continuously on the 12th and 13th of June 2020.

2.8.4 2020 pollinator observations

When all plots were in flower, bees were observed on calm, sunny days when air temperature was above 15°C for the duration of the observation period according to the Met Office weather forecast. As a result of travel restrictions during the COVID-19 pandemic and bad weather, observations were only possible on the 11th, 12th and 13th of June.

Two observation methods were evaluated. The first consisted of plot walks once every 15 minutes between 09:00 hrs and 16:00 hrs on the 11th of June. A slow walk was conducted down the 0.5 m gap between the two plots of every replicate. A walk took approximately 10 minutes depending on the number of pollinators observed. For any pollinator encountered on a walk the following information was recorded: pollinator type (honeybee or bumblebee) and pollinator behaviour (legitimate visit, robbing visit, extrafloral nectary visit, or searching). Pollinator behaviours are defined in **Figure 2.14**.

The second sampling method tested was continuous observation of a single pair of plots, highlighted in red in **Figure 2.13**. The length of the plots was deemed too long to be accurately observed, so a 0.5-meter boundary was cut out, reducing the size of the plots to 1.5 meters wide by 4 meters long. Continuous observations were collected from 09:00 hrs to 19:30 hrs on the 12th of June and from 06:00 hrs to 22:00 hrs on the 13th of June. The sampling period was divided into 15-minute intervals. For all pollinators entering the plot during each 15-minute period, pollinator type (honeybee or bumblebee) and pollinator behaviour was recorded.

After one day of plot walks on the 11th of June, it was decided that pollinator visits were massively underestimated using the plot walk method, and so continuous observations were made on the 12th and 13th of June.

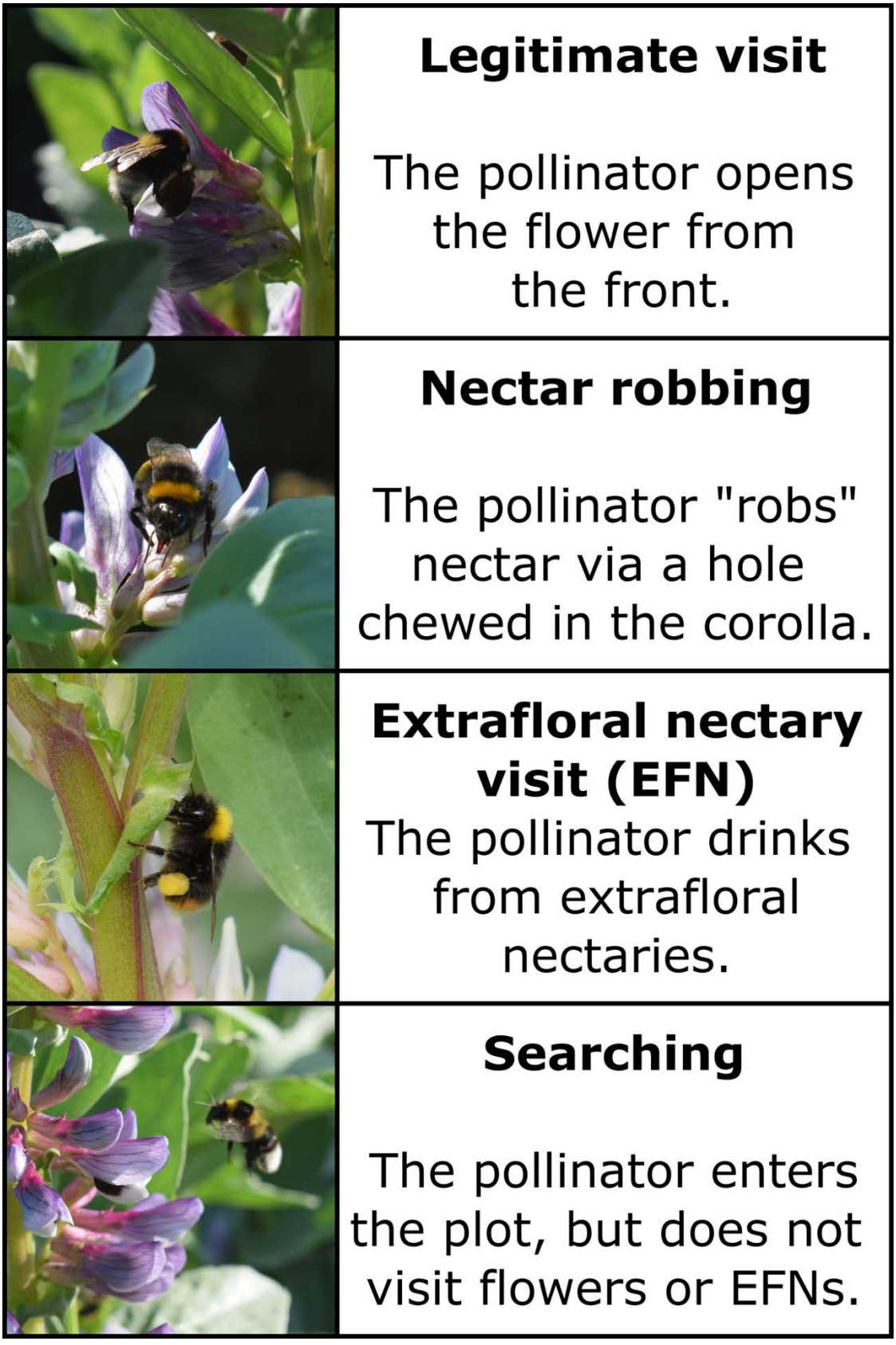


Figure 2.14 Definitions of pollinator behaviours recorded in the field. Pollinator visits to plots of *V. faba* were recorded as legitimate, robbing, extrafloral nectar visit or searching, as defined above.

2.8.5 2021 trial layout and drilling

Plots were drilled on the 1st of April 2021 in 1.5 meter by 4-meter plots planted in the Southwest corner of a field containing other PGRO field bean and pea trial plots. The southern edge of the field had a wildflower margin approximately 8 meters wide.

Seeds were drilled at a density of 34 plants per square meter in 10 rows. Plots were planted with Fuego, Maris Bead, NV100, and NV129, in an alternating pattern, surrounded by Tiffany. Tiffany was used to surround the other plots to avoid leaving gaps where weeds would grow. Due to poor soil conditions in the Northeast corner of the trial, the plan was rearranged at the time of drilling so that more plots of Fuego, NV100, NV129 and Maris Bead were planted in good soil (**Figure 2.15**). After seedlings had emerged, plot ends were hoed by hand to ensure all plots were equal in size. Populations were then recorded for each plot by counting the total number of plants that had emerged. Due to the poor soil conditions, plots in the Northeast corner of the trial emerged poorly and were discounted (**Figure 2.15**).

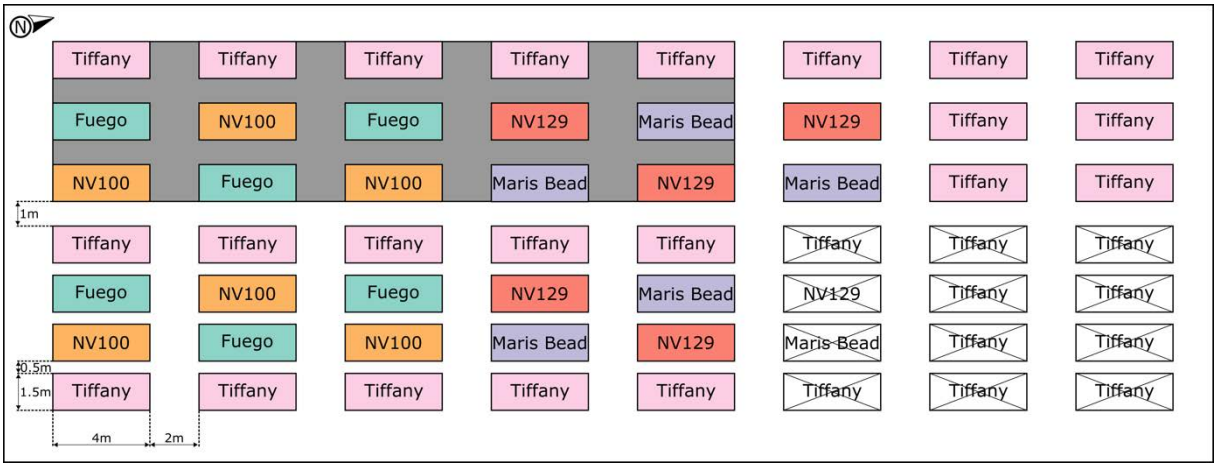


Figure 2.15 Field trial layout 2021. In total, 56 plots were drilled on the 1st of April 2021 in 1.5 meter by 4-meter plots at the PGRO field site, Stubton, Lincolnshire. The trial was in the corner of a field containing other PGRO field bean and pea trials. Seeds were drilled at an approximate density of 34 plants per square meter in 10 rows. Five *V. faba* lines were drilled; Fuego, Maris Bead, NV100, NV129, and Tiffany. Six plots of each line were planted in an alternating pattern, except for Tiffany, for which 32 plots were planted as "discard", used to surround the entire trial to prevent weed growth on bare soil. Due to poor soil conditions in the Northeast corner of the trial, the field plan was rearranged at the time of drilling so that more plots of Fuego, NV100, NV129 and Maris Bead were planted in good soil. Due to poor emergence, 12 of these plots were discounted and are shown crossed out. For the pollinator exclusion study, 15 plots were covered by insect proof netting, signified by the grey block. The rest of the plots in the trial were open pollinated.

2.8.6 2021 pollinator observations

When all plots were in flower, bees were observed on calm, sunny days when air temperature was above 15°C for the duration of the observation period according to the Met Office weather forecast (https://www.metoffice.gov.uk). The number of bees visiting field plots were recorded on the 16th, 19th, 22nd, 23rd and 24th of June 2021. Using methodology developed during the 2020 season, continuous observations were made, starting at 10:00 hrs and finishing at 15:00 hrs.

Volunteers assisted in data collection and were trained to identify bee behaviours (**Figure 2.14**) and bee types (**Figure 2.16**). Each volunteer observed 4 adjacent plots. The sampling period was divided into 15-minute intervals and for all bees entering the plot during that period, pollinator type and pollinator behaviour was recorded. Bees were identified to a "category", as opposed to each species due to difficulty identifying to species level by volunteers. Bees observed in the field were recorded as one of the four categories listed below. The categories named "white-tailed bumblebees" and "red-tailed bumblebees" each contained at least two species which could not be separated by volunteers. From this point forward, any mention of "white-tailed bumblebees" and "red-tailed bumblebees" refers to the descriptions provided below unless explicitly stated otherwise.

- **White-tailed bumblebees:** any bumblebee with a white tail. The species recorded in this category were most commonly *Bombus terrestris*, *Bombus lucorum*, and *Bombus hortorum*. Other less common species included *Bombus hypnorum* and *Bombus barbatellus*.
- **Red-tailed bumblebees:** any bumblebee with a red tail. The species recorded in this category were *Bombus lapidarius* and *Bombus pratorum*.
- **Carder bees:** Any bee orange in colour. One species of carder bee was observed during field trails and identified as *Bombus pascuorum*.
- **Honeybees:** One species of honeybee is present in the UK, *Apis mellifera*.

Volunteers were provided with identification sheets to assist in recording of bees during fieldwork (**Figure 2.16**). The species of bees recorded in each category were confirmed during the sampling period by capturing pollinators present in the field margin. A zig zag

transect was walked through the wildflower field margin at midday on a sunny warm day. Every bee encountered on the walk was captured. Bees were frozen and then identified using the Bumblebee Conservation Trust identification guide (Bumblebee Conservation Trust 2022).

To quantify the abundance of pollinators present in the wildflower margin, two 1.5 meter by 4-meter plots were marked out in the middle of the field margin closest to the trial. Pollinator observations were recorded in these plots on the 23rd and 24th of June for a total of 4.5 hours. As with *V. faba* plot observations, pollinator type (white tailed bumblebees, red tailed bumblebees, carder bees and honeybees) was recorded, but only legitimate and searching behaviour was recorded, as it was not possible for pollinators to rob or visit extrafloral nectaries of the wildflower species present in the margin. Plant species that were in flower during the time pollinator observations were made were recorded.

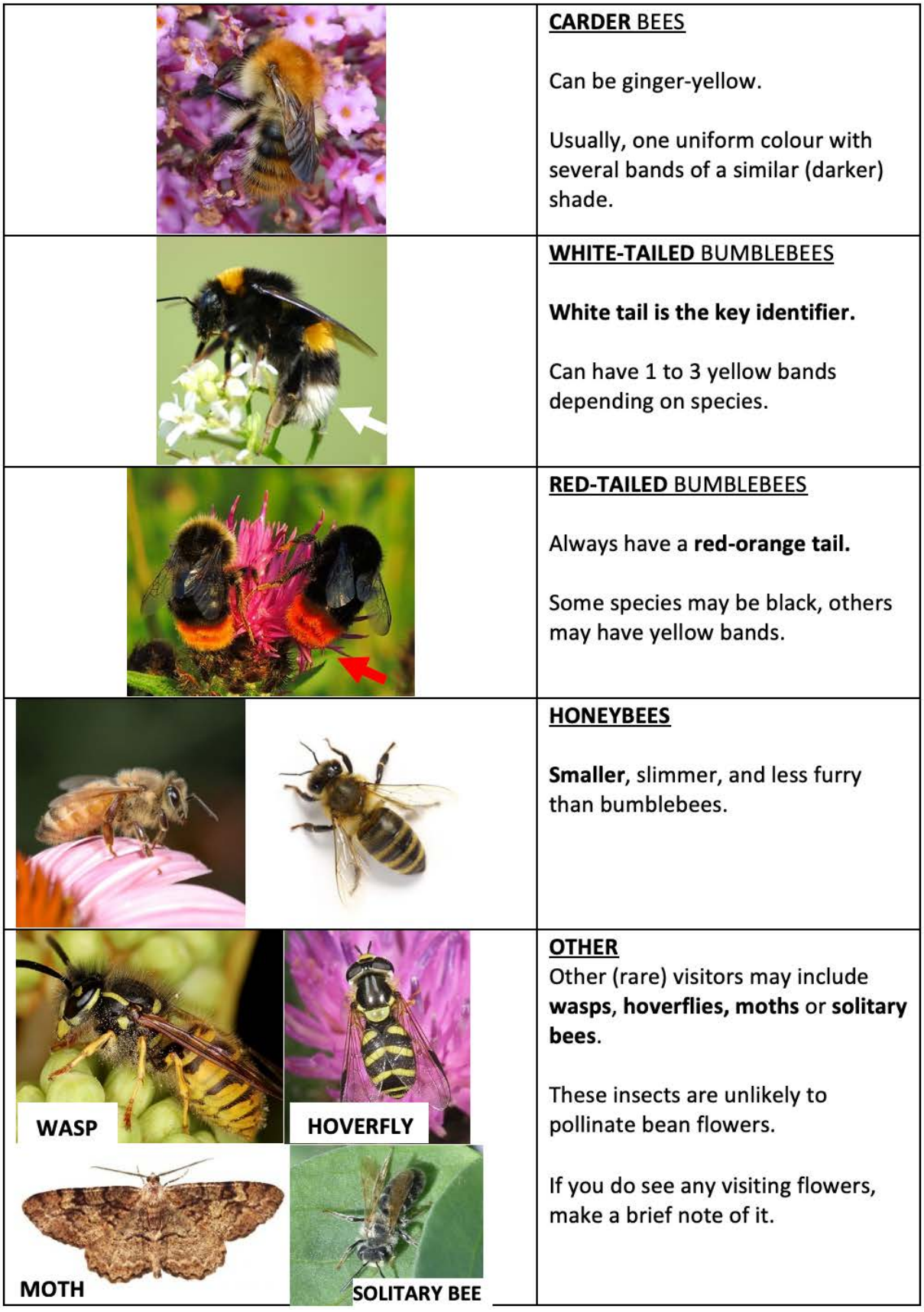


Figure 2.16 Bee identification sheet provided to volunteers for pollinator observations in 2021 and 2022. Bees were recorded as carder bees, white-tailed bumblebees, red-tailed bumblebees, or honeybees. Pollinators in the "other" category were not recorded.

2.8.7 2022 trial layout and drilling

Plots were drilled on the 23rd of March 2022 in 1.5 meter by 4-meter plots planted in the Northern end of a NIAB field with other field bean trial plots.

Seeds were drilled at a density of 35 plants per square meter in four rows. Plots were planted with Fuego, Lynx, Maris Bead, Tiffany, Vertigo and Yukon, in semi randomised pattern (**Figure 2.17**). After seedlings had emerged, plot ends were hoed by hand to ensure that all plots were equal in size. Populations were then recorded for each plot by counting the total number of plants that had emerged.

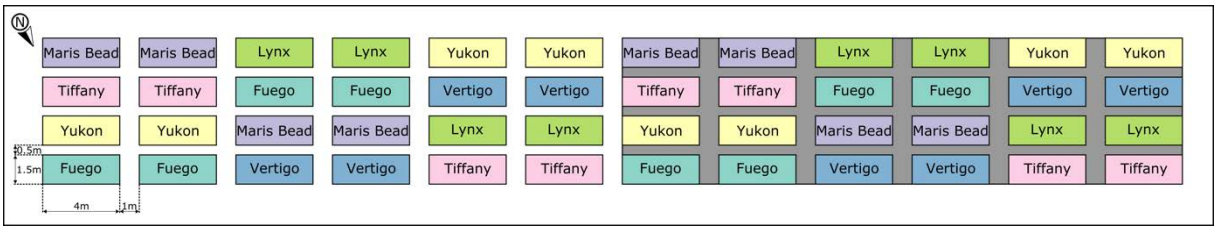


Figure 2.17 Field trial layout 2022. In total, 48 plots were drilled on the 23rd of March 2022 in 1.5 meter by 4-meter plots planted in the Northern end of a NIAB field with other field bean trial plots. For the pollinator exclusion study, 15 plots were covered by insect proof netting (grey block). The remaining plots were open pollinated.

2.8.8 2022 pollinator observations

When all plots were in flower, bees were observed on calm, sunny days when air temperature was above 15°C for the duration of the observation period according to the Met Office weather forecast (https://www.metoffice.gov.uk). The number of bees visiting field plots were recorded on the 2nd, 7th, 8th, 9th, and 10th of June 2022. Using methodology developed during the 2020 pilot study, continuous observations were made, starting at 10:00 hrs and finishing at 16:00 hrs with coffee and lunch breaks.

Volunteers assisted in data collection and were trained to identify bee behaviours (**Figure 2.14**) and bee types (**Figure 2.16**). Each volunteer observed 4 adjacent plots. The sampling period was divided into 15-minute intervals and for all pollinators entering the plot during that period, pollinator type and pollinator behaviour was recorded. Pollinator types were white tailed bumblebees, red tailed bumblebees, carder bees and honeybees (**Figure 2.16**). Pollinator types were chosen as opposed to species due to difficulty identifying to species level by volunteers. The species present in the field were confirmed during the sampling period by capturing pollinators present in the field margin. A zig zag transect was walked through the wildflower field margin at midday on a sunny warm day. Every bee encountered on the walk was captured. Bees were identified using the Bumblebee Conservation Trust identification guide (Bumblebee Conservation Trust 2022).

2.8.9 Environmental assessment and pollinator surveys

In both 2021 and 2022 a 1-mile radius around each trial was assessed to record predominant landscape type. All accessible areas within the radius were surveyed. See Appendix B for comparison of landscapes in 2021 and 2022.

In both 2021 and 2022, zig zag transects of the field margin closest to the trial was carried out to capture pollinators. Pollinators were captured with a sweep net and identified to species level where possible.

2.9 Pollinator exclusion experiments

2.9.1 2021 yield comparison

To investigate the effect of pollinator exclusion on *V. faba* yield, a cage covered by insect-proof netting was erected (**Figure 2.18**).The cage covered plots for the duration of flowering. The cage, supplied by PGRO, consisted of hollow aluminium poles, 2 meters in length. Vertical poles were sunk 20 cm into the ground. Cross bars were connected by push fittings. Standard insect proof HDPE netting was purchased from Wondermesh Ltd (Aberdeenshire), 1.33 mm mesh size, 65 g/m^2. The cage and net were erected on the 27th of May 2021 and removed on the 16th of July 2021.

Once pods had dried, 20 plants (approximately 10% of plot population) were hand-picked from each plot to record the number of pods per plant, number of seeds per plant, number of seeds per pod, number of pods and seeds per node position, and dry bean mas per plant for open pollinated and caged plants. Plants were randomly selected from each row of the plot using a random number generator. If plants were damaged or broken, the next intact plant was selected. Plants were stored in plastic bin bags and transported to NIAB Park Farm where they were processed. For pods per plant, only pods with seeds in were recorded. To obtain dry mass, seeds were dried in an oven at NIAB Park Farm at 82°C for 48 hours, as per NIAB protocol. Dry bean mass was recorded per individual plant.

After 20-plant samples had been collected, plots were harvested by combine on the 25th of August 2021 by PGRO staff. Harvested seed was dried and plot yield (kg) was supplied by PGRO.

Figure 2.18 Insect proof cage used to cover plots for pollinator exclusion experiment at Stubton, Lincolnshire in 2021. The cage and net were erected on the 27th of May 2021 and removed on the 16th of July 2021.

2.9.2 2022 yield comparison

To investigate the effect of pollinator exclusion on *V. faba* yield, a cage covered by insect-proof netting was erected (**Figure 2.19**).The cage covered plots for duration of flowering. The cage, supplied by NIAB, consisted of hollow aluminium poles, 1.5 meters in length. Vertical poles were sunk 20 cm into the ground. Standard insect proof HDPE netting, 1.33 mm mesh size, 65 g/m^2 (Wondermesh Limited, Aberdeenshire, Scotland) was used to cover the cage. The cage and net were erected on the 27th of May 2022 and removed on the 14th of July 2021.

Due to an extremely dry summer, plots were harvested by combine on the 15th of August 2021. Plot yield was given in kg alongside moisture content. Moisture content was then accounted for to produce final plot yield. Prior to combine harvest, 20 plants (10% of plot population) were hand-picked from each plot to record the number of pods per plant, number of seeds per plant, number of seeds per pod, number of pods and seeds per node position, and dry bean mass per plant for open pollinated and caged plants. Due to time constraints, these 20-plant samples could not be processed in time for thesis submission in September 2022. Samples will be processed after submission with the aim of publication.

Figure 2.19 Insect proof cage used to cover plots for pollinator exclusion experiment at NIAB, Histon, Cambridgeshire in 2022. The cage and net were erected on the 27th of May 2022 and removed on the 14th of July 2022.

2.10 Statistical analyses

All statistical analysis and modelling were undertaken using R (https://www.r-project.org). All p values ≤0.05 were considered to be statistically significant.

2.10.1 Variation in floral traits (Chapter 3)

Datasets were checked for normality using histograms, qqnorm plots and shapiro tests and were analysed using ANOVA tests to identify variation between means. If an ANOVA identified a significant difference between at least two groups, a post-hoc Tukey's HSD test for multiple comparisons was performed.

2.10.2 Bee experiments in the field (Chapter 4)

All bee visitation data was checked for normality and data were analysed using ANOVAs to test difference between mean visitation rate to plots of each *V. faba* line. Following a significant ANOVA result (p≤0.05), post-hoc Tukey's HSD tests were used to compare mean bee visitation rate between lines.

Mean values consistently appeared above median values for bee visitation rates. Examination of histograms and checks for normality found that the degree or departure from normality did not violate assumptions of ANOVA, Tukey's HSD and t-tests. For extra precaution, median values were also plotted on figures to show that comparison of median values for bee visitation did not change conclusions, with the most and least visited lines remaining the same when comparisons were performed using mean or median visitation rates.

2.10.3 Yield comparison (Chapter 4)

All plant level measures of yield were checked for normality using histograms, qqnorm plots and shapiro tests and were analysed using two-tailed t-tests.

2.10.4 Bee experiments in controlled conditions (Chapter 5)

All *Bombus terrestris* innate preference experiments were analysed using binomial tests to determine whether bumblebee preference significantly differed from a distribution expected by chance of 50:50 on the first choice. For all innate preference tests over first 10 visits, two-tailed t-tests were used to determine whether bumblebee preference significantly differed from a distribution expected by chance of 50:50.

For *Bombus terrestris* differential conditioning experiments, generalised linear models (GLMs) fitted on bees' choices in function of the number of visits were used to determine whether the probability of making a correct choice increased significantly with successive visits.

3 Variation in floral traits of *Vicia faba*

3.1 Introduction

Despite the reliance of *V. faba* on insect pollinators for maximal yield, breeders have not yet intentionally manipulated floral traits to enhance pollination. This has partly been due to lack of information on floral trait variation between *V. faba* lines. Only by recording variation between existing lines can we test the effect of trait variation on pollinator behaviour and *V. faba* yield. With this information, breeders will have the opportunity to develop lines which are more rewarding and attractive to bees due to their floral traits.

Variation of floral traits in *V. faba* has been poorly studied until recently, with Bailes (2016) completing the most comprehensive evaluation to date. Prior to that, work had been done to quantify the nectar production (Osborne et al. 1996; Pierre et al. 1996; Aredewa et al. 2004), pollen production (Kambal et al. 1976; Stoddard 1986b; Carré et al. 1994), and production of volatile organic compounds (Sutton et al. 1992; Griffiths et al. 1999; Salerno et al. 2017). However, only a small number of genotypes were evaluated, including few if any commercial lines. Bailes compared a panel of 30 commercial and "breeding" lines of *V. faba* to evaluate variation in multiple floral traits (Bailes 2016; Bailes et al. 2018). Between the panel of lines examined, variation was identified in flower and petal size, wing petal spot size, the volume and concentration of nectar produced by flowers, and the number of pollen grains produced by flowers. Bailes (2016) also compared the force required to open flowers and the volatile organic compounds produced by flowers between two lines: Fuego and Tattoo.

In this chapter, variation in multiple floral traits is presented for a panel of previously uncharacterised lines, with particular emphasis on uncharacterised commercial lines. Using the methods of Bailes (2016), nectar volume and concentration was measured, alongside number of pollen grains produced per flower. New methodology was developed to assess variation in floral morphology, pollen viability, force required to trip petals and the number of flowers produced per node. A summary of which lines were characterised for each trait can be seen in Appendix C. For methodology, refer to Chapter 2.

3.2 Results

3.2.1 Flower morphology

For methodology refer to section 2.2. Mean standard petal height ranged from 14.53 mm for LG Cartouche, to 18.23 mm for INRA29H (**Figure 3.1**). A one-way ANOVA revealed a statistically significant difference in mean standard petal height between at least two of the *V. faba* lines compared at $p \leq 0.0001$. A post-hoc Tukey's HSD test for multiple comparisons indicated that the mean standard petal height of INRA29H flowers was significantly larger than that of any other line, and that the mean standard petal height of LG Cartouche flowers was significantly smaller than that of any other line (Table 3.1). Standard petal height for flowers of Yukon, Vertigo and Fanfare and BPL10 was significantly greater than that of Victus, Fuego, Tiffany, Lynx and LG Cartouche.

Mean wing petal area ranged from 105.03 mm^2 for Yukon to 124.55 mm^2 for Victus (**Figure 3.2**). A one-way ANOVA revealed a significant difference in mean wing petal area between at least two of the *V. faba* lines compared at $p \leq 0.0001$. A post-hoc Tukey's HSD test for multiple comparisons indicated that the mean wing petal area of Victus flowers was significantly larger than that of any other line except Vertigo (**Table 3.2**). A post-hoc Tukey's test also indicated that the mean wing petal area of Yukon flowers was significantly smaller than that of any other line except Lynx, INRA29H and Tiffany (**Table 3.2**). Wing petal area of Victus, Vertigo, Fanfare and Tundra flowers was significantly greater than that of Lynx and Yukon.

Mean corolla tube length ranged from 11.38 mm for INRA29H to 13.45 mm for Tiffany (**Figure 3.3**). A one-way ANOVA revealed a statistically significant difference in mean corolla tube length between at least two of the *V. faba* lines compared at $p \leq 0.0001$. A post-hoc Tukey's HSD test for multiple comparisons indicated that the mean corolla tube lengths of Tiffany and BPL10 flowers were significantly larger than that of any other line (**Table 3.3**). A post-hoc Tukey's test also indicated that the mean corolla tube length of INRA29H flowers was significantly smaller than that of any other line (**Table 3.3**). Corolla tube length of Tiffany, BPL10, Vertigo, Fanfare, and Lynx flowers was significantly greater than that of Yukon, Tundra, LG Cartouche and INRA29H.

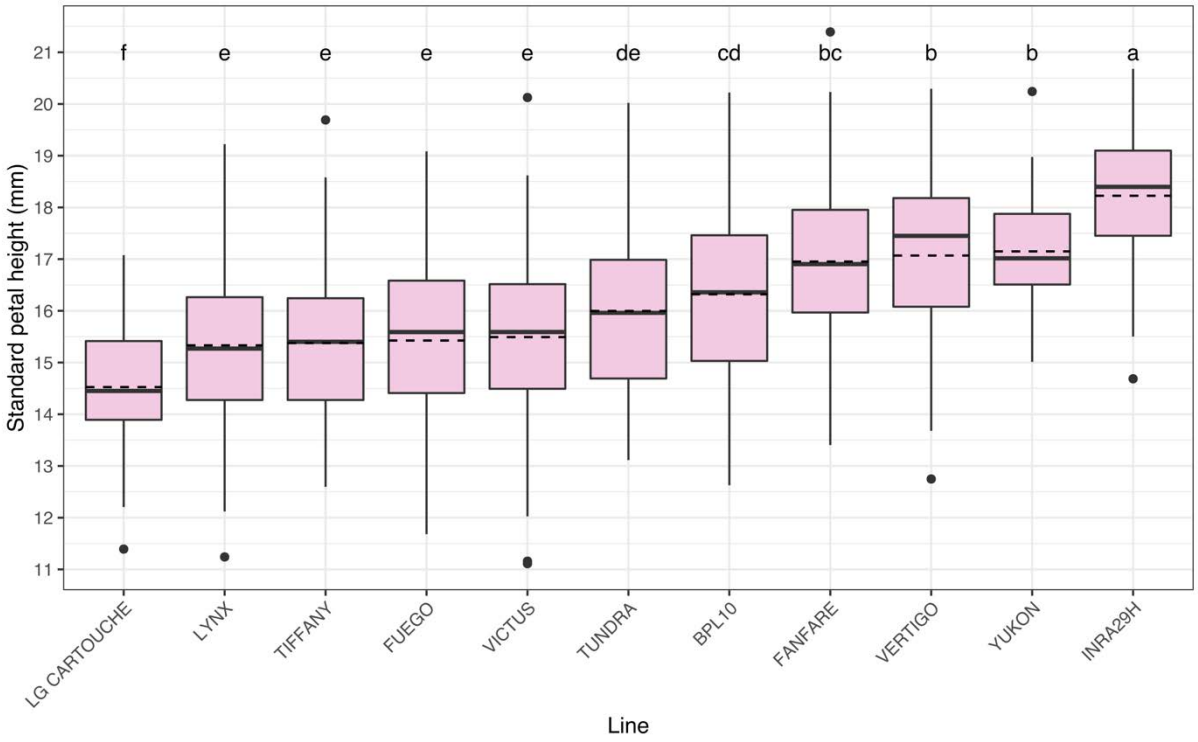


Figure 3.1 Standard petal height of *V. faba* flowers. Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the interquartile range). Dashed lines show means and solid lines show medians. Black dots show datapoints that fall outside of the calculated maxima and minima. Letters show Tukey significance groups. *V. faba* lines which do not share a letter have significantly different mean standard petal height ($p \leq 0.05$). Standard petal height ranged from 14.53 mm for LG Cartouche to 18.23 mm for INRA29H.

Line	n	Mean standard height (mm)	Tukey significance group					
INRA29H	46	18.23	A					
YUKON	70	17.15		B				
VERTIGO	70	17.07		B				
FANFARE	68	16.95		B	C			
BPL10	126	16.32			C	D		
TUNDRA	61	16.00				D	E	
VICTUS	72	15.49					E	
FUEGO	145	15.43					E	
TIFFANY	72	15.38					E	
LYNX	161	15.36					E	
LG CARTOUCHE	76	14.53						F

Table 3.1 The mean standard petal height for flowers of *V. faba* lines and Tukey significance groups. Mean standard petal height ranged from 14.53 mm for LG Cartouche to 18.23 mm for INRA29H. *V. faba* lines which do not share a letter have significantly different mean standard petal height ($p \leq 0.05$), n shows the number of flowers measured.

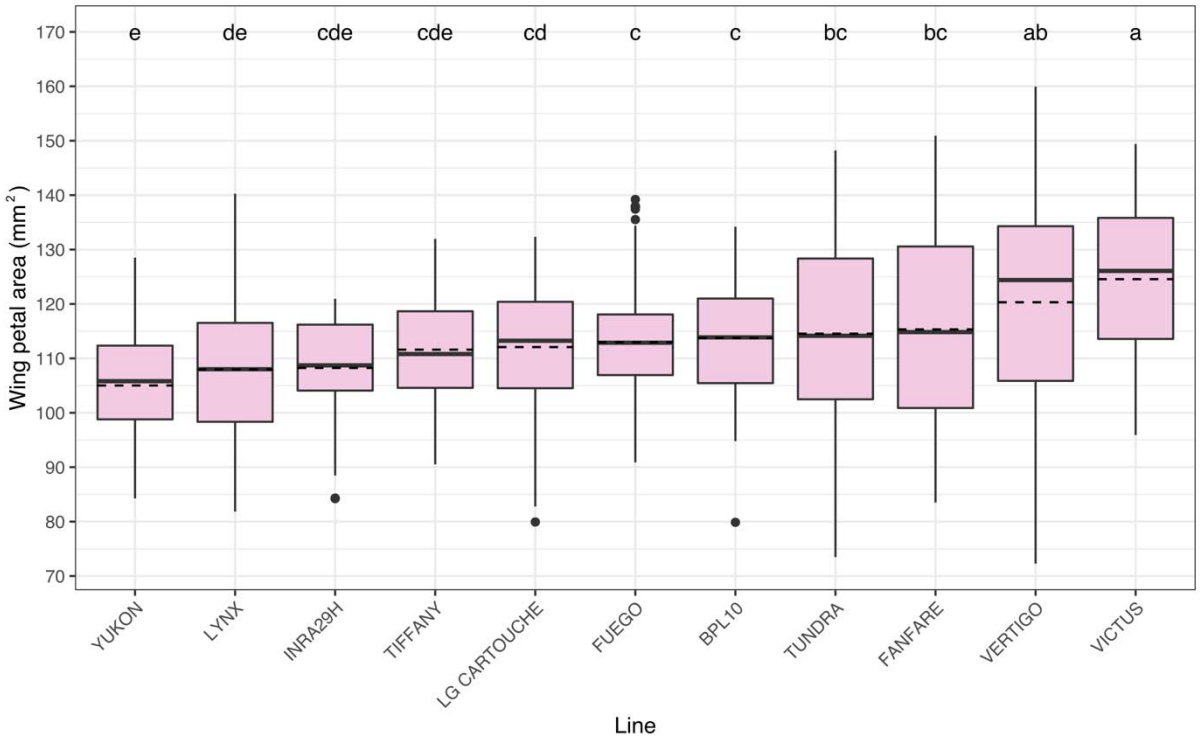


Figure 3.2 Wing petal area of *V. faba* flowers. Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the interquartile range). Dashed lines show means and solid lines show medians. Black dots show datapoints that fall outside of the calculated maxima and minima. Letters show Tukey significance groups. *V. faba* lines which do not share a letter have significantly different mean wing petal area ($p \leq 0.05$). Mean wing petal area ranged from 105.03 mm^2 for Yukon to 124.55 mm^2 for Victus.

Line	n	Mean wing area (mm^2)	Tukey significance group				
VICTUS	72	124.55	A				
VERTIGO	85	120.31	A	B			
FANFARE	68	115.31		B	C		
TUNDRA	70	114.54		B	C		
BPL10	126	113.74			C		
FUEGO	145	112.96			C		
LG CARTOUCHE	88	112.08			C	D	
TIFFANY	75	111.61			C	D	E
INRA29H	46	108.25			C	D	E
LYNX	160	107.96				D	E
YUKON	70	105.03					E

Table 3.2 The mean wing petal area for flowers of *V. faba* lines and Tukey significance groups. Mean wing petal area ranged from 105.03 mm^2 for Yukon to 124.55 mm^2 for Victus. *V. faba* lines which do not share a letter have significantly different mean wing petal area ($p \leq 0.05$), n shows the number of flowers measured.

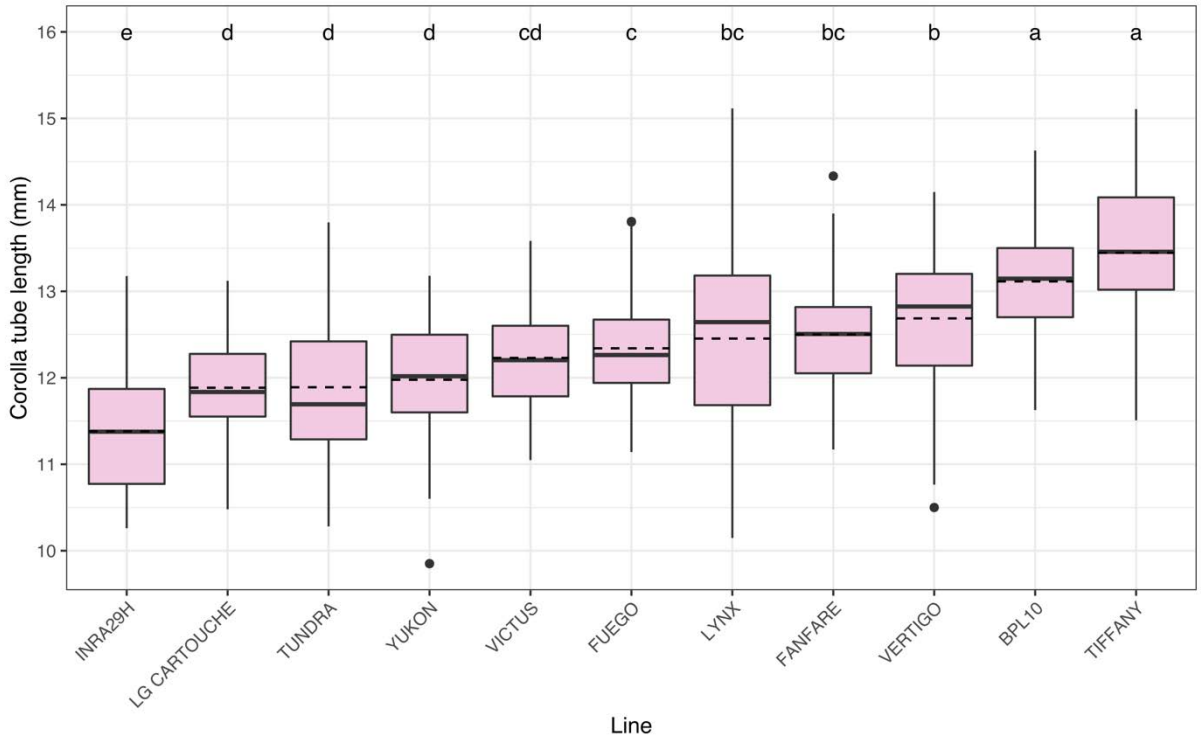


Figure 3.3 Corolla tube length of *V. faba* flowers. Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the interquartile range). Dashed lines show means and solid lines show medians. Black dots show datapoints that fall outside of the calculated maxima and minima. Letters show Tukey significance groups. *V. faba* lines which do not share a letter have significantly different mean corolla tube length ($p \leq 0.05$). Mean corolla tube length ranged from 11.38 mm for INRA29H to 13.45 mm for Tiffany.

Line	n	Mean corolla length	Tukey significance group				
TIFFANY	72	13.45	A				
BPL10	126	13.12	A				
VERTIGO	70	12.69		B			
FANFARE	68	12.50		B	C		
LYNX	160	12.42		B	C		
FUEGO	145	12.34			C		
VICTUS	72	12.23			C	D	
YUKON	70	11.98				D	
TUNDRA	61	11.89				D	
LG CARTOUCHE	76	11.89				D	
INRA29H	46	11.38					E

Table 3.3 The mean corolla tube length for flowers of *V. faba* lines and Tukey significance groups. Mean corolla tube length ranged from 11.38 mm for INRA29H to 13.45 mm for Tiffany. *V. faba* lines which do not share a letter have significantly different mean corolla tube length ($p \leq 0.05$), n shows the number of flowers measured.

3.2.2 Colour and patterning

For methodology refer to section 2.3.

Petal colour in bee colour space

Reflectance spectra were taken for standard petals, wing petal tips and wing petal spots of 11 *V. faba* lines: BPL10, Fanfare, Fuego, INRA29H, LG Cartouche, Lynx, Tiffany, Tundra, Vertigo, Victus, and Yukon (**Figure 3.4**). When processed with the Pavo package in R, reflectance spectra of standard petals and wing petal tips are predicted to excite blue and green receptors most whereas wing petal spots appear more achromatic to bees. This is apart from the wing petal spot of Yukon, which excites blue and green receptors, more like standard petals and wing petal tips. Apart from Yukon, little variation was measured in wavelengths between wing petal spots in bee colour space. Tiffany and INRA29H spots show greatest separation in bee colour space, with Tiffany being closer to the bee achromatic centre (**Figure 3.4 B**). Standard petals and wing petal tips form two clusters in bee colour space, with standard petals exciting blue receptors more strongly and wing petal tips providing slightly greater excitation to green receptors. For standard petal reflectance, there is little variation between lines in bee colour space. Fuego and BPL10 show the greatest separation (**Figure 3.4 C**). Again, for wing petal tips, there is little variation between lines in bee colour space, with INRA29H and BPL10 showing the greatest separation (**Figure 3.4 D**). Petal colour excitation values and hexagon coordinates can be seen in Appendix D.

During fieldwork in 2021 the corolla tubes of flowers had noticeably stronger colouration than was visible in the glasshouse. Reflectance spectra were measured for standard petals, wing petal tips, wing petal spots and corolla tubes for Fuego and Tiffany, which had also been sampled from the glasshouse. When plotted in bee colour space, reflectance spectra of Fuego and Tiffany standard petals differed by a similar extent between glasshouse and field conditions (**Figure 3.5, points 1, 2, 3, and 4**). The distance in bee colour space between Fuego wing petal tips differed greatly between glasshouse and field conditions (**Figure 3.5, points 5 and 6**), but Tiffany wing petal tips were very close in bee colour space (**Figure 3.5, points 7 and 8**). The reflectance spectra of both Fuego and Tiffany wing petal spots showed separation in bee colour space between glasshouse and field conditions, however separation was greater for Fuego (**Figure 3.5, points 9, 10, 11, and 12**). Between Fuego and Tiffany

grown in the field, corolla tube wavelengths showed noticeable separation in bee colour space (**Figure 3.5, points 13 and 14**).

Flowers of Maris Bead, NV100 and NV129 were also sampled from the field in 2021. The reflectance spectra of lines grown in field conditions showed greater separation in bee colour space within the petal area sampled than did lines grown in glasshouse conditions (**Figure 3.6**). Of areas sampled, corolla tubes excited blue receptors most strongly, with Tiffany and Maris Bead resulting in greatest excitation and Fuego being closest to the achromatic centre (**Figure 3.6 B**). Wing petal spots also showed greater separation, with the greatest distance in hexagon units between NV129 and Tiffany, Tiffany being closest to the achromatic centre (**Figure 3.6 B**). The reflectance spectra of standard petals and wing petal tips formed two clusters in bee colour space similar to that seen for glasshouse-grown plants, but showed substantial separation within each group. Fuego and NV100 showed the greatest separation in bee colour space for both standard petal and wing petal tip reflectance (**Figure 3.6 C and D**). Petal colour excitation values and hexagon coordinates for field-grown flowers can be seen in Appendix E.

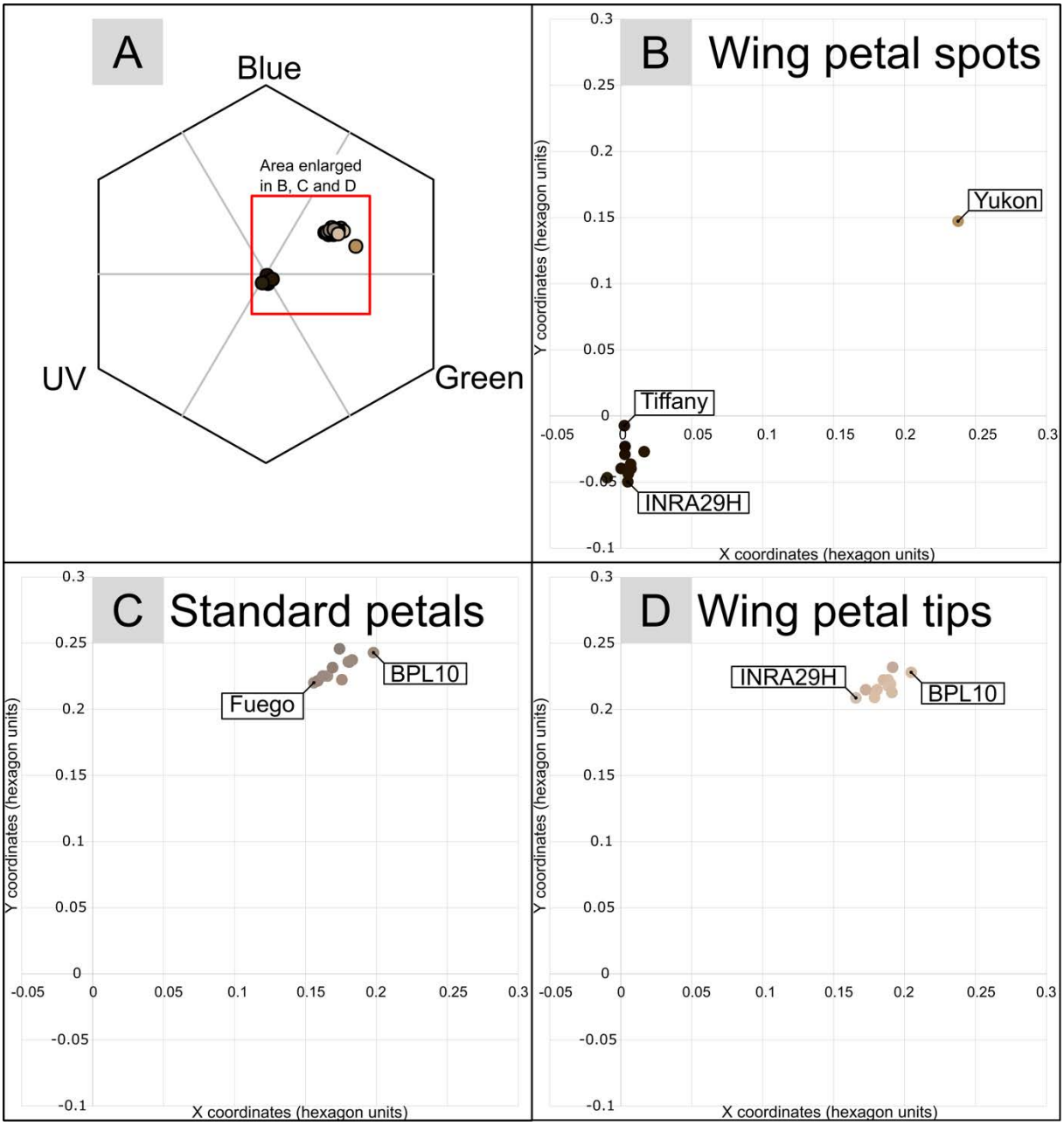
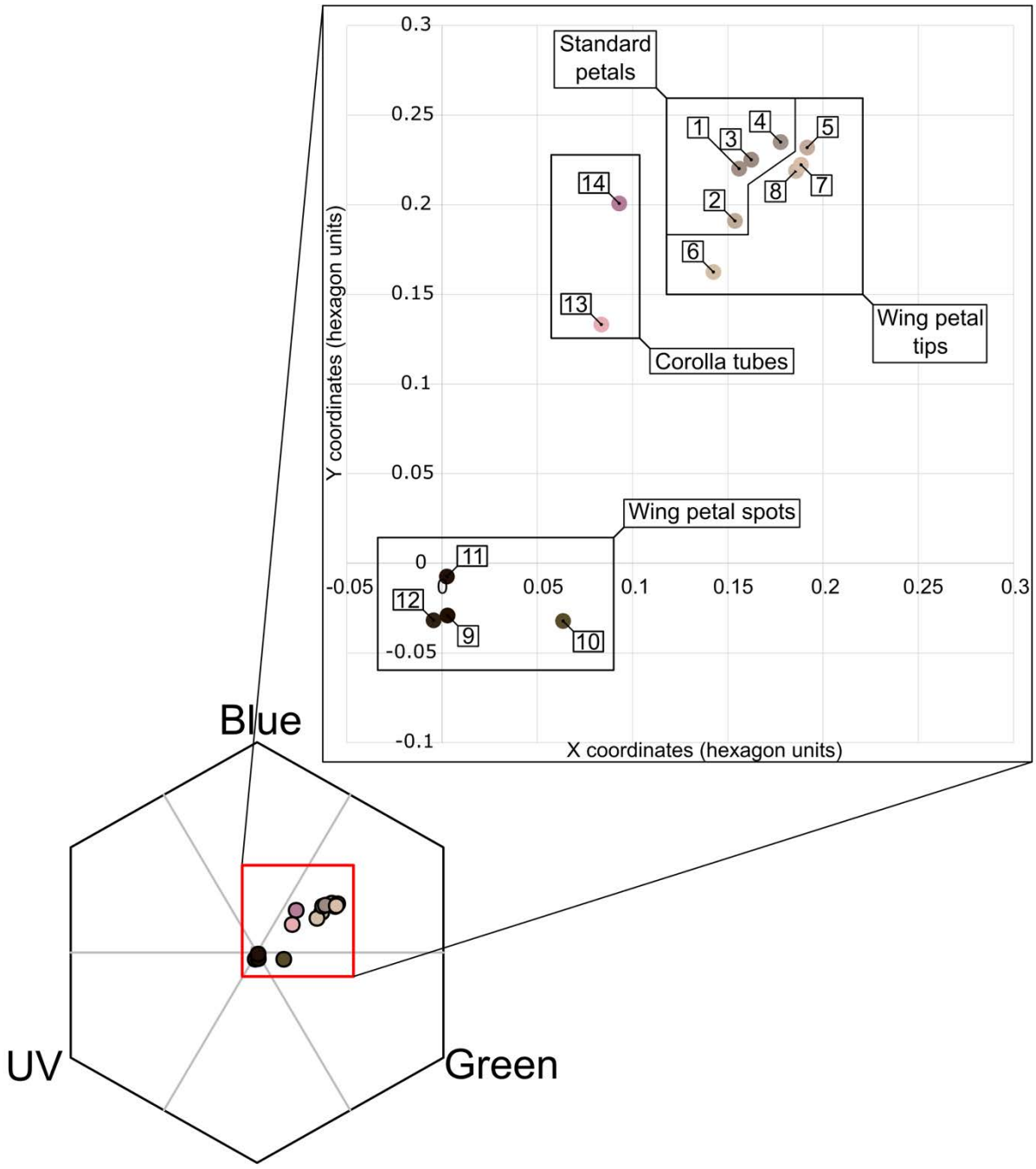


Figure 3.4 Bee hexagon plot for flowers of *V. faba* lines grown in glasshouse conditions. (A) Bee hexagon plot showing the reflectance spectra of petals in bee colour space, as a result of how they excite blue, green and UV receptors. Points closer to the centre of the plot excite receptors less strongly. Points closer to the edge of the plot excite receptors more strongly **(B)** Expanded view of bee colour space showing the colour of wing petal spots for a panel of *V. faba* lines. **(C)** Expanded view of bee colour space showing the colour of standard petals for a panel of *V. faba* lines. **(D)** Expanded view of bee colour space showing the colour of wing petal tips for a panel of *V. faba* lines. Plots are coloured according to the average colour of the point sampled after processing using the Pavo package in R.



Key		1.	Fuego Standard Glasshouse	2.	Fuego Standard Field	3.	Tiffany Standard Glasshouse	4.	Tiffany Standard Field
5.	Fuego Wing Glasshouse	6.	Fuego Wing Field	7.	Tiffany Wing Glasshouse	8.	Tiffany Wing Field	9.	Fuego Spot Glasshouse
10.	Fuego Spot Field	11.	Tiffany Spot Glasshouse	12.	Tiffany Spot Field	13.	Fuego Corolla Field	14.	Tiffany Corolla Field

Figure 3.5 Bee hexagon plot for flowers of Fuego and Tiffany grown in glasshouse and field conditions. Bee hexagon plot showing the reflectance spectra of petals in bee colour space,

as a result of how they excite blue, green and UV receptors. The red square is enlarged to show an expanded view. Plots are coloured according to the average colour of the point sampled after processing using the Pavo package in R.

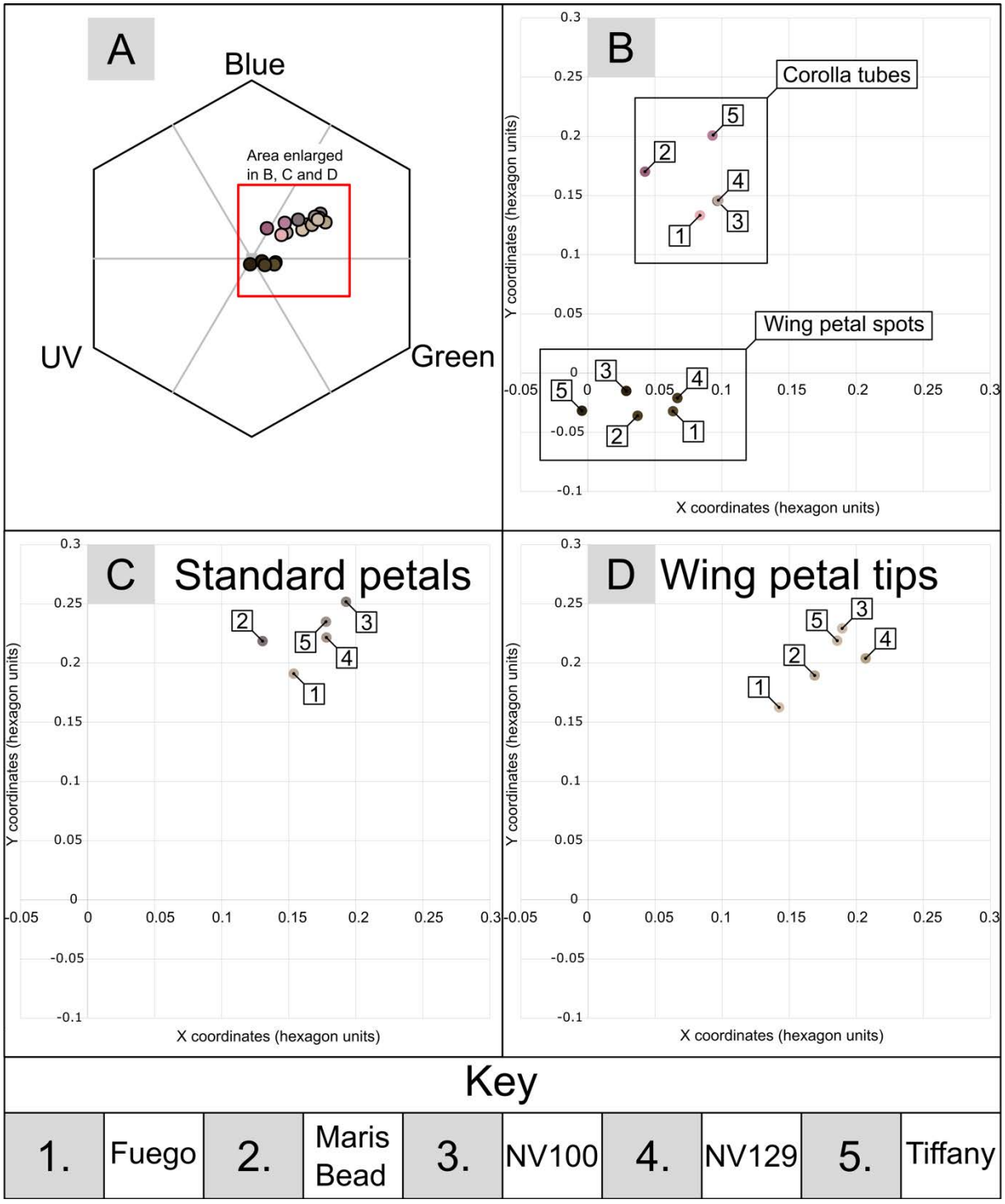


Figure 3.6 Bee hexagon for flowers of *V.faba* lines grown in field conditions. (A) Bee hexagon plot showing reflectance spectra of petals in bee colour space, as a result of how they excite blue, green and UV receptors. **(B)** Expanded view of bee colour space showing the colour of corolla tubes (above) and wing petal spots (below) for a panel of *V. faba* lines.

(C) Expanded view of bee colour space showing the colour of standard petals for a panel of *V. faba* lines. **(D)** Expanded view of bee colour space showing the colour of wing petal tips for a panel of *V. faba* lines. Plots are coloured according to the average colour of the point sampled after processing using the Pavo package in R.

Visible-spectrum colour

Flowers of *V. faba* lines grown in field conditions showed substantial variation in human-visible colour and extent of veins on the adaxial face of the standard petal (**Figure 3.7**). Quantification of the colour of standard petal veins was not possible as veins were too narrow to be sampled by the spectrophotometer beam. All lines appear human-white, however, Maris Bead shows more intense purple colouration of the standard petal and purple venation. Unlike all other lines, Yukon has human-yellow standard petal veins alongside yellow wing petal spots. All lines except NV100 and NV129 show varying degrees of human-pink colouration of the corolla tube.

UV patterning

Spectrophotometry and UV photographs of Maris Bead and Tiffany flowers revealed that flowers of both lines are highly UV absorbing across the entire flower surface (**Figure 3.8**). Both lines show slightly greater UV absorbance on wing petals than standard petals, however, this is only just noticeable (**Figure 3.8 C and D**). Flowers of Tiffany show greater UV reflectance at the base of the wing petals on the underside of the flower compared to Maris Bead, seen as a lighter area on the photographs.

Wing petal spot patterning

Wing petal spot size ranged from 37.3% of the total area of the wing petal for INRA29H to 48.4% for Vertigo (**Figure 3.9**). A one-way ANOVA revealed a statistically significant difference in mean wing petal spot size between at least two of the *V. faba* lines compared at $p \leq 0.0001$. A post-hoc Tukey's HSD test for multiple comparisons indicated that the mean wing petal spot size of Vertigo flowers was significantly larger than that of any other line except Tundra (**Table 3.4**). A post-hoc Tukey's test also indicated that the mean wing petal spot size of INRA29H flowers was significantly smaller than that of any other line (**Table 3.4**). The wing petal spot size of Vertigo, Tundra and Tiffany flowers was significantly greater than that of BPL10, Victus, Fanfare and INRA29H flowers. Significant differences in standard petal

height were also observed between other lines as shown in **Table 3.4** by lines which do not share Tukey significance letters.

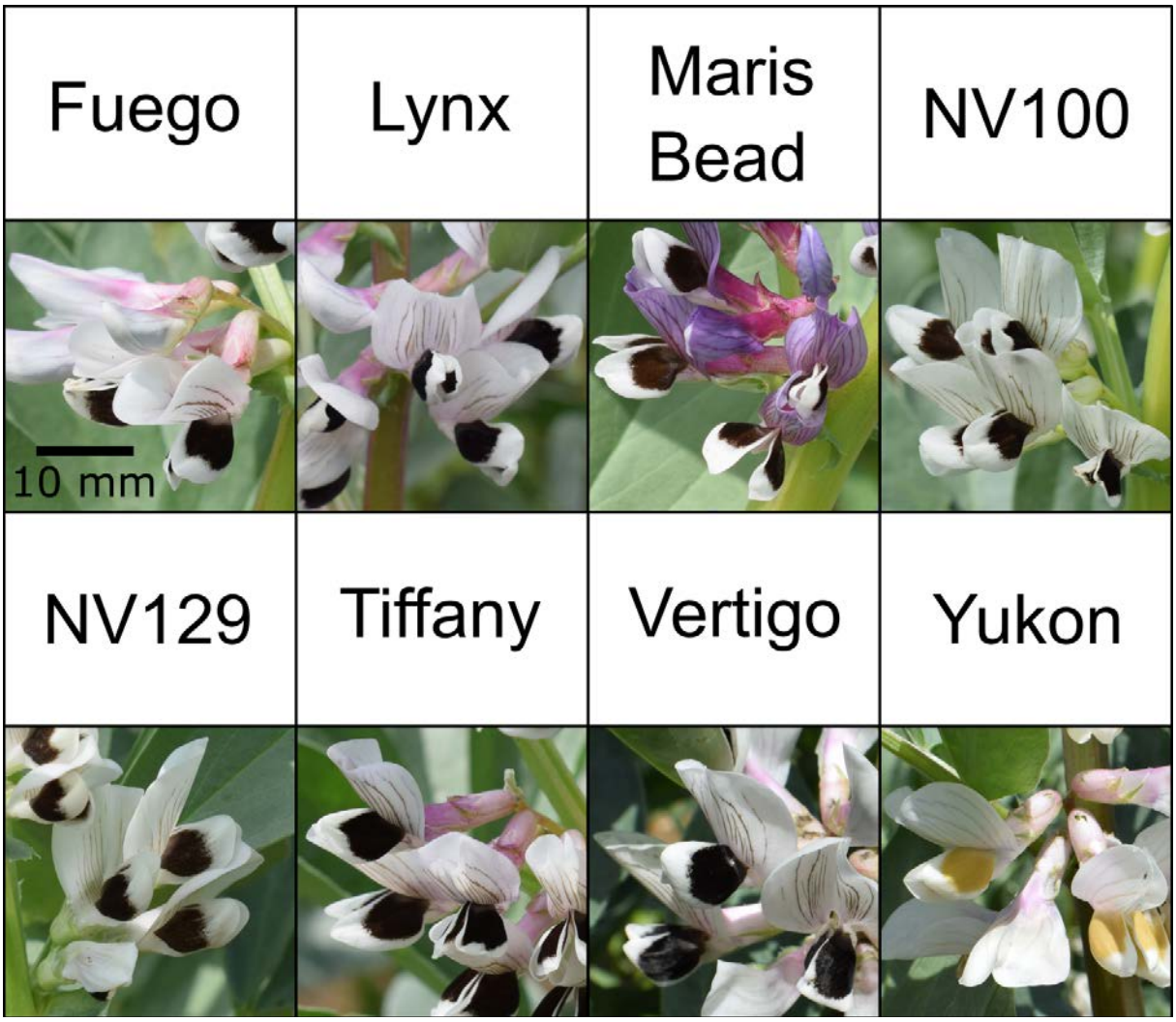


Figure 3.7 Visible-spectrum photographic comparison of *V. faba* lines grown in field conditions in 2021 and 2022. Lines showed great variation in human-visible colour of corolla tubes, standard petals, and standard petal veins.

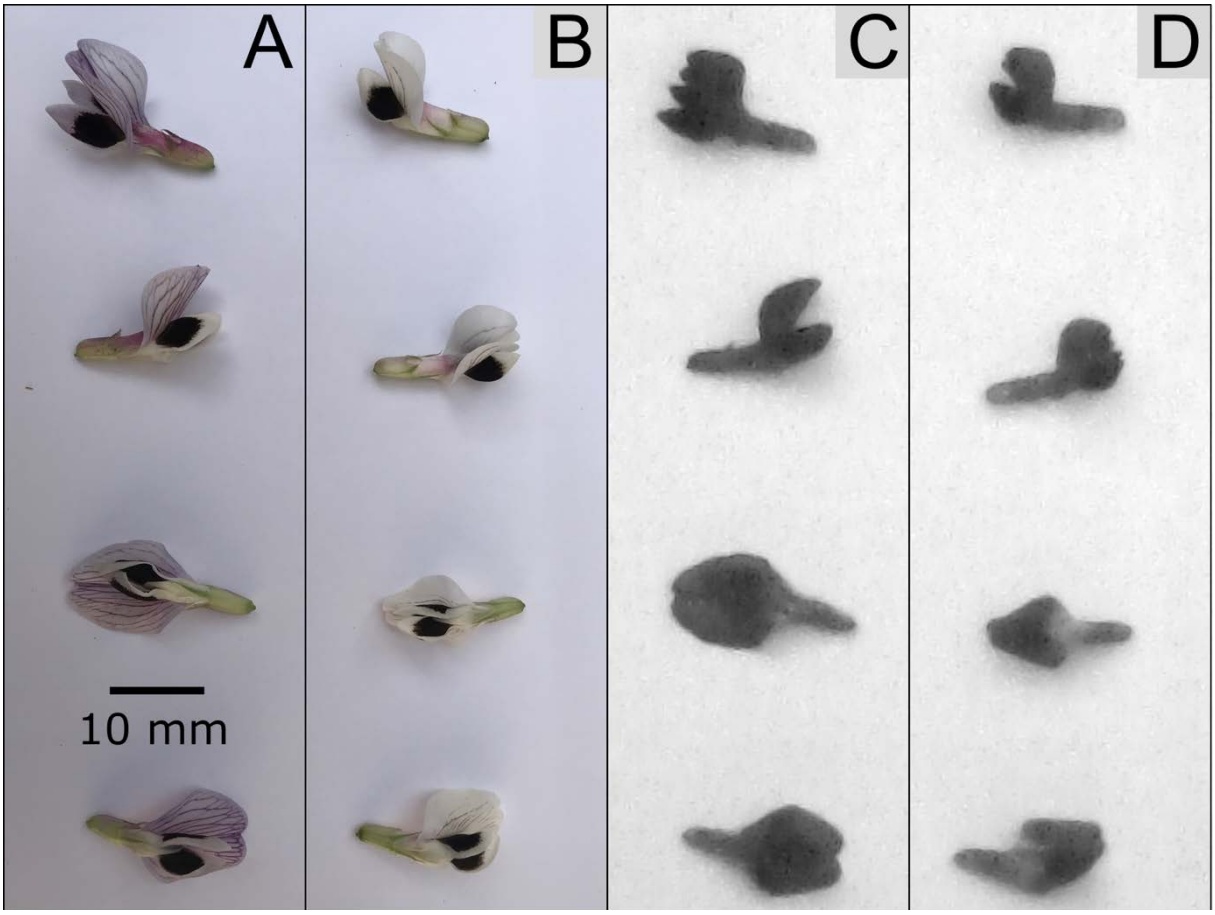


Figure 3.8 Visible and UV photographs of Maris Bead and Tiffany flowers grown in field conditions in 2022. Visible photographs were taken with an iPhone 7. UV photographs were taken with a Sunscreenr^{TM} UV camera for Android. Both visible spectrum and UV photographs were taken outdoors in the same lighting. In UV photographs, dark areas are the result of greater absorbance of UV light from a surface and lighter areas are the result of reflection of UV light. **(A)** Visible photographs of Maris Bead flowers. **(B)** Visible photographs of Tiffany flowers. **(C)** UV photographs of Maris bead flowers. **(D)** UV photographs of Tiffany flowers.

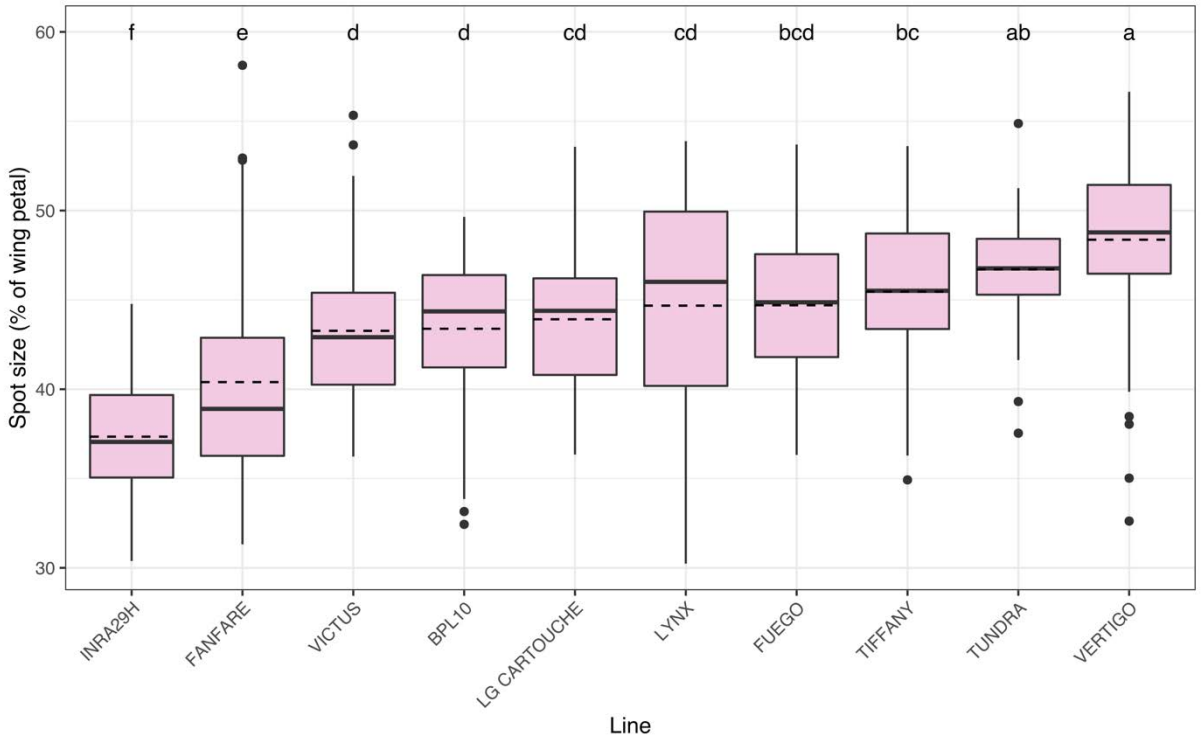


Figure 3.9 Wing petal spot size as percentage of the total wing spot area for flowers of *V. faba* lines. Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the interquartile range). Dashed lines show means and solid lines show medians. Black dots show datapoints that fall outside of the calculated maxima and minima. Letters show Tukey significance groups. *V. faba* lines which do not share a letter have significantly different mean wing petal spot size ($p \leq 0.05$). Spot size ranged from 37.3% for INRA29H to 48.4% for Vertigo.

Line	n	Mean spot size (%)	Tukey significance group					
VERTIGO	85	48.4	A					
TUNDRA	70	46.7	A	B				
TIFFANY	75	45.5		B	C			
FUEGO	145	44.7		B	C	D		
LYNX	161	44.7			C	D		
LG CARTOUCHE	88	43.9			C	D		
BPL10	126	43.4				D		
VICTUS	72	43.3				D		
FANFARE	68	40.4					E	
INRA29H	46	37.3						F

Table 3.4 Mean wing petal spot size and Tukey significance groups for flowers of *V. faba* lines gown in glasshouse conditions and Tukey significance groups. Spot size ranged from 37.3% for INRA29H to 48.4% for Vertigo. *V. faba* lines which do not share a letter have significantly different mean wing petal spot size ($p \leq 0.05$), n shows the number of flowers measured.

3.2.3 Size of floral display

For methodology refer to section 2.4.

The mean number of flowers produced per node ranged from 1.38 on plants of NV620 to 9.39 on plants of Tundra when grown in glasshouse conditions (Figure 3.10). After Tundra, the lines producing the most flowers per node were NV604, Kasztelan, Fuego and Tiffany. A one-way ANOVA revealed a statistically significant difference in the mean number of flowers produced per node between at least two of the *V. faba* lines compared at $p \leq 0.0001$. A post-hoc Tukey's HSD test for multiple comparisons indicated that the mean number of flowers produced per node on plants of Tundra was significantly greater than that of any other line (Table 3.5). A Tukey's test also indicated that the 10 lines producing the greatest mean number of flowers per node produced significantly more flowers than the 18 lines producing the least (Table 3.5). Plants of NV620 and BPL27 produced significantly fewer flowers per node than all other lines with 1.28 and 1.63 flowers per node respectively. The mean number of flowers produced per node for any line was 5.87. Out of the 31 lines examined, 17 produced more than six flowers per node on average, whereas five lines produced less than four flowers per node. Significant differences in the number of flowers produced per node were also observed between other lines, as shown in Table 3.5 by lines which do not share Tukey significance letters.

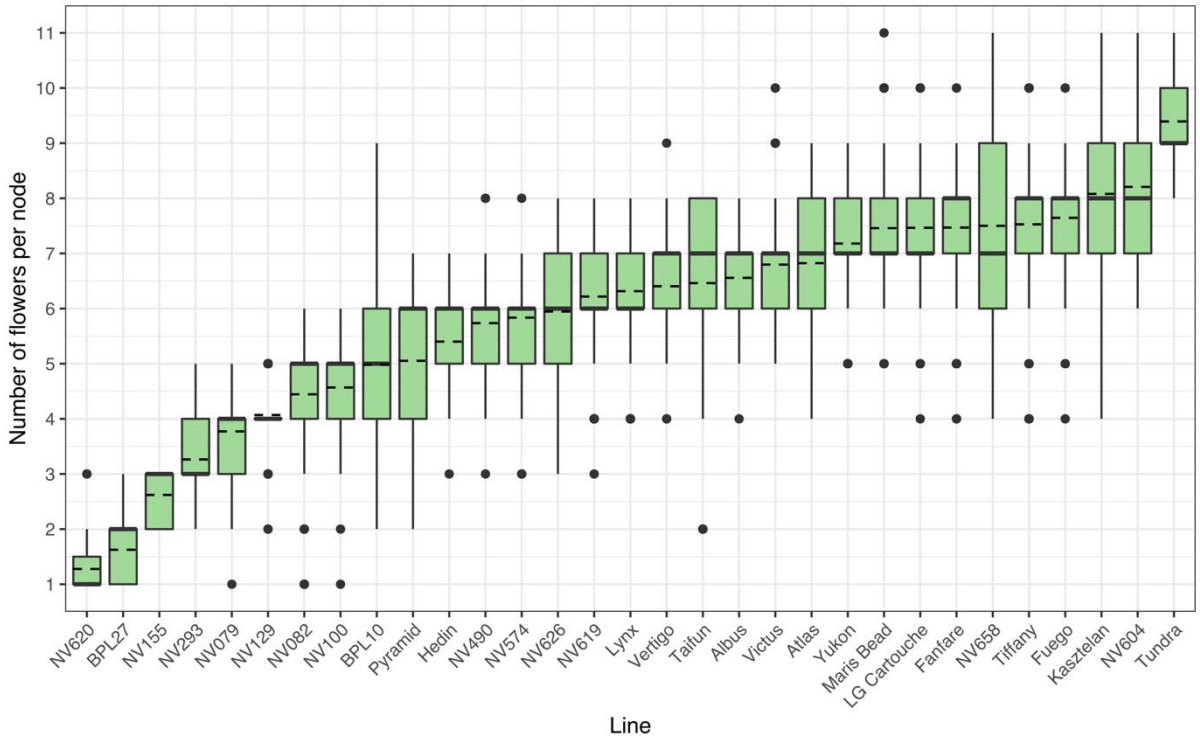


Figure 3.10 Number of flowers per node produced by lines of *V. faba*. Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the interquartile range). Dashed lines show means and solid lines show medians. Black dots show datapoints that fall outside of the calculated maxima and minima. Mean number of flowers per node ranged from 1.28 for NV620 to 9.39 for Tundra.

Line	n	Mean number of flowers per node	Tukey significance group															
Tundra	61	9.39	A															
NV604	53	8.21		B														
Kasztelan	51	8.08		B	C													
Fuego	192	7.65		B	C													
Tiffany	163	7.53			C	D												
NV658	64	7.50			C	D	E											
Fanfare	100	7.47			C	D	E											
LG Cartouche	86	7.47			C	D	E											
Maris Bead	87	7.46			C	D	E											
Yukon	172	7.18				D	E	F										
Atlas	62	6.82					E	F	G									
Victus	133	6.80						F	G									
Albus	52	6.56						F	G	H								
Taifun	65	6.46							G	H								
Vertigo	121	6.40							G	H								
Lynx	204	6.31							G	H								
NV619	60	6.22							G	H	I							
NV626	75	5.95								H	I	J						
NV574	55	5.84								H	I	J						
NV490	102	5.74									I	J						
Hedin	65	5.40										J	K					
Pyramid	75	5.05											K	L				
BPL10	115	4.98											K	L				
NV100	81	4.57												L	M			
NV082	106	4.44												L	M			
NV129	57	4.07													M	N		
NV079	185	3.77														N	O	
NV293	53	3.26															O	P
NV155	87	2.62																P
BPL27	72	1.63																Q
NV620	79	1.28																Q

Table 3.5 The mean number of flowers per node for lines of *V. faba* and Tukey significance groups. *V. faba* lines which do not share a letter have significantly different mean number of flowers per node ($p \leq 0.05$), n signifies number of nodes sampled. Mean number of flowers per node ranged from 1.28 for NV620 to 9.39 for Tundra, n shows the number of nodes measured.

3.2.4 Force required to open flowers

For methodology refer to section 2.5.

The operative force required to open *V. faba* flowers varied greatly between lines (**Figure 3.11**). Flowers of Yukon required the smallest amount of force to open on average (mean = 14.41 mN) and flowers of NV129 required the greatest force to open (mean = 36.07 mN) (**Table 3.5**).

A one-way ANOVA revealed a statistically significant difference in the mean operative force between at least two of the *V. faba* lines compared at $p \leq 0.0001$. A post-hoc Tukey's HSD test for multiple comparisons indicated that the amount of force required to trip flowers of NV129 was significantly greater than that required to trip flowers of any other line **(Table 3.6**). A post-hoc Tukey's test also indicated that the amount of force required to trip flowers of Yukon, NV155, LG Cartouche and Fanfare was significantly lower than the 17 lines requiring the most force to trip (**Table 3.6**). Out of the 27 lines examined, 15 required between 20 and 25 mN of force to trip their flowers, seven required between 14 and 20 mN, and four required between 25 and 27 mN. Only NV129 required more than 30 mN to trip flowers. Significant differences in standard petal height were also observed between other lines examined. These differences are shown in **Table 3.6** by lines which do not share Tukey significance letters.

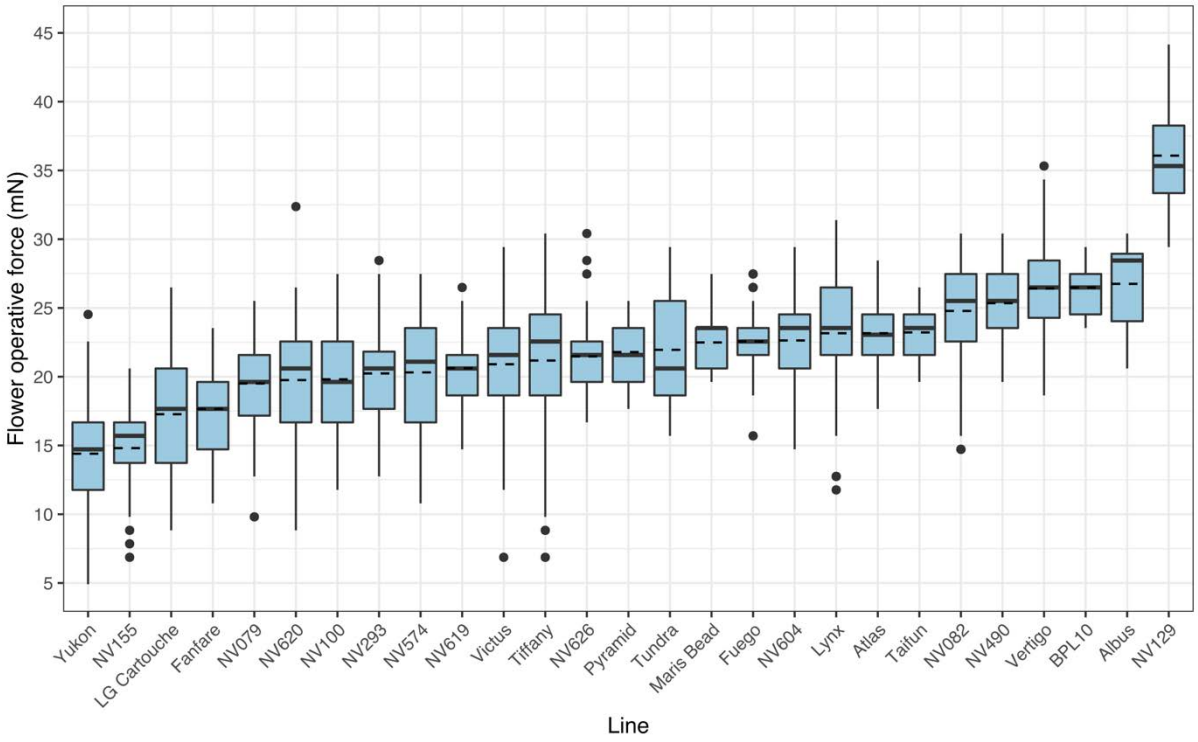


Figure 3.11 The force (mN) required to trip flowers of lines of *V. faba*. Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the interquartile range (IQR)). Dashed lines show means and solid lines show medians. Black dots show datapoints that fall outside of the calculated maxima and minima. Mean operative force ranged from 14.41 mN for Yukon to 36.07 mN for NV129.

Line	n	Mean operative force (mN)	Tukey significance group						
NV129	47	36.07	A						
Albus	11	26.75		B					
BPL10	13	26.49		B					
Vertigo	68	26.42		B					
NV490	64	25.35		B					
NV082	56	24.79		B					
Taifun	15	23.22		B	C				
Atlas	26	23.17		B	C				
Lynx	36	23.16		B	C				
NV604	52	22.64		B	C				
Fuego	28	22.56		B	C				
Maris Bead	13	22.49		B	C				
Tundra	24	21.95		B	C				
Pyramid	23	21.80		B	C				
NV626	39	21.48			C				
Tiffany	77	21.17			C				
Victus	74	20.91			C				
NV619	44	20.62			C	D			
NV574	52	20.32			C	D			
NV293	52	20.24			C	D			
NV100	53	19.81			C	D	E		
NV620	21	19.76			C	D	E		
NV079	63	19.51			C	D	E		
Fanfare	39	17.66				D	E		
LG Cartouche	43	17.27					E	F	
NV155	65	14.81						F	G
Yukon	57	14.41							G

Table 3.6 The mean operative force per flower for lines of *V. faba* and Tukey significance groups. *V. faba* lines which do not share a letter have significantly flower operative force ($p \leq 0.05$). Mean operative force ranged from 14.41 mN for Yukon to 36.07 mN for NV129, n shows the number of flowers measured.

3.2.5 Nectar production

For methodology refer to section 2.6.

Nectar volume

The mean volume of nectar produced ranged from 2.15 μl per flower from plants of INRA29H to 3.88 μl per flower from plants of Lynx (**Figure 3.12**). A one-way ANOVA revealed a statistically significant difference in mean nectar volume between at least two of the *V. faba* lines compared at $p \leq 0.0001$. A post-hoc Tukey's HSD test for multiple comparisons indicated that the volume of nectar produced by flowers of Lynx was significantly greater than that produced by any other line apart from Tundra (**Table 3.7**). The volume of nectar produced by flowers of Lynx, Tundra, Victus, Tiffany, Fanfare and Vertigo was significantly greater than that produced by flowers of Yukon and INRA29H. The volume of nectar produced by flowers of INRA29H was also lower than that of any other line except Yukon.

Nectar concentration

The mean concentration of sugar in nectar varied greatly between lines, ranging from 19.60% w/w for flowers of BPL10 to 51.46% w/w for flowers of LG Cartouche (**Figure 3.13**). A one-way ANOVA revealed a statistically significant difference in mean nectar concentration between at least two of the *V. faba* lines compared at $p \leq 0.0001$. A post-hoc Tukey's HSD test for multiple comparisons indicated that the sugar concentration of nectar produced by flowers of LG Cartouche was significantly greater than that produced by any other line, and the sugar concentration of BPL10 was significantly lower than any other line (**Table 3.8**). The nectar concentration of flowers of Yukon was also significantly lower than that of any other line except BPL10, and nectar concentration for Tiffany was significantly lower than any other line except BPL10 and Yukon. Out of the 11 lines examined, seven produced nectar with a sugar concentration of over 40% w/w. Only LG Cartouche produced nectar with a sugar concentration of over 50% w/w.

Nectar sugar mass

The mean mass of sugar produced per flower ranged from 3.14 mg from plants of Yukon to 5.70 mg from plants of Lynx (**Figure 3.14**). A one-way ANOVA revealed a statistically significant difference in the mean mass of sugar produced per flower between at least two

of the *V. faba* lines compared at $p \leq 0.0001$. A post-hoc Tukey's HSD test for multiple comparisons indicated that the mass of sugar produced by flowers of Lynx was significantly greater than that produced by any other line apart from Tundra, and the mass of sugar produced by flowers of Tundra was significantly different to that produced by any other line, apart from Lynx, Fanfare and LG Cartouche (**Table 3.9**). The mass of sugar produced by flowers of Yukon, BPL10 and INRA29H was significantly lower than that produced by any other line. Out of the 11 lines examined, eight produced over 4 mg of sugar per flower.

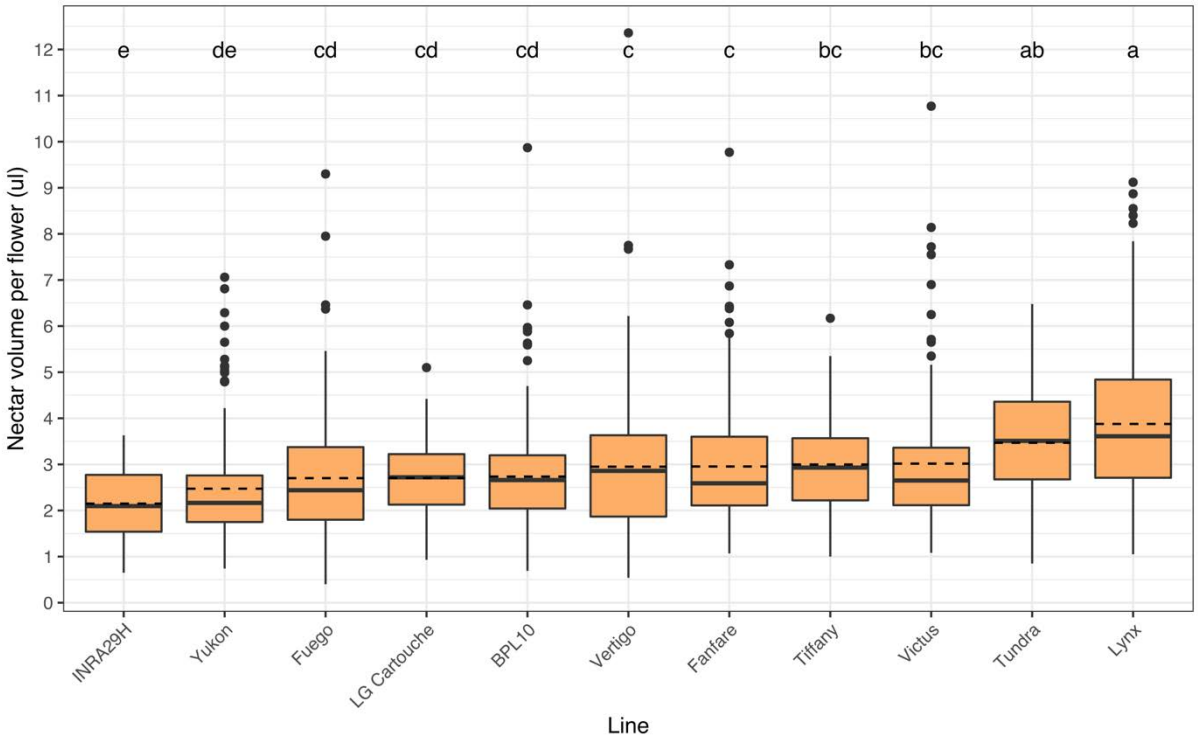


Figure 3.12 The volume of nectar produced per flower by *V. faba* lines. Boxplots show the interquartile range, whiskers show maxima and minima (1.5 x IQR). Dashed lines show means and solid lines show medians. Black dots show datapoints that fall outside of maxima and minima. *V. faba* lines which do not share a letter have significantly different mean nectar volume ($p \leq 0.05$). The mean volume of nectar produced ranged from 2.15 µl per flower from plants of INRA29H to 3.88 µl per flower from plants of Lynx.

Line	n	Mean nectar volume per flower (µl)	Tukey significance group				
Lynx	289	3.88	A				
Tundra	135	3.47	A	B			
Victus	140	3.02		B	C		
Tiffany	154	3.00		B	C		
Fanfare	157	2.95			C		
Vertigo	172	2.95			C		
BPL10	242	2.73			C	D	
LG Cartouche	182	2.70			C	D	
Fuego	299	2.70			C	D	
Yukon	152	2.47				D	E
INRA29H	80	2.15					E

Table 3.7 The mean volume of nectar produced per flower by *V. faba* lines and Tukey significance groups. *V. faba* lines which do not share a letter have significantly different mean nectar volume ($p \leq 0.05$), n shows the number of flowers measured.

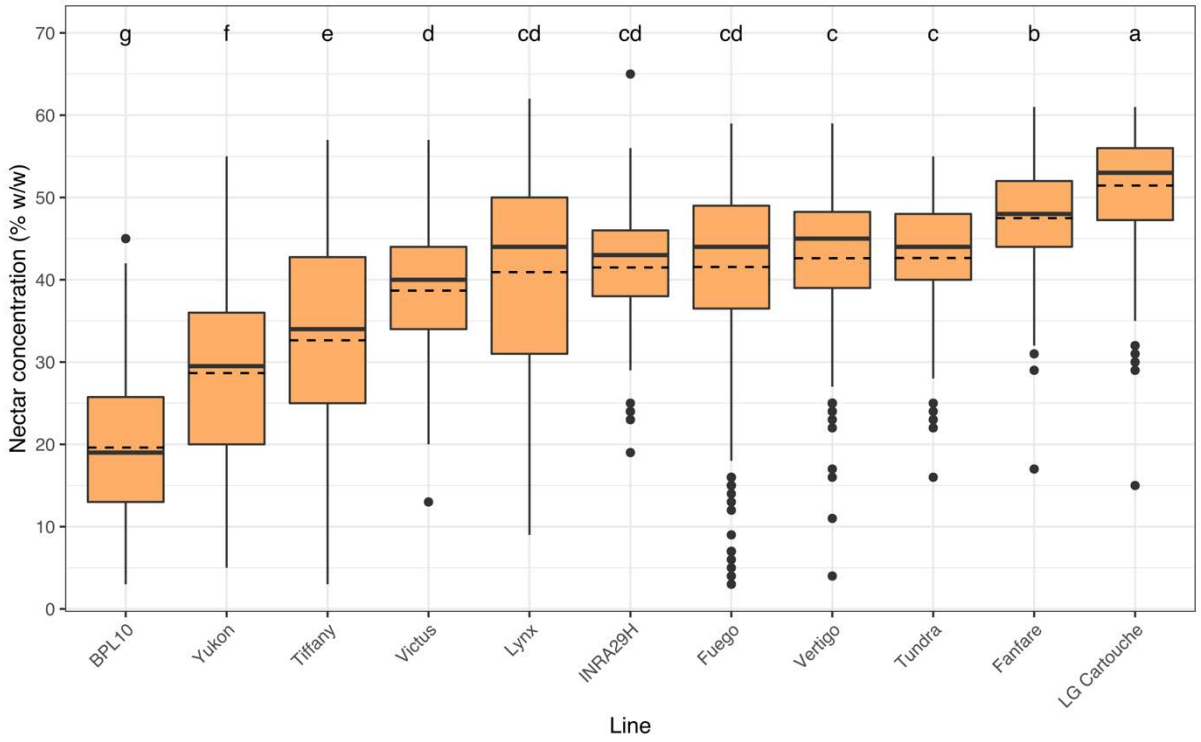


Figure 3.13 The concentration of nectar produced by *V. faba* lines. Boxplots show the interquartile range, whiskers show maxima and minima (1.5 x IQR). Dashed lines show means and solid lines show medians. Black dots show datapoints that fall outside of maxima and minima. *V. faba* lines which do not share a letter have significantly different mean nectar concentration ($p \leq 0.05$). Mean nectar concentration ranged from 19.6% w/w for flowers of BPL10 to 51.5% w/w for flowers of LG Cartouche.

Line	n	Mean nectar concentration (% w/w)	Tukey significance group						
LG Cartouche	182	51.46	A						
Fanfare	157	47.48		B					
Tundra	135	42.64			C				
Vertigo	172	42.62			C	D			
Fuego	299	41.55			C	D			
INRA29H	80	41.49			C	D			
Lynx	289	40.93			C	D			
Victus	140	38.69				D			
Tiffany	154	32.64					E		
Yukon	152	28.66						F	
BPL10	242	19.60							G

Table 3.8 The mean concentration of nectar produced by *V. faba* lines and Tukey significance groups. *V. faba* lines which do not share a letter have significantly different mean nectar concentration ($p \leq 0.05$), n shows the number of flowers measured.

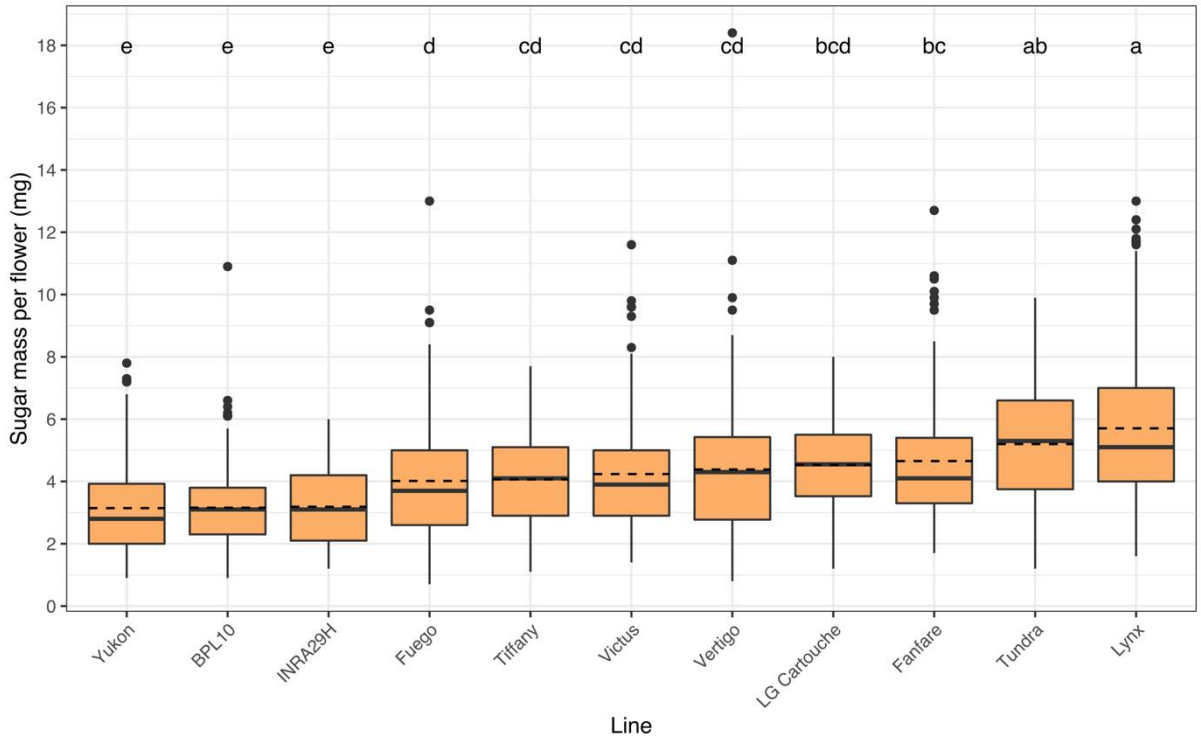


Figure 3.14 The mass of sugar in nectar per flower produced by *V. faba* lines. Boxplots show the interquartile range, whiskers show maxima and minima (1.5 x IQR). Dashed lines show means and solid lines show medians. Black dots show datapoints that fall outside of maxima and minima. *V. faba* lines which do not share a letter have significantly different mean sugar mass per flower ($p \leq 0.05$). The mean mass of sugar produced per flower ranged from 3.14 mg from plants of Yukon to 5.70 mg from plants of Lynx.

Line	n	Mean sugar mass per flower (mg)	Tukey significance group				
Lynx	289	5.70	A				
Tundra	135	5.20	A	B			
Fanfare	157	4.65		B	C		
LG Cartouche	182	4.53		B	C	D	
Vertigo	172	4.38			C	D	
Victus	140	4.24			C	D	
Tiffany	154	4.07			C	D	
Fuego	299	4.01				D	
INRA29H	80	3.19					E
BPL10	242	3.16					E
Yukon	152	3.14					E

Table 3.9 The mean mass of sugar in nectar per flower produced by *V. faba* lines and Tukey significance groups. *V. faba* lines which do not share a letter have significantly different mean sugar mass per flower ($p \leq 0.05$), n shows the number of flowers measured.

3.2.6 Pollen production

For methodology refer to section 2.6.

Quantity of pollen

The number of pollen grains produced per flower ranged between 58,559 for plants of BPL10, and 84,062 for plants of Yukon. Seven out of the 11 lines examined produced over 70,000 pollen grains. (**Figure 3.15**). A one-way ANOVA revealed a statistically significant difference in the mean number of pollen grains produced per flower between at least two of the *V. faba* lines compared at $p \leq 0.0001$. A post-hoc Tukey's HSD test for multiple comparisons indicated that the three lines producing the greatest number of pollen grains per flower (Yukon, INRA29H, and Fuego) produced significantly more pollen grains than the four lowest producing lines (BPL10, Victus, Tiffany and Tundra) (**Figure 3.10**). The number of pollen grains produced by flowers of BPL10 was also significantly lower than that of any other line except Victus and Tiffany.

Quality of pollen

The proportion of viable pollen grains produced by flowers was over 90% for all lines except Lynx (**Figure 3.16**). The lowest mean viability measured was for Lynx at 87.4% and the highest was for Yukon at 98.7%. A one-way ANOVA revealed a statistically significant difference in pollen viability between at least two of the *V. faba* lines compared at $p \leq 0.0001$. A post-hoc Tukey's HSD test for multiple comparisons indicated that Lynx had significantly lower pollen viability compared to all other lines except Fanfare (**Table 3.11**). There were no statistically significant differences between pollen viability for any other lines.

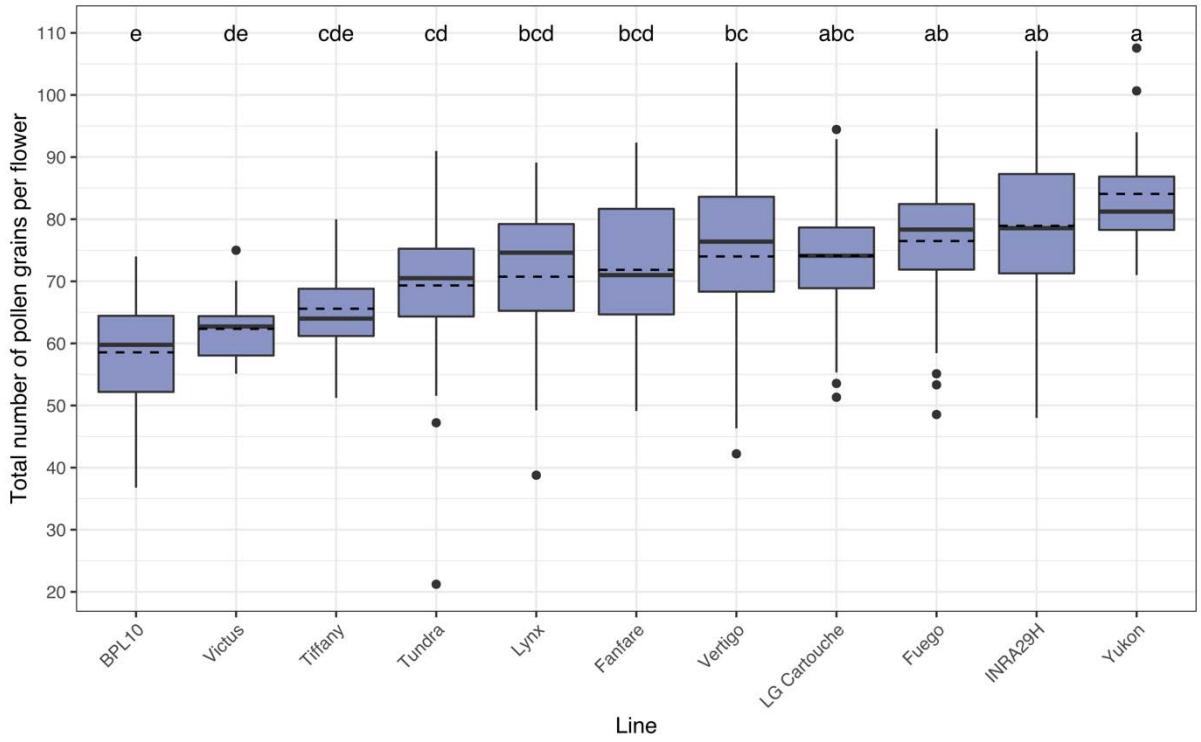


Figure 3.15 The total number of pollen grains produced by lines of *V. faba*. Boxplots show the interquartile range, whiskers show maxima and minima (1.5 x IQR). Dashed lines show means and solid lines show medians. Black dots show datapoints that fall outside of maxima and minima. *V. faba* lines which do not share a letter have significantly different mean number of pollen grains per flower ($p \leq 0.05$). The number of pollen grains produced per line ranged between 58,559 per flower for plants of BPL10, and 84,062 for plants of Yukon.

Line	n	Mean number of pollen grains per flower (thousands)	Tukey significance group				
Yukon	18	84.06	A				
INRA29H	27	78.93	A	B			
Fuego	57	76.49	A	B			
LG Cartouche	45	74.12	A	B	C		
Vertigo	50	74.00		B	C		
Fanfare	45	71.84		B	C	D	
Lynx	54	70.74		B	C	D	
Tundra	48	69.33			C	D	
Tiffany	18	65.58			C	D	E
Victus	18	62.35				D	E
BPL10	36	58.56					E

Table 3.10 The mean number of pollen grains per flower produced by *V. faba* lines and Tukey significance groups. *V. faba* lines which do not share a letter have significantly different mean number of pollen grains per flower ($p \leq 0.05$), n shows the number of flowers measured.

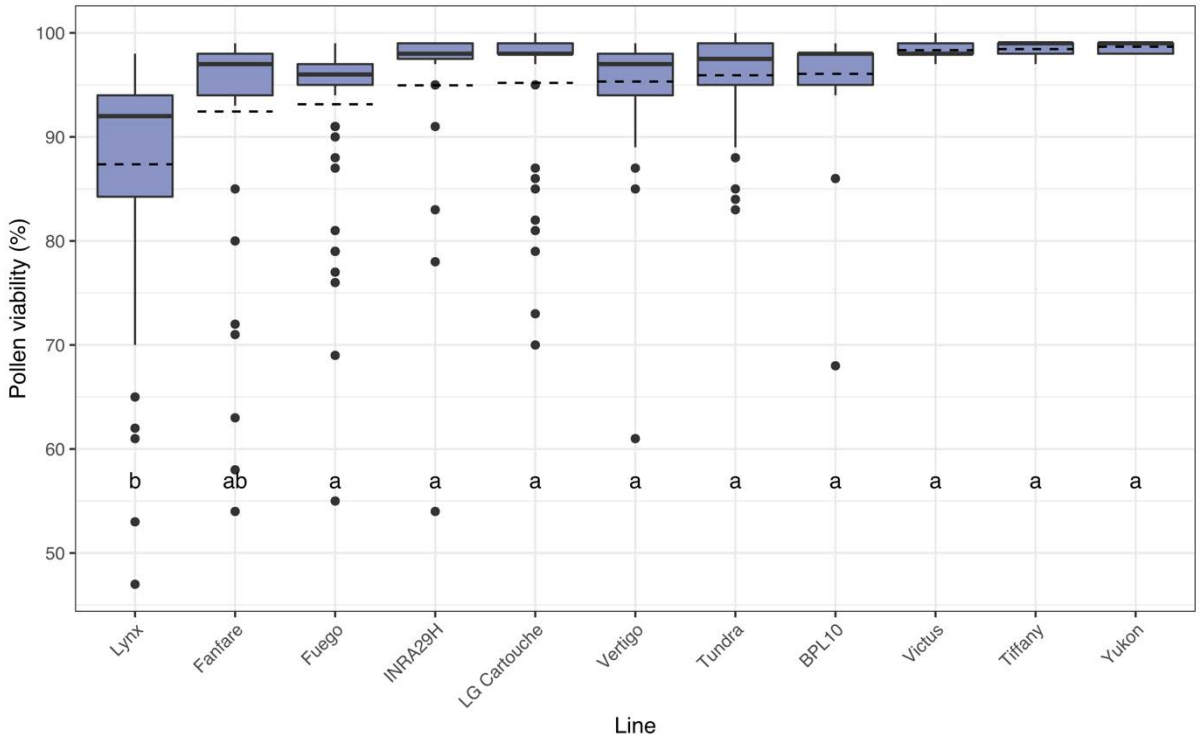


Figure 3.16 The proportion of viable pollen produced by flowers of *V. faba* lines. Boxplots show the interquartile range, whiskers show maxima and minima (1.5 x IQR). Dashed lines show means and solid lines show medians. Black dots show datapoints that fall outside of maxima and minima. *V. faba* lines which do not share a letter have significantly different mean pollen viability ($p \leq 0.05$). The proportion of viable pollen grains produced by flowers was over 90% for all lines except Lynx.

Line	n	Mean pollen viability (%)	Tukey significance group	
Yukon	18	98.7	A	
Tiffany	18	98.4	A	
Victus	18	98.3	A	
BPL10	36	96.1	A	
Tundra	48	95.9	A	
Vertigo	50	95.3	A	
LG Cartouche	45	95.2	A	
INRA29H	27	95.0	A	
Fuego	57	93.1	A	
Fanfare	45	92.4	A	B
Lynx	54	87.4		B

Table 3.11 The mean proportion of viable pollen from flowers of *V. faba* lines and Tukey significance groups. *V. faba* lines which do not share a letter have significantly different pollen viability ($p \leq 0.05$), n shows the number of flowers measured.

3.2.7 Relationships between traits

Six strong positive correlations (with a Pearson's r value of 0.5 or above) were found between floral traits, and five strong negative correlations (with a Pearson's r value of -0.5 or below) were found between floral traits (**Table 3.12**). The strongest positive correlation was apparent between mean nectar volume and mean flower sugar mass (Pearson's r = 0.890). This was the only statistically significant correlation at $p \leq 0.05$. The strongest negative correlation was seen between mean flower sugar mass and mean pollen viability but was not statistically significant at $p \leq 0.05$ (Pearson's r = -0.0610) (**Table 3.12**). The strongest correlations highlighted in **Table 3.12** are plotted in **Figure 3.17** with linear models shows by blue or orange lines, and 95% confidence ribbons shown by grey shading.

	Spot size	Corolla length	Wing area	Standard height	Pollen viability	Number of pollen grains	Sugar mass	Nectar concentration	Nectar volume	Operative force
Number of flowers	0.170	-0.500	-0.060	-0.160	0.120	0.330	0.390	0.530	0.150	-0.020
Operative force	0.570	0.520	0.410	0.001	-0.230	-0.570	0.110	-0.250	0.340	
Nectar volume	0.570	0.280	0.180	-0.490	-0.510	-0.410	0.890*	0.160		
Nectar concentration	-0.060	-0.480	0.190	-0.180	-0.400	0.330	0.600			
Sugar mass	0.450	0.010	0.200	-0.500	-0.610	-0.170				
Number of pollen grains	-0.230	-0.600	-0.570	0.420	-0.100					
Pollen viability	0.090	0.080	0.220	0.120						
Standard height	-0.490	-0.290	-0.180							
Wing area	0.330	0.230								
Corolla length	0.450									

Table 3.12 Pearson's correlation coefficients for floral traits quantified from flowers of *V. faba* lines grown in glasshouse conditions. The strongest positive correlation was observed between mean nectar volume and mean flower sugar mass. This was the only statistically significant correlation at $p \leq 0.05$, shown by an asterisk. The strongest negative correlation was observed between mean flower sugar mass and mean pollen viability. Blue shading highlights strong positive correlations with a Pearson's r value of 0.5 or above. Orange shading highlights strong negative correlations with a Pearson's r value of -0.5 or below. Pearson's correlation coefficients were calculated for lines from which data for all floral traits was collected (n = 11).

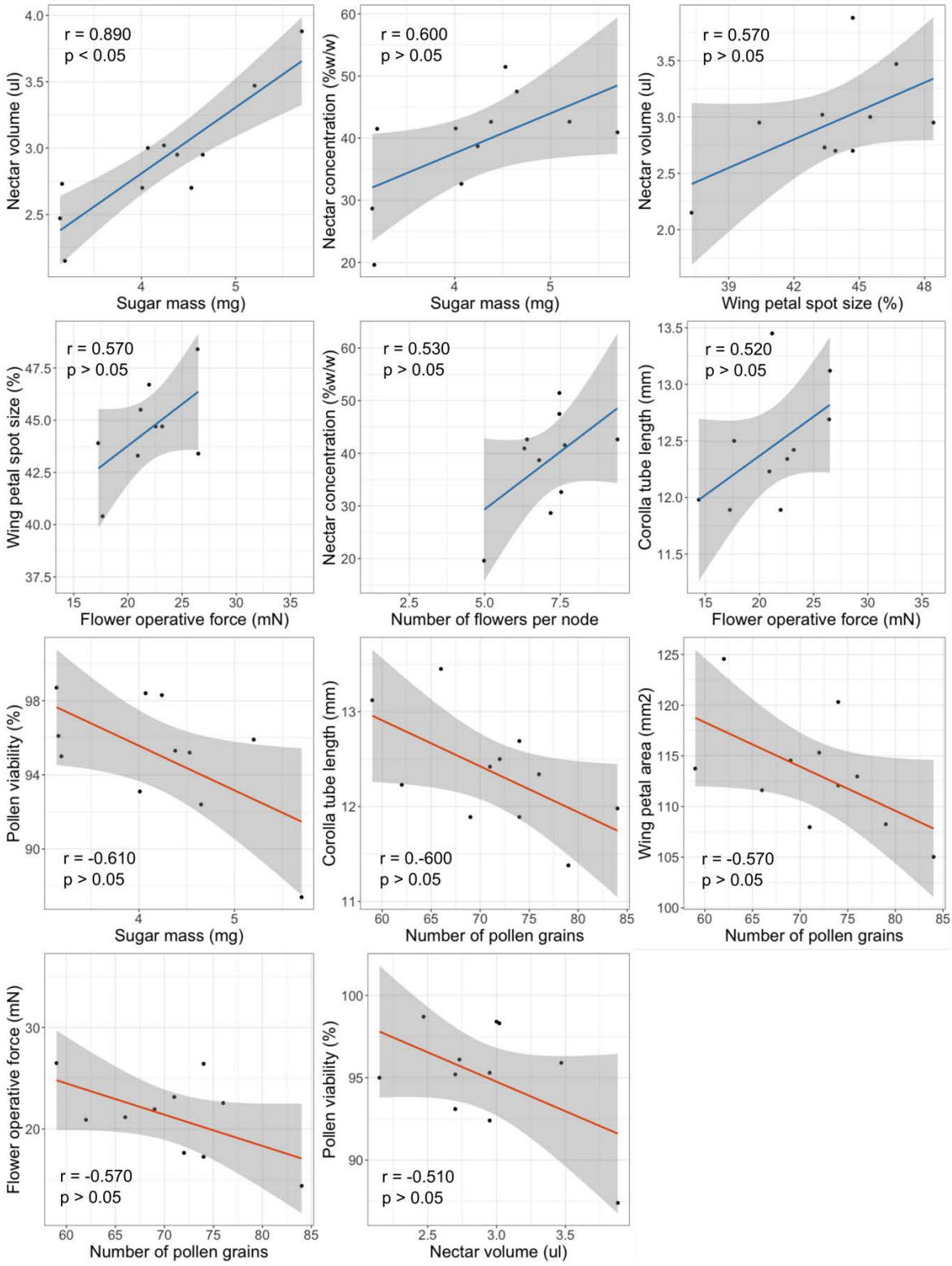


Figure 3.17 The strongest positive and negative correlations seen between *V. faba* floral traits measured in glasshouse conditions. Plots show datapoints for *V. faba* lines where data for both variables was collected in this study. Blue and orange lines show linear models fitted to datasets. Grey ribbons show 95% confidence intervals around linear models. The strongest positive correlation was observed between mean nectar volume and mean flower sugar mass (top left). The strongest negative correlation was observed between mean flower sugar mass and mean pollen viability (third row, left).

3.3 Discussion and conclusions

The objective of the work presented in this chapter was to assess floral trait variation in previously uncharacterised lines of *Vicia faba,* with an emphasis on modern commercial lines. Prior to this, only one study has extensively quantified multiple floral traits of different *Vicia faba* lines (Bailes 2016). Further exploration of floral traits of *Vicia faba* lines, especially modern commercial lines, is necessary to allow examination of the effect of biologically relevant differences in floral traits on bee behaviour and pollination of the crop.

In this chapter the hypothesis tested was that modern commercial lines of *Vicia faba* have significant differences in previously unexplored floral traits. The results presented in this chapter support this hypothesis, with statistically significant variation identified in flower morphology, petal patterning, size of floral display, operative force, nectar production and pollen production, and measurable differences present in flower colour, where statistical testing was inappropriate.

When considering the impact of this variation on pollinator behaviour, one should study the impact of all floral traits of a line simultaneously, as pollinators are likely to use multiple signals to inform their choices. So far, no studies have completed such experiments. This is therefore explored in Chapter 4. Some studies have investigated the effect of specific floral traits on pollination in *Vicia faba,* and where no information is present for the effect of *Vicia faba* floral trait variation on pollinator behaviour, one can look to other systems.

Visual traits, including the size of the floral display, petal size, and petal colour were all found to vary between the *V. faba* lines examined in this study. Multiple studies conclude that bee visitation rate, pollen movement and plant fitness increase with floral display size, as is the case in *Cirsium purpuratum*, and *Epilobium angustifolium* (Ohashi and Yahara 1998; Schmid-Hempel and Speiser 1988). However, larger displays may impose fitness costs through geitonogamous selfing, as larger displays can result in greater time spent on a plant and reduced bee movement between plants (Harder and Barrett 1995; Ohashi and Yahara 1998; Suso et al. 2005). To maximise outcrossing, plants should have many inflorescences, each with fewer flowers to encourage movement between plants (Suso et al. 2005). This study has found that many modern commercial *V. faba* lines, including Tundra, Fuego, Tiffany and

Maris Bead, produce more flowers and should therefore provide more floral resources and enhance bee attraction.

In this study, the commercial *V. faba* lines, Fanfare and Vertigo had particularly large standard and wing petals. Bees show preference for larger flowers in several plant species (Conner and Rush 1996; Elle and Carney 2003; Martin 2004). The most likely explanation being that larger flowers generally produce a larger reward (Ashman and Stanton 1991; Inoue et al. 1995). However, independent of greater reward, larger artificial flowers are located more rapidly by bees (Spaethe et al. 2001). Whether flower size affects bee behaviour in a complex flower like that of *V. faba* needs to be investigated, alongside any consequences for pollination.

This study identified measurable differences in the reflectance spectra of previously unstudied *V. faba* lines, however, it is unlikely that these differences will affect bee behaviour. This finding is like that of Bailes (2016), in which limited variation was reported between corresponding petals of human-white flowers. Previous studies examining bee behaviour in response to colour have found that honeybees can discriminate between colours as little as 0.008 hexagon units apart with considerable accuracy (Dyer and Neumeyer 2005). Bumblebees are less able to discriminate between colour distances of less than 0.07 hexagon units (Dyer 2006; Dyer et al. 2008). However, in the field, bumblebees generalise on colour distances of less than 0.1 hexagon units and only show high levels of flower constancy for colour distances greater than 0.2 hexagon units (Chittka et al. 2001). Therefore, it would be unlikely that the subtle differences found in this study would result in significant changes in bee behaviour of bumblebees, especially in field conditions. Future research should seek to quantify colour variation of flowers grown in the field, as this study found that reflectance spectra of flowers showed greater separation in bee colour space when grown in the field. Future research should also seek to quantify colour variation of corolla tubes in a greater panel of *V. faba* lines. In this study, the corolla tubes were found to be more strongly coloured than other flower parts, which may make flowers more conspicuous. Future work should also explore any variation in the UV patterning of *V. faba* flowers through high resolution UV photography or False colour images. Such techniques have proved a useful tool to visualise flower colour patterns as seen by bees, finding colour

patterning previously undetected by spectrophotometry (Vorobyev et al. 1997; Hempel De Ibarra et al. 2015; Verhoeven et al. 2018; Lunau et al. 2021).

In this study, variation in reward traits was also examined, encompassing nectar volume, nectar sugar concentration, pollen production, and pollen quality. Statistically significant variation was identified between lines for all these traits. A strong positive correlations were present between the nectar concentration of flowers and the total mass of sugar produced and between nectar volume and flower sugar mass, suggesting that, unsurprisingly greater concentration and volume equates to greater overall available amount of sugar for bees. Out of reward traits, use of nectar concentration and volume could provide a worthwhile and achievable way of enhancing plant-pollinator interactions both to support pollinators and enhance crop fitness (Prasifka et al. 2018). Previous research has shown that in field conditions bees prefer flowers with higher energetic reward in pepper, raspberry, watermelon, and onion (Wolf et al. 1999; Silva and Dean 2000; Roldán-Serrano and Guerra-Sanz 2015; Schmidt et al. 2015). However other research suggests that an optimum concentration for bees exists at 55% w/w due to greater viscosity at higher concentrations (Bailes et al. 2018). The same has been shown for hummingbirds, with preference reaching a limit at 55% (Tamm and Gass 1986). The 55% optimum may be due nectar offloading (vomiting) being more strongly affected by liquid viscosity than nectar uptake (Pattrick et al. 2020). One must not forget that temperature affects viscosity and thus, the optimum may move upwards if summer temperatures continue to rise die to climate change (Nicolson et al. 2013). Assuming 55% is the optimum concentration for bees, some *V. faba* lines are already highly optimised in their reward, including LG Cartouche (51.46%) and Fanfare (47.48%). These lines provide a good base to develop future genotypes with enhanced reward. Increasing nectar volume would also be beneficial for pollinator populations. However, plants with high nectar concentration and low volume would provide more energy for bees while encouraging greater movement between flowers and outcrossing. Future work may also look to explore variation in the types of sugars and non-sugar compounds of nectar which have been found to affect pollinator behaviour in other systems (Alm et al. 1990; Wright et al. 2013; Tiedge and Lohaus 2017; Broadhead and Raguso 2021).

The quantity of pollen produced by different *V. faba* lines varied greatly, reflecting the results of Bailes et al. (2018). However, a significant knowledge gap still existed in that the

viability (as a proxy for pollen quality) had never been assessed in commercial *V. faba* lines. This study found that pollen viability varied between lines from 87.4% for Lynx up to 98.7% for Yukon. Viable pollen was defined as pollen with intact cytoplasm, and non-viable pollen as pollen without cytoplasm. Cytoplasm-less pollen grains will not only be incapable of fertilisation but will also provide limited nutrition for bees. In *Mimulus*, bees have been found to discriminate between pollen based on quantity and quality (Robertson et al. 1999). Given that all lines except Lynx produced a high proportion of viable pollen, it may not be an efficient use of time to focus breeding efforts on increasing viability further for the sake of bee attraction. Although more pollen and greater viability would increase the chance of successful fertilisation, nectar traits provide more potential to increase pollinator attraction.

Lastly, variation in access traits between *V. faba* lines was studied, including corolla tube length and the force required to trip flowers. Significant variation in corolla tube length was reported (from 11.38 mm to 13.45 mm) comparable to that reported by Bailes (2016) (12 mm to 16 mm) and Suso et al. (2005) (13 mm). In *V. faba*, corolla tube length has been shown to significantly negatively correlate with outcrossing (Suso et al. 2005). The bee species commonly reported to visit *V. faba* vary in tongue length, with *A. mellifera* having the shortest (maximum 7 mm) and *Bombus hortorum* having the longest (13 mm maximum) (Goulson et al. 2005). Considering the variation found in this study, many bees may be unable to reach the full nectar reward at the back of the corolla tube. Longer corolla tubes may also promote nectar robbing (when a flower visitor obtains nectar without pollinating the flower, imposing a fitness cost on the plant) (Zhang et al. 2007; Irwin et al. 2010). A positive relationship exists between corolla tube length and incidence of robbing in *Polygala vayredae* and *Duranta erecta* (Castro et al. 2009; Navarro and Medel 2009). There are no published studies examining the effect of nectar robbing in *V. faba*, but evidence from other systems suggests that the fitness consequences would be negative. Selective breeding of *V. faba* to reduce corolla tube length could therefore lessen the incentive for nectar robbing. Future studies should better explore whether corolla tube length affects frequency of nectar robbing of *V. faba* flowers, the fitness consequences of nectar robbing in *V. faba,* and if manipulation of this trait can change bee behaviour.

This is the first study to report significant variation in the force required to trip *V. faba* flowers between more than two lines. The force required to trip flowers is likely to affect

how easily bees can access the reward and pollinate the crop. Tripping force varied greatly between lines (from 14.41 mN to 36.07 mN). Córdoba and Cocucci (2011) estimated the strength of honeybees at 26.3 mN meaning flowers of NV129, Albus, BPL10 and Vertigo may be impossible to open for honeybees. Work by Córdoba and Cocucci (2011) suggests that bumblebees should easily be able to trip flowers, but easier to trip flowers may still be more attractive to bumblebees. In alfalfa (*Medicago sativa*), easier to trip flowers set more seed in the field (Knapp and Teuber 1990). In this study, a positive association was present between operative force and corolla tube length (Pearson's r = 0.520). It is possible that corolla tube length or other aspects of floral morphology may affect operative force, as the size of contact surfaces will inevitably create friction. In other systems, weak correlations have been reported between operative force and measures of flower size including wing petal size in the legume *Collaea argentina* (Córdoba et al., 2015). However, no significant correlations have been reported across other legume species (Córdoba and Cocucci, 2011).

One may therefore hypothesise that breeding for easier to trip flowers should increase pollination, however, just as corolla tube length may filter out short-tongued pollinators, operative force of papilionaceous flowers may filter out weak insects and admit stronger, better pollinators (Córdoba and Cocucci 2011). Larger foragers of *B. terrestris* transport more pollen grains (Willmer and Finlayson 2014), and a positive relationship is seen between body size of *Osmia rufia* and oilseed rape yield in caged experiments (Jauker et al. 2016). Other research identifies bumblebees as more effective pollinators than honeybees, as they are hairier and actively collect less pollen, leaving more on their bodies (Willmer et al. 1994). When breeding for reduced flower operative force in *V. faba*, it may prove disadvantageous to attract more honeybees. Instead, breeding should aim to reduce work for bumblebees, so that they save energy and are discouraged from nectar robbing.

Conclusions

The results presented in this chapter have revealed that substantial variation exists in both reward and non-reward traits between previously uncharacterised *V. faba* lines. This study has identified nectar volume and concentration as promising traits that could be used to both enhance bee health and attractiveness of *V. faba* flowers. Size of floral display is also likely to increase attractiveness of *V. faba* plants through appearance and total reward

volume. Colour and patterning vary little between the lines examined, and more work is needed to properly quantify the variation present between *V. faba* lines in field conditions and explore the impact of variation in appearance on bee behaviour. This is the first study to quantify *V. faba* flower operative force and the number of flowers produced per node on a large scale. The influence of *V. faba* floral trait variation on bee visitation and *V. faba* yield is explored in Chapter 4. The effect of variation in specific floral traits on bee behaviour is explored in controlled conditions in Chapter 5.

In this study, LG Cartouche, Fanfare, Tundra and Lynx were identified as lines with superior nectar traits, providing the best energetic reward for bees. LG Cartouche and Fanfare require least force to open, but the greater force required for Tundra and Lynx may be a beneficial filter against ineffective pollinators. LG Cartouche and Tundra have shorter corolla tubes providing easier to access rewards for bees. These *V. faba* lines should be a focus of future research as they are likely to be more beneficial and attractive to pollinators.

4 Effects of floral trait variation on pollinator preference in the field and effect of pollinator exclusion on *V. faba* yield

4.1 Introduction

In Chapter 3, significant variation in multiple floral traits between *V. faba* lines was presented. Using the information gathered in Chapter 3 and data collected by (Bailes 2016), lines were selected which show contrasting floral characteristics. These lines were grown in side-by-side field plots to determine whether wild bee preference is influenced by floral trait variation. The effect of pollinator exclusion on multiple yield parameters was also investigated to determine the yield benefit of insect pollination.

The most common pollinators of *V. faba* are long-tongued bumblebees, including the buff tailed bumblebee, *Bombus terrestris* (Hanna and Lawes 1967; Garratt et al. 2014). The mechanisms of pollination of *V. faba* have been studied multiple times, as well as the effect of insect pollination on yield at a field scale (Riedel and Wort 1960; Bishop and Nakagawa 2020). However, preferences of bees between different lines of *V. faba* have never been studied in the field. All previous work studying *V. faba* pollinators at field scales have explored the influence of landscape complexity, flower margins, or the cultivation of *V. faba* on bee abundance and diversity in landscapes (Nayak et al. 2015; Beyer et al. 2020; Raderschall et al. 2022). This leaves a knowledge gap, both in what effect floral trait variation has on bee behaviour in agricultural settings, and in what variation in pollinator-attracting floral traits might mean for crop yield. In addition, there is no standard method for quantifying bee preferences at this scale in an agricultural setting.

Pollinator preferences in field conditions have been explored for some plant species. Honeybees prefer field plots of *Agastache* and *Pycnanthemum* over those of *Monarda* and *Salvia,* and hybrid varieties of *Agastache* attract more bees than other varieties (Wildrechner 1990). These findings are based on “counts” of bees in plots, to establish good forage plants for honeybees, however, methodological detail is lacking. In strawberries, mason bees (*Osmia bicornis*) prefer flowers of one variety, Sonata, over flowers of Honeoye in German strawberry fields, based on bee counts along transect walks (Klatt et al. 2013a). Other field

studies do not investigate bee preference on a scale directly comparable to the work carried out in this project. Many studies examine pollinator behaviour in natural habitats, finding preferences of flies between morphotypes of *Gorteria diffusa* (Ellis and Johnson 2009), preference of bees and hummingbirds between genotypes of *Mimulus lewisii* (Schemske and Bradshaw 1999), and preference of butterflies between *Phlox* species (Briggs et al. 2018). However, these approaches often use single or small clusters of plants, not easily comparable to the mass flower display of *V. faba* crops.

Despite there being few studies which examine bee preference at large scales, bee abundance has been explored in agricultural settings. Within these studies, transect walks are most commonly used to quantify pollinator abundance across multiple plants. For example, Lundin and Raderschall (2021) and Raderschall et al. (2021), found that transect walks were successful in quantifying bee abundance and bee behaviour in fields of *V. faba*, to explore effects of landscape complexity on bee abundance and behaviour. An earlier study by Nayak et al. (2015) used transects in a similar way to quantify bee abundance in *V. faba* fields, in conjunction with pan traps, allowing estimation of total species richness, despite not allowing for behaviour to be noted. Other published works detail the use of transect walks to estimate bee abundance in crops of *V. faba* (Cunningham and le Feuvre 2013; Raderschall et al. 2022), courgette (Knapp et al. 2019), oilseed rape (Lindström et al. 2016b), and cereal crops with wildflower strips (Geppert et al. 2020).

A minority of studies use alternative methods, including pan traps and sweep nets to estimate bee abundance in fields of oilseed rape (Perrot et al. 2018). One study reports use of bee counts within $1m^2$ and $10m^2$ observation plots of oilseed rape to estimate bee abundance, but does not state the duration of observation (Karise et al. 2007). Other attempts to estimate bee abundance and behaviour have used time lapse cameras on single plants, consequently obtaining a very small amount of data compared to other field studies (Smith-Ramírez et al. 2021).

The effects of pollination on yield in *V. faba* are better studied, with data published for multiple lines in various experimental designs. As a result, estimates for the yield benefit of insect pollination vary widely. A recent meta-analysis by Bishop and Nakagawa (2020) identified 22 papers that investigate the effects of pollination treatment on faba bean yield. Estimates of yield mass reduction when pollinators are excluded range from 6% (Bishop et al. 2020) to 60% (Varis and Brax 1990), bean number reduction from 5% (Suso and del Río 2015) to 61% (Varis and Brax 1990), pod number between 1% (Bishop et al. 2020) and 49% (Varis and Brax 1990) and number of beans per pod between 7% (Kendall and Smith 1975) and 14% (Varis and Brax 1990). Using their multi-level meta-analysis, Bishop and Nagawa (2020) conclude that, from currently published data, faba beans lose on average 32.9% of yield without biotic pollination and calculate that there is an 80% probability that a given farmer will see a yield benefit from biotic pollination for a *V. faba* crop. Most variation in pollinator dependence is due to genotype. Multiple works, including Bishop and Nakagawa (2020) and Lundin and Raderschall (2021), state that there is a clear need for more studies which compare the effects of pollinator exclusion across multiple *V. faba* genotypes. Without doing so, we currently have an incomplete view of pollinator dependency of the *V. faba* crop using data from multiple genotypes which are difficult to compare between studies. Understanding pollinator dependency of *V. faba* will add weight to the need to support wild pollinator populations but can also help tailor lines to environments with different pollination service capacities, whereby lines with lower dependence can still be grown in landscapes with damaged pollinator populations.

In this chapter results are presented from three field seasons: 2020, 2021 and 2022. In 2020 a pilot study was carried out to develop methodology, and compared bee visitation of two *V. faba* lines, Maris Bead and NV129. Using methodology developed in 2020, a larger trial was carried out in 2021 comparing bee visitation between five lines, Fuego, Maris Bead, NV100, NV129 and Tiffany. In 2022, another field trial compared bee visitation rate between six lines, Fuego, Lynx, Maris Bead, Tiffany, Vertigo and Yukon. Yield comparison experiments were carried out in 2021 and 2022, comparing seed set of lines when open pollinated, and when pollinators were excluded.

4.2 Results

4.2.1 A pilot study to compare bee visitation

Evaluation of data collection methods

Two experiments were compared to evaluate the most useful measure of bee preference: plot walks, and continuous observation. For methodology refer to section 2.8.

Plot walks over all replicates, once every 15 minutes, allowed a greater number of replicates to be covered by a single person. However, bees visiting plants outside the plot walk could not be observed and bees were disturbed by walking and flew away before accurate observations of behaviour or species could be made. Continuous observation allowed accurate recording of all bees entering the plots, without bees being disturbed. However, only one replicate could be observed by one person.

Using continuous observation, three types of data were contrasted to evaluate their capability to measure bee preference in the field. Total number of bees entering plots within 15-minute observation windows allowed accurate observation of all visitors, providing a relatively large amount of data. Number of flowers visited by individual bees provided a different measure of bee preference, on an individual scale, comparable to bee experiments in controlled conditions (see chapter 5). Recording the time that individual bees spent on flowers could be a useful means of comparing flower handling time between *V. faba* lines. All three data types required intense concentration, and only allowed for one type of data to be collected at a time. For both number of flowers visited by individual bees, and time that individual bees spent on flowers, only one bee could be observed at a time, and a small amount of data could be collected.

Bee activity

Continuous observations were made from 09:00 hrs to 19:30 hrs on the 12th of June and from 06:00 hrs to 22:00 hrs on the 13th of June, to evaluate methodology and establish when bees were active. Bees were active continuously from 09:00 hrs to 19:30 hrs on the 12th of June (**Figure 4.1A**). The sampling period was extended on the 13th of June from 06:00 hrs to 22:00 hrs. On the 13th of June, bees were most active between 08:00 hrs and 20:00 hrs (**Figure 4.1B**). Across both days, there was consistently more bee activity in the plot of Maris Bead compared to the plot of NV129.

Bee visitation

For two days, behaviour of bees visiting plots was recorded as legitimate, robbing, extra-floral nectary visits or searching. Plots containing Maris Bead received more legitimate, nectar robbing and searching visits than plots containing NV129. The rate of extrafloral nectary visits did not differ greatly between plots of Maris Bead and NV129 (**Figure 4.2**).

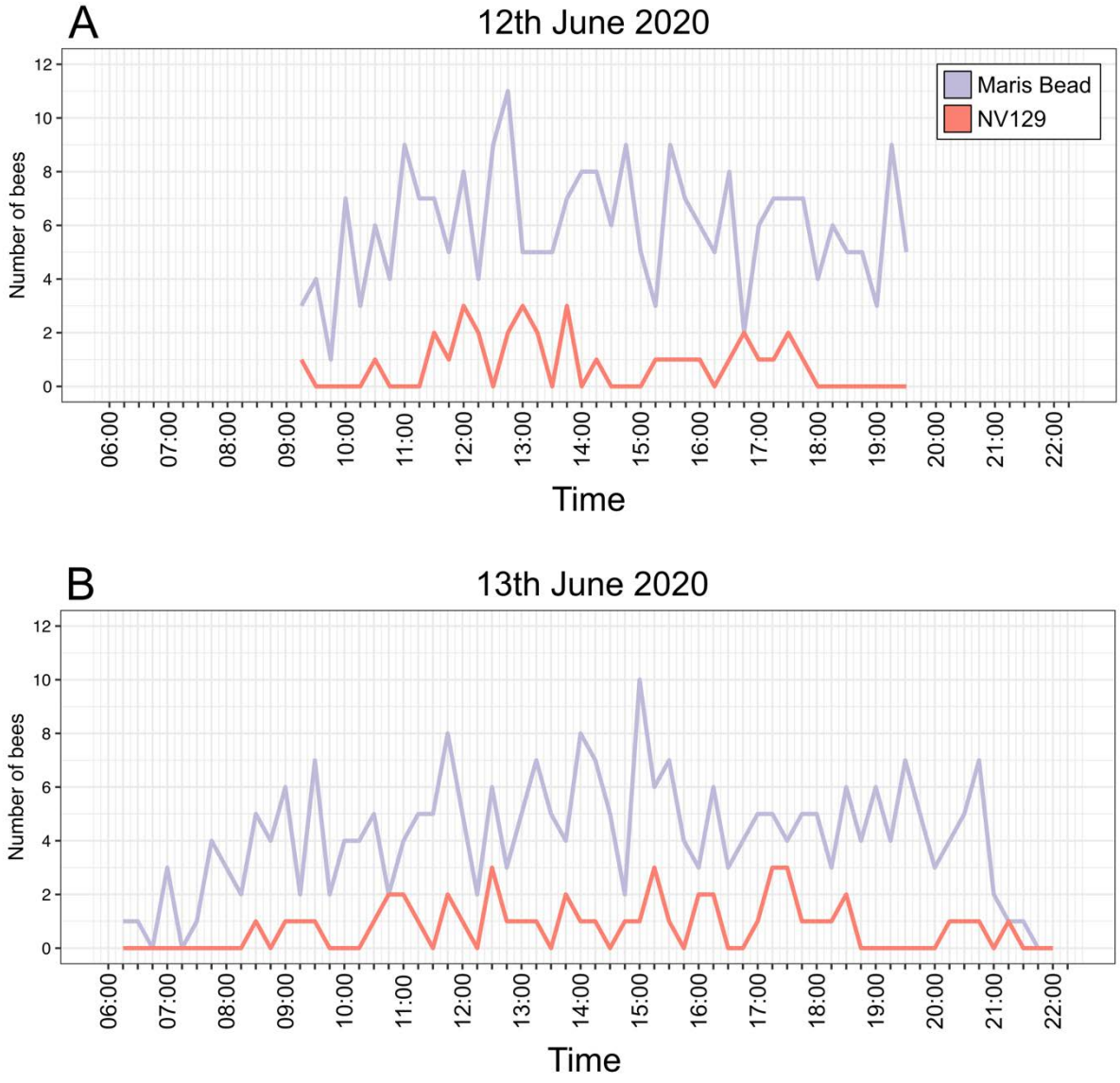


Figure 4.1 Bee activity over the course of a day in a single plot of Maris Bead and a single plot of NV129 in 2020. Each day was divided into 15-minute observation windows, within which the number of bees seen in each plot was recorded. In this instance, bees are defined as honeybees and bumblebees. **(A)** Bee activity from 09:00 hrs to 19:30 hrs on 12th June 2020. Bees were active continuously over the entire observation period. **(B)** Bee activity from 06:00 hrs to 22:00 hrs on 13th June 2020. Bees were most active between 08:00 and 21:00 hrs. On both days more bee activity was observed in the plot of Maris Bead compared to the plot of NV129.

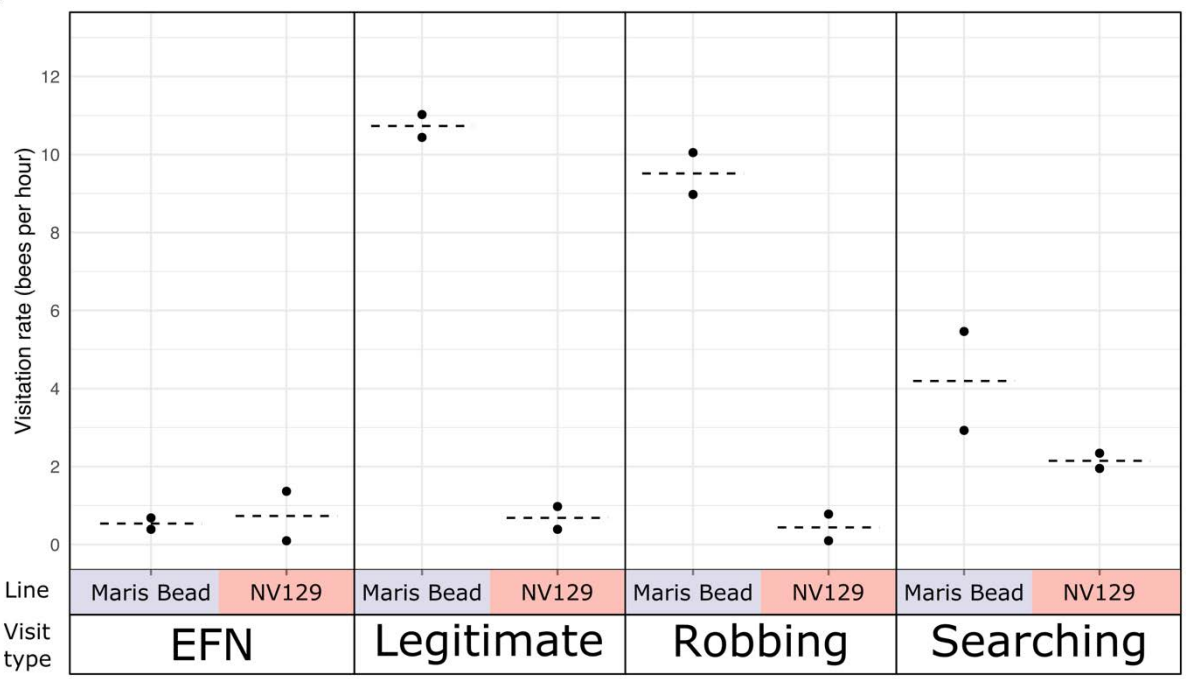


Figure 4.2 Bee visitation rate to plots of Maris Bead and NV129 on 12th and 13th of June 2020. Dotted lines represent mean values between the two days. Plots containing Maris Bead received more legitimate, nectar robbing and searching visits than plots containing NV129. The rate of extrafloral nectary (EFN) visits did not differ greatly between plots of Maris Bead and NV129.

4.2.2 Pilot study outcomes

The pilot study showed that of the two experimental methods compared, continuous observation was most feasible and provided the most accurate way of recording pollinator activity in the field, unlike plot walks. As the overall number of bees entering a 4 m by 1.5 m plot was low (no greater than 10.73 bees per hour on average for continuous observation, **Figure 4.2**), the volume of data generated by plot walks was too small to provide useful insights. This contrasts with most studies, which have used transect walks to estimate bee abundance and behaviour (Cunningham and le Feuvre 2013; Nayak et al. 2015; Raderschall et al. 2022) and is most like the methods of (Wildrechner 1990) and (Karise et al. 2007) in which plot counts of bees were used to study bee abundance. Studies which employ plot walks generate a relatively small amount of data. For the purposes of this work, continuous observation provided a much more comprehensive view of insect visitors to *V. faba* plots.

Of the three types of data collected using continuous observation, the number of individual bees entering the plot, alongside bee type and behaviour was most achievable and informative. Recording the number of flowers visited by individuals, and the time spent on flowers, produced a small amount of data and although could provide information on preference of individual bees, failed to capture wild bee preference on a large scale, which was the objective of fieldwork. Time spent on flowers could provide information on handling time, however, bees tended to hop between flowers making accurate recording unfeasible. The utility of using handling time to increase pollination and yield is also questionable, when compared to other floral traits like reward, colour, and number of flowers.

Continuous observations were made from 09:00 hrs to 19:30 hrs on the 12th of June and from 06:00 hrs to 22:00 hrs on the 13th of June to establish when bees were most active, in order to select appropriate observation periods in future field experiments. There were no substantial peaks or lulls in activity between 08:00 hrs and 20:00 hrs. Observations made anytime between 08:00 hrs and 20:00 hrs ought to be representative of bee activity.

Across both the 12th and 13th of June, bee activity was consistently greater in plots of Maris Bead compared to NV129 (**Figure 4.1**). The rate of legitimate, nectar robbing and searching visits was greater in plots of Maris Bead compared to NV129, with the largest difference in bee visitation seen for legitimate visits (Maris Bead mean = 10.73 bees per hour, NV129 mean = 0.68 bees per hour) (**Figure 4.2**). These differences indicate a strong preference for Maris Bead over NV129, suggesting that differences in floral traits affect wild bee attraction. Based on these results, Maris Bead and NV129 were compared again on a larger scale, alongside additional lines, in the 2021 field trial.

4.2.3 Bee visitation to Vicia faba lines in 2021

Pollinators observed in the field

When recording bee visitation rate in the field, bees were recorded under categories, as opposed to species, due to difficulty identifying to species level by volunteers. For methodology refer to section 2.8.

To verify which species were likely to have been recorded in each category by volunteers, bees were captured from the field margin and identified to species level (**Figure 4.3**).

- **White-tailed bumblebees:** The species recorded in this category were *Bombus terrestris/ lucorum*, and *Bombus hortorum*. *Bombus hypnorum* and *Bombus barbatellus* were also seen, but were rare. It was not possible to distinguish between *Bombus terrestris* and *Bombus lucorum*, and so they were recorded as *Bombus terrestris/ lucorum* .
- **Red-tailed bumblebees:** The species recorded in this category were *Bombus lapidarius* and *Bombus pratorum*.
- **Carder bees:** One species of carder bee was observed and identified as *Bombus pascuorum*.
- **Honeybees:** One species of honeybee (*Apis mellifera)* was observed.

A black bumblebee was identified as a melanic form of *Bombus ruderatus* but was not observed visiting field plots in 2021.

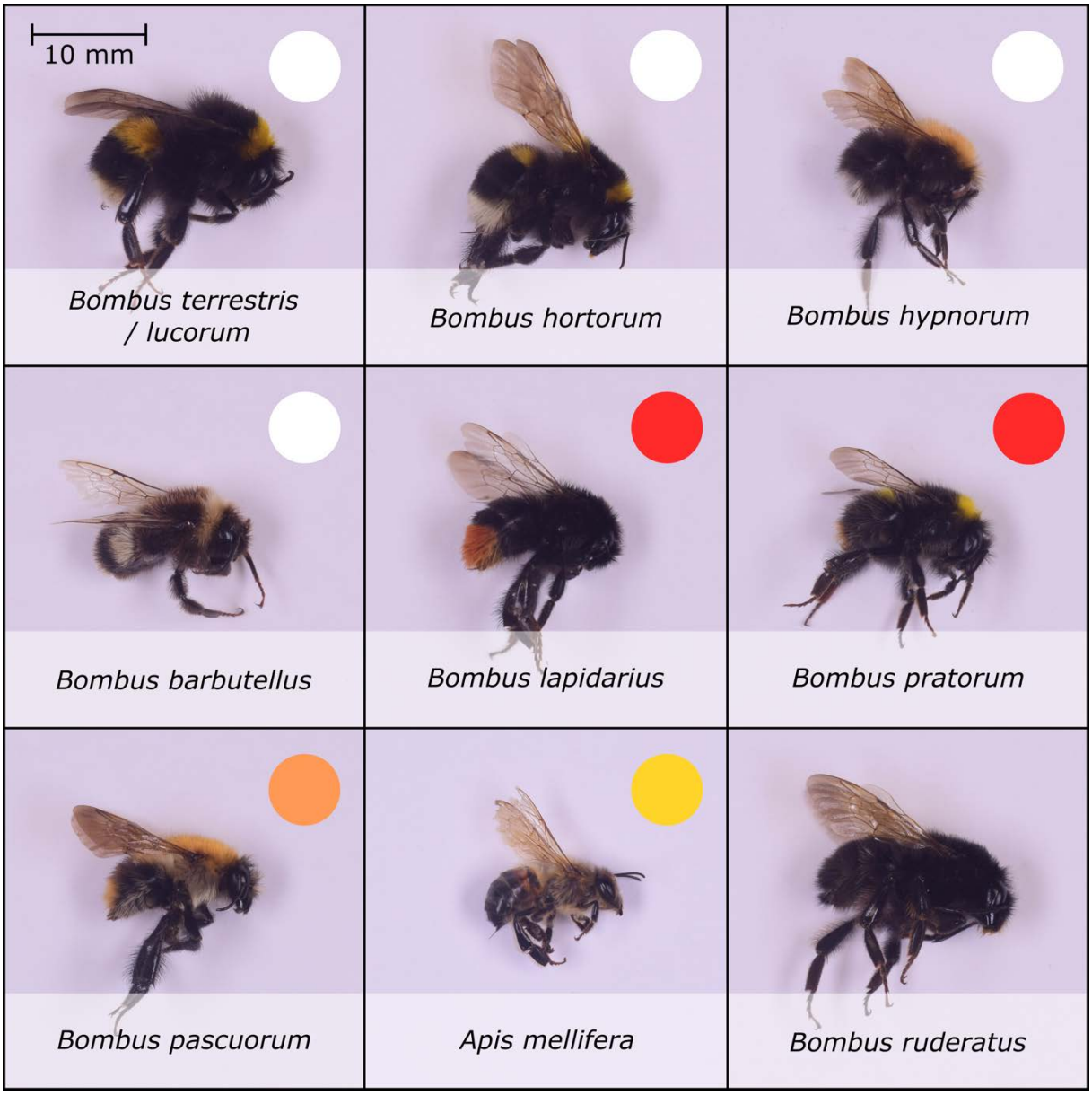


Figure 4.3 Bee species present in the field margin in 2021. The species of bee observed in the field margin, and the bee category they were recorded as during field observations. Nine species were observed, four species were recorded as "white-tailed bumblebees" (white circle), two species were recorded as "red-tailed bumblebees" (red circle), one species was recorded as a "carder bee" (orange circle), and one species was recorded as a "honeybee" (yellow circle). A black bumblebee was identified as a melanic form of *Bombus ruderatus* but was not observed in *V. faba* plots in 2021. *Bombus terrestris* and *Bombus lucorum* are categorised together, as workers of the two species are challenging to distinguish.

Bee visitation rate to *V. faba* lines

The number of bees visiting field plots was recorded on the 16th, 19th, 22nd, 23rd and 24th of June 2021. Plots were observed for a total of 202 hours and 901 visits by bees were noted in that time. For each day of observations, the mean number of bee visits per hour was calculated for each plot observed.

Total bee visitation

The total bee visitation rate in bees per hour for each *V. faba* line was calculated using visits made by all bee types (honeybee, carder bee, red-tailed bumblebee, and white-tailed bumblebee) for all behaviours (legitimate, robbing, extra-floral nectary visits and searching) (**Figure 4.4**). Mean visitation rate ranged from 8.83 bees per hour for Maris Bead to 1.45 bees per hour for NV129. A one-way ANOVA revealed a statistically significant difference in mean bee visitation rate between at least two of the *V. faba* lines compared ($F(4,47) = [6.518]$, $p = 0.00029$). A post hoc Tukey's HSD test for multiple comparisons found that the mean bee visitation rate for plots of Maris Bead was significantly different to every other line (Maris Bead – Tiffany $p = 0.03$, 95% CI = -0.21, -7.38). A Tukey's HSD test found that the mean bee visitation rate was not significantly different between plots of Fuego, NV100 and NV129 (Fuego – NV129 $p = 0.73$, 95% CI = 2.52, -6.4), (NV100 – NV129 $p = 0.67$, 95% CI = 2.37, -6.55). However, the mean bee visitation rate was significantly different between plots of Tiffany and NV129 (Tiffany – NV129 $p = 0.04$, 95% CI = 7.27, 0.104).

Bee visitation rate to the two plots marked out in the planted wildflower strip was significantly higher than to any *V. faba* line (**Figure 4.5**). The mean visitation rate to wildflower plots was 70.15 bees per hour, whereas the mean visitation rate to plots of Maris Bead was 8.83 bees per hour. Following a significant one-way ANOVA result, a post hoc Tukey test showed that the mean rate of bees visiting wildflower plots was significantly higher than to any *V. faba* plot (Wildflower – Maris Bead $p = 0.000$, 95% CI = 8.77, -12.95). For a full breakdown of bee visitation rates see Appendix F.

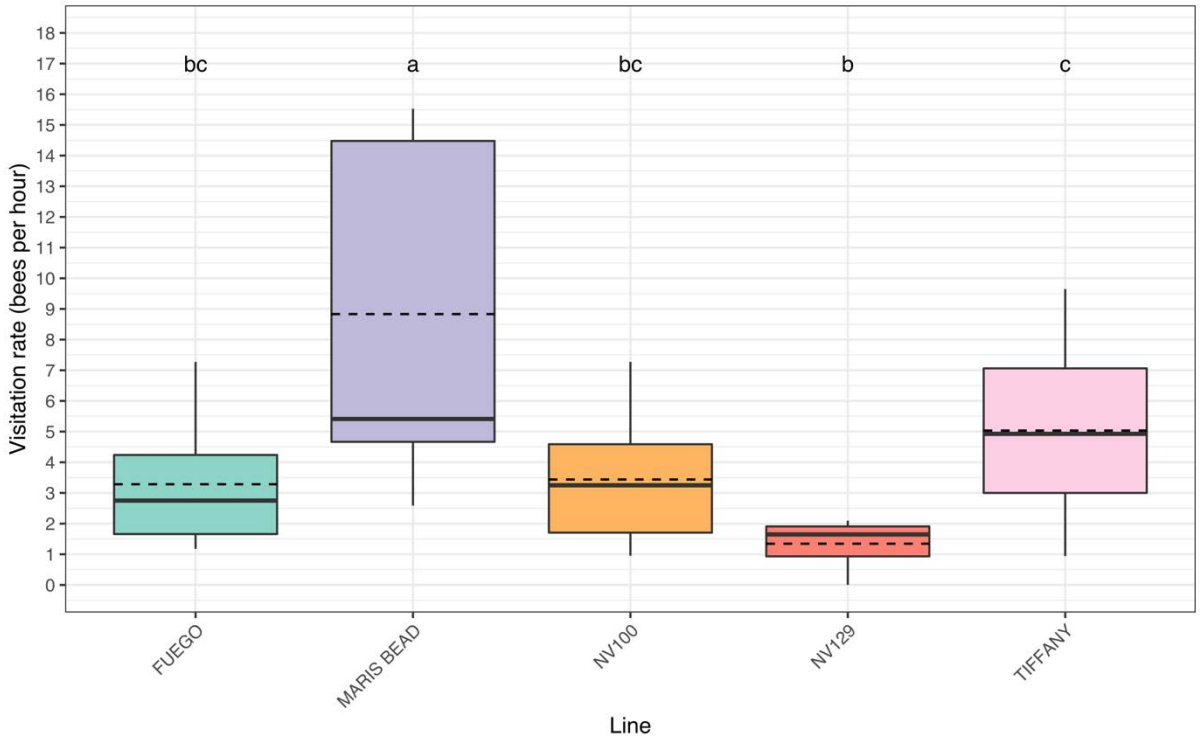


Figure 4.4 Visitation rate of bees to *V. faba* lines in 2021 (all bee types, all behaviours). Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the IQR). Dashed lines show means and solid lines show medians. Letters show Tukey significance groups. *V. faba* lines which do not share a letter have significantly different means at $p \leq 0.05$. Mean visitation rate ranged from 8.83 bees per hour for Maris Bead to 1.45 for NV129. A one-way ANOVA revealed a statistically significant difference in bee visitation rate between at least two of the *V. faba* lines compared at $p \leq 0.0005$. A post hoc Tukey test showed that the rate of bees visiting plots of Maris Bead was significantly higher than any other line at $p \leq 0.05$. The rate of bees visiting plots of Tiffany was significantly higher than plots of NV129, but not those of Fuego or NV100 at $p \leq 0.05$.

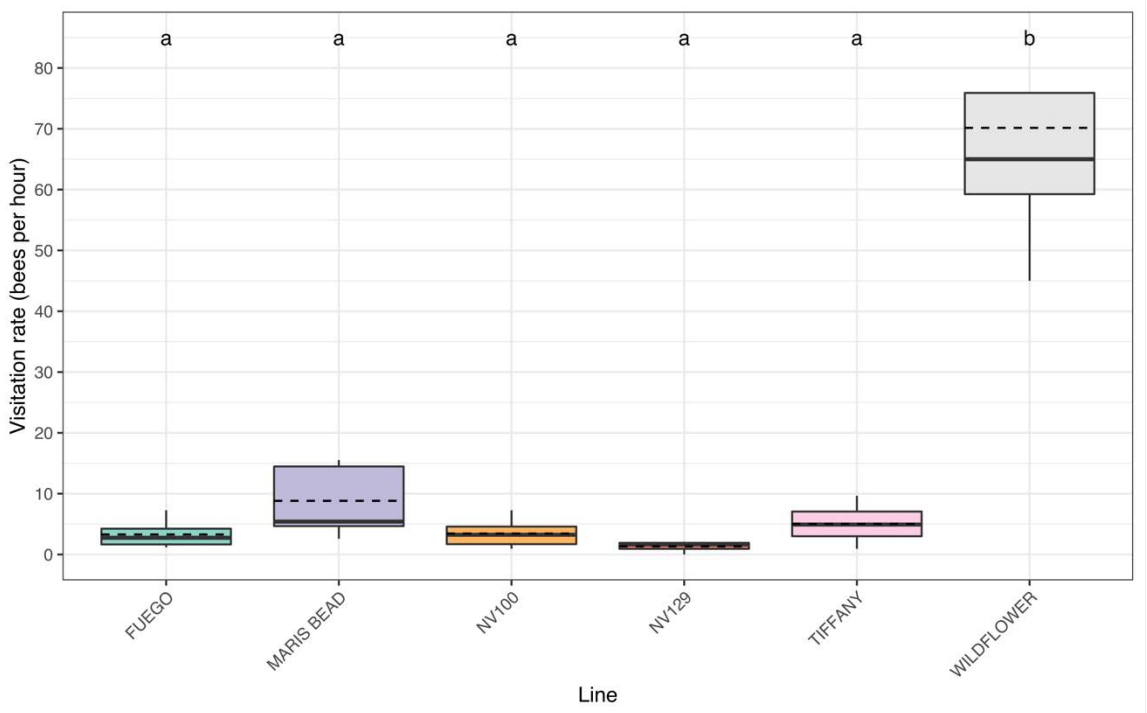


Figure 4.5 Visitation rate of bees to *V. faba* lines in 2021 including wildflower strip (all bee types, all behaviours). Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the IQR). Dashed lines show means and solid lines show medians. Letters show Tukey significance groups. *V. faba* lines which do not share a letter have significantly different means at $p \leq 0.05$. The mean visitation rate to wildflower plots was 70.15 bees per hour, whereas the mean visitation rate to plots of Maris Bead was 8.83 bees per hour. Following a significant one-way ANOVA result, a post hoc Tukey test showed that the rate of bees visiting wildflower plots was significantly higher than to any *V. faba* plot at $p \leq 0.0001$.

Bee visitation rate by visit type

For each bee observed in trial plots, the type of visit made was recorded. For every behaviour (legitimate, robbing, extra-floral nectary visits and searching), Maris Bead received the greatest mean rate of visits (**Figure 4.6**). Overall, legitimate visits were the most frequent behaviour observed across all lines (mean = 2.25 bees per hour) followed by robbing visits (mean = 1.01 bees per hour).

Extra-floral nectary visits (EFN)

Plots of Maris Bead received the greatest frequency of extra-floral nectary (EFN) visits (mean = 1.05 bees per hour), double that of any other line (**Figure 4.6**). A one-way ANOVA revealed a statistically significant difference in the frequency of extra-floral nectary visits between at least two of the *V. faba* lines compared ($F(4,47) = [3.065]$, $p = 0.0253$). A post hoc Tukey's HSD test for multiple comparisons found that the mean extra-floral nectary visitation rate for plots of Maris Bead was significantly different to plots of Fuego and NV129 (Maris Bead – NV129 $p = 0.0468$, 95% CI = -0.00852, -1.80).

Legitimate visits

Plots of Maris Bead received the greatest frequency of legitimate visits (mean = 4.42), followed by Tiffany (mean = 2.34) and NV100 (mean = 2.14) (**Figure 4.6**). A one-way ANOVA revealed that there was a statistically significant difference in the frequency of legitimate visits between at least two of the *V. faba* lines compared ($F(4,47) = [4.358]$, $p = 0.00444$). A post hoc Tukey's HSD test for multiple comparisons found that the mean legitimate visitation rate for plots of Maris Bead was significantly different to plots of Fuego and NV129 (Maris Bead – NV129 $p = 0.00204$, 95% CI = -1.11, -6.54).

Nectar robbing visits

Plots of Maris Bead again received the greatest frequency of nectar robbing visits (mean = 1.81), followed by Tiffany (mean = 1.38) (**Figure 4.6**). A one-way ANOVA revealed that there was a statistically significant difference in the frequency of nectar robbing visits between at least two of the *V. faba* lines compared ($F(4,47) = [9.068]$, $p = 1.63 \times 10^{-5}$). A post hoc Tukey's HSD test for multiple comparisons found that the mean nectar robbing visitation rates for

plots of Maris Bead and plots of Tiffany were significantly different to plots of Fuego, NV100 and NV129 (Tiffany – Fuego p = 0.0434, 95% CI = 1.74, 0.0176).

Searching

Plots of Maris Bead received the greatest frequency of bees searching (mean = 1.56), followed by Fuego (mean = 1.15) (**Figure 4.6**). A one-way ANOVA revealed that there was no statistically significant difference in the frequency of searching between the *V. faba* lines compared (F(4,47) = [1.503], p = 0.217).

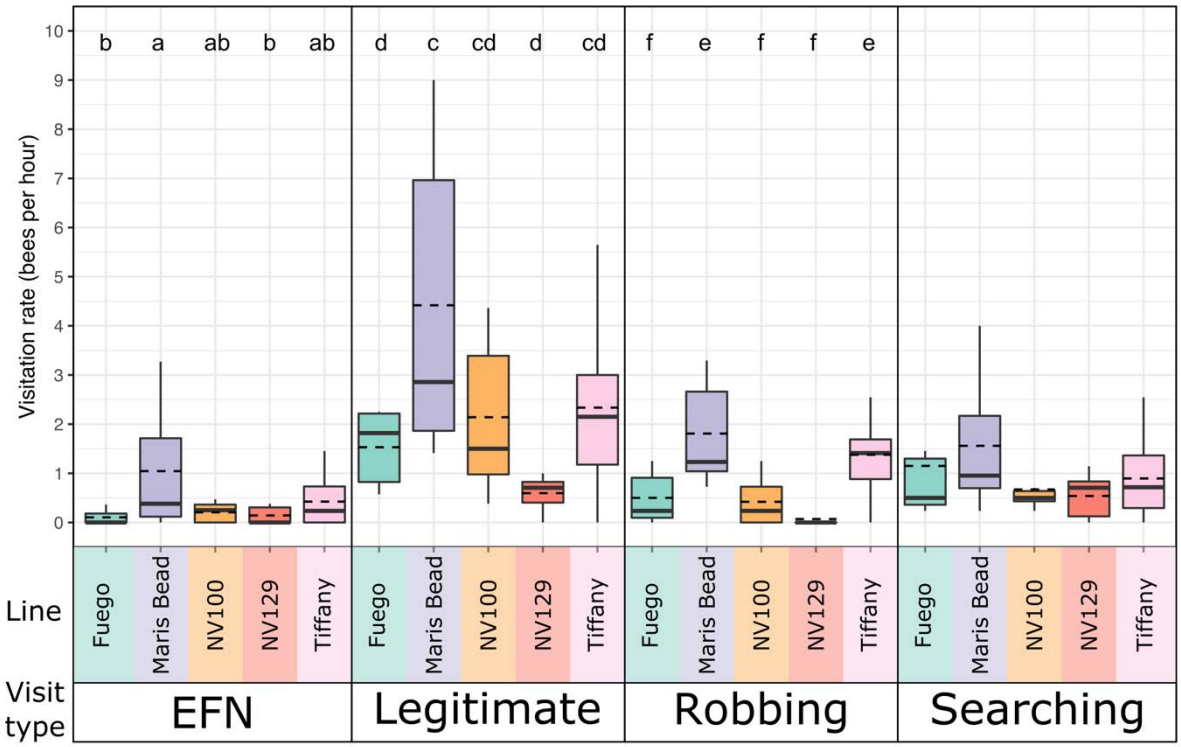


Figure 4.6 Visitation rate of bees to *V. faba* lines in 2021 behaviour breakdown. Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the IQR). Dashed lines show means and solid lines show medians. Letters show Tukey significance groups. *V. faba* lines which do not share a letter have significantly different means at $p \leq 0.05$. For all lines except NV129, most visits made by bees were legitimate. One-way ANOVAs were done within visit type groups. Following a significant ANOVA result, post hoc Tukey's HSD tests for multiple comparisons were done within visit type groups. A post hoc Tukey test showed that the rate of extra-floral nectary (EFN) visits to Maris Bead and Tiffany was greater than that to Fuego and NV129 at $p \leq 0.05$. Similarly, the rate of legitimate visits to Maris Bead was significantly greater than that to Fuego and NV129, but not to Tiffany and NV100. Maris Bead and Tiffany experienced a significantly higher rate of robbing visits than the other lines. A one-way ANOVA revealed that there was no statistically significant difference in the frequency of searching between the *V. faba* lines compared at $p \leq 0.05$.

The proportion of bees carrying out visit types

White-tailed bumblebees

White-tailed bumblebees were most frequently observed making legitimate visits overall, followed by robbing visits (**Figure 4.7**). The frequency of white-tailed bumblebees making legitimate visits was greatest in plots of Maris Bead (2.46 bees per hour) and lowest in plots of NV129 (0.18 bees per hour). Nectar robbing was the second most common behaviour seen for white-tailed bumblebees, with Tiffany and Maris Bead receiving most robbing visits (0.91 and 0.89 bees per hour respectively), and NV129 the least (0.07 bees per hour). White-tailed bumblebees were also seen searching most frequently in plots of Maris Bead (0.68 bees per hour). White-tailed bumblebees were rarely observed making extra-floral nectary visits.

Honeybees

Honeybees were most frequently observed searching and making legitimate visits (**Figure 4.7**). Maris Bead attracted the greatest frequency of honeybees for extra-floral nectary visits (0.46 bees per hour), and Tiffany and Maris Bead attracted the greatest frequency of honeybees for legitimate visits (0.51 and 0.39 bees per hour respectively).

Carder bees

Carder bees were most frequently observed making legitimate visits and were the second most common type of bee seen making legitimate visits after white-tailed bumblebees (**Figure 4.7**). Maris Bead and NV100 received the highest rates of legitimate visits by carder bees (1.25 and 1.03 bees per hour respectively) and NV129 received the lowest rate (0.29 bees per hour).

Red-tailed bumblebees

Red-tailed bumblebees were most frequently observed making legitimate visits and nectar robbing visits and were the second most common type of bee seen making nectar robbing visits after white-tailed bumblebees (**Figure 4.7**). Maris Bead attracted the highest rate of nectar robbing visits by red-tailed bumblebees (0.61 bees per hour) and NV129 received the lowest rate with no bees.

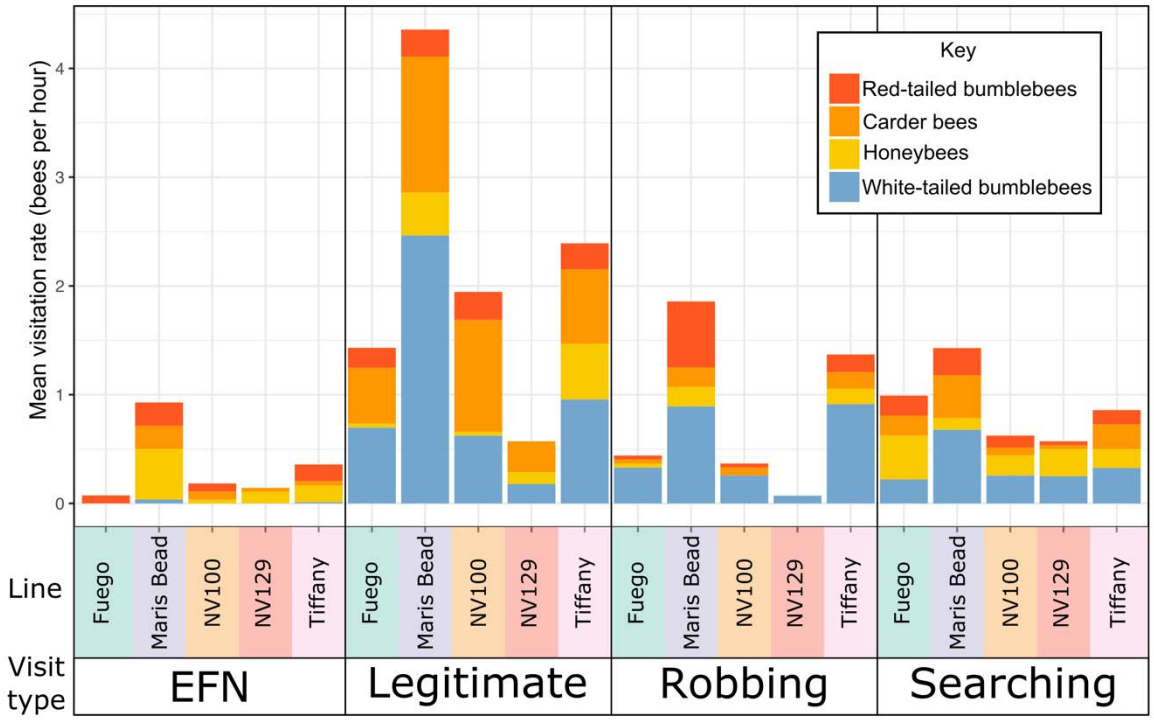


Figure 4.7 Mean visitation rate of bees to *V. faba* lines in 2021 with proportion of bees performing each behaviour. White-tailed bumblebees were most frequently observed making legitimate visits overall, followed by robbing visits. White-tailed bumblebees making legitimate visits were most often observed in plots of Maris Bead compared to other lines. Honeybees were most frequently observed searching and making legitimate visits. Tiffany and Maris Bead attracted the greatest frequency of honeybees for legitimate visits. Carder bees were most frequently observed making legitimate visits and were most observed making legitimate visits in plots of Maris Bead and NV100. Red-tailed bumblebees were most frequently observed making legitimate visits and nectar robbing visits. Red-tailed bumblebees were most often observed making robbing visits in plots of Maris Bead.

4.2.4 Bee visitation to *Vicia faba* lines in 2022

Pollinators observed in the field

When recording bee visitation rate in the field, bees were recorded under categories, as opposed to species, due to difficulty identifying to species level by volunteers. For methodology refer to section 2.8.

To verify which species were likely to have been recorded in each category by volunteers, bees were captured from the field margin and identified to species level. Two species fell into the category of "white-tailed bumblebees", these were *Bombus terrestris/lucorum* and *Bombus hortorum*. Two species fell into the category of "red-tailed bumblebees", these were *Bombus lapidarius* and *Bombus pratorum*). One species of "carder bee" was identified, *Bombus pascuorum*), and one species of honeybee was identified, *Apis mellifera*. A melanic form of *Bombus ruderatus* was identified and recorded twice in trial plots. In bee visitation data, *Bombus ruderatus* was classed as a "white-tailed bumblebee".

Bee visitation rate to *V. faba* lines in 2022

The number of bees visiting field plots were recorded on the 2nd, 7th, 8th, 9th, and 10th of June 2022. Plots were observed for a total of 328 hours and 3623 visits by bees were noted in that time. For each day of observations, the mean number of bee visits per hour was calculated for each plot.

Total bee visitation

The total bee visitation rate for each *V. faba* line was calculated including visits made by all bee types (honeybee, carder bee, red-tailed bumblebee, and white-tailed bumblebee) for all behaviours (legitimate, robbing, extra-floral nectary visits and searching) (**Figure 4.8**). Lynx received the highest frequency of visits (mean = 14.6 bees per hour), followed by Maris Bead (mean = 14.4 bees per hour). Fuego received the fewest visits (mean = 4.55 bees per hour) followed by Yukon (mean = 7.10 bees per hour). A one-way ANOVA revealed that there was a statistically significant difference in bee visitation rate between at least two of the *V. faba*

lines compared at $p \leq 0.05$. A post hoc Tukey's HSD test for multiple comparisons found that the mean bee visitation rate for plots of Lynx and Maris Bead were significantly different to plots of Fuego and Yukon (Maris Bead – Yukon p = 0.0244, 95% CI = -0.614, -14.1). A Tukey test found that the mean bee visitation rate was not significantly different between plots of Fuego, Tiffany, Vertigo and Yukon (Vertigo – Fuego p = 0.0918, 95% CI = 15.3, -0.675).

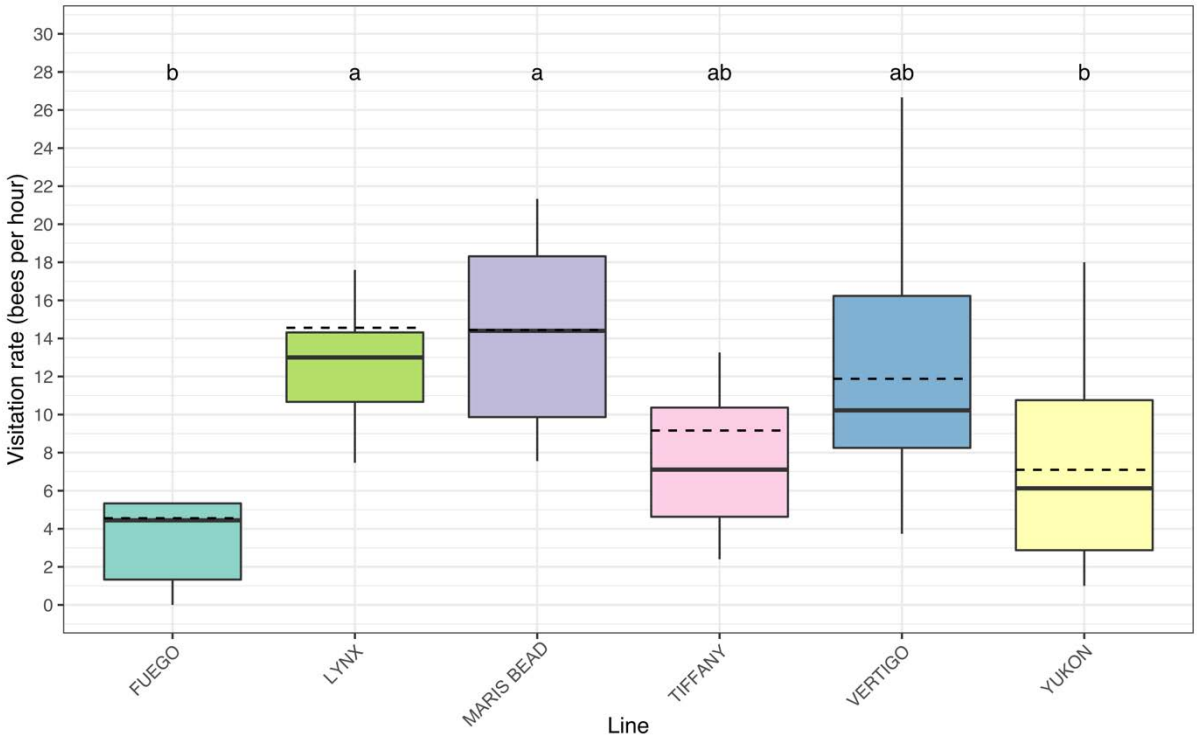


Figure 4.8 Visitation rate of bees to *V. faba* lines in 2022 (all bee types, all behaviours). Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the IQR). Dashed lines show means and solid lines show medians. Letters show Tukey significance groups. *V. faba* lines which do not share a letter have significantly different means at $p \leq 0.05$. A one-way ANOVA revealed that there was a statistically significant difference in bee visitation rate between at least two of the *V. faba* lines compared at $p \leq 0.05$. A post hoc Tukey's HSD test for multiple comparisons found that the mean bee visitation rate for plots of Lynx and Maris Bead were significantly different to plots of Fuego and Yukon at $p \leq 0.05$.

Bee visitation rate by visit type

For each bee observed in trial plots, the type of visit made was recorded. Overall, nectar robbing was the most frequent behaviour observed (mean = 4.5 bees per hour) followed by extrafloral nectary visits (mean = 2.23 bees per hour) and legitimate visits (mean = 2.22 bees per hour). For a full breakdown of bee visitation rates see Appendix G.

Extra-floral nectary visits (EFN)

Plots of Maris Bead received the greatest frequency of extra-floral nectary visits (mean = 3.38 bees per hour), followed by Lynx (mean = 3.03 bees per hour). Fuego attracted the lowest visitation rate (mean = 0.718 bees per hour) (**Figure 4.9**). A one-way ANOVA revealed that there was a statistically significant difference in the frequency of extra-floral nectary visits between at least two of the *V. faba* lines compared at $p \leq 0.05$. A post hoc Tukey's HSD test for multiple comparisons found that the mean extra-floral nectary visitation rate for plots of Maris Bead was significantly different to plots of Fuego (Maris Bead – Fuego p = 0.0227, 95% CI = 5.09, 0.242).

Legitimate visits

Plots of Maris Bead received the greatest frequency of legitimate visits (mean = 4.54 bees per hour), followed by Tiffany (mean = 2.42 bees per hour). Fuego attracted the lowest visitation rate (mean = 0.496 bees per hour) (**Figure 4.9**). A one-way ANOVA revealed that there was a statistically significant difference in the frequency of legitimate visits between at least two of the *V. faba* lines compared at $p \leq 0.05$. A post hoc Tukey's HSD test for multiple comparisons found that the mean legitimate visitation rate for plots of Maris Bead was significantly different to plots of Fuego and Yukon (Maris Bead – Yukon p = 0.0450, 95% CI = -0.0409, -5.90).

Nectar robbing visits

Plots of Lynx attracted the greatest frequency of nectar robbing visits (mean = 7.52 bees per hour), followed by Vertigo (mean = 5.85 bees per hour). Fuego attracted the lowest visitation rate (mean = 1.98 bees per hour) (**Figure 4.9**). A one-way ANOVA revealed that there was no statistically significant difference in the frequency of nectar robbing between the *V. faba* lines compared at $p \leq 0.05$.

Searching

Plots of Lynx received a marginally greater frequency of bees searching (mean = 2.32 bees per hour), and plots of Fuego attracted the lowest occurrence (mean = 1.36 bees per hour) (**Figure 4.9**). A one-way ANOVA revealed that there was no statistically significant difference in the frequency of searching between the *V. faba* lines compared at $p \leq 0.05$.

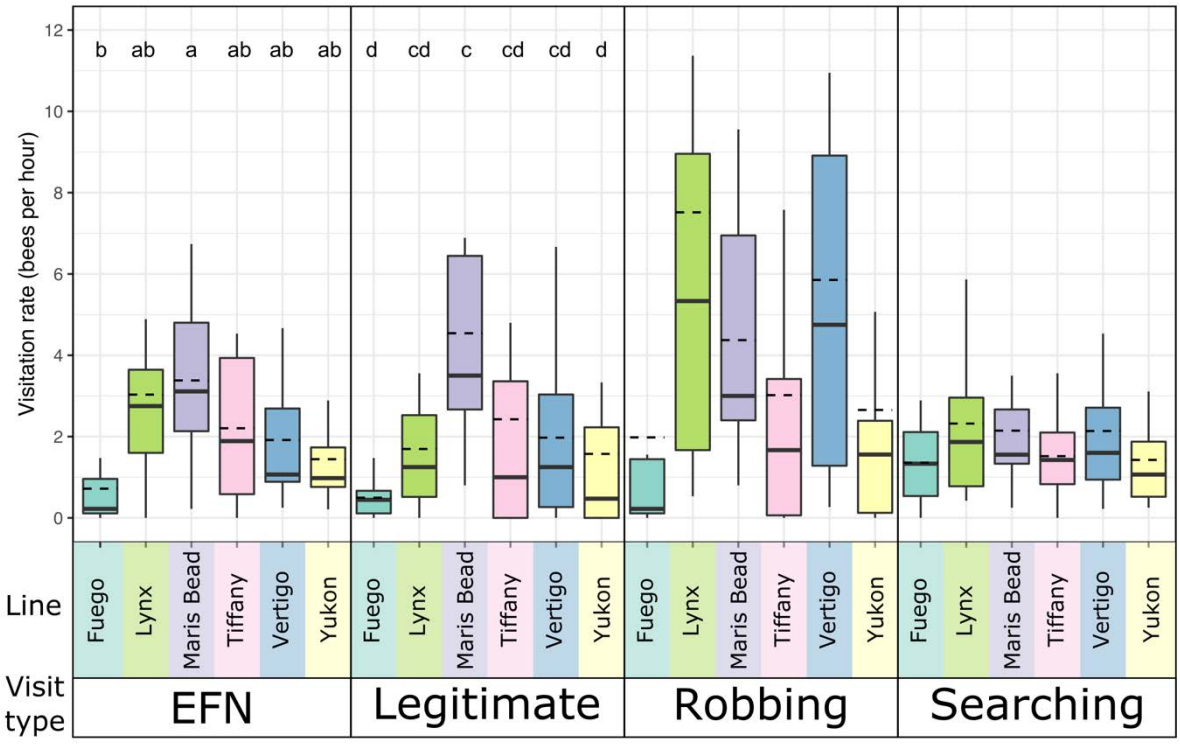


Figure 4.9 Visitation rate of bees to *V. faba* lines in 2022 behaviour breakdown. Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the IQR). Dashed lines show means and solid lines show medians. Letters show Tukey significance groups. *V. faba* lines which do not share a letter have significantly different means at $p \leq 0.05$. One-way ANOVAs were done within visit type groups. Following a significant ANOVA result, post hoc Tukey's HSD tests for multiple comparisons were done within visit type groups. Plots of Maris Bead attracted the highest rate of extra-floral nectary (EFN) visits. A post hoc Tukey test showed that the rate of extra-floral nectary visitation for plots of Maris Bead was significantly different to plots of Fuego at $p \leq 0.05$. Plots of Maris Bead also attracted the highest rate of legitimate visits. A post hoc Tukey's test found that the mean legitimate visitation rate for plots of Maris Bead was significantly different to plots of Fuego and Yukon at $p \leq 0.05$. Plots of Lynx attracted the highest rate of nectar robbing visits, followed by Vertigo and Maris Bead. A one-way ANOVA revealed that there was no statistically significant difference in the frequency of nectar robbing between the *V. faba* lines compared at $p \leq 0.05$. The rate of searching was greatest in plots of Lynx, followed by Maris Bead and Vertigo. A one-way ANOVA revealed that there was no statistically significant difference in the frequency of searching between the *V. faba* lines compared at $p \leq 0.05$.

The proportion of bees carrying out visit types

White-tailed bumblebees

White-tailed bumblebees were the most common type of bee observed in the trial plots and were most frequently observed making nectar robbing visits, followed by legitimate visits (**Figure 4.10**). The frequency of legitimate visits was greatest in plots of Maris Bead (3.80 bees per hour) followed by Tiffany (2.03 bees per hour). Lynx attracted the greatest rate of nectar robbing visits by white-tailed bumblebees (4.35 bees per hour), followed by Vertigo (3.54 bees per hour) and Maris Bead (3.14 bees per hour). Plots of Fuego attracted the lowest rate of both legitimate and nectar robbing visits (0.38 and 0.60 bees per hour respectively). White-tailed bumblebees were less commonly observed searching and performing extra-floral nectary visits.

Honeybees

Honeybees were the second most common type of bee observed in the trial plots and were most frequently observed making extra-floral nectary visits. Honeybees were the most common type of bee observed making extra-floral nectary visits (**Figure 4.10**). Maris Bead and Lynx attracted the greatest frequency of honeybees for extra-floral nectary visits (2.50 and 2.22 bees per hour respectively). Honeybees were also commonly seen nectar robbing and visited Lynx most frequently (2.55 bees per hour), followed by Vertigo (2.23 bees per hour). Honeybees were rarely observed making legitimate visits.

Carder bees

Carder bees were less common across plots of all *V. faba* lines (**Figure 4.10**). Carder bees were most often seen visiting flowers legitimately and were most frequently seen in plots of Maris Bead (0.53 bees per hour). Carder bees were also most frequently observed carrying out extra-floral nectary visits and searching in plots of Maris Bead (0.24 and 0.26 bees per hour).

Red-tailed bumblebees

Red-tailed bumblebees were most frequently observed nectar robbing, most often recorded in plots of Lynx and Maris Bead (0.73 and 0.35 bees per hour) (**Figure 4.10**). Red-tailed bumblebees made most legitimate visits to plots of Maris Bead (0.18 bees per hour) and

Vertigo (0.13 bees per hour) and made most extra-floral nectary visits to Maris Bead (0.22 bees per hour).

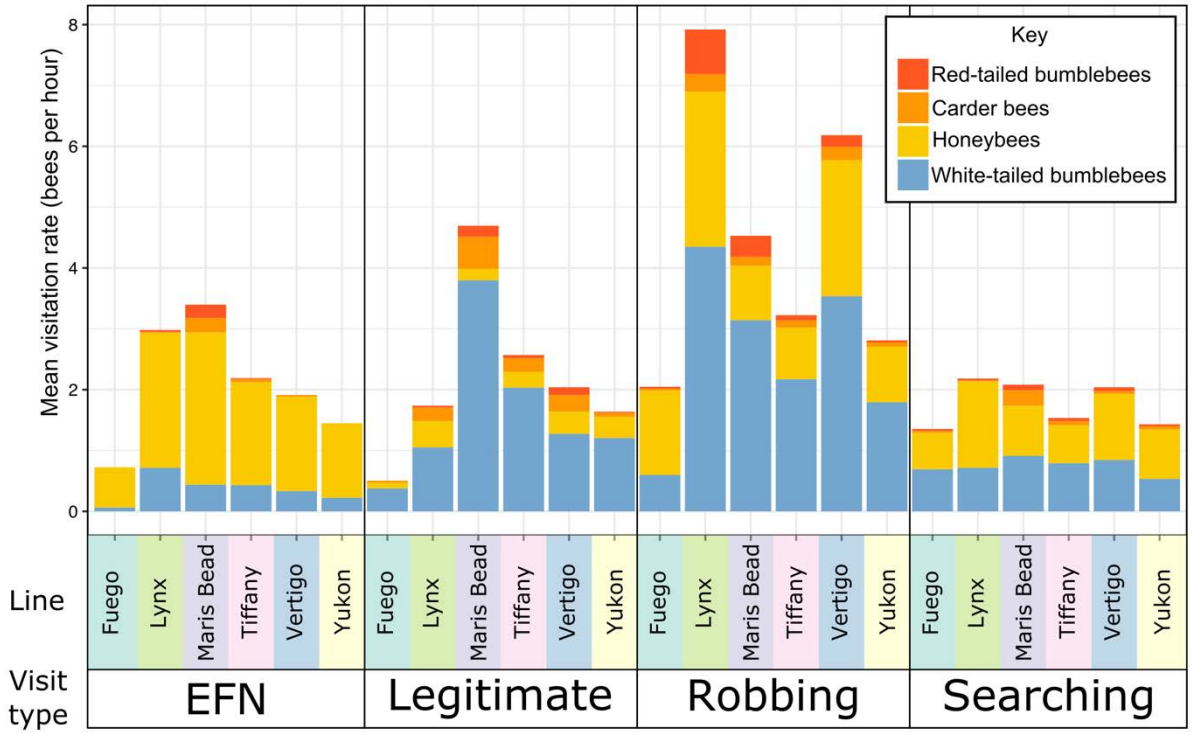


Figure 4.10 Mean visitation rate of bees to *V. faba* lines in 2022 with proportion of bees performing each behaviour. White-tailed bumblebees were most frequently observed nectar robbing overall, followed by legitimate visits. Most robbing visits made by white-tailed bumblebees were to Lynx and most legitimate visits were to Maris Bead. Honeybees were most frequently observed making extra-floral nectary (EFN) visits and robbing overall. Maris Bead attracted the greatest frequency of honeybees for extra-floral nectary visits and Lynx attracted the most robbing visits. Carder bees were most frequently observed making legitimate visits and were most observed making legitimate visits in plots of Maris Bead. Red-tailed bumblebees were most frequently observed nectar robbing. Red-tailed bumblebees were most often observed nectar robbing in plots of Lynx followed by Maris Bead.

4.2.5 Bee preference is consistent across years and locations

Pollinator visitation field trials were carried out in Stubton, Lincolnshire in 2021, and Histon, Cambridge in 2022. Three *V. faba* lines, Fuego, Maris Bead and Tiffany, were grown in both years to assess whether bee preference differed between the two locations and the two years. The number of bees visiting field plots were recorded in 2021 and 2022 using the same methodology.

Total bee visitation

In both 2021 and 2022, Maris Bead attracted the highest bee visitation rate when comparing these three lines (2021 mean = 8.83 bee per hour, 2022 mean = 14.4 bees per hour) (**Figure 4.11**). Tiffany received the second highest bee visitation rate across both years (2021 mean = 5.03, 2022 mean = 9.16), and Fuego received the lowest bee visitation rate across both years (2021 mean = 3.28, 2022 mean = 4.55). To account for the differences in the number of bees observed between the two years, the ratio of mean visitation rates for Maris Bead and Fuego compared to Tiffany were calculated, as Tiffany was the middle value in both 2021 and 2022 (**Table 4.1**). In 2021, the mean visitation rate to Fuego was 0.65 that to Tiffany, and in 2022 the mean visitation rate to Fuego was 0.50 that to Tiffany. In 2021, the mean visitation rate to Maris Bead was 1.75 times that to Tiffany, and in 2022 the mean visitation rate to Maris Bead was 1.58 times than that to Tiffany.

For both the 2021 results and 2022 results, one-way ANOVA tests revealed statistically significant differences in bee visitation rate between the *V. faba* lines compared. 2021 - ($F(2,35) = [5.493]$, $p = 0.00842$). 2022 - ($F(2,31) = [7.647]$, $p = 0.002$). For both the 2021 results and 2022 results, post hoc Tukey's HSD tests for multiple comparisons found that the mean bee visitation rates for plots of Maris Bead was significantly different to plots of Fuego and Tiffany. 2021 - (Maris Bead – Tiffany $p = 0.0273$, 95% CI = -0.367, -7.23). 2022 - (Maris Bead – Tiffany $p = 0.00494$, 95% CI = -0.0120, -10.5).

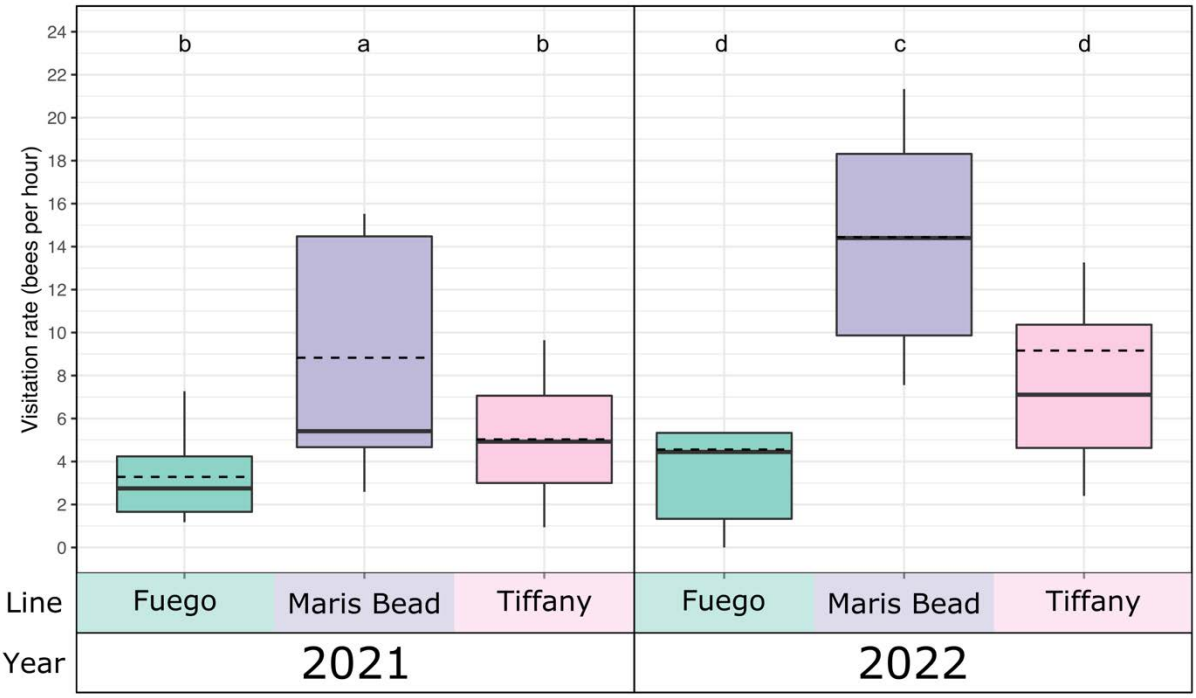


Figure 4.11 Visitation rate of bees to *V. faba* lines grown in both 2021 and 2022 (all bee types, all behaviours). Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the IQR). Dashed lines show means and solid lines show medians. Letters show Tukey significance groups. *V. faba* lines which do not share a letter within that year have significantly different means at $p \leq 0.05$. Fuego, Maris Bead and Tiffany were grown at both Stubton in 2021 and at Histon in 2022. In both years, Maris Bead attracted the highest bee visitation rate followed by Tiffany. Fuego attracted the lowest visitation rate across both years. For both the 2021 results and the 2022 results, one-way ANOVA tests revealed statistically significant differences in bee visitation rate between the *V. faba* lines compared at $p \leq 0.05$. For both the 2021 results and 2022 results, post hoc Tukey's tests found that the mean bee visitation rate for plots of Maris Bead were significantly different to plots of Fuego and Tiffany at $p \leq 0.05$.

Bee visitation rate by visit type

For all visit types apart from searching, the relationship in mean visitation rate between 2021 and 2022 was consistent, with Maris Bead attracting the highest visitation rate followed by Tiffany, and Fuego attracting the lowest visitation rate (**Figure 4.12**). This pattern was also true for searching in 2022, however, in 2021 Fuego experienced a higher mean searching rate than Tiffany. The prevalence of extra-floral nectary visits and nectar robbing was greater overall in 2022 compared to 2021. However, the rate of legitimate visits, although lower for Fuego, remained stable for Maris Bead and Tiffany between 2021 and 2022. The incidence of bees searching was also slightly elevated for every line in 2022 compared to the previous year.

The ratio of mean visitation rate for Fuego compared to Tiffany was consistent for both 2021 and 2022 in all visit types apart from searching (**Table 4.1**). In 2021 the searching visitation rate for Fuego was 1.29 times higher than that of Tiffany, whereas in 2022 the searching visitation rate for Fuego was 0.90 times as high as that of Tiffany. The greatest change in visit ratio was for legitimate visits to Fuego, with the rate for Fuego being 0.66 times that of Tiffany in 2021, and 0.20 times that of Tiffany in 2022. The ratio of mean visitation rate for Maris Bead compared to Tiffany was also very consistent for both 2021 and 2022. The largest change in visit ratio to Maris Bead was for extra-floral nectary visits, with the rate being 2.46 times that of Tiffany in 2021, and 1.53 times that of Tiffany in 2022.

One-way ANOVA tests revealed statistically significant differences in bee visitation rate between the *V. faba* lines compared for extra-floral nectary visits in 2021 and 2022, legitimate visits in 2021 and 2022 and nectar robbing visits in 2021 at $p \leq 0.05$. One-way ANOVA tests revealed no statistically significant differences in bee visitation rate between the *V. faba* lines compared for nectar robbing in 2022 and for searching in 2021 and 2022 $p \leq 0.05$. Post hoc Tukey's HSD tests for multiple comparisons found that the mean bee extra-floral nectary visitation rate for plots of Maris Bead were significantly different to plots of Fuego in both 2021 and 2022 (**Figure 4.12**). Post hoc Tukey's tests also found that the mean bee legitimate visitation rate for plots of Maris Bead were significantly different to plots of Fuego in both 2021 and 2022 (**Figure 4.12**).

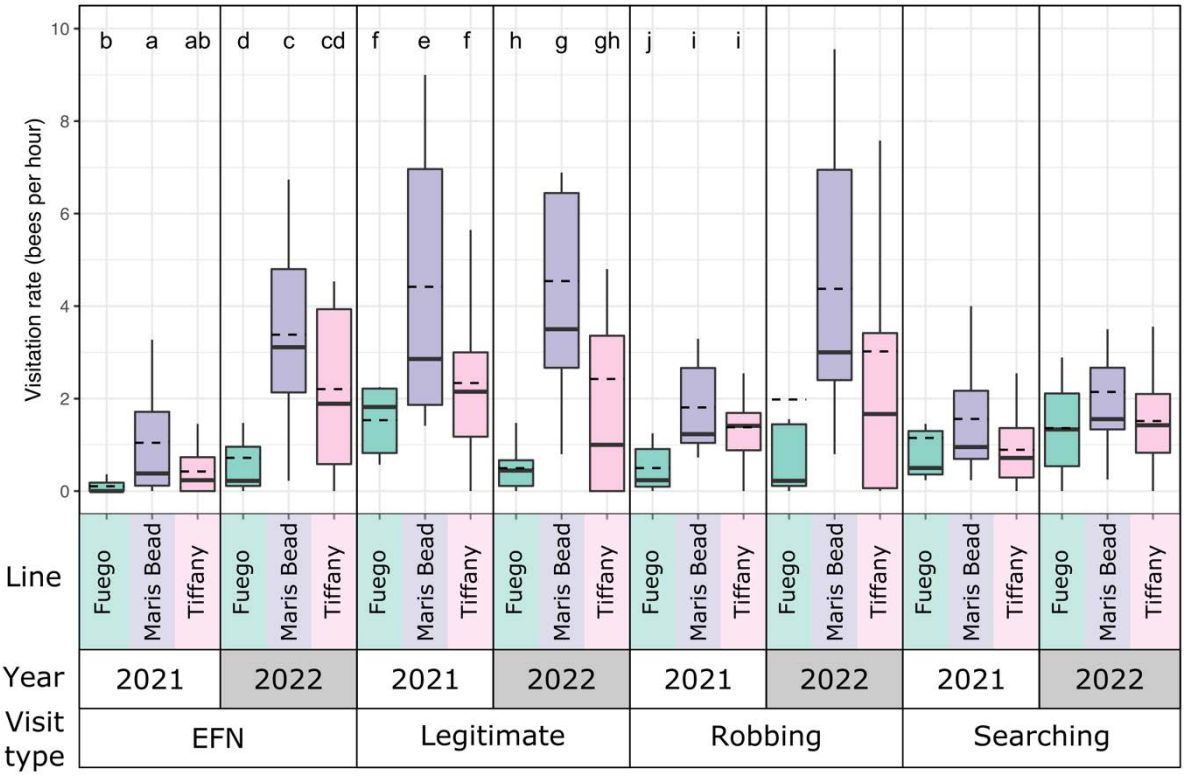


Figure 4.12 Visitation rate of bees to *V. faba* lines grown in 2021 and 2022 behaviour breakdown. Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the IQR). Dashed lines show means and solid lines show medians. Letters show Tukey significance groups. *V. faba* lines which do not share a letter within that year have significantly different means at $p \leq 0.05$. For all visit types apart from searching, the relationship in visitation rate between 2021 and 2022 was consistent, with Maris Bead attracting the highest mean visitation rate followed by Tiffany, and Fuego attracting the lowest mean visitation rate. This pattern was also true for searching in 2022, however, in 2021 Fuego experienced a higher mean visitation rate than Tiffany. One-way ANOVAs were performed within each year for each behaviour type. Following a significant AVOVA result at $p \leq 0.05$, post hoc Tukey tests were done within each year for each behaviour type. Post hoc Tukey's HSD tests found that the mean bee extra-floral nectary (EFN) visitation rate for plots of Maris Bead were significantly different to plots of Fuego in both 2021 and 2022 $p \leq 0.05$. Post hoc Tukey's tests also found that the mean bee legitimate visitation rate for plots of Maris Bead were significantly different to plots of Fuego in both 2021 and 2022 $p \leq 0.05$.

		Ratio of mean visitation rate relative to Tiffany		
Visit type	Line	2021	2022	Difference
Overall visitation rate	Fuego	0.65	0.50	-0.16
	Maris Bead	1.75	1.58	-0.18
Extra-floral nectary	Fuego	0.25	0.33	0.08
	Maris Bead	2.46	1.53	-0.93
Legitimate	Fuego	0.66	0.20	-0.45
	Maris Bead	1.89	1.87	-0.02
Robbing	Fuego	0.36	0.66	0.29
	Maris Bead	1.31	1.45	0.14
Searching	Fuego	1.29	0.90	-0.39
	Maris Bead	1.74	1.41	-0.33

Table 4.1 Ratios of mean bee visitation rates of Fuego and Maris Bead relative to Tiffany for 2021 and 2022. To account for differences in the number of bees observed between the two years, the ratio of mean visitation rates for Maris Bead and Fuego compared to Tiffany were calculated. The visit ratio of Maris Bead and Fuego relative to Tiffany were largely consistent between 2021 and 2022, with the change in ratio between years being less than 0.5 in all but one case. The most consistent visit ratio relative to Tiffany was for legitimate visits to Maris Bead with a change of -0.02 between 2021 and 2022. The largest change in ratio between 2021 and 2022 was for extra-floral nectary visits to Maris Bead with a change of -0.93. Colour shading shows size of ratio difference.

The proportion of bees carrying out visit types

White-tailed bumblebees

White-tailed bumblebees were the most common type of bee seen making legitimate visits in both 2021 and 2022 (**Figure 4.13**). This was also true for nectar robbing in both years, except for Fuego in 2022, where honeybees were most frequently observed nectar robbing. The rate of white-tailed bumblebees seen searching was relatively consistent between years, and although white-tailed bumblebees were very rarely seen making extra-floral nectary visits in 2021, they were more commonly observed making extra-floral nectary visits in 2022 for all lines.

Honeybees

Honeybees were far more common in 2022 compared to 2021 (**Figure 4.13**). Honeybees were the most frequent type of bee seen making extra-floral nectary visits on all lines in 2022, as they were for Maris Bead and Tiffany in 2021, however, honeybees making extra-floral nectary visits in plots of Maris Bead were on average 5.4 times more frequent in 2022 (2.50 bees per hour) compared to 2021 (0.46 bees per hour). Similarly, honeybees were more commonly observed making nectar robbing visits and searching in plots of all lines in 2022 compared to 2021. In contrast, honeybees were less commonly observed making legitimate visits in plots of Maris Bead and Tiffany in 2022.

Carder bees

Carder bees were less commonly seen in 2022 compared to 2021 for all behaviours (**Figure 4.13**). In 2021 carder bees were most often seen making legitimate visits compared to all other behaviours. This pattern was also present for Maris Bead and Tiffany in 2022. However, Fuego received a very low frequency of carder bee visits for legitimate, nectar robbing and searching in 2022.

Red-tailed bumblebees

Red-tailed bumblebees were less common in 2022 compared to 2021 (**Figure 4.13**). In 2021, red-tailed bumblebees were most often seen nectar robbing for Maris Bead and Tiffany, but were also seen making extra-floral nectary, legitimate visits and searching on all three lines. Nectar robbing was also the most common behaviour observed in 2022 for Maris Bead and

Tiffany and in plots of Fuego alongside searching. In both years, red-tailed bumblebees were more frequent in plots of Maris Bead than in any other line for all behaviours.

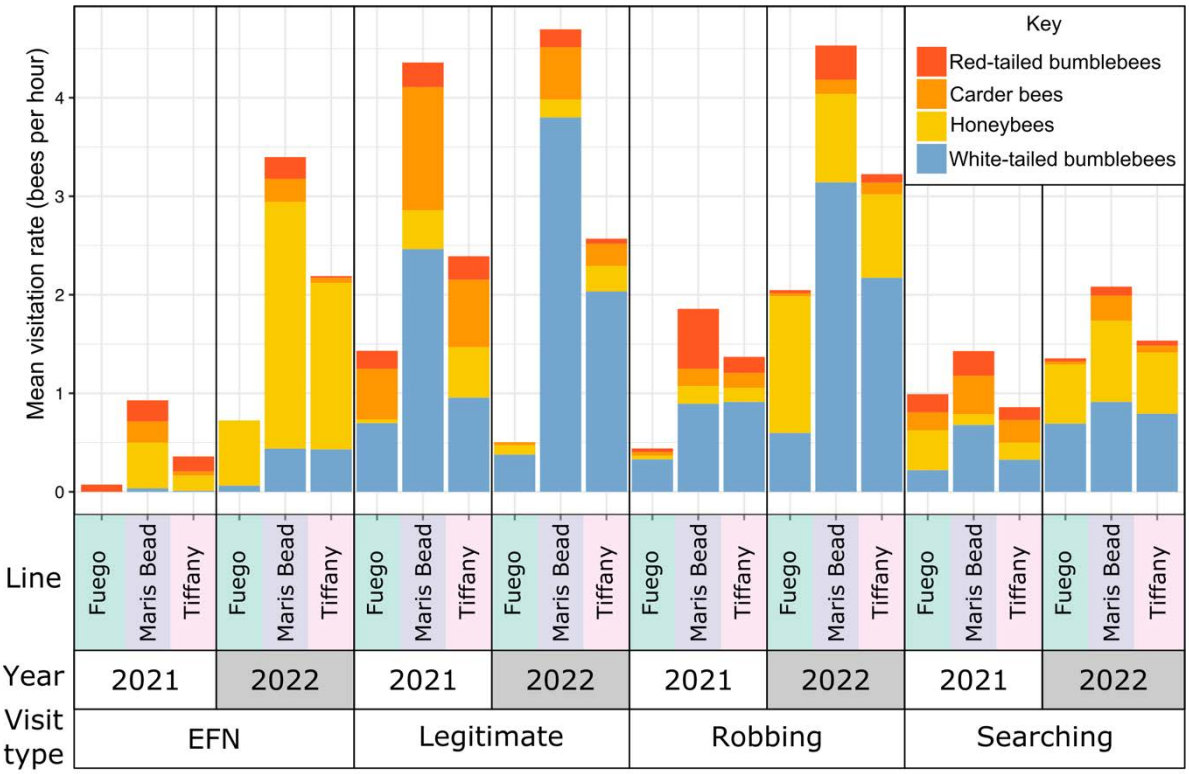


Figure 4.13 Mean visitation rate of bees to *V. faba* lines in 2021 and 2022 with proportion of bees performing each behaviour. White-tailed bumblebees were the most common type of bee seen making legitimate visits in both 2021 and 2022. Honeybees were far more common in 2022 compared to 2021. Honeybees were the most frequent type of bee seen making extra-floral nectary (EFN) visits on all lines in 2022, as they were for Maris Bead and Tiffany in 2021. Carder bees were less common in 2022 compared to 2021 for all behaviours. In 2021 carder bees were most often seen making legitimate visits, which was also true for Maris Bead and Tiffany in 2022. However, Fuego received a very low frequency of carder bees for legitimate, nectar robbing and searching in 2022. Red-tailed bumblebees were also less common in 2022 compared to 2021. In 2021, red-tailed bumblebees were most often seen nectar robbing on Maris Bead and Tiffany, but were also seen making extra-floral nectary, legitimate visits and searching on all three lines.

4.2.6 Effect of pollinator exclusion on *Vicia faba* yield - 2021

Number of pods per plant

The mean number of pods per plant was greater for open pollinated plants of Maris Bead, NV100 and NV129 compared to caged plants (**Figure 4.14**). The difference was significantly different for plants of Maris Bead ($t(98) = -2.79$, $p = 0.0006$) and NV100 ($t(118) = -5.80$, $p = 5.56\times10^{-8}$) when compared using an unpaired, two-tailed t-test. The number of pods per plant did not differ greatly between open pollinated and caged plants of Fuego and decreased slightly for open pollinated plants of Tiffany compared to caged plants. For tables containing all yield data and percentage changes in yield see Appendix H.

Number of seeds per plant

The mean number of seeds per plant was greater for open pollinated plants compared to caged plants for all lines (**Figure 4.15**). The difference was significantly different for plants of Maris Bead ($t(98) = -4.51$, $p = 1.79\times10^{-5}$), NV100 ($t(118) = -6.94$, $p = 2.27\times10^{-10}$) and NV129 ($t(98) = -4.47$, $p = 2.11\times10^{-5}$).

Number of seeds per pod

The number of seeds per pod was significantly greater for open pollinated plants compared to caged plants for all lines **(Figure 4.16**). Fuego ($t(1166) = -5.33$, $p = 1.16\times10^{-7}$), Maris Bead ($t(1339) = -12.09$, $p = 4.92\times10^{-32}$), NV100 ($t(1060) = -6.41$, $p = 2.25\times10^{-10}$), NV129 ($t(745) = -4.05$, $p = 5.55\times10^{-5}$) and Tiffany ($t(2154) = -13.36$, $p = 3.41\times10^{-39}$). The largest difference between the number of seeds per pod between open pollinated and caged plants was for Maris Bead, open mean = 3.18, caged mean = 2.49.

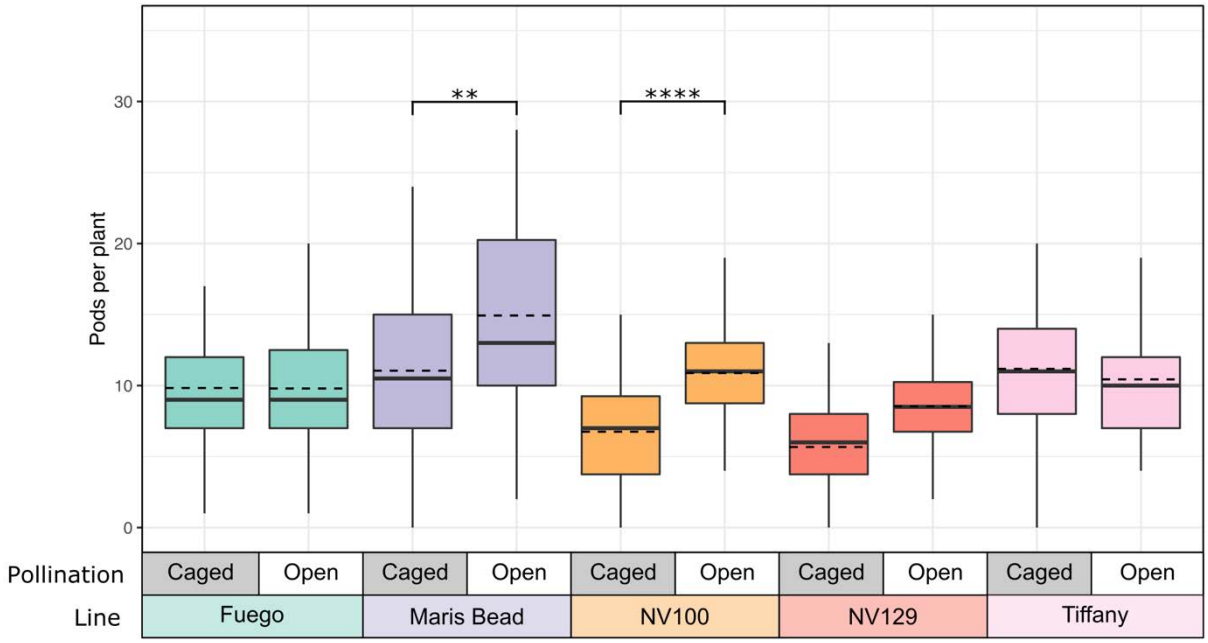


Figure 4.14 Number of pods per plant for open pollinated and caged *V. faba* plants 2021. Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the IQR). Dashed lines show means and solid lines show medians. When compared to caged plants, the mean number of pods per plant was significantly greater in open pollinated plants of Maris Bead at $p \leq 0.005$ and NV100 at $p \leq 0.0001$. The number of pods per plant did not significantly differ between open and caged plants of Fuego, NV129 and Tiffany. Number of asterisks indicate significance level between caged and open pollinated groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

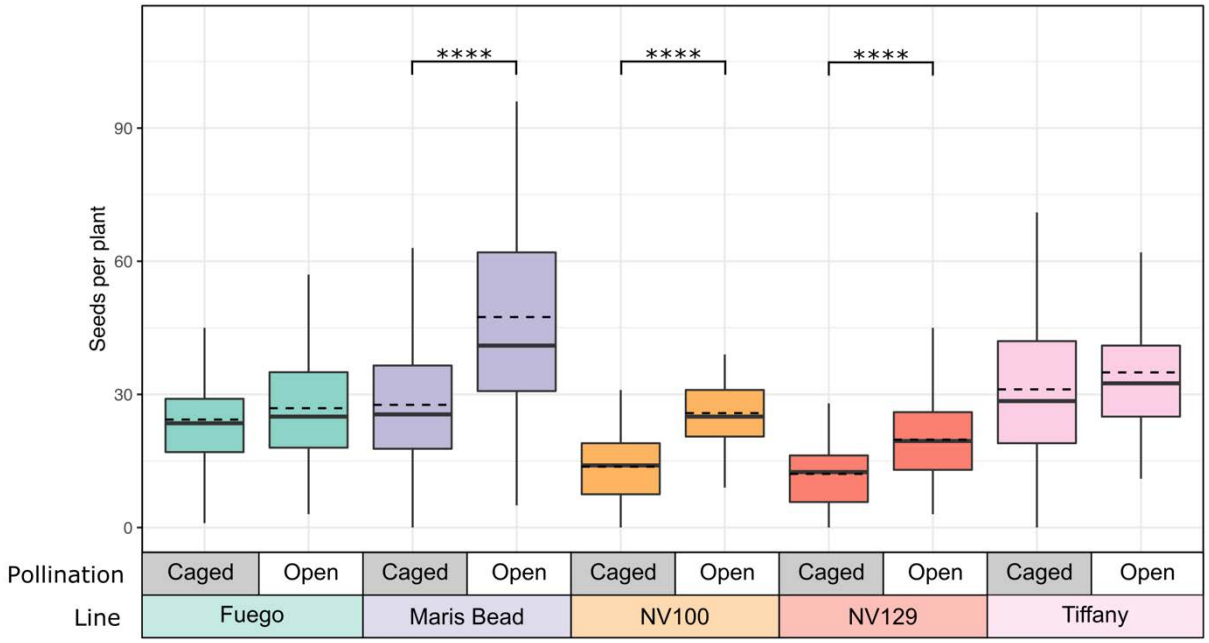


Figure 4.15 Number of seeds per plant for open pollinated and caged *V. faba* plants 2021. Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the IQR). Dashed lines show means and solid lines show medians. When compared to caged plants, the mean number of seeds per plant was significantly greater for open pollinated plants of Maris Bead at p≤0.0001, NV100 at p≤0.0001 and NV129 at p≤0.0001. The number of seeds per plant did not significantly differ between open and caged plants of Fuego and Tiffany. Number of asterisks indicate significance level between caged and open pollinated groups, * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001.

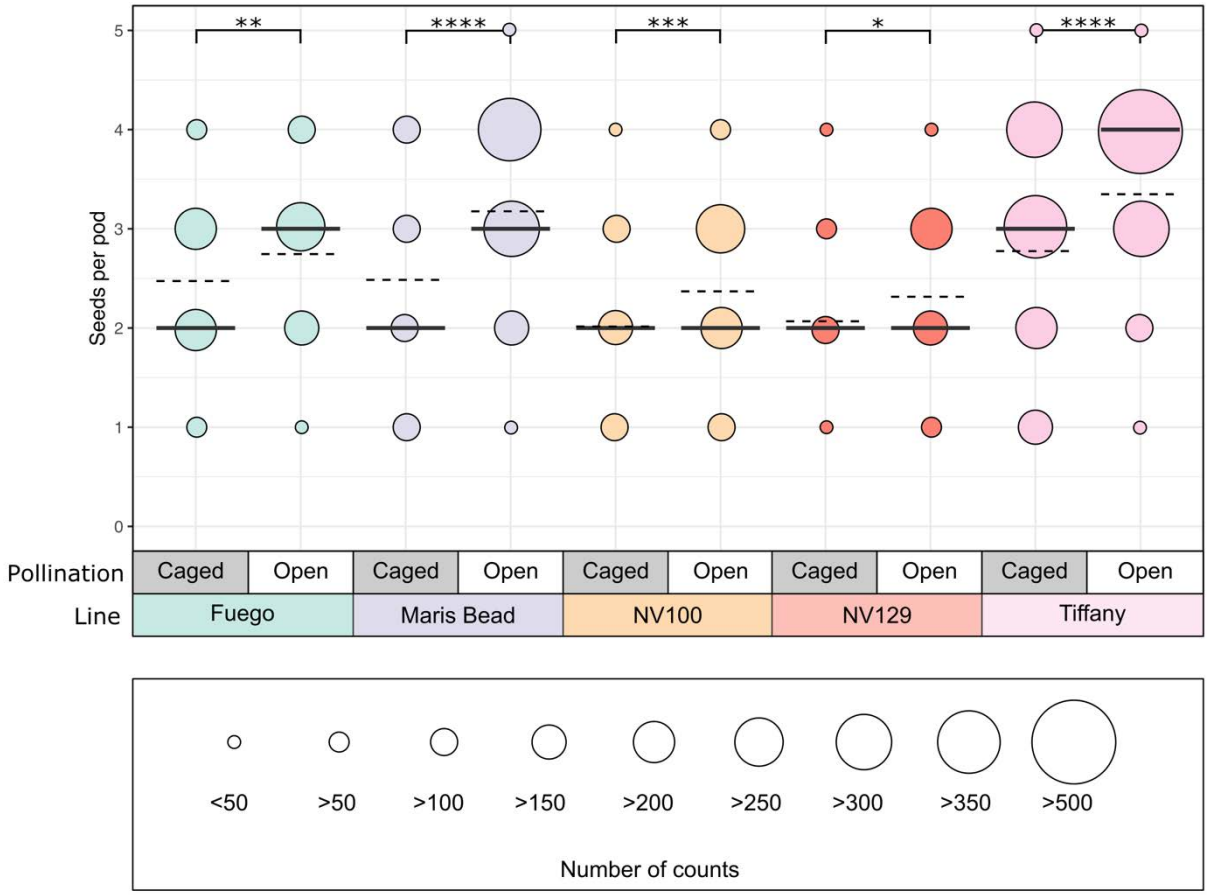


Figure 4.16 Number of seeds per pod for open pollinated and caged *V. faba* plants 2021. Bubble plots show the frequency that each number of seeds was observed in a pod. Dashed lines show means and solid lines show medians. When compared to caged plants, the mean number of seeds per pod was significantly greater for open pollinated plants of all lines at $p \leq 0.0001$. The largest difference between the number of seeds per pod between open pollinated and caged plants was for Maris Bead, caged mean = 2.49, open mean = 3.18. Number of asterisks indicate significance level between caged and open pollinated groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

Distribution of seeds and pods on *V. faba* plants

The node position of each pod and seed was recorded. Node position was counted from the bottom of the plant, a node was defined as the point where racemes are produced at the plant axil. The number of seeds produced at each node varied over the length of the plant for all lines, with most lines producing more seeds at lower node positions (**Figure 4.17**). Plants of Maris Bead produced seeds over the greatest range of nodes (14), followed by Tiffany (13), Fuego and NV100 (11), and NV129 (10).

Distribution of seeds differed little between open and caged plants of Fuego with both open and caged plants producing most seeds between nodes 4 and 8, and fewer seeds at higher node positions (**Figure 4.17 A**). Compared to caged plants, open pollinated plants of Maris Bead produced more seeds at nodes 6-12 but had similar quantities of seeds at higher node positions (**Figure 4.17 B**). Both open pollinated and caged plants of NV100 produced most seeds between node position 8 and 11, with fewer seeds at the lowest and highest nodes. Open pollinated NV100 plants had far more seeds on average at position 5, 6 and 16 than caged plants (**Figure 4.17 C**). Plants of NV129 set more seeds at lower node positions with a gradual decline in seed number between node 9 and 13. Open pollinated plants produced 6 seeds at node 14 on average, whereas caged plants produced none (**Figure 4.17 D**). Both open pollinated and caged plants of Tiffany produced most seeds at lower node positions, with seed number declining up the plant. Open pollinated plants produced more seeds at nodes 4-9 than caged plants, but similar quantities between node 10 and 17 (**Figure 4.17 E**).

The number of pods produced at each node reflected the pattern observed for seeds (**Figure 4.18**). The distribution of pods differed little between open pollinated and caged plants of Fuego (**Figure 4.18 A**). Plants of Maris Bead produced far more pods at nodes 6-12 when open pollinated (**Figure 4.18 B**). Open pollinated plants of NV100 produced more pods at all but one node position (**Figure 4.18 C**), and open pollinated plants of NV129 produced more pods at all node positions compared to caged plants (**Figure 4.18 C**). Plants of Tiffany produced more pods at nodes 4-11 when open pollinated (**Figure 4.18 C**).

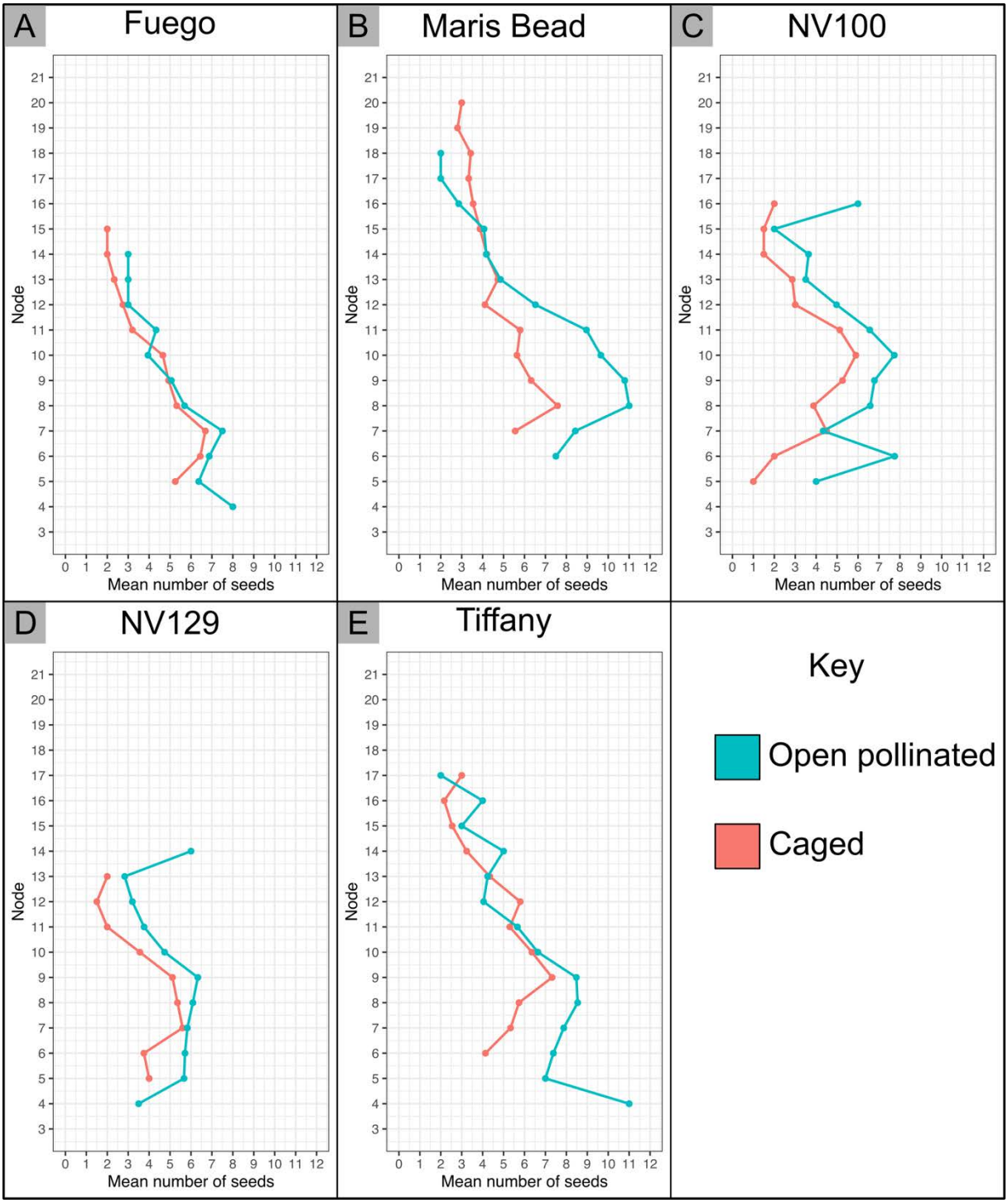


Figure 4.17 Distribution of seeds over *V. faba* plant nodes for open pollinated and caged plants 2021. The mean number of seeds produced at each node position for open pollinated (blue) and caged (red) plants. Node position is counted from the bottom of the plant. **(A)** Plants of Fuego produced most seeds on lower nodes. **(B)** Open pollinated plants of Maris Bead produced more seeds at nodes 6-12 than caged plants. **(C)** Open pollinated NV100 plants had more seeds at position 5, 6 and 16 than caged plants. **(D)** Plants of NV129 set more seeds at lower node positions. **(E)** Open pollinated plants of Tiffany produced more seeds at lower node positions than caged plants.

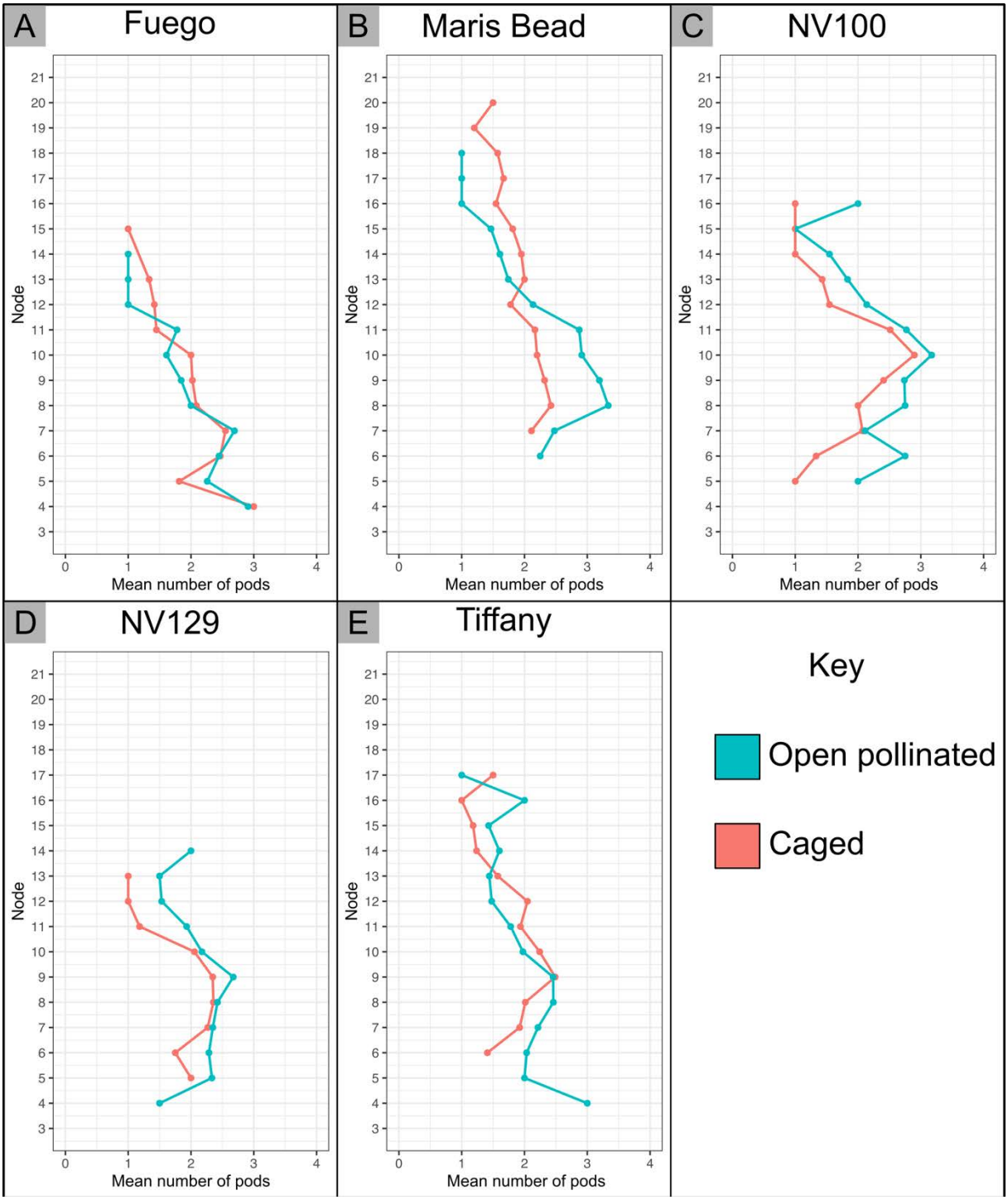


Figure 4.18 Distribution of pods over *V. faba* plant nodes for open pollinated and caged plants 2021. The mean number of seeds produced at each node position for open pollinated (blue) and caged (red) plants. Node position is counted from the bottom of the plant. **(A)** Pod distribution differed little between open and caged plants of Fuego. **(B)** Open pollinated plants of Maris Bead produced more pods at nodes 6-12 than caged plants. **(C)** Open pollinated NV100 plants produced more pods at most node positions as did NV129 **(D)**. **(E)** Open pollinated plants of Tiffany produced more pods at lower node positions 4-8 and 1-13.

Bean mass per plant

The bean mass per plant was significantly greater for open pollinated plants of Maris Bead ($t(95) = -3.42$, $p = 9.10\times10^{-4}$), NV100 ($t(114) = -6.09$, $p = 1.54\times10^{-8}$) and NV129 ($t(91) = -2.33$, $p = 2.20\times10^{-2}$) than caged plants. The largest difference in the mass of beans per plant between open pollinated and caged plants was for Maris Bead, caged mean = 8.01 g, open mean = 12.12 g (**Figure 4.19**).

Mean bean mass per pod

The mean bean mass per pod was significantly greater for open pollinated plants of Maris Bead ($t(95) = -4.15$, $p = 7.15\times10^{-5}$), Tiffany ($t(199) = -3.66$, $p = 3.20\times10^{-4}$), and NV100 ($t(114) = -2.90$, $p = 4.0\times10^{-3}$), than caged plants. The largest difference in mean bean mass per pod was between open pollinated and caged plants of Maris Bead, caged mean = 0.67 g, open mean = 0.80 g (**Figure 4.20**).

Mean bean mass

The mean mass per bean was significantly lower for open pollinated plants of Maris Bead ($t(95) = 2.20$, $p = 3.0\times10^{-2}$), NV129 ($t(91) = 2.30$, $p = 2.40\times10^{-2}$) and Tiffany ($t(197) = 3.80$, $p = 1.94\times10^{-4}$) than caged plants. The largest difference in the mean mass per seed between open pollinated and caged plants was for Tiffany, caged mean = 0.40 g, open mean = 0.35 g (**Figure 4.21**).

Plot yield

Plot yield was greater for open pollinated plots compared to caged plots for all lines (**Figure 4.22**). The largest absolute yield difference between open and caged plots was for Maris Bead (0.42 kg), followed by Tiffany (0.20 kg) and Fuego (0.12 kg).

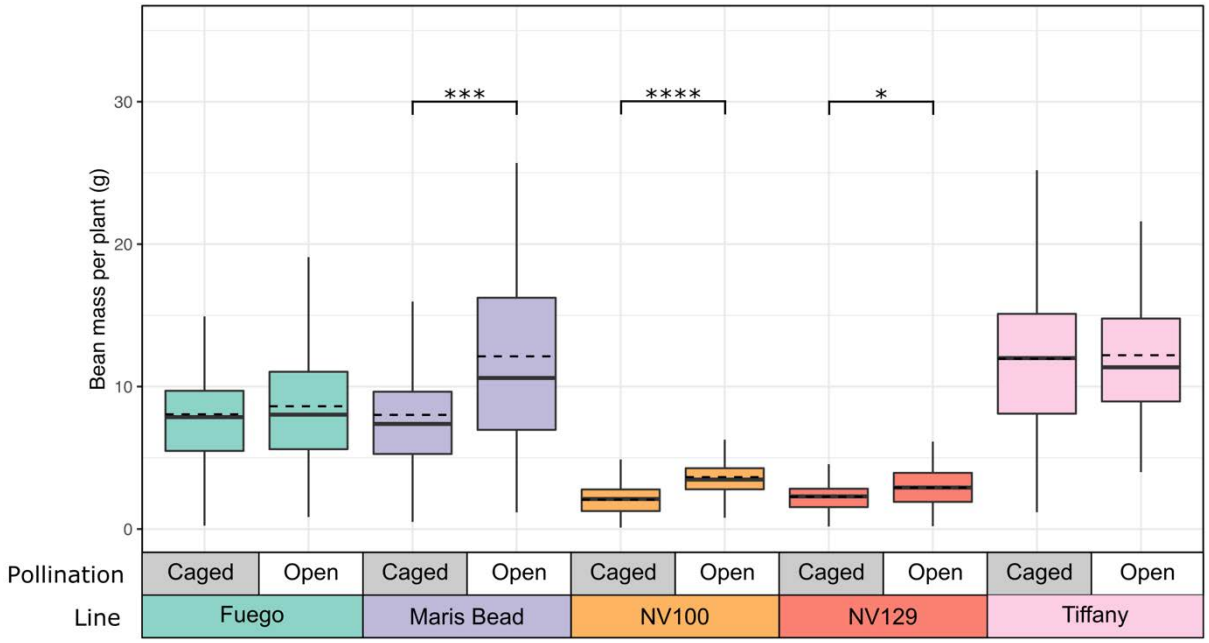


Figure 4.19 Bean mass per plant for open pollinated and caged *V. faba* plants 2021.

Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the IQR). Dashed lines show means and solid lines show medians. When compared to caged plants, the mass of beans per plant was significantly greater for open pollinated plants of Maris Bead at $p \leq 0.001$, NV100 at $p \leq 0.001$ and NV129 at $p \leq 0.05$. The largest difference in the mass of beans per plant between open pollinated and caged plants was for Maris Bead, caged mean = 8.01 g, open mean = 12.12 g. Number of asterisks indicate significance level between caged and open pollinated groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

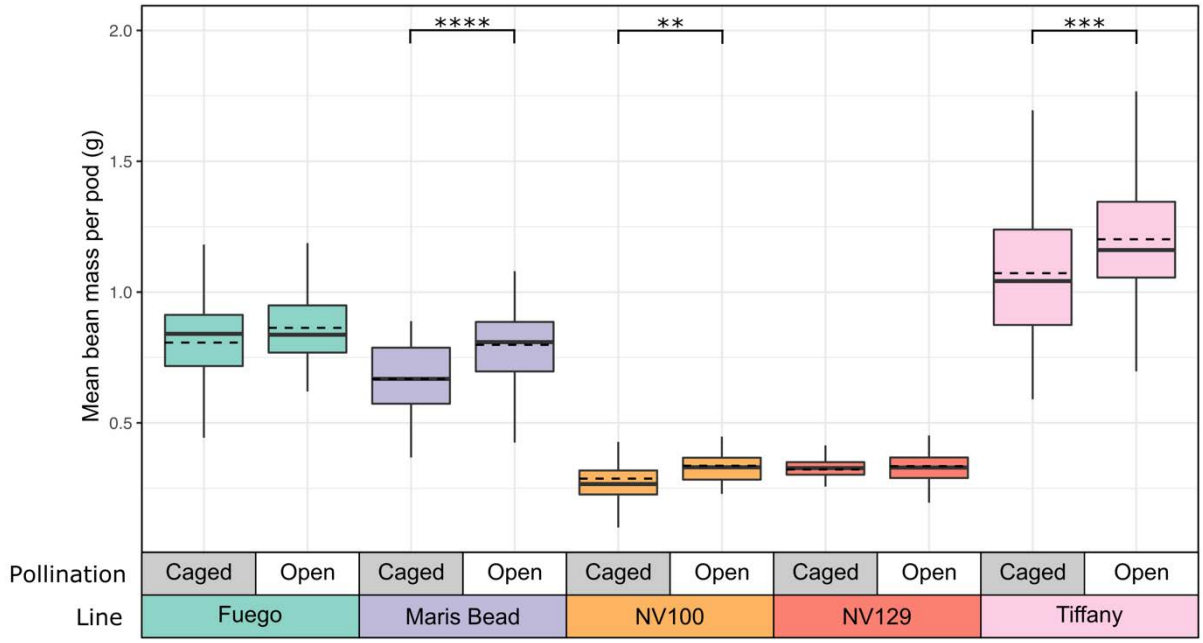


Figure 4.20 Mean bean mass per pod for open pollinated and caged *V. faba* plants 2021. Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the IQR). Dashed lines show means and solid lines show medians. When compared to caged plants, the mean mass of beans per pod was significantly greater for open pollinated plants of Maris Bead at $p \leq 0.0001$, NV100 at $p \leq 0.01$, and Tiffany at $p \leq 0.001$. The largest difference in the mean mass of beans per pod between open pollinated and caged plants was for Maris Bead, caged mean = 0.67 g, open mean = 0.80 g and Tiffany, caged mean = 1.07 g, open mean = 1.20 g. Number of asterisks indicate significance level between caged and open pollinated groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

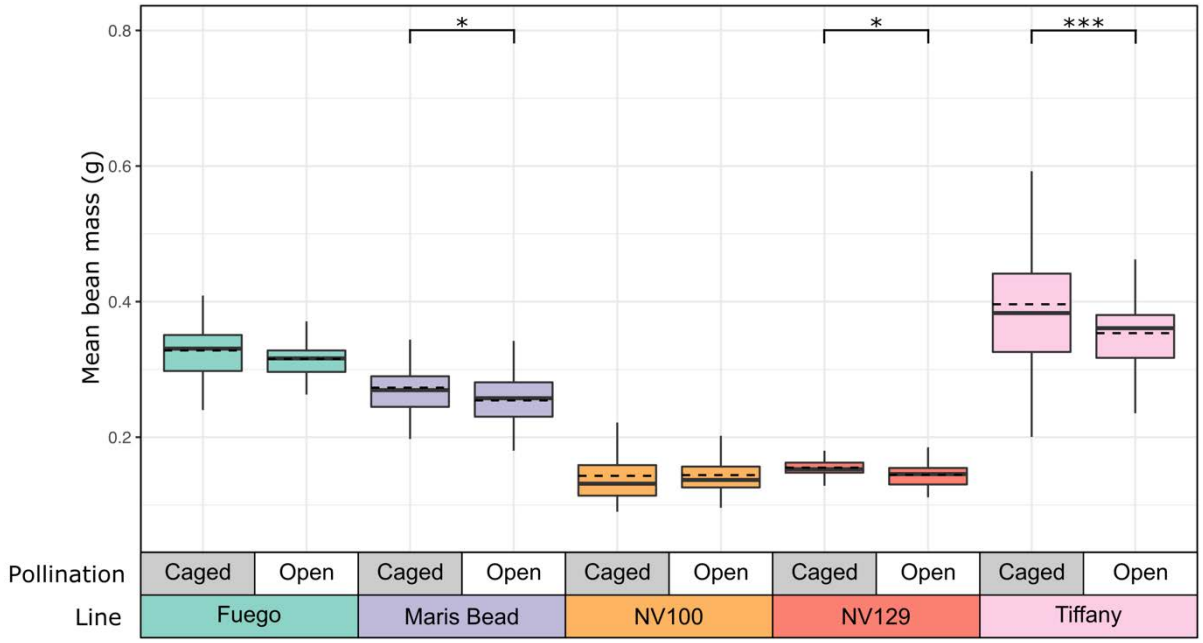


Figure 4.21 Mean bean mass per seed for open pollinated and caged *V. faba* plants 2021. Boxplots show the interquartile range for each line and whiskers show maxima and minima (calculated as 1.5 of the IQR). Dashed lines show means and solid lines show medians. When compared to caged plants, the mean mass per bean was significantly lower for open pollinated plants of Maris Bead at $p \leq 0.05$, NV129 at $p \leq 0.05$, and Tiffany at $p \leq 0.001$. The largest difference in the mean mass per seed between open pollinated and caged plants was for Tiffany, caged mean = 0.40 g, open mean = 0.35 g. Number of asterisks indicate significance level between caged and open pollinated groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

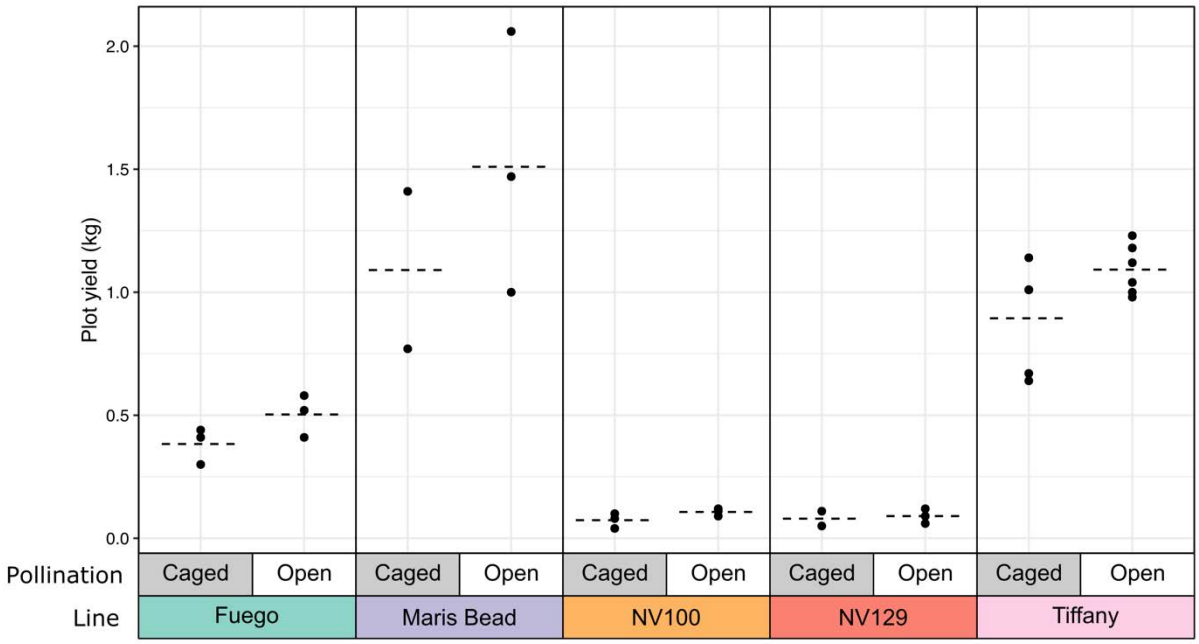


Figure 4.22 Plot yield for open pollinated and caged *V. faba* plants 2021. Black points are yield for individual plots. Dashed lines show means. Mean plot yield was greater for open pollinated plots compared to caged plots for all lines.

4.2.7 Effect of pollinator exclusion on *Vicia faba* yield - 2022

In 2022 plots were harvested by plot combine to obtain the overall yield per plot. A sample of 20 plants was also picked from every plot prior to combine harvest to count number of pods, seeds, and mass of seeds after thesis submission.

Plot yield

Plot yield was greater for open pollinated plots compared to caged plots for Lynx, Maris Bead, Tiffany, and Yukon (**Figure 4.23**). Plot yield was lower for open pollinated plots compared to caged plots for Fuego and Vertigo. The largest absolute yield difference between open and caged plots was for Maris Bead (0.23 kg), followed by Yukon (0.15 kg) and Tiffany (0.12 kg). Compared to 2021, plots produced far less seed in 2022. Open pollinated Maris Bead yielded highest in both years. In 2021 the mean yield was 1.51 kg per plot, whereas in 2022 it was 0.52 kg.

Percentage change in plot yield for 2021 and 2022

Across 2021 and 2022, 9 out of 11 lines experienced an increase in yield when open pollinated compared to when they were caged (**Figure 4.24**). The smallest positive change was for Lynx in 2022 (5.0%) and the largest was for Maris Bead in 2022 (79.5%). All lines grown in 2021 showed an increase in yield with open pollination, however, Fuego and Vertigo showed a decrease in yield in 2022 (-22.4% and -10.5% respectively). On average, lines experienced a plot yield benefit of 23.3% when lines were open pollinated compared to caged. For a breakdown of % changes for all yield parameters see Appendix H.

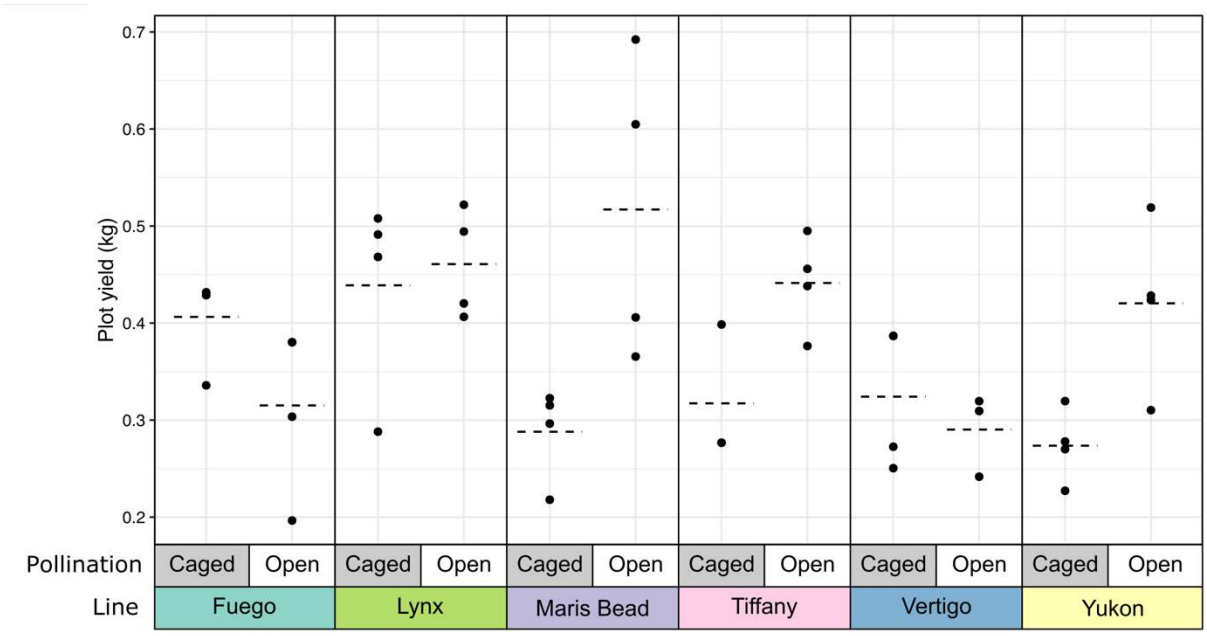


Figure 4.23 Plot yield for open pollinated and caged *V. faba* plants 2022. Black points are yield for individual plots. Dashed lines show means. Mean plot yield was greater for open pollinated plots compared to caged plots for Lynx, Maris Bead, Tiffany, and Yukon. Mean plot yield was lower for open pollinated plots compared to caged plots for Fuego and Vertigo.

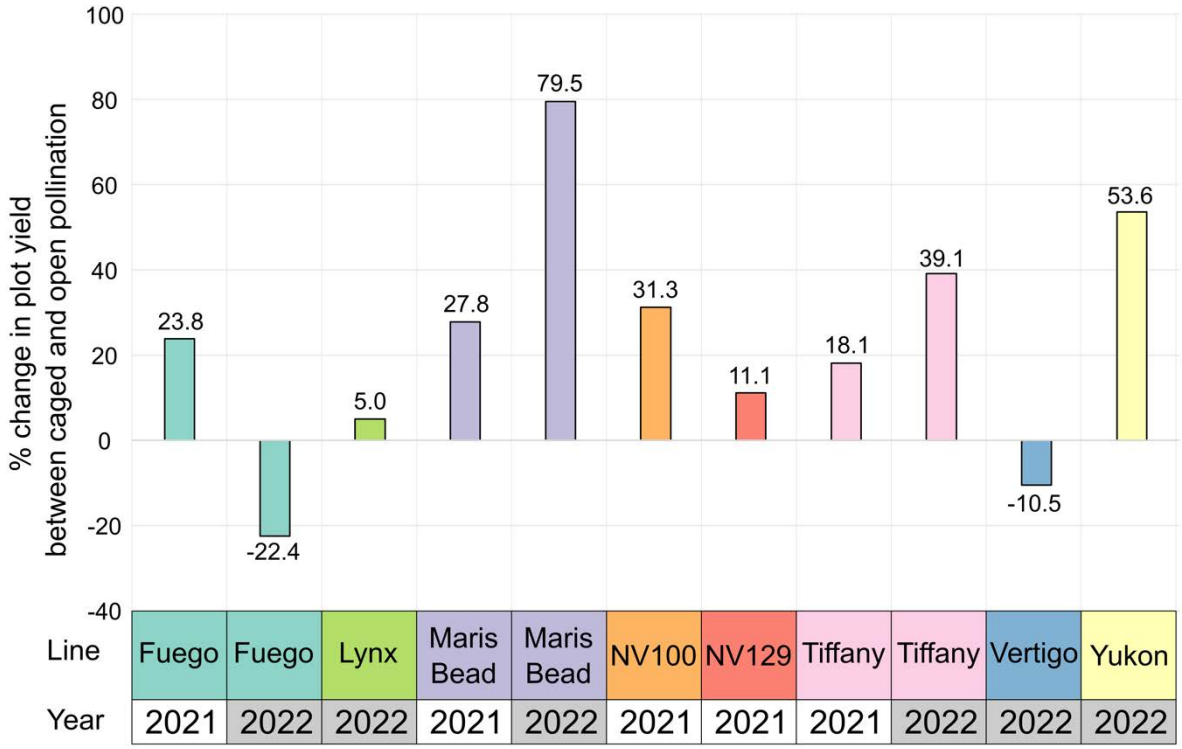


Figure 4.24 Percentage change in mean plot yield between caged and open pollinated plots for lines grown in 2021 and 2022. Across 2021 and 2022, 6 out of 8 lines experienced an increase in yield when open pollinated compared to when they were caged. The smallest positive change was for Lynx in 2022 (5.0%) and the largest was for Maris Bead in 2022 (79.5%). All lines grown in 2021 experienced an increase in yield with open pollination, however, Fuego and Vertigo showed a decrease in yield (-22.4% and -10.5% respectively). On average, lines experienced a plot yield benefit of 23.3% when lines were open pollinated compared to caged. For a breakdown of % changes for all yield parameters see Appendix H.

4.2.8 Relationship between bee visitation and plot yield

Strong positive correlations were found between the plot yield change with open pollination and both overall bee visitation rate, and legitimate visitation rate (**Figure 4.25**). A Pearson's correlation coefficient revealed a statistically significant positive correlation between plot yield change and overall bee visitation rate, r(4) = [0.83], p = [0.040] (**Figure 4.25 A**). There was also a strong positive correlation between plot yield change and legitimate bee visitation rate, but the correlation was not statistically significant at $p \leq 0.05$, r(4) = [0.80], p = [0.056] (**Figure 4.25 B**).

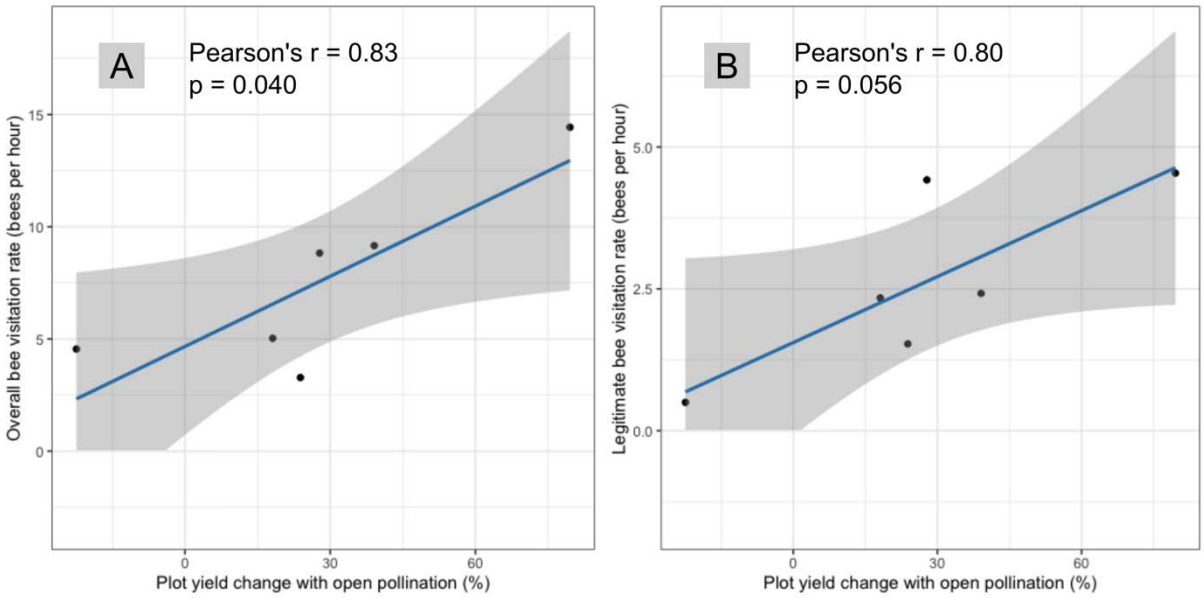


Figure 4.25 Relationship between yield benefit and bee visitation . Blue lines show linear models, grey bands show 95% confidence intervals around linear models. **(A)** A statistically significant strong positive correlation was found between plot yield change with open pollination and overall bee visitation rate r(4) = [0.83], p = [0.040]. **(B)** A strong positive correlation was found between plot yield change with open pollination and legitimate bee visitation rate, but was not statistically significant, r(4) = [0.80], p = [0.056].

4.3 Discussion and conclusions

The work presented in this chapter tested three hypotheses. The first hypothesis was that in field conditions, *Vicia faba* lines with floral traits which are theorised to be more attractive to pollinators will attract more pollinators than lines with floral traits which are theorised to be less attractive to pollinators. Across two field seasons it was found that bees most frequently visited the *V. faba* lines Maris Beas and Lynx, both of which have, among other traits, higher nectar content and more flowers per node than the other lines examined, supporting the hypothesis.

As each line differed in multiple floral traits, it is challenging to single out any one trait that was more important than any other for bee attraction. However, there are common features between the most visited lines. The main feature shared between Maris Bead and Lynx had in common was high nectar content. Maris Bead had the highest nectar concentration of any line, and Lynx had the highest nectar volume. Published research has established that bees prefer more concentrated nectar up to 55% w/w (Bailes et al. 2018). The results of this study show that in the field, nectar traits are likely to be key for pollinator attraction in *V. faba*. Flower appearance also contrasted greatly between lines, with those of Maris Bead having purple standard petals and pink corolla tubes, with intense purple veins on the adaxial side of the standard petal, unique among *V. faba* lines. Research has found that bees prefer purple hues, and highly saturated flowers (Raine and Chittka 2007; Reverté et al. 2016; Giurfa et al. 1995; Leslie et al. 2018). The strong venation of the Maris Bead standard petal may also act as a nectar guide, which may aid flower identification as has been shown in other systems (Leonard and Papaj 2011). The appearance of Maris Bead flowers may be innately more attractive to bees than those of the other lines. Experiments testing this hypothesis are presented in Chapter 5. Additionally, the more energy rich nectar of Maris Bead coupled with the distinctive appearance of flowers is likely to enhance innate preferences, through positive reinforcement.

During field trails, bee behaviour was also recorded, yielding interesting results. In 2021 most visits made to *V. faba* lines were legitimate. This is positive for the crop, as it is reasonable to assume that legitimate visits have the greatest probability of pollination and outcrossing (Kambal 1969). In 2022, most visits made were robbing, in part owing to the

greater abundance of honeybees in 2022 compared to 2021. Bee behaviour observations revealed that honeybees performed mostly extrafloral nectary visits and robbing, “white-tailed bumblebees” made legitimate visits and nectar rob, “red-tailed bumblebees” nectar rob, and “carder bees” made mostly legitimate visits. “Carder bees” and “white-tailed bumblebees” therefore have potential to be the most effective pollinators of *V. faba* as they perform most legitimate visits. This is the first study to demonstrate that bees show preference between *V. faba* lines in agricultural settings on this scale and that variation in floral traits does impact bee visitation and quality of pollination service.

The most-robbed lines were also those requiring the most force to open flowers. It is possible that, although bees were attracted to plots of Lynx, Vertigo and Maris Bead due to appearance or smell, nectar robbing was a common behaviour due to the effort required to open flowers. This may be the case particularly for honeybees, being the smallest bee type to visit *V. faba* flowers. Bee tongue length is also likely to be responsible for the observed rates of nectar robbing, with shorter tongued species like honeybees and red-tailed bumblebees carrying out more nectar robbing, but carder bees carrying out more legitimate visits. Although not possible on the scale of this trial, it would have been advantageous to identify bees recorded in the “white-tailed bumblebee” category to species level, as published data state that the short-tongued species *Bombus terrestris* and *Bombus lucorum* have been observed nectar robbing *V. faba*, whereas the long-tongued species, *Bombus hortorum*, is more likely to make legitimate visits (Gray 1993).

The second hypothesis was that in field conditions, *Vicia faba* plants will have lower yield when pollinators are excluded, compared to when pollinators are not excluded. In 2021, yield measures including the number of pods per plant, the number of seeds per pod, the mass of seeds per plant and the plot yield were lower when pollinators were excluded for most lines studied. In 2022 the plot yield was lower for four out of the six lines studied. Together, data from the two field seasons support the hypothesis, with the additional insight that the size of yield change due to pollinator exclusion varies greatly between *V. faba* lines. These results agree with the consensus of published literature that biotic pollination has a positive effect on *V. faba* yield, and that genotype is largely responsible for differences in the size of yield change (Bishop and Nakagawa 2020). This is the first study to compare the effect of pollinator exclusion and open pollination treatments on yield of field plots at this

scale. The results add to evidence that many *V. faba* lines being grown in the UK depend highly on wild pollinators for yield, and as such, more efforts need to be made to support wild pollinators.

Although only Maris Bead, NV100 and NV129 produced significantly fewer pods per plant with the caged treatment in 2021, the number of seeds per plant, and seeds per pod, was reduced for all lines when caged. The percentage change in number of seeds per plant ranged from 9.54% (Fuego) to 46.74% (Maris Bead), (Appendix H), in line with published data which range from 5 to 61% (Varis and Brax 1990; Suso and del Río 2015). The change in number of seeds per pod ranged from 9.94% (Fuego) to 21.75% (Maris Bead), above the range of published data (7 to 14%) (Kendall and Smith 1975; Varis and Brax 1990). Change in number of pods per plant ranged from 6.62% (Tiffany) to 37.98% (Maris Bead), within the range of published data (1 to 49%) (Varis and Brax 1990; Bishop et al. 2020).

As found by (Bishop et al. 2020), within-plant measures of yield can vary across the length of the stem. This was true within the 2021 trial, with lines showing greatest seed set in the lower two thirds of the stem, characteristic of *V. faba* (Suso and del Río 2015; Bishop et al. 2016a). The distribution of seed set along the stem differed between lines, with Maris Bead and Tiffany setting far greater numbers of pods at lower nodes when open pollinated. The same pattern was found in certain cases for Diana, Fuego, Fury and Vertigo when cross pollinated by (Bishop et al. 2020) and for L602 by (Stoddard 1986a). The results of the 2021 field trial reaffirm the need to sample whole plants to properly estimate yield effects of pollination treatments.

Mass parameters followed the same trend as pod and seed number parameters, with open pollinated plants producing a greater seed mass per plant and mean seed mass per pod. The mean mass per bean was lower for all lines when open pollinated, indicating that plants on average produced a greater mass and greater number of seeds when open pollinated, but individual seeds were smaller than when plants were caged. However, both have a positive outcome for production, with a greater mass of beans produced for consumption and a greater number of seeds for planting crops in subsequent years.

In 2021, plot yield was higher for every line when open pollinated, especially for Maris Bead. Differences were seen between lines, as expected, due to variation in yield independent of pollination treatments (Skovbjerg et al. 2020). In 2022, plot yield was again higher for Maris Bead, however, a loss of yield was seen for Fuego and Vertigo. For both Maris Bead and Tiffany, the yield benefit with pollination was greater in 2022 compared to 2021, and only Fuego saw a decrease in yield benefit into negative figures. The yield benefits observed, from 4.99% (Lynx 2022) to 79.53% (Maris Bead 2022) are greater than the range in current literature, from 6% to 60% (Varis and Brax 1990; Bishop et al. 2020). Yield loss due to pollination has been documented before but poorly discussed, with tissue damage due to over-pollination providing one explanation (Link 1990; Velthuis and van Doorn 2006; Bishop et al. 2020). However, considering the low bee visitation rate to Fuego, this seems unlikely. Results of the 2022 season may have been strongly affected by weather, with extreme drought and the highest summer temperatures ever seen in the UK (Met Office 2022). Weather appeared to have a large effect on the absolute plot yield of lines. Although open pollinated Maris Bead yielded highest in both 2021 and 2022, mean yield was 1.51 kg per plot in 2021, whereas in 2022 it was only 0.52 kg.

The third hypothesis was that in field conditions, *Vicia faba* plants with floral traits which are theorised to be more attractive to pollinators will receive a greater yield benefit with open pollination than *Vicia faba* plants with floral traits which are theorised to be more attractive to pollinators. Using plot yield of the three *V. faba* lines grow across both 2021 and 2022, a statistically significant positive correlation was found between the overall bee visitation rate to lines and the plot yield change between cage and open pollination treatments. A strong positive correlation was also found between the legitimate bee visitation rate to lines and the plot yield change between cage and open pollination treatments. This indicates that lines receiving more bee visits because of their floral traits also receive a better pollination service, resulting in a greater yield increase. Together these results support the hypothesis and make this the first study to demonstrate that differences in floral traits have direct consequences for *V. faba* yield.

Although bee visitation may be a significant factor affecting yield, the selfing ability (autofertility) of lines will have influenced the yield benefit received by bee pollination in this study. This may explain the plot yield for Lynx in 2022. Although open pollination had a

positive effect on Lynx plot yield, the change was small (+4.99% increase to 0.46 kg), as caged plants also produced a relatively high yield (0.44 kg). In contrast, caged plot yield was much lower for Maris Bead (0.29 kg), Tiffany (0.32 kg), and Yukon (0.27 kg), but each line experienced a much greater yield increase when open pollinated: Maris Bead (79.53% increase to 0.52 kg), Tiffany (39.09% increase to 0.44 kg), and Yukon (53.59% increase to 0.42 kg). Therefore, although bee pollination has a positive effect on yield overall, the benefit may differ between lines. More autofertile lines, like Lynx, may be better suited for environments where pollinator populations have been depleted. However, as shown by (Bishop et al. 2016b; Bishop et al. 2016a), heat stress can drive the dependence of *V. faba* yield from more selfing to outcrossing, and yield loss due to stress can be attenuated by bee pollination. Considering that the UK climate is likely to warm further due to climate change, it is essential that floral traits are optimised to increase bee attraction and outcrossing, as autofertile lines still benefit from bee pollination in stressful conditions.

Conclusions

The field trial experiments presented in this chapter revealed that the *V. faba* lines Maris Bead and Lynx attracted greater numbers of bees, most likely because they possess floral traits which are suggested to be more attractive to bees. The influence of variation in specific *V. faba* floral traits on bee behaviour is explored further in Chapter 5. Some bees, including those recorded as “white tailed bumblebees” and “carder bees”, have greater potential to pollinate *V. faba* flowers by making more legitimate visits, whereas honeybees and “red-tailed bumblebees” provide a limited pollination service and deplete plants of resources by nectar robbing and visiting extrafloral nectaries. The preference of bees is consistent between locations, showing that the findings of this project should be applicable regardless of location if similar bee species are present. Overall, *V. faba* plants receive a significant benefit from insect pollination, but the size of the benefit is dependent upon the line. The yield benefit experienced because of bee pollination positively correlates with bee visitation. It is therefore likely that efficacy of pollination service received, and the yield of *V. faba*, is directly affected by attractiveness of floral traits to bees.

Together these results strongly indicate that *V. faba* crops being grown in the UK highly depend on wild pollinators for their yield, and that floral traits have a significant impact on

bee visitation and yield. Consequently, more needs to be done to support wild pollinators, through farm management practises, and planting of *V. faba* lines which have good floral resources. Additionally, it is possible for farmers to increase the chance of good yield by growing *V. faba* lines which are more attractive to pollinators, like Maris Bead and Lynx.

5 Effect of floral trait variation on bumblebee behaviour in controlled conditions

5.1 Introduction

Bumblebees and other pollinating insects use a variety of sensory cues to identify flowers. The most easily detectable floral cues include volatile emissions, floral display size, colour, and patterning. A combination of these signals is likely to influence pollinator preference and signals may operate across different scales.

Floral scent has been shown to influence pollinator attraction over long distances and to work synergistically with visual traits at short range to attract pollinators (Raguso and Willis 2002). Manipulation of scent has recently been applied in agricultural scenarios to enhance honeybee visitation to sunflowers on field scales (Farina et al. 2020). Appearance of flowers is one of the most important attractants for pollinators (Chittka and Raine 2006), and different components of appearance may be important at different distances from flowers (Lehrer et al. 1995). At the furthest scale, pollinators are generally attracted to larger floral displays, with larger displays allowing easier identification of flowers. However, floral colour is particularly important for pollinator attraction and decision making (Heiling et al. 2003; Omura and Honda 2005), with bumblebees showing preference between colours (Raine and Chittka 2007). Alongside colour, floral patterning, including presence of nectar guides, is likely to aid flower identification, flower handling and direct bees towards the flower's reward, especially important with complex zygomorphic flowers. These patterns may therefore provide a means of increasing foraging efficiency (Waser and Price 1983; Dinkel and Lunau 2001).

By understanding the effect of floral traits on bee behaviour it may be possible to manipulate these traits to assist bees, allowing them to identify flowers more easily, and create flowers which are inherently more attractive to pollinators. When carried out in crops, and paired with enhanced reward, manipulation of attractive floral traits may provide a means of both ensuring adequate pollination of crops and providing more beneficial resources for wild pollinators.

In Chapter 3, variation was identified in several floral traits likely to be important in flower selection by bumblebees. Lines of *V. faba* exhibiting variation in these traits were then compared in Chapter 4 at a field scale to observe any effects on pollinator visitation in a realistic agricultural setting. The findings of both chapters helped to inform focused bee behaviour investigations presented in this chapter, which aims to shed light on specific floral traits which may influence bee preference between *V. faba* flowers.

5.2 Results

5.2.1 Responses of *Bombus terrestris* to extremes of wing petal spot size

For methodology refer to section 2.7.2.

Innate preference test

When presented with large and small spotted flower models, 56% of first choices made by 50 naïve foragers were to small spotted models and 44% to large spotted models (**Figure 5.1A**). The difference in the proportion of visits was not significant when examined using a binomial test (n = 50, p = 0.48), indicating that bees had no innate preference.

Across the first 10 choices, 52% of visits made by 50 naïve foragers were to small spotted models and 48% to large spotted models (**Figure 5.1B**). The difference in the proportion of visits was not significant when examined using a two-tailed t test (t(49) = 1.73, p = 0.12), indicating that bees had no innate preference.

Differential conditioning test

As bumblebees showed no significant innate preference between large and small spotted flower models, a differential conditioning experiment was performed to determine whether bumblebees can discriminate between large and small spotted flower models.

When a differential conditioning test was performed using large and small spotted flower models, the proportion of foragers visiting rewarded models significantly increased with successive visits ($p = 2.0\times10^{-16}$ when a GLM is fitted to the data set) (**Figure 5.2A**). When fitted to a generalised binomial linear model, the model showed that the probability of making a correct choice increased from 0.48 [95% CI: 0.44-0.52] after the 10th visit, to 0.70 [95% CI: 0.68-0.73] at the 50th visit, and to 0.89 [95% CI: 0.89-0.91] at the 100th visit. When considering only experiments in which large spotted flower models were rewarded, the GLM again showed that the proportion of foragers visiting rewarded models significantly increased with successive visits ($p = 2.0\times10^{-16}$). The probability of making a correct choice increased from 0.48 [95% CI: 0.42-0.53] after the 10th visit, to 0.71 [95% CI: 0.68-0.74] at the 50th visit, and 0.89 [95% CI: 0.86-0.96] at the 100th visit (**Figure 5.2B**). When considering only

experiments in which small spotted flower models were rewarded, the GLM again showed that the proportion of foragers visiting rewarded models significantly increased with successive visits ($p = 2.0 \times 10^{-16}$). The probability of making a correct choice increased from 0.49 [95% CI: 0.43-0.54] after the first 10 visits, to 0.70 [95% CI: 0.67-0.73] at the 50th visit, and 0.87 [95% CI: 0.83-0.90] after 100 visits (**Figure 5.2C**).

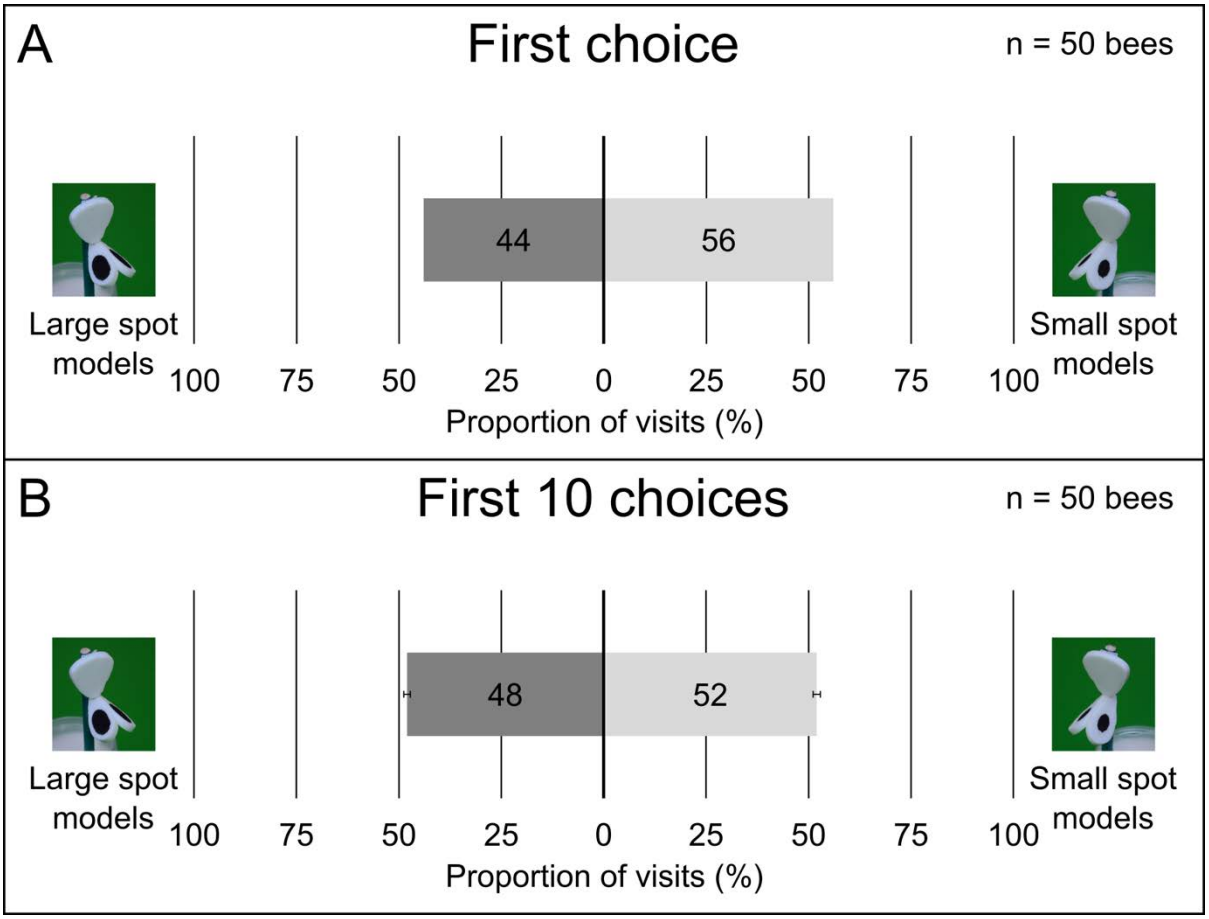


Figure 5.1 Bumblebees do not show innate preference between large and small spotted wing petal models. (A) The percentage of visits made on first encounter by 50 naïve foragers to large and small spotted wing petal models in an innate preference test where both models were equally rewarded. There was no significant preference between models (binomial test; n = 50, p = 0.48). **(B)** The percentage of visits made by 50 naïve foragers to large and small spotted wing petal models across their first 10 choices in an innate preference test where both models were equally rewarded. There was no significant preference between models (two-tailed t test; t(49) = 1.73, p = 0.12).

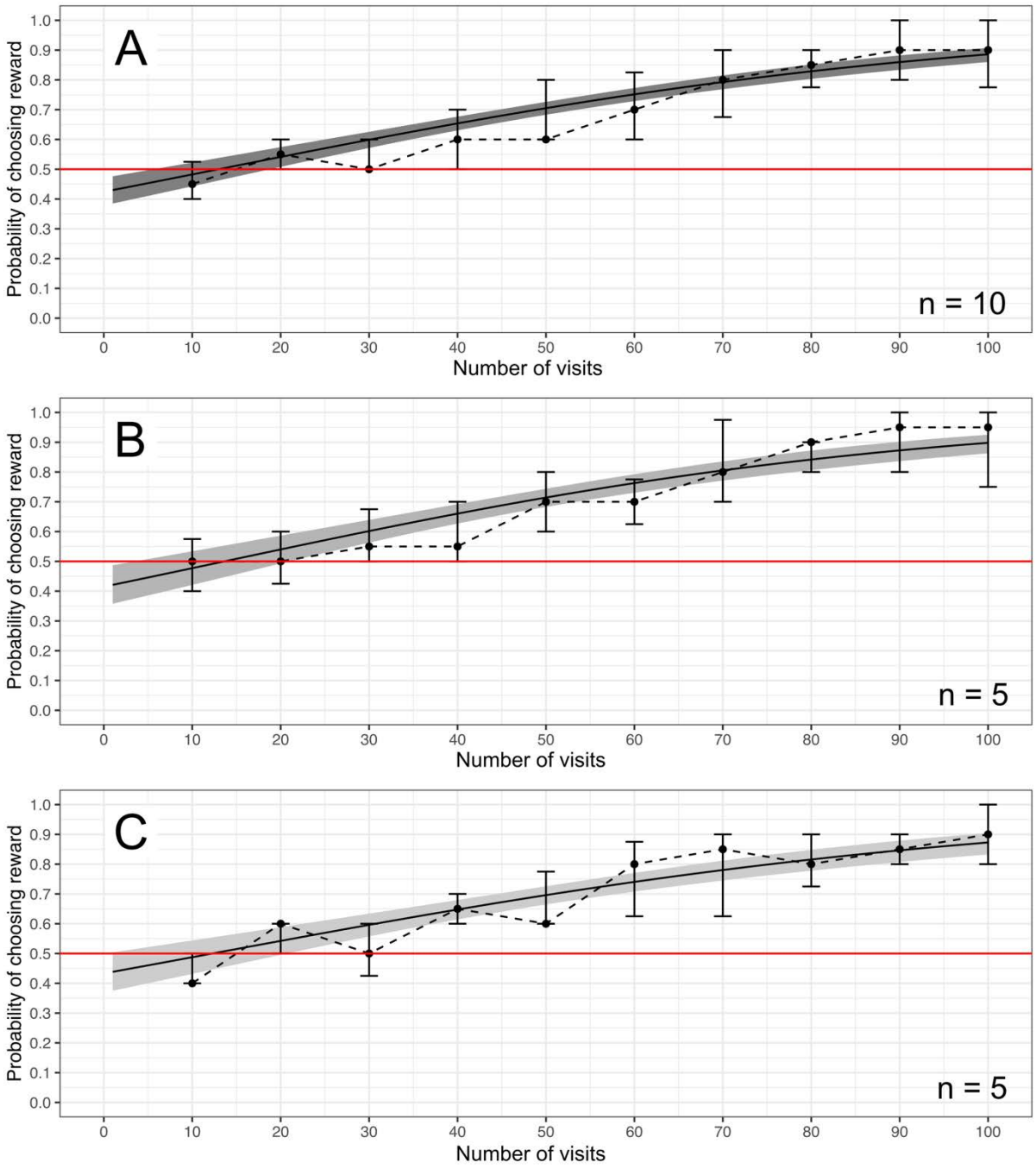


Figure 5.2 Bumblebees can perceive the difference between large and small spotted wing petal models in a differential conditioning experiment using 40% w/w sucrose solution to reward one choice and a 0.12% w/w quinine punishment with the other choice. For each pane, a black solid line shows the probability of choosing reward, estimated with a generalised linear model (GLM) fitted on bees' choices in function of the number of visits. Grey shading around the solid line represents 95% confidence intervals. Black dots joined by dashed lines indicate the mean proportion of correct choices, every 10 choices, for all bees, for 100 consecutive choices. The red horizontal line indicates the 0.5 probability value. Values above the 0.5 probability line represent increased success.

(A) The proportion of foragers (n=10) visiting rewarded models significantly increased with successive visits ($p = 2.0 \times 10^{-16}$ when a GLM is fitted to the data set). The GLM estimates that by the 10^{th} visit, the probability of a correct choice is 0.48 [95% CI: 0.44-0.52], by the 50^{th} visit, the probability of a correct choice is 0.70 [95% CI: 0.68-0.73], and by the 100^{th} visit, it is 0.89 [95% CI: 0.86-0.91].

(B) The proportion of foragers (n=5) visiting rewarded models significantly increased with successive visits when considering only experiments in which large spotted models were rewarded ($p = 2.0 \times 10^{-16}$ when a GLM is fitted to the data set). The GLM estimates that by the 10^{th} visit, the probability of a correct choice is 0.48 [95% CI: 0.42-0.53], by the 50^{th} visit, the the probability of a correct choice is 0.71 [95% CI: 0.68-0.74] and by 100 visits, it is 0.90 [95% CI: 0.86-0.93].

(C) The proportion of foragers (n=5) visiting rewarded models significantly increased with successive visits when considering only experiments in which small spotted models were rewarded ($p = 2.0 \times 10^{-16}$ when a GLM is fitted to the data set). The GLM estimates that by the 10^{th} visit, the probability of a correct choice is 0.49 [95% CI: 0.43-0.54], by the 50^{th} visit, the the probability of a correct choice is 0.70 [95% CI: 0.67-0.73], and by the 100^{th} visit, it is 0.87 [95% CI: 0.83-0.90].

5.2.2 Responses of *Bombus terrestris* to standard petal appearance

For methodology refer to section 2.7.3.

Innate preference tests

Experiment 1 – Maris Bead and NV129 standard petal images

When presented with images of Maris Bead and NV129 standard petals, 80% of first choices made by 20 naïve foragers were to Maris Bead and 20% to NV129 (**Figure 5.3A**). The difference in the proportion of visits was significant when examined using a binomial test (n = 20, p = 0.012).

Across the first 10 choices, 67.5% of visits made by 20 naïve foragers were to Maris Bead images and 32.5% of visits were to NV129 images (**Figure 5.3B**). The difference in the proportion of visits was significant when examined using a two-tailed t test (t = 7.13, p = 1.63×10^{-8}).

Experiment 2 – Maris Bead and NV129 standard petal average colour

When presented with printed discs the average colour of Maris Bead and NV129 standard petals, 55% of first choices made by 20 naïve foragers were to purple (Maris Bead) discs and 45% to grey (NV129) disks (**Figure 5.4A**). The difference in the proportion of visits was not significant when examined using a binomial test (n = 20, p = 0.82).

Across the first 10 choices, 53% of visits made by 20 naïve foragers were to purple (Maris Bead) discs and 47% of visits were to grey (NV129) discs (**Figure 5.4B**). The difference in the proportion of visits was not significant when examined using a two-tailed t test (t = -1.10, p = 0.28).

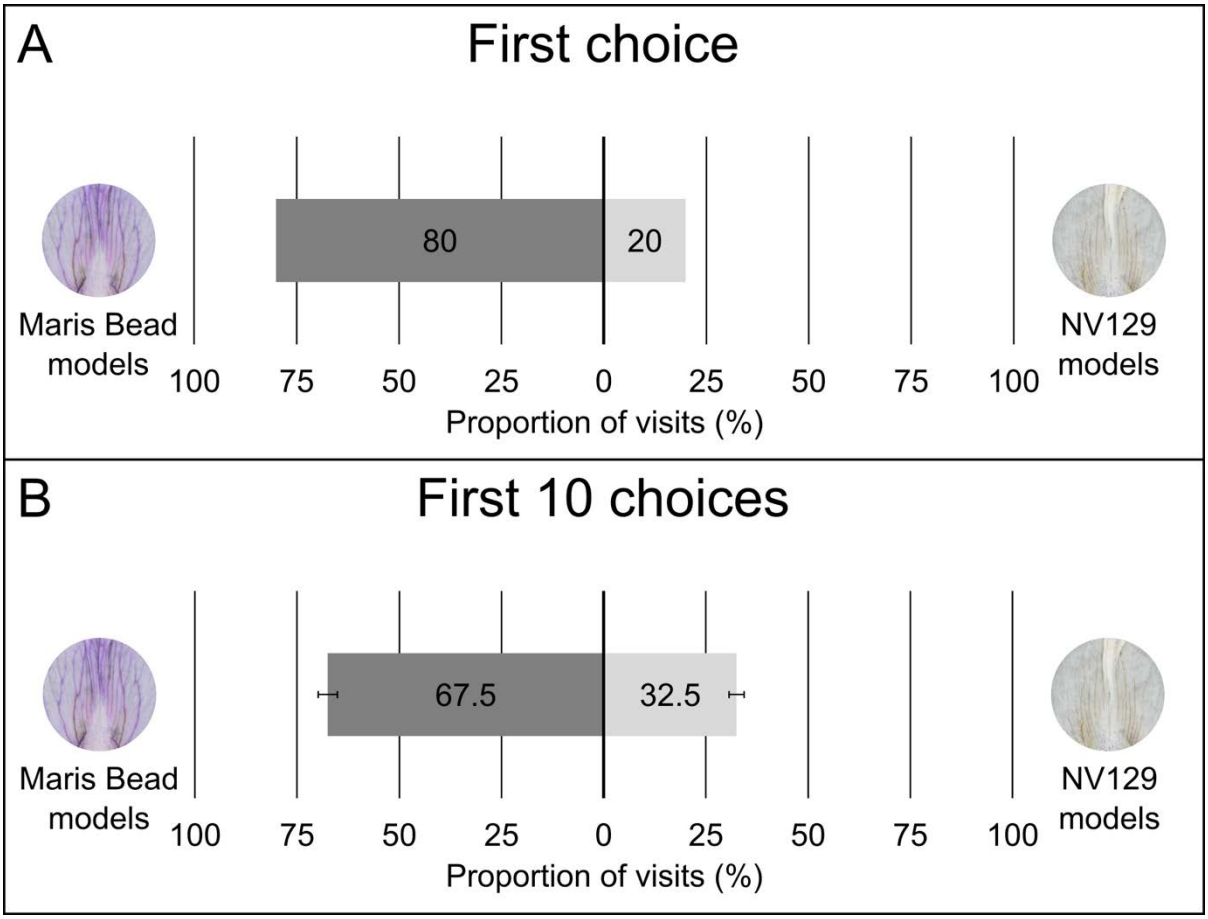


Figure 5.3 Bumblebees show innate preference for printed Maris Bead standard petal models over NV129 standard petal models. **(A)** The percentage of visits made by 20 naïve foragers to visible-spectrum printed models of Maris Bead and NV129 standard petals in an innate preference test where both models were equally rewarded. There was a significant preference for Maris Bead models over NV129 models (binomial test; n = 20, p = 0.012). **(B)** The percentage of visits made by 20 naïve foragers to models of Maris Bead and NV129 standard petals across their first 10 choices in an innate preference test where both models were equally rewarded (mean ± SE). There was a significant preference for Maris Bead models over NV129 models ($t(20) = 7.13$, $p = 1.63 \times 10^{-8}$).

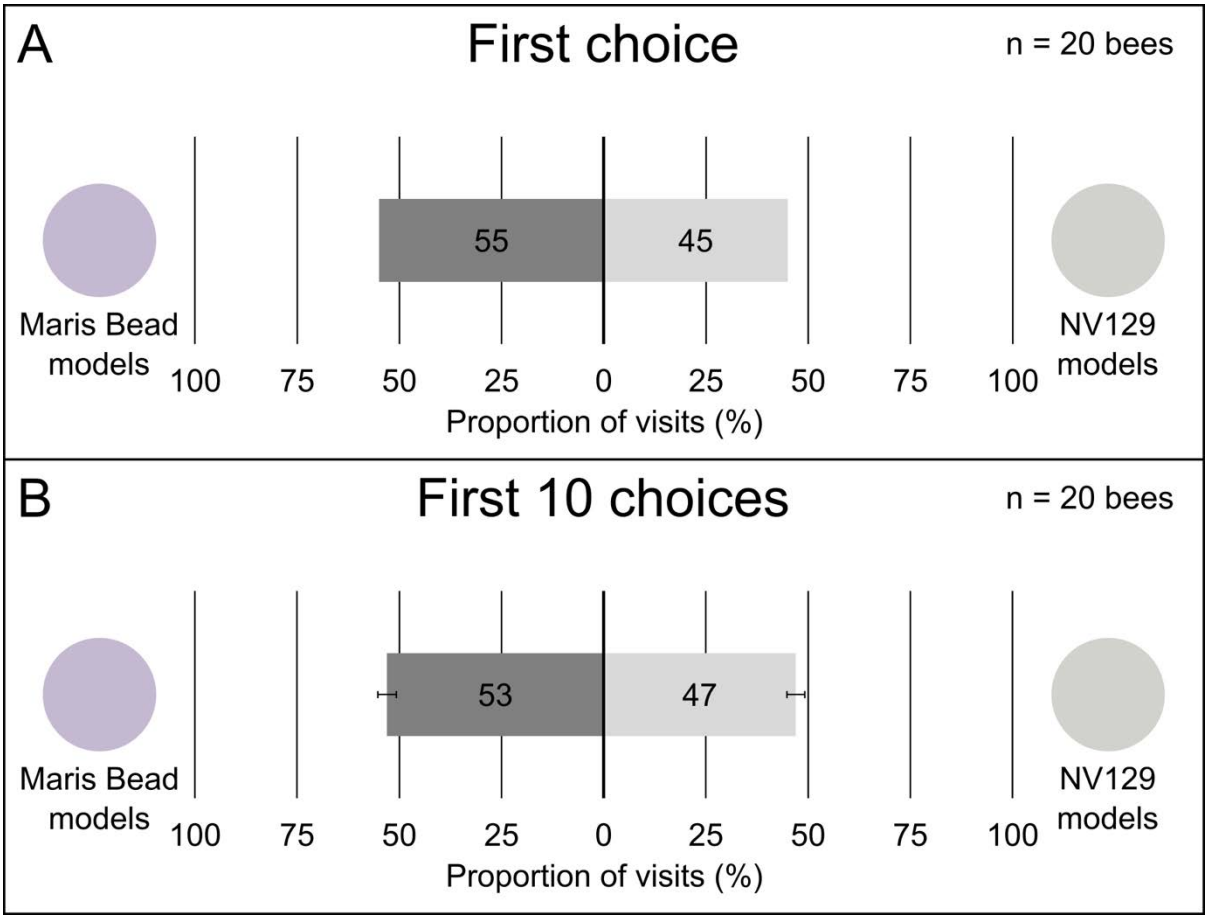


Figure 5.4 Bumblebees do not show innate preference between standard petal average colour models of Maris Bead and NV129. **(A)** The percentage of visits made by 20 naïve foragers to visible-spectrum standard petal average colour models of Maris Bead and NV129 in an innate preference test where both models were equally rewarded. There was no significant preference for either model (binomial test; n = 20, p = 0.82). **(B)** The percentage of visits made by 20 naïve foragers to standard petal average colour models of Maris Bead and NV129 across their first 10 choices in an innate preference test where both models were equally rewarded (mean ± SE). There was no significant preference for either model (t(20) = -1.10, p = 0.28).

Experiment 3 – Maris Bead and NV129 standard petal vein colour

When presented with printed discs the colour of Maris Bead and NV129 standard petal veins, 90% of first choices made by 20 naïve foragers were to purple (Maris Bead) discs and 10% to yellow/brown (NV129) discs (**Figure 5.5A**). The difference in the proportion of visits was significant when examined using a binomial test (n = 20, p = 0.000403).

Across the first 10 choices, 82% of visits made by 20 naïve foragers were to purple (Maris Bead) discs and 18% of visits were to yellow/brown (NV129) discs (**Figure 5.5B**). The difference in the proportion of visits was significant when examined using a two-tailed t test ($t = -9.34$, $p = 2.20 \times 10^{-11}$).

Experiment 4 – Maris Bead and NV129 standard petal vein patterning

When presented with Maris Bead and NV129 standard petal vein models, 45% of first choices made by 20 naïve foragers were to Maris Bead vein models and 55% to NV129 vein models (**Figure 5.6A**). The difference in the proportion of visits was not significant when examined using a binomial test (n = 20, p = 0.82).

Across the first 10 choices, 47% of visits made by 20 naïve foragers were to Maris Bead vein models and 53% of visits were to NV129 vein models (**Figure 5.6B**). The difference in the proportion of visits was not significant when examined using a two-tailed t test (t = -1.50, p = 0.14).

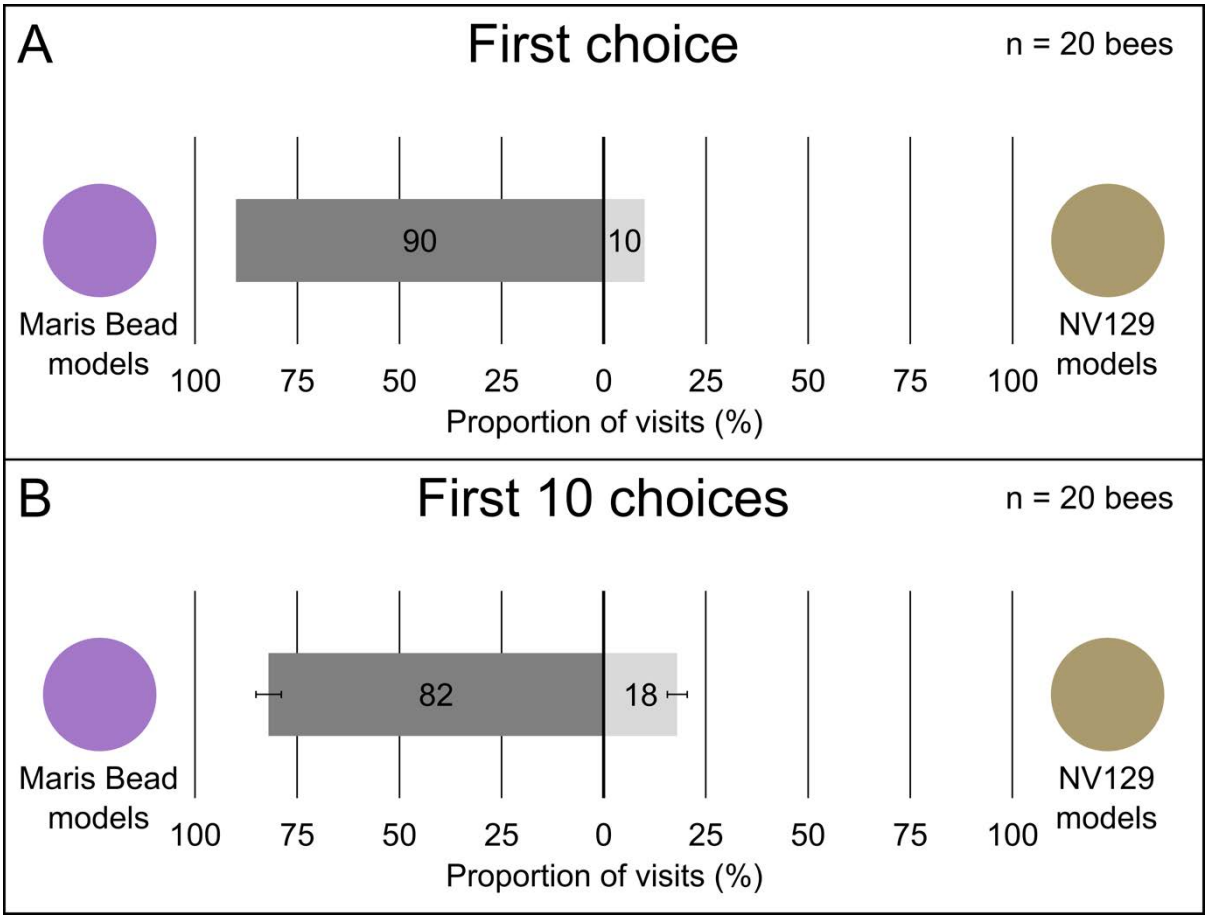


Figure 5.5 Bumblebees show innate preference for Maris Bead standard petal vein colour models over NV129 standard petal vein colour models. **(A)** The percentage of visits made by 20 naïve foragers to visible-spectrum standard petal vein colour models of Maris Bead and NV129 in an innate preference test where both models were equally rewarded. There was a significant preference for Maris Bead standard petal vein colour models over NV129 models (binomial test; n = 20, p = 0.000403). **(B)** The percentage of visits made by 20 naïve foragers to standard petal vein colour models of Maris Bead and NV129 across their first 10 choices in an innate preference test where both models were equally rewarded (mean ± SE). There was a significant preference for Maris Bead standard petal vein colour models over NV129 models ($t(20) = -9.34$, $p = 2.20 \times 10^{-11}$).

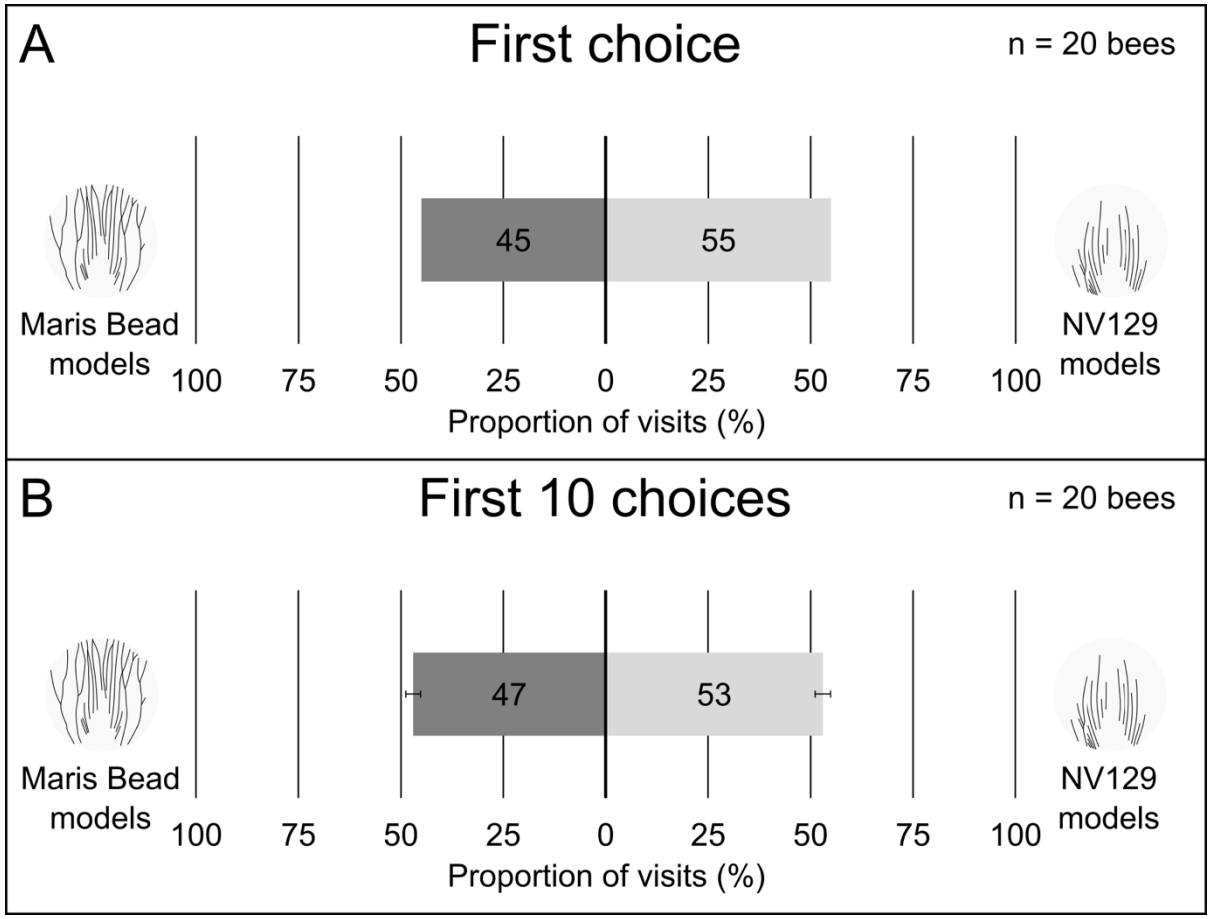


Figure 5.6 Bumblebees do not show innate preference between standard petal vein pattern models of Maris Bead and NV129. **(A)** The percentage of visits made by 20 naïve foragers to standard petal vein pattern models of Maris Bead and NV129 in an innate preference test where both models were equally rewarded. There was no significant preference for either model (binomial test; n = 20, p = 0.82). **(B)** The percentage of visits made by 20 naïve foragers to standard petal vein pattern models of Maris Bead and NV129 across their first 10 choices in an innate preference test where both models were equally rewarded. (mean ± SE). There was no significant preference for either model ($t(20) = -1.50$, $p = 0.14$).

Differential conditioning tests

Bumblebees showed no significant innate preference between Maris Bead and NV129 printed models for standard petal average visible-spectrum colour or standard petal vein pattern. Differential conditioning experiments were then performed to determine whether bumblebees can discriminate between the differences in petal visible-spectrum average colour and vein patterning used in the models. For methodology refer to section 2.7.3.

Maris Bead and NV129 standard petal average colour

When a differential conditioning test was performed using Maris Bead and NV129 standard petal visible-spectrum average colour models, the proportion of foragers visiting rewarded models significantly increased with successive visits ($p = 2.0\times10^{-16}$ when a GLM is fitted to the data set) (**Figure 5.7A**). When fitted to a generalised binomial linear model, the model showed that the probability of making a correct choice increased from 0.45 [95% CI: 0.40-0.52] after the 10th visit, to 0.80 [95% CI: 0.77-0.83] at the 50th visit, and to 0.97 [95% CI: 0.95-0.98] at the 100th visit. When considering only experiments in which Maris Bead average colour models were rewarded, the GLM again showed that the proportion of foragers visiting rewarded models significantly increased with successive visits ($p = 2.0\times10^{-16}$). The probability of making a correct choice increased from 0.43 [95% CI: 0.35-0.51] after the 10th visit, to 0.82 [95% CI: 0.78-0.86] at the 50th visit, and 0.98 [95% CI: 0.96-0.99] at the 100th visit (**Figure 5.7B**). When considering only experiments in which NV129 average colour models were rewarded, the GLM again showed that the proportion of foragers visiting rewarded models significantly increased with successive visits ($p = 1.14\times10^{-14}$). The probability of making a correct choice increased from 0.48 [95% CI: 0.40-0.56] after the first 10 visits, to 0.78 [95% CI: 0.74-0.82] at the 50th visit, and 0.95 [95% CI: 0.91-0.97] after 100 visits (**Figure 5.7C**).

Maris Bead and NV129 standard petal vein patterning

When a differential conditioning test was performed using Maris Bead and NV129 standard petal vein models, the proportion of foragers visiting rewarded models did not significantly increase with successive visits ($p = 0.62$ when a GLM is fitted to the data set) (**Figure 5.8A**). When fitted to a generalised binomial linear model, the model showed that the probability of making a correct choice was 0.52 [95% CI: 0.46-0.57] at the 10th visit, 0.53 [95% CI: 0.50-0.56] at the 50th visit, and 0.54 [95% CI: 0.48-0.60] at the 100th visit. When considering only experiments in which Maris Bead vein models were rewarded, the GLM again showed that the proportion of foragers visiting rewarded models did not significantly increase with successive visits ($p = 0.82$). The probability of making a correct choice was 0.53 [95% CI: 0.45-0.61] at the 10th visit, 0.54 [95% CI: 0.49-0.58] at the 50th visit, and 0.55 [95% CI: 0.46-0.63] at the 100th visit (**Figure 5.8B**). When considering only experiments in which NV129 vein models were rewarded, the GLM again showed that the proportion of foragers visiting rewarded models did not significantly increase with successive visits ($p = 0.63$). The probability of making a correct choice was 0.50 [95% CI: 0.43-0.58] at the first 10 visits, 0.52 [95% CI: 0.47-0.56] at the 50th visit, and 0.54 [95% CI: 0.45-0.62] after 100 visits (**Figure 5.8C**).

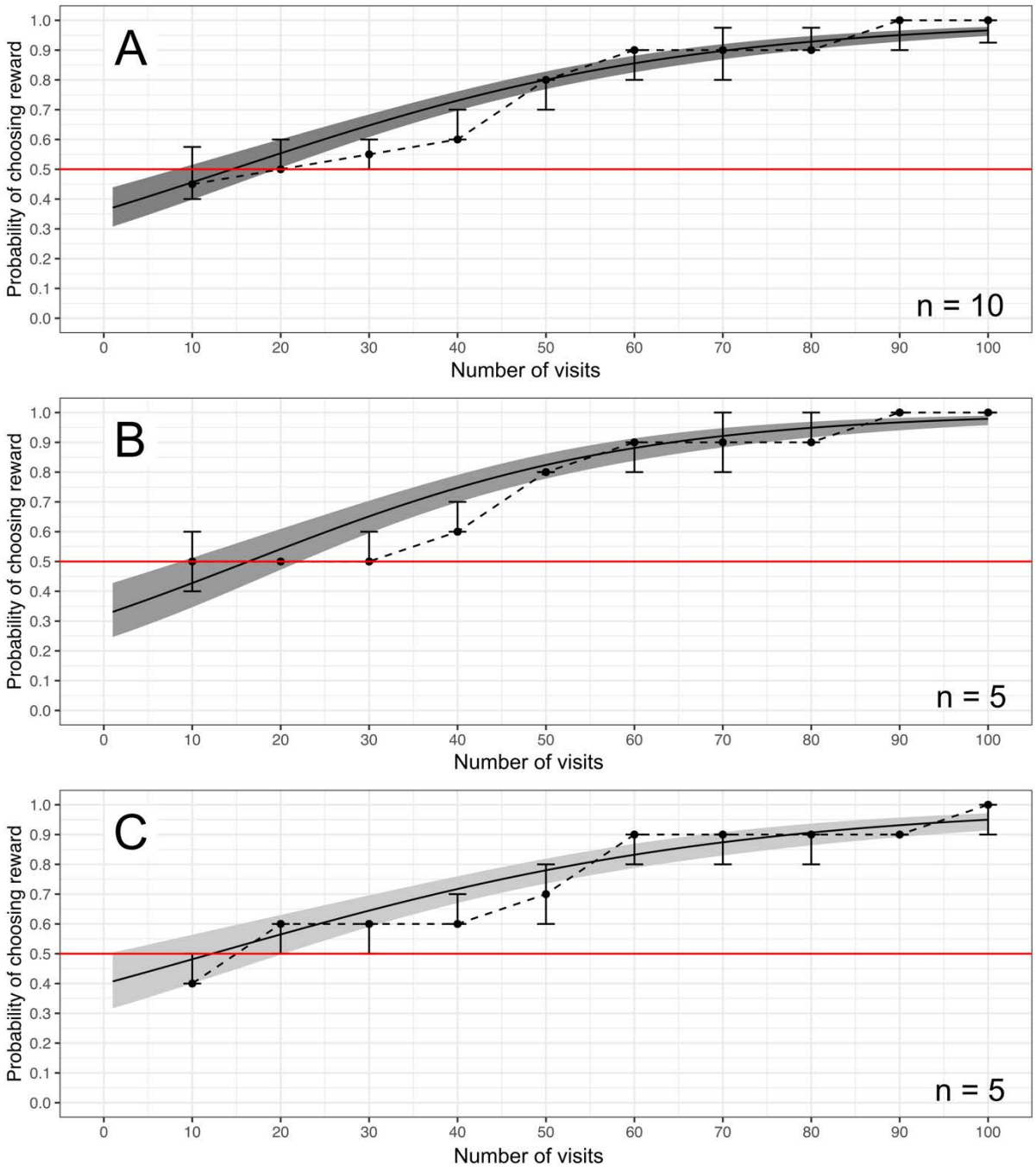


Figure 5.7 Bumblebees can perceive the difference between Maris Bead and NV129 standard petal average colour models in a differential conditioning experiment using 40% w/w sucrose solution to reward one choice and a 0.12% w/w quinine punishment with the other choice. For each pane, a black solid line shows the probability of choosing reward, estimated with a generalised linear model (GLM) fitted on bees' choices in function of the number of visits. Grey shading around the solid line represents 95% confidence intervals. Black dots joined by dashed lines indicate the mean proportion of correct choices, every 10 choices, for all bees, for 100 consecutive choices. The red horizontal line indicates the 0.5 probability value. Values above the 0.5 probability line represent increased success.

(A) The proportion of foragers (n=10) visiting rewarded models significantly increased with successive visits ($p = 2.0 \times 10^{-16}$ when a GLM is fitted to the data set). The GLM estimates that by the 10th visit, the probability of a correct choice is 0.45 [95% CI: 0.40-0.52], by the 50th visit, the probability of a correct choice is 0.80 [95% CI: 0.77-0.83], and by the 100th visit, it is 0.97 [95% CI: 0.95-0.98].

(B) The proportion of foragers (n=5) visiting rewarded models significantly increased with successive visits when considering only experiments in which Maris Bead average colour models were rewarded ($p = 2.0 \times 10^{-16}$ when a GLM is fitted to the data set). The GLM estimates that by the 10th visit, the probability of a correct choice is 0.43 [95% CI: 0.35-0.51], by the 50th visit, the probability of a correct choice is 0.82 [95% CI: 0.78-0.86], and by 100 visits, it is 0.98 [95% CI: 0.96-0.99].

(C) The proportion of foragers (n=5) visiting rewarded models significantly increased with successive visits when considering only experiments in which NV129 average colour models were rewarded ($p = 1.14 \times 10^{-14}$ when a GLM is fitted to the data set). The GLM estimates that by the 10th visit, the probability of a correct choice is 0.48 [95% CI: 0.40-0.56], by the 50th visit, the probability of a correct choice is 0.78 [95% CI: 0.74-0.82], and by the 100th visit, it is 0.95 [95% CI: 0.91-0.97].

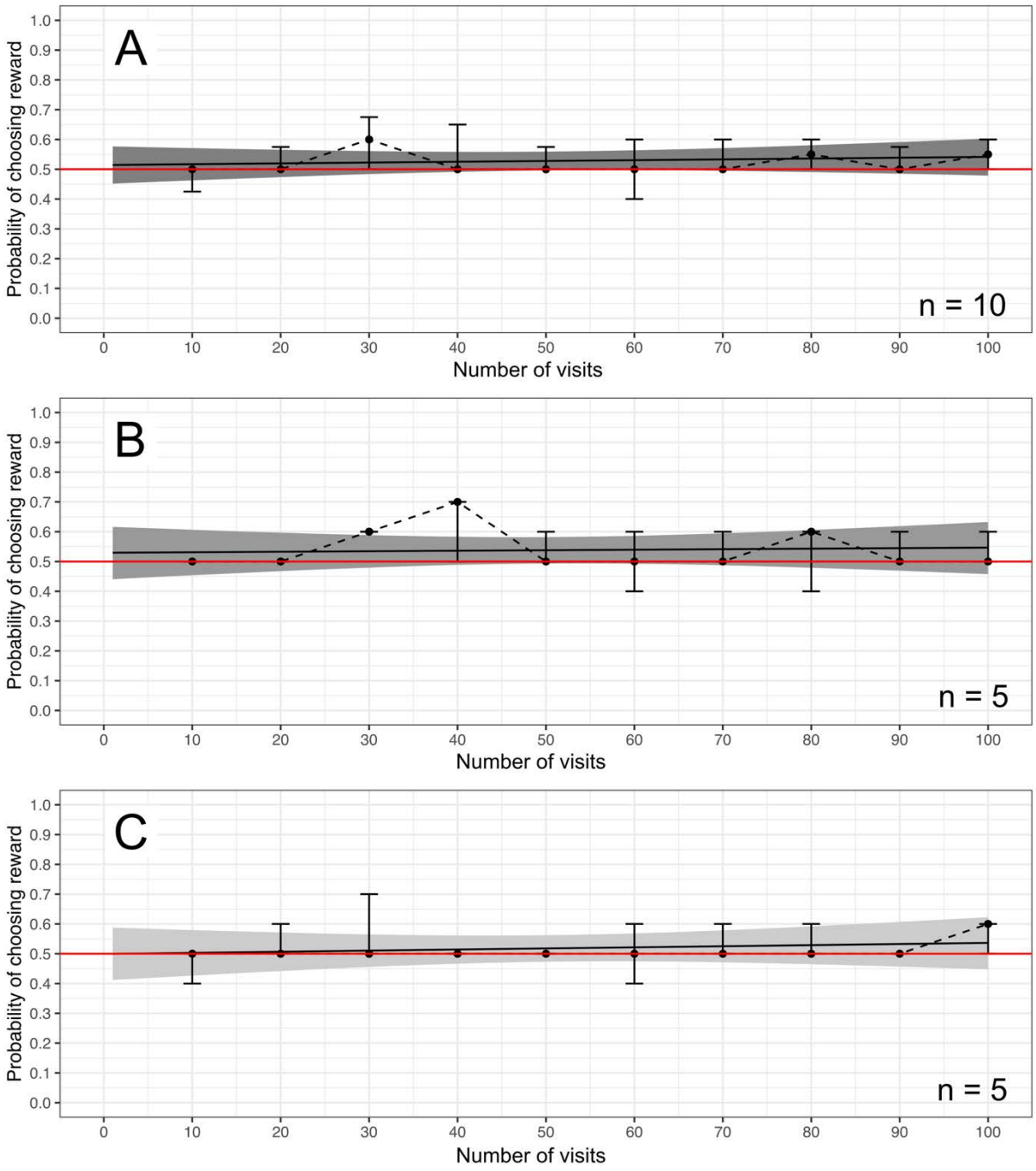


Figure 5.8 Bumblebees cannot perceive the difference between Maris Bead and NV129 standard petal vein models in a differential conditioning experiment using 40% w/w sucrose solution to reward one choice and a 0.12% w/w quinine punishment with the other choice. For each pane, a black solid line shows the probability of choosing reward, estimated with a generalised linear model (GLM) fitted on bees' choices in function of the number of visits. Coloured shading around the solid line represents 95% confidence intervals. Black dots joined by dashed lines indicate the mean proportion of correct choices,

every 10 choices, for all bees, for 100 consecutive choices. The red horizontal line indicates the 0.5 probability value. Values above the 0.5 probability line represent increased success. **(A)** The proportion of foragers (n=10) visiting rewarded models did not significantly increase with successive visits (p = 0.62 when a GLM is fitted to the data set). The GLM estimates that by the 10th visit, the probability of a correct choice is 0.52 [95% CI: 0.46-0.57], by the 50th visit, the probability of a correct choice is 0.53 [95% CI: 0.50-0.56], and by the 100th visit, it is 0.54 [95% CI: 0.48-0.60].

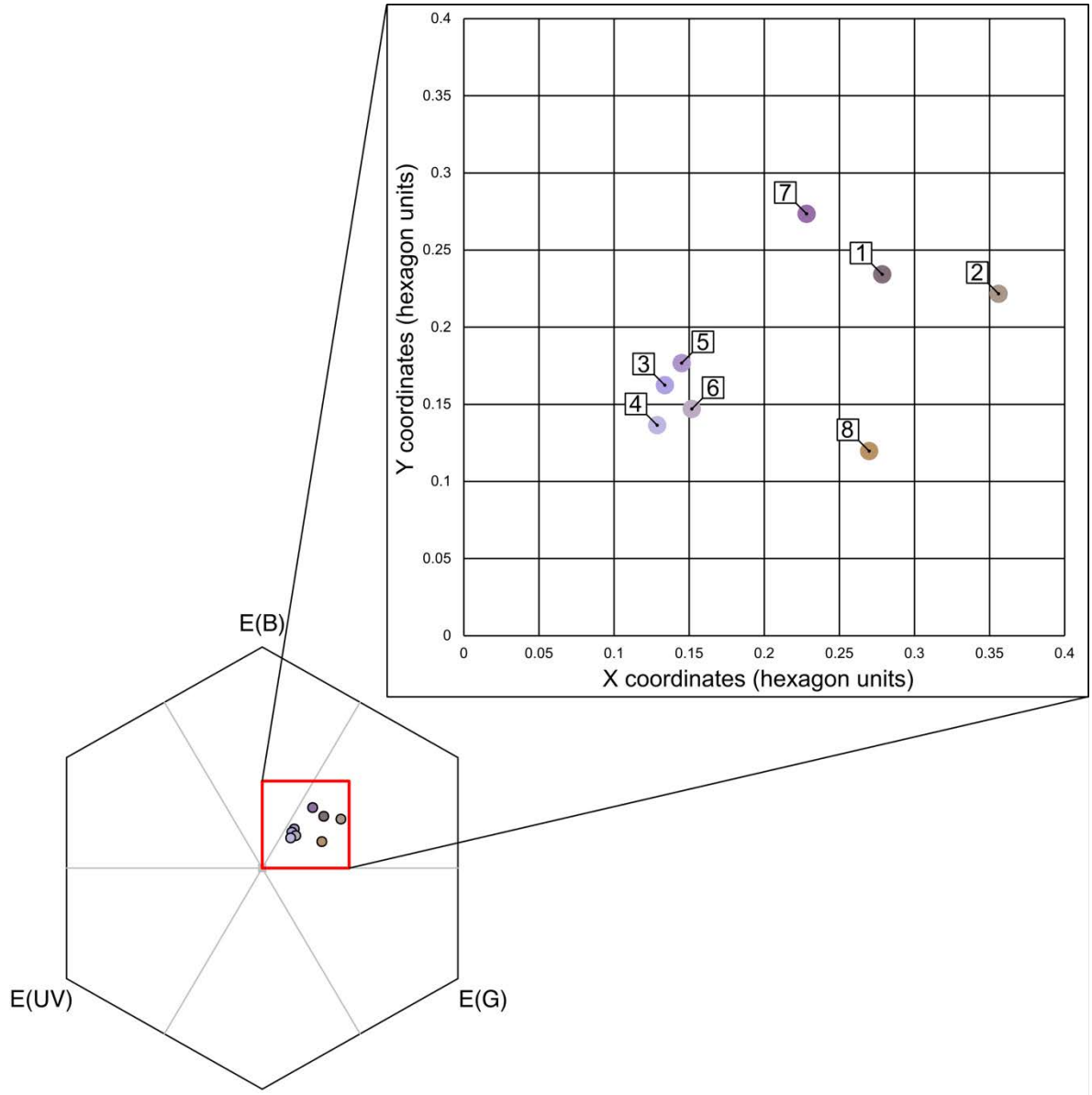
(B) The proportion of foragers (n=5) visiting rewarded models did not significantly increase with successive visits when considering only experiments in which Maris Bead vein models were rewarded (p = 0.82 when a GLM is fitted to the data set). The GLM estimates that by the 10th visit, the probability of a correct choice is 0.53 [95% CI: 0.45-0.61], by the 50th visit, the probability of a correct choice is 0.54 [95% CI: 0.49-0.58], and by 100 visits, it is 0.55 [95% CI: 0.46-0.63].

(C) The proportion of foragers (n=5) visiting rewarded models did not significantly increase with successive visits when considering only experiments in which NV129 vein models were rewarded (p = 0.63 when a GLM is fitted to the data set). The GLM estimates that by the 10th visit, the probability of a correct choice is 0.50 [95% CI: 0.43-0.58], by the 50th visit, the probability of a correct choice is 0.52 [95% CI: 0.47-0.56], and by the 100th visit, it is 0.54 [95% CI: 0.45-0.62].

5.2.3 The reflectance spectra of printed flower models

The reflectance spectra of printed flower models were measured using a spectrophotometer. Using the Pavo package in R, reflectance spectra were converted to co-ordinates in bee colour-space according to Chittka (1992). Reflectance spectra of the printed models could then be compared according to how they excite the photoreceptors of a bee eye.

As seen in **Figure 5.9**, the reflectance spectra of Maris Bead (1) and NV129 (2) flowers sampled between standard petal veins, as they would excite bee photoreceptors, were separated by 0.078 hexagon units. The reflectance spectra of Maris Bead (3) and NV129 (4) standard petal printed image models, sampled between veins, were separated by 0.026 hexagon units. The reflectance spectra of Maris Bead (5) and NV129 (6) standard petal average colour models were separated by 0.031 hexagon units. The reflectance spectra of Maris Bead (7) and NV129 (8) standard petal vein colour models were separated by 0.159 hexagon units (**Figure 5.9**). Hexagon coordinates for the reflectance spectra can be seen in Appendix I.



1	Maris Bead flower (between veins)
2	NV129 flower (between veins)
3	Maris Bead standard petal printed image model (between veins)
4	NV129 standard petal printed image model (between veins)
5	Maris Bead standard petal printed average colour model
6	NV129 standard petal printed average colour model
7	Maris Bead standard petal printed vein colour model
8	NV129 standard petal printed vein colour model

Figure 5.9 Hexagon plot of reflectance spectra sampled from printed flower models and real *V. faba* flowers. Reflectance spectra were converted to hexagon coordinates and plotted in a bee visual space hexagon using the Pavo package in R according to methods of (Chittka 1992). Zoom in shows plots and separation in hexagon units. It was not possible to measure reflectance spectra of real flower veins as they were too narrow for the spectrophotometer beam.

5.2.4 Responses of *Bombus terrestris* to floral scent

For methodology refer to section 2.7.4.

Maris Bead and NV129 floral scent innate preference test

When presented with towers containing flowers of Maris Bead and NV129, 60% of first choices made by 20 naïve foragers were to Maris Bead towers and 40% to NV129 towers (**Figure 5.10A**). The difference in the proportion of visits was not significant when examined using a binomial test (n = 20, p = 0.50).

Across the first 10 choices, 70% of visits made by 20 naïve foragers were to Maris Bead towers and 30% of visits were to NV129 towers (**Figure 5.10B**). The difference in the proportion of visits was significant when examined using a two-tailed t test (t = 4.95, p = 1.55×10^{-5}).

Maris Bead and NV129 floral scent differential conditioning test

When a differential conditioning test was performed using towers containing flowers of Maris Bead and NV129, the proportion of foragers visiting rewarded towers significantly increased with successive visits (p = 2.0×10^{-16} when a GLM is fitted to the data set) (**Figure 5.11A**). When fitted to a generalised binomial linear model, the model showed that the probability of making a correct choice increased from 0.61 [95% CI: 0.55-0.67] after the 10th visit, to 0.89 [95% CI: 0.86-0.91] at the 50th visit, and to 0.98 [95% CI: 0.97-0.99] at the 100th visit. When considering only experiments in which towers containing Maris Bead flowers were rewarded, the GLM again showed that the proportion of foragers visiting rewarded models significantly increased with successive visits (p = 2.04×10^{-10}). The probability of making a correct choice increased from 0.69 [95% CI: 0.60-0.76] after the 10th visit, to 0.92 [95% CI: 0.88-0.94] at the 50th visit, and 0.99 [95% CI: 0.97-0.1] at the 100th visit (**Figure 5.11B**). When considering only experiments in which towers containing NV129 flowers were rewarded, the GLM again showed that the proportion of foragers visiting rewarded models significantly increased with successive visits (p = 8.27×10^{-15}). The probability of making a correct choice increased from 0.54 [95% CI: 0.45-0.62] after the first 10 visits, to 0.86 [95% CI: 0.82-0.89] at the 50th visit, and 0.98 [95% CI: 0.96-0.99] after 100 visits (**Figure 5.11C**).

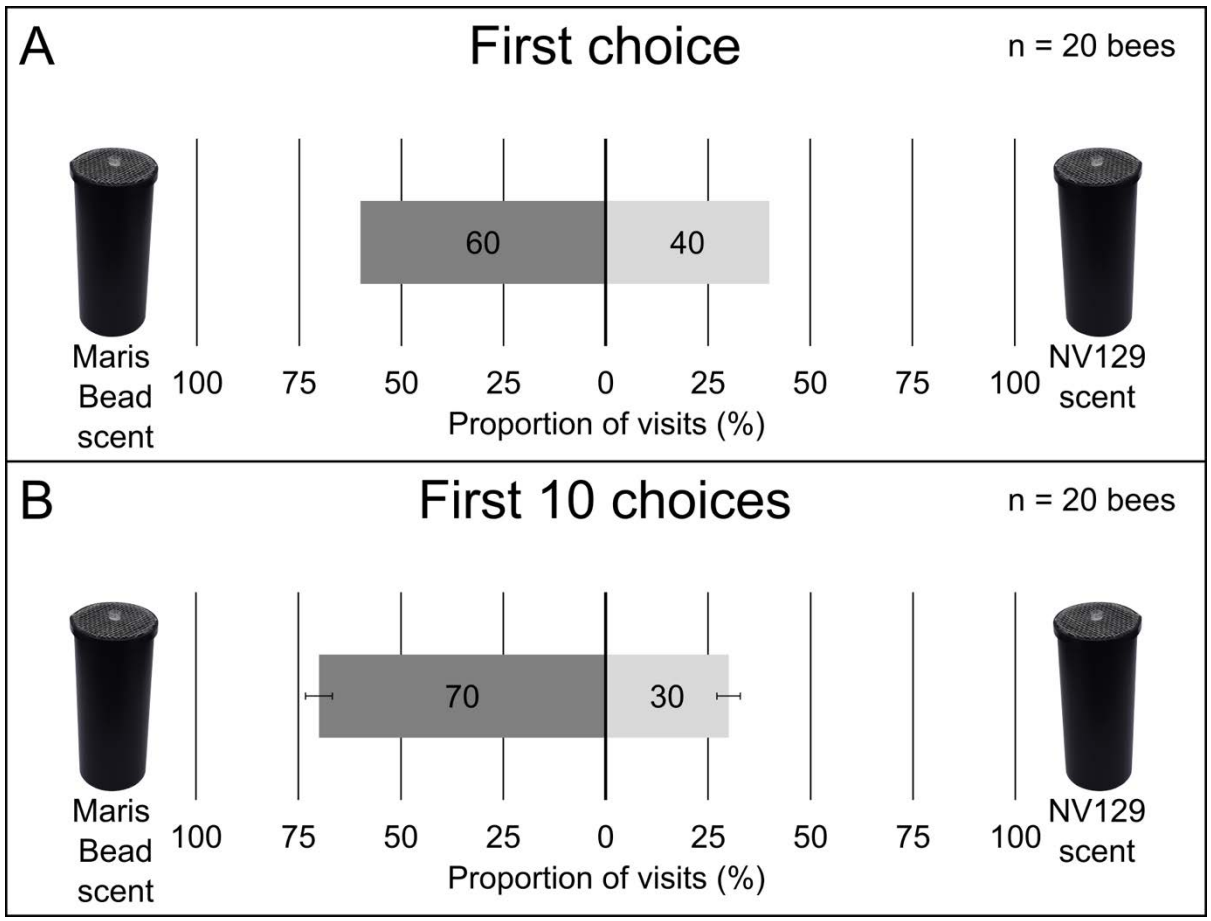


Figure 5.10 Bumblebees do not show preference for scent between Maris Bead and NV129 flowers on first choice but do over first 10 choices. (A) The percentage of visits made by 20 naïve foragers to tower feeders containing flowers of Maris Bead and NV129 in an innate preference test where both choices were equally rewarded. There was no significant preference for either scent (binomial test; n = 20, p = 0.50). **(B)** The percentage of visits made by 20 naïve foragers to tower feeders containing flowers of Maris Bead and NV129 across their first 10 choices in an innate preference test where both choices were equally rewarded (mean ± SE). There was a significant preference for Maris Bead scent over NV129 scent ($t(20) = 4.95$, $p = 1.55 \times 10^{-5}$).

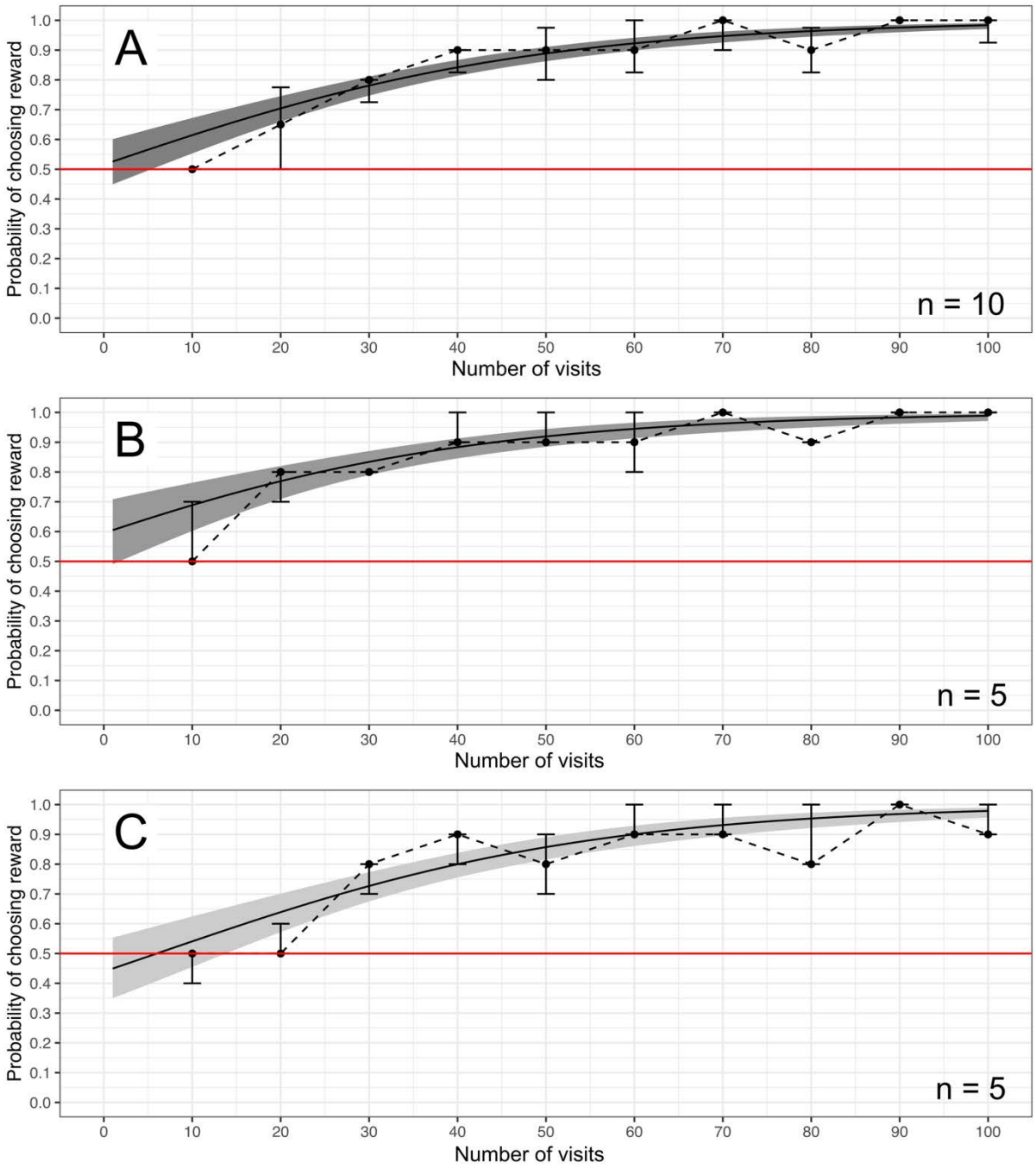


Figure 5.11 Bumblebees can perceive the difference between the scent of Maris Bead and NV129 flowers in a differential conditioning experiment using 40% w/w sucrose solution to reward one choice and a 0.12% w/w quinine punishment with the other choice. For each pane, a black solid line shows the probability of choosing reward, estimated with a generalised linear model (GLM) fitted on bees' choices in function of the number of visits. Coloured shading around the solid line represents 95% confidence intervals. Black dots joined by dashed lines indicate the mean proportion of correct choices, every 10 choices, for all bees, for 100 consecutive choices. The red horizontal line indicates the 0.5 probability

value. Values above the 0.5 probability line represent increased success. **(A)** The proportion of foragers (n=10) visiting rewarded scent towers significantly increased with successive visits ($p = 2.0 \times 10^{-16}$ when a GLM is fitted to the data set). The GLM estimates that by the 10^{th} visit, the probability of a correct choice is 0.61 [95% CI: 0.55-0.67], by the 50^{th} visit, the probability of a correct choice is 0.89 [95% CI: 0.86-0.91], and by the 100^{th} visit, it is 0.98 [95% CI: 0.97-0.99].

(B) The proportion of foragers (n=5) visiting rewarded scent towers significantly increased with successive visits when considering only experiments in which towers containing Maris Bead flowers were rewarded ($p = 2.04 \times 10^{-10}$ when a GLM is fitted to the data set). The GLM estimates that by the 10^{th} visit, the probability of a correct choice is 0.69 [95% CI: 0.60-0.76], by the 50^{th} visit, the probability of a correct choice is 0.92 [95% CI: 0.88-0.94], and by 100 visits, it is 0.99 [95% CI: 0.97-0.1].

(C) The proportion of foragers (n=5) visiting rewarded scent towers significantly increased with successive visits when considering only experiments in which towers containing NV129 flowers were rewarded ($p = 8.27 \times 10^{-15}$ when a GLM is fitted to the data set). The GLM estimates that by the 10^{th} visit, the probability of a correct choice is 0.54 [95% CI: 0.45-0.62], by the 50^{th} visit, the probability of a correct choice is 0.86 [95% CI: 0.82-0.89], and by the 100^{th} visit, it is 0.98 [95% CI: 0.96-0.99].

5.3 Discussion and conclusions

The objective of the work presented in this chapter was to assess the influence of extremes of variation in specific *V. faba* floral traits on bee behaviour in controlled conditions. Prior to this study, research has established that bees have preference for spotted *V. faba* wing petals over non-spotted wing petals, and that bees can distinguish between the scent of two lines (Bailes 2016). However, a knowledge gap remains concerning the effect of variation in other *V. faba* traits on bee behaviour, including colour and patterning. Such information is necessary, both to establish which floral traits are responsible for bee preferences observed in the field (presented in Chapter 4), and to demonstrate that manipulation of specific *V. faba* floral traits can be used to increase attractiveness of the crop.

The experiments presented in this chapter were informed by floral trait variation data presented in Chapter 3, and bee preference data from the field presented in Chapter 4. Three hypotheses were tested. The first was that *Bombus terrestris* foragers find the appearance of large wing petal spots more innately attractive than small wing petal spots. Data presented in Chapter 3 and data collected by Bailes (2016) shows that considerable variation exists in wing petal spot size between *V. faba* lines. Results of *B. terrestris* innate preference experiments did not support the hypothesis, as when presented with epoxy models representing extremes of wing petal spot size between *V. faba* lines (20 % petal area and 60 % petal area), foragers did not show innate preference between larger or smaller spots.

Following this result, a differential conditioning test revealed that foragers were able to distinguish between large and small petal spot models. Therefore, although *B. terrestris* foragers may not be innately attracted to *V. faba* lines with larger wing petal spots, the trait could be used as a tool to increase bee visitation to the crop when paired with enhanced reward.

Very little research has been carried out into the function of petal spots of *V. faba.* Looking to other systems, it is possible that they function as nectar guides or insect mimics. In *Gorteria diffusa*, dark spots on orange florets attract pollinating flies through sexual deception (Johnson and Midgley 1997; Ellis et al. 2014). In species of *Clarkia*, sweat bees

prefer spotted over non-spotted flowers, but only when spotted morphs are the majority (Jones 1996; Eckhart et al. 2006). Bee flies show preference for dark spotted model flowers over non spotted models of *Linum pubescens*, thought to be mate-seeking behaviour (Johnson and Dafni 1998), and inflorescences of *Daucus carota* attract fewer beetle visitors when the usual central purple umbellet "spot" is absent, which is hypothesised to act as an insect mimic (Goulson et al. 2009). In *V. faba* it is very unlikely that petal spots are sexually deceptive, given that foragers are all female. The configuration of the wing petal spots, being proximal to the nectary, may provide a directional cue to bees alongside the veins of the standard petal. In *V. faba*, wing petal spots may simply increase contrast, making flowers more easily detectable against the complex leafy background. Although *V. faba* flower spot size does not affect bee preference, future work should attempt to explore whether spot size and positioning can affect foraging speed, and if variation in this trait has measurable effects on bee behaviour and plant yield in the field.

In *V. faba*, non-spotted lines, which are associated with lower tannin content in the seed, are often lower yielding (Skovbjerg et al. 2020). As demonstrated by Bailes (2016), preference of *B. terrestris* foragers for spotted over non-spotted flowers could contribute to lower yield in such lines. Confirmation of this in the field would clarify the influence of wing petal spots on bee attraction and consequences for yield. An interesting avenue for future research on this trait would be to explore the effect of spot presence, absence, size, and colour (like the yellow spot of Yukon), on flower temperature due to light absorption. Bees can detect temperature differences, and increased floral temperature may benefit insects by making nectar less viscous (Nicolson et al. 2013; Harrap et al. 2017).

The second hypothesis tested in this chapter was that *Bombus terrestris* foragers find flower standard petal appearance, colour, and patterning of the *V. faba* line Maris Bead more innately attractive than that of the line NV129. Experiments carried out to test this hypothesis were designed to evaluate the relative importance of visual traits for bee attraction and were informed by field data. Innate preference tests using visible spectrum printed models of Maris Bead and NV129 standard petals photographs found that *B. terrestris* foragers showed innate preference for images of Maris Bead standard petals over NV129 standard petals, supporting the hypothesis that foragers find flower standard petal appearance, of Maris Bead more innately attractive than that of the line NV129. This result

suggests that the appearance of standard petals is likely to be a strong attractive feature of *V. faba* flowers, which is likely to have contributed to the high bee visitation to Maris Bead over other lines in field conditions.

The appearance of standard petals is made up of multiple visual components, including background petal colour, vein colour and vein patterning. Innate preference tests using visible spectrum printed models of Maris Bead and NV129 standard petal background colour found that *B. terrestris* foragers did not show preference between background colour of Maris Bead and NV129 standard petals, refuting the hypothesis that *B. terrestris* foragers find the background colour of Maris Bead more innately attractive than that of NV129. Following this result, a differential conditioning test showed that *B. terrestris* foragers could discriminate between the petal background colour models. Therefore, although bees do not find the background colour of Maris Bead standard petals more attractive than that of NV129, the colour may still enhance the ability of bees to identify Maris Bead flowers, especially considering Maris Bead has a high nectar sugar concentration, providing an incentive to learn.

The most striking difference between Maris Bead and NV129 standard petals observed in the field were in the veins. Unlike other *V. faba* lines, Maris Bead has highly saturated petal veins. This may provide a strong attractive cue for pollinators (Chittka and Raine 2006). Innate preference tests using visible spectrum printed models of Maris Bead and NV129 standard petal vein colour found that *B. terrestris* foragers showed strong preference for the vein colour of Maris Bead over that of NV129, supporting the hypothesis that *B. terrestris* foragers find the vein colour of Maris Bead flowers more innately attractive than that of NV129 flowers. These results suggest that the colour of Maris Bead standard petal veins is likely to provide a strong attractive cue which may have contributed to the high bee visitation to Maris Bead over other lines in field conditions. This result agrees with findings from other systems that suggest *B. terrestris* workers have a bias towards violet flowers (Raine and Chittka 2007; Reverté et al. 2016).

As well as the dominant wavelength of a flower, colour contrast and spectral purity (saturation) play significant roles in reward identification by bees (Koethe et al. 2018). The vein colour of models based on Maris Bead flowers have greater green colour contrast than

those of NV129 models (see Appendix I). It is therefore possible that flowers of Maris Bead, having a greater coverage of purple veins, appear more striking against a leafy green background than flowers of NV129 (and many other *V. faba* lines) with less widespread, yellow/brown-coloured veins. In the case of honeybees, brightness and green colour contrast have limited influence in decision making (Giurfa et al. 1995; Leslie et al. 2018). However, bumblebees have shown preference for colour models with greater spectral purity, perhaps because the spectral purity of flowers is often higher than that of their natural backgrounds, and this bee preference is likely adaptive (Lunau 1990). Similarly, attractiveness of a flower may be increased if its contrast against a background (often green in nature) is large, suggesting that attractiveness of a colour is related to its detectability against the background in nature (Lunau et al. 1996). In this project, the same standard green background was used for all choices. However, foliage colour does vary between *V. faba* lines, so much so that it is used to define commercial lines. In the field, differences in foliage colour and its contrast to flowers may provide an additional factor that influences bee attraction.

Alongside vein colour, Maris Bead and NV129 provide examples of extreme differences in vein patterning among *V. faba* lines. To examine the influence of differences in petal vein patterning on bee behaviour an experiment was devised using models representing the variation between Maris Bead and NV129 for vein cover, branching and area of the petal with highest density of veins. Innate preference tests found that *B. terrestris* foragers did not show preference between standard petal vein models of Maris Bead and NV129, refuting the hypothesis that *B. terrestris* foragers find the vein patterning of Maris Bead more innately attractive than that of NV129. In a differential conditioning test, foragers were not able to distinguish between vein models. Together these two experiments show that, in a black and white form, models of extremes of vein pattern do not provide an innately attractive, or discernible, cue for bumblebees.

The findings of this differential conditioning experiment also provided an additional control experiment, agreeing with those of Whitney et al. (2008) who demonstrated that bees cannot identify quinine at the concentrations used in this study based on any other cue than taste. This control experiment is a useful confirmation that other differential conditioning

experiments described in this thesis can be relied on since the quinine punishment is not discernible remotely.

The veins of *V. faba* flowers most likely function as nectar guides, as they point towards the nectaries at the centre of the flower. Vein-like floral markings are seen in actinomorphic flowers of geraniums, clematis and crocus, and zygomorphic flowers of violets, orchids, and nasturtiums. Studies most often conclude that nectar guides decrease nectar discovery time (Leonard and Papaj 2011; Goodale et al. 2014). Drone flies, *Eristalis tenax*, locate rewards faster on models with radial lines, however, colour of models greatly affects success (Dinkel and Lunau 2001). Some studies argue that bees do show preference for radial patterns of lines in model flowers, stemming from a preference for symmetry, "flower-like" shapes and dark centres (Lehrer et al. 1995; Biesmeijer et al. 2005).

The study of nectar-guides in zygomorphic flowers is less common. One system in which petal veins exist which are comparable to those of *V. faba* is *Antirrhinum.* White *Antirrhinum* flowers with pink venation (*Venosa* phenotype) attract more bee visits than solid white flowers or solid pink flowers (Shang et al. 2011). Outside of *Antirrhinum*, most other research has focused on the influence of nectar guides on bee behaviour in the context of floral symmetry(Giurfa et al. 1996; Horridge 1996). Currently there is no published data exploring effects of differences in extent of nectar guides, branching, coverage, or thickness.

Due to the complex nature of vein patterning it would be advantageous to investigate combinations of multiple characteristics on bee preference and foraging speed, including vein thickness, vein branching, orientation, and colour. Future bee behavioural experiments examining vein patterning require careful consideration of what choice is presented to the bee. A choice between thicker and thinner purple veins, for example, may also be a choice between a greater and lesser proportion of purple colour.

Considering the evidence produced by these bee experiments it is likely that the colouration of Maris Bead standard petal veins is the visual trait which most strongly contributes to the preference of bees for Maris Bead flowers. However, the widespread distribution of purple veins of Maris Bead flowers and the restricted distribution of yellow/brown veins of NV129 may also play a role in preference.

It must be noted that as with all artificial models, the printed models have caveats. Printed models excited bee photoreceptors less strongly that real flowers, as suggested by spectrophotometry data. In addition, printed models presented colour variation in the visible spectrum and not UV. Experiments using printed models in this study therefore provide evidence that bees can distinguish between and show preference between visible-spectrum colour and patterning akin to, but not identical to, that seen in real *V. faba* flowers. That said, other studies investigating the influence of colour on bee preference have never replicated the colour of real flowers in an artificial system and instead focus on fundamentals of how bees behave in response to artificially generated colours with different degrees of separation in bee colour space (Lunau et al. 1996; Spaethe et al. 2001; Raine and Chittka 2007). Additionally, I have found no published works that replicate UV colour in an artificial system, only manipulation of the area of UV patterning (Johnson and Andersson 2002; Horth et al. 2014; Koski and Ashman 2014). Future work to establish the influence of *V. faba* flower colour on bee behaviour should seek to genetically manipulate colour of real flowers. Only then can one explore the true effect of *V. faba* colour and patterning variation on bee behaviour. Until that is possible, artificial flower models provide the next best means to investigate fundamentals of bee behaviour in response to colour and patterning.

The final hypothesis tested in this chapter was that *Bombus terrestris* foragers find the floral scent of the *Vicia faba* line Maris Bead more innately attractive than that of the *Vicia faba* line NV129. Innate preference tests found that *B. terrestris* foragers did not show a statistically significant preference between the scent of Maris Bead and NV129 flowers on the first choice but did show statistically significant preference for the scent of Maris Bead flowers over the first 10 visits. Because foragers showed no significant preference on the first visit, a differential conditioning test was performed to verify that foragers could distinguish between the scents. The conditioning test found that foragers rapidly learned to distinguish between the two scents, within 30 visits. Together, the innate preference and conditioning test results support the hypothesis suggest that *B. terrestris* foragers prefer the scent of Maris Bead flowers over that of NV129 flowers, and that the difference in scent between the two lines provides a strong cue that bees can quickly learn to distinguish between. It is therefore likely that scent is a strong attractive cue that may have contributed to the increased bee visitation rate to Maris Bead plants in the field.

An explanation for the insignificant preference on first encounter may be a consequence of the experimental design. Towers containing flowers were placed 20 cm apart at the end of a flight arena (see section 2.7.4 for details). To train bees to visit towers, bees were fed on multiple randomly positioned towers containing no flowers prior to the experiment. Because of the training, bees may have indiscriminately selected towers based on previous memory of towers containing food on first encounter. After having experienced both towers in the experiment, it is possible that they then used scent to identify the reward, rather than just appearance. It is also possible that scent gradients could disperse in an uneven manner, making identification of the source difficult. However, towers were left for 10 minutes to allow scent to diffuse evenly before a bee was released into the arena, therefore this is unlikely.

Relatively little work has been published revealing the effect of scent variation within plant species on pollinator behaviour, despite their being calls to do so (Griffiths et al. 1999). The only work to examine the effect of *V. faba* floral volatiles on pollinator behaviour was done by Bailes (2016), showing that *B. terrestris* foragers have no innate preference between flowers of NV676 (Tattoo) or NV641 (Fuego) but can tell the difference between scents in a differential conditioning test. The only other studies concerning behavioural responses to *V. faba* volatiles found that *Aphis fabae* move towards leaf volatiles of the *V. faba* cultivar Sutton dwarf, and egg parasitoid wasps (*Trissolcus basalis*) prefer to lay eggs in stink bugs (*Nezara viridula*) on water stressed plants of the *V. faba* cultivar Aguadulce due to their volatile profile (Nottingham et al. 1991; Webster et al. 2008; Salerno et al. 2017). The only behavioural studies of floral volatiles in other crop flowers have found that bees prefer floral scent of the strawberry variety Sonata over the variety Elsanta, and strawberry blossom weevil, *Anthonomus rubi* show no preference between strawberry cultivars (Ceuppens et al. 2015; Mozūraitis et al. 2020)

Multiple studies have sought to identify and quantify the main VOCs present in the smell of *V. faba* flowers. The main VOC emitted by flowers of Maris Bead has been identified as (E)-β-ocimene alongside traces of α-pinene and limonene (Sutton et al. 1992). Other studies agree, identifying (E)-β-ocimene in the floral bouquet of Maris Bead alongside (Z)-β-ocimene, α-pinene, linalool, and β-myrcene (Griffiths et al. 1999). In the line Sutton Dwarf, (E)-Caryophyllene has been identified as the main VOC alongside linalool, limonene and α-

humulene (Bruce et al. 2011). As a result of specific compounds being identified in the volatile bouquets of flowers, many studies have used synthetic volatile compounds to investigate pollinator preferences. These studies enhance understanding of the molecules important for scent preference, but are less able to explore scent preference at biologically relevant quantities and real combinations. Linalool is frequently found attract pollinators and is a ubiquitous volatile compound in flowering plants (Raguso and Pichersky 1999). However, in some experiments, foragers of *Bombus impatiens* show no preference for linalool, but an innate preference for β-trans-bergamotene (Haber et al. 2021). Although not possible within this study, efforts should be made to quantify the volatile compounds present in the floral scent of Maris Bead, NV129 and the lines examined for other floral traits in this study. Doing so will allow evaluation of the importance of volatile compounds and their quantity in bee attraction.

Conclusions

Bee behavioural experiments presented in this chapter have shown that extremes of wing petal spot size of *V. faba* lines can be detected by *B. terrestris* foragers in a model system. *B. terrestris* foragers also prefer visible spectrum printed image models of Maris Bead standard petals over NV129 standard petal models, suggesting that standard petal appearance may contribute to the high bee visitation rate to Maris Bead flowers observed in field conditions. Preference for Maris Bead standard petals is likely to be due to a combination of strong purple vein colouration and the extent of the vein patterning as suggested by innate preference experiments. This work has identified the intense purple colouration of *V. faba* flowers as a trait which is likely to increase bee attraction to the crop. Innate preference experiments have also shown that floral scent is likely to strongly contribute to bee preference for Maris Bead as seen in field conditions.

Future studies should further investigate variation in *V. faba* floral volatile compounds and seek to genetically manipulate colour of *V. faba* flowers to explore the effect of colour manipulation on bee visitation and plant fitness in real plants in isolation from variation in other traits.

6 General Discussion

Summary

Studies have shown that *Vicia faba* benefits greatly from pollination by bees (Bishop and Nakagawa 2020). As a result of pollinator declines it is vital that the pollinator ecology of the crop is studied both to improve *V. faba* yield, and to support wild pollinators. The experiments of Bailes (2016) revealed that variation in floral traits exists between some *V. faba* lines, and that *Bombus terrestris* workers can distinguish between extremes of variation in corolla tube length, petal spot presence and absence, and nectar sugar concentration.

Following the work of Bailes (2016), I took the next step to establish whether floral trait variation exists in a wider panel of commercial *V. faba* lines, what effect this floral trait variation has on bee attraction in field conditions, what result this has on crop yield, and which floral traits are most likely to increase bee attraction to the crop. I formulated several hypotheses, the first being that novel floral trait variation exists between previously uncharacterised commercial *V. faba* lines. Data supported this hypothesis, with substantial variation documented in standard petal height, wing petal area, wing petal spot size, floral colour, nectar production, and pollen production, the number of flowers per node, and the tripping force of previously uncharacterised commercial *V. faba* lines.

Using these data, I tested a second hypothesis that *V. faba* lines with theoretically more attractive traits would attract more bees in field conditions that lines with less attractive traits. This hypothesis was supported by field data collected in 2021 and 2022, which revealed that the *V. faba* commercial lines Maris Bead and Lynx attracted more bee visits than other lines, most likely due to their higher nectar content and greater number of flowers per node. Field trials also tested my third hypothesis, that openly pollinated *V. faba* plants would have higher yield than caged plants where pollinators were excluded. Field data supported this hypothesis, with the additional insight that the size of yield change due to pollinator exclusion varied greatly between *V. faba* lines. Field data also supported the hypothesis that *V. faba* plants with more attractive floral traits would receive a greater yield benefit with open pollination than *V. faba* plants with less attractive floral traits.

Following field trials, I performed bee choice experiments in controlled conditions to identify floral traits most likely to increase bee attraction to the crop. *Bombus terrestris* preference tests revealed that bees do not find large wing petal spots more innately attractive than small wing petal spots, nor do they find the standard petal background colour, or vein patterning of the line Maris Bead more attractive than that of the line NV129. Preference tests did reveal that bees strongly prefer the purple colour of Maris Bead standard petal veins over the yellow standard petal veins of NV129. Data also showed that bees strongly prefer the scent of Maris Bead flowers over the scent of NV129 flowers. The results from bee experiments suggest that strong purple colouration unique to the flowers of Maris Bead is more attractive than the white and yellow colouration common in many other lines including NV129, and that alongside colour, scent is a trait that may be used to increase attraction to the crop.

Novel floral trait variation of *Vicia faba*

In Chapter 3 I presented data revealing previously undocumented variation in the floral traits of *V.faba* lines. Work by Bailes (2016) had previously documented variation in floral traits, but a knowledge gap remained in that many modern commercial lines were uncharacterised. To be able to investigate the effect of floral trait variation on bee behaviour, it was essential that data be collected from commercial lines available to farmers.

Floral traits were studied using a panel of up to 38 *Vicia faba* lines, complementing the work of Bailes (2016) (lines are summarised in Appendix C). Significant variation was found in reward traits, attraction traits and access traits (**Figure 6.1**). Of the reward traits measured, nectar concentration showed particularly high variation between lines. As nectar is the main reward sought out be bees visiting the crop, there is great potential for lines with a larger volume of more concentrated nectar to attract more bees than lines with inferior nectar. Considering the work of Bailes et al. (2018) and Pattrick et al. (2020) who proposed a nectar optimum concentration of 55% w/w, the results of this study suggest that some lines, including LG Cartouche, may already have an optimal nectar concentration. To support pollinator populations and improve *V. faba* pollination, breeding efforts would be best focused on producing flowers with ~50% w/w nectar concentration, but not increase volume, to encourage maximum movement between flowers and limit time spent on any

one flower. Considering the low variation in pollen quality, the overall large number of pollen grains produced, and field observation that bees did not actively collect pollen, it would be unlikely that pollen quantity and quality may be used to improve bee attraction and *V.faba* pollination.

Non-reward traits, which can be termed attraction traits, help bees to identify flowers with beneficial reward traits like high nectar sugar content. Of the attraction traits measured, petal size and the number of flowers per node may be particularly important for bee attraction, as larger floral displays are more easily located by bees (Spaethe et al. 2001). A large amount of variation was present in the number of flowers per node, representing a huge difference in the size of floral display, the potential quantity of floral volatiles, and reward available to bees. Although having a greater number of flowers increases the potential number of pods that can be set, multiple studies suggest that larger numbers of flowers per plant may impose fitness costs through geitonogamous selfing (Harder and Barrett 1995; Ohashi and Yahara 1998; Suso et al. 2005). To improve the chances of cross pollination in *V. faba*, breeders may seek to develop lines with fewer flowers per node but a longer flowering period to maximise bee movement between plants. Future work will need to establish the optimum number of flowers to maximise outcrossing but also attract more bees, assuming the two objectives are conflicting.

Two access traits were quantified, which affect how easily bees gain access to nectar and pollen. Flower operative force varied greatly between lines and based on research by Córdoba and Cocucci (2011), it is unlikely that honeybees can open flowers of Albus, BPL10 and Vertigo. However, developing future lines with easier to open flowers may not benefit crop yield, as operative force may filter out smaller, less effective pollinators (Jauker et al. 2016). The other access trait, corolla tube length, ranged from 11.38 mm to 13.45 mm between lines. Considering tongue length among common visitors to *V. faba* flowers ranges from 7 mm (*A. mellifera*) to 13 mm (*Bombus hortorum*) and is most commonly ~8 mm for most species of *Bombus*, bees may have difficulty reaching the nectar of many *V. faba* lines. Work by Suso et al. (2005) reported a negative correlation between corolla tube length and *V. faba* outcrossing. I hypothesize that this may be a consequence of nectar robbing. Therefore, breeding for shorter corolla tubes would still be advantageous, as it may reduce the incentive for robbing.

Floral trait variation has implications for pollination and yield of *Vicia faba*

In Chapter 4 I presented data from field trials which showed that bees preferentially visited *V. faba* lines with floral traits which are considered to be more attractive. Lines with greater nectar volume, more concentrated nectar and more flowers per node received significantly more visits than other lines, supporting the hypothesis. Bee preference was consistent between Lincolnshire in 2021 and Cambridgeshire in 2022, as shown by visitation rates to Maris Bead, Tiffany and Fuego which were grown in both years.

Alongside insights into how *V. faba* floral traits affect the preferences of bees, the field trials delivered additional insights into the effectiveness of different bees as pollinators of the crop. Behavioural observations revealed that carder bees and bees that fell into the "white-tailed bumblebee" category have greatest potential to be effective pollinators of *V. faba* plants as they perform most legitimate visits. In contrast, honeybees are least effective at pollinating the crop as they mostly performed extrafloral nectary visits, or nectar robbed, depleting flowers of resources without pollinating them. To improve pollination of the crop, it should therefore be a priority to support wild pollinator populations, rather than deploy managed honeybee colonies.

Pollinator exclusion experiments carried out in the field showed that open pollinated *V. faba* plants had a higher yield than caged plants where pollinators were excluded. This agreed with the consensus that bee pollination is beneficial for *V. faba* yield (Varis and Brax 1990; Suso and del Río 2015). The pollinator exclusion results also showed that the size of yield change due to pollinator exclusion varied greatly between *V. faba* lines. This result agreed with that of Bishop and Nakagawa (2020), in which their metanalysis suggested variation between yield benefit due to pollination was largely due to the use of different *V. faba* genotypes between studies. The results presented in Chapter 4 are the first time that the effect of open pollination on *V. faba* yield has been examined for multiple lines simultaneously in field conditions, which before now has been a hole in the literature (Lundin and Raderschall 2021). The results found that overall, plants produced more beans and a greater mass of seed when open pollinated compared to when caged, but individual seeds had lower mass on average. This is beneficial for growers, as a greater bean mass is produced for processing and consumption, and a greater number of seeds is produced for

planting in subsequent years. Unlike in 2021, two out of the six lines grown in 2022 (Fuego and Vertigo) showed a decrease in yield with pollination. The extreme heatwave of summer 2022 is most likely to have caused this effect, with the cage providing some protection from heat and evaporation.

The major finding of the field trials was that lines which received more bee visits because of their floral traits also receive a better pollination service, resulting in a greater yield increase. This finding was supported by a statistically significant positive correlation between the overall bee visitation rate to lines and the plot yield change between cage and open pollination treatments, and a strong positive correlation between the legitimate bee visitation rate to lines and the plot yield change between cage and open pollination treatments. The relationships demonstrate that the quality of pollination service received by *V. faba* plants and subsequently the yield of the plants is affected by attractiveness of floral traits to bees. This finding shows that through understanding of floral traits, the yield of *V. faba* can be improved in the field. This is the first study to directly link floral trait variation, bee visitation and yield in *Vicia faba.*

The floral traits most likely to improve pollination of *Vicia faba*

Presented in Chapter 5, the results of bee behavioural experiments in controlled conditions revealed that floral colour, patterning and scent were likely to be important factors that contribute to bee attraction to *V. faba* in the field. *Bombus terrestris* foragers found the purple colour of Maris Bead standard petal veins more innately attractive that the yellow colour of NV129 standard petal veins, and also found the floral scent of Maris Bead flowers more innately attractive than the scent of NV129 flowers. These results suggest that the purple colouration, and scent, of Maris Bead are likely to have contributed to the high bee visitation rate that the line received in the field, alongside it's high nectar sugar concentration and large number of flowers per node.

Other studies have reported bee preference for purple and strongly saturated colours in other systems (Raine and Chittka 2007; Reverté et al. 2016). The experiments presented in Chapter 5 contribute to the wider understanding of bee behaviour, but most importantly show that pollinators of *V. faba* have preferences between traits which are relevant to the

crop. Together, the results of field trials and controlled condition bee experiments suggest that *V. faba* lines with purple flowers, high nectar sugar concentration and a greater number of flowers per node should provide farmers with greater and more stable yield than other lines which have less attractive floral traits.

Applications and future work

The findings of this work demonstrate that selection of *V. faba* lines which possess specific attractive floral traits can be used to increase pollination and yield of the crop. Alongside floral trait data collected by Bailes (2016), the novel floral trait data collected in this study can be used by farmers to select lines which are more attractive to bees and are likely to have better yield in environments where pollinators are present. By growing lines which are also more rewarding to pollinators, farmers can use their *V. faba* crop to provide better floral resources for bees. In conjunction with other agricultural practices including reduced pesticide use, provision of wildflower forage in field margins, and habitat protection, planting of *V. faba* lines which have optimum reward traits have the potential to better support pollinator populations (**Figure 6.1**). Better provision of resources, including those provided by crop species can in turn help to improve the pollination of *V. faba* and other insect pollinated crops, by helping to rebuild wild pollinator populations. Considering the potential for *V. faba* floral traits to have benefits for both the crop itself and pollinator populations, there should be an incentive for crop breeders to consider pollinator-attracting traits as breeding targets in the future.

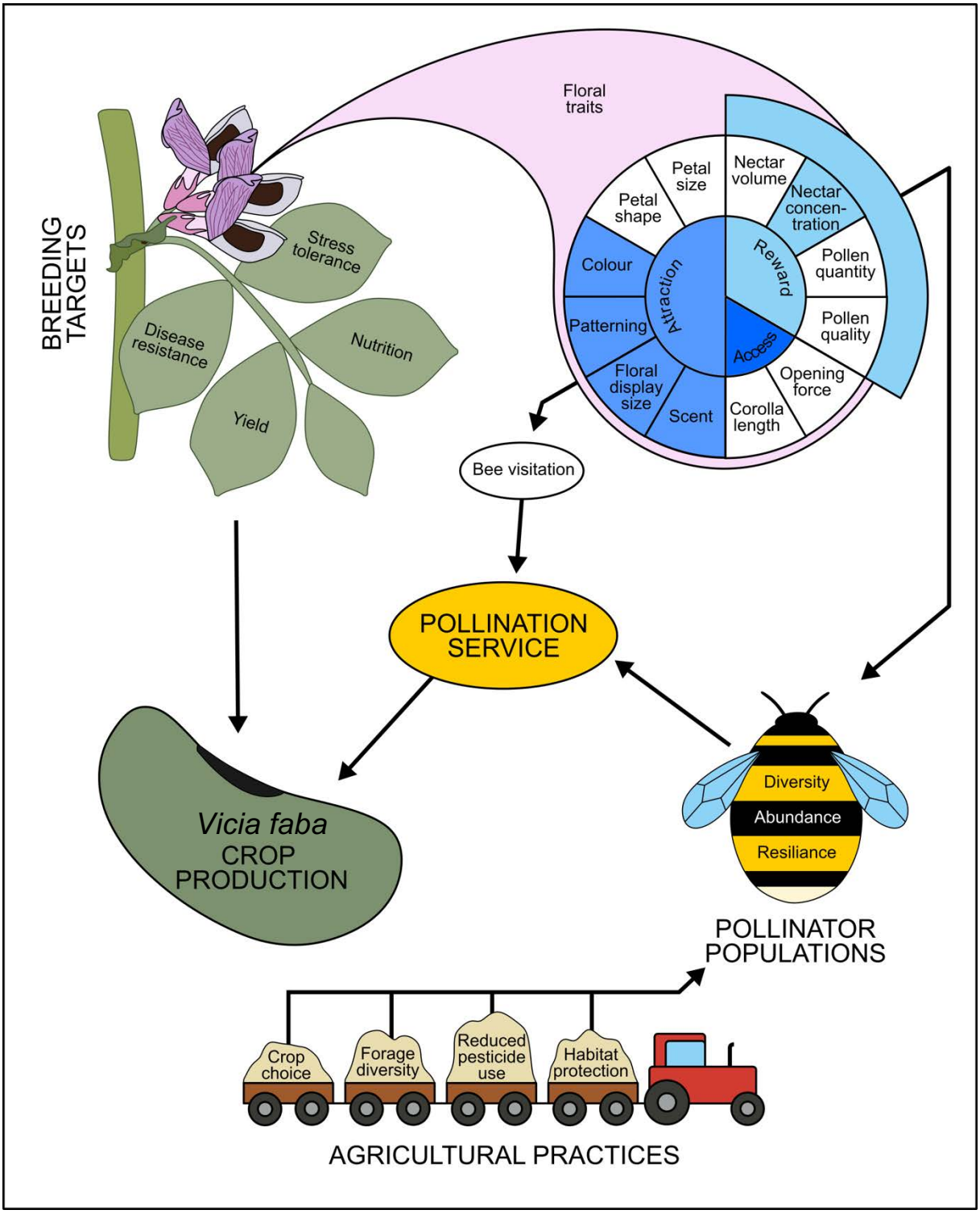


Figure 6.1 Floral traits can be used to optimise *Vicia faba* production through bee attraction and behaviour. This study found that variation in attraction, reward, and access traits of *V. faba* flowers significantly affects bee visitation to *V.* faba plants in the field, with consequences for crop yield. Floral colour, patterning, display size, nectar concentration and scent are traits showing greatest potential to increase *V. faba* pollination and yield. Growing *V. faba* lines with better floral rewards can be used to support pollinator populations and improve the pollination service, alongside use of better agricultural practices. Floral traits

should be considered as breeding targets, alongside traditional traits including disease tolerance, stress resistance, yield and nutrition.

For floral traits to be considered as breeding targets, substantial work is needed to further explore the utility of floral traits to improve crop yield, and the genetics of these traits. Although not possible within the scope of this project, I would seek to genetically manipulate colour and patterning of real flowers to further explore the effect of trait variation on bee preference. Elimination of colour from an attractive line e.g. Maris Bead would be an elegant demonstration of the power of floral colour to enhance pollination of the crop. I would hypothesise that eliminating colour from Maris Bead would reduce bee visitation in the field, and similarly enhancement of colour in a less attractive line like NV129 would increase bee visitation. Thanks to the work of Gutierrez and Torres (2019) we know that the *VfTTG1* gene influences flower colour and patterning in *V. faba*, and in many other species, flower colour is affected by expression of *MYB* genes (Shang et al. 2011). As shown by (Zanotto et al. 2020) it is likely that manipulation of *VfTTG1* could also be used to alter floral scent. Although exploration of floral volatile organic compound variation was not possible in this study, floral scent also shows promise to enhance bee attraction and would be beneficial to explore further.

I would also prioritise future research into the effects of nectar robbing on *V. faba* pollination, and how nectar robbing may be discouraged. I hypothesise that longer corolla tube length will contribute to greater rates of robbing, based on the work of Suso et al. (2005) that reported a negative correlation between corolla tube length and *V. faba* outcrossing, likely due to nectar robbing. Results presented in Chapter 3 highlighted great differences in the colouration of corolla tubes, which may provide strong attractive signals to nectar robbers. If time had permitted, I would have liked to carry out field experiments manipulating the corolla tube colour to explore the effect on rates of nectar robbing. Finally, further work to improve the pollination of *V. faba* should begin to take a wider landscape approach. Some studies have explored the effects of wildflower margins on crop pollination, but an absence of research remains for *V. faba* (Campbell et al. 2017; Carvell et al. 2017).

Conclusions

This study has identified substantial novel floral trait variation between *Vicia faba* lines, including many commercial lines grown by UK farmers. In field conditions, *Vicia faba* lines possessing floral traits considered to be more attractive to bees receive significantly more bee visits. Field and controlled condition experiments, together with published data suggest that floral colour, patterning, display size, nectar concentration and scent are traits most likely to increase pollinator attraction to the crop and should be considered as breeding targets in the future. Field experiments demonstrated that open pollination has an overall positive effect for *Vicia faba* yield and that lines with more attractive floral traits receive a larger yield benefit with open pollination due to the increased bee visitation they attract. In parallel with better agricultural practices, planting of existing *Vicia faba* lines with floral traits which are more attractive and beneficial for wild pollinators, and future development of optimised *Vicia faba* lines can help to increase crop yield, through bee attraction and supporting pollinator populations.

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Appendix A – Pollen staining

Modified Alexander stain was used to stain *V. faba* pollen grains as a means of estimating the proportion of viable and non-viable pollen grains, based on the work of (Alexander 1987) and adapted from further work by (Peterson et al. 2010).

Modified Alexander stain was made by adding the following solutions, in order, to a bottle wrapped in heavy duty Aluminium foil, to protect the contents from light. If kept in the dark, the solution is stable for many months.

- 10 ml 95% ethanol
- 1 ml Malachite green (1% solution in 95% ethanol)
- 50 ml Distilled water
- 25 ml Glycerol
- 5 ml Acid fuchsin (1% solution in distilled water)
- 0.5 ml Orange G (1% solution in distilled water)
- 4 ml Glacial Acetic acid
- Add distilled water (4.5 ml) to a total of 100 ml

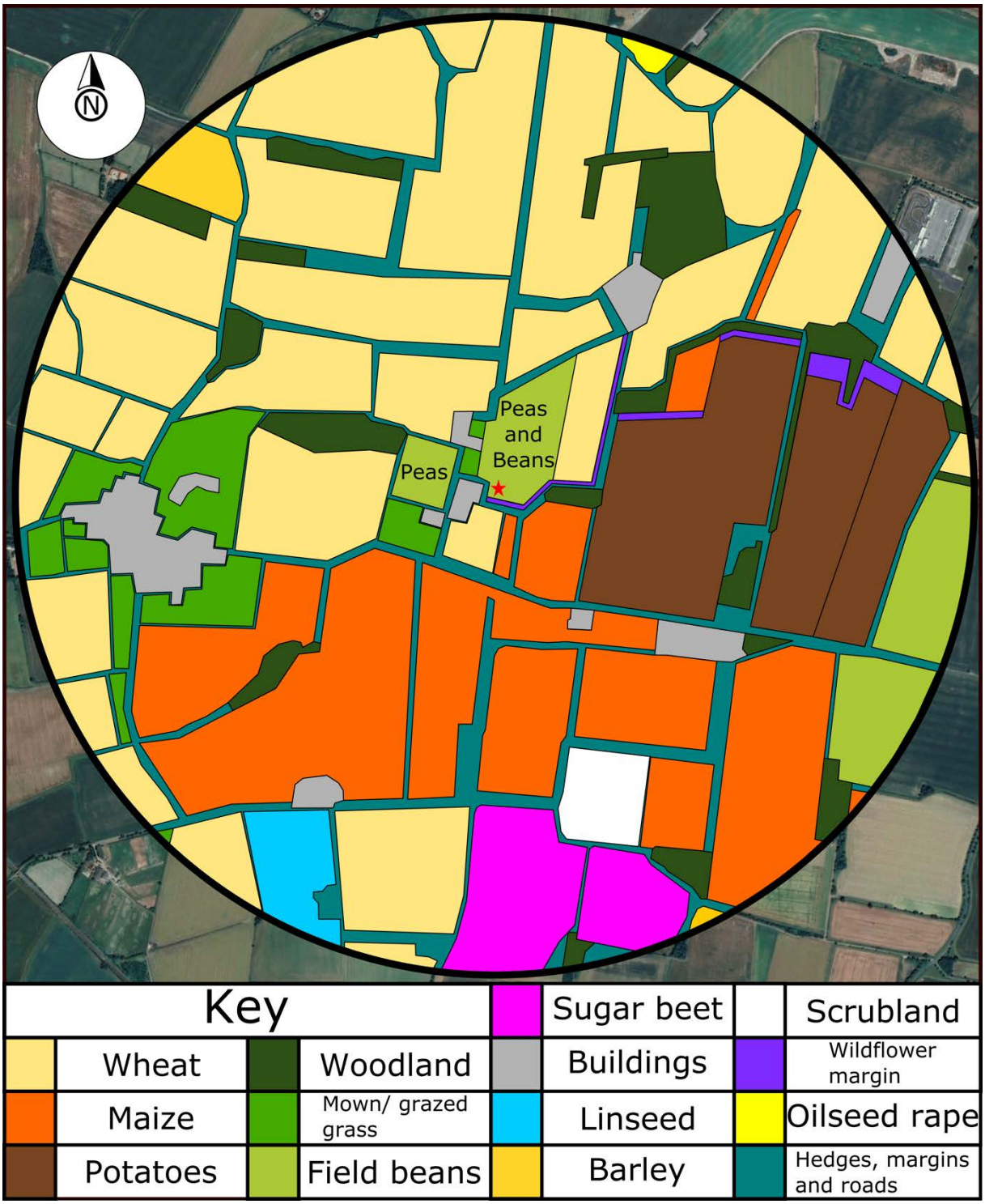
For use in pollen counting, Agar and Tween were added to Alexander stain. Agar makes the solution more viscous, enabling pollen grains to stay suspended for longer. Tween is a surfactant, ensuring pollen grains do not stick together to form clumps. A 1:1 preparation of Agar-Tween was made using 1% Agar and 1% Tween. Three parts of the Agar-Tween solution were mixed with one part modified Alexander stain to produce the final staining solution used for pollen counting. This ratio was tested and optimised to give the most intense staining of pollen grains.

A volume of 200 µl of staining solution was added to each sample. Once stained, pollen grains remained coloured and preserved for many months at room temperature. As an extra precaution, stained pollen grains were stored at -20°C until pollen could be counted. Freezing did not affect the staining or integrity of pollen grains.

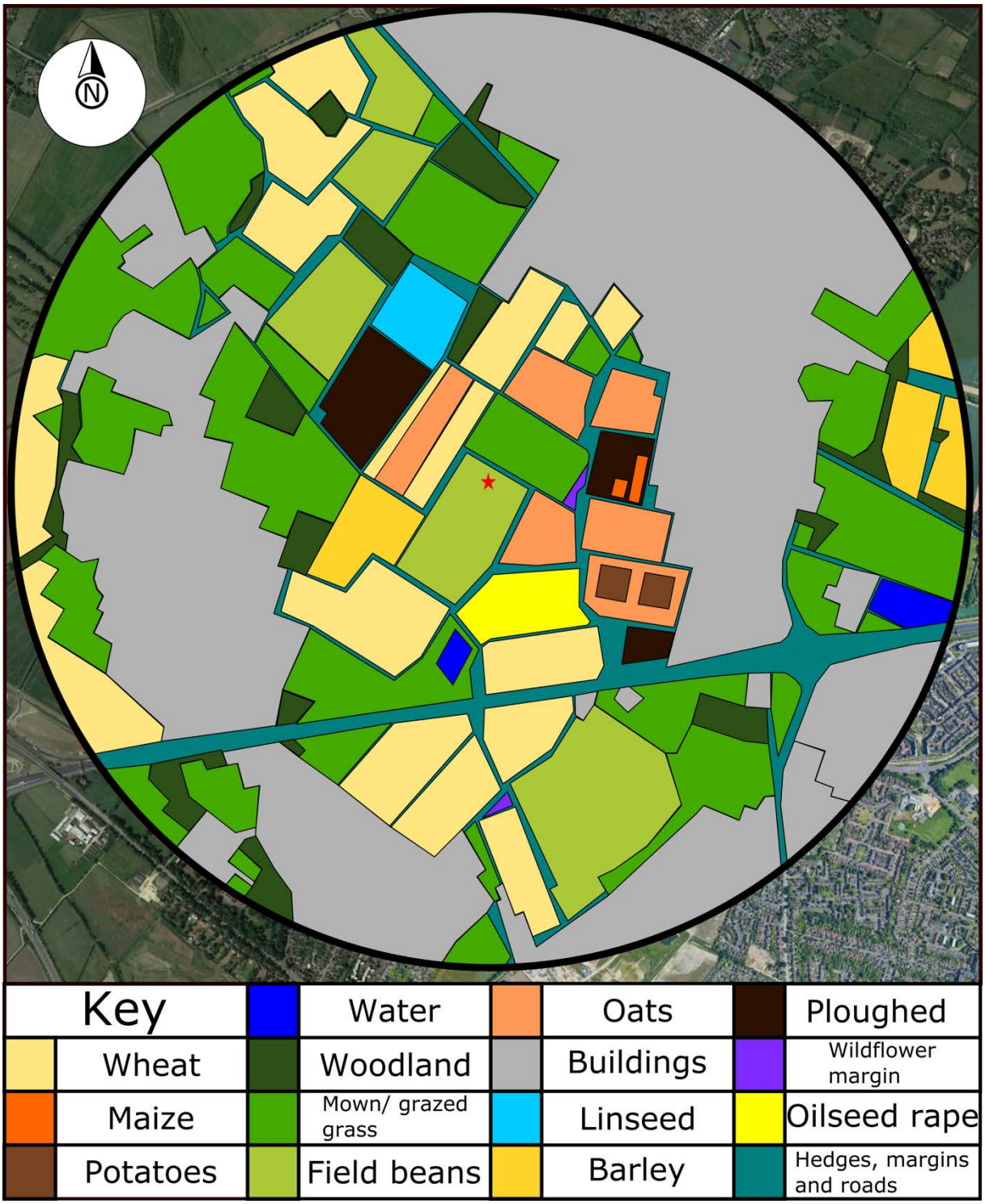
Appendix B – Comparison of field sites

Due to availability of trial ground, different sites had to be used for field trials in 2021 and 2022. The 2021 trial was carried out in Stubton, Lincolnshire. The 2022 trial was carried out at Histon, Cambridge. A 1-mile radius around each trial was assessed to compare the predominant landscape type, which may have influenced the pollinators present in the trials.

Survey on 2021 field site, Stubton, Lincolnshire, NG23 5DA, grid reference SK885490, What3Words those.consoles.holidays. Red star indicates position of the trial. Surrounding landscape was predominantly arable crops.



Survey of 2022 field site, NIAB trial ground, Histon Cambridge, grid reference TL432623, What3Words noon.chips.rift. Red star indicates position of the trial. Surrounding landscape was predominantly urban, mown/ grazed grassland or arable.



Appendix C - Summary of floral trait data for all lines examined

Green shading shows data collected in this study. Blue shading shows data collected by (Bailes 2016).

Line	Flower colour	Mean number of flowers per node	Mean operative force (mN)	Mean nectar volume per flower (µl)	Mean nectar concentration (% w/w)	Mean sugar mass per flower (mg)	Mean number of pollen grains per flower (thousands)	Mean pollen viability (%)	Mean standard height (mm)	Mean wing area (mm2)	Mean corolla length (mm)	Mean wing petal spot size (%)
Albus	Dark spot	6.6	26.8	1.3	42.0	0.6	26.0	NO DATA	17.0	107.0	13.0	NO DATA
Atlas	Dark spot	6.8	23.2	1.6	50.0	0.9	38.6	NO DATA	17.0	115.0	14.0	47.0
BPL10	Dark spot	5.0	26.5	2.7	19.6	3.2	58.6	96.1	16.3	113.7	13.1	43.4
BPL27	Dark spot	1.6	NO DATA	0.6	35.0	0.2	20.2	NO DATA	19.0	99.0	15.0	43.0
Fanfare	Dark spot	7.5	17.7	3.0	47.5	4.7	71.8	92.4	17.0	115.3	12.5	40.4
Fuego	Dark spot	7.6	22.6	2.7	41.6	4.0	76.5	93.1	15.4	113.0	12.3	44.7
Hedin	Dark spot	5.4	NO DATA	0.8	39.0	0.3	25.8	NO DATA	17.0	108.0	14.0	39.0
INRA29H	Dark spot	NO DATA	NO DATA	2.1	41.5	3.2	78.9	95.0	18.2	108.3	11.4	37.3
Kasztelan	Non-spotted	8.1	NO DATA	0.8	37.0	0.3	31.0	NO DATA	15.0	96.0	14.0	NO DATA
LG Cartouche	Dark spot	7.5	17.3	2.7	51.5	4.5	74.1	95.2	14.5	112.1	11.9	43.9
Lynx	Dark spot	6.3	23.2	3.9	40.9	5.7	70.7	87.4	15.4	108.0	12.4	44.7
Maris Bead	Dark spot	7.5	22.5	1.6	49.0	0.8	26.2	NO DATA	14.0	89.0	13.0	46.0
NV020	Dark spot	NO DATA	NO DATA	0.9	38.0	0.3	25.6	NO DATA	17.0	105.0	15.0	55.0
NV027	Dark spot	NO DATA	NO DATA	0.9	34.0	0.2	25.6	NO DATA	21.0	112.0	15.0	42.0
NV079	Dark spot	3.8	19.5	0.9	35.0	0.3	17.4	NO DATA	13.0	83.0	14.0	39.0
NV082	Dark spot	4.4	24.8	1.5	33.0	0.5	24.4	NO DATA	16.0	98.0	14.0	43.0
NV100	Dark spot	4.6	19.8	0.8	32.0	0.3	28.1	NO DATA	17.0	86.0	12.0	42.0
NV129	Dark spot	4.1	36.1	0.7	15.0	0.1	25.0	NO DATA	13.0	110.0	14.0	44.0
NV155	Dark spot	2.6	14.8	0.4	NO DATA	NO DATA	25.2	NO DATA	12.0	60.0	12.0	44.0
NV175	Non - spotted	NO DATA	NO DATA	2.3	33.0	0.8	NO DATA	NO DATA	22.0	148.0	16.0	NO DATA
NV293	Dark spot	3.3	20.2	1.1	36.0	0.3	22.8	NO DATA	19.0	104.0	14.0	44.0
NV490	Dark spot	5.7	25.4	0.9	43.0	0.4	9.8	NO DATA	15.0	80.0	15.0	41.0
NV574	Dark spot	5.8	20.3	1.6	51.0	0.8	NO DATA	NO DATA	18.0	105.0	14.0	24.0
NV604	Dark spot	8.2	22.6	0.6	34.0	0.3	30.1	NO DATA	15.0	107.0	13.0	41.0
NV619	Dark spot	6.2	20.6	3.8	37.0	1.6	34.2	NO DATA	18.0	97.0	14.0	38.0
NV620	Dark spot	1.3	19.8	0.5	21.0	0.1	13.9	NO DATA	12.0	67.0	14.0	51.0
NV626	Dark spot	5.9	21.5	1.4	51.0	0.7	40.2	NO DATA	16.0	115.0	14.0	48.0
NV649	Dark spot	NO DATA	NO DATA	0.9	30.0	0.2	31.3	NO DATA	21.0	107.0	15.0	55.0
NV650	Dark spot	NO DATA	NO DATA	1.4	26.0	0.3	38.5	NO DATA	22.0	158.0	14.0	60.0
NV658	Dark spot	7.5	NO DATA	NO DATA	NO DATA	NO DATA	NO DATA	NO DATA	NO DATA	NO DATA	NO DATA	NO DATA
Pyramid	Dark spot	5.1	21.8	1.8	46.0	0.9	30.4	NO DATA	16.0	95.0	14.0	48.0
Taifun	Dark spot	6.5	23.2	NO DATA	NO DATA	NO DATA	NO DATA	NO DATA	NO DATA	NO DATA	NO DATA	NO DATA
Tattoo	Non - spotted	NO DATA	NO DATA	2.7	42.0	1.3	31.3	NO DATA	18.0	109.0	15.0	NO DATA
Tiffany	Dark spot	7.5	21.2	3.0	32.6	4.1	65.6	98.4	15.4	111.6	13.4	45.5
Tundra	Dark spot	9.4	22.0	3.5	42.6	5.2	69.3	95.9	16.0	114.5	11.9	46.7
Vertigo	Dark spot	6.4	26.4	3.0	42.6	4.4	74.0	95.3	17.1	120.3	12.7	48.4
Victus	Dark spot	6.8	20.9	3.0	38.7	4.2	62.3	98.3	15.5	124.5	12.2	43.3
Yukon	Yellow spot	7.2	14.4	2.5	28.7	3.1	84.1	98.7	17.2	105.0	12.0	NO DATA

Appendix D - The colour of glasshouse-grown flowers in bee colour space

The average excitation UV, blue and green photoreceptors in bee colour space is shown alongside hexagon coordinates used to plot values in a bee colour space hexagon plot using the Pavo package in R, following the method of (Chittka 1992). The hexplot sector that coordinates relate to is also given and the colour contrast of petals against a human-green background.

Line	Petal	Photorecpetor excitation			Hexagon coordinates		Hexplot sector	Green colour contrast
		UV	BLUE	GREEN	x	y		
BPL10	Wing petal spot	0.07072653	0.03138101	0.07132594	0.000519099	-0.039645226	uvgreen	0.039648625
Fanfare	Wing petal spot	0.07900059	0.05054437	0.0950581	0.013906212	-0.036484973	uvgreen	0.039045307
Fuego	Wing petal spot	0.05757208	0.03182407	0.06429806	0.00582487	-0.029110998	uvgreen	0.029688033
INRA29H	Wing petal spot	0.09164161	0.04751317	0.10301036	0.009845627	-0.049812812	uvgreen	0.050776497
LG Cartouche	Wing petal spot	0.08245019	0.04478846	0.09481499	0.010708237	-0.043844125	uvgreen	0.045132844
Lynx	Wing petal spot	0.03686079	0.01734324	0.0439479	0.006137613	-0.023061109	uvgreen	0.023863886
Tiffany	Wing petal spot	0.06912051	0.06469659	0.07494578	0.00504483	-0.007336559	green	0.008903674
Tundra	Wing petal spot	0.0777751	0.04632628	0.09449633	0.014481007	-0.039809438	uvgreen	0.042361432
Vertigo	Wing petal spot	0.05108916	0.04317501	0.08927382	0.033068879	-0.027006482	green	0.042695443
Victus	Wing petal spot	0.16920869	0.11139384	0.1470948	-0.019151185	-0.046757901	uvgreen	0.050527905
Yukon	Wing petal spot	0.19030647	0.61212564	0.73929616	0.475439013	0.147324329	bluegreen	0.497741613
BPL10	Standard petal	0.2709623	0.74238694	0.72807814	0.395873934	0.24286672	bluegreen	0.464435588
Fanfare	Standard petal	0.36594592	0.78235325	0.74839512	0.33121072	0.225182729	bluegreen	0.400509429
Fuego	Standard petal	0.38880795	0.78900698	0.74895149	0.31189345	0.220127266	bluegreen	0.381750621
INRA29H	Standard petal	0.28529696	0.73211928	0.6872784	0.348126141	0.245831593	bluegreen	0.426174825
LG Cartouche	Standard petal	0.29078084	0.73935082	0.71324061	0.365860895	0.237340098	bluegreen	0.436101498
Lynx	Standard petal	0.35322965	0.75796959	0.71959152	0.317278685	0.221559006	bluegreen	0.386980823
Tiffany	Standard petal	0.36187172	0.77437653	0.73654214	0.324474102	0.225169601	bluegreen	0.3949491
Tundra	Standard petal	0.33311468	0.75836901	0.73880873	0.351341358	0.222407302	bluegreen	0.415819381
Vertigo	Standard petal	0.31074871	0.73771115	0.70138998	0.338305261	0.231641808	bluegreen	0.410010216
Victus	Standard petal	0.32515845	0.76964231	0.74284993	0.361731434	0.235638123	bluegreen	0.431711658
Yukon	Standard petal	0.31431542	0.75831439	0.73045991	0.360391707	0.235926725	bluegreen	0.430747725
BPL10	Wing petal tip	0.36455798	0.82897228	0.83734271	0.409443586	0.228021933	bluegreen	0.468655579
Fanfare	Wing petal tip	0.42357793	0.84700731	0.84083008	0.361350965	0.214803302	bluegreen	0.420374807
Fuego	Wing petal tip	0.37185781	0.82500302	0.81441554	0.383266238	0.231866345	bluegreen	0.447945321
INRA29H	Wing petal tip	0.4617797	0.86198394	0.84462167	0.331550875	0.208783259	bluegreen	0.391811731
LG Cartouche	Wing petal tip	0.42988185	0.84548756	0.84285	0.35764091	0.209121641	bluegreen	0.414293231
Lynx	Wing petal tip	0.42163356	0.83586892	0.82034409	0.345293443	0.214880091	bluegreen	0.406695236
Tiffany	Wing petal tip	0.41278292	0.85267508	0.84779247	0.376729322	0.222387387	bluegreen	0.437471292
Tundra	Wing petal tip	0.40319405	0.8417734	0.84230304	0.380279548	0.219024858	bluegreen	0.438844418
Vertigo	Wing petal tip	0.40712827	0.84328277	0.83477721	0.370354851	0.222330029	bluegreen	0.431964532
Victus	Wing petal tip	0.41157535	0.84702326	0.84721741	0.37727709	0.217626885	bluegreen	0.435545019
Yukon	Wing petal tip	0.39887502	0.83226381	0.84011354	0.382123772	0.212769532	bluegreen	0.437366495

Appendix E – The colour of field-grown flowers in bee colour space

The average excitation UV, blue and green photoreceptors in bee colour space is shown alongside hexagon coordinates used to plot values in a bee colour space hexagon plot using the Pavo package in R, following the method of (Chittka 1992). The hexplot sector that coordinates relate to is also given and the colour contrast of petals against a human-green background.

Line	Petal	Photorecpetor excitation			Hexagon coordinates		Hexplot sector	Green colour contrast
		UV	BLUE	GREEN	x	y		
NV100	Corolla tube	0.38308545	0.64022896	0.6062521	0.193268012	0.14556017	bluegreen	0.241951
NV129	Corolla tube	0.5842286	0.8427949	0.8092531	0.194876932	0.14605406	bluegreen	0.24353399
Fuego	Corolla tube	0.64105478	0.87080397	0.8341481	0.167223692	0.13320254	bluegreen	0.21379121
Maris Bead	Corolla tube	0.53715546	0.7566253	0.6358082	0.085435762	0.17014347	blue	0.19038926
Tiffany	Corolla tube	0.51692275	0.82509845	0.7317133	0.186014064	0.20078043	bluegreen	0.27370424
NV100	Wing petal spot	0.07907414	0.09728803	0.1454767	0.057506317	-0.0149874	green	0.05942725
NV129	Wing petal spot	0.15384753	0.20999058	0.3081682	0.133645592	-0.02101728	green	0.1352881
Fuego	Wing petal spot	0.27622397	0.31751147	0.4230075	0.127118292	-0.03210428	green	0.13110967
Maris Bead	Wing petal spot	0.24338293	0.25020381	0.3290978	0.074231263	-0.03603656	green	0.08251614
Tiffany	Wing petal spot	0.17803487	0.14122127	0.1678994	-0.008777554	-0.03174587	uvgreen	0.032937
NV100	Standard petal	0.26442225	0.73887961	0.7096343	0.385564932	0.25185134	bluegreen	0.46053166
NV129	Standard petal	0.35464861	0.78190414	0.7658255	0.356089659	0.22166707	bluegreen	0.41944742
Fuego	Standard petal	0.45463163	0.82320113	0.8096897	0.307489285	0.19104048	bluegreen	0.36200293
Maris Bead	Standard petal	0.35032791	0.71954744	0.6516261	0.260931874	0.21857044	bluegreen	0.34037991
Tiffany	Standard petal	0.34590655	0.78602117	0.7563011	0.355412061	0.23491737	bluegreen	0.42603275
NV100	Wing petal tip	0.40141323	0.84947627	0.8391662	0.379105211	0.22918654	bluegreen	0.442998
NV129	Wing petal tip	0.30763615	0.75056754	0.7856767	0.413995282	0.2039111	bluegreen	0.46148871
Fuego	Wing petal tip	0.51633502	0.84336727	0.8453156	0.284905578	0.16254194	bluegreen	0.32801078
Maris Bead	Wing petal tip	0.41659485	0.80119565	0.8067655	0.337897691	0.18951547	bluegreen	0.38741575
Tiffany	Wing petal tip	0.40392925	0.83728591	0.8328615	0.371466259	0.21889052	bluegreen	0.4311615

Appendix F - Bee visitation rates 2021

Overall bee visitation rate for *V. faba* lines gown in 2021. Mean and median rates are shown in bees per hour. Colour intensity is from highest to lowest values.

Overall visitation rate 2021 (bees per hour)		
Line	Mean visitation rate	Median visitation rate
FUEGO	3.28	2.75
MARIS BEAD	8.83	5.41
NV100	3.44	3.25
NV129	1.35	1.65
TIFFANY	5.03	4.93
WILDFLOWER	70.15	65.00

Visitation rate by visit type 2021 (bees per hour)			
Behaviour	Line	Mean visitation rate	Median visitation rate
EFN	FUEGO	0.10	0.00
	MARIS BEAD	1.05	0.38
	NV100	0.21	0.25
	NV129	0.14	0.00
	TIFFANY	0.42	0.24
LEGITIMATE	FUEGO	1.53	1.82
	MARIS BEAD	4.42	2.86
	NV100	2.14	1.50
	NV129	0.59	0.71
	TIFFANY	2.34	2.15
ROBBING	FUEGO	0.50	0.24
	MARIS BEAD	1.81	1.23
	NV100	0.42	0.24
	NV129	0.07	0.00
	TIFFANY	1.38	1.41
SEARCHING	FUEGO	1.15	0.50
	MARIS BEAD	1.56	0.95
	NV100	0.67	0.50
	NV129	0.54	0.71
	TIFFANY	0.90	0.72

Appendix G - Bee visitation rates 2022

Overall bee visitation rate for *V. faba* lines gown in 2022. Mean and median rates are shown in bees per hour. Colour intensity is from highest to lowest values.

Overall visitation rate 2022 (bees per hour)		
Line	Mean visitation rate	Median visitation rate
FUEGO	4.55	4.44
LYNX	14.56	13.00
MARIS BEAD	14.44	14.40
TIFFANY	9.16	7.11
VERTIGO	11.88	10.22
YUKON	7.10	6.13

Visitation rate by visit type 2022 (bees per hour)			
Behaviour	Line	Mean visitation rate	Median visitation rate
EFN	FUEGO	0.18	0.03
	LYNX	0.75	0.37
	MARIS BEAD	0.85	0.34
	TIFFANY	0.55	0.24
	VERTIGO	0.48	0.18
	YUKON	0.36	0.11
LEGITIMATE	FUEGO	0.13	0.06
	LYNX	0.43	0.33
	MARIS BEAD	1.17	0.36
	TIFFANY	0.64	0.24
	VERTIGO	0.51	0.32
	YUKON	0.41	0.21
ROBBING	FUEGO	0.51	0.31
	LYNX	1.98	1.64
	MARIS BEAD	1.13	0.62
	TIFFANY	0.81	0.48
	VERTIGO	1.55	1.23
	YUKON	0.70	0.49
SEARCHING	FUEGO	0.34	0.31
	LYNX	0.55	0.37
	MARIS BEAD	0.52	0.54
	TIFFANY	0.38	0.34
	VERTIGO	0.51	0.45
	YUKON	0.36	0.29

Appendix H - Change in yield measures with pollination treatment

Tables showing change in yield parameters between caged and open pollination treatments for 2021 and 2022. Colour intensity is from highest to lowest values.

1. Pods per plant 2021				
Line	**Treatment**	**Mean Pods**	**Difference**	**% change**
Fuego	CAGE	9.83	-0.04	-0.37
Fuego	OPEN	9.80		
Maris Bead	CAGE	11.05	3.88	26.00
Maris Bead	OPEN	14.93		
NV100	CAGE	6.75	4.13	37.98
NV100	OPEN	10.88		
NV129	CAGE	5.68	2.88	33.63
NV129	OPEN	8.55		
Tiffany	CAGE	11.18	-0.74	-6.62
Tiffany	OPEN	10.44		

2. Seeds per plant 2021				
Line	**Treatment**	**Mean Seeds**	**Difference**	**% change**
Fuego	CAGE	24.32	2.56	9.54
Fuego	OPEN	26.88		
Maris Bead	CAGE	27.65	19.78	41.71
Maris Bead	OPEN	47.43		
NV100	CAGE	13.73	12.05	46.74
NV100	OPEN	25.78		
NV129	CAGE	12.10	7.70	38.89
NV129	OPEN	19.80		
Tiffany	CAGE	31.14	3.82	10.93
Tiffany	OPEN	34.96		

3. Seeds per pod 2021				
Line	**Treatment**	**Mean Seeds**	**Difference**	**% change**
Fuego	CAGE	2.47	0.27	9.94
Fuego	OPEN	2.75		
Maris Bead	CAGE	2.49	0.69	21.75
Maris Bead	OPEN	3.18		
NV100	CAGE	2.01	0.35	14.96
NV100	OPEN	2.37		
NV129	CAGE	2.07	0.25	10.68
NV129	OPEN	2.32		
Tiffany	CAGE	2.77	0.57	17.12
Tiffany	OPEN	3.35		

4. Bean mass per plant 2021				
Line	**Treatment**	**Mean Mass (g)**	**Difference**	**% change**
Fuego	CAGE	8.06	0.56	6.55
Fuego	OPEN	8.62		
Maris Bead	CAGE	8.01	4.11	33.90
Maris Bead	OPEN	12.12		
NV100	CAGE	2.06	1.59	43.54
NV100	OPEN	3.65		
NV129	CAGE	2.27	0.64	14.96
NV129	OPEN	2.91		
Tiffany	CAGE	11.95	0.25	14.96
Tiffany	OPEN	12.21		

5. Mean bean mass per pod 2021				
Line	**Treatment**	**Mean Mass (g)**	**Difference**	**% change**
Fuego	CAGE	0.81	0.06	6.58
Fuego	OPEN	0.86		
Maris Bead	CAGE	0.67	0.13	16.37
Maris Bead	OPEN	0.80		
NV100	CAGE	0.29	0.05	14.70
NV100	OPEN	0.34		
NV129	CAGE	0.32	0.01	3.75
NV129	OPEN	0.33		
Tiffany	CAGE	1.07	0.13	10.76
Tiffany	OPEN	1.20		

6. Mean mass per bean 2021				
Line	**Treatment**	**Mean Mass (g)**	**Difference**	**% change**
Fuego	CAGE	0.33	-0.01	-3.84
Fuego	OPEN	0.32		
Maris Bead	CAGE	0.27	-0.02	-6.92
Maris Bead	OPEN	0.25		
NV100	CAGE	0.14	0.00	-0.73
NV100	OPEN	0.14		
NV129	CAGE	0.16	-0.01	-6.89
NV129	OPEN	0.14		
Tiffany	CAGE	0.40	-0.04	-0.37
Tiffany	OPEN	0.35		

7. Plot yield 2021				
Line	**Treatment**	**Mean mass (kg)**	**Difference**	**% change**
Fuego	CAGE	0.38	0.12	23.84
Fuego	OPEN	0.50		
Maris Bead	CAGE	1.09	0.42	27.81
Maris Bead	OPEN	1.51		
NV100	CAGE	0.07	0.03	31.25
NV100	OPEN	0.11		
NV129	CAGE	0.08	0.01	11.11
NV129	OPEN	0.09		
Tiffany	CAGE	0.89	0.20	18.11
Tiffany	OPEN	1.09		

8. Plot yield 2022				
Line	**Treatment**	**Mean mass (kg)**	**Difference**	**% change**
Fuego	CAGE	0.41	-0.09	-22.44
Fuego	OPEN	0.32		
Lynx	CAGE	0.44	0.02	4.99
Lynx	OPEN	0.46		
Maris Bead	CAGE	0.29	0.23	79.53
Maris Bead	OPEN	0.52		
Tiffany	CAGE	0.32	0.12	39.09
Tiffany	OPEN	0.44		
Vertigo	CAGE	0.32	-0.03	-10.47
Vertigo	OPEN	0.29		
Yukon	CAGE	0.27	0.15	53.59
Yukon	OPEN	0.42		

Appendix I - The colour of artificial colour models and field-grown flowers in bee colour space

The average excitation UV, blue and green photoreceptors in bee colour space is shown alongside hexagon coordinates used to plot values in a bee colour space hexagon plot using the Pavo package in R, following the method of (Chittka 1992). The hexplot sector that coordinates relate to is also given and the colour contrast of petals against a human-green background.

Number on figure	Line	Petal/model	Photorecpetor excitation			Hexagon coordinates		Hexplot sector	Green colour contrast
			UV	BLUE	GREEN	x	y		
1	Maris Bead	Flower (between veins)	0.3249709	0.7201531	0.6466958	0.27862189	0.23431972	bluegreen	0.3640548
2	NV129	Flower (between veins)	0.3546486	0.7819041	0.7658255	0.35608966	0.22166707	bluegreen	0.4194474
3	Maris Bead	Printed image model (between veins)	0.6707959	0.91053	0.8254355	0.13392187	0.16241434	bluegreen	0.2105077
4	NV129	Printed image model (between veins)	0.7015733	0.9124463	0.8501648	0.12868398	0.13657726	bluegreen	0.187651
5	Maris Bead	Printed average colour model	0.6300361	0.8906622	0.7976029	0.1451171	0.17684268	bluegreen	0.2287626
6	NV129	Printed average colour model	0.6502929	0.8849344	0.8255954	0.15181642	0.14699018	bluegreen	0.2113157
7	Maris Bead	Printed vein colour model	0.4318222	0.837223	0.6954187	0.22828128	0.27360257	bluegreen	0.3563295
8	NV129	Printed vein colour model	0.4376534	0.7131945	0.7492025	0.26980941	0.11976659	bluegreen	0.2951968