

Nutritional Resources for Pollinators from Mass-Flowering Crop Cultivars

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Abstract

The scarcity of flowers to provide dietary nectar and pollen is a key driver of recent declines in pollinators in agricultural areas, but the planting of mass-flowering crops enhances resources available to pollinators during parts of the year. This thesis investigates the nutritional resources provided for insect pollinators from various cultivars of two mass-flowering crops: short rotation coppice willow (*Salix* species) and oilseed rape (*Brassica napus* L.).

Willow cultivars vary in the numbers of flowers produced per plant, as well as in the quantity of nectar sugar secreted by those flowers. There were neither qualitative nor quantitative differences in pollen production between the cultivars. Foraging insect pollinators showed preferences for cultivars with more rewarding flowers.

Oilseed rape flowers of different cultivars produced a mass of nectar sugar that varied by up to three fold in mass when grown in a glasshouse. Cultivars differed in the size of their flowers, but neither flower size nor the seed yields they produced in industry trials were correlated with their nectar yields. When plants were grown in field conditions, differences between oilseed rape cultivars in nectar production were also present, although less pronounced. The weights of bumble bee (*Bombus terrestris* L.) colonies diverged after two weeks when restricted to foraging on plots containing either a high or a low nectar yielding cultivar.

The findings indicate that efforts to breed and to plant more widely the more rewarding cultivars of mass-flowering crops would enhance the resources available to pollinators in spring. As this is a critical time for pollinators, the extra resources could aid their survival and lead to more robust populations.

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Chapter 1. General Introduction

1.1 Introduction to Pollination and Pollinators

1.1.1 *The role of pollinators*

For sexual reproduction to occur, gametes containing genetic material from the male and female parents must fuse. In higher plants, the meeting of gametes is achieved in two stages: pollination and fertilisation. Pollination is the transfer of viable pollen grains containing the gametes from their source in the anthers of the male part of the flower, to their destination: the receptive stigma in the female part of the flower. Pollination may be achieved by abiotic means, with grains transferred by wind or water. However, in angiosperms, the transfer of pollen by animals is the ancestral and the most common method of pollination, used by around 88 % of angiosperm species (Ollerton *et al.* 2011).

Their role in angiosperm sexual reproduction makes pollinators vital for maintaining wild populations of the plant species with which they interact. Pollinators also provide a valuable service in agriculture, as their visits enhance yields and quality of 75 % of the crop species grown worldwide (Klein *et al.* 2007). The activity of pollinators is worth an estimated €153 billion annually to global agriculture (Gallai *et al.* 2009), and plays a vital part in producing the main sources of many nutrients essential for human health, including vitamin C, vitamin A and folic acid (Eilers *et al.* 2011).

1.1.2 *Pollinator species*

Animal pollinators include birds, bats, non-flying mammals, and most significantly, the insects (Proctor *et al.* 1996). Within the insects, various species of beetles (Coleoptera), flies (Diptera), wasps and ants (Hymenoptera), and butterflies and moths (Lepidoptera) act as pollinators. The dominant pollinators in most communities, however, are the bees (Hymenoptera: Anthophila), which depend on floral resources both as larvae and adults (Proctor *et al.* 1996).

For the pollination of crops, the European honey bee (*Apis mellifera* L.) is of great importance (Delaplane & Mayer 2000). Although it is not the most efficient pollinator of most crops, honey bees have a large work force, visit a wide variety of plants, and are easily managed and transported (vanEngelsdorp & Meixner 2010). In addition, honey bees produce honey and wax, for the purpose of which humans have spread this species around the world. Several non-*Apis* bee species are also managed for their pollination services, including bumble bees (*Bombus* species) (Velthuis & van Doorn 2006), the alfalfa leafcutter bee (*Megachile rotundata*) (Pitts-Singer & Cane 2011) and the red mason bee (*Osmia bicornis*) (Gruber *et al.* 2011). Unmanaged, wild insects also perform a large part of the pollination of crops (e.g. Breeze *et al.* 2011; Garibaldi *et al.* 2013; Ollerton *et al.* 2012). Flower visits by wild pollinators boost crop yields even when honey bees visit frequently (Garibaldi *et al.* 2013). Important wild pollinators include the various species of bumble bees, solitary bees and hoverflies (Syrphidae) (Biesmeijer *et al.* 2006; Jauker & Wolters 2008).

1.2 Pollinator Declines

1.2.1 Evidence of declining richness and abundance among pollinator species

On account of their importance to human food security, health and ecosystem services, recent evidence that insect pollinators have declined in abundance and richness has caused much concern (e.g. Gonzalez-Varo *et al.* 2013; Potts *et al.* 2010a; Vanbergen *et al.* 2013). There are several lines of evidence that indicate declines in pollinator species.

The best long-term data available are records of the numbers of commercial honey bee hives, which show a mixed picture. Worldwide, the number of hives increased by around 45 % between 1961-2006 (Aizen & Harder 2009). However, in the USA, colony numbers have dropped 61 % over a similar period (vanEngelsdorp & Meixner 2010), while in Europe, hive numbers declined by 16 from 1985-2005 (Potts *et al.* 2010b).

Unlike honey bee colony records, long-term datasets of abundance are not available for wild pollinator species. Declines of wild pollinators may be detected by comparing current distributions and diversity with historical records at the same location. Comparing recent and historical surveys of wild bumble bees, a pattern of declines in richness and relative abundance with some local extinction is seen in the USA (Bartomeus *et al.* 2013; Cameron *et al.* 2011; Gixti *et al.* 2009), Sweden (Bommarco *et al.* 2012a), Ireland (Fitzpatrick *et al.* 2007), Denmark (Dupont *et al.* 2011) and the UK (Williams & Osborne 2009). Declines are particularly evident in long-tongued species, while some short-tongued species remain abundant (Bommarco *et al.* 2012a; Williams *et al.* 2009).

Solitary bees are less well represented in historical comparison studies, but a survey in Illinois, USA, that recorded both solitary and social bees foraging in the understorey of a temperate forest in 2009 and 2010 found only 54 bee species remaining of the 109 that were observed there in the late 1800s (Burkle *et al.* 2013). Meanwhile, a comparison of observations made before and after 1980 in the UK and the Netherlands showed a general decline in species richness of all bee species at local scales in both countries, while communities of both bees and hoverflies are increasingly dominated by a smaller number of species (Biesmeijer *et al.* 2006).

1.2.2 Causes of pollinator declines

Pollinator declines have not been linked to a single cause, but a consensus has emerged that multiple interacting factors are responsible (Gonzalez-Varo *et al.* 2013; Potts *et al.* 2010a; Roulston & Goodell 2011; Vanbergen *et al.* 2013).

Pollinators face several general, underlying pressures, which act over a range of spatial and temporal scales, and can interact to buffer or amplify their individual effects on pollinators. These pressures and their effects may be summarised as:

1. Landscape alteration caused by urbanisation, pollution and the spread of agriculture, which causes the destruction, fragmentation and degrading of natural habitats. As a result, pollinator populations become isolated, and available nutritional and nesting resources are reduced (Winfree *et al.* 2009).
2. Agricultural intensification of land already in production, which entails the removal of hedgerows, field margins and arable weeds, along with increasing use of fertilisers and pesticides. Where this occurs, the number of nesting sites (Osborne *et al.* 2008) and the abundance and diversity of available sources of food decrease (Hautier *et al.* 2009; Schmitz *et al.* 2013), while insecticides have sub-lethal effects on pollinators (e.g. Gill *et al.* 2012; Whitehorn *et al.* 2012).
3. Non-native pollinator species that compete for food with local pollinators, diminishing its availability (Goulson & Sparrow 2009).
4. Climate change impacts, such as warming, extreme events, and mismatches between pollinators and the plants they feed on due to shifting geographical ranges and phenology (Memmott *et al.* 2007), (Kerr *et al.* 2015). Mismatches are likely to reduce the diet breadth of pollinators.
5. The spread of pathogens, for instance between managed and wild pollinators, which can impose large fitness costs on their hosts (Martin *et al.* 2012), (Fürst *et al.* 2014).

The impact of these pressures on pollinator populations is determined by species-specific traits, such as the pollinators' foraging range, dietary specialisation, life history and genetic variability (Roulston & Goodell 2011). While impacts on pollinators of some of these factors acting in isolation have been studied, the effects of more complex interactions between multiple pressures are not yet understood (Vanbergen *et al.* 2013).

1.2.3 *Evidence of limitation of pollinator populations by food availability*

Resource availability is a key factor determining population size. As described above, landscape alteration, agricultural intensification, competition with non-native species and climate change may all reduce the abundance and breadth of nutritional resources available to pollinators. Pollinator populations may, then, be limited by their ability to satisfy their dietary needs.

Recent evidence suggests that nutritional resource availability limits pollinator populations. Dietary specialists provide a simple means to detect relationships between the abundance of food and the size of pollinator populations (Roulston & Goodell 2011). Population size in the solitary bee *Andrena hattorfiana* in southern Sweden was strongly correlated with the population size of its main pollen source, *Knautia arvensis*, suggesting that bee populations are limited by the abundance of food (Larsson & Franzen 2007).

For pollinators with a broader diet, direct relationships between population size and nutritional resource availability are more difficult to show, due to the variety of resources that may be used. Instead, studies have inferred that resources limit population sizes by comparing pollinators foraging in landscapes that differ in their abundance of food. Williams *et al.* (2012) followed experimental colonies of *Bombus vosnesenskii* placed in 39 sites with varying abundances of floral resources. Colonies in the landscapes where food was scarce produced fewer males and workers, though there was no effect of resource abundance on queen production.

The limitation of pollinator populations by food abundance may also be inferred by the occurrence of competition between pollinator species.

Experimental colonies of bumble bees placed near to honey bee hives show lower foraging and reproductive success (Thomson 2004), and have a lower colony mass than colonies further from honey bees (Elbgami *et al.* 2014).

Additionally, workers of four bumble bees species had a smaller mean body size at sites where honey bees were present than sites without them (Goulson &

Sparrow 2009). These studies suggest that in the presence of honey bees, bumble bee colonies do not have sufficient food to grow as they would in the absence of honey bees, and that food availability is a limiting factor in their growth. However, the transmission of pathogens from honey bees to bumble bees could also explain the findings. Pathogen transmission between bumble bees can occur when infected and uninfected individuals visit the same flower (Durrer & Schmid-Hempel 1994; Fürst *et al.* 2014)

Lastly, food availability as a limiting factor to pollinator populations is demonstrated by the effects of food supplementation. Bumble bee colonies of the species *B. impatiens* and *B. ternarius* that were given sucrose solution and five grams of pollen every 12-15 days produced more workers and sexual offspring than controls (Pelletier & McNeil 2003). The meadow landscape in which colonies were placed provided nutritional resources insufficient for colonies to attain their maximum size and fitness.

Although other factors such as nest site availability, predation, disease and parasitism may all impose limits on pollinator populations, the examples above show the importance of nutritional resources to limiting pollinator populations. Any attempts to reverse the trend of pollinator declines must address the sources of nutrition available to them, and the formation of floral communities to enhance nutritional resources is often promoted (Carvell *et al.* 2007; Pywell *et al.* 2006). Most bees depend on floral nectar and pollen for both their adult and larval diets (Michener 2000), and the properties of these two resources are considered next.

1.3 Nectar

1.3.1 Components of nectar

Floral nectar is an aqueous, sugar-rich solution, with which flowers attract and reward the animals that transfer their pollen (Brandenburg *et al.* 2009). The importance of nectar in the diets of most pollinators is chiefly due to the energy

it provides in the form of sugars, which may be used to power flight, produce wax and incubate brood (Alford 1975). Sucrose is one of the three sugars found at significant concentrations in nectar; along with the monosaccharides glucose and fructose (Percival 1961; Wykes 1952a). The nectar produced by most species contains detectable amounts of all three of these sugars, but sometimes only one or two are found (Baker & Baker 1983). The enzyme invertase cleaves sucrose into glucose and fructose, but ratios of the monosaccharides in nectar can deviate from equal proportions, as the sucrose molecule is cycled through complex biochemical pathways in the nectary cells prior to secretion into the nectary lumen (Wenzler *et al.* 2008).

The relative proportions of the three main nectar sugars vary between plant families, but appear to be relatively consistent within them (Baker 1977; Percival 1961; Wykes 1952a; Wykes 1953). However, sometimes the ratios of the sugars can vary widely, even between nectaries in a single flower, where they have been altered by the action of yeasts (Herrera *et al.* 2013). Baker and Baker (1983) suggested that sucrose-rich nectars may have evolved in some plant species to suit the preferences of the animals that chiefly pollinate them, namely species of hummingbirds, butterflies and long-tongued bees, but this association has likely been overstated (Nicolson & Thornburg 2007). Many of the plants sampled were from a few families so did not evolve independently, and flowers favoured by those pollinators tend to have long corollas, which may also be linked to their sucrose-rich nectars, perhaps by limiting invasion by airborne microbes (Willmer 2011). Nonetheless, Wykes (1952b) reported that honey bees prefer sugar solutions with a balance between the three main nectar sugars to solutions of any other sugar or combination of sugars. Waller (1972), however, found that honey bees prefer sucrose solution to the balanced sugar solution. Sugar composition also affects crystallisation of solutions: a high proportion of glucose among the nectar sugars is associated with a tendency for rapid

granulation after nectar becomes concentrated when it is transformed into honey (Smanalieva & Senge 2009).

Sugars are by far the most concentrated solutes in nectar, though increasingly sensitive techniques have revealed a complex mix of more dilute solutes, for which adaptive roles have been suggested (Mitchell 2004). These minor components include amino acids, proteins, lipids, inorganic ions, volatile organic compounds and various secondary compounds (Nicolson & Thornburg 2007). The amino acids may influence the taste of nectar (Gardener & Gillman 2002), and the amino acid proline can be metabolised to power flight by the honey bee, though its importance as a source of energy is slight, relative to the sugars (Barker & Lehner 1972). The other minor nectar components may play a role in manipulating the behaviour of pollinators, deterring nectar robbing, preventing microbial spoilage of nectar, and defending plants from pathogens (Baker 1977; Carter & Thornburg 2004; Heil 2011).

1.3.2 Secretion and reabsorption of nectar

Floral nectar is produced by nectary glands, which are usually situated at the base of flowers (Willmer 2011). Nectaries have three main parts: 1) the epidermis that controls nectar release, 2) the parenchyma that produces or stores the nectar solutes, and 3) the vascular bundle that delivers water and nutrients to the parenchyma cells, and consists of either phloem, or both phloem and xylem (Pacini *et al.* 2003). Phloem sap is the raw material of nectar, which flows from the vascular bundle, through the parenchyma cells, and is exuded through the pores of modified stomata or unicellular glandular hairs in the epidermis (Nepi & Stpiczynska 2008; Pacini & Nepi 2007). The sugars secreted in floral nectar are produced by photosynthesis in either the nectar parenchyma or in other photosynthetic tissue in the plant, though they may be stored as starch molecules prior to secretion (Pacini *et al.* 2003).

The production and secretion of nectar sugar is costly to plants. In the common milkweed *Asclepias syriaca*, 4-37 % of the sugars produced in photosynthesis during flowering were expended in nectar (Southwick 1984) . A range of plant species have been found to reabsorb sugar from their nectar, which allows plants to recover part of the resources it has invested in nectar, and to have greater control over the properties of the nectar it presents (Nepi & Stpiczynska 2008).

Secretion and reabsorption can occur at the same time, so that increases or decreases in available nectar sugar are determined by the balance between the two processes (Burquez & Corbet 1991). Secretion of nectar can start before a flower opens (Pacini & Nepi 2007). Secretion may either a) continue for a flower's entire lifespan, b) cease during a period of pollinator inactivity, c) cease when a maximum volume is reached and resume only when nectar is removed, d) pause between sexual phases of the flower then continue, or e) follow a pattern of secretion at particular times of day (Pacini & Nepi 2007; Willmer 2011).

1.3.3 *Nectar properties*

The nectar secretion of flowers can be described by the standing crop and the secretion rate. The standing crop is the quantity of nectar in a flower at a particular instant; the secretion rate is the quantity of nectar secreted in a given time (Corbet 2003). Both the quantity of sugar and the volume in the standing crop fluctuate through time. Nectar sugar rises with secretion, but falls as it is removed by reabsorption or consumption by animals and microbes (Corbet 2003; Herrera *et al.* 2009). Nectar volume is also affected by secretion, reabsorption and consumption, but it also fluctuates with the gain or loss of water through precipitation, condensation and evaporation (Corbet 2003).

Nectar volumes are under conflicting selection pressures. From the perspective of a plant, the optimum volume is the smallest that will attract pollinators, as

this reduces its costs and forces animals to visit many different plants, transferring pollen as they go (Klinkhamer & Dejong 1993). It is in the interests of animals, however, to find high volumes of nectar that require minimal expenditure of time and energy to obtain. In consequence, the nectar volumes produced by different flowers range from below 0.05 μl in flowers typically pollinated by flies to over 5 ml in those pollinated by bats (Willmer 2011). Within plants, large variability in nectar volumes between flowers is commonly observed, even when animals are excluded (Cresswell 1998). The unpredictable nature of the rewards may encourage pollinators to forage elsewhere, having served the needs of the plant (Rathcke 1992). Nectar volumes are not solely determined by plants, however, but are also subject to microclimatic effects, and commonly decrease after secretion, due to evaporation (Nicolson & Thornburg 2007).

Nectar sugar concentration can range in extremes between 10-65 % (w/w), though a range of 20-50 % is more commonly found in temperate flowers (Willmer 2011). Nectar with low sugar concentrations is low in energy content for flower visitors, but the viscosity of nectar increases exponentially with sugar concentrations, and at high concentrations is too sticky to consume efficiently. The optimal concentration for nectar feeders depends on the drinking technique used; for most bees, which ingest nectar by dipping their tongues in and out of it, the optimum of 55 % has been calculated (Kim *et al.* 2011).

Nectar sugar concentrations generally show less variation than volumes between flowers within a plant (Real & Rathcke 1988). However, concentration of secreted nectar is also influenced by microclimate, increasing from its initial concentration when evaporation takes place. Higher concentrations are expected in circumstances where fast evaporation takes place, for instance in flowers with an open shape. Evaporation also occurs more quickly in small volumes of nectar than large volumes because of the greater surface area to volume ratios, and sucrose-rich nectar evaporates more quickly than nectar rich

in monosaccharides, because there are fewer solute molecules to lower the effective concentration of the water (Nicolson 2002). Reabsorption of nectar sugars can act to maintain concentrations when water loss occurs due to evaporation (Nepi & Stpiczynska 2008). Cnaani *et al.* (2006) found that bumble bees (*Bombus impatiens*) showed stronger discrimination in favour of more rewarding artificial flowers when choosing between rewards differing in concentration than rewards that differed in volumes.

The total sugar content of the nectar within a flower is the product of its volume and its concentration. From these measurements, the mass of sugar per flower is easily calculated, which allows comparisons between different flowers. The total sugar content very closely approximates the energetic value of nectar for pollinators, and the difference between the two, which is due to the minor non-sugar components of nectar, is never great (Willmer 2011).

1.3.4 Variability in nectar

A major challenge in studying nectar is the number of factors that influence secretion rate, and the properties of nectar after it is secreted. Environmental factors have a large influence on the nectar available in flowers. Relative humidity causes evaporation from nectar when low, and can dilute nectar when very high, particularly from more exposed nectaries (Corbet 2003). Nectar secretion is faster at higher temperatures in most species (Burquez & Corbet 1991; Pacini & Nepi 2007), but in some the rate can decrease with temperature, for instance *Trifolium repens* (Jakobsen & Kristjansson 1994). Greater water availability can increase nectar secretion; for instance, volumes but not concentrations were greater in watered plots than unwatered plots of *Asclepias syriaca* (Wyatt *et al.* 1992). Soil nutrients can influence nectar production. Baude *et al.* (2011) showed the total sugar content of nectar in *Lamium amplexicaule* increased when soil nitrogen levels were enhanced by litter amendment, but nectars of two other species investigated were not affected.

Nectar can also differ between the flowers on a plant. Flowers that occupy different positions on an inflorescence may vary in their nectar production. For instance Stpiczynska (2003) found that higher flowers on inflorescences of *Platanthera chlorantha* secreted smaller volumes of nectar than those lower. Flowers on larger inflorescences secrete less nectar sugar per flower than those on smaller inflorescences in *Asclepias quadrifolia* (Pleasant & Chaplin 1983). Flowers in the shade have a greater volume of more dilute nectar than those exposed to sun in *Aloe castanea* (Nicolson & Nepi 2005). The nectar in flowers may also differ according to flower age (Burquez & Corbet 1991), sexual phase (Klinkhamer & Dejong 1993) or after pollination has occurred (Gillespie & Henwood 1994).

1.4 Pollen

1.4.1 The pollen grain

From the perspective of a plant, pollen grains exist to transport hereditary information from the anthers to the stigma, and to deliver their genetic cargo to the ovule for fertilisation. They have evolved a complex structure to protect their nuclei from desiccation, overheating, UV radiation and microbial attack (Pacini & Hesse 2005). Pollen grain size ranges from 4 μm to 350 μm , though most in most plant species they are around 15-60 μm (Willmer 2011).

Pollen grains have four layers. The outer layer is a sticky, lipid-rich coating. In the majority of plant species this outer layer is a material called pollenkit. However, analogous materials formed by slightly different processes are present in some species – for instance, pollen in *Brassica* species is coated with a lipid-rich material called tryphine (Piffanelli *et al.* 1997). The extracellular lipid provides protection, attracts pollinators, and adheres grains to each other, to foragers and to a stigma (Edlund *et al.* 2004; Pacini & Hesse 2005).

Beneath this coating, two tough pollen walls provide mechanical protection, and store proteins and other substances that mediate the interaction with the

stigma and germination of the pollen grain (Raghavan 1997). The outer wall, the exine, is formed of intricately patterned layers of the complex carbohydrate sporopollenin, and within this, the intine is a non-patterned layer composed mainly of cellulose and pectin (Roulston & Cane 2000). Protected by these walls, angiosperm pollen grains contain a vegetative cell, which develops into the pollen tube, and a generative cell, which divides into two sperm cells, before or after germination (Stanley & Linskens 1974).

1.4.2 Pollen as a food reward

From the perspective of a pollen-feeding animal, pollen grains provide nutrients and energy. The list of species that consume pollen includes: some beetles, thrips, springtails, generalist flies, butterflies, wasps, many hoverflies, and virtually all bees, along with some birds and mammals (Willmer 2011). For these animals, pollen provides protein, amino acids, starch, lipids, vitamins and minerals (Roulston & Cane 2000). Pollen nutritional content varies between plant species (Roulston *et al.* 2000; Todd & Bretherick 1942), and the proportions of its constituents are largely determined by plant phylogeny (Roulston & Cane 2002). The protein content of pollen varies between 2.5 to 61 % (Roulston & Cane 2002). Starch content ranges between 0-22 %, and lipid content between 1-18 % (Roulston & Cane 2002).

The majority of these nutrients are contained beyond the pollen walls (Evans *et al.* 1991), and pollen consumers must break into the grains if they are to obtain them (Moritz & Crailsheim 1987). Few pollen consumers break or pierce grains with their mouthparts, but instead the grains burst during digestion, either by subjecting them to osmotic shock within their digestive tracts, or by providing conditions in which grains begin to germinate and exude their contents (Willmer 2011). Bees are able to digest pollen grains efficiently; adult honey bee workers aged 9 days emptied or partially emptied 93-97 % of pollen grains, though this ability decreased as workers aged (Crailsheim *et al.* 1992).

1.4.3 Comparison of pollens as nutritional resources

The majority of studies to compare the value of pollens from different plant species in terms of nutrition for pollinators have investigated their effects in bees. All bees require large amounts of pollen for larval development, and for muscle development after emergence as adults (Moritz & Crailsheim 1987). The nutritional value of pollen is usually evaluated by its crude protein content, which is related to development and reproduction in bees, although pollen lipids are also important (Vanderplanck *et al.* 2014). Only pollens rich in protein enable complete development of the solitary bee *Osmia lignaria* (Levin & Haydak 1957). Likewise, the number and size of offspring produced by groups of queenless workers in the bumble bee *Bombus terrestris* varies according to the protein content of the pollens they are fed (Genissel *et al.* 2002; Vanderplanck *et al.* 2014).

The total protein content of pollen does not distinguish between the individual amino acids from which proteins are formed. In addition to those bound in proteins, significant quantities of amino acids are also present in free form, and the proportion of each amino acid in the two states tends to correlate in a given pollen (Stanley & Linskens 1974). Proportions of amino acids vary between pollens, but similar proportions are found in closely related species (Stanley & Linskens 1974; Weiner *et al.* 2010). De Groot (1953) showed that ten amino acids are essential in the honey bee diet for growth (arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine and valine), and it is usually assumed that these requirements are shared by other bees since these are very similar to those of other insects (Roulston & Cane 2000). While most pollens contain all essential amino acids, some are present in extremely small quantities (Weiner *et al.* 2010). Pollen of the dandelion (*Taraxacum officinale*) lacks tryptophan, phenylalanine, and sufficient arginine, and neither honey bees (Herbert *et al.* 1970) nor bumble bees (*B. terrestris*) (Genissel *et al.* 2002) were able to produce offspring when fed on it alone.

Most bee species feed on pollen from a variety of plant species, but some are highly specialised and will only collect pollen from a narrow range (Proctor *et al.* 1996). When fed with a blend of pollen from multiple plant species, honey bees demonstrate stronger immune responses than those fed a monofloral diet (Alaux *et al.* 2010; Di Pasquale *et al.* 2013). The ability of bees to assess pollen quality and select those of the greatest value is poorly understood. Cook *et al.* (2003) found no innate preferences in honey bees between pollens of different quality, but after experience with both, the insects showed a preference for the pollen that had the greatest content of essential amino acids. However, bees frequently select pollens on which they fail to thrive (Horne 1995; Schmidt *et al.* 1995). The selection of pollens appears to be influenced by the presence of compounds that stimulate feeding, and may be unrelated to their nutritional contents (Schmidt & Hanna 2006).

1.5 Mass-Flowering Crops as Sources of Nutrition for Pollinators

1.5.1 Effects on pollinators of mass-flowering crop availability

Several crops produce floral nectar and pollen that are collected by pollinators. These mass-flowering crops create a large pulse of floral resources during the time that they are in flower. Patches of rewarding flowers attract pollinators, and some, like bumble bees are highly efficient at exploiting patches of resources by, for instance, preferentially visiting larger patches, spending more time in larger patches, and leaving patches when encountering low rewards (Goulson 2003). In landscapes where food availability is limiting, mass-flowering crops can affect pollinator populations. For example, reproduction in solitary bees increases with the availability of mass-flowering crops (Holzschuh *et al.* 2013; Jauker *et al.* 2012; Riedinger *et al.* 2015). Colony sizes of bumble bees also increase with the proportion of mass-flowering crops in the surrounding area (Westphal *et al.* 2003; Williams *et al.* 2012), although they can dwindle later in the year in areas where food becomes scarce (Persson & Smith 2013), so that

colony reproduction may not benefit from an early super-abundance of resources. (Westphal *et al.* 2009; Williams *et al.* 2012).

Another potential benefit of early-blooming mass-flowering crops to wild insect pollinators is that the abundance of food they offer may increase survival after winter. Although difficult to study, it seems likely that early in the year, pollinators are vulnerable to food shortages, and may show greater persistence where floral resources are enhanced by mass-flowering crops (Bohart & Knowlton 1952; Goulson 2003).

1.5.2 Differences in floral rewards between crop cultivars

Floral resources provided for insect pollinators may vary between different cultivars of a crop. Cultivar differences have been shown in nectar production in some crops. For instance, in field beans (*Vicia faba* L.) (Pierre *et al.* 1996) and cotton (*Gossypium hirsutum* L.) (Moffett *et al.* 1976), cultivars differed in nectar secretion rates. Differences in pollen production between crop cultivars have been little studied. Pollen protein content and amino acid composition may be expected to show little variation, as they are conserved between related species (Roulston *et al.* 2000; Weiner *et al.* 2010). However, the numbers of pollen grains per anther have been shown to differ in almond (*Prunus dulcis* Mill.) (Godini 1981), turnip rape (*Brassica rapa* L.) (Hinata & Konno 1975) and apple (*Malus domestica* Borkh.) (de Albuquerque Junior *et al.* 2010), and so the quantity of pollen produced per plant could vary between cultivars.

Cultivar differences in the floral rewards available for insect pollinators suggest that populations of pollinators limited by food availability could be enhanced by growing the most rewarding cultivars, or further diminished by planting those with lower rewards. In this thesis, the production of nectar and pollen are investigated in different cultivars of two crops, which are introduced next: short rotation coppice willow (*Salix* species) and oilseed rape (*Brassica napus* L.).

1.6 Short Rotation Coppice Willow

1.6.1 *Cultivation of willow*

Willows are woody plants belonging to the genus *Salix*. Since Roman times, willows have been fashioned into coracles and baskets (Newsholme 1992; Stott 1992). During the oil crisis of the 1970s interest developed in using willows as a source of sustainable and renewable energy (Karp *et al.* 2011). Willows are now grown as short rotation coppice (SRC) to produce biomass, which is burnt to generate heat or electricity. SRC willow is not widely grown at present. In 2014 the combined area of willow and poplar grown as short rotation coppice in England was 2849 hectares (Defra 2015a). Further planting of willows is likely to depend on the provision of financial incentives (Redman 2014).

Three characteristics make willows particularly useful as a biomass crop: the plants are fast-growing, are easy to propagate, and respond well to coppicing – that is, they regrow multiple stems when cut back (Keoleian & Volk 2005). These useful traits are probably adaptations to environments with high disturbance, as willows are often pioneer species (Keoleian & Volk 2005). Hybridisation between willow species occurs readily, and the genus represents a large pool of genetic diversity that can be used in breeding cultivars (Trybush *et al.* 2008). Many commercial cultivars are hybrids containing *Salix viminalis* (Karp *et al.* 2011).

The crop is grown from stem cuttings of around 20 cm planted in spring at densities of 10 000–20 000 per hectare (Karp *et al.* 2011). After the first year's growth is cut back to just above ground level, multiple stems branch from the stool and grow upwards. The stems are usually harvested at three year intervals, in which time they reach approximately five metres (Karp *et al.* 2011). The plants can remain in place for 20–25 years. As each cultivar is a clone propagated by cuttings, planting mixtures of cultivars is recommended to reduce the susceptibility of entire fields of willow to disease (Hilton 2002).

1.6.2 *Floral biology of willow*

Most cultivars of SRC willow are early flowering, with a blooming period of around three to four weeks between late February and early April, prior to leaf emergence. Willows are dioecious, each individual plant and cultivar producing only male or female flowers (Newsholme 1992). The flowers of willow are borne on catkins on the upper part of their branches. Each catkin is a cluster of several hundred small flowers without petals or sepals, arranged around a central stem.

Within the catkin, the individual flowers have a nectary at their base, and a floral bract that extends from the catkin stem below the flower. In female willows, the ovary adjoins the flower between the nectary and the bract, and it is surmounted by two stigmas (Figure 1.1a and b) (Newsholme 1992). Male willows have stamens in place of the ovary (Figure 1.1c and d), with two stamens per flower found in most willow species (Newsholme 1992). On female catkins, tiny seeds are formed that are dispersed by the wind 3–8 weeks after pollination (Karp *et al.* 2011), while male plants shed their catkins after dehiscence.

Willow flowers have characteristics of plants pollinated by insects and those pollinated by the wind. The production of nectar and floral scent are often adaptations for animal pollination, while the lack of petals and the production of copious small pollen grains before leaves develop are traits associated with wind pollination (Fægri & Van Der Pijl 1979). In fact, nearly all willow species investigated so far are pollinated by both insects and the wind, though the relative importance of each varies by species (Karrenberg *et al.* 2002). Willows attract many flower visitors, including butterflies and moths, honey bees and bumble bees, and range of species of solitary bees, many belonging to the genus *Andrena* (Füssel 2008).

1.6.3 *Nutritional value of willow for pollinators*

The floral resources of willows grown as short rotation coppice are yet to be investigated (Reddersen 2001). However, the value of native willows as early flowering sources of nutrition for wild (Kearns *et al.* 1998) and managed (Dalby 1999; Holmes 1974) pollinators has long been recognised.

Several studies have found that across a range of willow species, nectar from male flowers is richer in sucrose relative to other sugars than nectar from female flowers (Elmqvist *et al.* 1988; Füssel 2008; Katoh *et al.* 1985; Kay 1985; Percival 1961). The protein content of several willow pollens are reported at between 36.8-46.4 % of dry weight (Roulston *et al.* 2000). However, Auclair and Jamieson (1948) found that the essential amino acid tryptophan was absent from protein hydrolysate of willow pollen obtained from honey bee brood comb, and found only a small amount in its free form.

Honey bees (Campana & Moeller 1977) and bumble bees (*B. terrestris*) (Genissel *et al.* 2002; Vanderplanck *et al.* 2014) fed willow pollen produced an intermediate number of offspring in comparison to those fed other unifloral pollens. However, Aupinel *et al.* (2001) found a relatively high rate of oophagy among bumble bee micro-colonies fed *Salix* pollen, and suggested it may be deficient in some important component.

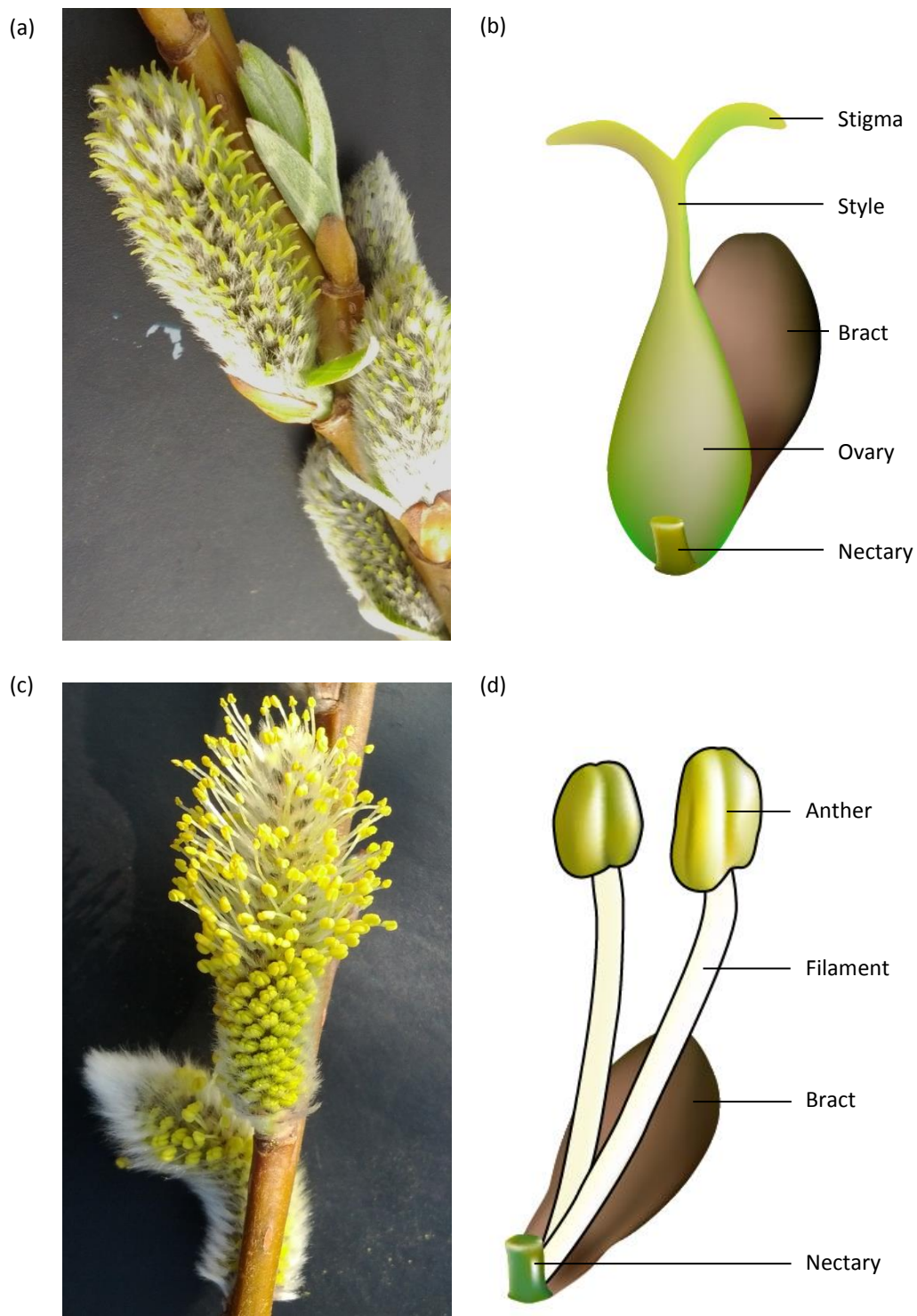


Figure 1.1. The structure of willow flowers. (a) A female catkin from the cultivar Resolution. (b) Drawing showing the form of an individual flower from the female catkin. (c) Male catkin from the cultivar Ulv, containing several hundred individual flowers. (d) Drawing showing a single male flower taken from the catkin.

1.7 Oilseed Rape

1.7.1 *Cultivation of oilseed rape*

Oilseed rape describes four crop species in the *Brassica* genus grown for their oil-rich seeds: *Brassica carinata*, *Brassica juncea*, *Brassica rapa* and *Brassica napus* (Bunting 1986). Cultivation for oil production of *B. carinata* is centred in Ethiopia, *B. juncea* in India and *B. rapa* in northern Europe (Warwick 2011). The *B. napus* subspecies *oleifera* dominates the oilseed rape crop in northern and central Europe (Williams 2010). There are spring-sown and autumn-sown varieties of *B. napus oleifera*, but the majority is autumn-sown (Williams 2010). In this thesis, autumn-sown *B. napus oleifera* is considered, and is referred to hereafter as oilseed rape.

In the UK, oilseed rape is the third most widely planted crop after wheat and barley (Defra 2015a). Oilseed rape is useful for farmers, as it is a break crop that reduces the build-up of weeds and pathogens in fields that are normally devoted to cereals. The seeds are crushed to extract the oil, which is used as vegetable oil for human consumption, as a component of biodiesel or for a range of industrial applications. The remaining seed meal produces a high protein animal feed.

The oilseed rape grown today is the result of an intensive, ongoing breeding effort. Prior to its recent improvement, high levels of erucic acid in the oil were associated with heart defects after consumption, and glucosinolates led to health problems in livestock fed the seed meal (Gupta & Pratap 2007). The vast majority of oilseed now grown produces seeds with no erucic acid and low glucosinolate levels, and is descended from the two cultivars in which these traits originated, namely the cultivars Liho and Bronowski, respectively (Friedt & Snowdon 2009). Due to these extreme selection bottlenecks, modern commercial cultivars of oilseed rape have only a limited degree of genetic diversity (Hasan *et al.* 2006).

Plants of autumn-sown oilseed rape form a rosette of leaves prior to winter (Daniels *et al.* 1986), and require vernalisation: a period of cold weather is needed to enable the production of flowers (Waalén *et al.* 2014). In spring, the main stems of the plants elongate. Flower buds develop, and start to open in April. Flowers open first on the main stem, which continues to extend upwards, with later flowers opening above the earlier ones to form the main raceme of the plant. Side branches with flower buds also extend, producing additional, secondary racemes (see Figure 1.2). The flowering period of the crop lasts approximately four weeks (Nedic *et al.* 2013). Once petals fall from a flower, the fertilised ovules develop into seeds within a pod. Pods grow and ripen, and are ready for harvest in July.

1.7.2 Floral biology of oilseed rape

An oilseed rape flower lives for around three days (Eisikowitch 1981). Each flower bears four yellow petals that open to reveal six stamens (Figure 1.3a). The stamens are arranged around the style, with two pairs of long stamens alternating with two single short stamens. Dehiscence occurs synchronously in the six anthers (Bell & Cresswell 1998). Two forms of nectary are found in oilseed rape, and flowers have a pair of each type. Outer (median) nectaries are located external to the filament bases of the pairs of long stamens, and are relatively exposed (Figure 1.3b). The supply of phloem to outer nectaries is limited (Davis *et al.* 1986) and they secrete only around 5 % of the nectar sugar produced by the flower (Davis *et al.* 1994). Inner (lateral) nectaries are positioned inside of the short stamens (Figure 1.3c). They have enhanced phloem vascularisation, and secrete the vast majority of floral nectar (Davis *et al.* 1986; Davis *et al.* 1994).

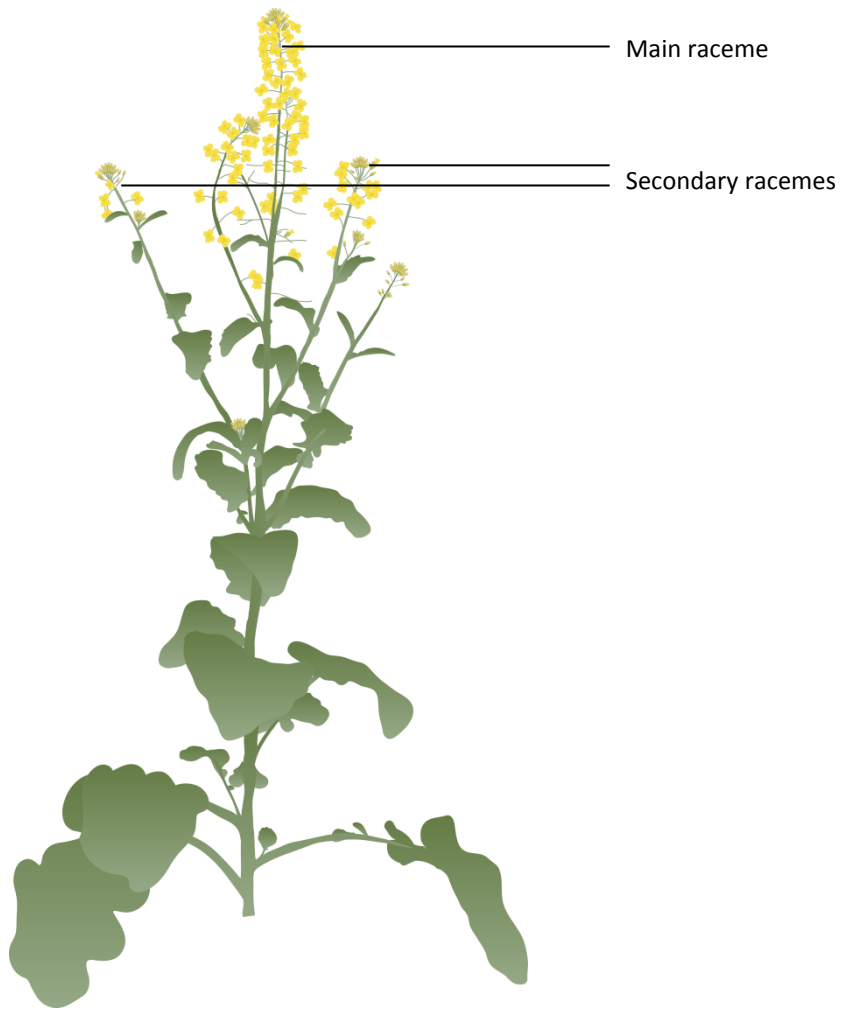


Figure 1.2. The position of the main and secondary racemes on an oilseed rape plant.

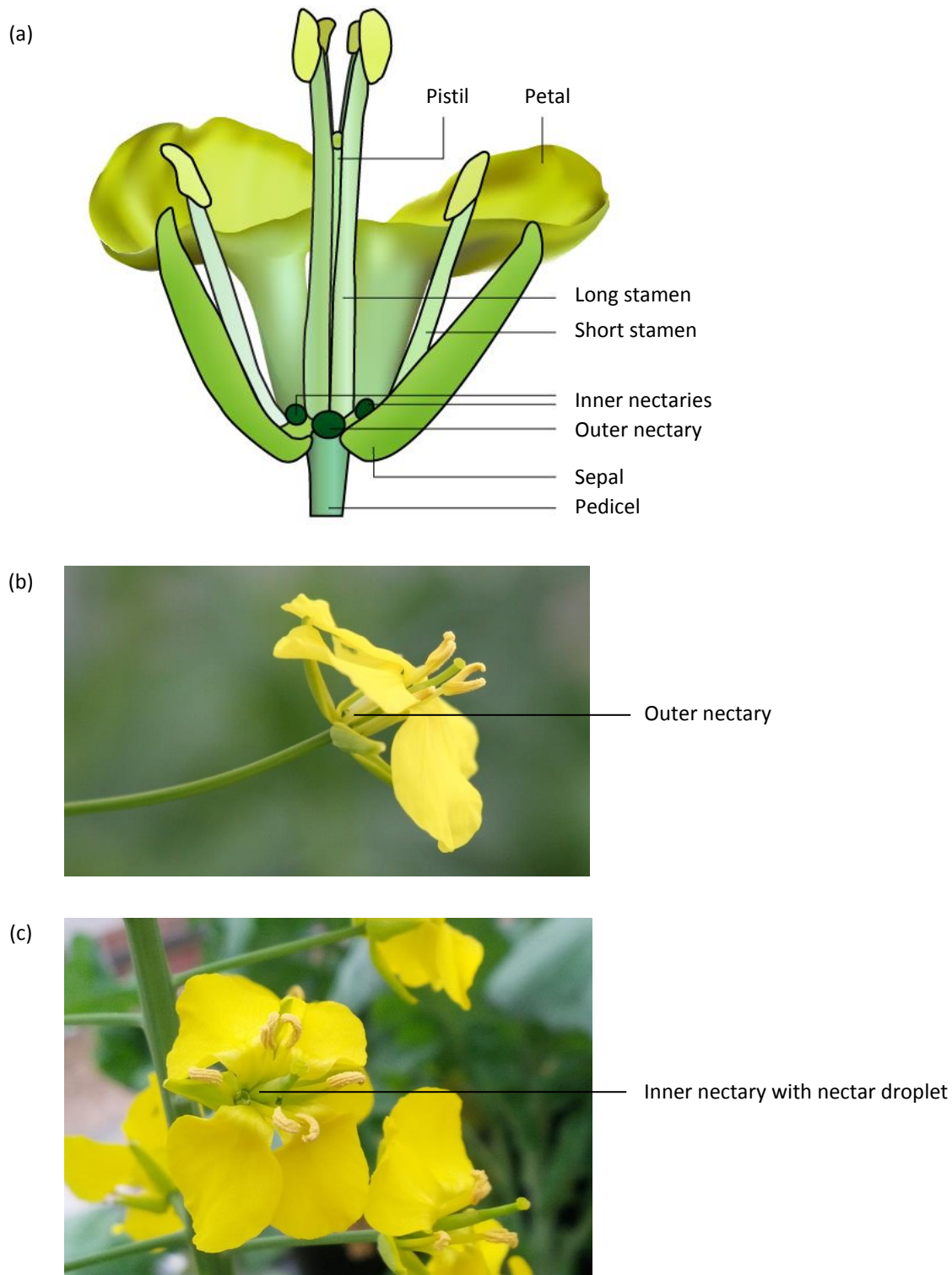


Figure 1.3. The oilseed rape flower. (a) Drawing showing floral parts with a sepal and two petals removed to expose the nectaries. (b) An outer nectary in a relatively young flower. (c) An older flower with an inner nectary and nectar visible.

The flowers of oilseed rape are capable of self-pollination. However, insect visitation is reported to increase seed yields (Hudewenz *et al.* 2014) and oil content (Bommarco *et al.* 2012b). Cultivars of oilseed rape have been shown to differ in the extent to which their seed yields enhanced by insect visitation (Marini *et al.* 2015). Oilseed rape fields are extremely attractive to honey bees (Free 1993). They also attract a range of bumble bees, solitary bees, hoverflies and butterflies (Stanley & Stout 2013).

1.7.3 Nutritional value of oilseed rape for pollinators

Oilseed rape nectar sugars are composed almost exclusively of glucose and fructose, with little, if any, sucrose (Farkas 2008; Kevan *et al.* 1991; Mesquida *et al.* 1988b) (Davis *et al.* 1994; Pernal & Currie 1998; Pierre *et al.* 1999).

Protein levels of 26.0 % (Evans *et al.* 1991) and 31.9 % (Roulston *et al.* 2000) of dry weight are reported in oilseed rape pollen. The ten amino acids essential to honey bee growth according to De Groot (1953) are all found, although methionine is present at only trace levels (Cook *et al.* 2003). Total lipid content is high, with values reported of 25.4 % (Evans *et al.* 1991) and 31.7 % (Evans *et al.* 1987) of dry weight, and includes high levels of myristic, stearic and arachidic acid, relative to other pollens (Manning 2001).

Schmidt *et al.* (1995) found that honey bees fed oilseed rape pollen had a longer median lifespan than those fed pollen of sesame, sunflower or a 15-species mix, and that oilseed rape pollen was preferred above the others. The authors suggest that bees restricted to oilseed rape pollen should receive adequate nutrition, although their assay failed to consider the requirements of brood. Bumble bee (*Bombus terrestris*) micro-colonies fed one of nine pollen mixes produced larvae with a high mean weight, and the greatest larval weight per gram of protein consumed when given a diet containing 95.8 % *Brassica* pollen by volume, suggesting the pollen is highly nutritious (Tasei & Aupinel 2008).

1.8 Objectives

The overall aim of this thesis is to compare cultivars of SRC willow and oilseed rape in the value of their floral resources for the nutrition of pollinators, to test whether cultivars vary, and if so, to identify cultivars that offer the greatest rewards. These rewarding cultivars can then be recommended to growers, and serve as breeding material for plant breeders to create improved cultivars in the future.

The main objectives of this thesis are to:

- Measure the production and quality of nectar and pollen in a range of SRC willow cultivars (Chapter 3).
- Compare attractiveness of SRC willow flowers of different cultivars to insect pollinators and relate this to their floral rewards (Chapter 3 and Chapter 4).
- Evaluate the production of nectar and the quality of pollen in a wide range of oilseed rape cultivars in glasshouse conditions (Chapter 5).
- Quantify the production of nectar and pollen among cultivars of oilseed rape in field conditions (Chapter 6).
- Test whether resource acquisition of bumble bee colonies varies when foraging exclusively on different oilseed rape cultivars (Chapter 6).

Chapter 2. General Materials and Methods

2.1 Introduction

Materials and methods that were used in more than one chapter of this thesis are described here. Included are the techniques used for collecting and analysing nectar and pollen, and details of the bumble bee colonies used in experimental work.

2.2 Collection and Analysis of Nectar

2.2.1 *Sample collection*

Nectar was collected in microcapillary tubes (Drummond Scientific Co., USA) by gently touching them against the surface of nectary glands, as shown in Figure 2.1 (Elmqvist *et al.* 1988; Mesquida *et al.* 1988a). For each sample of willow nectar, a 1 µl microcapillary tube was used to collect nectar, which was taken from several flowers within a single catkin. For each sample of oilseed rape nectar, a 5 µl microcapillary tube was used to collect the nectar from both inner nectaries of multiple flowers on a single plant. Outer nectaries were not used, as they produce only miniscule quantities of nectar (Davis *et al.* 1994).

For both crops, the number of flowers used to comprise the sample was recorded. After collecting nectar, microcapillary tubes were stored in 1.5 ml Eppendorf tubes. These were kept cool in a polystyrene freezer box filled with ice packs or crushed ice, in order to reduce evaporation and microbial growth within the nectar, prior to storing in a freezer set at -20°C as soon as possible after collecting samples.

(a)



(b)



Figure 2.1. Collection of nectar using microcapillary tubes. Nectar removal from (a) Nectaries of male willow flowers. (b) Inner nectaries of an oilseed rape flower.

2.2.2 *Measurement of nectar volumes*

To calculate volumes of nectar in a sample, the length of the column of nectar within microcapillary tubes was first measured using digital callipers under a magnifying lens. The length of this nectar column as a fraction of the length of the microcapillary tubes (32 mm) was multiplied by the total capacity of the microcapillary tube (1 or 5 μl) to find the volume of the sample. Sample volume was divided by the number of flowers from which it was taken to give the mean volume per flower.

2.2.3 *Analysis of nectar sugars*

High performance liquid chromatography (HPLC) was used to assess the sugar content of nectar. Samples were first diluted to one part in 2000 with HPLC grade water (Fisher Scientific, UK). A 10 μl volume of the diluted sample was introduced into the stream of 100 mM NaOH (flow rate 1 ml min⁻¹), and passed through a CarboPac PA100 column (Dionex, USA) to separate the sugars. Sugars were then detected with an ED40 electrochemical detector (Dionex, USA). Chromeleon software (ThermoFisher Scientific, USA) was used to determine sugar concentrations (see Figure 2.2 for an example) by reference to calibrations using sugar standards at 10 ppm that were run on each day of analysis. Sugars were identified by retention time with reference to standards. Glucose, fructose and sucrose were the only sugars detected in the nectar samples.

Concentrations of glucose, fructose and sucrose obtained from HPLC analyses in $\mu\text{mol l}^{-1}$ were multiplied by their molar mass (180.16 for the monosaccharides glucose and fructose, and 342.3 for the disaccharide sucrose) to calculate concentrations in $\mu\text{g g}^{-1}$. These quantities were multiplied by 2000 to account for the dilution made to nectar samples, and the resulting figure divided by 10^6 to give concentrations in $\mu\text{g } \mu\text{l}^{-1}$ (equivalent to g l^{-1}).

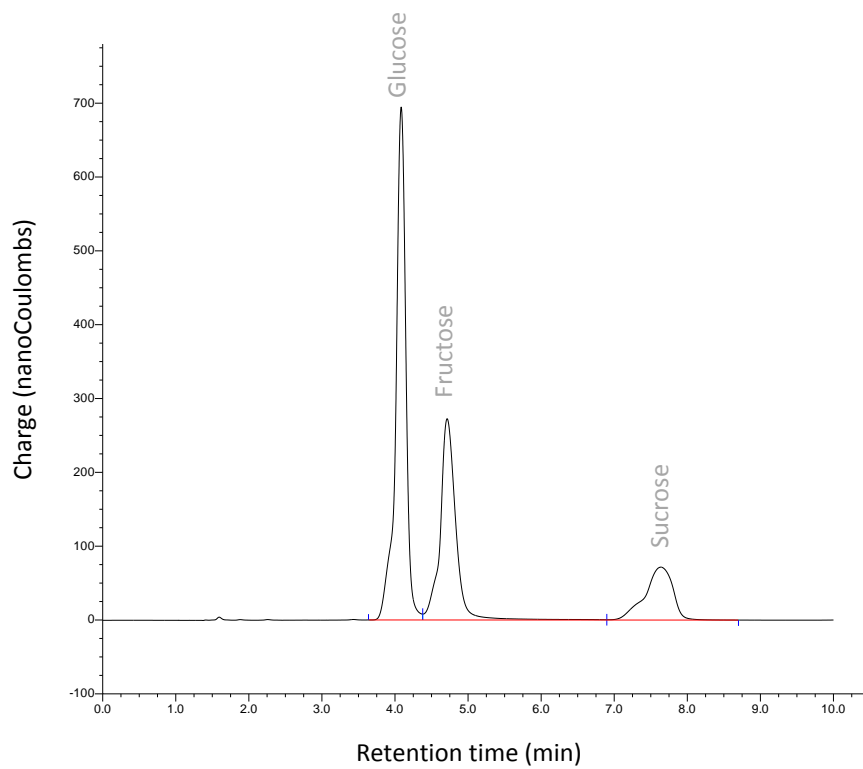


Figure 2.2. A chromatogram produced by HPLC analysis of nectar sugars from a willow cultivar (Ulv).

To calculate total sugar concentrations, the concentrations of the three sugars were summed. The total mass of nectar sugar per flower (in μg) for each sample was determined by multiplying the total sugar concentration (in $\mu\text{g } \mu\text{l}^{-1}$) by its mean volume per flower (in μl).

2.3 Collection and Analysis of Pollen

2.3.1 *Sample collection*

To collect pollen, anthers were brushed gently with the side of a 5 μl microcapillary tube to dislodge dehisced grains. A new microcapillary tube was used for each sample to avoid contamination. Anthers were brushed directly over the openings of glass vials (S. Murray & Co, UK) or polystyrene petri dishes (Sterilin, UK), and pollen was stored in these receptacles, in freezers set at -20 or -80°C .

2.3.2 *Preparation of samples for analysis of free amino acids*

For amino acid analyses, 2 mg of pollen was weighed into a 1.5 ml Eppendorf tube. Pollen from oilseed rape grown in the glasshouse trial discussed in Chapter 5 was weighed directly upon removal from the freezer without drying it first. As an improvement to the method, pollen from willow plants, discussed in Chapters 3, was first dried, before weighing out 2 mg as before. The samples were dried by placing the pollen in open glass vials covered with a filter paper and leaving in an oven set at 60°C for 24 hours. By drying pollen, comparisons could be made between samples in their composition of amino acids without variation due to differences in moisture content. Later, 18 samples of oilseed rape pollen were weighed before and after drying to estimate the amount of moisture lost. They lost 4.1–7.6 % of its original mass when dried, so the effect of differences in moisture content between samples that were not dried is likely to be small.

To remove free amino acids from the surface of pollen grains, 200 µl HPLC grade methanol was added to samples, and vortexed for 1 min, left for 10 mins and then vortexed for a further min (Cook *et al.* 2003). Samples were centrifuged for 30 mins at 148 000 rpm, and the supernatant containing free amino acids was transferred to a clean Eppendorf tube. The methanol was evaporated using a heat block at 70°C and free amino acids were recovered by adding 800 µl HPLC grade water and vortexing for 1 min. Free amino acids from oilseed rape pollen were analysed using HPLC at this concentration, but for willow pollen samples it was necessary to perform a 1:1 dilution with HPLC grade water due to the sensitivity of the detector.

2.3.3 *Preparation of samples for analysis of protein-bound amino acids*

To release protein-bound amino acids from the pollen grains, pellets formed from centrifugation (see above) were placed in a heat block to remove ethanol, and 200 µl of 6M hydrochloric acid was added. Sample tubes were sealed, vortexed briefly, then placed inside a sealed plastic container and irradiated for 20 mins using a domestic microwave oven (900 W, 2450 MHz) inside a fume hood. Excess radiation was absorbed by a beaker filled with 800 ml of tap water, also placed in the microwave oven. Sample tubes were allowed to cool, then opened and placed on a heat block at 70°C under a fume hood to evaporate the acid.

Hydrolysed amino acids were recovered in 800 µl of HPLC grade water, vortexed for 10 mins and left for 1 hour to dissolve. Tubes were centrifuged for 30 mins at 148 000 rpm, and though no pellet was visible, the supernatant was carefully removed and filtered through 0.45 µm syringe-tip filters (Whatman Puradisc 4, nylon) to remove any remaining pollen grains. The formerly protein-bound amino acids required a further dilution of 1:12 for willow pollen and 1:8 for oilseed rape pollen using HPLC grade water, prior to analysis.

2.3.4 *Analysis of pollen free- and protein-bound amino acid content*

High performance liquid chromatography (HPLC) was used to analyse the amino acid solutions prepared from both the free and protein-bound fractions of pollen samples, based on Stabler *et al.* (manuscript in preparation).

Derivatisation reactions were performed immediately prior to injection into the HPLC column to create fluorescent derivatives of the amino acids, which enabled their detection. The reactions were conducted by adding reagents to the sample vial with an autosampler (Ultimate 3000 Autosampler, Dionex, Thermo Fisher Scientific).

The vial initially contained 10 μl of the sample solution. To this, 15 μl was added of an aqueous solution of 7.5 mM *o*-phthaldialdehyde (OPA) and 225 mM 3-mercaptopropionic acid (MPA) in 0.1 M $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$ (adjusted to pH 10.2 with HCl). The reaction progressed for 1 min before adding 10 μl of 96.6 mM 9-fluorenylmethoxycarbonyl chloride (FMOC) in 1M acetonitrile. After a further min, 6 μl of 1 M acetic acid was added to terminate reactions. The reagents were drawn from their containers through a needle which was cleaned between each step with HPLC grade water.

To separate the amino acid derivatives, a 30 μl injection was performed onto a reversed-phase column (dimensions: 150 x 2.1 mm, stationary phase: Accucore RP-MS, particle size: 2.6 μm , pore size: 80 \AA , Thermo Scientific, USA). The temperature of the column was maintained at 40°C. The mobile phase contained an aqueous and an organic solvent maintained at a constant flow rate of 0.5 ml min⁻¹. The aqueous solvent was a solution of 10 mM Na_2HPO_4 , 10 mM $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ and 0.5 mM NaN_3 made with HPLC grade water and adjusted to pH 7.8 with concentrated HCl. The organic solvent contained acetonitrile, methanol and water in the ratio 9:9:2 (v/v/v). A programmed gradient adjusted the proportion of the organic solvent in the mobile phase (by volume) as follows: 10 % for 2 min, rising linearly to 57 % over 12 min, 100 % for 1 min, and

then dropping to 3 % for the remaining 4 min of the elution, based on Stabler *et al.* (manuscript in preparation)

Fluorescence detection was used to record amino acid derivatives in the mobile phase after separation, producing a chromatogram (see Figure 2.3 for an example) (Ultimate 3000 RS Fluorescence Detector, Dionex, Thermo Fisher Scientific, OPA: excitation at 330nm and emission at 450 nm, FMOC: excitation at 266nm and emission at 305nm). The concentrations of amino acids were quantified by automatic integration after calibrating the system with known standards. To ensure accuracy in peak identification despite variation in elution times for the amino acids, reference curves were produced on each day of analysis for all amino acids using calibration standards (Sigma-Aldrich, USA). The Chromeleon software package (Thermo Fisher Scientific) was used both to control the autosampler and solvent pumps, and to compile data. The software calculates solute concentrations (nmol ml⁻¹) based on pre-programmed reference curves for each amino acid at a range of dilutions.

All samples were run twice and their mean concentrations taken. To convert the concentrations of amino acids from nmol ml⁻¹ into ng per gram of pollen, the following calculation was performed:

$$\frac{c \times m \times v}{w} \text{ ng per gram}$$

where c is the concentration of the sample in nmol ml⁻¹, m is the molecular weight of the amino acid, v is the volume of water in which the amino acids were recovered in ml, and w is the weight of the pollen in g.

The technique described was able to quantify the concentrations of 20 amino acids, listed in Table 2.1. Concentrations of the amino acid proline could not be determined accurately, as the fluorescence of its derivatives was indistinguishable from background noise. The concentrations of tryptophan in

protein-bound amino acids could not be ascertained, as it is destroyed by the hydrolysis process.

2.4 Bumble Bee Colonies

The bumble bee *Bombus terrestris audax* (Harris) was used as a model pollinator to investigate responses to various cultivars of mass-flowering crops. The subspecies is native to Britain (Goulson 2010) but several *B. terrestris* subspecies exist in Europe, and have been transported widely outside of their native ranges (Goulson 2010). Colonies were obtained from Biobest (Belgium), and were supplied in a plastic box measuring 29 x 23 x 13 cm, contained within a cardboard outer layer. Nests arrived with a single queen, 20–30 workers, and brood of all stages (Figure 2.4) and were supplied with a source of sugar syrup, to which they were allowed *ad libitum* access before experiments began. Upon arrival and daily thereafter, nests were given 2 g of honey bee collected pollen (C. Wynne Jones, UK). A brief summary of the natural lifecycle of the species is given below.

2.4.1 Bumble bee lifecycle

Like other bumble bees, mated queens of *Bombus terrestris* L. are usually the only individuals of the species to survive winter. Typically, during the autumn after mating, queens excavate a cavity in the soil in which they overwinter in a quiescent state. When they emerge in February or March, queens search for a suitable nest site, often using abandoned rodent burrows and they must find flowers to obtain nectar and pollen (Goulson 2003).

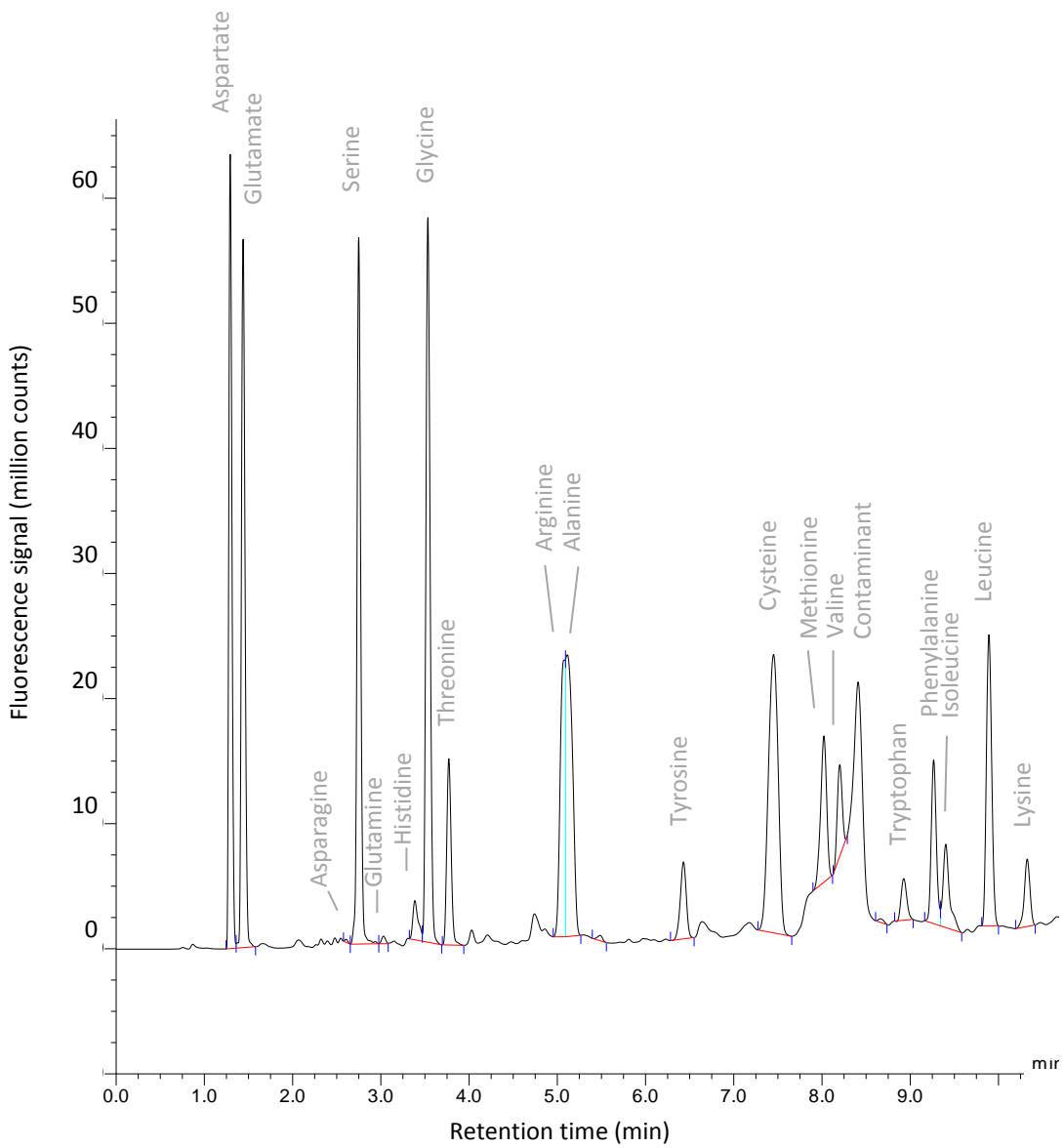


Figure 2.3. A chromatogram produced by HPLC analysis of protein-bound amino acids from an oilseed rape cultivar (Cash).

Table 2.1. Molecular weights of the amino acids quantified by HPLC. Amino acids are listed in the order of elution from the column.

Amino acid	Standard abbreviation	Molecular weight
Aspartic acid	Asp	133.1
Glutamic acid	Glu	147.13
Asparagine	Asn	132.12
Serine	Ser	105.09
Glutamine	Gln	146.14
Histidine	His	155.15
Glycine	Gly	75.07
Threonine	Thr	119.12
Arginine	Arg	174.2
Alanine	Ala	89.09
Tyrosine	Tyr	181.19
Cysteine	Cys	121.16
Valine	Val	117.15
Methionine	Met	149.21
Tryptophan	Trp	204.23
Phenylalanine	Phe	165.19
Isoleucine	Ile	131.17
Leucine	Leu	131.17
Lysine	Lys	146.19



Figure 2.4. A *Bombus terrestris audax* colony obtained for use in experiments.

The queen lays a clutch of eggs in the nest, which, upon hatching as larvae, feed on the pollen and nectar that she has collected. When not foraging, the queen incubates the developing brood. The larval stage lasts around two weeks, followed by a pupation of another two weeks or so, after which adult workers emerge (Alford 1975). The initial and subsequent offspring of the queen take over foraging while she remains in the nest laying more eggs. Some of the workers also remain inside the nest to tend the brood. If the colony reaches a sufficient size, it begins to produce new queens and males instead of workers between April and August (Goulson 2003), which then leave the colony to find mates. The rest of the colony becomes lethargic and eventually perishes.

2.5 Statistical analyses

All statistical analyses were performed using GenStat for Windows (2013, 16th Edition, VSN International Ltd, Hemel Hempstead, UK). Results were regarded as statistically significant where $P < 0.05$.

Chapter 3. Floral Resources for Nutrition of Insect Pollinators Varies in Cultivars of Short Rotation Coppice Willow (*Salix* species)

3.1 Abstract

Willows (*Salix* species), grown as a bioenergy crop, flower in early spring and provide a source of nutrition that may be valuable in the diets of insect pollinators. Like native willows, the crop produces flowers borne on catkins. Cultivars of the crop differ considerably in the appearance of their catkins, and in the numbers that adorn their plants. However, the amount and composition of the floral nectar and pollen that different willow cultivars produce has not previously been compared. Here, an existing field trial was used to measure the floral resources offered in one season by six willow cultivars: Loden, Olof, Ulv, Terra Nova, Stott-10 and Resolution.

The cultivars varied in the number of catkins on a plant, the number of flowers within catkins, and the volume, concentration, total sugar mass and proportion of sucrose of the nectar within their flowers. Differences were not seen in the mass of pollen produced per catkin by three male cultivars, or in the proportions of essential amino acids found in their pollens. The cultivars Ulv and Olof produced large quantities of both nectar and pollen, and insect pollinators were more frequently seen foraging on their flowers than on those of the other cultivars.

Overall, cultivars showed large differences in the value of their resources for, and use by, insect pollinators. The findings underscore the importance of considering the floral resources provided when selecting cultivars of this long-lived crop to maximise the benefits for pollinators while producing bioenergy.

3.2 Introduction

Willows are woody, deciduous plants that comprise the genus *Salix*, with approximately 400 species (Newsholme 1992). For centuries willows have been grown to supply rods of their light, flexible wood for basket-making (Stott 1992). Since the 1970s, willow has also been grown to create biomass as a source of renewable energy (Karp *et al.* 2011). Using willows instead of fossil fuels to generate heat and power reduces emissions of greenhouse gases (Heller *et al.* 2003), so the crop could help to mitigate climate change and its impacts on global biodiversity (Parmesan 2006; Thomas *et al.* 2004). However, to understand the implications on biodiversity of sourcing biomass energy from willows, the direct, local effects of its planting must also be considered (Dauber *et al.* 2010).

For biomass production, willows are grown in short rotation coppice (SRC) cycles. Plants are usually coppiced – cut back near to ground level – every three years (Karp *et al.* 2011), and new shoots grow from the stumps, known as ‘stools’. Willow plantations have low fertilizer and pesticide requirements, and as a perennial crop, the need for annual tillage is eliminated. In consequence, willows are expected to support greater biodiversity than arable crops (Powlson *et al.* 2005). The available studies show that biodiversity in SRC willow exceeds that in arable fields among birds, butterflies, earthworms and other invertebrates (Dauber *et al.* 2010; Haughton *et al.* In press).

Incorporating areas of SRC willow into farming landscapes may also enhance the abundance and diversity of insect pollinators (Reddersen 2001). Native willows such as sallow (*S. caprea* L.) are cherished by beekeepers as some of the earliest forage plants to bloom, and they attract a range of insects (Dalby 1999; Holmes 1974). The crop also flowers in early spring, with a hundred or more small flowers clustered in catkins that are produced on the upper part of willow stems (Figure 1.1). Breeding programmes to create new willow varieties specifically for biomass production have been underway in Sweden since the

1980s and in the UK since the 1990s, resulting in the release of many cultivars. However, the value of different cultivars of SRC willow as sources of nutrition for insect pollinators has not been considered.

There is considerable diversity among cultivars of the crop, which could affect the nutritional resources they offer to insect pollinators. A major reason that cultivars differ is that willows are dioecious, with male and female flowers borne on different plants. Individuals of a given cultivar are propagated from stem cuttings, so all shares the same genotype, and in consequence, each cultivar produces male or female flowers exclusively. While flowers of both sexes secrete nectar, only male flowers offer pollen, which is a requirement in the diets of some insect pollinators (Alford 1975). Among males, differences between cultivars in the quality and quantity of pollen produced have not yet been investigated. However, several previous studies have found that the nectar of male willow flowers has a greater proportion of sucrose relative to the other nectar sugars than the nectar of female willows (Elmqvist *et al.* 1988; Katoh *et al.* 1985; Kay 1985; Percival 1961).

Diversity among willow cultivars is also high because breeding programmes have purposely exploited a wide range of willow species in their crosses (Lindgaard & Barker 1997). As clones, individuals of a single cultivar planted together are susceptible to pathogen attack, but planting in mixtures reduces the risk, provided cultivars have a sufficient genetic distance (Karp *et al.* 2011). Willow species vary in the extent to which they rely for pollination on insects or the wind (Karrenberg *et al.* 2002). Species with the highest rates of pollination by the wind may produce less nectar, so that differences in the parent species of willow cultivars might influence the value of their floral rewards for insect pollinators.

The following hypotheses are tested here:

1. Cultivars vary in the number of catkins they produce, and the number of flowers within their catkins.
2. There are qualitative and quantitative differences between the pollen produced by the male cultivars.
3. The nectar produced by flowers of the cultivars differs in volume, the concentration of combined sugars, and total amount of sugar, while the percentage of nectar sugar comprised by sucrose varies between male and female cultivars.
4. Insect pollinators show differences in the number of visits they make to the various cultivars, and make more visits to the cultivars that offer greater rewards.

3.3 Methods:

3.3.1 *Plant material*

Six commercial cultivars of short rotation coppice willow were investigated. Cultivars were chosen that would come into flower at a similar time and represent a range of species and both sexes. The cultivars selected are presented in Table 3.1.

Table 3.1. Short rotation coppice willow cultivars compared in a field trial. '*S. vim.*' is *Salix viminalis* L.; '*S. sch.*' is *S. schwerinii* E. Wolf.

Cultivar name	Species	Sex
Loden	<i>S. dasyclados</i> Wimm.	Male
Olof	<i>S. vim.</i> x (<i>S. vim.</i> x <i>S. sch.</i>)	Male
Ulv	<i>S. vim.</i>	Male
Terra Nova	(<i>S. vim.</i> x <i>S. triandra</i> L.) x <i>S. miyabeana</i> Seemen	Female
Stott-10	<i>S. dasyclados</i> x <i>S. vim.</i>	Female
Resolution	((<i>S. vim.</i> x <i>S. vim.</i>) x (<i>S. vim.</i> x <i>S. sch.</i>)) x (<i>S. vim.</i> x (<i>S. vim.</i> x <i>S. sch.</i>))	Female

The plants were part of an existing field trial of 38 cultivars established in 2004 at Rothamsted Research in Hertfordshire, UK. The trial was arranged in a complete randomised block design with three blocks, shown in Figure 3.1a. Each block consisted of two adjacent rows of 19 plots of cultivars, laid out in an east-west direction, and surrounded by a row of willow plants from six different lines that act as a guard.

Plots measured 4.8 m x 2.5 m, and contained 24 stools of a single willow cultivar, arranged in four rows of six plants. Two central rows were planted 0.8 m apart with an outer guard row on each side, as shown in Figure 3.1b. Plants were coppiced in early 2005, 2007, 2010 and 2013. This study was performed in March 2014, a year after plants were last coppiced.

3.3.2 *The number of flowers produced by each cultivar*

In February 2014, prior to flowering, catkin buds were counted on all stems of 5-8 plants in each plot of the six cultivars. The eight plants in the central double-row, excluding the two at each end, were examined. Plants in the outer rows and the ends of the double-row were not used, in order to avoid the potential effect that the cultivar in the neighbouring plot might have on the number of catkin buds per plant (Langton 1990). In plots of cultivars Loden, Olof and Ulv, the catkins on all central eight plants were counted. In plots of Resolution, Stott-10 and Terra Nova, one stem had been removed from three of the central plants, so the catkins on the five intact plants only were counted. Catkin buds are easily distinguished from leaf buds as the latter remain much smaller and flatter than the former until flowering has started (Figure 3.2).

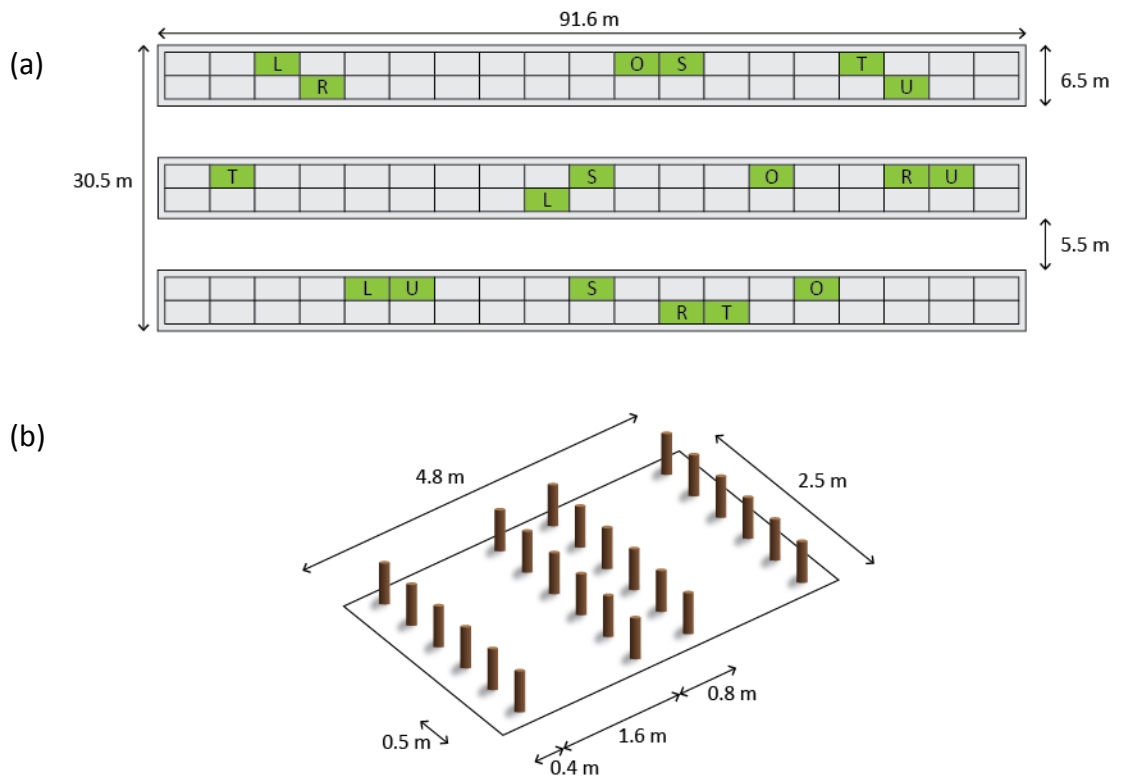


Figure 3.1. Design of short rotation coppice willow trial. (a) Three replicate blocks each arranged as two adjacent rows of 19 plots, with 24 plants in each plot. The plots of cultivars compared in this study are shaded green. The letters refer to the first initial of each cultivar name (L: Lodén, O: Olof, U: Ulv, T: Terra Nova, S: Stott-10, R: Resolution). (b) The layout of willow plants within each plot in four rows of six plants. Only the base of plants is shown.



Figure 3.2. Photo of stems of willow cultivar Stott-10, showing different forms of catkin and leaf buds. Scale bar shows 1 cm.

Once flowering had begun, catkins were removed from three different plants within each plot of the six cultivars, selected at random. The catkins were stored individually in plastic pots in a freezer set at -20°C . The number of individual flowers per catkin was later counted by removing flowers from the central stem of the catkin using forceps under a magnifying lens.

3.3.3 *Quantity and quality of pollen produced*

Pollen was collected from the three male cultivars. Willow pollen is easily dislodged from dehiscent anthers by the wind, so is difficult to collect in the field. To determine the mass of pollen produced by each catkin, three stems were collected from each plot of the three male cultivars, each stem bearing a catkin on which the stamen filaments were beginning to elongate but prior to dehiscence. Any surplus catkins were removed from the stem to leave just one. Stems were arranged as shown in Figure 3.3. Each stem was placed over a plastic petri dish (90 cm, Sterilin, UK) and held by inserting the cut end into a block of floral foam (Oasis, USA). To allow vascular flow to the catkins, the foam was placed in a dish of water prior to inserting stems, which was topped up daily. A cylinder formed from paper was placed around each petri dish in order to prevent air movement around the catkin from removing pollen. A small part of the cylinder was cut away to fit over the stem. Paper was also used to cover the cylinder and prevent dust accumulating in the petri dish. Pollen was collected in the petri dishes for three days, after which time any that remained on the anthers was dislodged by brushing gently with the side of a clean microcapillary tube. Petri dishes were weighed before and after collecting pollen to give the mass produced by each catkin. After weighing, petri dishes were stored in a freezer set at -20°C .

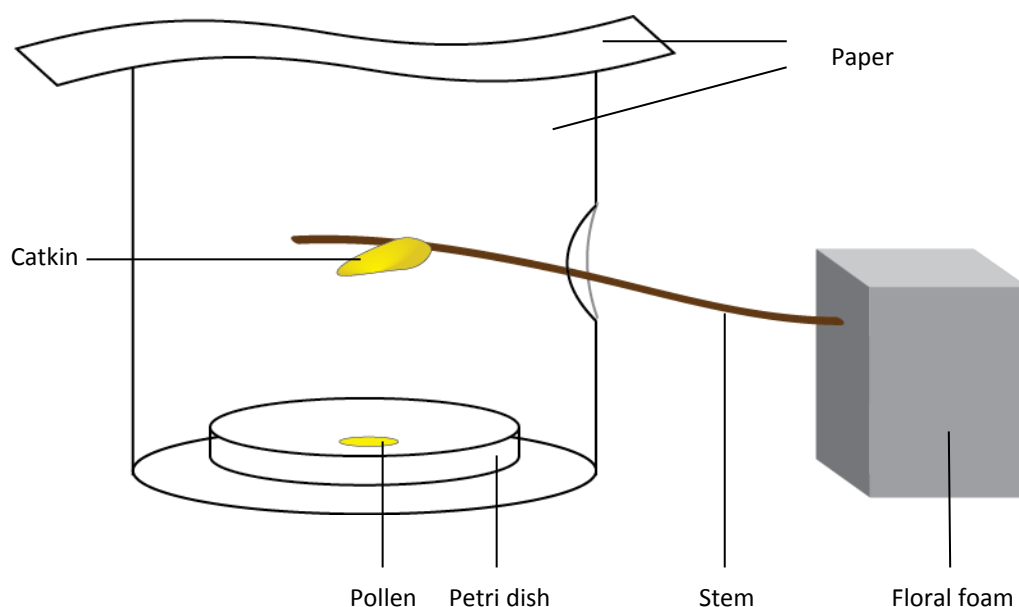


Figure 3.3. Arrangement of stems for collection of willow pollen in laboratory.

The three pollen samples from within each plot were bulked together to give one sample per cultivar from each of the three blocks. The composition of free and protein-bound amino acids in pollen samples was calculated using HPLC analysis as described in Chapter 2. The concentrations of amino acids that were found in the free and protein-bound form were combined for each sample to show the total concentrations that obtained by their consumption. Total concentrations were obtained of the amino acids found to be essential and non-essential in the honey bee by De Groot (1953). The cultivars were then compared according to the proportion of the mass of amino acids in their pollen that are essential.

3.3.4 *Nectar volume, sugar concentration and sugar mass*

Nectar secretion was measured in flowers over 24 hours. On 13th March 2014, all six cultivars were in flower. Five plants were chosen at random from the eight plants in the centre of plots of the six cultivars. On each of these a stem bearing unopened catkins was selected. A fine-mesh bag was tied around the stem with string in order to exclude insects from the catkins. The next day the stems were inspected and one catkin that had newly opened flowers was marked by

affixing coloured insulation tape to the stem adjacent to it. Flowers tended to open on one side of the catkin before the other rather than in concert, so a red marker pen was used to encircle a part of the catkin on which flowers were just opening. On male catkins these flowers had extended stamens and anthers still rounded at the initial stage of dehiscence, while flowers on female catkins had stigmas visibly forked from the style to form a 'Y' shape (see Figure 1.1). After marking the catkins and the regions containing new flowers, the mesh bags were replaced over the stem to cover the catkins. Within each block, the six plots were visited in each of five rounds to mark a new catkin, before moving on to the next block.

Nectar was removed from the flowers 24 hours after marking the new flowers. Plots were visited in the same order in which flowers were marked, with five rounds of nectar collection in each block in turn. On each plant, a 1 μ l microcapillary tube was used to take nectar, as described in Chapter 2, from 8-10 (mean: 9.6) of the flowers within the region previously marked. The nectar was taken from plants in the first block from 9:30-12:15, in the second block from 12:40-15:10, and in the third block from 15:20-17:30.

The volume of nectar secreted per flower over 24 hours was calculated as described in Chapter 2. To analyse nectar sugar composition, nectar was diluted using HPLC grade water. A rubber bulb was used to expel the nectar from the microcapillary tube into the Eppendorf tube in which it had been stored (1.5 ml capacity). For each nectar sample, the Eppendorf tube had been filled with the volume of the water necessary to make a 1:1999 solution. After expelling the nectar, the rubber bulb was manipulated to allow the solution to refill the microcapillary tube several times, removing all of the nectar. Nectar sugars were analysed using HPLC, and their concentrations and totals per flower were calculated.

3.3.5 *Insect visitation to willow catkins*

Insects foraging on catkins of the 12 plants in the central two rows of plots were recorded. The number of foraging insects was quantified using 'snapshot counts' following Garbuzov and Ratnieks (2014). Observations lasting approximately 10 seconds were performed by walking slowly alongside plants while looking at all catkins slightly ahead and noting numbers and the species of any insects that were foraging on them.

As flowering did not begin at the same time in all six cultivars, separate comparisons were made of three early (Resolution, Olof and Terra Nova) and three late (Loden, Stott-10 and Ulv) flowering cultivars. Observations on the early flowering catkins were made twice per day on four consecutive days (8th-11th March 2014). The later flowering catkins were observed three times per day over three consecutive days (18th-20th March 2014).

3.3.6 *Statistical analyses*

Analysis of variance was used to test for differences between the cultivars with respect to: the number of flowers on their catkins; the number of catkins produced per plant; the mass of pollen released per catkin; the proportion of their pollen amino acids that were essential amino acids; and the nectar volume, concentration, and mass of sugar per flower. Prior to analysis, the mass of pollen per catkin and nectar volumes were log-transformed (base 10), while the number of catkins per plant, the number of flowers per catkin and the mass of nectar sugar per flower were square-root transformed. The logit transformation was applied to the proportion of essential amino acids, where $\text{logit}(p) = \log_e(p / (100-p))$. Transformations were applied to reduce heteroscedasticity. Analyses were performed with a random model representing the nested structure of plots within blocks. In comparisons of nectar properties an extra nested level represented the rounds of nectar collection within plots.

Due to the large diversity between SRC willow cultivars and the small number of cultivars of each sex included in the trial, statistical comparisons between the sexes in specific traits measured here are likely to be misleading, and were generally avoided. However, as discussed above, a difference in the proportion of sucrose in the nectar sugar content has been found between males and females in a wide range of willow species. For this reason, nested contrasts were used to compare the proportion of nectar sugars comprised of sucrose within and between male and female willow cultivars, using analysis of variance. The percentages of sucrose among the nectar sugars were transformed using the logit transformation.

The mean numbers of flowers per catkin was calculated for each plot, and was multiplied by the mean mass of nectar sugar per flower from samples within that plot to give an estimate of total mass of nectar sugar per catkin. The estimate of total mass of nectar sugar per catkin was then multiplied by the mean number of catkins per plant in the same plot, to estimate the total mass of nectar sugar produced per plant. Analysis of variance was again used to compare the cultivars by their mass of nectar sugar per catkin, and by their mass of nectar sugar per plant. Both were transformed by taking logarithms (base 10) before analysis.

The total numbers of insects foraging on plots of each cultivar in all observation periods were calculated, and separate comparisons were made among the three early-flowering the three later-flowering cultivars using Chi-squared tests.

3.4 Results

3.4.1 *The number of flowers produced by each cultivar*

All plants that were inspected flowered, but the number of catkins each produced ranged from 1–683. There was a difference according to cultivar in the number of catkins per plant ($F_{5,10}=69.19$, $n=3$, average $SED=0.99$, $P<0.001$). The cultivar Ulv had the most catkins per plant (mean=344.5), and the cultivar

Loden produced the fewest (mean=14.3). Both male and female cultivars showed a wide range in their production of catkins (Table 3.2).

The dissected willow catkins contained 100-550 individual flowers. There was a difference in the number of flowers on catkins according to cultivar ($F_{5,10}=96.48$, $n=3$, $SED=0.57$, $P<0.001$). The catkins of cultivar Loden had the most flowers (mean=491.1), while those of cultivar Resolution had the fewest (mean=146.4). The male catkins had over twice as many flowers as female catkins (means of 385.6 and 168.7, respectively) (Table 3.2).

3.4.2 Quantity and quality of pollen produced

The male cultivars produced a mean of 13.6 mg pollen per catkin. A difference was not found between the three male cultivars in the mass of pollen produced per catkin ($F_{2,4}=1.11$, $n=3$, $SED=0.07$, $P=0.414$; Table 3.2).

From dried willow pollen samples, a mean of 4.0 mg per g of free amino acids and 52.8 mg per g of protein-bound amino acids were measured (0.4 % and 5.3 %, respectively). This gave a total of 56.8 mg per g of amino acids from the samples (5.7 %). The mean concentrations of essential and non-essential amino acids in pollens of the three male cultivars combined from the free and protein bound fractions are shown in Table 3.3 and Table 3.4. Of the amino acids in the pollen samples, essential amino acids comprised a mean of 44.7 % of the total. The cultivars did not vary in the proportion of their amino acids that are essential, by mass ($F_{2,6}=3.07$, $n=3$, $P=0.245$).

Table 3.2. Mean catkins per plant, flowers per catkin and pollen per catkin of six cultivars of short rotation coppice willow (n=3). Back-transformed means shown in parentheses.

Measurement	Cultivar (sex)						SED	F	P
	Loden (♂)	Olof (♂)	Ulv (♂)	Terra Nova (♀)	Stott-10 (♀)	Resolution (♀)			
Catkins per plant	3.5 (13)	10.5 (111)	18.2 (330)	13.0 (168)	7.9 (63)	4.9 (24)	1.00 [†]	F _{5,10} =69.19	<0.001
Flowers per catkin	22.2 (491)	17.8 (315)	18.6 (347)	14.0 (197)	12.7 (161)	12.1 (145)	0.57	F _{5,10} =96.48	<0.001
Pollen per catkin (mg)	-1.8 (14.6)	-1.9 (11.7)	-1.9 (12.4)	-	-	-	0.07	F _{2,4} =1.11	0.414

[†]Average SED

Table 3.3. Mean concentrations of essential amino acids (free and protein-bound) in pollen from three cultivars of short rotation coppice willow (n=3).

Cultivar	Amino acid concentration (mg per g of pollen)									
	Arginine	Histidine	Lysine	Tryptophan	Phenylalanine	Methionine	Threonine	Leucine	Isoleucine	Valine
Olof	6.8	1.5	6.0	0.0	2.0	2.3	1.7	3.4	0.8	1.3
Ulv	6.9	1.4	8.2	0.0	1.8	2.2	1.6	3.6	0.6	1.0
Loden	5.3	1.5	7.1	0.0	2.0	2.2	1.9	4.6	0.9	1.4

Table 3.4. Mean concentrations of non-essential amino acids (free and protein-bound) in pollen from three cultivars of short rotation coppice willow (n=3).

Cultivar	Amino acid concentration (mg per g of pollen)									
	Aspartate	Glutamate	Asparagine	Serine	Glutamine	Glycine	Alanine	Tyrosine	Cysteine	
Olof	6.1	8.1	0.0	4.4	0.0	4.9	2.6	1.5	3.5	
Ulv	6.7	8.0	0.0	4.5	0.0	4.5	2.2	1.4	4.3	
Loden	8.8	8.2	0.0	4.9	0.0	4.6	3.8	1.6	4.8	

3.4.3 *Nectar volume, sugar concentration and sugar mass*

Both male and female flowers produced nectar. Each willow flower produces a miniscule amount of nectar, with a mean volume of 0.03 μl found in the flowers sampled. The nectar volume per flower varied between cultivars ($F_{5,10}=6.47$, $n=3$, $P<0.006$; Figure 3.4a). The cultivar that produced the greatest mean volume of nectar per flower was Ulv (mean=0.06 μl), while the cultivar with the smallest volume of nectar per flower was Terra Nova (mean=0.01 μl) There was a range among both the male and female cultivars in the mean nectar volumes of nectar secreted.

The mean concentration of combined nectar sugars from the willows was 488 g/l (48.8 % w/w). There was a difference between cultivars in total nectar sugar concentration ($F_{5,10}=4.54$, $n=3$, $\text{SED}=23.31$, $P<0.02$; Figure 3.4b). The most concentrated nectar was found in cultivar Resolution (mean=531 g/l), and the least concentrated in cultivar Ulv (mean=439 g/l).

The composition of nectar sugars is represented in Figure 3.5. Sucrose comprised a greater proportion of the sugars in the nectar of the male cultivars than in female cultivars ($F_{1,10}=854.4$, $n=3$, $P<0.001$; mean (and back-transformed) sucrose percentage among nectar sugars in male cultivars: -1.1 (24.3 %), and female cultivars: -2.2 (9.7 %)). There was also a difference within the male cultivars ($F_{2,10}=39.4$, $n=3$, $P<0.001$) and within the female cultivars ($F_{2,10}=112.5$, $n=3$, $P<0.001$), shown in Figure 3.6.

Willow flowers had an average of 13 μg each of total nectar sugars. The mass of nectar sugar per flower also varied between cultivars ($F_{5,10}=7.17$, $n=3$, $P<0.004$; Figure 3.4c). The cultivar with the most sugar per flower was Ulv (mean=24.8 μg), while cultivar Terra Nova had the least (mean=5.1 μg).

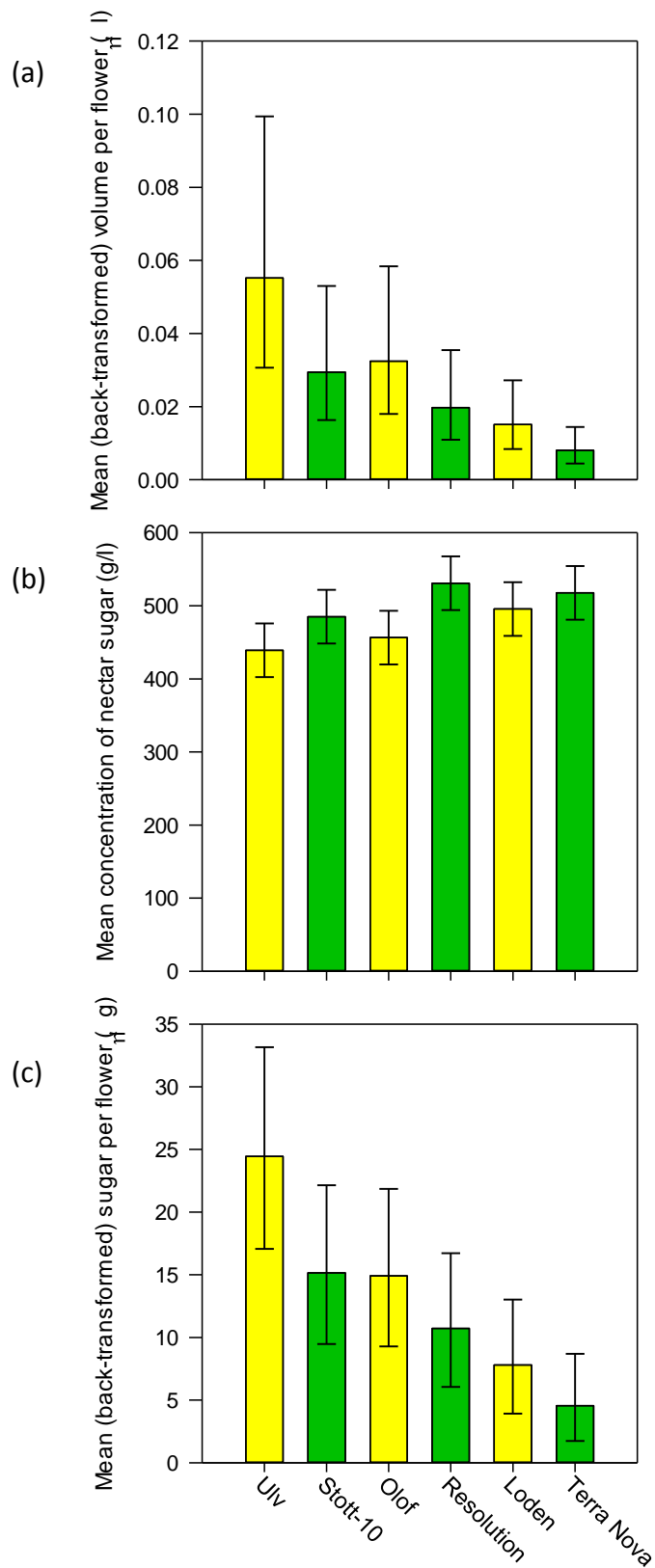


Figure 3.4. Mean nectar (a) volumes per flower, (b) sugar concentration and (c) mass of nectar sugar per flower of six cultivars of short rotation coppice willow (n=3). Error bars show 95 % confidence intervals. Male and female cultivars are represented by yellow and green bars, respectively.

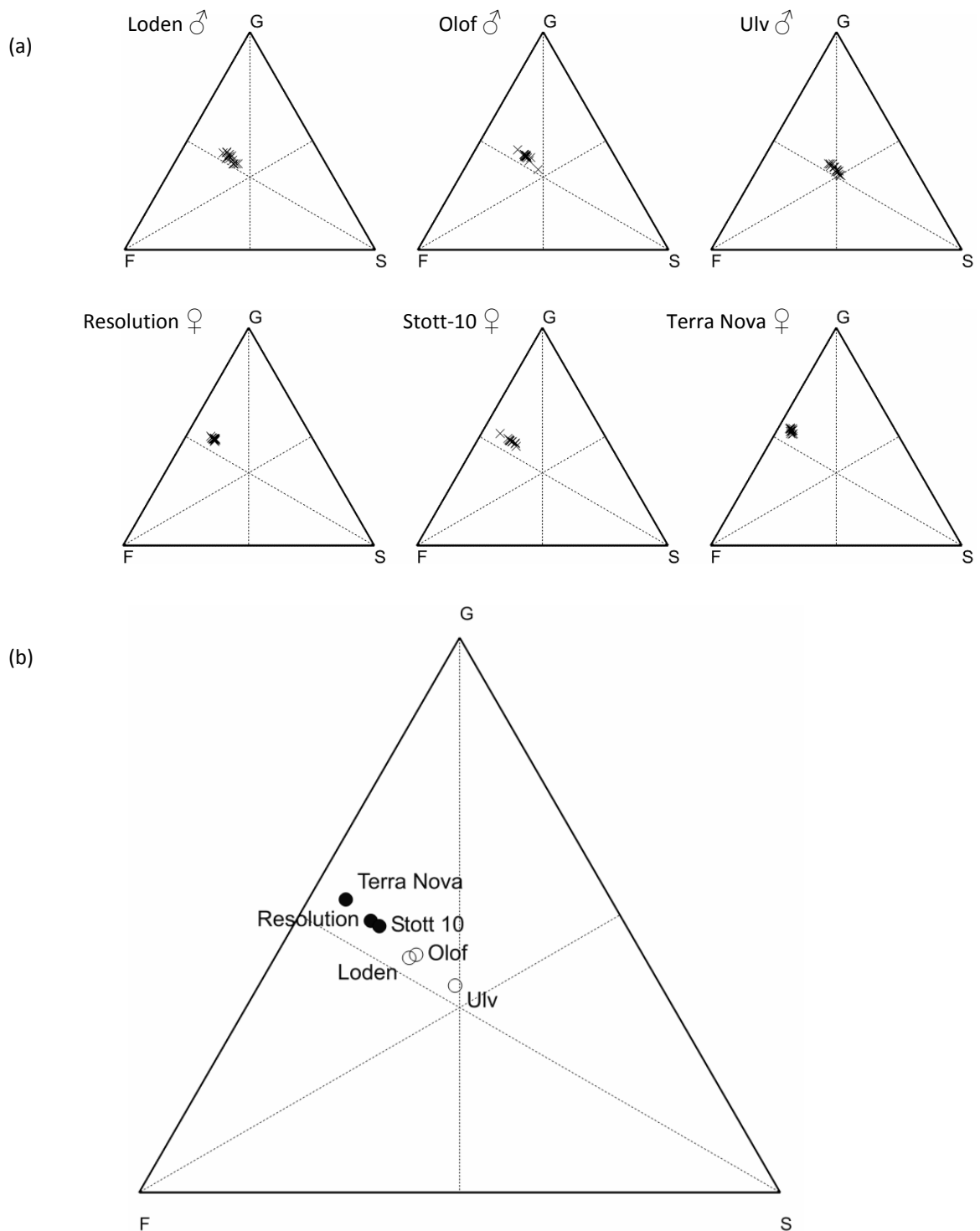


Figure 3.5. Ternary diagrams showing the proportions by mass of the sugars glucose (G), fructose (F) and sucrose (S) in nectar from six cultivars of short rotation coppice willow. Each corner represents 100 % of the sugar denoted by the label, while any point on the opposite edge of the diagram represents 0 % of that sugar. (a) Proportions of sugars in nectar. (b) Mean proportions sugars in nectar of each cultivar. Filled circles show female cultivars; open circles show male cultivars.

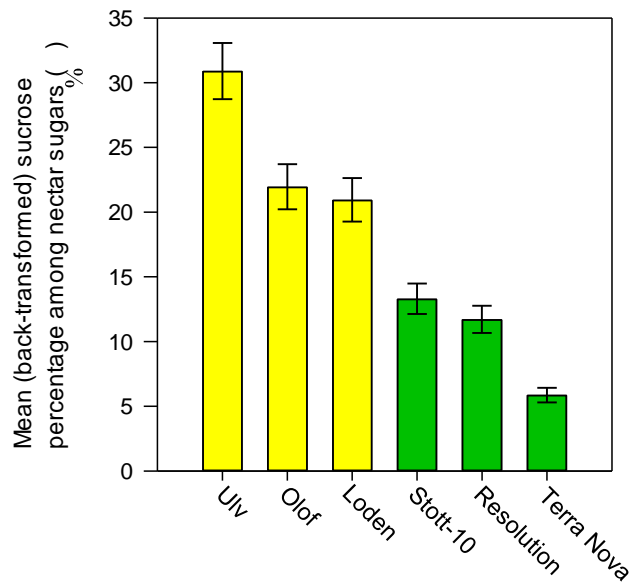


Figure 3.6. Mean percentage of nectar sugars comprised by sucrose in six cultivars of short rotation coppice willow (n=3). Error bars show 95 % confidence intervals. Male and female cultivars are represented by yellow and green bars, respectively.

The estimated mass of nectar sugar per catkin differed between cultivars ($F_{5,10}=19.35$, $n=3$, $P<0.001$; Figure 3.7). The most productive cultivar was Ulv, and the least productive was Terra Nova.

The per plant estimates of the mass of nectar sugar also differed between the cultivars ($F_{5,10}=68.54$, $n=3$, $P<0.001$; Figure 3.8). Plants with the greatest nectar production were again those of cultivar Ulv. However, the least productive plants were those of cultivar Resolution.

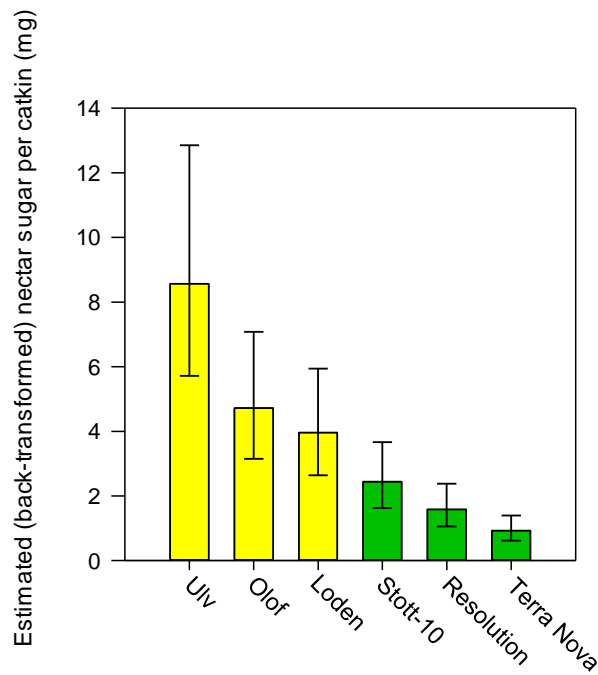


Figure 3.7. The estimated production of nectar sugar per catkin by six cultivars of short rotation coppice willow (n=3). Error bars show 95 % confidence intervals. Male and female cultivars are represented by yellow and green bars, respectively.

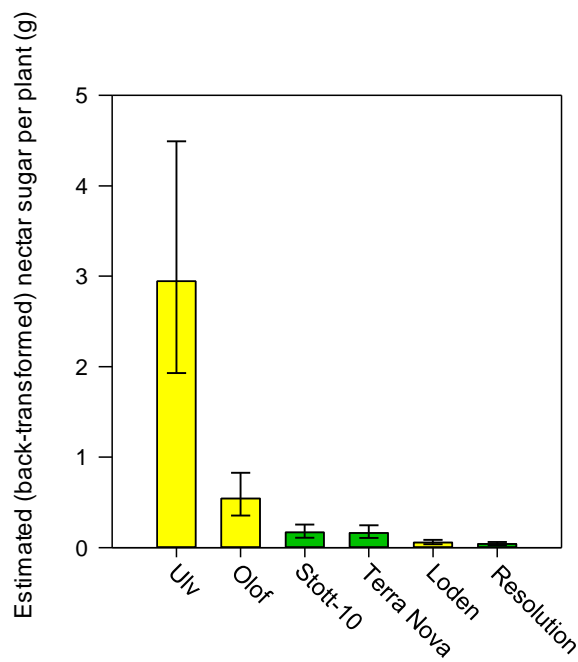


Figure 3.8. The estimated production of nectar sugar per plant by six cultivars of short rotation coppice willow (n=3). Error bars show 95 % confidence intervals. Male and female cultivars are represented by yellow and green bars, respectively.

3.4.4 *Insect pollinator visitation to willow catkins*

A range of insects were seen foraging on willows during the study, including honey bees (*Apis mellifera*), bumble bee queens (*Bombus terrestris/lucorum*, *B. lapidarius* L. and *B. bohemicus* Seidl.) solitary bees (*Anthophora plumipes* Pallas and *Andrena haemorrhoa* F.), butterflies (*Aglais io* L., *Aglais urticae* L. and *Polygonia c-album* L.) and bee flies (*Bombylius major* L.). In addition to these insects, a foraging blue tit (*Cyanistes caeruleus* L.) was also observed. However, during the snapshot observations, only honey bees and *Bombus terrestris/lucorum* queens were seen.

There were 72 observations made of plots with the three earlier flowering cultivars, during which 41 insects were seen foraging. Over half of these (26) were honey bees, and the remainder (15) bumble bees. There were 81 observations of plots with later flowering cultivars, during which 58 insects were seen foraging. In contrast to the earlier flowering cultivars, the majority of these (30) were bumble bees and the rest (19) were honey bees. Among both the earlier and later flowering willows, there were differences in the total numbers of insects foraging on the different cultivars ($X^2=20.83$, 2 d.f., $P<0.001$, and $X^2=78.03$, 2 d.f., $P<0.001$, respectively) (Figure 3.9). Cultivars Ulv and Olof had the highest numbers of visits, with means of 1.9 and 1.1 insects per observation, respectively. Meanwhile cultivars Terra Nova and Stott-10 had the fewest, with means of means of 0.2 and 0.07 insects seen per observation, respectively.

Sampling effort was not consistent between the early and late flowering cultivars, so correlations between insect visitation to willow cultivars and their flowering traits were not analysed statistically. However, for exploratory purposes, plots are shown in Figure 3.10 of the total numbers of insect visits to the various cultivars with their nectar sugar concentrations, number of catkins per plant, mass of sugar per flower, estimated mass of sugar per catkin.

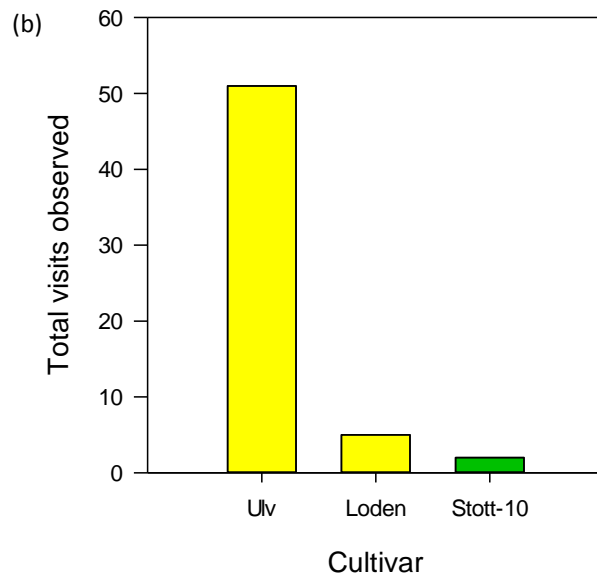
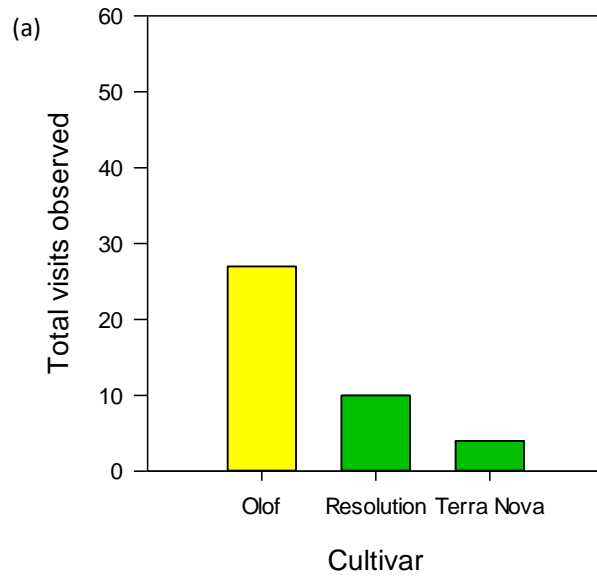


Figure 3.9. Total visits by insect pollinators observed on willow cultivars flowering in (a) early March 2014 and (b) mid-March 2014. Male and female cultivars are represented by yellow and green bars, respectively.

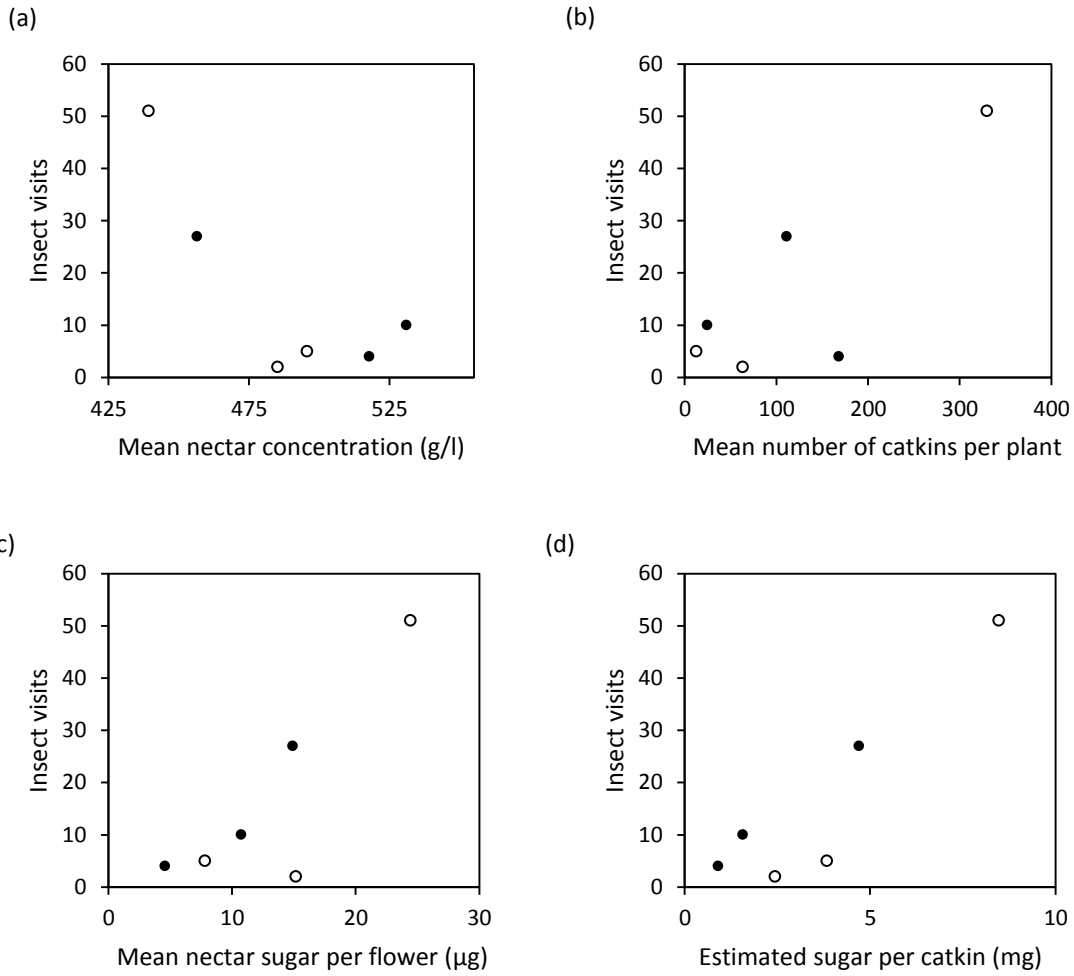


Figure 3.10. The number of insect visits observed to six cultivars of short rotation coppice willow in comparison with (a) the mean concentration of total nectar sugars, (b) the mean number of catkins per plant, (c) the mean mass of nectar sugar produced per flower, and (d) the estimated mass of sugar produced per catkin. Black circles show the early-flowering cultivars. White circles show the late-flowering cultivars.

3.5 Discussion

3.5.1 *The number of flowers produced by each cultivar*

The total resource provided for insect pollinators by each willow cultivar in a given year is a function of three values: the number of catkins produced by each plant, the number of flowers within those catkins, and the production of resources per flower. There was a 30-fold difference in the mean number of catkins produced per plant among the cultivars in the present study. The difference observed in the numbers of catkins produced per plant is most likely due to genetic differences between the cultivars. These differences may reflect contrasting reproductive strategies of the species prior to domestication. For example, cultivars that produce fewer catkins may be bred from species or populations that tended to delay allocating resources to reproductive rather than vegetative growth until a larger size is attained, while the ancestors of the more prolific catkin producers would reach sexual maturity more quickly (Fritz *et al.* 2006). Alternatively, the cultivars producing the most catkins may have evolved the trait due to a deficit of pollinators in their ancestral populations, leading either to competition and selection for more attractive floral displays, or a greater reliance on wind-pollination.

The plants considered here had a year's growth since they were last coppiced, but the numbers of catkins on plants of short rotation coppice willow increase in successive years after harvesting (Reddersen 2001). In consequence, the quantity of resources for insect pollinators from each plot will increase with each year following coppicing. However, the rank-order of cultivars in the numbers of catkins per plant in the second and third years post-harvest is unlikely to differ greatly from that found during their first year in the present study, as the stems bear catkins either sparsely or densely to an extent that appears characteristic of each cultivar (personal observation).

The numbers of flowers produced per catkin by male cultivars exceeded the numbers in female cultivars. While the cultivars selected in this study may not be representative of willows generally, the same trend has been observed in *Salix myrsinifolia-phylicifolia* (Elmqvist *et al.* 1988) and both *S. caprea* and *S. cinerea* (Kay 1985). Upon pollination, female willow flowers produce fruit containing seeds, requiring further investment. In contrast, after releasing pollen the male flowers die and catkins are dropped. Assuming that male and female plants have equal resources to invest in reproduction, the disparity in the cost per flower between the sexes could explain the greater number of flowers found in male catkins. Bateman (1948) explained that competition between males to fertilise the ovules on female flowers could explain the excessive production of pollen by males, and perhaps therefore the greater numbers of flowers per catkin.

3.5.2 *Quantity and quality of pollen produced*

Female cultivars do not produce pollen, which must be considered when assessing the nutritional value of their flowers for insect pollinators. The male cultivars did not show variation in the mass of pollen produced per catkin. However, the large difference in the number of catkins per plant between some of the male cultivars indicates that this strongly influences the expected amount of pollen produced by a plant of a given cultivar. In preliminary work it was found that collecting pollen from plants in the field was difficult, as some could be seen to be lost by the wind, so part of the total mass of pollen per catkin measured here will not be available to pollinators.

The total mass of amino acids recovered from willow pollen accounted for less than 6 % of its dry weight. Estimates of the protein content of willow pollen obtained by quantifying its total nitrogen are in the range of 37–46 % (Roulston *et al.* 2000), so hydrolysis of pollen proteins was presumably incomplete.

Assuming that the amino acids detected are representative of all those present

in willow pollen, the cultivars had similar in pollen amino acid concentrations. Cultivars also had similar proportions of essential amino acids in their amino acid complement. The finding is unsurprising, as pollen amino acid composition appears to be conserved between related species (Weiner *et al.* 2010). Among essential amino acids, tryptophan alone was not detected. Tryptophan in the protein fraction of pollen does not survive digestion with hydrochloric acids applied to hydrolyse amino acids, so the method used cannot determine its presence in protein-bound amino acids. However, it was not detected among the free-amino acids. The absence of tryptophan has previously been noted in willow pollen (Auclair & Jamieson 1948). Genissel *et al.* (2002) found that micro-colonies consisting of three adult workers of the bumble bees *Bombus terrestris* were able to rear an average of 8.2 male offspring when fed with willow pollen. However, the pollen was obtained from honey bee corbicular loads and separated by hand, so it may have been contaminated with tryptophan, or alternatively, the amino acid requirements of bumble bees may differ from those calculated for the honey bee by De Groot (1953). The mean proportion of essential amino acids in willow pollen quantified was 44.7 %, which compares favourably with that of some other early-flowering plants, for instance 35.0 % in dandelion (*Taraxacum officinale*) and 37.1 % in blackthorn (*Prunus spinosa*) (Weiner *et al.* 2010).

3.5.3 Nectar volume, sugar concentration and sugar mass

There were marked differences in all the measures of nectar collected from the six cultivars. The volume and mass of sugar per flower, and the concentration of nectar varied between the cultivars. The cultivars that produced the smallest volumes tended to have more concentrated nectar, which is likely to be because smaller droplets have a proportionally greater surface area from which evaporation can occur at the relatively exposed nectaries, as described by (Nicolson 2002).

Part of the difference between the cultivars in the mass of nectar sugar they produced per flower could be due to genetic differences between the cultivars. These differences may have arisen because of variation in the need to attract insects for pollination in ancestral populations. Willow species that evolved in communities with high densities of conspecific plants may achieve ample pollination from the action of the wind, and benefit from expending less energy in nectar sugar (Hesse & Pannell 2011). However, the observed difference between cultivars in the nectar produced may also be a result of variation in flowering phenology. Although cultivars that flowered at a similar time were selected, some of the cultivars were approaching the end of flowering when nectar was collected. The plants at the end of their flowering period may invest less in producing nectar in their flowers than those just coming into flower (Pierre *et al.* 1999).

The contrast in sugar composition of nectar between male and female cultivars is intriguing, and consistent with earlier studies. Like the present study, Percival (1961) found that nectar from male *Salix caprea* and *S. atrocinerea* plants was higher in sucrose than that from the females. The pattern has also been observed in *Salix myrsinifolia-phylicifolia* (Elmqvist *et al.* 1988) and a range of other willow species (Füssel *et al.* 2007; Katoh *et al.* 1985). The reason for the difference is unclear. Füssel (2008) speculates that the sugar composition of nectar produced by the females is preferred by pollinators over that of the males, following a study on preferences in the honey bee by Wykes (1952a), and that females are thus able to compete for insect visits with the pollen that male flowers provide. It seems likely that if their nectar sugar compositions were identical to males, female flowers would be at a disadvantage relative to the males in their attractiveness to insects, as pollen provided by males is not only a nutritional reward but also gives visual (e.g. Lunau 2000) and olfactory (e.g. Dobson 1987) cues to foragers. However, it is unclear why male flowers should

have evolved to produce less attractive nectar, since any male that matched the nectar sugar composition of females would attract foragers away from its rivals.

It is in the interests of male and female plants that foragers should alternate between visiting plants of each sex. The ideal system would appear to be one where the sexes provide two different resources and an insect visitor is motivated to obtain a balance between them. Worker bumble bees (*Bombus terrestris*) can moderate consumption of sources of protein and carbohydrate in order to meet an intake target (Stabler *et al.* 2015), but it seems unlikely that early spring pollinators would seek to obtain a balance of sugars. The nectar of males and females may vary in the composition of other nutritional compounds besides sugars with the effect of encouraging insects to seek both, for example in concentrations of amino acids that alter pH.

When the mass of nectar sugar per flower produced by each cultivar is scaled up to estimate total nectar reward provided per catkin and per plant (Figure 3.5 and 3.6), an extreme difference is apparent. The estimated mass of nectar sugar per plant in the cultivar Ulv is 15 times the mean of the other five cultivars.

3.5.4 *Insect pollinator visitation to willow catkins*

The numbers of insects observed foraging on plots of the various cultivars showed a difference that may suggest preferences for particular cultivars among them. The cultivars Olof and Ulv were visited far more than the other plots among the earlier and later flowering willows, respectively. Both are male cultivars that produce many catkins and copious nectar as well as pollen. The female cultivars received the fewest insect visitors in both time periods. As discussed above, the yellow anthers of the male catkins may serve as additional attractants to foragers visually and by their scent, as well as offering the additional reward of pollen (Dotterl *et al.* 2005). Foragers may also prefer the composition of nectar sugars offered by male willows.

The number of insect visitors appears to decrease with increasing nectar sugar concentration (Figure 3.10a), which is unsurprising, as the highest concentrations were found in flowers with small volumes of nectar, as previously discussed. The number of catkins per plant appears to have some influence on the number of insect visits (Figure 3.10b). The relationship could exist because plants with the most catkins have greater rewards on offer that induce insects to stay for a longer time (Dreisig 2012), and because the size of their floral display provides a signal that can be detected from a greater distance (Higginson *et al.* 2006). Although the catkins on a plant do not flower simultaneously, plants with higher numbers of catkins overall will tend to have a greater number in flower at a given time. The number of visits seems to increase with the mean nectar sugar per plant and per catkin (Figure 3.10c and Figure 3.10d), indicating that the insects spend more time foraging on the more rewarding flowers.

The proportion of bumble bees recorded on the later flowering cultivars was almost double that of the earlier flowering willows. As the two sets of observations were made about a week apart, the greater abundance of bumble bees is most likely due to the extra number of queens that emerged from hibernation and began to forage over that time. The recording of insect foraging on plots of willows in ‘snapshots’ proved to be a simple and quick way to compare the use of different cultivars. However, when using this approach, recording additional information – such as the species of bumble bees, or whether the insects were collecting pollen or nectar – becomes increasingly difficult in plots with many foragers. The additional time necessary to collect such information in highly visited plots is likely to bias the numbers counted, and so was not attempted here. Willow catkins tend to be located near the tops of their stems, making counts and identification, of insects difficult, and would be increasingly so in their second and third years after harvest when the plants are taller.

3.5.5 *Conclusions*

The ideal way to test the nutritional value to insect pollinator of cultivars of short rotation coppice willow would be to measure the reproductive success of a range of pollinators living in landscapes that differ only in the willow cultivar available. As such an experiment presents considerable practical difficulties, the study reported here instead attempted to determine which cultivars are most valuable to pollinators by measuring the resources that various cultivars provide and observing their use by insects.

The approach used here, however, is itself not without difficulties in the assessment of cultivars that differ in their flowering phenology. When comparing the nectar secretion rate of cultivars, sampling each cultivar on a different day is likely to yield inconsistent results, as the ambient conditions can cause large fluctuations in values observed (Shuel 1952). When comparing pollinator visitation to plots, observing each cultivar on a different day has further complications besides the effect of variation in the weather on plants and insects: there will be more foragers later in spring as insects emerge from hibernation, and the range of alternative nectar and pollen sources that the insects may visit will change over time. The cultivars in the present study had overlapping flowering periods, allowing comparison of their nectar production on a single day, with the caveat that they were at different points in their flowering period when sampled. Insect visits were recorded in two groups, and direct comparisons between the cultivars in different groups are not possible.

Among the cultivars assessed, Ulv produced large numbers of catkins, which provided both pollen and nectar, and the greatest number of foraging insects was seen on its flowers. The lack of pollen produced by the female cultivars is a major limitation to their value as sources of nutrition for insect pollinators.

Further, none of the female cultivars in the study combined large amounts of nectar sugar per flower with high numbers of catkins per plant. In spite of the limitations, the approach taken in the present study found that cultivars of SRC

willow varied both in the resources offered to insect pollinators, and in the insects' use of them. To compare a range of cultivars with flowering periods that diverge more, an alternative approach would be needed, perhaps using an indoor environment in which conditions can be controlled. Such an approach is used in the following chapter of this thesis.

Chapter 4. Preferences of bumble bees (*Bombus terrestris audax*) between two cultivars of short rotation coppice willow (*Salix*).

4.1 Abstract

Plants use visual and olfactory cues to attract pollinators to their flowers. The attractiveness of the flowers of two cultivars of short rotation coppice (SRC) willow to inexperienced bumble bees was compared in a bioassay. Individual naïve bumble bee workers were released in a flight-arena, and allowed to choose between a flowering catkin of the willow cultivars Tordis and Terra Nova. The cultivar initially chosen by bees was recorded, along with the duration of their first visits to both cultivars, and the number of visits and total time spent on each cultivar in a fifteen minute period. Bumble bees showed an innate preference for flowers of the cultivar Tordis in their initial choice and in the proportion of visits that were made to it. No difference was observed between the cultivars in the duration of the first visits or total time spent on catkins. The cultivar Tordis had flowers that were more attractive to inexperienced bumble bees than Terra Nova, which may be because of differences in their visual or olfactory characteristics.

4.2 Introduction

Animal-pollinated plants can manipulate the behaviour of potential flower visitors with a range of floral characteristics. Flowers may produce colours and scents that attract visitors likely to serve as pollinators (Fægri & Van Der Pijl 1979). Flowers may also have features that make them inconspicuous or repellent to visitors likely to consume their rewards without pollinating them (Kessler *et al.* 2008). Inexperienced flower visitors must detect visual and olfactory cues from flowers while foraging, and use innate rules to determine which to inspect. Innate preferences for particular colours and odours have been demonstrated among flower-visiting insects (Chen *et al.* 2009; Giurfa *et al.*

1995). In consequence, naïve flower visitors are likely to show innate preferences between flowers that differ widely in their appearance or scent.

Willow planted as short rotation coppice (SRC) produces flowers that offer nutrition for insect pollinators like bees (Reddersen 2001). Growers are recommended to plant a mix of SRC willow cultivars (Karp *et al.* 2011), and previous work has shown that cultivars of SRC willow are diverse, in both floral morphology and the quantities of nectar sugar they produce per flower (see Chapter 3).

When queens bumble bees emerge in spring, they must find sources of nectar quickly to avoid perishing (Goulson 2003). If cultivars vary in their attractiveness to inexperienced bumble bees, those that are the most appealing may be more easily found by queens looking for their first flowers in spring, and be detected from greater distances. After gaining experience, bees learn to associate particular features with floral rewards (Menzel 1993). Although foraging queen bumble bees are not naïve flower visitors, and may choose flowers on the basis of experiences prior to winter, they may still show preferences for flowers that display features more attractive or salient to inexperienced bees. The rate at which bees visit willow catkins varies between cultivars in field settings, as shown previously in this thesis (Chapter 3). However, determining whether the catkins of willow cultivars vary in their attractiveness to inexperienced bees cannot be determined in a field trial, because the previous experiences of these bees cannot be controlled.

In the present study, two cultivars that contrast in appearance were directly compared in a choice test under controlled conditions to test the hypothesis that they differed in their attractiveness to inexperienced bumble bee workers.

4.3 Methods

4.3.1 Preparation of willow flowers

Stems of two female willow cultivars, Tordis and Terra Nova, were cut from plants in the National Willow Collection, maintained at Rothamsted Research (Hertfordshire, UK). The cultivars were chosen as their catkins differ visually (Figure 4.1). Cuttings were made in January 2012, while buds were dormant, from plants that had been coppiced approximately 12 months previously. The stems, around 1.2 m in length, were sealed within a plastic bag to prevent desiccation, and stored in a freezer set at -4°C .

In September 2012, stems were removed from the freezer and trimmed to measure 80 cm from their topmost catkin bud to the base. One in four catkin buds was removed to enable the remaining buds to develop fully (W. Macalpine, pers. comm.). Following Macalpine *et al.* (2010), stems were placed upright in containers filled with 5 litres of water. Containers were covered by a black, plastic sheet with small holes through which stems protruded. By excluding light from the base of the stems, the sheeting encouraged the development of root tissue necessary for the uptake of water and the

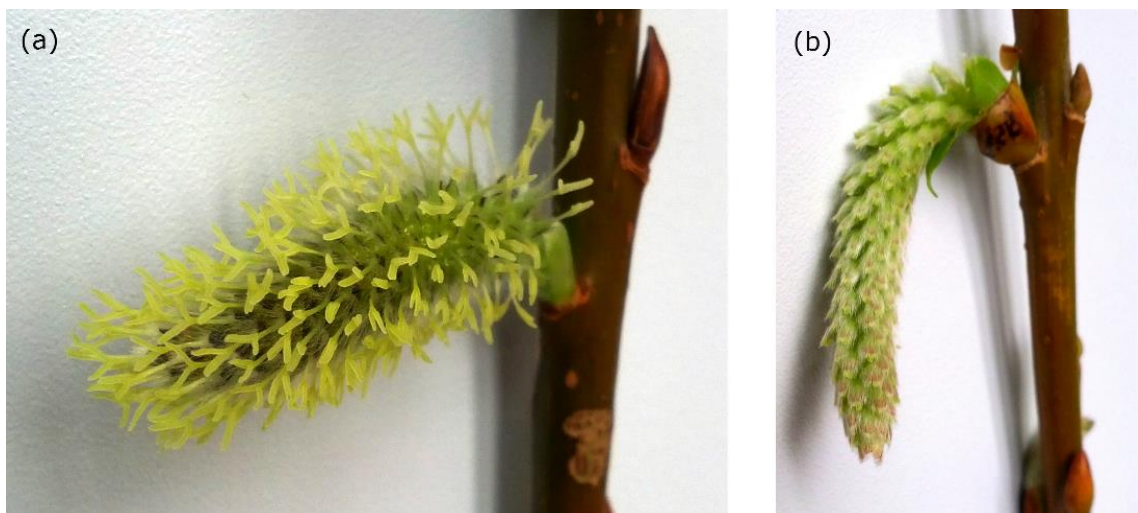


Figure 4.1. Catkins from two SRC willow cultivars. (a) Tordis and (b) Terra Nova

development of catkins. Stems were left in an unheated laboratory, receiving natural light from a north-facing window for 8-11 days, during which time catkins developed. To prevent bacterial blockage of the vascular tissue, 5 mm was cut from the base of the stems daily until roots had developed. Since prior testing showed that stems of Terra Nova required more time to develop catkins than those of Tordis, Terra Nova stems were removed from cold storage three days before those of Tordis to ensure that flowering coincided.

4.3.2 *Flight room and bumble bee training*

Tests were made in an indoor flight arena enclosed by plastic cloth netting within a windowless room (see Poppy & Williams 1999). The room was lit with fluorescent, white and UV lights with high flicker frequencies (>300 Hz). The arena had a square floor (3m x 3m) and a height of 1.7 m at the edges, curving up to 2.3 m in the centre.

A colony of *Bombus terrestris audax* (Biobest, Belgium) was kept in the centre of the arena. The colony was fed daily with 2 g of honey bee-collected pollen (C. Wynne Jones, UK), and allowed *ad libitum* access to the sugar solution with which it was supplied. An alternative entrance to the nest was created to provide control over the departure of bees. A clear plastic sample tube with a screw cap (Sterilin, 30 ml), cut at its base, was inserted through a hole made in the top of the nest box. A single bee could then be let out by unscrewing and replacing the cap. Two days before trials with willow flowers began, access to sugar solution was removed to motivate foraging. The day before testing their responses to willow flowers, bumble bees were allowed to visit two feeders positioned in line with the nest on two opposing sides, each at a distance of 40 cm from the nest. Feeders were constructed from a 1.5 ml Eppendorf tube affixed to a bamboo cane. The tubes were filled with a mixture of honey, sugar and water in the ratio 1:3:6 (by weight). This period of training was conducted for bees to perform orientation flights within the arena (Seguin & Plowright

2008), and to increase motivation to search for food within the arena during the trials.

4.3.3 *Comparing visits to willow flowers*

To test the responses of bumble bees to the two willow cultivars, one stem of each of the cultivars Tordis and Terra Nova was placed 40 cm from the colony, on opposing sides. Stems were placed in 1 litre conical flasks. Surplus catkins on the stems were concealed by fastening paper around them with staples, leaving just one exposed, and extra pieces of paper were fastened to the stem with fewer catkins in order to control for any influence the paper may have on bee behaviour.

Immediately prior to each test, a sample of nectar was collected and measured from 10 flowers on the exposed catkin of a stem of each cultivar for comparison. The nectar volumes were measured and samples were frozen for subsequent analysis of their sugar contents using HPLC (see Chapter 2 for details).

A bumble bee worker was released from the nest and allowed to forage on the two stems, observed by a video camera (Sony Handycam DCR) mounted above. Fifteen mins after leaving the nest, the bumble bee was removed from the arena, without returning it to the colony. The test was performed on 18 individuals over two consecutive days. Stems were replaced between trials, and the side of the colony on which stems of the two cultivars were positioned was randomised in each trial. In total, there were 10 trials in which stems of the cultivar Tordis were on one side of the colony, and eight trials in which they were on the other side.

Videos were analysed to record the number and duration of catkin visits. Visits were defined as any occasion on which a bee fully alighted on a catkin. Four features of the bees' behaviour were compared: the cultivar that bees first chose to visit, the duration of their first visits to both cultivars, the proportion of all

catkin visits that were made to each cultivar, and the proportion of the time spent on catkins that was accorded to each cultivar.

4.3.4 *Statistical comparisons*

The nectar volume, concentration, mass of sugar, and the percentage of sucrose among the sugars (by weight) were compared using ANOVA, with each trial as an experimental block. Percentages of sucrose were transformed using the logit transformation prior to analysis.

The first choice visits of the bees were analysed by Fisher's Exact test, to account for any bias that may arise due to differences between the position of stems on the left or right of the nest. The test was used to compare the total number of first choice visits by bees to the stem positioned on the left or right of the nest when the cultivar Terra Nova was positioned on the left or right. The time spent by bumble bees on their first visit to each cultivar was compared using ANOVA, with each trial as an experimental block. The proportion of the total number of visits made to the cultivars was compared using a one-sample binomial test, with a null-hypothesis of 0.5. The time that bees spent on each of the two cultivars within trials is not independent, so the proportion of time was calculated, using an offset of one to allow for a zero value, as follows:

$$\log_e \left(\frac{\text{time on Terra Nova} + 1}{\text{time on Tordis} + 1} \right)$$

Proportions were then compared with zero using a one-sample t-test.

4.4 **Results**

4.4.1 *Nectar*

The samples of nectar taken from 10 flowers showed a marked difference between the two cultivars (Table 4.1). Nectar volumes in the cultivar Tordis were approximately double those in Terra Nova ($F_{1,16}=23.03$, $n=17$, $SED=0.03$, $P<0.001$). Nectar from Terra Nova was slightly, but significantly, more

concentrated than that from Tordis ($F_{1,16}=59.48$, $n=17$, $SED=13.13$, $P<0.001$).

Tordis flowers secreted a greater total mass of sugar than Terra Nova ($F_{1,16}=11.96$, $n=17$, $SED=12.43$, $P=0.003$). The percentage of sucrose in the nectar also differed between cultivars, with that of Tordis nearly double that of Terra Nova ($F_{1,16}=78.68$, $n=17$, $SED=0.08$, $P<0.001$).

Table 4.1. Comparison of nectar collected from 10 flowers of two willow cultivars ($n=17$).

Nectar trait	Mean value	
	Tordis	Terra Nova
Volume (μl)	0.23	0.11
Concentration ($\mu\text{g}/\mu\text{l}$)	441	542.2
Sugar amount (μg)	100.0	57.0
Sucrose content (%)	-1.321 (21.1)*	-2.066 (11.2)*

*back-transformed means shown in parentheses

4.4.2 *Bumble bee movement in the arena*

Once released from the nest, bumble bees flew around the arena for a mean time of 81.6 s (range: 16 – 233 s) before alighting on a catkin. When landing on a catkin for the first time, bumble bees stayed for a mean time of 107.6 s and collected nectar. After leaving, bumble bees made a number of further visits to the catkins in which they again sampled the flowers (mean 6.6 visits per catkin). Subsequent visits were shorter than the initial visit on 31 of 34 catkins. Overall, bumble bees spent 41.4 % of the time in which they were observed on one of the two catkins. One of the 18 trials was not fully recorded by the camera. From that trial, the cultivar first chosen by the bumblebee was recorded, but comparisons of its first visit durations, numbers of visits and proportion of time spent on both catkins could not be included in the analyses.

4.4.3 *Bumble bee visits to catkins*

A graphical representation of the activity of the bees is shown in Figure 4.2. When presented with a flowering catkin from cultivars Tordis and Terra Nova,

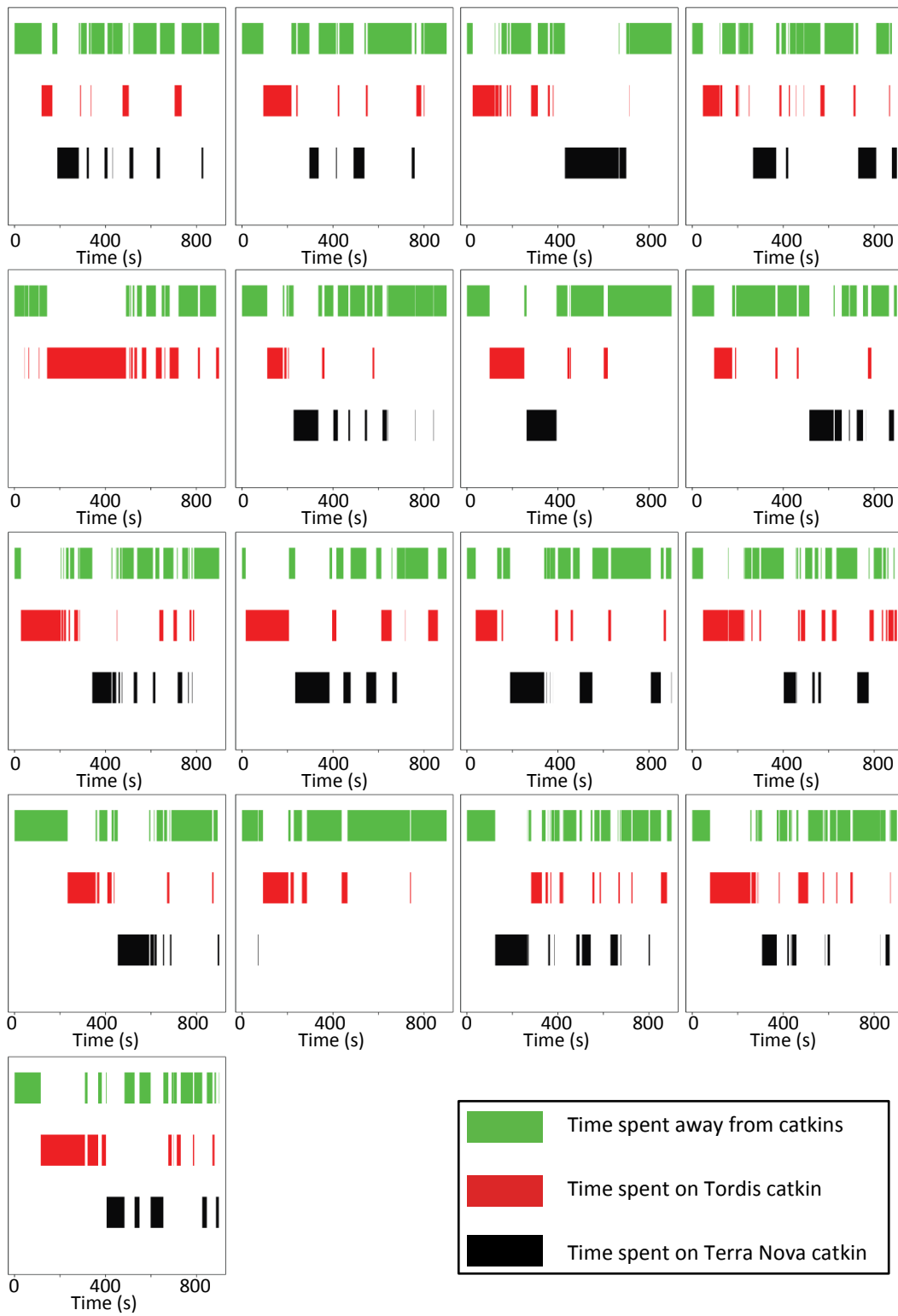


Figure 4.2. Activity of 17 inexperienced bumble bees in a flight room bioassay choice test with two SRC willow cultivars, Tordis and Terra Nova, observed for 15 mins.

inexperienced bumble bee workers first visited those of Tordis in 16 of 18 trials. The first choices of bees to cultivars on each side of the nest are shown in Figure 4.3. Bees were more likely to land first on Tordis catkins ($n=18$, one-tailed Fisher's Exact test $P<0.002$). However, the length of time bumble bees spent on their first visits to catkins did not vary between cultivars (mean: 107.6s, $n=17$, $F_{1,15}=0.34$, $P=0.57$).

Bumble bees made a greater number of total visits to Tordis catkins (mean=8.06) than to catkins of Terra Nova (mean=5.06). More than half of the total visits (223) made were to Tordis (proportion=0.61, $n=17$, one-sample binomial test, $P<0.001$). The proportion of time that bumble bees spent on the catkins of the two cultivars did not differ (mean: 186.3s, $n=17$, $t=-1.40$ on 16 d.f., $P=0.18$).

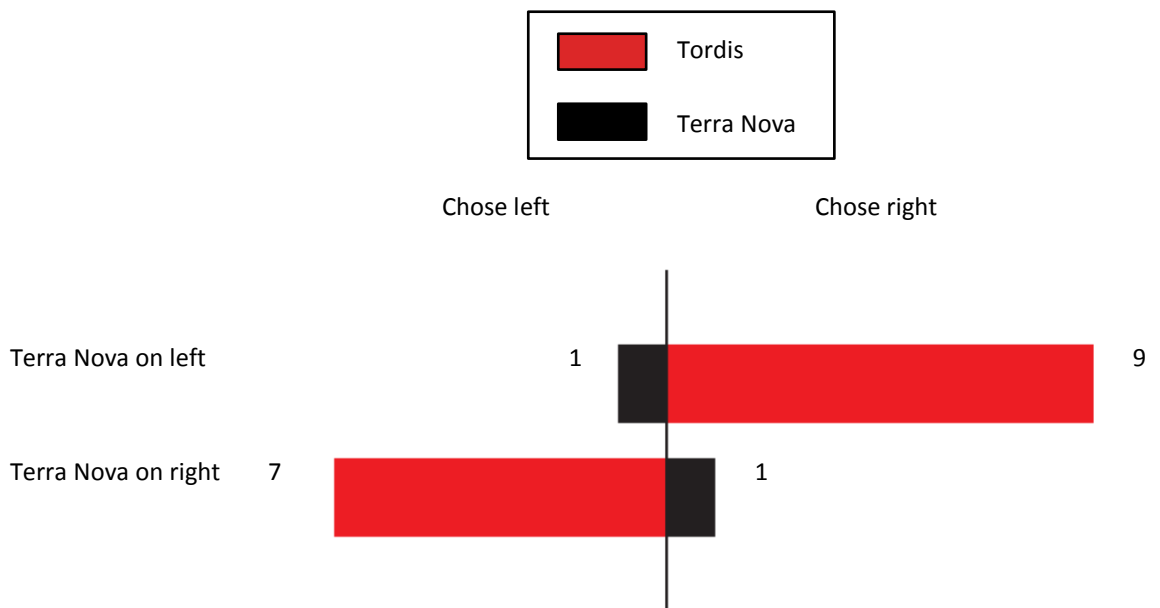


Figure 4.3. Total number of bumble bees landing first on Terra Nova or Tordis

4.5 Discussion

4.5.1 *Nectar*

Flowers of both Tordis and Terra Nova produced nectar, but there was a large disparity in the energetic value to pollinators of the rewards offered by the two willow cultivars. Tordis flowers had larger volumes of nectar and provided a greater amount of total sugar than those of Terra Nova. The nectar volumes and concentrations of Terra Nova measured from flowers on the cut stems were similar to those measured in field conditions (see Chapter 3), but the comparison could not be drawn in the cultivar Tordis, as nectar was not collected from Tordis plants in the field. The secretion of nectar by the cultivar Terra Nova appears to be particularly low. Terra Nova has a triploid genome, and like other willows with an odd number of chromosome copies, its unbalanced chromosome pairing during meiosis renders its flowers infertile (Karp *et al.* 2010). However, another infertile willow cultivar, Stott-10, which is pentaploid, secreted relatively large volumes of nectar in field conditions (Chapter 3), so the low production of nectar in Terra Nova cannot simply be explained as a result of its ploidy. Nectar from Terra Nova flowers was slightly greater in sugar concentration, which may be because its smaller droplets had a larger surface area for evaporation in proportion to their volume (Nicolson & Thornburg 2007).

4.5.2 *First choice of foraging bumble bees*

When released from the colony, around 90 % of bumble bees visited Tordis catkins before Terra Nova catkins. As they had no prior experience of any flowers, the cultivar that they visited first must have been determined by characteristics of the floral displays. It is not possible to distinguish whether bees visited Tordis catkins first because they were easier to detect than those of Terra Nova, or because, having detected both, they were the more appealing.

Flowers attract insects with both visual and olfactory cues (Fægri & Van Der Pijl 1979). Differences between the willow cultivars in either or both of these sensory modalities could explain the disparity in the attractiveness of their flowers to bumble bees.

Visually, the catkins of Tordis and Terra Nova differ in several respects (Figure 4.1). In Tordis flowers, the styles extend outward, almost perpendicular to the main shaft of the catkin, while in Terra Nova flowers styles protrude only slightly beyond the flower scales. In consequence, the stigmatic lobes are more prominently displayed in Tordis catkins, which makes them appear larger and greener, and exposes their nectar to a greater degree than Terra Nova catkins. To the bumble bees, Tordis catkins may have been more visually apparent or more attractive owing to these features, while Terra Nova catkins were more cryptic or less attractive. Giurfa *et al.* (1995) found that inexperienced bumble bees were strongly attracted to green, and speculated that this could be an adaptive behaviour to bring bees foraging for the first time close to foliage, where they are likely to find flowers.

Olfactory cues from the two cultivars were not compared in this study. However, in *Salix caprea*, Dotterl *et al.* (2014) found that honey bees were attracted in greater numbers to mixes of male and female catkins by their olfactory cues than by their visual cues, which indicates that floral scent is particularly important in pollinator attraction in willows. Tordis flowers may have attracted more bumble bees for their first visit by producing a more appealing or more concentrated scent than Terra Nova flowers. This hypothesis could be tested in further studies. Some flowers have scented nectar (Raguso 2004), and the greater amount of nectar, or the greater degree to which it is exposed in Tordis catkins may contribute to making them more easily detected or more alluring to bees.

Katzenberger *et al.* (2013) showed that initial choices of bumble bees to artificial flowers were strongly influenced by the salience of visual and olfactory cues operating both separately and together. A series of tests could measure the relative contributions of visual and olfactory cues from the willow flowers in rendering them attractive to bumble bees, by enclosing the flowers in either clear and closed containers, or opaque and open ones. An artificial flower could also be used to test the responses of bumble bees to particular flower and nectar volatiles identified in the two willow cultivars.

A limitation of the design of the present study is that surplus catkins on the stems were not removed, but were instead masked with paper to avoid providing additional visual cues. The superfluous catkins were retained to avoid the release of volatile organic compounds that occurs when plants sustain mechanical damage (e.g. Holopainen & Gershenzon 2010) and which could interfere with the bees' detection of floral scents. Olfactory cues from the additional catkins could have affected the bees' choices. However, as stems of Terra Nova, the less visited cultivar, had one catkin more than stems of Tordis on average, the number of catkins seems to have been insufficient to change the initial choices of the majority of bees.

4.5.3 *Duration of first visits*

The lengths of the bees' first visits to catkins were expected to relate more closely to the floral rewards produced by the two cultivars than subsequent visits, as nectar removal by the insects would reduce the amounts found when revisiting. In natural situations, bees often leave an inflorescence before visiting all flowers. It is hypothesised that bees move to new inflorescences when they encounter flowers with amounts of nectar below a threshold, as a means to maximise their overall collection of nectar (Dreisig 2012). The concept of the threshold led to the expectation that bees in the present study would spend less time on their first visit to the less rewarding cultivar, Terra Nova.

However, the bees' first visits to both cultivars were similar in duration, in spite of the differences in the nectar rewards they offered. The likely explanation is that in the present study, the only flowers available to bees were on the two catkins presented, and no advantage could be obtained from leaving the less rewarding catkin to search for more flowers. The similarity between cultivars in the lengths of the bees' first visits likely also indicates that the handling time required to remove nectar from both cultivars is equivalent. Comparisons between the lengths of time that bees spend on their first visits to each cultivar should be interpreted with caution, as bees may behave differently according to whether it is the first that they discover in the arena, or whether they arrived at it second.

4.5.4 *Bumble bee numbers of visits*

Bumble bees tended to return a greater number of times to Tordis catkins than Terra Nova catkins, so that the number of visits to the former accounted for around 60 % of total visits made to both. Bumble bees may have made more visits to Tordis catkins as they remained more appealing or apparent to the bees because of the visual and olfactory cues they provide (as when bees made their initial choice). Alternatively, having compared both catkins, bees may have formed a preference for the more rewarding one. Bumble bees learn which cues are associated with rewards after 3–5 consecutive trials (Goulson 2003). In the case of cultivars Tordis and Terra Nova, bees would not be expected to switch cultivars after learning, as the most rewarding cultivar was also the one initially preferred.

4.5.5 *Total duration of visits by bees to the catkins*

The combined time spent by bees on both cultivars was similar. The bees eventually stopped returning to catkins, presumably as their consumption of nectar led to a lower payoff for each visit, and as scent marks left on previous visits had a deterrent effect (Stout *et al.* 1998). After foraging ceased, bees either

flew around the arena, rested on the wall of the arena, or attempted to return to their nest. During some of the trials, bees stopped visiting the catkins during the observation period, while in others they continued to visit them.

The numbers of flowers in the catkins used in trials were not counted. Catkins of the cultivar Terra Nova that were collected from plants in the field (Chapter 3) had an average of around 200 flowers each, while the average numbers in the other female were 145 and 161. On the assumption that the catkins of both cultivars in the present study had a similar number of flowers, the average amount of nectar available to a bee in each trial from both cultivars was around 6.4 μl , whereas depending on the size of the individual, the honey stomach of a worker bumble bee holds 60-100 μl (Goulson 2003). Bumblebee foraging behaviour is subject to evolutionary forces, and when foraging, bumblebees typically spend more time on more rewarding flowers (e.g. Cresswell 1999). However, in the present study, the supply of flowers was limited, which may explain why bumblebees spent similar amounts of time on cultivars with different rewards. Differences may have been seen had more stems been used, with more catkins available for the bees.

4.5.6 Conclusions

In the flight arena, inexperienced bumble bee workers showed an innate preference between two willow cultivars in both the cultivar they visited first, in the number of visits made to each. Flowers of the preferred cultivar, Tordis, produced around double the nectar volume of the less visited cultivar, Terra Nova. However, bees consumed nectar from both cultivars, and spent similar amounts of time on each in total over the duration of the experiment.

When the two cultivars are grown as short rotation coppice, conditions contrast with those in the flight arena in several respects that are pertinent to the interpretation of these findings. In field conditions, the two cultivars flower in early to mid-March, before the emergence of worker bumble bees, but while

queen bumble bees and honey bees are foraging. The selective pressure to forage efficiently should apply at least as strongly to queen bumble bees as workers, since inefficient foraging by queens could easily cause starvation and failure of their nascent colony. Inexperienced queens in the field are therefore likely to discriminate against Terra Nova catkins at least as strongly as workers in the flight arena.

Unlike the present study, bees foraging in willow plantations are in competition with each other for nectar, and catkins may not be replete with nectar when first encountered. However, bees foraging in willow plantations have the opportunity to visit many more catkins, and to learn which are the most rewarding. In consequence, they should spend more of their time on Tordis than on Terra Nova catkins, in contrast to the bees studied indoors. The study also confirms that the cultivar Terra Nova produces small amounts of nectar in comparison to other cultivars, as found in field conditions.

Chapter 5. Nectar Secretion and Pollen Amino Acid Composition Varies Between Open-Pollinated and Hybrid Cultivars of Oilseed Rape (*Brassica napus*)

5.1 Abstract

Oilseed rape is widely grown in Europe and is an important source of nutrition for many insect pollinators. The development of new cultivars is ongoing, with many breeding programmes working to improve the crop. Plant breeders create open pollinated cultivars using classical line breeding techniques. They also produce hybrid cultivars by crossing genetically distinct parent lines, ensuring that cross-pollination occurs between them by using a male-fertile line to pollinate a male-sterile one. Male-sterile lines develop their peculiar phenotype as a result of genetic material located either in the mitochondria, which is known as cytoplasmic male-sterility (CMS), or in the cell nuclei, which is termed genic male-sterility (GMS). However, the value as dietary resources for insect pollinators of GMS hybrid cultivars has not been compared with that of CMS hybrid and open pollinated cultivars.

Several attributes of the three types of oilseed rape cultivar were compared by growing 24 cultivars in a trial within a glasshouse (7 – 9 of each cultivar type). The nectar volumes, mono- and di-saccharide concentrations and mass of sugar were measured per flower, along with the number of flowers per plant, and the sizes of flowers. Flowers of GMS hybrid cultivars were found to secrete larger volumes of nectar containing a greater mass of sugar than those of CMS hybrid and open pollinated cultivars, but the average concentration of nectar sugar did not vary between the three types. The number of flowers per plant did not differ between the three types of cultivar, but both CMS and GMS hybrid cultivars produced larger flowers than open pollinated ones. A difference was not seen in the composition of amino acids in pollen of the three cultivar types. The study suggests that in general, planting cultivars produced using a genic

male-sterility system will provide a greater source of nectar for insect pollinators in agricultural landscapes than planting open pollinated cultivars or hybrids derived from cytoplasmic male-sterility systems.

5.2 Introduction

Oilseed rape (*Brassica napus*) is among the world's most important sources of vegetable oil. The production of the crop in Europe has risen dramatically in the past three decades to meet growing demand for edible oil, biodiesel and industrial products (Juergens *et al.* 2010; Milazzo *et al.* 2013). One hectare of winter oilseed rape can contain 560 000 individual plants, which each produce an average of 375 flowers over a period of around four weeks in April and May, (Nedic *et al.* 2013). The flowers are a source of nectar and pollen, and are attractive to a range of insect pollinators (Riedinger *et al.* 2015). Flowering of oilseed rape substantially increases the food resources available for pollinators in landscapes where alternative sources may be inadequate (Carvell *et al.* 2006). Further, flowering of the crop coincides with the period when many bee species are starting to rear brood and are particularly vulnerable to scarcities of nectar and pollen (Goulson 2003). Proximity to this food supply has been shown to increase numbers of brood cells of the solitary bee *Osmia bicornis*, provided sufficient nesting sites are available (Holzschuh *et al.* 2013). Many cultivars of oilseed rape exist, but little is known about the variation in the nectar and pollen produced by different cultivars of the crop.

Oilseed rape is the focus of intensive breeding efforts. Many new cultivars are released and marketed to growers each year trumpeting improved agronomic traits, higher yields, greater seed quality and better resistance to diseases. However, the value to pollinators of new cultivars is not considered in the breeding process or in marketing. Traditionally, cultivars of the crop have been developed with classical line-breeding methods, which involve making a number of crosses and selecting the most promising genotypes over several

years to produce uniform open-pollinated cultivars (Friedt & Snowdon 2009). Breeders continue to create new open-pollinated cultivars to meet market demands for oilseed rape. However, in the mid-1990s the first hybrid cultivars of oilseed rape were registered (Frauen *et al.* 2003), and this type of cultivar is now widely grown in Europe (Friedt & Snowdon 2009). Hybrids are produced by crossing genetically distinct lines and often have seed yields greater than either parent – an effect known as ‘heterosis’ or ‘hybrid vigour’ – which makes them attractive to growers (Rai *et al.* 2007).

Hybrid seed is obtained from the pods of a male-sterile parent line, which is grown in rows. Interspersed with these rows are rows of a male-fertile parent line, which pollinate the male-sterile plants, and confer to the F1 offspring genes that fully restore fertility in their flowers. Plant breeders can create male-sterile plants by incorporating genes that induce this phenotype. They may opt to use genes that induce male-sterility located either in the cytoplasmic or nuclear genome (Delourme & Budar 1999). In lines with a cytoplasmic male sterility (CMS) system, a mutation in the mitochondrial genome inhibits the development of pollen. Several CMS systems have been developed in oilseed rape (Rai *et al.* 2007), but most CMS hybrid cultivars grown in the UK use a system called ‘Ogura’, which was transferred from radish (*Raphanus sativus*) (Delourme & Budar 1999). Instead of using CMS systems, some breeders induce male-sterility with the use of a genic male sterility (GMS) system, in which male-sterility develops due to the action of genes located in the nucleus. Most hybrid cultivars developed with GMS use the ‘Male Sterility Lembke’ (MSL) system developed through private research by the German breeding company NPZ Lembke (Friedt & Snowdon 2009), though an alternative developed and used by Syngenta is the ‘Safecross’ system (Stiewe 2008). Oilseed rape cultivars thus belong to one of three cultivar types according to the methods used in breeding: open pollinated, CMS hybrid or GMS hybrid.

The total resource for insect pollinators provided by an oilseed rape plant depends on both the average amount of nectar and pollen supplied by each flower and the total number of flowers. As a crop with an indeterminate growth habit (Wang *et al.* 2009), oilseed rape plants adjust the number of flowers they produce according to the conditions in which they grow (Cresswell *et al.* 2001). However, there may be an additional effect on the number of flowers produced due to cultivar or cultivar type that influences the overall value of a plant as a source of dietary nectar and pollen for insect pollinators. As plants have limited resources to invest, there may be a trade-off between the number of flowers on a plant and the average amount of nectar secreted per flower.

Nectar contains sugars that provide energy for insect pollinators. Little is yet known about the potential value to pollinators of the nectar produced by oilseed rape plants from open pollinated, CMS hybrid, or GMS hybrid cultivars. Pierre *et al.* (1999) found no differences between the nectar volumes and sugar concentrations when comparing three Ogura CMS hybrid lines with three open pollinated cultivars of oilseed rape. Likewise, Pernal and Currie (1998) detected no difference between the total nectar sugar content in flowers of six open pollinated cultivars and eight hybrid cultivars produced with the *pol* CMS system in spring oilseed rape. However, the nectar secretion of GMS hybrids has not been compared with CMS hybrids or open pollinated cultivars. The production of nectar by oilseed rape cultivars is of great interest to beekeepers that, in certain parts of UK and northern Europe, rely on nectar flow from the crop for honey yields. Honey produced from oilseed rape nectar crystallises quickly, and presents a problem for beekeepers if this occurs before honey is extracted from the comb (Kevan *et al.* 1991). A ratio of fructose to glucose below 1.11 in honey indicates a tendency to crystallise rapidly (Smanalieva & Senge 2009). The ratio of these sugars in oilseed rape nectar has been shown to vary in older cultivars of spring rape varieties (Kevan *et al.* 1991). However, differences

between modern cultivars and cultivar types of autumn-sown oilseed rape have not been investigated.

Nectar secretion can fluctuate with flowering phenology and ambient conditions (see Chapter 1), so variation arising due to differences between cultivars is difficult for plant breeders to assess. However, previous work on nectar secretion in a closely related species, *Brassica rapa* found that larger flowers produced more nectar (Davis *et al.* 1996). In a comparison between haploid ($n=10$), diploid ($2n=20$) and tetraploid ($4n=40$) plants, the average sizes both of petals and of the inner nectaries increased with ploidy – the number of sets of chromosomes in their nuclei (Davis *et al.* 1996). Larger flowers also produced a greater quantity of nectar sugar (Davis *et al.* 1994). If the size of flowers of an oilseed rape cultivar can reliably predict nectar production, flower size could be an index that breeders could use to produce cultivars that are more nectariferous for the benefit of pollinators, so long as their yields are not compromised.

Oilseed rape is self-fertile, but insect pollinators can facilitate outcrossing, which increases both the number of seeds per pod (Morandin & Winston 2005) and the weight of individual seeds (Bommarco *et al.* 2012b). Plants with greater nectar production attract more visits from insect pollinators (Pierre *et al.* 1996; Silva & Dean 2000). Oilseed rape cultivars with flowers that provide more copious nectar could therefore attract more pollinators and produce greater seed yields in variety tests.

In addition to nectar, flowers produce pollen, which serves both to transport male gametes of the plant, and as a reward for pollinators. Some adult insect pollinators consume pollen, including some flies, some beetles, and almost all bees (Willmer 2011). Bees also collect pollen to feed to their larvae (Proctor *et al.* 1996). The nutritional composition of pollen is an important aspect of the nutrition of pollen-feeding insect pollinators. Pollen contains amino acids,

protein, fatty acids, sterols, minerals, and other nutrients (Roulston & Cane 2000). One of the most important dietary components of pollen for bees is the protein found in pollen (Levin & Haydak 1957; Regali & Rasmont 1995; Schmidt *et al.* 1987), and the amino acids of which it is composed (Vanderplanck *et al.* 2014). De Groot (1953) showed that ten amino acids are essential in the honey bee diet for growth (arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine and valine). All ten have been reported in oilseed rape pollen, though tryptophan and methionine were found in very low concentrations (Cook *et al.* 2003). The concentration of pollen amino acids may vary between oilseed rape cultivars and cultivar types, but this is yet to be investigated.

In the present study the following hypotheses were tested:

- 1) The number of flowers per plant varies within and between the three cultivar types (under glasshouse conditions), and is correlated with the amount of nectar sugar per flower
- 2) The nectar volumes, concentrations, ratios of fructose to glucose, and mass of sugar produced by oilseed rape flowers over 24 hours varies within and between open pollinated, CMS hybrid and GMS hybrid cultivars.
- 3) Flower sizes vary according to the type of cultivar, and show a correlation with the amount of nectar sugar produced per flower.
- 4) There is a relationship between the amount of nectar sugar per flower produced by a cultivar and its seed yield.
- 5) The amino acid composition of pollen produced varies between open pollinated and the CMS and GMS hybrid cultivars.

5.3 Methods:

5.3.1 *Plant material*

Twenty four commercially available cultivars of oilseed rape were grown in a glasshouse. The cultivars included all 23 that were featured in the 2013 Recommended List for farmers in England and Wales (HGCA 2013). The cultivar SY Fighter was also included in the trial to increase the diversity of lines tested, as it is produced by a company not represented among the Recommended List cultivars (Syngenta). Of the 24 cultivars, eight were conventional open pollinated cultivars, seven were Ogura CMS hybrids, and nine were GMS hybrids, of which one was produced using the Safecross system, and the remainder with the MSL system. Details of the cultivars are shown in Table 5.1.

In March 2013, seeds were planted in seed trays using Rothamsted prescription mix compost with added nutrients. The compost contained:

- 75 % medium grade peat,
- 12 % screened sterilized loam,
- 3 % medium grade vermiculite,
- 10 % grit screened at 5 mm lime-free,
- 3.5 kg 'Osmocote Exact 3–4 month' per m³,
- 0.5 kg PG mix per m³ (Hydro Agri Ltd, UK),
- Approximately 3 kg per m³ lime to pH 5.5–6.0,
- 200 ml per m³ Vitax Ultrawet wetting agent.

Once germinated, seedlings were vernalised for eight weeks at 5°C with a 16 hour light : 8 hour dark cycle. In mid-May after vernalisation, seven plants from each cultivar were re-potted in 20 cm diameter pots using the same standard compost mix. Plants were moved to a ventilated glasshouse and arranged in a complete randomised block design with seven blocks (Figure 5.1).

Supplementary lighting was activated when necessary to ensure irradiance of at

least 100 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ from 05:00 to 21:00. Heaters were used to maintain a temperature of at least 14°C at night and 18°C during the day. Plants were watered twice daily by an automated system.

Table 5.1. Oilseed rape cultivars grown in glasshouse trial, with the breeding company and the cultivar type. Labels refer to Figure 5.1

Cultivar	Cultivar type	Breeder	Label
Cash	Open pollinated	KWS UK	CS
DK Cabernet	Open pollinated	DEKALB	CB
DK Camelot	Open pollinated	DEKALB	CM
Fashion	Open pollinated	Lantmännen SW Seed AB	FS
Quartz	Open pollinated	KWS UK	QZ
Rivalda	Open pollinated	KWS UK	RV
Sesame	Open pollinated	LS Plant Breeding	SS
Vision	Open pollinated	Lantmännen SW Seed AB	VS
Avatar	GMS hybrid, MSL system	LS Plant Breeding	AV
Compass	GMS hybrid, MSL system	DSV United Kingdom	CO
Cracker	GMS hybrid, MSL system	LS Plant Breeding	CR
Dimension	GMS hybrid, MSL system	DSV United Kingdom	DM
Marathon	GMS hybrid, MSL system	DSV United Kingdom	MR
Rhino	GMS hybrid, MSL system	KWS UK	RH
Thorin	GMS hybrid, MSL system	LS Plant Breeding	TH
Troy	GMS hybrid, MSL system	LS Plant Breeding	TR
SY Fighter	GMS hybrid, Safecross system	Syngenta	FG
DK Excalibur	CMS hybrid, Ogura system	DEKALB	EC
DK Expower	CMS hybrid, Ogura system	DEKALB	EP
Flash	CMS hybrid, Ogura system	DSV United Kingdom	FL
PR46W21	CMS hybrid, Ogura system	DuPont Pioneer	PW
PT-211	CMS hybrid, Ogura system	DuPont Pioneer	PT
DK Sequoia	CMS hybrid, Ogura system	DEKALB	SQ
PR45D05	CMS hybrid, Ogura system	DuPont Pioneer	PD

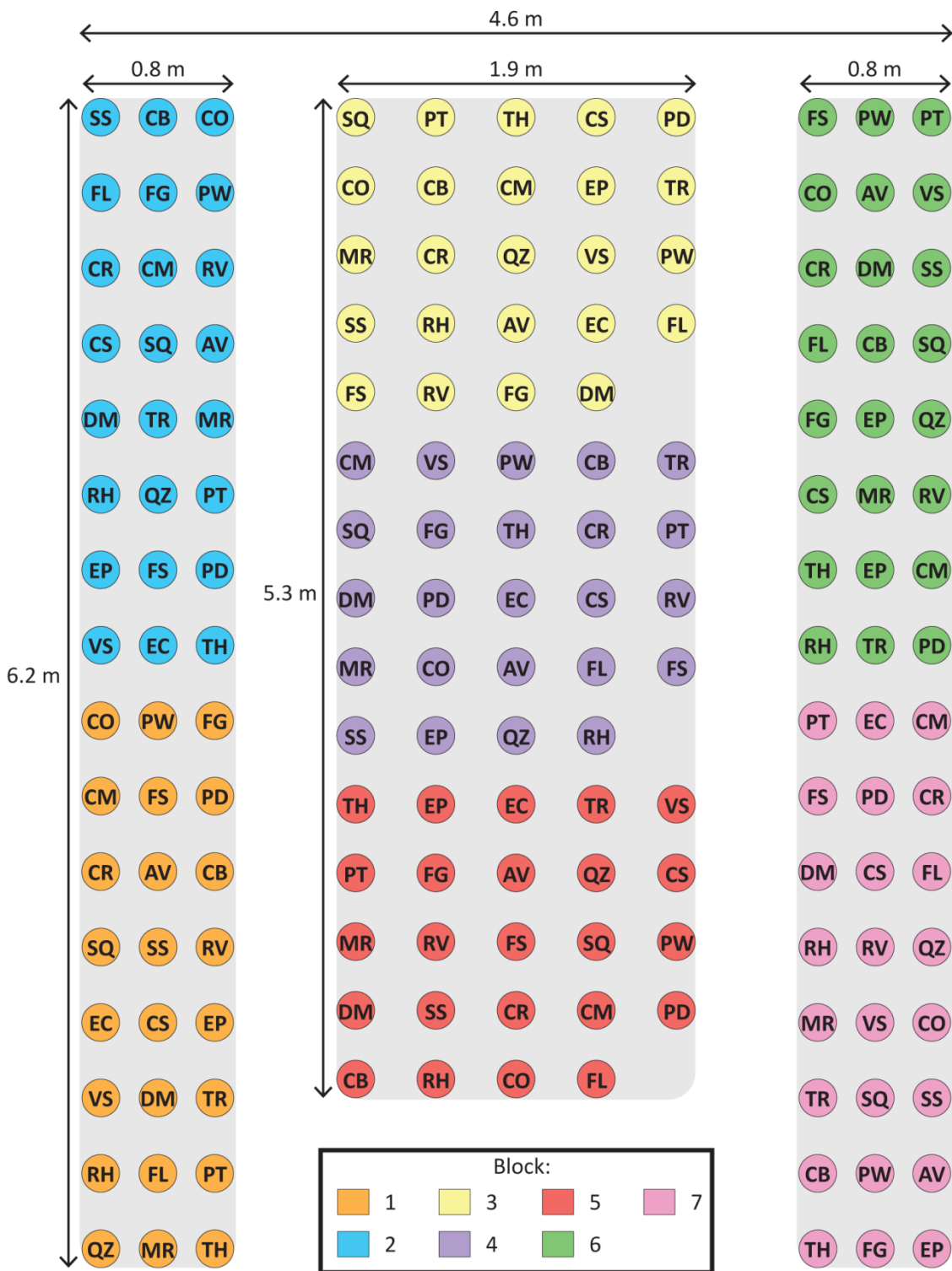


Figure 5.1. Layout of oilseed rape plants in seven blocks in a glasshouse. Letters refer to labels for each of 24 cultivars as shown in Table 5.1. The benches upon which the plants were maintained are shown in grey. The central bench could be moved to the left or right for access to the side benches.

5.3.2 *The number of flowers per plant*

The number of pods was counted as an assessment of the total number of flowers produced by each cultivar. After petals senesce and fall from the oilseed rape flowers, the ovary develops into a seed pod inside which the fertilised ovules ripen into seeds. Once flowering was completed (approximately four weeks), all stems with seed pods were carefully removed from five plants of each cultivar (those in experimental blocks 3-7). The stems from each plant were placed in a large perforated plastic bag and stored at 4°C. The pods on each plant were counted, including any that had become detached from the stem while in the bag, to determine the number of flowers produced by the plant.

5.3.3 *Collection of nectar*

Oilseed rape flowers bear two pairs of nectaries (see Figure 1.3). As the inner (lateral) pair produces 95 % of the total sugar from the flower (Davis *et al.* 1994), the outer (median) nectaries are not considered here.

The first flowers opened in early June. Plants were inspected daily to record when each had begun flowering. On the day that the first flowers opened, all open flowers were marked on their petals. The plant was revisited 24 hours later, and any new flowers that had opened in the intervening period were carefully drained of nectar from the inner nectaries. The nectar that accumulated in these flowers over the following 24 hours was then collected the next day for analysis. Microcapillary tubes (Drummond, USA) of 5 µl capacity were used to drain and to collect nectar. One nectar sample was collected from each plant; nectar from the inner nectaries was combined from multiple flowers present on the plant. The number of flowers from which nectar had been collected was recorded.

To control for effects on nectar production due to time of day, nectar from plants in each block was collected within a one-hour window, beginning with block 1 at 09:00, and ending after visiting plant in block 7 at 17:00. Nectar was

not collected between 13:00 and 14:00. Plants started flowering on different days, and as the nectar samples were collected on each plant's third day of flowering, the samples were collected over multiple days.

Microcapillary tubes were stored in 1.5 ml Eppendorf tubes placed on ice inside a cool box to reduce microbial growth and evaporation of nectar before being transferred to a freezer at -20°C later in the day. One cultivar (DK Sequoia) had not begun flowering by the time the other cultivars had, and consequently its nectar was not collected for analysis.

5.3.4 *Measurement of nectar volume, sugar concentration and sugar mass*

Volumes of nectar were calculated by measuring the proportion of the 5 μl microcapillary tube that was filled. Dividing by the number of flowers from which the sample was taken gave the mean volume per flower. To analyse sugars, nectar was expelled from microcapillary tubes using a rubber bulb into an Eppendorf tube (capacity 0.5 ml). From the pure nectar, 1 μl was taken and added to 29 μl of nano-pure water (Fisher) in a new tube (capacity 0.5 ml). After mixing thoroughly with a vortex, 1 μl of the diluted nectar was added to 65.6 μl of nano-pure water in a new tube (capacity 0.5 ml), to produce a solution with one part nectar in 2000 of water. Diluted nectar solutions were analysed with high performance liquid chromatography (HPLC; Dionex ICS-5000, Thermo Scientific, USA) as described in Chapter 2. The monosaccharides glucose and fructose, and the disaccharide sucrose were detected in oilseed rape nectar, and their concentrations were determined. Samples were run twice and mean concentrations used for subsequent analyses. The calculation of total sugar concentration and total mass of sugar per flower are also described in Chapter 2.

5.3.5 *Comparison of flower sizes*

One flower from the main raceme Figure 1.2 of each plant (seven plants per cultivar) was selected, and 1–4 (mean=2) petals were carefully removed at their

base. Petal size appeared to increase with floral age up to senescence, so all petals were collected when flowers had opened to an equivalent stage, when the petal laminas were perpendicular to the style (Figure 5.2). Petals were affixed to transparency film with clear tape (Sellotape, UK). Sheets of transparency film with affixed petals were scanned at 600 dpi, and the petal areas measured from the images produced using ImageJ version 1.44.

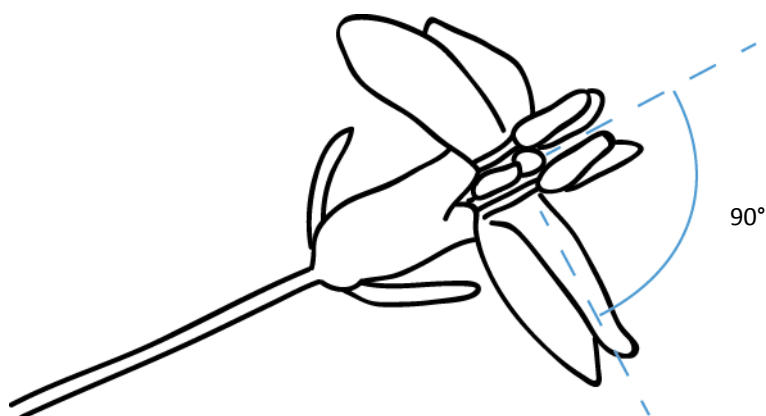


Figure 5.2. Drawing of the oilseed rape flower when petal laminas are perpendicular to the style. Petal sizes were compared when flowers had opened to this stage.

5.3.6 *Seed yields*

Yield data for the oilseed rape cultivars were obtained from the Recommended List for the East/West region in 2013-2014 (HGCA 2013). The Recommended List is produced annually by AHDB Cereals and Oilseeds, a division of the Agriculture and Horticulture Development Board that was known previously as the Home Grown Cereals Authority (HGCA). The Recommended List data are compiled from 11 separate field trials, each with three plots of each cultivar. Seed yields for each cultivar on the Recommended List are presented as a percentage of the mean of four control cultivars (DK Cabernet, DK Excalibur, Flash and Vision).

5.3.7 *Pollen amino acid composition*

A sample of pollen was taken from each plant in blocks 3–7 of the trial. The side of a microcapillary tube was used to dislodge dehiscid pollen from the anthers

of all open flowers into a glass vial. Vials were frozen at -20°C . For analysis of free- and protein-bound amino acids, 2 mg of the pollen was measured from each sample, being careful to exclude anthers. Quantification of amino acids was performed using HPLC, as described in Chapter 2.

5.3.8 *Statistical analyses*

Analysis of variance was used to compare the nectar volumes per flower, sugar concentrations, ratios of fructose to glucose, and mass of sugar per flower, as well as the number of flowers per plant, and the petal areas. Nested contrasts were included to compare cultivars within and between the three cultivar types: open pollinated, CMS hybrid and GMS hybrid. The overall difference between cultivar types was evaluated first, and the remaining treatment sums of squares and degrees of freedom were then partitioned into three parts due to differences between cultivars within each of the three types (equivalent to one-way comparisons within each group). Nectar volumes were square-root transformed, while both the sugar mass per flower and the number of flowers per plant were log-transformed (base 10), as these transformations reduced heteroscedasticity.

Pearson product-moment correlation coefficients were calculated to assess the relationships between the number of flowers and the nectar sugar mass per flower, between the petal area and nectar sugar mass per flower, and between the mean nectar sugar mass per flower of each cultivar and its seed yield in national trials.

The pollen amino acid composition was compared between cultivars and between the three cultivar types using canonical variates analysis (CVA). CVA is a method to separate known groups in multivariate data (Krzanowski 2000), by finding orthogonal, linear combinations of the data variates that maximise variation between groups. The contribution of each variate to the new linear combination is described by its loading (or latent vector). The analysis was

performed on the composition of amino acids in the pollen sample rather than on the absolute concentrations, so the mass of each amino acid as a percentage of the total within each sample was calculated. Box plots of the values of each amino acid showed that data were not skewed, so non-transformed data were used in the analyses.

5.4 Results

5.4.1 *The number of flowers per plant*

A mean number of 730 flowers per plant were produced across all cultivars. A difference was not found between the three types of cultivar ($F_{2,81}=2.53$, average $n=40$, $P=0.086$; Figure 5.3a), or within the CMS hybrids ($F_{6,69}=2.00$, $n=5$, $P=0.075$; Figure 5.3a). However, there was a difference within the open pollinated ($F_{7,81}=3.50$, $n=5$, $P=0.002$), and GMS hybrid cultivars ($F_{8,81}=2.39$, $n=5$, $P=0.023$), shown in Figure 5.3b. There was a negative correlation between the mean nectar sugar secreted per flower and the mean number of flowers per plant by the cultivars ($r=-0.45$, $P=0.03$), shown in Figure 5.4.

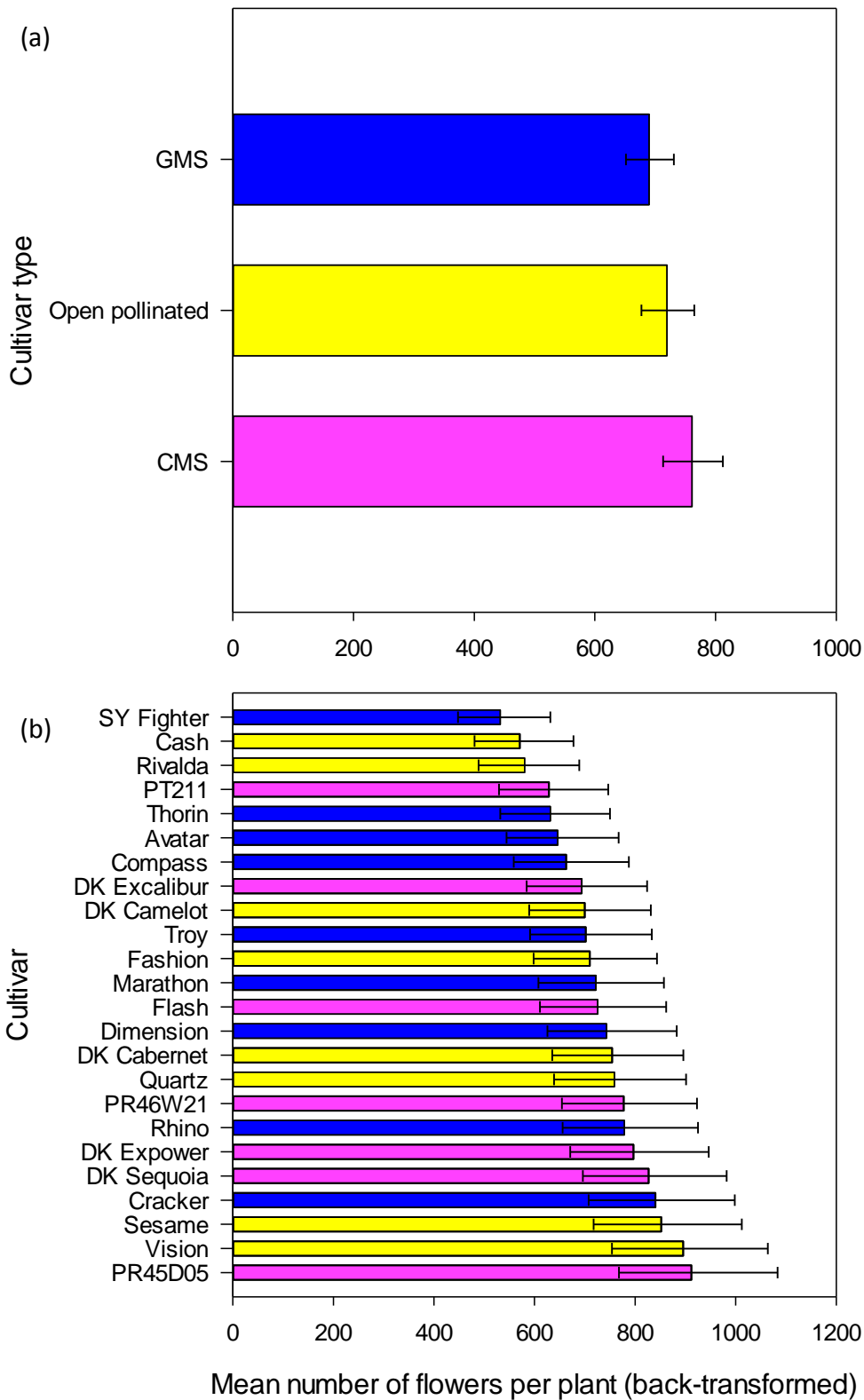


Figure 5.3. Mean number of flowers per plant produced by (a) three types of oilseed rape cultivars grouped according to their breeding system: hybrid cultivars produced using a genic male-sterility system (GMS) or a cytoplasmic male-sterility system (CMS), and open pollinated cultivars (average $n=40$). (b) 24 oilseed rape cultivars ($n=5$). Data are back-transformed to the normal scale. Error bars show 95 % confidence intervals. Yellow bars show open pollinated cultivars, pink bars show CMS hybrids, and blue bars show GMS hybrids.

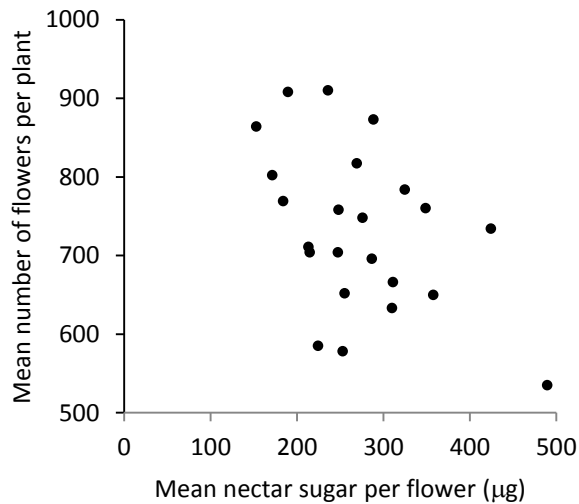


Figure 5.4. Relationship between the mean nectar sugar per flower and the mean number of flowers per plant in 23 cultivars of oilseed rape.

5.4.2 *Nectar volume, sugar concentration and amount*

In 24 hours, the mean volume of nectar secreted per flower by the inner nectaries across all oilseed rape cultivars was 0.88 µl. There was a difference between the three cultivar types ($F_{2,121}=23.66$, average $n=54$, $P<0.001$; Figure 5.5a). The GMS hybrids produced greater volumes of nectar per flower than CMS hybrids or open pollinated cultivars. There was also a difference in volumes of nectar per flower within the GMS hybrids ($F_{8,121}=2.45$, $n=7$, $P=0.017$), but not within open pollinated ($F_{7,121}=1.40$, $n=7$, $P=0.211$) and CMS hybrids ($F_{5,121}=2.19$, $n=7$, $P=0.059$; Figure 5.5b).

The majority of the sugar detected in oilseed nectar consisted of glucose (57.7 % by mass), followed by fructose (41.7 %) and sucrose (0.7 %). There was a difference between the three cultivar types ratio of fructose:glucose in their nectar ($F_{2,119}=5.03$, average $n=54$, $P=0.008$, average $SED=0.005$), as well as within the open pollinated cultivars ($F_{7,119}=5.37$, $n=7$, $P<0.001$, average $SED=0.005$), but not within the GMS hybrids ($F_{8,119}=1.16$, $n=7$, $P=0.032$ average $SED=0.005$) or within the CMS hybrids ($F_{5,119}=1.84$, $n=7$, $P=0.11$, average $SED=0.005$).

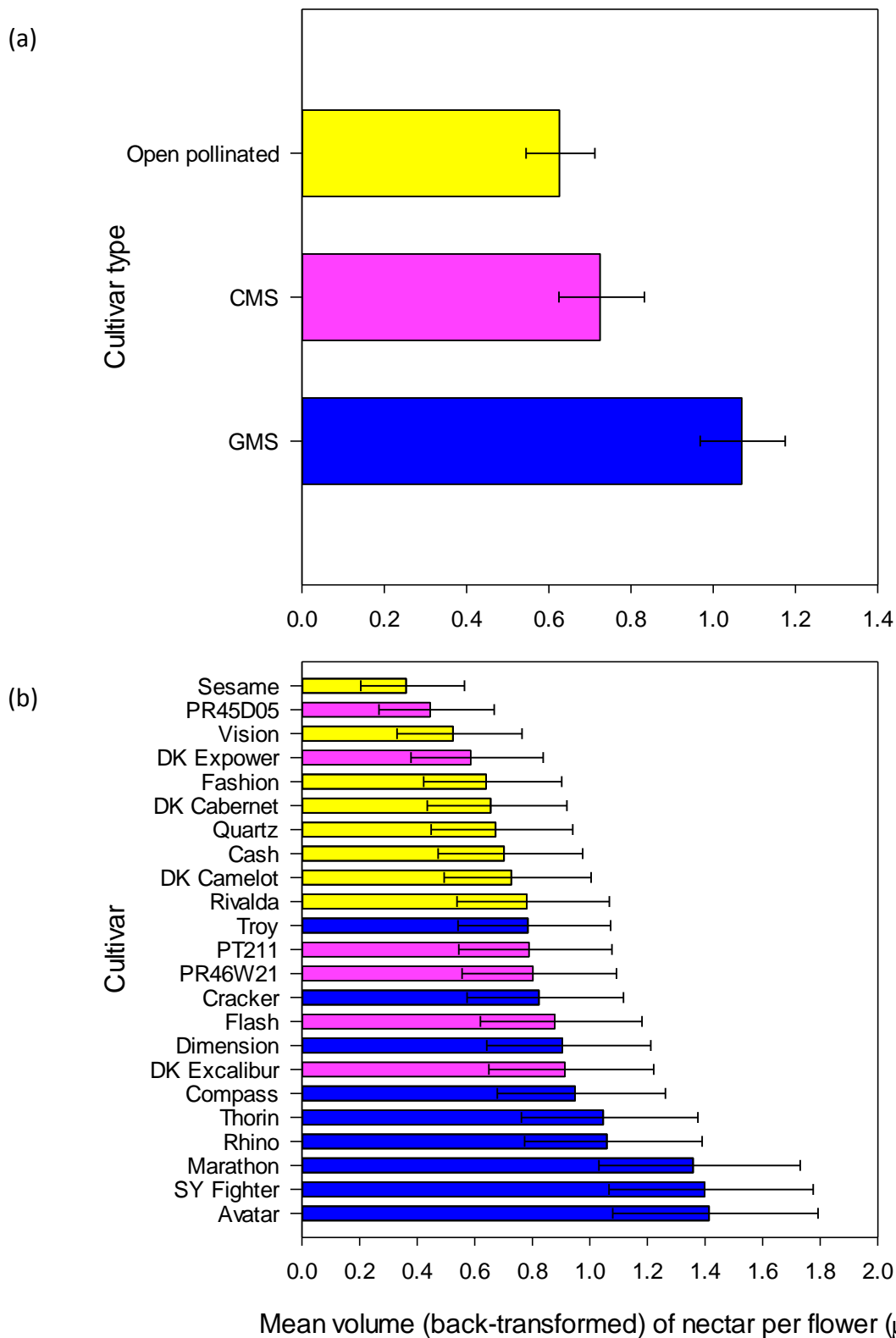


Figure 5.5. Mean volumes of nectar per flower secreted in 24 hours by (a) three types of oilseed rape cultivars grouped according to their breeding system: hybrid cultivars produced using a genic male-sterility system (GMS) or a cytoplasmic male-sterility system (CMS), and open pollinated cultivars (average $n=54$). (b) 23 oilseed rape cultivars ($n=7$). Error bars show 95 % confidence intervals. Means and error bars are back-transformed to the normal scale. Yellow bars show open pollinated cultivars, pink bars show CMS hybrids, and blue bars show GMS hybrids.

The ratios of fructose:glucose tended to be greater in the GMS hybrids and lower in the CMS hybrids (Figure 5.6a). The open pollinated cultivars had fructose:glucose ratios that were spread across the entire range of observed values (Figure 5.6b)

The mean concentration of all sugars in nectar from the inner nectaries was 324.32 g l^{-1} (32.4 % w/w). No difference was seen in nectar total sugar concentration between the three types of cultivar ($F_{2,119}=1.70$, average $n=54$, $P=0.187$, average $SED=14.637$), or within any of the types (open pollinated cultivars $F_{7,119}=1.34$, $n=7$, $P=0.236$, $SED=39.98$; CMS hybrid cultivars $F_{5,119}=1.5$, $n=7$, $P=0.196$, $SED=39.98$; GMS hybrid cultivars $F_{8,119}=1.38$, $n=7$, $P=0.212$, $SED=39.98$).

The mean mass of sugar per flower from all cultivars in the trial was $274.8 \mu\text{g}$. Between the three types of cultivar there was a difference in per flower sugar mass ($F_{2,117}=14.63$, average $n=54$, $P<0.001$;

Figure 5.7a). The GMS hybrid cultivars had a greater mean mass of nectar sugar within their flowers than the CMS hybrid and open pollinated cultivars. However, a difference was not found within the three cultivar types (open pollinated cultivars $F_{7,117}=1.38$, $n=7$, $P=0.218$; CMS cultivars $F_{5,117}=2.7$, $n=7$, $P=0.024$; GMS cultivars $F_{8,117}=1.45$, $n=7$, $P=0.181$;

Figure 5.7b).

5.4.3 Comparison of flower sizes

Mean petal size in oilseed rape flowers was 86.6 mm^2 . There was a difference between the three types of cultivar ($F_{2,130}=11.68$, average $n=56$, $P<0.001$, average $SED=1.687$; Figure 5.8a). The petal areas of CMS and GMS hybrid cultivars tended to exceed those of open pollinated cultivars. There was also a difference within open pollinated cultivars ($F_{7,130}=5.38$, $n=7$, $P<0.001$, $SED=4.749$), within the

CMS hybrids ($F_{6,130}=4.38$, $n=7$, $P<0.001$, $SED=4.749$), and within the GMS hybrids ($F_{8,130}=6.94$, $n=7$, $P<0.001$, $SED=4.749$), shown in Figure 5.8b.

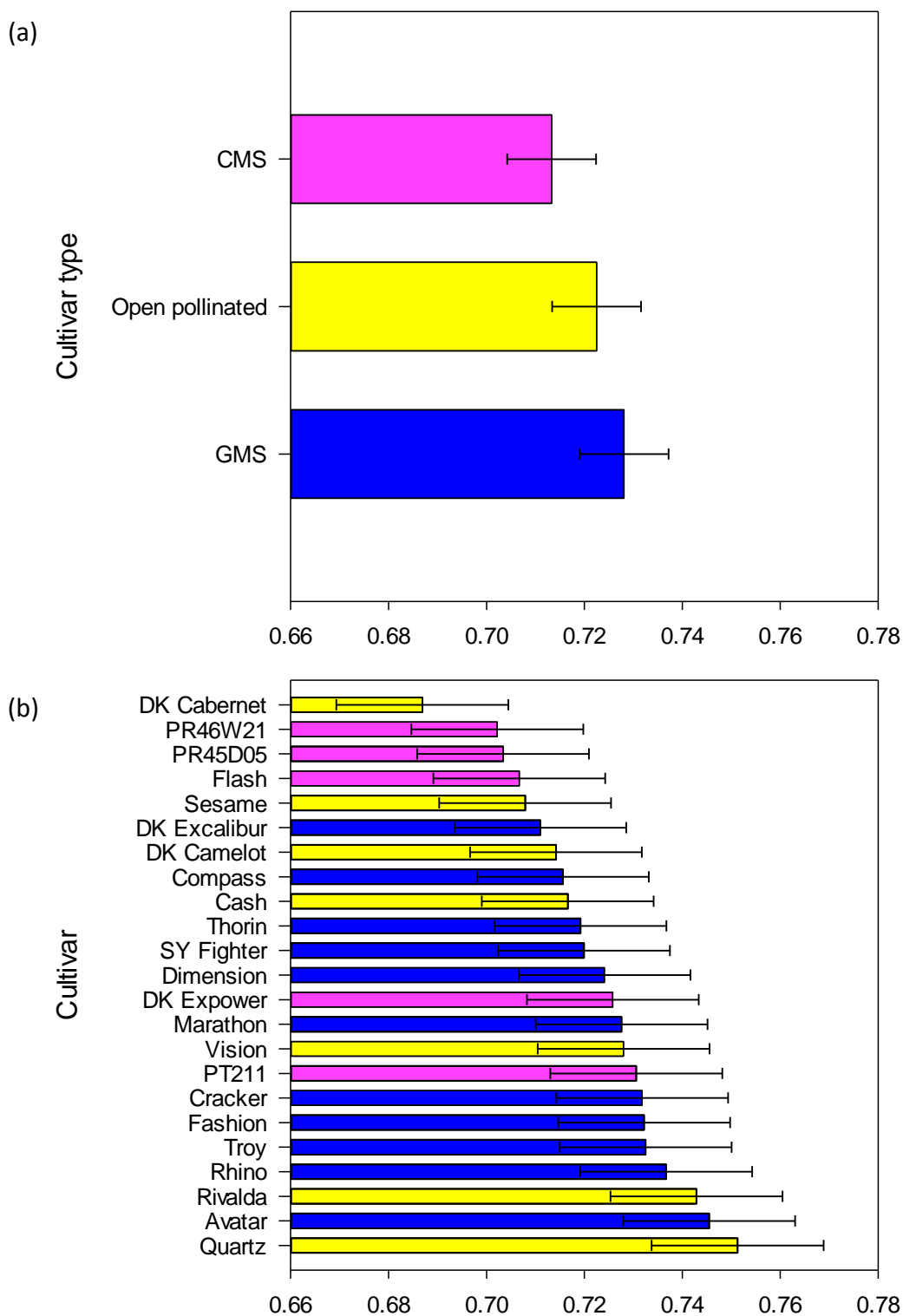


Figure 5.6. Mean ratio of fructose:glucose in the nectar of (a) three types of oilseed rape cultivars grouped according to their breeding system: hybrid cultivars produced using a genic male-sterility system (GMS) or a cytoplasmic male-sterility system (CMS), and open pollinated cultivars (average n=54). (b) 23 oilseed rape cultivars (n=7). Error bars show 95 % confidence intervals. Yellow bars show open pollinated cultivars, pink bars show CMS hybrids, and blue bars show GMS hybrids.

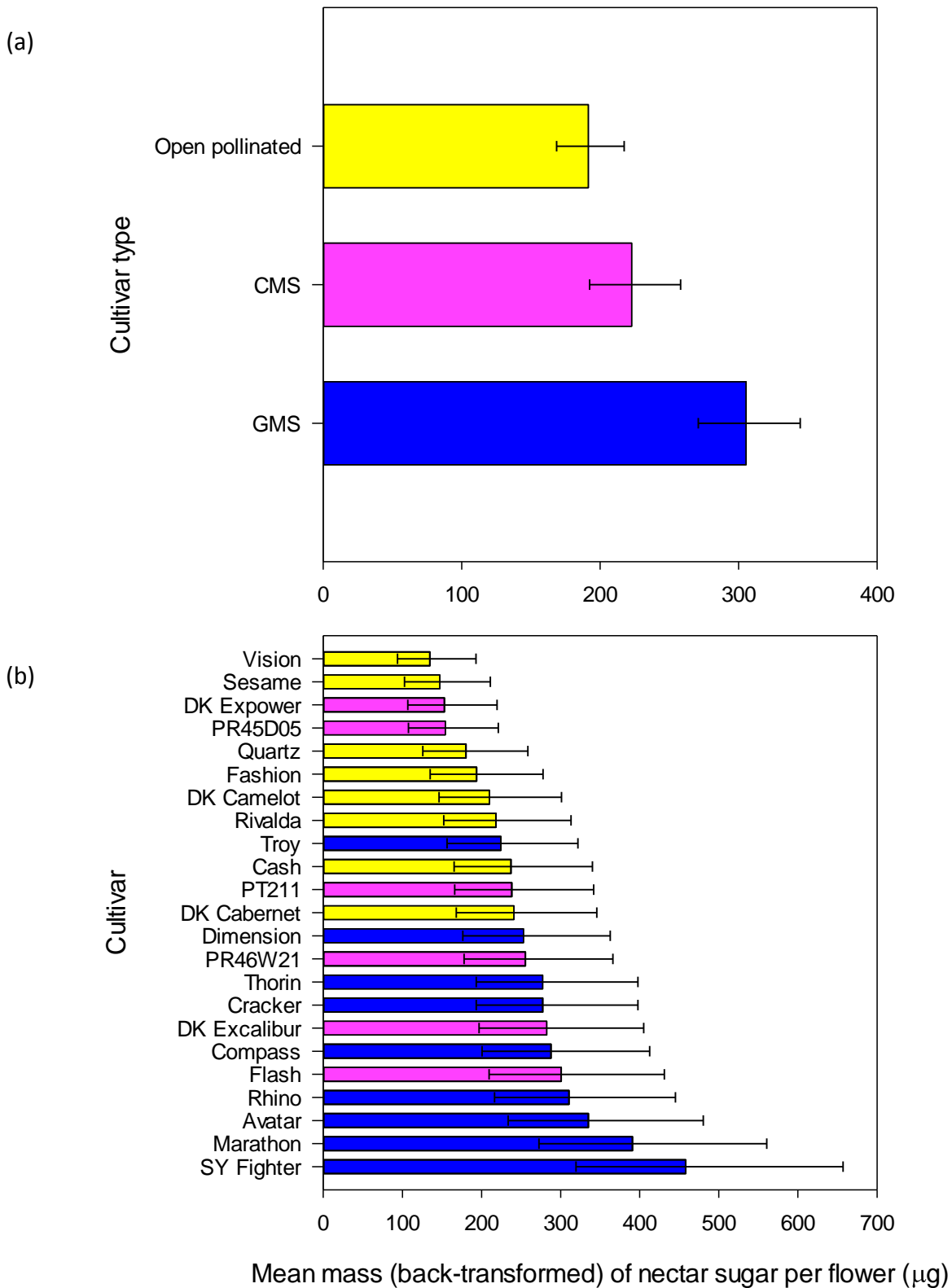


Figure 5.7. Mean mass of nectar sugar per flower secreted in 24 hours by (a) three types of oilseed rape cultivars grouped according to their breeding system: hybrid cultivars produced using a genic male-sterility system (GMS) or a cytoplasmic male-sterility system (CMS), and open pollinated cultivars (average $n=54$). (b) 23 oilseed rape cultivars ($n=7$). Data are back-transformed to the normal scale. Error bars show 95 % confidence intervals. Yellow bars show open pollinated cultivars, pink bars show CMS hybrids, and blue bars show GMS hybrids.

There was no correlation between petal area and the mass of nectar sugar per flower in the oilseed rape plants ($r=0.08$, $n=23$, $P=0.73$).

5.4.4 *Relationship between seed yields and nectar*

No correlation was found between seed yield and the mean mass of nectar sugar per flower among the oilseed rape cultivars ($r=0.17$, $n=23$, $P=0.45$).

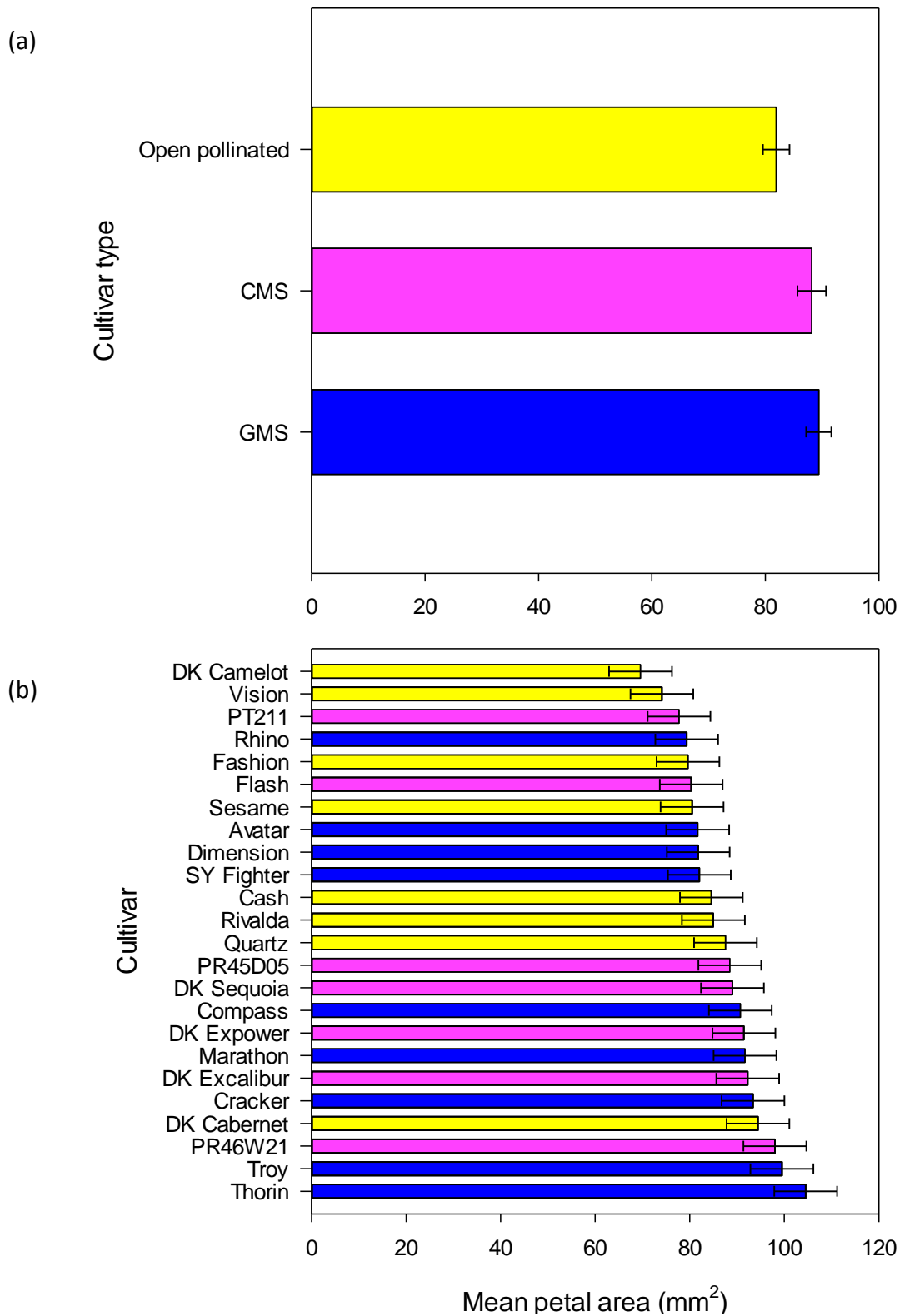


Figure 5.8. Mean area of a petal from (a) three types of oilseed rape cultivars grouped according to their breeding system: hybrid cultivars produced using a genic male-sterility system (GMS) or a cytoplasmic male-sterility system (CMS), and open pollinated cultivars (average n=56). (b) 24 oilseed rape cultivars (n=7). Error bars show 95 % confidence intervals. Yellow bars show open pollinated cultivars, pink bars show CMS hybrids, and blue bars show GMS hybrids.

5.4.5 *Pollen amino acid composition*

From samples of oilseed rape pollen, 3.6 mg/g of free amino acids and 57.8 mg/g of protein-bound amino acids were detected (0.36 % and 5.78 %, respectively), giving a total of 61.4 mg/g of amino acids in the pollen (6.14 %). The mean concentrations of the essential amino acids combined from the free and protein-bound fractions in pollen from the various oilseed rape cultivars are shown in Table 5.2. The mean concentrations of non-essential amino acids are shown in Table 5.3.

Canonical variates analysis showed that there was separation between some of the cultivars according to the composition of amino acids in their pollens. The first two canonical variates describe 24 % and 21 % of the variation, respectively. Variation between cultivars exceeds that within cultivars for the first two canonical variates, but not for subsequent dimensions (the latent roots, which describe the ratio of variance between groups to that within groups, of the first three canonical variates, are 1.40, 1.25 and 0.70, respectively).

Figure 5.9a shows the means of canonical variate scores for the cultivars with their 95 % confidence intervals, many of which are overlapping, indicating that a difference between those cultivars were not found. To aid interpretation, the same data are presented without confidence intervals in Figure 5.9b. Several cultivars appear to be separated to some extent by the two axes, though there is considerable variation around each mean. Loadings for each axis are shown in Table 5.4. Loadings are the coefficients by which the percentages of amino acids in pollen are multiplied in order to describe the position of the samples on the canonical variates, and so represent the contribution of each amino acid to the ordination. Amino acids with the greatest loadings (in magnitude) are proposed to have the most importance to the separation seen in the particular canonical variate dimension. Therefore, cultivars that were separated substantially on the first canonical variates differ in their associations with the amino acids phenylalanine, methionine and histidine. The second canonical variate

separated cultivars according to their associations with threonine, phenylalanine, serine and leucine.

There is some clustering of the cultivars in the three cultivar types in the analysis shown in Figure 5.9, albeit with a large overlap between them. To explore this, the proportions of amino acids were again analysed using a canonical variates analysis, grouped according to cultivar type. As this analysis tests whether any axes can separate three groups, there are only two canonical variates which together account for all of the variation. The means and 95 % confidence intervals of the canonical variate scores for the cultivar types, according to the first two canonical variates, are shown in Figure 5.10. The first canonical variate separates the mean score of the open pollinated cultivars from the means of the two types of hybrid cultivar, while the second canonical variate separates the mean score of the CMS hybrids from the GMS hybrids. Although the mean scores are separated with non-overlapping confidence intervals, there is substantial variation within these groups. Loadings for the analysis of cultivar types are shown in Table 5.5. The amino acids associated with the canonical variates differ from the previous analysis, as variation that was associated with additional dimensions is now projected onto the two canonical variates. The first canonical variate is associated with a difference between pollens in their content of isoleucine, serine, threonine and histidine, while the difference along the second canonical variate is associated with threonine, phenylalanine, methionine and alanine.

The mean percentages of the amino acids in pollens of each of the cultivars are shown in Table 5.6, with associated p-values following ANOVA partitioning variance first according to cultivar type, and then investigating the difference between cultivars, having accounted for the overall difference between types.

The ANOVAs show that histidine, alanine and leucine had significant (F-test, $P \leq 0.013$,) differences between cultivars having accounted for type of cultivar

(these three also being important in the CVA). There was also a strong overall effect of type ($F_{2,63}=8.56$, $P<0.001$) for histidine. Open pollinated cultivars Cash and Sesame had relatively high histidine, whereas DK Camelot and GMS hybrids Troy and Rhino had a high percentage of alanine. Some difference between cultivars was detected for arginine ($F_{21,63}=2.51$, $P<0.003$), an amino acid not deemed important from the CVA. This result looks to be largely due to Rhino having a particularly low percentage of arginine.

Table 5.2. Mean concentrations of essential amino acids in pollen from three types of oilseed rape cultivars: hybrid cultivars produced with a genic male-sterility system (GMS) or a cytoplasmic male-sterility system (CMS), and open pollinated cultivars. Totals of free and protein-bound amino acids are shown (n=5).

Cultivar	Amino acid concentration (mg per g of pollen)								
	Arginine	Histidine	Lysine	Phenylalanine	Methionine	Threonine	Leucine	Isoleucine	Valine
<u>Open pollinated</u>									
Cash	5.10	3.83	7.34	1.99	1.58	1.57	3.36	0.81	1.12
DK Cabernet	8.74	5.67	7.47	2.64	2.82	4.51	4.53	0.97	2.37
DK Camelot	5.13	4.38	6.11	2.56	2.66	4.40	4.19	0.86	1.85
Fashion	7.63	3.89	6.47	2.27	2.36	3.64	3.56	0.80	2.07
Quartz	5.40	2.98	6.18	2.15	2.09	3.06	3.58	0.72	1.50
Rivalda	4.82	3.43	6.52	1.95	1.97	2.53	3.18	0.85	1.68
Sesame	6.80	4.42	6.31	1.91	1.81	2.45	3.31	0.93	1.33
Vision	5.68	2.98	5.51	1.95	1.97	2.81	3.31	0.86	1.24
<u>GMS hybrids</u>									
Avatar	6.47	2.98	6.33	2.02	1.88	3.12	3.49	0.80	1.49
Compass	5.80	3.14	6.23	2.02	1.89	2.99	3.34	0.67	1.89
Cracker	6.70	2.65	8.37	2.06	1.78	1.74	3.81	1.07	1.65
Dimension	6.53	2.68	7.05	1.96	1.85	2.41	3.53	0.75	1.52
Marathon	7.17	3.07	6.00	1.98	1.76	2.87	3.46	0.71	1.71
Rhino	4.44	4.52	6.46	2.33	2.56	3.24	3.68	0.73	1.81
SY Fighter	5.48	3.17	5.73	1.95	2.01	2.41	3.25	0.80	1.68
Thorin	8.51	4.09	6.99	2.54	2.41	3.59	4.44	0.78	1.50
Troy	5.64	3.52	5.70	2.03	1.85	2.98	3.37	0.77	1.29
<u>CMS hybrids</u>									
DK Excalibur	6.41	3.27	6.60	2.37	2.36	3.62	3.92	0.82	1.71
DK Expower	4.33	2.93	5.76	1.52	1.62	1.46	2.55	0.75	1.39
DK Sequoia	6.21	3.19	5.27	1.82	1.92	2.36	3.24	0.66	1.25
Flash	5.72	2.49	6.20	2.11	2.07	2.48	3.43	0.73	1.67
PR45D05	5.69	2.96	5.97	1.75	2.13	2.64	3.21	0.73	1.59
PR46W21	5.32	3.37	5.22	1.75	1.61	2.28	2.83	0.80	1.45
PT211	7.86	2.62	6.71	2.01	1.95	2.32	3.59	0.87	1.53

Table. 5.3. Mean concentrations of non-essential amino acids in pollen from three types of oilseed rape cultivars: hybrid cultivars produced with a genic male-sterility system (GMS) or a cytoplasmic male-sterility system (CMS), and open pollinated cultivars. Totals of free and protein-bound amino acids are shown (n=5).

Cultivar	Amino acid concentration (mg per g of pollen)								
	Aspartate	Glutamate	Asparagine	Serine	Glutamine	Glycine	Alanine	Tyrosine	Cysteine
<u>Open pollinated</u>									
Cash	4.54	6.09	0.00	3.28	0.00	4.21	2.44	1.47	3.34
DK Cabernet	6.30	4.17	0.00	9.55	0.00	7.97	4.58	2.72	11.24
DK Camelot	6.55	4.91	0.00	8.68	0.00	7.31	6.15	2.43	10.44
Fashion	6.09	4.53	0.00	7.70	0.00	6.66	3.62	2.17	8.80
Quartz	5.27	3.94	0.00	6.50	0.00	5.43	3.52	1.57	8.18
Rivalda	4.79	5.64	0.00	5.30	0.00	4.94	4.10	1.57	6.88
Sesame	4.41	5.81	0.00	5.07	0.00	5.37	2.75	1.49	5.62
Vision	5.31	3.82	0.00	5.50	0.00	4.92	3.13	1.47	7.21
<u>GMS hybrids</u>									
Avatar	5.65	4.92	0.00	6.50	0.00	6.23	4.08	1.93	8.04
Compass	5.14	4.75	0.00	6.39	0.00	5.99	3.97	1.81	8.11
Cracker	4.69	7.34	0.00	4.24	0.00	4.82	2.14	1.53	3.46
Dimension	5.33	5.18	0.00	4.84	0.00	5.00	2.61	1.48	5.41
Marathon	4.33	3.69	0.00	6.17	0.00	5.50	2.64	1.50	7.06
Rhino	5.74	5.28	0.00	7.00	0.00	6.75	5.53	2.17	8.17
SY Fighter	4.50	5.59	0.00	5.46	0.00	5.23	3.14	1.51	6.02
Thorin	5.82	4.16	0.00	7.58	0.00	6.74	3.24	1.82	7.55
Troy	5.24	4.25	0.00	6.31	0.00	5.19	4.12	1.78	7.92
<u>CMS hybrids</u>									
DK Excalibur	5.78	4.82	0.00	7.75	0.00	6.93	4.70	2.03	8.73
DK Expower	4.03	6.13	0.00	3.17	0.00	4.09	2.51	1.41	3.29
DK Sequoia	5.26	5.06	0.00	5.28	0.00	5.32	2.73	1.72	6.29
Flash	5.14	5.68	0.00	5.08	0.00	5.26	2.80	1.61	5.43
PR45D05	4.20	5.28	0.00	5.89	0.00	6.48	3.67	1.65	5.42
PR46W21	4.72	4.78	0.00	4.61	0.00	4.76	3.36	1.64	5.69
PT-211	5.25	6.45	0.00	5.02	0.00	5.24	2.30	1.48	5.59

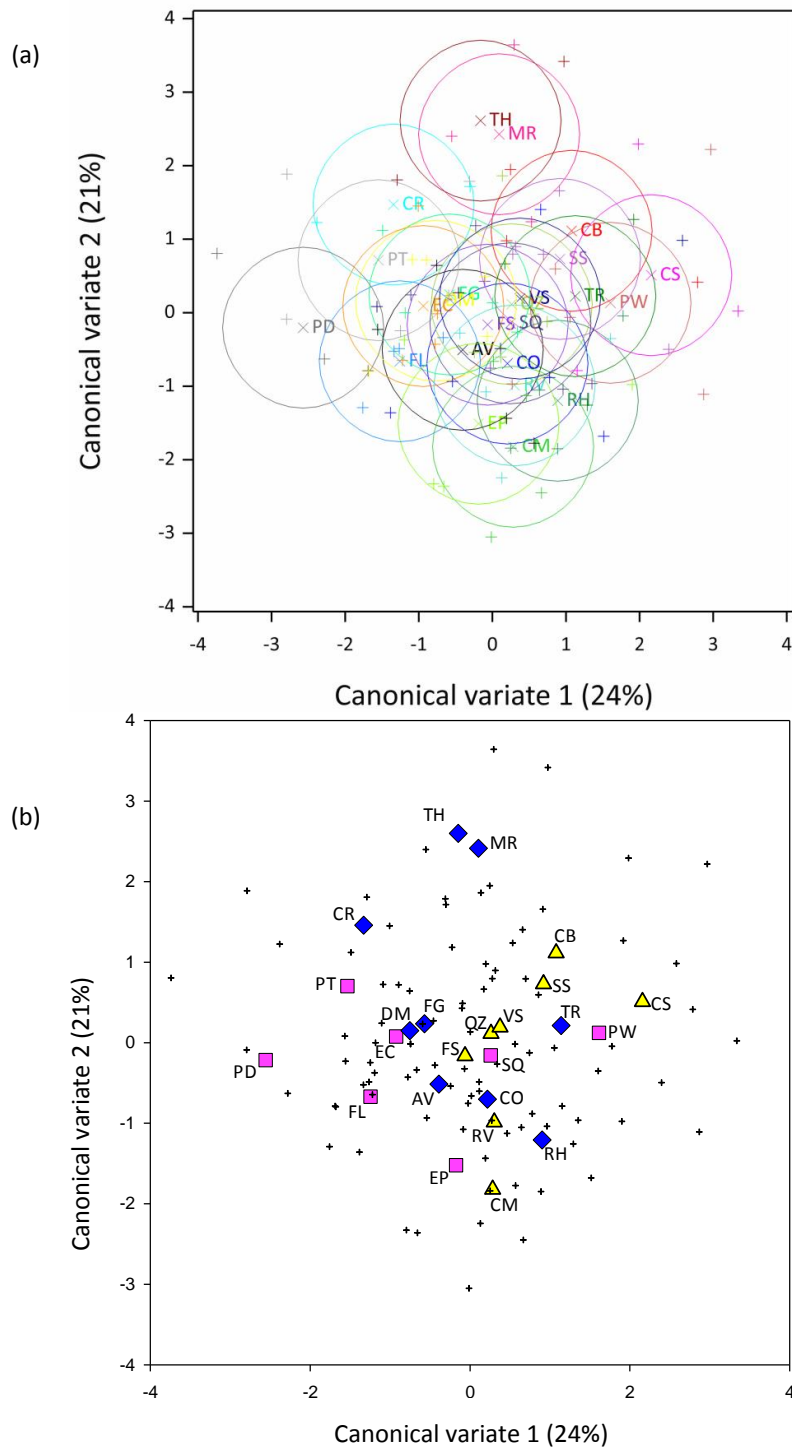


Figure 5.9. Canonical variate plot of the cultivar means and values of each sample in analysis of pollen amino acids from 24 oilseed rape cultivars. Cultivar means are labelled by their abbreviated names, as shown in . (a) Cultivar means are marked by X while the individual replicates are shown by the + symbol of the same colour. Circles show the 95 % confidence intervals around each mean. (b) Canonical variates plot as described in Figure 5.9a, shown without confidence intervals. Means of open pollinated cultivars are shown by yellow triangles, hybrids produced by cytoplasmic male-sterility systems (CMS) by pink squares, and hybrids produced by genic male-sterility systems (GMS) by blue diamonds. Crosses show the positions of individual samples to indicate the spread.

Table. 5.4 Loadings for the first two canonical variates from canonical variates analysis of pollen amino acid composition in 24 oilseed rape cultivars. Loadings in bold indicate corresponding amino acids important to the separation seen in the canonical variate dimension.

Amino acid	Loadings	
	Canonical variate 1	Canonical variate 2
Alanine	-0.03	0.47
Arginine	-0.08	0.61
Aspartate	0.03	-0.12
Cysteine	0.50	-0.18
Glutamate	-0.26	-0.33
Glycine	0.07	-0.28
Histidine	0.82	0.25
Isoleucine	0.16	0.78
Leucine	0.13	0.89
Lysine	0.05	-0.08
Methionine	-1.03	-0.37
Phenylalanine	1.57	0.99
Serine	-0.58	0.92
Threonine	-0.48	-1.60
Tyrosine	0.68	-0.19
Valine	0.43	0.24

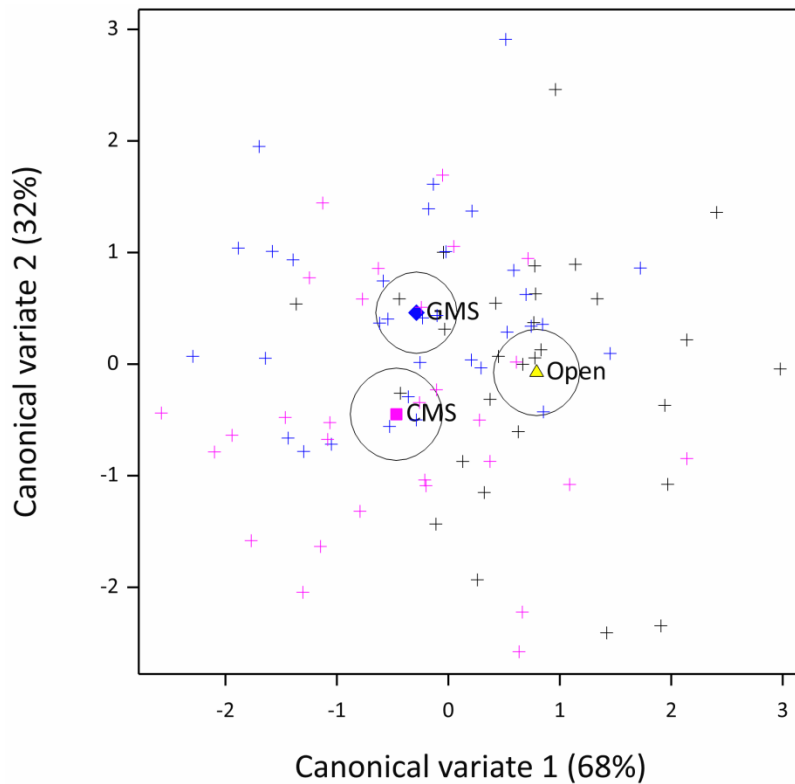


Figure 5.10. Canonical variate plot of the cultivar means and values of each sample in analysis of pollen amino acids from three cultivar types of oilseed rape. Means of the cultivar types are shown as large symbols. Circles show the 95 % confidence intervals around each mean. Crosses show the values of each sample, and are coloured according to the cultivar type (black crosses are open pollinated cultivars, pink are hybrids produced by cytoplasmic male-sterility systems (CMS), and blue are hybrids produced by genic male-sterility systems (GMS)).

Table 5.5. Loadings for the first two canonical variates from canonical variates analysis of pollen amino acid composition in 3 types of oilseed rape cultivars. Loadings in bold indicate corresponding amino acids important to the separation seen in the canonical variate dimension.

Amino acid	Loadings	
	Canonical variate 1	Canonical variate 2
Alanine	-0.59	0.67
Arginine	-0.43	0.30
Aspartate	0.03	-0.06
Cysteine	0.47	-0.06
Glutamate	-0.09	-0.38
Glycine	0.56	-0.23
Histidine	0.74	0.10
Isoleucine	1.25	-0.38
Leucine	0.63	0.10
Lysine	0.09	0.29
Methionine	-0.27	-0.70
Phenylalanine	0.18	0.84
Serine	-0.87	0.35
Threonine	0.99	-0.96
Tyrosine	0.05	-0.24
Valine	0.55	0.46

Table 5.6. Mean percentage of amino acids in pollen of 24 cultivars of oilseed rape. Standard abbreviations used for amino acid names are shown in Table 2.1. Standard errors of differences and p-values are shown following ANOVA in which variance was partitioned according to cultivar type, and then remaining variance was partitioned according to cultivar (type.cv). The least significant difference (LSD) between means for cultivars at the 5 % level of significance is given where there is a significant type.cv effect. (When significant, this supercedes the type effect.)

Cultivar	Mean percentage of each amino acid in oilseed rape pollen															
	Arg	His	Lys	Phe	Met	Thr	Leu	Ile	Val	Asp	Glu	Ser	Gly	Ala	Tyr	Cys
<u>Open Pollinated</u>																
Cash	9.8	7.4	13.8	3.8	3.1	3.0	6.4	1.6	2.2	8.7	11.7	6.3	8.2	4.8	2.8	6.5
DK Cabernet	10.8	6.5	9.1	3.0	3.3	4.8	5.3	1.2	3.1	7.2	6.0	10.4	9.2	4.9	3.2	12.0
DK Camelot	7.4	6.0	8.4	3.3	3.3	5.0	5.3	1.2	2.4	8.6	8.6	9.5	9.1	7.3	3.0	11.5
Fashion	10.7	6.0	9.6	3.1	3.3	4.4	4.9	1.1	3.2	8.5	8.2	9.7	9.4	4.6	3.1	10.3
Quartz	8.7	5.4	10.5	3.6	3.4	4.4	6.0	1.3	2.7	8.6	8.5	9.3	8.9	5.4	2.6	10.6
Rivalda	8.1	5.9	11.4	3.3	3.4	3.8	5.3	1.4	3.0	8.0	10.7	8.1	8.4	6.9	2.7	9.8
Sesame	11.6	7.8	11.1	3.2	3.1	3.7	5.6	1.7	2.2	7.3	10.9	7.7	8.9	4.4	2.5	8.3
Vision	10.4	5.5	10.6	3.5	3.3	4.3	6.0	1.7	2.1	9.5	8.4	8.4	8.5	4.9	2.6	10.4
<u>GMS Hybrid</u>																
Avatar	10.1	4.5	10.5	3.1	2.7	4.3	5.4	1.3	2.3	8.7	8.9	9.0	9.3	6.1	2.9	10.9
Compass	9.4	5.0	10.6	3.2	3.0	4.2	5.3	1.1	3.1	8.0	9.0	9.0	9.3	6.1	2.8	11.1
Cracker	11.2	4.6	14.7	3.7	3.1	3.1	6.7	1.9	2.7	8.2	12.5	7.3	8.1	3.8	2.6	6.0
Dimension	11.3	4.7	12.7	3.4	3.2	3.9	6.2	1.3	2.6	9.4	9.6	7.8	8.4	4.4	2.5	8.6
Marathon	12.4	5.4	11.1	3.3	3.2	4.3	6.0	1.3	3.3	6.7	7.3	9.6	9.2	4.2	2.6	10.3
Rhino	6.4	6.5	9.6	3.4	3.7	4.3	5.2	1.1	2.7	8.0	8.6	9.4	9.3	7.7	3.1	11.0
SY Fighter	9.2	5.7	10.4	3.5	3.5	3.8	5.7	1.5	3.0	7.6	11.0	8.9	8.9	5.5	2.5	9.4
Thorin	11.7	6.0	10.4	3.7	3.4	4.6	6.5	1.1	2.0	8.4	6.7	9.9	9.0	4.5	2.5	9.6
Troy	8.2	5.9	9.9	3.3	2.9	4.2	5.6	1.3	2.1	8.7	8.6	9.2	8.4	7.4	3.0	11.3
<u>CMS Hybrid</u>																
DK Excalibur	9.2	4.7	9.7	3.3	3.3	4.6	5.5	1.2	2.5	8.0	8.3	10.0	9.5	6.3	2.7	11.2
DK Expower	9.3	6.2	12.2	3.2	3.5	3.1	5.4	1.6	3.0	8.6	13.1	6.7	8.8	5.3	3.0	7.0
DK Sequoia	10.9	5.9	9.8	3.2	3.3	3.5	5.7	1.2	2.2	9.2	10.5	8.3	8.8	4.4	3.0	10.2
Flash	9.8	4.3	11.2	3.7	3.6	4.0	6.0	1.3	3.0	8.8	10.8	8.3	9.1	4.8	2.8	8.5
PR45D05	10.7	5.2	10.5	2.9	3.7	3.9	5.5	1.4	2.9	6.4	10.9	9.0	10.4	5.4	2.6	8.6
PR46W21	10.2	6.7	9.9	3.2	2.9	3.8	5.3	1.5	2.9	8.7	10.0	7.8	8.4	6.0	3.0	9.6
PT-211	12.8	4.4	11.5	3.4	3.2	3.6	6.0	1.5	2.6	8.7	11.4	7.8	8.3	3.8	2.4	8.7
P-value (type)	0.052	<0.001	0.403	0.526	0.287	0.786	0.427	0.650	0.953	0.842	0.429	0.964	0.331	0.639	0.807	0.944
P-value (type.cv)	0.003	0.006	0.251	0.270	0.722	0.094	0.013	0.332	0.284	0.088	0.479	0.139	0.426	0.001	0.522	0.286
Average SED*	1.30	0.80	1.46	0.26	0.38	0.47	0.42	0.24	0.36	0.83	1.69	1.01	0.76	0.87	0.37	1.73
LSD (5 %)	1.60	0.57					0.83							1.75		

*For comparing cultivars, on 21,63 degrees of freedom.

Discussion

5.4.6 *The number of flowers per plant*

No difference in the number of flowers per plant was observed between different cultivar types, though there was a difference between cultivars within types. The mean number of flowers on plants in the present study was over twice that estimated in oilseed rape grown in the field by Nedic *et al.* (2013). The tendency of oilseed rape plants to produce more flowers when insects are excluded was also found by Mesquida *et al.* (1988c). In addition to the reduced opportunity for insect and wind pollination experienced in the glasshouse, plants may have faced lower stress in competing for nutrients and water than those grown in field conditions. Oilseed rape plants are indeterminate, and the number of flowers they produce falls rapidly with increasing density of plants (Cresswell *et al.* 2001), so it may be expected that the numbers counted in this trial will differ from those grown in field conditions.

There was a negative correlation between the mean nectar sugar and the mean number of flowers among the cultivars. As nectar was collected at the start of flowering, this relationship could not be due to larger numbers of flowers shading the plants and decreasing their rate of photosynthesis. Flowers and floral nectar are costly for plants to produce and maintain (Pyke 1991), and even at the beginning of the flowering period, unopened flowers may represent a sink for photosynthate, so that plants on which more flowers would later open could not divert as much sugar to their nectar. The relationship will tend to reduce rather than amplify differences between plants in the value to flower visitors of the total nectar they produce over the blooming period. However, as discussed above, the numbers of flowers recorded in this study are unlikely to be representative of the number that plants would produce in field conditions.

5.4.7 *Nectar volume, sugar concentration and amount*

This study is the first to compare oilseed rape nectar production in hybrid cultivars created via genic male sterility (GMS) with hybrids made using a cytoplasmic male sterility (CMS) system and open pollinated cultivars. The mean nectar secreted in 24 hours, measured either by volume or by sugar mass, was around 50 % greater from GMS hybrid cultivars than from the other two types of cultivar. The finding that CMS hybrid cultivars and open pollinated cultivars produce a similar volume and sugar mass of nectar is consistent with previous studies (Pernal & Currie 1998; Pierre *et al.* 1999). However, the difference between GMS hybrid cultivars and the other types of cultivar was surprising, and has potential implications for pollinators.

Heterosis may explain why GMS hybrid cultivars produce more nectar per flower, perhaps endowing plants with larger or more active nectaries. The genetic basis for heterosis is poorly understood. The effect is usually attributed either to the suppression of homozygous recessive alleles that are deleterious from one line by the dominant alleles from the other (dominance model), or to an advantage obtained from bearing heterozygous alleles at particular loci (overdominance model) (Chen 2010).

If heterosis explains the high nectar production by GMS hybrid cultivars, what prevents CMS hybrid cultivars from sharing this trait? The CMS hybrid cultivars tested were all produced using the Ogura CMS system. Some studies have reported that male-sterile plants containing the Ogura cytoplasm from radish have less developed nectaries and lower production of nectar than male-fertile lines (Pelletier *et al.* 1987; Pham Délégue *et al.* 1991). The presence of this radish cytoplasm in the F1 hybrid offspring, even with male-fertility restored, may depress nectar production relative to hybrids without it. The F1 hybrid plants also contain a nuclear gene that restores fertility in the hybrid plant, named *Rfo*, inherited from the male-fertile parent (Bellaoui *et al.* 1999). The *Rfo* gene was also transferred to oilseed rape from radish, and was introgressed into

the oilseed rape nucleus along with unknown amounts of linked genes (Delourme *et al.* 1991) that could potentially influence the development and function of nectaries in the hybrids.

Among the GMS hybrid cultivars there was variation in the volumes of nectar produced, so that cultivars produced this way are not always more valuable for nutrition of insect pollinators than those produced by other methods. Variation between cultivars in genes associated with nectary development, nectar production or secretion may explain the difference detected within this type of cultivar (Bender *et al.* 2012).

The nectar of all cultivars contained greater concentrations of glucose than fructose. Honey in which the ratio of fructose to glucose is less than 1.11 crystallizes quickly, while honey in which the ratio is greater than 1.33 takes a long time to crystallize (Smanalieva & Senge 2009). All nectar samples collected in the study had ratios below 0.80, indicating that they will crystallize quickly.

Unlike the volume and total mass of nectar sugar produced per flower, the total concentration of sugars in nectar did not vary with cultivar or type of cultivar in this trial. Other studies have also found consistency in nectar sugar concentration between different lines of oilseed rape (Mesquida *et al.* 1991; Mohr & Jay 1990; Pierre *et al.* 1996), although fluctuations in concentration occur with weather conditions (Farkas 2008), time of day (Mohr & Jay 1990) and over the course of the flowering season (Pierre *et al.* 1999). There may be limited variation in genes that influence nectar sugar concentrations (Davis 2001).

A difficulty in making comparisons in nectar production between plants is that their flowering may not commence synchronously. As a consequence, comparisons must be made between plants at either a single point in time when many more flowers have opened on some plants than others, or over a range of times when each plant reaches a defined point in its flowering. The latter approach was taken in this study for several reasons. Firstly, previous studies

have suggested that oilseed rape nectar concentration can decrease between the earlier and later weeks of the flowering period (Mesquida *et al.* 1991; Mohr & Jay 1990; Pierre *et al.* 1996), so that comparisons between cultivars are only meaningful if they are made at equivalent time periods in their flowering phenology (Pernal & Currie 1998). Secondly, the relatively large number of cultivars to compare meant a wide expected range in times at which their flowering would commence, and consequently flowering might be beginning in some cultivars while it is coming to an end in others. Thirdly, the large number of plants involved made the prospect of simultaneously collecting nectar from all of them impractical.

However, the method used here, in which the nectar accumulated in 24 hours was taken from flowers of all plants on their third day of flower flowering, entails a notable weakness. The plants of each cultivar tended to begin flowering within a few days of one another, so comparisons between the earlier and later flowering cultivars were inevitably made on different days. The secretion of nectar is influenced by factors that can vary from day to day, such as air temperature, light intensity and humidity (Rathcke 1992). Conditions within the glasshouse were relatively consistent between days compared with field conditions, but conducting the study within a controlled environment chamber would be an improvement to the study design.

5.4.8 *Comparison of flower sizes*

The mean petal areas of both CMS and GMS hybrid cultivars were larger than those of open pollinated cultivars, suggesting that heterosis for flower size occurs in oilseed rape. The finding corroborates the visual impression when looking at some hybrid and open pollinated flowers, though there is a wide variation in flower sizes in cultivars within the three types compared. Cresswell *et al.* (2001) found that flower size was an attribute that was consistent when plants of one oilseed rape cultivar were grown under a range of conditions. The

effect of heterosis on flower size is not seen in *Arabidopsis* (Miller *et al.* 2012). In the present study, no relationship was observed between the flower size and the nectar sugar production of plants. Larger flowers often produce more nectar sugar where the sizes of nectaries and petals correlated (Davis 2001). However, in oilseed rape, the use of flower size to facilitate selection in breeding for lines with greater nectar yields is not supported by these data.

5.4.9 Relationship between seed yields and nectar

The mean mass of nectar sugar per flower of cultivars in this trial showed no relationship with their seed yields measured in the field trials coordinated to produce the Recommended List (HGCA 2013). This contrasts with lucerne (*Medicago sativa* L.), in which nectar sugar volume and sucrose concentration correlate with seed set (Holtkamp *et al.* 1992). If such a relationship exists in oilseed rape, it may have been undetected in this trial, as the two measures were made on different plants kept in different conditions. If nectar production does generally influence seed yield through enhancing pollinator visitation, the HGCA field trial data used here may not show such a pattern, as the trials flowered in spring 2012 when much of the UK experienced a prolonged period of exceptionally cold and wet weather. However, the production of nectar does have a cost for plants (Pyke 1991). It may be supposed that the benefits of extra insect visits are balanced by these costs, and the absence of any pattern in seed yields reflects the balance of this trade-off. If, as these data suggest, there is no tendency for cultivars that produce more nectar sugar in their flowers to have greater or lower seed yields, oilseed rape breeders and growers have an enormous opportunity to increase the supply of nectar in springtime without any penalty to their incomes.

5.4.10 Pollen amino acid composition

Potential differences in the proportions of the amino acids in oilseed rape pollen were investigated between cultivars and cultivar types. In an exploratory

analysis there was a suggestion that cultivar types and certain cultivars could differ in their proportions of several amino acids, but differences were very slight, and unlikely to be meaningful to pollen consumers. Oilseed rape has a small amount of genetic diversity (Hasan *et al.* 2006), and pollen amino acid concentrations tend to be highly conserved within plant genera (Weiner *et al.* 2010), so the lack of sizable differences is unsurprising.

The total amount of the amino acids quantified by the methods used in this study was low compared with other studies of oilseed rape pollen. For instance, Weiner *et al.* (2010) found a total of around 168 mg of free and protein-bound amino acids per gram of hand-collected pollen in oilseed rape, while Szczęsna (2006) reported around 229 mg per gram from bee-collected *Brassica* pollen, compared with 63 mg in the present study. The lower quantity of amino acids detected is partly lower than in other studies because proline could not be measured, due to the lack of fluorescence of the derivative formed with *o*-phthaldialdehyde. However, another reason for low detection of pollen amino acids might be that the hydrolysis of proteins was incomplete. Pollen proteins were hydrolysed by boiling with 6M for 20 minutes in the present study, while other studies have allowed 4 hours (Weiner *et al.* 2010) to 24 hours (Szczęsna 2006).

The amino acid compositions of oilseed rape pollen found here show similarities with those reported by Rayner and Langridge (1985) and Szczęsna (2006): for instance relatively large amounts of lysine, aspartate and glutamate, and smaller amounts of methionine, tyrosine and histidine were found in all cases. The rank order and percentages of amino acids are not identical between the studies. Differences may be due to the use of pollen collected by honey bees in previous studies, rather than pollen collected by hand, as in this study. Honey bees may contaminate the pollen grains with other pollen sources, and with the microflora from their honey stomach when they add nectar the pollen loads in order to adhere them to their corbiculae (Vasquez & Olofsson 2009).

Additionally, the incomplete hydrolysis of pollen proteins may have resulted in a profile of amino acids that was not reflective of all of those present in the protein fraction of the pollen. Of the amino acids measured here, those in Table 5.2 are essential amino acids in the honey bee diet (De Groot 1953). Among these, isoleucine comprised only around 1 % of the total amino acids, but is one of the three amino acids honey bees require in the largest amounts (De Groot 1953). Tryptophan is also required by honey bees, but in smaller quantities than other essential amino acids (De Groot 1953). Tryptophan was not detected among the free amino acids, and the method used here is unable to determine its concentration in pollen protein. However, Cook *et al.* (2003) detected small quantities of tryptophan among the free amino acids from oilseed rape pollen that was collected by honey bees. Oilseed rape pollen, therefore, is likely to offer a complete source of protein for insect pollinators.

5.4.11 Conclusions

Oilseed rape is particularly valuable for insect pollinators as a source of nectar. In observations, around 92 % of honey bees, bumble bees and solitary bees visiting flowers of the crop collected nectar (Woodcock *et al.* 2013). The present study provides evidence that the quantity of nectar provided by oilseed rape flowers is influenced by the cultivar grown. There was a threefold difference between the cultivar that yielded the most nectar sugar per flower (SY Fighter), and the cultivar with the least (Vision). In addition, hybrid cultivars that are offspring of a parent line in which male-sterility was induced by nuclear genes tended to have the greatest production of nectar.

These findings were obtained from plants grown in a glasshouse, and should be tested in field conditions to validate them. If the same results can be replicated in field conditions then more widespread planting of the most nectariferous cultivars would enhance the nectar available to insect pollinators in spring. As

this is a critical time for pollinators, the extra resources could aid their survival, and lead to more robust populations.

Chapter 6. Floral Resources for Nutrition of Insect Pollinators Varies in Cultivars of Oilseed Rape (*Brassica napus*) and Affects Colony Weight of Bumble Bees (*Bombus terrestris audax*)

6.1 Abstract

Winter oilseed rape (*Brassica napus*) is the most widely grown mass-flowering crop in the UK. It produces nectar and pollen that are consumed by insect pollinators. However, the relative value of the oilseed rape cultivars in the current UK landscape as sources of nutrition for pollinators is unknown.

The floral nectar and pollen produced by six commercially available oilseed rape cultivars was compared in a replicated field trial. The nectar secreted over 24 hours was collected from flowers of each of the cultivars, and its volume, sugar concentration and total mass of sugars were compared. The numbers of pollen grains produced by flowers of the six cultivars were estimated. Two cultivars were further compared in a bioassay in which colonies of bumble bees (*Bombus terrestris audax*) were kept in pollination cages with access only to plots of one of the cultivars, and changes in colony mass were recorded.

There was a difference between the cultivars in the volume and amount of sugar per flower produced, but not in their nectar sugar concentration.

Cultivars also showed a difference in the numbers of pollen grains produced per flower. Nests of bumble bees foraging on the cultivar SY Fighter were heavier than those foraging on the cultivar Sesame after two weeks.

The findings show that oilseed rape cultivars vary in their value to pollinators as sources of nutrition. As availability of resources can enhance the capability to reproduce, the choice of cultivar planted might impact colony growth in social species, and the pollination services provided in agro-ecosystems.

6.2 Introduction

Insect pollinators obtain nutrition from flowers, and require a supply of floral nectar and pollen throughout their foraging period. In intensively farmed landscapes, scarcity of wild flowers to provide nutrition may limit populations of pollinators (Roulston & Goodell 2011). However, pollinators can obtain nectar and pollen from some crop plants while they are in bloom, particularly mass-flowering crops (Stanley & Stout 2014). Previous work has shown that there is variation in nectar production between cultivars of certain crops such as sweet pepper (Roldan-Serrano & Guerra-Sanz 2004) and onion (Silva & Dean 2000), which suggests the possibility to increase the availability of nutritional resources for pollinators in a given locality by the selection of the cultivars providing the greatest floral rewards.

To compare the nectar produced by flowers, a measurement can be taken of either the standing crop (the amount available within a plant at a given instant) or the secretion rate (the amount produced during a given interval). Making fair comparisons of the nectar production in different plants by measuring either the standing crop or secretion rate is difficult, however, as many factors can influence nectar production (Cresswell 1998; Real & Rathcke 1988), which must be controlled or accounted for as far as possible. First, the standing crop of nectar can vary with the time of day, according to the daily patterns of nectar secretion and reabsorption through time, which vary between plant species (Burquez & Corbet 1991; Pernal & Currie 1998). The nectar standing crop is also influenced by ambient conditions, either losing water to the atmosphere by evaporation, or gaining it through condensation and precipitation (Corbet 2003). Second, flowers of different ages vary in their nectar secretion rate, frequently decreasing with age (Devlin *et al.* 1987; Real & Rathcke 1988). Third, removal of nectar can stimulate faster secretion, so the secretion observed may depend on the interval between measurements (Castellanos *et al.* 2002). Fourth, cultivars may begin flowering at different times, and flowers on plants just

coming into bloom can produce more concentrated nectar than later flowers on the same plants (Pierre *et al.* 1999), so it may be impossible to compare cultivars on the same day and at precisely the same stage in flowering.

Along with nectar production, the amount and desirability of pollen offered by flowers and plants is a major determinant of their value in the diets of pollinators. However, unlike the nectar reward, the amount of pollen produced by a flower is determined before it opens according to the number of pollen grains in its anthers, and is not subject to environmental variation. The number of pollen grains created by a flower is influenced by selective forces. Animal-pollinated plant species that produce large numbers of pollen grains tend to be those whose flowers have smaller stigmas, those with smaller pollen grains, and those whose main pollinators have a larger surface area that contacts the pollen (Cruden 2000). Pollen grain numbers are also higher in obligate out-crossing plants compared with those in which self-pollination is common (Cruden 2000). The number, size, and nutrient content of pollen offered by plants influence the total reward they offer to insect pollinators. In some crop species, the number of pollen grains per flower differs between cultivars, for instance in almond (Godini 1981), apple (de Albuquerque Junior *et al.* 2010) and turnip rape (Hinata & Konno 1975).

The most widely planted mass-flowering crop in the UK is winter oilseed rape (*Brassica napus*) (Defra 2015b). The presence of oilseed rape enhances populations of pollinator species that are active during the flowering period (Riedinger *et al.* 2015). Previous work identified cultivars that vary in the amount of nectar produced by their flowers in glasshouse conditions (Chapter 5 in this thesis). The present study aims to compare oilseed rape cultivars in field conditions, according to their provision of nectar and pollen, and by recording the collection of these resources from different cultivars by pollinators, using nests of the bumblebee *Bombus terrestris audax* as a model pollinator species. The following hypotheses are tested:

- a) The nectar volumes, sugar concentrations and sugar secreted per flower vary between oilseed rape cultivars in field conditions.
- b) The numbers of pollen grains per flower differ between oilseed rape cultivars.
- c) The difference between cultivars in the floral resources they provide influence the rate at which bumble bee colonies increase in mass when feeding on them.

6.3 Methods:

6.3.1 *Field trial establishment*

Six commercially available oilseed rape cultivars were planted in a replicated field trial at Rothamsted Research in Hertfordshire, UK in early September 2013. The trial contained the cultivars SY Fighter, PTPT-211211, Compass, Rivalda, Sesame and DK Expower. These cultivars were selected as they begin flowering at a similar time, and showed variation in the mass of nectar sugar produced by their flowers in earlier work in a glasshouse environment (Chapter 5).

Seeds of all cultivars were supplied with a coating of the insecticide Cruiser (Syngenta), containing the active neonicotinoid ingredient thiamethoxam. Seeds were drilled at a rate of 80 m⁻², and plots were treated with fertilizers, molluscicides, herbicides and fungicides in accordance with Rothamsted standard farm practice for the crop. Plots were covered with netting in the early stages of establishment to prevent damage by rabbits and pigeons.

Six plots of each cultivar were sown, and were arranged in a 6x6 Latin square design to control for variation within the field in soil or other conditions. Plots were 3 m long and 1.8 m wide, with gaps of 1.5 m between rows and 1.8 m between columns. The central column measured 3 m, to serve as a tractor spray path. The layout is shown in Figure 6.1.

6.3.2 Collection of nectar

In April 2014, nectar was collected from plants at an early stage in the flowering period. Separate nectar samples were taken from three plants, selected at random, on each plot. Only flowers on the main raceme were used. In order to compare flowers of a similar age, nectar was collected as follows. Between 15:00 and 18:00 on 7th April 2014, three plants with flowers were randomly selected on each plot, and a ring was drawn on their stems above the pedicels of any open flowers to distinguish them from flowers that would open later. A muslin bag was affixed over these racemes to prevent the removal of nectar by insect visitors. The bags were administered in a systematic order: each plot in the first row of the Latin square was visited (from left to right as shown in Figure 6.1), before repeated the process in each subsequent row in turn. Figure 6.2 shows the plots in part of the field trial with bags in place.

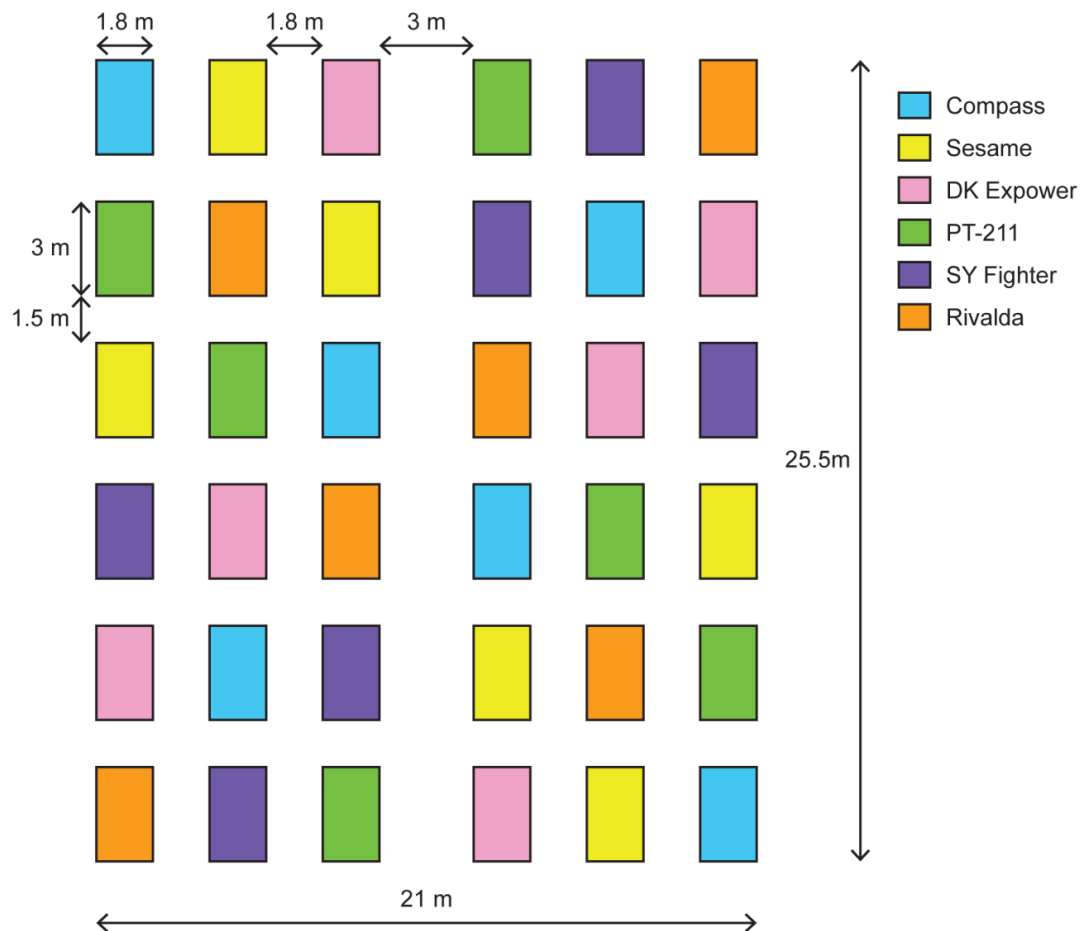


Figure 6.1. Design of oilseed rape field trial.



Figure 6.2. Plots in part of the oilseed rape field trial, in which flowers are protected by bags.

The following day (8th April 2014), between 11.30 and 18.30, all flowers that had opened since the bags were fastened (1-5 per plant) were counted and each was marked with a spot of permanent ink on their petals. While marking the newly opened flowers, the nectar from their inner (lateral) nectaries was carefully removed using 5 µl glass microcapillary tubes (Drummond, USA). Nectar removal on this day was not conducted to make comparisons, but in order to measure the nectar that would be secreted by each flower in the following 24 hours, removing variation due to the amount of time since each flower opened. Starting with the first row of the Latin square, a round of flower visits was made in which nectar was removed from one of the three marked plants on each of the six plots. Two further rounds were then made on this row of the Latin square, before moving on to the next row, so that three consecutive rounds of flower visits were completed on each row of the trial.

Twenty four hours later, on 9th April 2014, each plant was revisited in the same order as before. The nectar accumulated over that period in the inner nectaries was collected from the flowers that had been drained the preceding day. Nectar was collected with 5 µl microcapillary tubes, as before.

To provide a larger sample for more accurate sugar analysis, the nectar from multiple flowers was pooled together to give one sample for each plant. The samples from different plants within plots gave rise to three nested pseudo-replicate measurements from each plot. Nectar was collected from Row 1 between 11:34-12:34; from Row 2 between 12:56-13:33; from Row 3 between 14:06-14:56, from Row 4 between 15:00-15:47, from Row 5 between 16:08-17:30, and from Row 6 between 17:33-18:11. Microcapillary tubes were sealed in 1.5 ml Eppendorf tubes and placed in an ice box containing freezer packs to reduce microbial deterioration of nectar sugars during transportation to the lab, prior to storing at -20°C later that day.

During the period in which nectar was collected, hourly weather data were recorded by a weather station located approximately 1.5 km from the field site. Temperature, relative humidity, solar radiation and wind speed were recorded. The growth stage of plants within each plot was assessed using the BBCH scale (Lancashire *et al.* 1991). At the point of nectar collection, plots were at growth stages 61-64, when approximately 10-40 % of the flowers had opened on the main raceme.

6.3.3 *Nectar volumes*

The mean volumes of nectar produced per flower were determined using digital callipers and a magnifying lens to measure the length of the column of nectar within the microcapillary tubes. The length of nectar column as a proportion of the total length of the microcapillary tube was multiplied by 5 μ l (the total volume of the tube) and divided by the number of flowers from which the sample was obtained.

6.3.4 *Nectar sugar concentration and amount per flower*

All of the nectar was expelled from each microcapillary tube into the 1.5 ml Eppendorf tube that it was previously stored in, to which nano-pure water (Fisher) had been added in the appropriate volume to dilute the nectar to one part in 30. The dilution was allowed to run back into the microcapillary tube several times to ensure all of the nectar was removed. From this dilution, 3 μ l was combined with 197 μ l of nano-pure water in a new tube to produce a dilution of one part nectar in 2000 of water, suitable for analysis of sugars. High performance liquid chromatography (HPLC) was used to measure concentrations of glucose, fructose and sucrose within the samples, as described in Chapter 2. Each sample was analysed twice, and the mean values were taken. Concentrations of the three sugars were calculated, and the mean mass of sugar per flower was then determined.

6.3.5 *Pollen grains per flower*

On 11th April, an unopened flower bud from the main raceme was picked from a randomly selected plant on each plot. Following Takahata *et al.* (2008), each bud was placed in a 1.5 ml Eppendorf tube containing 1 ml of a 3:1 solution of ethanol and acetic acid, and stored at 4°C. All buds were collected within two hours of each other. Buds were carefully dissected, and all six undehisced anthers from each bud were placed in a new Eppendorf tube. To these tubes, 300 µl of a solution was added that contained water, glycerol and Fuchsin stain in the ratio 100:100:1. To release pollen grains, anthers were crushed with a micro-pestle, and tubes were agitated with a vortex machine for three mins. The micro-pestle was thoroughly cleaned with water and wiped dry between samples.

The solutions containing the pollen grains were again agitated momentarily with a vortex machine to ensure homogeneity prior to transferring a 10 µl sample to two chambers of a haemocytometer. Pollen grains were counted in four sections in both chambers (eight in total), each of which measured 1 mm x 1 mm x 0.1 mm (0.1 µl). For all samples, the mean number of grains counted in the eight 0.1 µl sections was multiplied by 3000 (the ratio between the volume of solution in the haemocytometer section and the total volume in which pollen grains from the flower were suspended) to give the estimated total pollen grains from the entire flower.

6.3.6 *Bumble bee colony assay*

Two cultivars that varied in their provision of floral rewards were compared to test whether there was a difference in the amount of resources that a bumble bee colony could collect from them. The cultivars SY Fighter and Sesame were selected for comparison, as it was possible to determine early in the flowering period that there was a difference in the nectar volumes secreted by the flowers of these cultivars. The other properties of nectar and pollen measured in the

present study could not be considered in selecting cultivars to compare as they were measured using collected samples after flowering had ceased.

On all six plots of each of the two cultivars SY Fighter and Sesame, an insect-proof pollinator cage was assembled. Cages were 1.83 m high with a 2.74 x 2.74 m footprint, and were enclosed by Tygan mesh with 0.78 x 0.38 mm gauge. Colonies of *Bombus terrestris audax* were obtained (Biobest, Belgium) at an early stage in development, with approximately 20-30 workers. Colonies were given 2 g of honey bee-collected pollen (C. Wynne Jones, UK) daily, and *ad libitum* access to the sugar syrup with which they were supplied, prior to their introduction into the cages.

Once flowering was underway with at least 30 % of flowers opened on all plots, one colony was placed in each cage. Access to the sugar syrup supplied with the nests was removed by sealing the plastic bottle in which it was contained. Each colony remained in the plastic and cardboard container in which it was supplied. Colonies were placed inside a larger cardboard box (34 x 30 x 34 cm) insulated with polystyrene (thickness 1 cm), with a hole for access. Further protection from the weather was provided with an upturned plastic tray as a base to elevate colonies from the ground, and a plywood roof to give shade and shelter. To prevent the wind from toppling the nest boxes, the capped bottles with sugar syrup were kept in each box, and the roofs were weighed down using a house brick.

Immediately prior to placing the colonies into the cages, and weekly for the following three weeks, each colony was weighed in order to provide a measure of the resources gathered from the two cultivars. The entrances to the nest boxes were adjusted in the evening prior to each occasion on which the colonies were weighed so that bees could enter the nest but were unable to leave. Setting the nest entrances in this way ensured that, as far as was possible, all individuals within the colony were included when its mass was recorded.

Colonies remained in the cages until flowering was almost finished, when over 90 % of flowers on the main raceme had opened. During this time, weekly counts of the numbers of open flowers in each cage were made for comparison, using a quadrat measuring 33 x 33 cm.

6.3.7 Statistical analyses

Analysis of variance (ANOVA) was used to compare the six cultivars with respect to: the volumes of nectar produced per flower in 24 hours, the concentrations of total nectar sugars and the total sugar mass per flower. The analyses accounted for the Latin square design by partitioning the variation within the observations that was due to the rows and columns. The remaining variation (associated with the combinations of rows and columns) was partitioned into the variation due to the cultivars and background variation. A further nested level accounted for the rounds of nectar collection within plots. Nectar samples were collected from within each row in turn, so the variation associated with rows comprises variation due to the difference in both space and time of sample collection. Prior to analyses, a log-transformation (base 10) was applied to nectar volumes and sugar masses, in order to reduce heteroscedasticity.

The estimated numbers of pollen grains per flower of the six cultivars was also compared using ANOVA, with the random model accounting for the rows and columns in the Latin square. Estimated numbers of pollen grains were transformed by taking their square roots before analysis to reduce heteroscedasticity.

The mass of bumble bee colonies and counts of flowers on plots of SY Fighter and Sesame were compared at each time-point using ANOVA. With only two cultivars, partitioning variation associated with both rows and columns is not possible. Since analyses of nectar properties and pollen grain numbers appeared to show a difference associated with rows, ANOVA with a blocking

structure accounting for rows was used to test for a difference in bumble bee colony mass and flower counts (equivalent to paired t-tests).

6.4 Results

6.4.1 Nectar volumes

The weather was settled during nectar collection, and is shown in Table 6.1. Across all cultivars, the mean volume of nectar per flower secreted during the 24-hour interval was 0.94 μ l. Nectar volumes per flower showed substantial variation, with a coefficient of variation of 50.6 %. There was a difference between the cultivars in the volumes of nectar per flower ($F_{5,20}=5.86$, $P=0.002$, $n=6$). The cultivars SY Fighter and Rivalda had the greatest volumes of nectar per flower, while the cultivar Sesame had the smallest, producing on average only around half the volume over the 24 hour period (Figure 6.3a).

The ANOVA table (Table 6.2) shows that a difference was not observed between columns in the Latin square design ($F_{5,20}=0.57$, $P=0.723$, $n=6$). However, there was a difference between rows ($F_{5,20}=10.00$, $P<0.001$, $n=6$), which accounts for variation in both the spatial layout of plots, and the times of day at which nectar was collected. There was a general increase in nectar volumes per flower from the first rows, where samples were collected in the morning, to the last rows, which were visited later in the day (Figure 6.3b).

Table 6.1. Local weather data for Rothamsted Research for the period in which oilseed rape nectar was collected

Time	Temperature (°C)	Relative humidity (%)	Solar Radiation (W m ⁻²)	Wind Speed (m s ⁻¹)
11:00	12.7	63.6	679.3	3.6
12:00	13.6	64.3	774.0	3.6
13:00	14.6	61.2	716.0	3.3
14:00	14.7	60.5	479.7	3.4
15:00	15.0	60.7	525.9	3.3
16:00	14.9	61.7	305.7	3.3
17:00	14.6	62.6	217.7	3.1
18:00	13.7	64.5	95.3	3.3

Table 6.2. ANOVA table for the Log10-transformed nectar volumes per flower from oilseed rape field trial with six cultivars

Source of variation	Degrees of freedom	Sums of squares	Mean squares	F	P
Row	5	0.095	0.019	0.57	0.723
Column	5	1.661	0.332	10.00	<0.001
Cultivar	5	0.974	0.195	5.86	0.002
Residual	20	0.665	0.033		
Round	72	2.330	0.032		
Total	107	5.724			

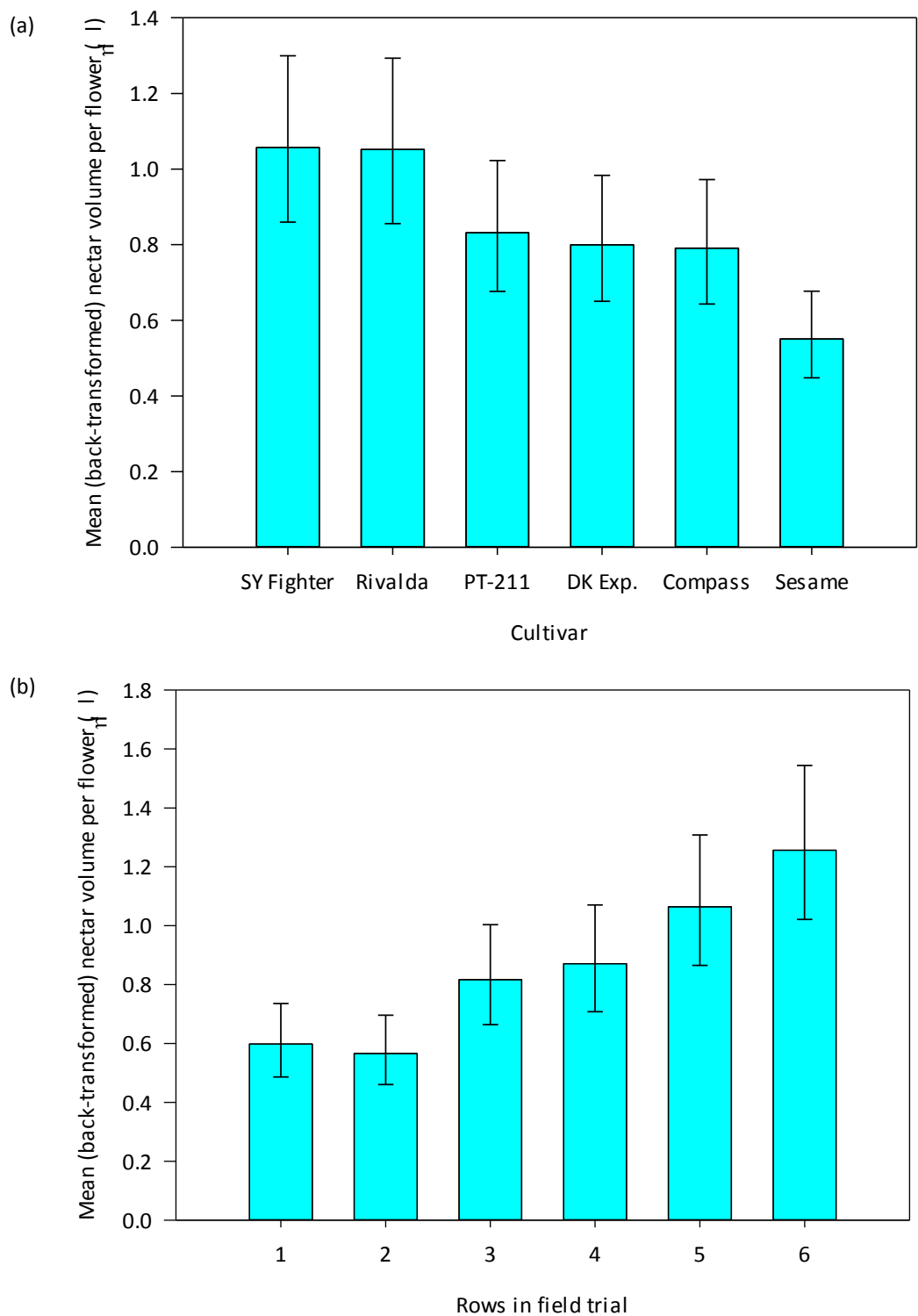


Figure 6.3. Mean (back-transformed) volumes of nectar per flower secreted in 24 hours by (a) six oilseed rape cultivars in the field trial (n=6), (b) six rows of the Latin square, shown in the order in which rows were visited to collect nectar (n=6). Bars show 95 % confidence intervals.

6.4.2 Nectar sugar concentration and amount per flower

Across all nectar samples, the mean concentration of sugar was 353 g per litre (35.3 % w/w). The nectar sugar concentration did not vary between different cultivars ($F_{5,20}=2.06$, $P=0.113$, $SED=26.02$, $n=6$). However, there was a difference between in sugar concentration between rows ($F_{5,20}=5.18$, $P=0.003$, $SED=26.02$, $n=6$; Figure 6.4). The nectar sampled from the first four rows had a similar mean sugar concentration, but there was a decrease in the sugar concentration of the nectar collected in the fifth and sixth rows. By weight, the mean composition of sugars in all nectar samples was 58.2 % glucose, 41.2 % fructose and 0.6 % sucrose.

The mean mass of sugar per flower measured across all cultivars was 304 μg secreted over 24 hours. There was a difference between cultivars in their mean sugar mass per flower ($F_{5,20}=7.17$, $P<0.001$, $n=6$; Figure 6.5a). Per flower, the cultivar that produced the greatest mass of nectar sugar was Rivalda, and the cultivar that produced the least was Sesame. There was also a difference in sugar mass per flower between the plants according to the row of the trial ($F_{5,20}=10.42$, $P<0.001$, $n=6$; Figure 6.5b). Flowers in rows 1 and 2, from which nectar was collected before 13:33, had a lower mean mass of nectar sugar than flowers in rows 3-6, from which the nectar was collected between 14:06 and 18:11.

6.4.3 Pollen grains per flower

Oilseed rape flowers had a mean of 145950 pollen grains per flower. The coefficient of variation was 16.8 %. There was a difference between the numbers of grains in flowers of different cultivars ($F_{5,19}=10.19$, $P<0.001$, $SED=11.01$, $n=6$; Figure 6.6). The cultivar Sesame had the highest mean number of pollen grains per flower, and DK Expower had the lowest.

As with nectar volumes, there was a difference in pollen grain numbers from flowers taken from plots in different rows ($F_{5,19}=5.06$, $P=0.004$, $SED=11.01$, $n=6$).

The mean values were similar in rows 1-4, but there was a smaller mean number of pollen grains in flowers from row 5, and a larger mean number of pollen grains in flowers from row 6. A difference was not observed in numbers of pollen grains per flower associated with columns in the field trial ($F_{5,19}=0.85$, $P=0.53$, $SED=11.01$, $n=6$).

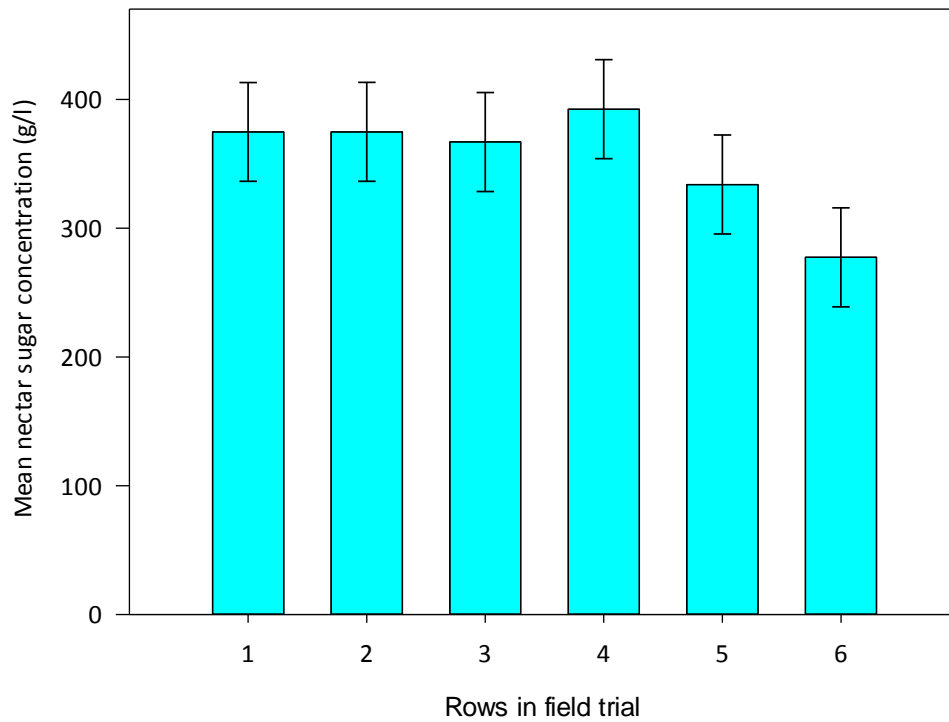


Figure 6.4. Mean sugar concentrations of oilseed rape nectar collected from six rows of the Latin square, shown in the order in which it was collected ($n=6$). Bars show 95 % confidence intervals.

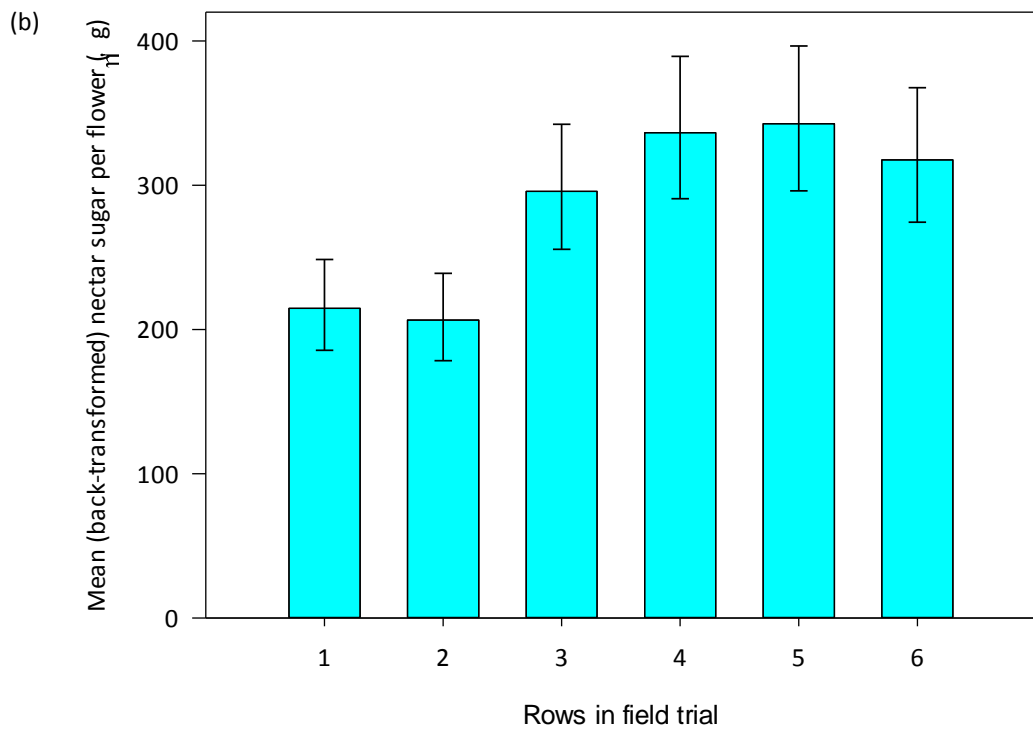
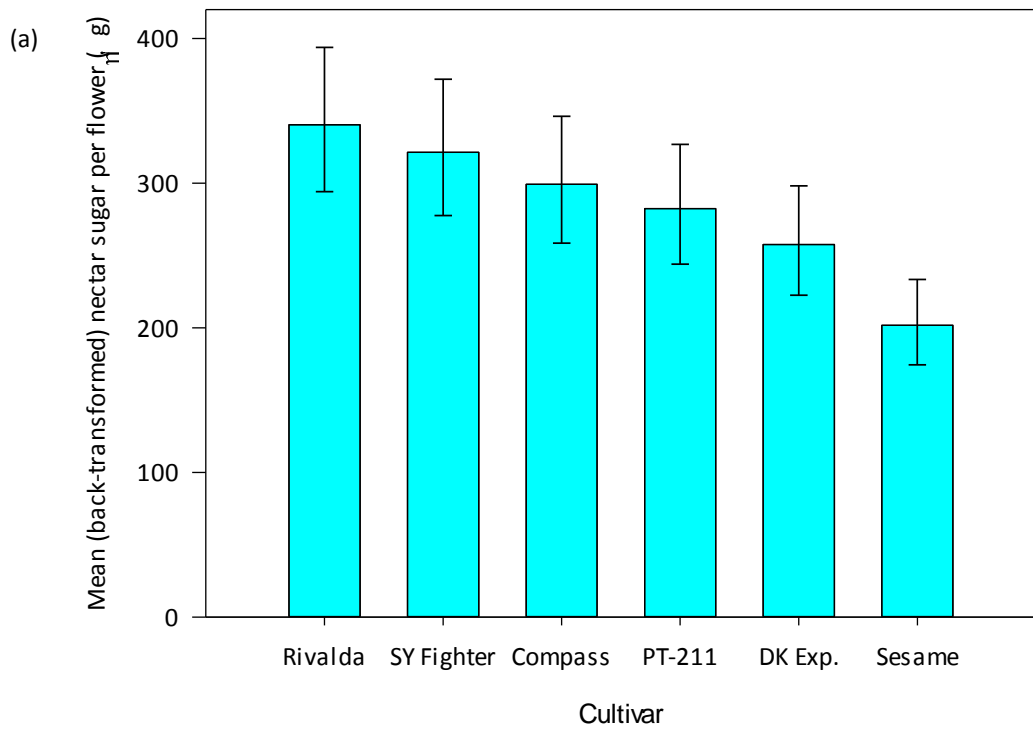


Figure 6.5. Mean total mass of nectar sugar per flower from (a) six oilseed rape cultivars (n=6). (b) Oilseed rape flowers from six rows of the Latin square. Bars show 95 % confidence intervals.

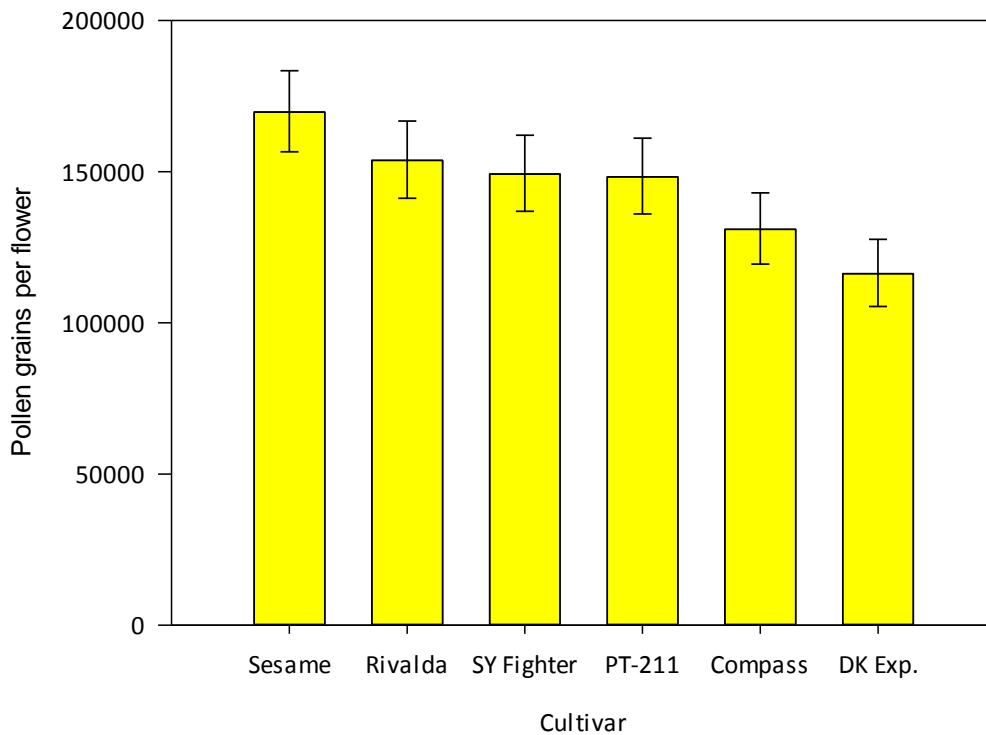


Figure 6.6. Estimated pollen grains per flower (square root) in six oilseed rape cultivars in the field trial (n=6). Bars show 95 % confidence intervals.

6.4.4 *Bumble bee colony assay*

The mean mass of the bumble bee colonies assigned to plots of oilseed rape cultivars SY Fighter or Sesame were not different at the beginning of the trial ($F_{1,5}=0.17$, $P=0.697$, $SED=2.67$, $n=6$). After one week, all colonies in both treatments had lost mass, but a difference in mass was not observed between colonies foraging on the two cultivars ($F_{1,5}=4.79$, $P=0.080$, $SED=4.85$, $n=6$). Colonies then gained mass, and after the second week there was a difference between the mass of colonies foraging on the two cultivars ($F_{1,5}=31.06$, $P=0.003$, $SED=1.82$, $n=6$). Colonies assigned to plots of cultivar SY Fighter heavier than those on cultivar Sesame. At the end of the third week, there was still a difference in the mass of colonies kept on plots of the two cultivars ($F_{1,5}=52.33$, $P=0.001$, $SED=1.67$, $n=6$). The mean mass of the colonies foraging on cultivar SY Fighter was 12.6 greater than that of the colonies foraging on cultivar Sesame (Figure 6.7a).

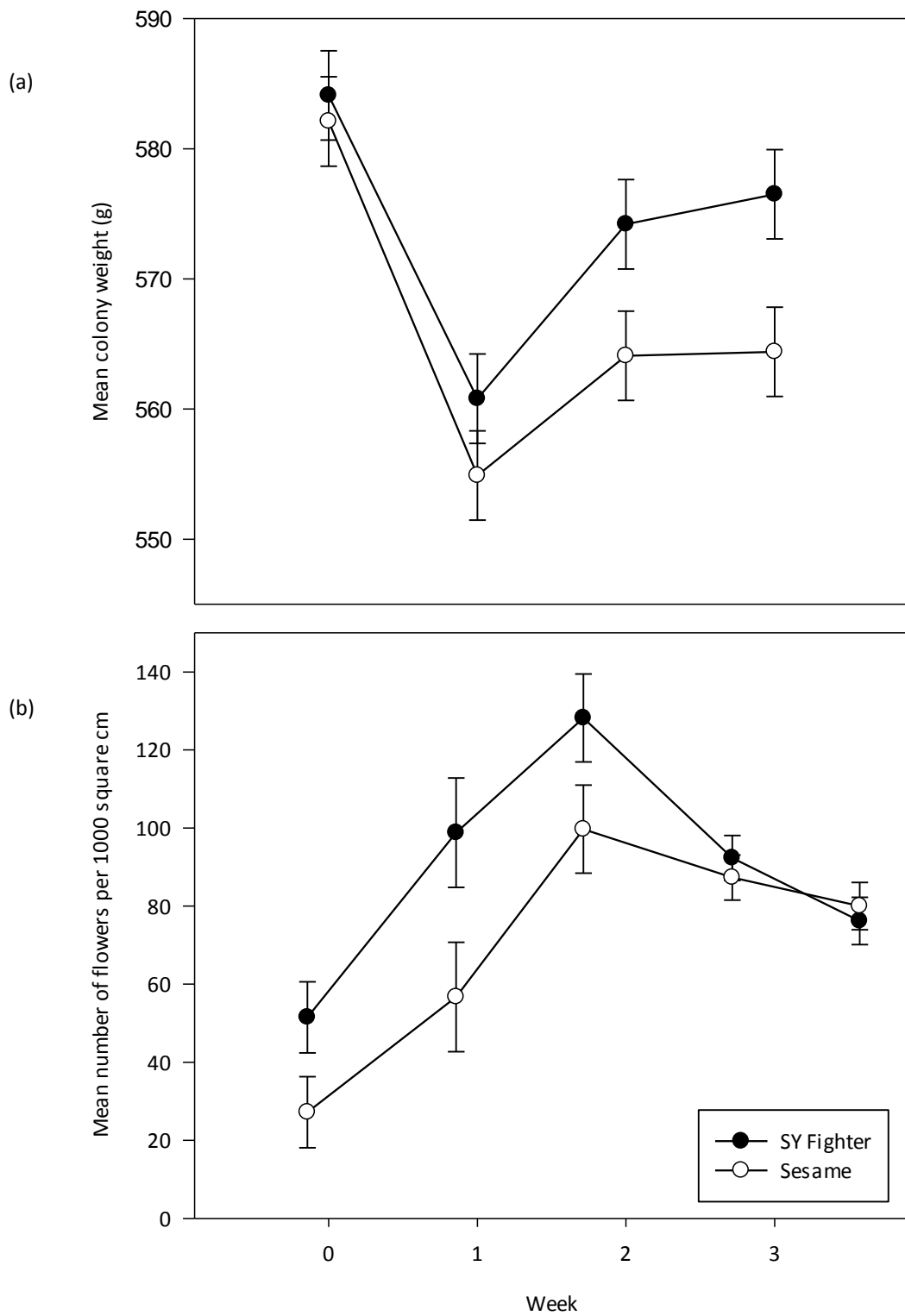


Figure 6.7. (a) Mean mass (\pm SEM) of *Bombus terrestris* colonies restricted to foraging on one of two oilseed rape cultivars – SY Fighter (black circles) or Sesame (white circles) for three weeks ($n=6$). **(b)** Mean numbers of oilseed rape flowers (\pm SEM) counted in 1000 square cm quadrats in plots of cultivars SY Fighter (black circles) and Sesame (white circles) ($n=6$).

During the trial, the cultivar SY Fighter tended to have more flowers than Sesame in the plots, though differences were not statistically significant in any comparison (Table 6.3) (Figure 6.7b).

Table 6.3. Mean numbers of flowers per 1000 square cm in plots of two oilseed rape cultivars

Date	Cultivar		SED	F _{1,5}	P
	SY Fighter	Sesame			
10 th April	51.5	27.2	12.90	3.56	0.118
17 th April	98.8	56.7	19.81	4.53	0.087
23 rd April	128.2	99.7	15.97	3.18	0.134
30 th April	92.3	87.3	8.14	0.38	0.566
6 th May	76.2	80.0	8.56	0.20	0.673

6.5 Discussion

6.5.1 Nectar volumes

There was a difference between the volumes of nectar per flower secreted over 24 hours by the six oilseed rape cultivars tested, with the cultivar Sesame producing smaller volumes than the others (Figure 6.3a). The finding supports an earlier study carried out in glasshouse conditions (Chapter 5), in which the cultivar Sesame also produced smaller volumes of nectar than some other cultivars. Previous studies of nectar production in oilseed rape have also shown that nectar volumes can vary between cultivars (Pierre *et al.* 1999).

There was almost a two-fold difference between the mean volume of nectar secreted by the cultivar Sesame (0.55 µl) and that of the cultivar with the greatest nectar volumes, SY Fighter (1.05 µl) (Figure 6.3a). However, between the individual plants from which nectar was taken, mean volumes per flower ranged from 0.15 µl to 2.58 µl per flower, a 17-fold difference. Had the nectar

volumes of individual flowers been recorded separately rather than pooled within plants, the range between the flowers with the smallest and the greatest volumes must have been greater still. Such wide variation is typical in studies of floral nectar production (e.g. Boose 1997; Real & Rathcke 1988; Zimmerman & Pyke 1986), and as a result, any cultivar differences may only be detected with large sample sizes. A literature survey of intraspecific variation in flowers found nectar properties to be the most variable by far of all floral characteristics included (Cresswell 1998), with a similar mean coefficient of variation (54 %) to that reported here (50.6 %). Variation in nectar rewards could be adaptive for plants, as pollinators visit fewer flowers when rewards are variable (Biernaskie *et al.* 2002), so that more pollen grains are transported to different plants, and stigmas avoid saturation with pollen from a single individual (Rathcke 1992).

In the present study, flowers in rows of the trial sampled later in the day tended to have greater volumes of nectar (Figure 6.3b). The nectar from the rows sampled later had lower mean sugar concentrations (Figure 6.4), but total amounts of sugar were higher (Figure 6.5b). It is not possible to determine whether the difference seen between rows were due to the time of day at which the nectar was taken, contrasting soil conditions between the rows, or a combination of both factors. Differences in soil moisture and nutrient levels can influence nectar production (Baude *et al.* 2011; Burkle & Irwin 2009), so a gradient in these conditions could explain the difference between rows. Alternatively, the time of nectar collection could explain the difference found in the nectar between rows. Temperature (Jakobsen & Kristjansson 1994) and relative humidity (Corbet *et al.* 1979) can influence nectar production, while wind speed could influence evaporation, but these were relatively consistent during nectar collection. Solar radiation that plants received while nectar was collected from the first row was eight times that which they received during nectar collection from the last row, yet total nectar sugar decreased during this

period. Therefore changes to environmental conditions over time do not explain the difference in nectar collected from different rows.

Pernal and Currie (1998) found that greater amounts of nectar sugar per flower were available in the afternoon than in the morning in undisturbed oilseed rape flowers across a range of cultivars. The finding could indicate that a peak of nectar sugar in the afternoons represents a diurnal pattern of nectar production in oilseed rape.

While all plants in the present study had an interval of 24 hours between the initial draining and subsequent sampling of their nectar, they may have replenished the nectar available relatively quickly rather than at a continuous, gradual rate, and might then have adjusted available nectar according to this diurnal pattern.

However, the increase in total nectar sugar with time of day observed by Pernal and Currie (1998) and in the present study contrasts with other findings. Burquez and Corbet (1991) showed that undisturbed oilseed rape flowers (cultivar Maris Haplona) over a day old had less available nectar sugar with time, due to reabsorption by the plants. Mohr and Jay (1990) also found that total nectar sugar per flower in undisturbed oilseed rape (cultivar Regent) flowers decreased after midday, though concentrations remained fairly constant, while volumes of nectar declined.

If the flowers in the present study did replenish nectar quickly after it was drained, and then adjusted nectar volumes according to their normal daily pattern, the precaution taken of draining all flowers of nectar before collecting samples may have been unnecessary. However, the difference observed between rows sampled at different times underscores the importance of taking groups of samples from each treatment as close together in space and in time as possible, in order to provide a fair comparison between treatments. Had the

study been performed with each row planted with a single cultivar, the difference found in their nectar would be exaggerated.

6.5.2 *Nectar sugar concentration and amount*

Nectar sugar concentration did not differ between cultivars (Figure 6.4). A previous study on oilseed rape nectar also found that sugar concentration was consistent between cultivars (Pierre *et al.* 1999). Although Szabo (1982) reported a difference in sugar concentration among fifteen lines of oilseed rape, the nectar was extracted by centrifugation, which was later shown to be an inappropriate technique for oilseed rape flowers, resulting in inaccurate estimates of sugar concentrations (Mesquida *et al.* 1988a). Real and Rathcke (1988) noted that variation in nectar concentrations generally appears smaller than variation in volume. In the few studies available (Campbell 1996; Mitchell & Shaw 1993), heritability in nectar sugar concentration was not detectable, suggesting there is little genetic variation in this trait, though large environmental variation can also result in lower heritability.

Total nectar sugar amount per flower is the product of volume and concentration. Since all cultivars had a similar concentration, the effect of cultivar on sugar amount closely resembles that on volumes, with a difference seen between cultivars (Figure 6.5a). The cultivar with the lowest sugar per flower, Sesame, had only around 60 % of that in the cultivar with the most, Rivalda. The findings suggest that pollinators foraging in a field planted with the cultivar Rivalda could collect more nectar sugar in a given time.

The protocol used to compare cultivars has the strengths that all flowers were: (a) of a similar age; (b) protected from insect visits and from which nectar had been removed only once at a fixed interval beforehand; (c) grown in replicate plots to prevent erroneous detection of variation between cultivars arising from differences between plots; and (d) visited close together in time for each cultivar. However, the results provide a single snapshot of nectar in the

cultivars near the start of the blooming season. Oilseed rape nectar sugar concentrations (Mesquida *et al.* 1991; Mohr & Jay 1990; Pierre *et al.* 1999) and sugar mass per flower (Pernal & Currie 1998) tend to decrease after the first two weeks of flowering, after which the differences between cultivars may not be consistent with those observed. In addition, the collection of nectar from a flower protected for 24 hours can underestimate its potential to produce nectar if visited more frequently (Burquez & Corbet 1991; Willmer 2011). Finally, the effect on nectar production by these cultivars of the different soils and geographic regions on which oilseed rape is grown cannot be predicted from this study.

6.5.3 *Pollen grains per flower*

The mean number of pollen grains per flower in this trial across all cultivars was 145 950. The number is consistent with work by Hinata and Konno (1975), who estimated the number of pollen grains per flower in *Brassica napus* (strain number N344), and found there were 110 000-120 000.

The cultivar Sesame had the highest mean number of pollen grains per flower, and DK Expower had the lowest (Figure 6.6). A possible explanation for the difference in pollen grains per flower between the cultivars could be the position of those flowers on the raceme. Hinata and Konno (1975) found that the earlier flowers at the basal part of the raceme had more pollen grains than those that opened later in several cultivars of *Brassica rapa* (*syn. campestris*), though the trend was not detected in some other *Brassica* species. The location on the raceme of flower buds used in the present study was not recorded, but an estimate of the advancement in flowering across each plot was made the day before buds were taken using the BBCH Growth Stage Classification (Lancashire *et al.* 1991). That assessment showed that flowering had progressed furthest in plots of DK Expower, the cultivar with the fewest pollen grains per flower, which lends some support to this idea. Were the study to be repeated, it

would be advisable to collect flowers at comparable growth stages from each plant, perhaps at several points during flowering, rather than on the same day.

Cresswell *et al.* (2004) used a particle counter to estimate that pollen grains of oilseed rape (cultivar Westar) have a mean mass of 1.57×10^{-8} g. Assuming that pollen grains counted represent reasonable estimates of the numbers per flower found in the different cultivars, and assuming the cultivars in the present study have pollen grains with a similar mass to cultivar Westar, it is possible to estimate the mass of pollen that might be produced by the cultivars at different scales. In previous work performed in a glasshouse (Chapter 5), the number of flowers per plant was counted for a range of cultivars. These numbers are likely to be greater than would be produced in field conditions, as the still air and absence of pollinators in the glasshouse may cause insufficient pollination in oilseed rape, which plants respond to with the production of more flowers (Sabbahi *et al.* 2006; Williams *et al.* 1986). With this caveat, the cultivar Sesame is estimated to produce a total of 2.3 g of pollen per plant over the blooming period, while the estimate for the cultivar SY Fighter is 1.2g (Table 6.4).

Only a part of this pollen will be available to pollinators. Although pollen grains are not easily dislodged from anthers by the wind in oilseed rape, on dry days the movement of insect visitors can release a cloud of pollen which is lost to the atmosphere (Eisikowitch 1981). Additionally, in a range of plant species, around 27 % of the total pollen from a flower is available to be collected at a given time, with the remaining part either still in the anthers, or having been removed by previous visitors. (Muller *et al.* 2006). By comparison with the pollen estimates in Table 6.4, when returning to the nest, pollen loads carried by workers in the bumble bee *Bombus vosnesenskii* were found to have a mean mass of 0.021 g (Allen *et al.* 1978), though nectar accounts for a part of this mass, as it is added by bumble bees to their pollen loads.

Table 6.4. Estimated production of pollen by oilseed rape cultivars

Cultivar	Grains per flower	Pollen per flower (g)	Flowers per plant	Pollen per plant (g)
Sesame	169 744	0.0027	852	2.3
PT-211	148 302	0.0023	629	1.5
DK Exp.	116 281	0.0018	797	1.5
Rivalda	153 742	0.0024	580	1.4
Compass	130 972	0.0021	663	1.4
SY				
Fighter	149 228	0.0023	532	1.2

6.5.4 *Bumble bee colony assay*

As the oilseed rape cultivar SY Fighter secreted greater volumes of nectar than cultivar Sesame, it was hypothesised that bumble bee colonies that foraged exclusively on the former cultivar would become heavier than those foraging on the latter. As expected, after two weeks, bumble bee colonies feeding only on cultivar SY Fighter had a greater mean mass than those feeding on cultivar Sesame. The difference in mass was maintained so that by the following week, it was around 12 g, which, after subtracting the plastic boxes containing each nest, accounted for around 16 % of the total mass of the colonies. The difference in mass between nests foraging on the two cultivars is likely due to the greater nectar production of cultivar SY Fighter than Sesame, as discussed above. The mean density of open flowers was also higher in plots of cultivar SY Fighter than Sesame, which, although differences were not statistically significant, may also have contributed to the disparity of colony mass between the two treatments.

The trial shows that the foraging success of bumblebee colonies is affected by the oilseed rape cultivar to which they had access. The result indicates that wild bumble bee colonies foraging in landscapes where more rewarding oilseed rape cultivars are planted, such as cultivar SY Fighter, are likely to experience greater colony growth than those in landscapes with resource-poor cultivars. Greater resource intake improves colony size and chances of reproduction (Thomson 2004).

It was shown previously that the cultivar Sesame produces high numbers of pollen grains per flower relative to the other cultivars. However, few workers were seen with pollen loads during the study. Plots in the trial were almost certainly too small to provide sufficient quantities of either nectar or pollen to meet the needs of the colonies, as weight gain was an order of magnitude smaller than in free-flying colonies (Goulson *et al.* 2002). The scarcity of resources may explain the limited foraging for pollen that was observed. Plowright and Silverman (2000) also found that, when deprived of both pollen and nectar, colonies of the bumble bee *Bombus impatiens* prioritised the collection of nectar over pollen, possibly because shortages of nectar immediately threaten the persistence of the colony (Cartar & Dill 1991).

Prior to placing colonies inside the pollination cages, all had access to the sugar syrup feeders provided by the suppliers, and had filled many of the honey pots in their nests with it. These stores were no longer visible when colonies were weighed after the first week, and their consumption explains why the mass of all colonies decreased in that period, as the workers learnt to forage. Some mass may also have been lost in the first week if the inexperienced bees were harmed by colliding with the cages.

The estimates of nectar secretion by the oilseed rape cultivars in the present study show the production of nectar by flowers over 24 hours while protected by bags. However, a single collection after 24 hours may give a lower estimate

of nectar production than the summed values of more frequent sampling of the same flowers over that time (Willmer 2011). The reason is that the nectar available in a flower can level off or even decrease over extended periods, as sugar is reabsorbed by the plant at the same rate, or a greater rate, than it is secreted (Burquez & Corbet 1991). As the bumble bees probably visited all open flowers in the pollination cages many times in 24 hours, the greater colony mass of those feeding on cultivar SY Fighter than Sesame is evidence that the rate at which nectar is secreted, rather than the maximum level in unvisited flowers, is greater in the former cultivar.

6.5.5 Conclusions

Fields of oilseed rape provide nectar and pollen that are valuable for pollinators, but cultivars of the crop vary in the rewards provided by their flowers. This study extends work that was performed in a glasshouse (Chapter 5) to show that in field conditions, pollinators that visit a highly rewarding cultivar, like the cultivar SY Fighter, may obtain twice the nectar sugar from each flower than those that visit a less resource rich cultivar, like the cultivar Sesame. The difference seen between cultivars in their nectar rewards translated into a measurable disparity in the mass of bumble bee colonies, which could increase colony size, and so enhance the provision of pollination and the chances of successful reproduction where more rewarding cultivars are grown.

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Chapter 7. General Discussion

Short rotation coppice (SRC) willow and oilseed rape bloom in spring, when insect pollinators visit their flowers to collect nectar and pollen. This thesis presents evidence that the rewards visitors find when they arrive at flowers of these crops depends, in part, on the cultivar grown. In particular, SRC willow cultivars Ulv and Olof secrete, on average, a much greater mass of nectar sugar per plant than those of the cultivars Stott-10, Terra Nova, Loden and Resolution (Chapter 3). The cultivars Ulv and Olof also produce a greater mass of pollen per plant than the cultivar Loden, while the female cultivars produce none (Chapter 3). In oilseed rape, the major difference between cultivars in the floral rewards offered was in the mass of nectar sugar secreted. Many cultivars offered similar masses of nectar sugar per flower, on average, but the cultivar SY Fighter tended to produce more than the cultivar Sesame, both in glasshouse (Chapter 5) and field (Chapter 6) conditions. The mass of bumble bee colonies that foraged exclusively the cultivar SY Fighter exceeded that of colonies feeding on Sesame after two weeks (Chapter 6). However, pollen amino acid composition showed little variation between different cultivars in either SRC willow or oilseed rape. This chapter discusses the wider implications of these findings.

7.1 Ecological effects of using rewarding mass-flowering crop cultivars

Declines of many pollinator species threaten production of many crops and reproduction in wild flowers (Garibaldi *et al.* 2013; Ollerton *et al.* 2011). Limited availability of nutritional resources is a major driver of pollinator declines (Carvell *et al.* 2006; Potts *et al.* 2010a; Scheper *et al.* 2014), especially in areas dominated by agriculture (Goulson *et al.* 2002). Mass-flowering crops produce floral resources and are attractive to insect pollinators, some of which, like bumble bees, quickly learn where the most rewarding flowers are and forage efficiently to maximise their collection of resources (Dreisig 2012; Goulson 2003).

The availability of floral nectar could be increased in landscapes in which SRC willow or oilseed rape is planted by selecting the most rewarding cultivars. To predict the effects of such a substitution, landscapes may be compared that vary in the area planted with mass-flowering crops, as this also creates a contrast in resource availability, albeit with potentially confounding differences in nest-site availability and the degree of habitat fragmentation. Landscapes in which a greater area is planted with mass-flowering crops have higher bee densities during and after flowering (Herrmann *et al.* 2007; Westphal *et al.* 2003), which increases rates of pollination in later-flowering crops and wild plants (Riedinger *et al.* 2014). High mass-flowering crop coverage in one year also leads to increased population density in the following year for solitary bees (Holzschuh *et al.* 2013; Jauker *et al.* 2012; Riedinger *et al.* 2015), though not for bumble bees (Westphal *et al.* 2009; Williams *et al.* 2012).

However, in landscapes with greater mass-flowering crop coverage, co-flowering wild plants may suffer pollination deficits due to competition with the crop for pollinators (Holzschuh *et al.* 2011). Also at a disadvantage from mass-flowering crops are the rarer pollinator species with specialist diets; they benefit less from the crop than generalist pollinators, and therefore encounter greater competition for food once blooming ceases in areas where mass-flowering crops are planted (Diekötter *et al.* 2010). Finally, mass-flowering crops expose foragers to potentially harmful agrochemicals, including neonicotinoid insecticides where they are used to treat seeds (Lundin *et al.* 2015; Rundlof *et al.* 2015).

These general impacts of mass-flowering crops result from the nutritional resources they provide for pollinators, and are likely to be enhanced by planting more rewarding cultivars. A consequence of planting those mass-flowering crop cultivars that offer greater rewards is that common, generalist pollinator species with the capacity to disperse and take advantage of additional food resources are likely to benefit, while rare, specialist pollinator

species are not (Scheper *et al.* 2013). The planting of highly rewarding cultivars can therefore help ensure that crops receive adequate pollination and meet their potential yields, but is not sufficient to conserve biodiversity among pollinators and the plants they visit (Bommarco *et al.* 2013).

7.2 Comparing the value to pollinators of floral resources from crops

The most rewarding cultivar of SRC willow assessed in this thesis, Ulv, produced an estimated 3.0 g of nectar sugar and 4.4 g of pollen per plant (Chapter 3). At a density of 15 000 plants per hectare, the estimated floral resources produced are 45 kg of nectar sugar and 66 kg of pollen per hectare. In the oilseed rape field trial (Chapter 6), the cultivars Rivalda and SY Fighter produced the greatest mass of nectar sugar per flower, and were among the three cultivars with the most pollen grains per flower. They produced around 330 µg nectar sugar and 2.4 mg of pollen per flower, using a mass of 1.57×10^{-8} g per pollen grain (Cresswell *et al.* 2004). Scaling up according with estimates of 375 flowers per plant, and 56×10^4 plants per hectare (Nedic *et al.* 2013), yields estimates of 69 kg nectar sugar and 500 kg pollen per hectare.

By these calculations, one hectare planted with oilseed rape provides more nectar sugar and far more pollen than the equivalent area planted with SRC willow. Would pollinators be better served by a hectare planted with oilseed rape than one planted with willow on this basis? Several other factors influence the value to a given area to insect flower visitors besides the quality and quantity of the nectar and pollen provided, and these must also be considered. Firstly, SRC willow typically flowers in March, while oilseed rape flowers in April. Species that begin foraging in March, as some bumble bee queens do (Alford 1975), may therefore benefit more from a field of willow than oilseed rape, with enhanced resources available in early spring increasing the survival and growth rates of incipient colonies (Goulson 2003). Secondly, the value to pollinators of enhanced nutritional resources depends on the availability of

alternative sources of nectar and pollen in the surrounding landscape (Carvell *et al.* 2015; Carvell *et al.* 2011; Scheper *et al.* 2015; Scheper *et al.* 2013). As willows are among the earliest-flowering plants visited by bees (Proctor *et al.* 1996), they are likely to have a high value to foraging pollinators. During oilseed rape flowering, many other plants are also in flower (Garbuzov *et al.* 2015) to offer nectar and pollen, not least other fields of oilseed rape. As SRC willow and oilseed have flowering periods that almost overlap, an area planted with both crops would provide nectar and pollen over a longer period than either in isolation, so a combination of both may be optimal for pollinator nutrition. Likewise, landscapes in which a mix of earlier and later flowering cultivars create patches of floral resources over an extended period may offer superior nutritional value for pollinators than a single recommended cultivar (Reddersen 2001). Alternative approaches, such as the modelling of honey bee foraging and colony dynamics (Becher *et al.* 2014), or the decoding of honey bee waggle dances (e.g. Couvillon *et al.* 2014), could further identify the optimal combination of floral resources for pollinator nutrition.

7.3 Increasing the consideration given to pollinator nutrition in agriculture

In the UK, annual estimates of the land area planted with various crops are published by the Department for Environment, Food and Rural Affairs (Defra), using data collected from growers using online surveys. However, information about which cultivars are planted is not collected. It is therefore unclear how much potential exists to increase nutritional resources for pollinators by using the most rewarding cultivars.

Several measures could be implemented that may increase uptake of the cultivars with the greatest floral rewards. Firstly, access to information is required on the relative value to pollinators of each cultivar at the time when planting decisions are taken. Crops are currently grown in standardised trials,

with information on their yield potential and agronomic characteristics obtained from these trials presented to growers in independent variety guides (e.g. AHDB Recommended Lists). Extending these trials and variety guides to include a simple measure of nectar and pollen production would allow growers to consider these traits when selecting cultivars. The work presented in this thesis indicates that the total concentrations of nectar sugar are less variable than the volumes of nectar secreted over a 24 hour period. As a result, analysis of nectar concentrations could be dispensed with, in the interests of providing a quick and simple means to compare the nectar production between cultivars. A realistic and informative procedure could involve bagging stems, collecting nectar the following day from a standard number of flowers with microcapillary tubes, and then scoring plants according to a visual assessment of the volume of nectar within the microcapillary tube. For the sake of simplicity, this approach ignores many factors that can influence nectar production (see Chapter 1), but there are two factors that should be controlled to make meaningful comparisons. Firstly, nectar available after 24 hours in oilseed rape flowers varies strongly with time of day at which it is collected (Chapter 6), so cultivars should be compared at a similar time. Secondly, the crops must not be exposed to precipitation during the period of nectar secretion and collection.

Differences in pollen composition were not detected among cultivars of SRC willow and were minor among cultivars of oilseed rape (Chapters 3 and 5). Quantitative differences in pollen production per plant were largely determined by the number of catkins per plant in SRC willow (Chapter 3), and the number of flowers per plant in oilseed rape (Chapter 6). While numbers of flowers per plant in oilseed rape is highly dependent on conditions (Cresswell *et al.* 2001), SRC willow cultivars display large and characteristic differences in the number of catkins per plant (Chapter 3), which are also relatively easy to quantify. The mean number of flowers found on SRC willow plants ranged

from 13 catkins per plant in cultivar Loden, to 330 in cultivar Ulv (Chapter 3). Clearly, the sex of SRC willow cultivars also determines their ability to produce pollen. For willow growers, then, a visual assessment of the number of catkins per plant, perhaps estimated to the nearest 100, along with the sex of the cultivar, provides a useful indication of its value to pollinators as a source of pollen. The number of catkins per plant increases with the number of years since it was coppiced (Reddersen 2001), so comparisons should be made between plants with a similar management history. For oilseed rape growers, the value of information on pollen production is unlikely to justify the effort of obtaining it, provided that plants of each cultivar are male-fertile, unlike some varietal associations.

Simple and standardised comparisons of nectar volumes in SRC willow and oilseed rape cultivars, like those described above, are feasible. Presented to growers, alongside the number of catkins per plant and the sex in SRC willow cultivars, these data would enable farmers, or others making planting decisions, to consider pollinator nutrition. In selecting cultivars, growers are likely to prioritise financial considerations, such as crop quality and yield, and to evaluate the suitability of cultivars for conditions in their fields. If no loss in revenue is incurred by selecting cultivars on the basis of their value to pollinators, some growers may opt to plant more rewarding cultivars, according to their personal values. Correlations between mass of nectar sugar per flower and seed yields in Recommended List trials were not detected in the oilseed rape cultivars studied in this thesis (Chapter 5).

With information routinely gathered on the floral resources provided by mass-flowering crop cultivars, a second measure may increase planting of the most rewarding cultivars: the use of incentives for growers. EU farmers receive payments administered through the Common Agricultural Policy (CAP), in return for which they must meet certain requirements (Allen *et al.* 2014). In addition to these payments, further funding is available for farmers who adopt

agri-environment schemes, which are intended to deliver benefits for wildlife and to enhance the provision of ecosystem services. Agri-environment schemes include creating flower strips to increase the biomass of male and queen bumble bees (Carvell *et al.* 2015). The specifications of agri-environment schemes are set individually by EU Member States, and in the UK, requirements are set by the devolved administrations for England, Scotland, Wales and Northern Ireland. The devolved administrations are therefore free to use part of the CAP budget to reward farmers for selecting crop cultivars offering the greatest floral rewards for pollinators.

A possible alternative to incentives provided by subsidising growers is to offer a higher price for products from cultivars with greater floral rewards. Oilseed rape cultivars that produce oils high in oleic and low in linolenic acid (HOLL cultivars) currently command a 10 % premium from seed crushers, on account of the purported health benefits of their oil (Jones 2015). As pollinators are charismatic insects, there is considerable public interest in their wellbeing (Ross & Wentworth 2010), and consumers may be willing to pay a premium for vegetable oil marketed as 'pollinator-friendly', as they do for products certified as organic or fair-trade.

A third approach to increasing the use of highly rewarding cultivars of mass-flowering crops is to encourage plant breeders to develop all varieties with greater potential for nectar and pollen production. Although Kamler (1984) showed that it was possible to increase nectar production in oilseed rape through breeding, previous recommendations for plant breeders to consider this trait (Allen-Wardell *et al.* 1998; Shuel 1989) have been ignored. Testing of cultivars and publication of their relative value as sources of nutrition for pollinators could increase the efforts of plant breeders to improve crops in this regard.

7.4 Concluding Remarks

Both SRC willow and oilseed rape provide valuable resources for insect pollinators in early spring. This work demonstrates that an opportunity exists to increase the benefits that these crops offer to insect pollinators. By choosing to plant the most rewarding cultivars identified in this thesis, growers can help to maintain vital pollination services and contribute to protecting biodiversity, while still using land productively.

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