IMPACTS OF OZONE POLLUTION ON NECTAR AND POLLEN QUALITY AND THEIR SIGNIFICANCE FOR POLLINATORS

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I hereby declare that this thesis is based on work conducted by myself and has not contributed to any other degree. Reference to ideas and work of others has been specifically acknowledged.

Abstract

This thesis explores the impacts of ozone pollution on the nutritional quality of nectar and pollen. We report the use of HPLC and HPIC to quantify the amino acids and carbohydrates in nectar and the development of a microwave-assisted acid hydrolysis to quantify the protein-bound amino acids available in pollen, allowing back-calculation to estimate total protein content.

Utilising ten cultivars of broad bean (*Vicia faba* L.), exhibiting considerable variation in response to ozone, we explored what parameters are meaningful when attempting to determine the impacts of ozone (reductions in biomass, seed yield etc.) and whether these measures are consistent with the influence of the pollutant on pollen quality. We found little correlation between impacts on pollen quality and any usually-measured plant traits affected by ozone. We concluded that ozone influences pollen qualities in two ways; (i) exposure to ozone during plant growth influences the allocation of proteins to pollen, and (ii) ozone may cause direct oxidative damage to pollen once dehisced from anthers.

We investigated the impact of ozone on the allocation of amino acid and carbohydrate resources to nectar and pollen, using broad bean (*Vicia faba* L.) as a convenient model. Plants grown in O_3 and exposed to charcoal/purafil® filtered air (CFA) at flowering allocated significantly more sucrose and amino acids into their nectar than plants in other fumigation treatments. We discovered a reduction in the amount of free amino acids recoverable from the outer surface of the pollen in all treatments subject to O_3 exposure, but most significant in plants maintained throughout in O_3. We also found a significant shift in the proportions of amino acids in the respective ozone treatments.

The final experimental thesis chapter explored the potential impact of ozone-induced changes in nectar quality on bee behaviour. Simulated ozone-induced changes in nutrient composition of nectar were employed in an olfactory conditioning assay using honeybees. Bees trained with nectar matching that of plants subject to O_3 and exposed to CFA at flowering demonstrated an initially improved rate of learning, but association with reward decreased rapidly, whereas those trained with nectar matching that of plants from CFA was sustained.

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Table of Contents

Publications

Abbreviations

µl	Microlitre
µm	Micrometre
AA	Amino acid
ANOVA	Analysis of variance
BSA	Bovine Serum Albumin
CCD	Colony collapse disorder
CDA	Canonical discriminant analysis
CFA	Charcoal filtered air
CHO	Non-structural carbohydrate
cm^3	Cubic centimetre
CO_2	Carbon dioxide
CS	Conditioned stimulus
d	Day
dm^3	Litre
dm^3	Cubic decimetre
EFN	Extrafloral nectar
FMOC	Fluoronylmethoxycarbonyl
FN	Floral nectar
g	Gram
g	Centrifugal force
h	Hour
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HPIC	High performance ion chromatography
HPLC	High performance liquid chromatography
ITI	Inter-trial interval
K	Allometric root/shoot coefficient
Logreg	Logistic regression
LSD	Least significant difference
m	Metre

M	Molar
MANOVA	Multivariate analysis of variance
MeOH	Methanol
mg	Milligram
min	Minute
mM	Millimolar
mol	Moles
MPA	3- mercaptopropionic acid
N	Nitrogen
$Na_2B_4O_7•10\ H_2O$	Sodium tetraborate decahydrate
Na_2HPO_4	Di-sodium hydrogen orthophosphate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
ng	Nanogram
NO_x	Nitrogen oxides
O_2	Oxygen
$‘O_2$	Peroxyl radical
O_2^-	Super oxide
O_3	Ozone
OPA	O-pthaldialdehyde
OTCs	Open top chambers
PCA	Principal components analysis
PER	Proboscis extension reflex
PLC	Programmable Logic Controller
ppb	Parts per billion
PPFD	Photosynthetic photon flux density
R	Plant relative growth rate
R%	Relative change in growth
R_{cfa}	Relative growth rate of plants in CFA
R_{O3}	Relative growth rate of plants in O_3
ROS	Reactive oxygen species
R_{root}	Relative growth rate of root

R_{shoot}	Relative growth rate of shoot
RT	Room temperature
RuBisCO	Ribulose-1,5-carboxylase/oxygenase
s	Second
SOG	Suboesophogeal ganglion
UHPLC	Ultra high performance liquid chromatography
US	Unconditioned stimulus
V	Volts
VOC	Volatile organic compound

1.0 General introduction

1.1 Background to ozone

Ozone (O_3) is a pungent, naturally-occurring three atom allotrope of atomic oxygen. It was its unique odour that led Swiss chemist, Schönbein, to its discovery and identification in the mid 19th century during electrolytic studies on the hydrolysis of water (Schönbein, 1841). He began detailed investigation and characterisation of the gas (he later named ozone) that was liberated at the positive electrode during his experimentation, noting that it could depolarise gold and platinum and suggesting that the gas could have negative impacts on organisms (Schönbein, 1844). He was correct and research subsequent to his discovery has identified ozone as an abundant and powerful oxidant, causing negative impacts to physical materials, animal health and vegetation (Ashmore, 2005).

Ozone is predominantly formed in the two atmospheric layers closest to the earth; the stratosphere, encompassing the air space 10 to 30 miles above the earth's surface, and the troposphere, the air space below the stratosphere, contacting ground level. Ozone in the stratosphere is formed by the photolysis of O_2 to atomic oxygen by UV light and is beneficial to life on earth by absorbing harmful UV radiation (Chameides and Lodge, 1992). Mixing of air between the stratosphere and troposphere results in a natural background concentration of ozone in the troposphere (reviewed by Vingarzan, 2004), but concentrations are influenced to a greater extent by anthropogenic influences which lead to unnaturally high ground-level concentrations of the pollutant *via* complex photochemical reactions involving oxides of nitrogen (NO_x) and volatile organic compounds (VOCs) released by a myriad of sources, particularly the burning of fossil fuels, which are favoured by meteorological conditions i.e. high levels of irradiation, favourable temperatures, dry and stagnant air masses (Figure 1.1).

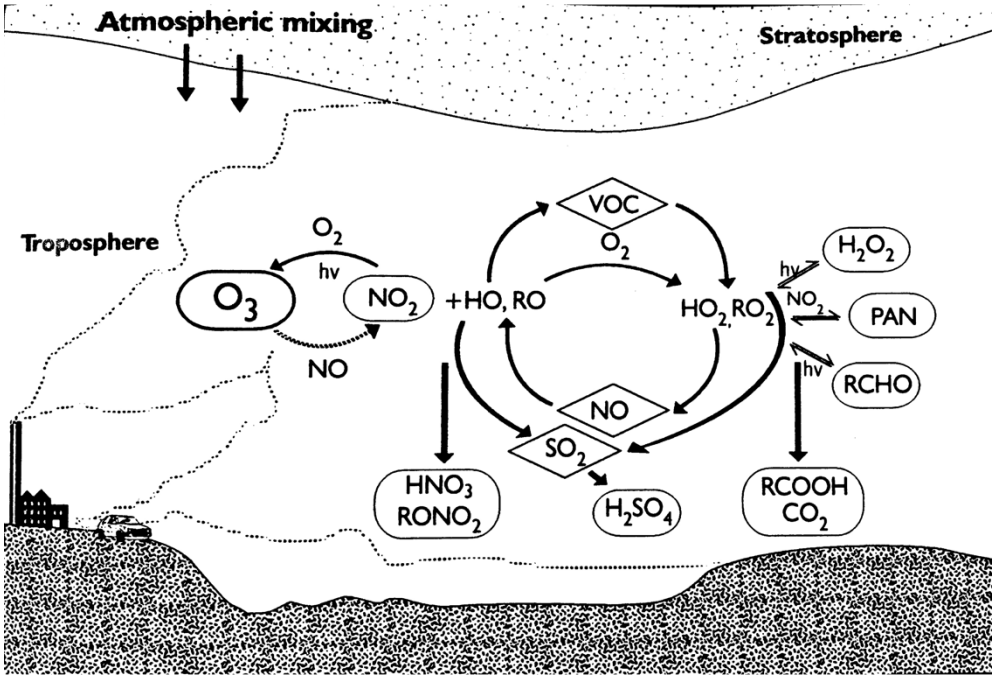

Figure 1.1. Schematic of ozone-production and cycling in response to anthropogenic sources of precursor emissions. Diamond boxes represent primary pollutants and round boxes represent secondary pollutants formed by atmospheric reactions. (From Barnes and Wellburn, 1998).

1.2 Ozone formation and distribution

The nature of ozone pollution in Europe and North America is changing (see The Royal Society, 2008). High emissions of NO_x and VOCs, associated with industrial development, have resulted in the recognition of ozone as a serious pollutant and growing problem since the 1970's. However, policy-driven abatement strategies, in a deliberate effort to curb precursor emissions *via* the burning of fossil fuels, have led to a successful reduction in peak ozone concentrations (Jenkin, 2008). However, efforts to reduce ozone precursor emissions in the Northern hemisphere are not sufficient to account for increased industrial development in other parts of the world (Ashmore, 2005; Fuhrer, 2009) and thus due to long distance transport of precursors from the growing economies in the Southern hemisphere, background concentrations of tropospheric O_3 have been, and will continue to rise, steadily for the foreseeable future (The Royal Society, 2008; RoTAP, 2012). There is general agreement that background ozone concentrations have increased annually by ~0.2 ppb per year over the last 20-25 years (Figure 1.2). Despite the decrease in peak episodes of ozone pollution, including the UK, when conditions are favourable for ozone formation, short-term average hourly

ground-level O_3 concentrations can exceed 110 ppb during the summer months in the South of England (RoTAP, 2012).

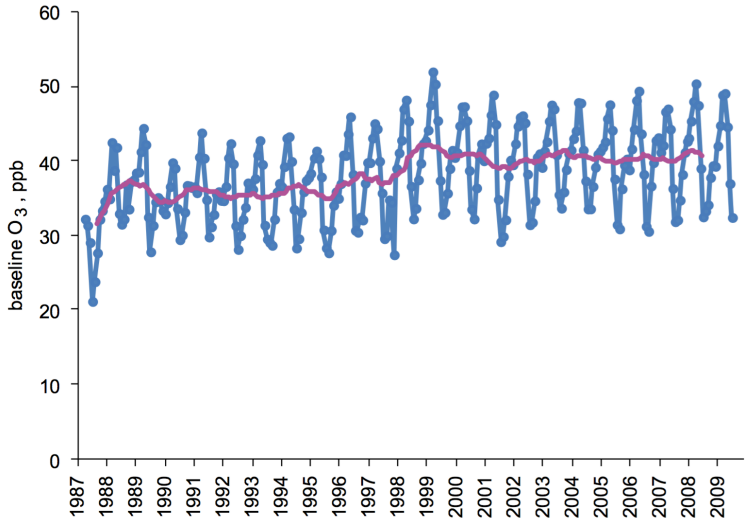

Figure 1.2 Evidence of increasing baseline ozone levels over 20-year period from measurements made at Mace Head, West coast of Ireland between 1986 and 2006. The dotted line represents monthly values and the solid line indicates the running annual mean. Data reveal an average increase in ground level ozone concentration of ~5 ppb (From RoTAP, 2012).

Ground-level concentrations of ozone are highly dependent on spatial location and climate (The Royal Society, 2008; Fuhrer, 2009). Episodes of high ozone pollution develop when weather conditions allow precursors to accumulate. Although concentrations of precursor emissions are highest in urban areas (generally the site of intense emissions), transport of ozone and its precursors to rural areas leads to damaging levels of pollution in regions far from emission sources (Stockwell, 1997). Lowland environments are more likely to experience a strong diurnal cycling in ozone concentrations, due to dry deposition at ground level, whereas, exposed upland habitats experience more stable and longer episodes of ozone exposure because O_3 supply from higher elevations surpasses the rate of dry deposition (Figure 1.3).

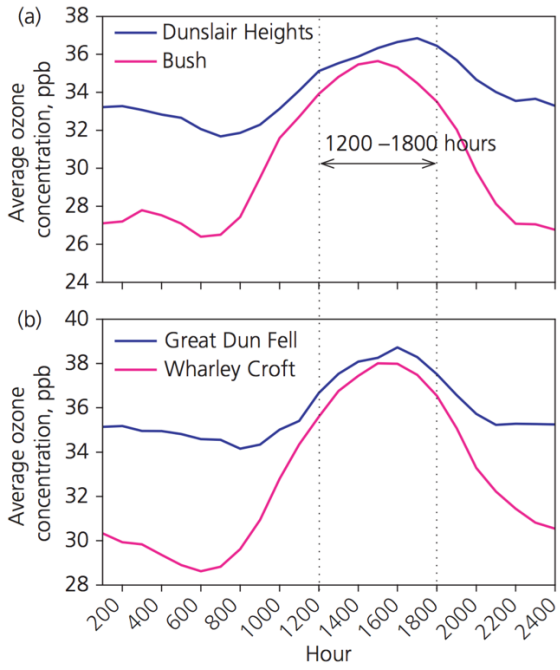

Figure 1.3. Evidence of increased diurnal exposure in higher altitude environments (blue lines) compared to lowland, valley environments (pink lines) (From The Royal Society, 2008).

1.3 Ozone phytotoxicity and plant responses

Ozone is particularly phytotoxic and enters plant tissue through open stomata. Once inside the leaf ozone dissolves in the leaf apoplast; intercellular space measurements are close to zero (Laisk *et al*., 1989). Ozone is a powerful oxidising agent (oxidising potential = 2.07 V) and the primary oxidation targets in plant tissue are metabolites (Plöchl *et al*., 2000), lipids/unsaturated fatty acids (Pryor *et al*., 1991) and proteins (Mudd *et al*., 1969; Fordham, 1994) in cell membranes and cell walls (Itiri and Faoro, 2009). Primary oxidative reactions and dissolution in the apoplast lead to the production of a suite of reactive oxygen species (ROS) (including, but not limited to, hydrogen peroxide (H_2O_2), superoxide (O_2^-) and peroxyl radicals ('O_2)), which further intensify the oxidative burden on cell membrane components (Fiscus *et al*., 2005; Sarkar *et al*., 2010) and result in marked shifts in the expression of stress-related genes (Kangasjarvi, 1994; Sharma and Davis, 1997; Cho *et al*., 2008) including rbcS (the gene(s) encoding the small subunit of ribulose-1,5-carboxylase/oxygenase (RubisCO)) which results in a decline in photosynthetic capacity (Cooley and Manning, 1987). Ozone can also result in visible blemishes on foliage and major changes in assimilate distribution between and within plant organs (Davison and Barnes, 1998; Booker *et al*., 2009). The

ultimate manifestation of all these impacts is reduced crop yield *via* negative impacts on plant biomass, numbers of flowers and flower abortion (Fuhrer, 2009; Jaggard *et al*., 2010).

1.4 Risks posed by ozone to agriculture

The first identified crop damage resulting from exposure to ambient levels of ozone resulted from observations made in highly polluted regions of Southern California in the 1940's (Thomas, 1951). Since then, visible symptoms of tissue damage (often referred to as 'flecking') have been identified as a common feature associated with ozone-stress around the globe. More importantly, it is now recognised that current and predicted ground level concentrations of ozone pose a very real threat to global food security (Avnery *et al*., 2011a,b) and yield reductions have been reported in many major crops, some exposed to long-term daily average concentrations as low as 30 ppb (Jaggard *et al*., 2010). It is recognized that present-day concentrations of the pollutant may be responsible for >5% reduction in yield of some sensitive crops including cotton, lettuce, pulses, soybean, peas, rice, onion, tomato, potatoes, strawberries, watermelon and wheat (Mills *et al*., 2013). Other crops have been proven to be more tolerant to ozone-stress, these include barley, plum and Brassicaceae (Mills *et al*., 2013). Current estimates of the global costs to the agricultural economy are between $11 and $26 billion (Van Dingenen *et al*., 2009; Avnery *et al*., 2011a). However, by the year 2030, losses to agriculture as a result of rising O_3 concentrations are expected to increase to between $19 and $35 billion per annum (Avnery *et al*., 2011b).

Increases in the human population are putting unprecedented pressures on successful agricultural practice, with global crop demands predicted to increase by 100% by the year 2050 (Tilman *et al*., 2011). There is particular concern over food security in rapidly developing parts of the world such as India and China where ozone concentrations are expected to spike rapidly in response to increased industrialisation (Ashmore, 2005; Fuhrer, 2009).

1.5 Impacts of ozone on plant reproductive processes

Agricultural productivity is compromised, in part, by the impacts of ozone pollution on plant reproductive biology. Any part of the plant that is directly exposed to air is a potential site for oxidative damage by ozone. It has been acknowledged that the timing of exposure to ozone during reproductive development can be significant (Wolters and Martens, 1987; Black *et al*., 2000) with implications for knock-on effects of crop success/yield when damage occurs at flowering/anthesis (Soja, 1997; Vandermeiren and De Temmermann, 1996; Pleijel *et al*., 1998; Gonzalez-Fernandez *et al*., 2010) (Figure 1.4).

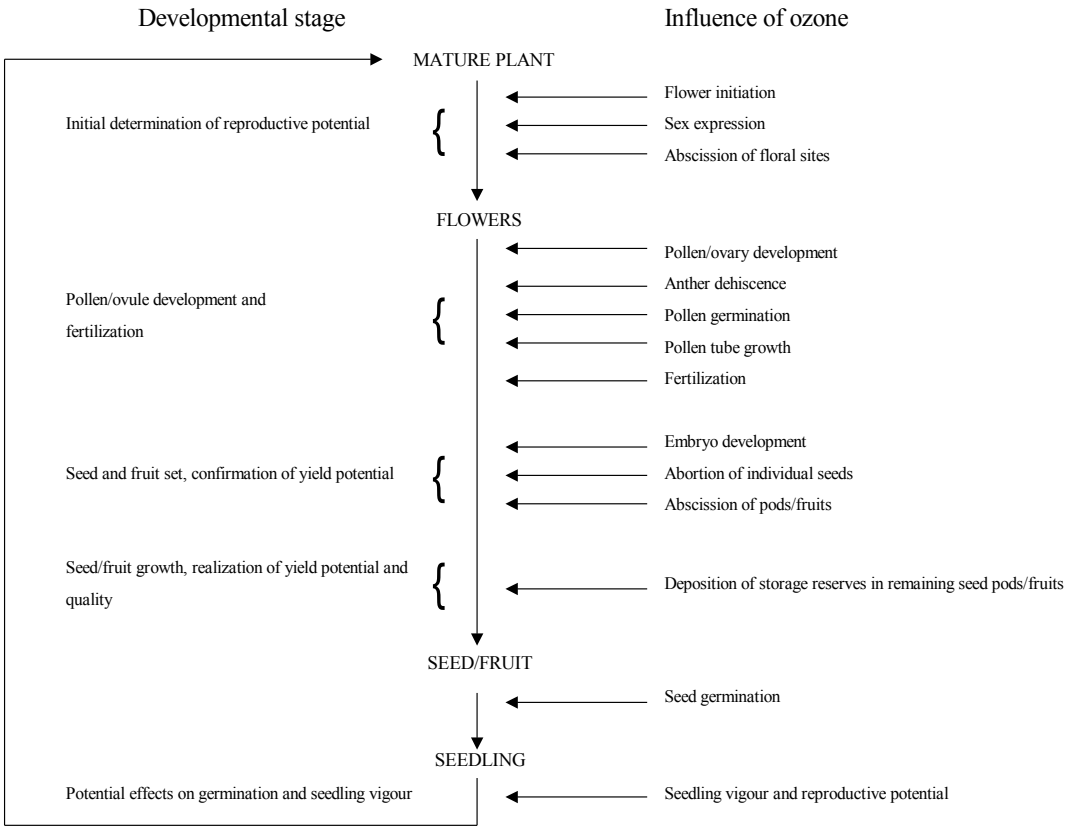

Figure 1.4 Potential targets where exposure to ozone may influence fruit development/reproductive success of higher plants (Redrawn from Wolters and Martens, 1987).

1.5.1 Nectar

Impacts of ozone pollution on nectar production and composition have rarely been studied. Nectar[1] is secreted by plants for the purpose of supplying visiting animals with a reward in exchange for a reproductive service to the plant. Floral nectar is produced by angiosperms for the sole purpose of attracting and rewarding floral visitors (Simpson and Neff, 1981; Pacini and Nicolson, 2007; González-Teuber and Heil, 2009), in return for the transfer of pollen to conspecifics. Nectar provides a major dietary source of nutrition to insects, birds and mammals and the qualities of floral nectar are thought to be shaped by the animals that feed and pollinate certain guilds (Nicolson, 2007). In the case of eusocial bees such as honeybees (*Apis mellifera*) and bumblebees (*Bombus spp.*), nectar is the primary source of carbohydrate to nourish not only the individual insect, but the entire colony, where nectar is stored and condensed to form honey.

Nectar is secreted by nectaries, a collective name for a diverse set of specialised structures that generally share the following traits: (i) epidermis (stomata present or absent), (ii) parenchyma, which store nectar substances, and (iii) vascular bundles, that provide water and/or nutrients to parenchyma (Fahn, 2000; Pacini *et al*., 2003). Furthermore, nectaries are found at all structures of a plant, excluding the roots (Pacini, *et al*., 2003; Nicolson *et al*., 2007; Heil, 2011) and can be classified as floral or extrafloral. Floral nectaries supply a nutritive reward to a pollinating animal, whereas extrafloral nectaries are usually involved in providing a reward to animals, such as ants, for herbivore deterrence (Pacini and Nicolson, 2007).

Genetic mechanisms governing nectar production, composition and secretion have, for the most part, been overlooked (Kram and Carter, 2009). There is an exceptional level of understanding of the genetic basis of some model plant species, such as *Arabadopsis thaliana* L. but this species does not easily lend itself to the study of nectar because it has very small nectaries and produces low volumes of nectar (Kram and Carter, 2009). One of the consequences of this is that we have a rather poor understanding of the genetic controls over nectar production. Plants that have large

[1] In Greek: '*the drink of the gods*'

nectaries and produce greater volumes of nectar, such as ornamental tobacco (*Nicotiana spp.*) (Carter *et al*., 1999; Ren *et al*., 2007) constitute an ideal model for nectary and nectar studies. However, the genetic resources for such species are not as advanced as for *A. thaliana*, for example, and so identification of specific genes allied to nectary and nectar control is problematic (Kram and Carter, 2009).

It is generally accepted that phloem sap constitutes the primary substance that becomes floral nectar (Fahn, 2000; Pacini and Nepi *et al*., 2007). 'Pre-nectar' is unloaded from phloem sieve elements to adjoining parenchyma *via* companion cells. There are then two suggested pathways in which pre-nectar is transported to the excretion sites at floral nectaries; the symplast or apoplast route (Figure 1.5). In the symplastic route pre-nectar travels from the phloem parenchyma to the nectary parenchyma through plasmodesmata. In the apoplastic route, pre-nectar in transferred from sieve elements to the secretory cells *via* cell walls and intercellular space (Pacini and Nepi, 2007).

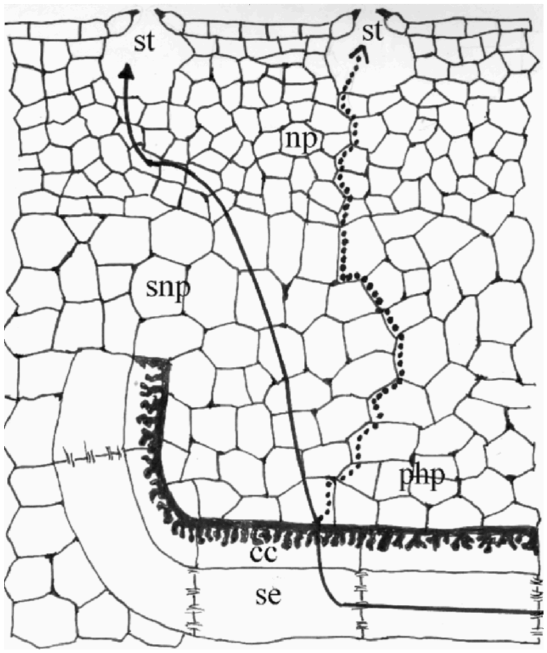

Figure 1.5 Potential pathways in which phloem sap may be transported as 'pre-nectar' to nectary stomata (st). Phloem sap from the sieve element (se) is transported to the phloem paranchyma (php) by ingrowths on companion cells (cc). It is then transported to subnectary parenchyma (snp) and on to the nectary parenchyma (np) where it is converted to nectar via symplast (dotted line) and/or apoplast (solid line) (From Pacini and Nepi, 2007).

A recent study observed that starch accumulated in the nectaries of *Anemopaegma album* Mart. prior to nectar secretion and that starch depleted from parenchyma surrounding the nectary during active nectar secretion (Guimarães *et al*., 2016). The authors concluded that starch is the primary source of nectar constituents after initial depletion of nectar from the flower, ensuring that the nutritive quality of nectar is maintained throughout the lifetime of the flower (Guimarães *et al*., 2016). Starch accumulation in the nectaries of ornamental tobacco has also been reported during nectary development, with rapid depletion after anthesis (Ren *et al*., 2007), suggesting conversion of starch to hexoses is necessary to supply nectar with simple sugars.

The proposition that "pre nectar" from phloem sap is the basis of floral nectar is generally supported (Davis *et al*., 1988; Ren *et al*., 2007; Wenzler *et al*., 2008; Kram and Carter, 2009). Yet, nectar constituents do not necessarily reflect the constituents of phloem. A study that compared the carbohydrate and amino acid composition of phloem sap and nectar in the same plant species found significant differences in composition (Lohaus and Schwerdtfeger, 2014). Nectar *of Maurandya barclayana* Auct.*, Lophospermum erubescens* D. Don*, and Brassica napus* L. contain sucrose, glucose and fructose, yet only sucrose is present in phloem sap. This suggests invertase enzymes in the nectary convert sucrose to monosaccharides (Rhulmann *et al*., 2010). Total amino acids are consistently reported to be present at lower concentrations in nectar than in phloem sap, suggesting the preservation of nitrogenous compounds within the plant (Lohaus and Schwerdtfeger, 2014). Furthermore, the proportions of individual amino acids also vary between phloem sap and nectar (Lohaus and Schwerdtfeger, 2014), suggesting that there is greater control over nectar production than simple passive flow from phloem sap. Few studies have focussed on mechanisms controlling nectar quality. However, a recent publication reported the study of sucrose production in nectar and identified the expression of *SWEET9*, a gene highly expressed in nectary parenchyma, to be essential for sucrose control in nectar (Lin *et al*., 2014). The authors also report that mutation of the *SWEET9* gene causes nectar secretion to cease. There is a significant gap in published literature on mechanisms controlling the allocation of amino acids to nectar. Yet, these nutrients are seemingly important given their universal presence in

floral nectars; concentrations of which have been shaped by their consumers (Baker, 1977; Baker and baker, 1973, 1977; Lanza and Kraus, 1984; Carter *et al*., 2006).

To date, no studies have been reported on the influence of ozone on floral nectar composition. However, it has been reported that long-term exposure of lima beans (*Phaseolus lunatus* L.) to 120 and 160 ppb O_3 induces production of extrafloral nectar (EFN), while exposure to lower concentrations of 80 and 100 ppb did not (Blande *et al*., 2010). The authors suggest that production of EFN at high concentrations of O_3 may be linked to strategy favouring plant defence (Blande *et al*., 2010). Ozone-stress is known to reduce phloem loading from source leaves (Grantz, 2003), so we hypothesise that nectar production is directly related to phloem content and thus ozone exposure may alter the amount of carbon allocated to sugars in nectar.

1.5.2 Pollen

Pollen is the gametophyte produced by vascular plants as a means to safely transport the male genetic material to conspecifics. Development of angiosperm pollen usually follows the following details. Pollen develops in pollen sacs on the anthers of a flower. Microsporocytes within the pollen sack undergo meiosis until haploid microspores are developed. Each microspore contains a nucleus which then divides by mitosis to produce a two-celled pollen grain; the generative cell within the vegetative cell (Vasil, 1967). Pollen that meets the stigma of a suitable conspecific then germinates and the pollen tube, carrying the male genetic material, travels down the style to the ovules where fertilisation takes place.

Aside from its obvious role in plant reproduction, pollen also constitutes a vital nutrient source which is consumed by a diverse range of animal taxa, including insects, birds and mammals (Roulston and Cane, 2000). The nutritional content of pollen is diverse and macronutrient concentrations range from ~2.5 to ~61% protein (Buchmann, 1986), ~2 to 20% fats (Roulston and Cane, 2000) and ~0 to 22% carbohydrate (Todd and Bretherick, 1942; Roulston and Cane, 2000). Pollen also provides a diverse range of micronutrients including vitamins, minerals and other secondary metabolites (reviewed by Puerto *et al*., 2015). Pollen cytoplasm is well protected by the exine, and so the

question of how pollen consumers gain access to nutrients has been widely speculated. Roulston and Cane (2000) compared the digestibility of pollen between animals and highlighted the diverse range of taxa that consume it. They also noted that in general, honeybees (*Apis mellifera*) were able to digest pollen better than mammals (Crailsheim *et al*., 1992; Van Tets, 1997; Law, 1992b; Herrera and Martinez del Rio, 1998; Turner, 1984; Quin *et al*., 1996), and birds (Paton, 1981; Wooler *et al*., 1988; Grant, 1996; Brice *et al*., 1989).

The influence of ozone pollution on pollen has received a little more attention than that of the gas on nectar, but in-depth knowledge of pollen-ozone interactions is far from exhaustive. The topographic structure of pollen exine has been demonstrated to be influenced by environmentally-relevant levels of O_3, this results in changes in the autofluorescent spectra, a phenomenon believed to be due to damage to lipofuscin-like compounds in carotenoids (Roshchina and Karnaukhov, 1999; Roshchina and Mel'Nikova, 2001). The composition of pectin-like material in the exine of the pollen of ragweed (*Ambrosia artemisiifolia* L.) has also been reported to be altered by exposure to O_3 (Kanter *et al*., 2013). Commonly observed impacts of ozone on pollen are a reduction in viability due to impaired pollen germination and germ tube growth (Black *et al*., 2007; Leisner and Ainsworth, 2012; Gillespie *et al*., 2015). The mechanisms governing such detrimental impacts are not understood. A further reported impact of exposure to ozone is a reduction in the soluble protein content of pollen (Ribeiro *et al*., 2013) and increased NADPH oxidase activity in the cell walls suggestive of an increase in oxidative burden (Pasqualini *et al*., 2011).

1.6 Pollinators

Animal pollination is essential for reproductive success in wild plant communities (Kearns *et al*., 1998; Larson and Barret, 2000; Ashman *et al*., 2004) and domesticated crops (McGregor, 1976; Klein *et al*., 2007). It is estimated that yield of around 84% of European crops depend on some form of animal pollination (Williams, 1994) and the most important pollinators, from an agricultural perspective, are bees. Bee pollination is essential for the success of many crops and reductions in yield of more than 90% are reported when some fruit, seed and nut crops go without bee visitation (Southwick and

Southwick, 1992; Kearns *et al*., 1998). Furthermore, it is estimated that around one third of all food consumed by humans is dependent on bee pollination (McGregor, 1976; Klein *et al*., 2007).

Pollination by commercially-kept honeybees is an important agricultural service. However, a combination of factors including pesticide exposure, parasites and disease transmission are resulting in commercial beekeepers reporting unprecedented and alarming losses (of between 20 and 40%) of their colonies annually since 2006 (Neumann and Carreck, 2010; Lee *et al*., 2015) – a phenomenon commonly referred to colony collapse disorder (CCD). A further critical stress that bees are faced with is a significant reduction in the diversity of food on which they can forage (Williams, 1986). Monoculture is an ever increasing tool in modern agriculture, and where bees would once have access to forage on a diverse flora, in many parts of the world agricultural land is now dominated by individual crops.

All animals have a specific requirement for nutrients at any given time in their life (Simpson and Raubenheimer, 2012). Both honeybees and bumblebees regulate their intake of protein, essential amino acids and carbohydrate in order to achieve nutritional homeostasis (Altaye, *et al*., 2010; Pirk *et al*., 2010; Paoli *et al*., 2014; Stabler *et al*., 2015). When bees are restricted to diets outside of the range in which they can balance their nutrient intake, early mortality results (Altaye *et al*., 2010; Stabler *et al*., 2015). There is a significant knowledge gap regarding the nutritional quality of nectar and pollen of individual crops for bee pollinators, and so the manner in which bees are able to regulate their intake of nutrients when foraging on monoculture crops is unknown. It is believed that when bees are restricted to forage on a single crop that does not allow them to maintain the correct balance of nutrient intake, this contributes to the observed premature mortality of honeybee colonies associated with CCD.

Foraging bees are exceptional at learning and remembering floral cues including odour (Menzel and Giurfa, 2001; Spaethe *et al.,* 2007; Wright *et al*., 2007), floral morphology (Laverty, 1994; Chittka *et al*., 1999) and colour (Gumbert, 2000; Nicholls and Hempel de Ibarra, 2014). When foraging, bees assess the quality of nectar rewards

from available plants and preferentially forage from plants that provide the greatest food resource (Ribbands, 1949; von Frisch, 1967; Waser, 1986). Upon returning to the colony, forager bees dance to communicate a lucrative food resource to nest-mates which, in turn, recruits more bees to forage from the same resource (von Frisch, 1967). It is interesting to consider how bees would respond to the cues given by plants if the nectar rewards were not consistent. Would an unreliable cue for a reward reduce the pollination service to that plant?

Foraging bees can assess and learn pollen quality, in terms of its protein content, and associate the information with the colour cues of flowers (Nicholls and Hempel de Ibarra, 2014). Pollen is a source of essential nutrients for the development of honeybee colonies. However, the ability of honeybees to digest pollen and protein declines as they age (Crailsheim 1986, 1990; Moritz and Crailsheim 1987; Crailsheim *et al*., 1992; Lass and Crailsheim 1996; Paoli *et al*., 2014), and studies have repeatedly demonstrated that when bees are limited to feed from diets in which the protein concentration exceeds their regulatory range, this is reflected in increased mortality (Altaye *et al*., 2009; Pirk *et al*., 2010; Paoli *et al*., 2014; Stabler *et al*., 2015).

1.7 Project outline

Background concentrations of ozone in the Northern hemisphere are increasing and are predicted to continue on this trajectory for the foreseeable future. Considerable effort has been made to identify the risks posed by ozone pollution to wild and domestic vegetation, but the impacts of the pollutant on plant-pollinator interactions has been largely overlooked. Bees are responsible for delivering ≈30% of the food that we eat. Yet, in spite of this, their nutritional requirements have been largely overlooked when considering environmental impacts. The experiments described in this thesis were designed to explore the allocation of protein-bound and free amino acids, non-structural carbohydrates and free fatty acids to nectar and pollen under defined ozone-exposure conditions, employing broad bean (*Vicia faba*) as a convenient model plant. Seed set of this major crop plant is reliant on insect pollination and bees foraging on broad bean collect both pollen and nectar from the flower.

There is a significant gap in published literature accounting for the influence of shifts in resource allocation within a plant and how demands for resources in some tissues may alter allocation to others (i.e. leaves/pollen/nectar). To bridge the gap, these novel studies explore how plants exposed to long and short term ozone-exposure regimes allocate protein, free amino acids and carbohydrates to nectar and pollen. The study of ozone exposure on the chemical composition and volume of nectar is entirely novel and the experiments described in chapter 5 exploit this. The influence of ozone on the amino acid composition of pollen is novel, however, reductions in protein concentration have been reported (Ribeiro *et al*., 2013). There are currently no reported studies comparing the presence of nectar-like compositions of carbohydrates and amino acids on learning and memory in honeybees. We exploit this in Chapter 6 and report and novel role for amino acids in nectar.

The aims of the research described in this thesis include:

- Development of novel methods for the quantification of protein-bound and free amino acids in pollen using microwave-assisted acid hydrolysis utilising small amounts of pollen.
- Exploration of methods for the reliable extraction and quantification of amino acids and carbohydrates in nectar from low-volume nectar flowers.
- Exploration of whether existing measures for the assessment of plant 'sensitivity' to ozone constitute suitable surrogates for ozone-induced changes in pollen quality.
- Investigation of the impacts of sustained- and short-term exposure to ozone on resource allocation within plants and to nectar and pollen.
- Assessment of how ozone-induced changes in amino acid and carbohydrate composition of nectar may influence honeybee olfactory learning.

2.0 Chapter 2: Exploration of methods to quantify the amino acid qualities of pollen

2.1 Introduction

Pollen is produced by vascular plants to carry the male gamete, facilitating safe transfer of genetic material and leading to sexual out-crossing in plant reproduction. Wind transport accounts for the majority of successful pollination in gymnosperms and is also a common mechanism for angiosperms. However, angiosperms also rely heavily on animal pollinators for sexual reproduction. The evolution of multimodal cues provided by flowers (diverse colours, sizes, shapes and odours) enables pollinating animals to more accurately collect and deliver pollen to the appropriate recipient (Gegear, 2005; Ghazoul, 2006; Raguso, 2008 (review); Wright and Schiestl, 2009), giving rise to complex and diverse mutualisms.

Most pollinating insects visit flowers to collect pollen as a source of food (Roulston *et al*., 2000). The main dietary components of pollen include enzymatic proteins, lipids, starch, vitamins and free amino acids, held within pollen cytoplasm (Stanley and Linkens, 1974). Pollen grains are protected by a robust exine and intine that form the pollen wall (Roulston and Cane, 2000). The pollenkitt on the outer surface of the exine contains free amino acids, lipids, fatty acids, sterols and small amounts of carbohydrate (Dobson, 1987; Dobson and Bergström, 2000; Pacini and Hesse, 2005; Piskorski *et al*., 2011). It is hypothesised that pollinators may use components of the pollenkitt as phagostimulatory cues with which they can detect and discriminate between nutritional resources (Dobson, 1987).

Honeybees are important pollinators of wild (Ashman *et al*., 2004) and domesticated (Klein *et al*., 2007) plant species. They rely on pollen and nectar as their main source of nutrition to feed developing larvae and nurse bees. Bees utilise pollen as a source of proteins, amino acids, fatty acids, vitamins and sterols and achieve their carbohydrate requirements by feeding on nectar. On average, the concentration of protein provided by different species of plants ranges between ~2.5 and 60 % of the dry

weight (Buchmann, 1986; Roulston *et al*., 2000). However, very little is known about variations in the distribution of specific amino acids contained in the pollen of different plant species. Understanding the constituents of pollen (and nectar) is important when considering the nutritional requirements of pollinators and assessing whether or not they are being met by the landscapes in which they forage. Assessing the quality of pollen could also be a useful tool in developing agri-environment schemes and managing both urban and rural areas to provide pollinators with reliable and nutritious floral resources or substitutes.

The study of pollen chemistry poses several challenges. Standard methods for protein analysis require sample sizes of raw material of between 60-150 mg to 1 g for combustion and micro-Kjeldahl methods, respectively (Roulston *et al*., 2000). However, the amount of pollen produced by anthers of angiosperms is generally orders of magnitude lower than this. For example, Denisow (2008) described pollen production in six Brassicacae species and found the maximum weight of pollen produced by one species hybrid (Aubrieta x hybrida) was 66.8 μg of pollen per anther and the lowest recovered weight was 11.6 μg of pollen per anther (*Iberis semprevinems* L.). An additional challenge is that the physical structure of pollen provides limitations to its study. Free amino acids can simply be washed from the pollen surface with solvent (Mondal *et al*., 1998; Cook *et al*., 2003), but protein-bound amino acids are well protected by the exine, and so this robust structure must be ruptured effectively when protein-quantification is necessary.

Three main methods for determining protein concentration of pollen are currently in use; the Bradford assay, micro-Kjeldahl digestion and combustion. The Bradford assay (Bradford, 1976) is used to estimate total soluble protein concentration. The assay involves the binding of Coomassie blue dye reagent to soluble proteins and the absorbance is read at 595nm using a spectrophotometer. Although straightforwardly implemented, the method suffers a number of limitations. Proteins are held within the pollen exine, so pollen-dye interactions will not bind if the exine is in place. Pollen proteins are not all soluble (Zársky *et al*., 1985; Evans *et al*., 1992; Shahi *et al*., 2008) and so the assay may significantly underestimate insoluble protein

concentration (Gotham *et al*., 1988). Moreover, in the acidic state of the reagent, Coomassie dye binds with basic amino acid side chains such as arginine, histidine and lysine (Schaffner and Weissmann, 1973; Tal *et al*., 1985; Syorvy and Hodny, 1991). Proteins lacking amino acids with such side chains are likely to be underestimated (Sapan *et al*., 1999). However, a potential benefit of the Bradford assay is that it requires 1000- and 100-fold less sample tissue to quantify protein than required for Kjeldahl and combustion methods, respectively (Roulston *et al*., 2000).

The Kjeldahl method involves protein hydrolysis using a strong acid, whereas combustion involves heating samples to ~900 ℃ without the use of chemicals. Both Kjeldahl and combustion methods rely on digestion of protein to absolute nitrogen (N), with the application of a correction factor to back calculate from absolute nitrogen to crude total protein concentration.

Roulston and colleagues (2000) compared the Bradford assay with combustion and micro-Kjeldahl methods for the analysis of protein in pollen by measuring N with a carbon and nitrogen analyser for combustion experiments and a Technicon Autoanalyser II for micro Kjeldahl digestion. They found that all three methods produced similar concentrations of protein after the application of a multiplication factor of 6.25 for micro kjeldahl and combustion methods. This suggests that, although all methods produce similar estimates for crude protein concentration, none are adequate for measurement of the individual protein-bound amino acids in pollen. An advantage of the Bradford assay over combustion and micro-Kjeldahl methods is that a Bradford assay can be applied reliably using 1 mg of pollen, whereas combustion and micro-Kjeldahl require ~100 mg and ~1000 mg of plant material, respectively. Moreover, estimating crude protein concentration from absolute nitrogen content can be unreliable. Firstly, nitrogen may not all be of protein-origin. Secondly, the multiplication factor applied to nitrogen values may be variable leading to a misinterpretation of actual protein concentration; there are several views on which correction factors to use for different protein types (Rabie *et al*., 1983; Buchmann, 1986; Roulston *et al*., 2000). A correction factor of 6.25 is commonly adopted for Kjeldahl analyses. However, this estimation is based on the assumed N content of animal derived proteins, which average ~16%

(100/16 = 6.25) (Jones, 1941) and may not be applicable to determining protein concentration from nitrogen in plant tissue (Conklin-Brittain *et al*., 1999). Milton and Dintzis (1981) suggested overall lower correction factors for plant tissue and concluded that correction factors of 3.9 for floral tissue, 4.0 for ripe fruit and 4.2 for young leaves were more appropriate. Although the Bradford assay, combustion and micro-Kjeldahl methods produce similar estimates of protein concentration (Roulston et al., 2000), the major limitation of these methods is the lack of information on the amino acid profiles in the proteins, meaning that quantification of the nutritional quality of protein is unachievable.

Protein bound amino acids can be cleaved by subjecting proteins to acid hydrolysis. Acid hydrolysis has been used to allow quantification of protein-bound amino acids/peptides in a number of recent publications (Gonzalez-Paramás *et al*., 2006; Human and Nicolson, 2006; Nicolson and Human, 2013; Vanderplank *et al*., 2014; Somme *et al*., 2015). Standard methods for hydrolysis of proteins to enable amino acid quantification involve acidic digestion with 6 M HCl, boiled at 110°C for 24 h (Blackburn, 1978; Fountoulakis and Lahm, 1998). Amino acids in the hydrolysate can then be detected and quantified by HPLC (Nicolson and Human, 2013). Amino acids can be derivatized either pre- or post-column (Fountoulakis and Lahm, 1998) and detected by UV or fluorescence.

Analysis of the amino acid properties of pollen is critically important in the study of nutritional quality to consumers. This chapter describes a series of experiments designed to investigate the reliability of adopting the Bradford assay to estimate the protein concentration of pollen and compares the outcomes to a method involving acid hydrolysis, back calculating amino acid concentrations to represent crude protein. We also aimed to identify the minimum amount of pollen needed to carry-out amino acid analyses in a robust, quick and inexpensive manner.

2.2 Methods

2.2.1 Pre-treatment regimes: Estimation of protein content of pollen

To test how solubility affected measurements made in the Bradford assay for bee-collected pollen, four preparation treatments were applied to pollen before determining protein content: (1) untreated, (2) methanol washed, (3) ground and (4) methanol washed and ground. Honeybee-collected rock rose pollen (*Cistus* spp. mixed source) was used as a standard pollen-type (Kiki Ltd. Rock rose pollen, Norfolk, UK). Pollen was dried at 65°C for 48 h, ground using a pestle and mortar, then 1 mg of the ground pollen weighed in to 1.5 ml microcentrifuge tubes (N = 10) before adding 1 ml nanopure H_2O to untreated pollen and mixing using a vortex for 15 min (1). Untreated pollen was used as a control to compare the binding efficiency of Bradford reagent to pollen proteins from other pre-treatments. To test if solubility caused by fats in pollen affected the Bradford assay (2), 200 μl of methanol was added to 1 mg of pollen. The tube was vortexed for 1 min, and left for 10 min at room temperature and mixed again for 1 min. The sample was centrifuged at 13,249 x *g* for 30 min and then the methanol decanted. The remaining pollen pellet was dried at 60°C prior to the addition of 1 ml of nanopure water and mixing using a vortex for 15 min. To test if the cell walls of pollen affected the Bradford assay (Roulston and Cane, 2000) (3), 1 ml H_2O was added to the dried pollen along with 20 mg of 0.7 mm diameter zirconia beads (Bio Spec Products Inc. Cat No. 11079107zx). The sample was homogenised in a tissue lyser (frequency 30 Hz) for 5 min (TissueLyser 2, Qiagen). The tube was briefly centrifuged to settle the beads before protein content was measured. In treatment 4, pollen was methanol-washed and dried, as in treatment 2, then 1 ml of nanopure H_2O and 20 mg of zirconia beads were added to the dried pollen and the sample homogenised in a tissue lyser for 5 min. The tube was briefly centrifuged (13,249 x *g*) to settle the beads before protein analysis.

Following pollen pre-treatments, 50 μl of the pollen/protein solution was removed and pipetted into a 2 ml micro-centrifuge tube. To this, 1.5 ml of Bradford reagent (Sigma Aldrich, Cat. Ref. B6916) was added and the protein-Bradford mixture was vortexed for 30 s. Samples were left to incubate at room temperature for 15 min before being mixed on a vortex for a further 30 s. The protein-Bradford mixture was

then added to a cuvette and absorbance recorded at 595 nm on a spectrophotometer (Genesys 10 VIS, Thermo Scientific). Protein concentration was quantified from a graph of standards (developed using bovine serum albumin (BSA) at five dilutions; 0, 0.25, 0.5, 0.1 and 1.4 mg ml^{-1} (Appendix A)).

2.2.2 Efficacy of Bradford assay with different proteins

Twelve protein sources were compared using the spectrophotometric assay (Bradford, 1976). Protein isolates used were: (1) sodium caseinate, (2) bovine serum albumin (BSA), (3) rock rose pollen, (4) calcium caseinate, (5) egg white albumin, (6) brown rice protein, (7) hemp protein, (8) impact whey isolate, (9) isopro whey, (10) honeybee royal jelly, (11) soy protein isolate and (12) pea protein isolate (Table 2.1). Each protein was weighed out to 1 mg into separate 1.5 ml micro-centrifuge tubes. To each, 1 ml of deionised water was added, then samples were vortexed at room temperature for 10 min to allow maximum hydration of the proteins. Each protein solution was then subject to Bradford assay, as described in Section 2.2.1.

2.2.3 Impact of protein hydrolysis on Bradford assay

Bovine serum albumin (BSA) was weighed out to 1 mg and added to 100 µl of 6M HCl prior to vortexing for 30 s. The samples were placed in a 900W microwave with a glass beaker containing 600 ml water (for safety and to absorb excess radiation (Zhong et al., 2005)). The samples were then hydrolysed in the microwave at full power (900W) for 20 min. Once finished, samples were left to cool, then tubes were moved to a heat block and the lids opened. The acid was evaporated at 100 °C. Once dry, 1 ml nanopure water was added and the sample was mixed using a vortex for 15 min. A Bradford assay was then carried-out on hydrolysed BSA and un-treated BSA (1 mg ml^{-1}) as described in Section 2.2.1.

2.2.4 Optimisation of protein hydrolysis for HPLC analysis

A series of experiments was conducted to optimise a reliable and convenient method for hydrolysing protein in pollen. Three experiments were carried out to test (1) how the volume of HCl affected the hydrolysis of the sample; (2) how the amount of sample affected hydrolysis efficiency and (3) to identify whether the efficiency of

hydrolysis was affected by sample size at a specific ratio of HCl to sample (1 : 100 mg : μl).

Experiment 1: The amount of sample was constant (1 mg) but volume of acid varied (10, 50, 100, 150 and 200 μl HCl) (Appendix B).

Experiment 2: Amount of HCl (6M) was constant (100 μl) but the amount of sample varied (Appendix B)

Experiment 3: Variable amounts of BSA were used in a hydrolysis experiment in which the volume of acid used was proportional to the amount of protein present. A fixed ratio of 1 : 100 (mg : μl) of protein to acid was used (Appendix B) .

In all above experiments samples were hydrolysed at full power (900W) for 20 min using the microwave method described in Section 2.2.3. Acid was evaporated at 100°C in a heat block in a fume hood and samples were recovered in HPLC gradient grade H_2O (Fisher Scientific. Product code: 10221712). Tubes were vortexed for 15 min and then centrifuged at 13,249 x *g* for 30 min. Supernatant was removed with a sterile 1 ml syringe (Tuberculin luer) and passed through a 0.45 μm syringe filter (Whatman Puradisc 4 syringe filter, 0.45 μm, nylon) to remove any remaining particulates. Filtrate was then centrifuged at 13,249 x *g* for a further 10 min before HPLC analysis.

2.2.5 Optimisation of pollen protein hydrolysis

Amino acid composition was determined in pollen prepared as described in experiment 3, Section 2.2.4 following microwave-assisted acid hydrolysis. A range of weights of pollen were added to sealed tubes; low weights included 0.1, 0.2, 0.3 0.4 (n=5) and 0.5 mg (n=20), high weights included 1, 2, 3, 4 and 5 mg (n=20). To each tube, 200 μl of HPLC gradient grade methanol was added prior to vortexing for 1 min. Samples were then left to stand at room temperature for 10 min prior to re-vortexing for 1 min, then subject to centrifugation for 30 min at 13,249 x *g* and the supernatant decanted. The remaining pollen pellet was retained for analysis of protein-bound amino acids (see below). The methanol extract was dried at 70°C in a heat block prior to recovery in 300

μl HPLC gradient grade H_2O. Samples were passed through a 0.45 μm syringe filter to remove particulates prior to storage at -20°C until HPLC analysis.

Following methanol-extraction, the remaining fraction of the pellet was used for the analysis of protein-bound amino acids. Amino acids were hydrolysed from proteins using microwave-assisted acid hydrolysis (See Section 2.2.3). The volume of acid added was maintained in a relative volume for each weight of pollen used (see appendix B. 1 mg pollen: 100 μl 6M HCl). Samples were vortexed briefly prior to microwave-assisted hydrolysis (as described in Section 2.2.3).

2.2.6 Amino acid analysis

Ultra high performance liquid chromatography (uHPLC) was used to measure the concentration of 21 amino acids: aspartic acid (asp), glutamic acid (glu), asparagine (asn), serine (ser), glutamine (gln), histidine (his), glycine (gly), threonine (thr), arginine (arg), alanine (ala), tyrosine (tyr), cysteine (cys), valine (val), methionine (met), gamma-aminobutyric acid (GABA), tryptophan (trp), phenylalanine (phe), isoleucine (ile), leucine (leu), lysine (lys) and proline (pro).

Immediately before injection, using an automated pre-column derivitization programme for the autosampler (Ultimate 3000 Autosampler, Dionex, Thermo Fisher Scientific Inc.), 10 μl of sample was treated for 1 min with 15 μl of 7.5 mM o-phthaldialdehyde (OPA) and 225 mM 3- mercaptopropionic acid (MPA) in 0.1 M sodium tetraborate decahydratre ($Na_2B_4O_7 \cdot 10\ H_2O$), pH 10.2 and for 1 min with 10 μl of 96.6 mM 9-fluroenylmethoxycarbonyl chloride (FMOC) in 1 M acetonitrile. This was followed by the addition of 6 μl of 1M acetic acid. After pre-treating, 30 μl of the amino acid derivatives were then injected onto a 150 x 2.1 mm Accucore RP-MS (Thermo Fisher Scientific Inc.) uHPLC-column. Elution solvents were: A = 10 mM di-sodium hydrogen orthophosphate (Na_2HPO_4), 10 mM $Na_2B_4O_7 \cdot 10\ H_2O$, 0.5 mM sodium azide ($NaN_3$), adjusted to pH 7.8 with concentrated HCl, and B = Acetonitrile/Methanol/Water (45/45/10 v/v/v). Elution of the column occurred at a constant flow rate of 500 μl min^{-1} using a linear gradient of 3 to 57% (v/v) of solvent B over 14 min, followed by 100% solvent B for 2 min and a reduction to 97% solvent B for the remaining 4 min. The

derivatives were detected by fluorescence (Ultimate 3000 RS Fluorescence Detector, Dionex, Thermo Fisher Scientific, OPA: excitation at 330 nm and emission at 450 nm, FMOC: excitation at 266 nm and emission at 305 nm) and quantified by automatic integration after calibration of the system with amino acid standards. Reference calibrations (for all amino acids) were conducted following the processing of each batch of 20 samples by injecting calibration standards (a pre-made solution of 17 amino acid standards for fluorescence detection (Sigma-Aldrich)). The missing four amino acids (available in solid form from Sigma-Aldrich) were added manually prior to system calibration; mean concentration= 25 mM. Elution profiles (see example provided in Appendix C) were analysed using Chromeleon software v. 6.8 (Thermo Fisher Scientific Inc). Amino acid peaks were automatically detected based on pre-calibrated elution times *via* Chromeleon software, with all peaks checked to ensure correct identification. If amino acid peaks were wrongly assigned by the software, then they were manually re-assigned by selecting the peak area of the correct peak, identified by retention time of the standard. Chromeleon output (micromoles per litre (μM)) was converted to a standardised unit (μg mg^{-1}) in order to compare methods.

2.2.7 Statistical analysis

Data were first checked for normality of distribution, then subject to one-way analysis of variance (ANOVA) to compare the effect of pre-treatment on the protein content of pollen, and the binding efficiency of Bradford reagent to different proteins. The expected values for each protein (%) were compared to median measured values in a one-sample non-parametric test. Low and high weights of BSA and pollen used in hydrolysis experiments were analysed separately. Total amino acids recovered from hydrolysis of both BSA and rock rose pollen were compared in a generalised linear model, comparing source and weight of the protein. To compare the distribution of amino acids quantified in hydrolysis experiments, values for each amino acid were square root transformed ($\sqrt{(x+1)}$) and used in a factor analysis (principal components) to reduce variables into significant factors with similar correlations. Tryptophan and GABA were removed from the data set prior to analysis because they were present at values < 0.1 ng mg^{-1} of pollen. Factors produced from the PCA were then used as dependent variables in a multivariate analysis of variance (MANOVA) using protein

weight as a main effect. The total free- and protein-bound amino acids recovered following the hydrolysis of rock rose pollen were compared in a MANOVA using pollen weight as a main effect and total free amino acids and total protein-bound amino acids as dependent variables.

2.3 Results

2.3.1 Pre-treatment regimes: Estimation of protein content of pollen

Figure 2.1 illustrates the impact of four ways of pre-treating pollen on the protein content recovered (estimated by Bradford assay). Pre-treatment of pollen with a methanol wash or mechanical disruption significantly ($P < 0.001$) affected the amount of protein recovered. Homogenisation of pollen (no wash treatment) resulted in the highest protein return (6%), whereas a simple methanol-wash returned the lowest value (0.5%).

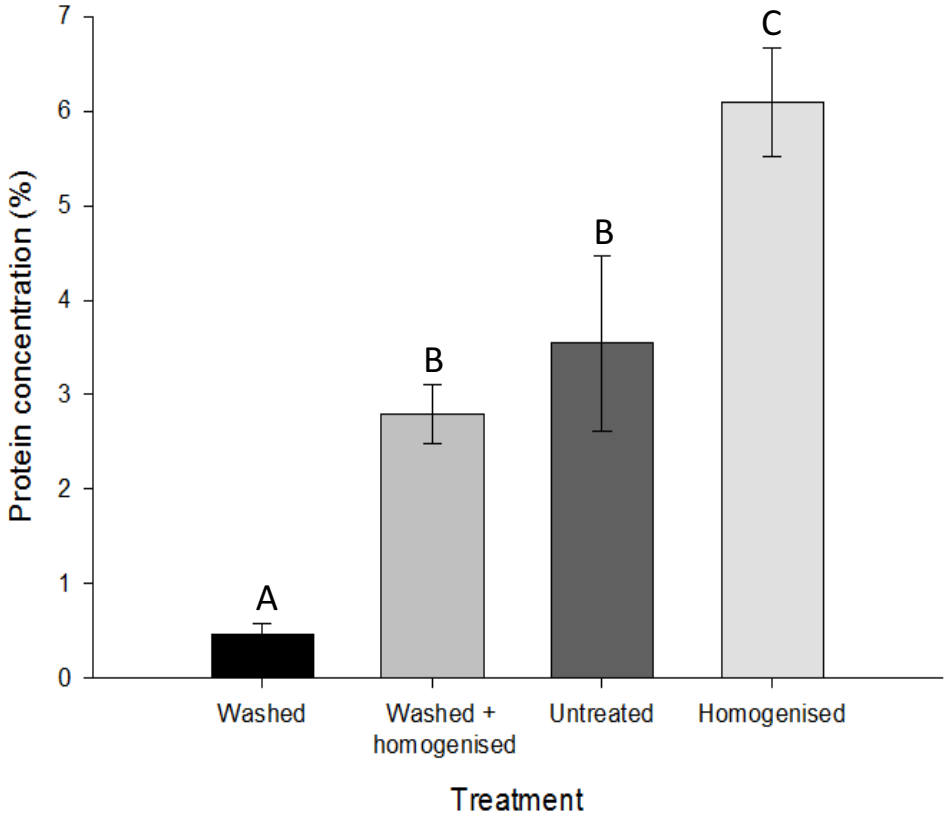

Figure 2.1. Estimated protein concentration of pollen following four pre-treatments; washed (methanol), washed and homogenised, untreated, and homogenised. Different letters represent significant difference at 5% level, ± standard error of mean.

2.3.2 Efficacy of Bradford assay with different proteins

Twelve protein fractions were used in an equivalent assay to compare binding efficiency to Bradford reagent. Data revealed a significant ($P< 0.05$) difference between measured and expected protein content of different isolates (Table 2.1). Assay of sodium caseinate, BSA, and calcium caseinate produced results anticipated. However, the Bradford assay significantly ($P< 0.05$) underestimated protein concentration of all other protein.

Table 2.1. Protein isolates used in a protein quantification assay using Bradford reagent. Estimated percentage of protein is presented with ± standard error of mean. Expected protein concentrations are presented with significance values from a one sample non-parametric test comparing measured values to the expected

Protein isolate	Mean (%) ± St. Error	Expected value (%)	P-value
BSA	109.9 ± 4.7	100	**0.059**
Sodium caseinate	92.8 ± 6.5	99	**0.333**
Calcium caseinate	86.4 ± 6.3	90	**0.114**
soy	38.7 ± 2.7	95	0.005
Isopro whey	37.7 ± 3.0	78	0.005
Egg white albumin	31.3 ± 2.9	97	0.005
Hemp protein	29.4 ± 3.8	50	0.005
Impact whey isolate	27.4 ± 1.6	92	0.005
Pea protein isolate	20.0 ± 1.2	80	0.005
Royal jelly	18.7 ± 1.0	12.5	0.005
Rock rose pollen	3.5 ± 0.9	14.2	0.005
Brown rice protein	2.0 ± 1.0	79	0.005

2.3.3 Optimisation of pollen protein hydrolysis

The total protein-bound amino acids ($\mu g\ mg^{-1}$) returned from a hydrolysis of BSA were a function of the amount of sample (Linear regression, $r^2 = 0.842$, , $P< 0.001$). This relationship was also observed for the hydrolysate of pollen, but the relationship was not as strong (Linear regression, $r^2 = 0.134$, $P< 0.001$). The relative increase in amount of protein-bound amino acids with weight of sample was similar for low weights of pollen

and BSA. For sample weights ≥ 1 mg of BSA, the total protein bound-amino acids rendered by hydrolysis increased as a function of the sample size ($P< 0.001$), the same was not true for pollen (Figure 2.2). For BSA, the largest sample sizes tested, 4 and 5 mg, yielded similar concentrations of protein-bound amino acids. All pollen samples between 1 and 5 mg returned similar amounts of protein-bound amino acids (Figure 2.2).

For BSA, the mean total amino acids rendered by hydrolysis ceased to change between the 4 and 5 mg samples. The mean concentration was 97.5 $\mu g\ mg^{-1}$, with the highest relative amount rendered by the hydrolysis method. The expected value should have been close to 1000 $\mu g\ mg^{-1}$ from hydrolysis of the pure protein. This indicated that the efficiency of hydrolysis in this assay was ~9.75% of the available protein. For the pollen samples, the total mean amount of amino acids (22.7 $\mu g\ mg^{-1}$) rendered by hydrolysis ceased to change for samples ≥ 1 mg (Figure 2.2). This indicated that the efficiency of the hydrolysis was stable within the designated sample range. Assuming that the hydrolysis method worked with the same level of efficiency for both pollen and BSA, returning ~10% of the protein as lysate when the amount rendered as lysate stabilised, then the total protein concentration of rock rose pollen should be ~23.2%. Because efficiency of hydrolysis for pollen weights ≥ 1 mg was stable, a 'correction' factor of 10.3 was applied to these weights. Efficiency of hydrolysis was not stable for low weights of pollen. We therefore developed separate multiplication factors for lower weights of pollen. The correction factors were regressed against sample size; the best fit to the data was a first order inverse function (Curve estimation, $r^2 = 0.506$, $F_{1,58} = 59.4$, $P< 0.001$). The equation of this line (Figure 2.3, $y=14.4074+(4.8282/x)$) was then used to deduce a specific adjustment factor for a given starting weight of pollen, to allow the estimation of the protein concentration of the pollen.

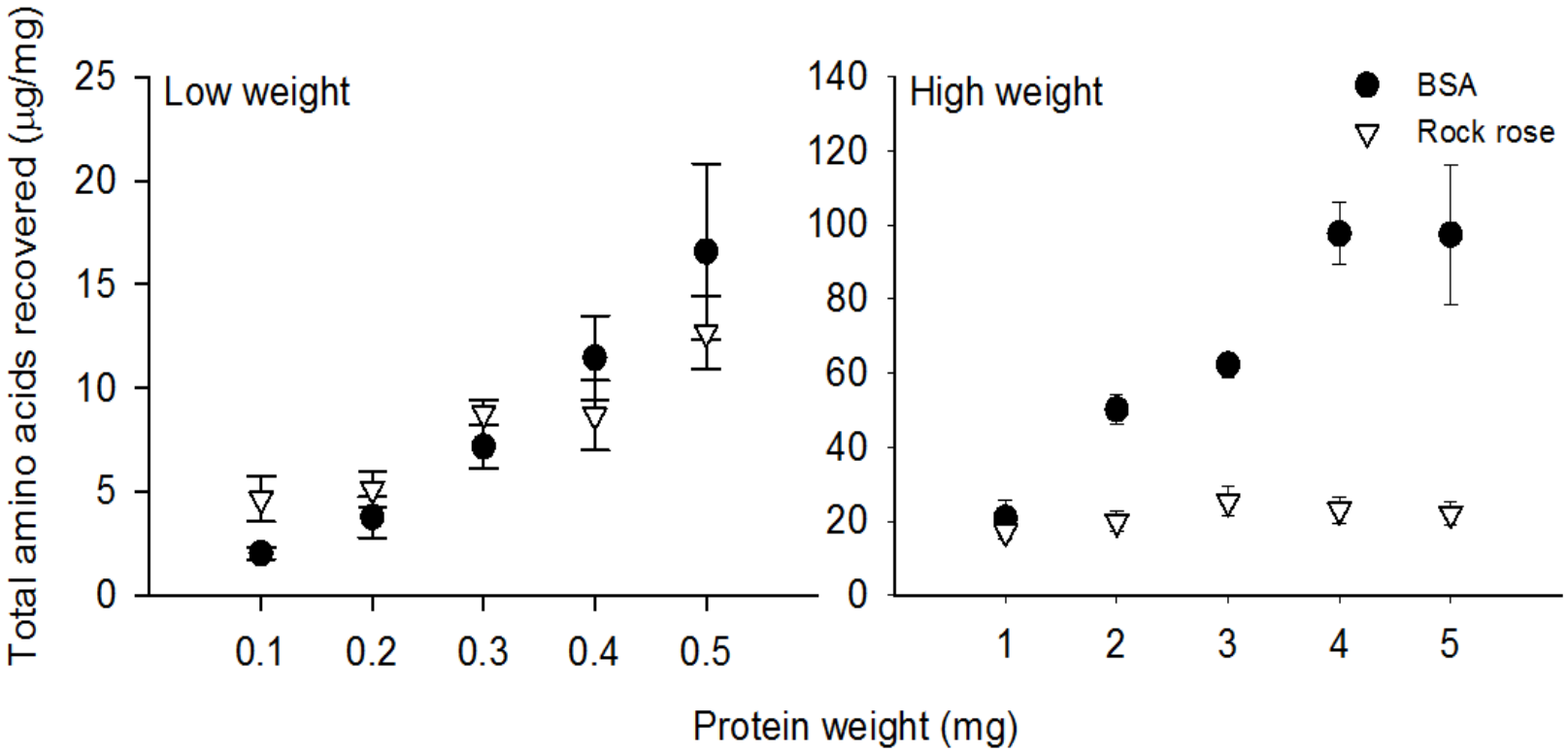

Figure 2.2. Total amino acids (µg/mg) recovered from a microwave-assisted acid hydrolysis of low weight (0.1 - 0.5 mg) and high weight (1 - 5 mg) BSA and Rock rose pollen. Error bars represent ± SEM.

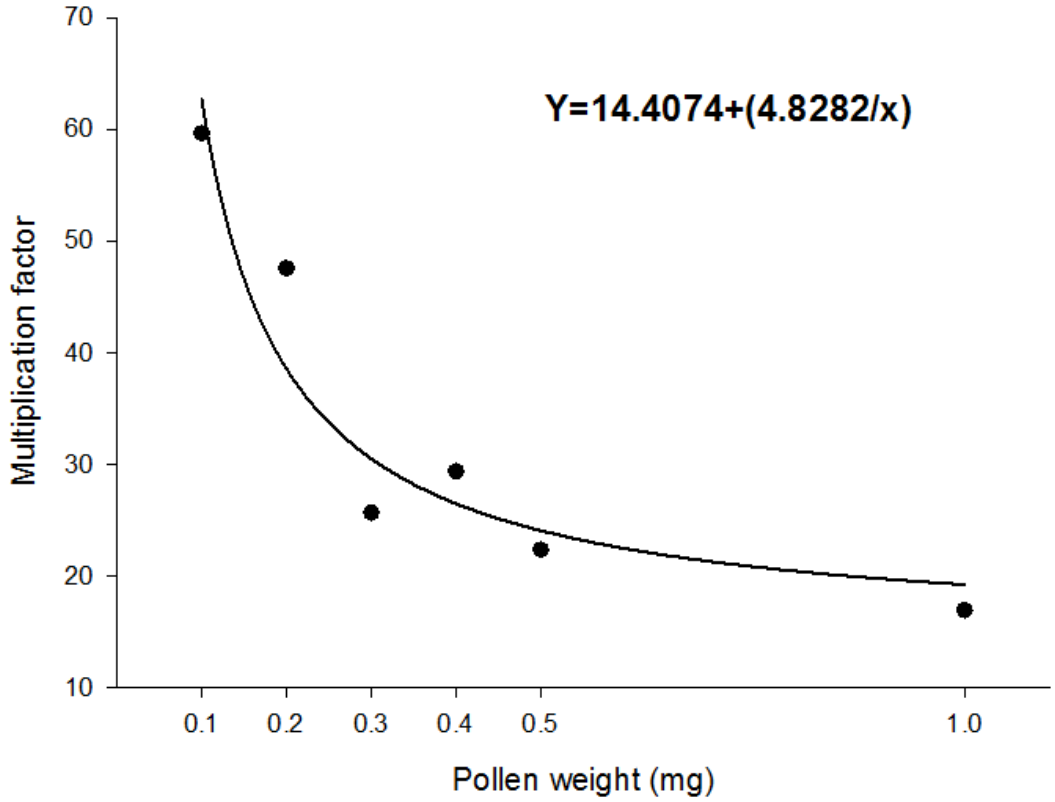

Figure 2.3. Inverse first order line fitted to the correction factors of low weights of pollen (0.1-0.5 mg). The correction factor for the 1 mg weight is included as a reference point for weights between 0.5 and 1mg.

Factor analysis (principal components) was used to test whether weight of sample used influenced the distribution of protein-bound and free amino acids recovered from hydrolysed pollen. Factor analysis was applied to the free and protein-bound amino acids for sample sizes between 0.1 - 0.5 mg (low) and separately to sample sizes between 1 – 5 mg (high).

2.3.4 Protein-bound amino acids – low weights

Each protein-bound amino acid from low weights of pollen was represented by one of four significant factors explaining 84% of the variance in the data (protein bound amino acids, Table 2.2). Factor 1 covered the greatest variance of the data and was represented by positive correlations between 4 amino acids (asp, asn, his and met) and a negative correlation with ser. Factor 2 represented 6 amino acids with positive

correlations (leu, val, phe, ile, and lys) with pro negatively correlation. Factor 3 represented by positive correlations between all 5 amino acids represented (glu, thr, ala, tyr and cys). The fourth factor only represented a positive correlation between two amino acids (gly and arg). Pollen weight was responsible for a change in the profile of amino acids in Factors 1 and 3 but did not influence factors 2 and 4 (Table 2.2, MANOVA). The distribution of amino acids from the 0.5 mg samples were different to that of the 0.1 - 0.4 mg weights ($P< 0.001$), which were all similar to each other. Each weight of pollen produced a different distribution of amino acids, represented by factor 3, to at least one other weight ($P< 0.05$).

2.3.5 Free amino acids – low weights

Free amino acids in the low weights of pollen were also reduced to four significant factors, accounting for 77.9% of the variance in the data (free amino acids, Table 2.2). Factor 1 represented positive correlations between 9 amino acids (asn, ser, his, thr, ala, tyr, cys, phe and lys) and represented the greatest variation in the data. Factor 2 was characterised by positive correlations between 5 amino acids (asp, leu, arg, val and met) and a negative correlation between these and glu. Two amino acids were represented in the third factor (gly and pro) and only ile in the fourth Factor. Pollen weight significantly influenced the distribution of amino acids in Factor 2 and 4 but not in Factor 1 and 3 (Table 2.2, MANOVA). Distribution of amino acids in 0.5 mg samples were different to all other weights of pollen in Factor 2 ($P< 0.001$), whereas distribution of amino acids represented in factor 4 were similar in 1 and 5 mg weights and 2, 3, 4 and 5 mg weights.

Table 2.2 PCA and MANOVA for amino acid distribution in low weights of pollen

Protein-bound amino acids					Free amino acids				
	Factors					Factors			
	1	2	3	4		1	2	3	4
Eigenvalue	4.83	4.59	3.49	2.20	Eigenvalue	6.28	4.50	1.70	1.56
Variance %	26.8	25.5	19.4	12.2	Variance %	34.9	25.0	9.4	8.6
Amino acids					Amino acids				
Asp	**0.777**	0.408	0.024	0.231	Asp	0.009	**0.881**	0.088	0.415
Glu	-0.035	-0.088	**0.830**	0.050	Glu	0.485	**-0.638**	-0.341	0.089
Asn	**0.851**	0.458	0.014	-0.069	Asn	**0.914**	0.042	0.253	0.026
Ser	**-0.773**	-0.365	0.412	0.141	Ser	**0.956**	-0.096	0.150	0.047
His	**0.795**	0.155	0.085	-0.124	His	**0.704**	-0.654	-0.117	-0.081
Leu	0.441	**0.750**	0.389	-0.008	Leu	0.471	**0.589**	0.062	0.491
Gly	0.589	0.143	0.120	**0.690**	Gly	0.236	0.182	**0.688**	0.439
Thr	0.540	0.233	**0.696**	-0.184	Thr	**0.853**	0.186	0.219	0.122
Arg	-0.031	-0.269	-0.043	**0.928**	Arg	0.322	**0.729**	0.188	0.005
Ala	0.382	0.293	**0.627**	-0.555	Ala	**0.671**	-0.415	0.226	0.277
Tyr	0.139	0.214	**0.755**	-0.382	Tyr	**0.884**	-0.008	-0.16	-0.051
Cys	-0.200	0.097	**0.842**	0.337	Cys	**0.549**	0.004	0.343	-0.156
Val	0.340	**0.800**	0.011	-0.239	Val	0.428	**0.763**	0.218	0.034
Met	**0.820**	0.336	0.234	0.128	Met	-0.212	**0.779**	-0.105	0.200
Phe	0.232	**0.927**	0.066	-0.110	Phe	**0.695**	0.318	0.017	-0.034
Ile	0.355	**0.646**	-0.013	-0.266	Ile	-0.102	0.249	-0.109	**0.849**
Lys	0.160	**0.837**	0.302	0.035	Lys	**0.653**	-0.623	-0.118	-0.083
Pro	-0.485	**-0.683**	0.384	-0.049	Pro	-0.076	-0.136	**-0.792**	0.210
Test stat F	$21.999_{4,35}$	$2.488_{4,35}$	$4.252_{4,35}$	$0.457_{4,35}$	Test stat F	$1.238_{4,35}$	$20.421_{4,35}$	$0.207_{4,35}$	$3.477_{4,35}$
P value	<0.001	0.061	0.007	0.767	P value	0.313	<0.001	0.933	0.017

2.3.6 Protein-bound amino acids – higher weights

The distribution of amino acids in high weights of pollen was not the same as found previously for the low weights. Protein-bound amino acids were reduced down to four significant factors (75.9% of variance), (protein bound amino acids, Table 2.3). Factor 1 covered the greatest variation in the data and was characterised by positive correlations between 9 amino acids (asp, glu, asn, leu, thr, tyr, cys, val and met). Factor 2 represented 4 amino acids (phe, ile, lys and pro). The third Factor was characterised by three amino acids (gly, arg and ala) and the fourth being led by strong correlations between ser and his. Unlike the lower weights of pollen, weight of pollen used in the higher weight experiments did not influence the distribution of protein-bound amino acids in any factor (Table 2.3).

2.3.7 Free amino acids – higher weights

The distribution of free amino acids in the large sample sizes was reduced to three significant factors. The majority of amino acids loaded on the first factor, which covered most of the variation in the data (asp, asn, ser, his, leu, gly, thr, arg, ala, tyr, cys, met, ile and pro). Factor 2 represented positive correlations between phe and lys, whereas the third factor was influenced by glu and val. Similar to the protein-bound

amino acids, weight of pollen did not change the distribution of free amino acids in the higher weight experiments (free amino acids, Table 2.3 MANOVA).

Table 2.3. PCA and MANOVA for amino acid distribution in high weights of pollen

Protein-bound amino acids					Free amino acids			
	Factors					Factors		
	1	2	3	4		1	2	3
Eigenvalue	5.957	3.696	2.742	1.703	Eigenvalue	8.603	3.616	1.437
Variance %	33.1	20.5	15.2	9.5	Variance %	47.8	20.1	8.0
Amino acids					Amino acids			
Asp	**0.617**	0.28	0.442	0.178	Asp	**0.892**	0.201	-0.205
Glu	**0.708**	-0.020	0.002	0.111	Glu	-0.102	0.545	**0.559**
Asn	**0.835**	0.256	-0.05	-0.384	Asn	**0.762**	0.549	0.149
Ser	-0.153	0.17	0.155	**0.820**	Ser	**0.861**	0.438	0.175
His	0.246	-0.033	-0.048	**0.835**	His	**0.849**	0.242	-0.073
Leu	**0.697**	0.559	0.042	0.147	Leu	**0.753**	0.529	0.073
Gly	0.323	-0.219	**0.798**	0.094	Gly	**0.822**	0.06	0.152
Thr	**0.849**	0.112	-0.042	0.123	Thr	**0.751**	0.486	0.198
Arg	0.035	-0.104	**0.904**	0.088	Arg	**0.737**	0.563	-0.076
Ala	0.492	0.081	**-0.761**	0.086	Ala	**0.728**	0.033	0.447
Tyr	**0.615**	-0.006	-0.581	0.050	Tyr	**0.814**	0.182	0.103
Cys	**0.837**	0.376	0.092	-0.126	Cys	**0.810**	0.467	0.067
Val	**0.616**	0.496	-0.259	0.107	Val	0.168	0.029	**0.757**
Met	**0.802**	0.480	-0.062	0.061	Met	**0.698**	0.626	-0.055
Phe	0.469	**0.811**	-0.190	0.112	Phe	0.553	**0.643**	0.099
Ile	0.453	**0.827**	-0.149	0.113	Ile	**0.676**	0.477	-0.031
Lys	0.428	**0.712**	-0.076	0.106	Lys	0.165	**0.771**	0.027
Pro	0.171	**-0.815**	0.033	0.098	Pro	**0.501**	0.093	-0.388
Test stat F	$1.18_{4,95}$	$0.224_{4,95}$	$0.014_{4,95}$	$1.447_{4,95}$	Test stat F	$0.431_{4,95}$	$0.118_{4,95}$	$0.473_{4,95}$
P value	0.325	0.924	1	0.225	P value	0.786	0.976	0.755

Relevant multiplication factors were applied to the total protein-bound amino acids for each weight of pollen (Table 2.4). The protein-bound amino acids were then plotted against the free amino acids in a bivariate plot (Figure 2.4). Low weights of pollen produced similar concentrations of free amino acids, and applying relevant multiplication factors, we produced similar concentrations of protein-bound amino acids. Higher weights of pollen also produced similar concentrations of free and protein-bound amino acids.

Table 2.4 Multiplication factors for protein-bound amino acids for each weight of pollen used in hydrolysis experiments

pollen weight (mg)	correction factor
0.1	62.60
0.2	38.25
0.3	30.13
0.4	26.07
0.5	23.63
1	10.26
2	10.26
3	10.26
4	10.26
5	10.26

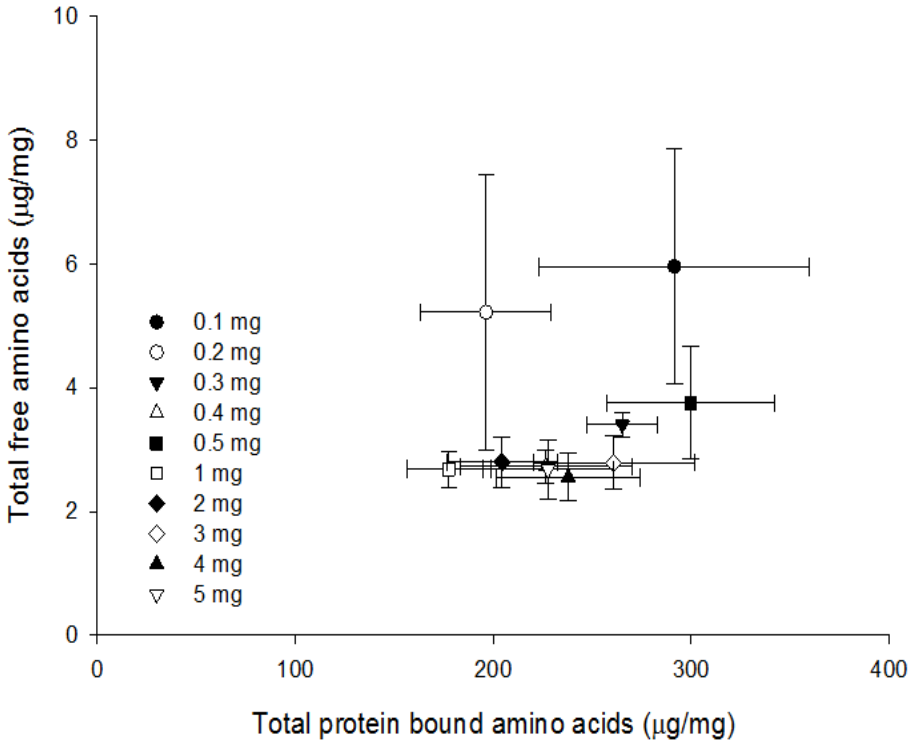

Figure 2.4. Bivariate plot of mean total protein-bound (after correction factor) and mean total free amino acids recovered following hydrolysis of a range of weights of pollen.

2.4 Discussion

2.4.1 Estimation of protein concentration from amino acids in pollen hydrolysate

Our analyses indicated that 1 mg represents the minimum amount of pollen needed to reliably estimate protein concentration after acid hydrolysis. Even though the results were variable, the total amino acid/protein concentration returned from the lysate of low sample sizes (0.1 - 0.5 mg) still represented a function of the amount of pollen used in the hydrolysis. As pollen sample size increased, hydrolysis was more complete, delivering more reliable values (Figure 2.2).

Previously, studies which employed Bradford, micro-Kjeldahl, and combustion methods used quantities of pollen between 1 and 1000 mg (Roulston *et al.*, 2000), far greater than is usually achievable when hand-collecting pollen from individual plants. Both micro-Kjeldahl digestion and combustion methods rely on reducing proteins to absolute nitrogen and back-calculating these values with fixed correction factors to give a protein concentration (Roulston *et al*., 2000). The standard factor for estimating protein from nitrogen concentrations is 6.25. Our method indicated a correction factor of 10.3 was more applicable to weights of pollen ≥ 1 mg. Both Kjeldahl and combustion methods are limited in that crude protein concentration can be calculated. However, these methods do not provide information about the amino acid composition of protein in pollen. The method developed in this chapter provides a partial hydrolysis, from which total protein content is estimated based on the total amino acids rendered versus an expected value of a pure protein (BSA). The efficiency of the hydrolysis can be improved by increasing the ratio between protein and acid from 1 : 100 to 1 : 400 (mg : µl) (Appendix B). However, using higher volumes of acid is unsafe, increasing the risk of the seal on tubes breaking under pressure while in the microwave.

Development of current methodolgy to allow the reliable nutritional study of pollen is of considerable importance, though the collection of sufficient quantities of pollen to allow appropriate analysis remains a recurrent problem to be addressed. Common practice is to exploit pollen that is collected on the corbicula, or 'pollen basket', of foraging bees (Cook *et al*., 2003; Gonzalez-Paramas *et al*., 2006; Höcherl *et al*., 2012;

Nicolson and Human, 2013; Vanderplanck *et al*., 2014; Somme *et al*., 2015). Yet, exploration of free amino acid and carbohydrate concentration of such pollen is erroneous because up to 40% of the dry weight of such pollen pellets may contain sugar and amino acids from regurgitated nectar (Roulston and Cane, 2000). Researchers who elect to analyse hand-collected pollen are invariably limited by the amount of sample that can be collected, so pollen is commonly 'pooled' to provide sufficient amounts for analysis (Weiner *et al*., 2010; Human and Nicolson, 2013). Pooling hand-collected pollen masks any information on variation within populations and makes collecting enough material for sufficient biological replicates problematic. In addition, replication can be difficult if composite samples have to be made in order to obtain sufficient samples for analysis. Our method allows reliable quantification of free and protein-bound amino acids in samples as low as 1 mg; this is 100- and 1000-fold lower than that needed to carry-out reliable combustion and micro-Kjeldahl approaches, respectively (Roulston *et al*., 2000).

Hydrolysis of protein followed by amino acid analysis is a commonly used technique. The major advantage of hydrolysing proteins and quantifying amino acids over other methods of protein quantification (Bradford assay, Kjeldahl or combustion etc.) is that while crude protein concentration can still be estimated, amino acids can be quantified thus providing essential information on nutritive value. Quantification of amino acids in pollen is useful when assessing the quality of pollen as a food source for pollinators, assuming greater nutritive value with a greater proportion of essential amino acids to non-essential (Wathelet, 1999). Microwave-assisted acid hydrolysis has greatly improved the speed at which hydrolysis can be performed, reducing the standard method (6M HCl boiled at 110°C for 24 h) by between 1 and 30 min (Fountoulakis and Lahm, 1998). Microwave-assisted hydrolysis is also beneficial in that it allows samples to be hydrolysed in batches, making treatments more comparable.

Currently, there is no protein hydrolysis method that allows successful quantification of all protein-bound amino acids. Fountoulakis and Lahm (1998) highlight that under the conditions of acid hydrolysis, amino acids themselves can be hydrolysed and so correction factors need to be applied for accurate quantification and estimation

of overall protein content. Sulphur amino acids (methionine and cysteine) benefit from oxidation prior to hydrolysis. However, oxidation reduces the measureable tyrosine (Bech-Andersen *et al*., 1990). Tryptophan is entirely destroyed by acid hydrolysis and so samples must be subject to alkaline hydrolysis in a separate representative sample (Fountoulakis and Lahm, 1998), while asparagine and glutamine are readily deaminated to aspartic acid and glutamic acid, respectively.

Previous publications have suggested methods for correcting amino acid values measured in hydrolysate claiming to compensate for losses in amino acids during acid hydrolysis. Robel and Crane (1972) hydrolysed a purified protein (lysozyme) after 4, 24, 50, 72 and 141 h hydrolysis. Plotting the amino acid residues detected at each time point, they proposed a non-linear least-squares equation to account for hydrolysis and loss rates for individual amino acids. A similar approach by Darragh and colleagues (1996) also applied a non-linear least-squares equation but they highlighted the need for sample replication and raised the issue that unknown contaminants may influence hydrolysis efficiency. They suggested a formula correcting for the time needed to cleave amino acids from proteins, the time at which amino acids are available for detection and the time amino acids are stable before acid degradation. It has been suggested that separate correction factors for individual amino acids are necessary based on the different individual rates of degradation during hydrolysis (Rees, 1946; Darragh *et al*., 1996; Bunka *et al*., 2009). Increasing the duration of hydrolysis can also increase amino acid loss (Simpson *et al*., 1976; Bunka *et al*., 2009). Hydrolysis efficiency may also be influenced by protein structure; complex proteins require longer hydrolysis times (Blackburn, 1978). However, a balance must be made between how long to hydrolyse and how much amino acid loss is acceptable. Glutamine and tryptophan were not detected in lysate of any hydrolysed pollen samples. There is evidence that glutamine is rapidly deaminated to glutamic acid under hydrolysis conditions (Salo-väänänen and Koivistoinen, 1996). Tryptophan is well documented as being entirely destroyed by acid hydrolysis (Blackburn, 1978).

It is interesting that the efficiency of hydrolysis of BSA improves with overall amount of protein and acid when the ratio between the two components is maintained.

The same relationship was not observed when hydrolysing proteins in pollen. One reason for this could be that pollen is a very different substrate to that of pure protein. Another is that there simply is no more protein available in the pollen to be hydrolysed and so the rate of hydrolysis plateaus. Protein-bound amino acids of pollen are held within the pollen exine and are also expected to be considerably lower in concentration than in a pure protein. We found that efficiency of hydrolysis of BSA was greatly improved when the ratio of protein to acid was closer to 1 : 400 (mg : μl) (Appendix B). However, using this volume of acid becomes unsafe: tubes began to burst open during microwave irradiation. Our goal was to develop a method that is cost effective and convenient to ecologists, and avoid the requirement for specialist equipment which is invariably costly and unsuitable for high throughput experiments.

2.4.2 Protein quantification using a Bradford assay depends on solubility of the protein

In this study a variety of pollen pre-treatments were explored to enquire whether this would influence protein/reagent binding. We found that using the regularly-employed Bradford assay (Bradford, 1976) resulted in the underestimation of protein concentration in all treatments employed. The highest concentration returned was for pollen that was homogenised, but this value was still significantly short of the expected value. This suggests that Bradford reagent is not suitable for accurate quantification of protein in small sample sizes of pollen. The solubility of the proteins within pollen may influence the binding efficiency of protein to the Bradford reagent, as we discovered that pollen washed in methanol showed a significant reduction in the estimated protein concentration as compared to the untreated controls. This suggests that either proteins are being removed from the pollenkitt and so are no longer available for protein-dye binding, or that the Bradford reagent is actually binding to free amino acids present in the pollenkitt, and thus not actually representing the protein of the pollen. It is also well documented that Bradford reagent readily binds to the basic structures of amino acids. A protein-dye complex is formed when positively charged amino acids (arginine, histidine and lysine) mix with the acidic dye (Schaffner and Weissmann, 1973: Tal *et al*., 1985: Syorvy and Hodny, 1991), and so proteins lacking amino acids with basic side chains may be less likely to be represented in the

colorimetric reading. One method that could improve the accuracy of using a Bradford assay to quantify protein concentration of pollen is to use a pollen of known protein concentration to calibrate the standard curve (Roulston *et al*., 2000; Yeamans *et al*., 2014) instead of a vastly different protein 'standard' such as BSA (Roulston *et al*., 2000; Yeamans *et al*., 2014).

2.4.3 Free amino acids are reliably measured in pollen weights ≥ 1 mg

It is important to understand the profile of amino acids within the pollenkitt as pollinators may use olfactory and gustatory cues provided to make assessments of pollen quality based on either pre- or post-ingestive feedback (Dobson, 1987). Using the method described by Cook and co-workers (2003), free amino acids were washed from pollen and not exposed to hydrolysis. Pollen weights ≥ 1 mg produced similar concentrations of free amino acids. However, there was significantly greater variation in smaller sample sizes, so we recommend using larger sample sizes if sufficient amounts of pollen can be collected. An extraction buffer preventing any further amino acid loss may provide more reliable extraction, but this would need to be tested.

2.4.4 Conclusion

Using HPLC to analyse a lysate of pollen proteins allowed the determination of total protein content of pollen more accurately than using a Bradford assay. The highest measured protein concentration for rock rose pollen was ~6% whereas quantifying protein bound amino acids in the lysate produced ~23% protein. We demonstrated that free amino acids and protein-bound amino acids can be reliably quantified from pollen weights ≥ 1 mg. Weights of pollen lower than this are prone to result in unacceptable degrees of error.

3.0 Chapter 3: Analysis of nectar from low-volume flowers: a comparison of collection methods for carbohydrates and free amino acids

3.1 Introduction

Nectar is produced by flowering plants as a nutritional reward for animal pollinators. Pollinating animals learn to associate the multimodal cues provided by flowers (colour, size, odour etc.) with nectar so that they can identify other conspecific flowers to obtain nectar. Pollinator fidelity to the flowers of a particular plant species results in successful pollination. The carbohydrate and amino acid properties of nectar have a great influence on visitation rates and nectar preferences by insect pollinators (Alm *et al*., 1990; Chitka and Shürkens, 2001; Simcock *et al*., 2014) and how well pollinators will form lasting associations between floral features and a nectar reward.

Nectar is a water-based secretion mainly composed of a broad range (7% to 70% w/w) of simple sugars (glucose, fructose and sucrose) (Nicolson *et al*., 2007). Amino acids are the second most abundant nutrients in nectar (Baker and Baker, 1973; Petanidou *et al.*, 2006), yet a thousand times less concentrated than sugars (Gottsberger, 1984; Petanidou *et al*., 2006). For pollinators that do not eat pollen, nectar is an important source of dietary amino acids (Baker and Baker, 1973; Baker, 1977). Amino acids have also been shown to affect pollinator behaviour (Inouye and Waller, 1984; Gardener and Gillman, 2002; Simcock, 2014; Hendricksma *et al*. 2014). The study of nectar chemistry has focused primarily on carbohydrates and amino acids and other nectar constituents have been less well studied (Nepi, 2014).

The study of nectar chemistry has been subject to some limitations. Many flowers are very small and/or produce very low volumes of nectar (~1 µl). Plants naturally vary in the volumes of nectar secreted (< 1 µl to > 20 ml), so most studies have focussed on those that produce greater volumes due to the ease of sample collection. There are a number of commonly used methods for nectar collection. Raw nectar can be extracted from flowers using microcapillary tubes. This method involves inserting the capillary tube into the flower and precisely placing the tube over the nectaries. Nectar is drawn into the tube by capilliary action. This method allows the volume of nectar

available in the flower to be measured directly and the nectar can be expelled from the tube to allow further analysis (Corbet, 2003; Morrant *et al*., 2009). Alternatively, filter paper can be cut into wicks and used to absorb nectar from flowers. To allow further analysis of nectar, the filter paper must be soaked in water. This method is limited in that estimations of the volume of nectar extracted from flowers cannot be calculated (McKenna and Thomson, 1988; Kearns and Inouye, 1993; Morrant *et al*., 2009). A further method to extract nectar from flowers, referred to as 'wash' is to excise the flower from the plant and immerse the floral tissue in a specific volume of water (Käpylä, 1978; Grunfeld *et al*., 1989; Morrant *et al*., 2009). It has also been reported that nectar can be collected by 'rinsing' a known volume of water directly over the nectaries and collecting the nectar-water solution for analysis (Núñez, 1977; Mallick, 2000; Morrant *et al*., 2009).

A study comparing nectar-collecting techniques in low-volume flowers (*Eucalyptus* spp.) reported that sampling method (microcapillary tubes, filter paper, washing and rinsing) influenced the ratio and overall mean concentration of carbohydrates recovered from flowers (Morrant *et al*., 2009). The authors recommended washing and rinsing flowers (immersion or addition of 2 ml of water to a flower to dilute and extract nectar) to estimate quantities available to pollinators because these methods recovered a higher concentration of carbohydrates. However, it is not clear whether these rinse/wash methods provide a realistic measure of the total concentration of carbohydrates available to a flower visitor. Samples collected by rinsing or washing could include dried carbohydrates on the flower surfaces which are not obtainable by a flower visitor. Furthermore, a major problem with the use of rinsing or washing is that pollen could contaminate the samples leading to an overestimation of solutes including amino acids or toxins (Gottsberger *et al*. 1984; Gottsberger *et al*., 1990).

The experiments in this chapter report the use of high performance ion chromatography (HPIC) and uHPLC (Ultra High Performance Liquid Chromatography) to compare and identify how different methods of nectar collection may affect the carbohydrate and amino acid compositions of low-volume nectar flowers, using *Calluna vulgaris* as a model species.

3.2 Methods

3.2.1 Nectar collection

Five methods of nectar collection were compared for sugar and amino acid recovery using flowers collected from *Calluna vulgaris* L. (Ericaceae). Plants of the same age and condition were bought at a garden centre in Newcastle Upon Tyne, United Kingdom. To reduce variation based on flower age, plant and time of sampling (Nicolson *et al.*, 2007), four flowers of similar maturity (open and showing no signs of senescence) were taken from each of the three plants at the same time for each collection method. Nectar was sampled from these flowers using the following methods: (1) microcapillary tubes (McKenna and Thomson, 1988; Kearns and Inouye, 1993; Corbet, 2003; Morrant *et al.*, 2009); (2) filter paper wicks (McKenna and Thomson, 1988; Kearns and Inouye, 1993; Morrant *et al.*, 2009); (3) washing in 2 ml of water (Morrant *et al.*, 2009); (4) rinsing with 2 ml of water (Morrant *et al.*, 2009); and (5) rinsing with 2 µl of water. The first four methods were compared by Morrant and colleagues (2009) in terms of suitability for sugar recovery in low-nectar flowers, in addition here, a fifth method has been explored as suitable for sugar and amino acid recovery. The details of each collection method are given below.

(1) Microcapillary tubes (raw nectar): This method allows the estimation of the volume of nectar obtained from individual flowers. In the experiments decribed in this chapter, nectar was sampled from 12 individual flowers using 1 µl microcapillary tubes (Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany). Nectar was drawn into the tubes by capillary action. This was done to avoid damage to floral tissue and to prevent uptake of pollen grains into the sample. The volume of withdrawn nectar was quantified by measuring the length of the tube (mm) and calculating the proportion of the tube that was filled with nectar. Each nectar sample was diluted with de-ionised uHPLC gradient grade water (Fisher Scientific UK Ltd., Loughborough, United Kingdom) to meet the minimal sample volume requirements for HPIC and uHPLC analysis (uHPLC amino acid dilution: 10 µl requiring 1 : 65 dilution; HPIC carbohydrate dilution: 30 µl requiring 1 : 2,000 dilution).

(2) Filter paper: Nectar was sampled from 12 flowers using filter paper wicks, *sensu* Morrant and colleagues (2009). Twelve strips of Fisherbrand QL100 filter paper (Fisher Scientific UK Ltd., Loughborough, United Kingdom) with dimensions (5 x 42 mm, tapered to 1 mm width tip at one end) were cut using sterile blades. Using sterile forceps, the edges of one filter paper strip were applied to the nectaries of one flower. Each strip was then placed in a sealed sterile vial (20 ml) containing 2 ml of de-ionised uHPLC gradient grade water, soaked for 15 min and then agitated for 1 min.

(3) Wash 2 ml: Nectar was sampled from 12 flowers using a washing method *sensu* Morrant and colleagues (2009). Each flower was cut from the plant and placed in a sealed sterile vial (20 ml) containing 2 ml of de-ionised uHPLC gradient grade water. The vial was agitated for 1 min.

(4) Rinse 2 ml: Nectar was sampled from 12 flowers using a rinsing method *sensu* Morrant and colleagues (2009). A flower was inverted over a 2 ml sterile vial and four successive rinses (0.5 ml) of de-ionised uHPLC gradient grade water were expelled over the floral nectaries using a sterile pipette. It was not necessary to remove the flowers from the plant for this method.

(5) Rinse 2 µl (micro-rinse): Nectar was sampled from 12 flowers. Using a sterile pipette, 2 µl of de-ionised uHPLC gradient grade water was expelled into a flower over the nectaries. The water was left to remain in the flower for 1 min then the nectar-water solution was drawn into a 10 µl microcapillary tube (Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany). This was done with care to avoid damaging floral tissue, preventing uptake of pollen grains into the sample. No floral tissue was removed prior to rinsing. The volume of withdrawn nectar-water solution was quantified by calculating the proportion of the tube that was filled with solution. Each sample was diluted further with de-ionised uHPLC gradient grade water to meet minimal sample volume requirements for HPIC and uHPLC analysis (see above). It was not necessary to remove the flowers from the plant for this method. The 2 µl volume of water added to the nectary was chosen because it was a sufficient-sized volume to cover the nectary but not the anthers.

(6) Filter paper control: To determine if filter paper leached amino acid contaminants into the nectar sample, ten filter paper wicks of similar type and

dimensions as used in the above method were dipped using sterile forceps in ten sterile 2 ml microcentrifuge tubes containing 1 µl of de-ionised uHPLC gradient grade water. This procedure was designed to emulate nectar extraction from 12 flowers. Each strip was then placed in a sealed sterile vial (20 ml) containing 2 ml of de-ionised uHPLC gradient grade water, soaked for 15 mins and then agitated for 1 min.

When using filter paper, washing or rinsing methods, separate estimates of the average standing crop (nectar volume per flower) had to be made so that the mass of solutes in nectar per flower could be calculated. To obtain an average standing crop, the volume of nectar in 12 flowers was recorded and averaged using the microcapillary method. In addition, previous studies have used distilled water to dissolve nectar solutes from filter paper or wash and rinse nectar from flowers (McKenna and Thomson, 1988; Mallick, 2000; Petanidou *et al.*, 2006; Morrant *et al.*, 2009). In the present study de-ionised uHPLC grade water was used which is free from amino acids and other ionic contamination.

3.2.2 Nectar sample preparation and analysis

3.2.2.1 Filtration

For the analysis of sugars *via* high performance ion chromatography (HPIC), 30 µl of sample volume was required while for uHPLC amino acid analysis, 10 µl of sample was required. Some nectar collection methods produced enough sample volume for analysis (e.g. filter paper, wash 2 ml, rinse 2 ml methods and filter paper control). These samples were filtered using a sterile 0.45 µm 4 mm nylon Whatman Puradisc syringe filter to remove particulates and plant material (Note: filtering caused the loss of a significant amount of sample). Low volume (< 100 µl) samples were not filtered.

3.2.2.2 Centrifugation

Microcapillary and rinse 2 µl samples were diluted 2,000-fold for sugar analysis and 65-fold for amino acid analysis using de-ionised uHPLC gradient grade water. These dilution factors were derived by diluting nectar so that sugar and amino acid

concentrations matched those of the sugar and amino acid standards used to calibrate the chromatography instruments. Low-volume samples were centrifuged for 10 min at 13,249 x *g* to remove soluble sugars and amino acids (supernatant) from any residual plant material.

3.2.3 Carbohydrate analysis

The concentrations of glucose, fructose, sucrose, sorbitol and mannitol were measured by HPIC. The HPIC analysis was conducted by injecting 20 µl of each sample *via* a Rheodyne valve onto a Carbopac PA-100 column (Dionex, Sunnyvale, California, USA) fitted with a Dionex Carbopac PA-100 BioLC guard (4 x 50 mm). Approximately 30 µl of sample was inserted into an analysis vial to ensure optimal immersion of the autosampler syringe. Sample components were eluted from the column isocratically using 100 mM NaOH (de-gassed by helium gas) employing a flow rate of 1 ml min^{-1} for 10 min at room temperature (RT). The chromatographic profile was recorded using pulsed amperometric detection with an ED40 electrochemical detector (Dionex, Sunnyvale, California, USA). Elution profiles were analysed using Chromeleon v.6.8 software (Thermo Fisher Scientific Inc., MA, USA) which automatically calculated solute concentrations based on a range (different dilutions) of pre-programmed reference curves for each sugar and/or sugar alcohol. The HPIC was calibrated at least twice every 24 h period for glucose, fructose, sucrose, sorbitol and mannitol by injecting calibration standards (Appendix D). Standard solutions were made from the solid form of each sugar available (Sigma-Aldrich, St. Louis, MO, USA). The dual calibration each day ensured accuracy of peak identification in the event of daily drift in elution times for sugars. The optimal dilution of nectar : water required for this HPIC method was 1 : 2,000, requiring approximately 0.05 µl of raw nectar (to make 100 µl of solution).

3.2.4 Amino acid analysis

The concentrations of 21 amino acids in nectar were measured using uHPLC: aspartic acid (*asp*), glutamic acid (*glu*), asparagine (*asn*), serine (*ser*), glutamine (*gln*), histidine (*his*), glycine (*gly*), threonine (*thr*), arginine (*arg*), alanine (*ala*), tyrosine (*tyr*), cysteine (*cys*), valine (*val*), methionine (*met*), gamma-aminobutyric acid (*GABA*), tryptophan (*trp*), phenylalanine (*phe*), isoleucine (*ile*), leucine (*leu*), lysine (*lys*) and

proline (*pro*). The method employed for amino acid quantification with uHPLC is described in Section 2.2.6. The optimal dilution of nectar : water required for this HPLC method was 1 : 65; requiring at least 0.25 µl of raw nectar (to make 16.25 µl of solution).

3.2.5 Derivation of values

After each compound was identified in each chromatogram, values produced by the Chromeleon software were scaled up to their original concentrations in nectar based on how much the nectar was diluted by each method. Microcapillary method: the raw nectar extracted directly from the flower was diluted to make enough sample for the chromatography instruments. Therefore, the measured sugar/amino acid concentrations were multiplied by the dilution factor used to dilute the raw nectar before analysis (2,000 for sugars and 65 for amino acids). Filter paper, wash and rinse 2 ml methods: these methods yielded volumes of 2,000 µl. To estimate how much the raw nectar was diluted, the total amount of water added to extract the nectar (2,000 µl) was divided by the average standing crop per flower. The mean standing crop per flower recovered using microcapillary tubes was 0.5 µl (± 0.06 SE). The standing crop was used as an estimate of how much nectar was in the flower at the time of sampling.

Equation 1:

$$D = \frac{W}{S}$$

Where: D = final dilution factor; W = amount of water (µl) added to flower during nectar sampling; S = standing crop (µl). The final dilution factor was multiplied by the Chromeleon sample measurement for each sugar/amino acid to obtain approximate raw nectar values.

The 2 µl rinse method: in this method, the raw nectar was first diluted by adding 2 µl to the flower to extract the nectar. The mean recovery of nectar solution from this treatment was 2 µl (± 0.1 SE). The nectar solution was then diluted a second time to increase the sample volume for optimal machine operation. Therefore, calculations

were as follows: the 2 µl added to the flower was divided by the average standing crop per flower (similar to above). This value was then multiplied by the initial dilution factor used to prepare the samples for the machine (1 : 2,000 for sugars and 1 : 65 for amino acids).

Equation 2:

$$D = \left(\frac{W}{S}\right) \times I$$

Where: D, W and S as for Equation 1; I = initial dilution factor (the value for carbohydrates was 2000 and for amino acids it was 65). The final dilution factor (D) derived from this calculation was multiplied by the Chromeleon sample measurement to obtain approximate raw nectar values for each sugar/amino acid.

3.2.6 Statisitical analysis

Statistical analysis was carried out using SPSS (Version 2.1). Glucose and fructose were significantly more concentrated in the nectar of this plant than sucrose, sorbitol and mannitol and so were analysed separately. Mannitol was only present in samples from the 2 ml wash method so was excluded from the analysis. All sugar values were log transformed (ln(x + 1)) prior to analysis. Carbohydrate concentration was analysed using a 2-way analysis of variance (ANOVA) with sampling method and carbohydrate as main effects. Sidak's *post hoc* tests were used for pairwise comparisons.

Essential amino acids (arginine, threonine, phenylalanine, isoleucine, leucine, lysine, methionine, valine, histidine, tryptophan) and non-essential amino acids (proline, aspartic acid, alanine, cysteine, glutamic acid, glycine, serine, tyrosine, glutamine, and GABA) were analysed separately. Proline was analysed independently because the dataset contained extreme outliers. Total amino acid concentrations (excluding proline) were log transformed (ln(x + 1)); concentrations of proline were square root transformed (√(x+1)). Total amino acids were analysed in a 2-way ANOVA with method and amino acid group as main effects. Data were subject to one-way ANOVA to test the effect of method on proline concentration. Sidak's *post hoc* tests were used for pairwise comparisons.

Differences in the distribution of individual amino acids (except proline) were explored using a factor analysis (principle components analysis) for all 5 nectar collection methods. The factor scores produced by the factor analysis were compared with a one-way ANOVA using nectar collection method as the main effect.

3.3 Results

3.3.1 Carbohydrates

Glucose and fructose were the most abundant sugars found in nectar, with sucrose, sorbitol and mannitol 100-fold less concentrated than glucose and fructose (Figure 3.1, Table 3.1). Sampling method had a strong effect on the estimated concentrations of carbohydrates. Glucose, fructose, sucrose and sorbitol were significantly ($P< 0.001$) higher in concentration in the rinse 2 ml and wash 2 ml samples than in the microcapillary, filter paper and rinse 2 µl methods. Recovery of glucose and fructose did not depend on sampling method, but the method adopted did significantly (sugar*method $P< 0.001$) affect the proportions of sorbitol and sucrose recovered.

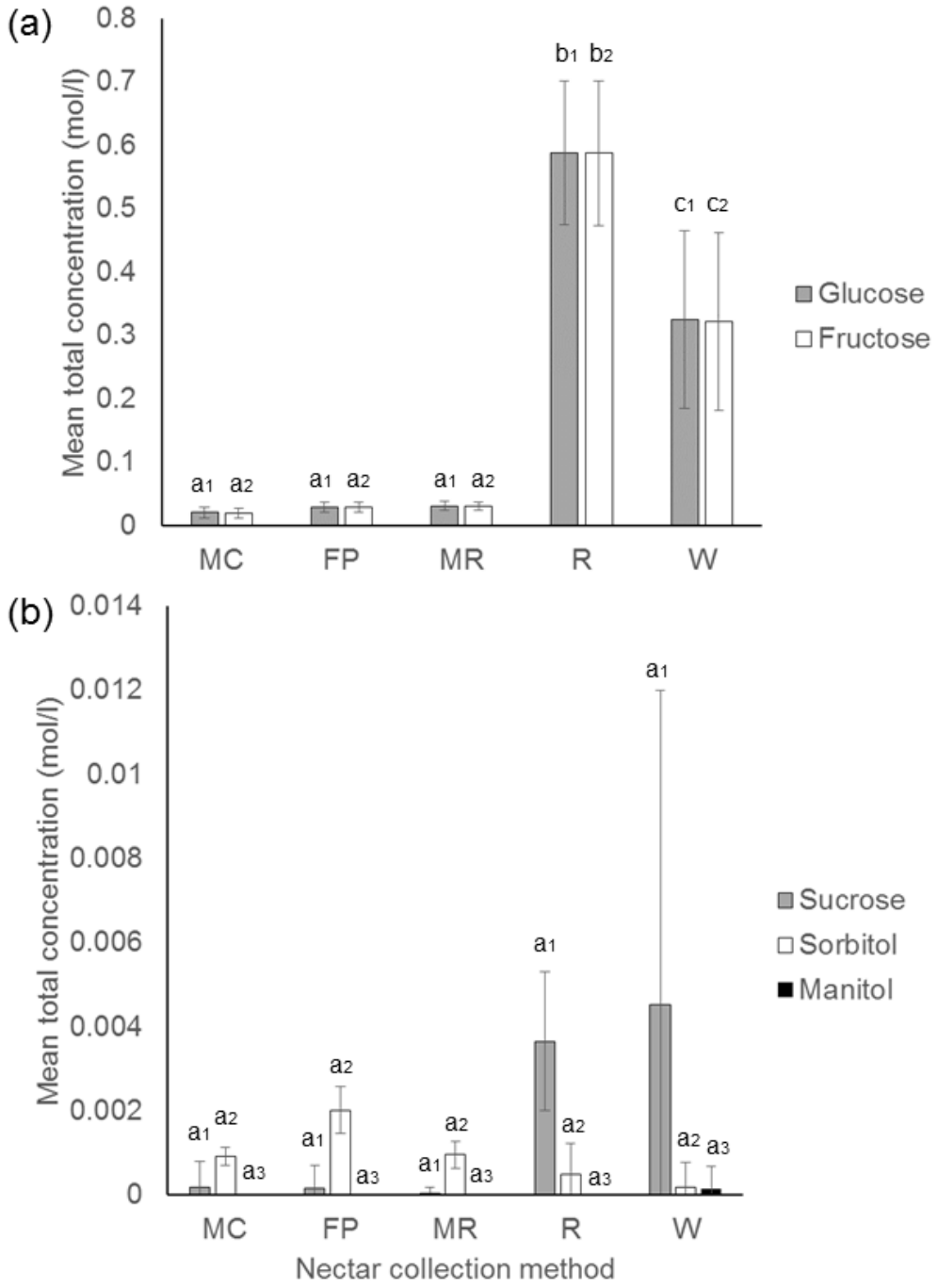

Figure 3.1. The mean concentrations of glucose and fructose (a) and sucrose, sorbitol and mannitol (b) from nectar collected by five methods: MC = microcapillary; FP = filter paper; MR = rinse 2 µl; R = rinse 2 ml and W = wash 2 ml. Letters denote significant differences between methods (P< 0.05). Bars represent standard error of mean.

Table 3.1. Mean (± SE) sugar concentration (mM) in samples from five nectar collection methods

Method	Sugars				
	Glucose Mean (±SE)	Fructose Mean (±SE)	Sucrose Mean (±SE)	Sorbitol Mean (±SE)	Mannitol Mean (±SE)
Microcapillary	20±10	20±10	0.2±0.2	0.9±0.06	0±0
Filter paper	30±10	30±10	0.2±0.2	2±0.2	0±0
Rinse 2µl	30±10	30±10	0.03±0.03	1±0.09	0±0
Rinse 2ml	590±110	590±110	3.7±0.5	0.5±0.2	0±0
Wash 2ml	320±14	320±140	4.5±2.2	0.2±0.2	0.2±0.2

3.3.2 Amino acids

Amino acids were present at concentrations that were orders of magnitude lower than glucose and fructose in nectar (Figure 3.2, Table 3.2). Figure 3.2 shows that the total average essential and non-essential amino acid concentrations depended on the method of sampling (amino acids*method $P< 0.001$). The microcapillary method, which it was predicted would be the most reliable indicator of the actual values of the solutes found in nectar, returned the lowest total amino acid concentrations (Figure 3.2). The filter paper, rinse 2 ml and wash 2 ml recovered total average values for both essential and non-essential amino acids that were 10 - 50 times greater than the rinse 2 µl and microcapillary methods.

Proline quantification from these samples seemed to vary greatly and it is possible that there was contamination of this peak (from non-amino acid solutes in nectar). In general, the concentration of proline was greater than any other amino acid (Figure 3.2, Table 3.2) but there was large variation between samples. The filter paper method recovered more 'proline' ($P< 0.001$) in the sample than any of the other methods.

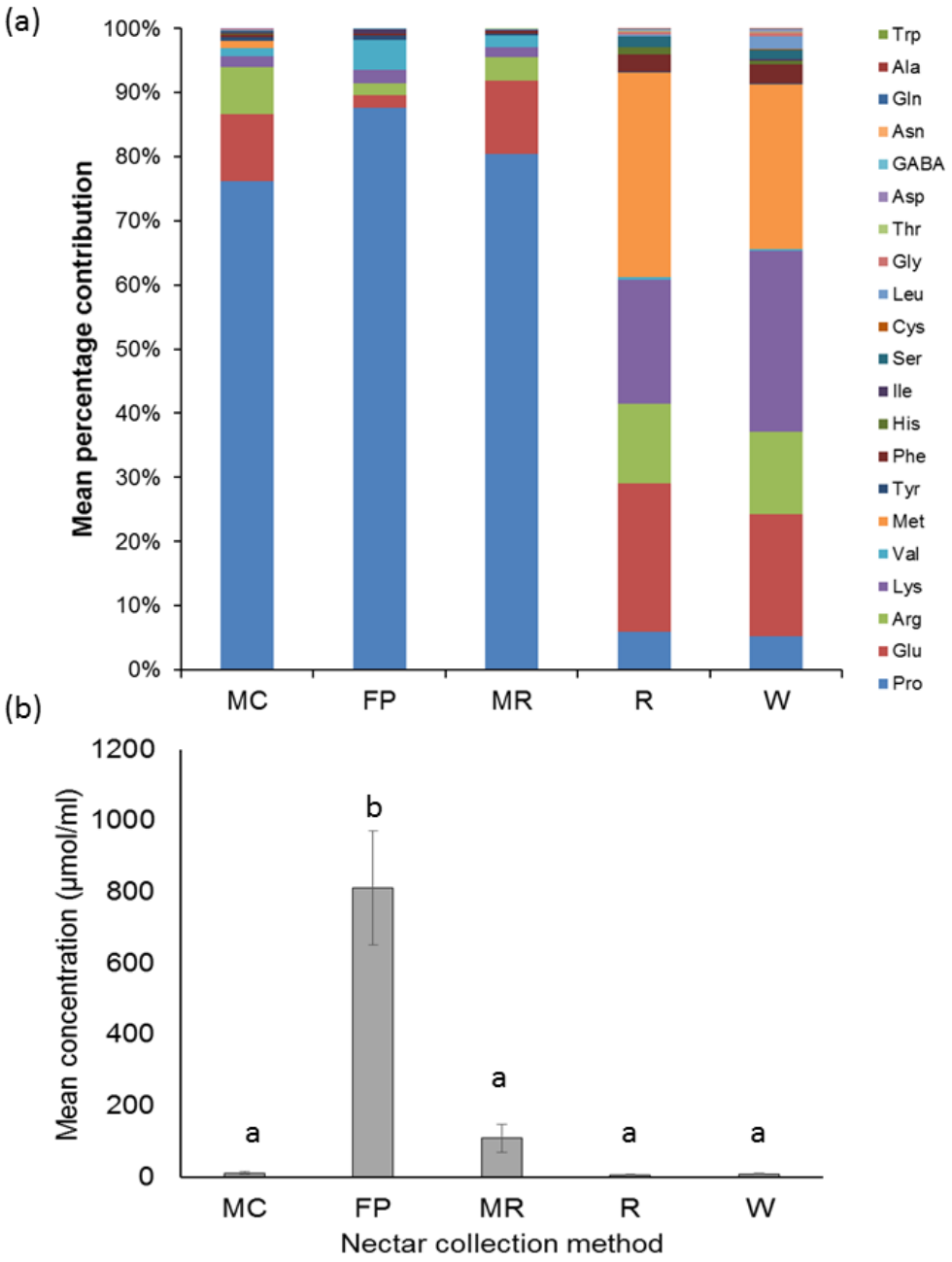

Figure 3.2 (a): mean percentage contribution of all amino acids to nectar samples collected by five methods: MC = microcapillary; FP = filter paper; MR = rinse 2 µl; R = rinse 2 ml and W = wash 2 ml. Note: the dominance of proline in the microcapillary, filter paper and rinse 2 µl methods. (b) The mean concentration of proline (µmol/ml) in nectar samples collected by five methods. Columns bearing same letter are not significantly different at the 5% level.

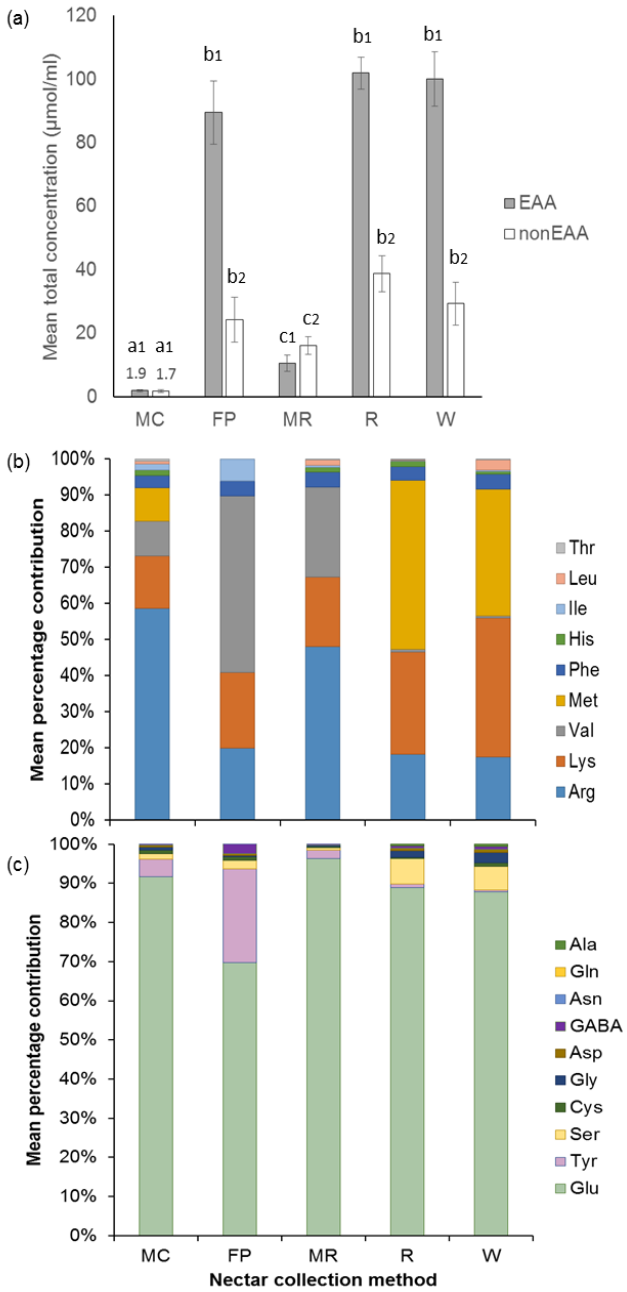

Figure 3.3 (a): Mean (± SE) total essential and non-essential amino acid concentration in nectar samples collected by five methods: MC = microcapillary; FP = filter paper; MR = rinse 2 µl; R = rinse 2 ml and W = wash 2 ml. The filter paper, rinse 2 ml and wash 2 ml methods recovered a significantly higher concentration of essential than non-essential amino acids whereas the microcapillary and rinse 2 µl methods recovered relatively equal concentrations of both. Letters denote significant differences between methods and amino acids ($P < 0.05$). Analysis was done using Sidak post-hoc tests. (b) Mean percentage contribution of essential amino acids to nectar samples collected by the five methods employed. (c) The mean percentage contribution of non-essential amino acids to nectar samples collected by the five methods employed.

Table 3.2: Mean (±SE) amino acid concentration in samples from five nectar collection methods and a filter paper control

Amino Acids	Microcapillary (µmol/ml)	Filter paper (µmol/ml)	Rinse 2µl (µmol/ml)	Wash 2ml (µmol/ml)	Rinse 2ml (µmol/ml)	Filter paper control (µmol/ml)
Aspartic acid	0.01±<0.01	0.12±0.04	0.01±<0.01	0.23±0.03	0.19±0.03	0
Glutamic acid	1.60±0.38	17.35±6.81	15.51±2.83	26.02±6.55	34.68±5.31	<0.01±<0.01
Asparagine	<0.01±<0.01	<0.01±<0.01	<0.01±<0.01	<0.01±<0.01	<0.01±<0.01	0
Serine	0.02±<0.01	0.55±0.25	0.10±0.03	1.78±0.60	2.52±0.94	<0.01±<0.01
Glutamine	0	0	0	<0.01±<0.01	<0.01±<0.01	0
Histidine	0.03±0.01	0	0.15±0.08	0.81±0.24	1.64±0.51	<0.01±<0.01
Glycine	0.01±<0.01	0.06±0.32	0.05±0.01	0.77±0.09	0.71±0.09	<0.01±<0.01
Threonine	0.01±<0.01	0	0.03±0.02	0.18±0.09	0.16±0.09	0
Arginine	1.12±0.27	17.82±10.66	5.07±1.68	17.55±0.95	18.56±2.33	<0.01±<0.01
Alanine	0	0	0	0.16±0.05	0.16±0.04	<0.01±<0.01
GABA	0.01±<0.01	0.62±0.02	0.03±<0.01	0.25±0.04	0.28±0.04	<0.01±<0.01
Tyrosine	0.08±<0.01	5.98±0.04	0.35±0.01	0.14±0.09	0.32±0.02	0
Cysteine	0.02±0.01	0.22±0.15	0.04±0.01	0.25±0.08	0.13±0.06	<0.01±<0.01
Valine	0.18±0.08	43.78±0.96	2.62±0.04	0.40±0.12	0.78±0.13	0.01±<0.01
Methionine	0.18±0.04	0	0	35.27±0.59	47.67±0.17	0
Tryptophan	0	0	0	0	0	<0.01±<0.01
Phenylalanine	0.06±0.02	3.64±0.14	0.45±0.13	4.00±1.48	3.90±1.00	<0.01±<0.01
Isoleucine	0.03±0.02	5.46±1.06	0.04±0.04	0.46±0.26	0.01±0.01	<0.01±<0.01
Leucine	0.02±0.01	0	0.16±0.10	2.78±0.83	0.33±0.16	<0.01±<0.01
Lysine	0.28±0.11	18.76±5.84	2.02±0.89	38.56±5.74	28.87±3.26	<0.01±<0.01
Proline	11.73±3.51	812.23±159.27	109.19±38.24	7.18±1.28	8.86±0.82	<0.01±<0.01

To compare how nectar sampling method influenced the available amino acids that could be quantified, each amino acid was applied to a factor analysis (principal components). Amino acids were reduced to six principal components (F1 – 6) accounting for 83% of the variation in the data set (Table 3.3). The majority of amino acids were significantly positively correlated with the first four factors with the exception of cysteine (F5) and asparagine (F6). Sampling method influenced the amino acid profiles represented in the factor analysis for F1, F2, and F4. Importantly, the microcapillary and the rinse 2 µl methods did not significantly differ in their amino acid profiles (Table 3.3). However, the filter paper, rinse 2 ml and wash 2 ml treatments exhibited significantly different amino acid profiles compared to the microcapillary method (Table 3.3, Figure 3.5). Specifically, the filter paper method contained a higher proportion of *val*, *il*e and *tyr* (F1) than the microcapillary method (Figure 4 (b) and (c)). The rinse 2 ml and wash 2 ml methods contained higher proportions of *met, ser* and *lys* (F2 and F4) than the microcapillary method (Figure 4 (b) and (c)). Some amino acids did not differ depending on the method, and those were represented by F3, F5, and F6 (Table 3.3).

To identify whether the filter paper method introduced amino acid contamination, a simple rinse of the filter paper was analysed by uHPLC. Filter paper added very low amounts (0.1% of nectar concentration) of 15 of the amino acids measured (Table 3.2).

Table 3.3: Factor analysis of amino acids. Top panel: eigenvalues and percentage variance for six factors (F1 – 6) extracted from all data (excluding proline). Values for each amino acid represent Pearson correlation coefficients (*r*) with each factor. Amino acids significantly associated with each factor are denoted with asterisks: *P< 0.05; **P< 0.01; ***P< 0.001. Middle panel: one-way ANOVA comparing methods. Bottom panel: Sidak *post-hoc* pairwise comparisons of each method against the microcapillary method.

	Factor					
	1	2	3	4	5	6
Eigenvalue	6.16	3.84	1.82	1.51	1.33	1.06
% variance	19.9	18.1	17.2	10.9	9.8	7.1
Amino acids						
Aspartic acid	0.086	0.735***	0.343**	0.097	0.089	-0.069
Glutamic acid	-0.016	0.187	0.209	0.741***	0.313*	0.169
Asparagine	-0.100	-0.013	0.106	-0.026	0.016	0.966***
Serine	-0.036	0.130	0.875***	0.286*	0.183	-0.121
Glutamine	-0.130	0.396**	0.790***	-0.060	0.058	-0.227
Histidine	-0.130	0.239	0.818***	0.127	-0.023	0.326**
Glycine	-0.225	0.807***	0.239*	0.208	0.227	-0.038
Threonine	-0.106	0.073	0.813***	0.229	0.032	0.269*
Arginine	0.108	0.056	0.096	0.309*	0.845***	0.175
Alanine	-0.215	0.474***	0.083	0.600***	0.038	-0.146
GABA	0.889***	0.302*	-0.095	0.231	0.073	-0.022
Tyrosine	0.959***	-0.155	-0.085	0.012	0.107	-0.031
Cysteine	0.103	0.175	0.059	-0.003	0.914***	-0.128
Valine	0.957***	-0.176	-0.087	-0.032	0.123	-0.037
Methionine	-0.230	0.743***	0.141	0.476***	0.017	0.145
Phenylalanine	0.301*	0.186	0.372**	0.679***	0.031	-0.143
Isoleucine	0.881***	-0.049	-0.060	-0.130	-0.063	-0.060
Leucine	-0.126	0.524***	0.325	-0.219	0.284*	-0.086
Lysine	0.172	0.841***	0.023	0.219	-0.022	0.081
1-way ANOVA						
Test stat (F_{df})	$209_{4,59}$	$31.2_{4,59}$	$1.32_{4,59}$	$4.01_{4,59}$	$0.69_{4,59}$	$1.56_{4,59}$
P value	<0.01	<0.01	0.27	0.01	0.60	0.20
Post-hoc tests						
Filter paper	<0.01	0.25	1.00	0.90	0.93	1.00
Rinse 2 µl	0.98	1.00	1.00	1.00	1.00	0.96
Rinse 2 ml	0.01	<0.01	0.71	<0.01	1.00	0.37
Wash 2 ml	0.01	<0.01	0.73	0.87	0.84	1.00

3.4 Discussion

The method used to collect nectar considerably affected the mean total carbohydrate (glucose and fructose) and amino acid concentrations measured by HPIC and HPLC in nectar from *C. vulgaris*. Samples obtained using the wash 2 ml and rinse 2 ml methods reported much higher carbohydrate concentrations than the microcapillary, filter paper and rinse 2 µl methods. Amino acid concentrations followed a similar pattern with the wash 2 ml, rinse 2 ml and filter paper methods containing higher concentrations and significantly different amino acid composition compared to the microcapillary and rinse 2 µl methods.

Matching the data presented in this chapter, Morrant and colleagues (2009) and Petit and colleagues (2011) found that nectar collected using microcapillaries returned lower measurements of carbohydrates than nectar collected using rinse or wash methods. This is probably because washing dissolves carbohydrates that have dried on the inner petal surfaces. These dried carbohydrates are unlikely to be available to floral visitors. Moreover, most insect pollinators have very short visits to flowers, in order to maximize collection rates when competing with other pollinators. For example, bumblebees are reported to spend between 0.5 and 3 s per flower on a variety of plant species (time spent foraging was correlated with corolla length) (Inouye, 1980). Two bumblebee species' nectar removal rates have also been estimated to be between 0.3 - 0.4 µl s^{-1} in two high-nectar producing plant species (Graham and Jones, 1996). For this reason, rapid licking or sucking near the nectary is unlikely to involve much ingestion of solutes present in crystallised form across the entire flower surface. The microcapillary method recovers only nectar around the nectary (liquid and crystallised carbohydrates, amino acids, etc.).

The microcapillary method exhibits the lowest risk of contamination from non-nectar sources such as pollen. Pollen is a common source of contamination of nectar by free amino acids (Gottsberger *et al*., 1990). Contamination of the microcapillary samples happens less frequently because the microcapillary tubes are narrow and can be positioned more directly to the nectary. However, the microcapillary method is not always effective in extracting nectar from flowers with low-nectar volumes because the

nectar around the nectaries may be too viscous to be removed by capillary action. Thus, other methods for nectar collection are required for low nectar volume flowers.

Of all the methods in our study, the rinse 2 µl method returned the most similar results to the microcapillary method. The rinse 2 µl method recovered the same carbohydrate concentrations as the microcapillary method and a similar amino acid profile (albeit a higher mean total of amino acids). Like the microcapillary method, the rinse 2 µl method recovers mainly nectar around the nectary (liquid and crystallised solutes) as this is a sufficient volume of water to cover only the nectary of *C. vulgaris*. In addition, contamination was kept to a minimum since the pipette tips and microcapillary tubes used in this method were narrow, and so contact with anthers and other floral parts were reduced. Techniques that emulate the microcapillary method allow for easier comparison between low- and high-nectar volume flowers where the microcapillary method is most commonly used. The rinse 2 µl method is the next best method, besides the microcapillary method, for carbohydrate and amino acid recovery from low-nectar flowers.

Other studies have suggested that filter paper is useful in extracting nectar from low-nectar-flowers (McKenna and Thomson, 1988; Petanidou *et al*., 2006). In these experiments, the filter paper method returned similar carbohydrate concentrations to the microcapillary method and may be a suitable method if carbohydrates are the sole purpose of investigation. However, in comparison to carbohydrates, filter papers worked less well for reliably measuring amino acids. Significantly higher concentrations of amino acids (particularly essential amino acids) were reported in the filter paper samples, and the amino acid composition was significantly different to that of the microcapillary method. It is probable that this difference arises from contamination by pollen. Filter paper wicks can easily collect pollen; when they come into contact with the nectary, they most likely touch the surrounding flower (especially when the flowers are very small such as in *C. vulgaris*). Therefore, it is not recommended to use the filter paper method to analyse any nectar solutes other than carbohydrates due to the risk of sample contamination.

The wash 2 ml and rinse 2 ml methods recovered significantly higher mean total concentrations of carbohydrates and amino acids than the microcapillary method, and the proportion of amino acids recovered from the samples was affected by these methods. In terms of carbohydrates, these findings are similar to that of Morrant and colleagues (2009) and Petit and colleagues (2011), where these methods returned higher values for carbohydrates on average. The wash 2 ml and rinse 2 ml methods cover the entire floral structure in water and, in the case of the wash 2 ml method, the entire flower and cut stem are immersed and agitated in water, which is then analysed. Possible contaminants may include vascular fluid (from damaged floral tissue), cellular fluid (de-ionised water may cause leaching of carbohydrates from the flower's cells as a result of osmotic pressure), crystallised nectar or pollen. Phloem carbohydrates consist mainly of sucrose (Fukumorita *et al*., 1982; Pate *et al*., 1985; Lohaus *et al*., 2014). It is thought that sucrose is hydrolysed to glucose and fructose in the nectary by invertase prior to or during nectar secretion (Pate *et al*., 1985). If phloem contents leak from damaged floral tissue (i.e. cut stem, etc.) then one would expect an increased sucrose concentration in the nectar sample. The nectar of our model species almost entirely consisted of glucose and fructose but there were slightly raised levels of sucrose in the wash 2 ml and rinse 2 ml methods. This may indicate minor contamination by phloem sap but does not explain the significantly higher concentrations of glucose and fructose in the wash 2 ml and rinse 2 ml methods compared to the other three methods. These values are probably inflated due to contamination of wash 2 ml and rinse 2 ml samples by crystallised carbohydrates, or because of back-calculation errors that overestimate the amount present in the sample due to the large wash volume.

The wash 2 ml and rinse 2 ml methods also affected the amount and composition of amino acids. For example, methionine, serine, and lysine were present in high amounts. Gottsberger and colleagues (1990) found that damaging flowers altered the amino acid profile of nectar, particularly in terms of asparagine. It is likely that, if amino acid leakage occurred from the phloem, the amino acids involved would be species specific. Amino acid concentrations in phloem have been measured in the region of 121 - 300 mM for plants like alfalfa and spinach, and cytosolic concentrations have been found to be 121 mM (Girousse *et al*, 1996; Riens *et al*, 1991). These values are

approximately 2,000 times more concentrated than the amino acids found in our nectar samples. Given the low concentrations of amino acids in nectar in our model plant species *versus* that reported in phloem for other species, it would take only a small quantity of leaked cellular or vascular fluid to significantly alter nectar amino acid composition. It is possible that amino acids are also present at higher concentrations in the wash 2 ml and rinse 2 ml samples because they were obtained from nectar that dried across the floral surface. The greatest source of amino acid contamination in these methods, however, is pollen. The amount of contamination caused by pollen in nectar probably does not reflect what floral visitors retrieve when they visit for nectar. While one could remove anthers with tweezers prior to sampling, this runs the risk of vascular fluid leaking into the sample. Sealing the cut surface with wax or surgical glue may prevent fluid leakage (Morrant *et al*., 2009) but would be extremely time consuming and difficult to accomplish with small flowers.

There is no available information on which amino acids are transported from the phloem into nectar and this is likely to be species specific due to the varying nature of nectar amino acid content between plant species (Baker, 1977). It is clear from this study that the wash 2 ml and rinse 2 ml methods do not emulate nectar extraction methods by most insect visitors. Further research is needed to elucidate the relationship between cellular and vascular carbohydrate or amino acid content and nectar secretion so that the high variation between nectar sampling methods may be more easily understood. When analysing nectar for amino acids it is important to reduce environmental contamination of samples. For example, proline values for some samples were erratic, particularly in the filter paper method. However, no amino acids were found to have leached from the filter paper itself. Pollen can be a source of proline contamination (Gottsberger *et al*., 1990) but the filter paper is less likely to be contaminated with pollen compared to the wash 2 ml or rinse 2 ml methods. Contamination reduction in nectar analysis is critically important as amino acids are low in concentration in nectar but are widespread in the environment.

There are drawbacks associated with different nectar collection methods because their efficacy is influenced by floral morphology, nectar characteristics,

sampling regime, nectar volume and the intended chemical analysis (Bolten and Feinsinger, 1978; Kearns and Inouye, 1993; Lloyd *et al.*, 2002; Morrant *et al.*, 2009). This study demonstrated that nectar carbohydrate and amino acid recovery from low-volume flowers differs significantly depending on collection method. There is no perfect method of nectar extraction from low-volume flowers because nectar can be viscous, preventing uptake by microcapillary tubes and the original nectar volume may need to be estimated if using a rinse method. However, microcapillaries, a micro-rinse (rinse 2 µl) or filter paper can provide reliable methods of nectar extraction for carbohydrate analyses. Only microcapillary or micro-rinse methods are suitable when amino acids are to be quantified.

4.0 Chapter 4: Impacts of ozone on growth and/or yield components: are they a surrogate for impacts on the quality of pollen?

4.1 Introduction

Ozone (O_3) is a phytotoxic gas that is directly responsible for global losses in agricultural productivity amounting to ~$26 billion per annum (Avnery *et al*., 2011a) and the situation is expected to worsen with predicted losses amounting to a value of ~$35 billion p.a. by the year 2030 (Avnery *et al*., 2011b). Many crop plants are negatively affected by present-day ground-level O_3 concentrations, with impacts varying in a species-specific manner from transient visible symptoms of 'injury' to substantive losses in yield (Lefohn, 1991). A commonly-reported symptom of ozone-induced impacts is visible injury to foliage (Vollenweider *et al*., 2003). It can be reasoned that foliage harvested and sold fresh may be subject to economic losses under such circumstances, but if visible injuries were the only symptom present in a root crop, then the detrimental effect would be minimal (Ashmore, 2005). Indeed, it is generally argued that measuring visible injury to leaf tissue is not a reliable measure of plant sensitivity to the pollutant, and represents no more than a reaction to the pollutant at the cellular level (Ashmore and Davison, 1996; Davison and Barnes, 1998).

Visible injury is only one of many physical impacts of exposure to environmentally-relevant levels of O_3. The phytotoxicity of this pollutant is primarily caused by the dissolution of the gas in the mesophyll cell walls bounding the sub-stomatal cavity following uptake *via* open stomata (Turcsanyi *et al*., 2000) which initiates an oxidative cascade resulting in enhanced levels of maintenance respiration, changes in the distribution of assimilate and reduced photosynthetic activity (Barnes *et al*., 1999; Burkey *et al*. 2012). In crop plants this is ultimately manifested in reduced yield (Ashmore, 2005).

Plant species vary in their sensitivity to environmentally-relevant levels of O_3 and responses within-species (i.e. between cultivars or varieties of the same species) can also show significant variation (Barnes *et al*., 1990; Barnes *et al*. 1997; Lyons *et al*., 1997; Burkey and Carter 2009; Saitanis *et al*., 2014). For a domesticated crop plant the most

detrimental impact that O_3 exposure could exert would be a reduction in yield. When studying the impacts of O_3 on plants a variety of parameters are often measured as key performance-related indicators. In this study we question which of these parameters are relevant to the assessment of O_3 impacts, and explore whether any commonly-related measures of 'ozone sensitivity' relate to impacts on parameters associated with plant-pollinator interactions (including the free amino acid, protein and non-structural carbohydrate content of pollen). Insect pollination improves the quality and yield of many agricultural crops (Klein *et al*., 2007; Klatt *et al*., 2013) including broad bean (*Vicia faba*), a globally important crop known to be 'sensitive' to O_3 and reliant on insect pollination (Bartomeus *et al*., 2014). Foraging insects, like bumblebees and honeybees, visit flowers to collect nectar and pollen as a source of nutrition. Pollen proteins are essential for bee survival and changes in protein content are reported to influence reproduction, physiology, immunity and larval development of honeybees (Alaux *et al*., 2010; Cardoza *et al*., 2012; Di Pasquale *et al*., 2013; Génissel *et al*., 2002; Human *et al*., 2007; Li *et al*., 2012; Tasei and Aupinel, 2008).

In this study we employed ten cultivars of broad bean exhibiting a range of 'sensitivity' to environmentally-relevant levels of O_3. We chose broad bean as a convenient model for study since it is considered sensitive to O_3, lends itself to chamber-based studies, pollen can be readily collected, and it is *per se*, a globally important crop used as food, feed and green manure (Crépon *et al*., 2010; Jensen *et al*., 2010). The crop returns an average annual yield in the field equating to 1.8 tonnes ha^{-1} (FAOSTAT, 2008; Jensen *et al*., 2010) and almost 50% of the global crop is grown in Asia (FAOSTAT, http://faostat3.fao.org/); a part of the world where O_3 pollution poses a growing problem (Ashmore, 2005; Fuhrer, 2009).

4.2 Materials and Methods

4.2.1 Plant material

Dwarf broad bean (*Vicia faba L.*) cultivars were acquired from UK-based suppliers (Appendix E). Seeds were imbibed with tap water for 24 h prior to potting in John-Innes No.2 (JI2) (supplied by East Riding Horticulture) compost in 48-cell plug trays (each cell containing 32 cm^3 compost). Seed trays were incubated in growth chambers, fumigated with particulate/charcoal/Purafil®-filtered air for 24 h d^{-1} and subject to 300 µmol m^{-2} s^{-1} PPFD (at tray height) administered as an 8 h photoperiod *via* metal-halide floodlights (full growth cabinet details in Barnes *et al.,* 1995). Two weeks after germination, plants were potted-up into pots containing 1.5 dm^3 JI2 compost and placed back into the 'incubator chambers'. One week after being potted-on, plants were transferred to six glass-cladded open top chambers (OTCs) situated at Close House Field Station (Heddon-on-the-Wall, Northumberland, UK [NZ 128658]). For each of the 10 cultivars, 20 plants were divided across two OTC treatments; particulate/charcoal filtered air (CFA) or particulate/charcoal filtered air plus 110 ppb ozone 24 h d^{-1} (O_3). Both treatments were replicated in three separate randomised OTCs. Details of the OTCs and ozone delivery / monitoring systems are provided elsewhere (Gonzalez-Fernandez *et al*., 2008). Plants were maintained in the OTCs for ~8 weeks until flowering. When flowers were mature, pollen was collected from 50% of the plants, using a 200 µl PCR tube to collect pollen from flowers on one raceme (this constituted one sample for HPLC analysis). Following collection, pollen was frozen at -20 °C until further analysis. One week after pollen was collected, the same subset of plants was harvested, root separated from shoot at soil level and then all soil was washed free from the root. Plant components were then dried to constant mass at 60 °C, before determining the dry weight.

The remaining plants were maintained in the OTCs until seed set. Bees were observed in all chambers and so it was assumed that all plants had a similar pollination service, and differences in seed set would thus be a result of either cultivar or response to treatment. Once seed pods had developed and begun to dry, all pods were harvested. Numbers of pods per plant and seeds per pod were recorded, along with pod fresh weight. Pods and seed were then dried to constant mass and re-weighed.

4.2.2 Growth analysis

Once dry weight of root, shoot and pods had been recorded, relative growth rates were calculated. Total relative growth rate (RGR) included the biomass of roots (RGR_r) and shoots (RGR_s), excluding pod weight (RGR_{pod}). The method described in Hunt (1990) was used to calculate plant resistance (R%) and root to shoot allometry (K%).

$RGR = (\log W_2 – \log W_1)/(t_2-t_1)$

W = dry weight (g)

t = time (weeks)

The ratio of resource allocation between root and shoot allometry was also calculated as:

$K\% = RGR_r / RGR_s \times 100$

Resistance to O_3 (R%) was calculated as the relative change in biomass of plants grown in O_3 compared to those grown in CFA:

$R\% = (RGR_{O3}/RGR_{CFA}) \times 100$

Relative change in number of seeds (seed%), number of pods (pod%), pod fresh weight (pod_{fresh}%), pod dry weight (pod_{dry}%) and pod moisture content ($pod_{moisture}$%) were also calculated.

4.2.3 Pollen analysis

For pollen analyses, the frozen pollen was dried at 60°C for 48 h and then 1 mg weighed in to a 1.5 ml microcentrifuge tube. Pollen was then subject to a series of steps in order to separate the free amino acids and non-structural carbohydrates in the outer coating of pollen from the protein-bound amino acids held within the pollen exine. Detailed methods employed for pollen analyses are described in Sections 2.2.4 - 2.2.6.

4.2.4 Statistical analysis

Biomass of roots, shoots and pods from 10 broad bean cultivars were compared using a generalised linear model (GLM) with cultivar and treatment (CFA or O_3) as key factors. Pairwise comparisons were made between each cultivar and treatment using least significant difference (LSD). RGR, K, number of seeds and pods produced and the water content of pods were analysed using GLM, with LSD at the 5% level applied *post hoc* to compare the effect of treatment on each cultivar. Linear regressions were applied to correlate plant resistance (R%) against the number of seeds produced, number of pods produced, pod fresh weight, pod dry weight and pod moisture content. Total protein-bound amino acids and total free amino acids were compared using a two-way analysis of variance (ANOVA) using cultivar and treatment as factors. The distribution of protein-bound and free amino acids were compared using a canonical discriminant analysis (CDA). Total non-structural carbohydrate was compared in a two-way ANOVA and individual carbohydrates were compared in a two-way multivariate analysis of variance (MANOVA).

4.3 Results

The impacts of ozone pollution on various key performance indicators of ten dwarf broad bean cultivars were assessed; biomass of root, shoot and pod, number of pods/seeds produced (crop yield), water status and the protein/amino acid and non-structural carbohydrate content of pollen.

4.3.1 Plant growth and resource allocation

Ozone treatment and cultivar both significantly ($P< 0.001$) influenced total biomass (root, shoot and pod dry weight) (Figure 4.1). Impacts of ozone on each of the contributing measures to overall biomass (dry root, shoot and pod weight) varied between cultivars (treatment*cultivar; root $P= 0.008$; shoot $P= 0.003$; pod, $P= 0.019$, respectively). The total biomass of cultivars 1, 2, 3, 7, 8 and 10 was significantly ($P\leq 0.01$) reduced by O_3 exposure, with the greatest overall reduction in total biomass recorded in cultivar 7 where plants exposed to O_3 weighed 58% less than the controls grown in CFA. Total biomass of cultivars 4, 5, 6 and 9 was not significantly influenced by exposure to O_3.

Ozone impacts on plant RGR (root and shoot biomass, excluding pod) depended on cultivar (treatment*cultivar $P< 0.001$): plant RGR of cultivars 1, 2, 3 and 7 was significantly ($P\leq 0.01$) decreased by exposure to O_3 whereas plant RGR of cultivars 4, 5, 6, 8, 9 and 10 was unaffected by O_3 (see Figure 4.2). Figure 4.3 shows that exposure to O_3 also caused a significant shift in resource allocation to support root growth relative to shoot growth (K) in all cultivars, with the impacts dependent on the cultivar (treatment*cultivar, $P\leq 0.04$).

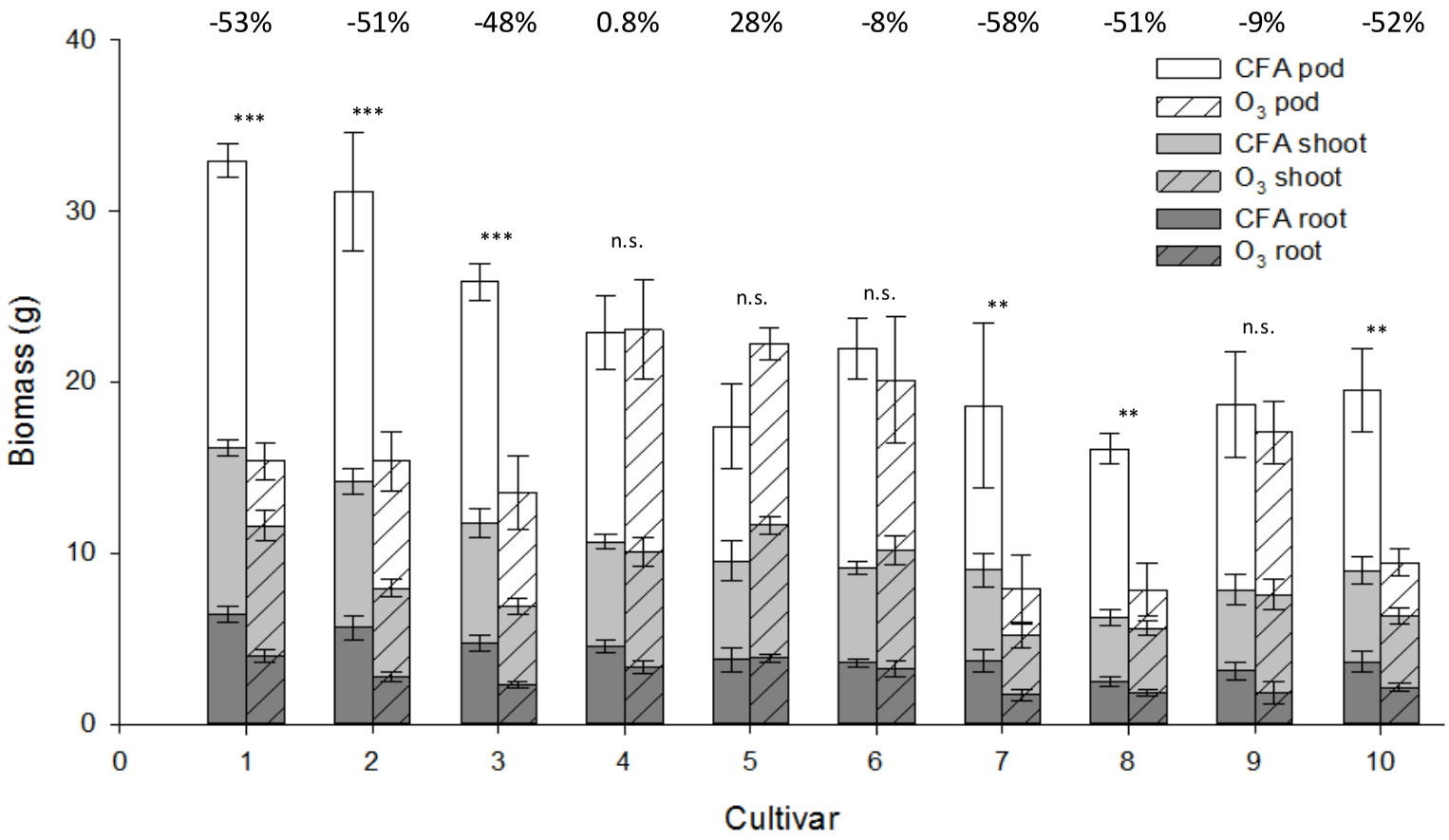

Figure 4.1. Biomass of root, shoot and pod of ten cultivars of broad bean (*Vicia faba* L.) grown in either CFA (CFA) or CFA + 110 ppb O_3 24 h d^{-1} (O_3). Bars represent standard error of mean (n = 15). Significant differences in total biomass are noted using: * P≤ 0.05, ** P≤ 0.01, *** P≤ 0.001, n.s. indicates no significant difference at 5% significance level. Overall percentage change presented above bars for each cultivar.

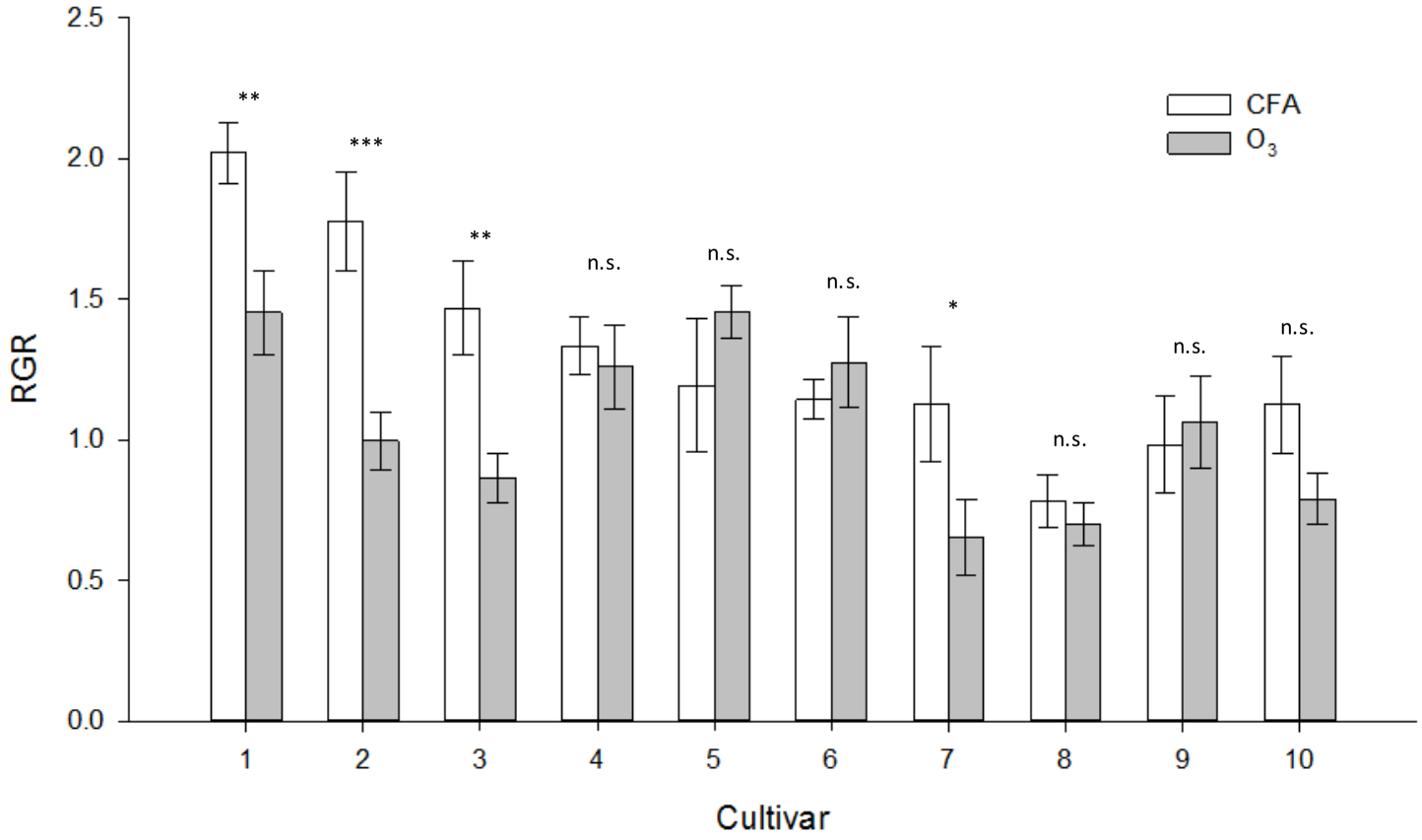

Figure 4.2. Impact of ozone on relative growth rate (RGRplant plant not pod) of 10 cultivars of broad bean. Plants were exposed to either CFA (CFA) or CFA + 110 PPB O_3 24 h d^{-1} (O_3). Bars represent standard error of mean. Significant differences in K are noted using: * P≤ 0.05, ** P≤ 0.01, *** P≤ 0.001, n.s. indicates no significant difference at 5% significance level.

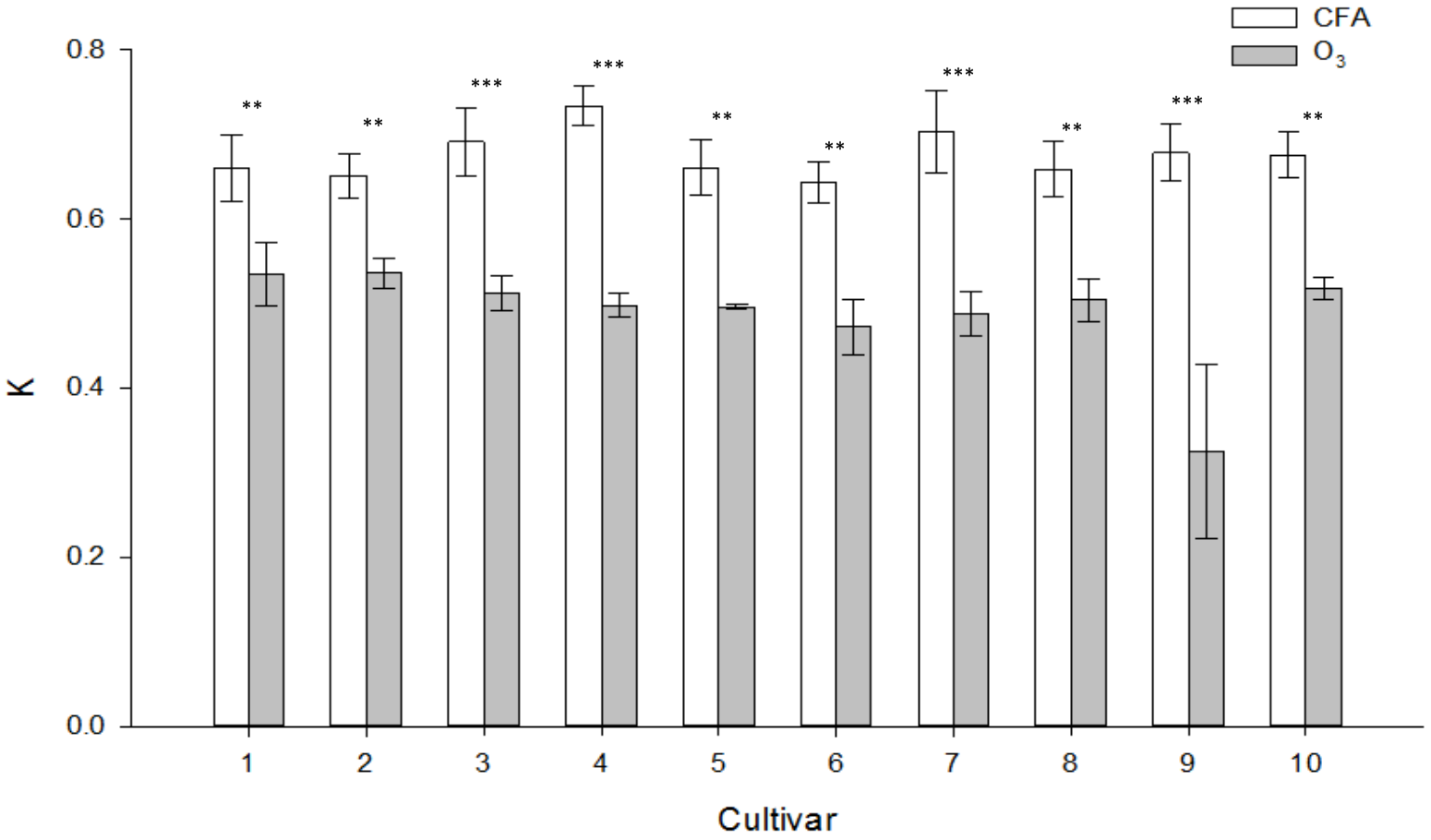

Figure 4.3. Impacts of ozone exposure on allometric root:shoot growth (K; RGR_{root}/RGR_{shoot}) of ten cultivars of broad bean grown in either CFA (CFA) or CFA + 110 ppb O_3 24 h d^{-1} (O_3). Bars represent standard error of mean. Significant differences in total biomass are noted using: * P≤ 0.05, ** P≤ 0.01, *** P≤ 0.001.

Figure 4.4(a) shows there was significant variation between cultivar responses to O_3 in the number of pods produced (treatment*cultivar $P< 0.001$). The number of pods produced by cultivars 1 and 2 in O_3 was reduced significantly ($P< 0.05$) by ~61 and ~38%, respectively. In contrast, the number of pods produced by cultivars 4 and 5 increased ($P\leq 0.02$) in response to O_3 exposure, by ~77 and ~130 %, respectively. The number of pods produced by all other cultivars was not significantly affected by exposure to O_3.

Figure 4.4(b) shows the number of seeds produced per plant varied between cultivars and was differentially affected by exposure to O_3 (treatment*cultivar $P< 0.001$). Cultivars 1, 2 and 8 produced ~65, ~36 and ~61 %, respectively, fewer seeds per plant ($P\leq 0.04$) when exposed to O_3 than control plants raised in CFA. Conversely, cultivars 4 and 5 produced ~60 and ~97 % more seeds per plant under O_3 than those grown under CFA ($P\leq 0.04$). All other cultivars produced similar numbers of seeds per plant under both control (CFA) and O_3 treatment.

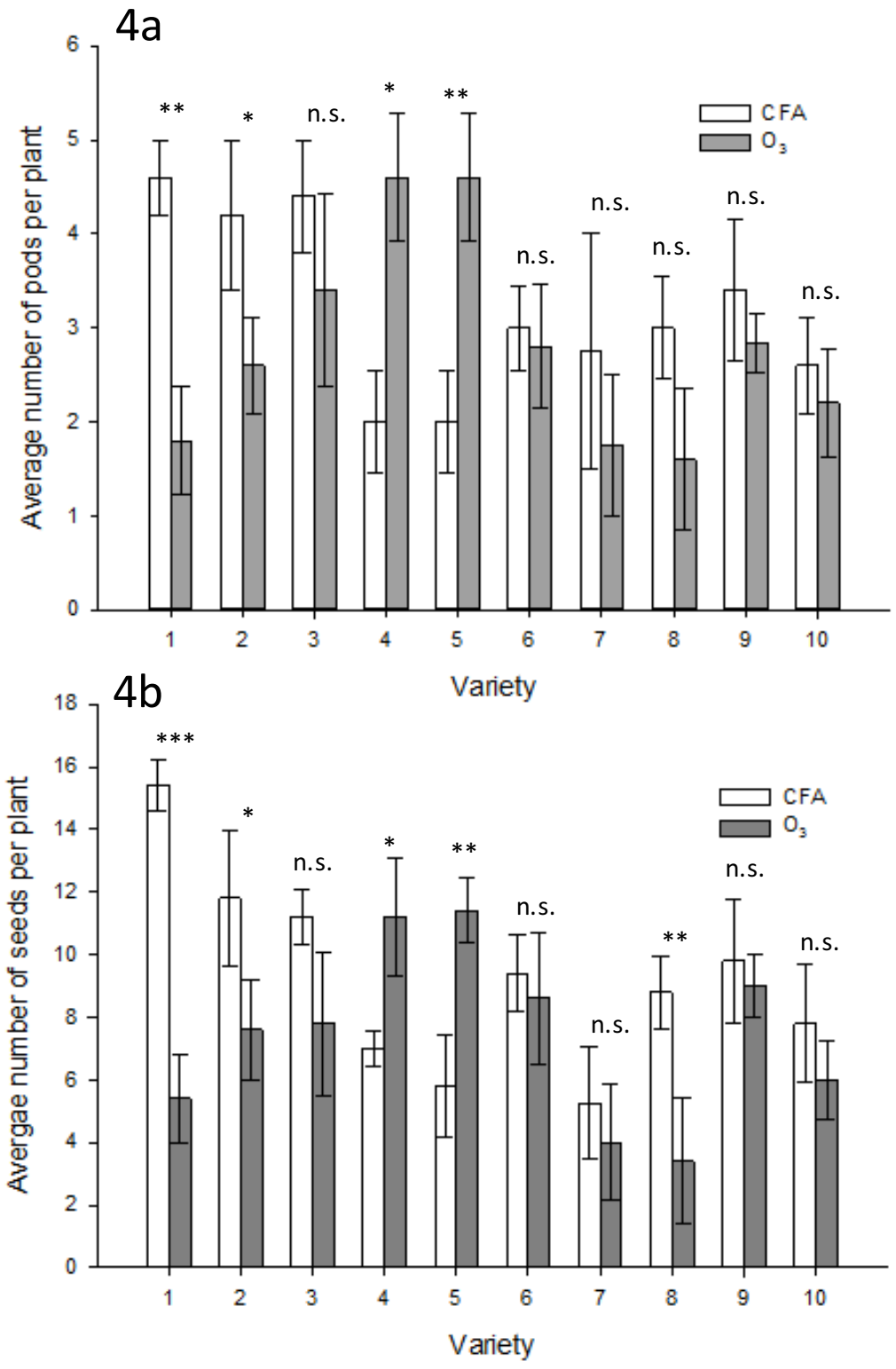

Figure 4.4 Impacts of exposure to CFA (CFA) or CFA + 110 ppb O_3 24 h d^{-1} (O_3) on (a) the number of pods and (b) number of seeds produced per plant in ten cultivars of broad been. Bars represent standard error of mean. Significant differences in biomass are noted using: * P≤ 0.05, ** P≤ 0.01, *** P≤ 0.001. n.s. indicates no significant difference at 5% significance

4.3.2 Relationship analyses

Regressions were used to explore whether the impact of O_3 on growth, in terms of impacts on RGR_{plant} (R%), constituted a reliable surrogate for prediction of the influence of O_3 on other ecologically- and agriculturally-important parameters.

Plant resistance to ozone (R%) afforded a significant predictor of the impacts of O_3 on the number of seeds (seed%) that were produced by broad beans (Table 4.1. P= 0.039). Out of 10 cultivars, 7 demonstrated reductions in both R% and seed% (Figure 4.5).

The number of pods produced also followed a similar trend to number of seeds. A clear relationship existed between impacts of O_3 on the number of pods and seeds produced. Similarly, 7 out of the 10 cultivars that demonstrated a reduction in R%, also exhibited decreased pod%, as a direct response to ozone exposure (Table 4.1. P= 0.022, Figure 4.6a).

Pod fresh weight was also correlated with R% (Table 4.1, P= 0.027). Seven of the 10 broad bean cultivars produced pods with significantly (P< 0.05) lower fresh weight than those grown in CFA (Figure 4.6b). Pod dry weight showed a similar trend to fresh weight, and 7 cultivars that showed increased sensitivity to ozone, reflected in reduction in R%, produced pods which weighed less than plants grown in CFA (Table 4.1, P= 0.024, Figure 4.6c).

Table 4.1. Regression analyses for measured traits of broad beans exposed to CFA (CFA) or CFA + 110 ppb O_3 24 h d^{-1} (O_3).

X axis	Y axis	N	r^2	P value	Regression
%seed	**%R**	**10**	**0.430**	**0.039**	**y = 53.7644 + 0.2966 x**
%Pod	**%R**	**10**	**0.502**	**0.022**	**y = 54.0180 + 0.2725 x**
%podfreshweight	**%R**	**10**	**0.477**	**0.027**	**y = 60.2568 + 0.3864 x**
%pod weight	**%R**	**10**	**0.493**	**0.024**	**y = 55.9345 + 0.4088 x**

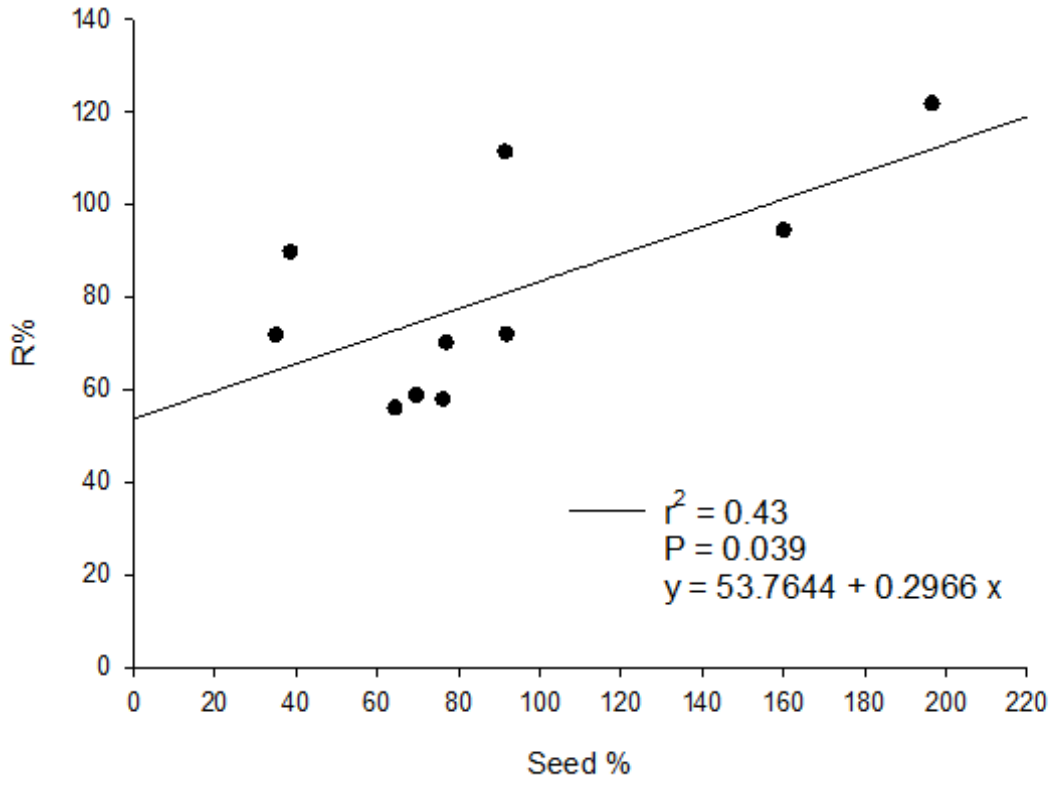

Figure 4.5. Correlation between ozone-induced change in number of seeds produced per plant (Seed %) and relative change in growth (R%) across ten cultivars of broad bean (*Vicia faba* L.) exposed in OTCs to either CFA (CFA) or CFA + 110 ppb O_3 24 h d^{-1} (O_3)

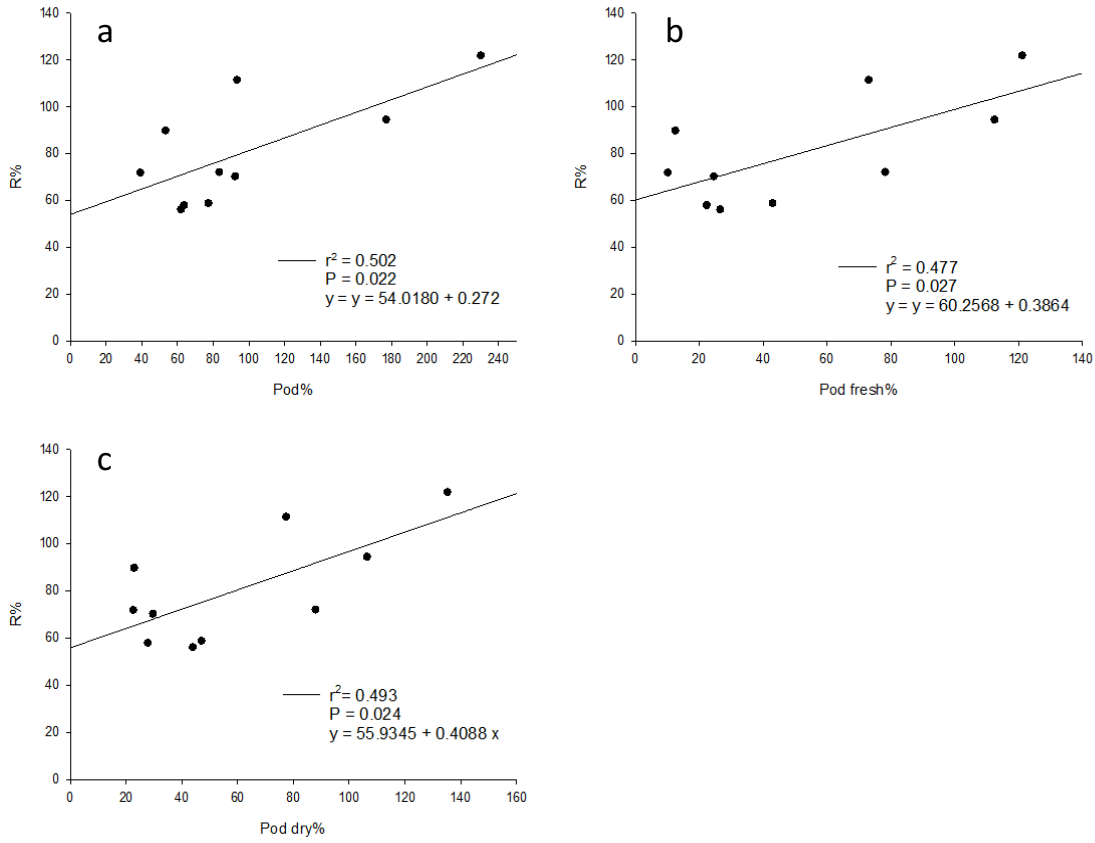

Figure 4.6. Correlation between ozone-induced change growth (R%) and (a) the relative change in number of pods produced per plant, (b) the change in fresh weight of pods and (c) the change in dry weight of pods.

4.3.3 Pollen qualities

Pollen from three cultivars exhibiting a range of sensitivities to O_3 (based on impacts on seed yield) was collected and the protein-bound and free amino acid, plus non-structural carbohydrate qualities quantified. Total protein-bound amino acids recovered *via* a microwave-assisted acid hydrolysis of broad bean pollen was not significantly influenced by exposure to O_3 (Figure 4.7a). There was no significant treatment*cultivar and interaction on free amino acid composition, but overall exposure to O_3 significantly (P= 0.003) reduced the total free amino acid content of pollen (Figure 4.7b).

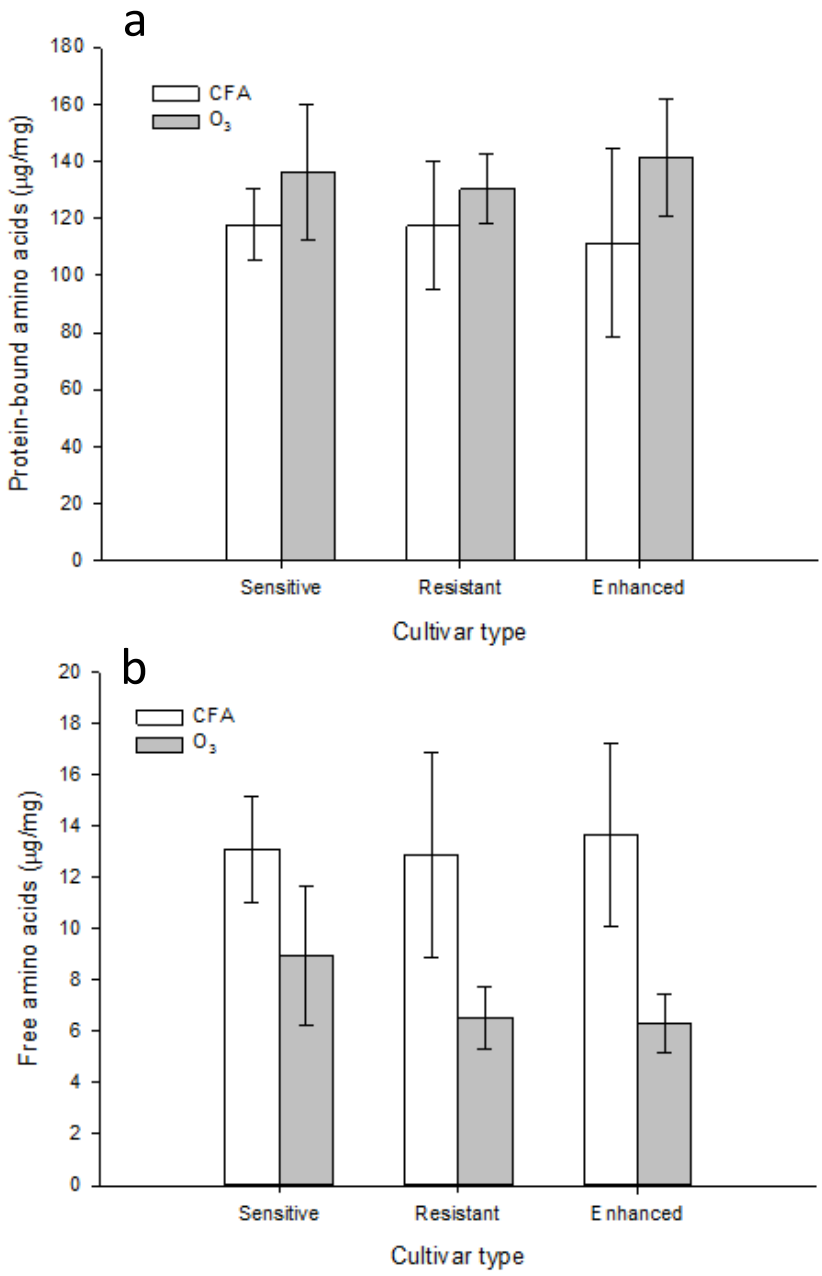

Figure 4.7. Total protein-bound (a) amino acids and (b) total free amino acids from the pollen of three cultivars exhibiting contrasting responses to exposure to CFA (CFA) or CFA + 110 ppb O_3 24 h d^{-1} (O_3). Bars represent standard error of mean (n = 10).

A canonical discriminant analysis (CDA) was used to identify major changes in the amino-acid composition of proteins in pollen. Pollen from plants that showed enhanced seed yield when exposed to O_3, had significantly (P= 0.033) greater amounts of histidine, leucine, methionine, phenylalanine and isoleucine, and less threonine, arginine, valine, and lysine (Tables 4.2, 4.3 Appendix F).

Table 4.2. CDA table for pollen protein-bound essential amino acids

	Essential amino acids			
	Canonical discriminant function statistics			
Function	Eigenvalue	% Variance	test stat	P value
Sensitive function	3.589	100	x_9^2=14.5	0.106
Resistant function	16.085	100	x_8^2=11.4	0.182
Enhanced function	56.20	100	x_9^2=18.2	0.033

Pooled within-groups correlations

Function					
Amino acid	Sensitive	Amino acid	Resistant	Amino acid	Enhanced
Methionine	0.434	Lysine	-0.325	Methionine	-0.088
Lysine	-0.427	Isoleucine	0.311	Lysine	0.087
Valine	-0.283	Phenylalanine	*	Histidine	-0.076
Isoleucine	-0.204	Valine	-0.118	Valine	0.04
Phenylalanine	-0.183	Histidine	0.114	Leucine	-0.024
Threonine	-0.105	Threonine	-0.099	Threonine	0.013
Arginine	0.087	Leucine	0.046	Arginine	0.011
Leucine	-0.043	Arginine	-0.037	Isoleucine	-0.006
Histidine	0.007	Methionine	0.037	Phenylalanine	-0.003

	Canonical discriminant function coefficients		
	Function		
Treatment	Sensitive	Resistant	Enhanced
CFA	-1.195	-3.587	7.428
O_3	2.629	3.587	-6.19

Table 4.3. CDA table for pollen protein-bound non-essential amino acids

	Non-essential amino acids			
	Canonical discriminant function statistics			
Function	Eigenvalue	% Variance	test stat	P value
Sensitive function	1.131	100	x_8^2=6.81	0.557
Resistant function	5.49	100	x_6^2=11.2	0.082
Enhanced function	5.21	100	x_7^2=10.0	0.186

Pooled within-groups correlations

Function					
Amino acid	Sensitive	Amino acid	Resistant	Amino acid	Enhanced
Proline	0.765	Proline	*	Glycine	-0.322
Aspartic acid	-0.177	Glycine	0.13	Proline	0.201
Glycine	-0.165	Cysteine	*	Aspartic acid	0.135
Alanine	0.098	Alanine	-0.034	Glutamic acid	0.122
Cysteine	-0.052	Glutamic acid	0.03	Alanine	*
Serine	0.037	Tyrosine	-0.011	Cysteine	-0.005
Glutamic acid	-0.034	Aspartic acid	0.01	Tyrosine	-0.005
Tyrosine	0.026	Serine	0.006	Serine	-0.001

	Canonical discriminant function coefficients		
	Function		
Treatment	Sensitive	Resistant	Enhanced
CFA	-0.597	2.322	-2.262
O_3	1.642	-1.935	1.885

The free essential amino acids of pollen collected from broad beans identified as 'sensitive' and 'resistant' to O_3 differed significantly (Table 4.4 and Appendix G). In the sensitive cultivar, the amount of histidine, leucine, threonine, arginine, valine, and isoleucine in pollen was significantly (P= 0.03) lower in O_3-treated plants than that from plants grown in CFA. However, the amount of free methionine and lysine was higher in plants sampled under O_3 compared to those in CFA. When exposed to O_3, the amounts of arginine, threonine, histidine, leucine, valine and isoleucine were lower (P= 0.002) in pollen of the resistant cultivar than the equivalent plants grown in CFA. Yet, lysine and methionine content was higher and similar to the profile of protein-bound amino acids of plants in the sensitive cultivar (Table 4.2 and Appendix G). The CDA was unable to categorise the free essential amino acids based on treatment for the seed yield-

enhanced cultivar. A CDA of the free non-essential amino acids was also unable to extract any significant changes in profile of amino acids for either cultivar of broad bean (Tables 4.5 Appendix G).

Table 4.4. CDA table for pollen free essential amino acids

	Essential amino acids			
	Canonical discriminant function statistics			
Function	Eigenvalue	% Variance	test stat	P value
Sensitive function	4.457	100	x_8^2=14.5	0.03
Resistant function	399.2	100	x_8^2=11.4	0.002
Enhanced function	22.38	100	x_8^2=18.2	0.126

Pooled within-groups correlations

Function					
Amino acid	Sensitive	Amino acid	Resistant	Amino acid	Enhanced
Methionine	0.312	Arginine	-0.043	Lysine	0.209
Histidine	-0.152	Threonine	-0.038	Methionine	0.16
Arginine	-0.135	Lysine	0.034	Arginine	-0.119
Valine	-0.121	Methionine	0.031	Histidine	-0.108
Lysine	0.085	Histidine	-0.022	Leucine	0.075
Leucine	-0.058	Leucine	-0.013	Isoleucine	0.067
Threonine	-0.056	Valine	-0.002	Threonine	-0.054
Isoleucine	-0.027	Isoleucine	-0.002	Valine	0.04

	Canonical discriminant function coefficients		
	Function		
Treatment	Sensitive	Resistant	Enhanced
CFA	-1.331	-17.87	-4.232
O_3	2.929	17.87	4.232

Table 4.5. CDA table for pollen free non-essential amino acids

	Non-essential amino acids			
	Canonical discriminant function statistics			
Function	Eigenvalue	% Variance	test stat	P value
Sensitive function	1.473	100	x_8^2=9.05	0.338
Resistant function	1.608	100	x_7^2=4.31	0.743
Enhanced function	4.187	100	x_8^2=6.59	0.582

Pooled within-groups correlations

Function					
Amino acid	Sensitive	Amino acid	Resistant	Amino acid	Enhanced
Proline	0.416	Tyrosine	0.192	Aspartic acid	0.13
Glycine	0.374	Aspartic acid	0.179	Glutamic acid	-0.111
Tyrosine	0.268	Glycine	-0.148	Tyrosine	-0.1
Alanine	0.249	Cysteine	0.08	Alanine	0.091
Cysteine	0.248	Serine	0.053	Glycine	0.084
Serine	0.174	Alanine	0.048	Serine	0.072
Aspartic acid	0.136	Glutamic acid	0.03	Proline	0.041
Glutamic acid	-0.055	Proline	*	Cysteine	-0.002

	Canonical discriminant function coefficients		
	Function		
Treatment	Sensitive	Resistant	Enhanced
CFA	0.765	1.134	1.83
O_3	-1.684	-1.134	-1.83

The non-structural carbohydrates washed from pollen were quantified. Total non-structural carbohydrate content was not influenced by cultivar or treatment, and there was no significant cultivar*treatment interaction. (Figure 4.8).

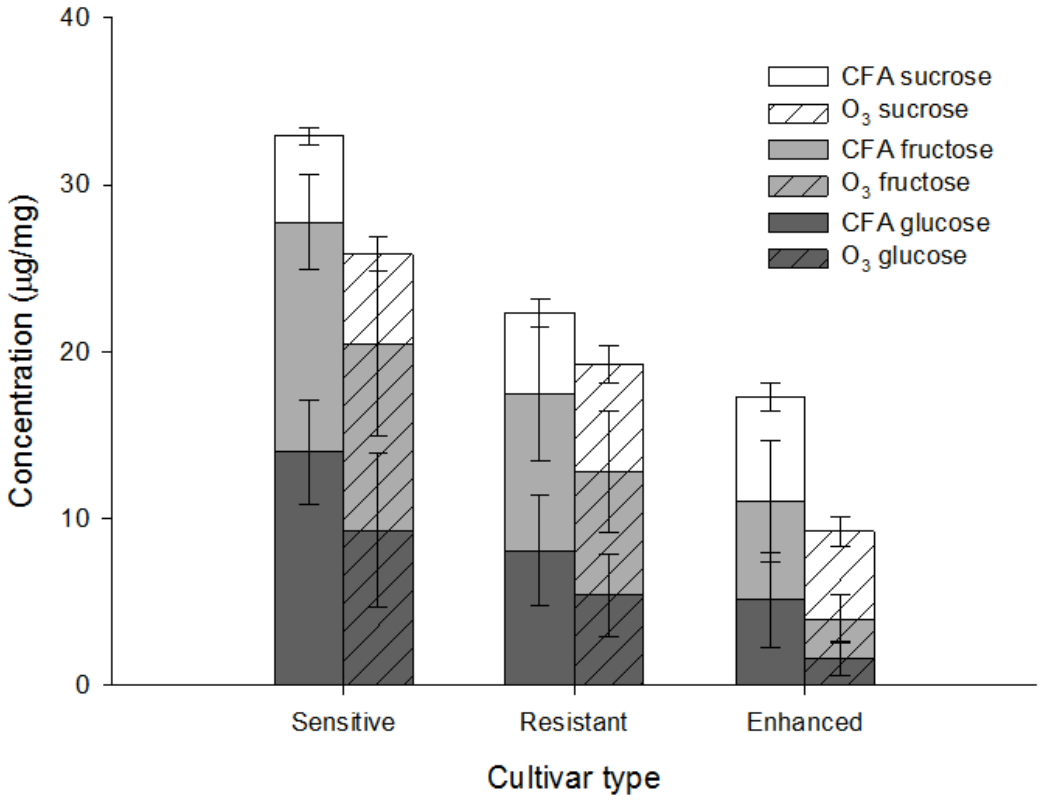

Figure 4.8. Concentration of key soluble non-structural carbohydrates washed from the pollen of three cultivars of broad bean exhibiting contrasting growth responses when exposed to CFA (CFA) or CFA + 110 ppb O_3 24 h d^{-1} (O_3). Bars represent standard error of mean (n = 10).

4.4 Discussion

The 'ozone-sensitivity' of broad beans was assessed using a variety of commonly-adopted parameters. The study demonstrated that impacts on growth rate (R%) of roots and shoots constitute a reliable predictor of impacts on seed yield of broad bean. The measured changes in the relative growth rate (RGR) of broad beans, in response to exposure to O_3, were cultivar-dependent; only 4 of the 10 cultivars tested showing a statistically significant reduction in RGR. Intraspecific variation in responses to O_3 are well documented in other species including wheat (Barnes *et al*., 1990), *Plantago major* (Lyons *et al*., 1997) and *Centaurea jacea* (Bassin *et al*., 2004). A reduction in the root to shoot allometric coefficient (K) has been demonstrated repeatedly in many species as a result of both short-term (Davison and Barnes 1998) and long-term exposure to O_3 (Grantz *et al*., 2006). Herein, all 10 cultivars of broad bean tested exhibited reduced resource allocation to roots, in comparison to that of shoots, under O_3 stress. This finding supports numerous other publications that demonstrate a reduction in K in both wild species and crop plants subject to environmentally-relevant levels of O_3 pollution (Barnes *et al*., 1990; Davison and Barnes, 1998; Grantz *et al*., 2006; Chaudhary and Agrawal, 2015). Although the mechanisms behind O_3-induced reductions in resource allocation to root growth are not fully elucidated, it is generally assumed that the cause is due to a disruption in phloem loading mechanisms, which result in reduced ability to translocate assimilate from source leaves (Spence *et al*., 1990; Chiou and Bush, 1998; Fuhrer and Booker, 2003) and therefore reduces assimilate allocation to sinks (Anderson, 2003).

It can be reasonably argued that seed and pod production (yield) is the most valuable measure of ozone-sensitivity in crop plants such as broad bean. As a short-lived, crop plant, any influences of ozone on biomass and physical injury may be irrelevant if seed production is not negatively impacted. Ozone is well documented to reduce crop yield (Black *et al*., 2000; Feng *et al*., 2003; Fuhrer and Booker, 2003; Fiscuss *et al.,* 2005; Ainsworth *et al*., 2008; Fuhrer, 2009a) and in this experiment it was found that ozone reduced the number of pods produced per plant in 2 out of the 10 cultivars tested and reduced the total number and weight of seeds in 3 cultivars. However, potentially

interestingly from a breeding perspective, two cultivars actually produced significantly more pods and seeds in response to ozone exposure. An O_3-induced increase in seed yield has been previously reported in some other cultivars of the *Fabaceae* family including soy bean (Endress and Grunwald, 1985) and french bean (Sanders *et al*., 1992). Cultivars producing more pods per plant may respond to O_3 stress by investing greater resources into reproduction; something that is especially important in short- lived plants like broad bean.

As found in previous studies (Barnes *et al*., 1990; Barnes *et al*. 1997; Lyons *et al*., 1997; Burkey and Carter 2009; Saitanis *et al*., 2014) cultivars of the same species exhibited marked variation in their responses to O_3 pollution; some cultivars even showing enhanced yield in response to exposure to O_3. However, O_3 impacts on growth and yield proved a poor predictor of effects on the quality of pollen; the present study shows that the protein and amino acid composition of pollen may be significantly influenced by long-term exposure to ozone with no correlation to impacts on growth or yield. Pollen from 3 cultivars identified as 'ozone sensitive', 'ozone resistant' and 'ozone enhanced' was analysed in terms of protein, free amino acid and non-structural carbohydrate content. Ozone is acknowledged to inhibit pollen viability and germ tube growth and it is thought that most of its detrimental impact arises during pollen development (Black *et al*., 2000; Gillespie *et al*., 2015). However, studies on the pollen of *Acer negundo, Quercus rober* and *Plantanus. spp.* exposed to ozone after collection reveal a significant reduction in soluble protein content. This suggests the pollutant may also exert direct effects on pollen after dehiscence (Ribeiro *et al*., 2013). In the present study no impact of O_3 was identified on protein content of broad bean pollen. However, we did observe that the suite of protein-bound essential amino acids quantified in the 'enhanced' cultivar was altered compared to that of plants grown in CFA. No changes were observed in the 'sensitive' or 'resistant' cultivars. Changes in the types of proteins present in pollen induced by ozone exposure have previously been reported; usually associated with increased allergens such as the *PR-10*, *Bet v 1* and *Cup a 3* proteins (Cortegano *et al*., 2004; Suárez-Cervera *et al*., 2008; Pasqualini *et al*., 2011; Beck *et al*., 2013; Frank and Ernst, 2016). Interestingly, in the present study, we observed a marked reduction in the free amino acids washed from the external coating of pollen (also

known as the pollenkitt) subject to O_3, in all three cultivars tested – regardless of 'ozone sensitivity' based on impacts on growth and yield. The distribution of free essential amino acids was also altered in the sensitive and resistant cultivars, but not in the enhanced cultivar. These data suggest two modes in which the pollutant influences the quality of broad bean pollen. Firstly, that during pollen development, exposure to ozone may cause the plant to allocate different resources to pollen (reflected in a change in the distribution of protein-bound amino acids), and secondly that exposure to ozone after anther dehiscence may cause direct oxidation of the free amino acids in the pollenkitt and also disruption in the suite of essential amino acids.

Ozone pollution is recognised to induce shifts in natural and semi-natural plant communities (Davison and Barnes, 1998; Fuhrer *et al*., 1997). Ozone-sensitive species are thought to be outcompeted by ozone-resistant species under long term exposure to the pollutant. However, the way in which species are classified as ozone-sensitive and/or –resistant has failed to measure the response of pollen to ozone and its contribution to species fitness. Changes in the nutrient qualities in pollen will impact on consumers, as will changes in leaf composition for herbivorous insects (Mills *et al*., 2013). It is important that when defining a species as 'sensitive' or 'resistant' to O_3 pollution, that all qualities that could induce significant downstream affects are considered. As such, meta analyses of the relative sensitivities of plants based on ozone-induced changes in biomass (such as that presented by Hayes *et al*., 2007) may be extremely misleading.

The findings in this chapter highlight the need to consider additional traits when assessing ozone sensitivity. Widely used traits such as relative change in biomass and yield in response to ozone were correlated in broad bean, however these traits were not suitable indicators of impacts on pollen quality – a major determinant of pollinator interactions. Changes in plant community structure and biodiversity as a consequence of exposure to ozone have not investigated the role of ozone-induced changes in pollen quality in governing changes in populations. Carry- over effects of detrimental changes to pollen may influence reproductive success (Black *et al*., 2000; Gillespie *et al*., 2015). However, as a primary nutrient source to pollinators, changes in pollen quality could put

nutritional stress on the insect, impacting pollinator visitation rates (Somme *et al*., 2015). A reduction in pollinator visitation and therefore pollination success could lead to species with ozone-sensitive pollen being outcompeted by resistant species. Further work should explore plant species variation in terms of ozone sensitivity/resistance of pollen. If ozone pollution causes direct negative oxidative effects, as we report, then changes to pollinator nutrition may be widespread in polluted regions.

5.0 Chapter 5: The influence of ozone pollution on nutrient allocation to nectar and pollen

5.1 Introduction

Tropospheric ozone (O_3) concentrations are not constant. Ground-level concentrations peak when conditions favour the photolytic generation of the pollutant in the troposphere ie. high levels of NO_x and VOCs, bright sunshine, elevated temperatures and low humidity (RoTAP, 2012). Diurnal variations are more pronounced in lowland and urban areas, correlating with times of particularly heavy anthropogenic precursor emissions (Marr and Harley, 2002) and subsiding in response to lower NO_x and VOC emissions (Simon *et al.,* 2014), with levels commonly accumulating over several days during peak episodes (Chameides *et al.*, 1994). Climate has a strong impact on ground-level ozone concentrations (Ashmore, 2005). Generally, the greatest concentrations are measured at the hottest part of the day (Fiscus *et al*., 2005), though rainfall and high humidity can diminish ground-level O_3 concentrations *via* the removal of precursors and the dissolution of gaseous O_3 (Varotsos *et al*., 2013; Smith and Tirpak, 1988). Wind can also strongly influence ground level O_3 concentrations dependent on the location (Notario *et al*., 2012). As a consequence, the atmospheric concentrations of O_3 to which vegetation is exposed can change rapidly and often.

Ozone is highly reactive and almost exclusively enters plant tissue through open stomata (Fuhrer *et al*., 1997). The primary oxidation targets in plant tissue are generally believed to be lipids/unsaturated fatty acids (Pryor and Church, 1991) and protein components of cell membranes. Dissolution of O_3 in the cell walls of mesophyll and palisade cells, and reaction with constituents of the apoplast, lead to the synthesis of reactive oxygen species (ROS) (incl. H_2O_2, O_2^-, 'O_2), which may oxidise cell membrane constituents when the titre of ROS exceeds the intrinsic ROS scavenging capability of the apoplast/symplast (Lyons *et al*., 1999; Plöchl *et al*., 2000). The oxidative burden resulting from the uptake of ozone results in marked shifts in gene expression, which displays commonalities with responses at the molecular level induced by a range of other abiotic and biotic stresses (Kangasjärvi *et al*., 1994). Consequences include a marked increase in maintenance respiration (to fuel cellular repair and detoxification of ROS) (Amthor,

1988), a commonly substantive decline in carbon assimilation (as result of a decline in RubisCO activity) (Heath, 1994) and significant shifts in the allocation of newly-produced assimilates within and between plant tissues (Davison and Barnes, 1998; Fuhrer and Brooker, 2003) ultimately resulting in often pronounced reductions in biomass and crop yield (Ashmore, 2002; Jaggard *et al*., 2010).

Little is known about how changes in resource allocation within the plant, resulting from exposure to O_3, may influence supply and support for plant reproductive processes (in particular nectar and pollen). There appears to be no literature pertaining to the impacts of O_3 on floral nectar quality, though a few studies have probed impacts, in a rather superficial manner, on pollen. With regard to impacts on nectar quality and composition it may be prudent to consider the impact of other stress factors on resource allocation to nectar. Soil water deficit has for example been shown to result in a three-fold reduction in the volume of nectar collected from fireweed flowers (*Epilobium angustifolium* L.) though carbohydrate concentration of the nectar remained unaffected (Carroll *et al*., 2001). Conversely, supplemental watering of field-grown milkweed (*Asclepias syriaca* L.) results in an increase in the volume of nectar produced and also the concentration of carbohydrates in the nectar (Wyatt *et al*., 1992). High temperatures have been shown to increase nectar secretion in thyme (*Thymus capitatus* Hoff. *et* Link.) and result in relative increases in carbohydrate concentration, although at temperatures above 38 °C nectar flow subsided (Petanidou and Smets, 1996).

Pollen, on the other hand, has been shown to be negatively affected by exposure to O_3 (Black *et al*., 2000). Exposure to environmentally-relevant concentrations of O_3 at critical developmental times slows maturation rate of pollen by inhibiting starch accumulation (Schoene *et al*., 2004) and the pollutant has been shown to inhibit germ-tube growth in several studies (Wolters and Martens, 1987; Black *et al*., 2007; Pasqualini *et al*., 2011; Gillespie *et al*., 2015). Interestingly, there are also data indicating a negative effect of O_3 pollution on mature pollen; pollen of an O_3-sensitive strain of tobacco (*Nicotiana tabacum* L. Bel W-3) showing both reduced pollen viability and germ tube growth upon exposure to O_3 following collection from plants grown in clean air (Feder, 1968).

Plants and pollinators have a mutualism that relies on the exchange of nectar in return for pollination services. Some pollinators, such as bees, also collect pollen as a source of food. The quality of nectar and pollen offered by a plant strongly influences the visitation rate by insect pollinators (Seeley *et al*., 1991; Seeley, 2009); plants providing nectar with low concentrations of carbohydrate are less likely to receive multiple floral visits from insect pollinators when there are more nutrient-rich sources available (Von Frisch, 1965; Richter and Waddington, 1993; Seeley, 2009; Waddington, 1998; Scheiner *et al*., 2004). Amino acids are the second most abundant component in nectar and the majority of floral nectars contain amino acids (Baker and Baker, 1973). Honeybees will preferentially feed from nectar-like solutions that contain amino acids (Alm *et al.,* 1990), and more specifically, solutions that contain the essential amino acids over non-essential amino acids (Hendriksma *et al*., 2014). However, some amino acids can be repellent to bees, including glycine, glutamic acid, serine, alanine and arginine when amino acid concentrations exceed 35 - 80 mM in 50% sucrose (Hendriksma *et al*., 2014; Roubik *et al*., 1995).

The experiments described in this chapter were designed to assess the impact of both long- and short-term exposure to O_3 on carbohydrate and amino acid/protein allocation to nectar and pollen. It is important to ascertain how quickly changes in nutrient allocation may occur when considering the impact on the available nutrition to pollinating animals and also how this may be reflected in plant reproductive success. Because ozone concentrations are highly variable and microclimate-specific, it is important to identify whether short-term exposures can influence the nutrient qualities of the rewards and food sources utilised by the plants' pollinators. In this study, I exposed plants to clean air or O_3 for extended periods during their development as well as conducting transfer experiments to identify short-term as well as long-term impacts of exposure to O_3.

5.2 Methods

5.2.1 Plants and growth chambers

Seeds of an ozone-sensitive variety of dwarf broad bean (identified in Chapter 4) ('The Sutton', B&Q, UK) were germinated in 48 cell trays, each cell filled with 32cm^3 of John Innes No.2 compost (JI2: East Riding Horticulture, Sutton Upon Derwent, UK). Five days after germination, seedlings were potted-up in to pots containing 1 dm^3 of JI2. Seven days' post germination, plants were moved into their respective treatment chambers. Plants were grown in purpose-built fumigation chambers (internal volume 660 dm^3) designed and described by Barnes and colleagues (1995). Air was supplied to growth chambers from an air-handling unit, making two complete air changes per minute in each chamber (Zheng *et al*., 1998). Lighting was provided using metal-halide luminaires (Siemens HR400H units fitted with 400-W HQI-T lamps, Osram, St. Helens, Merseyside, UK). This created a photosynthetic photon flux density (PPFD) of 250 µmol m^{-2} s^{-1} (at canopy height) delivered as a 16 : 8 h light/dark cycle.

5.2.2 Fumigation treatments

Plants were subjected to one of four treatments: Charcoal/Purafil® filtered clean air (CFA), CFA plus 110 ppb ozone for 8 hours per day (O_3), transfer from CFA to 110 ppb O_3 for 8 h d^{-1} at flowering (CFA-O_3), and transfer from 110 ppb O_3 for 8 h d^{-1} to CFA at flowering (O_3-CFA). Plants were exposed to each treatment in duplicate chambers with 10 plants in each chamber. Plants in reciprocal transfer treatments (CFA-O_3 and O_3-CFA) were moved when all flowers on a raceme were mature, usually within three days of the first flower bud opening. Plants in permanent treatments (CFA and O_3) were also moved to the duplicate chamber to control for any potential impact on physical movement on nutrient allocation to nectar and pollen. A schematic of the treatment regimes is shown in Figure 5.1. The entire experiment was repeated and results combined.

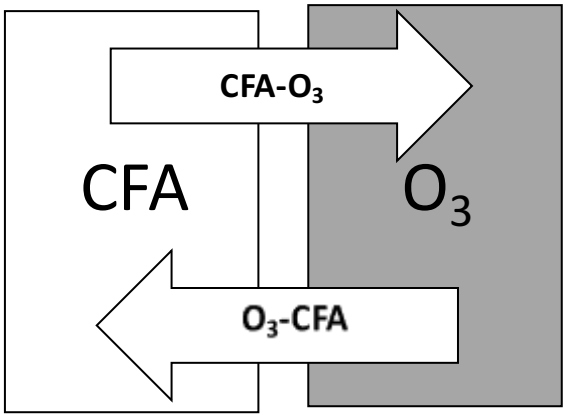

Figure 5.1. Diagram showing O_3 treatments. Plants were exposed in controlled environment chambers to charcoal/Purafil® filtered air 24 h d^{-1} (CFA) or 110 ppb O_3 8 h d^{-1} (O_3). Some plants were transferred from CFA to O_3 (CFA-O_3) at flowering, others were transferred from O_3 to CFA (O_3-CFA) at flowering.

5.2.3 Biomass

A subset of plants (5 plants/chamber) were destructively harvested. Shoots were cut from roots where the shoot met the soil and soil was carefully washed from the roots. Roots and shoots were then placed in labelled paper bags and dried in an oven at 60°C to constant mass. When dry, roots and shoots were weighed separately. A subset of plants (10 plants/chamber) was used for analysis of nectar and pollen at the same time as plants used in the destructive harvest. The number of racemes on each plant was recorded along with the number of flowers contributing to each raceme.

5.2.4 Nectar collection and analysis

Three days after all flowers on an individual raceme were open, the raceme was removed and the flowers were sampled for nectar. Nectar was collected from each flower on a raceme and composited to constitute a sample, so that each raceme constituted a surrogate for a plant in the analyses. Nectar was extracted from flowers by inserting a 5 µl microcapillary tube (McKenna and Thomson, 1988; Kearns and Inouye, 1993; Corbet, 2003; Morrant *et al.*, 2009) in to the intact flower to reach the nectaries at the base of the corolla. Available nectar was then drawn in to the capillary tubes and the capillary tube was placed in to a 2 ml microcentrifuge tube until analysis. Nectar volume was calculated by measuring the length of the nectar in the capillary

tube. The maximum volume of the tube was 5 µl and the length of the tube was 30 mm, allowing a conversion value of 6. Measured length of nectar was divided by 6 to convert the distance of nectar in the tube to µl (eg. 30 / 6 = 5 µl). Nectar samples were stored at -20°C until HPLC analysis. Composite samples were made after volume of nectar from each flower had been measured. Nectar was ejected from each capillary tube by squeezing air into the capillary tube or spinning the microcentrifuge tubes in a centrifuge to draw the nectar out of the tubes.

To prepare samples for amino acid analysis, raw nectar was diluted 1 in 30 in HPLC gradient grade H_2O (Fisher Scientific UK Ltd., Loughborough, United Kingdom Fisher). The solution was then mixed on a vortex for 1 min before being further diluted for carbohydrate analysis (1 : 2,000). Pilot studies had previously optimised the dilution of nectar for carbohydrate and amino acid analyses. Carbohydrates were separated and quantified using high performance ion chromatography (HPIC). Thirty microliters of sample were required for each injection on to the column.

5.2.5 Carbohydrate chromatographic method

Concentrations of glucose, fructose and sucrose were measured using HPIC. Thirty µl of sample was pipetted in to a 200 µl glass insert held in a snap-cap vial that was placed in the auto sampler tray. Twenty µl of the sample was injected on to a Carbopac PA-100 column (Dionex, Sunnyvale, California, USA) fitted with a Dionex Carbopac PA-100 BioLC guard (4 x 50 mm) *via* a Rheodyne valve. Sample components were eluted from the column isocratically using 100 mM NaOH (de-gassed using helium) employing a flow rate of 1 ml min^{-1} for 10 min at RT. The chromatogram profile was recorded using pulsed amperometric detection with an ED40 electrochemical detector (Dionex, Sunnyvale, California, USA). Elution profiles were analysed using Chromeleon v 6.8 software package (Thermo Fisher Scientific Inc., MA, USA) which automatically calculates solute concentrations based on a range (different dilutions) of pre-programmed reference calibrations for each sugar. The HPIC was calibrated at least twice every 24 h period for glucose, fructose, and sucrose by injecting calibration standards with concentrations of 10 ppm each. Standard solutions were made from the solid form of each sugar available (Sigma-Aldrich, St. Louis, MO, USA). The dual

calibration each day ensured accuracy of peak identification in the event of a daily drift in elution times. After each compound was identified in each chromatogram, the values produced by the Chromeleon software were scaled up to their original concentrations in nectar based on how much the nectar was diluted.

5.2.6 Amino acid analysis method

Raw nectar was diluted to 1 in 30 for amino acid analysis, then 10 µl of solution was added to a 200 µl tapered glass insert and held in the same autosampler vials as with carbohydrate HPIC. The chromatographic method for amino acid separation and detection is described in Section 2.2.6.

5.2.7 Pollen collection and analysis

Pollen from all flowers on one raceme was collected in a 200 µl microcentrifuge tube. Pollen that had not fallen from the anthers was collected by lightly tapping the anther in to the microcentrifuge tube. Pollen was stored at -20°C until analysis. Pollen was dried to constant mass at 60°C in an oven and then 1 mg weighed out. Free amino acids, non-structural carbohydrates and fatty acids were extracted by washing pollen in 200 µl Methanol (MeOH). The sample was centrifuged at 13,249 × *g* for 20 min and the MeOH extract was removed to a clean microcentrifuge tube. The remaining pollen pellet was subject to a microwave-assisted acid hydrolysis. Detailed methods of extraction, hydrolysis and quantification using HPLC are provided in Section 2.2.4 and 2.2.5. Carbohydrates were quantified using the same isocratic method described for nectar carbohydrate quantification (see Section 3.2.3). Fatty acids were quantified using GC/MS. A 50 µl aliquot of the MeOH extract was removed to a clean 2 ml microcentrifuge tube. The lid was opened and the tubes were placed in a heat block in a fume hood at 35 °C until dry. To the dried extract, 250 µl of hexane containing an internal standard (20 µg ml^{-1} tridecanoic acid) was added and the sample was left to extract overnight. Fatty acids were analysed using a Hewlett-Packard HP 6890 series gas chromatograph paired with a Hewlett-Packard HP 5973 mass spectrometry detector. Fatty acids were eluted through a DB-FFAP column using helium as the carrier gas.

5.2.8 Leaf tissue analysis

The first mature leaf and an immature leaf from each plant was removed, weighed, and then snap frozen in liquid nitrogen in a labelled foil packet. Mature leaves were identified as the first leaf on the main stem of the plant. The immature leaf was identified as a leaf that was not yet fully extended and usually the 5th leaf from the growing tip. Leaves were stored at -80°C until analysis.

Non-structural carbohydrate content was profiled and quantified using the HPIC method described in Section 3.2.3. Carbohydrates were extracted from leaves by grinding frozen samples in liquid nitrogen and aliquoting the dry powder in to pre-weighed 2 ml microcentrifuge tubes. To each tube, 1.5 ml 80% MeOH was added and each tube was incubated at 75°C for 1 h. Tubes were then centrifuged for 30 min at 13,249 × *g* and the supernatant was removed to a clean 2 ml microcentrifuge tube. The MeOH extract was dried at 70°C in a block heater using a concentrator that blew compressed air over the sample until dry. The sample was re-suspended in 200 µl nanopure water. The leaf extract was then de-salted prior to HPLC analysis. To desalt the extract, columns were prepared by placing a small piece of glass wool inside a 5 ml column, then ~0.7 cm^3 Amberlite was placed on top of the glass wool, and ~0.7 cm^3 Dowex, on top of that. The column was washed with Nanopure water prior to samples being washed through it and collected in a clean 5 ml plastic sample tube. Dowex and Amberlite were prepared as follows: 30 g of Dowex (AG50W X4 - 200) was washed in 95% ethanol with one change in 30 min to remove most of the colour. This was washed repeatedly with deionised water until clear. The 30 g of Amberlite (IRA-67) was washed with 5 volumes of 1M NaOH for 30 min, stirring every 5 min. This was rinsed with deionised water until neutral, as indicated using litmus paper. From each sample, 200 µl was placed on to the column and allowed to soak in. This was then washed through with 3 ml of HPLC gradient grade H_2O, and the eluent collected, centrifuged and then analysed using HPIC as described in Section 3.2.3.

5.2.9 Statistical analysis

Statistical analyses were carried out using SPSS v. 22. Prior to combining the data from the repeated experiment, the influence of growth chamber and round of

experiment were tested in a 2-way analysis of variance (ANOVA). The were no confounding impacts of experimental replicate and growth chamber and so data were combined. Biomass of root, shoot, root to shoot ratio, number of racemes, number of flowers and volume of nectar were all compared using a one-way ANOVA with significance determined using least square difference (*LSD*) *post hoc* analysis at the 5% level. Concentration of nectar carbohydrates were compared in a multivariate analysis of variance (MANOVA) with significance at the 5% level determined using LSD. Total essential and non-essential amino acids were analysed with a MANOVA with significance at the 5% level determined using LSD. The distributions of amino acids in nectar were analysed using a canonical discriminant analysis (CDA), having first removed tryptophan, GABA and glutamine data (which were more than 10-fold lower than other amino acid concentrations). Pollen weight was compared using MANOVA. Free and protein-bound amino acids were also analysed using CDA. Treatment effects on pollen carbohydrates and fatty acid composition were compared using MANOVA. Mature and immature leaf weights were compared in a one-way ANOVA.

5.3 Results

5.3.1 Growth characteristics

Exposure to ozone had a significant (P= 0.007) influence on the dry weight of shoots (Figure 5.2A). Transfer from O_3 to CFA (O_3-CFA) caused a significant (P< 0.05) reduction in shoot biomass compared to that of plants subject to sustained exposure to 110 ppb O_3 for 8 h d^{-1}. Whereas shoots from plants maintained in O_3 throughout weighed significantly (P< 0.05) more than those of plants in both transfer treatments (CFA-O_3 and O_3-CFA) but were similar to plants maintained in charcoal-filtered air (CFA) throughout. Ozone exposure resulted in no significant change in the dry weight of roots nor the ratio between root and shoot weight (Figure 5.2B and 5.2C).

Sustained exposure to 110 ppb O_3 for 8 h d^{-1} (O_3) resulted in no significant influence on the number of flowers produced on each raceme (Figure 5.3A) nor on the number of racemes produced (Figure 5.3B). Sustained exposure to O_3 had no significant influence on the volume of floral nectar produced by broad beans compared to plants raised throughout in CFA. Interestingly, the volume of nectar collected from both transfer treatments i.e. plants transferred from O_3 to CFA and *vice versa*, was significantly (P< 0.001) increased by more than 2-fold that of plants maintained in permanent conditions (Figure 5.3C). There was a significant interaction between treatment and leaf age on the fresh weight of broad bean leaves (treatment*age, P<0.001) (Figure 5.4). Plants grown under CFA (CFA and CFA-O_3) produced mature and immature leaves of similar weights (P= 0.132). Plants maintained in O_3 produced leaves with the lowest weight ($P \leq 0.001$) and those in the O_3-CFA treatment were of a similar weight to those in the CFA-O_3 treatment (P=0.378), but weighed significantly less than those sustained in CFA (P=0.018) and more than those sustained in O_3 (P=0.001).

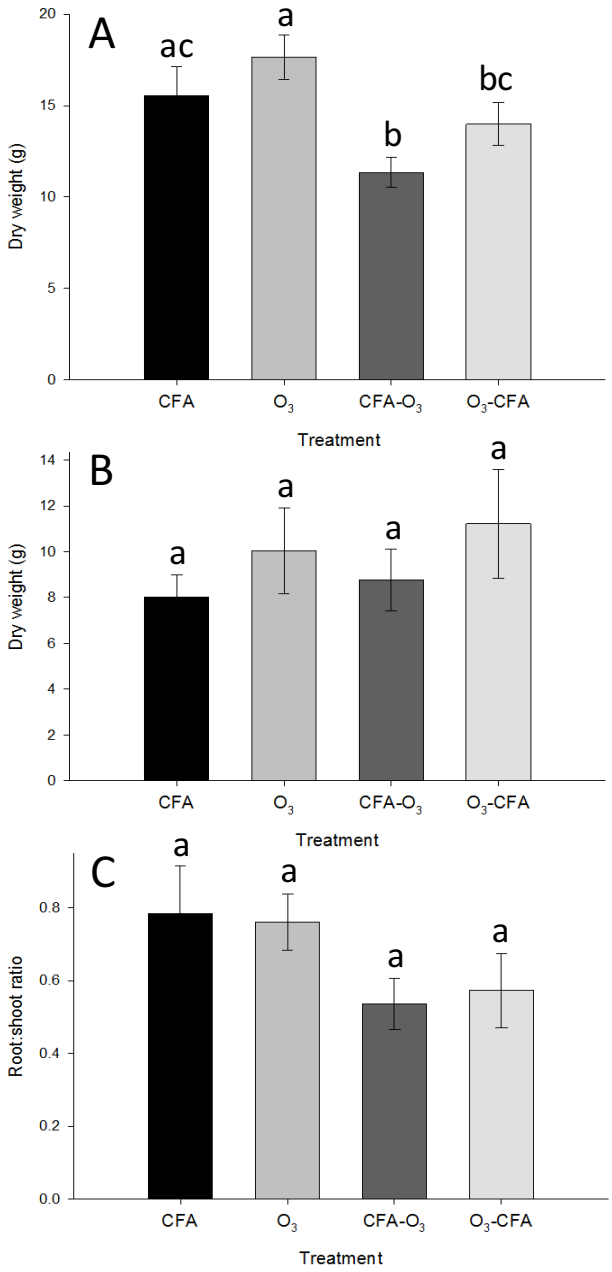

Figure 5.2. Biomass (dry weight) of shoots (A), roots (B) and root:shoot ratio (C) of broad beans (*Vicia faba*) grown from seed and exposed to four fumigation treatments; charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 pbb O_3 8 h d^{-1} at flowering (CFA-O_3) and grown in CFA + 110 pbb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA). Different letters indicate significant differences at the 5% level. Error bars represent +/-SEM.

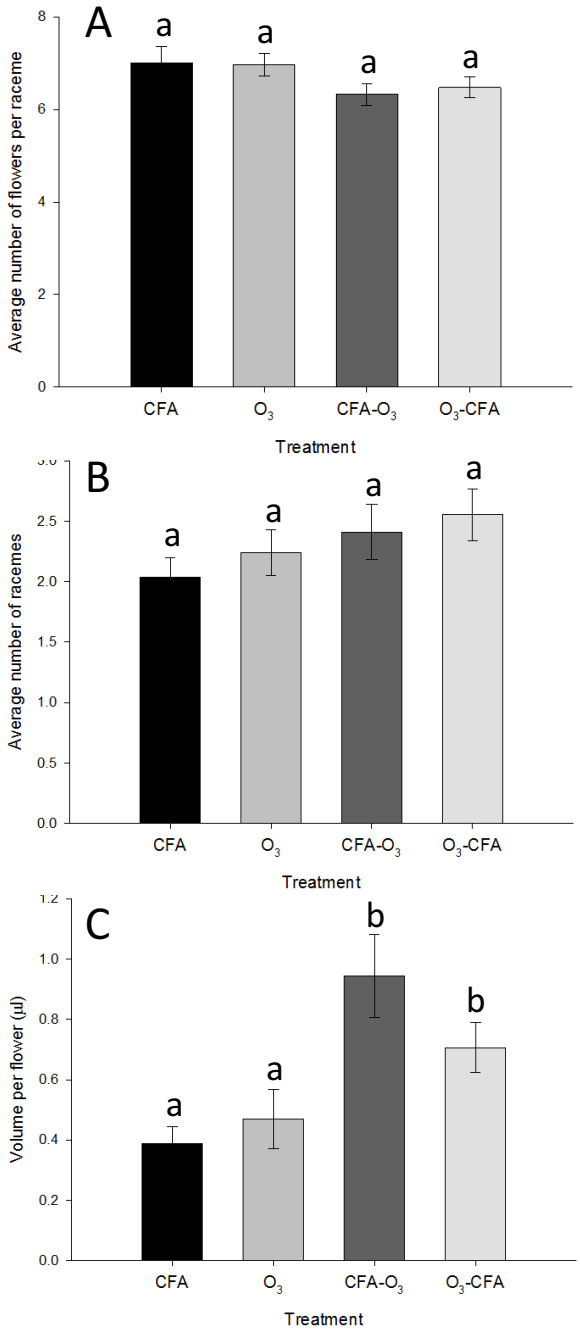

Figure 5.3. Average number of flowers present per raceme (A), number of racemes (B) and volume of nectar per flower (C) from broad bean (*Vicia faba*) grown under four treatments; charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 ppb O_3 8 h d^{-1} at flowering (CFA-O_3) and grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA). Different letters indicate significant differences at the 5% level. Error bars represent +/-SEM.

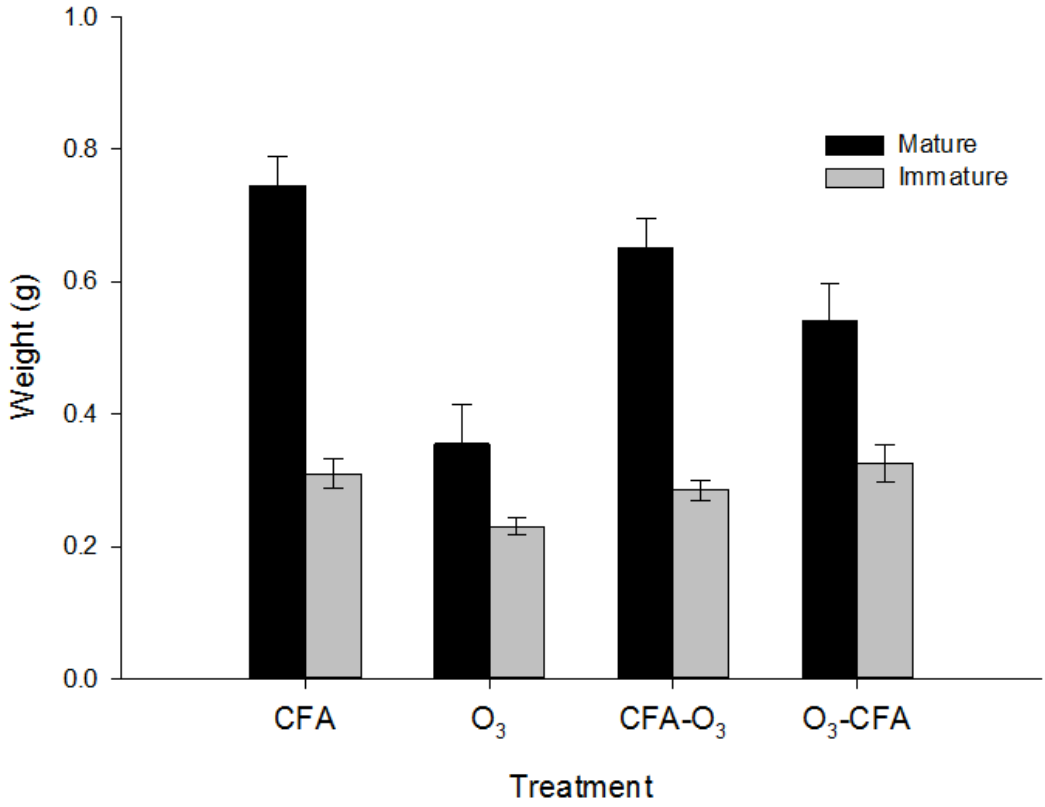

Figure 5.4. Mature and immature leaf fresh weight from broad bean (*Vicia faba*) plants grown under charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 ppb O_3 8 h d^{-1} at flowering (CFA-O_3) or grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA). Different letters indicate significant differences at the 5% level. Error bars represent +/-SEM.

5.3.2 Leaf non-structural carbohydrates

The non-structural carbohydrate content of mature and immature broad bean leaves was quantified (Figure 5.5A and 5.5B). The concentrations of glucose and fructose in mature leaves did not differ in response to exposure to O_3. However, there was a significant ($P< 0.05$) effect of O_3 on the concentration of sucrose in mature leaves. Sucrose accumulated to similar concentrations of ~7.8 μg g fwt^{-1} and ~5.4 μg g fwt^{-1} in plants grown in O_3 and O_3-CFA, respectively, but was significantly ($P\leq 0.02$) higher in plants raised in O_3 throughout than in plants subject to CFA (CFA and CFA-O_3). Conversely, in immature leaves glucose ($P< 0.001$) and fructose ($P< 0.02$) content of

leaves was significantly influenced by treatment, whereas effects on sucrose concentration was on the borderlines of statistical significance ($P< 0.08$). Plants in the O_3-CFA treatment exhibited the lowest ($P\leq 0.005$) concentration of glucose in foliage. Plants grown in CFA (CFA and CFA-O_3) had similar concentrations of glucose in immature leaves (2.9 and 2.2 μg/g fwt^{-1} CFA and CFA-O_3, respectively), as did plants sustained in O_3 and those transferred from CFA to O_3 at flowering. Transfer from O_3 to CFA at flowering induced a reduction in the amount of fructose in immature leaves compared to plants grown in CFA (CFA and CFA-O_3) ($P\leq 0.016$). Plants sustained in O_3 had similar concentrations of fructose to all other treatments.

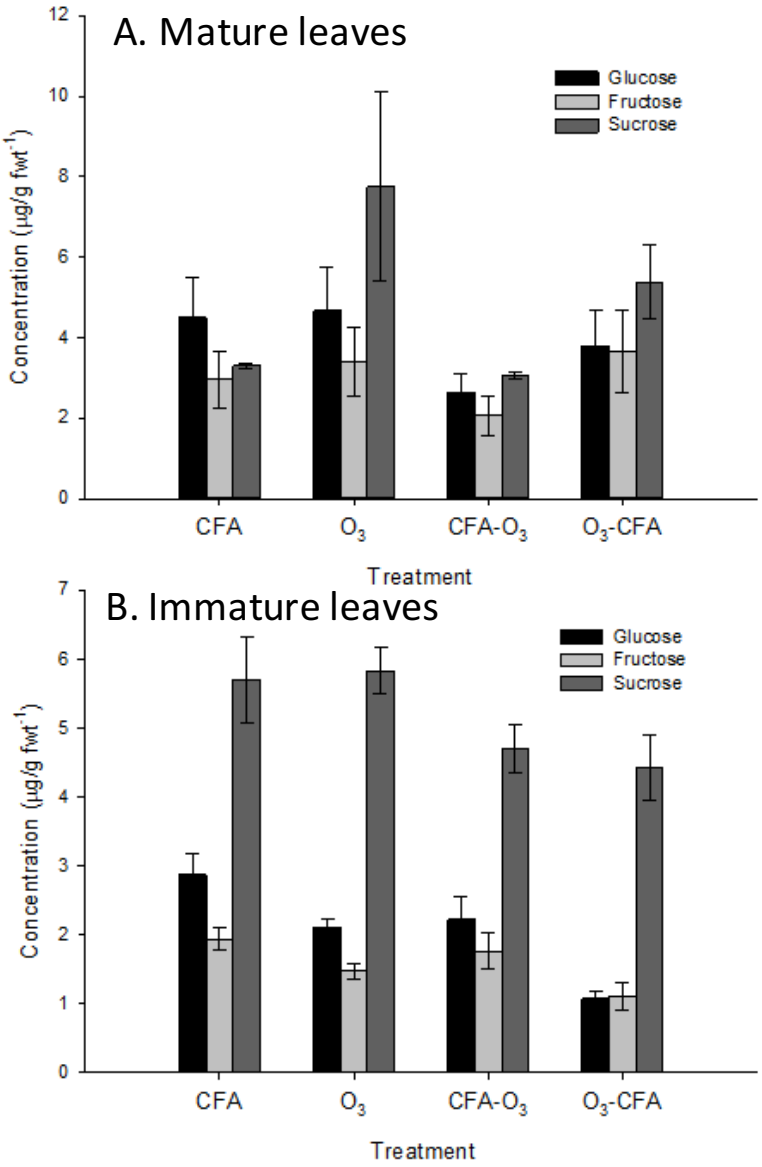

Figure 5.5. Leaf non-structural carbohydrate composition of mature (A) and immature (B) leaves of broad bean (*Vicia faba*) grown in charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 ppb O_3 8 h d^{-1} at flowering (CFA-O_3) and grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA). Error bars represent +/-SEM.

5.3.3 Nectar composition

5.3.3.1 Carbohydrates

The concentration of glucose and fructose in nectar of broad bean flowers was similar in all treatments. However, plants grown in O_3 and moved to CFA at flowering (O_3-CFA) produced nectar with 2-fold more concentrated sucrose than all other treatments ($P \leq 0.01$) (Figure 5.6).

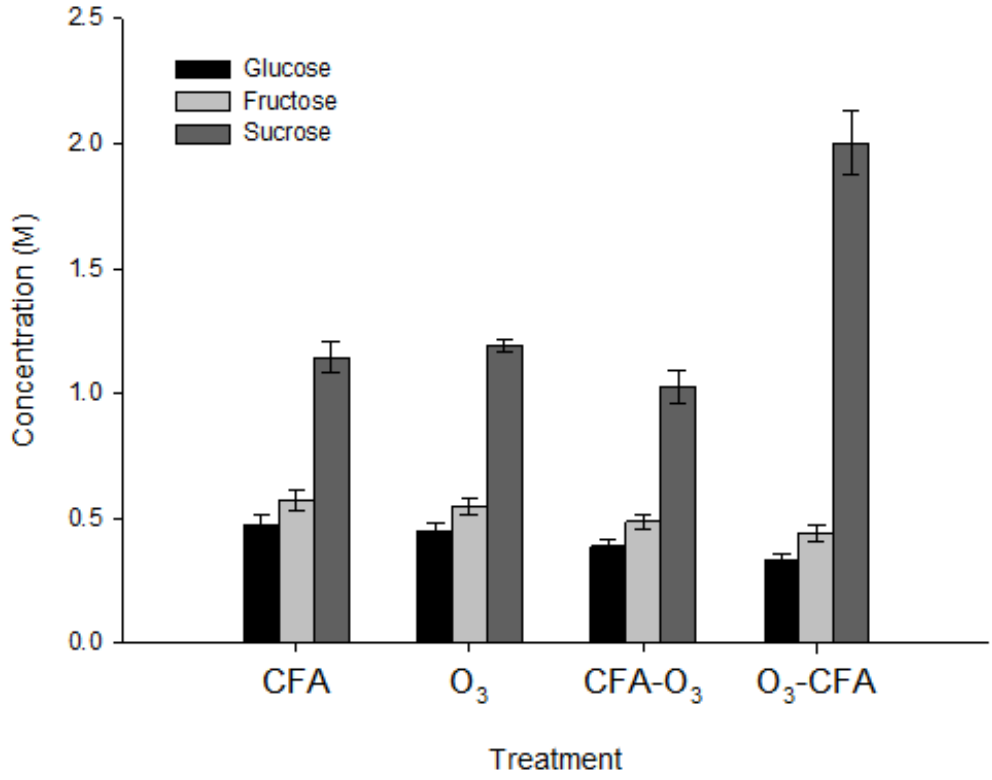

Figure 5.6. Nectar concentrations of glucose, fructose and sucrose collected from flowers of broad bean (*Vicia faba*) grown under four treatments; charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 ppb O_3 8 h d^{-1} at flowering (CFA-O_3) and grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA). Error bars represent +/-SEM.

5.3.3.2 Amino acids

The total amino acid content of nectar was significantly influenced by ozone exposure (P= 0.01) (Figure 5.7). However, the total essential amino acid content of nectar was not significantly influenced by exposure to ozone. The total concentration of non-essential amino acids (P= 0.004) was the main factor influencing the change in total concentration of amino acids in nectar (Figure 5.8). Nectar of plants grown in O_3 then transferred to clean air (CFA) at flowering contained significantly ($P \leq 0.01$) more non-essential amino acids than any other treatment.

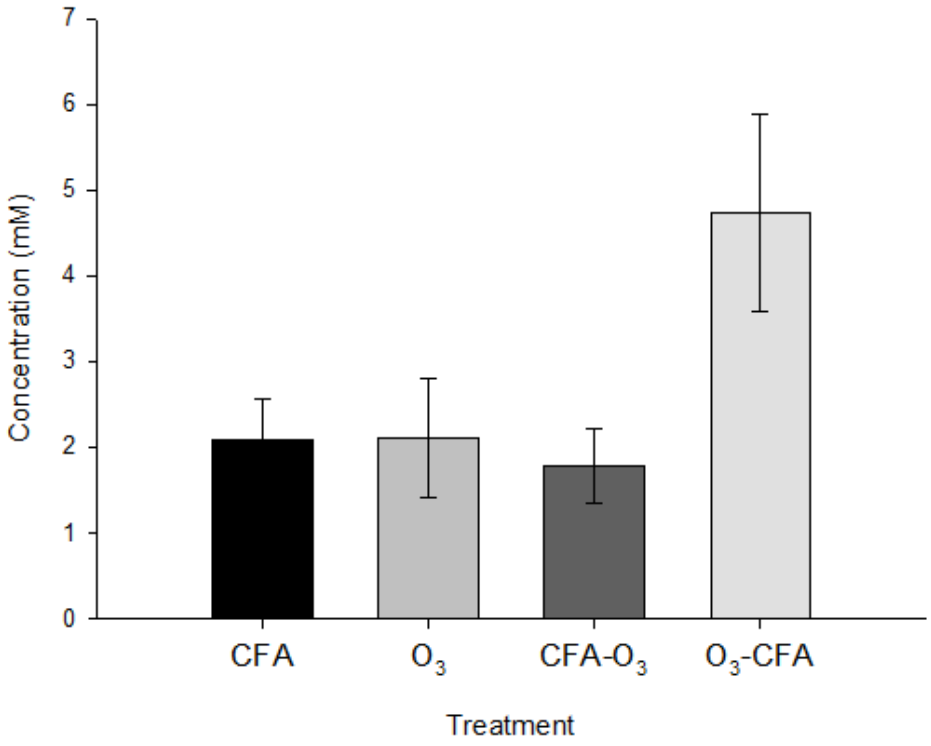

Figure 5.7. Nectar concentrations of total free amino acids collected from broad bean (*Vicia faba*) flowers grown under four fumigation treatments: charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 ppb O_3 8 h d^{-1} at flowering (CFA-O_3) and grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA). Error bars represent +/-SEM.

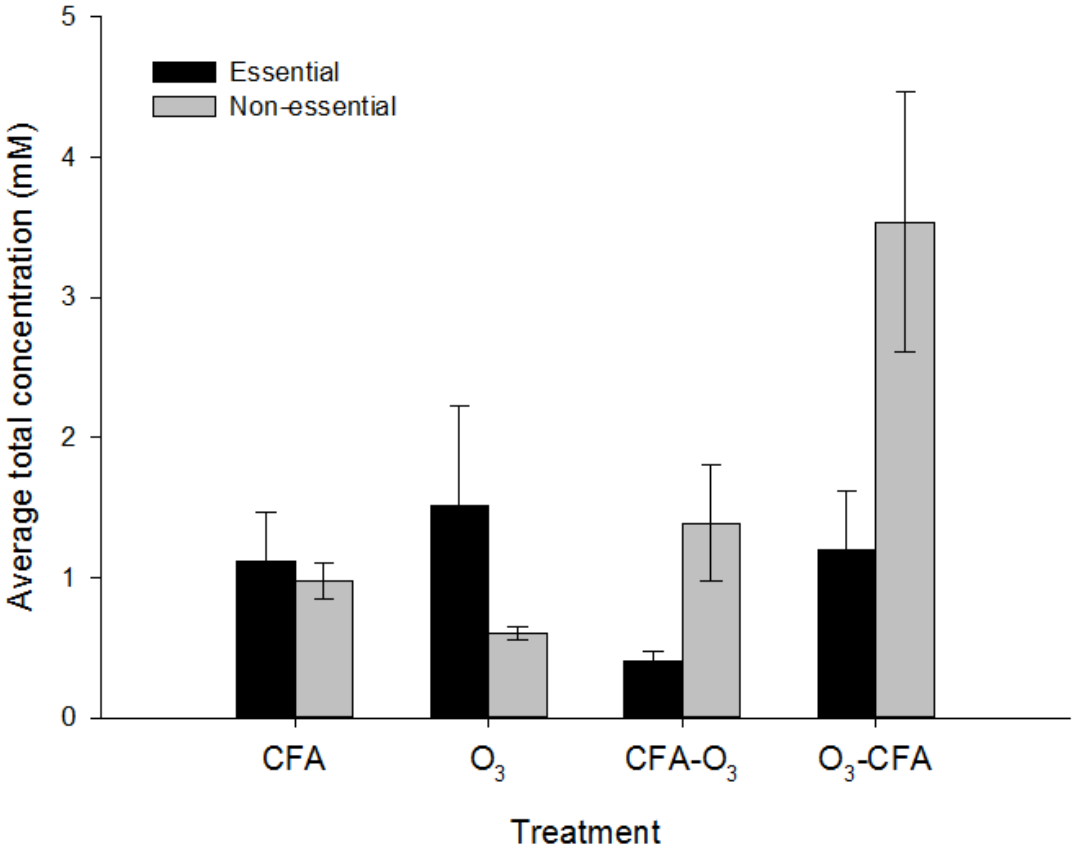

Figure 5.8 Nectar essential and non-essential amino acids quantified from broad bean (*Vicia faba*) flowers under four growth treatments: charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 ppb O_3 8 h d^{-1} at flowering (CFA-O_3) and grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA). Different letters indicate significant differences at the 5% level. Error bars represent +/-SEM.

5.3.3.3 Distribution of essential and non-essential amino acids in nectar

Canonical discrimination analysis (CDA) was used to identify whether exposure to ozone influenced the distribution of amino acids in nectar. Essential and non-essential amino acids were analysed in two separate CDAs.

Essential amino acids were reduced to two significant functions accounting for 90% of the variation in the data. The first discriminant function separated plants that were maintained in O_3 and those grown in O_3 and transferred to CFA at flowering from the other treatments (canonical discriminant function coefficients, Table 5.2). Threonine

in nectar of plants subject to sustained O_3 accumulated to ~2.3-fold· that of the concentration in nectar from plants sustained in CFA. However, when plants were moved from O_3 to CFA at flowering, the concentration of threonine dropped to zero (Table 5.1 and pooled within-groups correlations, Table 5.2). The second discriminant function separated plants grown in CFA-O_3 from those in the O_3-CFA treatment (canonical discriminant function coefficients, Table 5.2). Plants that were grown in O_3 and moved to CFA at flowering exhibited higher concentrations of histidine, isoleucine and methionine than plants grown in CFA and transferred to O_3 at flowering (Tables 5.1 and 5.2). The third discriminant function was unable to significantly distinguish between treatment groups.

A CDA on non-essential amino acids also produced two significant functions (Table 5.2). The first discriminant function distinguished the difference between plants that were grown in O_3 and transferred to CFA at flowering from those maintained in CFA or O_3 throughout (canonical discriminant function coefficients, Table 5.2). Transfer of plants from O_3 to CFA at flowering resulted in increased concentrations of cysteine, proline, asparagine and glutamic acid in nectar compared to plants maintained in either condition (CFA or O_3) throughout (Tables 5.1 and 5.2). The second significant function distinguished plants grown in CFA and transferred in to O_3 at flowering (CFA-O_3) from the other treatment groups (canonical discriminant function coefficients, Table 5.2). Nectar of plants grown in CFA and moved to O_3 (CFA-O_3) at flowering contained more aspartic acid and serine, but had lower concentrations of glycine, in comparison to plants grown under O_3 and transferred to CFA at flowering (O_3-CFA) (Tables 5.1 and 5.2).

Table 5.1. Nectar essential and non-essential amino acid concentrations from broad bean (*Vicia faba*) in 4 growth treatments; charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 ppb O_3 8 h d^{-1} at flowering (CFA-O_3) and grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA). +/-SEM.

Essential amino acids										
	μM									
Amino acid	Histidine	Leucine	Threonine	Arginine	Lysine	Valine	Methionine	Tryptophan	Phenylalanine	Isoleucine
CFA	14.7 ±6.5	59.5 ±23.7	530.7 ±250	83.8 ±36.5	124.1 ±53.5	187.5 ±46.5	47.4 ±10.2	0.02 ±0.0	72.3 ±15.9	1.24 ±1.2
O_3	8.99 ±2.8	7.00 ±4.2	1218 ±698	21.7 ±7.0	56.8 ±21.5	97.3 ±21.6	60.9 ±12.7	0.05 ±0.0	38.1 ±7.8	8.16 ±5.8
CFA-O_3	9.05 ±6.1	38.0 ±8.05	25.8 ±6.4	85.7 ±19.7	61.1 ±15.5	124.6 ±27.0	33.0 ±6.0	0.02 ±0.0	25.7 ±5.4	0.47 ±0.3
O_3-CFA	344.4 ±88.3	108.0 ±48.7	0.00 ±0.0	123.5 ±42.6	126.5 ±64.1	291.4 ±115.3	43.8 ±9.0	0.04 ±0.0	81.9 ±30.1	82.5 ±39.5

Non-essential amino acids											
	μM										
Amino acid	Aspartic acid	Glutamic acid	Asparagine	Serine	Glutamine	Glycine	Tyrosine	Cysteine	GABA	Alanine	Proline
CFA	28.3 ±11.8	124.3 ±35.6	0.12 ±0.03	114.3 ±35.1	0.03 ±0.01	386.9 ±73.8	23.9 ±9.26	25.3 ±3.29	3.02 ±1.38	34.6 ±17.6	238.1 ±66.7
O_3	16.1 ±9.98	62.4 ±15.4	0.06 ±0.02	58.9 ±19.8	0.01 ±0.00	327.6 ±68.9	4.96 ±1.85	16.0 ±2.86	0.41 ±0.25	3.69 ±2.62	110.8 ±27.1
CFA-O_3	83.0 ±23.6	198.1 ±35.0	0.95 ±0.45	641.2 ±376	0.12 ±0.06	39.1 ±3.56	30.1 ±6.12	32.8 ±4.29	0.09 ±0.03	42.6 ±16.0	320 ±56.2
O_3-CFA	21.4 ±4.50	1395 ±747	2.16 ±0.78	388 ±116	0.40 ±0.16	60.5 ±14.9	21.8 ±3.91	99.3 ±13.8	0.77 ±0.22	139.0 ±72.1	1405 ±261

Table 5.2. Nectar essential and non-essential amino acids canonical discriminant analysis (CDA) output from broad bean (*Vicia faba*) grown under four treatments: charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 ppb O_3 8 h d^{-1} at flowering (CFA-O_3) and grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA).

	Essential amino acids			
	Canonical discriminant function statistics			
Function	Eigenvalue	% Variance	test stat	P value
1	0.970	68	x^2_{27}=109	<0.001
2	0.311	21.8	x^2_{16}=40.9	0.001
3	0.146	10.2	x^2_7=13.7	0.057

	Pooled within-groups correlations		
	Function		
Amino acid	1	2	3
Threonine	**0.290**	0.171	0.181
Histidine	-0.406	**0.631**	-0.268
Isoleucine	-0.206	**0.363**	-0.105
Methionine	0.146	**0.249**	0.038
Phenylalanine	-0.066	0.249	**-0.445**
Leucine	-0.174	0.148	**-0.321**
lysine	-0.048	0.095	**-0.302**
Valine	-0.136	0.180	**-0.278**
Arginine	-0.189	0.007	**-0.272**

	Canonical discriminant function coefficients		
	Function		
Treatment	1	2	3
CFA	0.881	-0.243	-0.612
O_3	**1.327**	0.456	0.432
CFA-O_3	-0.467	**-0.768**	0.271
O_3-CFA	**-1.056**	**0.524**	-0.096

	Non-essential amino acids			
	Canonical discriminant function statistics			
Function	Eigenvalue	% Variance	test stat	P value
1	1.40	75.6	x^2_{27}=127	<0.001
2	0.396	21.4	x^2_{16}=38.9	0.001
3	0.055	3	x^2_7=5.37	0.615

	Pooled within-groups correlations		
	Function		
Amino acid	1	2	3
Cysteine	**0.592**	-0.370	0.295
Proline	**0.498**	-0.348	0.222
Asparagine	**0.263**	-0.027	0.026
Glutamic acid	**0.190**	-0.148	0.045
Aspartic acid	0.009	**0.584**	0.169
Glycine	-0.498	**-0.567**	0.443
Serine	0.104	**0.264**	0.040
Tyrosine	0.095	0.304	0.858
Alanine	0.177	-0.095	0.182

	Canonical discriminant function coefficients		
	Function		
Treatment	1	2	3
CFA	**-1.199**	-0.312	0.354
O_3	**-1.219**	-0.383	-0.358
CFA-O_3	0.081	**1.018**	-0.014
O_3-CFA	**1.531**	**-0.409**	0.004

5.3.4 Pollen composition

Exposure to ozone at flowering was directly responsible for a significant ($P< 0.001$) reduction in the fresh weight of pollen collected from broad bean flowers (Figure 5.9). The weight of pollen collected from plants sampled in CFA (CFA and O_3-CFA) was similar. However, the weight of pollen produced by plants sampled in O_3 (O_3 and CFA-O_3) was significantly ($P< 0.02$) less than that of plants sampled in CFA.

Total protein-bound essential amino acids were significantly ($P< 0.001$) reduced in plants exposed to O_3 by ~75% in the sustained treatment. In fact, all treatments involving exposure to ozone (O_3, CFA-O_3 and O_3-CFA) resulted in the recovery of significantly ($P< 0.001$) less total protein-bound essential amino acids from pollen compared to plants subject to sustained CFA. Pollen from plants in reciprocal transfer treatments exhibited similar concentrations of total protein-bound essential amino acids (Figure 5.10). Total protein-bound non-essential amino acids were also significantly ($P< 0.001$) reduced in plants exposed to O_3. Like protein-bound essential amino acids, all plants subject to O_3 (O_3, CFA-O_3 and O_3-CFA) exhibited less ($P< 0.001$) total free essential amino acids in pollen than plants subject to sustained CFA (Figure 5.10).

The total free essential amino acids reduced by ~75% in plants sustained under O_3 ($P< 0.001$); however, plants in all other treatments (CFA, CFA-O_3 and O_3-CFA) had similar total concentrations of free essential amino acids in their pollen (Figure 5.10). Total free non-essential amino acids were also influenced by ozone exposure ($P< 0.001$). Plants sustained in O_3 had ~17% more free non-essential amino acids than plants sustained in CFA ($P= 0.031$). Plants grown in CFA and transferred in to O_3 at flowering had ~24% less ($P\leq 0.01$) free non-essential amino acids than those sustained in CFA (Figure 5.10).

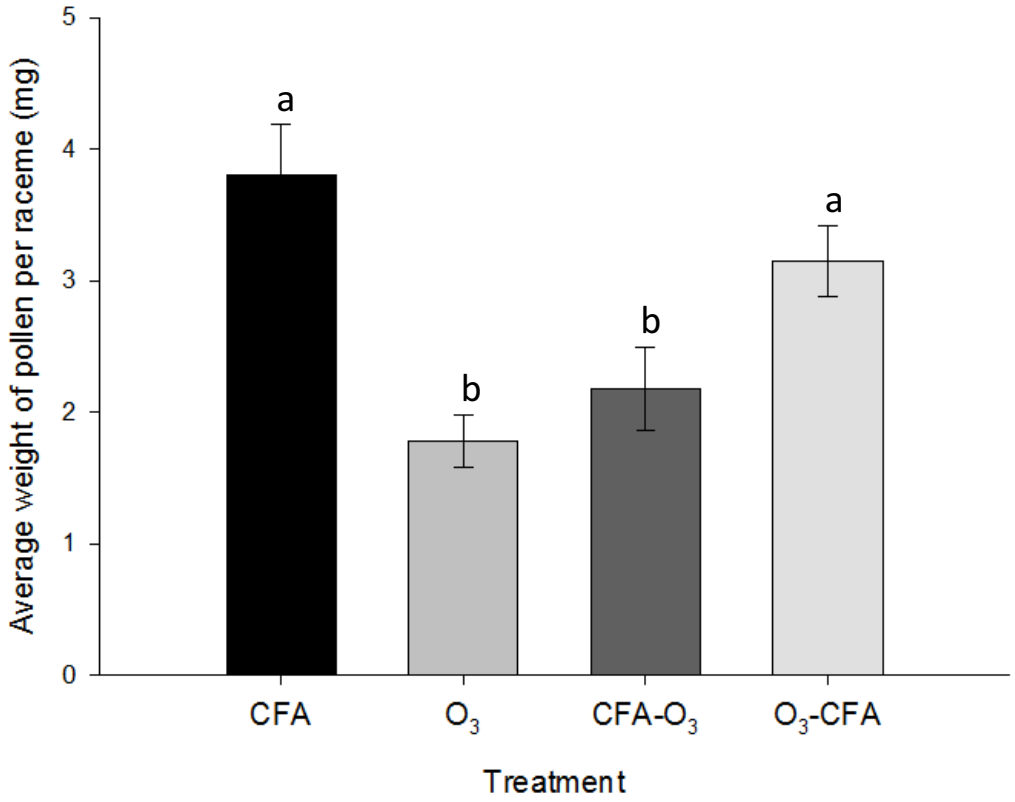

Figure 5.9. Weight of pollen per flower collected from broad bean (*Vicia faba*) from four growth treatments: charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 ppb O_3 8 h d^{-1} at flowering (CFA-O_3) and grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA). Different letters indicate significant differences at the 5% level. Error bars represent +/-SEM.

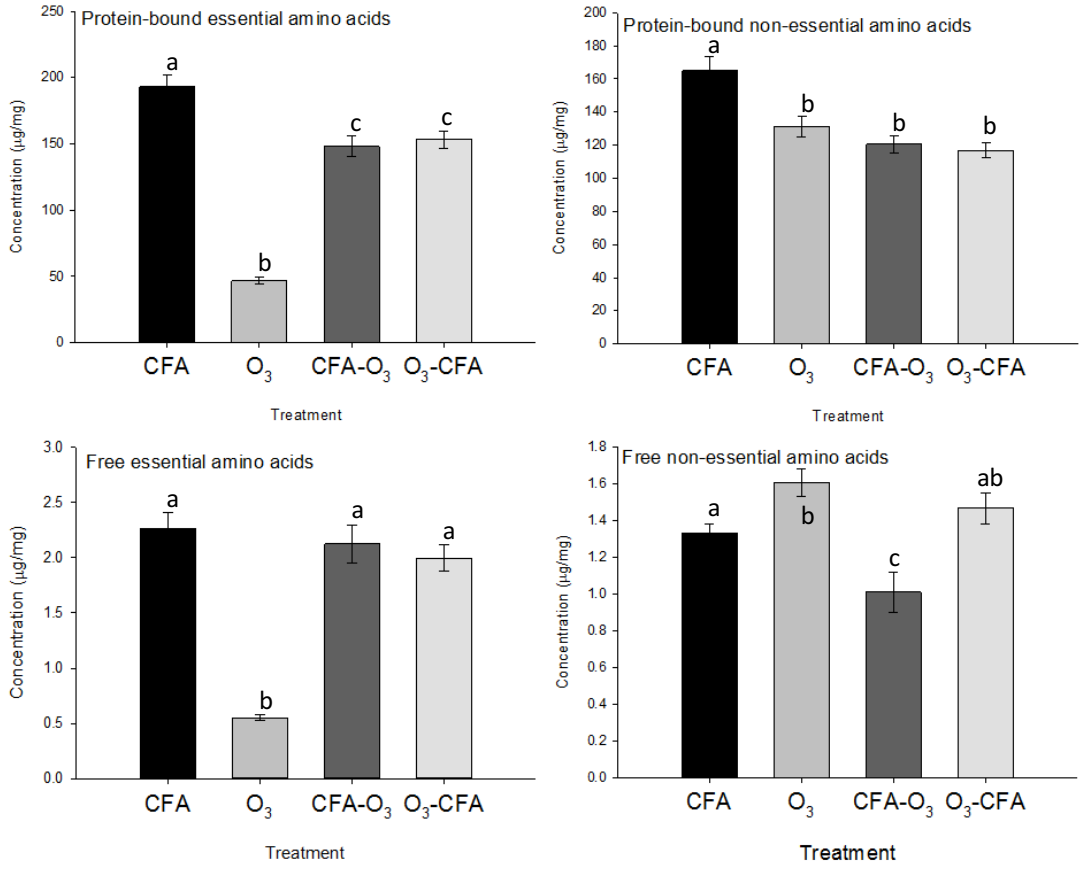

Figure 5.10 Pollen total protein-bound and free essential amino acids from broad bean (*Vicia faba*) from four growth treatments: charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 ppb O_3 8 h d^{-1} at flowering (CFA-O_3) and grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA). Different letters indicate significant differences at the 5% level. Error bars represent +/-SEM.

5.3.5 Protein-bound amino acid composition

The distribution of protein-bound essential amino acids were analysed using a CDA to identify ozone-induced changes to the distribution of amino acids in pollen. Three significant functions were produced from nine essential amino acids, (tryptophan was not included in the analysis). The first function represented the change in amino acids between plants maintained in CFA and those grown in CFA and moved to O_3 at flowering (canonical discriminant function coefficients, Table 5.4). All nine essential amino acids were able to be predicted based on treatment (pooled within-groups correlations, Table 5.4). The amount of histidine, leucine, arginine, methionine, phenylalanine and isoleucine decreased as a direct impact of plants being moved to O_3

at flowering. However, plants that were moved from CFA to O_3 exhibited a greater amount of protein-bound threonine, lysine and valine than those subject to CFA throughout (Tables 5.3 and 5.4). The second discriminant function separated reciprocal transfer treatments (CFA-O_3 and O_3-CFA) from sustained exposure to either O_3 or CFA (canonical discriminant function coefficients, Table 5.4). Pollen of plants grown in CFA contained ~two-fold more threonine than those grown in O_3 (Tables 5.3 and 5.4). The third discriminant function was able to separate plants that had been grown under O_3 and transferred in to CFA from those sustained in O_3 (canonical discriminant function coefficients, Table 5.4). When plants were moved from O_3 in to CFA, the amount of protein-bound methionine, arginine, lysine, leucine, valine, histidine, phenylalanine and isoleucine increased in pollen compared to that of plants subject to sustained O_3 (Tables 5.3 and 5.4).

Ozone-induced shifts in protein-bound non-essential amino acids were also successfully explained by three significant functions. The first function extracted the difference between plants maintained throughout in CFA and those maintained throughout in O_3 (canonical discriminant function coefficients, Table 5.4). Protein-bound glycine was ~79% higher in pollen from plants maintained in O_3 compared to those maintained in CFA (Tables 5.3 and 5.4). The second function highlighted the effect of transferring plants from CFA to O_3 at flowering (canonical discriminant function coefficients, Table 5.4). Plants that were moved into O_3 at flowering exhibited increased levels of protein-bound aspartic acid and serine in pollen (Tables 5.3 and 5.4). The third significant function explained differences between plants grown in O_3 and moved in to CFA at flowering with those sustained in CFA (canonical discriminant function coefficients, Table 5.4); plants grown in O_3 and moved to CFA at flowering exhibited significantly lower levels of protein-bound tyrosine, alanine, asparagine, glutamic acid, cysteine and proline in pollen compared to plants maintained throughout in CFA (Tables 5.3 and 5.4).

Table 5.3. Pollen mean essential and non-essential, protein-bound amino acids from broad bean (*Vicia faba*) in four growth treatments; charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 ppb O_3 8 h d^{-1} at flowering (CFA-O_3) and grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA). +/-SEM.

Essential amino acids										
	µg/mg									
Amino acid	Histidine	Leucine	Threonine	Arginine	Lysine	Valine	Methionine	Tryptophan	Phenylalanine	Isoleucine
CFA	44.8 ±3.5	24.3 ±1.2	9.18 ±1.3	73.1 ±3.2	21.3 ±1.1	3.41 ±0.2	5.26 ±0.3	0.00 ±0.0	10.3 ±0.4	1.72 ±0.3
O_3	10.8 ±0.6	5.99 ±0.4	5.62 ±0.4	15.7 ±1.1	1.73 ±0.1	2.28 ±0.2	0.87 ±0.1	0.00 ±0.0	3.51 ±0.2	0.50 ±0.1
CFA-O_3	29.3 ±2.0	9.67 ±0.5	11.41 ±1.1	49.47 ±3.3	36.2 ±2.4	4.05 ±0.2	1.91 ±0.1	0.00 ±0.0	5.50 ±0.4	0.74 ±0.1
O_3-CFA	47.5 ±3.6	12.2 ±0.5	5.70 ±0.4	44.2 ±1.6	27.4 ±1.9	7.03 ±0.3	3.26 ±0.1	0.00 ±0.0	4.90 ±0.2	1.37 ±0.1

Non-essential amino acids											
	µg/mg										
Amino acid	Aspartic acid	Glutamic acid	Asparagine	Serine	Glutamine	Glycine	Tyrosine	Cysteine	GABA	Alanine	Proline
CFA	39.6 ±5.4	56.7 ±4.9	0.14 ±0.0	0.19 ±0.1	0.01 ±0.0	3.02 ±0.5	7.12 ±1.1	33.8 ±2.6	0.04 ±0.1	14.1 ±0.9	9.39 ±0.8
O_3	24.9 ±1.1	38.9 ±2.1	0.06 ±0.0	0.22 ±0.0	0.00 ±0.0	14.3 ±0.7	8.73 ±0.6	18.4 ±1.2	0.01 ±0.0	19.7 ±1.2	5.95 ±0.7
CFA-O_3	26.9 ±2.2	43.3 ±3.0	0.08 ±0.0	0.08 ±0.0	0.00 ±0.0	3.31 ±0.2	6.20 ±0.5	24.0 ±1.9	0.00 ±0.0	9.64 ±0.6	6.99 ±0.6
O_3-CFA	37.1 ±2.3	37.4 ±3.0	0.09 ±0.0	0.26 ±0.1	0.00 ±0.0	2.85 ±0.2	2.37 ±0.2	22.0 ±1.7	0.04 ±0.1	8.37 ±0.4	6.58 ±0.9

Table 5.4. Pollen essential and non-essential protein-bound amino acids canonical discriminant analysis (CDA) output from broad bean (*Vicia faba*) grown under four treatments: charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 ppb O_3 8 h d^{-1} at flowering (CFA-O_3) and grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA).

	Essential amino acids			
	Canonical discriminant function statistics			
Function	Eigenvalue	% Variance	test stat	P value
1	8.924	56.1	x^2_{27}=655	<0.001
2	4.354	27.4	x^2_{16}=369	<0.001
3	2.625	16.5	x^2_7=160	<0.001

	Pooled within-groups correlations		
	Function		
Amino acid	1	2	3
Threonine	0.008	**0.224**	0.141
Methionine	-0.386	-0.099	**0.725**
Arginine	-0.227	0.183	**0.717**
Lysine	0.168	0.181	**0.711**
Leucine	-0.443	0.044	**0.603**
Valine	0.174	-0.408	**0.594**
Histidine	-0.072	-0.149	**0.531**
Phenylalanine	-0.340	0.177	**0.436**
Isoleucine	-0.120	-0.087	**0.260**

	Canonical discriminant function coefficients		
	Function		
Treatment	1	2	3
CFA	**-5.176**	0.752	0.905
O_3	-0.106	-0.372	**-2.805**
CFA-O_3	**2.854**	**2.645**	0.664
O_3-CFA	1.489	**-2.846**	**1.119**

	Non-essential amino acids			
	Canonical discriminant function statistics			
Function	Eigenvalue	% Variance	test stat	P value
1	10.454	90.6	x^2_{27}=410	<0.001
2	0.670	5.8	x^2_{16}=107	<0.001
3	0.416	3.6	x^2_7=43.3	<0.001

	Pooled within-groups correlations		
	Function		
Amino acid	1	2	3
Glycine	**-0.548**	0.347	0.498
Aspartic acid	0.080	**0.283**	-0.028
Serine	-0.010	**0.152**	-0.115
Tyrosine	-0.123	-0.076	**0.817**
Alanine	-0.246	0.375	**0.778**
Asparagine	0.178	0.467	**0.553**
Glutamic acid	0.059	0.114	**0.513**
Cysteine	0.115	0.170	**0.507**
Proline	0.060	0.112	**0.300**

	Canonical discriminant function coefficients		
	Function		
Treatment	1	2	3
CFA	**2.718**	**0.793**	**0.865**
O_3	**-5.543**	0.203	0.111
CFA-O_3	1.152	**-1.299**	0.13
O_3-CFA	1.618	0.444	**-0.922**

5.3.6 Free amino acid composition

Impacts of O_3 on free essential amino acids were explained by three significant functions *via* CDA (Table 5.6). The first function separated plants that were maintained in CFA and O_3 from those transferred between treatments at flowering (canonical discriminant function coefficients, Table 5.6); sustained exposure to O_3 resulting in ~43% more free valine in pollen, but ~75% less free lysine compared to plants maintained in CFA (Tables 5.5 and 5.6). The second function separated the treatments that were sampled in CFA (CFA and O_3-CFA) from the other treatments (canonical discriminant function coefficients, Table 5.6). The amount of methionine was 100% higher, arginine was ~29% higher and leucine was ~70% higher in plants moved from O_3 to CFA at flowering compared to those subject to sustained CFA. However, the amount of threonine and phenylalanine was reduced, by ~78% and ~30%, respectively (Tables 5.5 and 5.6). The third function extracted the differences between plants subject to reciprocal transfers (CFA-O_3 and O_3-CFA) from the other treatments (canonical discriminant function coefficients, Table 5.6), analyses revealing histidine and isoleucine were lower in plants transferred from O_3 to CFA at flowering compared to plants grown in CFA and transferred to O_3 at flowering (Tables 5.5 and 5.6).

Impacts of O_3 on free non-essential amino acid composition were again explained by three significant functions *via* CDA (Table 5.6). The first function separated the plants maintained throughout in CFA or O_3 from the other treatments (canonical discriminant function coefficients, Table 5.6). Plants exposed to O_3 exhibited ~98% higher levels of free alanine but ~81% lower levels of aspartic acid compared with plants maintained in clean air (CFA) throughout (Tables 5.5 and 5.6). The second function highlighted the impact of transferring plants from CFA to O_3 at flowering (canonical discriminant function coefficients, Table 5.6); transfer to O_3 at flowering causing a ~92% increase in free glycine compared to plants maintained in clean air (CFA) throughout (Tables 5.5 and 5.6). The third function separated plants in the reciprocal transfer treatments from the permanent treatments (canonical discriminant function coefficients, Table 5.6). Transfer from O_3 to CFA at flowering resulted in increased levels of free proline, tyrosine and glutamic acid by ~96%, ~50% and ~36%, respectively. In contrast, serine and cysteine levels decreased by ~53% and 48%, respectively, compared

to pollen from plants grown in CFA and transferred to O_3 at flowering (Tables 5.5 and 5.6).

Table 5.5. Pollen mean essential and non-essential, free amino acids from broad bean (*Vicia faba*) in four growth treatments; charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 ppb O_3 8 h d^{-1} at flowering (CFA-O_3) and grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA). +/-SEM.

Essential amino acids										
	ng/mg									
Amino acid	Histidine	Leucine	Threonine	Arginine	Lysine	Valine	Methionine	Tryptophan	Phenylalanine	Isoleucine
CFA	341.9 ±26.2	5.7 ±1.0	435.9 ±111	1048 ±53.1	244.8 ±50.8	110.4 ±2.6	0.00 ±0.0	0.00 ±0.0	76.1 ±14.9	5.00 ±3.0
O_3	71.8 ±5.0	13.7 ±2.3	121.5 ±10.0	31.0 ±19.0	61.7 ±8.5	193.6 ±11.4	0.20 ±0.1	0.00 ±0.0	55.2 ±7.2	4.60 ±2.4
CFA-O_3	76.6 ±22.5	14.5 ±2.1	184.7 ±33.3	1411 ±126.1	197.5 ±17.2	113.4 ±7.7	9.00 ±3.2	0.00 ±0.0	56.7 ±5.6	62.3 ±7.6
O_3-CFA	74.6 ±5.1	19.3 ±1.4	95.8 ±15.2	1477 ±97.8	54.1 ±8.0	123.3 ±9.9	65.1 ±8.5	0.00 ±0.0	52.7 ±5.2	35.9 ±3.5

Non-essential amino acids											
	ng/mg										
Amino acid	Aspartic acid	Glutamic acid	Asparagine	Serine	Glutamine	Glycine	Tyrosine	Cysteine	GABA	Alanine	Proline
CFA	552.7 ±34.4	362.2 ±23.2	1.80 ±0.2	2.20 ±0.3	0.10 ±0.0	5.40 ±2.4	4.40 ±0.9	13.6 ±2.0	7.90 ±0.6	7.8 ±0.7	374.6 ±12.2
O_3	103.3 ±5.7	754.1 ±64.2	0.80 ±0.1	7.40 ±2.5	0.10 ±0.0	30.9 ±1.1	56.8 ±3.8	25.5 ±2.7	0.10 ±0.0	570.2 ±32.6	56.4 ±16.3
CFA-O_3	423.8 ±44.5	374.2 ±37.6	0.90 ±0.1	8.10 ±2.6	0.10 ±0.1	71.5 ±11.0	16.8 ±2.8	89.1 ±68.9	0.50 ±0.0	14.3 ±1.7	10.6 ±7.03
O_3-CFA	421.3 ±34.7	585.0 ±40.5	1.20 ±0.1	3.80 ±0.7	0.10 ±0.0	96.6 ±7.5	40.2 ±6.2	46.0 ±5.1	0.10 ±0.0	19.5 ±2.9	252.5 ±46.8

Table 5.6. Pollen essential and non-essential free amino acid canonical discriminant analysis (CDA) output from broad bean (*Vicia faba*) grown under four treatments: charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 ppb O_3 8 h d^{-1} at flowering (CFA-O_3) and grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA).

	Essential amino acids			
	Canonical discriminant function statistics			
Function	Eigenvalue	% Variance	test stat	P value
1	3.787	50.9	x^2_{27}=443	<0.001
2	2.660	35.7	x^2_{16}=248	<0.001
3	1.000	13.4	x^2_7=86.3	<0.001

	Pooled within-groups correlations		
	Function		
Amino acid	1	2	3
Valine	**-0.324**	0.140	-0.065
Lysine	**0.259**	0.161	-0.145
Methionine	-0.038	**-0.526**	0.494
Arginine	0.431	**-0.477**	0.080
Leucine	-0.109	**-0.255**	-0.031
Threonine	0.157	**0.179**	0.092
Phenylalanine	0.060	**0.081**	0.055
Histidine	0.366	0.458	**0.515**
Isoleucine	0.166	-0.396	**-0.514**

	Canonical discriminant function coefficients		
	Function		
Treatment	1	2	3
CFA	**2.187**	**1.983**	0.839
O_3	**-2.967**	1.299	-0.268
CFA-O_3	1.298	-0.691	**-1.436**
O_3-CFA	-0.387	**-2.081**	**0.959**

	Non-essential amino acids			
	Canonical discriminant function statistics			
Function	Eigenvalue	% Variance	test stat	P value
1	9.135	76.9	x^2_{24}=487	<0.001
2	2.253	19	x^2_{14}=197	<0.001
3	0.489	4.1	x^2_6=49.8	<0.001

	Pooled within-groups correlations		
	Function		
Amino acid	1	2	3
Alanine	**0.875**	0.073	-0.173
Aspartic acid	**-0.268**	0.148	0.022
Glycine	-0.086	**-0.502**	0.439
Proline	-0.136	0.424	**0.765**
Tyrosine	0.227	-0.225	**0.523**
Glutamic acid	0.182	-0.086	**0.421**
Serine	0.035	-0.096	**-0.202**
Cysteine	-0.017	-0.074	**-0.092**

	Canonical discriminant function coefficients		
	Function		
Treatment	1	2	3
CFA	**-2.001**	**2.596**	-0.086
O_3	**5.245**	0.145	-0.067
CFA-O_3	-1.751	**-1.310**	**-0.882**
O_3-CFA	-1.348	-0.947	**0.987**

5.3.7 Non-structural carbohydrate composition

Non-structural carbohydrate (CHO) composition was quantified by HPLC. Sucrose was the dominant component, followed by fructose, with glucose being the least abundant CHO (Figure 5.11). Treatments significantly influenced the carbohydrate composition of broad bean pollen (glucose $P< 0.001$, fructose, $P< 0.001$, sucrose, $P< 0.001$). The amount of glucose was significantly ($P\leq 0.05$) higher in pollen from plants grown under O_3 (O_3 and O_3-CFA) compared to those grown in CFA (CFA and CFA-O_3). The amount of fructose in pollen appeared to be a product of the treatment in which plants were sampled during flowering; plants sampled in CFA (CFA and O_3-CFA) exhibited no significant difference in concentrations of fructose recovered from pollen, whereas plants maintained in O_3 exhibited the highest ($P\leq 0.001$) levels of fructose and plants grown in CFA and transferred to O_3 at flowering exhibited the lowest ($P\leq 0.001$) levels of fructose. Sucrose content of pollen was similar in plants subject to CFA, O_3 or O_3-CFA. However pollen from plants raised in CFA and transferred to O_3 at flowering exhibited significantly ($P\leq 0.001$) lower levels of sucrose in pollen than other treatments (Figure 5.11).

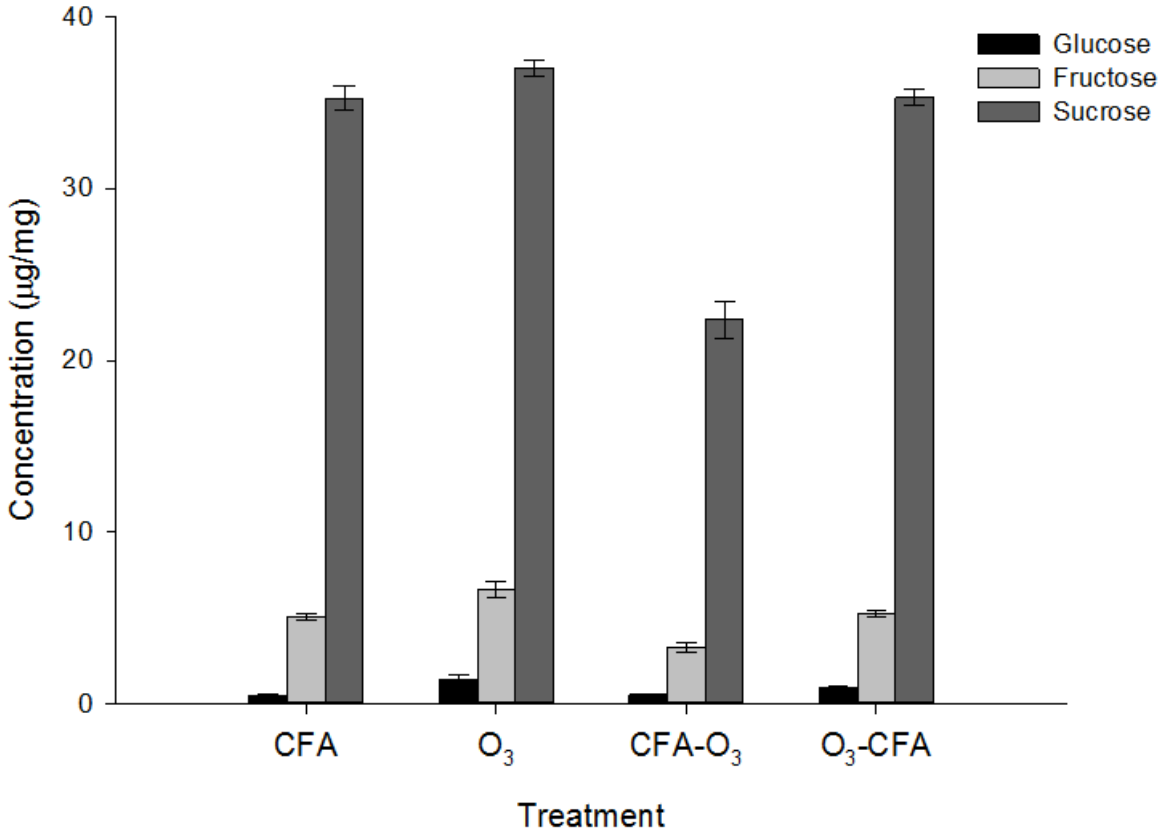

Figure 5.11. Non-structural carbohydrates extracted from pollen of broad bean (*Vicia faba*) in four growth treatments; charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 ppb O_3 8 h d^{-1} at flowering (CFA-O_3) and grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA). +/-SEM.

5.3.8 Fatty acid composition

Fatty acid composition of pollen was largely unaffected by exposure to O_3 (Table 5.7). However, growth in O_3 resulted in a significant ($P< 0.024$) increase in the proportion of the essential fatty acid, α-linoleic acid (omega 6) in pollen and this altered the ratio of α-linoleic : α-linolenic acid, from 1 : 3 in clean air-grown plants (CFA) to 1 : 1, and 1 : 2 in O_3 and O_3-CFA treatments, respectively.

Table 5.7. Concentrations of fatty acids (± SEM) washed from pollen collected from broad bean *(Vicia faba)* flowers from four to one of four growth treatments; charcoal filtered air (CFA), CFA + 110 ppb O_3 8 h d^{-1} (O_3), grown in CFA and moved to CFA + 110 ppb O_3 8 h d^{-1} at flowering (CFA-O_3) and grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA). MANOVA output is presented with F statistic.

	µg/mg			ng/mg	
Treatment	**Tetradecanoic acid**	**Hexadecanoic acid**	**Octadecanoic acid**	**Linoleic acid**	**Linolenic acid**
CFA	3.65±1.06	1.64±0.50	2.80±0.78	12.0±1.99	32.5±4.21
O_3	14.6±6.42	3.93±1.28	8.16±2.01	47.7±10.8	56.4±7.61
CFA-O_3	11.8±5.74	4.17±1.31	12.5±6.34	37.0±9.78	72.7±13.2
O_3-CFA	4.58±1.42	1.76±0.36	1.71±0.68	27.9±7.63	62.3±23.9
Test stat (F)	$1.49_{3,76}$	$1.98_{3,76}$	$2.18_{3,76}$	$3.32_{3,76}$	$1.42_{3,76}$
P value	0.223	0.124	0.097	0.024	0.244

5.4 Discussion

The present study has shown that exposure to transient, field-relevant levels of ozone during flowering significantly affects nutrient allocation to nectar and pollen.

Nectar quality appeared to be highly sensitive to environmental perturbations and was clearly influenced by short-term, as well as long-term, exposure to elevated levels of ozone pollution. The main influences of O_3 on pollen seemed to be the direct oxidation of free-essential amino acids in the pollenkitt, and a reduction in total protein content within the pollen. Furthermore, the total weight of pollen available to collect from plants maintained under O_3 was ~50% lower than plants maintained in CFA. A reduction in the amount of food available could have significant implications for plant-pollinator interactions. It is likely that there are at least two ways that transient O_3 exposure affects the nutrients measured in nectar and pollen. First, that the nutrient requirements for somatic maintenance of plant tissue increase in response to oxidative stress and so allocation of resources to reproductive processes (nectar and pollen) are altered to accommodate this shift in nutrient requirements. Second, O_3 may also influence the nutrients available in pollen as a result of direct oxidation.

5.4.1 Impacts of O_3 on plant performance and resource allocation

The biomass of broad bean was unaffected by long-term exposure to ozone in this experiment. However, a direct response to short-term exposure was evident in terms of reduced shoot weight; plants grown in CFA and exposed to O_3 at flowering exhibited a reduction in shoot dry weight after only three days' exposure to O_3 compared with plants maintained in clean air throughout (CFA). Interestingly, plants that were grown under O_3 and exposed to CFA at flowering showed an equivalent reduction in shoot biomass compared to plants sustained under O_3. It may be that plants in the sustained O_3 treatments had acclimatised to the O_3-induced oxidative stress during growth, so, when exposed to clean air at flowering, this allowed the mechanisms underlying tolerance to be diminished, changing assimilate distribution within the plant (Wang *et al*., 2003).

There were no ozone-induced changes in the number of racemes or flowers produced by broad beans. This was not unexpected since it is unlikely that short-term exposure would influence the number of flowers because most assimilate investment in floral development has already taken place. Likewise, Black and co-workers (2007) reported that the number of flowers and racemes produced by *Brassica campestris* were unaffected by short-term (two day) exposure to O_3 at the time of flowering. However, the literature shows that long-term exposure to ambient O_3 can reduce the number of flowers produced by plants (Bergweiller and Manning, 1999; Black *et al*., 2000). In the present experiment, no evidence of this was found but Black and colleagues (2000) do highlight that this effect of O_3 on flowering may be concentration and species-specific.

5.4.2 Nectar quality

The most abundant nutrients in nectar are carbohydrates; primarily glucose, fructose and sucrose (Nicolson *et al*., 2007), though amino acids are also prominent constituents (Baker and Baker, 1973). Nectar appears to be predominantly derived from phloem constituents (Fahn, 2000; Nicolson *et al*., 2007), but there may be many other factors contributing to its composition, including degradation of stored starch and possibly the degradation of nectary parts (Nicolson 2007; Pacini *et al.,* 2003). Nectary tissue itself can be capable of photosynthesis, and chloroplasts have been reported as present in the nectary parenchyma of *Vicia faba* (Davis *et al.,* 1988), suggesting that phloem content may not be the only factor governing nectar nutritive quality.

Long-term O_3 exposure had no influence on the volume of nectar produced by broad bean nor on the non-structural carbohydrate content of nectar. However, plants in transfer treatments (CFA-O_3 or O_3-CFA) responded by changing the composition and volume of the nectar available in their flowers. In both transfer treatments (CFA-O_3 or O_3-CFA) the volume of nectar in flowers increased compared to that of plants in sustained treatments (CFA or O_3). The concentration of carbohydrates in nectar from plants in the transfer between CFA and O_3 (at flowering), was similar to that of plants in the CFA and O_3 treatments. But, plants grown under O_3 and exposed to CFA at flowering provisioned nectar with double the concentration of sucrose. It is also worth noting that in both transfer treatments the volume of nectar in flowers increased. This correlates

with a reduction in the measured biomass of shoots, suggesting that resource investment was partitioned towards reproduction as opposed to somatic maintenance.

An interesting finding of this study is that long-term exposure to O_3 caused sucrose to accumulate in mature leaf tissue, yet this increased demand for sucrose was not reflected in a reduction in the sucrose present in nectar. However, when plants were grown under O_3 and exposed to CFA at flowering, the amount of sucrose in leaf tissue decreased compared to plants subject to sustained O_3, and this corresponded with a marked increase in the amount of sucrose in the nectar of these plants. Interestingly, the demand for sucrose in leaf tissue did not cause any detrimental impact on the quality of the plants' nectar. This could suggest that nectar composition is tightly conserved and that minimum thresholds of nectar quality are maintained so as to ensure pollinator visitation (Shuel, 1952, Petanidou *et al*. 1999; Davis, 2003). Sucrose is the main vector for assimilate transport in plants (Ziegler, 1975). Nutrient allocation to cell maintenance increases when plants are exposed to ozone (Amthor, 1988; Grantz, 2003) and assimilates are accumulated in foliage (Friend and Tomlinson, 1992; Robinson and Rowland, 1996). Despite the lack of O_3 impacts on biomass accumulation or visible symptoms of injury (leaf flecking, reduced biomass), an O_3-induced oxidative burden on cells is still likely to have taken place. I hypothesize that the demand for carbon in stressed leaf tissue increases when plants are subject to O_3 and so more of the nutrient is allocated to leaf tissue (Guy et al., 1992; Ashmore, 2005). When plants are grown under O_3 but exposed to CFA at flowering, oxidation by O_3 in leaves is less of a burden and the demand for extra carbon in leaf tissue decreases. The accumulated sucrose in leaf tissue is then freely available to be allocated to nectar, improving nectar quality (increased volume and sucrose concentration).

The proportions of total essential and non-essential amino acids in the nectar of plants grown in CFA were almost equal. Ozone exposure at flowering resulted in a reduction in the total essential amino acids in the nectar of plants grown in CFA. Similarly, plants that were grown under O_3 but exposed to CFA at flowering allocated far more non-essential amino acids to nectar than any other treatment.

A striking finding of the analysis conducted on nectar was that plants in both transfer treatments (CFA-O_3 and O_3-CFA) provisioned less threonine to their nectar than plants subject to sustained CFA or O_3. Threonine protein phosphatases have been identified as important markers of stress responses in plants (País *et al.,* 2009). They are also involved in the transmission of jasmonic acid signalling (País *et al.,* 2009; Dammann *et al.,* 1997), a reaction well documented as a response to ozone exposure (Kangasjarvi *et al*., 1994; Zadra *et al.,* 2006). The demand for threonine phosphatases in plant tissue may increase in response to a perceived change in air quality, so the availability of threonine for partitioning in to nectar may be compromised. This observation suggests further evidence that 'sudden' changes in air quality may be as, if not more, stressful, than sustained daily exposure to elevated levels of O_3.

Proline was one of most concentrated amino acids in nectar. This finding supports previous studies in which proline has been found to be a dominant amino acid in the nectar of a range of plant species (Carter *et al*., 2006; Baker, 1978; Gardener and Gillman, 2001a; Kaczorowski et al. 2005). In the present study, I found that the concentration of proline in nectar was heavily dependent on O_3 treatment; plants subject to sustained levels of elevated O_3 produced the lowest concentration of proline, but when O_3-stress subsided (ie plants were transferred to CFA at flowering) plants responded by allocating ~12-fold more proline to their nectar than plants that maintained in O_3. This may indicate that proline is accumulated elsewhere in response to exposure to O_3. Indeed, proline accumulation has been well documented as a marker for plants under stress, including drought, osmotic stress and high temperature (Szabados and Savouré 2009; Mafakheri *et al.,* 2010; Sairam *et al.,* 2002; Zhang *et al*., 2013).

Glutamic acid followed a similar trend to proline. When plants grown in O_3 were exposed to CFA at flowering, the concentration of glutamic acid was ~22-fold higher than that in plants subject to sustained in O_3. Glutamic acid (glutamate) is highly important in its function of donating both its α-amino group and carbon skeleton to the production of other amino acids (Forde and Lea, 2007), and therefore is essential for nitrogen metabolism in plants. This could suggest that enhanced nitrogen metabolism

is required to combat oxidative stress at the plant tissue level and so less is partitioned in to nectar (Singh *et al*., 1973; Hare and Cress, 1997; Di Martino *et al*., 2003). Moreover, glutamic acid is a precursor for both arginine and proline, so observed impacts of O_3 exposure on arginine and proline levels may be linked (Miflin, 2014).

Non-essential amino acids can be synthesised in the bodies of animals, so dietary consumption is not essential. However, some amino acids are utilised in greater proportions than can be synthesized in the body. For example, proline is utilised by bees in the initial stages of flight (Barker and Lehner, 1972; Carter et al., 2006; Micheu et al., 2000; Mollaei et al., 2013) and is used as a rapid energy source, yielding a similar amount of ATP as glucose in a shorter reaction time (Carter *et al.,* 2006).

5.4.3 Pollen

There were two critical findings. Firstly, that sustained exposure to O_3 significantly reduced the protein content of pollen compared to that of plants subject to sustained clean air (CFA). Short-term exposure to O_3 also induced a reduction in pollen protein content. This could reflect the fact that nutritional investment in pollen is still ongoing in the late stages of pollen development (Pacini, 2000). Pollen is dehisced from anthers after nutrient absorption and dehydration of the locular fluid in the anther (Clement *et al*., 1998; Pacini, 2000). It is possible that the availability of nutrients to pollen can be influenced right up to the point of dehiscence. Further evidence to support this is illustrated in the increase in total protein observed in plants transferred from O_3 to CFA at flowering compared with that of plants maintained in O_3 throughout. Interestingly, not only did the total amount of pollen protein change, but also the distribution of protein-bound amino acids, potentially suggesting that a different suite of proteins maybe present in the pollen of plants that have experienced short-term ozone exposure.

The detrimental effects of ozone on pollen are generally agreed upon. Previous publications have reported impaired pollen germination and germ tube development (Wolters and Martens, 1987; Black *et al*., 2007; Pasqualini *et al*., 2011). Interestingly, Gillespie and co-workers (2015) found that pollen from tomato plants exposed to

elevated O_3 were as negatively affected when crossed in to stamens of plants in clean air, suggesting that detrimental impacts on pollen were long-term and influenced viability after exposure to the pollutant ceased. The data reported herein lend additional support to the view that O_3 exposure negatively influences pollen viability since I report that exposure to ozone reduced the total amount of protein in pollen and negatively affected protein-bound amino acid composition.

5.4.4 Fatty acids

The composition of fatty acids was largely unaffected by transient exposure to ozone. However, a potentially important finding was that pollen sampled from plants raised in O_3 exhibited a higher proportion of the essential fatty acid α-linoleic (omega 6) than those raised in CFA, altering the ratio of α-linoleic to α-linolenic acid (omega 3). A recent publication has highlighted the importance of the balance between omega 3 and omega 6 (Arien *et al*., 2015). These authors reported that bees fed a low omega 3 diet suffer cognitively, and perform poorly at olfactory learning tests. They also found that bees on low omega 3 diets produced smaller hypopharyngeal glands. It is not known what causes the increase in omega 6 in pollen of plants exposed to O_3, but lipid peroxidation could be taking place, releasing greater proportions of the free fatty acid from triacylglycerides, the most abundant form of lipid in pollen (Cresti *et al.,* 1986; Dumas, 1977; Walters-Arts *et al*., 1998). A further interesting function of lipids in pollen is their essential requirement for pollen germination and penetration of the stigma. Pollen from tobacco plants did not germinate without lipids present on the pollenkitt, and would even penetrate leaf tissue when lipids were present (Walters-Arts *et al*., 1998).

5.4.5 Conclusion

Ground-level concentrations of O_3 fluctuate greatly (RoTAP, 2012). However, the short term impacts of O_3 exposure on vegetation are rarely studied. The experiments described in this chapter have highlighted that detrimental changes in nutrient qualities of nectar and pollen in response to O_3-exposure can occur rapidly. Furthermore, long-term exposure to the pollutant also causes significant detrimental effects to pollen protein and fatty acid qualities. The implications of such rapid changes in the available

nutrition to nectar and pollen feeders is therefore likely to be a common occurrence in the natural environment. There is therefore potential for severe negative implications for both plant and animal fitness and this should be explored in future studies.

6.0 Chapter 6: Ozone-induced changes in nectar quality reduce honeybee learning

6.1 Introduction

The ability to learn and remember floral traits is essential for pollinators to forage efficiently. Eusocial pollinators like honeybees are unique in that they forage not only to supply nutrition to themselves, but their foraging choices also influence the nutrition of the colony (Von frisch, 1967). Bees must discriminate between flowers and their reward quality and adapt their foraging behaviour to exploit the most nutritionally rewarding food source. To do this, they learn which floral traits (odour, flower size, colour and shape) signal a flower with high quality nectar and those that indicate a flower with low quality nectar or one that is nectarless. Honeybees are capable of forming long-lasting memories of floral cues and reward quality (Wright *et al*., 2007) and share their memory of a high quality food resource with hive-mates *via* a waggle dance in the colony (von Frisch, 1967).

Bee learning has been studied in numerous ways. Free-flying experiments have been useful in identifying colour preferences and floral visitation rates of bees (Gegear and Laverty, 2004; Hempel de Ibarra *et al*., 2000). However, laboratory studies on individual restrained bees have allowed greater control over variables such as the timing that the insect is exposed to certain experimental conditions; something that is difficult with free-flying animals. The learning ability of restrained honeybees has been exploited since the mid 1940's when their ability to associate odours with rewards was explored (Frings, 1944). Techniques for studying bee learning were developed along with robust methods for studying the olfactory learning of honeybees (Bitterman *et al*., 1983). Associative learning is a form of classical conditioning, the principles of which are demonstrated in pavlovian conditioning (Pavlov, 1927). In appetitive learning, animals associate a conditioned stimulus (CS) (odour, sound, colour etc.) with an unconditioned stimulus (US) (nutritional reward) (Pavlov, 1927). The response of the animal is then measured as an indication of its level of association. Pavlov's study involved pairing a sound (CS) with feeding (US) using dogs and measuring their salivary secretions as the response. The same principle can be applied to bees in olfactory conditioning, where

odour is used as a conditioned stimulus. When sucrose contacts chemoreceptors on the antenna, feet or mouthparts of bees (Bitterman *et al*., 1983) the proboscis is extended to initiate feeding (proboscis extension reflex [PER]; unconditioned stimulus [US]). Conveniently, this feeding reflex can be paired with cues such as odour or colour, and bees will elicit PER in response to the presentation of a paired stimulus. The probability that a cohort of bees will respond to a CS after subsequent training trials with a food US can be used as an indication of how well the animal has learnt the association between the odour and the reward (Menzel *et al*., 1974).

Sucrose has been used as a rewarding stimulus in olfactory conditioning of PER in numerous insect species including flies (*Drosophila melanogaster*) (Chabaud *et al*., 2006), moths (*Manduca sexta*) (Daly and Smith, 2000) and honeybees (Menzel *et* al., 1974; Wright *et al*., 2007, 2013). Honeybees can learn an association between an odour and a sucrose reward after one reinforcing trial (Smith, 1991) and the majority of honeybees will learn an association by the third reinforcing trial (Menzel *et al*., 1974). Most studies employing olfactory conditioning of PER using honeybees use sucrose as the reward. This is justified as sucrose is a common compound in floral nectar (Nicolson *et al*., 2007) and the probability that bees will elicit PER is positively correlated with concentration of sucrose used to stimulate receptors on the antenna (Menzel *et al*., 1996; Pankiw and Page, 2000; Haupt and Klemt, 2005).

Sucrose is used so commonly that there are few studies reporting the role of amino acids as reinforcers for pollinators feeding on nectar and there are no publications assessing true 'nectar like' solutions containing full suites of amino acids and carbohydrates on the learning rate of honeybees. A free-flying experiment demonstrated that honeybees would consume significantly more of a sucrose-amino acids solution than a sucrose-only solution (Alm *et al*., 1990). However, the concentrations of amino acids used in the study did not match those of nectar that would normally be accessible to short-tongued bees. There are also suggestions that some amino acids are initially deterrent to bees, in particular glycine (Inouye and Waller, 1984; Hendriksma *et al*., 2014). However, at specific concentrations amino acids are preferred over sugar-only solutions (Inouye and Waller, 1984). Also, some individual

amino acids improve olfactory conditioning of PER (Kim and Smith, 2000; Simcock *et al*., 2014) compared to sucrose-only solutions and the study by Simcock *et al*., (2014) highlighted that prior feeding of amino acids can influence their learning ability; bees pre-fed with proline and isoleucine were more likely to learn an association when a reward also contained amino acids (Simcock *et al*., 2014).

This chapter utilises an olfactory conditioning of the honeybee's PER to assess how O_3-induced changes in nectar quality affect the way in which honeybees learn and remember odours associated with reward. Broad bean plants subjected to a transient change in air quality following long-term O_3 exposure provisioned their nectar with greater concentrations of carbohydrates (sucrose) and amino acids. Simulating this influence, nectar equivalent in carbohydrate and amino acid qualities quantified from broad bean flowers, described in Chapter 5, was tested to see if the impacts influenced the nectar preferences of honeybees, as reflected in their motivation to form an olfactory association with the nectars differing in amino acid composition.

6.2 Methods

6.2.1 General

Returning forager honeybees (*Apis mellifera*) (Ridley Building 2, Newcastle University) were collected from one colony in plastic ventilated vials. No more than 5 bees were collected in each vial. Vials containing bees were placed on ice to induce anaesthesia (usually < 3 min). Anaesthetised bees were placed in to a plastic harness using fine forceps. Harnesses were made from modified 1 ml plastic pipette tips with the tip end cut off and a small groove cut into the remaining structure to allow the abdomen of the bee to be exposed to air, allowing the bee to breathe. The bee was positioned so that its head was free-moving and black tape was used to secure the bee to the harness over the back of the head. After checking that movement of the proboscis was not inhibited by positioning in the harness, bees were left to recover from cold anaesthesia for ~15 min. Bees were then fed 0.7 M sucrose until satiety, by delivering the solution directly to the mouthparts with a pipette. Once fed, bees were left 24 h before being used in the experiment.

6.2.2 Training

Bees were trained with 5 different treatment solutions. A 1 M sucrose solution was used as the control. Treatment solutions simulated the amino acid and carbohydrate compositions of nectar from broad beans exposed to charcoal-Purafil® filtered air (CFA nectar) or from plants that were exposed to O_3 during growth and then CFA at flowering (O_3 nectar). To control for the presence of amino acids in solutions, treatments were used in which only the carbohydrate profiles of nectar were present; amino acids were not (CFA carbs and O_3 carbs). Detailed solution composition is presented in Table 6.1.

Table 6.1. Carbohydrate and amino acid composition of treatment solutions used in olfactory conditioning assays with honeybees.

	Treatment solution			
	CFA_{nectar}	**O_3-CFA_{nectar}**	**CFA_{carbs}**	**O_3-CFA_{carbs}**
Carbohydrates (M)				
Sucrose	1.0	2.0	1.0	2.0
Glucose	0.5	0.5	0.5	0.5
Fructose	0.5	0.5	0.5	0.5
Essential Amino acids (µM)				
Threonine	530.7	0		
Valine	187.5	291.4		
Lysine	124.1	126.5		
Arginine	83.8	123.5		
Phenylalanine	72.3	81.9		
Leucine	59.5	108		
Methionine	47.4	43.8		
Histidine	14.7	344.4		
Isoleucine	1.24	82.5		
Tryptophan	0.02	0.04		
Non-essential amino acids (µM)				
Glycine	386.9	60.5		
Proline	238.1	1405		
Glutamic acid	124.3	1395		
Serine	114.3	388		
Alanine	34.6	139		
Aspartic acid	28.3	21.4		
Cysteine	25.3	99.3		
Tyrosine	23.9	21.8		
GABA	3.02	0.77		
Asparagine	0.12	2.16		
Glutamine	0.03	0.4		
Total amino acids (mM)	2.10	4.74		

6.2.3 Olfactory conditioning

Olfactory condition experiments using honeybees were designed based on the methods of Bitterman and colleagues (1983). All bees were tested for their sensitivity to sucrose by stimulating the antenna with 1 M sucrose with a pipette. Bees that did not elicit a proboscis extension reflex (PER) were not used in the olfactory conditioning experiment. The odour used in training was 1-hexanol (98% purity, Sigma-Aldrich). To present the odour, 5 μl of pure 1-hexanol was pipetted on to a strip of filter paper and placed in to a glass tube that was connected to an air supply, pre-programmed and controlled by a 2-way valve connected to a Programmable Logic Controller (PLC) (Automation Direct, Cumming, GA, USA). Air was directed through the odour tube by pressing a button connected to the PLC which was programmed to exert a 4 s flow of air through the odour tube. Individual bees were positioned 3 cm in front of the odour tube. Air was constantly drawn over the bee and odour was removed by a fixed exhaust system positioned behind the bee. Bees were conditioned to the odour by pairing a food reward (unconditioned stimulus) with the odour pulse (conditioned stimulus). The food reward was treatment-dependent (Table 6.1). Following an odour pulse, the antenna of the bee was stimulated with 1 M sucrose to elicit a PER response. The bee was then fed a 0.4 μl droplet of the treatment solution 2 s after the odour pulse had started but before the odour stimulus had ceased (Figure 6.1). Bees were then left for 5 min and the process was repeated. In each trial, the PER response of the bee was recorded. If bees elicited a PER in response to the odour stimulus the response was recorded as '1'. However, if antennal stimulation with 1 M sucrose was needed to induce a PER, then the response was recorded as '0'. Bees were trained for 6 consecutive trials, each with a 5 min inter-trial interval (ITI).

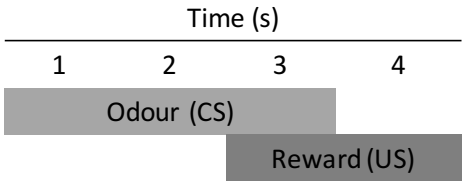

Figure 6.1. Schematic diagram illustrating the timing of odour stimulus and reward presentation. An odour pulse (conditioned stimulus) is delivered for 3 s. A reward is presented to the subject after 2 s so that the animal can associate the odour with reward.

6.2.4 Testing

After training for 6 trials, bees were left for 10 min. To test for memory of the association between the treatment reward and the odour stimulus, the trained odour was presented to the bee and the PER response was recorded as '1' or '0' for a positive PER or no response, respectively. After testing, bees were fed to satiety with 0.7M sucrose and left for 24 h. After 24 h, PER response to the odour was tested again.

6.2.5 Statistical analysis

Data collected for olfactory conditioning trials were binary and so treatments were compared using a two-way binary logistic regression using treatment and trial number as factors. Comparisons between treatment and trial number were made using least significant difference (*LSD*). Short term (10 min) and long term (24 h) memory of association were also analysed using a binary logistic regression with significance determined using LSD.

6.3 Results

Bees were trained to associate an odour (1-hexanol) with different nectar-like rewards, simulating the amino acid and carbohydrate composition of broad bean nectar collected from plants subject to (i) charcoal/Purafil® filtered, clean air (CFA nectar) or (ii) 110 ppb O_3 for 8 h d^{-1} until flowering and then exposed to CFA (O_3-CFA nectar). To control for the presence of amino acids in nectar, bees were also trained with diets matching only the carbohydrate composition of these nectars (CFA carbs and O_3-CFA carbs).

6.3.1 Influence of ozone-induced changes in nectar quality on honeybee learning

The reward treatment exerted a significant (P= 0.002) influence on the rate at which honeybees learned to associate the odour with reward (Figure 6.2). The probability that honeybees exhibited a conditioned response on the second training trial was 30% higher when bees trained with O_3-CFA nectar than those trained with CFA nectar (P= 0.027). The acquisition of association between odour and reward reached ~73% in trials 3 and 4 in both treatments. However, by the fifth trial bees reinforced with O_3-CFA nectar were ~23% less likely to elicit PER than those trained with CFA nectar (P= 0.023). The probability of response continued to decrease in the sixth trial, and bees trained with O_3-CFA nectar were ~40% less likely to respond to the odour than bees trained with CFA nectar (P= 0.001) (Figure 6.2). Bees were tested for their memory of the association between odour and reward at 10 min and 24 h after training trials (Figure 6.2). There was no significant interaction between treatment and time of test. However, there was a significant (P= 0.017) change in memory development between the 10 min test and the 24 h test. In bees trained with CFA nectar, memory at 24 h was significantly (P= 0.004) higher than the 10 min test. There was no difference in response between 10 min and 24 h tests for bees trained with O_3-CFA nectar.

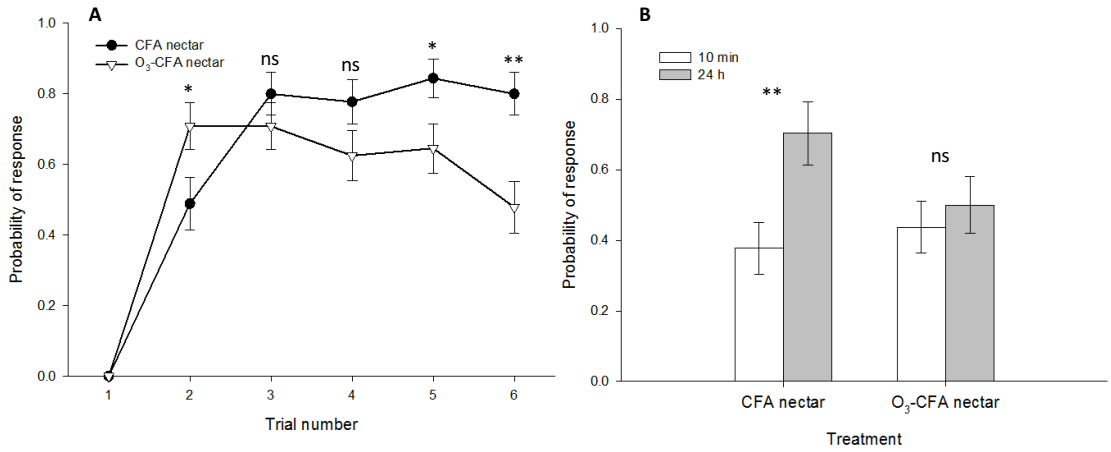

Figure 6.2. (A) Olfactory conditioning of nectar simulating that of nectar collected from flowers of broad beans grown under two ozone-exposure treatments; charcoal filtered air (CFA) and grown in CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA). Significant differences between CFA and O_3-CFA responses are indicated by asterisks (* P< 0.05, ** P< 0.01, *** P< 0.001) ns indicates no significant differences by *LSD* at 5% level. (B) Olfactory memory tests 10 min and 24 h after conditioning. Error bars represent +/-SEM.

6.3.2 Amino acids in floral nectar enhance honeybee learning

Bees were trained to associate 1-hexanol with solutions simulating the amino acid and carbohydrate profiles of nectar from broad bean flowers grown in CFA or O_3 and transiently exposed to CFA at flowering. Additional bees were also trained to associate the odour with the same solutions lacking amino acids. Presence of amino acids in CFA nectar significantly (P< 0.001) influenced the rate at which bees learned to associate the training odour with reward (Figure 6.3). Bees trained with CFA nectar (containing amino acids) were 33% more likely to elicit PER on the second trial than those trained with CFA carbs (P= 0.017). The acquisition rates in trials 3 and 4 were similar for both treatments (CFA nectar and CFA carbs) with between 60% and 80% of bees eliciting PER. However, by trial 5 bees were ~46% more likely to respond to the odour (PER) when the training solution contained amino acids (CFA nectar) (P≤ 0.001). Similarly, by trial 6 bees were ~29% more likely to respond to a solution that contained amino acids (CFA nectar) (P= 0.015). The olfactory memory of bees trained with CFA

carbs and CFA nectar were not influenced by treatment. However, 24 h test memory was significantly ($P< 0.001$) higher than 10 min tests in both treatments.

Presence of amino acids in O_3-CFA nectar also influenced the acquisition behaviour of bees, but there was no significant treatment*trial interaction, though there was a significant ($P= 0.003$) treatment effect (Figure 6.3). Bees that were trained with O_3-CFA nectar and O_3-CFA carbs solutions learnt the association of odour with reward at a similar rate, for the first four trials. However, by trials 5 and 6, bees were more likely to elicit PER when trained with O_3-CFA nectar than those trained with O_3-CFAcarbs ($P\leq 0.05$). Bees trained with O_3-CFA carbs and O_3-CFA nectar showed no significant differences in their 10 min test or 24 hr test for olfactory memory.

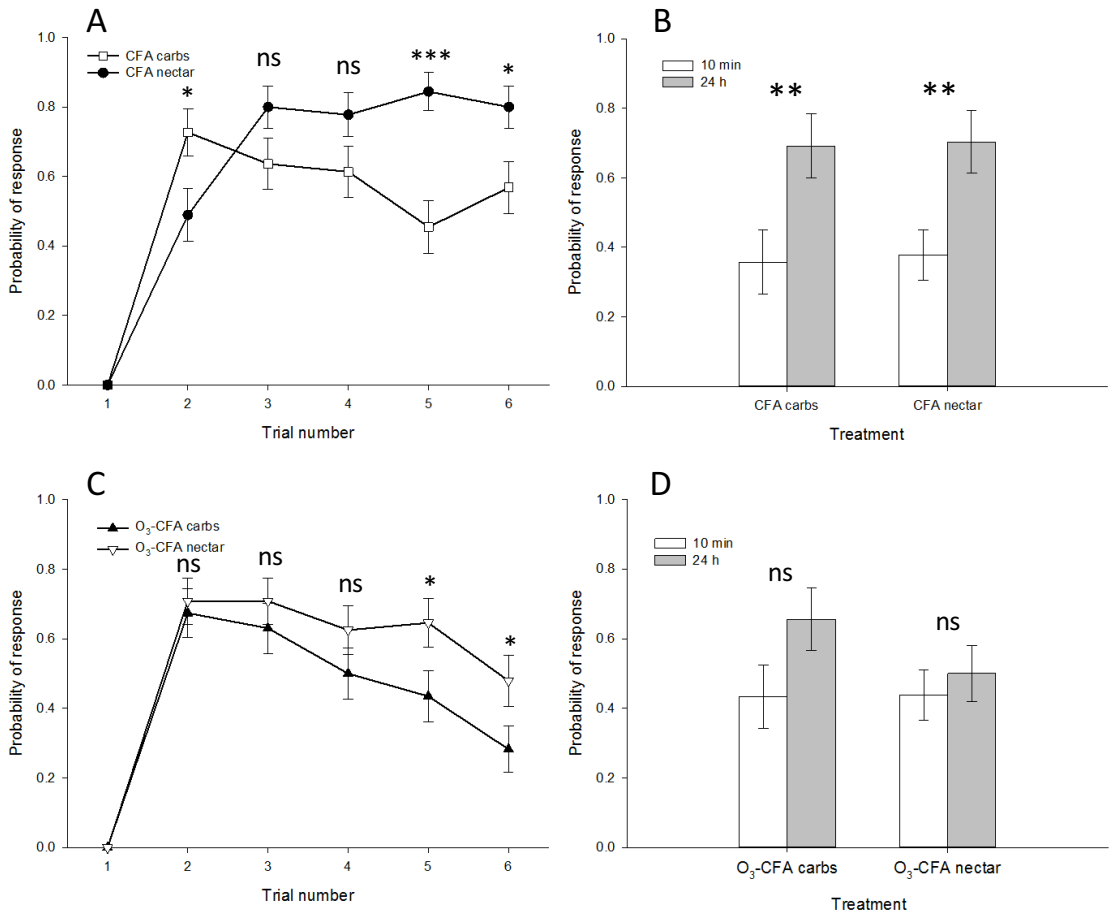

Figure 6.3. (A) Olfactory conditioning of synthesised nectar matching carbohydrate (CFA carbs) and the amino acids and carbohydrate (CFA nectar) composition of nectar collected from flowers of broad beans under two growth treatments; charcoal filtered air (CFA). (C) Olfactory conditioning of synthesised nectar matching carbohydrate (O_3-CFA carbs) and the amino acids and carbohydrate (O_3-CFA nectar) composition of nectar collected from flowers of broad beans grown under CFA + 110 ppb O_3 8 h d^{-1} and moved to CFA at flowering (O_3-CFA). (B and D) Olfactory memory tests 10 min and 24 h after conditioning. Significant differences between responses are indicated by asterisks (* P< 0.05, ** P< 0.01, *** P< 0.001), ns indicates no significant differences by *LSD*.

6.4 Discussion

Amino acids in nectar are not thought to be major nutritive sources to bees, in particular because the primary source of protein and amino acids for bees is pollen (Hendriksma *et al*., 2014). The present study yields evidence that amino acids in nectar may play a role in controlling pollinator behaviour and floral visitation rate. My data clearly demonstrate that the presence and concentration of amino acids in nectar can cause pollinators to positively modify their behaviour in favour of learning to associate an odour with the reward when amino acids are present. In agreement with other publications (Kim and Smith, 2000; Carter *et al.,* 2006; Hendriksma *et al*., 2014), it is demonstrated that honeybees may show a foraging preference for some amino acids, evidenced by the increased motivation to maintain an association of odour and reward when amino acid concentrations are higher (O_3-CFA nectar) than when trained with a diet of lower amino acid concentration (CFA nectar).

The initially slower rate of acquisition for nectars that contain amino acids (CFA nectar and O_3-CFA nectar), over those without (CFA carbs and O_3-CFA carbs), could suggest that preliminary nutritive assessment of a nectar is made on the perception of its sweetness. There is some evidence that some amino acids such as proline may stimulate salt cell receptors in insect labella (Goldrich, 1973; Hansen, 1998), which could inhibit the perceived sweetness of a sugar solution containing amino acids (Carter *et al*., 2006). Glycine has been reported to be initially aversive to honeybees (Hendriksma *et al*., 2014), and in our treatment solution where glycine was minimal (O_3 nectar) bees were observed to demonstrate improved association. Conversely, presence of glycine in a sucrose reward has also been reported to enhance olfactory conditioning in honeybees (Kim and Smith, 2003). Possible discrepancy between these findings may be attributed to experimental design. The study by Hendriksma and co-workers (2014) involved free-flying bees, whereas the study by Kim and Smith (2000) involved harnessed bees that had been food deprived. Phenylalanine has been described as phagostimulatory amino acid to honeybees and when given the choice, bees will consume greater proportions of sucrose solutions containing phenylalanine than a sucrose-only solution (Hendriksma *et al*., 2014). Phenylalanine may be

phagostimulatory to bees as it has been identified to stimulate sugar-sensing neurons in flesh flies and blow flies (Shiraishi and Kuwabara, 1970).

The observed increase in the olfactory association in subsequent trials, when amino acids were present compared to those trained without amino acids in the nectar, could indicate that amino acid detection and nutritive assessment is more likely to be based on post-ingestive feedback, and less on an initial taste assessment of the food source (Wright *et al*., 2007). A few studies have begun to investigate the role of post-ingestive feedback between the gut and brain and its ability to regulate appetitive learning. Bees that were injected with individual amino acids (2 μl of 10^{-6} M per amino acid) prior to an olfactory conditioning assay showed both improved and inhibited short- and long-term memory depending on the amino acid injected (Chalisova *et al*., 2011). Short-term memory was enhanced by the injection of arginine, glutamic acid, aspartic acid, tyrosine, valine and threonine, but inhibited by the injection of lysine and tryptophan (Chalisova *et al*., 2011). Long-term memory was enhanced by the injection of fewer amino acids; alanine, asparagine, aspartic acid and isoleucine, but inhibited by the injection of lysine, proline, cysteine and valine (Chalisova *et al*., 2011). A study in which honeybees were pre-fed isoleucine, phenylalanine, proline or methionine 24 h before an olfactory conditioning assay reported that bees that were pre-fed amino acids were less likely to learn an association when trained with sucrose-only (Simcock *et al*., 2014). However, pre-feeding bees with isoleucine or proline improved associative learning when bees were rewarded with solutions containing amino acids (Simcock *et al*., 2014).

It is possible that the amino acids and sucrose in the training solutions used in the present study could pass through the gut and into the haemolymph in the 5 min ITI used in the olfactory conditioning trials influencing appetitive decisions in subsequent trials. A study on the transport of sugars in honeybees identified that after feeding bees 1 M glucose, 50% of it had reached the haemolymph 5 min after feeding (Crailsheim, 1988a; 1988b). Once nutrients have reached the haemolymph it is likely that the brain can then detect nutrient qualities in the haemolymph and a recent study has suggested that an initial pre-ingestive deterrent taste of proline is overcome by a post-ingestive

response (Simcock *et al*., 2014), enhancing a memory of reward. Furthermore, honeybees actively regulate their dietary consumption of essential amino acids to meet specific intake targets (Paoli *et al*., 2014), delivering further evidence that post-ingestive feedback is important in making foraging decisions.

An ozone-enriched climate is highly likely in the coming decades and average ground-level concentrations are predicted to continue to steadily increase for the foreseeable future in the Northern hemisphere (Ashmore, 2005). We have demonstrated that transient, short-term changes in exposure to O_3 are enough to influence the carbohydrate and amino acid qualities of nectar produced by broad bean flowers, and in turn, have demonstrated that such changes have direct impacts on the foraging behaviour of honeybees, reducing the likelihood of forming a lasting olfactory association with plants. Further studies are required to test the response of free-flying bees. Real world implications of reductions in visitation rates to pollinator-dependent crops could have a huge impact on crop success. Not only should the influence of environmental stressors such as ozone pollution, higher temperatures, soil water deficit, rising [CO_2] and increased nitrogen be studied for their influence on nectar, but assessments of nectar quality could be an important tool in the targeted developmental breeding of new varieties to produce crops that will attract and maintain a pollination service by meeting the nutrient demands of the pollinators.

7.0 General discussion

The intention of this thesis was to explore the interactions between ozone pollution and the allocation of nutrients to nectar and pollen; essential sources of nutrition to pollinating insects. The initial experiments explored and refined methodology to allow chemical analyses of amino acids and carbohydrates in nectar and pollen. These methods were then applied in experiments exploring intraspecific variations in 'ozone sensitivity' and the protein and free amino acid qualities of pollen. A 'sensitive' genotype was then selected and used to probe impacts on resource allocation, exploring how plants responded to long-term and short-term exposure to ozone. Changes in nectar composition induced by ozone pollution were then applied to a honeybee learning experiment to assess how the impacts of the pollutant on reward quality, particularly amino acids, influence bee learning and memory.

7.1 Methods for nectar and pollen analysis

Pollen and nectar are important ecological drivers of both plant and pollinator fitness. However, methods for studying their nutrient composition are largely unsuitable for the analysis of amino acids; either because of the large sample size needed or because of the loss of amino acids during the hydrolysis/combustion procedures adopted for sample preparation. The method developed for the hydrolysis of pollen proteins (see Chapter 2) allows the quantification of free and protein-bound amino acids. Previous methods commonly used for quantifying protein in pollen have required much larger samples than are easily collected from plants, for example 20 mg (Nicolson and Human, 2013). Also, methods such as micro-kjehldahl and combustion rely on degrading the sample to absolute nitrogen and back-calculating to estimate crude protein content (Roulston and Cane, 2000). There are arguments over the reliability of these methods, as the correction factor used to estimate protein content are subject to differences depending on the tissue used (Roulston and Cane, 2000) and also because not all of the nitrogen measured may be off protein-origin. Hydrolysis of small amounts of pollen (≥ 1 mg) in a microwave-assisted acid manner provides a partial hydrolysis, liberating free amino acids that can be quantified using HPLC. The measured values of amino acids can then be used to back-calculate (i.e. estimate) total protein content by

multiplying by 10.3. Lower weights of pollen (< 1 mg) can be used in this assay, but a specific multiplication factor must be applied to correct for the efficiency of the hydrolysis associated with lower sample sizes (See Chapter 2). Collection techniques were compared for their reliability in determining carbohydrate and amino acid composition of nectar. We established that using a microcapillary tube to carefully extract nectar from flowers was the most reliable collection method; techniques such as filter paper, wash 2 ml and rinse 2 ml methods are likely to incorporate various contaminants in the sample, such as amino acids and carbohydrates from phloem, xylem and pollen. The microcapillary method was applied to nectar sampling in subsequent thesis Chapters (Chapters 4 and 5).

7.2 Measures of ozone-sensitivity

Broad bean cultivars demonstrated considerable variation in their physiological response to O_3 exposure. The one shared trait among all cultivars tested was a reduction in resource allocation to roots compared to shoots (K), but there was considerable variation in the impacts of ozone on biomass accumulation and the number of pods and seeds produced per plant. Interestingly, ozone-induced changes in biomass constituted a reliable indicator of pollutant impacts on seed and pod production. The number of seeds and pods produced by 7 out of 10 cultivars was negatively impacted by ozone-exposure, but two cultivars demonstrated significantly enhanced seed and pod yield in response to ozone-exposure. Similar observations have been reported previously when numerous cultivars of the same species are screened for their response to environmentally-relevant levels of ozone pollution, but these positive responses remain largely unexplained (Endress and Grunwald, 1985; Sanders *et al*., 1992). Perhaps this represents a common stress response favouring reproductive success; a particularly important feature for short-lived, annual plants.

Interestingly, ozone impacts on growth and resource allocation were not representative of the impacts of the pollutant on pollen. It seems likely that ozone results in direct oxidative damage to the free amino acid components on the outer surface of pollen, the 'pollenkitt'. Consistent with our findings, Benoit and colleagues (1983) found no correlation between ozone-induced foliar symptoms of 'injury' and

impacts on pollen viability. The results of the present study suggest that the parameters used to assess a species 'sensitivity' to ozone need to be viewed with caution. Effects of ozone on reproductive structures and features could have serious carry-over effects to following generations. The viability of pollen and seed was not explored in this thesis, but this would be a valuable addition to future datasets. Potential carry-over effects would have a huge impact on wild plant community structure. From an agricultural and horticultural perspective, unsuccessful pollination would have a detrimental impact on productivity and commercial value.

7.3 Ozone exposure and resource allocation

An interesting finding in the present study was that the greatest impacts on the volume and nutrient composition of nectar was observed in the short-term ozone transfer treatments. Plants that were grown in either CFA or O_3 but then exposed to the alternate treatment at flowering (CFA-O_3 and O_3-CFA) responded by producing increased volumes of nectar compared to plants sustained in either condition (CFA or O_3). The weight of pollen produced by plants exposed to O_3 for sustained and short periods was significantly lower than plants maintained in CFA. It is potentially very important to study the short-term effects of ozone, as evidenced in this work, exposure to O_3 for 3 days caused a reduction in pollen weight. Short-term exposures to O_3 clearly have rapid impacts on plant response and in turn the nutrient qualities of nectar and pollen. The vast majority of prior research on the influence of ozone on plant growth compares plants raised from seed or seedlings in a constant daily ozone environment against counterparts raised in clean air (controls). However, in reality, plants are rarely exposed to long periods of ozone-free air, and similarly, when O_3 is present, concentrations tend to fluctuate on an episodic basis from day-to-day and week-to-week in many low-lying agricultural areas. This raises the question of what is a true ozone control treatment? The conditions that are used as control treatments should probably match real-world exposures of the pollutant and future studies need to pay greater heed to the influence of natural diurnal and spasmodic variations in O_3 exposure on plant performance. The study in Chapter 5 reports the effect of long-term and short-term exposure to O_3, but it seems important to also understand how growth and

nutrient allocation is affected if plants are exposed to long- term fluctuations in air quality versus constant exposure.

7.4 Impact of ozone to pollinators

Ozone can influence the available nutrition to pollinating animals in three main ways; changes to nectar quality, pollen quality and floral availability. Firstly, as novelly described in this thesis, the amino acid and carbohydrate composition as well as volume of nectar produced by plants can be influenced by long- and short-term ozone exposure.

There are numerous reports on the influence of ozone causing a detrimental impact to pollen viability in terms of germination and tube growth (Summarised in table 7.1). The pollen of almond, apple, apricot, nectarine, peach, pear (Hormaza *et al*., 1996), white pine (Benoit *et al*., 1983), petunia (Feder and Sullivan, 1969b; Harrison and Feder, 1974; Krause *et al*., 1975; Feder, 1981; Feder *et al*., 1982; Feder, 1968), tobacco (Feder and Sullivan, 1969a,b; Krause *et al*., 1975), tomato (Feder *et al*., 1982; Gillespie *et al*., 2015), maize (Mumford *et al*., 1972), greater plantain, Wisconsin Fast Plants (Stewart, 1998) and Ragweed (Pasqualini *et al*., 2011) all demonstrated reduced pollen viability in terms of inhibited germination and/or tube growth. However, Stewart (1998) noted that different populations of greater plantain demonstrated variability in their response to ozone and some exhibited increased pollen tube growth. *In vivo* experiments have given evidence to suggest that the pollen of tobacco (Feder, 1968), tomato (Feder *et* al., 1982), greater plantain and Wisconsin Fast Plants (Stewart, 1998) also demonstrate reduced pollen viability due to exposure to ozone. However, fewer studies have quantified the protein content of pollen in response to ozone. A significant reduction in protein was reported in the pollen of Boxelder maple, English oak, sycamore (Ribeiro *et al.*, 2013), Hophornbeam (Cuincia *et al*., 2014) and as demonstrated in this thesis, broad bean. However, there was no noticeable change in the amount of protein in ragweed pollen (Pasqualini *et al*., 2011). Furthermore, free amino acids of pollen have been reported to increase in maize pollen in response to ozone exposure (Mumford *et al*., 1972). In the work conducted in this thesis, the proportion of free amino acids of broad bean pollen was significantly reduced (Table 7.1).

Ozone can cause changes in available nutrition by influencing the flowering behaviour of plants and also causing changes in species composition. Without flowers, pollinating animals would have no food source. Therefore, influences of ozone on flowering are of great importance. It has been acknowledged that the timing of flowering can be influenced by exposure to O_3 and that there is great species diversity in this response to the pollutant (summarized in Table 7.2). Delayed flowering has been reported in numerous species including soybean (Amundson, *et al*., 1986), cotton (Oshima *et al*., 1979), duckweed (Feder and Sullivan, 1969b; Feder, 1970), geranium (Feder, 1970); carnation (Feder and Campbell, 1968), buddleia (Findley *et al*., 1997), and spreading dogbane (Bergweiler and Manning, 1999), Harebell and Tufted vetch (Rämö *et al*., 2007). Conversely, O_3 exposure is reported to accelerate flowering of *Lotus corniculatus* L. (Hayes *et al*., 2012). Aside from altering the timing of flowering, ozone has also been reported to cause a reduction in flowers produced by many species including harebell (Hayes *et al*., 2012; Rämö *et al*., 2007; Franzaring *et al*., 2000; Gimeo *et al*., 2004), meado buttercup (Witton, 2013; Wedlich *et al*., 2012), watermelon and muskmelon (Fernandez-Bavon *et al*., 1993), duckweed (Feder and Sullivan, 1969b; Feder, 1970), carnation (Feder and Campbell, 1968; Feder, 1970), begonia, petunia and snapdragon (Adedipe *et al*., 1972; Reinert and Nelson, 1979), Wisconsin Fast Plants (Stewart, 1998), Buddleia (Findley *et al*., 1997) and spreading dogbane (Bergweiler and Manning, 1999). However, ozone exposure had no influence on the flowering of broad bean in the experiments described in Chapters 4 and 5.

Studies into the species composition of an open air fumigation experiment at Keenley Fell reported significant reductions in the biomass of *Rhinanthus minor* L. after 3 years of ozone exposure, compared to control plots (Wedlich *et al*., 2012). Changes in floral resource availability could have huge impacts on the available nutrition to foraging animals.

Table 7.1 Effects of ozone exposure to pollen viability/germination, protein content and free amino acids. Arrows indicate an increase or decrease in the response variable and = indicates no measured changes (adapted from Black *et al*., 2000).

Response	Effect	Species	Common name	References
In vitro				
Germination and/or tube growth	↓	*Prunus dulcis*	Almond	Hormaza *et al*., 1996
		Malus domestica	Apple	Hormaza *et al*., 1996
		Prunus armeniaca	Apricot	Hormaza *et al*., 1996
		Prunus persica	Nectarine	Hormaza *et al*., 1996
		Prunis persica	Peach	Hormaza *et al*., 1996
		Pyrus communis	Pear	Hormaza *et al*., 1996
		Pinus strobus	White pine	Benoit *et al*., 1983
		Petunia hybrida	Petunia	Feder and Sullivan, 1969b
				Harrison and Feder, 1974
				Krause *et al*., 1975
				Feder, 1981
				Feder *et al*., 1982
				Feder, 1968
		Nicotiana tabacum	Tobacco	Feder and Sullivan, 1969a,b
				Krause *et al*., 1975
		Lycopersicon esculentum	Tomato	Feder *et al*., 1982
				Gillespie *et al.,* 2015
		Zea mays	Maize	Mumford *et al*., 1972
		Plantago major	Greater plantain	Stewart, 1998
		Brassica camperstris	Wisconsin Fast Plants	Stewart, 1998
		Ambrosia artemisiifolia	Ragweed	Pasqualini *et al*, 2011
Tube growth	↑	*Plantago major*	Greater plantain	Stewart, 1998
In vivo	↓	*Nicotiana tabacum*	Tobacco	Feder, 1968
		Lycopersicon esculentum	Tomato	Feder *et al*., 1982
		Plantago major	Greater plantain	Stewart, 1998
		Brassica campestris	Wisconsin Fast Plants	Stewart, 1998
Protein content	↓	*Acer negundo*	Boxelder maple	Riberio *et al*., 2013
		Quercus robur	English Oak	Riberio *et al*., 2013
		Platanus spp.	Sycamore	Riberio *et al*., 2013
		Ostrya spp.	Hophornbeam	Cuinica *et al*., 2014
		Vicia faba	Broad bean	Stabler, 2016
	=	*Ambrosia artemisiifolia*	Ragweed	Pasqualini *et al*., 2011
Amino acids	↑	*Zea mays*	Maize	Mumford *et al*., 1972
	↓	*Vicia faba*	Broad bean	Stabler, 2016

Table 7.2 Effects of ozone exposure on flowering, including rate of flowering, and floral production. Arrows indicate an increase or decrease in the response variable (adapted from Black *et al*., 2000).

Response	Effect	Species	Common name	References
Flowering rate	delayed	*Glycie max*	Soybean	Amundson *et al*., 1986
		Gossypium hirsutum	Cotton	Oshima *et al*., 1979
		Lemma perpusilla	Duckweed	Feder and Sullivan, 1969b
				Feder, 1970
		Pelegonium × hortorum	Geranium	Feder, 1970
		Dianthus caryophyllus	Carnation	Feder and Campbell, 1968
				Feder, 1970
		Buddleia davidii	Buddleia	Findley *et al*., 1997
		Apocynum androsaemifolium	Spreading dogbane	Bergweiler and Manning, 1999
		Campanula rotundifolia	Harebell	Rämö *et al*., 2007
		Vicia cracca	Tufted vetch	Rämö *et al*., 2007
	Increased	*Lotus corniculatus*	Birdsfoot trefoil	Hayes *et al*., 2012
Flower production	↓	*Campanula rotundifolia*	Harebell	Hayes *et al*., 2012
				Rämö *et al*., 2007
				Franzaring *et al*., 2000
				Gimeno *et al*., 2004
		Ranunculus acris	Meadow buttercup	Witton, 2013
				Wedlich *et al*., 2012
		Citrullus lanatus	Watermelon	Fernandez-Bayon *et al*., 1993
		Cucumis melo	Muskmelon	Fernandez-Bayon *et al*., 1993
		Lemna perpusilla	Duckweed	Feder and Sullivan, 1969b
				Feder, 1970
		Dianthus caryophyllus	Carnation	Feder and Campbell, 1968
				Feder, 1970
		Begonia semperflorens	Begonia	Adedipe *et al*., 1972
		Petunia × hybrida	Petunia	Adedipe *et al*., 1972
		Antirrhinum majus	Snapdragon	Adedipe *et al*., 1972
		Begonia × hiemalis Elatior	Begonia	Reinert and Nelson, 1979
		Brassica camperstris	Wisconsin Fast Plants	Stewart, 1998
		Buddleia davidii	Buddleia	Findley *et al*., 1997
		Apocynum androsaemifolium	Spreading dogbane	Bergweiler and Manning, 1999
	↑	*Conopodium majus*	Pignut	Witton, 2013
		Dactylis glomerata	Orchard grass	Witton, 2013

Ozone also has a profound influence on the VOCs emitted from plants (Heiden *et al*., 1999; Pinto *et al*, 2010) and degradation of VOCs can reduce the ability of herbivorous insects to locate the flowers of their hosts eg. the striped cucumber beetle (*Acalymma vittatum*) struggles to locate the flowers of its host *Cucurbita foetidissima* (Fuentes *et al*., 2013). Of particular importance is the influence of VOC degradation on pollinator behaviour. Reliable floral odours are essential for floral discrimination and olfactory conditioning in foraging bees (Wright and Shiestl, 2009). The influence of ozone pollution on floral scent trails was modelled by McFrederick and colleagues (2008). They suggest that reactions between the O_3 and VOCs in polluted regions reduce the distance that volatiles travel from their floral source from kilometres to less than 200 m (McFrederick *et al*., 2008). The significance of such modelling studies is revealed by studies on the degradation of VOCs in the floral scent of *Brassica nigra* L. by exposure to O_3 (Farré-Armengol *et al*., 2016). These authors went on to illustrate the influence of reduced floral scent of *B. nigra* on pollinator (*Bombus terrestris*) attraction; bees were more likely to orient to flowers that had a floral scent than clean air. However, when this scent was mixed with O_3 bees were more likely to orient to a floral source at 0 m from the odour source and the attraction decreased over 4.5 m (Farré-Armengol *et al*., 2016). The degradation of floral VOCs may change the profile of compounds within the suite of VOCs released as floral odour (McFrederick *et al*., 2008; Farré-Armengol *et al*., 2016). Honeybees are capable of discriminating specific odour compounds (Wright *et al*., 2005) and so an unstable odour plume would be expected to produce unreliable plant cues to foraging bees. This requires further experimentation in the field.

Bees actively regulate their protein, carbohydrate (Altaye *et al*., 2010; Paoli *et al*., 2014; Stabler *et al*., 2015) and fatty acid (see Appendix I) intake. Ozone has been shown to reduce the protein content of pollen (see Chapter 4; Ribeiro *et al*., 2013). As the sole source of protein to eusocial bee colonies, negative impacts of O_3 on the nutrient qualities of pollen could put greater foraging demands on the colony, which in turn would put greater nutritional demands on the colony. When honeybees forage from low quality pollen, the number of pollen foragers from the colony increases (Pernal and Currie, 2001). It could be assumed that an increase in the number of foraging bees could actually improve the pollination service to the plant. However, ozone can cause

significant detrimental impacts on pollen viability and so increased pollination, or transfer of pollen, may not actually improve plant fitness.

Ozone concentrations in the Northern hemisphere are increasing and vegetation is exposed to longer periods of elevated air pollution (The Royal Sociey, 2008). Of course, ozone is not an exclusive air pollutant. There is evidence that other pollutants such as NO_x can degrade floral VOCs (Girling *et* al., 2013) and such changes reduce honeybee's olfactory memory of a conditioned stimulus when the odour profile changes (Lusebrink *et al*., 2015). Ozone pollution clearly has the potential to cause numerous detrimental impacts to plant-pollinator interactions, evidenced by the degradation of floral scent (McFrederick *et al*., 2008; Farré-Armengol *et al*., 2016), changes to the timing of flowering and floral senescence (Rämö *et al*., 2007; Hayes *et al*., 2012), and as described in this thesis, profound influences on the only food source to bees; nectar and pollen.

7.6 Research limitations

The results pertaining to the nutrient composition of nectar and pollen in Chapter 4 and 5 give robust evidence that both long and short term exposure to ozone, during development and at a critical time, such as flowering, can cause detrimental impacts to the available nutrition to floral visitors. However, caution must be taken when extrapolating such findings to possible field conditions. The findings described in this thesis compare the influence of 110 ppb O_3 to CFA. However, it is unlikely that vegetation would be exposed to 0 ppb O_3 in the natural environment (The Royal Society, 2008), therefore, it would be beneficial to further study the influence of O_3 on nutrient allocation to nectar and pollen versus non-filtered air. However, it was necessary to filter the air in the reported experiments due to the city centre location and the high concentrations of ozone precursors such as NO_x and diesel fumes. A further limitation is that episodes of peak O_3 exposure are not likely to sustain the length of time (~7 weeks) that plants were exposed to the pollutant in the long term treatment. It would be valuable to study the impact of long term exposure to background O_3 concentrations (~30-40 ppb) with peak episodes of exposure to higher concentrations (110 ppb) and determine how this may influence resource allocation to nectar and pollen.

7.7 Future research suggestions

The influence of O_3 on plant-pollinator interactions remains a largely understudied topic, with potentially vast consequences for plants, pollinators and agricultural productivity. Further studies that would continue the findings reported in this thesis include:

- Measured changes in pollen induced by environmentally-relevant exposure to O_3 should be applied to behavioural assays in which pollinators are assessed for their pollen preferences. Field studies quantifying visitation rates to plants with different pollen qualities would expose any real-world influence of pollen quality on the fitness of plants exposed to ozone. We were planning to undertake such experiments in the final year of this study using the OTCs that were at Close House but the facility was destroyed before this could be achieved.
- A screening style experiment should be carried-out on a diverse range of plant species for their response in terms of nutrient allocation to nectar and pollen. If ozone-resistant cultivars of crop plants can be identified, in terms of their resistance to changes in nectar and pollen quality, then this trait could be exploited in agricultural practice to maintain nutrient availability to pollinators.
- Secretion of sucrose is dependent on *sweet9*. Without the gene, nectaries do not secrete sucrose. It would be interesting to investigate whether there are any genetic markers for stress that could be assessed to identify whether nectar control at a molecular level is influenced by ozone. If ozone influences the secretion of sucrose in nectar, as it did in these experiments, then perhaps the expression of *sweet9* is influenced. Expression may be enhanced in order to improve the quality of the nectar in order to attract more pollination visitation ensuring seed set.
- This work has not elucidated whether nectaries are controlling the quality of nectar, or if indeed changes in the phloem sap are responsible for the nectar end product. Further study should sample phloem sap from stem to nectary and trace the nectar components.

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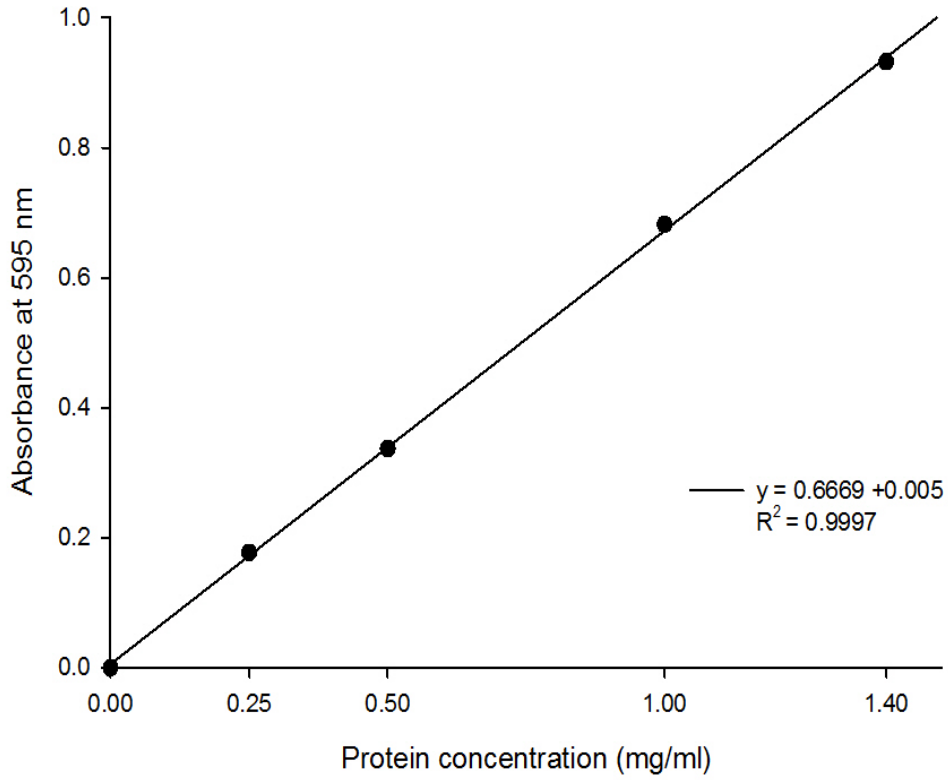
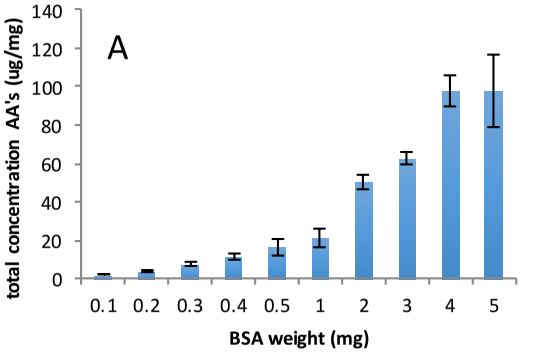
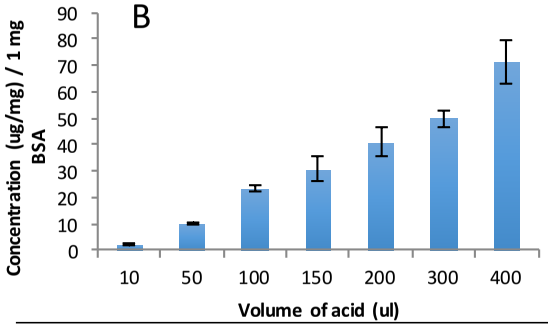
Appendix A: Bradford assay standard curve



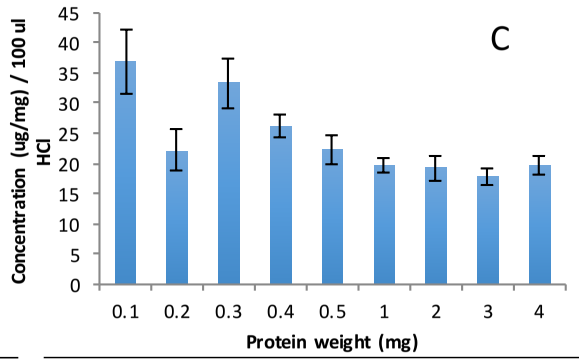
Appendix A. Bradford assay standard curve used to estimate protein concentration from unknown samples. Absorbance values are applied to the equation of the line to calculate protein concentration (mg/ml).



Protein weight (mg)	Volume of acid (ul)	Mean (ug/mg)	total recovered (ug)	Expected (ug protein)	multiplication factor
0.1	10	2.03	0.20	100	492.25
0.2	20	3.78	0.76	200	264.68
0.3	30	7.17	2.15	300	139.57
0.4	40	11.45	4.58	400	87.33
0.5	50	16.57	8.29	500	60.34
1	100	20.90	20.90	1000	47.85
2	200	50.24	100.48	2000	19.91
3	300	62.30	186.90	3000	16.05
4	400	97.63	390.53	4000	10.24
5	500	97.38	486.91	5000	10.27



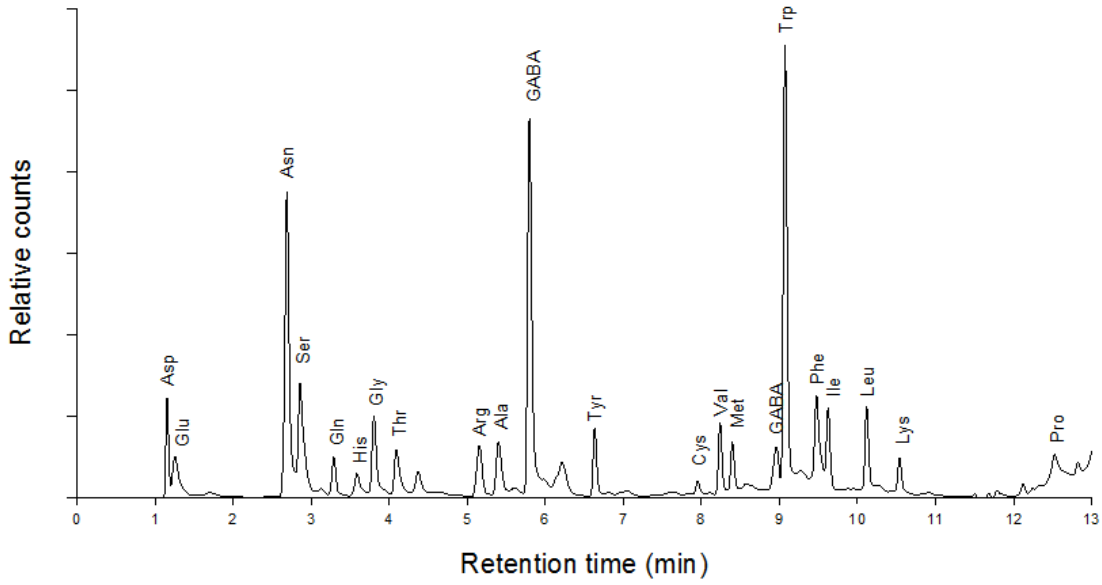
Protein weight (mg)	Volume of acid (ul)	Mean (ug/mg)	Total recovered (ug)	Expected (ug)	Multiplication factor
1	10	2.31	2.31	1000	432.08
1	50	10.51	10.51	1000	95.15
1	100	23.35	23.35	1000	42.83
1	150	30.84	30.84	1000	32.43
1	200	41.12	41.12	1000	24.32
1	300	49.78	49.78	1000	20.09
1	400	71.46	71.46	1000	13.99



Protein weight (mg)	Volume of acid (ul)	mean (ug/mg)	total (ug)	expected (ug)	multiplication factor
0.1	100	36.92	3.69	100	27.09
0.2	100	22.14	4.43	200	45.16
0.3	100	33.29	9.99	300	30.04
0.4	100	26.18	10.47	400	38.20
0.5	100	22.32	11.16	500	44.80
1	100	19.54	19.54	1000	51.18
2	100	19.24	38.48	2000	51.98
3	100	17.81	53.44	3000	56.13
4	100	19.70	78.82	4000	50.75

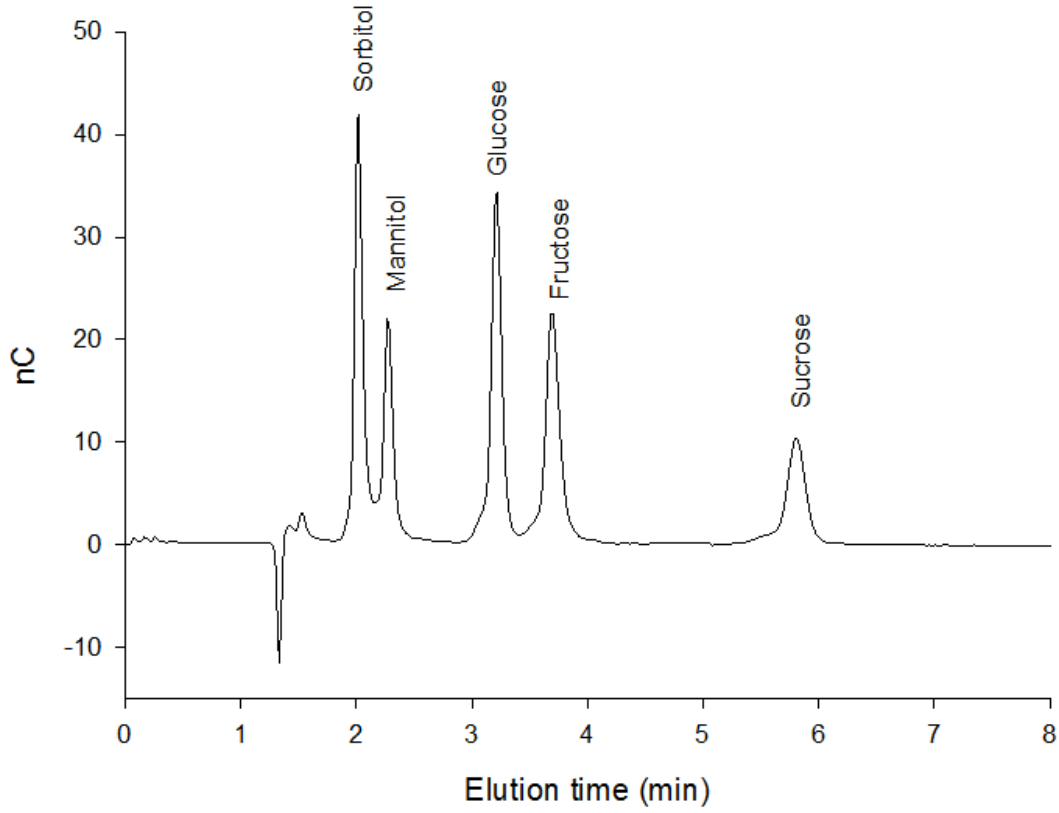
Appendix B: Total recovered amino acids from three hydrolysis experiments of BSA with ratio between protein weight and acid volume maintained (1 mg : 100 µl) (A), protein weight maintained and acid volume varied (B) and protein weight varied and acid volume maintained (C). Tables below each figure demonstrate the required multiplication factors dependent on treatment based on the expected values and measured values of total amino acids rendered from hydrolysis.

Appendix C



Appendix C. Example chromatogram showing retention times for 21 amino acids quantified. Note that GABA elutes as two peaks.

Appendix D



Appendix D. Example chromatogram showing retention times for 5 sugars quantified.

Appendix E

Appendix E Populations of broad bean (*Vicia faba* L.) used to screen for sensitivity to ozone (See Chapter 4).

Cultivar number	Supplier	Location
1	B&Q	Chandlers Ford, Hampshire
2	Sowseeds.co.uk	Cuddington, Cheshire
3	Thomson and Morgan	Ipswich, Suffolk
4	Unwins	Huntington, Cambridshire
5	Johnsons	Newmarket, Suffolk
6	Sutton Seeds	Paignton, Devon
7	Country Value	Newmarket, Suffolk
8	Mr. Fothergills	Kentford, Suffolk
9	Wilko	Worksop, Nottinghamshire
10	Homebase	Avebury, Milton Keynes

Appendix F. Protein-bound essential and non-essential amino acid content of pollen collected from three cultivars of broad bean exhibiting contrasting growth responses following exposure to CFA (CFA) or CFA + 110 ppb O_3 24 h d^{-1} (O_3). Values ± standard error of mean

Essential amino acids

		µg/mg									
Cultivar	Treatment	Histidine	Leucine	Threonine	Arginine	Valine	Methionine	Tryptophan	Phenylalanine	Isoleucine	Lysine
Sensitive	CFA	4.85±0.74	5.20±0.82	1.36±0.32	7.87±1.38	2.83±0.25	10.9±3.72	0.00±0.00	4.45±0.43	2.16±0.27	2.16±0.36
	O_3	4.94±2.02	4.76±1.18	0.99±0.21	9.49±2.49	1.86±0.47	42.7±13.5	0.00±0.00	3.35±0.84	1.49±0.36	0.48±0.15
Resistant	CFA	3.72±1.11	3.58±0.91	1.12±0.23	7.66±1.85	2.73±0.52	19.3±11.5	0.00±0.00	4.85±0.57	1.38±0.35	1.60±0.29
	O_3	6.06±1.43	4.29±0.10	0.76±0.23	6.45±2.23	1.66±0.62	26.3±12.4	0.00±0.00	2.96±0.57	1.13±0.21	0.47±0.11
Enhanced	CFA	3.56±0.70	4.13±1.02	1.16±0.38	11.1±5.00	2.14±0.25	1.09±0.55	0.00±0.00	3.52±0.63	1.39±0.38	0.84±0.12
	O_3	6.79±1.61	4.93±1.07	1.04±0.21	9.70±2.80	1.77±0.31	26.13±11.5	0.00±0.00	3.58±0.65	1.45±0.27	0.49±0.13

Non-essential amino acids

		µg/mg										
Cultivar	Treatment	Aspartic acid	Glutamic acid	Asparagine	Serine	Glutamine	Glycine	Alanine	Tyrosine	Cysteine	GABA	Proline
Sensitive	CFA	1.74±0.59	23.7±4.05	0.00±0.00	16.0±2.34	0.00±0.00	8.15±1.22	8.51±1.22	4.26±0.54	10.9±1.78	0.00±0.00	2.88±0.49
	O_3	1.05±0.24	22.6±8.35	0.00±0.00	16.7±4.93	0.00±0.00	6.37±1.28	9.46±2.55	4.37±1.08	10.2±3.14	0.00±0.00	8.38±3.00
Resistant	CFA	4.02±2.51	21.1±6.62	0.00±0.00	13.0±3.75	0.00±0.00	9.69±3.07	6.79±2.04	3.30±0.87	8.76±2.83	0.00±0.00	4.89±1.08
	O_3	3.82±1.81	19.2±5.91	0.00±0.00	12.8±3.86	0.00±0.00	6.46±1.98	7.53±2.21	3.39±0.92	9.36±2.71	0.00±0.00	6.70±2.38
Enhanced	CFA	2.06±0.44	17.5±9.41	0.00±0.00	18.1±5.04	0.00±0.00	16.5±4.95	9.23±2.59	4.33±1.07	12.1±3.01	0.00±0.00	2.70±0.37
	O_3	3.03±0.88	26.9±6.72	0.00±0.00	18.1±4.54	0.00±0.00	6.00±1.46	9.30±2.39	4.28±1.08	11.9±3.35	0.00±0.00	6.20±2.28

Appendix G. Free essential and non-essential amino acids washed from the pollen from from three cultivars of broad bean exhibiting contrasting growth responses following exposure to CFA (CFA) or CFA + 110 ppb O_3 24 h d^{-1} (O_3). Values ± standard error of mean.

Essential amino acids

		ng/mg									
Cultivar	Treatment	Histidine	Leucine	Threonine	Arginine	Valine	Methionine	Tryptophan	Phenylalanine	Isoleucine	Lysine
Sensitive	CFA	93.3±14.9	351.5±85.0	189.2±38.6	9061±1743	817.7±147.6	104.5±32.6	1.50±0.25	2.90±1.23	208.7±39.7	193.9±34.1
	O_3	59.1±26.7	286.7±96.2	159.8±48.9	5764±2486	566.7±211.5	249.5±49.9	1.30±0.33	0.80±0.42	192.8±28.7	230.8±28.7
Resistant	CFA	96.1±42.2	253.6±103.5	149.3±51.9	11589±3192	485.9±179.7	120.5±36.6	1.60±0.52	0.10±0.07	155.4±44.3	163.0±63.0
	O_3	42.1±16.1	167.6±60.9	35.7±11.6	3575±740	458.0±138.1	243.1±58.5	1.40±0.33	0.30±0.21	149.3±24.3	303.2±38.9
Enhanced	CFA	34.8±8.83	81.8±39.9	39.8±7.52	9666±3851	395.1±75.8	150.1±32.3	1.00±0.28	0.30±0.21	97.4±12.3	129.4±12.3
	O_3	19.5±5.94	189.1±99.3	32.3±7.02	3395±872.3	499.3±176.9	335.9±79.5	1.00±0.14	0.40±0.36	157.9±64.9	413.0±100

Non-essential amino acids

		ng/mg										
Cultivar	Treatment	Aspartic acid	Glutamic acid	Asparagine	Serine	Glutamine	Glycine	Alanine	Tyrosine	Cysteine	GABA	Proline
Sensitive	CFA	51.6±12.7	170.0±86.7	0.20±0.05	298.1±44.5	0.10±0.02	168.0±33.4	686.9±124.8	212.8±32.8	314.6±60.4	0.20±0.05	174.0±25.5
	O_3	39.0±10.5	204.9±70.1	0.20±0.07	234.2±70.0	0.10±0.04	77.5±25.9	448.8±144.9	141.6±48.4	203.7±58.0	0.10±0.04	99.0±14.0
Resistant	CFA	121.3±79.3	206.2±98.7	0.30±0.10	301.5±71.5	0.10±0.04	132.5±34.4	518.6±185.5	149.5±47.5	212.4±88.0	0.10±0.04	112.7±22.6
	O_3	67.5±27.8	193.6±64.8	0.10±0.04	277.5±105.2	0.10±0.05	174.8±71.9	474.4±174.1	110.3±31.9	179.4±74.0	0.10±0.04	78.3±17.0
Enhanced	CFA	126.2±42.1	218.9±33.4	0.10±0.06	219.3±35.4	0.10±0.03	107.4±22.6	367.4±97.5	90.9±18.7	106.0±54.7	0.10±0.02	95.1±26.3
	O_3	82.8±39.5	288.6±103.2	0.10±0.09	191.8±56.3	0.10±0.04	89.7±28.3	298.9±86.5	115.8±38.7	106.9±43.4	0.10±0.05	88.3±10.1

Appendix H

Exposure to environmentally-relevant levels of ozone negatively influence pollen and fruit development

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Exposure to environmentally-relevant levels of ozone negatively influence pollen and fruit development

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ABSTRACT

A combination of *in vitro* and *in vivo* studies on tomato (*Lycopersicon esculentum* Mill. *cv.* Triton) revealed that environmentally-relevant levels of ozone (O_3) pollution adversely affected pollen germination, germ tube growth and pollen-stigma interactions – pollen originating from plants raised in charcoal-Purafil® filtered air (CFA) exhibited reduced germ tube development on the stigma of plants exposed to environmentally-relevant levels of O_3. The O_3-induced decline in *in vivo* pollen viability was reflected in increased numbers of non-fertilized and fertilized non-viable ovules in immature fruit. Negative effects of O_3 on fertilization occurred regardless of the timing of exposure, with reductions in ovule viability evident in O_3 × CFA and CFA × O_3 crossed plants. This suggests O_3-induced reductions in fertilization were associated with reduced pollen viability and/or ovule development. Fruit born on trusses independently exposed to 100 nmol mol^{-1} O_3 (10 h d^{-1}) from flowering exhibited a decline in seed number and this was reflected in a marked decline in the weight and size of individual fruit – a clear demonstration of the direct consequence of the effects of the pollutant on reproductive processes. Ozone exposure also resulted in shifts in the starch and ascorbic acid (Vitamin C) content of fruit that were consistent with accelerated ripening. The findings of this study draw attention to the need for greater consideration of, and possibly the adoption of weightings for the direct impacts of O_3, and potentially other gaseous pollutants, on reproductive biology during 'risk assessment' exercises.

1. Introduction

Ozone (O_3) is considered to be the most common phytotoxic air pollutant to which agricultural, horticultural and wild vegetation is exposed, and is known to be responsible for substantial losses in crop yield and shifts in the composition of wild plant communities (The Royal Society, 2008; RoTAP, 2011). Losses to the agricultural economy are estimated to be between $11 and $26 billion (Van Dingenen et al., 2009; Avnery et al., 2011a), with predicted economic losses rising to between $19 and $35 billion per annum by 2030 (Avnery et al., 2011b). Tomato is generally considered to be sensitive (Mills et al., 2007) and in the U.S. alone the pollutant is believed to be responsible for losses amounting to ≈7% of production (Mutters and Soret, 1998; Grantz and Shrestha, 2005).

The adverse effects of ozone on yield and/or vegetative growth are the net result of a myriad of changes in gene expression (Kangasjärvi et al., 1994) that ultimately manifest in a decline in assimilation, and increase in respiration and pronounced shifts in assimilate distribution (Davison and Barnes, 1998; Booker et al., 2009; Burkey et al. 2012). Impacts on yield manifest through the direct effects of ozone on reproductive processes are poorly understood and have rarely been considered in isolation (Thwe et al., 2015). The reproductive phase is a critical stage in the plant life-cycle and given the known impacts of O_3-induced oxidative stress on plant metabolism there is a high likelihood that reproductive success may be negatively affected (Drogoudi and Ashmore, 2000, 2001; Morgan et al., 2003; Zhang et al., 2014). Either directly through the direct impact of the pollutant on the reproductive organs themselves – about which little is known and documented (see Stewart et al., 1996) or indirectly *via* effects on the vegetative organs and the resulting consequences of these effects on assimilate distribution and the timing of flowering (Barnes et al., 1999; Wedlich et al., 2012). Impairment of any steps during reproductive development, such as plant–pollinator interaction, physical events associated with pollination, fertilization and seed development may have significant implications for reproductive success,

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and thus yield determinants, in many agricultural and ecologically-important plant species.

The sensitivity of the key stages of the plant reproductive cycle to air pollutants is known to vary considerably (see Supplementary Fig. from Wolters and Martens, 1987). Pollen is considered particularly sensitive; direct exposure to various air pollutants, including O_3, resulting in reduced pollen germination and germ tube development (Wolters and Martens, 1987; Black et al., 2007; Pasqualini et al., 2011), but neither the degree of damage nor the mode of action are understood. There are reports of shifts in the topography of the stigmatic surface and pollen exine induced by direct exposure of pollen to O_3, which are associated with a reduction in the soluble protein content of pollen of different species (Roshchina and Karnaukhov, 1999; Roshchina and Mel'Nikova, 2001; Ribeiro et al., 2013) but the consequences of these observations have not been explored.

It is well documented that the timing of exposure to O_3 in relation to the plant life-cycle is an important determinant of the net impact of the pollutant on plant growth and yield (Lyons and Barnes, 1998), with many crops exhibiting enhanced sensitivity when exposure occurs during or after flowering/anthesis (Soja, 1997; Vandermeiren and De Temmermann, 1996; Pleijel et al., 1998; Gonzalez-Fernandez et al., 2010). It is important to better understand the extent of these impacts and to discriminate between indirect and direct effects of pollutants, such as ozone, on plant reproductive biology in order that consideration may be given to the potential need to adjust flux-based risk assessment approaches to better accommodate such impacts. In this manuscript we report a series of purposely-designed investigations employing tomato (*Lycopersicon esculentum* Mill.) as a model to discriminate between effects on fruit yield attributable to direct effects of O_3 on reproductive biology *versus* indirect effects mediated *via* damage to vegetative organs (e.g. reduced CO_2 fixation and shifts in resource partitioning).

2. Materials and methods

2.1. Plant material

Individual seeds of tomato (*L. esculentum* Mill. *cv.* Triton) were sown into plugs (2.5 cm^2) containing Levington M3 compost and placed in a controlled environment chamber ventilated with charcoal/Purafil®-filtered air (CFA: <5 nmol mol^{-1} O_3). Chamber details are described elsewhere (see Barnes et al., 1995).

2.2. Impacts of O_3 on pollen viability and fertilization

Following germination, 10-d-old seedlings were transplanted individually into pots containing 5 dm^3 Levington M3 compost and transferred to duplicate controlled environment chambers ventilated with CFA (<5 nmol mol^{-1} O_3) or CFA plus 75 nmol mol^{-1} O_3 7 h d^{-1}. Plants were watered three-times-per-day as required, and fed every 10 d with Phosphogen tomato food (Phosphogen Tomato Food, Monsanto, UK). Chambers were constructed from melamine-clad tubular steel and vented by a dedicated air conditioning system providing almost 2 air changes per minute in each chamber. Each chamber was supplied with particulate/charcoal/purafil®-filtered air into which ozone produced by electric discharge from pure oxygen was introduced *via* a mass flow PC-controlled system. Monitors were serviced regularly by an experienced technician and cross-calibrated against EPA standards using an annually-calibrated Dasibi 1008PC monitor. Details of the controlled ozone exposure system are provided elsewhere (Zheng et al., 1998).

Flowers were emasculated prior to dehiscence, by removing the anthers and connecting cone, so as to prevent self-fertilization. Tomato flowers exhibit diurnal opening and closing patterns, with pollen release occurring naturally around midday, when the petals are fully reflexed. Pollen removed from flowers, prior to full petal reflex exhibit low germination (see Picken, 1984), therefore pollen was removed on the second day of opening, when petals attained full reflex (between 11:00 and 13:00). All pollen was obtained from fully open flowers born on the 3rd truss of plants exposed to either CFA (<5 nmol mol^{-1} O_3) or plants exposed to ozone (75 nmol mol^{-1} O_3 7 h d^{-1}) since they were 10-d-old.

For crosses, pollen (collected and applied with the aid of a camel hair brush) was placed on the stigma of emasculated flowers borne on the 3rd truss of another plant. Methodological controls were performed to investigate the intrinsic effects of flower emasculation and self *versus* cross-pollination. These controls revealed no effects of flower emasculation or self *versus* cross-pollination. Consequently, all recipient flowers were emasculated and individual trusses were used to host both donor and recipient flowers (i.e. donor and recipient flowers on plants exposed to CFA (<5 nmol mol^{-1} O_3) or + O_3 (75 nmol mol^{-1} O_3 7 h d^{-1} from 10-d-old).

2.3. Impacts of O_3 on pollen viability in vitro

Pollen harvested from plants exposed to CFA (<5 nmol mol^{-1} O_3) or + O_3 (75 nmol mol^{-1} O_3 7 h d^{-1}) was sown on 0.1% agar fortified with 0.6 mM $CaNO_3$ and 10% sucrose (sensu Brewbaker and Kwack, 1963). Agar plates were partitioned in to 1 cm^2 segments and pollen distributed within each segment.

Seeded plates were incubated in duplicate mini-controlled environment chambers ventilated with either charcoal/Purafil-filtered air or O_3-enriched CFA at a rate sufficient to achieve 0.4 air changes min^{-1} in each chamber. The fumigation system was housed in a walk-in temperature controlled growth-room maintained at 22 ± 2 °C using an air conditioning system supplied by Troldahl Ltd. (Gateshead, UK). Ozone was generated by passing oxygen through a dielectric generator (model SGA01 Pacific Ozone Technology Inc., Brentwood, CA, USA) and diluted with CFA prior to injecting into individual mini-chambers at a rate of 2 dm^3 min^{-1}. Sampled air was continuously drawn from each mini-chamber *via* a multi-channel sampling unit (ICAM Ltd., Worthing, Sussex, UK), which diverted sample lines to an O_3 monitor (model 450, Advanced Pollution Instrumentation Inc., San Diego, CA, U.S.A. supplied and serviced by EnviroTechnology Ltd, Stroud, U.K.). Chambers were illuminated by four 250 W daylight fluorescent tubes (GE Lighting, General Electric Corporation, USA) providing a PPFD of 160 μmol m^{-2} s^{-1} (at the position in the chamber occupied by the plates) supplied as a 14 h photoperiod.

Pollen tube development was assessed periodically by removing three agar segments at intervals from duplicate plates, and then studying 20 pollen grains per segment under a light microscope (×400).

2.4. Impacts of O_3 on pollen viability in vivo

Provisional experimentation indicated that germ tube development was best-studied *in vivo* 48 h after pollination. At this stage, gynoecia (comprising carpels, stigma, style and ovaries) were removed from all treatment × cross combinations and fixed according to Wedderburn and Richards (1990). The procedure involved the fixing of gynoecia for 24 h in 3:1 absolute ethanol:-glacial acetic acid, prior to transfer to 80% ethanol and storage at −20 °C. To undertake visual assessments, gynoecia were softened in 1 M NaOH for 40 min, transferred to 2% parosaniline for 15 min then counter-stained for a further 15 min in 0.1% aniline blue made up in 0.1 M K_2HPO_4. Gynoecia were mounted on to a

slide and gently squashed in 50% glycerol and examined (at ×400) using a Nikon SF fluorescence microscope employing dark ground illumination. Non-germinated pollen was observed in the mounting medium. This suggested some unattached pollen grains became dislodged during slide preparation, so pollen germination scores may be under-estimated. We have no reason to believe that displacement was treatment-related in any way. Development of all pollen on the surface of the stigma was recorded and a pollen viability index[1] derived. Tomato flowers exhibited a robust style and stigma, and tangled pollen tubes. Consequently, it was not possible to track pollen germ tube development within the gynoecial tissues. However it was possible to derive a pollen tube development index.[2] This was calculated by recording the number of pollen tubes that had reached the bottom of the style, approaching the ovary.

2.5. Impacts of O_3 on ovule fertilization

Following germination, 10-d-old seedlings were transplanted to pots (5 dm^3) containing Levington M3 compost and transferred to duplicate controlled environment chambers ventilated with either CFA (<5 nmol mol^{-1} O_3) or O_3-enriched CFA (75 nmol mol^{-1} O_3 7 h d^{-1}). Plants were watered as required, and fed every 10 d with Phosphogen tomato food (Phosphogen Tomato Food, Monsanto, UK).

Following cross-pollination, fruit were allowed to expand for 3–4 weeks until they reached a standard circumference of 11 cm. The immature fruit were harvested and 20 ovules per fruit randomly sampled using a microscope (×20) to assess state of ovule fertilization. At this stage of fruit development, 3 types of ovule were clearly discernible: non-fertilized embryos (under-developed, appearing withered and coloured green/brown), fertilized non-viable seed (clear, and containing no embryo) and fertilized viable seed.

2.6. Direct effects of O_3 on fruit yield

Plants were grown in controlled environment chambers ventilated with CFA until the first flowering truss emerged, then they were transferred to a cubicle in a glasshouse at the University's Field Station (Close House, Northumberland, UK [NZ 128658]), maintained at a temperature of 25 ± 2 °C day, 18 ± 2 °C night and supplementally-illuminated with 5 metal-halide lamps (Siemens HR400H housing fitted with 400 W HQI-T lamps, Osram, St. Helens, Merseyside, UK), providing a minimum PPFD of ≈500 μmol m^{-2} s^{-1} mid-canopy, delivered in a manner designed to extend the natural photoperiod to 14 h (07:00–21:00).

Once the flower buds comprising the 3rd truss began to emerge, the whole truss was placed inside a purpose-built fumigation chamber constructed from 6 mm clear perspex (see Plate 1). This procedure was repeated for ten independent plants. Individual fumigation chambers (each 0.28 m^3 internal volume) were connected *via* ¼″ Teflon® tubing to a diaphragm pump (Air Supply Ltd, Washington, Co. Durham, UK) supplying CFA air at a flow rate sufficient to achieve 0.4 air changes min^{-1} in each chamber.

Charcoal/Purafil®-filtered air was injected in to each of the truss-holding chambers directly below a fan (12 V, 2700 rpm fan, RS Ltd, UK), which provided turbulence inside each fumigation chamber. Ozone was generated by pumping Charcoal/Purafil®-filtered air through an electric discharge generator (BA. 023 laboratory ozone generator, Wallace and Tiernan, Tunbridge, Kent, UK) and subsequently filtering through a cold water trap (replaced every couple of days). The airstream entering each chamber was diluted with CFA and injection in to individual chambers to attain two target treatments CFA (<5 nmol mol^{-1} O_3) and O_3 (CFA + O_3: <5 nmol mol^{-1} O_3 overnight rising to a maximum between 08:00–18:00 of 100 nmol mol^{-1} O_3).

Sampled air was continuously drawn from each chamber *via* a multi-channel ICAM sampling unit (ICAM Ltd., Worthing, Sussex, UK), which diverted each of the lines to an O_3 monitor (Monitor Labs 8810, serviced and supplied by EnviroTechnology Ltd., Stroud, Gloucs, UK). Each sample line was monitored for 600 s, with 5 s values over the final 30 s of the recording period averaged and logged. An internal mixing fan maintained the temperature within each chamber at 0.5 ± 0.2 °C higher than outside.

Flowers were purposely self- and cross-pollinated (using pollen derived from a different plant in the same treatment), to maximise pollination efficacy and mimick bee assisted-pollination; a common practice in commercial glasshouses.

Fruit were harvested when they achieved full maturity, ripening on the truss to a commercially-marketable Class 1 grade[3] (*sensu* Slack et al., 1988). Fruit size and fresh weight were recorded, then fruit were dried to constant weight in an oven at 70 °C prior to recording the number of seed.

2.7. Analysis of non-structural carbohydrate content

The non-structural carbohydrate content of the pericarp was analysed using the phenol-sulphuric method of Dubois et al. (1956). A two-stage extraction procedure was adopted. Fresh pericarp was cut finely and incubated in hot 80% ethanol. The supernatant was decanted for later analysis. The remaining plant material was used forstarch determination. Tissue was rinsed several times in distilled water then ground (using a pestle and mortar) in 2.4 ml acetate buffer (0.1 M sodium acetate and 0.1 M acetic acid pH 4.5) and autoclaved for 30 min at 120 °C to assist the solubilisation of starch. Extracts were cooled, then 0.5U α-amylase and 5U of amyloglucosidase (Sigma–Aldrich) added and samples incubated overnight at 45 °C. After centrifugation at 12,000 *g* for 10 min the supernatant was assayed for glucose equivalents. For the assay of glucose, 10 μl of extract was added to 0.5 ml H_2O, 1 ml 5% phenol and 5 ml conc. sulphuric acid. The mixture was agitated occasionally and allowed to cool for 15 min. The absorbance of duplicate samples was subsequently read at 483 nm using an automated UV/visible spectrophotometer (Pye-Unicam SP8700) and carbohydrate content determined from a standard curve constructed using glucose standards.

2.8. Analysis of ascorbate (vitamin C) content and redox state

Analyses were performed on *c.* 1.0 g of fresh pericarp. Plant material was homogenized in 1 ml of buffer (2% metaphosphoric acid, 2.5 mM EDTA), then transferred to 1.5 ml Eppendorf tubes and centrifuged for 3 min at 11,000 *g* at 4 °C. The supernatant was decanted and kept on ice. All assays were duplicated and performed within 20 min of the preparation of crude extracts.

The assay contained 100 μl of extract in 1 M potassium phosphate buffer (pH 6.1). The absorbance was measured at 265 nm, then 5 μl of ascorbate oxidase (AO; E.C. 1.10.3.3) added. The residual absorbance was recorded following complete oxidation of ASC

[1] Pollen viability index – germinated *versus* non-germinated pollen on the stigmatic surface.

[2] Pollen tube development index – number of pollen tubes at the base of the papillia *versus* the number of germinated pollen on the surface of the stigma.

[3] Class 1 fruit – round, uniformly-coloured, free from blemish and ripening disorders with a maximum diameter of 40–57 mm.

Plate 1. Truss fumigation system. The 3rd truss of *Lycopersicon esculentum* (Mill.) *cv.* Triton was isolated and developed within fumigation chambers ventilated with CFA (<5 nmol mol^{-1} O_3) or CFA plus O_3 (100 nmol mol^{-1} O_3 10 h d^{-1}). Plants were grown in 'clean air' in a glasshouse cubicle – the 3rd truss, independent of the rest of the plant, was exposed to a controlled level of ozone from flower initiation to fruit ripening.

(2 min). Ascorbate content was determined from the difference between these measurements. Total ascorbate [ASC + DHA] content was estimated in a parallel sample, following the reduction of DHA to ASC by DL-dithiotheritol (DTT). The assay mix contained 100 μl of extract in 1 M potassium phosphate buffer (pH 6.1), following the complete reduction of DHA to ASC (12 min) the absorbance was determined at 265 nm. An extinction coefficient of 14.3 mM^{-1} cm^{-1} for ASC at 265 nm was employed in calculations to determine ASC content (*sensu* Takahama and Oniki, 1992). Absorbance was registered using a Pye-Unicam UV/visible spectrophotometer (Model SP 8700) fitted with an eight-cell carriage. All assays were performed at 25 °C employing matched quartz cuvettes.

2.9. Statistical analysis

Statistical analysis was performed using SPSS (SPSS Inc, Chicago, USA). Data were first subjected to ANOVA, to determine whether there was significant chamber-to-chamber variation within treatments. No evidence to support block effects was detected, so data for individual treatments were re-analysed using multivariate ANOVA (MANOVA) to investigate the significance of O_3, time and interactions with crosses/plant history. All percentage data were arcsine transformed prior to ANOVA. Significant differences were determined using either a t-test or the least significant differences (LSD), calculated at the 5% level.

3. Results

3.1. Impacts of O_3 on pollen viability in vitro

Effects of *in vitro* exposure to O_3 on pollen germination and subsequent germ tube development are shown on Fig. 1. Germination of pollen from O_3-grown plants incubated in an O_3-enriched environment (O_3/O_3) was significantly ($P < 0.001$) retarded in comparison with plants raised in CFA and incubated in clean air (CFA/CFA). However, the final extent of pollen germination (assessed after 33 h) was not significantly different from the control. Germination of pollen originating from plants exposed to O_3, but incubated in NFA (i.e. O_3/CFA), was significantly ($P < 0.001$) greater than that of O_3/O_3 pollen. This suggests direct effects of O_3 on pollen germination, rather than 'memory' effects resulting from the exposure of flowers and vegetative organs to the pollutant. This conclusion is substantiated by the observation that pollen germination from CFA-grown plants was significantly ($P < 0.05$) decreased by O_3 exposure *in vitro* (i.e. CFA/O_3 *versus* CFA/CFA). Germ tube development was significantly ($P < 0.001$) reduced in O_3/O_3 pollen compared with controls (CFA/CFA pollen), and a major contributor to this effect appeared to be the 'direct' impact of the pollutant on pollen viability – germ tube development in CFA/O_3 pollen was also significantly ($P < 0.001$) less than that of controls (i.e. CFA/CFA pollen), while there was no significant difference in germ tube development between O_3/O_3 and O_3/CFA.

3.2. Impacts of O_3 on pollen viability in vivo

The effects of parental O_3 history on pollen germination and germ tube development *in vivo* are illustrated in Fig. 2. Pollen originating from plants grown in O_3 exhibited reduced ($P < 0.05$) germination when placed on the stigma of flowers borne on other plants subjected to the same treatment (i.e. $O_3 \times O_3$). Pollen germination in $O_3 \times$ CFA and CFA $\times O_3$ crosses was not significantly different from CFA controls and substantially higher (*c.* 25%) than that of $O_3 \times O_3$ crosses. Interestingly, the fraction of germ tubes reaching the base of the papilla was significantly ($P < 0.05$) reduced in $O_3 \times O_3$ and CFA $\times O_3$ crosses compared with CFA $\times$ CFA or $O_3 \times$ CFA crosses.

3.3. Impacts of O_3 on ovule fertilization

Fertilization success for reciprocal crosses performed on plants subjected to CFA or O_3 are shown in Fig. 3. The proportion of fertilized viable ovules contained in immature tomato fruit was reduced ($P < 0.05$) substantially (*c.* 26%) in $O_3 \times O_3$ crosses compared with CFA $\times$ CFA. This effect appeared to be independent of pollen exposure to the pollutant (i.e. the proportion of viable fertilized ovules was reduced to a similar extent in $O_3 \times$ CFA, CFA $\times O_3$ and $O_3 \times O_3$ crosses). In addition the proportion of non-fertilized ovules was highest ($P < 0.05$) in fruit resulting from $O_3 \times O_3$ crosses compared with controls (i.e. those resulting from CFA $\times$ CFA crosses). Also, embryo abortion was 29% less in fruit originating from CFA $\times$ CFA crosses, compared with those from plants exposed to O_3 prior to, or following, pollen transfer (i.e. $O_3 \times O_3$, CFA $\times O_3$ and $O_3 \times$ CFA crosses).

3.4. Direct effects of O_3 on fruit yield

The impact of O_3-exposure on the 3rd truss of tomato (i.e. isolated exposure of the 3rd truss independent of the rest of the plant) is shown in Table 1. Ozone exposure resulted in a 'direct' reduction ($P < 0.05$) in fruit size, fresh and dry weight plus the average number of seeds per fruit. Moreover, the number of fruit reaching maturity was also consistently reduced by 'direct' exposure to O_3 – though the effect did not attain a statistically significant level. Notably, for plants in which the isolated 3rd truss was exposed to ozone, there were no significant effects of the treatment on fruit size, number or weight (or seed number per fruit) in truss 2.

Plants were grown in a glasshouse cubicle (<5 nmol mol^{-1} O_3) under natural daylight, with supplemental lighting. From initiation, the 3rd flowering truss was isolated and exposed in specially-

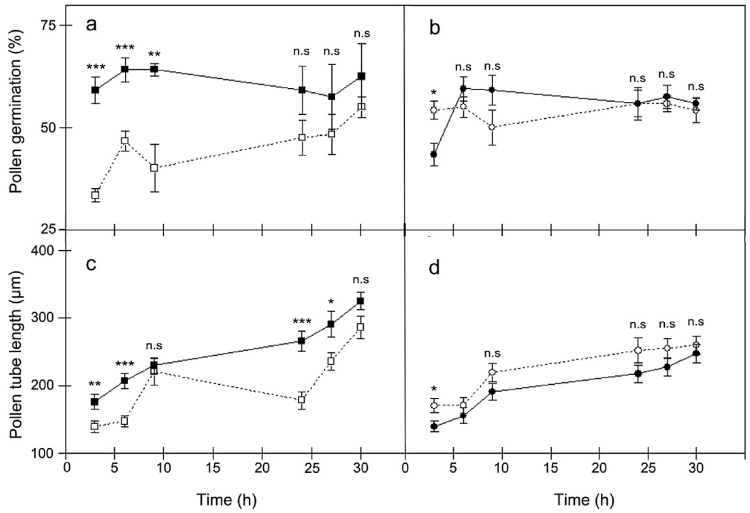

Fig. 1. Impacts of O_3 on pollen germination (a,b) and germ tube development (c,d) *in vitro*. Pollen was harvested from flowers borne on the 3rd truss of tomato (*Lycopersicon esculentum* (Mill.) *cv.* Triton) plants raised from 10-d-old in CFA (<5 nmol mol^{-1} O_3) or CFA plus O_3 (75 nmol mol^{-1} O_3 7 h d^{-1}). Harvested pollen was sown on fortified agar and exposed *in vitro* to CFA (<5 nmol mol^{-1} O_3) or O_3-enriched CFA (100 nmol mol^{-1} O_3 7 h d^{-1}). Treatment combinations: pollen from CFA-grown plants incubated in CFA (CFA/CFA; —■—), pollen from O_3-grown plants incubated in an O_3-enriched atmosphere (O_3/O_3; —□—), pollen from CFA-grown plants incubated in an O_3-enriched atmosphere (CFA/O_3; —●—) and pollen from O_3-grown plants incubated in CFA (O_3/CFA; —○—). Values represent mean of 120 observations ± SE of three agar segments per duplicate plate. Significant differences denoted: *P < 0.05, **P < 0.01, ***P < 0.001. n.s. = no significant difference at 5% level.

constructed chambers to CFA (<5 nmol mol^{-1} O_3) or CFA plus O_3 (100 nmol mol^{-1} O_3 8 h d^{-1}). Values represent mean ± SE of between 24 and 21 fully-ripe 'Class 1' fruit per treatment. Values bearing the same superscript are not significantly different at the 5% level.

A linear relationship was found between the number of viable seed contained within mature fruit and fruit size/weight; the greater the seed count, the larger the fruit (see Supplementary Material)

Isolated exposure of the 3rd truss to O_3 significantly ($P < 0.05$) reduced the starch content of 'Class 1' tomato fruit, but resulted in no change in soluble carbohydrate content (Table 1).

Direct exposure of developing fruit to controlled levels of O_3 resulted in a significant ($P < 0.05$) decrease in the total ascorbate content (ASC + DHA) of fruit (Table 1). Despite a slight (but statistically significant) improvement in ascorbate redox status, the level of the reduced form (i.e. vitamin C) was reduced significantly ($P < 0.05$) by *c.* 15% in O_3-treated fruit.

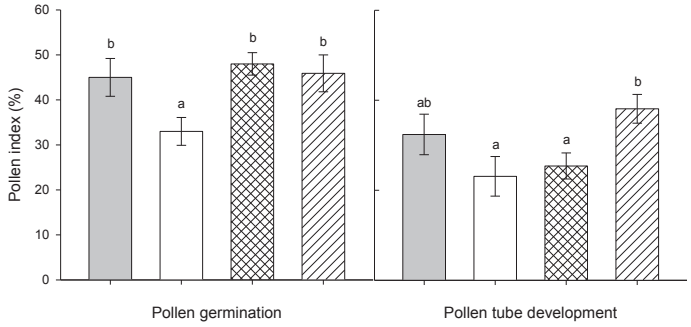

Fig. 2. Impacts of O_3 on pollen viability *in vivo*. (a) pollen germination index for pollen on the surface of the stigma and (b) development index for germ tubes successfully reaching the base of the stigma in *Lycopersicon esculentum* (Mill.) *cv.* Triton. Pollen from donor flowers was transferred to the stigma of recipient flowers and the plants returned to CFA (<5 nmol mol^{-1} O_3) or CFA + O_3 (75 nmol mol^{-1} O_3 7 h d^{-1}). Crosses: pollen from CFA plants was used to pollinate flowers borne on another CFA-grown plant (CFA × CFA; ■), pollen from plants raised in O_3 was used to pollinate flowers borne on another O_3-grown plant (O_3 × O_3; □), pollen from CFA plants was used to pollinate flowers borne on a plant raised in O_3 (CFA × O_3; ▨) or pollen from a plant raised in O_3 was used to pollinate flowers borne on a plant raised in CFA (O_3 × CFA; ▨). Values represent the mean ± SE of 15 independent observations. Values bearing the same superscript are not significantly different at the 5% level.

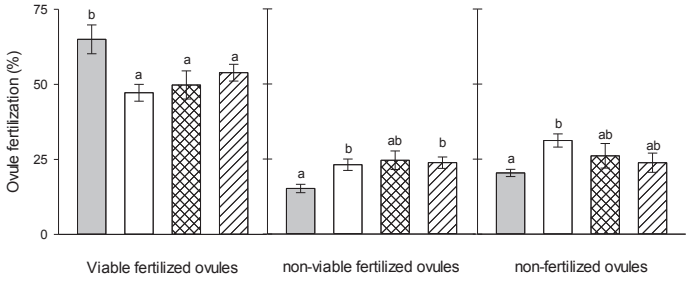

Fig. 3. Impacts of O_3 on ovule fertilization. Proportion of (a) viable fertilized ovules, (b) non-viable fertilized ovules and (c) non-fertilized ovules in immature fruit of *Lycopersicon esculentum* (Mill.) *cv.* Triton resulting from crosses between plants raised in controlled environment chambers ventilated with CFA (<5 nmol mol^{-1} O_3) or O_3 (75 nmol mol^{-1} O_3 7 h d^{-1}). Pollen was harvested from the 3rd truss of donor plants raised in CFA or O_3 and transferred to the stigma of a flower borne on another plant subject to the same or different treatment. Crosses: pollen from CFA-grown plant used to pollinate flowers borne on another plant subject to the same treatment (CFA × CFA; ■), pollen O_3-grown plant used to pollinate flowers borne on a different plant subject to the same treatment (O_3 × O_3; □), pollen from CFA-grown plant used to pollinate flowers borne on a plant exposed to O_3 (CFA × O_3; ▨) or pollen from an O_3-treated plant used to pollinate flowers borne on a plant raised in CFA (O_3 × CFA; ▨). Values represent the mean ± SE (n = 15). Values bearing the same superscript are not significantly different at 5% level.

Table 1
'Direct' effects of O_3-exposure on fruit yield and development of tomato (*Lycopersicon esculentum* (Mill.) *cv.* Triton).

	CFA	O_3
Fruit yield parameters		
Final fruit diam. (mm)	48.9 ± 10.9[a]	46.6 ± 0.5[b]
Fruit fresh weight (g)	67.0 ± 2.0[a]	54.0 ± 2.0[b]
Fruit dry weight (g)	3.3 ± 0.2[a]	2.7 ± 0.1[b]
Seed count per fruit	74.9 ± 3.1[a]	64.4 ± 3.7[b]
Total number of fruit	24	21
Carbohydrate content (mg $[CH_2O]_6$ g^{-1} fwt)		
Soluble sugars	273.5 ± 14[a]	306.4 ± 27[a]
Starch	47.8 ± 4[a]	31.9 ± 5[b]
Ascorbate content (nmol g^{-1} fwt.)		
Total ascorbate	448.8 ± 25[a]	373.2 ± 18[b]
Redox state (%)	89.7 ± 2[a]	95.4 ± 1[b]

Values bearing the same superscript are not significantly different at the 5% level.

4. Discussion

In vitro experimentation plus the isolated exposure of the 3rd truss of tomato to environmentally-relevant levels of O_3 demonstrated clearly that the pollutant exerts 'direct' adverse effects on flower-related reproductive biology and results in detrimental effects on fruit yield and quality.

A combination of *in vitro* and *in vivo* studies on pollen viability revealed that exposure to environmentally-relevant levels of O_3 retards pollen germination and decreases germ tube development in tomato. Agar-based studies performed *in vitro* indicated direct effects on pollen, while studies conducted *in vivo* reflect effects on pollen-stigma interactions as well as impacts of the environment. The combination of approaches is important, since pollen viability determined *in vitro* does not necessarily reflect that *in vivo* (Dempsey, 1970). Our finding that exposure to O_3 results in adverse effects on pollen viability are in general agreement with the available literature – sparse though it is (reviewed by Black et al., 2000). However, virtually no information is available as to the mode of action of O_3. Some authors suggest that O_3 exposure may damage pollen and cause shifts in pollen exine composition – thus affecting adhesion and recognition at the surface of the stigma (Roshchina and Karnaukhov, 1999; Cuinica et al., 2013; Taia et al., 2013). Others suggest that O_3 may weaken pollen to the extent that the metabolite levels required to fuel germination fall below a critical threshold (Black et al., 2000).

Germination occurs following the adherence and hydration of pollen on the stigma. The germ tube emerges and enters the protection of the style, growing down the transmitting tract of the pistil towards the ovary where the male gametes are eventually delivered to the ovule (reviewed by Franklin-Tong, 1999; Shi and Yang, 2010). Consequently, the developing germ tube is protected by the style from direct contact with gaseous pollutants. However, germ tube development is heavily dependent on the topography of the stigma (Feder and Shrier, 1990), the supply of water, carbohydrates and amino acids provided *via* the style of the recipient (reviewed in Lord, 2000, 2003) and is controlled through signals passed between pollen and stigma (Brugière et al., 2000; Dickinson, 2000). There is thus an intimate relationship between the pollen tube and the epidermal cells of the transmission tract in the pistil. Impacts of O_3 on the complex interaction between pollen and stigma may well have contributed to our observation that O_3-exposure substantially reduced germ tube development *in vivo* as well as *in vitro* and are consistent with our finding that germination and development of O_3-exposed pollen recovered (to control levels) when placed on the stigma of CFA-grown plants. Interestingly, some authors have suggested that impacts of O_3 on pollen germination and development may reflect the tolerance of the parent to O_3 stress (Feder, 1968), and genotype screening programmes involving the *in vitro* screening of pollen under varying environmental conditions such as temperature and air pollutants have confirmed a strong genotype–environment interaction with regard pollen success (Hormaza and Herrero, 1996; Hedhly et al., 2005).

The reduced performance of O_3-exposed pollen was reflected in higher numbers of non-fertilized and fertilized non-viable ovules in fruit resulting from O_3 × O_3 crosses compared with CFA × CFA, as well as a decline in the proportion of seed attaining maturity. Interestingly, the adverse effects of O_3 on fertilization persisted prior to or following crossing (i.e. adverse effects were notable in O_3 × CFA and CFA × O_3 crosses), suggesting the main impact of O_3 to be on pollen development (i.e. *via* reduced pollen viability) and/or ovule development.

Initial and continued cell division within developing fruit is closely dependent on the success of ovule fertilization (Johnson et al., 1992; Gillaspy et al., 1993; Vivian-Smith et al., 2001). Although tomato can be parthenocarpic, so a seed threshold is unnecessary for initiating fruit swelling, the fruit produced are generally smaller and of lower quality (Varoquaux et al., 2000;

Vergara and Fonseca-Buendía, 2012). There is increasing optimism that modern approaches will be capable of engineering parthenocarpic fruit to surpass the yield of fruit fertilized in a conventional sense (Pandolfini et al., 2002; Gorguet et al., 2005). Nevertheless, the number of seed within a tomato fruit strongly influences final size with seed number and fruit weight generally correlated within cultivars (Reviewed by Picken, 1984; Vergara and Fonseca-Buendía, 2012). This finding is supported by the strong relationship noted in the present study between seed number and yield parameters (i.e. fresh and dry weight and final fruit size). Although the precise role of seed in fruit development is poorly understood, it is known that the developing seed and embryo constitute a rich source of hormones (with gibberellin and auxin believed most important) which stimulate cell division and fruit swelling (reviewed by De Jong et al., 2009). In the present study, there was no effect of O_3 on fruit fresh: dry weight ratio, indicating both initial cell division and cell expansion were jointly affected by O_3 exposure. Considering the key role played by fertilized seed in fruit development and given the adverse effects shown on pollen germination and development, it is perhaps not unsurprising to find that O_3-exposure resulted in 'direct' effects on fruit yield – in terms of impacts on weight, number and quality-related characteristics. Our findings in this regard are consistent with previous reports of O_3 on the yield of tomato, though the authors did not seek to differentiate between 'direct' and 'indirect' impacts on fruit, but noted that O_3-induced reductions in yield were more substantial when exposure to the pollutant co-incided with flowering and early fruit development (Tenga et al., 1990; Younglove et al., 1994; Thwe et al., 2015).

Removal or damage of immature trusses on mature tomato plants induces a compensatory shift in yield within trusses located directly above and below those damaged (Slack and Calvert, 1977; Horridge and Cockshull, 1998). Similarly, removal of individual flowers within a truss can initiate compensatory weight increases within the remaining fruit (Cockshull and Ho, 1995). No evidence was found in the present study to suggest that depression in yield components resulting from exposure of the 3rd truss to O_3 were offset by compensatory increases in the weight or number of fruit borne on the 2nd truss.

Interestingly, direct exposure of the 3rd truss to O_3 resulted in a decline in the starch and ascorbate (vitamin C) content of fruit i.e. quality-related characteristics. Both starch and ascorbate content correlate with ripening. Starch levels within a tomato fruit are relatively low decreasing to almost trace levels as the fruit ripens (Cuartero and Fernández-Muñoz, 1998; Gao et al., 1998). In contrast, ascorbate levels generally attain optimum levels immediately prior to full ripening (Islam et al., 1996) and then gradually decline (Yahia et al., 2001). The pattern of changes induced by O_3 exposure are consistent with the accelerated ripening of tomato fruit under the influence of the pollutant. A similar conclusion was drawn by Crisosto et al. (1993) when studying the effects of O_3 on plum (*Prunus salicina* Lindel., 'Casselman').

The results of the present study draw attention to the potential need to give greater consideration to the impacts of O_3 on plants during the reproductive phases of growth both from a mechanistic perspective as well as from the point of view of risk assessment. Indeed, there are numerous studies on a range of crops that suggest greater impacts of O_3 on crop yields where exposures co-inside with anthesis, grain fill or fruit development (Younglove et al., 1994; Soja, 1997; Vandermeiren and De Temmermann, 1996; Pleijel et al., 1998; Mina et al., 2010; Thwe et al., 2015; Zhang et al., 2014). The differential impacts of pollutants (including O_3) on pollen could, and probably should, be explored as a potential opportunity for rapid, relatively straightforward, large-scale screening programmes aimed at breeding of crops with enhanced tolerance to ozone, and prospectively other air pollutants.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.08.003.

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Appendix I

Bumble bees regulate their intake of the essential protein and lipid pollen macronutrients
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Bumble bees regulate their intake of the essential protein and lipid pollen macronutrients

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Summary Statement
Bumble bees selectively feed among synthetic diets to acquire proteins and lipids ideal for survival. Optimal protein:lipid ratios are similar to the values of pollen from their preferred host-plant species.

Abstract
Bee population declines are linked to reduction of nutritional resources due to land-use intensification, yet we know little about the specific nutritional needs of many bee species. Pollen provides bees their primary source of protein and lipids, but nutritional quality varies widely among host-plant species. Therefore, as with other animal species, bees may be adapted to assess resource quality and adjust their foraging behavior to balance nutrition from multiple food sources. We tested the ability of two bumble bee species, *Bombus terrestris* and *B. impatiens*, to regulate protein and lipid intake. We restricted *B. terrestris* adults to single synthetic diets varying in protein:lipid ratios (P:L). The bees overate protein on low fat diets and overate lipid on high fat diets to reach their targets of lipid and protein respectively. The bees survived best on a 10:1 P:L diet; the risk of dying increased as a function of dietary lipid when bees ate diets with lipid contents greater than 5:1 P:L. Hypothesizing that P:L intake target of adult worker bumble bees was between 25:1-5:1, we presented workers from both species unbalanced but complementary paired diets to determine if they self-select their diet to reach a specific intake target. Bees consumed similar amounts of proteins and lipids in each treatment and averaged a 14:1 P:L for *B. terrestris* and 12:1 P:L for *B. impatiens*. These results demonstrate that adult worker bumble bees select foods that provide them with a specific ratio of P:L. These P:L intake targets could affect pollen foraging in the field and help explain patterns of host-plant species choice by bumble bees.

Introduction

Bee population declines are linked with many interacting factors associated with anthropogenic land-use intensification (Goulson et al., 2015; Ollerton et al., 2014). Intensification often dramatically reduces host-plant abundance and diversity, which may lead to nutritional stress for some bee species (Biesmeijer et al., 2006; Carvell et al., 2006; Potts et al., 2010). Bees depend entirely on floral resources for nutrition, but nutritional quality and quantity of nectar and pollen differ widely among plant species (Nicolson et al., 2007; Roulston and Cane, 2000). Differences in resource quality can have direct effects on bee development, reproduction, immunocompetence, resilience to stress, and survival (Vaudo et al., 2015). Therefore, if bees are limited to suboptimal resources within their foraging range, then adults, developing larvae, and colonies could suffer negative health consequences. To address the problem of nutritional deprivation in the landscape, it is crucial to develop a comprehensive understanding of the nutritional requirements of bees.

Bees obtain their macronutrients (carbohydrates, proteins, and lipids) from both nectar and pollen. Macronutrients are considered the most important nutrients required for fitness and survival, and obtaining them is considered a main driver of foraging strategies of animals (Behmer, 2009). Bees primarily obtain carbohydrates from nectar to fuel energetically costly foraging efforts, and adults cannot survive without a continuous carbohydrate source (Brodschneider and Crailsheim, 2010). Bees obtain proteins and lipids from pollen. Differences in protein in bee diets can influence adult reproduction, physiology, and immunity, and larval development (Alaux et al., 2010; Cardoza et al., 2012; Di Pasquale et al., 2013; Génissel et al., 2002; Human et al., 2007; Li et al., 2012; Tasei and Aupinel, 2008a). Lipids fulfill many functions in insects; for bees, they play important roles in production of cuticular hydrocarbons and wax, behavioral maturation in adults (through the reduction in lipid stores), diapause, learning, and development of glands that produce brood food (Canavoso et al., 2001; Fliszkiewicz and Wilkaniec, 2007; Toth et al., 2005). Essential sterols obtained exclusively from pollen are precursors for molting hormone, which is essential for larval development (Feldlaufer et al., 1986; Roulston and Cane, 2000; Vanderplanck et al., 2014). Moreover, the lipid-dominant pollenkitt on the exterior of pollen is an important discriminative stimulus and phagostimulus of pollen for bees (Dobson and Bergström, 2000; Pacini and Hesse, 2005).

Although bees can obtain protein and lipids from most pollen sources, the nutritional quality and quantity of these resources varies across plant species. Pollen protein (including essential amino acids) and lipid (including essential fatty acids and sterols) concentrations vary considerably among plant species (pollen contains ~2-60% protein and ~2-20% lipid; (Roulston and Cane, 2000). Inequality of nutrients among plant species suggests that bees may prefer certain species whose resource quality meets their nutritional demands. Generalist bee species, such as *Bombus terrestris* (Hymenoptera: Apidae) in Europe, North Africa, and and *B. impatiens* in North America, which are active throughout the growing season, forage on a variety of different plant species during their lives. A handful of studies have suggested that bumble bees preferentially forage on flowers that have high sugar concentrations in nectar (Cnaani et al., 2006; Somme et al., 2014), and high protein (Cardoza et al., 2012; Hanley et al., 2008; Konzmann and Lunau, 2014) or amino acid and sterol content in pollen (Somme et al., 2014).

Although foraging bumble bees collect pollen mainly to feed developing larvae, adult workers eat pollen as well (Brodschneider and Crailsheim, 2010; Roulston and Cane,

2000), when they assess nutritional stores in pollen pots (Dornhaus and Chittka, 2005), while they feed pollen to larvae (Pereboom, 2000; Pereboom et al., 2003), or when they eat pollen to develop their own ovaries for male-egg laying (Amsalem et al., 2015; Tasei and Aupinel, 2008a). Note that in three-worker queenless microcolonies, workers ate between 0.4-0.9g of pollen in the five days prior to egg laying, which would average ~25-60mg/pollen/day by worker egg-layers (Tasei and Aupinel, 2008a; Tasei and Aupinel, 2008b).

However, it remains to be determined the extent to which bumble bees selectively forage from floral resources to balance their diet and meet specific macronutrient intake targets. This could be an important skill, however, because nutritional intake can influence reproductive success. For example, *B. terrestris* microcolonies fed low protein pollen diets had higher larval abortion rates and those fed higher protein diets had higher larval weight (Tasei and Aupinel, 2008a). Indeed, recent studies demonstrated that *B. impatiens*–both when foraging for colonies with brood or isolated from brood–preferentially forage for pollen with high protein:lipid ratios and their consumption of pollen diets depended on protein and lipid concentrations (Vaudo et al., submitted). This indicates that bees are sensitive to nutritional components and ratios and exhibit nutrient regulation that affects their feeding behavior.

Many studies have demonstrated that insects regulate their consumption of food around optimal proportions of macronutrients in ways that reflect their age, somatic needs, and reproductive status (Behmer, 2009; Simpson and Raubenheimer, 1993; Simpson et al., 2004). The geometric framework (GF) for nutrition is a method for examining the mechanisms and constraints that govern how animals regulate feeding to achieve specific macronutrient optima, or "intake targets." It employs an approach wherein individuals self-select diets or alter food consumption when confined to diets comprising specific ratios of macronutrients (Raubenheimer and Simpson, 1999; Simpson and Raubenheimer, 1993). This approach has made it possible to characterize simultaneous regulation of protein and carbohydrate in arthropods, fish, reptiles, birds, and mammals (Behmer, 2009; Simpson and Raubenheimer, 1997; Simpson and Raubenheimer, 2001; Simpson and Raubenheimer, 2012).

The GF has been successfully used to characterize nutrient balancing for protein and carbohydrate in worker honey bees (Altaye et al., 2010; Paoli et al., 2014; Pirk et al., 2009) and bumble bees (Stabler et al., 2015). Workers, especially foragers, have a high demand for carbohydrates, as reflected in their measured intake targets (for bumble bees, this is ~1:150 protein:carbohydrate or P:C ratio). Moreover, their tolerance of dietary protein (or essential amino acids) is relatively low, as they have reduced survival when forced to ingest surplus protein (Altaye et al., 2010; Paoli et al., 2014; Pirk et al., 2009; Stabler et al., 2015).

None of the previous studies using the GF have tested whether bees or other social insects regulate their dietary intake of fats. Indeed, the few studies that have investigated protein and fat regulation in insect herbivores have been limited to lepidopteran larvae, but were not clear assessments using the GF of simultaneous regulation of protein and fat. The tobacco hornworm (*Manduca sexta*) did not regulate lipid intake but preferred high fat diets (Thompson and Redak, 2005). Gypsy moth (*Lymantria dispar*) males switched from high-protein to high-fat diets in later instars, potentially to store energy for flight for adulthood (Stockhoff, 1993). In contrast, arthropod predators clearly regulate both protein and fat

simultaneously. For example, the ground beetle *Agonum dorsale*, when reared on food deficient in protein or fat adjusts its consumption of complementary foods to meet an intake target (Mayntz et al., 2005). Furthermore, the intake target of *A. dorsale* was biased toward lipid following diapause, but switched to a protein-biased target as it aged (Raubenheimer et al., 2007). Similar to *A. dorsale*, the wolf spider *Pardosa prativaga* was able to regulate its diet by eating flies that complemented a previous diet that was higher in protein or fat (Mayntz et al., 2005), and also overate protein on lipid poor diets to reach an intake target for lipid (Jensen et al., 2011).

Here, we use the GF methodology to test and measure regulation of protein and lipid intake in bumble bee foragers of two species, *B. terrestris* and *B. impatiens* (both important crop pollinators and commercially available in their respective geographic range [Velthuis and van Doorn, 2006; (Amsalem et al., 2015; Velthuis and van Doorn, 2006)]). In our first experiment, we restricted *B. terrestris* individuals to single synthetic diets differing in P:L ratios that spanned the realistic and extreme possibilities found in pollen, and measured their food consumption and survival. Then to determine if the two species indeed regulate protein and lipids to a specific intake target, we presented individual *B. terrestris* and *B. impatiens* two diets differing in their P:L ratios, while providing a separate and constant carbohydrate source. We expected that the species would regulate their P:L intake to a target at which they survived best. Furthermore, given the importance of P:C regulation in insects, we expected that the bumble bees would defend a specific P:C intake target in both experiments. Our results characterize the specific macronutrient requirements of these two species and provide insights into the ability of bumble bees to regulate lipids in their diet, suggesting nutritional quality may drive pollen foraging preferences.

Methods

General bee rearing conditions

We purchased mature research colonies of *Bombus terrestris* ("Single P:L diet assay" and "Paired P:L diets assay") and *B. impatiens* ("Paired P:Ls diet assay") from Koppert Biological Systems (Havervill, Suffolk, UK for *B. terrestris*; Howell, MI, USA for *B. impatiens*). Each colony contained approximately 100 workers and the natal queen. During the course of the study, we stored colonies at ambient temperatures and provided them sugar water *ad libitum*. For each assay, we collected foragers as they exited their colonies and placed individual bees in their own 11 × 11 × 10-cm plastic cages kept in a 24-hr dark incubator at 28°C and 40% humidity. We provided all diets to bees in 2-mL microcentrifuge tubes with four holes drilled in the tube from which the bees could feed. The tubes were suspended halfway up and at opposite sides of each cage such that the bees could perch on the tube and feed through the holes.

Single P:L diet assay

Individual forager bumble bees (15 bees/treatment, 4 colonies) were given access to food tubes containing 0.5 M sucrose solution or 0.5 M sucrose solution containing a specific protein:lipid ratio (P:L). We tested eight different dietary ratios of P:L (Protein-only, 50:1, 25:1, 10:1, 5:1, 1:1, 1:5, and 1:10; Table 1). We chose these particular P:L diets to include possible ranges of P:L ratios in pollen (Roulston and Cane, 2000) as well as values outside of the reported range of P:L in pollen. Nutrient sources were sucrose (Sigma-Aldrich, St. Louis, MO, USA) for carbohydrates, casein sodium salt from bovine milk (Sigma-Aldrich) for protein, and soy lecithin (Optima Health & Nutrition, Bradford, UK) for lipids, which contains essential fatty acids (32% ω-6/linoleic acid, 4% ω-3/alpha-linolenic acid).

Experiments lasted seven days, and we replaced each food tube daily. We weighed food tubes each day prior to placement in the cage and 24 hr later. Cages with three tubes of each diet with no bees served as controls to measure evaporation rate for each diet. Amounts of solution (g) consumed by bees were adjusted by the mean amount of solution that had evaporated from the "control" cages prior to analysis. We calculated the mass of each nutrient (carbohydrate, protein, lipid) consumed from the total mass consumed from each diet tube each day. We measured the thorax width of each individual bee as a covariate in data analyses to control for the effect of size on diet consumption. We recorded the number of days each bee survived in the assay with a maximum of seven days.

Paired P:L diets assay

To test our hypothesis that bumble bee intake targets lie within the 25:1-5:1 P:L range (see "Results-Single P:L diet assay"), we measured survival and nutrient consumption of *B. impatiens* and *B.terrestris* foragers presented with paired P:L diets encompassing this range. As in the "Single P:L diet assay," we collected *B. impatiens* and *B. terrestris* foragers as they exited their colonies and caged them individually (20 bees/treatment; 2 colonies for each species).

For each treatment, we provided a bee with one of four paired P:L diets and with a sucrose-only food tube. These diet pairings were: 1) 25:1 and 5:1, 2) 50:1 and 5:1, 3) 75:1 and 5:1, and 4) 100:1 and 5:1 P:L (diets prepared as above; Table 1). We measured daily consumption of each diet and nutrient (accounting for evaporation rate) and survival of bees over seven days (see "Single P:L diet assay"). Prior to placement in cages, we cold anaesthetized and weighed foragers to use their weight as a covariate in data analyses to control for effects of size on diet consumption (note thorax width and bee weight are correlated (Stabler et al., 2015)], and we measured thorax width in the "Single P:L diet assay").

Statistical analysis

Single P:L diet assay

We conducted survival analyses with Cox-regression proportional hazards, and used the Protein-only treatment as reference to determine the effect of adding lipid to the diet on bee survival. To determine whether bumble bees ate randomly among diet sources, we analyzed differences in daily consumption of diet sources among treatments by 2-way ANOVA and *post-hoc* Tukey-HSD pairwise comparisons with treatment, diet source (treatment diet or sucrose-only), and the interaction of treatment and diet source as independent variables and thorax width as a covariate. To analyze differences in daily consumption of nutrients among treatments, we used MANCOVA with *post-hoc* Tukey-HSD pairwise comparisons with nutrient (carbohydrate, protein, or lipid) as the dependent variable and thorax width as a covariate. Finally, for bees that survived on the diets for all seven days, we analyzed differences in cumulative consumption of carbohydrate, protein, and lipid with MANCOVA and *post-hoc* Tukey-HSD pairwise comparisons with nutrient (carbohydrate, protein, or lipid) as the dependent variable and thorax width as a covariate. After reviewing the data, it was apparent that there were differences in amounts of nutrients consumed between bees that died and survived in the 1:10 P:L treatment. We compared their cumulative consumption of nutrients on day three, using MANOVA and *post-hoc* t-tests for each nutrient.

Paired P:L diets assay

We analyzed differences in survival among treatments with the Kaplan-Meier test. To determine daily differences in mass of diets consumed among treatments, we conducted 2-way ANOVA and *post-hoc* Tukey-HSD pairwise comparisons, using treatment, diet source (5:1, treatment diet, and sucrose-only), and the interaction of treatment and diet source as independent variables with colony and bee weight as covariates. Finally, for bees that survived all seven days, we analyzed cumulative nutrient consumption among treatments with MANCOVA with *post-hoc* Tukey-HSD pairwise comparisons with nutrient (carbohydrate, protein, or lipid) as the dependent variable and colony and bee weight as covariates. If consumption of each nutrient among treatments was similar, we could conclude that the bumble bees were regulating their nutrients equally. We determined P:C and P:L ratios consumed by bees using the average cumulative consumption of each treatment. All statistical analyses were conducted with JMP Pro v.12 (SAS Institute; SPSS Statistics [IBM] was used for Cox-regression).

Results

Single P:L diet assay

For seven days, we fed *B. terrestris* foragers with sucrose only and one of the P:L diets. The bees consumed similar quantities of total food each day across treatments ($F_{7,1321}$ = 1.99, P = 0.053), except that foragers in the "protein only" treatment ate more each day than bees on the high fat 1:5 P:L treatment at $P < 0.05$ (Figure 1). Bees differed in the relative amounts of each diet (treatment diet versus sucrose only) consumed (treatment x solution; $F_{7,1321}$ = 16.0, $P < 0.001$) (Figure 1). Notably, bees consumed much less of the treatment diet than sucrose-only diet in the highest lipid treatments (1:5, 1:10 P:L) (Figure 1).

The only significant difference in daily consumption of carbohydrates was between protein-only and 1:5 treatments ($F_{8,666}$ = 5.32, $P < 0.001$; Table 2), but bees across treatments differed significantly in amounts of protein and lipid consumed (MANCOVA: $F_{21,1640}$ = 13.7, $P < 0.001$). Bees on the highest fat diets (1:5 and 1:10 P:L) consumed much less protein than the other treatments ($F_{8,663}$ = 14.7, $P < 0.001$; Table 2), suggesting that they ceased eating the diet after having reached or exceeded their lipid intake target, and therefore did not reach their protein target. Finally, bees across treatments differed significantly in amounts of lipids consumed; specifically, bees consumed more lipids as lipid content of the treatment diet increased ($F_{7,573}$ = 20.4, $P < 0.01$; Table 2).

For the bees that survived all seven days of the experiment, there were significant differences among treatments in cumulative amount of nutrients consumed (MANCOVA: $F_{21,164}$ = 5.03, $P < 0.001$; Figure 2). Though there were no differences in cumulative carbohydrates consumed across treatments ($F_{7,59}$ = 1.13, P = 0.36; Figure 2a,c), bees on different diets consumed significantly different amounts of cumulative protein and lipids over seven days; similar to the daily consumption data, bees on the highest lipid treatments (1:5 and 1:10 P:L) consumed significantly less protein ($F_{7,59}$ = 3.86, P = 0.002; Figure 2a,b).

For cumulative lipids consumed, surviving bees in the 1:10, 1:5, and 1:1 treatments consumed significantly more lipids than bees on the remaining treatments ($F_{7,59}$ = 10.2, $P <$ 0.001, Figure 2b,c). Furthermore, bumble bee foragers consumed on average ~3.5mg protein on 1:1, 5:1, 10:1 and 25:1 P:L diets, while consuming ~5.1mg protein on the 50:1

P:L diet ($F_{1,59}$ = 2.86, P < 0.1), suggesting that bees compensated for low lipids by overeating the 50:1 diet to reach an intake target for lipid (Figure 2b). These data also indicate that *B. impatiens* foragers regulated their protein intake eating similar amounts of proteins (~4.0mg) except on the highest lipid diets of 1:5 and 1:10 (~0.6mg).

Bombus terrestris foragers were more likely to die when they consumed diets high in lipid (Table 3). Additionally, there tended to be higher mortality of bees over seven days as lipid content of diets increased or decreased relative to the 10:1 P:L treatment (Table 3). Although bees in the high fat treatment (1:5 P:L) appeared to survive well in the first days of the study, their mortality increased sharply over the remainder of the week and ended with the second highest mortality and a nearly equal hazard ratio (Figure 1, Figure 3). Interestingly, by day three on the 1:10 P:L diet, surviving bees had eaten significantly less of their treatment diet (protein and lipid) than those bees that died (t_{14} = 2.29, P < 0.02), but living and dead bees ate equal amounts of carbohydrates (t_{14} = 0.64, P = 0.27; Figure 4). These data suggest that high lipid consumption leads to toxicity and increased mortality.

Bombus terrestris foragers 1) overate lipids to defend their protein intake, 2) had increased mortality as lipid content of diets increased or decreased away from 10:1 P:L), and 3) increased protein consumption on the 50:1 P:L diet to potentially defend a lipid target. Therefore, we hypothesized that the bumble bees' P:L intake target lies within the 25:1 – 5:1 range. We performed a "Paired P:L diets assay" to identify the actual intake target for P:L of *B. terrestris*, and to compare it to the intake target of *B. impatiens*.

Paired P:L diets assay

For seven days, we fed *Bombus impatiens* and *B. terrestris* workers a single sucrose-only diet, a 5:1 P:L diet, and a complementary treatment P:L diet (25:1, 50:1, 75:1, or 100:1). Each diet pairing of 5:1 P:L and treatment P:L created a protein and lipid nutrient space encompassing the hypothesized P:L intake target. The bees consumed significantly different amounts of total food consumed across treatment (*B. impatiens*: $F_{3,1446}$ = 5.65, P < 0.001; *B. terrestris*: $F_{3,1178}$ = 4.75, P < 0.003), diet sources (*B. impatiens*: $F_{2,1446}$ = 23.7, P < 0.01; *B. terrestris*: $F_{2,1178}$ = 30.7, P < 0.001), and the relative amounts of each diet source consumed among treatments (treatment × diet source interaction: *B. impatiens*: $F_{6,1446}$ = 3.55, P = 0.0017; *B. terrestris*: $F_{6,1178}$ = 3.31, P = 0.003; Figure S1). Importantly, daily consumption differed between treatment diet (25:1, 50:1, 75:1, 100:1) and 5:1 diet for both *B. impatiens* and *B. terrestris*, indicating that these diets were not being consumed randomly (Figure S1).

Surviving *B. impatiens* and *B. terrestris* foragers regulated their carbohydrate, protein, and lipid intake, consuming equal amounts of the three macronutrients and total nutrients across treatments (carbohydrate: *B. impatiens:* $F_{3,52}$ = 2.20, P = 0.10; *B. terrestris*: $F_{3,47}$ = 1.50, P = 0.23; protein: *B. impatiens*: $F_{3,52}$ = 2.63, P = 0.06; *B. terrestris*: $F_{3,47}$ = 1.02, P = 0.39; lipid: *B. impatiens:* $F_{3,52}$ = 1.78, P = 0.16; *B. terrestris*: $F_{3,47}$ = 0.02, P = 0.99; total nutrients: *B. impatiens*: MANCOVA: $F_{9,122}$ = 1.35, P = 0.22; *B. terrestris*: MANCOVA: $F_{9,110}$ = 1.07, P = 0.39; Table 4, Figure 5, Figure S2). Therefore, *B. impatiens* and *B. terrestris*, foragers regulated their P:L intake to within our hypothesized range, averaging 12:1 P:L for *B. impatiens* and 14:1 P:L for *B. terrestris* (Table 4, Figure 5, Figure S2). The P:C intake targets regulated by both species averaged 1:85 P:C for *B. impatiens* and 1:67 P:C for *B. terrestris* (Table 4, Figure 5,Figure S2). Both bee species survived equally well

on the various diets (*B. impatiens*: $\chi^2 = 3.98$, df = 3, $P = 0.26$; *B. terrestris*: $\chi^2 = 0.39$, df = 3, $P = 0.94$; Figure S1).

Discussion

Our experiments revealed that *B. terrestris and B. impatiens* regulated their protein and lipid intake to an average of 14:1 and 12:1, respectively, with *B. terrestris* preferring a diet slightly lower in fat than *B. impatiens*. Also, bees limited to diets high in lipids had increased risk of mortality (Table 3, Figure 3). Taken together, this study provides the first evidence that pollinators (specifically *Bombus* spp. bees) regulate fat intake. Coupled with our previous study that demonstrated that bumble bees foraging preferences were significantly correlated with protein:lipid ratios in pollen (Vaudo et al., submitted), these results suggest that pollinators adjust their foraging to achieve specific macronutrient targets.

The protein and lipid regulation of bumble bee adults appears more similar to predaceous arthropods than herbivorous ones. *Manduca sexta* caterpillars, within a similar design as our "Paired P:L diets assay," failed to regulate lipid intake but preferred diets high in fat (Thompson and Redak, 2005). In contrast, both *B. terrestris* and *B. impatiens* workers regulated their intake of fat, and preferred diets with specific P:L ratios. This difference is likely due to the vastly different life histories between lepidopteran larvae, which are typically constrained to specific food sources, and hymenopteran adults, which can forage among many sources. Both predaceous species (i.e., the wolf spider and ground beetle) ate protein excessively on low fat diets, apparently to reach a lipid intake target (~4:1 P:L for wolf spider; or ~2:1 P:L in for ground beetle; [(Jensen et al., 2011; Mayntz et al., 2005; Raubenheimer et al., 2007)]). In our work, *B. terrestris* generally ate more protein on the low-fat diet (50:1 P:L) than the other treatments, including those that provided only protein. This behavior indicates that workers may also overeat protein to reach their lipid intake; indeed, lipid intake did not differ across the groups fed 50:1, 25:1, 10:1 and 5:1 diets. Finally, the web building spider *Stegodyphus lineatus* having no control over the nutrient composition of prey captured in its web selectively extracted dietary protein from prey based on previous feeding history (Mayntz et al., 2005). Few studies have shown that bee larvae assimilate pollen protein and lipids efficiently (Roulston and Cane, 2000), but it remains to be tested if the sedentary and dependent bee larvae can regulate their nutrient intake or if they are completely dependent upon adults to sense and select an appropriate diet for them.

In contrast to *A. dorsale,* the predatory ground beetle, which stopped eating when it reached its lipid intake target in high fat diets (Raubenheimer et al., 2007), *B. terrestris* overate lipid in high-fat diets (1:1, 1:5, and 1:10 P:L), potentially to reach their protein target. This overconsumption of lipid to reach a protein target may have led to increased mortality. For example, bees survived when they ate less of the high fat diet 1:10 P:L (Figure 4). And although the bees in the 1:5 P:L treatment ate significantly less of the treatment diet than the sucrose-only diet, their high lipid consumption in the first days of the study likely lead to their rapid death (Figure 1-3). Thus, surviving bees were able to eat enough to meet their nutritional needs, sense the toxicity of the diet, and cease feeding, while the others did not. What caused this individual variation in behavior remains to be determined; the bees used in this study were not age-controlled, and thus there may have been physiological differences associated with age, social status, or behavioral task.

The exact mechanism underlying the toxicity of high-fat diet consumption is unclear. One possibility is a deficiency in protein intake, though this seems unlikely because adult bees can survive quite well on sugar diets alone (Brodschneider and Crailsheim, 2010; Paoli et al., 2014). Another possibility is that high intracellular concentrations of lipids is toxic; with too much fat in the diet, insufficient amounts could be converted into storage triacylglycerols or expelled from the body (Canavoso et al., 2001). The ratio of the essential fatty acids ω-6:ω-3 in our diets was 8:1. Excessive amount of ω-6 in diets (i.e., ω-3 deficiency) has been linked to chronic diseases in humans (Simopoulos, 2002; Simopoulos, 2008), and impaired learning and physiology in honey bees (Arien et al., 2015). Moreover, high polyunsaturated fatty acids (including essential fatty acids) in the diet may lead to lipid peroxidation and cell damage, and cell membrane composition has been linked to the vast difference in maximum lifespan between honey bee queens (highly monounsaturated) and workers (highly polyunsaturated) (Haddad et al., 2007).

Although not the focal test of the study, bees consistently ate similar amounts of carbohydrates across all treatments in both the single and paired diets assays. The protein:carbohydrate ratio (P:C) intake target averaged 1:69 P:C for *B. terrestris* and 1:85 for *B. impatiens*. These are carbohydrate-biased as expected, but significantly lower than previously found for *B. terrestris* in studies that did not include lipid intake (Stabler et al., 2015). It may be that the energy otherwise obtained from carbohydrates (e.g., for flight) was metabolized from the lipids ingested in our study, resulting in reduced feeding from the sucrose only solution (Canavoso et al., 2001).

The results of this study may provide insights into the nutritional ecology of foraging bees. First, the high requirement of carbohydrates for bumble bees is likely met by nectar foraging, which explains the attraction of bees to flowering species with high volumes and high sugar concentrations of nectar (Cnaani et al., 2006; Somme et al., 2014). Because carbohydrate concentrations in pollen are fairly low, bees appear to forage on pollen to meet their protein and lipid needs. Our results suggest that bumble bees forage to obtain pollen that allows them to achieve a dietary ratio of 12:1 - 14:1 P:L. Notably, in previous work, *B. impatiens* exponentially increased their foraging rates to the plant species with the 5:1 P:L ratio; moreover, using assays with caged bees and nutritionally modified pollen, *B. impatiens* was most attracted to 5:1 and 10:1 P:L diets (Vaudo et al. submitted). These preferred diets matched the results from the current study, which found that bumble bee workers survive best on, and regulate their diets to, approximately 10:1 P:L. Because the pollen P:L ratio in the previous work (Vaudo et al., submitted) had an upper limit of 5:1, it is unclear whether bumble bees can reach 10:1 P:L from pollen in the field. Even if the target P:L ratio cannot be met, the predisposition of bumble bees to prefer protein-biased pollen may explain host-plant preferences in natural environments (Cardoza et al., 2012; Hanley et al., 2008; Somme et al., 2014); Vaudo et al., submitted).

It must be noted that in the current study, we evaluated feeding preferences of isolated bumble bee workers. It is unknown whether bumble bee foragers adjust their nutritional and foraging preferences depending on the colony needs, and specifically presence of larvae (Hendriksma and Shafir, 2016). Information on pollen quality and its availability in the colony may be accessible to workers via pollen pots (Dornhaus and Chittka, 2005; Kitaoka and Nieh, 2008) allowing the colony to make informed foraging decisions. Indeed, in our other studies, attraction of bumble bees to pollen with 5:1 and 10:1 P:L ratios remained

intact for both bees foraging for colonies or foraging in cages, suggesting that these dietary preferences are conserved across a variety of scenarios (Vaudo et al., submitted).

Our study demonstrated that two bumble bee species, which occupy separate geographic ranges, regulate their protein and fat intake and converge on similar intake targets, likely due to their relatedness, similar life histories, and foraging behavior (Amsalem et al., 2015). Notably, their ability to regulate protein and lipids is more similar to arthropod predators than herbivores, perhaps because pollen is more nutritionally similar to prey (versus leaf tissue) with high protein and lipid concentrations (Jensen et al., 2011; Raubenheimer et al., 2007). Because bees are a monophyletic group evolved from predatory wasps (Danforth et al., 2013), it is likely that bees maintained their protein and lipid biases when making the transition to pollen feeding. There may be taxa-specific P:L intake targets across bee families, genera, or species that could explain the patterns of foraging behavior and pollen preferences observed among host-plant species in field-based studies (Behmer and Joern, 2008). Knowing these particular intake targets can guide decisions for targeted habitat restoration protocols by matching nutritional intake targets of bee species to pollen quality of host-plant species (Vaudo et al., 2015).

List of Symbols and Abbreviations
GF – Geometric framework for nutrition
P:C – Protein to carbohydrate ratio
P:L – Protein to lipid ratio

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Competing Interests
No competing interests declared.

Author Contributions
Vaudo: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing – Original Draft Preparation, Writing – Review & Editing, Visualization, Funding Acquisition
Stabler: Conceptualization, Methodology, Formal Analysis, Investigation
Patch: Conceptualization, Validation
Tooker: Conceptualization, Validation, Resources, Writing – Review & Editing
Grozinger: Conceptualization, Validation, Resources, Writing – Review & Editing, Funding Acquisition
Wright: Conceptualization, Methodology, Validation, Resources, Writing – Review & Editing, Funding Acquisition

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Figures

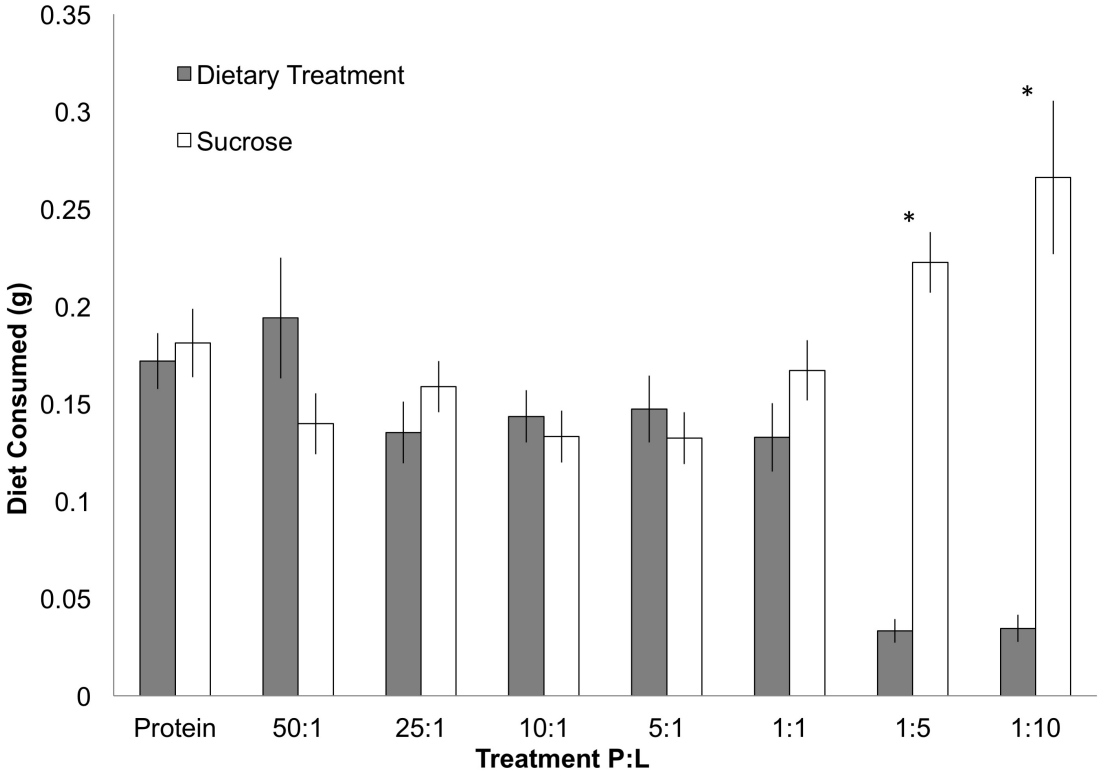


Figure 1. Mean (± SE) daily consumption of diets across treatments for *B. terrestris* foragers in "Single P:L diet assay." Treatments are represented by their protein:lipid (P:L) treatment diet ratio, including protein-only diets. Diets are represented as sucrose-only and diet associated with each treatment. Asterisks represent significant differences ($P < 0.05$) in diet consumed within treatment (N = 15 bees/treatment).

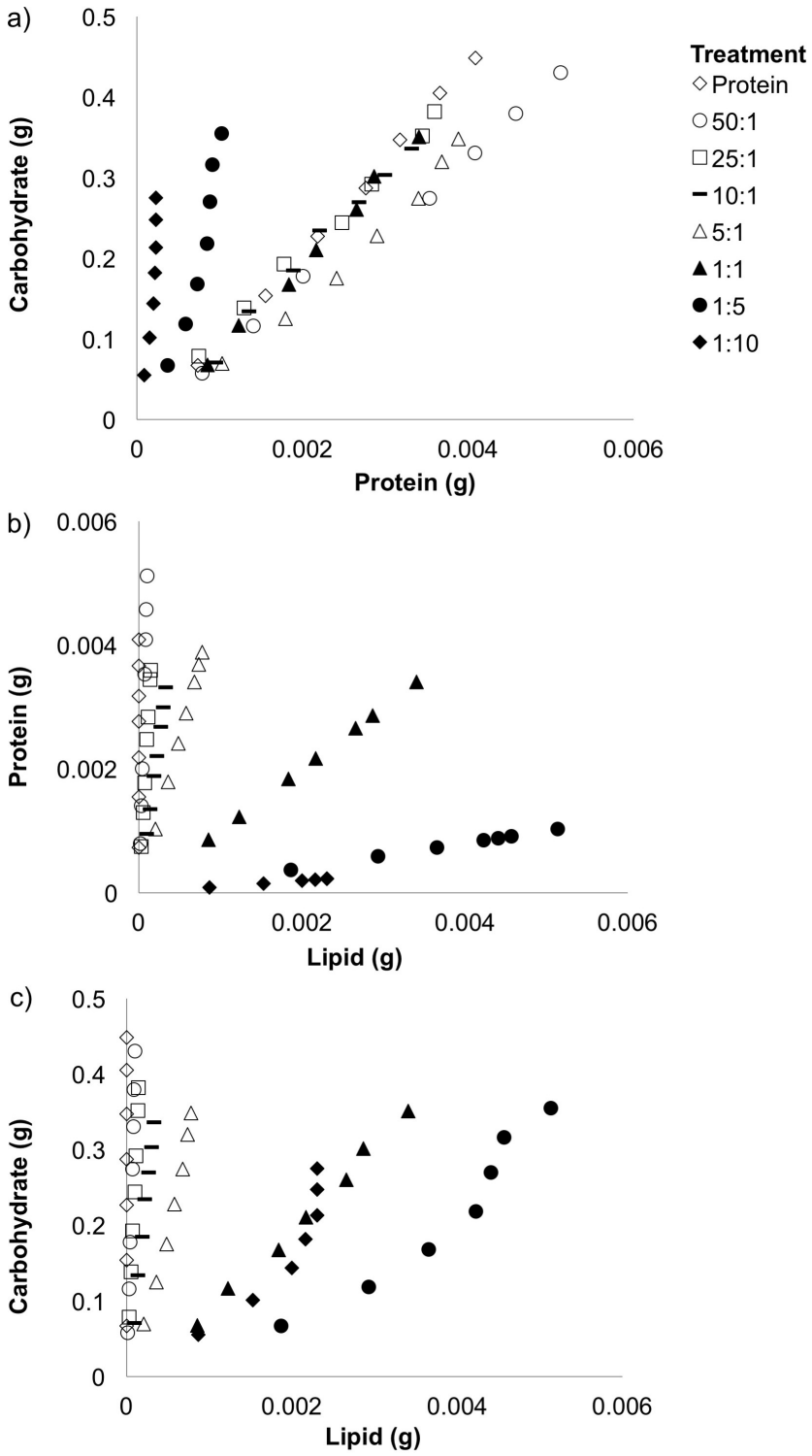


Figure 2. Nutritional arrays of *B. terrestris* foragers surviving seven days in "Single P:L diet assay." Treatments are represented by their protein:lipid (P:L) diet ratio, including protein-only diet. Markers of each treatment represent mean cumulative consumption of each nutrient for each successive day up to seven days forming daily trajectories. a) carbohydrate and protein array, b) protein and lipid array, c) carbohydrate and lipid array ($N_{Protein} = 10$, $N_{50:1} = 9$, $N_{25:1} = 9$, $N_{10:1} = 11$, $N_{5:1} = 10$, $N_{1:1} = 8$, $N_{1:5} = 7$, $N_{1:10} = 4$).

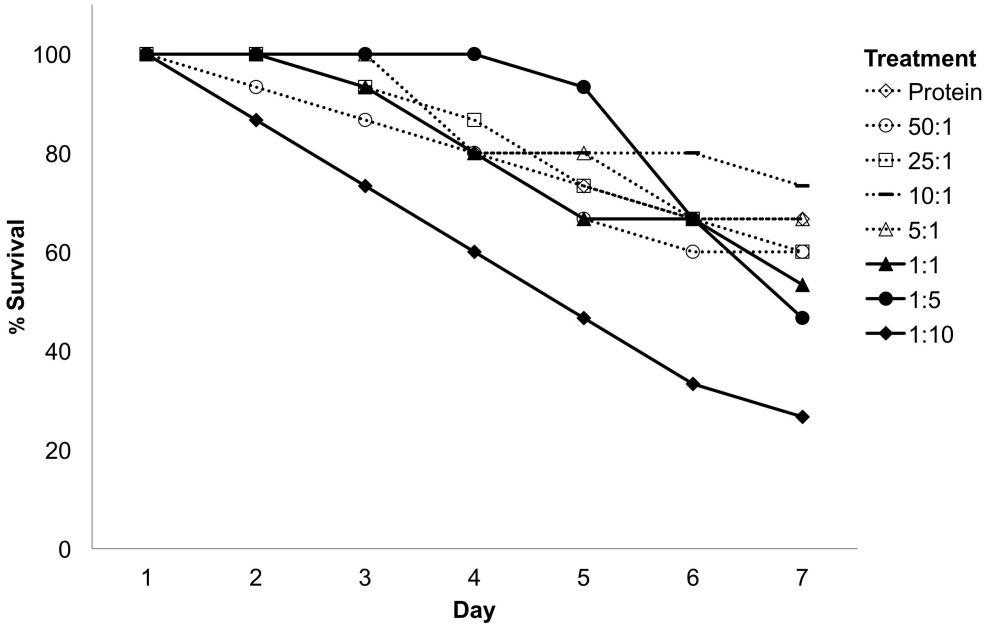


Figure 3. Survival curve of *B. terrestris* foragers in "Single P:L diet assay." Treatments are represented by their protein:lipid (P:L) treatment diet ratio, including protein-only diet. Note that mortality increased as the lipid content of the diets increased (N = 15 bees/treatment).

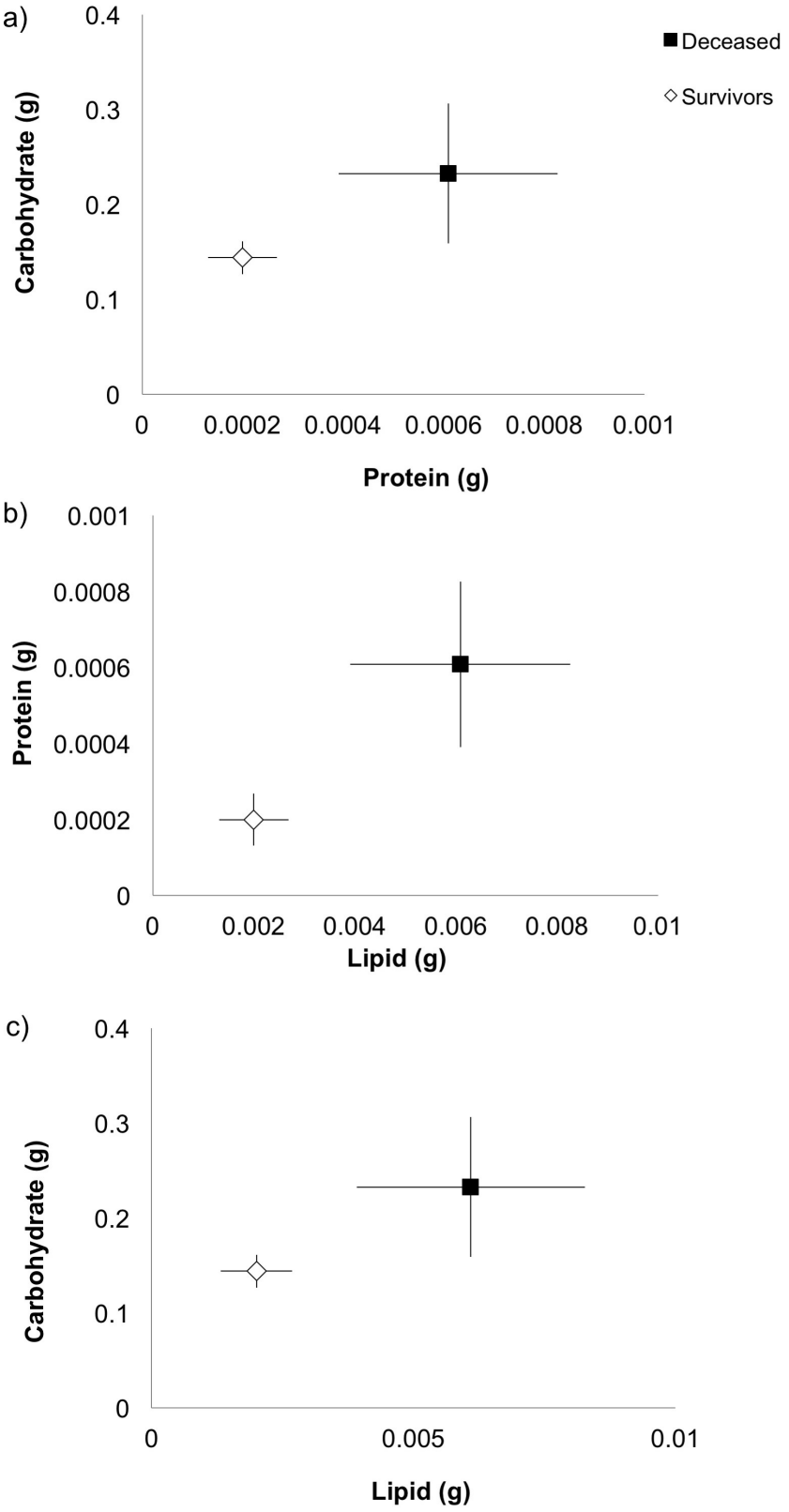


Figure 4. Mean (± SE) cumulative consumption of nutrients by deceased (N = 11) and surviving (N = 4) *B. terrestris* foragers in 1:10 P:L treatment on Day 3 of "Single P:L diet assay": a) carbohydrate and protein, b) protein and lipid, c) carbohydrate and protein. Note that surviving bees ate significantly less protein and lipid than the deceased bees.

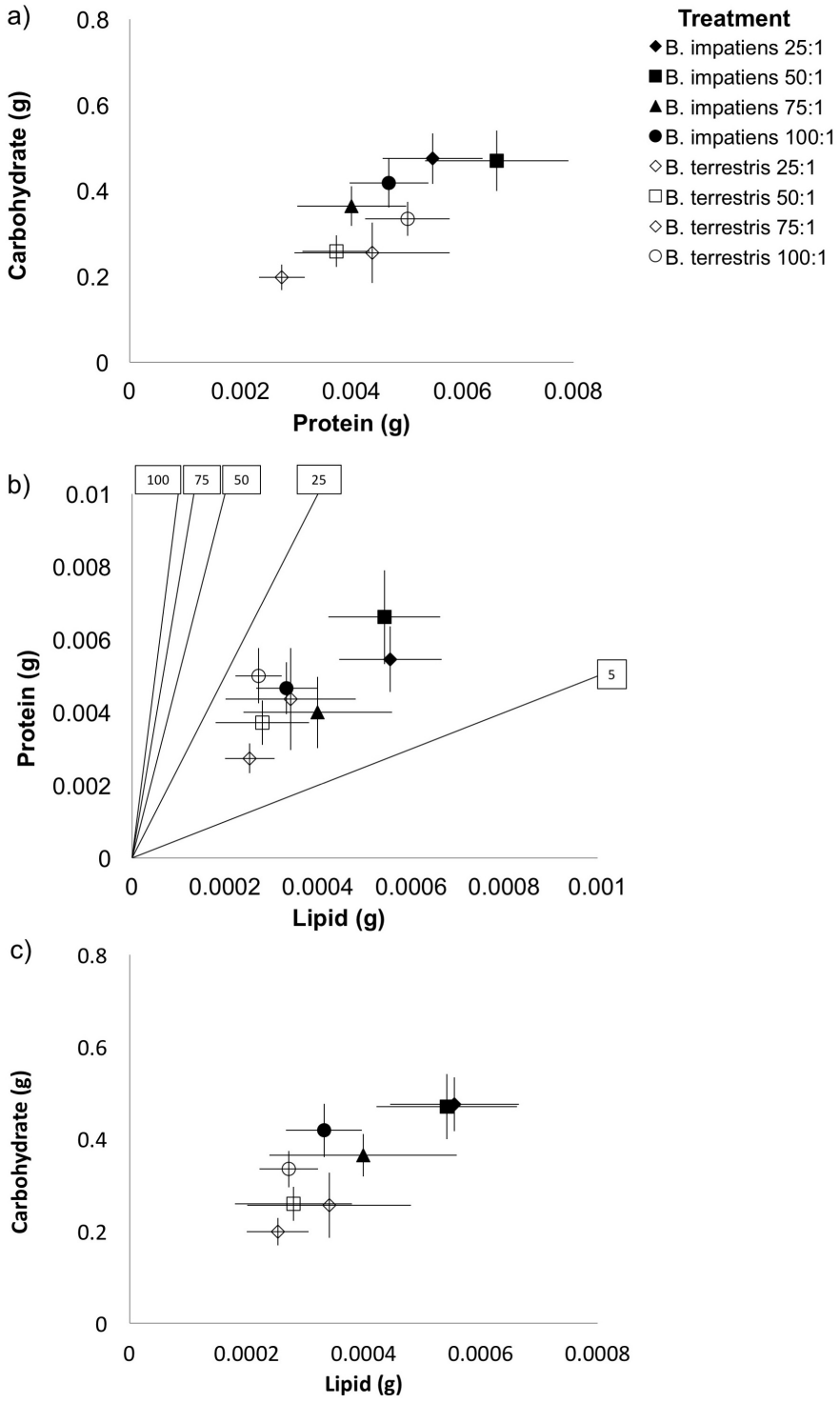
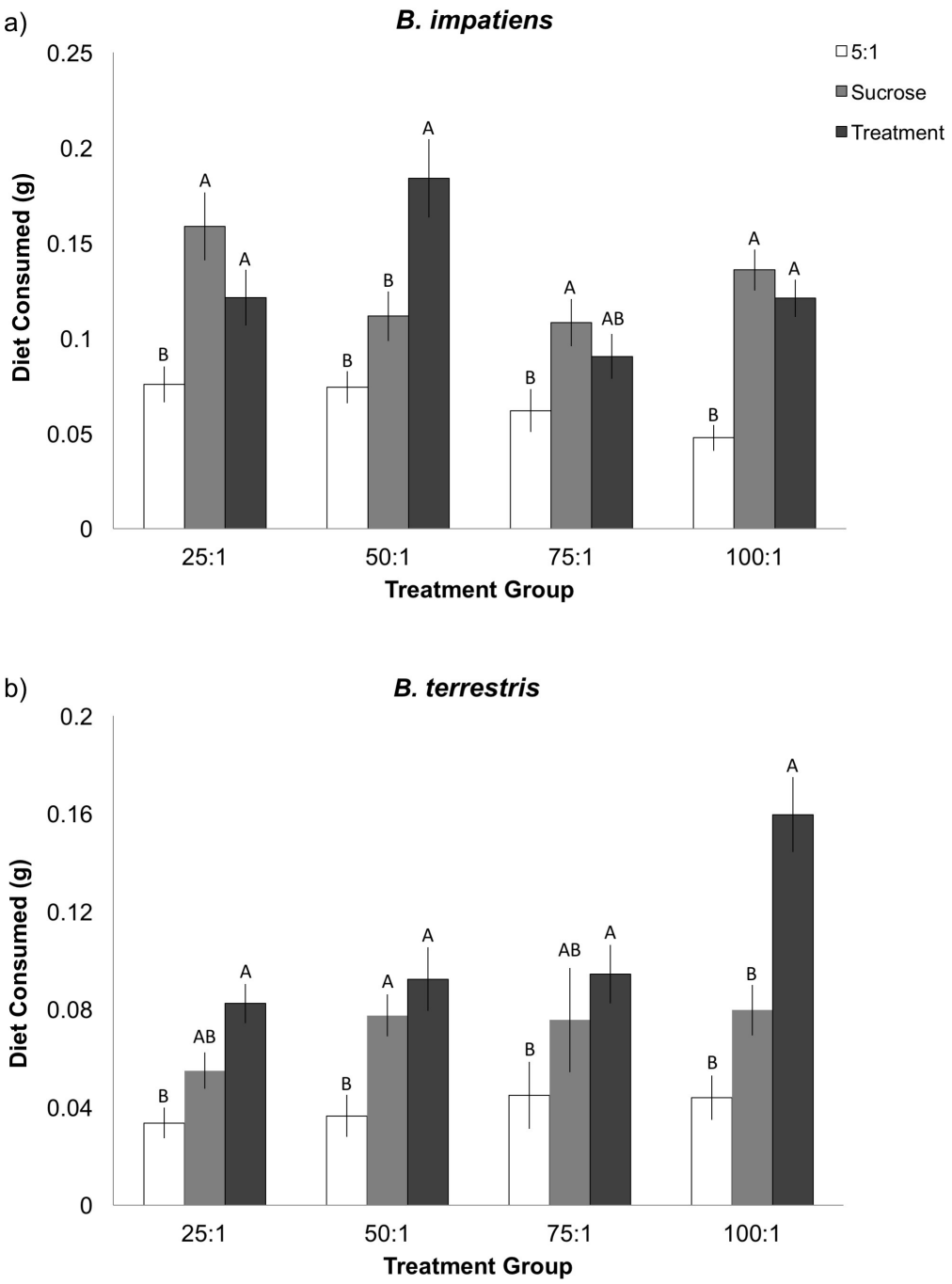
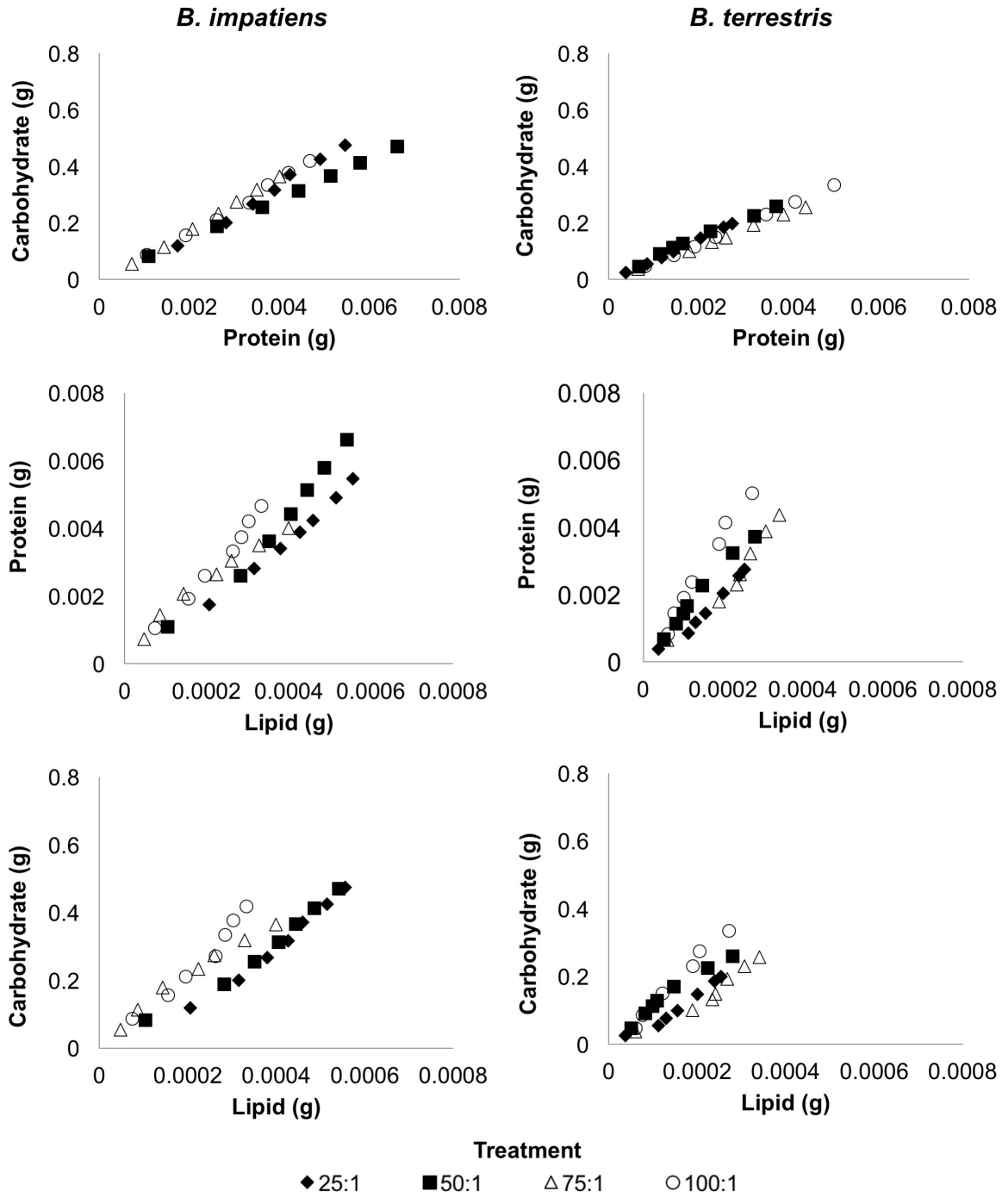


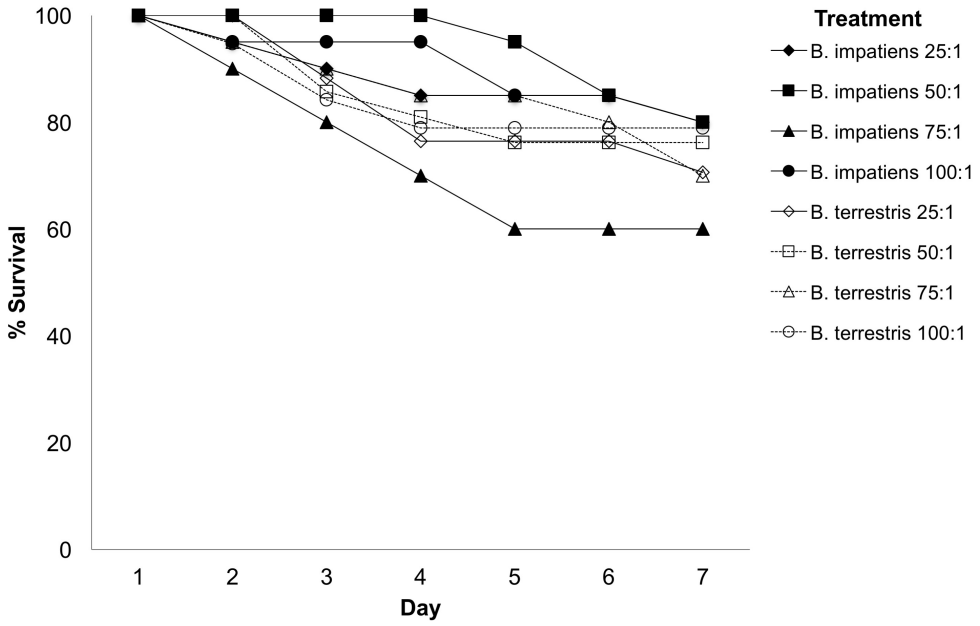
Figure 5. Mean (± SE) cumulative consumption nutrients of *B. impatiens* and *B. terrestris* foragers in "Paired P:L diets assay" that survived for seven days. Note for both species there were no significant differences in carbohydrate, protein, or lipid consumption across treatments. Treatments are represented by protein:lipid diet ratio (P:L) paired with 5:1 P:L diet: a) carbohydrate and protein, b) protein and lipid. Lines represent the different diet rails, emphasizing that across treatments all P:L intake targets lie within our expected 25:1-5:1 P:L range, c) carbohydrate and protein (*B. impatiens*: $N_{25:1} = 16$, $N_{50:1} = 16$, $N_{75:1} = 12$, $N_{100:1} = 16$; *B. terrestris*: $N_{25:1} = 12$, $N_{50:1} = 16$, $N_{75:1} = 14$, $N_{100:1} = 14$).



Supplementary Figure 1. Mean (± SE) daily consumption of diets across treatments for a) *B. impatiens* and b) *B. terrestris* foragers in "Paired P:L diets assay." Diets are represented as 5:1 P:L, sucrose-only, and the treatment P:L diet (25:1, 50:1, 75:1, and 100:1). Bars marked with different letters are statistically different ($P < 0.05$) within treatment (N = 20 bees/treatment).



Supplementary Figure 2. Daily trajectories of *B. impatiens* (a-c) and *B. terrestris* (d-f) in "Paired P:L diets assay." Treatments are represented by their protein:lipid diet ratio (P:L) paired with 5:1 P:L diet. Markers within each diet represent mean cumulative consumption of each nutrient for each successive day up to seven days: a,d) carbohydrate and protein trajectories, b,e) protein and lipid trajectories, c,f) carbohydrate and lipid trajectories (*B. impatiens*: $N_{25:1} = 16$, $N_{50:1} = 16$, $N_{75:1} = 12$, $N_{100:1} = 16$; *B. terrestris*: $N_{25:1} = 12$, $N_{50:1} = 16$, $N_{75:1} = 14$, $N_{100:1} = 14$).



Supplementary Figure 3. Survival curve of *B. impatiens* and *B. terrestris* foragers in "Paired P:L diets assay." Treatments are represented by their species and protein:lipid diet ratio (P:L) paired with 5:1 P:L diet (N = 20 bees/treatment).

Table 1. Diet recipes. Diets are represented by their protein:lipid (P:L) ratios or sucrose-only and protein-only diets. Sucrose was used as the carbohydrate source, soy lecithin was used as the lipid source, and casein as a protein source.

Nutrient source	**Sucrose-only**	**Protein-only**	**100:1**	**75:1**	**50:1**	**25:1**	**10:1**	**5:1**	**1:1**	**1:5**	**1:10**
Sucrose	171g	17.1g	17.1g	17.1g	17.1g	17.1g	17.1g	17.1g	17.1g	17.1g	17.1g
Casein	-	0.342g	0.342g	0.342g	0.342g	0.342g	0.342g	0.342g	0.342g	0.342g	0.342g
Lecithin	-	-	0.00342g	0.00456g	0.00685g	0.0137g	0.0342g	0.0685g	0.342g	1.71g	3.42g
H_2O	1000mL	100mL	100mL	100mL	100mL	100mL	100mL	100mL	100mL	100mL	100mL

Table 2. Mean (± SE) daily consumption (mg) of nutrients for *B. terrestris* foragers in "Single P:L diet assay." Treatments are represented by their protein:lipid (P:L) diet ratio, including protein-only diet. Means marked with different letters within each column are statistically different ($P < 0.05$).

Treatment	Carbohydrate	Protein	Lipid
1:10	50 ± 7 ab	0.12 ± 0.02 b	1.20 ± 0.23 a
1:5	44 ± 3 b	0.11 ± 0.02 b	0.57 ± 0.10 b
1:1	50 ± 4 ab	0.44 ± 0.06 a	0.44 ± 0.06 bc
5:1	47 ± 3 ab	0.50 ± 0.06 a	0.11 ± 0.012 cd
10:1	47 ± 3 ab	0.49 ± 0.05 a	0.05 ± 0.005 d
25:1	50 ± 3 ab	0.47 ± 0.05 a	0.02 ± 0.002 d
50:1	57 ± 5 ab	0.66 ± 0.11 a	0.01 ± 0.002 d
Protein-only	60 ± 4 a	0.60 ± 0.05 a	-

Table 3. Cox – regression of survival for *B. terrestris* foragers in "Single P:L diet assay." Treatments are represented by their protein:lipid (P:L) diet ratio, including protein-only diet. Protein-only diet (no lipid) was used as reference to test the effect of adding lipids to the diet. Note that likelihood of mortality (B) decreased for 10:1 treatment, and increased as the lipid content of the diet increased. Model: $\chi^2 = 10.52$, df = 7, p = 0.161

							95.0% CI for Exp(B)	
Treatment	**B**	**SE**	**χ^2**	**df**	**Sig.**	**Exp(B)**	**Lower**	**Upper**
Protein			9.667	7	0.208			
50:1	0.266	0.606	0.193	1	0.661	1.305	0.398	4.275
25:1	0.186	0.606	0.094	1	0.759	1.204	0.367	3.946
10:1	-0.256	0.671	0.146	1	0.703	0.774	0.208	2.884
5:1	-0.019	0.632	0.001	1	0.976	0.981	0.284	3.389
1:1	0.375	0.586	0.410	1	0.522	1.455	0.462	4.584
1:5	0.372	0.570	0.425	1	0.514	1.451	0.474	4.436
1:10	1.136	0.540	4.424	1	**0.035**	3.113	1.080	8.970

Table 4. Consumption (g; mean ± SE) by *B. impatiens* and *B. terrestris* foragers in the "Paired P:L diets assay" and protein:carbohydrate (P:C) and protein:lipid (P:L) intake ratios over seven days. Each treatment was paired with a 5:1 P:L diet. Within each species, there were no statistical differences in total carbohydrate, protein, or lipid consumed.

	Treatment	**Carbohydrate**	**Protein**	**Lipid**	**P:C**	**P:L**
B. impatiens	25:1	475 ± 58.5	5.46 ± 0.90	0.56 ± 0.11	1:87.01	9.84
	50:1	470 ± 70.2	6.62 ± 1.29	0.54 ± 0.12	1:71.05	12.22
	75:1	344 ± 46.7	3.84 ± 0.90	0.37 ± 0.15	1:89.55	10.49
	100:1	398 ± 51.9	4.34 ± 0.66	0.29 ± 0.06	1:91.69	14.83
B. terrestris	25:1	199 ± 29.5	2.74 ± 0.41	0.25 ± 0.05	1:72.41	10.83
	50:1	248 ± 36.1	3.47 ± 0.62	0.26 ± 0.09	1:71.39	13.29
	75:1	264 ± 65.4	4.09 ± 1.32	0.32 ± 0.13	1:64.61	12.98
	100:1	335 ± 39.5	5.01 ± 0.76	0.27 ± 0.05	1:66.86	18.40

Appendix J

Nutrient balancing of the adult worker bumblebee (*Bombus terrestris*) depends on the dietary source of essential amino acids

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RESEARCH ARTICLE

Nutrient balancing of the adult worker bumblebee (*Bombus terrestris*) depends on the dietary source of essential amino acids

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ABSTRACT

Animals carefully regulate the amount of protein that they consume. The quantity of individual essential amino acids (EAAs) obtained from dietary protein depends on the protein source, but how the proportion of EAAs in the diet affects nutrient balancing has rarely been studied. Recent research using the Geometric Framework for Nutrition has revealed that forager honeybees who receive much of their dietary EAAs from floral nectar and not from solid protein have relatively low requirements for dietary EAAs. Here, we examined the nutritional requirements for protein and carbohydrates of foragers of the buff-tailed bumblebee *Bombus terrestris*. By using protein (sodium caseinate) or an equimolar mixture of the 10 EAAs, we found that the intake target (nutritional optimum) of adult workers depended on the source and proportion of dietary EAAs. When bees consumed caseinate-containing diets in a range of ratios between 1:250 and 1:25 (protein to carbohydrate), they achieved an intake target (IT) of 1:149 (w/w). In contrast to those fed protein, bees fed the EAA diets had an IT more biased towards carbohydrates (1:560 w/w) but also had a greater risk of death than those fed caseinate. We also tested how the dietary source of EAAs affected free AAs in bee haemolymph. Bees fed diets near their IT had similar haemolymph AA profiles, whereas bees fed diets high in caseinate had elevated levels of leucine, threonine, valine and alanine in the haemolymph. We found that like honeybees, bumblebee workers prioritize carbohydrate intake and have a relatively low requirement for protein. The dietary source of EAAs influenced both the ratio of protein/EAA to carbohydrate and the overall amount of carbohydrate eaten. Our data support the idea that EAAs and carbohydrates in haemolymph are important determinants of nutritional state in insects.

KEY WORDS: Carbohydrate, Protein, Geometric framework, *Apis*, Bee, Forager

INTRODUCTION

Animals obtain essential amino acids (EAAs) by the consumption of plant or animal proteins. Proteins are digested into amino acid (AA) units, which are absorbed and then used to produce new proteins, generate ATP, make other amino acids or used as signals between cells. Because the need for AAs continues throughout an animal's lifespan, protein intake is actively regulated around a nutritional optimum that is determined by age, physiological state and reproductive capacity (Simpson and Raubenheimer, 2012). Animals regulate their protein intake by altering quantities of food eaten (Simpson et al., 2004) or by consuming a mixture of foods with the correct balance of protein and other macronutrients (Raubenheimer and Simpson, 1993; Simpson and Raubenheimer, 1993, 2012; Simpson et al., 2004). How the regulation of protein intake is accomplished by the body's ability to detect the need for essential amino acids (EAAs) is largely unknown (Morrison et al., 2012).

The protein source determines the proportion and types of AAs produced by its digestion (Boisen et al., 2000) and can affect macronutrient balancing (Lee, 2007; Altaye et al., 2010). The amount of protein consumed in the diet directly affects the concentration of free AAs in the blood/haemolymph (Zanotto et al., 1996; Abisgold and Simpson, 1988). For this reason, several authors have hypothesized that blood/haemolymph levels of AAs are a potential means by which the body detects AA nutritional sufficiency (Sanahuja and Harper, 1963; Peters and Harper, 1985; Simpson and Raubenheimer, 1993; Morrison et al., 2012; Solon-Biet et al., 2014). For example, haemolymph AA titre can directly influence feeding behaviour, as seen when injection with AA solutions reduces meal size and increases the time between meals in locusts (Abisgold and Simpson, 1988). Haemolymph EAA composition can also modulate gustatory sensitivity to AAs in taste neurons (Simpson and Simpson, 1992) and could interact with feeding circuits in the brain to regulate protein feeding. In mammals, neurons in the hypothalamus, which govern food intake and are sensitive to carbohydrate levels in the blood, also respond to specific AAs, including leucine (Karnani et al., 2011), and direct injection with AAs can reduce meal size (Jordi et al., 2013). However, few AAs have been identified that interact with these neurons and additional brain structures could also be involved (Schwartz, 2013).

The Geometric Framework for Nutrition is a modelling method that works on the principle that all animals need specific proportions of macronutrients for optimal performance (Simpson and Raubenheimer, 2012). This optimum, called the 'intake target', can be determined experimentally for a species with a given set of traits (sex, age, reproductive status) (Simpson and Raubenheimer, 1993; Raubenheimer and Simpson, 1997; Simpson et al., 2004). This is accomplished by either confining individuals to diets composed of specific proportions of macronutrients or by giving animals a choice of two diets with different macronutrient ratios and measuring the amount of food they consume as well as other performance indicators including lifespan, digestion efficiency, weight and health (Raubenheimer and Simpson, 1993; Simpson and Raubenheimer, 2012).

Adult workers of eusocial insects such as honeybees and ants are unusual because their requirements for dietary protein are very low (Pirk et al., 2010; Altaye et al., 2010; Paoli et al., 2014a,b; Dussutour and Simpson, 2009). For example, a recent study using

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the Geometric Framework estimated that foraging worker honeybees need 250 times less dietary EAAs than bee larvae (Paoli et al., 2014a,b); broodless honeybee 'nurses', in contrast, required five times more dietary EAAs than foragers (Paoli et al., 2014a). However, few other social insect species have been studied using the Geometric Framework. The buff-tailed bumblebee *Bombus terrestris* is a generalist pollinator that lives in eusocial colonies of a few hundred individuals. It is an important wild pollinator but has recently been domesticated and is now used extensively in commercial pollination systems. In comparison to honeybees, its biology is more similar to other wild bee species in that it does not store much food; instead pollen brought back to the colony is consumed quickly by colony residents and fed to brood, and only nectar is stored. In comparison to other wild pollinators, commercially reared colonies make it easy to study this species under lab conditions. At present, we know very little about the dietary requirements of bee species other than honeybees, and whether the low requirement for dietary protein is common to other species of eusocial insect workers. Furthermore, because workers mainly require protein for somatic maintenance, bees could be ideal models to test how the dietary intake of EAAs is regulated in the absence of sexual reproduction. Foraging worker bees are unusual because they derive a portion of their dietary EAAs from free AAs found in floral nectar (Gardener and Gillman, 2002; Petanidou et al., 2006; Nicolson and Thornburg, 2007) and readily consume solutions containing AAs.

Here, we use the Geometric Framework to identify the nutritional optimum for dietary EAAs and carbohydrates of the adult worker bumblebee (*B. terrestris*). Previous studies of its nutrition have shown that microcolonies compensate for protein levels in pollen: bumblebees eat relatively more pollen when its protein content is low (measured as %N) (Tasei and Aupinel, 2008). At present, however, very little is known about the macronutrient requirements of this bee species, in spite of the fact that it is widely used in commercial pollination and is an important model in laboratory studies. In these experiments, the IT for carbohydrates and a dietary source of EAAs (either the 10 EAAs or a protein, caseinate), was determined using an experimental design where bees were allowed to choose between a diet containing sucrose and a source of EAAs and sucrose alone. Using this design, we were also able to test whether the dietary source of EAAs influenced the IT. To gain insight into the mechanisms of nutrient regulation (Simpson and Raubenheimer, 1993), we also measured how the amount of AAs present in bumblebee haemolymph depended on the dietary protein source and concentration.

RESULTS

Nutrient balancing depends on the source of EAAs

The intake target of worker bumblebees depended on the dietary source of EAAs (Fig. 1A, B). Three of the diet solutions in each of the treatments (protein or EAAs) allowed bees to achieve their intake target. Bees fed with caseinate achieved an intake target of 1:149 (w/w) when given the option to eat from tubes containing 0.5 mol l^{-1} sucrose paired with the 1:100, 1:75 and 1:50 (w/w) caseinate and sucrose diets (Fig. 1A). Bees fed with both 0.5 mol l^{-1} sucrose and sucrose containing free EAA achieved an intake target of ~1:255 (mol/mol) when fed with the 1:90, 1:75 and 1:50 (mol/mol) diets (Fig. 1B), which translates into an intake target of 1:560 w/w. Bees fed with the caseinate diets consumed approximately twice as much carbohydrate as those fed with the free EAA solutions when they were feeding on diets within the range over which they could achieve their intake target (1:50, 1:75, 1:100 w/w).

We calculated the intake target by measuring the total amount of food consumed by adult worker bumblebees over the course of the 7 day experiment. The proportion of protein to carbohydrate (P:C) or EAA:C in the diet solution had a strong effect on the amount of food eaten (Fig. 1). Bees fed with sucrose paired with sucrose-caseinate solutions (Fig. 1A) ate significantly more caseinate when they were given the 1:25 and 1:10 (w/w) diets than the bees fed with any of the other diets (Table 1, Šidák's *post hoc*, $P<0.05$). These diets had a much higher concentration of protein than the bees' intake target. In contrast, bees fed with the diet pairs in which it was not possible for them to eat enough to achieve their intake target for protein (the 1:500 and 1:250 diets) ate less on average (Fig. 1A) but also ate significantly less carbohydrate than bees on the other caseinate diets (Table 1, Šidák's *post hoc*, $P<0.05$). Like the bees fed diets containing a high proportion of caseinate, bees fed the diets made of free EAAs ate significantly more EAAs when fed the diets with high EAA:C proportions (1:10 and 1:25 mol/mol) (Fig. 1B, Table 1, Šidák's *post hoc*, $P<0.05$). These data show that bees have a set mean requirement for daily carbohydrate (supplementary material Fig. S1; ~45 mg day^{-1}) and prioritize their intake of carbohydrate over their intake of protein. Unlike the bees fed the dilute caseinate diets, however, bees fed the most dilute EAA:C diet (1:100) ate significantly more carbohydrate than those fed with the other diets (Table 1, Šidák's *post hoc*, $P<0.05$).

In our analysis, we controlled for the colony of origin of the bees and found that it affected the amount of carbohydrate consumed in both sets of experiments (Table 1). The colony also influenced the amount of caseinate but not the amount of EAAs eaten (Table 1). In addition, we controlled for bee size and found that it influenced the amount of carbohydrate consumed when bees were fed the caseinate diets (Table 1). For both diet treatments, larger bees ate more carbohydrate (Pearson's correlation coefficient: caseinate, $r=0.496$, $P<0.001$; EAA, $r=0.221$, $P=0.043$).

The source of EAAs in diet also influenced the mean daily volume of each diet solution consumed by bees (Fig. 1C,D). Bees fed with the caseinate diets altered their intake to consume more sucrose-only solution when it was paired with a high-protein solution (e.g. 1:50 diet) but ate less of the sucrose-only solution when it was paired with a dilute protein source (e.g. 1:250) (Fig. 1C, two-way ANOVA, treatment×solution, $F_{6,234}=7.99$, $P<0.001$). Bees fed with caseinate diets on the extreme ends of the range we tested (1:500, 1:25, 1:10) did not compensate in this way. Furthermore, larger bees ate a greater volume of the diet solutions on average [two-way ANOVA, weight (cov), $F_{1,249}=5.85$, $P=0.016$]. The amount of food eaten also varied as a function of colony [two-way ANOVA, colony (cov), $F_{1,249}=9.07$, $P=0.003$]. In contrast, bees fed with diets containing the 10 EAAs in 0.5 mol l^{-1} sucrose solution always ate more of the sucrose solution (Fig. 1D), and the amount of the EAA diet solution they consumed depended on the proportion of EAA:C (two-way ANOVA, trt×solution, $F_{5,223}=7.07$, $P<0.001$). There was no effect of bee size on this relationship (two-way ANOVA, weight covariate, $F_{1,224}=1.05$, $P=0.306$). The volume eaten varied as a function of colony [two-way ANOVA, weight (cov), $F_{1,249}=6.49$, $P=0.012$].

High concentrations of EAA in food increase the risk of mortality

Bees fed diets composed of caseinate had very low rates of mortality and their survival was largely unaffected by caseinate concentration in the diet (Coxreg, $\chi^2_1=2.79$, $P=0.095$) (Fig 1E). However, the bees fed the highest concentration of caseinate (1:10) had a 3.9 times greater risk of dying than those fed the most dilute diet (1:500). Bees fed diets

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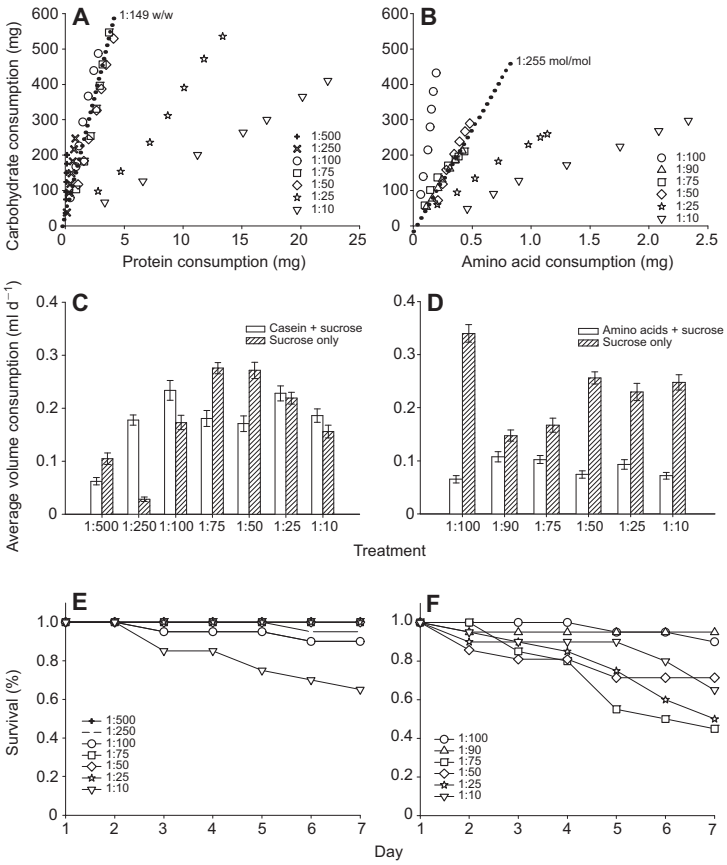

Fig. 1. Nutrient balancing towards an intake target depends on the dietary source of EAAs. (A) Bees fed a choice of diets containing caseinate and 0.5 mol l^{-1} sucrose and 0.5 mol l^{-1} sucrose alone balanced their intake of protein and carbohydrate to an intake target of 1:149 w/w (P:C). (B) Bees fed with diets containing free EAAs and 0.5 mol l^{-1} sucrose and 0.5 mol l^{-1} sucrose alone achieved an intake target of 1:255 mol/mol (1:560 w/w). The dotted line in both panels illustrates the putative intake target. (C) The proportion of caseinate-sucrose diet to sucrose-only diet depended on the diet pair. (D) Bees fed the EAA-sucrose to sucrose-only diet consistently ate more of the sucrose-only diet than the diets containing free EAAs. (E) Bees fed with diets high in caseinate had a lower risk of mortality than bees fed diets high in free EAA (F). The data are for the same individuals in all panels. Error bars indicate s.e.m. *N*=20 bees per diet pair per panel.

high in EAA, however, were more likely to die during the course of the experiment than those fed with caseinate (Fig. 1F, Coxreg, χ^2_1=5.78, *P*=0.016). The risk of dying increased as a function of the amount of EAAs in the diet; bees fed diets with EAA:C ratios less than 1:90 (mol/mol) had a 3–7 times greater risk of dying than those fed diets higher in carbohydrates (e.g. >1:90).

Dietary source of EAAs influences the amount and proportion of sugars and AAs in haemolymph

We confined bees to a specific diet and measured how diet influenced haemolymph nutrient composition. The ratio of P:C or EAA:C in the diet influenced the amount and proportion of sugars and amino acids in the bee haemolymph (Fig. 2). The main sugars we found in bumblebee haemolymph were trehalose, glucose and fructose (Fig. 2A); sucrose was also present, but at concentrations ≥2 orders of magnitude lower than the other sugars (0.380 mmol l^{-1}; data not shown). The amount and proportion of sugars in bee haemolymph depended on the diet (GEE, treatment×sugar, χ^2_{12}=58.1, *P*<0.001). Of all the sugars we measured, trehalose was present in the haemolymph in the highest concentration, except in bees fed the low EAA diet (1:600 mol/mol). In these bees, glucose was at a higher concentration than trehalose (Šidák's *post hoc*,

Table 1. MANOVA of total amount of each macronutrient eaten over 7 days

		Casein diet		Free amino acid diet	
Effect	Dependent	Test stat (d.f.)	*P*-value	Test stat (d.f.)	*P*-value
Diet	Protein/EAA	$F_{6,113}$=32.4	<0.001	$F_{5,75}$=15.8	<0.001
	Carbohydrate	$F_{6,113}$=2.56	0.023	$F_{5,75}$=15.7	0.026
Colony (cov)	Protein/EAA	$F_{1,113}$=4.56	0.016	$F_{1,75}$=1.20	0.275
	Carbohydrate	$F_{1,113}$=1.40	0.005	$F_{1,75}$=13.4	<0.001
Bee size (cov)	Protein/EAA	$F_{1,113}$=1.36	0.185	$F_{1,75}$=2.04	0.090
	Carbohydrate	$F_{1,113}$=1.45	0.004	$F_{1,75}$=0.36	0.148

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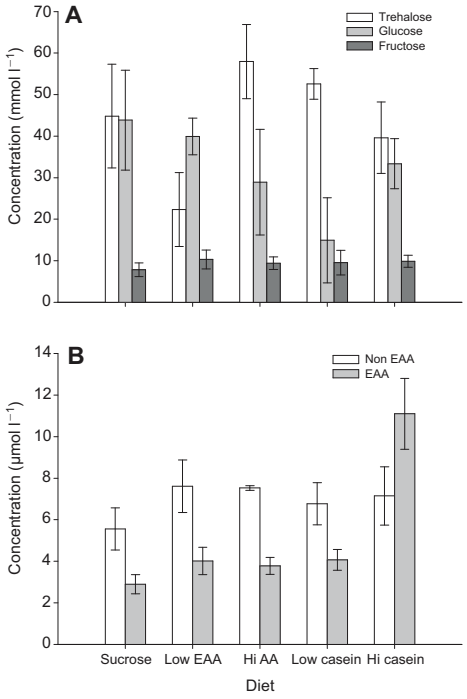

Fig. 2. Haemolymph sugars and amino acids depended on the concentration of protein or EAAs in diet. (A) The sugars, glucose and trehalose, varied according to diet, but fructose did not. (B) Bees fed diets high in caseinate had almost twice the total average amount of EAAs in haemolymph as bees fed sucrose alone or any of the other diets. The mean amount of non-EAAs did not vary as a function of diet. Error bars indicate s.e.m. N_{suc}=9, N_{lowAA}=4, N_{hiAA}=10, N_{lowcas}=8, N_{hicas}=6.

P<0.05). In all of the bees we sampled, fructose was present at an average concentration of ∼9.2±0.8 mmol l^{-1}; in contrast to trehalose and glucose, fructose concentration did not vary as a function of the diet treatment (Fig. 2A, Šidák's *post hoc*, all P>0.05).

Haemolymph amino acid concentrations were also influenced by diet (Fig. 2B). The proportion of total EAAs to non-EAAs depended on diet (GEE, diet×AA class, χ^2_4=24.4, P<0.001). In all the diet treatments except the high caseinate diet (1:20 w/w), the bees had lower concentrations of EAAs than non-EAAs in haemolymph (Fig. 2B). The bees fed the high caseinate diet had almost three times the level of haemolymph EAAs compared with bees fed the other diets; in fact, six of the 10 EAAs (leucine, isoleucine, valine, methionine, threonine and lysine) were elevated in haemolymph when bees were fed this diet (Table 2). Total haemolymph EAAs were not significantly different for any of the other diet treatments (Šidák's *post hoc*, P>0.05). Interestingly, with the exception of bees fed sucrose, the mean concentration of non-EAAs was not strongly affected by diet treatment (Fig. 2B, Šidák's *post hoc*, P>0.05); the sucrose-only fed bees had significantly lower non-EAAs than those fed with the high EAA diet (Šidák's *post hoc*, P=0.042).

To identify whether specific amino acids signalled protein/EAA sufficiency, it was necessary to identify whether diet influenced the amino acid profile of haemolymph. To do this, we tested whether the proportion of specific EAAs and non-EAAs in bumblebee haemolymph could predict the diet the bees were fed using canonical discriminant analysis (CDA). A CDA for the EAAs revealed that diet influenced the specific profile of AAs in bee haemolymph (Table 3). The first canonical discriminant function (function 1) separated the bees fed diets high in protein (high caseinate diet, 1:20 w/w) and the bees fed diets high in EAAs (high AA diet, 1:30 mol/mol) from all other groups (canonical discriminant function coefficients, Table 3). The main haemolymph AAs used to separate the bees fed the high caseinate diet from the other groups were leucine, threonine and valine (pooled within-group correlations, Table 3). In fact, these bees had ∼10× as much leucine in their blood as the bees fed the sucrose-only or low caseinate diets. Bees fed the high AA diet, however, had the lowest concentration of leucine of all the diets. The second discriminant function distinguished the bees fed sucrose only and the low AA diet from those fed with the low caseinate diet; the bees fed the low caseinate diet had relatively elevated levels of tryptophan and low levels of phenylalanine (Tables 2 and 3). The third and fourth discriminant functions did not significantly distinguish the groups.

A second CDA was performed for the non-essential AAs (Table 3). Three significant functions were produced. The first function distinguished the bees fed the sucrose-only diet from those fed caseinate based on the quantities of cysteine and glutamine (Tables 2 and 3). Cysteine was highest in concentration in the low caseinate diet and glutamine was highest in concentration in the sucrose-only diet. The second discriminant function distinguished the bees fed the low caseinate diets and those fed the high AA diets from the others based on the concentration of cysteine and tyrosine (Tables 2 and 3). The third distinguished the low AA diet and the high caseinate diet from the high AA diet; the low AA diet and the high caseinate diet had relatively greater concentrations of alanine and lower concentrations of GABA (Tables 2 and 3).

DISCUSSION

Our experiments show that, like honeybees (Paoli et al., 2014a,b), bumblebee workers prioritize their intake of carbohydrates over the ingestion of dietary EAAs. The source of dietary EAAs influenced nutrient balancing: when bees were fed with a protein (caseinate), they ate a relatively higher proportion of P:C (intake target, 1:149 w/w) than bees fed with the equimolar, free EAA solutions (intake target, 1:255 mol/mol or 1:566 w/w). Interestingly, bees fed with caseinate also consumed almost twice as much carbohydrate as those fed with the free EAA solutions, even though proportionally their diets were skewed towards protein. The bees fed solutions of free EAAs required less of the EAA solution, consumed less carbohydrate and regulated their intake of the solution over a wider range of concentrations. Like other social insect workers studied previously (Dussutour and Simpson, 2009; Pirk et al., 2010; Paoli et al., 2014a,b), diets high in EAAs caused higher rates of mortality in adult worker bumblebees.

Potential mechanisms for adjustment of protein/EAA intake

One of the most striking results of our study was that the amount and proportion of EAAs in food affected the regulation of EAA intake by individual bees. Bees fed the caseinate diet consumed ∼4× more of the sucrose-caseinate diet to meet their needs for EAAs than bees fed with diets containing equimolar concentrations of free EAAs. This could be as a result of incomplete digestion of the casein by the bees, resulting in a greater demand for the substrate, but we were unable to test the frass of the bees to confirm this. Furthermore, the

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Table 2. Mean concentration of amino acids in bumblebee haemolymph 3 days after feeding

	Sucrose only	Low AA	High AA	Low casein	High casein
Essential amino acids					
Arginine	177.7	340.3	514.2	1624	834.6
Histidine	190.6	362.4	333.8	140.3	373.2
Isoleucine	478.9	342.5	465.7	356.4	1282
Leucine	45.82	72.17	32.07	44.23	401.2
Lysine	218.5	310.3	421.6	150.9	624.6
Methionine	864.7	580.4	877.0	858.1	3597
Phenylalanine	92.67	86.64	2.278	13.73	50.75
Threonine	100.1	204.9	123.2	126.1	722.9
Tryptophan	0.0357	0.0435	0.1629	0.1361	0.0715
Valine	724.8	1712	1012	759.2	3211
Total	2893	4011	3782	4073	11,097
Non-essential amino acids					
Alanine	267.4	599.9	184.7	318.4	1110
Asparagine	12.71	16.81	14.21	10.44	12.65
Aspartic acid	211.6	373.4	243.1	429.1	460.5
Cysteine	162.7	249.7	191.6	422.1	182.7
GABA	0.5793	0.2933	0.5346	0.5846	0.1840
Glutamine	0.1873	0.1388	0.1794	0.0746	0.1400
Glutamic acid	263.6	775.1	240.2	309.1	484.6
Glycine	148.8	175.8	403.3	326.6	440.6
Proline	4049	4834	5566	4536	3974
Serine	356.6	467.8	335.9	262.8	290.3
Tyrosine	84.52	119.5	349.2	155.2	193.3
Total	5557	7612	7528	6770	7148

Grey highlights indicate the highest concentration (nmol l^{-1}) in the five treatments for each AA.

costs of production of enzymes to digest casein might also cause a greater demand for EAAs. The bees fed with diets dilute in caseinate also exhibited difficulty in regulating their intake to compensate for the low amount of protein, suggesting that they might not be capable of post-ingestively detecting protein in the diet when it is present at concentrations less than 1:250 w/w.

The main difference between the caseinate-sucrose diet and the free EAA-sucrose diets was the proportion of EAAs (Table 4). The fact that the bees had to eat ∼4× more caseinate implies that some of the less abundant EAAs produced by the digestion of caseinate were important for the regulation of protein intake. With the exception of isoleucine and phenylalanine, the free EAA diet had greater proportions of all the other EAAs than caseinate. Two of these, threonine and valine, were ∼4× less concentrated in the caseinate diet (Table 4); the close match of their relative concentration to the factor by which the bumblebees ate more caseinate could imply that these two are particularly important for the regulation of EAA intake. Only lysine was less concentrated than threonine and valine; it was ∼13× less concentrated in the caseinate diet. Furthermore, phenylalanine and isoleucine were more concentrated than any of the other EAAs, perhaps indicating that they are less important in protein regulation. Our caseinate digest data also show that hydrolysis of caseinate yielded non-EAAs, which were not present in our equimolar, free EAA diet. The fact that bees had to ingest more caseinate to meet their needs for dietary EAAs – in spite of the fact that the caseinate diet also provided non-EAAs – indicates that non-EAAs play only a minor role in the regulation of food intake.

The mechanisms that give rise to the regulation of protein intake are largely unknown (see Morrison et al., 2012 for a review). Dietary protein can affect signalling by peptides such as insulin (Buch et al., 2008), and this could indirectly provide a way of determining that protein has been eaten. A few studies have identified that an 'over-abundance' of specific amino acids can limit feeding behaviour (Purpera et al., 2012; Jordi et al., 2013). Leucine, for example, and the other branched-chain EAAs, isoleucine and valine, which activate the cellular target TOR (target of rapamycin), suppress feeding when they are present in abundance in diets fed to vertebrates (Peters and Harper, 1985) or injected directly into the brain centres involved in feeding, such as the hypothalamus (Blouet and Schwartz, 2010; Karnani et al., 2011; Laeger et al., 2014). In our experiments, only valine matched the predictions that branched-chain EAAs are important for protein regulation because it was four times less concentrated in the caseinate-sucrose diet than in the free EAA-sucrose diet; leucine was ∼30% less concentrated than in the free EAA diet and isoleucine was six times more concentrated (Table 4). These data imply either that valine is more important in protein regulation in insects or more than one EAA is necessary for the body to determine protein sufficiency. At present, there are few data to support the idea that the abundance of a single amino acid (e.g. valine or threonine) is used as a signal of protein sufficiency (Laeger et al., 2014). The more likely explanation is that more than one EAA is necessary for regulation of protein/EAA intake. Previous studies in locusts and rats have shown that the ingestion of several amino acids simultaneously often has a stronger effect on feeding than individual amino acids (Simpson et al., 1990; Karnani et al., 2011). It is possible that the ratios of the branched-chain amino acids to each other – or to combinations of other EAAs – are what affect the body's signals for protein sufficiency. Future studies that test how much each of the branched-chain AAs contributes to the intake of protein when they are present in a mixture of other EAAs will be necessary to identify whether all EAAs must be present in specific proportions to signal protein sufficiency.

Dietary source of EAAs influences AAs in haemolymph

Our study is the first to establish that the dietary source of EAAs has a direct influence on haemolymph levels of AAs, especially EAAs, in

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Table 3. Canonical discriminant analysis of amino acids in bee haemolymph

	Essential amino acids					Non-essential amino acids			
	Canonical discriminant function statistics					Canonical discriminant function statistics			
Function	Eigenvalue	% Var	Test stat	*P*-value	Function	Eigenvalue	% Var	Test stat	*P*-value
1	11.8	77.6	χ^2_{40}=131	<0.001	1	3.95	52.6	χ^2_{44}=107	<0.001
2	2.41	15.7	χ^2_{27}=27	<0.001	2	1.75	23.3	χ^2_{30}=62.6	<0.001
3	0.57	3.70	χ^2_{24}=16	0.090	3	1.38	18.4	χ^2_{18}=34.2	0.012
4	0.45	3.00	χ^2_{7}=7	0.153	4	0.42	5.70	χ^2_{8}=9.99	0.265
	Pooled within-groups correlations					Pooled within-groups correlations			
	Function					Function			
AA	1	2	3	4	AA	1	2	3	4
Leu	**0.518**	0.149	0.025	−0.349	Tyr	0.175	**0.607**	0.358	0.089
Thr	**0.454**	0.175	−0.092	−0.172	Cys	**0.311**	**−0.448**	0.222	0.323
Val	**0.372**	0.102	−0.309	0.175	Gln	**−0.248**	0.285	0.019	−0.152
Phe	0.081	**−0.586**	0.420	0.242	Gly	0.211	0.245	0.019	−0.177
Trp	−0.097	**0.437**	−0.401	−0.176	Ala	0.117	0.056	**−0.541**	−0.133
Arg	−0.001	0.498	**0.634**	0.115	GABA	−0.078	−0.155	**0.475**	−0.132
His	0.084	0.001	**−0.575**	0.165	Asp	0.148	−0.079	−0.159	0.047
Lys	0.103	0.046	**−0.399**	−0.119	Ser	−0.220	0.078	−0.125	**0.673**
Met	0.390	0.171	−0.021	**−0.591**	Glu	0.021	−0.026	−0.306	**0.468**
Ile	0.289	0.058	−0.128	**−0.514**	Asn	−0.073	0.147	−0.065	**0.343**
					Pro	0.026	0.138	0.165	0.230
	Canonical discriminant function coefficients					Canonical discriminant function coefficients			
	Function					Function			
Diet	1	2	3	4	Diet	1	2	3	4
Sucrose	−1.103	**−2.122**	0.402	−0.446	Sucrose	**−2.76**	−0.741	0.026	−0.439
Low AA	0.766	**−1.215**	−0.281	1.697	Low AA	−0.908	−0.311	**−1.242**	1.571
High AA	**−2.039**	0.677	−0.98	−0.234	High AA	0.127	**1.612**	**1.063**	0.111
Low casein	−1.873	**1.746**	0.933	0.159	Low casein	**2.236**	**−1.618**	0.719	0.002
High casein	**7.039**	0.536	−0.027	−0.285	High casein	**1.552**	0.789	**−1.941**	−0.577

Pooled within-groups correlations in bold indicate the highest correlation of each amino acid with each discriminant function. Canonical discriminant function coefficients indicate how the CDA split each treatment in distinct groups; treatments with the highest magnitude (in bold) for significant functions indicate how the classification separated the groups based on the weighting of each amino acid to each discriminant function.

an insect. In this experiment, we confined bees to diets that we predicted were either near the intake target or strongly unbalanced towards protein/EAA, with the expectation that we would be able to identify differences in the unbalanced diets that predicted the signal for protein sufficiency. As might be expected, our data show that over-ingestion of caseinate (arising from the need of the bees confined to this diet to ingest sufficient carbohydrate) resulted in ∼3–4× greater concentrations of 7 of the 10 EAAs in haemolymph. Notably, two of the branched-chain EAAs (leucine and valine) were elevated in haemolymph in spite of the fact that they were not the most abundant AAs present in the caseinate digest. A recent study also showed that rats chronically fed diets high in protein had elevated levels of the branched-chain AAs (leucine, isoleucine and valine) in their blood (Solon-Biet et al., 2014). Furthermore, in this same study, all other plasma AAs were negatively correlated with protein intake or not correlated at all. The selective elevation of the branched-chain EAAs in haemolymph when diets are high in protein could indicate that all other AAs from protein: (1) do not pass across the gut wall as readily; (2) are used more quickly by corporeal cells; or (3) are selectively excreted when they are in excess in haemolymph.

An interesting aspect of our study was that bumblebees did not accurately regulate their intake of EAAs when diets were high in protein or EAAs, as they over-ate EAAs in these diets, in spite of the fact that they were also given access to a sucrose-only diet. This suggests several possible explanations. The first is that bees cannot easily taste differences in the concentration of protein or EAAs in the diet, such that they passively over-ingest protein/EAAs in sugar solution. Few studies have examined the ability of bees to taste amino acids (Inouye and Waller, 1984; Roubik et al., 1995; Carter et al., 2006; Simcock et al., 2014; Hendriksma et al., 2014) and none have reported whether *B. terrestris* or other bees have the appropriate gustatory receptor neurons to detect them. The second is that post-ingestive mechanisms for the regulation of protein/EAA intake may be tuned to a specific range of concentrations of these amino acids, and if the concentration of protein/EAA is too high, the bees cannot adjust by reducing their intake.

Bees prioritize carbohydrates over protein intake

We also observed that carbohydrate regulation depended on the dietary source of EAAs and the amount of protein eaten. Bees fed sucrose could only clearly regulate their intake around a specific daily quantity of carbohydrates (∼45±4 mg sucrose day^{-1}, supplementary material Fig. S1). Bees fed with the free EAA-sucrose diets also regulated their intake of carbohydrates to ∼47±2 mg sucrose day^{-1}; when caseinate was very dilute in the diet, as in the 1:250 and 1:500 caseinate-sucrose diets, bees regulated their intake to a similar amount (36±3 mg sucrose day^{-1}). However, when caseinate was present at concentrations greater than the 1:250 diet, the bees not only consumed proportionally more caseinate (∼5±0.1 mg day^{-1}), they also increased their total intake of carbohydrates to twice that of the bees on all the other diets (75±2 mg sucrose day^{-1}). Our data could indicate that the brain integrates information about nutritional state using carbohydrates and EAAs simultaneously

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Table 4. Proportion of amino acids found in sodium caseinate and in the equimolar EAA diet

	Casein diet (μg g^{-1})	Proportion of total EAA in casein	Free EAA diet (mg ml^{-1})	Proportion of total EAA in EAA diet	Casein/EAA diet
Essential amino acids					
Arginine	855.2±118.1	0.06	1.74	0.11	0.55
Histidine	622.9±76.4	0.04	1.55	0.10	0.40
Isoleucine	7235±909	0.52	1.31	0.09	5.8
Leucine	779.2±93.4	0.05	1.31	0.09	0.67
Lysine	145.0±16.7	0.01	1.82	0.12	0.08
Methionine	589.5±85.3	0.04	1.49	0.10	0.40
Phenylalanine	3015±351	0.22	1.65	0.11	2
Threonine	226.5±23.2	0.02	1.19	0.08	0.25
Tryptophan	0.1874±0.0188	<0.01	2.04	0.13	0.08
Valine	336.6±22.1	0.02	1.17	0.07	0.28
Non-essential amino acids					
Alanine	13.65±1.74				
Asparagine	0.032±0.014				
Aspartic acid	97.64±7.95				
Cysteine	711.5±102.4				
GABA	1.467±0.330				
Glutamine	0.0839±0.006				
Glutamic acid	1674±77.8				
Glycine	248.8±29.9				
Proline	58.93±5.06				
Serine	678.3±78.3				
Tyrosine	1211±163.2				

Sodium caseinate was digested using acid hydrolysis to render EAA and non-EAAs. AAs with the greatest concentrations are highlighted in grey. Note that tryptophan is often destroyed by acid hydrolysis and these data may not reflect actual values rendered by bee digestion. *N*=6 samples.

(Simpson and Raubenheimer, 2012), and that this calculation is done independently from an evaluation of the sufficiency of individual EAAs in the diet. Like other proteins, caseinate does not provide the same proportions of all EAAs (Table 4); to obtain sufficient specific EAAs, the bumblebees probably had to consume more caseinate (w/w) than the free EAA solution (w/w). However, they did this whilst also maintaining their intake target for a specific proportion of P:C and this forced them to eat, in total, more carbohydrates than the bees fed with the free EAA diets. Thus, our data show that the proportions of EAAs (as determined by the EAAs produced when protein is digested) can also influence the total dietary intake of carbohydrates, perhaps through two different mechanisms.

Our study is the first to examine in detail the nutritional needs for protein/EAA and carbohydrates of the adult worker bumblebee. The bumblebees in our experiments strongly regulated their daily intake of carbohydrate to achieve a minimum of 45 mg sucrose day^{-1}. Our previous work has also shown that adult worker honeybees prioritize their intake of carbohydrate over EAAs/protein (Altaye et al., 2010; Paoli et al., 2014a,b; Archer et al., 2014), and that the need for carbohydrate increases when honeybee workers become foragers (Paoli et al., 2014a,b). In our studies with honeybees, we estimated that the IT for newly-emerged honeybees fed the free EAA diets was 1:50 mol/mol. The IT we observed for bumblebees fed the free EAA diets was 1:255 mol/mol – a value that is very similar to our estimate of the IT for honeybee foragers (~1:250 mol/mol, Paoli et al., 2014a,b). Worker bees have significant demands for carbohydrates to fuel flight (Joos et al., 1997; Suarez et al., 1996; Harrison and Roberts, 2000; Darveau et al., 2014) and also have high resting metabolic rates (Harrison and Roberts, 2000). Their main dietary source of carbohydrates is floral nectar: a solution that contains free AAs but whose composition is largely sucrose, glucose and fructose (Baker and Baker, 1982; Petanidou et al., 2006; Nicolson and Thornburg, 2007). Thus, unlike herbivorous or carnivorous insects, by consuming a nectar-only diet it is possible for foraging bees to selectively consume carbohydrates without being required to eat high concentrations of protein/EAAs at the same time. In this way, they can obtain their carbohydrate needs first, and secondarily consume other substrates (e.g. pollen or glandular secretions from other nest mates) to meet their needs for dietary EAAs.

Honeybees regulate their intake of carbohydrates to maintain haemolymph trehalose titres (Blatt and Roces, 2002a,b). In spite of changes in the quality and quantity of sugar solutions fed to honeybees, the trehalose concentration in haemolymph is tightly regulated to a constant level (Blatt and Roces, 2001, 2002a,b) because trehalose is the main sugar, along with glucose, used to produce glucose-6-phosphate as a substrate for ATP production to fuel flight muscles (Beenakkers et al., 1984). In contrast, we found that in bumblebees, trehalose concentration varied with diet composition, but fructose concentration remained constant. If the maintenance of a storage carbohydrate in haemolymph facilitates flight, we predict that the diet-invariant nature of haemolymph fructose indicates that bumblebees use fructose rather than trehalose to fuel flight. Interestingly, enzymatic studies of bumblebee flight muscles have shown that bumblebees are unique among insects because they rely on fructose-6-phosphate and fructose-1,6-diphosphate as cycling substrates for flight muscles (Staples et al., 2004; Clark et al., 1973; Beenakkers et al., 1984) rather than glucose and glucose-6-phosphate produced from trehalose used by other insects (Beenakkers et al., 1984). Fructose-6-phosphate and fructose-1,6-phosphate can be produced from both glucose and fructose, but the production of fructose-1,6-phosphate – one of the substrates for ATP production in a fructose-6-phosphate/fructose-1,6-phosphate cycle – requires fewer enzymatic steps than it would if trehalose was used as a substrate (Beenakkers et al., 1984; Berg et al., 2012). For this reason, it would be faster and require less ATP for *Bombus* sp. to use fructose than trehalose as a haemolymph storage carbohydrate. Future research on this topic may reveal that fructose plays an important role in the diet of

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foraging bumblebees for this reason. We also provide the first report we know of that shows sucrose in insect haemolymph, but the significance of this is unknown.

Diets high in protein lead to eusocial worker mortality

In addition to requiring diets high in carbohydrates to fuel flight, the bumblebees in our study, as well as worker honeybees and ants, also exhibit high rates of mortality when fed diets high in protein or EAAs (Pirk et al., 2010; Paoli et al., 2014a,b; Dussutour and Simpson, 2012) but can survive on a diet of sucrose alone for several days (Paoli et al., 2014a,b). In fact, in addition to having modest demands for dietary protein, bees have significant diversification of genes encoding enzymes necessary for sugar metabolism (Kunieda et al., 2006). Most animals fed diets higher in protein than their actual IT can convert dietary AAs into fuel via gluconeogenesis; we have been unable to find many accounts where diets high in protein kill animals outright, although an abundance of some amino acids, such as methionine, has been associated with toxicity or a reduction in lifespan (Harper et al., 1970; Grandison et al., 2009). These studies, in combination with our data, suggest that the need for diets high in carbohydrates is a general trait of social Hymenoptera workers and could suggest that workers in these lineages have undergone a metabolic trade-off that has perhaps enhanced their ability to use carbohydrates but at the cost of being able to use EAAs efficiently as substrates for energy production.

MATERIALS AND METHODS

Experimental animals

Fourteen commercially reared bumblebee (*Bombus terrestris terrestris* Linnaeus 1758) colonies (Koppert Ltd, UK and Syngenta Bioline) were kept in a temperature controlled room or incubators maintained at 28°C and 60% relative humidity at Newcastle University (UK). Prior to the experiment, each colony had access to a liquid food source supplied with the colonies and ~3 g of honeybee collected pollen was provided daily to each colony. Female worker bees were removed from the colony by opening the flight holes and catching individual bees in plastic vials; bees that emerged from the colony exit were used in the experiments. Bees were briefly cold-anaesthetized on ice until activity was reduced to transfer them into the feeding chambers. Only female bees were used; to identify females, genitals were inspected during cold anaesthesia for the presence of male claspers (Hannan et al., 2012). Workers of all sizes were captured and used in the experiments and care was taken to distribute them randomly across treatments.

Experimental chambers

Bees were housed individually in a plastic box (16.5×11×6.5 cm) with 20 holes (2 mm) drilled at each end of the lid for ventilation. In three sides of the box, a hole was cut to insert a 2 ml microcentrifuge tube; each tube had four holes (2 mm) drilled in a line in one side of the tube to facilitate feeding by the bees. Two of the tubes were filled with food solution; the remaining tube was filled with deionized water. A piece of absorbent laboratory paper was added to the housing box, covering the base. After being placed in the box, bees were left to acclimatize at room temperature before the feeding solutions were added. Bees were then moved into the 28°C controlled temperature room or incubator and kept in darkness for 7 days, through the course of the experiment. After use in treatments, bees were killed by freezing at −20°C.

Nutrient balancing experiments and diets

To test how the dietary source of EAAs influenced the intake target of adult worker bumblebees, each bee was presented with a choice of two solutions: a 0.5 mol l^{-1} sucrose solution and another solution that contained 0.5 mol l^{-1} sucrose with protein (sodium caseinate, Sigma-Aldrich, C8654) or the 10 EAAs at equimolar concentrations (Table 5). The AAs used were: methionine, tryptophan, leucine, lysine, valine, arginine, isoleucine, phenylalanine, threonine and histidine (all from Sigma-Aldrich). These AAs are essential for many insect species and were identified as 'essential' for honeybees by de Groot (1953). Both of the EAA sources were dissolved in a 0.5 mol l^{-1} sucrose solution made with deionized water. Diets were made to specific protein to carbohydrate ratios (P:C), where the carbohydrate concentration remained constant (0.5 mol l^{-1} sucrose) (Tables 4 and 5). The caseinate solutions were based on weight-to-weight proportions; the EAA solutions were based on the molar ratio of the EAAs-to-sucrose as in Paoli et al. (2014a,b). Our diets did not have the same proportion of EAAs: upon acid hydrolysis (see below), caseinate was digested to a specific proportion of EAA and non-EAA that was dominated by isoleucine, phenylalanine, glutamic acid and tyrosine (Table 4). Furthermore, the most concentrated amino acids were in some cases three or four orders of magnitude higher than the least concentrated amino acids. In contrast, our EAA diet was nearly equimolar with a similar proportion w/w.

Table 5. Ratios of dietary source of EAA:C

Dietary EAA source	EAA:C (w/w)	EAA:C (m/m)
Casein	1:500	–
	1:250	–
	1:100	–
	1:75	–
	1:50	–
	1:25	–
	1:10	–
Essential amino acids	1:230	1:100
	1:206	1:90
	1:153	1:75
	1:115	1:50
	1:57	1:25
	1:23	1:10

Diet tubes were weighed and replaced every 24 h. To adjust for evaporation, evaporation rates for each solution were measured in boxes containing the solutions (without bees). The average value for each solution was subtracted from the final weights for the consumption of each diet solution. Values for the amount of carbohydrate or protein and EAAs consumed were determined by dividing the weight of the consumed solution by its density (1.06) to obtain the volume. The amount of each solute in the solution was then obtained for the volume of solution consumed; this amount was combined to give a single value for consumption of protein and carbohydrate for each day. Total consumption was a measure of the total amount eaten over the 7 day period.

Effect of diet on haemolymph composition

We measured haemolymph sugars and AAs with the aim of identifying how diets of caseinate or EAAs influenced nutritional state and hence nutrient balancing (Simpson and Raubenheimer, 1993). To do this, we restricted individual bees for 3 days to one of the following diets: sucrose only, low caseinate (1:140 w/w), high caseinate (1:20 w/w), low EAAs (1:600 mol/mol), or high EAAs (1:30 mol/mol) using the protocol described above. After 3 days, haemolymph was collected from each bee. Bees were cold-anaesthetized, and a hypodermic needle was used to cut an incision in the back of the head posterior to the ocelli. Haemolymph from individual bees was collected using 10 µl capillary tubes and expelled into a 0.2 ml microcentrifuge tube with an equal volume of 0.1 mol l^{-1} perchloric acid to haemolymph. Each sample represented haemolymph from one bee. The average volume of haemolymph collected from an individual was 6.16 µl (the volume of haemolymph did not differ between treatment groups). Samples were kept frozen at −20°C until HPLC analysis. For HPLC analysis, 4 µl of haemolymph-perchloric acid mixture from each bee was diluted to 1:30 with HPLC gradient grade H_2O. Each sample was passed through a 0.45 µm syringe-tip filter (Whatman Puradisc 4, nylon, 4 mm) prior to analysis.

In each sample, we used HPLC to measure glucose, trehalose, fructose and sucrose and a suite of EAAs and non-EAAs. Sugars were quantified using a Dionex DX500 HPLC system with an ED40 electrochemical

detection unit. The mobile phase was 100 mmol l^{-1} NaOH. A separate aliquot of the original haemolymph-perchloric acid sample was diluted to 1:200 with distilled, deionized water. Twenty microlitres of this sample was injected on to a Carbopac PA-100 column (Dionex, Sunnyvale, California, USA). Sugars were eluted isocratically with 100 mmol l^{-1} NaOH with a flow rate of 1 ml min^{-1}. Elution profiles were analysed with Chromeleon software (Thermo Fisher Scientific).

We quantified 21 AAs in the samples using a Dionex Ultimate 3000 RS system fitted with a 150×2.1 mm Accucore RP-MS (Thermo Scientific) column. Before being injected onto the column, 10 µl of diluted sample was pre-treated for 1 min with 15 µl of 7.5 mmol l^{-1} o-phthaldialdehyde (OPA) and 225 mmol l^{-1} 3-mercaptopropionic acid (MPA) in 0.1 mol l^{-1} sodium borate ($Na_2B_4O_7{\cdot}10H_2O$, pH 10.2), then with 10 µl of 96.6 mmol l^{-1} 9-fluroenylmethoxycarbonyl chloride (FMOC) in 1 mol l^{-1} acetonitrile for 1 min, followed by the addition of 6 µl of 1 mol l^{-1} acetic acid. A final volume of 30 µl of the treated sample was then injected into the HPLC system. Elution solvents used were: A, acetonitrile/methanol/water (45/45/45 v/v/v) and B, 10 mmol l^{-1} Na_2HPO_4, 10 mmol l^{-1} $Na_2B_4O_7{\cdot}10H_2O$, 0.5 mmol l^{-1} sodium azide (NaN_3), adjusted to pH 7.8 with concentrated HCl. Elution of the column occurred at a constant flow rate of 500 µl min^{-1} with a linear gradient of 3 to 100% (v/v) eluent A and 97 to 0% eluent B. Amino acid derivatives were fluorometrically detected (Ultimate 3000 RS Fluorescence Detector, Dionex, Thermo Fisher Scientific) and elution profiles were analysed using Chromeleon software (Thermo Fisher Scientific).

Amino acid composition of sodium caseinate

To identify the AAs produced by the digestion of caseinate, we digested sodium caseinate in HCl. Sodium caseinate (1.7 mg) was first washed in 200 µl of methanol to extract free AAs. The samples were vortexed for 1 min and then left for 10 min and vortexed a second time for 1 min (Cook et al., 2003). Each sample was centrifuged for 30 min at 134,000 r.p.m. The supernatant was removed and placed in a new microcentrifuge tube. The remaining pellet and the supernatant sample were dried down in a heat block at 70°C. Dry samples from the methanol extract were then recovered in 200 µl HPLC gradient grade water and vortexed for 1 min. To the dried caseinate pellet, we added 170 µl of 6 mol l^{-1} hydrochloric acid (HCl) and the sample was briefly vortexed. Sealed tubes were placed in plastic microcentrifuge tube boxes, sealed, and placed in a domestic 900 W (2450 MHz) microwave oven inside of a fume hood. A Pyrex beaker containing 800 ml of cold tap water was also placed in the microwave oven to absorb excess radiation (Zhong et al., 2005). Samples were irradiated for 15 min on full power and then left to cool. Cooled samples were then moved to a heat block within a fume hood, unsealed and heated at 70°C to evaporate the acid. Once dry, 200 µl of deionized UHPLC gradient grade water was added to each sample. Both free AA (supernatant) and hydrolysed protein-bound AA samples (digested pellet) were centrifuged for 1 min and filtered through 0.45 µm syringe-tip filters (Whatman Puradisc 4, nylon, 4 mm). Ten microlitres of each filtered sample was analysed using the HPLC method for AA analysis above.

Statistical analyses

Analyses were carried out using IBM SPSS v19. The amount of food consumed (mg) was analysed using multivariate analysis of variance (MANOVA) and the volume analysed using two-way analysis of variance (ANOVA). Both analyses included colony and bee size as covariates. Šidák's *post hoc* tests were used for multiple comparisons. Data were natural log transformed prior to analysis. (Note that we used bee weight as a proxy for bee size based on a factor analysis that identified that bee weight had the strongest correlation with four other measured parameters: abdomen and thorax width, head length, total bee length.) The intake targets were determined by the *post hoc* comparisons of the amount of protein/EAAs and carbohydrates eaten on each dietary treatment; diet treatments that were not significantly different in both were averaged to determine the intake target P:C or EAA:C ratio. Survival data were analysed using a Cox regression (Coxreg) analysis with diet treatment as a covariate; comparisons between groups were evaluated using the 'indicator' contrasts. The hazard ratio (HR) was calculated for each comparison against the indicator group, which was always the most dilute EAA:C or P:C treatment. Generalized estimating equations (GEEs) were used to test for differences in haemolymph sugars and total EAA and non-EAA. A canonical discriminant analysis (CDA) was used to identify differences in the treatments in the amount and proportion of specific AAs in haemolymph.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

D.S. performed and designed the experiments, analysed the data and revised the manuscript; P.P. performed the experiments; S.W.N. helped to design the experiments and revised the manuscript; G.A.W. designed the experiments, analysed the data and wrote the manuscript.

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Supplementary material

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Appendix K

Caffeine in floral nectar enhances a pollinator's memory of reward

Caffeine in Floral Nectar Enhances a Pollinator's Memory of Reward

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Plant defense compounds occur in floral nectar, but their ecological role is not well understood. We provide evidence that plant compounds pharmacologically alter pollinator behavior by enhancing their memory of reward. Honeybees rewarded with caffeine, which occurs naturally in nectar of *Coffea* and *Citrus* species, were three times as likely to remember a learned floral scent as were honeybees rewarded with sucrose alone. Caffeine potentiated responses of mushroom body neurons involved in olfactory learning and memory by acting as an adenosine receptor antagonist. Caffeine concentrations in nectar did not exceed the bees' bitter taste threshold, implying that pollinators impose selection for nectar that is pharmacologically active but not repellent. By using a drug to enhance memories of reward, plants secure pollinator fidelity and improve reproductive success.

Many drugs commonly consumed by humans are produced by plants as a form of toxic defense against herbivores (*1*, *2*). Although plant-derived drugs like caffeine or nicotine are lethal in high doses (*3*–*5*), at low doses they have pharmacological effects on mammalian behavior. For example, low doses of caffeine are mildly rewarding and enhance cognitive performance and memory retention (*6*). Caffeine has been detected in low doses in the floral nectar and pollen of *Citrus* (*7*), but whether it has an ecological function is unknown.

Two caffeine-producing plant genera, *Citrus* and *Coffea*, have large floral displays with strong scents and produce more fruits and seeds when pollinated by bees (*8*, *9*). If caffeine confers a selective advantage when these plants interact with pollinators, we might expect it to be commonly encountered in nectar. We measured caffeine in the nectar of three species of *Coffea* (*C. canephora*, *C. arabica*, and *C. liberica*) and four species of *Citrus* (*C. paradisi*, *C. maxima*, *C. sinensis*, and *C. reticulata*) using liquid chromatography–mass spectrometry (*10*) (fig. S1A). When caffeine was present, its concentration ranged from 0.003 to 0.253 mM. The median caffeine concentration in both genera was not significantly different (Fig. 1A, Mann-Whitney, $Z = -1.09$, $P = 0.272$). Caffeine was more common in the nectar of *C. canephora* than in that of *C. arabica* or *C. liberica* (*Coffea*: logistic regression $\chi_2^2 = 11.1$, $P = 0.004$); it was always present in *Citrus* nectar. The mean total nectar sugar concentration ranged from 0.338 to 0.843 M (Fig. 1B; see fig S1B for individual sugars). Caffeine concentration in nectar did not correlate with total sugar concentration (Pearson's $r = 0.063$, $P = 0.596$).

We hypothesized that caffeine could affect the learning and memory of foraging pollinators. To test this, we trained individual honeybees to associate floral scent with 0.7 M sucrose and seven different concentrations of caffeine and tested their olfactory memory. Using a method for classical conditioning of feeding responses (proboscis extension reflex) (*11*), we trained bees for six trials with 30 s between each pairing of odor with reward. This intertrial interval approximated the rate of floral visitation exhibited by honeybees foraging from multiple flowers on a single *Citrus* tree (see methods). The presence of low doses of caffeine in reward had a weak effect on the rate of learning (Fig. 2A), but it had a profound effect on long-term memory. When rewarded with solutions containing nectar levels of caffeine, three times as many bees remembered the conditioned scent 24 hours later and responded as if it predicted reward (Fig. 2B, logistic regression, $\chi_7^2 = 41.9$, $P < 0.001$). Twice as many bees remembered it 72 hours later (Fig. 2C). This improvement in memory performance was not due to a general increase in olfactory sensitivity resulting from caffeine consumption (fig. S2A). Indeed, the effect of caffeine on long-term

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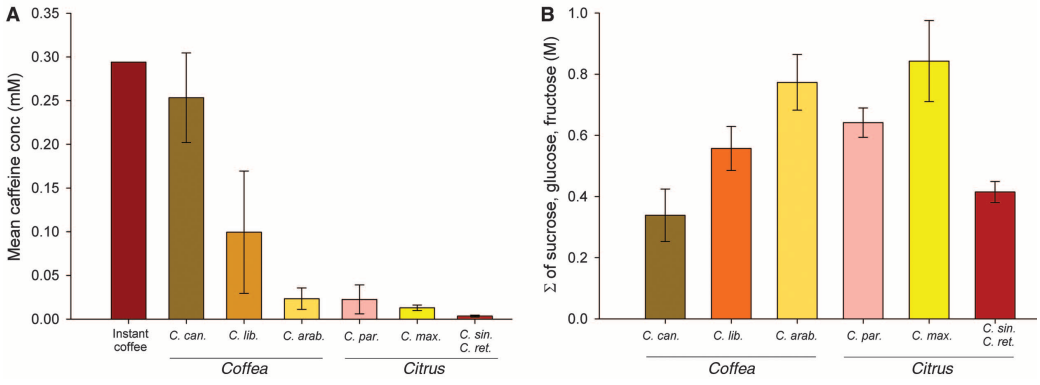

Fig. 1. (**A**) Caffeine concentration in *Coffea* and *Citrus* spp. and a cup of instant coffee. Caffeine concentration depended on species within each genus (*Coffea*: Kruskal-Wallis, $\chi_2^2 = 28.1$, $P < 0.001$; *Citrus*: Kruskal-Wallis, $\chi_2^2 = 6.98$, $P = 0.030$); *C. canephora* had the highest mean concentration of all species sampled. (**B**) The sum of the concentration of sucrose, glucose, and fructose (total nectar sugars) depended on species (one-way analysis of variance: $F_{5,\ 161} = 4.64$, $P < 0.001$) and was greatest in *Citrus maxima* and hybrids (citron, lemons, clementines). [*C. can.*, *Coffea canephora*, $N = 34$; *C. lib.*, *Coffea liberica*, $N = 31$; *C. arab.*, *Coffea arabica*, $N = 27$; *C. par.*, *Citrus paradisi* and hybrids, $N_{cp} = 17$; *C. max.*, *Citrus maxima* and hybrids, $N = 5$; *C. sin.* and *C. ret.*, *Citrus sinensis* and *Citrus reticulata*, $N_{CS} = 7$, $N_{CR} = 5$ (data for these two species were pooled).] Mean responses ± SE.

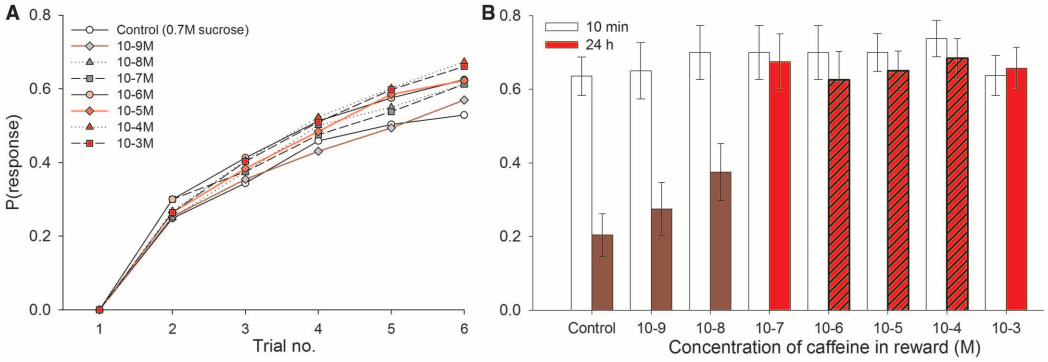

Fig. 2. (**A**) The rate of learning of bees conditioned with an odor stimulus paired with a 0.7 M sucrose reward containing caffeine. The rate of learning was slightly greater for the bees fed caffeine in reward during conditioning (logistic regression, $\chi_1^2 = 4.85$, $P = 0.028$). $N \geq 79$ for all groups. (**B**) Memory recall test for odors at 10 min (white bars) or 24 hours (red bars) after bees had been trained as in (A). Bright red bars indicate that the response at 24 hours was significantly different from the control (0.7 M sucrose) (least-squares contrasts: $P < 0.05$); dark red bars were not significantly different. Nectar levels of caffeine are indicated by hatching. $N > 79$ for each group. (**C**) Bees fed 0.1 mM caffeine in sucrose (orange bars) were more likely to remember the conditioned odor than sucrose alone (white bars) (logistic regression, $\chi_1^2 = 9.04$, $P < 0.003$) at 24 hours and 72 hours after conditioning. $N = 40$ per group.

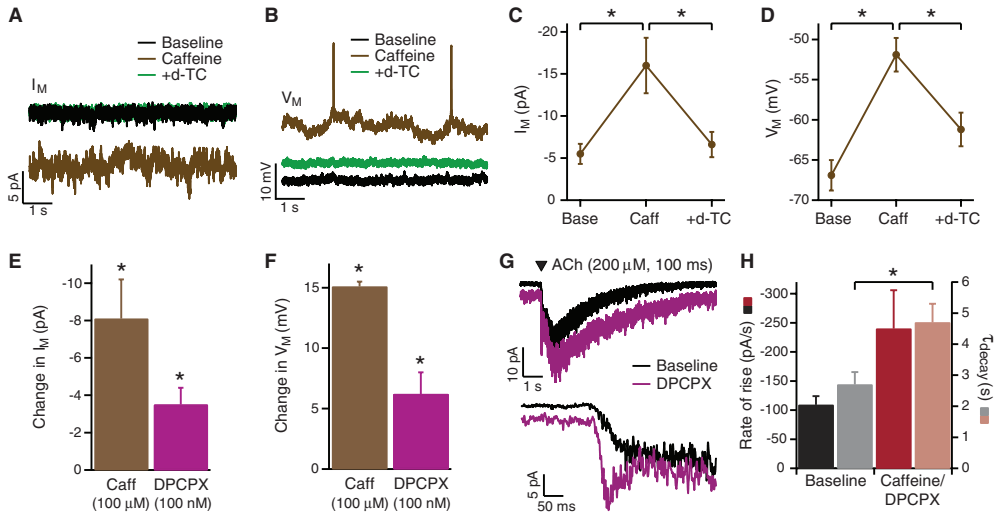

Fig. 3. The effect of caffeine on Kenyon cells. (**A** and **B**) Example traces from a KC in intact honeybee brain recorded under voltage-clamp [(A), $V_H = -73$ mV) and current-clamp [(B), at resting V_M), showing the increase in I_M and depolarization evoked by bath application of caffeine (100 µM) and subsequent reversal by the nAChR antagonist d-TC (500 µM). (**C** and **D**) Mean data showing the reversal by d-TC (500 µM) of the effect of caffeine (Caff; 100 µM) on I_M [(C); $N = 6$, $t_5 = 4.03$, $P = 0.010$; $t_5 = 4.07$, $P = 0.010$] and V_M [(D); $N = 6$, $t_5 = 34.1$, $P < 0.001$; $t_5 = 12.0$, $P < 0.001$]. (**E** and **F**) Comparison of the mean effects of caffeine and DPCPX on I_M [(E); Caff: $N = 10$, $t_9 = 3.84$, $P = 0.004$; DPCPX: $N = 6$, $t_5 = 4.04$, $P = 0.010$] and V_M [(F) Caff: $N = 6$, $t_5 = 34.1$, $P < 0.001$; DPCPX: $N = 6$, $t_5 = 3.39$, $P = 0.019$]. (**G** and **H**) Example traces [(G); rising phase shown on an expanded time scale below] and mean data [(H); rate of rise: $N = 6$, $t_5 = 2.20$, $P = 0.079$; τ_{decay}: $N = 9$, $t_8 = 3.54$, $P = 0.008$] showing that DPCPX (100 nM) and caffeine (100 µM) slowed the decay and, in six of nine KCs, potentiated the fast component of the response evoked by exogenous ACh. (Student's paired t test used in all comparisons.) Mean responses ± SE.

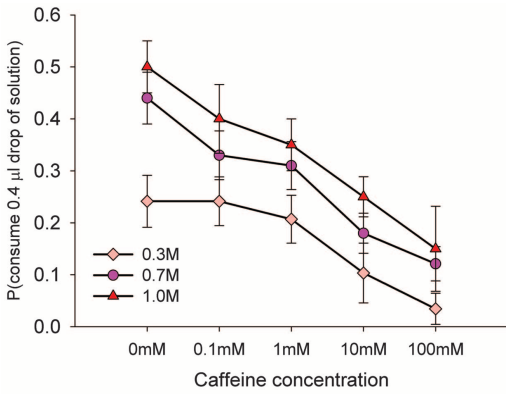

Fig. 4. Bees are more likely to reject sucrose solutions containing caffeine at concentrations greater than 1 mM (logistic regression, χ_4^2 = 23.4, $P < 0.001$; for 0.7 and 1.0 M, 1 mM caffeine versus sucrose post hoc, $P < 0.05$; for 0.3 M, 100 mM caffeine versus sucrose post hoc, $P < 0.05$). Bees were less likely to drink 0.3 M sucrose (pale pink diamonds) than 0.7 M (pink circles) or 1.0 M solutions (red triangles) (logistic regression, $\chi_2^2 = 8.69$, $P = 0.013$). Mean responses ± SE. $N_{0.3M} = 29$, $N_{0.7M} = 100$, $N_{1.0M} = 20$.

olfactory memory in bees was greater than that produced by high concentrations of sucrose when the same experimental methods were used (e.g., 2.0 M, fig. S2B).

Caffeine's influence on cognition in mammals is in part mediated by its action as an adenosine receptor antagonist (*6*). In the hippocampal CA2 region, inhibition of adenosine receptors by caffeine induces long-term potentiation (*12*), a key mechanism of memory formation (*13*). The Kenyon cells (KCs) in mushroom bodies of the insect brain are similar in function to hippocampal neurons: They integrate sensory input during associative learning, exhibit long-term potentiation, and are involved in memory formation (*14*–*16*). To determine whether nectar-caffeine doses affect mushroom body function, we made whole-KC recordings in the intact honeybee brain. Caffeine (100 µM) evoked a small increase in the holding current (I_M) and depolarized KC membrane potential (V_M) toward the action potential firing threshold, by increasing nicotinic acetylcholine receptor (nAChR) activation (Fig. 3, A to D). To determine whether the observed effects of caffeine were due to interactions with adenosine receptors, we applied the adenosine receptor antagonist DPCPX and observed that it similarly increased I_M and depolarized V_M, but to a lesser extent (Fig. 3, E and F). Both caffeine and DPCPX affected KC response kinetics evoked by brief, local application of ACh, increasing the activation rate and slowing the decay (Fig. 3, G and H). Our data show that caffeine modulates cholinergic input via a postsynaptic action, but could act via presynaptic adenosine receptors to potentiate ACh release (*17*). The resulting increase in KC excitability should lead to an increased probability of action potential firing in response to sensory stimulation (*18*), thereby facilitating the induction of associative synaptic plasticity in KCs (*19*). The enhanced activation of KCs may also facilitate plasticity at synapses with mushroom body extrinsic neurons (*20*), which exhibit spike-timing–dependent plasticity (*21*). In this way, a "memory trace" could be formed for the odor associated with reward during and after conditioning (*22*, *23*).

Caffeine is bitter tasting to mammals and is both toxic (*24*) and repellent to honeybees at high concentrations (*25*, *26*). If bees can detect caffeine, they might learn to avoid flowers offering nectar containing it (*27*). We found that honeybees were deterred from drinking sucrose solutions containing caffeine at concentrations greater than 1 mM (Fig. 4); they also have neurons that detect caffeine in sensilla on their mouthparts (fig. S3). However, nectar concentrations did not exceed 0.3 mM (0.058 mg/ml), even though levels of caffeine in vegetative and seed tissues of *Coffea* have been reported to be as great as 24 mg/ml (*28*). This implies that pollinators drive selection toward concentrations of caffeine that are not repellent but still pharmacologically active.

Our data show that plant-produced alkaloids like caffeine have a role in addition to defense: They can pharmacologically manipulate a pollinator's behavior. When bees and other pollinators learn to associate floral scent with food while foraging (*29*), they are more likely to visit flowers bearing the same scent signals. Such behavior increases their foraging efficiency (*30*) while concomitantly leading to more effective pollination (*31*, *32*). Our experiments suggest that by affecting a pollinator's memory, plants reap the reproductive benefits arising from enhanced pollinator fidelity.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/339/6124/1202/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S3
References (*33*–*36*)

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Editor's Summary

Bees Get That Caffeine "Buzz"

Caffeine improves memory in humans, millions of whom find that their daily dose enhances clarity, focus, and alertness. The human relationship with caffeine is relatively recent, however, and thus its impact on our brains is likely a by-product of its true ecological role. Caffeine occurs naturally in the floral nectar of *Coffea* and *Citrus* plants. **Wright *et al.*** (p. 1202; see the Perspective by **Chittka and Peng**) found that caffeine presented at naturalistic levels significantly improved the ability of bees to remember and locate a learned floral scent and potentiated the responses of neurons involved in olfactory learning and memory.

Appendix L

Nutritional balance of essential amino acids and carbohydrates of the adult worker honeybee depends on age

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ORIGINAL ARTICLE

Nutritional balance of essential amino acids and carbohydrates of the adult worker honeybee depends on age

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Abstract Dietary sources of essential amino acids (EAAs) are used for growth, somatic maintenance and reproduction. Eusocial insect workers such as honeybees are sterile, and unlike other animals, their nutritional needs should be largely dictated by somatic demands that arise from their role within the colony. Here, we investigated the extent to which the dietary requirements of adult worker honeybees for EAAs and carbohydrates are affected by behavioural caste using the Geometric Framework for nutrition. The nutritional optimum, or intake target (IT), was determined by confining cohorts of 20 young bees or foragers to liquid diets composed of specific proportions of EAAs and sucrose. The IT of young, queenless bees shifted from a proportion of EAAs-to-carbohydrates (EAA:C) of 1:50 towards 1:75 over a 2-week period, accompanied by a reduced lifespan on diets high in EAAs. Foragers required a diet high in carbohydrates (1:250) and also had low survival on diets high in EAA. Workers exposed to queen mandibular pheromone lived longer on diets high in EAA, even when those diets contained 5× their dietary requirements. Our data show that worker honeybees prioritize their intake of carbohydrates over dietary EAAs, even when overeating EAAs to obtain sufficient carbohydrates results in a shorter lifespan. Thus, our data demonstrate that even when young bees are not nursing brood and foragers are not flying, their nutritional needs shift towards a diet largely composed of carbohydrates when they make the transition from within-hive duties to foraging.

Keywords Honeybee · Amino acid · Nutrition · Protein-to-carbohydrate ratio · *Apis mellifera* · Diet

Introduction

All animals require a dietary source of essential amino acids (EAAs) which are used for growth, somatic maintenance, and reproduction. EAAs are obtained by consuming the protein found in other animals or plants and are in greatest demand during periods of growth early in life (Behmer 2009; Tigreros 2013). In contrast to juvenile animals, adults mainly require amino acids for basic somatic functions (e.g. production of enzymes, peptide or amine signalling, tissue repair, immune function) or reproduction, and their needs for EAA decline with age (Millward et al. 1997; van de Rest et al. 2013). Reproduction, in the form of allocation of resources to eggs or offspring by females (O'Brien et al. 2002) or the donation of nuptial gifts and the production of sperm by males (Voigt et al. 2008), also places demands on the acquisition of amino acids in adult diets, which is often manifested as a trade-off between lifespan and protein/amino acid

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ingestion (Fanson et al. 2012; Grandison et al. 2009; Maklakov et al. 2008).

Adult eusocial insects such as ants and bees live in colonies of closely related, largely sterile adults that engage in a division of labour that includes caring for the queen and brood. In honeybees, behavioural caste correlates with age within the colony. After eclosion, adult worker bees perform within-colony activities such as cleaning, food storage, rearing larvae, and attending the queen (Winston 1987). These bees continue to perform within-hive behavioural tasks as a function of their exposure to the queen's mandibular pheromone (QMP); bees near the queen remain more 'nurse-like' whereas bees exposed to less of the queen's pheromones become foragers (Pankiw et al. 1998). Honeybees are unique, even among social insects, because nurses provision larvae, other workers, and the queen with glandular secretions as food—royal jelly—which is the main source of protein that larvae receive for the first three instars after hatching (Winston 1987). Because most adult bees or ants are sterile, their nutritional requirements for amino acids should be considerably less than those of queens or drones. However, the production of royal jelly is likely to place metabolic demands on young bees (e.g. nurses) for dietary amino acids (Crailsheim 1990): how much that demand exceeds their own requirements for somatic maintenance is unknown. As young bees mature and pass through their behavioural ontogeny, they stop eating pollen and lose the ability to digest solid proteins (Crailsheim 1986, 1990; Lass and Crailsheim 1996; Moritz and Crailsheim 1987); they also stop producing glandular secretions and tending larvae and start building wax comb, packing food into the cells, guarding the colony entrance and eventually become foragers (Winston 1987). Furthermore, during this period, their physiology changes substantially: their mandibular glands and ovaries atrophy (Winston 1987) and their fat body reduces (Seehuus et al. 2007; Ament et al. 2008). How much their nutritional optimum depends on their behavioural caste and age has not yet been tested.

The Geometric Framework (GF) for nutrition is a modelling method developed to identify an animal's optimal intake of key nutrients such as protein and carbohydrate, and the regulatory priorities for different nutrients and performance consequences when animals are confined to suboptimal diets (Simpson and Raubenheimer 1993, 2012). The GF is based on the principle that animals require multiple nutrients simultaneously. The requirement to achieve the optimal proportion of nutrients in their diet forces them to consume a varied diet and/or make trade-offs by overeating or undereating specific nutrients in available foods (Raubenheimer and Simpson 1997). When animals are restricted to a diet containing a set proportion of nutrients, the amount they eat should reflect a 'rule of compromise' that is governed by homeostatic mechanisms tuned to regulate feeding behaviour (Raubenheimer and Simpson 1997; Simpson et al. 2004). These rules are dictated both by nutrient requirements, but also by costs associated with over or under-consuming nutrients relative to requirements (Simpson et al. 2004). The optimal amounts and ratio of specific nutrients, or the 'intake target' (IT), for an animal can be identified in various ways, including by examining the food intake of animals when they are confined to one of several foods composed of different proportions of two or more nutrients (Simpson et al. 2004) or by offering different pairwise choices of nutritionally complementary foods (Chambers et al. 1995).

Here, we tested how caste determined the adult worker honeybee's nutritional requirements for EAAs using the principles in the GF (Simpson and Raubenheimer 1993; Simpson et al. 2004). By confining cohorts of workers to single diets composed of specific proportions of sucrose and the ten EAAs required by honeybees (i.e. protein), we identified the ITs of young bees (days 0–14 from emergence within the colony) and foragers (collected from outside the colony). Diet was limited to carbohydrates (sucrose) and amino acids because these are the main components of honey (Anklam 1998; Hermosin et al. 2003): one of the foods eaten by young workers within the colony and the only food consumed by foragers (with the exception of glandular secretions received during trophallaxis, see Crailsheim 1998). To identify costs associated with over consumption of specific nutrients, we also measured how dietary intake of EAAs influenced survival. To confirm that the costs associated with overeating EAAs was a result of physiological changes associated with age and behavioural caste, we exposed newly enclosed workers to synthetic QMP and measured food consumption and survival on diets high in EAA. This study is the first to show that the nutritional requirements of honeybees change as a function of age and behavioural role, and has implications for dietary intervention strategies designed to improve honeybee health.

Materials and methods

Animals

Frames of newly emerged workers were removed from two colonies of *Apis mellifera* "Buckfast" hybrid honeybees kept in outdoor colonies at Newcastle University. Each frame was placed in a box in a controlled temperature room at 34 °C and 60 % relative humidity. Newly emerged bees were brushed off the frame each day; foraging workers were collected daily at the hive entrance by capturing individuals in plastic sample tubes. For each cohort, 20

bees were placed in a Perspex box (11 × 6 × 20 cm) with five feeding tubes consisting of 2-ml microcentrifuge tubes (four 3-mm holes were drilled along the top of each tube). Four feeding tubes were filled with a treatment solution; each box also had a water tube. The boxes were placed in a constant temperature room at 34 °C and 60 % RH. Experiments continued for 14 days for newly emerged workers (i.e. nurse bees), and 7 days for foraging workers (or as long as the bees survived in both cases). The number of dead bees in each box was counted daily.

Diets

Each of the ten EAAs needed by honeybees (de Groot 1953; methionine, tryptophan, arginine, lysine, histidine, phenylalanine, isoleucine, threonine, leucine, valine) was added to a 1.0 M sucrose solution (Table S1). Ratios of amino acids to carbohydrates (sucrose) were calculated on a molar–molar basis as the following: 1:750, 1:500, 1:250, 1:100, 1:75, 1:50, 1:10, 1:5. All ten amino acids were was added at the same concentration: for example, for the 1:10 diet, the total final concentration of the amino acids was 0.1 M, with each amino acid present at a concentration of 0.01 M.

To measure food consumption, each tube containing nutrient solution or water was weighed, placed in the box, and then reweighed 24 h later. Each tube was replaced with a new, full tube daily. The difference in weight was a measure of the amount consumed in a 24-h period. A control for the evaporation rate of the solution from each tube was performed for each diet by monitoring weight loss in feeding tubes in empty boxes daily for a 5-day period. The final figure for total consumption from each tube was adjusted for evaporation rate by subtracting the amount of solution evaporated from the control boxes with no bees; changes in concentration were also estimated (Table S2). Total daily consumption represented the sum of the adjusted weight of all four feeding tubes; this figure was then divided by the number of live bees remaining on that day. Total carbohydrate and amino acid consumption was calculated by multiplying the amount consumed per bee by the weight of sucrose and the weight of amino acid in 1 ml of solution.

For the sucrose only experiments, cohorts of 20 bees were confined to feeding on 1.0 M sucrose solution for 14 days for young bees and 7 days for foragers. The total volume of solution was measured each day. The volume was adjusted by the evaporation rate in each tube as above.

Queen mandibular pheromone

Cohorts of 20 newly emerged honeybees were kept in the same conditions as described previously. QMP was administered to the treatment group by placing a 2-cm strip of BeeBoost QMP substitute (Pherotech) in each box. Honeybees without QMP were maintained in a separate incubator to avoid contamination with QMP, as were the diets. The diets were administered as described previously; the two diets chosen were sucrose only and the 1:5 diet. Experiments continued for 14 days, with survival and consumption recorded each day for each box (10 per treatment).

Statistics

Daily and total consumption data were analysed using generalized linear models or repeated-measures ANOVA using SPSS (IBM SPSS Statistics v.19) with diet as a main effect. *Post hoc* comparisons were made using least squares difference (LSD) analysis with significance at $P \leq 0.05$. The impact of diet on survival was analysed using a Cox regression (Coxreg) analysis to calculate the hazard ratio (HR) or by Kaplan–Meier analysis. Diets were compared per day over the experimental period using log-rank, pairwise comparisons for each strata, and LSD post hoc tests.

Results

Nutritional intake target of worker bees shifts towards carbohydrate with age and caste

The GF model described by Simpson and Raubenheimer (1993, 2012) predicts that animals make compromises when they are confined to unbalanced diets that reflect the fitness costs associated with over-ingesting and under-ingesting specific nutrients relative to the IT (Fig. 1a, b). In Fig. 1a, the case is illustrated where the underlying fitness landscape is symmetrical about the IT, i.e. the fitness costs of over-ingestion equal the costs of under-ingestion for a given nutrient relative to the IT (and in the case illustrated in Fig. 1a, the costs are also symmetrical between the two nutrients). Here, the intake array across an array of unbalanced food rails forms a smooth arc (Simpson et al. 2004). In Fig. 1b, by contrast, the costs of ingesting excesses are less than those of ingesting deficits of either or both nutrients (Simpson et al. 2004). In this case, the intake array across a range of food rails in not a smooth arc, but rather it can be inferred that the IT lies at the hinge point of the array—identified as a red dot in Fig. 1.

By confining bees to specific proportions of EAAs and sucrose, we were able to identify the IT and the rule of compromise for diets composed of EAAs and carbohydrates (EAA:C). The IT of queenless, newly emerged (young) worker honeybees shifted towards carbohydrates as the workers aged and transitioned to the forager caste (Figs. 2, 3). The intake arrays recorded in the present

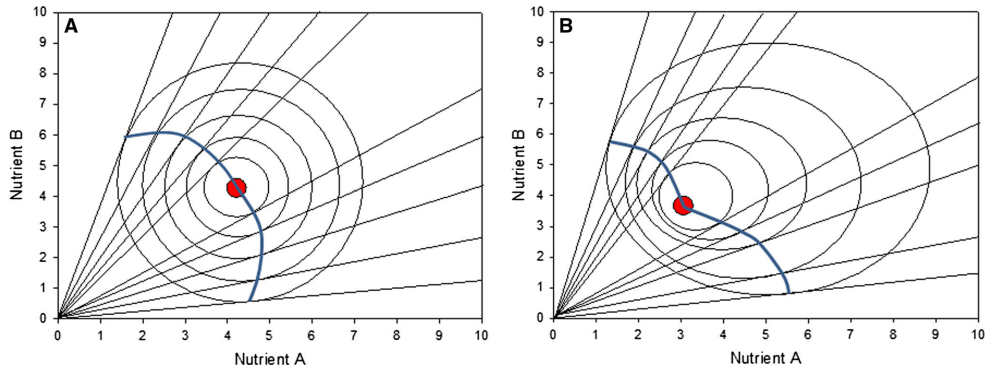

Fig. 1 Model of dietary regulation when animals are confined to diets of specific proportions of two macronutrients. When unable to reach an optimal diet composition (the IT, indicated as *red dot*) they must trade-off overeating the nutrient in excess against undereating the nutrient in deficit. Across an array of diets, these trade-off points form an intake array, the shape of which reflects the underlying fitness landscape describing the costs of ingesting nutrient excess and deficits. Fig. 1a, b showcases whether the costs of eating excesses are less than the costs of ingesting deficits relative to the IT. Figure 1a, b indicates cases for quadratic cost functions with these being symmetrical for the two nutrients in **a** and asymmetrical in **b**. Note how in **b** the array hinges outwards at the IT, and hence the hinge point in an array of this form can be used to infer the position of the IT (colour figure online)

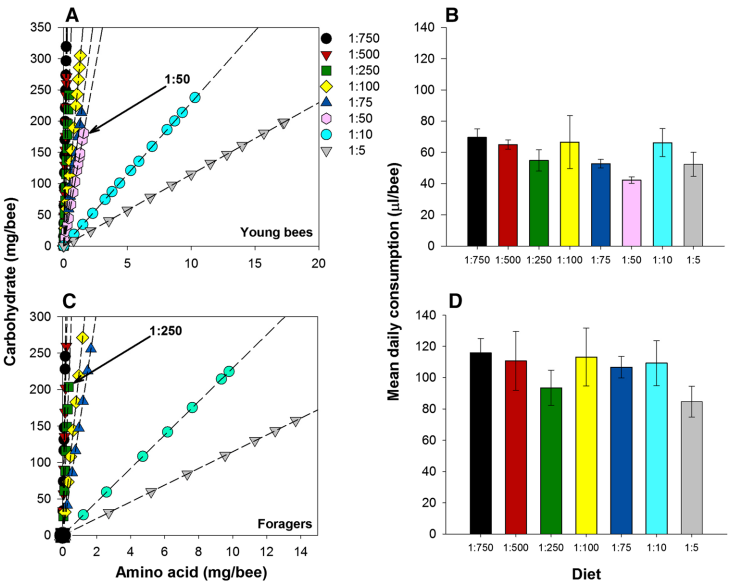

Fig. 2 Nutritional regulation in young honeybees and foragers. The demand for EAAs decreases when worker honeybees become foragers ($n = 10$ cohorts of 20 bees each per rail). **a** Newly emerged workers confined to diets of specific proportions of EAAs and sucrose regulated their intake around an IT of 1:50. **b** The total volume of diet solution consumed by young bees depended on the proportion of EAAs and carbohydrates in the diet (one-way ANOVA, 1:50 × all other diets, all $P < 0.033$). **c** Foragers use an asymmetric rule of compromise when regulating their intake, such that intake is always skewed towards carbohydrates; the IT was estimated to 1:250. **d** The average daily volume consumed by foragers did not depend on diet

experiment tended to hinge at a point of least consumption on one diet and open outwards as bees ingested more on diets diverging from this ratio, indicating that the bees followed an asymmetrical quadratic rule of compromise as in Fig. 1b (Simpson et al. 2004).

Young bees consistently overate diets high in EAAs to obtain sufficient carbohydrate (Fig. 2a, GLZM, main effect, diet: $\chi^2_6 = 182$, $P < 0.001$), but would also overeat diets containing a high proportion of carbohydrates to obtain sufficient EAAs (GLZM, main effect, diet:

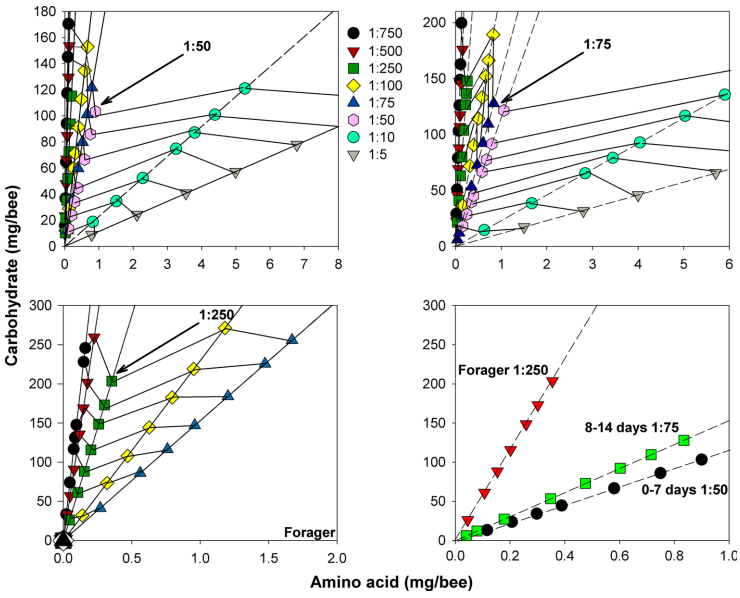

Fig. 3 ITs of honeybees shift towards carbohydrates as bees age. The IT of newly emerged bees **a**, young worker bees **b**, and foragers **c** is re-plotted from a subset of data in Fig. 2. **d** The trajectory for bees fed the diet closest to the IT of all three age groups is shown; foragers require ~5-fold less dietary EAAs than young bees

$\chi^2_6 = 182$, $P < 0.001$). The hinge point in this graph, when compared to Fig. 1, indicates that young, adult worker honeybees have an IT of 1:50 (Compared to previous work using protein in diet instead of amino acids, our 1:50 diet is equivalent to a weight-for-weight diet of 1:115, amino acid-to-carbohydrate, Table S1). The daily average volume of each diet solution consumed by the cohorts of young bees was not significantly different (Fig. 2b, GLZM, main effect, diet: $\chi^2_7 = 10.8$, $P = 0.145$).

Foragers died rapidly when confined to cages within the lab; for this reason, we evaluated the survival and consumption of foragers over 7 days rather than 14 days. Using the same logic as above in Fig. 1, we estimate that the IT of foragers was 1:250 (Fig. 2c). The foragers defended their intake of carbohydrates at the expense of overeating EAAs: the cumulative quantity of EAAs consumed depended on the diet (Fig. 2c, GLZM, main effect, diet: $\chi^2_6 = 182$, $P < 0.001$), but the amount of carbohydrates was constant (GLZM, main effect, diet: $\chi^2_6 = 10.7$, $P = 0.097$). Again, the daily average volume of each diet solution consumed was not significantly different (Fig. 2d, GLZM, main effect, diet: $\chi^2_6 = 4.83$, $P = 0.565$).

The shift towards a diet high in carbohydrates between young bees and foragers is most obvious when a subgroup of the rails around the IT for newly emerged bees, young bees, and foragers is plotted separately (Fig. 3). Since we noticed a large change in the ability to survive on diets high in EAAs, we compared whether there was an effect of diet on the IT of nurses aged 0–7 days (Fig. 3a), nurses aged 8–14 days (Fig. 3b), and foragers (Fig. 3c). The amount of diet consumed per day depended on the age group and the dietary ratio of EAA:C (three-way interaction: repeated-measures ANOVA, day × age × diet, $F_{8,702} = 6.44$, $P < 0.001$).

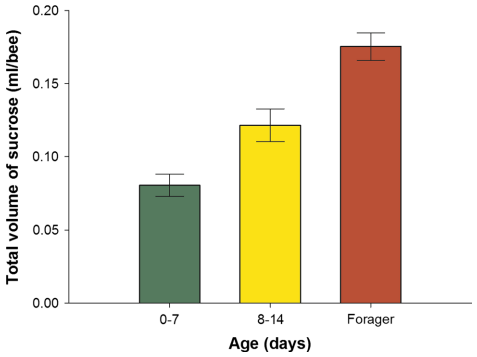

Fig. 4 Dietary demand for carbohydrates increases with age ($n = 20$). Bees aged 0–7 days consumed the least amount of 1.0 M sucrose solution (pairwise LSD: vs. 8–14 days, $P = 0.002$; vs. foragers: $P < 0.001$); foragers consumed the most (8–14 day-old bees vs. foragers, pairwise LSD, $P < 0.001$)

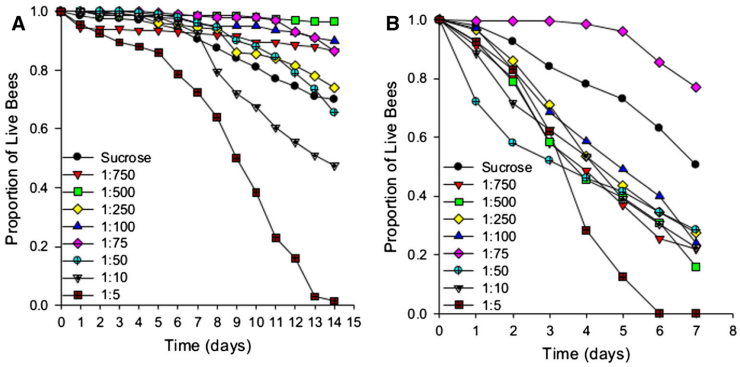

Fig. 5 Survival of young bees and foragers is compromised by high concentrations of EAA. Survival on the amino acid diets for nurses over 14 days **a** and foragers **b** over 7 days. Each rail is represented as the total proportion of live bees/day ($n = 10$ cohorts of 20 bees each per rail). **a** Young bees fed on diets high in EAAs died at a faster rate than those on diets low in EAAs or sucrose alone (GLZM, Preg, two-way interaction, diet × day, $\chi^2_{73} = 3.5 \times 10^{14}$, $P < 0.001$). **b** Foragers fed diets containing EAAs also died at a faster rate (GLZM, Preg, two-way interaction, diet × day, $\chi^2_{56} = 752.45$, $P < 0.001$)

Young bees during days 0–7 had an IT closest to 1:50, whereas bees aged 8–14 days had an IT that had shifted towards 1:75. When the ITs of all three groups are compared (Fig. 3d), it is clear that as adult worker bees age, their IT shifts away from a diet relatively high in EAAs towards a diet that has 5 times less EAAs.

This change in the need for dietary EAAs was also accompanied by a shift towards a greater demand for carbohydrates (Fig. 4). In a separate experiment, we found that foragers ate 60 % more 1.0 M sucrose solution than young bees (Fig. 4, GLZM: age, $\chi^2_2 = 50.3$, $P < 0.001$). Thus, the shift in the IT with changes in caste reflected not only a reduced demand for EAAs, but also an increase in the overall amount of carbohydrates needed by foragers.

Diets high in amino acid concentration result in poor longevity

To identify costs associated with over consumption, we also compared the survival of each behavioural caste of the bees when fed each diet. Both young bees and foragers exhibited shorter lifespans on diets high in EAAs (Fig. 5). For the first 7 days, newly emerged bees had a lower proportional hazard of dying regardless of diet (Fig. 5a, Coxreg, days 0–7, sucrose × all other diets, HR = all <0.9 [95 % CI (confidence interval) 0.366–2.403], all $P = 1.000$). For bees aged 8–14 days, however, the risk of dying on diets high in EAAs increased dramatically such that there was a 6.5-fold increased risk when they were fed the 1:5 diet compared to sucrose (Coxreg, days 8–14, sucrose × 1:5 diet, HR = 6.065 [95 % CI 2.402–15.312], $P < 0.001$).

The proportional hazard of dying for foragers was also greater when they consumed diets containing EAAs (Fig. 5b, Coxreg, sucrose × 1:750–1:75, HR = 1–2.333 [95 % CI 0.202–9.03], $P = 0.220$). Their risk was ~3-fold greater when they were fed the 1:10 diet, and 8.3-fold greater when they were fed the 1:5 diet compared to sucrose (Coxreg, 1:10 × sucrose, HR = 3.00 [95 % CI 0.812–11.1], $P = 0.067$; 1:5 × sucrose, HR = 8.333, [95 % CI 2.52–27.6], $P = 0.001$).

Young bees tolerate diets high in EAAs longer when exposed to QMP

To identify whether EAAs affected survival of bees regardless of caste, we maintained bees in the 'nurse-like' caste using QMP and measured their rate of survival when fed a diet high in EAAs (1:5) or sucrose alone (Fig. 6a). Bees exposed to QMP had lower proportional hazard of dying over the 14-day period, regardless of diet (Coxreg, +QMP vs −QMP, HR = 0.688 [95 % CI 1.01–1.71], $P = 0.045$). As we observed in Fig. 4, when fed the 1:5 diet, bees had a greater risk of dying if they were reared in the absence of QMP (Coxreg, sucrose vs 1:5, HR = 6.50 [95 % CI 2.75–15.4], $P < 0.001$). When bees were exposed to QMP, the diet high in EAA (1:5) did not increase their risk of death during days 0–10 (Fig. 6a, Coxreg, sucrose vs 1:5, HR = 1 [95 % CI 0.791–1.27], $P = 1.00$). However, in days 11–14, regardless of QMP exposure, the 1:5 diet reduced survival compared to sucrose only (Coxreg, sucrose vs. 1:5, HR = 1 [95 % CI 1.932–11.27], $P = 0.001$). To verify this result, we also tested whether these curves were different fitting a Kaplan–

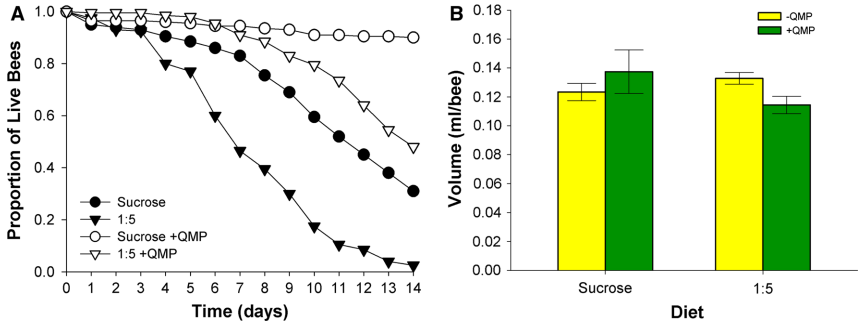

Fig. 6 QMP exposure increases tolerance for dietary EAA. **a** Bees exposed to QMP have a higher rate of survival than same-aged queenless bees when fed a diet high in EAAs. Cohorts of 20 bees were confined to either sucrose or a 1:5 diet of EAAs and sucrose for 14 days. In the absence of QMP, young bees die faster when fed the 1:5 diet indicating that QMP is required to maintain them in the nurse-caste nutritional physiology. **b** Daily average consumption was not significantly different between bees exposed to QMP and bees that were not exposed to QMP. $n = 10$ cohorts per treatment

Meier model; comparison of these treatments revealed that QMP-exposed bees fed the 1:5 diet died at a faster rate in the second week of the experiment (Kaplan–Meier, log-rank pairwise comparison, sucrose × 1:5, $\chi^2_1 = 23.9$, $P < 0.001$). Bees exposed to QMP did not consume significantly more of either the sucrose or the 1:5 diets than the unexposed bees (Fig. 6b, 2-way GLZM: QMP trt × diet, $\chi^2_1 = 3.58$, $P = 0.058$).

Discussion

Our data demonstrate four important findings: (1) adult worker honeybees prioritize their dietary intake of carbohydrates over EAAs; (2) nutritional requirements for EAAs are greater for young bees, but shift as they age and become foragers; (3) workers show reduced survival on diets high in EAAs, but no cost is associated with eating diets composed only of carbohydrates; and (4) the risk of death associated with consumption of diets high in EAAs is a function of age and caste.

We expect that the IT for young bees (EAA:C of 1:50) mainly represents the IT required for somatic maintenance without the demands of producing glandular secretions or wax. Honeybees are unusual among insects, because adult workers have a specialisation that allows them to produce glandular secretions (e.g. royal jelly) as a form of care for offspring and other nest mates. Like mammalian milk, the glandular secretions produced by adult nurse bees are composed of proteins, fatty acids, and carbohydrates (Garcia-Amoedo and de Almeida-Muradian 2007; Kanbur et al. 2009; Peixoto et al. 2009) and are likely to place great nutritional demands on nurse bees during their production. In our experiments, young bees probably produced much less of their glandular secretions because they were not caring for larvae or exposed to brood pheromone. In addition to the demand for nutrients to produce royal jelly, young bees also sequester nutrients by making hexamerin proteins that are stored in the fat body and later used as a source of amino acids (Martins et al. 2008, 2010). In our experiments, the demand for EAA was high in broodless young bees, but perhaps not as great as if they had been in contact with brood or the queen in the colony. In this case, we predict that the young adult worker's IT will be near the P:C ratio found in royal jelly (~1:1) (Garcia-Amoedo and de Almeida-Muradian 2007; Kanbur et al. 2009; Peixoto et al. 2009; Schmitzova et al. 1998).

In honeybees, behavioural caste is confounded with age: young bees are nurses, comb builders or guards and older bees are foragers (Robinson 1992). The transition to foraging is affected in part by proximity to the queen and exposure to QMP (Pankiw et al. 1998) as well as brood pheromone (Pankiw 2007). Workers that have little or no exposure to the queen or her pheromones undergo substantial physiological changes orchestrated by juvenile hormone (Robinson 1992) and begin to behave like foragers (Pankiw et al. 1998). Furthermore, in the absence of the queen, workers lose the ability to digest protein after their eighth day post-eclosion (Moritz and Crailsheim 1987). After the eighth day in our experiments with queenless workers, we also observed that the IT of our young bees shifted towards more carbohydrates. Using synthetic QMP as a tool to prevent young bees from transitioning to the forager caste, we were able to show that workers continue to tolerate high levels of dietary EAAs, even at levels exceeding their IT. In spite of having better survival early on, however, the QMP-exposed nurse bees fed with the 1:5 diet still died at a faster rate than those fed with sucrose in

the final days of the experiment. It is possible that in this case, even though they were exposed to QMP, their lifespan was affected by the continued consumption of diets high in amino acids (Grandison et al. 2009) or that synthetic QMP alone was not sufficient to keep them in the 'nurse-like' caste (Maisonnasse et al. 2010).

The carbohydrate and lipid metabolism of bees is also affected by QMP (Fischer and Grozinger 2008). QMP maintains the abdominal fat of young bees and increases their resistance to starvation (Fischer and Grozinger 2008). Our data indicate that this resistance to starvation may be due to the fact that the presence of QMP maintains their physiological state such that they require much less carbohydrate. Thermal stress is another factor that could influence the within-hive bees' demand for carbohydrates. In the presence of brood, young bees keep the brood warm using their flight muscles to produce heat when the temperatures outside of the colony drop (Simpson 1961; Fahrenholz et al. 1992; Stabentheiner et al. 2010). Our bees were neither exposed to brood nor did we investigate the influence of temperature on the IT, but we predict that young, within-hive bees performing endothermy would exhibit a greater demand for dietary carbohydrates. Based on our data, we also estimate that the workers' demands for carbohydrate increase from 2 to 5 times when they become foragers. Foraging honeybees fly to and from the nest to collect food and water for the colony: an activity with high demands for energy (Suarez and Darveau 2005; Suarez et al. 2005). Indeed, the mass-specific metabolic rate of flying foragers is the highest of any animal recorded (Suarez et al. 1996). If placed under the demands of flight and carrying pollen loads, the energetic demands of foragers increase to +50-fold greater than at rest (Joos et al. 1997). For this reason, we predict the IT of flying foragers to be skewed even further towards the intake of carbohydrates than we were able to measure in our cohorts of bees confined to cages.

Previous studies of worker honeybee and ant nutrition also showed that workers die at a faster rate when they are forced to consume diets high in protein (Pirk et al. 2010; Dussutour and Simpson 2012). In our experiments, the foragers fed diets high in EAAs (1:10 and 1:5 diets) had much reduced survival because they were required to over-ingest EAA to obtain sufficient carbohydrates. Their premature mortality was not a function of a refusal to eat diets high in dietary EAA, indicating that consumption of the EAA was the cause of mortality. Even bees exposed to QMP and fed the 1:5 diet for 14 days had greater mortality during days 11–14 than those fed sucrose alone. The fact that honeybee workers are more likely to die as a function of eating EAAs may indicate that bees are not efficient at converting EAAs into energy via gluconeogenesis. Diets high in EAAs could cause metabolic stress and require that excess is excreted. Locusts fed diets high in protein excrete higher concentrations of amino acids in their faeces than those fed diets low in protein (Zanotto et al. 1994). In the cohorts fed diets high in EAAs, we also observed more defecation within the boxes. This could indicate that bees accumulated waste associated with excess amino acids or uric acid and had difficulty eliminating it.

A previous study showed that the IT of isolated cohorts of young worker honeybees was 1:12 (wt/wt) when they were fed solid diets based on proteins such as casein (Altaye et al. 2010). This is an almost 10-fold lower IT for EAAs compared to the IT of our young bees ($\sim$1:115 wt/wt, Table S1). The digestion of protein requires the production of proteases, which could place a greater demand on bees for EAAs (Moritz and Crailsheim 1987). Furthermore, proteins such as casein are composed of unequal ratios of amino acids when they are digested (Moritz and Crailsheim 1987; Szolderits and Crailsheim 1993), whereas our liquid diets were composed of all the EAAs at the same concentration. While we did not specifically test this, the fact that our IT was strongly skewed towards carbohydrates in comparison to an IT calculated for solid proteins (Altaye et al. 2010) implies that the ratios of EAAs in diet strongly affect dietary regulation of protein/EAA intake. This could be a general feature of protein regulation in animals, but has yet to be tested. In support of this, Altaye et al. (2010) found that diets rich in digestible protein such as royal jelly yielded a ratio skewed more towards carbohydrate (royal jelly: 1:14), whereas artificial diets that are likely to be more difficult for bees to digest predicted an IT of 1:11.

Previous studies have found that starvation and social isolation induce the transition to the foraging caste (Marco Antonio et al. 2008; Pankiw et al. 1998). The young worker bees in our study were not socially isolated and were given *ad libitum* access to food sources, yet they still continued on a trajectory towards a nutritional IT biased towards carbohydrate—consistent with them undergoing physiological changes that accompany foraging. Our data demonstrate that caste determines dietary needs of workers in the honeybee colony. Worker honeybees fail to survive if they do not consume sufficient dietary carbohydrates, and will over-ingest EAAs to obtain carbohydrate, even if it reduces their long-term survival. The influence of the queen, combined with feedback about the amount of food in the colony (Marco Antonio et al. 2008) and the presence of other foragers (Leoncini et al. 2004), is all likely to be the factors that affect the worker bee's caste and hence its nutritional optimum.

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Conflict of interest The authors declare that they have no conflict of interest.

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