Using metabolic fingerprints to study insect-plant interactions

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Abstract

Metabolic fingerprinting is a biochemical method that takes an untargeted approach to measure a large number of metabolites and gain a 'snapshot' of an organism's metabolome at a specific time. This thesis explores how metabolic fingerprinting can be used to study plant-insect interactions using *Pieris rapae* and its larval host plant species as model systems, and investigates how biotic and abiotic factors shape plant and insect metabolomes. I found that different Brassicales host plant species, as well as *P. rapae* larvae feeding on these plant species, had different metabolic fingerprints. A group of very abundant metabolites in the host plant *Cleome spinosa* were present in larvae feeding from this plant species, documenting a new occurrence of metabolite transfer between plants and insect herbivores. There was some evidence that the metabolic fingerprints of plants predicted the performance of insects, implying that the presence or absence of specific metabolites in host plants may determine the success of herbivores. Changes in metabolites measured in three host plant species following herbivory by *P. rapae* showed that herbivory changed the metabolic fingerprints of plants but there was little overlap in metabolites that were induced. I conclude that plants respond in a species-specific manner to herbivory, which implies that the evolution of plant defences has varied among the three species resulting in no similarities in induced metabolites. The metabolic fingerprints of the host plant *Brassica oleracea* as well as *P. rapae* larvae were changed by elevated temperature and to a lesser extent by elevated carbon dioxide (CO_2). The larvae developed more quickly under elevated temperature but larval performance was not affected by elevated CO_2 despite the diet of *B. oleracea* leaves grown under elevated CO_2 containing less nitrogen. These findings provide a unique metabolite perspective of insects and plants and were facilitated by the wide breadth of metabolites studied using metabolic fingerprinting.

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Declaration

I declare that the work in this thesis is my own work and where other work is included this has been properly acknowledged.

Chapter 3 has been prepared for publication as a co-authored paper with the following collaborators: M.V.L. Perera, H.V. Florance, L.A. Robinson, S.D. Penfield and J.K. Hill and therefore repeats information in the materials and methods chapter.

Chapter 1 General Introduction

1.1 Plant-insect relationships

The kingdom Plantae and the class Insecta encompass a large number of organisms with an estimated 390,800 species of plants and around 1 million species of insects described (Chapman 2009). Consequently the number of plant-insect interactions is considerable (Schoonhoven *et al.* 2005). Such interactions include insects which are essential for the pollination of plants (De Luca & Vallejo-Marin 2013), plants which provide shelter and protection from insect predators (Diniz *et al.* 2012), and the half of all insect species that are estimated to use plants as a food source (Wu & Baldwin 2010). Improving our knowledge of these latter plant-herbivore interactions is important for several reasons. First, if an insect's host plant is a crop plant and the insect is a pest it may be a threat to our food security (Gregory *et al.* 2009). Secondly, conserving species usually requires an understanding of an insect's relationship with its host plants (Moir *et al.* 2012). Thirdly, plants and insects interact with a range of environmental factors that are altered by climate change (Robinson *et al.* 2012). If we are to predict and mitigate the effects of climate change it will be important to have information on how plant physiology is affected (Fuhrer 2003) and how pest performance and populations of insects may change (Bale *et al.* 2002; Newman 2004).

Some insects have evolved to develop on a restricted range of host plants. The alternative insect strategy is to be polyphagous and have the ability to develop on a variety of plant species (Schoonhoven *et al.* 2005). If multiple plant species can be consumed, advantages of a polyphagous strategy may include increased resource availability (Bernays & Minkenberg 1997) or increased fitness by consuming different host plants that are optimal for different life stages of the larvae (Rodrigues & Freitas 2013). The range of plant species that are included as hosts of an insect is thought to be determined by co-evolution of plants and insects and by plant-insect arms races involving chemical plant defences (Ehrlich & Raven 1964;

Janz 2011). An arms race between plants and herbivorous insects involves the plant evolving new defences as the insect evolves immunity to existing defences (Mithofer & Boland 2012). These co-evolution and arms race theories explain the observations that taxonomically related butterfly species tend to feed on taxonomically related host plants and that different species of host plants consumed by a butterfly species often share common chemicals (Ehrlich & Raven 1964; Janz 2011). An alternative theory proposed to explain the chemical commonality among host plants is that as plants evolved, insects have tracked certain plant chemicals which the insect has either utilised as signals for locating the correct host plant or has been sequestering for its own defence (Bernays 2001). It is possible that both mechanisms contribute to determining the final suite of host plants consumed by a species of insect.

The performance of polyphagous insects can vary between different host plants (see Gripenberg *et al.* 2010 for examples). A number of plant characteristics have been proposed to determine the performance of an insect on a plant, including water and nitrogen content, and chemical defences in leaves (Slansky & Feeny 1977; Scriber & Feeny 1979). Some studies have correlated the performance of insect herbivores on different host plant species with the characteristics of the plants. Whiteflies (*Bemisia tabaci*) were found to perform better on plant species containing more nitrogen and lower defence compounds (Jiao *et al.* 2012), whereas nutrition of four host plant species was independent of the performance of larvae from two species of Pierid butterflies (Hwang *et al.* 2008). The defences of oak trees (*Quercus* sp.) in the form of condensed tannin content were found to negatively correlate with the performance of a tussock moth (*Orgyia vetusta*) as did the mechanical defence of leaf toughness (Pearse 2011). Another mechanical defence, the presence of trichomes, as well as the presence of chemical defences in host plants were found to reduce the performance of sawfly (*Athalia rosae*) (Travers-Martin & Müller 2008). However, in addition to characteristics of the host plants the performance of whitefly (*Bemisia tabaci*) has been found to be affected by the insect population's previous experience of host plants (Shah & Liu 2013). These examples demonstrate that there is unlikely to be a single factor which is

responsible for the majority of variation observed in the performance of insects on different species of host plant and that these factors may be species specific.

1.2 Induced plant responses to herbivory

Plants are not defenceless against insect herbivores. They possess defences that can be either constitutive, where the compound is maintained at a constant abundance in the plant, or induced upon herbivore attack (Bezemer & van Dam 2005). In order to mount a defence against an insect herbivore, a plant must first detect that it is under attack and one indication can be the physical wound a herbivore inflicts on a plant (Heil 2009). The resulting disintegration of plant cells releases molecules such as systemin which has been shown to ultimately result in the transcription of defence related genes (Pearce *et al*. 1991). Defence genes can also be activated by ATP which escapes the cell upon plant cell destruction and which is known to have a signalling function when it is extracellular (Roux & Steinebrunner 2007).

Inflicting physical damage to a plant using, for example a blade, may release indicative molecules but does not replicate a herbivore attack exactly (Mattiacci *et al*. 1994; Korth & Dixon 1997). This is because mechanical damage does not involve herbivore derived signals which have been termed Herbivore Associated Molecular Patterns (HAMPs) (Mithöfer & Boland 2008). HAMPs contribute to the perception of an attack and can be derived from the oral secretions or regurgitants from insects. HAMPs include FACs (fatty acid-amino acid conjulates) which are transferred to the plant while the insect is chewing (Mithöfer & Boland 2008). Applications of artificially produced FACs have been shown to increase the circulating levels of phytohormones and prompt changes in plant transcripts (Halitschke 2001). Another chemical indicator that has been found in insect regurgitant is β-glucosidases. This has been shown to initiate the release of volatile chemicals from the plant (Mattiacci *et al*. 1995). Such volatile chemicals are employed as indirect plant defences by attracting to the plant predators or

parasitoids of the attacking insect herbivore (Kessler & Heil 2011). It has also been shown that indirect plant volatile defences can be triggered by the oviposition of herbivore eggs on plants (Hilker & Meiners 2006). For example, following oviposition of eggs from a bug (*Nezara viridula*) on leaves of broad bean (*Vicia faba*) the plant releases volatiles that attract the egg parasitoid wasp *Trissolcus basalis* (Collaza *et al*. 2004). In addition to attracting egg parasitizing enemies it is possible that ovipositional signals could prime the direct defences of a plant before the larvae hatch so that herbivore performance is reduced (Hilker & Meiners 2011).

Phytohormones are the link between the perception of a herbivore attack and the resulting expression of plant genes that generate a defensive response (León *et al.* 2001). The cascades of phytohormone signals triggered after an attack are not simple linear pathways but series of multiple, concurrent reactions that can have inhibitory effects on each other or work synergistically (Kunkel & Brooks 2002, Rojo *et al*. 2003). Among the known phytohormones, jasmonic acid (JA), ethylene and salicyclic acid are primarily associated with defence responses (Rojo *et al*. 2003). Attack by chewing and biting insects will mainly activate the JA biosynthesis pathway (termed the octadecanoid pathway) while piercing-sucking insects (such as aphids) induce a reaction more akin to pathogen attack which mainly features salicyclic acid (Walling 2000). The octadecanoid pathway employs enzymes to catalyse the modifications of a string of precursors to produce JA and associated compounds (collectively named jasmonates) (Furstenberg-Hagg *et al*. 2013; Schaller *et al*. 2005). The genes that code for such enzymes are themselves JA inducible. This suggests a positive feedback mechanism in JA synthesis (Wasternack 2007). The presence of JA allows transcription factors to produce RNA from JA-responsive genes (Furstenberg-Hagg *et al*. 2013). Application of JA to plants or insect attack on plants has successfully increased the transcription of defence related genes or increased levels of defensive compounds (Reymond *et al*. 2004; van Dam *et al*. 2004; van Dam & Oomen 2008). These findings confirm the role of JA in the defence of the plant against chewing insects.

Defensive responses to herbivore attack can be found at or near the attacked area, however, these local responses can be accompanied by systemic responses in other parts of the plant (Gatehouse 2002). JA is one of the signals produced in response to herbivore attack that is thought to travel in the phloem of plants to unwounded leaves where it can stimulate the transcription of defensive genes and ultimately produce a defensive response (Furstenberg-Hagg *et al*. 2013). For instance, JA increases were recorded in the leaf of a plant (*Mentha aquatic*) attacked by a beetle (*Chrysolina herbacea*) but there were also increases in the undamaged, younger leaves (Occhipinti *et al*. 2011). As well as signalling between leaves, the systemic communication between aboveground and belowground plant tissues has been revealed. In two Brassica plant species JA applied to the roots to simulate attack by a belowground herbivore, resulted in increases in defensive compounds in the aboveground plant shoot (van Dam *et al*. 2004). Systemic responses have not only been demonstrated using the application of signalling hormones and quantification of defensive compounds. Effects on the performance of insects raised on the leaves of plants that have been attacked either belowground or aboveground have been recorded for a number of plant and insect species (Johnson *et al*. 2012).

Plants can exhibit physical defences such as thick leaves which can have detrimental effects on insects such as causing mandibular wear (Raupp 1985). Some of these physical defences can be induced by herbivore attack. Elevated lignin content which reduces plant palatability (Wardle *et al*. 2002) has been recorded in *Alternanthera* species following herbivory by a grasshopper (*Atractomorpha sinensis*) (Fan *et al*. 2013). A mechanism by which lignification is induced by herbivory has been suggested in tobacco plants (*Nicotiana attenuata*). In these plants the biosynthesis of lignin could be promoted by the biosynthesis of phenolamides which are induced by tobacco hornworm (*Manduca sexta*) herbivory (Gaquerel *et al*. 2013). As well as the leaves, lignification has also been found to protect the plant roots from underground herbivores (Johnson *et al*. 2010). Trichomes (hairs) can also deter insect herbivores (Agren and Schemske 1993) and in some instances can be induced. For example, new leaves on willows (*Salix*

cinerea) were found to have a greater trichome density if the plant had recently experienced a beetle (*Phratora vulgatissima*) attack (Bjorkman *et al*. 2008).

Another type of defence that plants can employ against herbivores are defensive proteins which include protease inhibitors (Bowles 1990). Protease inhibitors restrict the insect from obtaining nutrients from the ingested host plant. The proteases of the insect gut are used by the insect to digest plant proteins. However, the plant protease inhibitors interrupt this enzymatic process which can result in slower growth, development and reproduction of the insect herbivore (Lawrence & Koundal 2002). Other defensive proteins include enzymes such as arginase and threomine deaminase. These break down the plant-derived amino acids in the insect gut reducing the number of essential amino acid nutrients the insect obtains from its food (Chen *et al*. 2005; Gonzales-Vigil *et al*. 2011).

1.3 Defensive metabolites

In addition to the indirect, volatile plant metabolites previously mentioned which are employed as defences by attracting predators or parasitoids of the attacking insect herbivore (Kessler & Heil 2011), there are a wide variety of metabolites that have a role as a direct defence against herbivores by making the food unpalatable, unattractive or toxic (Furstenberg-Hagg *et al.* 2013). There are a large number of different classes of defensive metabolites some of the most commonly studied classes are alkaloids which include compounds familiar to us such as caffeine, nicotine and morphine; cyanogenic glucosides such as tyrosine and phenyalanine; phenolics which include flavonoids and tannins; terpenoids which include some volatile compounds and a class of compounds called glucosinolates (Mithöfer & Boland 2012). The glucosinolates are characteristic of the plant family Brassicaceae (Kos *et al*. 2012) and are well studied (Kroymann 2011). They are therefore a good example with which to explain the mechanisms that result in a plant producing defensive metabolites following herbivory. There have been around 120 different glucosinolates described (Fahey *et al*. 2001) all of which share a common core

structure plus a part referred to as the R-group that is characteristic of the specific glucosinolate (Hailker & Gershenzon 2006). The biosynthesis of glucosinolates can be considered in three stages. First of all specific amino acids are elongated by adding methylene to their side chains. Then these amino acids are reconfigured into the common core structure of a glucosinolate. Finally the side chains of this core structure can be modified to produce further glucosinolates (Halkier & Gershenzon 2006; Grubb & Abel 2006). Many of the enzymes that catalyse the steps in the biosynthesis of glucosinolates as well as the genes encoding those enzymes have been characterised in the model plant *Arabidopsis* (Grubb & Abel 2006). Such genes can be activated by the presence of jasmonates as described above.

Glucosinolates are stored in plant cells but in a separate compartment to myrosinase enzymes that hydrolyse them. This means that only when a chewing insect eats a plant do the two chemicals combine to produce toxic compounds (Bones & Rossiter 1996). There are five classes of compounds that can be produced when glucosinolates are hydrolysed: isothiocyanates, nitriles, epithionitriles, oxazolidine-2-thione and thiocyanates. Isothiocyanates are the most common hydrolysis products from glucosinolates (Halkier & Gershenzon 2006) and have been associated with the reductions in growth rates of generalist insect species (Li *et al.* 2000; Müller *et al.* 2010). However, some insect species which have specialised on glucosinolate-containing plants are known to use a gut protein to divert the hydrolysis of glucosinolates into harmless nitriles (Wittstock *et al.* 2004). Furthermore, glucosinolates are used to the advantage of some insect species to aid recognition and to locate host plants for oviposition or to stimulate larval feeding (Hopkins *et al.* 2009).

The chemical diversity and vast number of secondary metabolites in plants is frequently commented upon (Gershenzon *et al.* 2012) although no one knows exactly how many metabolites there are, or which metabolites are present in which species. One estimate of the number of metabolites within the plant kingdom is in excess of 200,000 (Dixon & Strack 2003) though this may be an underestimation of the true number (Pichersky & Lewinsohn 2011). Of this total number,

approximately 50,000 secondary metabolites are known and recorded in databases (De Luca & St Pierre 2000; Mithofer & Boland 2012). It has only been relatively recently that metabolomic technology has advanced to a point where most metabolites can be recorded easily (Allwood *et al.* 2008). Thus, the large number of metabolites present in plants combined with newly emerging technology to measure multiple metabolites means there are still many unknowns surrounding plant metabolites, such as how they have evolved (Kliebenstein 2008), why metabolites are so diverse (Moore *et al.* 2014), and what their functions are (Theis & Lerdau 2003).

1.4 The metabolome

The metabolome is the collective term for the full suite of metabolites within an organism (Oliver *et al.* 1998; Fiehn 2001; Hall 2006). These metabolites, which are generally thought of as chemicals that are less than 1500 Daltons in size (Hall 2006), encompass primary and secondary metabolites. Primary metabolites refer to organic and amino acids, sugars and sugar alcohols, fatty acids and sterols (Kral'ova *et al.* 2012) while secondary metabolites may refer to phytohormones (Wasternack 2007) and defensive metabolties such as those groups of chemicals discussed above, along with their chemical precursors, intermediates and derivatives (Kaplan *et al*. 2004). The metabolome can be thought of as the end result of gene expression (Sumner *et al.* 2003; Fig. 1.1). However, between the “instructions” in the genome and the resulting metabolome there are many influences that shape the metabolome, which is constantly changing (Kooke & Keurentjes 2012). The plant metabolome has been shown to change over time, for example diurnal changes in the abundance of sugars and amino acids in *Arabidopsis* (Espinoza *et al.* 2010) and differences between metabolic fingerprints of juniper berries (*Juniperus communis*) harvested in different seasons (Falasca *et al.* 2014) have been found. There are also spatial variations in metabolomes, for example, glucosinolate composition and quantities vary between the seeds, flowers, fruits, roots and

young and old leaves of *Arabidopsis* (Brown *et al.* 2003). Individual metabolites are influenced by abiotic and biotic factors therefore it follows that the metabolome as a whole is also influenced by such factors (Kral'ova *et al.* 2012) for example nutrient availability (Lubbe *et al.* 2011), water availability (Lavoir *et al.* 2009), ozone (Cho *et al.* 2008), CO_2 (Levine *et al.* 2008) and temperature (Gray & Heath 2005). The reported effects of these abiotic factors mean that any large changes in the environment such as climate change will have consequences for plant metabolomes. In addition to abiotic factors, there are biotic factors such as pathogens (Vikram *et al.* 2006), mycorrhizal fungi (Kogel *et al.* 2010) and herbivores both below and above ground (Kutyniok & Müller 2012) that alter the metabolome.

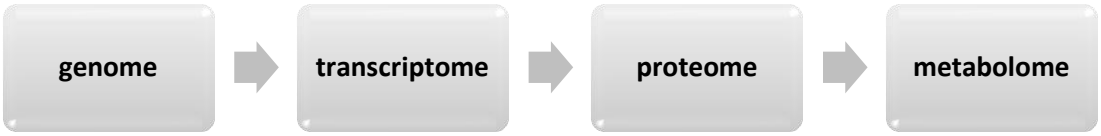


Fig. 1.1 The multiple levels of an organism. The metabolome is shaped by the expression of the genome.

Compared to plants, the metabolomes of insects have not been as extensively studied especially using techniques that measure multiple metabolites simultaneously. One area of research that has recently expanded our knowledge of the insect metabolome is the investigation of diapause (Colinet *et al.* 2012b; Zhang *et al.* 2012b) and cold tolerance (Colinet *et al.* 2012a; Kostal *et al.* 2012; Teets *et al.* 2012) in insects. Studies have also examined the metabolome of insects in response to heat shock (Malmendal *et al.* 2006; Verberk *et al.* 2013) in addition to other stresses such as desiccation and salinity (Michaud *et al.* 2008; Laparie *et al.* 2012; Malmendal *et al.* 2013). These studies have shown that insect metabolomes undergo major changes when entering diapause or in response to stress, for example, within the insect tissue high concentrations of proline are thought to enable *Drosophila* to survive cold temperatures (Kostal *et al.* 2012). There are a few global metabolite studies that have examined insect metabolomes in the context of

plant-insect interactions. One study tested the effects of jasmonic acid on the metabolites in *P. rapae* fed on *B. oleracea* plants. System-wide changes in the insect metabolome were not detected although increases in a group of plant-derived metabolites were found within the insects fed plants that had jasmonic acid applied to their shoots (Jansen *et al.* 2009). Another plant-insect study profiled multiple metabolites to find the mechanism underlying the accumulation of lactate in leaf beetle (*Gastrophysa atrocyanea*) and butterfly (*Papilio machaon*) larvae feeding on dock weeds (*Rumex obtusifolius*) and fennel (*Foenicolum vulgare*) respectively (Miyagi *et al.* 2013). As more research is published on insect metabolomes, there has been more contemplation on the methods used. For example, one paper emphasises the value of measuring metabolites from different insect body parts following demonstration of large spatial variation in the metabolome of *Drosophila* (Chintapalli *et al.* 2013). In addition to these studies which measure multiple insect metabolites, information on the insect metabolome is also provided by the large number of targeted studies which have focused on specific metabolites. These studies have included investigating sequestration of metabolites from host plants (Opitz & Müller 2009), detoxification of plant defences (Ferguson *et al.* 1985; Snyder *et al.* 1994; Stauber *et al.* 2012) and investigating insect hormones (Ohta *et al.* 1977; Robinson *et al.* 1987; Cangialosi *et al.* 2012). Although such targeted studies are important in uncovering functions of metabolites, they ignore the rest of the insect metabolome. A wider look at the metabolome of an insect using metabolic fingerprinting could reveal the effects of unexplored factors such as pathogens, parasites or host plants on insect metabolites and point towards unconsidered metabolites of interest.

1.5 Metabolomic methods and metabolic fingerprinting

The term ‘metabolite profiling’ was first coined in research published in the early 1970s (Sumner *et al.* 2003) although it was not until 1998 that the term ‘metabolome’ was introduced and used in the same way as the terms genome,

proteome and transcriptome (Oliver *et al.* 1998; Fiehn 2001). The number of metabolomic-related publications has been much lower compared to other 'omic' (genomic, proteomic and transcriptomic) research, although at the start of the 21st century metabolomic-related publications began to increase (Sumner *et al.* 2003; Schripsema 2010). In 2004, interest had risen sufficiently for the Metabolomics Society to be established which has the aim of advancing the use and understanding of metabolomics in the life sciences (www.metabolomicssociety.org) and the following year the first issue of a journal dedicated to metabolomics, '*Metabolomics*', was published.

Metabolomic studies aim to analyse all the metabolites within a system's metabolome (Fiehn 2001). Approaches to analysing these metabolites include targeted metabolic profiling, which measures a small number of specific metabolites (Dettmer *et al.* 2007), and untargeted metabolic fingerprinting which measures a large number of metabolites and allows samples to be classified according to the metabolic patterns obtained (Fiehn 2001). Compared to metabolic profiling, which is usually hypothesis driven, metabolic fingerprints can be used as an initial exploratory analysis that may highlight metabolites of further interest (Zhou *et al.* 2012) and for this reason has been described as a hypothesis generator (Hall 2006).

A range of technologies has been used to carry out metabolomic analyses, including mass spectrometry (MS) based techniques, Nuclear Magnetic Resonance (NMR) and Fourier Transform-Infrared (FT-IR) spectroscopy (see Allwood *et al.* 2008 and Nakabayashi & Saito 2013 for reviews). However, no single method will measure all metabolites present within a metabolome due to the diversity of chemical structures and properties of metabolites (Sumner *et al.* 2003; Hall 2006). The technologies capable of measuring the widest range of metabolites are NMR, GC-MS (Gas Chromatography-Mass Spectrometry) and LC-MS (Liquid Chromatography-Mass Spectrometry) (Sardans *et al.* 2011). NMR provides more structural information and so leads to better identification of metabolites, although this approach has lower sensitivity in terms of the number of metabolites it can

measure compared to MS-based approaches (De Vos *et al.* 2007; Schripsema 2010). GC-MS is generally cheaper compared to LC-MS but is limited to measuring volatile metabolites. GC-MS can also measure non-volatile metabolites that can be derivatised, to make them less polar although this makes sample preparation more complex (De Vos *et al.* 2007; Bedair & Sumner 2008). Thus, LC-MS can measure a wider range of metabolites compared to GC-MS, although metabolite identification using databases can be more difficult (Bedair & Sumner 2008), as explained below.

In this thesis, the specific LC-MS method used was HPLC-MS (High Performance Liquid Chromatography coupled to Mass Spectrometry) with ESI (Electrospray Ionisation) as the ionisation method, which is the most common ionisation technique used with LC-MS (Bedair & Sumner 2008). This is a 'soft' ionization technique which keeps more of the molecular ions present within samples intact, with fewer ions breaking up into fragments (Zhou *et al.* 2012). ESI applies a high electrical charge to the sample as it is sprayed out of a needle which provides the energy to ionise (charge) the molecules before they enter the mass spectrometer. ESI can be used to create either positive or negative ions (Allwood & Goodacre 2010). In these studies, I used a Q-ToF (Quadrupole - Time of Flight) mass spectrometer. The quadrupole filters out a proportion of the ions in the sample so that only a narrow mass range of ions is passed into the mass spectrometer at any one time (Allwood *et al.* 2008). In the ToF mass spectrometer, the mass of the ion determines how long it takes to pass through the flight tube and reach the detector (Guilhaus *et al.* 1997), and therefore the mass of each feature can be calculated.

A disadvantage of untargeted metabolomic studies is that the majority of features (peaks or potential metabolites) cannot be definitively identified from databases of metabolite masses (Fiehn *et al.* 2000; Kind & Fiehn 2006). A number of metabolomic databases have been complied (see Tohge & Fernie 2009 for lists of databases; Aliferis & Chrysayi-Tokousbalides 2011) where molecular weights of metabolites are recorded along with other information such as chemical structures or properties, nomenclature information, MS/MS spectra or associated biochemical pathways (Dettmer *et al.* 2007). The difficulty in trying to identify a feature

obtained by LC-MS using these databases is that the retention time is unhelpful because this can be very variable, often varying between runs on the same machine (Zhou *et al.* 2012). By contrast, with GC-MS databases, a more definite identification of a feature is possible because a retention index derived from the retention time, in addition to the molecular mass, can be used to match metabolites measured on different GC-MS machines (Schauer *et al.* 2005).

From the mass of an unknown feature, a list of possible compounds can be obtained from databases. However, these preliminary matches are far from sufficient to provide an identity to the feature (Kind & Fiehn 2006), although they do give a starting point for choosing standards to analyse. Fragmentation of the unknown metabolite using tandem MS (MS/MS) can provide masses and abundances of the fragments which can then be compared to spectral data in databases (such as MassBank or METLIN) or to fragments from a standard (Bedair & Sumner 2008). Fig. 1.2 shows the fragmentation of a standard of oleanolic acid and fragmentation of an unknown metabolite from samples collected in Chapter 3. Despite the two compounds having the same molecular mass the fragmentation pattern does not match and thus rules out the possibility that the unknown compound is oleanolic acid. The Chemical Analysis Working Group of the Metabolomics Standard Initiative (MSI) recommend that to validate the identification of a feature there should be at least two independent pieces of information which should be orthogonal to each other and relate to a standard of the chemical which is analysed under the same experimental conditions as the unknown metabolite (Sumner *et al.* 2007). If these criteria are not met the metabolites can only be described as tentatively annotated chemicals or as unknown compounds if they are not identified in any way (Sumner *et al.* 2007). To identify just one metabolite with certainty can be time consuming and costly even if a standard is available and realistically can only be done for a limited number of unknown metabolites (Schauer *et al.* 2005; Zhou *et al.* 2012).

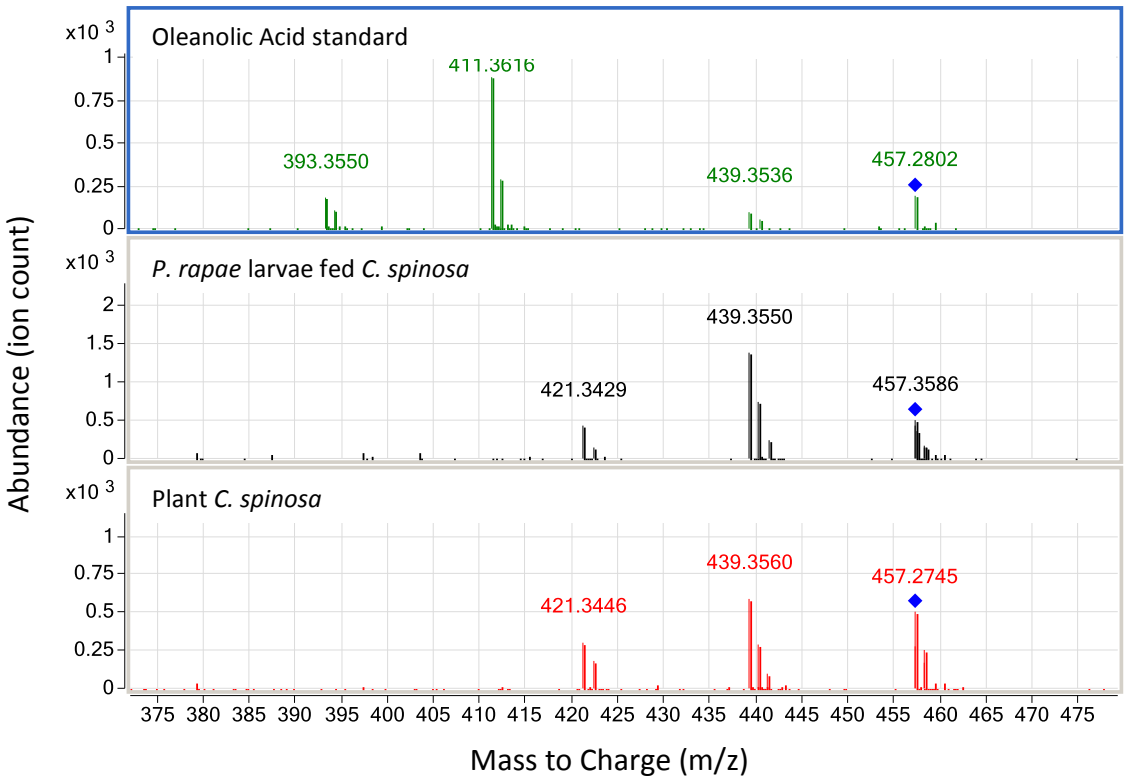


Fig. 1.2 The tandem MS fragmentation of an oleanolic acid standard (top) and an unidentified metabolite in samples of *Pieris rapae* larvae (middle) and of *Cleome spinosa* (spider flower) that larvae had fed from (bottom). The fragmentation patterns (peaks on the graphs) are different between the standard and the samples therefore confirming that the unknown metabolite in the larvae and plant is not oleanolic acid. The blue diamond denotes the original metabolite molecules that have not been fragmented. For the full experiment see Chapter 3.

As metabolomic techniques have improved there have been new opportunities to gain insights into organisms and ecosystems (Sardans *et al.* 2011). The use of metabolomics in ecology (termed 'ecometabolomics') (Penuelas & Sardans 2009) has answered questions in many ecological situations, for instance investigating the effect of an abiotic stress such as salinity on the metabolomic reactions of plants (Sanchez *et al.* 2008), and defining how a fungal pathogen suppresses plant defence systems during infection (Parker *et al.* 2009). Other examples include, revealing the metabolites that enable a fish embryo (*Austrofundulus limnaeus*) to survive in oxygen deprived water (Podrabsky *et al.* 2007), and examining if metabolites explain why marsupials prefer one eucalyptus over another as a food resource

(Tucker *et al.* 2010). This thesis employs metabolomics to discover more about plant-insect relationships.

1.6 Thesis rationale and outline

The main aim of this thesis is to investigate relationships between an insect herbivore and its larval host plants using an untargeted metabolic fingerprinting method. This metabolomic method has not previously been used extensively in ecology despite its potential to gain new insights, therefore I attempt to demonstrate how metabolic fingerprints can be useful in furthering ecological knowledge.

Chapter 2 describes *Pieris rapae,* its host plants studied and rearing protocols. There is a detailed account of the metabolic fingerprinting method and data analysis used, including the reasoning behind the specific methods chosen.

In **Chapter 3** I quantify the metabolic fingerprints of *P. rapae* larvae fed on different species of host plants. The main hypotheses tested were:

• The metabolome of the larvae is not dependent on the species of host plant eaten.

• The similarities between plant metabolic fingerprints reflect the taxonomy of the plant species.

• The metabolic fingerprints of host plants are related to insect performance on those host plants.

• The variation in insect performance among host plants is explained by the nitrogen content of plants.

The results opened up a further question and allowed the testing of one further hypothesis:

• The group of abundant metabolites found in one group of larvae originated from the host plant.

In **Chapter 4** I examine the metabolites induced by *P. rapae* herbivory in three host plants using metabolic fingerprinting to investigate the evolution of plant metabolites in related host plants. The main hypotheses addressed are:

• The metabolic fingerprints of host plants are changed by herbivory.

• There are induced metabolites that are common to all three species of host plant following herbivory.

In **Chapter 5** I evaluate the consequences of climate change by examining metabolic fingerprinting of *P. rapae* larvae feeding on *B. oleracea* growing under ambient and elevated temperature and CO_2 concentrations. This allowed me to address the following hypotheses:

• The effects of elevated temperature and CO_2 can be detected in the metabolic fingerprints of both the plants and their insect herbivores.

• Elevated temperature increases and elevated CO_2 decreases the performance of insects.

• Plants reared under elevated CO_2 have lower concentrations of nitrogen.

In **Chapter 6** I give a critical evaluation of the metabolic fingerprinting method used and discuss how this method can contribute to the study of plants and insects. I consider my findings from all three experimental chapters to make conclusions regarding *P. rapae* and its host plants and outline areas of further work.

Chapter 2 Materials and methods

2.1 Abstract

All experiments in the current thesis used *Pieris rapae* (Lepidoptera: Pieridae) and at least one of its host plants as study systems. Descriptions of *P. rapae* and host plant species and the methods used to rear and grow them are provided. In addition, the metabolic fingerprint methods used in all three of the experiments and statistical analyses are described.

2.2 Study systems

2.2.1 *Pieris rapae* background

Pieris rapae can be found in Europe, North West Africa, Asia, Japan and has been introduced to North America and Australia (Asher *et al.* 2001). It is commonly known in Britain as the small white butterfly or as one of the cabbage white butterflies and in America as the imported cabbage worm. Butterflies in Britain are the European subspecies *P. rapae rapae* (Fukano *et al.* 2012) and are a common and widespread butterfly.

In Britain *P. rapae* usually has two generations except in warm summers when it can have three. The second generation is more numerous, boosted in numbers by immigrants from the continent. The pupae of this second generation overwinter and emerge the following year. Eggs are laid singly on host plants usually on the underside of leaves (Asher *et al.* 2001). As with many insects the development time of the larvae from hatching to pupation is dependent on temperature and the experiments described in this thesis found that at a constant 21 ^{o}C they will take an average of 16 days to pupate. Females usually have a darker second spot on the dorsal side of the forewing. The black markings on the adults of the second

generation have a more intense colour compared to the adults of the first generation of the year (Tolman & Lewington 2008).

Fig. 2.1 a) male b) female c) larva and d) pupa of *P. rapae*. Photos used with permission from ukbutterflies.co.uk copyright Vince Massimo.

Adult butterflies feed on the nectar of several plant species and are especially attracted by white flowers. Larvae are considered polyphagous being able to develop on several plant species many of which can be found in the family Brassicaceae (Stevens 2001 onwards; Beilstein *et al.* 2008; Eeles 2012). A well studied group of defensive compounds called glucosinolates characterise this group of plants (Kos *et al.* 2012). Glucosinolates have been found to have a negative effect on a number of lepidopteran herbivores (Li *et al.* 2000; Arany *et al.* 2008; Müller *et al.* 2010). However, the Pieris group of butterflies use glucosinolates as oviposition cues and as phagostimulants (Huang & Renwick 1994; Renwick & Lopez 1999).

2.2.2 Suitability of *P. rapae* as a study species

P. rapae was chosen for these experiments for several reasons. First it is a well studied insect. Second, its host plants are related to, and include *Arabidopsis thaliana*. *A. thaliana* is a model plant species and therefore was more likely to have a greater number of metabolites characterised and saved in metabolite databases. It was thought this would increase the chance of identifying metabolites found in related host plants. Third, *P. rapae* is considered a pest species (Ahuja *et al.* 2010) and therefore studying its relationship with plants could bring advances in pest management. And finally, *P. rapae* can be reared in the laboratory. The disadvantage of *P. rapae* is that it is a specialist species. Specialists are adapted to avoid some of the plant defences so that there is less impact on the performance of the insect. For example, *P. rapae* can avoid the hydrolysis of glucosinolates into toxic isothiocyanates (Wittstock *et al.* 2004). This means that relating the changes in the plant metabolomes to the performance of *P. rapae* is not as meaningful compared to a plant-generalist model where the generalist performance may be more reflective of the changes in plant metabolite defences.

2.2.3 Host plants background

The plants used in these studies are shown in Table 2.1 along with their scientific and common names. They are all in the order Brassicales and four are in the family Brassicaceae. Descriptions of each follow.

Scientific name	Common name(s)	Photograph
Arabidopsis thaliana	thale cress	
Barbarea vulgaris	wild rocket/winter cress	
Brassica oleracea	cabbage	
Cleome spinosa	spider flower	
Lunaria annua	honesty/money plant	
Reseda lutea	wild mignonette	
Tropaeolum majus	nasturtium	

Table 2.1 Host plants of *P. rapae* used in this thesis. Scientific name, common name(s) and photographs of the young plants as used in the experiment in Chapter 3.

A. thaliana is a small plant which is a model organism used in many laboratories due to its fast growth, copious seed production and small genome. It is native to Britain and widely distributed (Preston *et al.* 2002). In the wild *A. thaliana* is not usually a host plant of *P. rapae* because the time of year the plant is in leaf and the time of year the larvae is active, do not overlap (Harvey *et al.* 2007). Seeds used in this experiment were obtained from the Penfield lab, University of Exeter.

The species *B. oleracea* encompasses the wild cabbage found in Britain (var. *oleracea*) as well as many crop plants including cultivated cabbage (var. capitata), broccoli and cauliflower (var. *botrytis*), kale (var. *viridis*) and brussels sprouts (var. *gemmifera*) (Stace 2010). *B. oleracea* var. *capitata* (cultivar 'stonehead') (Groves Nurseries, Dorset, UK) which is a white summer cabbage grown for food, was used in all the experiments described here.

B. vulgaris is a native plant found growing in the wild across Britain (Stace 2010) and is also cultivated for use in salads. The variety used here is *variegate* (Thompson and Morgan, Suffolk, UK).

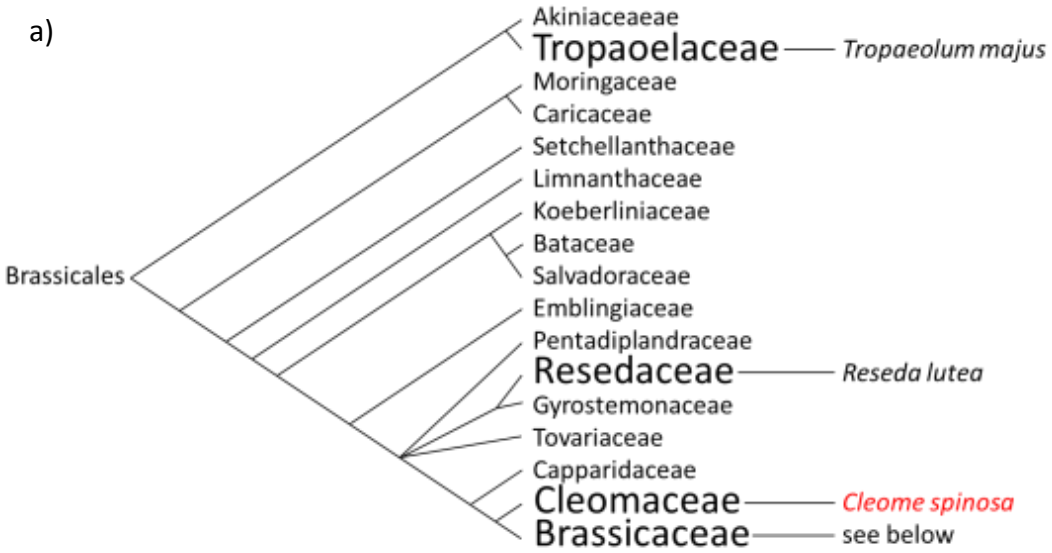
C. spinosa is widely distributed in the world but is not native to Britain (http://www.cabi.org). It is grown in Britain as an ornamental garden plant. It is listed as a host plant for the Asian subspecies of small white butterfly *P. rapae crucivora* but not for the European *P. rapae rapae* (Robinson *et al.* 2001). Seeds used here were obtained from Chiltern Seeds, Oxfordshire, UK.

L. annua has been grown in British gardens for centuries and can be found growing wild in Britain (Preston *et al.* 2002). Seeds used here were from Chiltern Seeds, Oxfordshire, UK.

R. lutea is native to Britain and grows in the wild (Preston *et al.* 2002). Seeds were from Emorsgate Seeds, Norfolk, UK.

T. majus is a common garden plant with many cultivars (Stace 2010). It is well known as a host for *P. rapae*. Seeds were from Chiltern Seeds, Oxfordshire, UK.

In Chapter 3 host plants were primarily chosen to achieve a spread of plants across the phylogeny. In addition to this, plant characteristics such as the ability to grow easily and quickly in the laboratory and having large leaves that could sustain larvae eating them, contributed to the decision of which host plants were used.



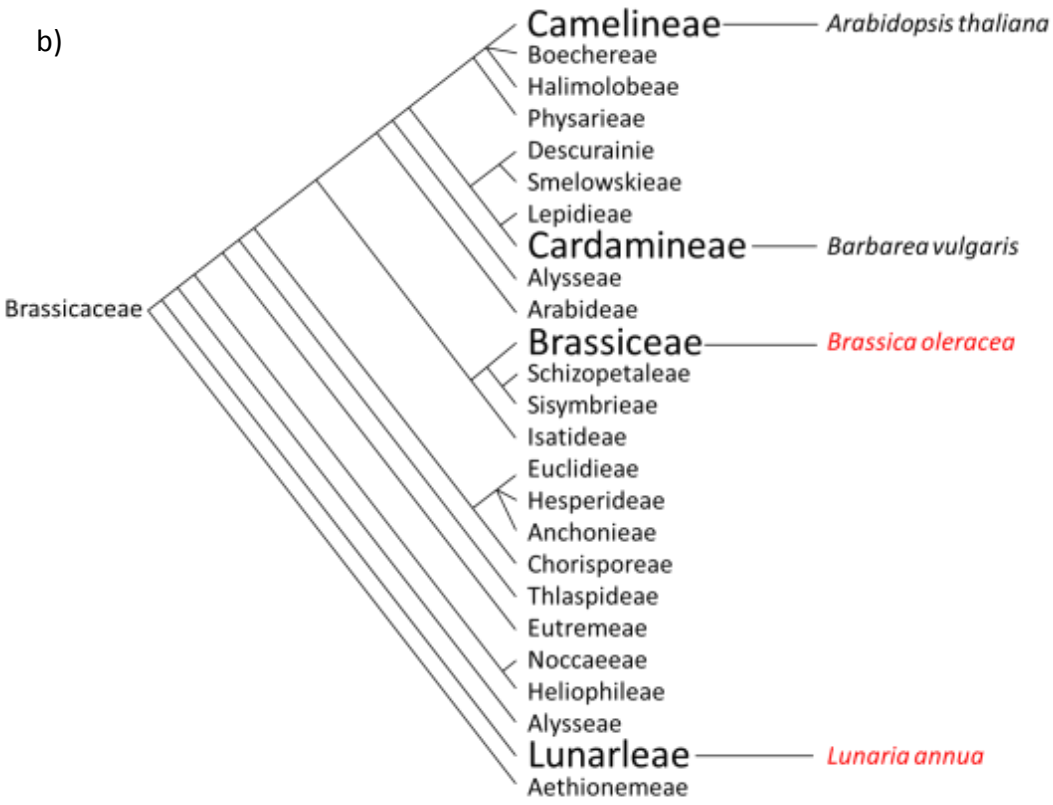


Fig. 2.2 The taxonomic relationships between the seven species of host plants used in Chapter 3. The three species in red were used in Chapter 4 and *B. oleracea* was used in Chapter 5. Plant species used encompass four families within the order Brassicales shown in the large font in a) with species in italics. The other four species are in the family Brassicaceae shown in b) where tribes are in a larger font size. Cladogram constructed using information from Beilstein *et al*. (2008) and Stevens (2001 onwards).

2.3 Rearing of *P. rapae* and plants

2.3.1 *P. rapae* rearing

Female *Pieris rapae* were caught in gardens or allotments in York, UK (53.95^{o}N, 1.08^{o}W) each year during the last week of July and the first week of August when the adults of the second generation of butterflies are active. Butterflies were kept in a greenhouse in individual 31 x 42 cm keep nets and provided with a honey

solution on cotton wool and a potted *B. oleracea* plant on which to oviposit (Fig. 2.3).

Fig. 2.3 Adult *P. rapae* in keep nets in the greenhouse.

After the young larvae of *P. rapae* had been transferred to plants using a paintbrush and developed on an experimental host plant (see individual Chapters) the pupae were left where they had pupated for at least 24 hours to harden before being cut off and weighed. Pupae were then put in an individual plastic pot with the lid on loosely and with a small piece of damp paper towel before being placed in a cabinet (Sanyo MLR-350) set to a relevant temperature. Pupae were checked for emergence every twelve hours (Chapter 3) or twenty-four hours (Chapter 5). These pupation and emergence times were noted for each individual. After emergence from the pupae, butterflies were killed by placing in a freezer then sexed, placed in an envelope and dried in a 60 ^{o}C oven for three days before being weighed to obtain an adult dry weight.

2.3.2 Plant rearing

Seeds of *A. thaliana* and *C. spinosa* were put in a fridge for three weeks before sowing. Seeds were sown in trays 35 x 21 cm using Levington F2+S seed and modular compost and germinated in a greenhouse in summer (about 10 to 30 ^{o}C) for at least two weeks. Plants were then potted into 10 cm diameter pots and grown in temperature controlled cabinets (details of the different experimental designs are described in the individual Chapters).

2.4 Metabolic fingerprinting methods

The metabolomic approach used in this thesis is outlined in the flow diagram in Fig. 2.4. The identification of metabolites using standards is included in the diagram, although in this thesis I attempted to identify only one metabolite in Chapter 3 using standards. Unless otherwise stated, the metabolomic methods used in all the experiments were as described below.

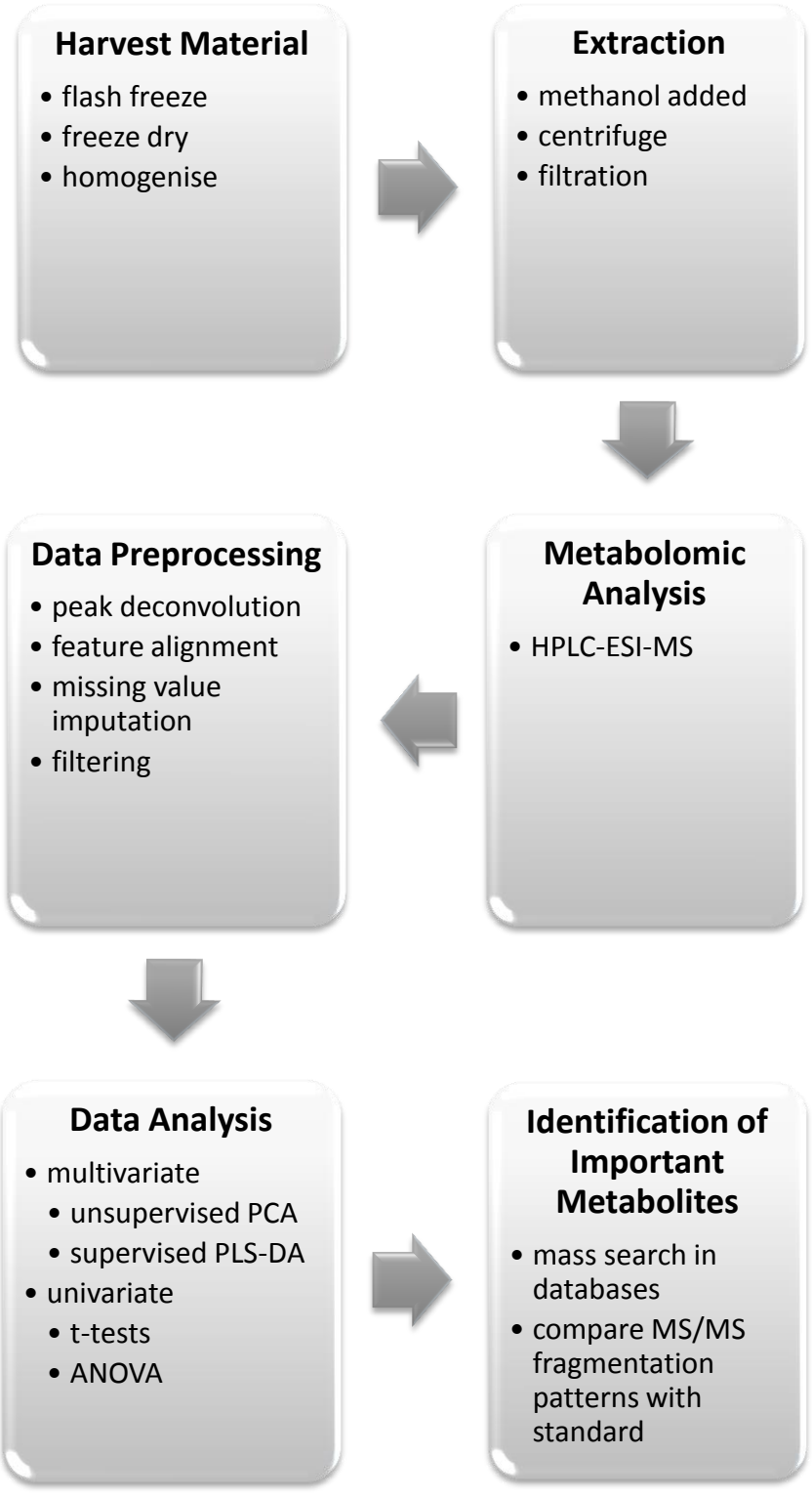


Fig. 2.4 Flowchart summarising the main steps taken in this thesis in the metabolomic analysis of organisms using LC-MS. The details of each step are given below.

2.4.1 Harvesting material and extraction of metabolites

Leaf and insect material was immediately flash frozen after harvesting and was stored at -80 ^{o}C before being freeze dried for 16 hours. Material was ground for two minutes at 20 rpm and then 10 mg samples were kept cool while 400 µl of 80% methanol containing an internal standard of umbelliferone, was added to each sample. The extraction protocol consisted of samples placed in blocks that had been pre-cooled in a -80 ^{o}C freezer and shaken in a ball mill at 20 rpm for 30 s and then stood on ice for 10 min, and this process repeated three times. Samples were then centrifuged at 16 rpm at 4 ^{o}C for 10 min and the supernatant removed. This process was repeated so that each sample was extracted twice with the only difference being that the methanol in the second extraction did not contain the standard umbelliferone. The two supernatants from the two extractions were combined and filtered through a 0.4 µm (PVDF) syringe filter.

Using the same extraction techniques for insects and plants meant that metabolomic features (potential metabolites) were easier to align and compare. However, by not using an insect specific extraction technique, the proportion of the metabolome detected may be lower for insects than plants although this was not examined. Therefore where the total number of detected metabolites is lower for insects than plants (as was found in Chapter 3 and 5), this should not necessarily be interpreted as insects containing fewer metabolites compared to plants. An optimised extraction process for lepidoptera larvae that detects the maximum number of metabolites has not yet been developed.

2.4.2 HPLC-MS metabolic analysis

Metabolite profiling was performed using a QToF 6520 mass spectrometer coupled to a 1200 series Rapid Resolution LC system. 5 µl of sample extract was loaded onto a Zorbax StableBond C18 1.8 µm, 2.1 x 100 mm reverse phase analytical column (LC-MS and column, Agilent Technologies, Palo Alto, USA). Metabolomic features were detected in positive and negative ionisation mode in Chapter 5, but only in positive ionisation mode in Chapters 3 and 4. This was because experiments in

these Chapters examined multiple plant species so the number of features detected was greatly increased and made datasets difficult to align due to their size. Mobile phase A comprised 5% acetonitrile with 0.1% formic acid in water, and mobile phase B was 95% acetonitrile with 0.1% formic acid in water. The following gradient was used: 0 min – 0% B; 1 min – 0% B; 5 min – 20% B; 20 min – 100% B; 30 min – 100% B; 31 min – 0% B; 7 min post time. The flow rate was 0.25 ml min^{-1} and the column temperature was held at 35 ^{o}C for the duration. The source conditions for electrospray ionisation were as follows: gas temperature was 325 ^{o}C with a drying gas flow rate of 9 l min^{-1} and a nebuliser pressure of 35 psig. The capillary voltage was 3.5 kV. Skimmer and fragmentor voltages were 115 V and 70 V respectively. Data in Chapter 3 were also run using tandem MS (MS/MS) to gain the fragmentation pattern of the metabolites.

2.4.3 Data pre-processing

The Molecular Feature Extractor (MFE) in the MassHunter software (Agilent Technologies, Palo Alto, USA) was used to deconvolute the many peaks in the chromatogram to identify individual features. The MFE algorithm picks out the peak of a potential metabolite which will be $[M+H]^+$ in positive ionisation mode and $[M-H]^-$ in negative ionisation mode. The algorithm will then 'recognise' any isotopes and adducts of this potential metabolite, examples of adducts being, $[M+Na]^+$ or $[M+K]^+$ in positive ionisation mode, and in negative ionisation mode $[M+Br]^-$ or $[M+Cl]^-$. The ion counts calculated from all these peaks are then added together to give the ion count (abundance) for each feature. Any feature that had abundance lower than 100 ions was excluded from the dataset because measurements below this concentration are unreliable. MFE calculates the neutral mass i.e. [M] accurate to 10 ppm, which for the majority of features that have molecular mass in the 100s of Daltons will be accurate to two decimal places. A retention time for each feature (in each sample) is obtained. In addition, the 'ion' number is given, which is the number of adducts and isotopes of each metabolomic feature detected by the MFE.

Features eluting within the first minute are contained within the 'dead' volume, and thus excluded. Features obtained after 27.9 minutes are within the re-equilibration period and thus also excluded.

Due to machine drift, the same metabolite is unlikely to have exactly the same mass and retention time in two samples, and therefore alignment of data is necessary. This machine drift is not uniform or regular and therefore cannot be corrected using calibration curves or formulae. Thus each feature needs to be matched across samples using information on retention times and molecular masses. An alignment program (see below) was used to decide the acceptable error range for retention times and molecular mass for a particular dataset.

The alignment of features across samples was performed using a Kernel based Feature Alignment (KFA) programme developed in-house (Perera 2011). The programme took a feature in one sample and searched for matching features in all other samples in turn according to retention times and molecular masses. The internal standard was used to check the accuracy of the alignment. This process resulted in a matrix with the abundance (ion count) of each feature for each sample it was detected in. For each metabolomic feature detected, a mass and retention time was provided along with their associated errors calculated from the mass and retention times of all the samples in which the feature was detected.

Not all the features detected in the analysis represent a metabolite and removal of this 'noise' without removing features that truly represent metabolites is necessary for sound statistical analysis. Metabolomic data can be described as 'patchy' in that features are rarely detected in all the replicates in a sample. Thus, a threshold value was used to determine how many replicates a feature must be detected in for the feature not to be considered noise. In this study, metabolites had to be in ≥50% of replicates to be scored present. Thus, if a treatment had ten replicates then a feature would need to be detected in at least five or more replicates to be included in the final dataset. However, for data in Chapter 5 this threshold was increased because the data was of poor quality due to a technical error on the machine,

resulting in samples containing more 'noise' than usual, and so the threshold was raised to 60%.

In the raw data not every feature will be detected in every sample, as mentioned above. Metabolites may not be detected in a sample because; 1) it is genuinely not present in the sample, 2) it is present but below detectable abundance, or 3) it is present but not detected due to a technical error. Therefore, Missing Value Imputation (MVI) was performed on the data sets to make subsequent statistical analysis easier and more reliable. This is an essential manipulation of the data because some statistical programmes will exclude any variables with missing values, which in the case of metabolomic data will be a high proportion of features and therefore analyses are carried out on fewer features reducing statistical power (Hrydziuszko & Viant 2012). In addition, failure to apply MVI may result in some features incorrectly appearing more important than other features when models are fitted to the data. The MVI method used here was part of the alignment programme and used an algorithm to calculate and insert an appropriate value (Perera 2011).

2.4.4 Multivariate statistical analysis

Prior to multivariate statistical analysis all data were log transformed and centred. Metabolic fingerprints were summarised with Principal Component Analysis (PCA), using either the prcomp function in R (R Core Team 2013) or Simca-P software (Umetrics UK Ltd, Windsor, UK). Plots of the Principal Component (PC) scores were used to compare samples within treatments and between treatments.

Failure to detect differences among metabolic fingerprints in a PCA may occur if other high loading variables or the large number of variables mask any differences. For this reason, further analyses of metabolic fingerprints were carried out in Chapter 3 by fitting data to a Partial Least Squares Discriminant Analysis (PLS-DA) model. Also in Chapter 3, to identify features that were driving the separation of the larvae fed *C. spinosa*, an Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) was performed. These two analyses were both

carried out in Simca-P software (Umetrics UK Ltd, Windsor, UK) and are described in detail in Chapter 3.

2.4.5 Correction for false discovery rate after univariate statistical analyses

In order to assess differences in the abundance of each metabolite between treatments, various univariate tests were carried out which are described in individual Chapters. When one univariate test is carried out for each of the thousands of metabolites in a dataset simultaneously, this large number of tests will have a large number of false positives which means a correction for the False Discovery Rate (FDR) is required (Benjamini & Hochberg 1995; Storey 2002). Therefore the FDR was accounted for by converting all p values to q values using an R function (obtained from http://www.public.iastate.edu/~dnett/microarray/multtest.txt) based on Benjamini and Hochberg's (1995) approach for correcting the FDR. This approach will find the same proportion of false positives as a Bonferroni correction when all tests are significant but otherwise is not as conservative as a Bonferroni approach (Benjamini & Hochberg 1995).

Chapter 3 Metabolic fingerprints reveal how an insect herbivore is affected by its larval host plants.

3.1 Abstract

Polyphagous insects can consume a wide range of different host plant species, but how these host plants vary in their metabolite compositions and whether this variation affects the biochemistry of the insect herbivores is unknown. We studied the interactions between the polyphagous insect herbivore *Pieris rapae* (Lepidoptera: Pieridae) and five species of its larval host plants (family Brassicaceae and Cleomaceae) by examining untargeted metabolic fingerprints of the plants and their larval herbivores. Metabolic fingerprints of the five host plant species were highly distinctive, and larvae could also be distinguished based on the species of host plant they fed on. The fingerprints of larvae feeding on *Cleome spinosa* plants were most distinctive due to a large group of abundant metabolites also found in high abundance in *C. spinosa,* but not in the other host plants examined. We conclude that host plants influence the biochemistry of their larval herbivores, and that some metabolites are conserved from one trophic level to the next.

3.2 Introduction

Plants are used as a food source by nearly half of all insect species (Wu & Baldwin 2010), and there is a large body of research examining factors that affect these herbivore-plant interactions (Schoonhoven *et al.* 2005). When insect herbivores consume host plants they take in nutritious primary plant metabolites as well as defensive secondary metabolites which can be toxic. The number of metabolites an insect consumes is generally unknown although a single species of plant is estimated to contain several thousand metabolites (Davies *et al.* 2010). Due to this large number of metabolites and the difficulties in measuring all these small compounds within an organism (Allwood *et al.* 2008) our knowledge is incomplete as to which metabolites occur within which plants, the concentrations of them or, critically, their effects on the herbivores consuming the plants. Thus, wider examination of the composition and abundance of metabolites in plants and their insect herbivores could provide further information on how plant metabolites affect herbivore performance, leading to better understanding of insect-plant interactions.

We investigated the effects of host plant metabolite composition on the polyphagous butterfly *Pieris rapae* (Lepidoptera; small white butterfly). Many larval host plants used by *P. rapae* belong to the plant family Brassicaceae (Asher *et al.* 2001; Stevens 2001 onwards; Beilstein *et al.* 2008). This family contains glucosinolates which are toxic to some herbivore species (Li *et al.* 2000; Rohr *et al.* 2009) but are used by other herbivores as ovipositional cues and phagostimulants (Huang & Renwick 1994; Renwick & Lopez 1999). Glucosinolates are well studied (Rask *et al.* 2000; Kroymann 2011) however the focus on one group of metabolites may have led to less familiar groups of compounds being ignored despite evidence that other important compounds in the Brassicaceae family exist (Schroeder *et al.* 2006). Therefore widening the range of metabolites examined in these host plants may bring new, important compounds to our attention and metabolic fingerprinting, which can measure the abundances of hundreds of metabolites simultaneously (Fiehn 2001), has the potential to do this.

Plant factors which have been shown to determine the success of insects on different host plants include nitrogen and water content (Matsuki & Maclean 1994; Honek *et al.* 2002; Coley *et al.* 2006), and the abundance of specific metabolite groups (Slansky & Feeny 1977; Matsuki & Maclean 1994; Poelman *et al.* 2008). A metabolic fingerprint of a host plant aims to quantify a large number of primary metabolites as well as defensive secondary metabolites (Fiehn 2001) and so fingerprinting should provide a good representation of the metabolites within a herbivore's diet. However, no previous studies have used metabolic fingerprints to examine variation in insect performance on different host plants.

Assessment of the herbivore in an insect-plant relationship usually considers factors such as growth rate, mortality and fecundity, whereas quantifying the metabolome of an insect (the metabolome being all the metabolites within an organism) (Fiehn 2001) is less common (but see Jansen *et al.* 2009). As with plant metabolomes (Bidart-Bouzat & Imeh-Nathaniel 2008), insect metabolomes are affected by abiotic factors such as temperature (Malmendal *et al.* 2006; Michaud *et al.* 2008; Colinet *et al.* 2012a; Verberk *et al.* 2013). However, little is known about the effect of diet on insect metabolomes, specifically whether insect metabolomes are affected by the species of host plant the insect feeds on. Evidence that the insect metabolome may be changed by the insect's diet comes from studies which have focused on single plant metabolites or specific metabolite groups (Opitz and Müller 2009), but studies that investigate a broader spectrum of metabolites from the insect metabolome are lacking.

In this paper, we use untargeted metabolomics to address two questions. First, we examine whether or not the metabolic fingerprints of *P. rapae* vary across different larval host plants, by rearing larvae on five species of Brassicales host plants. Second, we test the hypothesis that there is a relationship between the metabolic fingerprints of host plants and larval performance (growth rate).

3.3 Methods

3.3.1 Plant and insect rearing

We studied *P. rapae* on five of its host plants. Four plant species were from the family Brassicaceae, namely *Arabidopsis thaliana* (thale cress), *Barbarea vulgaris* (yellow rocket), *Brassica oleracea* (cabbage) and *Lunaria annua* (honesty) and one from the related family Cleomaceae, *Cleome spinosa* (spider flower). The metabolic fingerprints of two additional plant species from two further families (*Tropaeolum majus,* nasturtium, family Tropaoelaceae; *Reseda lutea,* wild mignonette, family Resedaceae) were studied but insect data are lacking due to insufficient *R. lutea* plant material for insect rearing and rejection of *T. majus* by many early instar larvae transferred from oviposition plants. Therefore there were no corresponding insect metabolic fingerprints for these two host plants, and these were excluded from most analyses. The host plants were chosen to span a wide phylogenetic range of host plants that *P. rapae* larvae have been reported to feed on, and so allowed us to examine a range of plants likely to have contrasting chemical compositions. Data for *T. majus* and *R. lutea* were included in the phylogenetic analyses to increase their statistical power.

Plant seeds were sown in trays using Levington F2+S seed and modular compost (added N:P:K 150:200:200 mg/litre) over a two week period in an unheated glasshouse in July and August which varied in temperature between 10-30 °C. Plants were grown under natural daylight and watered daily. Four to six week old plant material was flash frozen for metabolomic analysis. Leaves were taken from 13 individuals per plant species to make 13 biological replicates per host plant. Due to the variation in size of the plants, and in order to obtained sufficient plant material for analysis, samples comprised the single youngest leaf of *B. oleracea*, *L. annua* and *T. majus*, the three youngest leaves of *B. vulgaris*, *C. spinosa* and *R. lutea*, and the whole rosette of *A. thaliana*. These samples also reflected the parts of the plants that the larvae were observed typically to consume.

P. rapae larvae were the F1 offspring of 16 adult female butterflies caught in York, UK (53°95'N, 1°08'W) in August. Female butterflies were kept individually in a glasshouse, provided with honey solution and potted *B. oleracea* plants for oviposition. Five days after hatching, second instar larvae were transferred using a paint brush and randomly assigned to different host plants. Larvae were placed in plastic boxes (175 x 116 x 52 mm; 10 larvae per box; 20 boxes per plant species) with fine netting taped over the front to reduce condensation. Cut plant leaves were placed in vials of water inside boxes, except for *A. thaliana* plants (which quickly wilted using this method) which were left in soil in small pots. Plant material was replaced daily as required and thus larvae were provided with excess fresh leaves during the experiment. Larvae in boxes were kept in temperature-controlled cabinets (Sanyo, MLR 350) at 21 °C under a photoperiod of 16L:8D. When the majority of larvae in a box had developed to fifth-instar, four larvae from each box were pooled and flash frozen for metabolomic analysis after the head, gut and heamolymph were removed to avoid analysing undigested plant material. A total of 13 pooled larval samples was analysed for each of the five host plant species.

3.3.2 Insect performance and leaf measurements

Boxes containing the remaining larvae were checked every twelve hours for pupae. Larval development time was computed as the time (in days) between placing second-instar larvae on plants and pupation. The day after pupation, pupae were weighed and placed in a small pot with a piece of damp paper towel until adult emergence. Within twenty-four hours of emergence, adults were killed by freezing then dried at 60 °C in an oven for two days before being weighed. Larval growth rate was calculated by dividing larval development time (days) by adult dry mass (mg). Leaf carbon and nitrogen content were determined using 1.5 mg of a sample on an analyser (Elemental Combustion System CHNS-O, Costech Instruments). Equivalent leaves to those sampled for metabolomic analysis were flash frozen, dried in an oven at 60 °C overnight then homogenised in a ball mill. There were five replicates per plant species with each replicate from a different plant.

3.3.3 Metabolic fingerprint analyses

Leaf and insect material was stored at -80 °C and then freeze dried for 16 hours. Material was ground for two minutes at 20 rpm in a ball mill. 10 mg samples were extracted twice with 400 µl of 80% methanol on ice, using umbelliferone as an internal standard. Samples were sonicated, vortexed and the supernatant removed. The two supernatants were combined and filtered through a 0.4 µm (PVDF) syringe filter.

Metabolite profiling of leaf and insect material was performed using a QToF 6520 mass spectrometer coupled to a 1200 series Rapid Resolution LC system. 5 µl of sample extract was loaded onto a Zorbax StableBond C18 1.8 µm, 2.1 x 100 mm reverse phase analytical column (LC/MS and column, Agilent Technologies, Palo Alto, USA). Features were detected in positive ionisation mode. Mobile phase A comprised 5% acetonitrile with 0.1% formic acid in water, and mobile phase B was 95% acetonitrile with 0.1% formic acid in water. The following gradient was used: 0 min – 0% B; 1 min – 0% B; 5 min – 20% B; 20 min – 100% B; 30 min – 100% B; 31 min – 0% B; 7 min post time. The flow rate was 0.25 ml min^{-1} and the column temperature was held at 35 °C for the duration. The source conditions for electrospray ionisation were as follows: gas temperature was 325 °C with a drying gas flow rate of 9 l min^{-1} and a nebuliser pressure of 35 psig. The capillary voltage was 3.5 kV. Skimmer and fragmentor voltages were 115 V and 70 V respectively. All samples were run in MS/MS.

3.3.4 Metabolic fingerprint data pre-processing

The Molecular Feature Extractor (MFE) in MassHunter software (Agilent Technologies, Palo Alto, USA) identified features (potential metabolites) from peaks produced by the LC/MS. Features eluting within the first minute are contained within the ‘dead’ volume, and features post 27 minutes are within the re-equilibration period. Therefore features eluting before one minute and after 27 minutes were excluded. The alignment of features across samples, filtering out noise, and missing value imputation were performed using an in-house alignment

algorithm, 'Kernel Feature Alignment' (Perera 2011). Plant and insect data were aligned separately due to the large number of samples examined. Features that were not detected in at least seven out of the thirteen replicates were excluded from the dataset, reducing the number of features detected in plants from 50,958 to 12,023 and in insects from 25,479 to 2,209. Principal Component Analyses (PCA) were performed on the datasets before and after the removal of these features to ensure their exclusion did not qualitatively alter the conclusions of the analyses, and we only present the more conservative analyses based on their removal. Prior to data analysis, missing value imputation (MVI) was applied in those cases where metabolites were detected in more than seven but fewer than 13 replicates (Hrydziuszko & Viant 2012). Data were log transformed and centred before multivariate statistical analyses.

3.3.5 Statistical analyses

To assess differences among host plants in terms of insect growth rates, one-way ANOVAs followed by post hoc Tukey tests were performed in R after assumptions of parametric testing were met (R Core Team 2013). To measure similarities among plant metabolic fingerprints, a hierarchical cluster analysis was carried out based on Euclidean distances between individual plant metabolic fingerprints. Relationships among clusters were visualised with a dendrogram using the mean Euclidean distance for each of the five main plant species examined and compared with insect growth rates on different host plants. To test if the metabolic fingerprints of plant species reflected the phylogenetic relatedness of those species, the metabolic fingerprints of all seven species of host plant were used to calculate Euclidean distances, and phylogenetic distances among plant species (in million years ago) were taken from a cladogram (Beilstein *et al.* 2010). Relationships between these two data sets were analysed using a Mantel test (Mantel 1967; Hardy & Pavoine 2012).

Multivariate analyses were carried out in Simca-P software (Umetrics UK Ltd, Windsor, UK). Principal Component Analysis (PCA) was used to summarise the

metabolomic data in order to compare patterns of metabolites among plants and among insects. Plant and insect data from each of the five plant species studied were analysed in two separate PCAs. Outliers falling outside Hotelling's T^2 (a generalization of Student's t-distribution applied in multivariate situations) (Prokhorov 2011) were identified and excluded to prevent their having a disproportionate influence on the analysis (one *B. vulgaris* plant sample and one *B. oleracea* insect sample were excluded). To visualise the data, plant and insect replicates were plotted according to their Principal Component (PC) scores.

A Partial Least Squares Discriminant Analysis (PLS-DA) was fitted to the insect metabolomic data (in Simca-P software). A PLS-DA is a supervised model which uses the identity of samples (i.e. identity of larval host plant species) to maximize discrimination among groups. To avoid over-fitting the data, the PLS-DA model was cross-validated by excluding a seventh of the data in turn and testing the predictive ability of a model fitted to the remaining data. If a PLS-DA model has low predictive ability, the model cannot be validated implying that there are no differences among the groups being examined. Simca-P software presents the predictive ability of the model as a Q^2 value, ranging between 0 and 1, with values closer to 1 indicating better predictive ability.

3.3.6 Isolating important metabolites

To identify metabolites responsible for distinguishing larval samples feeding on *C. spinosa*, an Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) was performed on the insect metabolic fingerprint data. This supervised analysis explains the maximum amount of variation between two chosen groups of samples in this case, insects on *C. spinosa* and insects on all other host plants. The OPLS-DA model was performed on a balanced dataset (13 *C. spinosa* fed larval replicates versus 13 replicates from a mixture of the other insect groups) so that the larger dataset would not skew the analysis. This group of 13 larval replicates from the other four larval groups was chosen by performing a PCA on the data and

selecting replicates which reflected the full range of PC 1 scores whilst ensuring each of the four host plant groups were represented.

Statistical differences in metabolite abundances in insects were analysed using ANOVAs carried out in R (R Core Team 2013) with host plant species as a factor, once parametric assumptions were met. The p values from each analysis were converted to q values to correct for false discovery rates (Benjamini & Hochberg 1995). In order to compare how abundant a metabolite was in an insect fed *C. spinosa*, fold differences in abundance were calculated using the average abundances of *C. spinosa* fed larvae and the larval group with the next highest average abundance. Fold differences in abundance were also calculated for equivalent metabolites found in *C. spinosa* plant samples.

3.4 Results

In order to understand the effect of host plants on the metabolome of insects reared on them HPLC-MS was used to analyse the metabolic fingerprints of host plants, and of the *P. rapae* larvae fed on those plants. Untargeted metabolic fingerprints were obtained from plants and insects reared under controlled conditions (see methods) and analysed for similarity and differences using supervised PCA and unsupervised PLS-DA. In total, plant fingerprints were obtained for seven species and fingerprints for insects reared on these plants were obtained for a subset of five of these plants. For the other two plants there was insufficient insect material (see methods).

3.4.1 Host plants and insects fed on them have distinct metabolic fingerprints

The metabolic fingerprints of the five species of host plants were distinguished by the first two Principal Component (PC) scores in a PCA analysis (Fig. 3.1). More closely related plant species were more likely to have similar metabolic fingerprints (based on analysis of the seven plant species; Mantel test Z-statistic= 419366,

p<0.05) and similarities between fingerprints reflected known phylogenetic relationships (Fig. 3.2). An estimate was made of the relative importance of glucosinolates compared to other metabolites in discriminating the plant species by metabolic fingerprint. We obtained the molecular weights for glucosinolate compounds (Fahey *et al.* 2001) from databases and 19 metabolites in our plant samples were tentatively identified as possible glucosinolates. To visualise the influence of these glucosinolate metabolites compared to all other measured metabolites the related loadings were highlighted in loading plots of the PCA performed on the plant metabolic fingerprints, but none of the 19 metabolites had high loadings (Fig. 3.3). Thus, we conclude that variation in glucosinolates were unlikely to be important in distinguishing among host plant species based on their metabolic fingerprints.

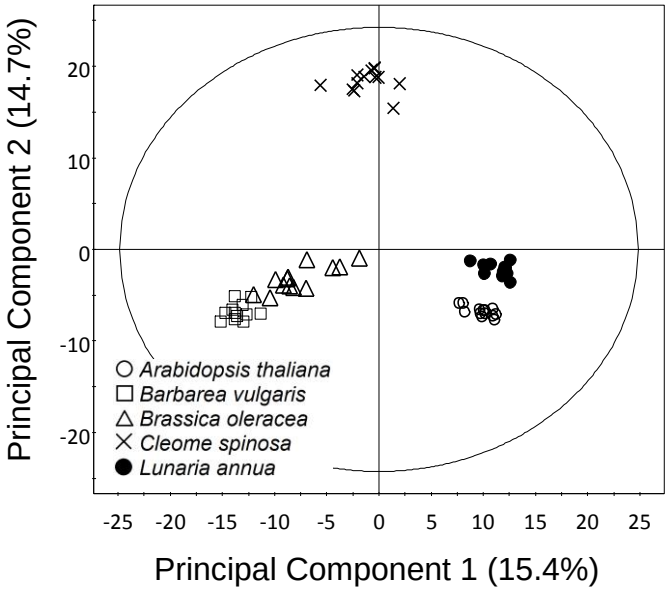


Fig. 3.1 Scores from a PCA model fitted to the metabolic fingerprints of five host plant species demonstrate that those plant species can be distinguished by their metabolic fingerprints. A total of 51.5% of the variation in the plant metabolic fingerprints was explained by five axes of a PCA fitted to the data. The variation explained by the first two PC scores is shown in brackets on the axes.

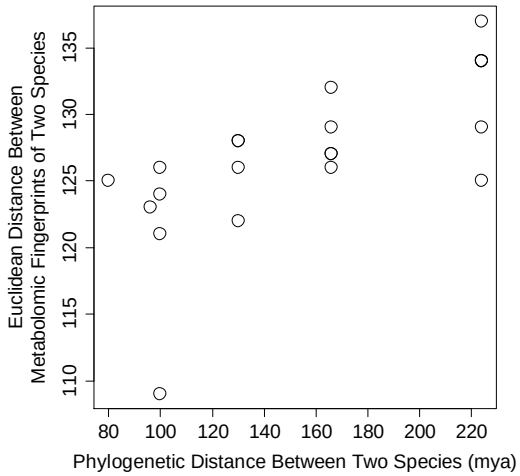


Fig. 3.2 There is a correlation (Mantel test Z-statistic= 419366, $p<0.05$) between the phylogenetic relatedness of plants (x-axis) and similarity of their metabolic fingerprints, as measured in Euclidean distances (y-axis). The outlier at the bottom left is the distances between *Brassica oleracea and Barbarea vulgaris.*

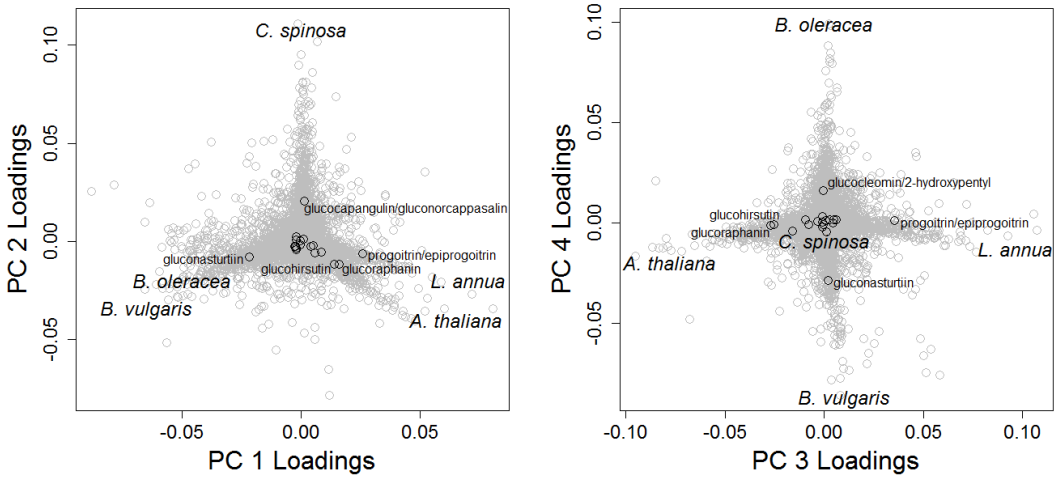


Fig. 3.3 Loading plots of a PCA analysing the metabolic fingerprints of plants. The 19 metabolites that matched the molecular weights of 29 glucosinolates (due to glucosinolate isomers) have their loadings highlighted in black. For some metabolites the name is shown of the glucosinolate with the same molecular mass as the metabolite. The loadings of other metabolites are shown in grey. The position of the five host plants according to PCA scores is indicated by the names of the plant species. Compared to other metabolites, the metabolites putatively identified as glucosinolates do not have a huge influence on discriminating the different host plant species.

Next we analysed the metabolite content of the insect fat bodies. Compared to the distinctiveness of metabolic fingerprints from different plant species (Fig. 3.1), PCA revealed less differentiation in metabolic fingerprints amongst larvae raised on different host plants (Fig. 3.4). This demonstrates that differences among the insect metabolomes are smaller than among the plant metabolomes. In the PCA analysis, only those larvae that fed on *C. spinosa* could be distinguished from other larvae (Fig. 3.4), and when these larvae were excluded from the analysis, we still found no differentiation among larvae fed on the other four host plants. A supervised PLS-DA confirmed that larvae fed *C. spinosa* could be distinguished from other larvae (first latent variable of the PLS-DA; Fig. 3.5), but unlike the unsupervised PCA, the next three latent variables of the PLS-DA were able to discriminate among larvae reared on the other host plants (Fig. 3.6). Thus, host plants influence the metabolic

fingerprint of *P. rapae* larvae and these changes are especially large in larvae feeding on *C. spinosa*.

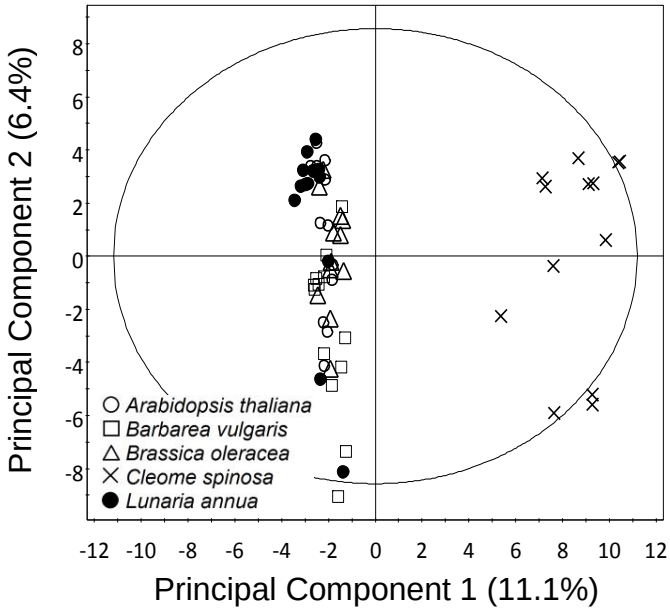


Fig. 3.4 Scores from PCs 1 and 2 from a PCA model fitted to larval metabolic fingerprints. Larvae fed on *C. spinosa* are distinguished from other larvae feeding on other host plants. The PCA was fitted to four axes which together explained 25.9% of the total variation in the dataset. The variation explained by the first two PCs is shown in brackets on the axes.

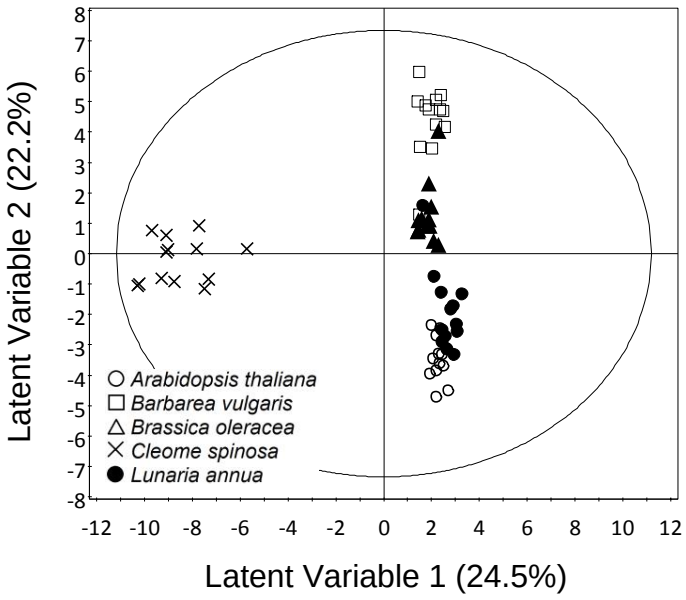


Fig. 3.5 Latent variable 1 versus latent variable 2 scores from a PLS-DA model fitted to the metabolic fingerprints of the larvae. Latent variable 1 shows that the metabolic fingerprints of the larvae fed *C. spinosa* were different from the other larvae. The % of the modelled Y variation is shown in brackets on the axes labels. 6 latent variables explained 97.2% of the Y variation in the model. The Q^2 value was 0.836.

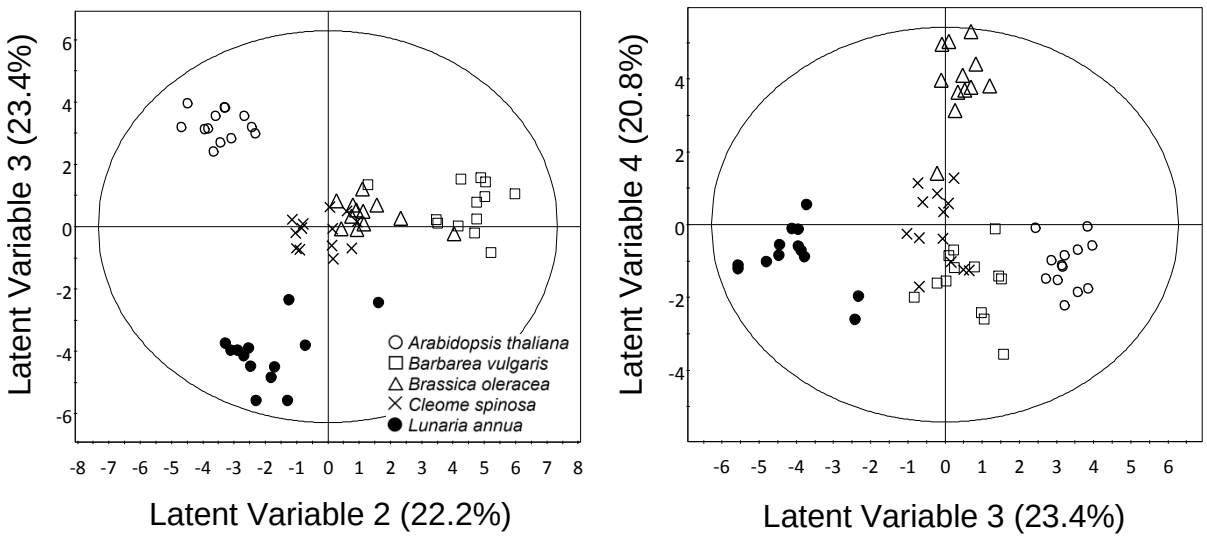


Fig. 3.6 Latent variables 2, 3 and 4 scores from a PLS-DA model fitted to the metabolic fingerprints of the larvae emphasize the differences among larvae feeding on different host plants. The % of the modelled Y variation is shown in brackets on the axes labels. 6 latent variables explained 97.2% of the Y variation in the model. The Q^2 value was 0.836.

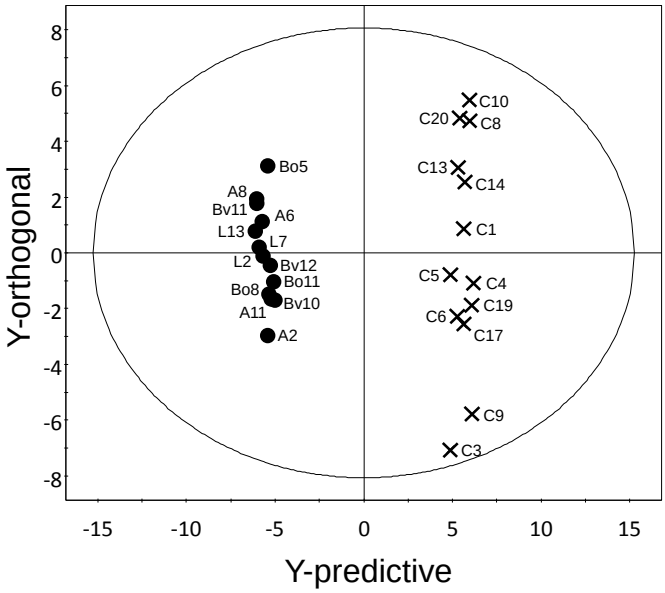
3.4.2 Identification of abundant metabolites in larvae feeding on *C. spinosa*

In order to understand why the metabolic fingerprints of *C. spinosa* fed larvae were different, those metabolites that distinguished these larvae from other larvae were isolated by performing an OPLS-DA model (Fig. 3.7). The OPLS-DA produced an 'S-plot' which was used to visualise the relative importance of different metabolites and to extract metabolites that were highly abundant in those insects feeding on *C. spinosa*. We focused on metabolites that were at least four times more abundant in *C. spinosa* fed larvae than in other larvae, to ensure that any selected metabolites represented a major difference in abundance compared to other larvae. Using this criterion 44 metabolites were found to be highly abundant in *C. spinosa* fed larvae (Table 3.1). Comparisons between the molecular masses and fragmentation patterns of these metabolites with reference information in metabolomic databases as well as the fragmentation of the samples alongside a standard, all failed to determine the identities of these metabolites.

In order to establish whether the metabolites that were abundant in larvae feeding on *C. spinosa* could have originated from the host plant, the plant metabolic

fingerprints were examined for metabolites with the same masses and retention times. Of the 44 metabolites most influential in distinguishing the metabolic fingerprints of the larvae fed *C. spinosa* from other larvae*,* all but two matched metabolites in the plant fingerprint data, with equivalent mass and retention time. Furthermore, the abundances of these metabolites were many times higher in *C. spinosa* plants compared with other plant species (Table 3.1). We conclude that metabolites detected in larvae feeding on *C. spinosa* plants which distinguished them from other larvae, are likely to have originated from the *C. spinosa* host plant.

a)



b)

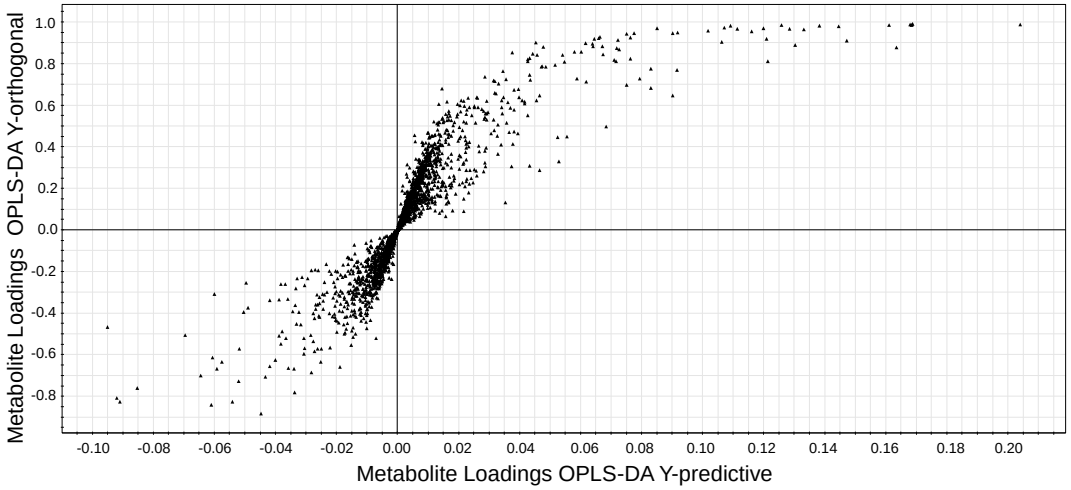


Fig. 3.7 a) A plot of the OPLS-DA analysis carried out on larvae fed *C. spinosa* (x) and larvae fed other plant species (•). b) An S-plot produced by plotting loadings from the OPLS-DA analysis on the samples of larvae fed *C. spinosa* and larvae fed other host plants. Individual metabolites are denoted by solid triangles. Metabolites at the top right of the plot are very different in abundance in larvae fed *C. spinosa* compared to other larvae.

Mass (Da)	RT (min)	q value	Insect FD	Plant FD
57.26	20.33	0.00005	176	67
409.35	23.73	0.00003	72	34
355.3	21.70	0.00005	75	6
145.1	3.26	0.00005	68	3.2
383.34	23.47	0.00004	57	38
395.34	23.13	0.00005	55	33
456.35	22.36	0.00006	99	114
369.32	22.86	0.00025	58	21
331.25	19.59	0.00006	56	65
381.32	22.08	0.00004	32	43
970.72	22.39	0.00003	30	21
333.26	20.70	0.00002	26	53
416.31	20.35	0.00006	20	3.2
341.29	21.04	0.00172	36	19
407.34	22.49	0.00014	32	68
393.32	21.95	0.00029	23	21
319.25	18.35	0.00005	20	34
343.25	18.09	0.00005	18	39
367.3	21.48	0.00003	16	28
533.41	22.40	0.00004	15	no match
99.1	3.26	0.00004	13	8.7
496.35	22.40	0.00021	23	13
438.35	22.40	0.00004	23	9.4
388.11	17.45	0.00004	10	66
314.07	14.86	0.00005	10	28
358.1	17.09	0.00005	8.2	41
432.3	16.50	0.00003	7.3	no match
411.37	25.77	0.00001	6.9	12
355.25	19.27	0.00423	16	53
327.27	19.84	0.00095	11	2.8
303.22	17.82	0.00000	7.2	38
881.71	22.47	0.00006	6.7	22
344.08	14.21	0.00002	4.5	4.2
313.26	19.08	0.00006	6.7	1.7
359.28	21.39	0.00000	7.3	37
397.35	24.51	0.00004	5	6.2
397.35	24.94	0.00003	5.2	6.2
173.13	2.11	0.00077	4.6	27
390.29	19.59	0.00003	4.9	1.6
358.1	16.75	0.00004	5.1	41
289.2	15.62	0.00003	5.2	19

405.32	21.47	0.00000	4.4	7.6
291.21	16.44	0.00006	4.6	15.2
286.08	14.10	0.00001	4.4	6.5

Table 3.1 List of 44 metabolites in larvae feeding on *C. spinosa* which showed high abundances in insect and plant fingerprints. All these metabolites were extracted from an OPLS-DA 'S plot', have a significant q value and are at least 4 times more abundant than in other larvae. Mass and RT are the observed molecular weight and retention time from larval data as recorded from the LC-MS. Fold Difference (FD) is calculated from the average abundance of the *C. spinosa* fed larval replicates compared to the group with the next highest average abundance. Plant FD is calculated in the same way. No match under plant FD indicates that no match was found between plant metabolites and the mass and retention time of the insect metabolite. The q values are p values from one-way ANOVAs corrected for false discovery rate.

3.4.3 Insect growth rates reflect similarities in metabolic fingerprints of plants

In order to determine if the variation in insect performance could be explained by plant metabolic fingerprints or by the macro nutrient content, we measured the growth rate of larvae on each of the plants and the nitrogen and carbon content of each plant. Larvae reared on *B. oleracea*, *B. vulgaris* and *C. spinosa* (mean = 1.899 mg day) had growth rates which were 1.4 times faster than those reared on *A. thaliana* and *L. annua* plants (mean = 1.405 mg day; ANOVA of growth rates by plant species, $F_{4,81} = 33.67$, $p<0.001$; Fig. 3.8a). Leaf nitrogen content did not vary among plant species (ANOVA $F_{4,20} = 1.23$, $p=0.331$), but carbon to nitrogen ratio (C:N) was significantly higher in *C. spinosa* (C:N = 6.9, C = 40.9%, N = 6.0%) than in *A. thaliana* (C:N = 5.4, C = 37.1% N = 6.9%) with C:N measurements from the other plants having intermediate values (ANOVA $F_{4,20} = 3.41$, $p=0.028$; post hoc Tukey test). A hierarchical cluster analysis summarised the similarities among the metabolic fingerprints of the plant species and this pattern of similarity matched variation in the high or low growth rates of insects on those same plant species (Fig. 3.8). This apparent association between variation in plant metabolic fingerprints

and insect performance implies that a suite of metabolites in host plants influence the performance of insect herbivores on host plants.

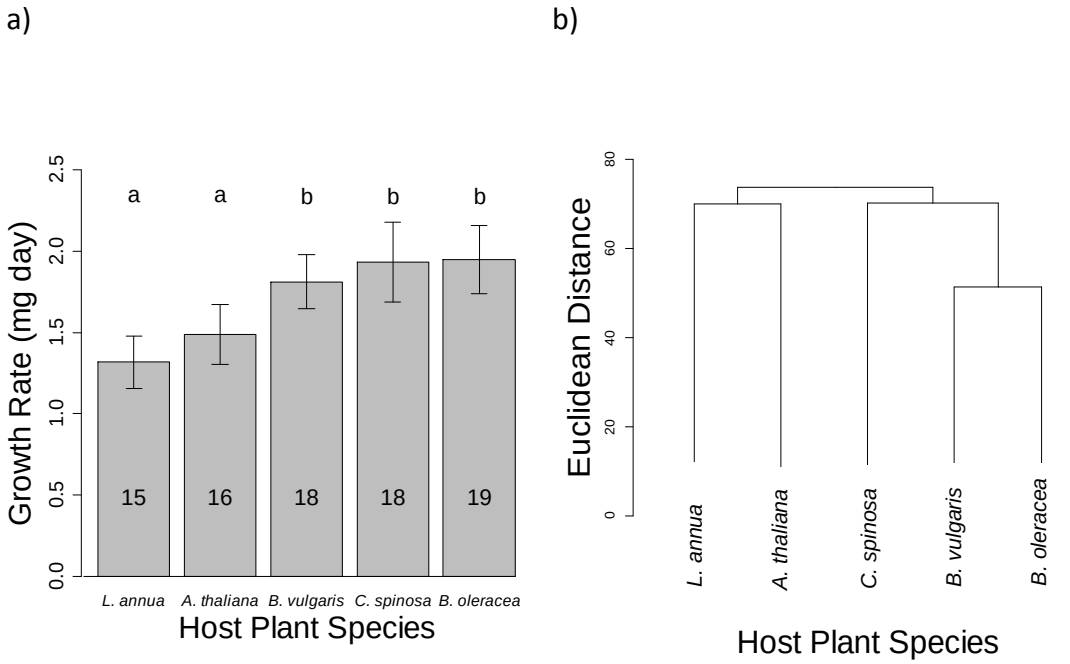


Fig. 3.8 a) Growth rates of *P. rapae* larvae on different host plants. Larvae fed *B. vulgaris*, *C. spinosa* and *B. oleracea* had significantly higher growth rates than larvae fed *L. annua* or A. thaliana. Bars with the same letters were not significantly different at the 5% level following ANOVA and post hoc Tukey tests. Numbers on bars indicate the number of replicates. b) Visualisation of the similarities among metabolic fingerprints of host plants according to hierarchical cluster analysis. The split into two groups corresponds to differences in insect growth rates on these plant species.

3.5 Discussion

The advantage of untargeted metabolomics is that a wide range of metabolites are examined providing a better overview of changes to metabolomes. By applying this approach to the insect herbivores as well as their host plants we could evaluate the effect of the plant species on the insect metabolome. Our discovery of the transfer of a group of metabolites from the host plant *C. spinosa* to *P. rapae* larvae was

because we used an untargeted approach with both plant and insect fingerprints rather than focusing primarily on a single group of known metabolites.

One of our aims was to investigate the extent to which the metabolome of an insect herbivore is determined by the host plant it feeds upon. We conclude that the larval host plant influences metabolite composition in the fat-body of *P. rapae* because larval metabolic fingerprints differed according to the host plant they fed on. Larvae feeding on *C. spinosa* were particularly distinctive and larvae feeding on other host plants could only be distinguished using a supervised multivariate analysis indicating that these host plants had much smaller effects on insect metabolomes compared to *C. spinosa* plants. Other studies support the idea that larval diet affects the metabolites found within the insect. For example, the concentration of cardenolide compounds in dogbane tiger moths (*Cycnia tenera*) are dependent on the species of *Asclepias* plant larvae feed on (Cohen & Brower 1983), and *P. rapae* were found to contain pinoresinol at the end of the larval glandular hairs only if they fed on *B. oleracea* (Schroeder *et al.* 2006). Our study is the first to examine the wider insect metabolome to demonstrate the effects of host plant diet on the insect herbivore.

Nitrogen content of plants can limit larval growth (Slansky & Feeny 1977) but we found little variation in nitrogen or C:N ratios among host plants, and no effect on insect growth rates. We found some evidence that variation in larval growth rates was associated with differences in metabolic fingerprints, but with only five plant species studied this association pattern could have arisen by chance. Further investigation of whether variation in metabolites among host plants determines insect performance deserves further study and might reveal better understanding of why insect performance varies among host plants.

Several metabolites that occurred in high abundance were responsible for observed differences between the metabolic fingerprints of larvae fed excised *C. spinosa* leaves and larvae fed other plant species. Metabolites with similar molecular mass and retention times were also found in high abundance in the leaves of *C. spinosa*

plants from the glasshouse (Table 1). Despite the differences in the growing conditions of the glasshouse plants sampled for the plant metabolomes and those fed to the insects, we conclude that these abundant metabolites originated from the *C. spinosa* plants. This certainty stems from the presence and exceptionally high abundance of the chemicals in the *C. spinosa* plants compared to the virtual absence of them in the other host plant species.

The finding that some metabolites can transfer from *C. spinosa* plants into larvae of *P. rapae* with unchanged chemical structures has not been shown before. Other studies have shown many examples of sequestration of metabolites by insects from their host plants (Opitz & Müller 2009) including the cardenolide metabolites from milkweed host plants (Asclepiadaceae) sequestered for defence by monarch butterflies (*Danaus plexippus*) (Brower *et al.* 1967). Therefore, active sequestration of *C. spinosa* metabolites by *P. rapae* larvae is one possibility. Alternatively, these metabolites may have no function in the insects but be passively absorbed and bioaccumulate because larvae are unable to digest, break down or excrete the compounds. *C. spinosa* differs from the other host plant species under test in that it originates from Asia occurring only in this country as a garden ornament plant or garden escapee (http://www.cabi.org). This could indicate that *P. rapae* has not evolved alongside *C. spinosa* for any significant period of time and therefore lacks the ability to process all of the metabolites within *C. spinosa*. If further study confirmed this inability of the insects, it would provide an explanation as to why insect species are not able to fully exploit alien plant species as hosts.

We found no effect of the abundant metabolites present in *C. spinosa* host plants on *P. rapae* growth rates, and larval growth rates did not differ between *C. spinosa, B. oleracea* and *B. vulgaris* plants (Fig. 3.8a). This contrasts with other studies that have shown that concentrated extracts of *Cleome arabica* were toxic to larvae of *Spodoptera littoralis* and *Cleome deoserifolia* caused mortality in the first instar larvae of *Phthorimaea operculella* (Soliman 2012; Ladhari *et al.* 2013). As we used second instar larvae it is possible *C. spinosa* is toxic to the first instar of *P. rapae*. In addition, a superior method of measuring insect performance which could involve

regularly assessing growth of the larvae throughout development would give a more informative insight into how the species of host plant affects the growth of the insect. Such an approach could reveal that the effect of host plant is reliant on the stage of larval development.

The host plants of polyphagous insect herbivores have not previously been examined in relation to their metabolic fingerprints. We showed that host plants of *P. rapae* have very different metabolomes (Fig. 3.1), and that more closely related plants have more similar metabolic fingerprints (Fig. 3.2). Since the metabolome is essentially the end product of the genome (Sumner *et al.* 2003) and more closely related species are more similar genetically, this pattern is expected but has not been shown before using multiple plant species.

Much research has focussed on differences between Brassicaceae host plants in terms of glucosinolates (Rodman & Chew 1980; Hasapis *et al.* 1981; Koritsas *et al.* 1991), but we estimate that other metabolites are more important in distinguishing host plant species (Fig. 3.3), and deserve further study when considering insect-Brassicaceae interactions. In summary, we have provided new perspectives on interactions between plants and insects; discovered metabolites transferring between trophic levels and found evidence that the plant metabolome may be associated with insect performance. This study raises the possibility that further understanding of plant-insect interactions may be possible by comparing the metabolomes of organisms at different trophic levels.

Chapter 4 Metabolic fingerprints reveal idiosyncratic responses in three host plant species to herbivory by *Pieris rapae* larvae.

4.1 Abstract

Studying the biochemical responses of different plant species to herbivory may help improve our understanding of the diversity of metabolites found in the host plants of insects. Untargeted metabolic fingerprints were used to compare metabolite reactions in three host plant species (*Cleome spinosa*, *Brassica oleracea* and *Lunaria annua*; order Brassicales) to larval herbivore attack (*Pieris rapae*; Lepidoptera). Principal Component Analyses of metabolic fingerprints were able to distinguish among the three plant species, as well as between infested plants that had been eaten and uneaten control plants. A large number of metabolites (1186, 13% of all measured metabolites) were common to the three plant species. The abundances of metabolites in control and infested plants were compared in order to isolate metabolites induced by herbivory. Although *B. oleracea* and *C. spinosa* had many species-specific metabolites that were induced by herbivory (after correction for false discovery rate; *B. oleracea* = 87 metabolites, *C. spinosa* = 68), only three metabolites were induced in both plant species. By contrast, *L. annua* only had one metabolite induced by herbivory, and this was not found to be induced in the other two plant species. Thus metabolites induced by herbivory were idiosyncratic in the three host plant species.

4.2 Introduction

Plants contain thousands of metabolites, some of which are secondary metabolites that have a defence function against herbivores. Metabolite defences against herbivores can be either 'constitutive', which means high levels of the metabolite are maintained in the plant, or 'induced' which means the metabolite is changed in abundance by herbivore attack (Bezemer & van Dam 2005). For example, chlorogenic acid which acts as a feeding deterrent to *Lochmaea capreae* (leaf beetle) feeding on *Salix* sp. (willow), is thought to be maintained at consistent levels in host plants (Ikonen *et al.* 2001). By contrast in *Nicotiana sylvestris* (tobacco plants), herbivory by *Manduca sexta* (moth) larvae has been shown to increase levels of the defensive metabolite nicotine (McCloud & Baldwin 1997). Using induced metabolites as a defence is thought to allow the plant to incur the cost associated with producing defensive metabolites only when attacked (Heil & Baldwin 2002).

Some of the plants that Pierid butterflies feed on are related (Braby & Trueman 2006). For example, many of the host plants of the polyphagous *P. rapae* (small white butterfly; Lepidoptera) are within the family Brassicaceae (Asher *et al.* 2001; Stevens 2001 onwards; Beilstein *et al.* 2008). Groups of structurally similar secondary metabolites tend to be common to plant species which are phylogenetically related (Wink 2003). For example, the plant family Brassicaceae is characterised by glucosinolates (Fahey *et al.* 2001) and plants in the family Solanaceae are characteristically rich in tropane alkaloids (Shimomura *et al.* 1991). Metabolites in both the glucosinolates and tropane alkaloid groups are known to have defensive functions against herbivores (Ziegler & Facchini 2008; Ahuja *et al.* 2010). Common metabolites among related plants would suggest that the related host plants of a polyphageous insect would employ some of the same specific metabolites for defence. As with similarities in genomes, species that are more closely related may share a greater number of defensive metabolites. On the other hand, the evolutionary arms race believed to happen between plants and their herbivore attackers means there is a continual cycle of negative effects of defence

chemicals being overcome by the insect and the plant evolving new defence chemicals (Mithofer & Boland 2012). This arms race means it is possible that any successful defence chemical originating in a common ancestor would be overcome by the insect during evolutionary time and lost, leaving few common chemical defences among related modern day plant species.

The vast diversity of metabolites in plants (Kroymann 2011) can be taken as evidence of the continual evolution of new metabolites. Mechanisms used to achieve new metabolites may involve gene duplication and point mutations of genes which lead to novel enzymes able to make new metabolites from precursor metabolites in the biosynthesis pathways of existing metabolites (Kliebenstein *et al.* 2001; Kampranis *et al.* 2007; Kliebenstein 2008). These processes enable the maintenance of a large diversity of metabolites (Moore *et al.* 2014) which suggests plants may be using a 'screening' strategy whereby maintaining a large pool of metabolites increases the probability that some will prove to have a defensive function (Jones & Firn 1991). In this way plants would be able to adapt to evolutionary selection pressures which include insect herbivores (Rask *et al.* 2000).

There is little evidence to gain an idea of the extent to which plant metabolite defences have been shaped by a shared evolutionary history and the extent to which each plant species has evolved its own set of unique metabolites. This question may not have been addressed before because of the difficulty in measuring a lot of metabolites in a plant at once. Now metabolic fingerprinting, which is an untargeted, high-throughput method, can measure a large number of metabolites to gain a 'snapshot' of an organism's metabolome (Fiehn 2001; Overy *et al.* 2005). Metabolic fingerprints have already been used to assess the effects of herbivory on plants. For example, the metabolic fingerprints of *Plantago lanceolata* differed in response to different stresses (including herbivory) yet no difference was observed using a parallel targeted analysis (Sutter & Müller 2011). An untargeted method revealed new metabolites induced by *Spodoptera littoralis* (moths) in *Zea mays* (Marti *et al.* 2013), and analysis of metabolic fingerprints of *Arabidopdsis thaliana* revealed differences in the metabolome of leaves attacked by *Brevicoryne*

brassicae (aphids) compared to the metabolome of roots attacked by *Heterodera schachtii* (nematodes) (Kutyniok & Müller 2012). However, none of these studies have looked at multiple plant species and therefore the number of induced metabolites common or unique among species has never been compared. Such interspecific measurements could uncover the evolution of metabolites in plants and if there are particular defensive compounds that are very successful against herbivory and have therefore been retained in multiple plant species throughout evolutionary time.

Although untargeted studies in this area are lacking, targeted analyses have compared metabolites among host plants and in the Brassicaceae plants have focused on the group of metabolites called glucosinolates which are the most studied and defining secondary metabolites of the family Brassicaceae (Rask *et al.* 2000; Kroymann 2011). Some studies have compared multiple host plants after insect attack to show induced differences in the abundances and presence of glucosinolates between plant species (Koritsas *et al.* 1991; van Dam & Raaijmakers 2006). From these studies we know some metabolites (namely glucosinolates) vary qualitatively and quantitatively among host species. However, it is unknown if these differences in glucosinolates could be extrapolated to the rest of the metabolome.

In this study, I investigate three larval host plant species (*B. oleracea* and *L. annua* in the family Brassicaceae and the more distantly related *C. spinosa* in family Cleomaceae) that are all eaten by *P. rapae* in order to examine whether herbivore induced metabolites are common to these host plants. By studying metabolites that are induced by insect feeding, I focus on those metabolites that are likely to have a defensive role. A search for induced compounds likely to be glucosinolates was also made in order to enable comparisons with previous work on induced metabolites in Brassicaceae. Quantifying the shared and unique induced metabolites among the three species provides information on the evolution of metabolites in plants, and common metabolites which are induced by multiple host plants may suggest metabolites that have a vital function in the *P. rapae*-plant interaction.

4.3 Methods

4.3.1 Insect and plant rearing

The three species of host plant that were investigated were *Brassica oleracea* (cabbage), *Cleome spinosa* (spider flower) and *Lunaria annua* (honesty). These plants are taxonomically relatively far apart and provide two study species *B. oleracea* and *L. annua* that are in the same family (Brassicaceae) and one species *C. spinosa* in another family (Cleomaceae) which is more distantly related thereby enabling a comparison between plant relatedness and the number of shared metabolites.

Plants were grown from seeds sourced from Groves Nurseries, Dorset, UK (*B. oleracea* 'stonehead F1') and Chiltern Seeds, Oxfordshire, UK (*C. spinosa* 'Cherry Queen' and *L. annua*). Seedlings were grown in a greenhouse in trays for two weeks and were then potted into 10 cm pots and grown in temperature controlled cabinets (Sanyo MLR 350) at constant temperature (21 ^{o}C); photoperiod of 16L:8D and approximate light 60 µmol m^2 s^{-1}. Pots were randomised among different growth cabinets until the plants had been growing for seven weeks, at which point each plant was assigned as either a control (n = 10) or infested (n = 10) plant and kept in different cabinets to prevent plant volatile organic compounds affecting other plants.

Pieris rapae larvae were the offspring of four adult butterflies caught in York, UK in July. Female butterflies were kept in a greenhouse in 31 cm x 42 cm keep nets, fed a honey solution soaked on cotton wool and given potted *B. oleracea* seedlings to oviposit on. Larvae laid over a period of two days were allowed to feed from the three host species until they reached fourth and fifth instar (14 days after hatching) when they were transferred with a paintbrush to experimental plants. There was one larva per plant and ten infested plants per plant species. Larvae were placed on a middle aged leaf in all replicates. Larvae were confined to leaves using organza

bags and hair crocodile clips to prevent the plant stem being crushed. Empty bags and clips were also placed on the equivalent leaves of control plants. Larvae were left on *B. oleracea* and *L. annua* plants for 67 hours but were taken off *C. spinosa* plants after 44 hours due to its smaller leaves and to ensure some of the leaf was left for sampling. The remains of the eaten leaves in the infested treatment and the equivalent leaves in the control plants were cut at the stem and put in eppendorf tubes immediately after removal of larvae, flash frozen and stored at -80^{o}C.

4.3.2 Metabolomic analysis

Leaves were flash frozen immediately after harvesting, homogenised while being kept cool and extracted using methanol containing an internal standard. After filtering, the metabolites in the samples were measured using a QToF Mass Spectrometer coupled to a High Performance Liquid Chromatography system (HPLC-MS). Full details of sample extraction and metabolomic analysis are described in Chapter 2.

4.3.3 Data pre-processing

Features were aligned between all samples using a Kernel based Feature Alignment programme (KFA) (Perera 2011). Features which were considered noise were then filtered out if they were not recorded in at least five out of ten replicates in any group and missing value imputation (MVI) performed on the dataset. After alignment of the data there were abundances for 36,432 features. After filtering out noise in the data, this was reduced to 11,649 features that were considered metabolites. PCAs (Principal Component Analysis) were performed on both datasets to ensure that exclusion of these features had no effect on the results. Full details of pre-processing of data are described in Chapter 2.

4.3.4 Statistical analysis

To determine if the three different plant species could be distinguished by their metabolic fingerprint, the data were analysed using a PCA. To investigate differences between infested and control plants, each plant species was analysed

separately by PCA, followed by t-tests of Principal Component (PC) scores. The lists of metabolites recorded in each species of host plant were cross-referenced and Venn diagrams used to summarise numbers of metabolites that plant species had in common. This was done for control plants and repeated for infested plants.

To determine metabolites induced by herbivory in the three plant species, it was first established whether or not a metabolite differed in abundance between the infested and control plants. Data were tested for normality (Anderson Darling test) and equality of variance (Levene's test) with data transformation if necessary (natural log, inverse or square root transformation) followed by t-tests, otherwise non-parametric Mann-Whitney U tests were used. These p values were corrected for False Discovery Rates (FDR) by conversion to q values (Benjamini & Hochberg 1995). For each metabolite, the difference in abundance was quantified using mean abundance in infested plants divided by the mean abundance in control plants which indicated if the metabolite had increased or decreased. This process produced lists of significantly increased and decreased metabolites according to $q<0.05$, for each plant species. These were then cross-referenced to find common metabolites induced by *P. rapae* herbivory. The numbers of common and unique metabolites were summarised in Venn diagrams.

4.3.5 Tentative glucosinolate identification

Molecular weights for glucosinolate compounds (Fahey *et al.* 2001) were obtained from databases for 99 out of 120 glucosinolates and a search made for metabolites with these same molecular weights as glucosinolates accurate to two decimal places. Identification using only molecular weight is considered a tentative identification (Sumner *et al.* 2007). Due to the low abundances of these metabolites the raw values of the metabolites were used before MVI (Missing Value Imputation, see Chapter 2) was performed. This slightly different approach was taken in this analysis because some of the metabolites detected had abundances below the MVI values which misrepresented the presence and abundance of these metabolites in the samples. To test for significant differences in these glucosinolate-matching

metabolites between control and infested plants a t-test was used or a Mann-Whitney U test when assumptions of normality could not be met.

4.4 Results

Thousands of metabolites were measured by HPLC-MS from foliar samples (n = 60) of three species of host plant both infested (eaten by *P. rapae*) and control (not eaten) to obtain metabolic fingerprints. This allowed me to compare the effect of herbivory among the three species using PCA and to find the number of induced metabolites that were common among the species using univariate statistics. Some metabolites were putatively identified as glucosinolates and abundances statistically tested to evaluate if any were induced by herbivory.

4.4.1 Metabolic fingerprints are changed by herbivory in all three plants

Regardless of whether or not a plant had been eaten, the three plant species had different metabolic fingerprints when analysed in a PCA (Fig. 4.1a), meaning effects of *P. rapae* herbivory on the metabolic fingerprints were smaller than the differences among metabolic fingerprints of the plant species. Subsequent PCAs performed separately on the three plant species showed that control plant samples and infested plant samples were distinguished in PC score plots (Fig. 4.1b, c and d), and there were statistically significant differences in the scores of either the first or second PC (t-tests *B. oleracea* PC 2 scores $t_{18} = 5.52$, $p<0.001$; *C. spinosa* PC 1 scores $t_{18} = -4.62$, $P<0.001$; *L. annua* PC 2 scores $t_{18} = 3.66$, $p<0.01$). This demonstrated that in all three species of plant the metabolic fingerprint had been changed by *P. rapae* herbivory.

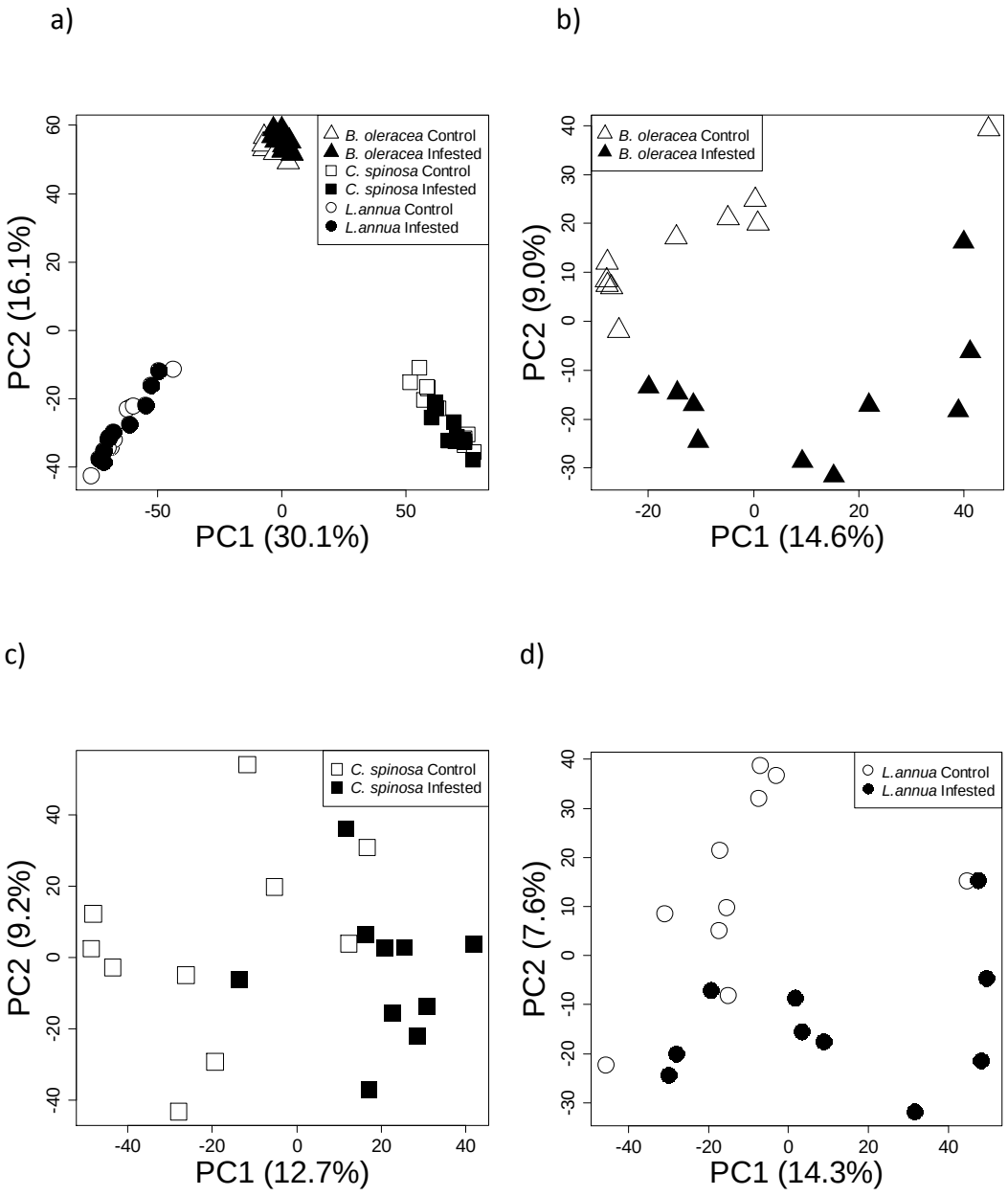


Fig. 4.1 PC (Principal Components) 1 and 2 from PCA models fitted to metabolic fingerprints of a) all three host plants, and separately for b) *B. oleracea* c) *C. spinosa* and d) *L. annua*. The variation explained by each PC is shown in brackets on axes labels. In a) different species cluster together but the control and infested plants within a species are indistinguishable. A total of 51.8% of the variation in this PCA was explained by four principal components. In panels b), c) and d) the infested and control plants are distinguishable indicating differences in metabolic fingerprints.

4.4.2 Common metabolites exist between the three plant species but not common induced metabolites

When the metabolites detected in the three plant species were cross-referenced there were a large number of metabolites (1186; 13% of measured metabolites) which were found in the control plants of all three species (Fig. 4.2a). There was a similar number of metabolites (1198; 12% of measured metabolites) that were common to infested plants from all three plant species (Fig. 4.2b). However, the majority of metabolites (74% of measured metabolites in control and 75% in infested) were unique to only one plant species (Fig. 4.2a and b).

a) b)

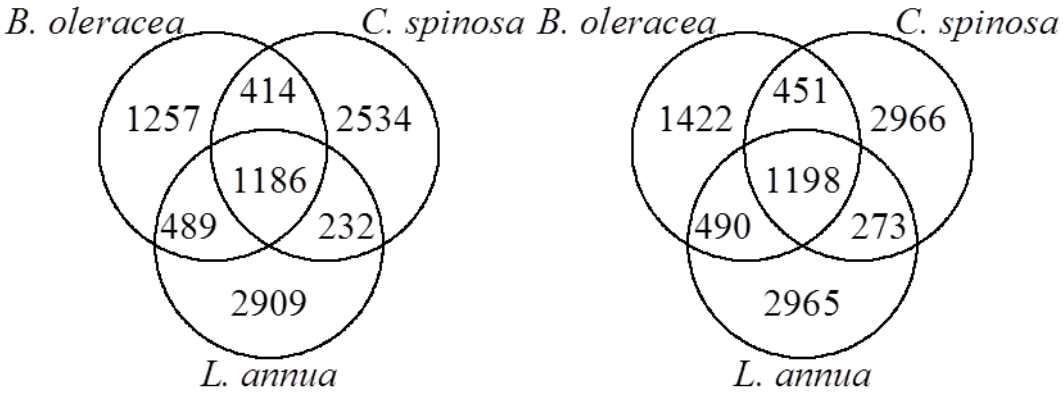
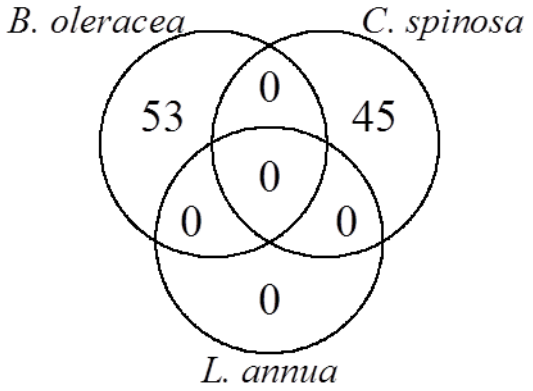


Fig. 4.2 Venn diagrams summarising the number of shared and unique metabolites. a) Metabolites measured in control plants and b) metabolites measured in plants infested with *P. rapae*. In control plants 74% of all metabolites measured were unique to one plant, 13% shared by two species and 13% shared by three species. In infested plants the equivalent percentages were 75% unique, 12% shared by two and 12% shared by three plants.

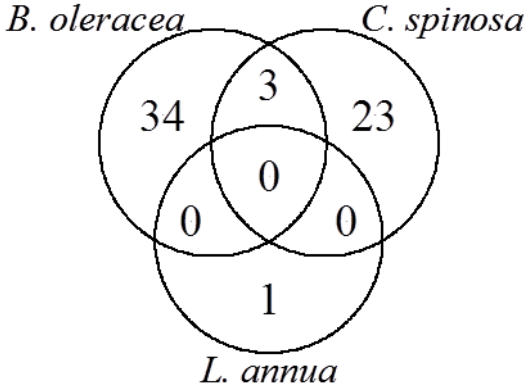
Of those metabolites that were considered to have significantly changed in abundance following herbivory, there were no metabolites that were common to all three host plant species (Fig. 4.3a and b). This result is partly due to the low number of metabolites (one) found to be changed by herbivory in *L. annua* (after

correction for FDR). However, even before FDR correction there were only two metabolites in common among the three plant species (Fig. 4.3c and d; 0.07 % of all the metabolites found to change according to $p<0.05$). Thus I conclude that metabolite responses to herbivory are idiosyncratic in these plant species.

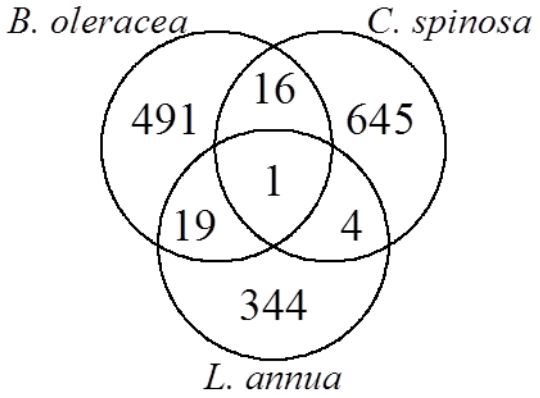
a)



b)



c)



d)

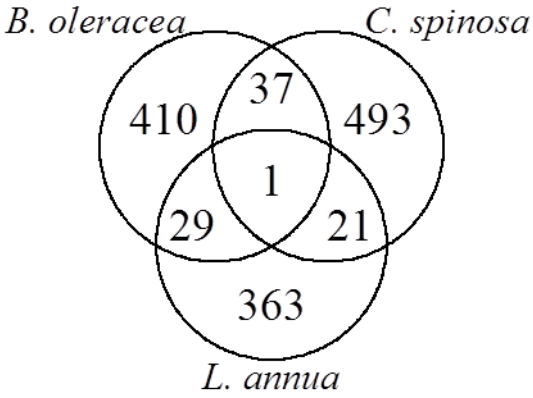


Fig. 4.3 The number of metabolites that a) increased and b) decreased in infested plants according to q<0.05 (p values corrected for FDR). *L. annua* only had one metabolite decrease after *P. rapae* herbivory under these criteria. The numbers of metabolites found to have changed in more than one plant species are indicated by the circle overlaps. Also shown are the number of c) increased and d) decreased metabolites which significantly changed according to p<0.05 (before FDR). This confirms that the results are not primarily due to FDR.

4.4.3 Two glucosinolate-like metabolites are induced in *B. oleracea*

A total of 13 metabolites detected in this study had the same molecular weight as 12 glucosinolates (multiple metabolites matched some glucosinolates and some

metabolites matched glucosinolate isomers). The average abundances as measured on the HPLC-MS and the number of plant replicates in which the metabolites were detected in are shown in Fig. 4.4, along with the tentative glucosinolate identification. The Metabolomics Standards Initiative recommends that metabolites are described as being only 'tentatively identified' (Sumner *et al.* 2007) when the identification is based only upon the molecular mass of the metabolites, as in this analysis. Only two glucosinolate-matching metabolites, both in *B. oleracea*, were found to have significantly increased in abundance between control and infested plants (11-(Methylsulfinyl)undecyl and the isotopes progoitrin and epiprogoitrin; p values in bold in Fig. 4.4). Some of these metabolites were recorded in only one plant species and not the other two (for example metabolites with the same molecular mass as 3-Methoxycarbonylpropyl, 2-Hydroxypentyl, glucobrassicin, glucoibarin and 10-(Methylsulfonyl)decyl (Fig. 4.4). However the tentative identifications means conclusions regarding effects of herbivory on glucosinolate abundances or the variation of glucosinolates among host plants are unreliable.

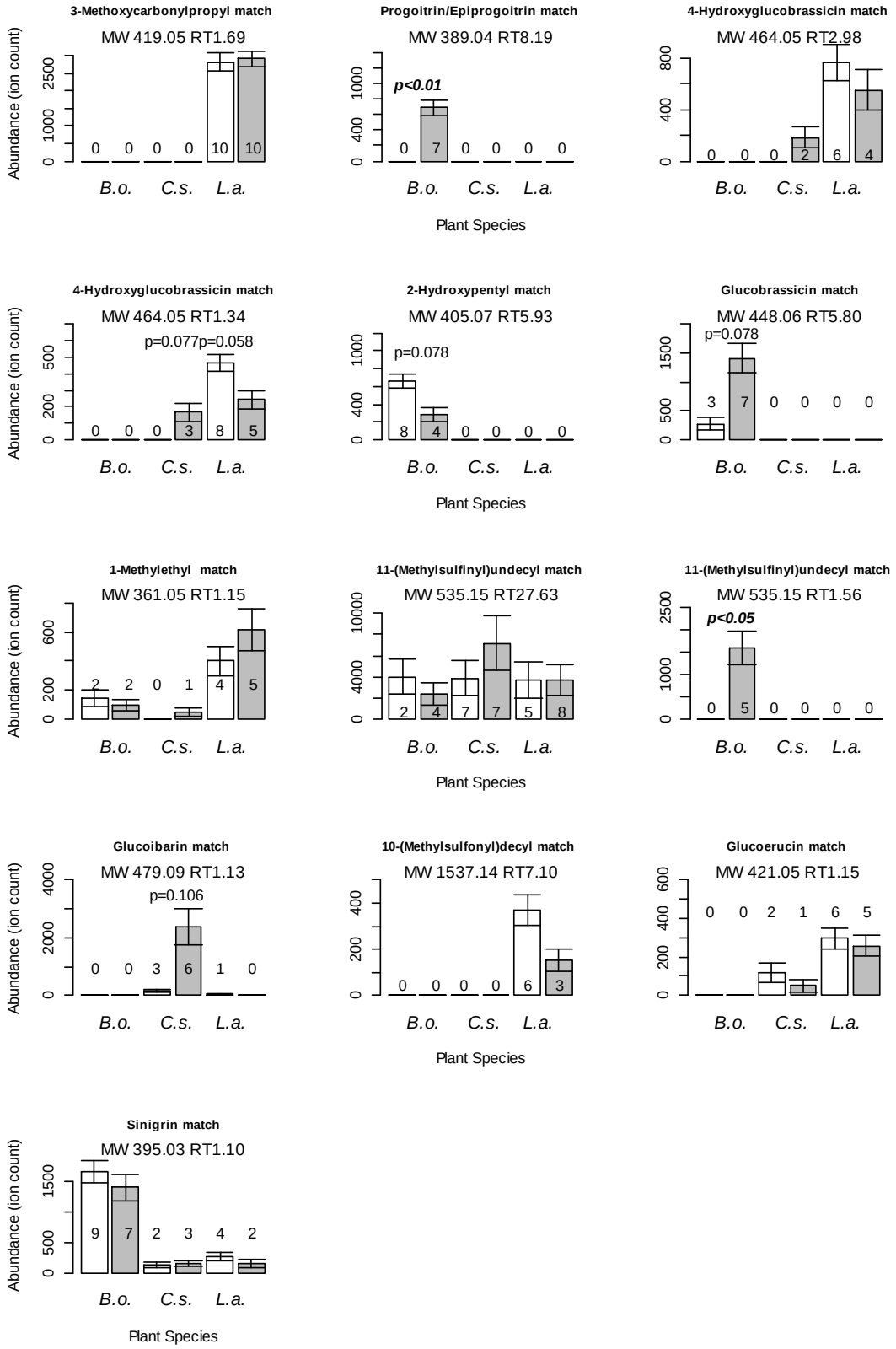


Fig. 4.4 Average abundances of 13 metabolites with the same molecular weights as glucosinolates. White bars are control plants, grey bars are infested plants. *B. oleracea*, *C. spinosa* and *L. annua* are indicated by *B.o.*, *C.s.* and *L.a.* respectively. Means and SDs are

plotted. The shared molecular weight (MW) of the glucosinolate and metabolite and retention time (RT) of the metabolite is shown above the graphs. In instances where the abundance between control and infested plants is significantly different or approaching significance p values from a t-test or a Mann-Whitney U test are given in bold. Numbers on the bars are the number of samples out of ten replicates that the metabolite was detected in.

4.5 Discussion

The metabolic fingerprints of all three plant species were altered by insect herbivory (Fig. 4.1b-d). Cross-reference of the metabolites across plants revealed a number of metabolites which were common to all three plants (Fig. 4.2). Despite this, when metabolites which showed differences in abundance between infested and control plants were cross-referenced, there were no measured metabolites that were common in all three host plants (Fig. 4.3). These results will be discussed in terms of the response of the plants to herbivory by *P. rapae* and plant species-specific responses to herbivory.

4.5.1 Responses induced by *P. rapae* in plants

Previous studies have shown for a wide range of plant species that herbivory elicits a change in the concentration of specific metabolites (for examples see reviews by Textor & Gershenzon 2009; Pavarini *et al.* 2012; Zhang *et al.* 2012a). Far fewer studies have shown changes in the overall metabolic fingerprints of plants induced by herbivory (Widarto *et al.* 2006; Sutter & Müller 2011; Kutyniok & Müller 2012; Plischke *et al.* 2012; Marti *et al.* 2013). Herbivore induced changes in metabolic fingerprints have not been shown before for *B. oleracea, C. spinosa* and *L. annua*. Previous herbivory research on *B. oleracea* had gone as far as showing that *P. rapae* induces genome wide changes in *B. oleracea* by inducing the transcription of a number of genes (Broekgaarden *et al.* 2007) and with this current study we can now confirm that those transcriptional changes are translated into changes in the metabolite composition.

Herbivory by *P. rapae* causes increases in glucosinolate metabolites in *Arabidopsis thaliana*, *B. nigra*, *Lepidium virginicum*, *Raphanus raphanistrum*, *R. sativus* and *B. oleracea* plants (Agrawal *et al.* 2002; Traw 2002; Agrawal & Kurashige 2003; Shelton 2005; Mewis *et al.* 2006) therefore an attempt was made to find evidence of glucosinolates being induced by *P. rapae* in the three plant species used in the current study. Evidence that two glucosinolate metabolites increased in infested *B. oleracea* was found. This concurred with a study where glucosinolates had been found to increase after *P. rapae* herbivory in *B. oleracea* (Agrawal & Kurashige 2003). However, identification of the glucosinolates was tentative due to only the molecular mass being used (Sumner *et al.* 2007). Therefore, measurement of glucosinolates using a more conclusive method would be more informative as to the affect of *P. rapae* herbivory on glucosinolate abundance in *B. oleracea*. Similarly, more definitive glucosinolate identification and measurement could explore glucosinolates in the other host plants. To date few studies have measured glucosinolates in the leaves or seeds of *C. spinosa* and *L. annua* (Daxenbichler *et al.* 1991; Griffiths *et al.* 2001; Vaughn *et al.* 2006) and none have been in relation to herbivory.

4.5.2 Plants respond idiosyncratically to herbivory

The large number of metabolites (over 1100) present in all three species regardless of herbivory (Fig. 4.2) demonstrates that there are chemicals in plants that are common across multiple species. One would expect a degree of metabolite similarity considering metabolites are the end expression of the genome (Sumner *et al.* 2003) and all three plants are genetically related being in the order Brassicales. However, the extent of the similarities (13% of all metabolites measured in all three control plants) has not been shown before using an untargeted method such as metabolic fingerprinting. The difficulty in identification of metabolites from untargeted analyses means these common metabolites are chemically unknown. Primary metabolites will account for some of these shared 1000 metabolites since primary metabolites are common to nearly all plant species (Pichersky & Lewinsohn 2011) due to them being fundamental for key functions essential to life. However,

there is no easy way of estimating the proportions of primary metabolites and secondary metabolites represented by these shared metabolites.

The focus on induced metabolites in this study was because an induced metabolite is likely to have a defensive function. However, some plant defences are constitutive (Kempel *et al.* 2011), therefore it is possible that some of the metabolites common to the three plants are constitutive defences which could play an equally large role in the defence of the plant. Unfortunately, confirming which metabolites are constitutive, and the ratio of constitutive to induced metabolites, would be difficult requiring each metabolite to be chemically identified or isolated and tested for negative effects against insects. For these reasons it is difficult to determine the importance of induced versus constitutive metabolites in plant-insect interactions, although other experimental methods have been used to achieve this (Gutbrodt *et al.* 2012).

In comparison to the shared metabolites found in the three plants (Fig. 4.2), the number of shared metabolites induced by herbivory was very low (Fig. 4.3). One explanation for the lack of common induced metabolites could be that the reaction to herbivory in plants is species-specific. A previous study showed that in *B. oleracea* (cabbage) and *Tropaeolum majus* (nasturtium) the release of volatile organic compounds (gases that attract parasitoids) in reaction to herbivory by *P. rapae* is plant species-specific (Geervliet *et al.* 1997). And species-specific transcriptions of genes have been found in *Nicotiana attenuata* (tobacco) and *Solanum nigrum* (nightshade) following attack by *Manduca sexta* (moth) (Schmidt *et al.* 2005). The same may be true for foliar secondary metabolites. Species specificity at this level is a possibility assuming that plants are continually producing new metabolites (Jones & Firn 1991), through mechanisms such as gene mutations and duplication (Kliebenstein *et al.* 2001; Kampranis *et al.* 2007; Kliebenstein 2008), which enable them to compete successfully in arms races with their insect herbivores (Mithofer & Boland 2012). Therefore three plant species competing in separate arms races and evolving many defensive metabolites over time may no longer contain a large proportion of shared metabolites. This is

conceivable considering that *C. spinosa* is in the family Cleomaceae which split from the family Brassicaceae around 65 million years ago, and *B. oleracea* and *L. annua* although both in the family Brassicaceae are in different tribes which split around 50 million years ago (Beilstein *et al.* 2010). It is possible that there are more induced metabolites found shared between plants that are more closely related than species studied here, at the family level for example, in the same genus, or within a species. This suggestion is supported by a transcription study on two cultivars of *B. oleracea* which found induced transcription of the same genes in both plants in reaction to herbivory by *P. rapae* (Broekgaarden *et al.* 2007).

4.5.3 The suitability of a specialist herbivore

The reactions of the three plants are specific to herbivory by *P. rapae.* This species is a good model to use because it feeds from a wide range of hosts therefore enabling the comparisons among taxonomically far apart plants. *P. rapae* is considered a specialist with known abilities to avoid the defences of hosts for example by neutralising glucosinolates (Wittstock *et al.* 2004). Compared to generalist species which are thought to suppress the induced responses of plants, as found in mustard (*Sinapis alba*) and tobacco plant (*Nicotiana attenuata*) attacked by the generalist armyworms *Spodoptera frugiperda* and *S. exigua* respectively (Travers-Martins & Müller 2007; Diezel *et al*. 2007), specialists are not thought to suppress plant responses (Ali & Agrawal 2012). Therefore, a specialist is more suitable than a generalist for an experiment where defence responses are purposely invoked so that they can be studied among plant species. It is unclear whether specialists and generalists induced different reactions in plants. Some studies have found differences in glucosinolate abundance, gene transcript levels (Mewis *et al.* 2006; Voelckel & Baldwin 2004) or trichome increases (Traw & Dawson 2002) whereas other studies comparing specialists and generalists have observed no differences in the transcript or glucosinolate responses in plants (Bidart-Bouzat & Kliebenstein 2011; Reymond et al. 2004). A better predictor of the plant defence response than the level of specialisation of an insect appears to be the feeding mode of the insect herbivore (Ali & Agrawal 2012; Bidart-Bouzat &

Kliebenstein 2011). It is unknown whether a phloem-feeding herbivore such as an aphid would induce different metabolite reactions among the plant species as the leaf chewer *P. rapae* has in the current experiment.

4.5.4 Conclusion

It is apparent from these results that host plants of *P. rapae* share some of the same metabolites but also have a large number of unique metabolites and that their metabolomes do not respond to herbivory in the same way. This is the first study to compare a large number of metabolites among species and now that the technology to measure large portions of the metabolome is available other research could extend investigations of the commonality of metabolites. This could create further revelations concerning the evolution of metabolite diversity and how that diversity is related to the pressures of insect herbivore attack.

Chapter 5 The metabolic fingerprints of an insect herbivore and its host plant are altered under elevated CO_2 and temperature.

5.1 Abstract

Plant-insect interactions may be affected by future climate changes and studies incorporating more than one climate variable are important in predicting these effects. *Pieris rapae* (small white butterfly) and its host plant *Brassica oleracea* (cabbage) raised under elevated temperature and CO_2 were examined by measuring insect performance, plant nitrogen content and the untargeted metabolic fingerprints of plants and insects. Higher temperatures decreased the development time of larvae and altered larval and plant metabolic fingerprints. In contrast, the performance of insects was not affected by elevated CO_2 although elevated CO_2 changed the metabolic fingerprints of insects and plants, and decreased the nitrogen content of host plants. Metabolic fingerprints of plants and insects could also be distinguished under the combined effects of elevated temperature and CO_2 and plants responded more to these treatments than did insects. In both insects and plants, elevated temperature had more effect than elevated CO_2 on metabolic fingerprints, and there were combined effects of temperature and CO_2 on some metabolites that were not apparent when either temperature or CO_2 were altered separately. These metabolomic data provide unique insights into the effects of changing climates on plants and insects.

5.2 Introduction

Atmospheric CO_2 has increased by 40% compared to pre-industrial levels with current concentrations at 397 ppm. Global climates have warmed by 0.85 ^{o}C since 1880 (IPCC 2013; Tans & Keeling 2014). Many species are responding to these changes (Hickling *et al.* 2006) and improving our understanding of how future environments may affect species and the interactions among species, may help to improve predicted impacts of climate change on ecological systems (Hunter 2001). Predicting how climate change will affect the interactions between plants and insects is especially important given the respective roles plants play as crops and insects as pests of these crops (Gregory *et al.* 2009).

Many studies have evaluated the effects of single climate factors on insects and plants, however researchers are realising that multi-factorial experiments are necessary to determine how abiotic factors may interact (Ziska *et al.* 2011). For example, a study on chrysomelid beetles (*Phratora vitellinae*) feeding on willow (*Salix myrsinifolia*) under elevated temperature and CO_2 found that insect growth rates increased under elevated temperature, decreased under elevated CO_2 but when the factors were combined the elevated temperature compensated for the reduced growth under elevated CO_2 resulting in no overall difference in growth rates (Veteli *et al.* 2002). Therefore studies of multi-factorial experiments are important for understanding the consequences of future climate changes.

Plant responses to CO_2 and temperature have been well studied. Numerous effects on plants grown under elevated CO_2 have been reported including changes in the rate of photosynthesis, increases in starch and sugar content and increases in biomass and yield (Long *et al.* 2004; Li *et al.* 2007). Other biochemical effects of CO_2 on plants that are important to their insect herbivores include changes in the abundance of defensive proteinases (Zavala *et al*. 2009; Zavala et al. 2008) and secondary defence metabolites (Klaiber *et al*. 2013). This is brought about by the repressive effect CO_2 has on the expression of genes encoding for signalling hormones such as jasmonates (Casteel *et al*. 2008; Zavala *et al*. 2008). In addition,

insects are potentially affected by the decrease in N (nitrogen) which can be observed in leaves grown under elevated CO_2 (Bezemer & Jones 1998). N is thought to decrease in plants growing under elevated CO_2 because the assimilation of soil nitrate into organic nitrogen in the plant is inhibited by higher concentrations of CO_2 (Bloom *et al.* 2010). Furthermore, the N that is present in plant tissues is diluted by the accumulation of non-structural carbohydrates in plants grown under elevated CO_2 (Taub & Wang 2008) creating a high C:N (carbon to nitrogen) ratio in these plants. Although elevated temperature is recorded as combining with CO_2 to affect the abundances of secondary metabolites, temperature is not thought to interact with the effects of elevated CO_2 on N in plants (Robinson *et al.* 2012).

These effects of CO_2 on the concentration of N in host plants will affect their insect herbivores (Coviella & Trumble 1999) because nitrogen is a limiting factor for insect growth (Slansky & Feeny 1977). In a meta-analysis of insect-host plant studies under elevated CO_2, 51% of studies where a decrease in plant N was recorded found a significant decrease in the performance of insects (Ryan *et al.* 2010). However, there were also 38% of insects in the same meta-analysis that showed no change in performance and 57% of studies recorded insects eating increased amounts of host plant tissues to compensate for the reduction in N (Ryan *et al.* 2010). One species in which this compensatory feeding has been observed is *Pieris brassicae* fed on *Brassica oleracea* grown under elevated CO_2 where the larvae consumed up to 58% more leaf tissue compared to plants grown under ambient CO_2 (Klaiber *et al.* 2013). There may be processes within larvae associated with compensatory feeding that enable the insect to maintain the same level of performance as under ambient CO_2. This could mean that even if insect performance is not apparently affected by a rise in CO_2, an effect could manifest biochemically through changes in the insect metabolome.

A ‘snapshot’ of an organism’s metabolome can be taken using metabolic fingerprinting (Overy *et al.* 2005) which is the untargeted measurement of a large number of metabolites (Fiehn 2001). Metabolic fingerprinting has been used to investigate the direct effects of temperature on insects. Michaud *et al*. (2008) found

metabolic fingerprints changed in an Antarctic midge exposed to heat shock and freezing, and Malmendal *et al*. (2006) found changes in the metabolic fingerprint of *Drosophila melanogaster* associated with increases in the abundances of amino acids in response to heat shock. Therefore, it is likely that changes in temperature will affect the metabolomes of insects in addition to the performance of insects. These effects of temperature on insect performance are well recorded with a typical response to elevated temperatures being accelerated growth and development (Bale *et al.* 2002). In addition to the effects of temperature, the metabolome could also potentially change under elevated CO_2 as outlined above. However, metabolic fingerprints of insects exposed to elevated CO_2 and elevated temperature have not previously been examined, and there is no information on how the presence and abundance of insect metabolites change under these altered conditions.

In contrast to insects, the metabolomes of plants have received more attention, with metabolomic methods used in a number of studies to examine how the quantity and quality of metabolites change in plants exposed to higher levels of CO_2 (Levine *et al.* 2008). Investigation of metabolomic changes in plants in relation to temperature have concentrated on cold and heat shock effects or cold acclimation (Gray & Heath 2005; Shulaev *et al.* 2008; Kral'ova *et al.* 2012) rather than temperature changes related to climate change. Metabolic fingerprinting of plants has also been used to evaluate the reactions of plants to biotic stresses such as herbivory and fungal pathogens (Sutter & Müller 2011; Kutyniok & Müller 2012) and abiotic stresses such as high salinity (Johnson *et al.* 2003; Lugan *et al.* 2010). However, changes to the metabolomes of plants grown under elevated temperature and CO_2 have not been examined using metabolic fingerprinting. The advantage of using an untargeted metabolomic approach to study the effects of abiotic factors is that the reaction of a larger number of metabolites can be summarised, providing a better representation of the overall reaction of a plant metabolome to climate change compared with targeted studies. For example, the magnitude of the effect of elevated CO_2 on a plant metabolome can be quantified and compared to that of elevated temperature to determine which climate change

factor has a greater effect on the biochemistry of the plant. Currently there is little information on this.

Here I examined metabolic fingerprints of *Pieris rapae* (small white butterfly) feeding on *Brassica oleracea* (cabbage) reared under elevated temperature and CO_2 to investigate how an altered climate would affect the metabolomes of these plants and insects. A large change in these climate factors was used (approximate doubling of CO_2 and 5 ^{o}C increase in temperature) to assess if organisms would respond to increases. The performance of *P. rapae* and the N content of *B. oleracea* plants were also measured to determine if insect performance is affected by the changes in climate factors and to establish if the larvae under elevated CO_2 were likely to be eating host plants containing low N. The metabolic fingerprints are analysed to gain information on which climate change factor will have the biggest effect on organisms' metabolomes in the future.

5.3 Methods

5.3.1 Plant and insect rearing

B. oleracea seeds (variety capitata, cultivar stonehead; grovesnurseries.co.uk) were sown in compost (John Innes No.2) in trays and germinated in a greenhouse for two weeks. Seedlings were transferred to individual 10 cm diameter pots and randomly assigned to one of four treatments. Plants were reared in growth cabinets (Jumo LPF200, Snijders Scientific; 1 cabinet per treatment, 39 plants per treatment) under the following conditions: (1) ambient CO_2 (373 ppm) and temperature (18 ^{o}C); (2) ambient CO_2 (373 ppm) and elevated temperature (23 ^{o}C); (3) elevated CO_2 (700 ppm) and ambient temperature (18 ^{o}C); (4) elevated CO_2 (700 ppm) and elevated temperature (23 ^{o}C). In all cabinets humidity was 70%, light levels were kept at 300 µmols/m^2/s and the light/dark cycle was 16L:8D. To minimise cabinet effects, cabinet temperature treatments were switched and plants moved between cabinets every two weeks until larvae were added when plants were eight weeks

old. Plants were watered every day and fertilized twice during the growing period with 30 ml per pot of plant food (Phostrogen N 14.0%, P 4.4%, K 22.4%) made up to packet instructions. A group of ten caterpillars required the young leaves of three plants to develop into pupae therefore within a treatment plants were spilt up into 13 groups of three plants to provide sufficient food for larvae, and caterpillars were transferred to a new plant within the group of three plants before all the young leaves had been eaten. Thus all larvae were provided with excess young leaves during their development. Before larvae were added to the plants, a five mm leaf disc was taken from between the mid vein and the leaf edge of the two youngest fully-formed leaves. These plant samples provided information on the chemical diversity of plants resulting from changes in CO_2 and temperature but without any confounding herbivory effects. The leaf discs from the three plants comprising the group of plants that insects would feed from were pooled for biochemical analysis. The leaf samples were flash frozen in liquid nitrogen and stored at -80 ^{o}C for metabolomic analysis. Leaf discs for carbon and nitrogen analysis were taken from the other side of the leaf vein of the same leaves, flash frozen and stored.

Insect rearing is described in detail in Chapter 2 and is described here briefly with specifics to this chapter. Fifteen wild-caught, female butterflies collected from around York, UK (53^{o}95'N, 1^{o}08'W) laid eggs on *B. oleracea* plants and 2nd instar larvae were transferred with a paint brush to eight week old experimental plants. Between 10 -12 caterpillars laid by the same female were placed on a plant, a white net bag was placed over the plant and secured with an elastic band around the pot rim (Fig. 5.1a). Females contributed eggs to all four treatments to generate a split-brood design. Once insects had developed to 4th instar, plants were checked every day for pupae. When the first 5th instar larvae on a plant pupated or was preparing to pupate (Fig. 5.1b), five caterpillars from that plant were flash frozen for metabolomic analysis after the gut and haemolymph were removed. Growth rate was calculated for each individual larva by dividing the adult dry weight on emergence by the number of days between placing the 2nd instar larva on the plant and pupation (development time).

a) b)

Fig. 5.1 a) *B. oleracea* plants growing in cabinets. The plants at the back covered with net bags have larvae on them. b) A *P. rapae* larva preparing to pupate.

5.3.2 Statistical analyses of insect performance data

All statistical analyses were performed in R (R Core Team 2013). A split-brood design was implemented with the intention that the effect of butterfly family on insect performance would be tested, however, family effects were included in analyses but only as a random effect. This was because a large number of pupae diapaused under ambient temperature treatments and these diapaused insects had significantly different performance parameters compared to non-diapaused insects and had to be excluded from all analyses, leaving some family-treatment combinations with no replicates. To test the effects of CO_2 and temperature on insect performance, insect growth rates (mg day) and adult dry weights (mg) were each fitted to a linear mixed effects model (function 'lmer' in R) and development times (days) were fitted to a generalized linear mixed model (function 'glmer' in R) while controlling for non-normality by fitting a poisson error distribution. CO_2 and temperature with an interaction effect were included as fixed effects and butterfly family was a random effect. The model with the lowest AICc value was deemed the model of best fit.

5.3.3 Carbon and nitrogen analysis

To obtain sufficient material for carbon and nitrogen analysis leaf discs from six different plants were pooled and four of these samples per treatment were analysed. Samples were dried in a 60 ^{o}C oven for eight hours, weighed to obtain a dry weight then homogenised in a ball mill. 80 mg of each sample were analysed on a vario MACRO element analyser (elementar Analysen systeme GmbH) along with glutamic acid blanks to correct for drift. Two-way ANOVAs examined the effects of CO_2 and temperature and their interaction on N and C:N ratio of plants. N data were arcsine square root transformed and C:N ratio data were ln transformed to meet normality assumptions of ANOVA.

5.3.4 Metabolomic analysis

To obtain metabolic fingerprints six out of the 13 plant groups from each of the four treatments were sampled for both *B. oleracea* and *P. rapae* (in total 24 plant samples and 24 larval samples). For plant samples six leaf discs were pooled from each group of three plants that a group of insects had fed from and five larvae from this group of larvae were pooled to form a sample. Thus I analysed the metabolic fingerprints of larvae as well as the metabolic fingerprints of the plants the larvae had fed on.

Sample extraction and metabolomic analysis are described in full detail in Chapter 2 and briefly here. Leaves and larvae were flash frozen immediately after harvesting, freeze dried, homogenised while being kept cool and extracted using methanol containing an internal standard. After filtering, the metabolites in samples were measured using a QToF Mass Spectrometer coupled to a High Performance Liquid Chromatography system (HPLC-MS) in both positive and negative ionisation mode.

5.3.5 Metabolomic data pre-processing

Features were aligned across all samples, filtered and Missing Value Imputation (MVI) performed on the data using a Kernel based Feature Alignment programme (KFA) (Perera 2011). Samples analysed on the LC-MS machine were shown to have a

larger amount of noise in the data than usual for unknown technical reasons and therefore the filtering criterion was higher than the usual 50% and a feature was included in the filtered data only if it was found to occur in at least four out of six of the replicates in at least one of the four treatment groups. Before filtering the datasets contained measurements of 17,814 features and 8,331 features in positive and negative ionisation mode respectively. A total of 68.5% and 47.4% of features were considered noise and excluded during filtering leaving 5,606 and 2,503 metabolites in the positive and negative ionisation mode datasets, respectively. PCAs were performed on both positive and negative ionisation mode datasets to ensure that exclusion of features had no qualitative effect on the results.

5.3.6 Statistical analyses of metabolomic data

When using HPLC-MS, the varying chemical structures of the metabolites mean some are more easily ionised in positive or negative ionisation mode, therefore positive and negative ionisation modes will measure a different suite of metabolites. There is likely to be overlap between the two modes as some molecules are ionised by both methods. Therefore data collected in positive and negative ionisation modes were analysed separately. The 'prcomp' function in R was used to perform PCAs (Principal Component Analyses) on *B. oleracea* and *P. rapae* data to compare the magnitude of any differences between metabolic fingerprints of insects and plants. Separate PCAs on *B. oleracea* and *P. rapae* were then performed to examine if the different CO_2 and temperature treatments had affected the plant and larval metabolic fingerprints.

To quantify the number of metabolites that significantly changed in abundance in response to CO_2, temperature, and the interaction of the two factors, two-way ANOVAs were carried out on every metabolite. For a small number of metabolites (ten in plants and six in insects), assumptions of normality could not be met even after transformation of the data and so non-parametric Scheirer-Ray-Hare tests were carried out instead. Three p values (CO_2, temperature and their interaction) for each metabolite were obtained and converted to q values to correct for FDR. Chi

squared tests were used to examine if a similar number of metabolites were changed by CO_2 as by temperature.

5.4 Results

5.4.1 Insect performance is affected by temperature but not CO_2 and there is less foliar nitrogen in plants under elevated CO_2.

Insect performance was the net effect of direct and plant-mediated indirect effects on larvae. Models fitted to the growth rate and development times of larvae indicated that temperature improved and CO_2 reduced these insect performance parameters. However, there was no effect of CO_2 or temperature or their interaction on adult dry weights (indicated by the best fit null model) therefore the decrease caused by temperature and the increase caused by CO_2 in growth rate (composed of adult dry weight divided by development time) was primarily due to effects on development time. At higher temperatures *Pieris rapae* had shorter development times by about 12 days (23 ^{o}C mean = 13.82 ±2.40 days and 18 ^{o}C mean = 26.03 ±4.34 days; Fig. 5.2a) and faster growth rates by 0.64 mg per day (23 ^{o}C mean = 1.41 ±0.30 mg day and 18 ^{o}C mean = 0.77 ±0.17; Fig. 5.2c). Whereas the increase in development rate between ambient and elevated CO_2 was only two days longer (development time: ambient CO_2 mean = 15.12 ±5.36 and elevated CO_2 mean = 17.42 ±5.77) and 0.24 mg per day less in growth rate (ambient CO_2 mean = 1.40 ±0.40 and elevated CO_2 mean = 1.16 ±0.31). Furthermore, there was a temperature gradient in the ambient CO_2 cabinets because air was not mixed as effectively as in the elevated CO_2 cabinets where CO_2 enriched air was pumped in. Thus the majority of insects developed in the warmer areas of the ambient CO_2 cabinets (Fig. 5.2d), and I conclude that these apparent small effects of CO_2 on insect development are probably artefacts. The slight temperature gradient was unlikely to have affected the metabolic fingerprints of the insects because those samples were from the middle of the cabinets where temperatures were the same as those in the elevated CO_2 treatments. Similarly, all plant samples were taken

after plants had been moved regularly around the cabinets although this randomisation stopped when larvae were added to plants to prevent confusion while recording data from plants.

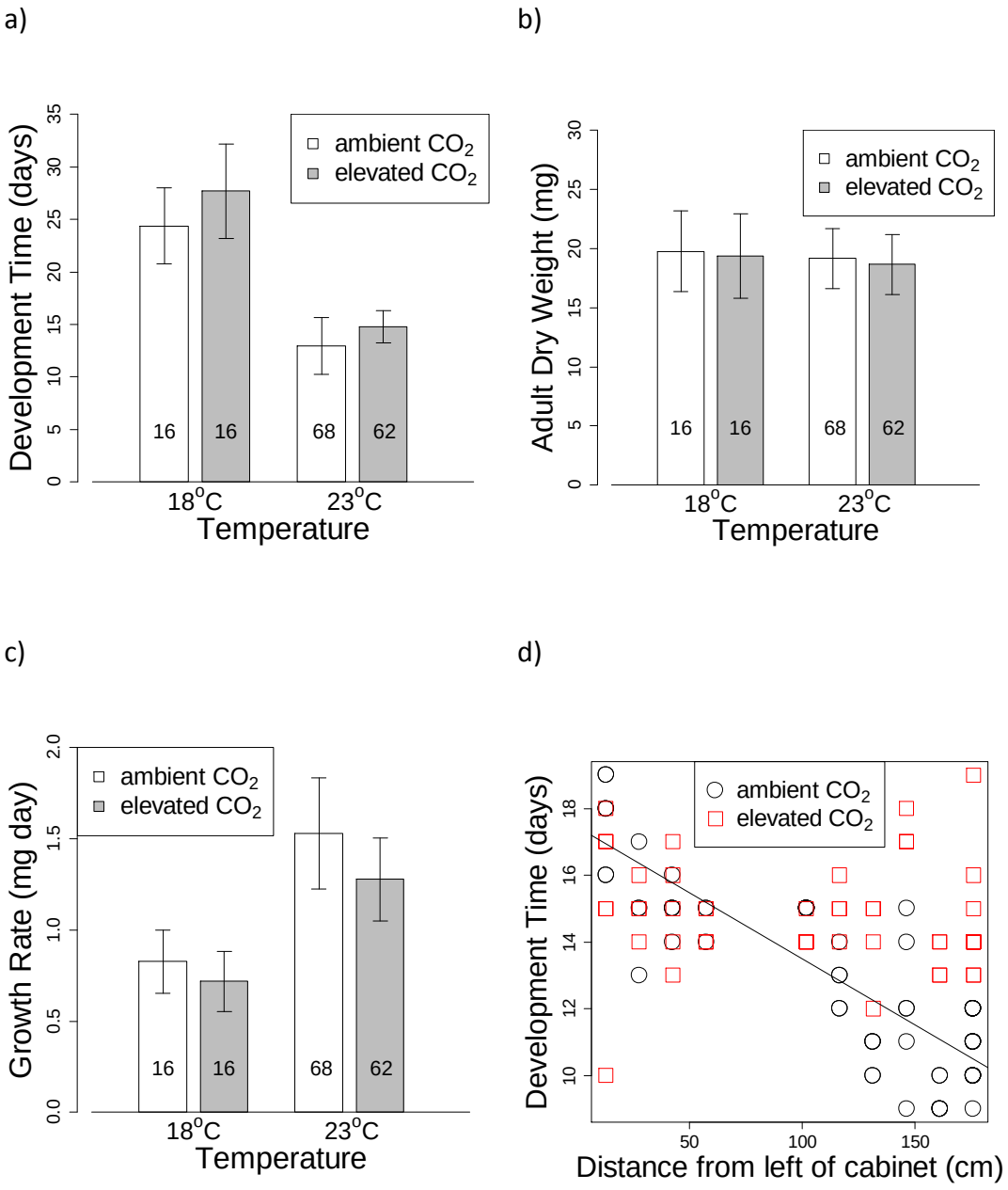


Fig. 5.2 The performance of *P. rapae* under elevated temperature and CO_2 a) development time b) adult dry weight c) growth rate. Means and SDs are plotted. Replicate numbers are shown on bars. d) The development time of insects at 23 °C in elevated CO_2 cabinets (red squares) and ambient CO_2 cabinets (black circles and black line). Development time in ambient CO_2 cabinets, but not elevated CO_2, was related to pot position in the cabinet because of a slight temperature gradient in those cabinets.

Foliar nitrogen was measured to determine if the level of nitrogen in the plants would explain any differences in insect performance. Plants grown under elevated CO_2 had 32.8% less N than those under ambient CO_2 (two-way ANOVA, $F_{1,12} = 52.30$,

p<0.001; ambient, mean = 1.40% ±0.18; elevated, mean = 0.94 ±0.10), but there was no significant effect of temperature (although it approached significance; two-way ANOVA, $F_{1,12}$ = 4.18, P = 0.063) and no interaction effects (two-way ANOVA, $F_{1,12}$ = 2.0, p = 0.182; Fig. 5.3a). The C:N ratios were higher under elevated CO_2 treatments (two-way ANOVA, CO_2, $F_{1,12}$=46.34, p<0.001; ambient, mean = 29.85 ±4.10; elevated, mean = 43.71 ±4.90), but there was no significant effect of temperature or any interaction effects (two-way ANOVA, temperature, $F_{1,12}$ = 2.92, p = 0.113; interaction, $F_{1,12}$ = 1.51, p = 0.243; Fig. 5.3b). Thus plants grown under elevated CO_2 contained less N but insect performance was not lower on these plants with less N.

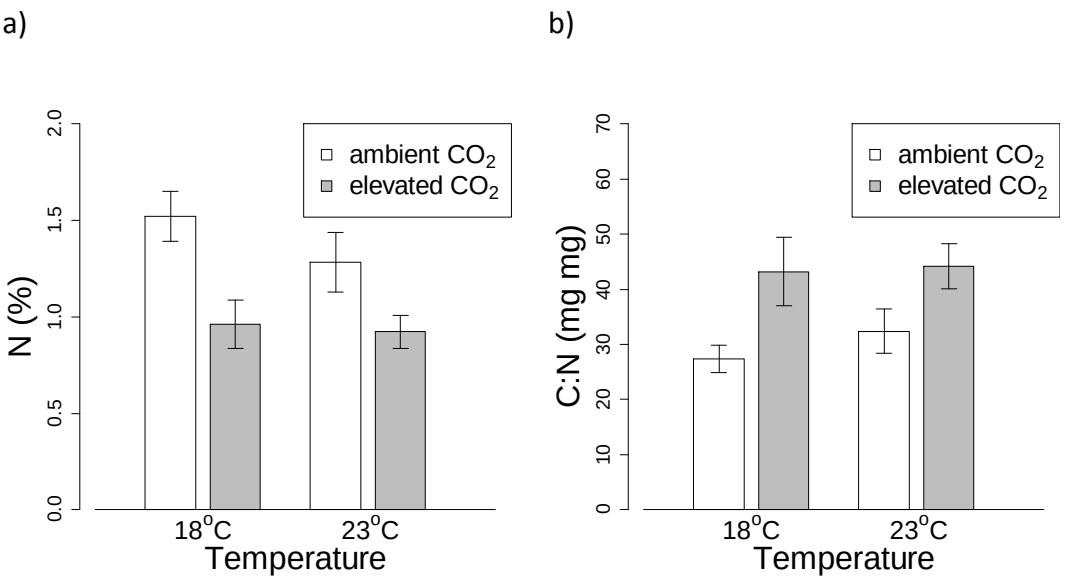


Fig. 5.3 a) Nitrogen (N) content and b) carbon:nitrogen ratio (C:N) of leaves from plants grown under four experimental treatments. Mean and standard deviations are plotted. N = 4 samples per treatment.

5.4.2 More metabolites are detected in plants than in insects

There were more metabolites detected only in plants (positive 2471 and negative 1161) than only in insects (positive 1954 and negative 853) (Fig. 5.4), and only a few metabolites (positive 1181 and negative 489) were detected in both insects and plants (grey in Fig. 5.4). This was evident for both positive and negative ionisation mode datasets.

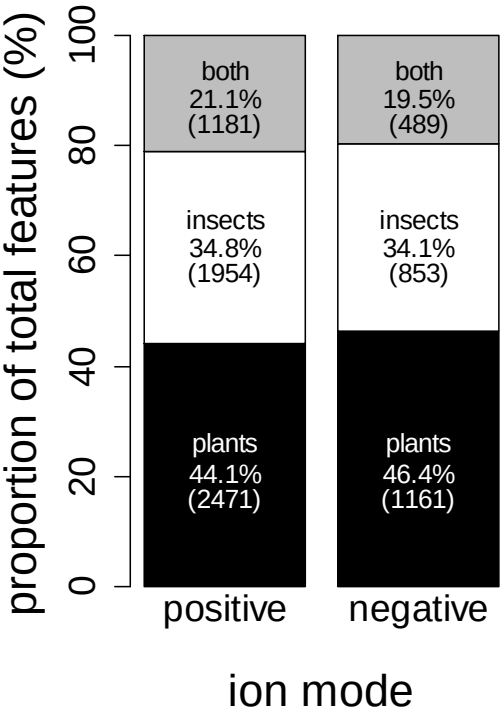


Fig. 5.4 The proportions of metabolites that were detected in plants only, insects only and both plants and insects for datasets collected in positive and negative ionisation modes. The numbers of metabolites are in brackets.

5.4.3 Plant fingerprints are more distinctive among treatments than insect fingerprints

PCA scores were similar between positive and negative ionisation mode datasets. The metabolic fingerprints of plants and insects were distinguished by PCAs fitted to metabolic fingerprints of *B. oleracea* and *P. rapae* samples (Fig. 5.5). PCA plots showed that PC 1 separated insects from plants. PC 1 explained a large proportion of the variation in both data sets (positive ionisation mode, PC 1 47.9%; negative, PC 1 47.4%) compared to the proportion explained by the next 4 PCs (positive PC 2-5 18.1%, negative PC 2-5 18.0%) reflecting the larger difference between plant and insect metabolic fingerprints than between treatments within either of the organisms. PC 2 distinguishes the fingerprints of plants grown under different

treatments, but did not distinguish among insect data. This indicated that the metabolic differences among plants in different treatments were much larger than metabolic differences among the insects.

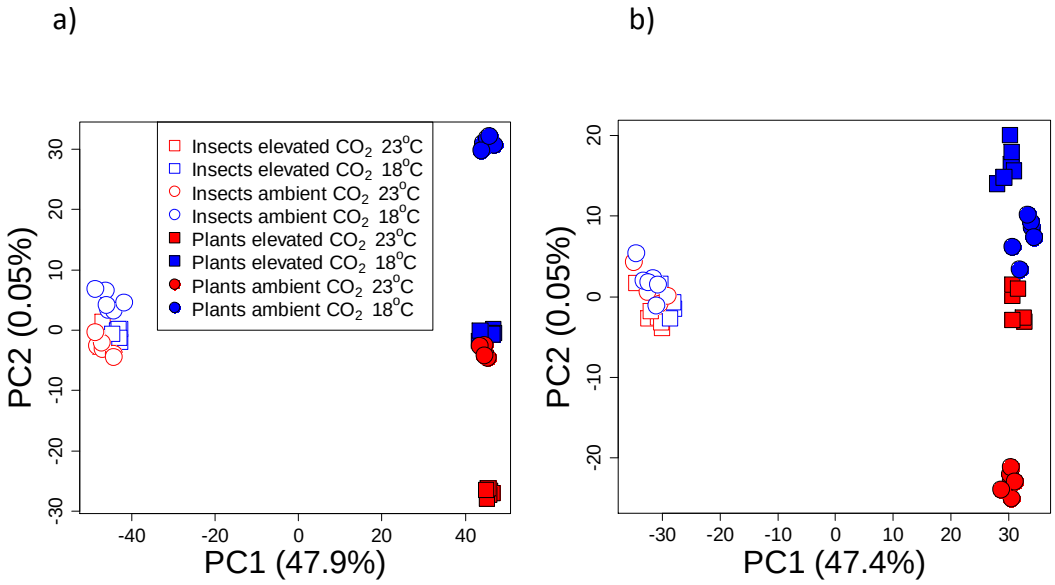


Fig. 5.5 Score plots from PCAs performed on a) positive and b) negative ionisation mode plant and insect metabolic fingerprints. Variation explained by each PC is shown in brackets on the axes. The insect samples (hollow symbols) and plant samples (filled symbols) are distinguished by PC 1 scores which account for a large proportion of the variation in the data. PC 2 distinguishes between plants grown under different treatments although the small size of the variation explained by PC 2 (0.05%) compared to PC 1 (47.9%) shows that the differences among plants are much smaller than the differences between plants and insects.

5.4.4 Metabolic fingerprints of *B. oleracea* and *P. rapae* are changed by CO_2 and temperature treatments

A scatterplot of scores from PC 1 and 2 from PCAs performed on plant metabolic fingerprints collected in positive and negative ionisation mode showed similar patterns in terms of effects of CO_2 and temperature on plant metabolic fingerprints (Fig. 5.6). There was little effect of elevated CO_2 on insect development rates (see above) yet metabolic fingerprints of insects were distinct among treatments (Fig. 5.7). There was a greater amount of variation within treatments observed in insect

samples (Fig. 5.7; replicates within a group are spread out) compared to plant data (Fig. 5.6; replicates are tightly clustered).

a) b)

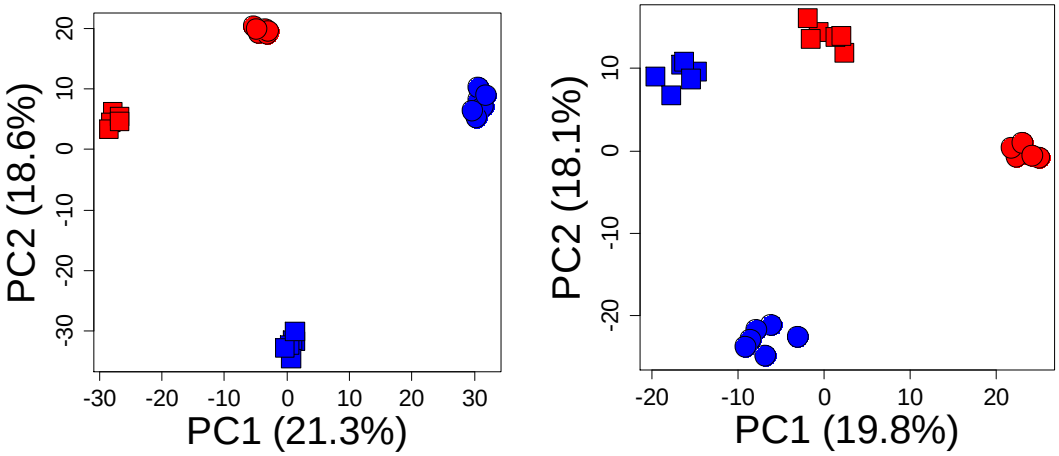


Fig. 5.6 Score plots from PCAs performed on a) positive and b) negative ionisation mode plant metabolic fingerprints. Plants under different temperature and CO_2 treatments have distinct metabolic fingerprints. Temperature treatments are denoted by colour (18 ^{o}C in blue and 23 ^{o}C in red) with CO_2 as shapes (elevated CO_2 square symbols and ambient CO_2 circles). Variation explained by each PC is shown in brackets on the axes.

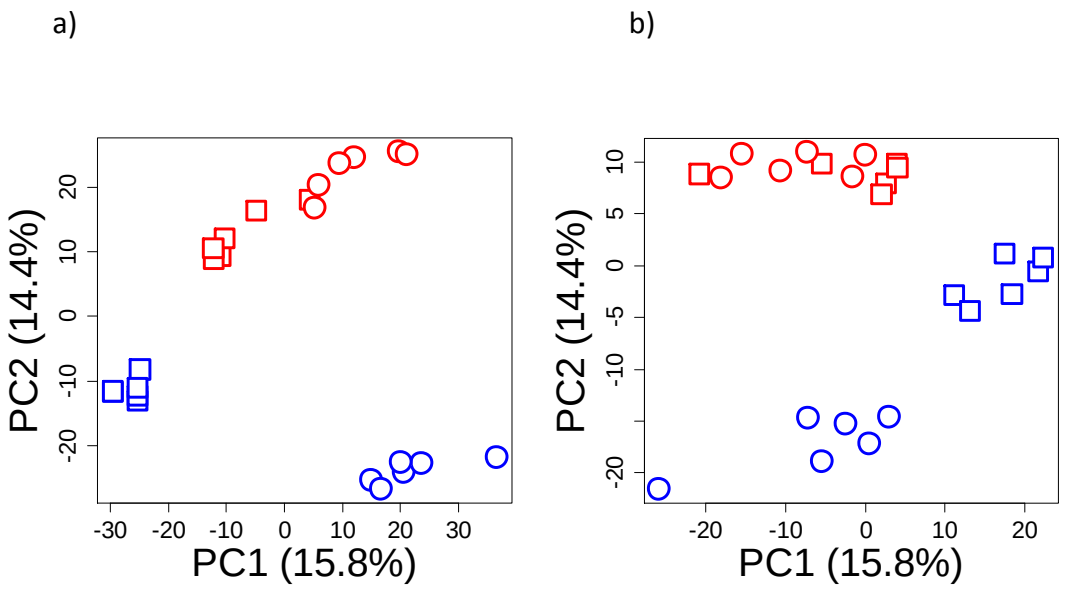


Fig. 5.7 Score plots from PCAs performed on a) positive and b) negative datasets of insect metabolic fingerprints. Variation explained by each PC is shown in brackets on the axes. Temperature treatments are denoted by colour (18 °C in blue and 23 °C in red) with CO_2 as shapes (elevated CO_2 square symbols and ambient CO_2 circles). Insects under different temperatures and CO_2 have distinct metabolic fingerprints.

5.4.5 The metabolome is affected more by temperature than CO_2

There were more metabolites significantly changed in abundance (according to FDR corrected $q<0.05$) by temperature than by CO_2 in both plant datasets (positive dataset, temperature = 402 metabolites and CO_2 = 244 metabolites; $\chi^2= 38.64$, $p<0.001$; negative dataset, temperature = 108 metabolites and CO_2 = 37 metabolites; $\chi^2= 34.77$, $p<0.001$; sum of the numbers in the temperature or CO_2 circles in Fig. 5.8a and b). This suggests the plant metabolome was more affected by the rise in temperature than the rise in CO_2. Similarly, for insects there were more metabolites changed by temperature than CO_2 in the positive dataset (temperature = 75 metabolites and CO_2 = 40 metabolites; $\chi^2= 10.65$, $p<0.01$; temperature and CO_2 circles in Fig. 5.8c) although this was not evident in the negative dataset (temperature = 18 metabolites and CO_2 = 24 metabolites; $\chi^2=0.86$, $p=0.50$; Fig. 5.8d).

There were some patterns that were the same for both positive and negative datasets of the plants and insects. For example, some metabolites changed in abundance in the elevated temperature treatments as well as the elevated CO_2 treatments (numbers in bold in the overlap between temperature and CO_2 Fig. 5.8). There were also metabolites that did not respond to either temperature or CO_2 but were changed in abundance by an interaction between temperature and CO_2 (numbers italicised in the CO_2 x Temp Interaction circles in Fig. 5.8).

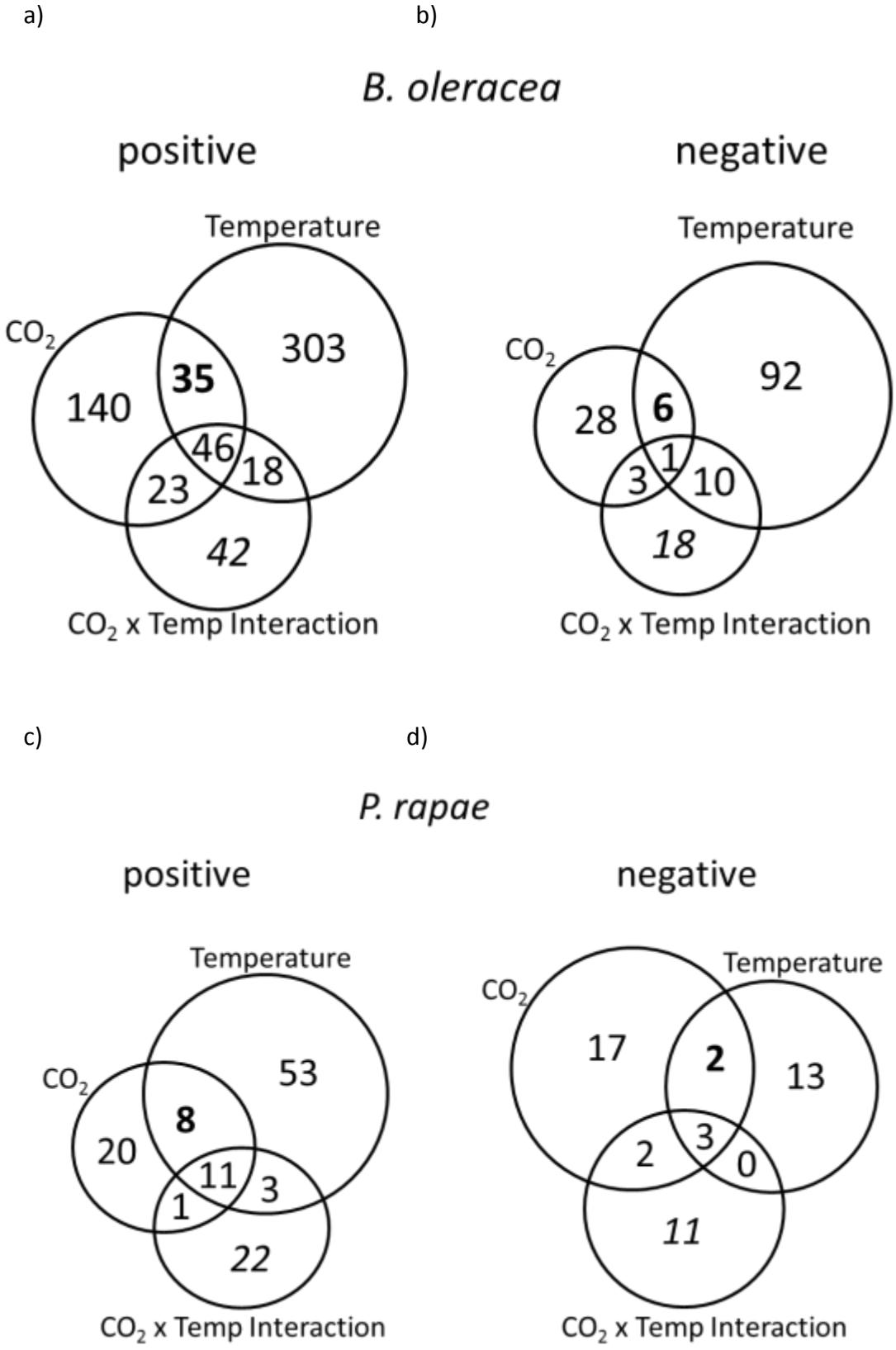


Fig. 5.8 Venn diagrams summarising the number of measured metabolites found to have a statistically significant change in abundance (according to q<0.05) in *B. oleracea* plants (a) positive (b) negative ionisation modes and in *P. rapae* larvae (c) positive and (d) negative

ionisation modes. Circle areas are proportional within a diagram to the number of metabolites. The numbers of metabolites that are changed by both the temperature and CO_2 treatments are in bold. The number of metabolites that were only changed by the interaction but not by either temperature or CO_2 alone is in italics.

5.5 Discussion

The metabolic fingerprints of insects and plants were different (Fig. 5.5). This is expected because the metabolome is the end result of the genome (Sumner *et al.* 2003) and these two organisms have very different genomes. The differences among the metabolic fingerprints of plants under different experimental treatments were larger compared to the differences among insects under the treatments (Fig. 5.5) and variation within a treatment was smaller for plants (Fig. 5.6) than for insects (Fig. 5.7). These patterns suggest that the plant metabolome is more affected than the insect metabolome by changes in CO_2 and temperature, with more plant metabolites changed in abundance and those changes of a greater magnitude. Previous studies comparing the reaction of plant and insect metabolomes to abiotic factors do not exist making the cause for this pattern difficult to interpret. One explanation could be that plants contain more metabolites than insects as suggested by the greater number of metabolites that were measured in plants than in insects using the same methods (Fig. 5.4). This is conceivable considering plants are thought to contain thousands of metabolites (Davies *et al.* 2010). However, caution needs to be taken with this interpretation because it is possible that more metabolites were measured in plants than insects because the methanol extraction method used has been optimised for plants (see Chapter 2) and therefore plants only appeared more affected by temperature and CO_2 compared to insects. Nonetheless, using the same extraction technique for the two taxa is beneficial because it allows these direct comparisons between plants and insects under the same experimental treatments.

5.5.1 Metabolic fingerprints of *B. oleracea* and *P. rapae* are changed by CO_2 and temperature treatments

The effect of rising temperatures decreasing the development time of insects is well known (Bale *et al.* 2002) and has been recorded in *P. rapae* (Gilbert 1984; Kingsolver 2000). However, the effect of rising temperature on the metabolome of an insect has not been measured and here I found that the metabolic fingerprint of *P. rapae* was changed by rearing temperature. Direct effects and plant-mediated effects were not separated in this experiment however it is likely that at least some of the changes in *P. rapae*, if not all, are due to the direct effect of temperature. Previous metabolomic analyses of other species of insect have found metabolites change in response to temperature albeit in response to heat shocks and cold tolerance (Malmendal *et al.* 2006; Michaud *et al.* 2008; Colinet *et al.* 2012a; Malmendal *et al.* 2013; Verberk *et al.* 2013). Nonetheless, changes in metabolites such as amino acids (Malmendal *et al.* 2006) increases in α-ketoglutarate, putrescine and decreases in glycerol, glucose and serine (Michaud *et al.* 2008) and sugars such as lactate and succinate associated with energy metabolism (Verberk *et al.* 2013) have been recorded in association with heat shocks or higher temperature therefore these could be the starting points for establishing which metabolites are responsible for the changes in *P. rapae* metabolomes between different temperatures in the current study. Future work could focus on this.

The reactions of plants to abiotic factors have been researched for decades although there is still a lot we do not know about the changes in plant molecular processes (Rodziewicz *et al.* 2014). A rise of 5 ^{o}C in the current study was shown to alter the metabolic fingerprint of *B. oleracea*. Studies on the effect of temperature on the metabolome, although often targeting certain groups of metabolites and focussed on heat shock rather than sustained elevated temperature, show increases in primary metabolites such as sugars and amino acids in *Arabidopsis* (Kaplan *et al.* 2004; Rizhsky *et al.* 2004). Through meta-analysis, general responses of carbon based secondary metabolites in plants to rises in temperature have been shown to be decreases in phenolics and increases in terpenoids (Zvereva & Kozlov 2006; Robinson *et al.* 2012) with general patterns for responses to temperature of

other secondary metabolites not clear (Bidart-Bouzat & Imeh-Nathaniel 2008). Such changes are likely to be driven by signalling hormones that change in abundance as environmental temperature increases (Wahid *et al*. 2007). Considering these phytohormones are also metabolites they could themselves account for the differences in plant metabolomes observed for plants grown under the different environments. In consideration of this previous research, it is likely the changes in the metabolic fingerprints of the *B. oleracea* grown under elevated temperature in the current study are due to alterations in both primary and secondary metabolites.

5.5.2 Elevated CO_2 affected the insect metabolome though not the performance of insects

Despite the lower level of N in the host plants under the elevated CO_2 treatments, insect performance was not affected by CO_2 suggesting that insects may have been employing compensatory feeding as observed in other insect species (Bezemer & Jones 1998; Ryan *et al.* 2010). Although compensatory feeding in *P. rapae* has not been studied under elevated CO_2, there are reports of *P. rapae* carrying out compensatory feeding when fed either plants manipulated to contain different amounts of N (Loader & Damman 1991; Hwang *et al.* 2008) or artificial diets differing in N status (Morehouse & Rutowski 2010). This compensatory feeding may be the reason for the change in the metabolites in insects under elevated CO_2 even when there is no effect on insect performance, and it is possible that in compensating for the lack of N in the host plant, changes were made in the insect metabolome. For example, metabolome changes may be the result of changes in the biochemical pathways involved in the process of extracting nutrients from the food. Similarly, the metabolite changes in the insect could be caused by the changes in the plant defences. Elevated CO_2 can change the quantities of defensive metabolites as well as non-metabolite compounds such as defensive proteins, due to the effect of CO_2 on the controlling phytohormone signals (Zavala *et al*. 2009; Zavala *et al*. 2008; Klaiber *et al*. 2013). These changes in plant defences could in turn change the insect metabolome. Alternatively, since the design of the experiment did not allow for the separation of direct and plant-mediated effects,

the differences in metabolic fingerprints of insects could also be due to the direct effect of elevated CO_2.

This is the first time an insect metabolome has been studied under elevated CO_2 at this concentration. A previous study which quantified the metabolic fingerprint of *Drosophila melanogaster* when anaesthetised by CO_2 found that after twenty-four hours any initial differences in the metabolic fingerprint were no longer evident (Colinet & Renault 2012). This return to a normal metabolic fingerprint is support for there being no long-term effect of CO_2 on the insect metabolome, although comparisons are difficult because in Colinet & Renault (2012) one dose of highly concentrated CO_2 was applied to the insect as opposed to the constant elevated CO_2 in this study. Knowledge of the insect metabolome under elevated CO_2 is currently insufficient to understand what drives changes in metabolites, although further research could separate the direct and indirect effects of CO_2 on insects and could investigate if there is an underlying mechanism whereby compensatory feeding necessitates a change in the metabolome.

5.5.3 A multi factor approach allows unique conclusions on the effects of temperature and CO_2 on metabolites.

Some studies have profiled multiple metabolites in plants exposed to higher temperatures (a heat shock treatment) (Kaplan *et al.* 2004; Rizhsky *et al.* 2004) or to elevated CO_2 (Li *et al.* 2006; Li *et al.* 2008; Kaplan *et al.* 2012) but this study is the first to examine metabolic fingerprints under both elevated temperature and CO_2 simultaneously. This multi-factor approach has been championed by a number of previous studies (Williams *et al.* 2000; Villalpando *et al.* 2009; Murray *et al.* 2013b; Scherber *et al.* 2013) and has allowed a comparison of the size of the temperature effect compared to the CO_2 effect on the plant metabolome: 116% and 229% more metabolites changed under elevated temperature than under elevated CO_2 in positive and negative modes respectively. Since the chemical identify and function of each of these metabolites is unknown it is difficult to ascertain why the plant metabolome is changed more by elevated temperature than elevated CO_2. It is also unknown if the number of metabolites changed by a factor is related to the size of

change in the factor, for example if a rise of 5 ^{o}C would change more metabolites than a 2 ^{o}C rise. Furthermore, there were a number of metabolites in both the plants and insects that changed due to an interaction effect between CO_2 and temperature but did not respond to either factor separately and therefore would not have been apparent in a single factor study. Experimental designs should strive to incorporate multiple factors if we are to learn more about the consequences of climate change for organisms.

5.5.4 Conclusion

The current study is the first to use metabolic fingerprinting to demonstrate the effects of elevated temperature, elevated CO_2 and both simultaneously on the metabolomes of a plant and its insect herbivore. In order to expand on the findings from this study, metabolic profiling could be employed to identify the specific metabolites that are affected by climate change factors. Realising the identity and function of metabolites affected by elevated temperature and CO_2 is essential in understanding the consequences of a changing climate.

Chapter 6 General Discussion

6.1 Summary of thesis findings

The main aim of my thesis was to use metabolic fingerprinting to improve understanding of plant-insect interactions. To achieve this I measured the effects of biotic and abiotic factors on the metabolic fingerprints of the herbivore *Pieris rapae* and several species of its larval host plants.

In **Chapter 3** I examined interactions between the polyphagous insect *Pieris rapae* and its Brassicales host plants by characterising the biochemistry of the insects as well as the plants. Larvae of *P. rapae* were fed on seven different species of host plants in order to examine how larval diet affects the metabolic fingerprint of *P. rapae* larvae. Host plants that were more distantly related were selected for study to encompass a wide taxonomic range of larval host plants used by *P. rapae*. The metabolic fingerprints of *P. rapae* larvae varied according to the host plant species the larvae had eaten. Larvae fed *Cleome spinosa* were the most distinct from other larvae because of a group of abundant metabolites that were likely to have originated in the host plant. Similarities between the metabolic fingerprints of the host plants reflected the taxonomic relationships between the host plants. Contrary to prediction, the nitrogen content of the different host plant species did not explain the performance of the insects on those hosts, although there was some evidence that plants with more similar metabolic fingerprints were associated with more similar levels of herbivore performance. Thus I conclude that the species of host plant an insect uses affects the biochemistry of the insect and that the metabolites within those host plants may influence larval performance.

In **Chapter 4** I examined the metabolites induced by *P. rapae* herbivory on three host plant species to compare plant reactions to herbivory and to examine evidence for common defensive metabolites among plant hosts. The metabolic fingerprints of control (i.e. uneaten) plants and plants attacked by *P. rapae* were compared to

assess if herbivory by *P. rapae* had an effect on the metabolite fingerprints of plants. The abundances of metabolites from control and attacked plants were compared to isolate metabolites that had been induced by herbivory. It was predicted that these induced metabolites would include some that were common to the three plant species and would therefore indicate important plant defence compounds. As hypothesised, herbivory by *P. rapae* altered the metabolic fingerprints of host plants. Contrary to prediction, metabolites that were induced by herbivory differed among the three plant species demonstrating the extent to which reactions to herbivory were plant species–specific. One possibility is that these host plants have had different assemblages of insect herbivores associated with them that apply different selection pressures resulting in the divergent evolution of plant defence responses.

In **Chapter 5** I evaluated how plant-insect interactions might alter in future environments by rearing *P. rapae* and one of its host plants *Brassica oleracea* under elevated temperature and CO_2. The performance of larvae was predicted to increase under elevated temperature and decrease under elevated CO_2. The N (nitrogen) content of plants under elevated CO_2 is known to decrease (Bezemer & Jones 1998) and N is a limiting factor of insect growth (Slansky & Feeny 1977), therefore the N content of plants was measured to assess if changes in N arising from different CO_2 rearing conditions might explain any observed changes in insect performance. The metabolic fingerprints of organisms under conditions where temperature or CO_2 or both factors were elevated were predicted to be different from metabolic fingerprints under ambient treatments. Insects developed faster under elevated temperature. As predicted the leaves of plants that insects fed upon under elevated CO_2 contained less N, however, insects did not show any difference in performance under elevated CO_2. As hypothesised the metabolic fingerprints of plants and insects were changed by elevated temperature and CO_2 and more metabolites were found to change under elevated temperature compared with elevated CO_2. The multi-factorial design of the experiment highlighted that some metabolites were changed by a combination of elevated temperature and elevated CO_2 but were not affected by either factor alone. I conclude that under future

climates *P. rapae* larvae may develop faster due to increased temperatures, whereas the lower N in its host plants as a consequence of elevated CO_2 may have little impact on insect performance, although insects may eat more to compensate for lower N in plants. The specific chemical identities of metabolites that changed in plants grown under elevated temperature and CO_2 are not known although they are likely to include both primary and secondary metabolites, and may help to maintain the functioning of plants in the face of a changed environment. Similarly, the metabolic changes in insects may be the consequence of biochemical systems that enable increased growth of insects under elevated temperatures and maintain larval performance by compensatory feeding when faced with less nutritious food under elevated CO_2. Further investigation of these metabolic fingerprints could reveal the underlying mechanisms that allow organisms to adapt to changes in the environment.

Overall this thesis found that different plant species have distinct metabolic fingerprints which are altered by *P. rapae* herbivory and by abiotic changes in the environment such as elevated temperature and CO_2. I found that the metabolic fingerprints of insects are influenced by the species of host plant they feed on and by environmental conditions (temperature and CO_2 concentration) during development. I also found that some host plant metabolites transfer chemically intact into insect tissues. These findings demonstrate that new information can be revealed by extending the use of metabolic fingerprinting to include interacting species, and enabling host plant metabolomes to be compared with those of their insect herbivores. An assessment of metabolic fingerprinting as a method, the contributions of this thesis to the study of plant-insect interactions and future work are discussed below.

6.2 Critical evaluation of the metabolic fingerprinting method

Metabolomics is a new method compared to the other 'omics' approaches such as proteomics or genomics (Wishart 2007), therefore standard practices, procedures and methods are still developing and improvements are regularly suggested (Perera

2011; Hrydziuszko & Viant 2012). Metabolic fingerprinting has the unique capability of measuring thousands of metabolites simultaneously which makes it a useful tool for investigating metabolomes, although the method does have some drawbacks which I discuss below.

6.2.1 Metabolome coverage of a fingerprint

Metabolic fingerprinting measures thousands of metabolites, but no single metabolomic method is capable of measuring all the metabolites within a metabolome (Hall 2006). The sample of metabolites measured from the metabolome is biased towards certain types of chemical compounds by the extraction and LC-MS methods used (Sanchez *et al.* 2008). For example, a methanol extraction will extract non-polar metabolites but is likely to leave polar metabolites behind and GC-MS (Gas Chromatography-Mass Spectrometry) will measure more volatile, low molecular weight compounds compared with a LC-MS method (Allwood *et al.* 2008; Macel *et al.* 2010). It is difficult to assess what proportion of the entire metabolome is measured because the total number of metabolites within a plant species is unknown, although estimates are in the region of several thousand metabolites per species (Davies *et al.* 2010). This raises the possibility that additional metabolites of interest may exist in the study systems considered in this thesis that were beyond the scope of the methods used in this fingerprinting study. If it is supposed that the majority of the metabolome was not measured in this study, this implies that in addition to those metabolites found in larvae fed *C. spinosa* there could be other plant metabolites that are transferred chemically intact into insects (Chapter 3) but were not included in the range of metabolites extracted by the techniques used in this study. Similarly, the full range of metabolites that are induced by *P. rapae* herbivory is unlikely to have been measured in the three host plant species attacked (Chapter 4). Therefore it is possible that some induced metabolites might have been missed in my study, and that these metabolites might have shown a common response to herbivory in the three study species of plant, implying more consistent responses to herbivory than I detected. Nonetheless, I conclude there was little evidence of a common response

to herbivory by different plant species based on the large number of metabolites measured in this study.

Having acknowledged that I cannot assess what proportion of the metabolome the metabolic fingerprint represents, the following reasons suggest that the proportion is sufficiently large to address the questions posed in this thesis. For instance, I wished to examine the metabolites of insects feeding on different host plant species (Chapter 3), and the coverage of the metabolome by fingerprinting was sufficiently large to detect metabolites in *P. rapae* larvae feeding on *C. spinosa* that were previously unknown. Such metabolites would probably not be revealed in a targeted analysis that measures a much smaller number of metabolites. In Chapter 4, the study of metabolites induced by herbivory in the three host plant species was able to include a wide range of metabolites because the metabolic fingerprints encompassed > 3000 metabolites per plant species. This represents a far greater proportion of the plant metabolome than could be measured by a targeted analysis, which usually focusses on fewer than 50 metabolites. Therefore I conclude that failure to find metabolites that are induced in common among the study species is likely to be a robust finding. For these reasons, I conclude that metabolic fingerprinting is a good method for revealing plant compounds that move between trophic levels and for comparing effects of herbivory among plant species.

6.2.2 Pre-processing and statistical analyses of metabolic fingerprint data

Metabolomic data can be described as 'noisy' with many features that do not represent real metabolites, and also real metabolites that are not recorded in every replicate due to technological failings (Hrydziuszko & Viant 2012). In this thesis, these problems were circumvented by filtering out the 'noise' so that any remaining features are more likely to represent metabolites. In instances where a metabolite was not recorded in all replicate samples, these zero values in the data were managed by applying MVI (Missing Value Imputation) to enable more robust statistical analyses (Hrydziuszko & Viant 2012). In addition, the relatively large number of biological replicates had been maximised to enable better filtering of the

data and to increase the robustness of the metabolite measurements. The techniques used in this thesis are expected to be surpassed in the near future because better technology to measure metabolites and to pre-process the data are currently being developed (Wishart 2007; Scheubert *et al.* 2013) which will likely result in metabolic datasets becoming more accurate and more reliable to use in future.

Data sets from metabolic fingerprinting contain thousands of data points and require various statistical and descriptive analyses to summarise this information. Appropriate statistics include specialist methods such as PLS-DA (Partial Least Squares-Discriminant Analysis) or a combination of different methods to interpret the data. With noisy data, a univariate test requires a large difference in metabolite abundances or a large number of replicates in order to detect a change, whereas Principal Components Analysis (PCA) examines changes in the full data sets, including small changes in individual metabolites that may be too small to be significantly different in a univariate test. This means that using both univariate and multivariate approaches together can give a clearer picture of an organism's metabolome, and how the metabolites in an organism change in response to biotic and abiotic factors. This is illustrated in Chapter 4 where a PCA suggested the metabolic fingerprint of *Lunaria annua* was altered by herbivory whereas multiple separate univariate analyses found that the abundance of only a single metabolite was significantly altered by herbivory (after correction for FDR). This demonstrates how careful consideration should be given to the statistical and descriptive analyses employed to fully explore the questions being asked.

6.2.3 Barriers to identification of metabolites

A disadvantage of metabolic fingerprinting and of metabolomic studies in general is the difficulty in obtaining chemical identities of unknown metabolites. Despite the number of metabolic databases which aim to gather together identifying parameters of metabolites (Tohge & Fernie 2009), the information in these databases was not sufficient to obtain even tentative identities for the majority of

the metabolites measured in this thesis. Identification using only the molecular weight of the metabolite (as used for identifying glucosinolate compounds in Chapters 3 and 4) is possible but is always a tentative identification, making conclusions drawn less reliable. Comparisons between the MS/MS spectrums of abundant *C. spinosa* metabolites (Chapter 3) with MS/MS spectrums in databases apparently matched one of the unknown metabolites to oleanolic acid. However, a standard of oleanolic acid failed to confirm this identification which highlights the uncertainty of identifications based on molecular weights and database MS-MS spectrums alone. As more metabolites are characterised and more contributions to metabolomic databases are made, the task of identifying metabolites of interest that are highlighted by metabolic fingerprinting should become easier (Scalbert *et al.* 2009; Bowen & Northen 2010), further advancing the benefits of fingerprinting methods.

6.3 Contributions to the knowledge of plant-insect interactions

6.3.1 Can metabolic fingerprinting further our knowledge of plants and insects?

Metabolic fingerprinting evaluates the metabolites of an organism in a way that targeted analyses cannot because of the large number of metabolites measured which provides a more holistic view of the metabolome. In this thesis, metabolic fingerprinting has enabled questions to be answered about which biotic and abiotic factors influence the quality and quantity of metabolites that constitute the metabolome. By comparing *P. rapae* larvae feeding on different species of host plants (Chapter 3), metabolic fingerprinting revealed that diet affected the insect metabolome. Similarly the metabolome of insects was altered by environmental rearing conditions (Chapter 5). There is a lack of metabolomic data on insects, with previous studies defining the insect in terms of growth, fecundity and/or mortality. However, the findings from this thesis examining which factors influence the metabolomes of insects can now be built on in future to investigate the underlying

biochemical mechanisms that result in observed differences in insect performance reported in the literature.

In plants, the fingerprinting method used in this thesis also revealed new findings. Temperature was found to have more of an effect on plants than CO_2 (Chapter 5). The methods used here where all the metabolites changed by a specific environmental factor were summed to determine which climate factor had the larger effect on plants in terms of the number of metabolites affected, could be applied in other multi-factorial fingerprinting studies. This method which quantified the magnitude of metabolic change following abiotic stress could be used to assess which elements of climate change (for example temperature, greenhouse gases and/or drought) might have stronger effects on plants. This approach assumes that if a greater number of metabolites are observed being changed by a factor, this implies that a greater number of biochemical pathways are changed in a plant as it responds to altered abiotic conditions. Since these pathways control many plant functions such as photosynthesis, growth or defence, the overall change in the plant as a consequence of environmental change would be measured. By comparison, a targeted study measuring fewer metabolites would be less likely to evaluate the overall state of the plant but instead focus on metabolites associated with one particular aspect of the plant's response such as photosynthesis. Thus analysing the metabolic fingerprints of plants can give a more holistic view of plant responses.

6.3.2 The suitability of a plant as an host plant

It is not clear what the defining qualities of a plant species are that result in it being incorporated into the diet of a polyphagous insect (Janz 2011). Neither is it known the specific properties of individual plants within a species that affect the performance of insect herbivores (Scriber & Feeny 1979). In this thesis I have contributed to addressing these questions by measuring the performance of insects on different species of host plant (Chapters 3), and on the same species of plant in different environmental treatments (Chapter 5). Nitrogen (N) is thought to be an

important limiting factor for insect growth (Slansky & Feeny 1977), however, the N content of plants measured in this study (Chapter 3 and 5) did not explain the performance of *P. rapae* larvae on the host plants studied. It is likely this is because, even if N is limiting, *P. rapae* larvae may compensate for the lack of N by eating more leaf material (Loader & Damman 1991; Hwang *et al.* 2008).

The N content of plants did not apparently explain insect growth rates in this study, which suggests that there were other factors determining the success of insects on host plants. Therefore I investigated whether metabolic fingerprints of the host plants could explain any differences in insect performance on these host plants. I found that five host plant species (Chapter 3) divided into two groups according to how similar their metabolic fingerprints were, and plants divided into the same two groups according to whether plants were categorised as good or poor hosts of *P. rapae* larvae. This provides some evidence that metabolic fingerprints could explain the performance of *P. rapae* larvae on different host plant species. Thus plant metabolic fingerprints may yet reveal new information for understanding the performance of polyphagous insects on different host plants and help understand mechanisms underlying evolution of host plant choice by insects.

6.4 Future Work

6.4.1 Determining the function of abundant metabolites detected in *C. spinosa*

Approximately 44 metabolites were found in *P. rapae* larvae which were thought to have originated in the host plant *C. spinosa* (Chapter 3). There are no previous records of compounds transferring intact between *P. rapae* larvae and *C. spinosa*, probably because it is rarely used as a study system. Such chemically intact metabolites in larvae are interesting because they suggest that either larvae are unable to digest the compounds yet absorb them into their tissues, or that larvae actively sequester them. Screening for such metabolites through the examination of the insect metabolic fingerprint, as done here, could prove a productive research

strategy for the following reasons. Extracts of other *Cleome* sp. are known to be toxic to Lepidoptera larvae (Soliman 2012; Ladhari *et al.* 2013). In trials, I observed high mortality of first instar larvae of *P. rapae* on *C. spinosa*, although the larvae used in the experiments described in the thesis were second instar or older and showed no adverse performance effects. Therefore there is a possibility that *C. spinosa* metabolites identified in this study are toxic to young larvae and have the potential to be developed into new pesticides. The first steps in developing such compounds as new pesticides would involve feeding larvae artificial diets spiked with these metabolites to verify any detrimental effects of the compounds. Another consideration is the large number (44) of abundant *C. spinosa* metabolites that were found in larvae. This allows an opportunity to examine if any toxic effects of these metabolites (if validated) are dependent upon a complex mixture of all these metabolites or whether each of these metabolites on their own is equally harmful.

6.4.2 Studying insects and plants under future climates

Previously, insect responses to climate factors have been evaluated using measurements of their performance, whereas in this study I showed that insects also change chemically as a consequence of both elevated temperature and CO_2 (Chapter 5). The insect metabolome has already been explored in relation to abiotic stressors to reveal how insects enter diapause or deal with heat shock or salinity increases (Michaud *et al.* 2008; Zhang *et al.* 2012b; Hidalgo *et al.* 2013). In a similar manner, the metabolome could be used to investigate potential biochemical mechanisms underpinning climate change impacts on insect performance. For example, changes in the metabolome could hold the key to understanding the mechanisms determining increased insect growth rates under elevated temperature, or the ability of insects to process more food when employing compensatory feeding on host plants low in N under elevated CO_2. Such experiments would involve measuring metabolites from insects raised under elevated temperature and fed diets low in N. This would determine the metabolites that are increased or decreased under these different treatments. Through the use of metabolomic databases that can suggest the pathways some metabolites are

associated with, the mechanisms that help the insect adapt to the change in the environment could be uncovered.

The temperature and CO_2 study in this thesis did not attempt to separate the direct and plant-mediated indirect effects of climate factors on insects. In reality, the direct and indirect effects will occur simultaneously therefore measuring the net effect on insects is a realistic depiction of the likely future impacts. However, an experiment that assigned effects as either direct or indirect would be beneficial because direct effects may apply to all individuals of an insect species whereas indirect effects may be specific to insects feeding on particular species of host plants. A previous study assessing the direct versus indirect effects of temperature and CO_2 fed larvae on excised leaves from plants grown under different treatments did not find any direct effects of CO_2 on the performance on insects (Murray *et al.* 2013a). A similar experiment could establish if CO_2 directly affects the insect metabolome, or whether all changes in the insect metabolome under elevated CO_2 are attributable to the changes caused by elevated CO_2 in the host plant.

6.4.3 Isolating metabolites that determine insect performance

The grouping of plants that had similar metabolic fingerprints as well as similar rates of insect growth (Chapter 3) suggested that plants on which insects developed better may have had certain metabolites present (or absent) that determined insect performance. Whether these common metabolites are beneficial chemicals promoting growth and stimulating feeding or harmful metabolites inhibiting growth is unknown. Establishing if such chemicals exist and finding out the chemical identities of such metabolites would help to answer questions surrounding the suitability of one plant species over another as a host plant. Such an experiment could first establish if the apparent relationship between metabolic fingerprints of plants and insect performance proved robust when a wider range of species are included in the analysis. The plant metabolic fingerprints could be used to distinguish the metabolites that plants with more similar insect performance have in common. To do this, different plant species could be grouped according to the

level of insect performance so that an OPLS-DA method (as used in Chapter 3) could be employed to identify metabolites that discriminate these groups of plants. The identification of such indicated metabolites or 'biomarkers' would be time consuming, but if successful would enable standards of the compounds to be added to artificial diets and tested for their beneficial or harmful effects on the performance of insects.

6.4.4 Use of metabolomics with other 'omics' techniques

Metabolomics has been used in other areas of research in conjunction with transcriptomics and proteomics to piece together the underlying biochemical systems in plants and insects (Cho *et al.* 2008; Colinet *et al.* 2012b; Rodziewicz *et al.* 2014). In this way, metabolites can be associated with a known biochemical pathway by determining if a metabolite is increased or decreased in abundance at the same time as up or down regulated genes or proteins that have known functions. Combining metabolomics with another 'omics' technique could identify if those metabolites that react to temperature or CO_2 (Chapter 5) are associated with genes known to react to stress or changes in plant processes such as photosynthesis or growth. In plants that have been attacked by a herbivore (Chapter 4) measuring gene transcripts or changes in protein abundance could point towards specific signalling pathways or pathways that synthesize known defensive metabolites. Through this approach, functions could potentially be assigned to some of the plant metabolites that do not currently have a known role, providing new knowledge of how crop plants defend themselves against herbivores and adapt to biotic factors.

6.5 Overall conclusions

The breadth of metabolites that metabolic fingerprinting can measure means it can address ecological questions that a targeted approach cannot. In this thesis I have demonstrated how metabolic fingerprinting can provide new information on the

metabolomes of plants and insects, such as how diet and climate factors influence metabolites. I have revealed plant metabolites that were previously unknown to move structurally intact into *P. rapae*, and I have shown that the metabolite reaction of plants to herbivory is species-specific. These findings demonstrate the specific ways in which metabolic fingerprinting can be used to increase our knowledge of plant-insect interactions and contribute to establishing metabolic fingerprinting as an essential tool for ecologists.

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