Plants signalling to herbivores: is there a link between chemical defence and visual cues?

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Summary

The use of visual cues by insect herbivores is likely to be an important component of plant-herbivore interactions in the wild, yet has until recently received little attention from researchers. In the last decade, however, interest in this topic has intensified following Hamilton & Brown's (2001) autumn colouration hypothesis, which proposes that the intensity of colouration of trees at autumn time is a signal of their defensive commitment to potential herbivores. This idea remains controversial and to date robust empirical data linking colouration with chemical defence and herbivory have been lacking.

This thesis begins with a meta-analysis, in which I synthesize and analyse previously published data to determine the evidence for the use of host plant colouration by herbivores. I then move to explore the relationship between chemical defences and colouration in a classic plant-herbivore system: the wild cabbage (*Brassica oleracea)* and its herbivores the cabbage white butterfly (*Pieris rapae)* and the cabbage aphid (*Brevicoryne brassicae*). Both species have colour vision, and I use spectral sensitivity data to model the colour of the host 'through the eyes' of the herbivores. First, I present data from a field study of wild cabbage populations showing significant relationships between herbivory, plant colouration and levels of glucosinolates defensive compounds. These results suggest that plant colouration could be used by herbivores to gain information about plant chemical defence. I then show colouration has a fixed genetic component in a common garden experiment; a necessary requirement for evolution of a colour signal. I explore the use of colouration in host choice by herbivores in more detail in a series of behavioural experiments. I show that cabbage aphids do not use leaf brightness as a cue when selecting among plants, but they do respond to different leaf colours. I also show that cabbage white butterflies do not choose hosts based on particular colour cues, even though this colouration potentially provides important information about host defence levels, which are shown to impact upon offspring fitness.

Together, these results provide a clear demonstration of a link between plant chemistry and colouration in the wild cabbage system. However, the data presented in this thesis indicate that the use of colouration as a guide to host defence is limited, and I conclude by discussing possible reasons why this might be the case.

Declaration

The model of *Pieris rapae* and *Brevicoryne brassicae* spectral sensitivities and the software to run the models were designed by Dr Lucas Wilkins at the University of Sussex. The common garden was established by Dr Dave Hodgson at the University of Exeter. CN samples collected in the field surveys were run by Debbie Coldwell at the University of York. However, the analysis and interpretations drawn from the data in all chapters are my own.

I certify that, with the above qualifications, the work carried out in this thesis is entirely my own, and that any help by other individuals with data collection and analysis is fully acknowledged.

In addition, I certify that this thesis has not been, and will not be, submitted in whole or in part to another university for the award of any other degree.

Signature:

Rosie Foster

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Table of contents

Chapter One. Introduction

1.1. Plant herbivore interactions

Herbivory – the consumption of plants by animals – occurs in every ecosystem worldwide and so the study of plant-herbivore interactions has applications across the whole globe (Schoonhoven et al. 2005). From an ecological perspective, the study of plant chemical defence and its interaction with herbivory can explain why the majority of terrestrial plant biomass is not eaten by herbivores or literally "why the world is green" (Hartley and Jones 1986). Interactions between plants and their herbivores underpin community dynamics, as bottom-up effects on herbivore numbers affect animals higher up in the food chain (Harvey et al. 2003). Insect herbivory is particularly illuminating in this respect because insect herbivore life-cycles are usually more tightly bound to specific host plants than vertebrate herbivores due to their small size and high levels of specialization. For these reasons, plants and insect herbivores often have intimate coevolutionary relationships (Berenbaum and Zangerl 1998). The study of plant-insect herbivore interactions has considerable implications economically and for agriculture as pest outbreaks can be extremely costly: without insecticides or other methods of control, between 10% - 100% of crop production is lost (Schoonhoven et al. 2005).

Plant-herbivore interactions are characterised by antagonistic pressures: for plants, defence against herbivory; for herbivores, how to identify suitable hosts and overcome these defences. I now discuss these conflicting pressures.

1.1.1. Plant defence

Herbivores reduce plant fitness (Marquis 1984), leading to selection for plant defences against herbivores (Rausher and Simms 1989). As a first level of defence, a plant's physical structure can provide a barrier to herbivores. There are many types of physical defence, including wax, trichomes and leaf toughness (Schoonhoven et al. 2005). For example, the waxy surface of *Eucalyptus globules* reduces the fitness of one of its herbivores, *Ctenarytaina spatulata* (a psyllid bug) by preventing it from adhering to the plant (Brennan and Weinbaum 2001). Trichomes are plant hairs; these provide a physical barrier and often have associated defensive chemicals, and can therefore prevent small insects from reaching the epidermis and feeding (Schoonhoven et al. 2005). Leaf toughness can be increased through the deposition of cellulose, which is difficult to digest and thus limits the consumption of plants (Abe and Higashi 1991). Materials can also be deposited in leaves to protect from herbivory. For example, silica in grasses can be taken up from the soil, and high levels of silica has been shown to reduce the growth rate of *Spodoptera exempta* (African armyworm moth) through decreased efficiency of food utilization and mandible wear (Massey and Hartley 2009).

There is an enormous variety of defensive chemicals in plants (Schoonhoven et al. 2005). These include nitrogen-based compounds, such as alkaloids (McKey 1974) and cyanogenic glycosides (Jones 1962), terpenoids such as the cardenolides (Rasmann et al. 2009), phenolics and tannins (Feeny 1970), and the polyacetates (Guillet et al. 1997). Each class of compound is generally widespread, but particular compounds within these classes may be specific to particular plant groups. For example, terpenoids are the largest group of secondary metabolites but digitoxin, a type of cardenolide within the terpenoids, is only found in foxgloves (*Digitalis*), a genus comprising about 20 species (Agrawal et al. 2012). There is debate about what drives the diversification of secondary chemicals (Hartley and Jones 1986). One theory is that coevolution between a plant and its herbivore(s) (Ehrlich and Raven 1964), drives diversification by chemical "matching" between a herbivore and its host plant (Berenbaum and Zangerl 1998), whereby the evolution of novel chemical defence to deter herbivores selects in turn for novel detoxification strategies by the herbivore to overcome the plant defences. There is an excellent case study of matching in the diversification of furanocoumarins in the wild parsnip and detoxification in the parsnip webworm (Berenbaum and Zangerl 1998), but this is a rare example, and many other hypotheses have been explored. One of these, the sequential evolution hypothesis (Jermy 1976), suggests that plant evolution is more markedly influenced by other factors (e.g. climate, soil, competition, mutualisms, pathogens) than herbivores; insects track plant defence changes but do not strongly influence their evolution. Yet another hypothesis is the screening hypothesis (Jones and Firn 1991), which posits that most plants have a large range of secondary metabolites, but that the majority of these are inactive because there is a low probability of producing an active compound. Inactive compounds are retained and high diversity is selected because this increases the probability of possessing an active compound (Jones and Firn 1991).

For plants, the production of defence chemicals is thought to be costly (Strauss et al. 2002), and although the evidence is mixed, the allocation of energy to chemical defence may be an important decision for plants. The induction of chemical defences can help to reduce these costs by ensuring defence compounds are only produced after herbivore attack (Karban and Baldwin 1997). In some cases, chemical induction occurs in response to specific cues, including the particular type of mechanical damage caused by a herbivore (Baldwin 1990). Remarkably, it has been shown that chemical response can be specific to the life-stage of a particular herbivore. For example, the induction of chemical defences by *Brassica rapa* (field mustard) is more pronounced in response to second-instar larvae of two lepidopteran species than to fourth-instar larvae (Widarto et al. 2006). As the second-instar larvae are less mobile

and so potentially more destructive, this appears to be a logical deployment of resources in response to costs of herbivory (Widarto et al. 2006).

Previous studies have suggested that there is a trade-off between constitutive defences (those always present in the plant) and induced defences (Koricheva et al. 2004). Constitutive defences are costly as they continuously consume resources, but they are advantageous as they offer immediate defence against herbivores (Karban et al. 1999). Karban and Myer (1989) have argued that when the probability of attack is high, a plant should rely on constitutive defences; conversely, where the likelihood of herbivory is low, defences should be induced. This idea is supported by research into a variety of plant species. For example, Traw (2002) found that chemical induction in *Brassica nigra* (black mustard) was negatively correlated with levels of constitutive defence. Across the literature as a whole a meta-analysis by Koricheva et al. (2004) has found strong support for this hypothesis.

A second hypothesis – the 'optimal defence' hypothesis (McKey 1974) – has been proposed to explain variation in the levels of chemical defence within different parts of a plant. This predicts that those parts which are most valuable to the plant will be preferentially defended where resources are limited (McKey 1974). Accordingly, new leaves, which have higher growth rates and therefore a higher nitrogen content than older leaves, should be better defended than older leaves (Stamp 2003). Moreover, new leaves are more vulnerable to attack as they have less mechanical protection than older leaves, and for this reason are also expected to enjoy greater levels of chemical defence (McKey 1974). One case where these predictions hold is in *Cynoglossum officinale* (houndstongue), where pyrrolizidine alkaloids were found to be at least 50 times higher in new leaves than in old leaves (Van Dam et al. 1996). A recent meta-analysis supports the optimal defence theory, finding that plant tissues across a range of species that are assumed to be of high value also have higher levels of defensive chemicals (McCall and Fordyce 2010).

Volatiles are airborne chemicals released by a plant. All plants emit volatiles as a by-product from metabolic processes, but their release can also serve an adaptive function. Following herbivore damage, chemicals within the plant volatilize on contact with air, leading to higher volatile release by the plant (Schoonhoven et al. 2005). Plant volatiles can defend plants by deterring herbivores (Himanen et al. 2010) and in some cases may also attract parasitoids that will prey upon insect herbivores (Turlings et al. 1990, Mattiacci et al. 1995, Thaler 1999).

1.1.2. Host selection

Herbivores may be classified according to whether they are specialists or generalists. Specialist herbivores are restricted to plants within a single family whereas generalists feed on a broader range of hosts (Schoonhoven et al. 2005). Specialists as a group are more species rich than generalists: 80% of British herbivorous insects are regarded as specialists (Schoonhoven et al. 2005). The degree of specialisation shown by a herbivore has obvious implications for host finding and selection. In particular, the ability of herbivores to deal with plant defences will vary according to their degree of speciality. Specialists often have detoxification systems that are specific to the chemicals produced by the host plant group (Wittstock et al. 2004). Generalists, in contrast, have less specific detoxification systems (Francis et al. 2005). For this reason, specialists are often better able to exploit their hosts than generalists and in doing so often cause more severe damage (Cole 1997). The theme of specialist versus generalist host plant choices will be revisited in Chapter Five.

Many insects have chemosensory systems that detect plant volatiles; specialists, in particular, are highly attuned to specific volatiles as an essential means of host location (Schoonhoven et al. 2005). The specificity of volatiles may arise either through specific chemicals or else more commonly through unique ratios of common chemicals (Visser 1986, Bruce et al. 2005). Using techniques such as electroantennograms, it is possible to determine not only the sensitivity of insects to different chemicals but also the precise chemicals within bouquets of volatiles to which they respond (van Loon et al. 2002). Olfactory responses have been recorded for species in many insect orders, including the Lepidoptera, Diptera, Hemiptera and Coleoptera (see Table 1.1).

Table 1.1. Examples of olfactory responses of different orders of insects to volatiles.

	Reference example	Insect	Volatile	Response
Lepidoptera	(Van Loon et al. 1992)	*Pieris rapae* *Pieris brassicae* (cabbage butterflies)	23 volatiles purified from *Brassica* and *Sinapis* species	Positive response to volatiles from head space of host plant, including green leaf volatiles and crucifer-specific volatiles.
Diptera	(Judd and Borden 1989)	*Delia antique* (onion fly)	Specific volatile dipropyl-disulphide purified form *Allium* plant	Can detect bait containing volatile from over 100m away.
Hemiptera	(Nottingham et al. 1991)	*Aphis fabae* (black bean aphid)	Various hosts including *Vicsa faba* (bean) and non hosts e.g. *Ocimum basilicum* (basil).	Aphid walked towards odours from damaged but not undamaged leaves. Isothiocyanates were found to be repellent. Non-host odours could mask host odours.
Coleoptera	(Tommeras and Mustaparta 1987)	*Ips typographus* (bark beetle)	Bark extracts from hosts *Picea abies, Pinus silvestri* and non-host *Betula pubescens* (spruce, pine and birch trees)	Excitation to host odours and some non-host odours.

Insects can use a variety of visual cues to find their host plant. The physical structure of the plant provides important cues for herbivores. For instance, *Battus philenor* (Pinevine Swallowtail) butterflies discriminate their host *Aristolochia* (birthworts) by leaf width, and show flexible preferences for wide or narrow leaves depending on prior experience (Rausher 1978). Plant size can also be an important visual cue, with insects generally preferring larger plants (Johnson and Agrawal 2005). Size was found to be a more important cue than chemical composition for the butterfly *Melitaea cinxia* (Glanville Fritillary), with higher rates of oviposition recorded on larger plants (*Plantago lanceolata*) (Reudler Talsma et al. 2008). Finally, and most importantly for this thesis, some studies have pointed to the possibility that herbivores use colour in host selection. For example, Degen and Stadler (1997) showed that the carrot fly (*Psila rosae)* was found to oviposit preferentially on plant models with a high reflectance in the yellow region of the spectrum. The evidence for herbivore use of colour cues will be explored in greater depth in Chapter Two.

The vast majority of research on the use of olfactory and visual cues in host selection by herbivores has explored the cues used to discriminate between host and non-host species (e.g. Nottingham et al. 1991, Miles et al. 2005, Doring and Skorupski 2007). In contrast, the use of cues to choose between suitable hosts remains poorly understood (e.g. Bullas-Appleton et al. 2004). In particular, little is known about how insect herbivores use visual cues when selecting among conspecific hosts.

1.2 Autumn colouration

Autumn colours are among the most dramatic displays made by plants. If host choice is governed by colour, the large differences at autumn time should make it simpler to detect interactions based on plant colouration. The beautiful array of red and yellow leaves we see on trees in autumn is caused by the production of chemicals and the breakdown of chlorophyll. Yellow colouration is caused by carotenoids which are present in the leaf throughout the year and are revealed when the green chlorophyll is removed. The red colouration is caused by anthocyanins, which are newly synthesized prior to abscission, and are not a product of leaf senescence (Lee et al. 2003). Why would trees pump newly synthesized anthocyanins into leaves that are about to fall? It is, moreover, a common situation, with approximately 70% of trees species producing anthocyanins (Lee et al. 2003). The production of these chemicals is costly, involving many stages, each requiring different enzymatic steps (Winkel-Shirley 2001). Currently, two main hypotheses attempt to explain this adaptive investment in senescing leaves. The first posits that the anthocyanins have a physiological role in protecting the leaf against abiotic factors such as UV light. The second hypothesis argues that anthocyanins function as a signal of a plant's defensive commitment against insect herbivores. In the following section, I introduce each in turn.

1.2.1. Physiological hypothesis

Many anthocyanins are photoprotectants. They have been described as a "red sunscreen" and shield chloroplasts from excess solar energy (Lee and Gould 2002). In autumn, when temperatures fall and carbon fixation is reduced, the degree of protection offered by anthocyanins increases (Lee and Gould 2002). This has led researchers to suggest that the additional anthocyanins placed within the leaves in autumn serve a protective function (Wilkinson et al. 2002, Gould 2004). The benefit, however, of investing in photoprotection for leaves that are senescing and soon to fall is unclear. Addressing this point, Lee and Gould (2002) suggest that anthocyanins protect the nutrient dismantling process, thereby enabling more nitrogen to be transferred back into the tree. In addition, anthocyanins may protect plant tissues from chlorophyll breakdown products, many of which have oxidative effects, especially free radicals (Lee and Gould 2002). Free radicals (molecules with an unpaired electron) are very reactive due to the presence of the extra electron, and will oxidise and damage molecules they come into contact with, including cell membranes, proteins and DNA (Lee and Gould 2002).

The potential role of anthocyanins as a photoprotectant in young leaves has also been emphasised (Coley and Barone 1996). Delayed greening, whereby the greening of young leaves is delayed until maturity, is observed in a number of species especially in the tropics. High levels of anthocyanins are often present in young leaves (Kursar and Coley 1992). The photoprotective benefits of anthocyanins are likely to be greater for young leaves than for mature or senescent leaves, for two reasons (Lev-Yadun et al. 2012). First, as noted above, investment in young leaves would yield more long term benefits. Second, young leaves are neither as tough nor as thick as mature ones, and so would probably require more protection from solar energy (Kursar and Coley 1992).

Given the complexity of colour production and the many roles pigments can serve in a plant, it is unlikely that a single mechanism can fully account for the presence of the pigments that are the basis of autumn colouration. While the benefits proposed by physiological hypotheses for autumn colouration appear logical, clear evidence for a photoprotective effect of anthocyanins is lacking (Zeliou et al. 2009). In addition, the large variation of autumn colouration found both between and within species is not adequately explained purely by physiological mechanisms (Lev-Yadun and Gould 2007). Consequently, other hypotheses need to be considered.

1.2.2. Signalling hypothesis

The autumn colouration signalling hypothesis (henceforth ACH) was first proposed by Hamilton and Brown in 2001. Prior to the publication of Hamilton and Brown's paper, Archetti (2000) presented a game-theoretic model showing how tree colour and insect herbivory could coevolve based on Hamilton's verbal arguments of the ACH. As stated in Hamilton and Brown (2001), the ACH posits that tree colouration functions as a signal of defensive commitment to herbivores colonising trees in the autumn. An important focus of Hamilton and Brown's study is herbivory by aphids, which colonise trees in autumn and then proliferate in the spring causing significant damage (May and Carlyle 2003). The intensity of the colour is the active signalling component, such that the most strongly coloured trees are the most chemically defended. By signalling a high level of defence, Hamilton and Brown argue that the tree is seeking to deter insects from colonising it, thereby avoiding the damage caused by aphid proliferation in the spring. Herbivores such as aphids are argued to benefit from attending to the signal as they are able to avoid the best-defended trees (Archetti 2000). Hamilton and Brown (2001) propose that the signal is a handicap (Zahavi 1975) because only the highest quality trees can afford the cost of signalling at a high intensity. Although the ACH originally focused on aphids colonising trees in the autumn time, the idea that host colouration may

signal chemical defence could potentially be applied to many plant-herbivore interactions, as the authors themselves suggest (Hamilton and Brown 2001).

The ACH is one of the most influential hypotheses of plant colouration and insect herbivore host choice. But what makes it so influential, and how does it differ from other ideas on plant defence and herbivore choice? Most ideas about plant-herbivore interactions originate from plant biologists (Berenbaum and Zangerl 1998) and so to have a hypothesis put forward by an evolutionary theoretician is exceptional. This is the only hypothesis of plant-herbivore interactions to involve strategic signalling, a concept normally applied to animal interactions (Maynard Smith and Harper 2003). A fundamental tenet of the ACH is that plants signal to animals, which in of itself is of course not unusual (Schaefer et al. 2004; see also below). What is exceptional, however, is the idea that the signal represents an ostentatious display, the cost of which ensures the honesty of the signal, consistent with Zahavi's notion of handicaps (Zahavi 1975). Under this scheme, only those plants of high quality (and therefore presumably a high level of defence) can afford the costs or 'waste' associated with producing a strong signal. Another unusual feature of the ACH is its focus on visual channels of communication, which contrasts with previous experimental work on host location in aphids and other herbivores that has focused largely on olfactory cues. Overall, the novelty of the ACH compared with previous work on plant-herbivore communication, combined with its broad approach linking ecological, evolutionary and physiological mechanisms and the current lack of convincing experimental support (see below) have meant that the ACH has remained controversial. In the following sections, I provide brief introductions to two areas that are central to the ACH: insect vision, and colour vision in particular, and strategic signalling. Finally I review the current evidence for the ACH.

1.2.2.1. Vision in host choice

Light

Light is used to tell us where and what an object is. A light quantum is known as a photon, the interaction of photons with substrates, for example if the light is reflected, absorbed or transmitted, provides us with information about that substrate. Humans are able to see light in the violet to red region (c. 400-650nm); however, many other animals such as insects and birds can also detect UV (Land and Nilsson 2002). A few animals can sense wavelengths in the infrared region, for example *Melanophila acuminata* (fire-chaser) beetles, whose larvae depend on freshly-burnt wood, are able to detect forest fires from great distances, which they then approach to lay their eggs (Schmitz and Bleckmann 1998).

Insect eyes

Sensory systems allow animals to gain information about the environment – in the case of vision, this is from the interaction of light energy with the physical world. Most insects have apposition compound eyes (Land and Nilsson 2002). The units in a compound eye are called ommatidia and are arranged in a hemisphere. The number of ommatidia in an eye can vary, but large insects (e.g. *Pieris rapae*) can have several thousand. The ommatidium consists of receptor cells, which form the rhabdom, and these are surrounded by pigment cells. The pigment cells prevent light from entering multiple ommatidia. Each ommatidium focuses light from a region about 2^{o} across to form a 'pixel' in the eye's visual image .The light is focused onto microvilli of photoreceptor cells which form a rhabdom and act as an optical waveguide (Stavenga and Arikawa 2006). All butterflies so far tested, have nine photoreceptor cells in each ommatidium (Qiu and Arikawa 2003). The light is absorbed by visual pigments within the rhabdom, where phototransduction is initiated and an electrical response results (Stavenga and Arikawa 2006).

The nine photoreceptors in each ommatidium of *Pieris rapae* differ in their spectral sensitivities (Stavenga and Arikawa 2006). By summing and comparing the outputs of the different receptor types the butterfly eye can (potentially) produce neural signals for sensing colour and brightness (Osorio and Vorobyev 2005). Although originally defined for human vision I now give a brief account of these terms as they can be applied to insects (Kelber and Osorio 2010).

Brightness

Brightness depends primarily on the number of photons that can stimulate photoreceptor cells (in one or more receptor types) in the eye. The brightness of an object depends on how reflective a substrate is and how much light is available (i.e. the intensity of the light); a leaf appears duller in the shade than in bright sunlight because less light is available to reflect from the surface. Brightness is an achromatic measure: it does not include information about colour composition. Brightness detection differs from chromatic vision, which is achieved by comparing receptor responses. Achromatic cue use is explored in detail in Chapter Five.

Colour

The colour sensed by an animal is determined by which wavelengths are reflected from a substrate, and especially the relative reflectance at different wavelengths (Land and Nilsson 2002). Colour vision is important because it allows identification and discrimination of objects that could not be achieved by comparing aspects of their physical appearance (for instance,

their shape) e.g. ripe versus unripe fruit. Colour vision requires at least two visual pigments with different spectral sensitivities so that comparisons can be made between the levels of stimulation each receives. Most arthropods have at least two different spectral sensitivities, but most commonly have three (Kelber 2006). Butterflies, however, often have more – six in the case of *P.rapae* (Stavenga and Arikawa 2006*)*. Incredibly, some mantis shrimps (Stomatopoda) have 15 different visual pigments, including four types of UV receptor (Marshall and Oberwinkler 1999).

Individual photoreceptors cannot distinguish between different wavelengths. So how is colour vision achieved? It occurs because stimulation thresholds of different types of photoreceptor vary with wavelengths i.e. the different types of photoreceptors are said to have different spectral sensitivities. Spectral sensitivity is given by the probability that a photon of a given wavelength elicits a neural response. By comparing inputs from differently sensitive photoreceptor cells in the brain the colour can be determined.

There is some evidence that colour may be used in host selection by insect herbivores, consistent with the ACH. Lower rates of herbivore damage are associated with leaf variegation in *Hydrophyllum virginianum* (Virginia waterleaf) (Campitelli et al. 2008) and with red abaxial colouration in *Columnea consanguinea* (Wong and Srivastava 2010*)*, which suggests that leaf colour may play a role in reducing herbivory in these species. To determine whether these and other reported effects are due to colour-based host selection, it is necessary to separate visual and olfactory cues, which many of the studies looking at colouration have unfortunately failed to do, particularly when using real leaves (see Chapters Five and Six). For example, the herbivores studied by Campitelli et al. (2008) could have selected the non-variegated leaves based on an odour correlated with the colour rather than the colour itself. One example of how colour and olfactory cues can be disentangled is provided in the extensive work on the use of colour by butterflies when selecting oviposition sites (Kelber 1999). Much of this work is carried out with coloured card, thereby eliminating the effect of olfaction. For example, by wetting coloured paper with an oviposition stimulant, Traynier (1979) was able to determine the colour preference of oviposition sites for *P. rapae* (green<yellow<violet<blue<orange<red). Unfortunately, Traynier did not know the spectral sensitivities of the butterfly's photoreceptors, and so defined his stimuli in terms of human colour vision.

1.2.2.2. Signalling

Maynard-Smith and Harper (2003) define a signal as "any act or structure which alters the behaviour of other organisms, which evolved because of that effect and which is effective because the receiver's response has also evolved." An example of a plant signal is the chemical

volatiles released by a plant under attack by a herbivore, which function to attract parasitoids of that herbivore (De Moraes et al. 1998). The signaller (the plant under attack) benefits from the signal as herbivory will be reduced by the parasitoid, and the receiver (the parasitoid) benefits from the signal because it is able to find its host. A signal differs from a cue because a cue is a feature which can be used to guide behaviour, but did not evolve because of its effect on the receiver. An example of a cue is the use of plant size by herbivores as an indicator of resource abundance (Reudler Talsma et al. 2008). Here, plant size is considered a cue rather than a signal as size conveys information to the herbivore about the plant but has not evolved for that purpose. Signals are thought to have evolved from cues and over time have subsequently been modified to enhance the desired effect on the receiver (Maynard Smith and Harper 2003). In practice it can be very difficult to distinguish between a signal and a cue.

An important challenge in understanding the evolution of a signal is to determine how reliability in the signal is maintained. Signals need not be honest all the time – for example, in Batesian mimicry, harmless species mimic the aposematic signals of harmful species. However, dishonest signals must be at a low frequency compared with honest signals or else it would not pay the receiver to believe the information being signalled. A signal that becomes stable in a population may be termed an evolutionary stable strategy (an ESS) (Maynard Smith and Price 1973). An ESS is a strategy, which, if adopted by most members of the population, is resistant to invasion by a mutant strategy (Maynard Smith and Price 1973). The ACH proposes that trees signal their defensive commitment to herbivores and that only those individuals of high quality can signal at a high intensity. In ESS terms, the strategic cost of the signal is thought to prevent invasion by a mutant that is poorly defended but that signals a high level of defence. Without this cost, such a mutant would invade at the expense of individuals honestly signalling a high level of defence as it does not pay the costs associated with defence. Given the benefits of cheating, honest signalling can occur only under certain circumstances, most notably when there is a cost to signalling to deter weak individuals from signalling strongly. As well as having strategic costs, signals can also be honest because they physically cannot be faked; in this case, the signal is known as an index. Though the ACH was originally conceived in terms of strategic signalling, it is possible that plant colouration may alternatively constitute an index of defensive commitment. In the following sections, I provide a brief introduction to strategic signals and indices.

Strategic signalling, the handicap principle and indices

The handicap hypothesis proposes that signals are honest because the costs they incur are quality-dependent: only the best quality individuals can afford to signal because of the

associated costs: the cost is condition dependent (Zahavi 1975). For a signal to be a handicap it needs to be shown that it is possible for the signal to be dishonest as the signal could vary independently of the signaller's quality. In practice, however, this does not occur because the signal has a strategic cost and so it does not pay the signaller to be dishonest. In addition, costs are differentially felt according to quality: for the evolution of a handicap, the ratio of costs to benefits must be lower for high quality individuals (Grafen 1990). The costs could be associated with the production of the signal, for example in terms of the resources required to make the signal; alternatively, costs may be associated with the consequences of producing the signal, for example, signalling at a high intensity may incur a higher predation risk. Strategic signalling is central to the ACH as originally proposed by Hamilton and Brown (2001): the bright colouration of the tree is costly therefore only good quality trees which can produce high levels of chemical defence are able to pay these costs (Hagen et al. 2004). Whether Hamilton and Brown (2001) were justified in treating leaf colourations as a strategic signal depends on whether there is a cost to colour production over and above the cost to defence which deters low-quality plants that are poorly-defended from signalling at a high intensity (Maynard Smith and Harper 2003).

Indices, in contrast, are signals that do not impose a cost that is differentially felt according to condition. Rather, honesty is maintained purely by physical constraint. A classic example is a tiger marking its territory by scratching as high as it can on a tree. The scratch marks signal the tiger's size and are an index because only large tigers are capable of scratching high on the tree(Maynard Smith and Harper 2003). If the production of plant colouration is not costly (as required by the ACH) it may nonetheless function as an index of plant quality if it is correlated with some aspect of quality.

Plants signalling to animals

Most work on signalling has been done with animals, probably because animals rather than plants have sense organs more like humans, meaning that many displays using visual, olfactory or auditory stimuli are apparent to us. In addition, animal communication often involves clear behavioural traits, whereas plant communication is more covert. It is irrefutable, however, that plants too are able to signal. Plants signal to fungi via root exudates to alert them that they are a potential host for colonisation to produce mycorrhiza (Giovannetti et al. 1994). Plants are also able to signal to animals as well as fungi. The volatiles emitted by damaged plants have found to be a specific signal to parasitoids of the herbivore attacking the plant (Mattiacci et al. 1995, De Moraes et al. 1998, Thaler 1999). In this study by De Moraes et al. (1998), the plant volatiles are so distinct that the parasitoid can distinguish between two

closely related host species. β-glucosidase from gut regurgitate of *Pieris brassicae* larvae is a specific elicitor of volatiles from damaged cabbage plants which signal to attract the parasitoid *Cotesia glomerata* (Mattiacci et al. 1995).

Plant colouration that is thought to function as a warning signal to potential herbivores has been entitled aposematic colouration in plants (Lev-Yadun 2009). Aposematism is a process whereby unpalatable or dangerous animals advertise these qualities to other animals (Gittleman and Harvey 1980). Aposematic animals are often boldly coloured, most commonly red or yellow and black but also purple, yellow and orange (Savage and Slowinski 1992). Lev-Yadun (2001) has investigated aposematism in thorny, spiny and prickly plants, as physical structures such as spines clearly render such plants unpalatable to most herbivores. The author found that 80% of cacti had brightly coloured spines, which could be taken as evidence of aposematism as the colouration could warn herbivores of the unpalatable spines (Lev-Yadun 2001). It is also possible, however, that spine colour functions as an amplifier, drawing a receiver's attention to the spines rather than providing additional information about the palatability of the plant (Harper 2006) .

Production of plant signals

In order to assess the feasibility of the ACH as an explanation for variation in leaf colouration, it is necessary to consider the biochemical pathways underlying production of chemical defences and in particular the relationship between chemical defences and leaf colour. Red colouration of autumn leaves is mainly the result of anthocyanins and yellow colouration of autumn leaves is the result of carotenoids.

Anthocyanins are the main pigments responsible for red colouration of leaves (Schoonhoven et al. 2005). Anthocyanins are a type of flavonoid. Basic structures for flavonoids are produced on the phenylpropanoid pathway where phenylalanine is modified and then joined to malonyl-CoA (produced from fatty acids) (Winkel-Shirley 2001). This molecule may then progress to produce many kinds of flavonoid, but in the production of anthocyanins, it goes through the flavanol production pathway before being converted into anthocyanins (Winkel-Shirley 2001) (see Figure 1.1). There are 11 separate enzyme reactions required for the production of anthocyanins from phenylalanine, which consequently appears a costly and lengthy process (Winkel-Shirley 2001). Additional evidence for such production cost comes from transgenic plants that over-express anthocyanins: these plants were smaller than wild types, perhaps because metabolites were diverted from plant growth to production of anthocyanins (Malone et al. 2009).

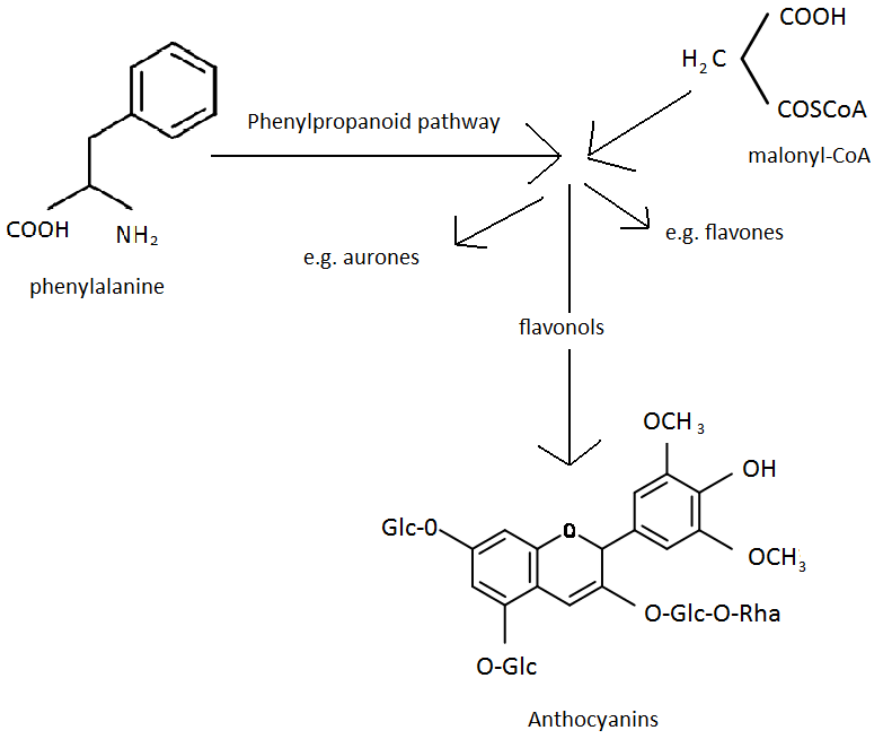

Figure 1.1. Production of anthocyanin, with important pathways and molecules highlighted. Produced from Winkel-Shirley (2001).

Carotenoids are a type of terpenoid and have a very different structure to anthocyanins. They are built from isoprene units which are synthesised along the DOXP pathway, so-called because the DOXP enzyme (1-deoxyxylulose 5-phosphate synthase) catalyses the reaction between pyruvate and glyceraldehyde-3-phosphate to produce the isoprene unit (Hirschberg 2001). These units are joined together to form a long chain, which becomes a type of carotenoid e.g. β-carotene (see Figure 1.2) (Hirschberg 2001). Carotenoids are found in chloroplasts and are thought to provide protection from the damaging effects of light.

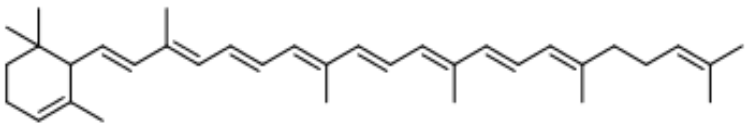

Figure 1.2. Structure of β-carotene. Produced from Hirshberg (2001).

Linking chemical defence and colour

Anthocyanins and some defence chemicals share common biosynthetic pathways, so a mutation exerting an effect in the these pathways could affect both the production of colour pigments and defensive chemicals (Fineblum and Rausher 1997). There is some evidence for this; for example, Simms and Bucher (1996) found that larvae of a specialist tortoise beetle

(*Charidotella bicolor*) performed better on *Ipomoea purpurea* (Common Morning Glory) with white flowers rather than on those with dark-coloured flowers, which are rich in anthocyanins. *Charidotella bicolor,* however, consumes the leaves, not the petals, indicating that the effect of the anthocyanins was not a direct one. Other studies have investigated this further by looking at chemical defence within the plant. Within *Raphanus sativus* (wild radish), there are four morphs differing in petal colour. Two morphs (bronze and pink) contain anthocyanins, while the other two (white and yellow) lack anthocyanins. In herbivore preference and performance trials, almost all leaf-eating herbivores were found to prefer and perform better on plants with flowers lacking anthocyanins (Irwin et al. 2003). In the same study, the morphs containing anthocyanins were found to have higher levels of induced glucosinolates than the morphs lacking anthocyanins (Irwin et al. 2003). The correlation between glucosinolates and anthocyanins may be due to pleiotropic effects of genes influencing both classes of chemical or to genetic linkage between glucosinolate and anthocyanin production and/or expression, both of which may result in correlated selection on either defence or colouration as a consequence of direct selection on the other (Strauss et al. 2004).

Further evidence for linkage between colour and defensive chemistry in brassicas has been revealed by genetic manipulation. Hemm et al. (2003) produced an *Arabidopsis* mutant with reduced levels of phenylpropanoid pathway-derived products. Anthocyanins and other coloured flavonoids are produced by this process (see Figure 1.3). The mutant was found to have reduced levels of aliphatic glucosinolates and increased indole glucosinolates, suggesting that there is a metabolic link between glucosinolate biosynthesis and products from the phenylpropanoid pathway, probably due to an aldoxime-oxidising enzyme which is active in both pathways (Hemm et al. 2003) (see Figure 1.3). The first step in glucosinolate metabolism and in the phenylpropanoid pathway is conversion of amino acids to aldoximes using aldoxime enzymes in the CYP families (see Figure 1.3) (Feldmann 2001, Halkier and Gershenzon 2006). As both pathway have aldoximes as intermediates and use many aldoxime-oxidising enzymes, the mutual use of a specific enzyme could influence the products from both pathways (Hemm et al. 2003).

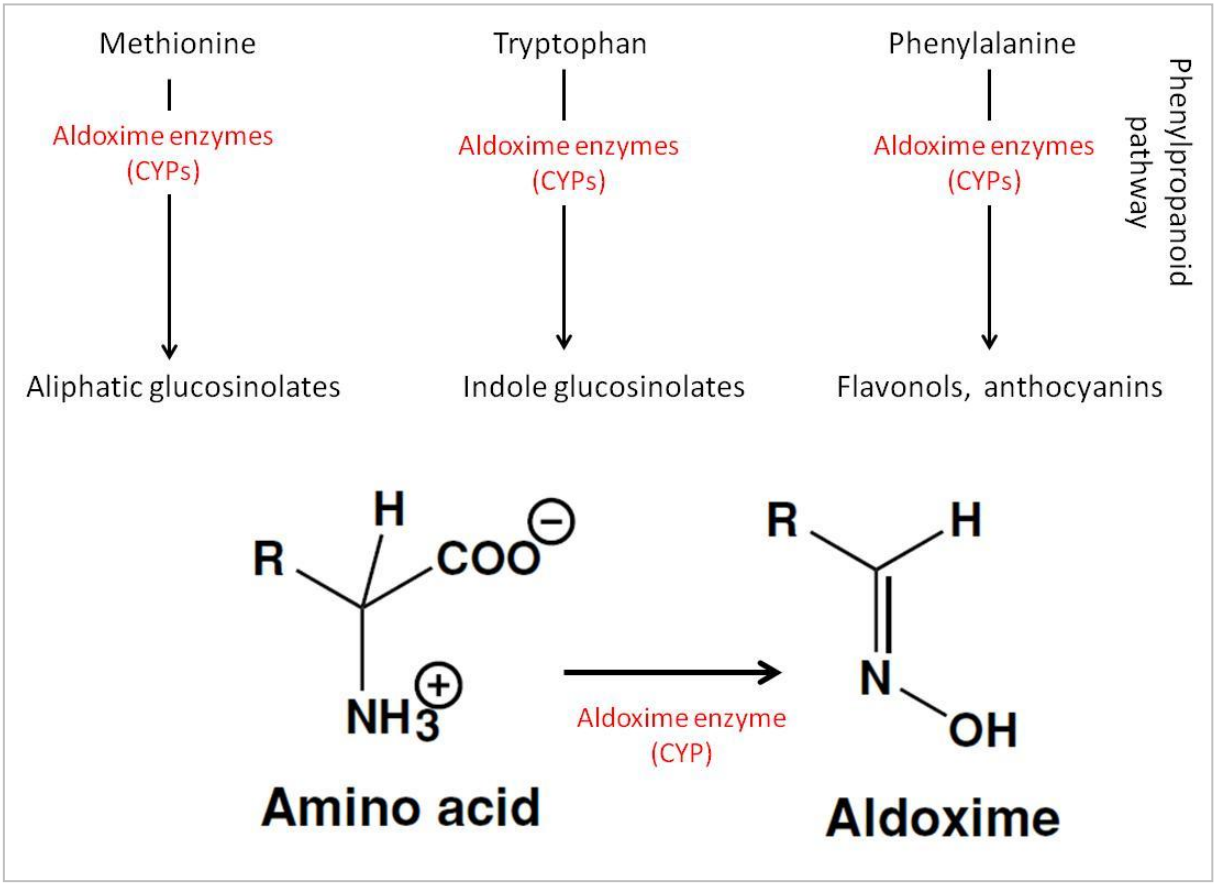

Figure 1.3. Aldoxime enzymes in the production of glucosinolates (top left) and in the phenylpropanoid pathway (top right). Pathway at the bottom is the structure of an amino acid and it oxidation to produce an aldoxime by an aldoxime enzyme. CYPs = Cytochromome P450s.

An alternative way that colour and chemistry could be linked is through allocation costs (Mole 1994). All organisms have a finite amount of resources and must allocate these optimally among competing functions (Herms and Mattson 1992). As chemical defence is usually costly (Strauss et al. 2002), this may have important implications for the amount of resources available for signalling. The cost of producing a colour signal may be threefold. First, the allocation of resources to the production of the signal may divert resources that could be used elsewhere by the plant e.g. growth (Close and Beadle 2003). Second, the presence of any non-green pigment in leaves is expected to lead to some reduction in photosynthesis given that there will consequently be a smaller volume of chlorophyll; the amount of light reaching the chlorophyll may also be reduced if it is absorbed or scattered by colour pigments (Soltau et al. 2009). Third, if the colour is conspicuous it may actually attract herbivores (Stefanescu et al. 2006). Costs of producing leaf colouration above and beyond the costs associated with chemical defence of these same leaves is a crucial element of the ACH, as it is this additional strategic cost that would stabilise leaf colouration as an honest signal of herbivore defence.

1.2.2.3. Review of ACH

There are 97 citations of Hamilton and Brown's (2001) ACH (Web of Science 26/3/2012). Of these, however, only 13 investigate the relationship between colouration and herbivory in the field, while only eight look specifically at autumn colouration and herbivory. The remaining articles supply reviews (Ougham et al. 2008), alternative hypothesis (Yamazaki 2008) or criticisms of Hamilton and Brown's hypothesis (Wilkinson et al. 2002). Of the few studies purporting to test the autumn colouration hypothesis empirically, none have demonstrated all of the steps necessary for the hypothesis to work: first, that there is natural variation in colour intensity among plants (and at least some of this is due to fixed genetic effects), and that this variation reflects differences in defensive capabilities; second, that potential herbivores are able to discern differences in colour intensity; third, that these herbivores choose between hosts based on differences in intensity; fourth, that herbivores reduce the fitness of host plants, and fifth, that herbivores themselves suffer reduced fitness as a consequence of attacking intensely-coloured hosts. Satisfying these criteria represents an experimental challenge and requires collaboration between several disciplines: evolutionary ecologists and behaviourists, plant physiologists, chemists and sensory ecologists.

Nonetheless, some evidence for the ACH has been provided by modelling and experimental work. A game theoretic model of signalling by Archetti (2000) has argued that the ACH is evolutionary stable (an ESS) under reasonable conditions. The stability of the signal was found to collapse if the environment is too rich or poor i.e. too few resources to produce the signal or else such a great abundance of resources that costs become unimportant (Archetti 2000). For the signal to be reliable the production and maintenance of the colouration must also be costly, but this cost is lower than the cost incurred by herbivory. Finally, honest signalling is only possible if the cost of producing the signal is relatively greater for lower quality plants than for high quality plants, consistent with the handicap principle (Archetti 2000; see also Zahavi 1975). Nine studies have sought to test the ACH empirically; of these, five report a negative correlation between red colouration and herbivory (Hamilton and Brown 2001, Archetti and Leather 2005, Rolshausen and Schaefer 2007, Ramirez et al. 2008, Archetti 2009), while one study found no relationship between red leaf colouration and the number of aphids on the plant (Schaefer and Rolshausen 2007). A further study reports a negative correlation between yellowness of leaves and insect damage (Hagen et al. 2003). More recently, however, this result has been challenged by studies by Holopainen et al. (2009) and Sinkkonen et al. (2012) which report a positive correlation between leaf yellowness and aphid number. A more complete description of these studies, including their main findings and limitations, are provided in Table 1.2 (overleaf).

Table 1.2. Studies investigating the ACH. Principal findings, focal herbivores and hosts, and criticisms are presented.

Study	Finding	Host species	Herbivore	Limitations
(Hamilton and Brown 2001)	More intense redness and yellowness resulted in a greater diversity of specialist aphids	262 species of tree	Specialist aphids	Brightness was analysed using a field guide and relies on the author of the guide to select a tree which is representative of the species. Tree damage was estimated as the diversity of specialist aphids. Given that a single aphid species can cause severe defoliation (May and Carlyle 2003), the usefulness of aphid diversity as a measure for herbivore damage is questionable. Colouration was compared between tree species, whereas to fully address the question intraspecific differences need to be examined. Intensity of signalling by an individual relative to its conspecifics is what is most important in competition; traits evolve because they confer an advantage in gene transmission over competitors of the same species (Maynard Smith and Harper 2003).
(Archetti and Leather 2005)	Fewer aphids were found on trees with a higher percentage of yellow and red leaves.	*Prunis padus* Bird Cherry Tree	*Rhopalosiphum padi* Bird cherry – Oat aphid	Red and yellow leaves are assumed to be conveying the same information, even though the pigments responsible for these colours are synthesised in separate pathways. Colour was measured visually as a percentage of red and yellow, and intensity was not measured. Too few aphids survived to measure growth rate in the spring, so the fitness consequences of aphid preferences remain unknown.
(Archetti 2009)	Aphids were more abundant on green and yellow autumn leaves than on red leaves and this is reflected in aphid survival. Trees with high disease susceptibility had red leaves. Trees with red leaves produced fruits that were small and tangy.	*Malus pumila* Apple tree	*Dysaphis plantaginea* Rosy apple aphid	Cultivated trees were used, which potentially conflate the processes of natural and artificial selection on leaf colouration. Colour estimates were very approximate e.g. a tree was scored as 'green' if > 80% of leaves were green and intensity was not measured. Colour estimates were also made through photographs from locations that were not visited. Again, this relies on the photographer to select a tree which is representative of the species. Data on apple size and taste were obtained from an online database; the various sources are potentially inconsistent. Autumn colours in cultivated areas in the UK were compared with wild plants in Kazakhstan to investigate whether colours were less common under domestication. There are many variables which would affect results between two such large ranging regions including temperature variation which could potentially confound results.
(Hagen et al. 2003)	A negative correlation was found between the percentage of yellow leaves and leaf damage by herbivores. Positive correlations were found between herbivore damage and fluctuating asymmetry (a measure of tree	*Betula pubescens* Downy birch	Unknown (not an aphid)	Leaves grazed by unknown herbivore(s) in the summer time were investigated rather than counting aphids in the autumn. It is unknown whether the herbivore(s) colonise(s) in the autumn time and therefore whether autumn colouration influences host choice. Colouration was recorded as the percentage of yellow vs. green leaves and intensity was not measured.

	quality) and number of seeds.			
(Holopainen et al. 2009)	More aphids were found on yellow leaves than on green leaves	*Betula pendula* Silver birch	*Euceraphis betulae* Silver birch aphid	Aphids were monitored on 31st July, meaning that the yellow colouration is likely to reflect poor tree health rather than autumn colouration. The confounding factor of age was not controlled for when analysing yellow colouration. Some of the foliage of the trees dies due to anoxia, suggesting that high stress levels may have produced confounding affects. Colour measured by eye rather than more accurately using a spectrophotometer and intensity was not measured.
(Ramirez et al. 2008)	More aphids were found on green trees (and leaves within trees) than on red trees. Herbivores preferred to spend more time in green rather than red illuminated regions on a petri dish in the absence of olfactory cues. No fitness differences were recorded in the spring between aphids on green vs. red trees.	*Nothofagus alessandrii*	*Neuquenaphis staryi* Southern Beech aphid	The authors photographed each leaf to measure RGB, using R/G values in the analysis. While this is more accurate than measuring colour by eye, this approach does not indicate what spectra can be seen by the insects. Intensity was not measured. Leaves within trees were compared, but the non-independence was not taken into account in the analysis.
(Rolshausen and Schaefer 2007)	Fewer aphids were found on trees with stronger colouration. The number of fruit (of the current but not the following year) negatively correlated with percentage of red leaves. In populations in cooler regions, trees had a lower percentage of red leaves.	*Sorbus aucuparia* Rowan	winged aphids	Trees were categorised as pale medium or strong according to the percentage of red leaves and colour and intensity was not measured. The number of aphids at one site was extremely low, which indicates that the selection pressure on colour-based signals in this location may not be particularly strong.
(Schaefer and Rolshausen 2007)	Aphid numbers were not influenced by tree colour (tree colours were manipulated by spraying with paint.) Aphid numbers strongly correlated with fruit production – trees with higher reproductive investment were preferentially attacked.	*Sorbus aucuparia* Rowan	*Dysaphis sorbi* Mountain ash aphid and *Rhopalosiphum insertum* Apple-grass aphid	The study recorded the percentage of leaves with the colouration and did not measure intensity. There was no grading of colour – trees were simply labelled as either red or green. Aphid numbers dropped after manipulation but in the absence of a suitable control it is not possible to rule out an effect of the painting method on aphid numbers. The size of the tree and number of leaves were not controlled for in the analysis.
(Sinkkonen et al.	There was a positive correlation between	*Betula*	*Euceraphis betulae*	The arrival of most winged female aphids did not correspond with the variable

2012)	yellow reflectance and aphid abundance. The authors showed that the intrapopulation variation in autumn leaf colouration has a genetic component.	*pendula* Silver birch	Silver birch aphid	colouration period. This suggests the evolution of autumn colouration more likely relates to timing of tree colours than intensity of the colouration in this system.

Around half of the studies (5/9) present a negative correlation with colouration and herbivory, which is consistent with the ACH. There are a number of significant flaws with studies of the ACH, among which are the use of artificially selected cultivars and failure to control for the age of the leaf (Archetti 2009, Holopainen et al. 2009; see Table 1.2). More serious is the failure to consider the colour from the herbivore's perspective (i.e. using the herbivore's spectral sensitivities rather than our own) and the absence of detailed chemical analyses of the plants needed to determine the correlation between chemical defence and colour intensity. I address each of these problems in turn below, before moving to address some of the other difficulties with testing the ACH.

How does chemistry relate to colour?

Some studies have examined plant chemistry indirectly by examining herbivore fitness in the spring or by carrying out herbivore performance tests (Table 1.2). To date, however, no studies investigating colouration at autumn time have looked directly at the correlation between leaf colour and chemistry.

It is important that different colours such as red and yellow are analysed separately as they may potentially convey different information, particularly if they are produced by different chemicals and show different patterns of linkage. Indeed, red and yellow colouration at autumn time may be under different selection pressures (Lev-Yadun and Keasar 2012) and it is therefore important that we consider these separately.

Classifying the signal

The accurate measurement of the intensity of leaf colouration through the eyes of insect herbivores is one of the outstanding challenges of testing the autumn colouration hypothesis. It is important to note that the original ACH explicitly focuses on the intensity of colour; thus the hypothesis is not concerned with how herbivores choose *between* colours but how they react to varying levels of a single colour. To consider the choice between two different colours is thus inappropriate.

A review of the empirical studies of the ACH suggests that researchers have been confused on this point, focusing on herbivore preferences for different colours rather than different levels of intensity. In their original paper, Hamilton and Brown (2001) rank trees 1, 2, or 3 for dull, medium or intense colouration. Subsequent studies citing Hamilton and Brown (2001), however, have not followed suit. In a recent paper, Archetti (2009) notes that "it has been difficult to compare green and red trees because it is difficult to find a species with a clear polymorphism. Ideally mutants for leaf colour should be used." This remark serves to

underline the misunderstanding surrounding the original hypothesis. Contrary to the above suggestion, a discrete colour polymorphism is not necessary to test the ACH; rather, intraspecific variation in the intensity of a single colour is required. Many of the studies described in Table 1.2 looked at the percentage of leaves of a particular colour (e.g. red or yellow) within a tree, arguing that a tree with a high percentage of red (or yellow) leaves is signalling at a high intensity. This approach, however, is of limited use when testing the ACH as no distinction is made between a plant with a high proportion of bright red leaves and one with a similar proportion of dull red leaves. To repeat, a plausible test of the ACH as proposed by Hamilton and Brown (2001) requires that we look at intensity or brightness of a single colour, not differences between two colours.

It is necessary to illustrate the above mentioned signal intensity, in particular the two possibilities of differing intensity: colour and brightness. Figure 1.4 shows the two possibilities for a weak red signal compared to a strong red signal. Figure 1.4a shows a weak signal. Figure 1.4b shows a leaf with increased redness: the stimulation of the red receptor compared to the other receptors (e.g. the red to green ratio) is higher than in Figure 1.4a. Only the part of the spectrum in the red region has increased. This differs from Figure 1.4c where the whole leaf is brighter, therefore the level of red receptor stimulation is higher than in Figure 1.4a) but so are all the other receptors. Both 1.4b and 1.4c have the same amount of red; it differs only in the ratio with other receptors. Figure 1.4a and 1.4c have the same ratios but 1.4c has higher brightness. It was not specified in the ACH which of these two processes are to be studied when looking at red intensity, so both will be investigated throughout the course of this thesis.

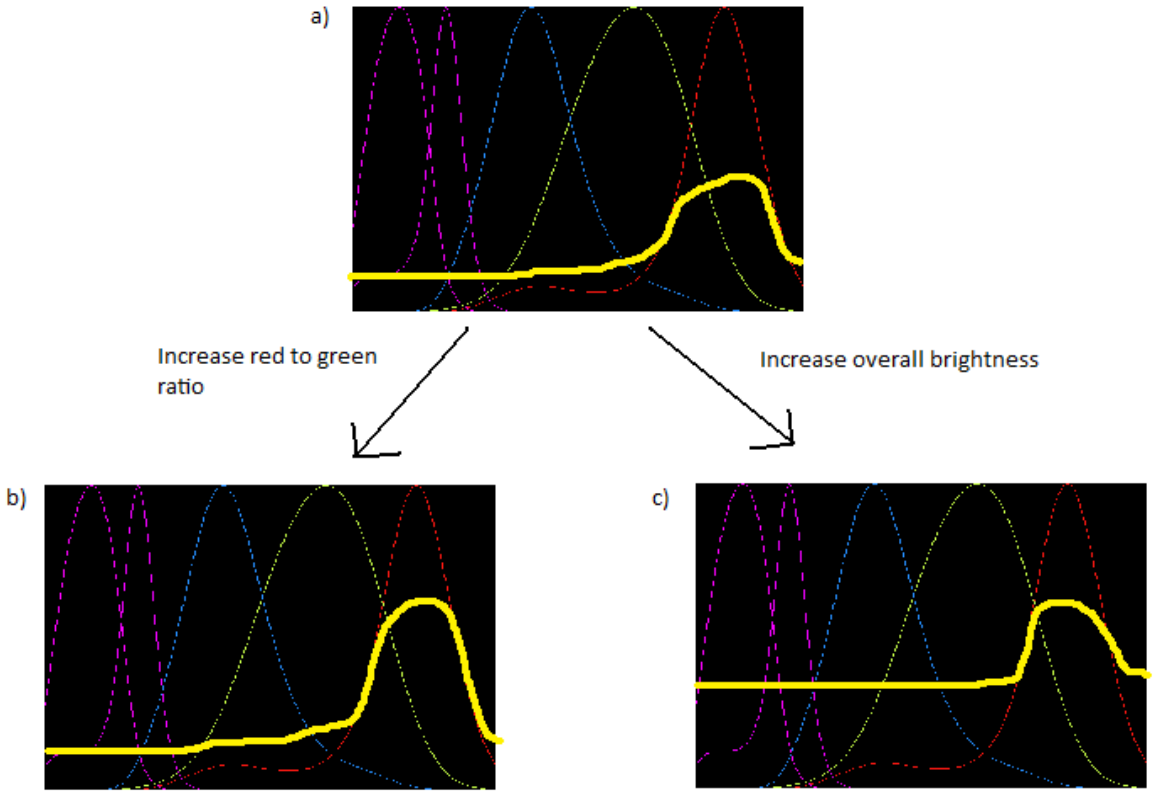

Figure 1.4. Possible mechanisms for increasing the intensity of the red signal. The coloured peaks are *Pieris* spectral sensitivities (Shimohigashi and Tominaga 1991). The yellow line shows spectra of a hypothetical leaf differing in redness: (a) a weakly signalling leaf, (b) a strongly signalling leaf in terms of red ratio (chromatic signal) and (c) a strongly signalling leaf in terms of brightness (achromatic signal). See text for details.

Colour from the insect's perspective

Of the studies that investigate the ACH in Table 1.2, all except Sinkkonen et al. (2012) classify colour according to human categories. Aphid and human colour vision is very different as aphids lack the red receptor found in humans but have a unique UV-sensitive receptor (Kirchner et al. 2005). It is therefore uncertain whether human classifications of colour have any meaning for aphid vision, and it is important to consider this when measuring leaf colour.

The problem with trees

Although focusing in particular on autumn colouration in trees, Hamilton and Brown (2001) were careful to state that their hypothesis may apply to other plant groups and their herbivores. Given this, it seems surprising that nearly all studies to date have focused on trees, which pose serious challenges due to their large size and longevity, as well as the potential for the fitness effects of herbivory to carry over a number of years. In addition, leaf age will be confounded with colouration when studying colour in autumn. For instance, do aphids avoid red leaves because they are highly defended or because red is a cue that the leaf is soon to fall

from the tree? In order to investigate whether plants signal their defensive capabilities to insect herbivores through leaf colouration, as predicted by the ACH, we therefore require the following:

- A study system practically easier to study than trees at autumn time where leaf colour is not affected by leaf age and where host-herbivore interactions are well-described;
- A study system in which host leaf chemistry is thoroughly described, allowing for a detailed analysis of specific defence compounds to be undertaken, and
- A way of measuring colour as seen by the insect herbivores.

In this thesis, I address these problems using wild *Brassica oleracea* and its herbivores. Intraspecific colouration is present in this species throughout the year, not only at autumn time. As *B. oleracea* is a small shrub, it is easier to grow than a tree and also easier to search among its leaves for herbivores. *Brassica oleracea* has many herbivores, including a variety of specialists, which are well studied in terms of their visual perception. *Brassica oleracea's* defensive chemistry is well documented. These reasons make it an excellent system for which to investigate the ACH.

1.3. The *Brassica oleracea* L. system

1.3.1. *Brassica oleracea* natural history

Brassica oleracea (in its uncultivated form: wild cabbage; in its cultivated form: the cabbage) is a perennial forb in the order Brassicales (Mitchell and Richards 1979). The wild form has a strong tap root and a single stem that can grow to 7cm thick depending on the age, which can be as much as 30 years. *Brassica oleracea* can grow over 1m in height or else may spread along the ground. The leaves are broad and rounded in shape, and are glabrous and glaucous in appearance. Around 10 flowering stems are produced, which produce 10-100 yellow flowers (Mitchell and Richards 1979). These form long cylindrical seed pods later in the season (these can be seen in Figure 1.5a). *Brassica oleracea* is located along maritime cliffs of the south coast of England, typically along chalky cliffs (Wichmann *et al.* 2008) (see Figure 1.5b). Wild *B. oleracea* populations in Britain vary in size: in some areas plant numbers can be very high (in the tens of thousands) while in others there are just a few hundred plants (Mitchell and Richards 1979). *Brassica oleracea* is tolerant of the harsh weather and exposed windy, salty conditions found on cliff tops.

Figure 1.5. *Brassica oleracea* and its habitat. (a) *B. oleracea* plant – note the rounded leaves and seed pods on the flowering stems; (b) *B. oleracea's* typical habitat on a chalk cliff on the south coast of England (Old Harry in Dorset).

1.3.2. *Brassica oleracea* defence

The main defensive chemicals in brassicas are the glucosinolates. Glucosinolates are also known as the mustard oil glycosides and are responsible for the sharp, bitter taste that we favour in cultivated forms. As with many crop plants that have undergone artificial selection, the amount of defensive chemical present in the plant has been reduced to improve the taste. Our extensive knowledge of glucosinolates can be partly attributed to their presence in the model plant *Arabidopsis* (Kliebenstein 2004, Grubb and Abel 2006).

The structure of a glucosinolate consists of a beta-thioglucose moiety, a sulfonated oxime moiety and a variable r-group which is derived depending on the type of amino acid used (Halkier and Gershenzon 2006) (Figure 1.6). Glucosinolate compounds can be classified according to the amino acid precursor and modification to the r-group. There is a huge diversity of in the structure of glucosinolates between and within species (Halkier and Gershenzon 2006). A simplified diagram showing the production of the glucosinolate core structure is shown in Figure 1.6. Amino acids are converted to aldoximes by chytochrome p460s in the CYP79 family (Halkier and Gershenzon 2006). Via several intermediates, these are then converted to thiodroximic acid molecules by the aldoxime-metabolising enzyme CYP83 (Halkier and Gershenzon 2006). Finally, the acid molecules are converted into the core glucosinolate structure. After this step, a wide range of modifications to the side chain can take place. The production of the enzymes involved in the biosynthesis regulates the type and concentration of glucosinolates (Grubb and Abel 2006).

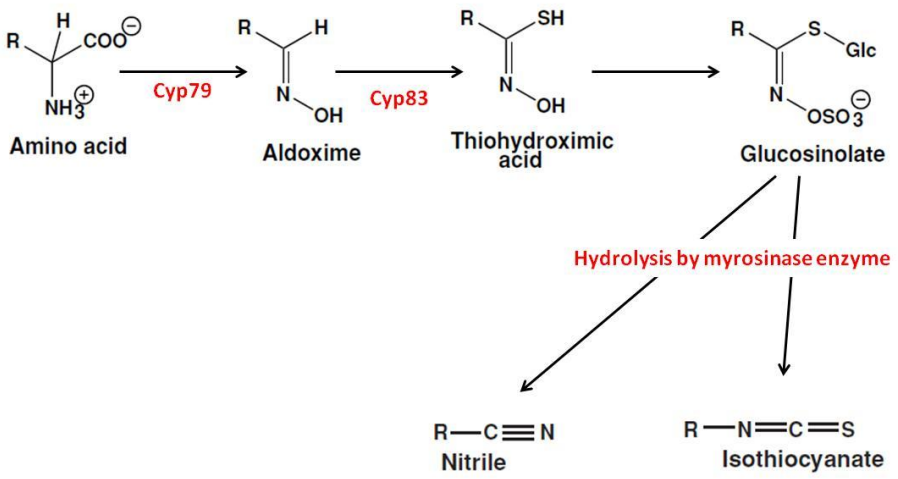

Figure 1.6. Glusosinolate biosynthesis and hydrolysis (produced from Halkier and Gershenzon (2006)). See text for details.

There are three types of glucosinolate: aliphatic, indole and aromatic glucosinolates. The differences between these are highlighted in Table 1.3.

Table 1.3. Differences between the three types of glucosinolate.

	Aliphatic	**Indole**	**Aromatic**
Amino acid derivative (Halkier and Gershenzon 2006)	Methionine	Tryptophan	Phenlyalanine/ tyrosine
% of known structures (Hopkins et al. 2009)	50%	10%	10%
Influence (Raybould and Moyes 2001)	Strong genetic control	Genetic control and environmental factors	Genetic control and environmental factors
Induction (Textor and Gershenzon 2006)	Induced at low levels	Induced to high levels	Induced to low levels
Main product formed upon hydrolysis	Isothiocyanates and nitriles	Nitriles	Isothiocyanates and nitriles
Volatile release on hydrolysis?	No	Yes	Yes
Examples	Glucoiberin, sinigrin, glucoraphanin, gluconapin, progoitrin.	Glucobrassicin	Benzyl glucosinolate

The toxic effects of glucosinolates come from hydrolysis products. Hydrolysis is initiated by tissue disruption, so that when an insect attacks it is faced with the 'mustard oil bomb' (Ratzka et al. 2002). The hydrolysis reaction is catalysed by the enzyme myrosinase and occurs because the enzyme and glucosinolate are stored in separate cells or separate areas within cells, which are brought together when the cells are broken by herbivore damage. Myrosinase hydrolyses the glucosinolate to different end products depending on the r-group of the glucosinolate and presence of cofactors. The end product is most commonly an isothiocyanate but can also be a nitrile, which is favoured by a pH of less than 3 and the presence of Fe^{2+} ions (see Figure 1.6) (Halkier and Gershenzon 2006).

Glucosinolates play an important role in brassica-herbivore interactions (Newton et al. 2009a). As they are the main defence in brassicas, any potential signal of chemical defence is most likely to involve glucosinolates. While it is known that some of the red colouration in *B. oleracea* is caused by anthocyanins (Lo Scalzo et al. 2008), there is little evidence that anthocyanins themselves are toxic to herbivores (Hughes et al. 2010) and I will not consider them further in this thesis.

1.3.3. Herbivores of *B. oleracea*

A variety of herbivores feed on *B. oleracea*, many of which will be known to gardeners as serious garden pests (Kirk 1992). Generalist molluscs feed on the leaves of *B. oleracea*, as do a large number of more specialised herbivores that possess specific defences to overcome the toxic effects of glucosinolates. These include many beetle species, in particular members of the Chrysomelidae (e.g. the cabbage stem flea beetle (*Physlliodes chrysocephala*; Figure 1.7b)

and cabbage small striped flea beetle (*Phyllotreta undulata*) and Curculionidae (e.g. cabbage seed weevil (*Ceutorhynchus quadridens*) (see Figure 1.7a) and cabbage stem weevil (*Ceutohynchus assimilis*)). Seeds weevils feed on pollen and lay their eggs in the seed pod, whereas flea beetles feed on the leaves. Many lepidopterans can be found on *B. oleracea*, including *Plutella xylostella* (diamond-back moth), *Evergestis forficalis* (garden pebble moth) (Figure 1.7d), *Pieris rapae* (small cabbage white), *Mamestra brassicae* (cabbage moth), and *Pieris brassicae* (large cabbage white) (see Figure 1.7c). The adults of these species oviposit on *B. oleracea* and the emerging larvae feed on the leaves. Some species of Hemiptera are also pests of *B. oleracea*. *Aleryrose protella* (cabbage whitefly) is a very common herbivore of *B. oleracea*, as are the aphid species *Brevicoryne brassicae* (the cabbage aphid) and *Myzus persicae* (the peach-potato aphid), which feed on phloem sap from the plant.

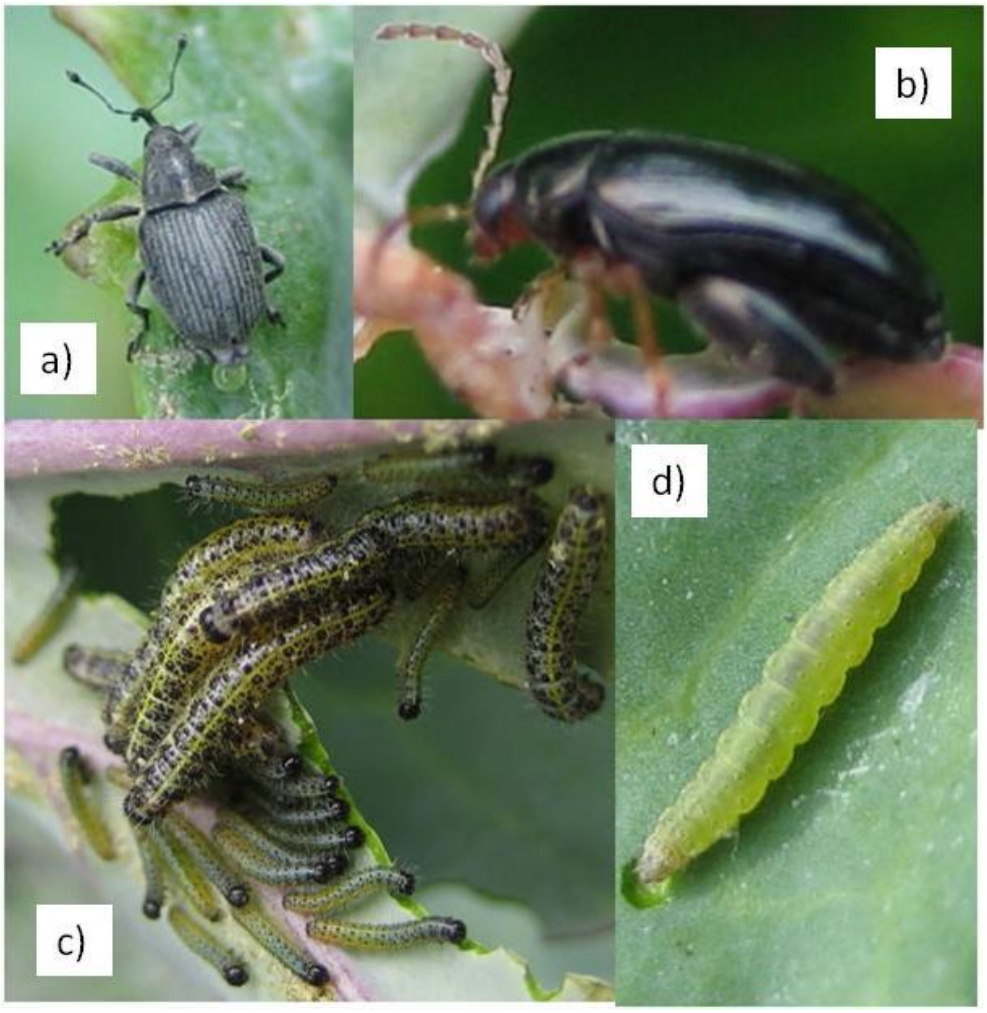

Figure 1.7. Common herbivores of *B. oleracea* (a) cabbage seed weevil, (b) cabbage stem flea beetle, (c) large cabbage white larvae and (d) garden pebble moth larvae.

In the remainder of the thesis I focus in particular on three of these herbivores - *Pieris rapae, Brevicoryne brassicae* and *Myzus persicae*. Of the many herbivores of *B. oleracea*, *P.*

rapae, B. brassicae and M. persicae are the only ones for whom spectral sensitivities are known (Qiu and Arikawa 2003, Kirchner et al. 2005), meaning that it is possible to explore visual responses to leaf colouration in these species. In addition, specialist pests are most important to look at in terms of the ACH (Hamilton and Brown 2001). Both *P. rapae* and *B. brassicae* are specialist herbivores, whereas *M. persicae* is a generalist and is included as a comparison to the specialists. In the following sections I provide a more detailed introduction to each of these species.

1.3.3.1 Pieris rapae (Linneaus 1758)

Natural history

Pieris rapae is in the family Pieridae. The females lay eggs singly, normally on the underside of a cabbage leaf (see Figure 1.8). The eggs are ridged, cone-shaped and slightly yellowish in colour. An adult can lay hundreds of eggs during her life. The larvae take a few days to emerge from the egg (see Figure 1.8). The larvae are green, with a faint yellow line down the side, hairy and slow moving and they feed on the leaves of brassicas. The larvae have five different instars, taking about three weeks to turn into pupae, with the adults emerging approximately one week later (Richards 1940) (see Figure 1.8). The adults are strong, fast fliers and can live up to one month, during which time they feed on nectar. The females can easily be distinguished from the males by the presence of two spots on their upper wing, while the males have one or no spots (see Figure 1.8). There are usually three generations per year (Richards 1940).

Figure 1.8. Stages of the *P. rapae* life cycle. Clockwise from top left: eggs on the underside of a leaf; larvae feeding on a leaf; fresh pupae; male (right) and female (left) adult butterflies.

Reaction to glucosinolates

As I describe above, the main defence of *B. oleracea* is provided by the hydrolysis of glucosinolates to form highly toxic isothiocyanates. In order to feed extensively on a brassica, herbivores must either prevent this reaction from happening or divert the hydrolysis of the glucosinolate into a less toxic product than an isothiocyanate. In the case of *P. rapae*, the larvae secrete a gut protein known as the nitrile specifer protein (NSP), so-called because it redirects the hydrolysis reaction of glucosinolates to form nitriles, which are less toxic than isothiocyanates (Wittstock et al. 2004). This process is presumably costly due to the production of the protein.

Glucosinolates are strong oviposition stimulants for *P. rapae*. Oviposition behaviour has profound consequences for offspring fitness; *Pieris rapae* larvae are relatively immobile and their survival therefore depends on host selection by the parent (Renwick & Chew 1994). Both aliphatic and indole glucosinolates can act as oviposition stimulants. For example, butterflies were found to oviposit preferentially on wild type *Arabidopsis thaliana*, compared to mutants expressing low levels of indole glucosinolates, indicating that indoles act as a stimulant to oviposition (de Vos et al. 2008). The effect of oviposition stimulation by glucosinolates is so strong that even sinigrin applied to paper can stimulate oviposition (Traynier and Truscott 1991).

Although glucosinolates are strongly attractive for oviposition, there is evidence that glucosinolates can have a negative effect on *P. rapae* fitness. While larvae are able to detoxify the glucosinolate hydrolysis products, there is nonetheless evidence that isothiocyanates can act to reduce larvae survival and growth (Agrawal and Kurashige 2003). Plants with elevated (induced) levels of glucosinolates are not only less attractive to ovipositing females but also adversely affect larval growth compared to uninduced plants (Agrawal and Kurashige 2003, Bruinsma et al. 2007). There is also evidence that those glucosinolates present at low concentrations within plants have a relatively stronger effect on *P. rapae* fitness, presumably because *P. rapae* is well adapted to the most abundant glucosinolates. For example, larval development was found to be more strongly affected by the level of neoglucobrassicin (an indole) than by total glucosinolates levels (Gols et al. 2008b). Similarly, ovipositing butterflies have been shown to avoid transgenic *Arabidopsis* engineered to produce high level of nitriles rather than isothiocyanates (de Vos et al. 2008).

Vision

P. rapae has nine photoreceptors in each ommatidium of its compound eye. The rhabdom in each ommatidium is tiered so that receptors 1-4 (R1-4) make up the distal tier, R5-8 the proximal tier and R9 is at the basal part of the rhabdom (Qiu et al. 2002). R1-2 express either ultraviolet, violet or blue rhodopsins which have peak sensitivity at 360nm, 425nm and 453nm respectively. R3-8 express a green rhodopsin which peaks at 563mm. In the distal part of the rhabdom, a red screening pigment surrounds the rhabdom and shifts this sensitivity of the R5-8 to red (Wakakuwa et al. 2004). To complicate matters, there are three different types of ommatidia, two of which have a pale red sensitivity (peaking at 620nm) and one with a dark red sensitivity (peaking at 640nm) due to different red pigment filters(Qiu and Arikawa 2003) (see Figure 1.9 for a summary of sensitivities). This large range of sensitivities could have arisen because *P. rapae* has many visual tasks. Sensitivity in the UV range could assist with mate recognition (Arikawa et al. 2005), whereas medium wavelengths could be used to locate flowers for feeding (Kolb and Scherer 1982) and sensitivity in the long wavelength region may be important for host selection (Qiu and Arikawa 2003), although these regions are expected to overlap in function.

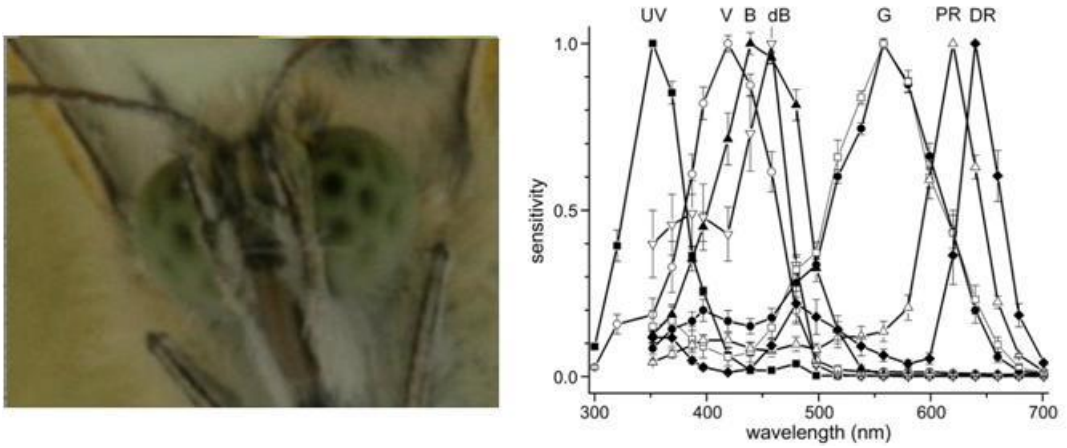

Figure 1.9. *Pieris rapae vision*. The photograph on the left shows *P. rapae* eyes, with individual ommatidia visible. The figure on the right shows spectral sensitivity of *P. rapae*, the peaks show peak spectral sensitivities (UV=ultraviolet, V=violet, B=blue, G=green, PR=pale red, DR=dark red). dB= double blue and is only relevant for *P. rapae crucivora* so should be ignored here (taken from Stavenga and Arikawa (2006))

There is a wealth of evidence indicating that *P. rapae* detects brassicas through olfaction (Van Loon et al. 1992), but also much to suggest that visual cues are important. *P. rapae* can associate coloured cues with the presence of sinigrin, such that in further experiments colour alone in the absence of sinigrin was sufficient to promote oviposition (Traynier 1984). Most oviposition experiments have used artificial coloured substrates, either lights (e.g. Scherer and Kolb 1987) or coloured paper (e.g. Traynier 1979). A concern with these approaches is how closely such substrates resemble natural leaves in terms of the wavelengths reflected – UV reflectance in particular is very difficult to produce accurately with coloured paper. The colours used in the laboratory may also be more extreme than that experienced in the field, (e.g. a bright red piece of card compared to a red tinted leaf). Using real leaves can overcome these problems. A study by Snell-Rood and Papaj (2009) found that *P. rapae* displays an innate bias to green rather than red cultivated *B. oleracea,* but with experience butterflies are able to learn to locate red plants just as efficiently as green plants.

1.3.3.2. Brevicoryne brassicae (Linneaus 1758) and Myzus persicae (Sulzer 1776)

Aphid natural history

Aphids (order Hemiptera, family Aphididae) are small, soft bodied insects that feed from the plant phloem. *Brevicoryne brassicae* and *M. persicae* are approximately 2-5mm. Aphids are unusual because they alternate periods of asexual reproduction, producing apterae (wingless) forms, with sexual reproduction, producing alate (winged) forms, which then disperse (Dixon 1985). Aphids generally produce alate forms if the colony becomes too crowded or if host quality decreases (Dixon 1985). Aphids are well-known for their ability to reproduce via parthenogenesis with telescoping generations (Dixon 1985). Many aphids undergo host

alternation, whereby two different hosts are used: a primary host on which it overwinters (normally a tree) and a secondary host on which it feeds during the summer months. The switching between the two occurs when soluble nitrogen levels change (Dixon 1985).

Brevicoryne brassicae is covered in a waxy coating which gives the aphid a powdery-bluish appearance. *Brevicoryne brassicae* only infests members of the brassica family and in large numbers can be serious pests (Hughes 1963). *Brevicoryne brassicae* overwinters on perennial brassicas and may then migrate to other brassica crops in the spring. *Myzus persicae* is a very well-known aphid because of its worldwide distribution and broad host range (Van Emden et al. 1969). It is green and has longer legs than *B. brassicae*. In the summer it can feed on an enormous variety of secondary host plants, including brassicas and members of the Solonaceae (e.g. potatoes).

Reactions to glucosinolates

B. brassicae is a specialist of brassicas and is able to fully exploit the plant's defence by sequestering the brassica's toxic chemicals. *Brevicoryne brassicae* has a glucosinolate-myrosinase system; it sequesters the glucosinolate from the plant and is able to produce its own myrosinase enzyme to break the glucosinolates down (Kazana et al. 2007). This means *B. brassicae* can become a "walking mustard oil bomb," capable of producing toxic isothiocyanates on attack by predators (Kazana et al. 2007). Interestingly, winged aphids sequester less glucosinolate than wingless aphids. Winged aphid defence lies in flight whereas the less mobile wingless aphids need chemical defence: energy for flight is suggested to be traded off for energy for sequestration (Kazana et al. 2007). This trade-off suggests there is a cost associated with this mechanism of biochemical defence.

M. persicae is unable to sequester glucosinolates and unlike the specific detoxification system of *P. rapae,* has a more general system which includes detoxification enzymes, for example Glutathione S-Transferases (GST) (Francis et al. 2003), as well as the classic detoxification system the cytochrome P-450 system (Schuler 1996). GST is found in many insects including both lepidopterans and dipterans (Francis et al. 2005). GST is induced when feeding (Francis et al. 2003) which may indicate that maintaining a reservoir of the chemical is costly to the aphid; if such a cost exists, there may be a selective advantage in identifying and targeting less well-defended hosts.

There is good evidence that both aphid species are attracted to glucosinolates, which presumably facilitates host identification (Nottingham et al. 1991, Visser et al. 1996). Clear evidence for an effect of glucosinolates on aphid fitness is lacking, however. It is likely that different glucosinolates have different effects, and that this also varies between brassicas. For

example, Mewis et al. (2005) found that numbers of *B. brassicae* and *M. persicae* were negatively correlated with glucosinolate concentration in *Arabidopsis*. The opposite result was obtained by Cole (1997), who found that *B. brassicae* grew faster on host species containing higher levels of glucosinolate.

Vision

Brevicoryne brassicae and *M. persicae*, like most other insects, have compound eyes. As their eyes are very small, resolution is probably very poor, although they do have colour vision (Land and Nilsson 2002, Doring and Chittka 2007). *Brevicoryne brassicae* and *M. persicae* have three spectral sensitivity receptor types: – ultraviolet (peak sensitivity at 320-330nm), blue (peak sensitivity at 440-480nm) and green (peak sensitivity at 530nm) (Kirchner et al. 2005) (see Figure 1.10). As *Myzus persicae* is the only aphid to have been physiologically tested for spectral sensitivity (Kirchner et al. 2005), I will make use of these sensitivities when modelling colour vision for both of the aphid species. Thomas Doring at The Organic Research Centre (Berkshire, UK) has carried out preliminary tests on *B. brassicae* spectral sensitivity, which appears to be very similar to that of *M. persicae* (T. Doring pers comms. 2010).

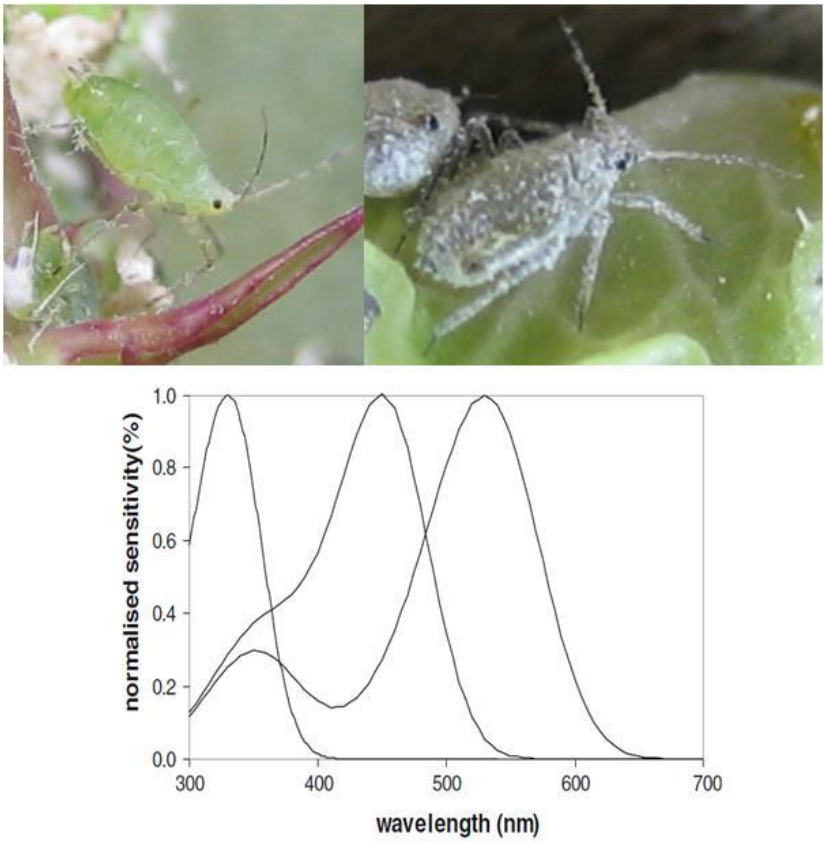

Figure 1.10. (Top left) *M. persicae* and (Top right) *B. brassicae*. (Below) Spectral sensitivity of *M. persicae* (reproduced from Doring and Chittka (2007)).

Aphid preference for yellow has been extensively studied, especially in terms of pest control; indeed many sticky traps are yellow in order to attract aphids (for a review see Doring and Chittka (2007)). Yellow leaves, most commonly either young or dying leaves, are characterised by high nitrogen translocation levels; a preference for yellow may therefore have evolved in order to exploit nutrients (Prokopy and Owens 1983). On a mechanistic level, the preference for yellow is still not fully understood. It has been suggested that yellow is preferred because it functions as a "super-normal foliage-type stimulus" by over-stimulating the green receptor (Prokopy and Owens 1983). An opponent channel of positive input from the green receptor coinciding with a negative input from the blue and UV receptor may, however, also explain this preference (Doring and Chittka 2007). As well as this preference for yellow, there is a general pattern of avoidance of red colouration. For example, the aphid *Neuquenaphis staryi* was found to prefer to occupy areas lit by green light rather than red light (Ramirez et al. 2008). With the exception of autumn colouration (see above), there has been very little work done on the response of aphids to natural colours as opposed to artificial substrates, and to my knowledge none using *M. persicae* or *B. brassicae*.

1.4. Thesis objectives and outline

In this thesis, I aim to test the assumptions and predictions of Hamilton and Brown's Autumn Colouration Hypothesis. Specifically, I aim to investigate whether host colouration functions to signal levels of defensive commitment to insect herbivores. In Chapter Two, I begin by assessing the strength of the current evidence for colour-based host selection by herbivores using a meta-analysis. In Chapters Three to Six, I then investigate the potential for colour-based signalling of chemical defence in the *B. oleracea* system. In Chapter Three, I explore the relationship between *B. oleracea* colouration, glucosinolate levels and insect herbivory in wild populations in the field, which forms the foundations of subsequent experimental work. In Chapter Four, I use a common garden design to determine firstly to what extent leaf colouration is under genetic versus environmental control and secondly to what extent genetic variation in colouration predicts herbivory. In Chapter Five, I then move to focus in detail on herbivory of *B. oleracea* by the specialist aphid *B. brassicae* and the generalist aphid *M. persicae*. For both aphid species, I use a combination of preference and performance experiments together with glucosinolate analysis to determine whether achromaticity functions as a cue or signal of chemical defence. In Chapter Six, I move to explore *B. oleracea* host selection by the butterfly *P. rapae*. Again using a combination of preference and performance experiments in conjunction with glucosinolate analysis, I test whether chromatic reflectance functions as a cue or signal of chemical defence. In Chapter Seven, I conclude with a discussion of the results presented in the previous chapters and determine the overall level of support for the Autumn Colouration Hypothesis.

Chapter Two. The influence of plant colouration on host selection by insect herbivores: a meta-analysis

2.1 Introduction

2.1.1. The importance of cues based on colour

Understanding the role of plant cues in host selection by insect herbivores is of fundamental importance to behavioural ecology, as well as having significant implications for the provision of key ecosystem services such as pollination and agricultural pest control (see Chapter One). Traditionally, studies of herbivore host selection behaviour have focused on olfactory cues (Bruce et al. 2005), while comparatively little is known about the use of visual cues (Doring and Chittka 2007), although their use has been explored in the context of flower choice by pollinators (Goulson and Cory 1993, Kandori and Ohsaki 1996). A number of insect groups have well-developed visual systems (Qiu and Arikawa 2003), and their use of vision has been well explored in other areas, for example mate choice (Bybee et al. 2012), so the relative lack of information on the visual mechanisms underpinning host selection by insect herbivores is a surprising gap in our knowledge.

The spectral properties of a plant potentially provide the insect herbivore with a colour cue. In the majority of leaves, the green appearance of the leaf is caused by the dominance of chlorophyll (Prokopy and Owens 1983), which gives the reflectance of leaves a characteristic peak at 500-580nm. Despite this dominance of chlorophyll, leaf colour varies dramatically, most obviously in variegated leaves, which lack chlorophyll in certain areas of the leaf (Campitelli et al. 2008). Additionally, plants may vary in colour due to the presence of other pigments. One example is a group of pigments known as anthocyanins, which are responsible for the reds and purples of leaves (Gould 2004). Often young leaves in the tropics undergo a process called delayed greening, whereby they delay the greening of their leaves until mature. During delayed greening, young leaves are often flushed with anthocyanins, which may protect them from light damage and herbivores (Coley and Barone 1996, Dominy et al. 2002). Another well-studied group of pigments are the carotenoids, such as carotenes, which colour leaves and other plant tissues yellow and orange (Zheng et al. 2010).

Leaf colour has the potential to inform insects about some aspect of plant quality, allowing them to select between hosts. The autumn colouration hypothesis (ACH) (Hamilton and Brown 2001) proposes that leaf colour signals the level of plant chemical defence (for a fuller account see Chapter One), but for such a signal to be effective, the insect herbivore must be able to detect the signal (i.e. the colouration). Aphids and butterflies have colour vision (see Chapter One), suggesting a selective advantage for this ability (Land and Nilsson 2002). Aphids have UV, blue and green receptors (Kirchner et al. 2005), thus they sense UV but see more poorly in the red region than humans. Butterflies commonly have five spectral photoreceptors

(red, green, blue, violet and UV) (Shimohigashi and Tominaga 1991), so their potential for colour discrimination is greater than our own.

There are a variety of insect herbivore responses to colour documented in the literature, making generalisations about the role of such visual cues in host selection difficult. For example, Wong and Srivastava (2010) found that the presence of red axial spots on the leaves of *Columnea consanguinea* in Costa Rica had a negative effect on the leaf area consumed by herbivores, while Lempa et al. (2001) studied delayed greening in mountain birch trees in the subarctic and found that leaf redness and amount of herbivory were positively correlated. This demonstrates how difficult it is to compare results: Wong and Srivastava (2010) looked at a colour phenotype that is expressed permanently in the tropics, whereas Lempa et al. (2001) examined delayed greening in the subarctic. It is clear from these results that responses are context dependent, with outcomes potentially dependent on the plant-herbivore system under study, its geographic location, and whether leaf colours change during development. Even looking within very similar systems, researchers have found contrasting results. For example, Hughes et al. (2010) found that the amount of red pigment on leaf margins of *Veronica* had no association with herbivory. In contrast, Cooney *et al.* (2012), while still studying red leaf margins, found that the widest red margins had the least herbivory in the field. In order to determine the strength and direction of herbivore colour preferences across these different studies we therefore require a meta-analytical approach. I provide a short introduction to this technique below.

2.1.2. Meta-analyses

The first meta-analysis was carried out in 1977 to investigate whether psychotherapy was an effective treatment (Smith and Glass 1977). There were hundreds of studies on psychotherapy with disparate results. This first meta-analysis standardized these results to demonstrate that there was an overall positive effect of psychotherapy (Smith and Glass 1977). Originally meta-analyses were used in medicine, but now their use is widespread, encompassing all areas of biology including behavioural ecology (e.g. Koricheva et al. 2004, Nakagawa et al. 2007)

Quantitative meta-analyses are a powerful and informative tool used to combine and compare results of experiments in order to identify underlying general patterns in the face of context dependency (Lipsey and Wilson 2001). By combining many studies with small sample sizes, it is possible to detect an effect that is not apparent when each study is considered in isolation. Meta-analysis requires the calculation of effect sizes in order to combine results. An effect size is the measure of the strength of the relationship between two variables, which provide standardized, directional measures of mean change in the dependent variable in each

study (Nakagawa and Cuthill 2007). Probability estimates (i.e. p-values) do not provide information about the magnitude or precision of the effect size; instead, the use of effect sizes with their confidence intervals permits accurate assessment of the strength of relationship between variables (Johnson 1999).

Effect sizes can be weighted so that studies with a lower variance (and larger sample size) are given more weight in the meta-analysis (Lipsey and Wilson 2001). Statistics are then applied to the effect sizes and their variance. In a meta-analysis, statistical analysis examines the effect size variation between and within studies and investigates how much of this can be attributed to moderator variables, which are the details recorded about each study: e.g. location and plant type (Harrison 2011). Moderator variables are factors about each study that are recorded because they may cause variation in effect size. In the meta-analysis, overall variation in effect size can be partitioned into variation explained by the moderator variables and variation that is unexplained. If the moderators explain a lot of the variation it means the effect size (here, the response to the colouration) can be effectively predicted by the factors recorded.

Publication bias is a serious problem in biology, whereby non-significant results (usually studies with small sample sizes and hence large standard errors or small effect sizes) are not published (Cassey et al. 2004). This can have serious implications for a meta-analysis because if data are missing the estimate of overall effect-size is skewed. Consequently, a meta-analysis is usually accompanied by a measure of publication bias to investigate the reliability of the result. The measure of publication bias is often an analysis of the correlation between effect size and sample size: the correlation will be zero if there is no bias because studies with small and large sample sizes will be equally represented, but there will be a correlation if the number of studies with small sample sizes that are published is smaller than expected. Funnel plots can be drawn as a visual representation of this (see Figure 2.1).

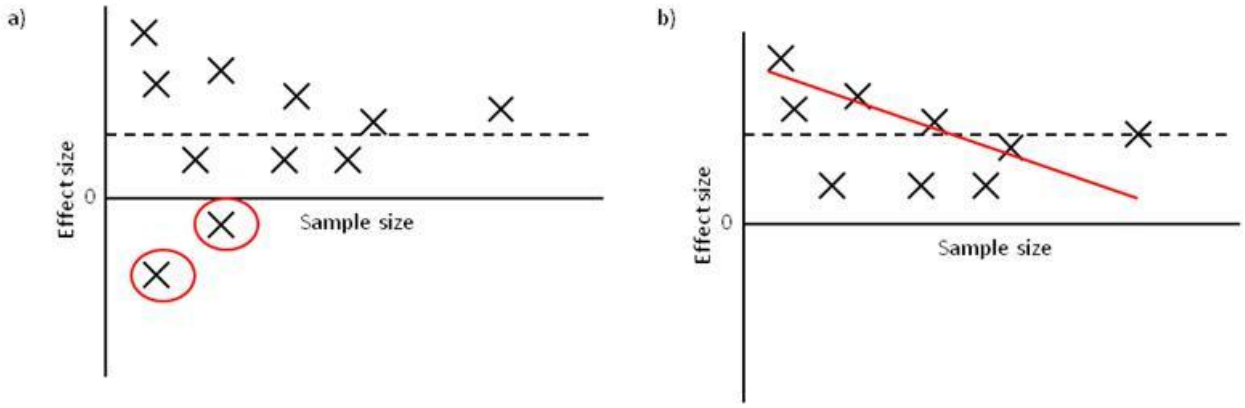

Figure 2.1. Funnel plots show how publication bias can be investigated. The dashed line shows the estimated mean effect size from a meta-analysis. It is called a funnel plot because studies with a large sample size have a more reliable estimate of effect size; hence they become funnelled towards the end of the plot. Figure 2.1a) shows an analysis where there is no evidence of bias – the effect sizes are symmetrically distributed such that there is no correlation between sample size and effect size. The studies circled in red show studies that are likely to have non-significant results as they have a small sample size and estimated effect sizes close to zero: these are the studies that are more likely not to be published and so be responsible for publication bias. Figure 2.1b) shows what the effect would be if these studies were not included in the meta-analysis. The funnel plot becomes asymmetrical, the effect size estimation is shifted and the red line indicates the correlation that becomes apparent.

2.1.3. Aim of meta-analysis

The aim of this meta-analysis is to quantify the effect of plant colouration on host choice by insect herbivores across the literature as a whole. Previous reviews on host choice and colouration have been entirely qualitative and only focused on colour use in a single context, e.g. autumn colouration (Archetti et al. 2009b), and as a result have not taken into account the strength of the relationship between variables or the variation in sample size. This meta-analysis thus represents a substantial contribution to the field as it seeks to quantify the relationship between colouration and host choice across all contexts. These different contexts will be dealt with through the use of moderator variables in the analysis (see below).

The ACH suggests that insects should avoid the most intensely coloured trees, i.e those most different from green (Hamilton and Brown 2001, Archetti and Leather 2005). In a green leaf, the dominant pigment is chlorophyll; however, in leaves of other colours (i.e. non-green colours), other pigments might be cues for herbivores. Any colour differing from green may be conveying information about chemical defence or some other aspect of host quality. There are a lot of different colourations in the literature that differ from green – for example, blue colouration, red colouration, variegation and delayed greening. In this analysis, I will consider all these as being in the opposite direction to green, that is, the effect of "non-green". The different colours and contexts (e.g. location, herbivore type) will be included in the meta-analysis as moderator variables to test whether different types of non-green colouration affect host choice. A positive effect size in this analysis would indicate a positive influence of green

plant colouration on host selection i.e. a preference for green rather than non-green colouration, whereas a negative effect size would indicate that green colouration is avoided in preference for non-green colouration. Large effect sizes demonstrate a strong influence of plant colouration on herbivore host choice and a small effect size a small influence. An effect size of r=0.1 is considered small, 0.3 medium and 0.5 a large effect size (Cohen 1988).

The strength and/or direction of any effect are likely to vary with a number of factors, including herbivore type, host type and differences in experimental design between studies. In all, eight potential sources of variation (see moderator variables below) were included in the analysis, based upon the following five questions:

1) Does herbivore response to green versus non-green colouration vary in different situations? From previous work, herbivore responses to non-green colouration are predicted to be negative across a range of contexts. In particular, insects are expected to respond negatively to red and yellow colouration in the autumn (Hamilton and Brown 2001), and to colouration of young leaves in plants exhibiting delayed greening (Kursar and Coley 1992). Red or blue colouration is also predicted to have a negative influence on oviposition (Kelber 1999).

2) Do different types of herbivores respond differently? Colour perception will differ between the insect groups, so potential variation in their responses to colouration is important to consider. The response of aphids is of particular interest as the ACH was formulated specifically with aphids in mind, and depends on aphids' ability to perceive and avoid autumn colours (Hamilton and Brown 2001), for this reason, aphids were considered separately from other Hemiptera in the meta-analysis. In general, it is expected that insects with more spectral types of photoreceptors, especially the Lepidoptera (Kelber 1999), will be more responsive to colour as they can discriminate a greater range of colours. In addition, specialist herbivores are likely to enjoy a closer co-evolutionary relationship with their host (Berenbaum and Zangerl 1998), and so may be more likely to respond to variation in host phenotype (see Chapter One).

3) Do herbivore responses to leaf colour vary with plant type? In its original formulation, the ACH explicitly considered the evolution of signalling between trees and their herbivores (aphids). While it is therefore clearly of interest to look at how insects use colour when selecting among host trees, it will also be important to consider herbivory of smaller plants such as herbs and forbs. This is because the fitness effects of herbivory on trees may well be relatively small and accrue over longer periods (Haukioja 2006). As a consequence, selection for defensive signals to deter herbivores and the associated costs of herbivory may be stronger for smaller, shorter-lived plants, where fitness effects of herbivory may be stronger.

4) Does the type of non-green colouration affect herbivore responses? The importance of considering different colour cues separately has been recently highlighted (Lev-Yadun and

Keasar 2012). Evidence for avoidance of red colouration by aphids is stronger (Ramirez et al. 2008) than for yellow colouration (Holopainen et al. 2009), indicating that anthocyanins and carotenoids are likely to influence herbivores to differing degrees. Variegated leaves versus non-variegated leaves differ in chlorophyll content as variegated leaves have white patches that lack chlorophyll. Variegation has been suggested to deter herbivores (Campitelli et al. 2008) and so it is predicted that herbivores will avoid variegated leaves lacking chlorophyll.

5) Does the experimental design deployed by the study affect herbivore responses to colouration? An important factor is whether researchers performed an observation or a manipulation. In many cases, colour stimuli used in manipulations (e.g. coloured cards in oviposition trials) contrast more strongly than leaf colours recorded in a field study, suggesting that the influence of colouration may be greater in manipulations than in observational studies. This question will also consider the effect of the location of the experiment on the relationship between colouration and herbivory, and finally whether the comparisons of herbivore host choice are made within host species (i.e. intraspecifically) or between species (interspecifically).

2.2. Methods

2.2.1 Literature search

A literature search was carried out using Web of Science from 4th March 2012 to the 20th April 2012, and included studies from 1950 until this date. Originally searches were carried out using: Topic=(host) AND Topic=(colour OR color) AND Topic=(insect herbivore). More specific searches were then performed: "delayed greening" AND herbivor*, "red leaves" AND herbivor*, oviposition AND colour OR color, aphid AND colour OR color AND host, "autumn colouration". References within articles and citations of the articles found were also followed up. For details of all studies included in the meta-analysis see Table 2.1.

Oviposition represents a special case of herbivory where the ovipositing female must choose the best host for her offspring. Given that the choice of leaf can have important consequences for the success of the offspring (Rausher 1979), I included studies exploring the effect of colouration on oviposition behaviour. Very little experimental work (only two studies to my knowledge) has been performed with natural leaves (Snell-Rood and Papaj 2009, Zheng et al. 2010) so it was necessary to include studies looking at oviposition on paper as well. The use of paper as a substrate is an advantage because it means that chemical cues can be completely controlled.

As a specific question was being considered, strict criteria were used when selecting studies to include in the analysis. First, I restricted the analysis to leaf colour only and omitted studies describing preference for coloured flowers or fruits, except in the case of oviposition, where studies using paper were included used in addition to leaves. Second, as I was interested only in colour cues, studies exploring other visual cues such as leaf shape and size were omitted. Third, I restricted the analysis to effects of leaf colour on herbivores, and omitted studies looking at responses of parasites or predators. Fourth, I considered only those colour cues made by the plant itself, and omitted cases where leaf colour resulted from pathogens such as leaf mines or fungi.

Only one effect size per study can be used in a meta-analysis in order to ensure the effect sizes are independent (Lipsey and Wilson 2001). Strict criteria were therefore also used in cases where studies presented results from multiple tests. First, where herbivory was quantified in a number of ways, I selected data on the most appropriate measure of herbivory. For example, where a study presented data on the number of leaves attacked and the amount of the leaf that was eaten, I chose the results for the number of leaves attacked as this is likely to relate more immediately to any initial choice made by the herbivore in response to colour cues. Second, where data were presented from field observations and lab experiments, I

preferentially selected the data from the field observations, regardless of sample size. This is because host use of colour in natural settings is more relevant to testing ecological theories such as the ACH (Hamilton and Brown 2001). Third, where data were presented from multiple tests in different contexts (e.g. upland and lowland sites), I selected the data set with the largest sample size. An exception to this rule was made in the case of the study by Spicer et al. (1995), who present two data sets from different years, with results for herbivory on green trees, red trees with permanent phenotypes and trees with delayed greening. The data could be separated into pair-wise comparisons (green trees versus permanent red trees and green trees versus delayed greening) for the different years, enabling herbivory in two separate and independent contexts to be analysed. Fourth, if a study reported colour preferences for wingless and winged aphids, then the results for the latter were selected as winged aphids are able to more disperse to different hosts.

The literature includes hundreds of studies on aphid preference of coloured sticky traps. The preference of aphids for yellow has been explored extensively in the context of pest control, and numerous studies have confirmed this preference (e.g. Doring and Chittka 2007). As the aim of this meta-analysis is to look specifically at leaf colour choice by herbivores, data on preference for sticky traps were not included here.

2.2.2. Moderator Variables

In order to determine possible sources of variation in the relationship between colour and herbivory, the following moderator variables were included in the analysis:

1) Situation – this moderator variable considers the situation in which the colour is produced (i.e. delayed greening (young leaves that delay their greening until mature), permanent phenotypes (plant differences that are not transient) or autumn colouration) and the situation in which host selection occurs (i.e. herbivory or oviposition).
2) Intra-or-interspecific comparisons i.e. whether herbivory was compared within or between plant species.
3) Plant type – tree, forb (any herbaceous plant other than a grass) or artificial.
4) Herbivore type – aphid, Coleoptera, Hemiptera, Lepidoptera or unknown (in cases where damage was measured but the herbivore was not identified).
5) Specialism – this describes whether the herbivore is a specialist, generalist or unknown (in cases where damage was measured but the herbivore was not identified).
6) Design – this concerns whether the studies were conducted as a manipulated experiment or responses observed in natural settings.

7) Location – the location where the study was carried out: tropics, temperate, Mediterranean or subarctic.
8) Chemical – this describes the chemical that is responsible for the signal: carotenoids (yellows and oranges), anthocyanins (red and purples) or chlorophyll (greenness).

Table 2.1. Studies included in the meta-analysis.

Study	n	Situation	Plant type	Herbivore type	Chemical responsible for colouration
(Lempa et al. 2001)	28	Delayed greening	Tree	Lepidoptera	anthocyanins
(Coley et al. 2005)	340	Delayed greening	Tree	Unknown	Chlorophyll
(Numata et al. 2004)	8	Delayed greening	Tree	Unknown	anthocyanins
(Kursar and Coley 1992)	5	Delayed greening	Tree	Unknown	Chlorophyll
(Karageorgou and Manetas 2006)	26	Delayed greening	Tree	Unknown	anthocyanins
(Raymond 1998)	18	Delayed greening	Tree	Coleoptera	anthocyanins
(Spicer et al. 1995)	39	Delayed greening	Tree	Coleoptera	anthocyanins
(Wong and Srivastava 2010)	300	Permanent phenotypes	Forb	Unknown	anthocyanins
(Hughes et al. 2010)	10	Permanent phenotypes	Forb	Unknown	anthocyanins
(Cooney et al. 2012)	98	Permanent phenotypes	Tree	Lepidoptera	anthocyanins
(Malone et al. 2009)	264	Permanent phenotypes	Forb	Lepidoptera	anthocyanins
(Campitelli et al. 2008)	394	Permanent phenotypes	Forb	Coleoptera	Chlorophyll
(Soltau et al. 2009)	3143	Permanent phenotypes	Forb	Lepidoptera	Chlorophyll
(Yue and Liu 2000)	18	Permanent phenotypes	Forb	Aphid	anthocyanins
(Prokopy et al. 1983)	40	Permanent phenotypes	Forb	Diptera	anthocyanins
(Rowe et al. 2002)	23	Permanent phenotypes	Tree	Coleoptera	anthocyanins
(Harris et al. 1995)	40	Permanent phenotypes	Tree	Lepidoptera	anthocyanins
(Spicer et al. 1995)	31	Permanent phenotypes	Tree	Coleoptera	anthocyanins
(Bullas-Appleton et al. 2004)	50	Permanent phenotypes	Forb	Hemiptera	anthocyanins
(Campbell 1991)	6176	Permanent phenotypes	Forb	Aphid	carotenoids
(Hamilton and Brown 2001)	262	Autumn	Tree	Aphid	anthocyanins
(Archetti and Leather 2005)	30	Autumn	Tree	Aphid	anthocyanins
(Archetti 2009)	80	Autumn	Tree	Aphid	anthocyanins
(Hagen et al. 2003)	24	Autumn	Tree	Unknown	carotenoids
(Holopainen et al. 2009)	418	Autumn	Tree	Aphid	carotenoids
(Ramirez et al. 2008)	16	Autumn	Tree	Aphid	anthocyanins
(Rolshausen and Schaefer 2007)	25	Autumn	Tree	Aphid	anthocyanins
(Schaefer and Rolshausen 2007)	29	Autumn	Tree	Aphid	anthocyanins
(Traynier 1984)	83	Oviposition	Artifical	Lepidoptera	carotenoids
(Snell-Rood and Papaj 2009)	60	Oviposition	Forb	Lepidoptera	anthocyanins
(Zheng et al. 2010)	60	Oviposition	Forb	Lepidoptera	Chlorophyll
(Stefanescu et al. 2006)	18	Oviposition	Forb	Lepidoptera	Chlorophyll
(Castrejon and Rojas 2010)	25	Oviposition	Artifical	Lepidoptera	carotenoids
(Ilse 1937)	65	Oviposition	Artifical	Lepidoptera	anthocyanins
(Hirota and Kato 2001)	11	Oviposition	Artifical	Lepidoptera	anthocyanins
(Hirota and Kato 2004)	16	oviposition	Artifical	Lepidoptera	Chlorophyll
(Traynier 1986)	177	Oviposition	Artifical	Lepidoptera	anthocyanins
(Degen and Stadler 1997)	11	Oviposition	Artifical	Diptera	carotenoids
(Kuhnle and Muller 2011)	17	Oviposition	Forb	Coleoptera	carotenoids
(Mercader and Scriber 2007)	81	Oviposition	Artifical	Lepidoptera	carotenoids
(Traynier 1979)	20	Oviposition	Artifical	Lepidoptera	anthocyanins

2.2.3. Statistical analysis

2.2.3.1. Effect sizes

In the analysis I define the directionality such that a positive effect size reflects a positive influence of green colouration i.e. the herbivore prefers green to non-green colouration, and a negative effect size indicates a negative response to green, i.e. the herbivore prefers the non-green colouration to the green colouration. The effect sizes and variances were calculated with online macros ((Lipsey and Wilson 2001), retrieved April 24th 2012 from http://mason.gmu.edu/~dwilsonb/ma.html). Effect sizes used in a meta-analysis must be consistent across the analysis (i.e. expressed as the same statistic throughout). Here, the effect size used was r (the correlation coefficient). In cases where the weighted mean difference (d) was calculated this was then transformed into r using the Excel macros ((Lipsey and Wilson 2001), retrieved April 24th 2012 from http://mason.gmu.edu/~dwilsonb/ma.html").

2.2.3.2. Meta-analysis

The meta-analysis was carried out in R (version 2.10.0) using the metafor package (Viechtbauer 2010). To meet the assumptions of normality, the effect size r was converted to Fisher's Zr (the transformed correlation coefficient).

A reduced major axis regression analysis (rma function; metafor package) was performed, which uses a weighted maximum likelihood method. It is important for the model to be weighted so that less precise effect size estimates influence the model less than more precise ones. This is done by weighting studies according to the variance in results, such that results with a high variance carry less weight. In the model, the effect size (Zr) was the response variable and the variance the predictor. Model simplification proceeded by backwards deletion of non-significant terms until further removals lead to significant ($p<0.05$) increases in deviance assessed using likelihood ratio test values. Significance levels are reported on the addition of non-significant terms and removal of significant terms from the minimum adequate model and models compared with likelihood ratio tests (LRT). The model provides measurements of heterogeneity (Q) in the output, which partition variation in effect size into variation that is explained and unexplained by the model.

To investigate publication bias a regression test (regtest function; metafor package) was carried out to look at the asymmetry around the funnel plot (see Figure 2.1).

2.3. Results

Results of the meta-analysis showed that herbivores preferred green plants. There was an overall positive effect of green colouration on herbivore choice of host plants (Zr = 0.231, 95% CI = 0.0468 to 0.4156, p=0.014) (Zr = transformed correlation coefficient: the effect size) (see Figure 2.3). This can be classified as a small effect size (Cohen 1988), indicating that plant colouration has only a small influence on host choice.

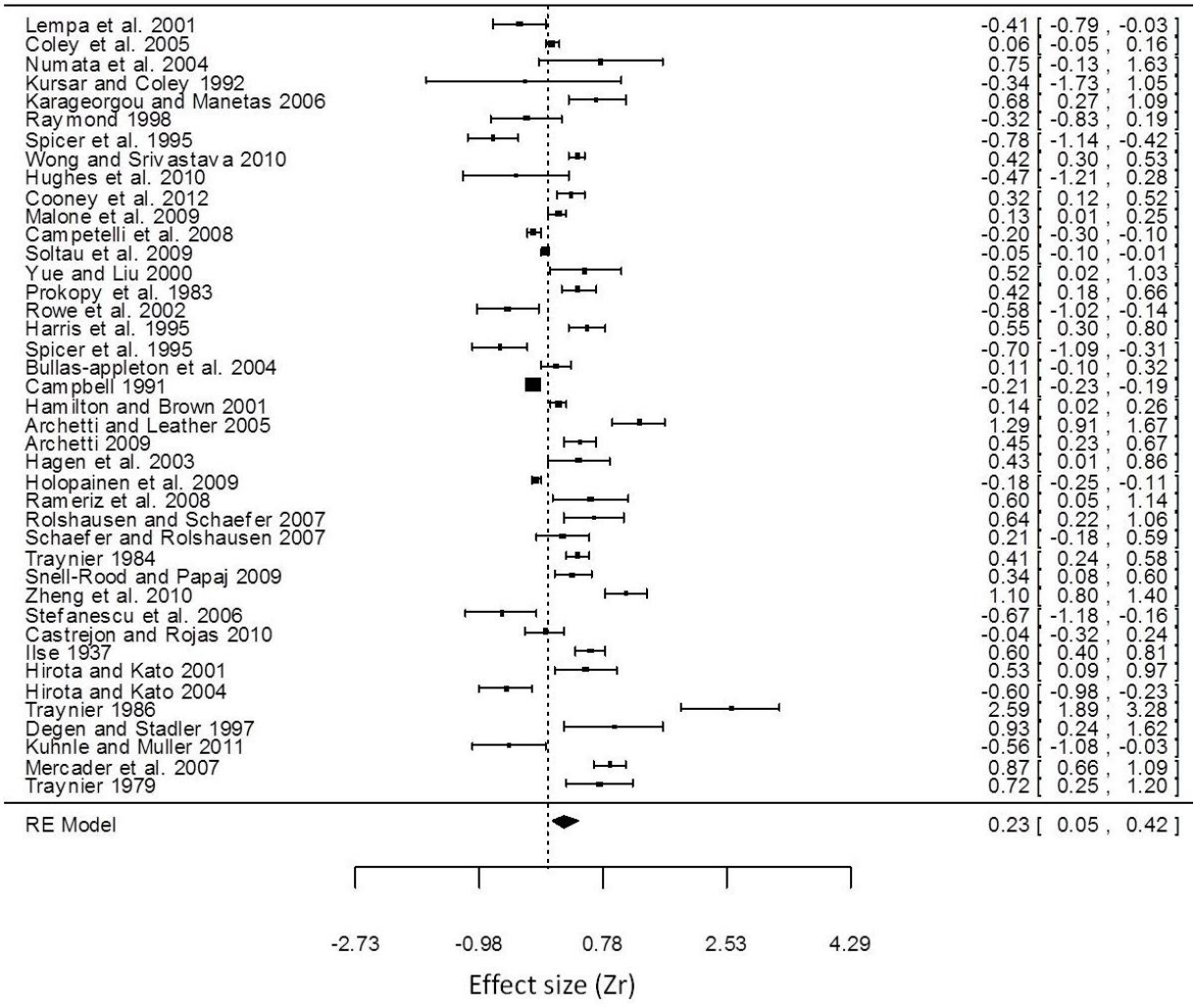

Figure 2.3. Forest plot showing the influence of colouration on herbivory (effect size (Zr)) in each of the 41 studies included in the meta-analysis. The plot is based on a random-effects model with no moderators. The numbers to the right are the effect size estimates with 95% confidence intervals. The dashed line shows a zero effect size. In the left hand column of the forest plot the list of studies can be seen. The central column displays their corresponding effect size (small black box) for each study with the confidence intervals (whiskers to the box). Most studies show a tendency towards favouring green, since most points lie to the right of the dashed line (zero effect), and many of the individual studies show a significant bias in this direction since their confidence intervals do not overlap the dashed line. The polygon at the bottom of the plot shows the mean effect size as calculated from the meta-analysis; as the points of the polygon do not overlap the dashed line of zero effect the result can be said to be different from no effect at the given level of confidence.

2.3.1. Publication bias

To check for bias in the effect size estimate, sample size was plotted against effect size (see Figure 2.4). The resulting funnel plot produced the characteristic funnel shape, indicating that studies with small sample sizes are more variable about the mean effect size, and studies with a large sample showed less variance about the mean. The test for funnel plot asymmetry was not significant (Z=-0.729, p=0.47; Fig 7). There was thus no evidence for publication bias, which might otherwise have skewed the mean effect size estimate.

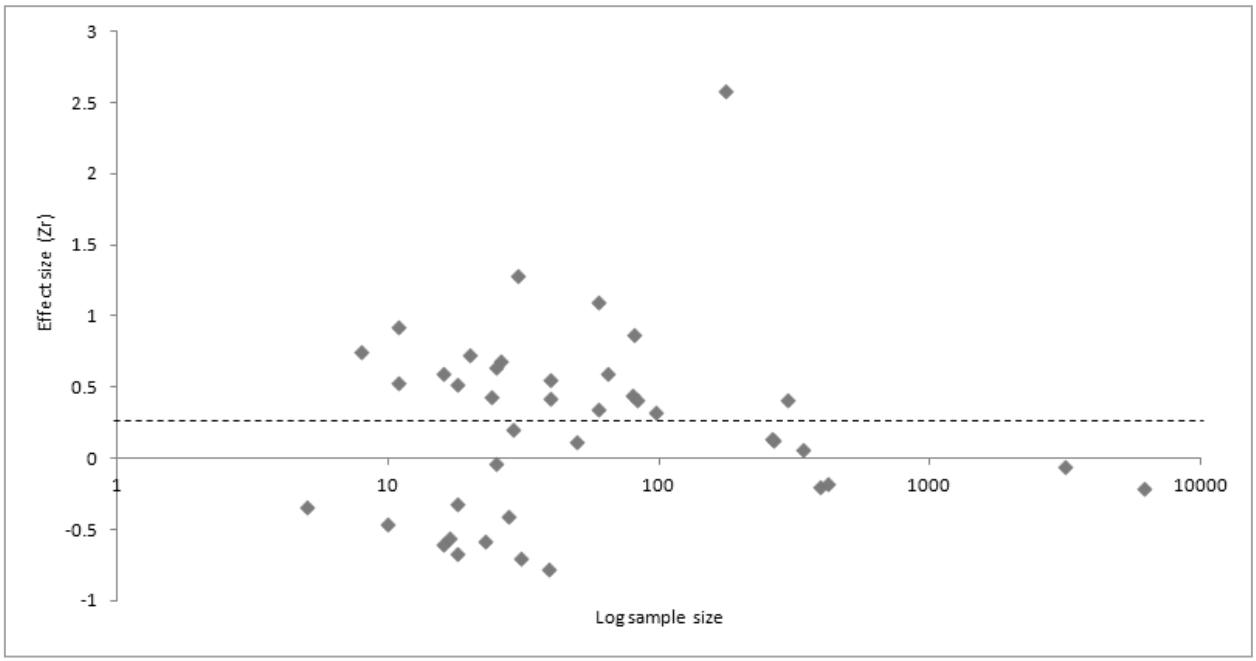

Figure 2.4. Funnel plot to search for publication bias. The dotted line shows the estimated effect size from the meta-analysis. The x-axis is on a log scale to show the critical points near the origin of the x-axis.

2.3.2. Moderator variables

Only 12.9% of the variation in the model was explained by the moderator variables. There is significant heterogeneity among experiments indicating that other, unexplored factors may influence the effect of plant colouration on the behaviour of insect herbivores (Q=171, df= 21, p <0.001). This seems to be a common problem in meta-analyses with many other studies having large amounts of unexplained heterogeneity (e.g. Barto and Rillig 2010).

All types of non-green colouration were found to produce the same negative response (LRT=2.53, df=5, p=0.28), suggesting that even very different non-green colours (e.g. red or orange colouration or variegated patterning) will generally be avoided. Herbivore responses to green versus non-green colouration did not vary according to the type of colouration produced (autumn, delayed greening, permanent phenotype) or between herbivory and oviposition (LRT=3.08, df=6, p=0.38). Responses to colouration, however, did vary significantly between insect orders (LRT=11.4, df=8, p=0.043; Fig 2.5a). In particular, coleopterans are attracted to

non-green colouration whereas aphids are attracted to green colouration. Specialists and generalists both preferred green colouration over non-green colouration (LRT=1.94, df=5, p=0.38). Plant type did not influence herbivore response to colouration (LRT=3.68, df=5, p=0.16), which indicates that the response is consistent across all plant types. The only aspect of experimental design found to have a significant effect on herbivore response was whether colouration was compared within or between plant species (LRT=5.00, df=3, p=0.025; Fig 2.5b). Interestingly, studies of herbivory within host species found a positive relationship between herbivory and green colouration while among those looking at herbivory between host species a preference for non-green colouration was preferred. The location where the study was carried out did not influence the response to colouration (LRT=3.72, df=6, p=0.29) suggesting that the overall negative response to colouration is consistent across all areas tested (sub-arctic to tropical). Finally, the type of experiment (manipulation or observation) also had no significant effect on impact on the response of herbivores to leaf colour (LRT=1.76, df=4, p=0.18).

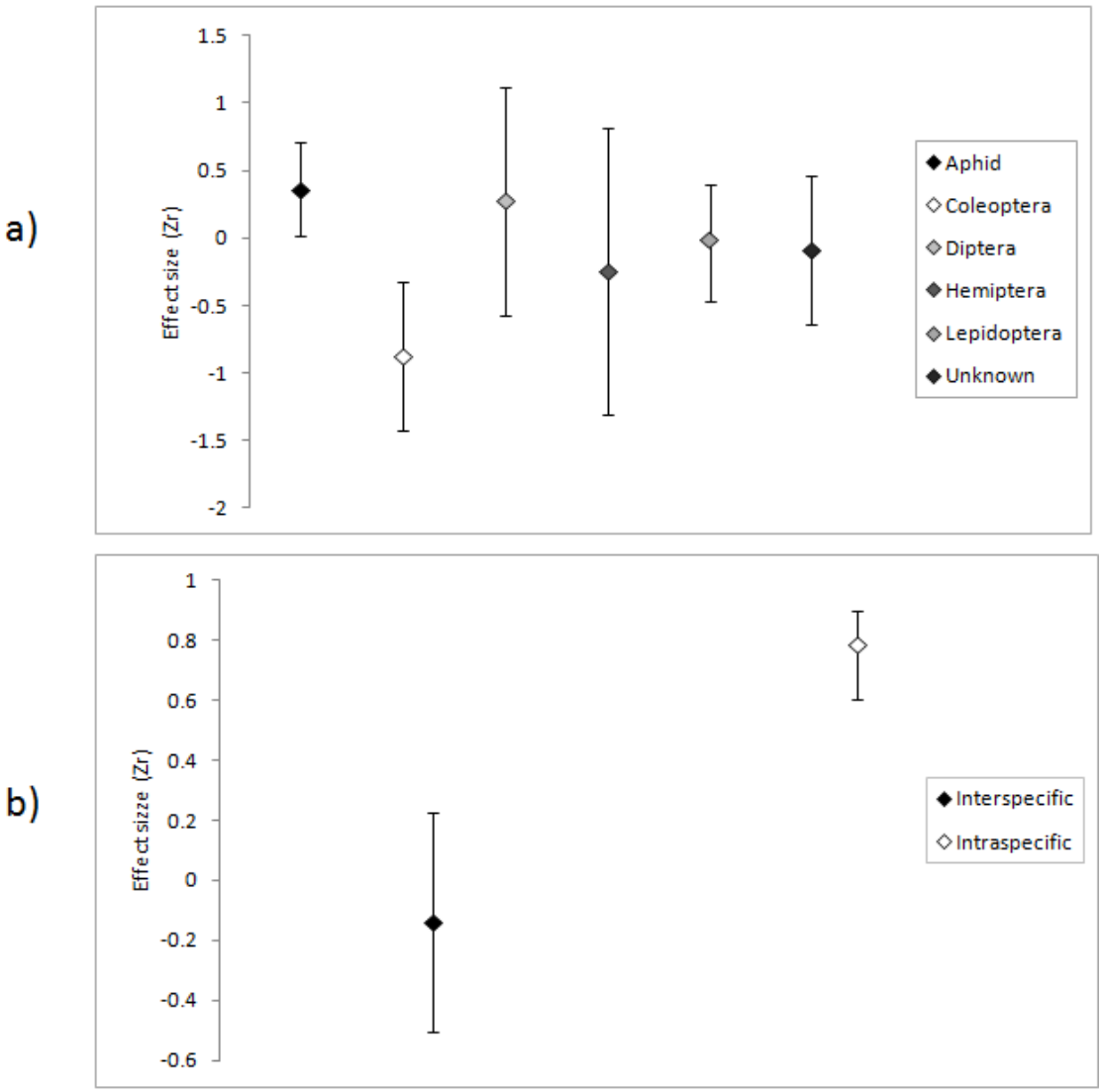

Figure 2.5. a) The influence of herbivore type on colour preference b) the influence of study type on colour preference. Points show predicted effect sizes, tails from the points represent the 95% confidence intervals, as estimated from the model.

2.4. Discussion

2.4.1 What is the effect of leaf colouration?

The meta-analysis presented in this chapter provides the first overall quantitative assessment of the effect of plant colouration on host selection by herbivores. There was a small but significant overall positive effect of green colouration on host selection by insect herbivores. Moreover, this effect was consistent over a range of plant and herbivore types and in a range of contexts, which suggests that leaf colouration is a general and widespread phenomenon in herbivore host selection. Indeed, these results indicate that leaf colour may provide valuable information to herbivores, either as a cue or a signal, in a broad range of contexts, for example across a range of plant types, environments and colour forms, including both transient, seasonal phenotypes and phenotypes that are fixed throughout the plant's (or leaf's) lifetime.

2.4.2. Does herbivore response to green versus non-green colouration vary in different situations?

Herbivores avoided non-green colouration, regardless of whether this was associated with transient phenotypes such as autumn colouration and delayed greening or permanent phenotypes such as leaf variegation. Previous work on autumn colouration has predicted that herbivorous insects should respond negatively to intense autumn colouration (Hamilton and Brown 2001, Hagen et al. 2003), a prediction that is supported by the meta-analysis. However, the analysis also found evidence that insects avoid non-green colours expressed in young leaves. This is perhaps surprising, given that the selection pressures on the colouration of leaves in autumn and of young leaves are predicted to differ (Lev-Yadun et al. 2012). Lev-Yadun et al. (2012) have shown that the colour of various species of young tree leaves do not always predict their colouration in autumn, suggesting that colour is unlikely to be a reliable cue across seasons. Further evidence for a difference between autumn colouration and delayed greening comes from Karageorgou et al. (2008) who showed the relationship between colouration and levels of chemical defence (phenolics) varied between young and old leaves, again suggesting that colouration may not offer a reliable, year-round guide to defence.

Additionally, there is stronger evidence of a photoprotective role for delayed greening than for autumn colouration (Gould 2004). Young leaves are less tough than mature leaves and so require more protection (Dominy et al. 2002). The photoprotective roles of anthocyanins at autumn time are much debated, however (Archetti et al. 2009b).

2.4.3. Do different types of herbivores respond differently?

The strength and direction of the association between colouration and herbivory was found to differ between major herbivore groups. Coleopterans appear to respond negatively to green colouration, while other herbivore groups respond positively. The preference for non-green colouration in the Coleoptera seems puzzling, but it is important to note that this result is based on relatively few studies that investigated responses of beetles to colour and should therefore be interpreted with caution. Some beetles have colour vision (Land and Nilsson 2002), but very few herbivorous species have been tested for spectral sensitivity. Spectral sensitivities have been obtained for a number of pest species, for example *Leptinotarsa decemlineata* (Colorado potato beetle), which has peaks in the UV, blue and green (Doring and Skorupski 2007). Very little work, however, has explored the potential visual cues used by *L. decemlineata* (Otalora-Luna and Dickens 2011), despite its enormous economic impacts (Liu et al. 2012). Evidence from this meta-analysis indicates that vision may be important in host choice for beetles and so colouration should not be overlooked as a potential cue for beetles during host selection (Reeves 2011).

Surprisingly, specialist and generalist herbivores did not differ significantly in their response to host colouration. It was predicted that specialists may respond more strongly as they enjoy a stronger co-evolutionary relationship with their host than do generalists (Berenbaum and Zangerl 1998), and this would be expected to lead to stronger reciprocal selection pressures on specialists and their hosts. Stronger selection pressures may make signalling more likely to evolve between specialists and their hosts, where the benefits of signalling defensive commitment to the plant outweigh the costs imposed by the herbivore. The absence of a stronger effect on colouration on specialists in this study refutes this idea, and may indicate that the strength of avoidance of colouration depends on a more subtle interplay of factors, including the relative costs and benefits to specialists vs. generalists of attending to the colour cue, as well as to the host plant of producing and advertising both general and specific herbivore defences. Specialist and generalist host choice is a theme that will be returned to in Chapter Five.

2.4.4. Does the type of plant result in different responses to colour?

The type of host plant was found to have no effect on the influence of colouration on host choice by insect herbivores. It was predicted that a colour signal would be more likely in a forb where the fitness costs of herbivory for a small, short-lived plant would be greater than for a tree, and therefore there would be greater pressure to single defence (Haukioja 2006). It is therefore somewhat surprising that the effect of colouration on herbivory was similar between

forbs and trees. This may indicate that the costs of herbivory to trees are in fact similar to those incurred by forbs. In support of this idea, the defoliation of trees by herbivores can result in mortality either directly or indirectly through other pathogens (Kulman 1971). Alternatively, it is possible that the costs accrued over a tree's lifetime are sufficient to favour the evolution of signals of defensive commitment, even if the damage sustained by herbivores each year is relatively low.

2.4.5. Do variations of non-green colouration produce different responses?

All non-green colours were found to give the same negative response, whether they were produced by carotenoids, anthocyanins or variations of chlorophyll presence. This result is surprising; while there is good evidence that anthocyanin-based red colouration deters insects (Hagen et al. 2003, Wong and Srivastava 2010, Cooney et al. 2012), previous research has reported an attraction by insects, in particular aphids, to the yellow colouration produced by carotenoids (Doring and Chittka 2007, Holopainen et al. 2009). Many of the studies in this meta-analysis that explored yellow colouration did so, however, in the context of oviposition behaviour, focusing in particular on the Lepidoptera. While aphids are attracted to yellow (Doring & Chittka 2007), butterflies are not and instead show a strong preference for green (Kelber 1999). As most butterflies have a red receptor, there will be stronger discrimination between yellow and green than for aphids (Kelber 1999). Thus as most preference for yellow were tested with butterflies rather than aphids this may explain the surprising preference for non-green within the carotenoids.

Potential signalling systems based on anthocyanins and carotenoids are likely to differ in the underlying biochemical pathways and the precise relationship between colour and defense compounds, though evidence from this meta-analysis suggests that the overall effect on herbivores may be similar. Colouration by anthocyanins and carotenoids involves laying down pigment whereas non-green colouration by chlorophyll is found in variegated leaves where there is a lack of this pigment. Avoidance of colouration based on anthocyanins and carotenoids supports the idea that such colouration may function as a strategic signal of chemical defence (Hamilton and Brown 2001). There may be, however, a different mechanism for the avoidance of variegated leaves (Campitelli et al. 2008, Zheng et al. 2010). It has been suggested that this colouration is avoided because it mimics leaf mining, thereby resembling a host with low level of resources (Soltau et al. 2009), rather than functioning as a strategic signal, as is argued in the case of autumn colouration (Hamilton and Brown 2001).

2.4.6. Does experimental design affect the result recorded?

Whether the study examined intraspecific (within a species) or interspecific (between species) plant colourations had a significant impact on herbivore choice. Within a single host species, herbivores presented with a choice of differently coloured plants preferred those with the greenest leaves, whereas when given the choice between hosts of different species, herbivores preferred those hosts with the least green colouration. This result highlights the need to consider the type of choice that a herbivore faces in the wild, i.e. do herbivores actively choose between hosts of the same species or of different species, or both? For instance, when considering leaf colour as a signal of plant defence, it is necessary to consider how herbivores respond to variation in colouration among hosts of the same species as the evolution of such a signal, as with any trait, is expected to proceed via selection among competing conspecifics rather than between heterospecifics (Maynard Smith and Harper 2003). Therefore, when testing whether plant colouration functions as a signal of defensive commitment to herbivores, researchers should take care to examine herbivore responses to variation in colouration within, rather than between, host species.

Herbivore responses to plant colouration did not vary with geographic location. This provides further evidence that the effect of colouration on herbivory is likely to be a widespread phenomenon, and may be a defining feature of many plant-herbivore interactions in a range of habitats. Herbivory in the tropics is higher than in temperate regions (Coley and Aide 1991); however, there is no evidence from this analysis that colouration affects herbivores more strongly in the tropics, which in turn implies that colour-based signalling is no more likely in the tropics than in other regions. Finally, the type of experimental design utilised by studies did not influence the response of the herbivores to host colouration, indicating that there is no substantial difference in herbivore responses between studies based on observations and those based on manipulations

2.4.7. Implications for the Autumn Colouration Hypothesis

The ACH predicts that aphids should avoid the brightest coloured trees as they provide an honest indication of the tree's chemical defence levels. This meta-analysis provides evidence for the first condition for this hypothesis: that insects respond negatively to non-green plant colouration.

Two main findings from the meta-analysis are directly relevant to the study of autumn colouration, and in particular to tests of the ACH. Firstly, the negative impact of non-green colouration on herbivory is widespread among plant types and is seen among both transient and permanent phenotypes. This implies that the ACH can be tested in a plant-herbivore

system that is practically easier than those involving trees, for example a smaller plant which is easier to search for insects. In addition, a plant species with permanent phenotypic differences in colour can be used, meaning that experimental work need not be restricted to autumn time. This suggests I am valid in using the *Brassica oleracea* system of which colour variations are present permanently. Indeed, Hamilton and Brown (2001) stress that the ACH can be applied to other plant-herbivore systems beyond aphids and trees (Hamilton and Brown 2001). Secondly, with the exception of beetles, it was found that most herbivores respond positively to green colouration. Aphids and butterflies, the two groups that I will focus on in the reminder of this thesis, both respond negatively to leaf colours other than green, suggesting they are a good choice of herbivore to investigate use of colouration on host plant choice.

2.4.8. Conclusions

In general, non-green leaf colouration is associated with lower levels of herbivory than green leaves. The chemical basis of this colouration, together with the circumstance in which the colouration is expressed, had no effect on herbivores' responses, nor did the degree of specialisation of the herbivore on the host plant or the type of environment inhabited by the herbivore and its host. This suggests that the negative response of insect herbivores to leaf colours other than green is a potentially widespread effect in plant-herbivore interactions. These results therefore provide some support for the ACH, which may plausibly be extended to all cases where herbivores select between individual hosts of a single species based on the spectral properties of leaves. To explore this possibility further, a more detailed study of the responses of herbivores to colour cues is required. This will be pursued using the *B. oleracea* system (see Chapter One). The next chapter will investigate the relationships with colouration, chemical defence and herbivory in the field, providing the foundation for further experimental work in controlled conditions (Chapters Five and Six) and investigation into the genetic variation of colouration (Chapter Four). From the data presented here, it is expected that plants that differ from green colouration will be avoided in the field.

Chapter Three. The relationship with *Brassica oleracea* colouration, glucosinolates and invertebrate herbivory in the field

3.1. Introduction

Herbivores use a range of cues to find their host plant (Schoonhoven et al. 2005). Information about the host plant needs to be obtained to determine if its suitability in comparison to other available hosts (Renwick and Chew 1994). The use of olfactory cues in host choice has been extensively studied in insects (e.g.Visser 1986); however, the use of visual cues has received less attention (Reeves 2011).

A number of studies have nonetheless demonstrated a plant's colouration is important in host choice across a range of insect herbivores (Traynier 1984, Doring and Chittka 2007, Kuhnle and Muller 2011) and a number of studies have linked plant colouration and herbivore damage in the field (Spicer et al. 1995, Soltau et al. 2009). The results of the meta-analysis presented in Chapter Two suggested that there is a general tendency across herbivore groups to avoid non-green leaf colouration. An important contribution to this topic has been the autumn colouration hypothesis (ACH) of Hamilton and Brown (2001) (see Chapter One for a review). This hypothesis posits that the intensity of colouration displayed by trees in autumn is a signal of their investment to chemical defence against insect herbivores, specifically autumn-colonising aphids. Under this hypothesis, the most intensely coloured trees are those with the highest levels of defence, and thus should be avoided by herbivores. While there is some empirical support for the ACH (Archetti and Leather 2005, Ramirez et al. 2008), it nonetheless remains a controversial idea (Wilkinson et al. 2002) and the necessary studies linking colour, chemical defence and herbivory in the field are still lacking.

An important requirement of Hamilton and Brown's ACH is that herbivore fitness is negatively affected by host chemical defences. Glucosinolates are a class of defensive compounds that provide an effective defense against a wide range of herbivores (Hopkins et al. 2009). For example, the glucosinolate gluconapin is known to affect *Ceutorhynchus assimilis* (seed weevil) herbivory (Moyes and Raybould 2001), while the extent of herbivory by *Brevicoryne brassicae* varies with the presence of sinigrin in hosts (Newton et al. 2009b). Determining the precise effects of glucosinolates on herbivores can be problematic, however, because these effects are often context dependent and vary according to the host species and herbivore species. Furthermore, different glucosinolates also vary in their toxicity to different herbivores, particularly in the case of indole compared to aliphatic glucosinolates (Cole 1997, Gols et al. 2008a). For example, while higher levels of sinigrin are associated with reduced herbivory by *B. brassicae*, they are also associated with increased levels of herbivory by snails (Newton et al. 2009b). To date, there have been surprisingly few field-based studies on glucosinolate-herbivore interactions (Mithen et al. 1995b, Moyes et al. 2000, Moyes and

Raybould 2001, Newton et al. 2009b, Staley et al. 2010). Detailed research into the effect of glucosinolates upon herbivores in the field thus represents an important contribution to the understanding of herbivore dynamics, the evolution of chemical defence, and the possible control of pest species.

A second requirement of the ACH is that there is a reliable link between some aspect(s) of a plant's colouration and its investment in chemical defence. The ACH does not specify what kind of chemical defence should be linked with colouration, but intuitively this should be specific defence compounds as these often have the strongest effect on herbivores (Schoonhoven et al. 2005). While a number of studies have investigated the link between colour and chemistry, most have explored the relationship between colouration and whole classes of defensive compounds (in particular phenols) rather than looking at specific compounds (e.g. Lempa et al. 2001, Hughes et al. 2010; but see, Cooney et al. 2012). Therefore, this is a gap in the literature that needs to be addressed. The correlation between levels of defence and colouration must be reliable if colouration is to evolve as a signal of defensive commitment to herbivores. Reliable links between colouration and chemistry could occur through allocation costs. Given that resources are finite, plants must allocate these resources optimally among competing functions (Herms and Mattson 1992). Given that colouration and chemical defence is required to be costly for the handicap argument of the ACH (see Chapter One), resources allocated to defence may have consequences for the resources available for colouration production, and therefore colouration and plant defence may be linked through allocation costs (Zahavi 1975, Herms and Mattson 1992, Hamilton and Brown 2001). Alternatively, genes coding colour and defensive chemistry may be pleiotropically linked, thus providing a reliable link between these two factors (Strauss et al. 2004). There is some evidence that flower colouration and glucosinolate concentration is pleiotropically linked in brassicas (Irwin et al. 2003), raising the possibility that leaf colouration and glucosinolate levels may be similarly linked.

3.1.1. The study system

As a first step in determining whether plant colouration has evolved to signal chemical defence to herbivores we require information about the relationship between plant secondary metabolites, colouration and herbivory in natural plant communities. In this chapter, these questions are addressed using the wild cabbage (*Brassica oleracea*) and its herbivores (see Chapter One for a review of this study system). *Brassica oleracea* grows along maritime cliffs, particularly along the south coast (Mitchell and Richards 1979). Wild *B. oleracea* populations exhibit remarkable variation in colouration, both within and between populations.

Glucosinolates are the main defensive chemicals in *B. oleracea*, where they have been well-studied (e.g. van Dam et al. 2004, Halkier and Gershenzon 2006). Herbivores encountered include the small cabbage white butterfly (*Pieris rapae*), the large cabbage white butterfly (*Pieris brassicae*), the cabbage aphid (*Brevicoryne brassicae)*, the cabbage stem flea beetle (*Psylliodes chrysocephala*), the cabbage small striped flea beetle (*Phyllotreta undulata*), the cabbage seed weevil (*Ceutorhynchus quadridens*), the cabbage stem weevil (*Ceutorhynchus assimilis*) and the cabbage whitefly (*Aleyrodes proletella*), all of which are specialist feeders on brassicas (Kirk 1992). *Brassica oleracea* is also vulnerable to attack by generalist feeders, including various molluscs (slugs and snails) (Kirk 1992). While the herbivores of *B. oleracea* are well-described, very little, however, is known about how herbivory varies with plant colouration in the field. Moreover, there are, to my knowledge, no data linking levels of glucosinolate defence and leaf colouration among wild plants. In this study, I provide the first detailed analysis of the relationships between colour, chemistry and herbivory in wild *B. oleracea* populations as a first step in addressing whether cabbage colouration can provide herbivores with information about levels of chemical defence.

3.1.2. Aims

This study is separated into two main questions:

1) ***What features of wild B. oleracea affect colour?***

Site

It is expected that plant colouration will vary between different sites. This is because abiotic factors will differ between locations and these may affect plant colouration. For example soil nutrients are expected to vary between sites, and it has been shown that nitrogen levels can influence colouration (Sinkkonen 2008). Other abiotic factors such as sun exposure and stresses e.g. low water levels, may also influence colouration differences between sites. In addition, if fixed genetic effects are an important component of colouration, restricted gene flow between sites may result in colouration differences.

Plant factors

If colour is a signal of plant quality, correlations with other indicators of plant quality (plant size and the number of flowers) are expected. If colouration was negatively correlated with other plant parameters, it may suggest that the expression of colouration is constrained by the expression of other phenotypes.

Nutrient status

If plant defence and colouration are related through allocation costs it is expected that there will be a correlation between plant colouration and the CN ratio. A negative correlation may indicate that colouration is constrained by nutrient availability. In addition, the pattern between glucosinolate concentrations and CN ratio will also be investigated, because this may likewise suggest that glucosinolate content is limited by nitrogen availability. In previous studies, relationships with nitrogen availability and glucosinolate levels have been found. For example, Staley et al. (2010) found that the glucosinolate concentration of brassicas varied under different fertiliser treatments, with the highest levels found with organic fertilisers.

Chemical defence

This final section moves to consider the relationship that is most fundamental to the ACH, namely that between plant colouration and chemical defence (here, glucosinolate concentration). The first step in establishing if colouration is a signal of chemical defence will be to determine whether correlations between chemical defence and colouration exist. If such relationships are found, this indicates information about glucosinolate levels could be conveyed by plant colouration.

2) How do herbivores respond to this information?

While a positive correlation between plant defence and colouration would support the idea that colour functions as a signal of chemical defence, it is not sufficient to demonstrate that this occurs. Rather, we also require a demonstration of herbivore responses both to colour and glucosinolate defence. To do this, it will be necessary to look at the factors affecting patterns of herbivory. Though this section focuses largely on *B. brassicae* and *P. rapae*, the section begins with a brief investigation of the other herbivores mentioned in the Introduction (see above).

For the presence of each herbivore, the influence of site and plant factors (size and flowers) were analysed. It is expected that herbivores will vary in abundance between sites (Newton et al. 2009b). Plant size has been shown to be important to herbivores (Reudler Talsma et al. 2008) and since some herbivores lay eggs in seed pods (Moyes and Raybould 2001), the numbers of flowers is likely to have an impact on these herbivores.

A positive correlation between plant nitrogen content and herbivory is expected. Nitrogen is an essential component in the diet of animals, and makes up approximately 10% of their biomass. This proportion is considerably lower in plants, with phloem consisting of around 0.25% nitrogen and leaves around 3%, the highest levels being found in young and

actively growing tissues (Mattson 1980). Herbivores can compensate for low nitrogen levels in their diet by eating larger quantities of plant material (Slansky and Feeny 1977) but they must also adopt strategies for locating hosts with high levels of available nitrogen, meaning that herbivores will be more likely to be found on these plants.

It is predicted that glucosinolate concentrations within the plant will affect herbivore colonisation. Various studies have found negative impacts of glucosinolates on herbivory (Gols et al. 2008b, Newton et al. 2009a, Kos et al. 2012), though others have reported positive associations (Moyes and Raybould 2001, Harvey et al. 2007), suggesting findings are variable and often context-dependent. Therefore, patterns that are found within this system are of interest.

Unlike previous studies, this field survey analyses colour from the herbivore's perspective as human colours are not what the insects see, and therefore using human colours in insect host choice may be misleading (Archetti et al. 2009a). This process involves taking reflectance measurements from the plant and modelling these to the herbivore's visual system. The colours of interest were blueness and redness, for which colour ratios were calculated (see Methods for more details) and brightness. According to the ACH and the results of the meta-analysis in Chapter Two, intense colours that differ from green should be avoided by insect herbivores. Of these colours, the avoidance of red leaves is most well documented (Traynier 1986, Ramirez et al. 2008, Archetti 2009). There is very little work on the effect of blueness on herbivore host choice; oviposition experiments using coloured cards suggest that green is preferred to blue (Traynier 1979). Brightness is important in host choice (Stefanescu et al. 2006) and according to the ACH, the brightest plants should be avoided.

For the focal herbivores (*P. rapae* and *B. brassicae*), this section included modelling both the variation in colouration among hosts and the correlations between different colours according to the herbivores' spectral sensitivities. If the colours are very variable, this suggests they may be useful in host choice because the range of information that could be conveyed would be large. The correlations between colours were also investigated: if colours are highly correlated, this suggests that the use of both colours in host choice will be limited because they will convey similar information. Of particular interest are the correlations between redness and blueness for *P. rapae*. The reason for the evolution of the red receptors is debated (Kelber 1999), but if these were uncorrelated to the output of the blue receptors it would suggest they will be useful in host choice because they provide extra information that the blue receptor cannot.

3.2. Methods

3.2.1. Field survey methods

Twelve spatially distinct populations of wild cabbage were studied along the south coast of England: Prussia Cove 1 (50°10N, 5°42W), Prussia Cove 2 (50°12N, 5°42W), Prussia Cove 3 (50°10N, 5°41W) in Cornwall; Watcombe Bay (50°50N, 3°51W), Wall's Hill (50°46N, 3°49W) and King's Wear (50°34N, 3°56W) in Devon; Old Harry (50°64N, 1°92W), Winspit (50°59N, 2°03W) and Kimmeridge (50°60N, 2°13W) in Dorset, and Margaret-at-Cliff 1(51°28N, 1°56W), Margaret-at-Cliff 2 (51°26N, 1°55W) and King's Down (51°28N, 1°46W) in Kent (see Figure 3.1).

Figure 3.1. Location of the *B. oleracea* populations surveyed. The red crosses mark the locations of populations studied (three populations in each of the four counties).

Each population was surveyed twice, once in early summer (20th June -8th July 2010) and again in late summer (30th August- 17th Sept 2010). All plants were marked with a labelled plastic stick and photographed. A GPS reading was taken for each plant (Garmin, etrex, Legend HCx) to aid identification of the same plant in the second survey. A transect was laid at each site so that 50 *B. oleracea* plants could be randomly sampled (except for Watcombe Bay, where 20 were surveyed and Prussica Cove 3 where 30 were surveyed). A total of 550 plants were sampled across the 12 sites in the first survey.

3.2.1.1. First summer survey

For plants identified in the early summer survey, a number of morphological characteristics were recorded. Two measures of plant size were recorded: basal stem diameter measured using callipers, and rosette area, which was estimated using measurements of the width and breadth of the rosette (made to the nearest centimetre with a ruler). The number of leaves and flower heads were counted (if present). The number of other *B. oleracea* plants in the surrounding $2m^2$ of the focal plant was also recorded as a measure of density. In order to estimate herbivory, each plant was thoroughly searched for invertebrate (insects and

molluscs) herbivores and these were identified according to Kirk (1992). In cases where caterpillar larvae were too small to be identified, these were removed and reared to permit identification of later instars.

To record the colour of the plant, a leaf sample was taken from each plant. Leaf samples were taken at the end of the day in order to remove any variation in colour throughout the day associated with changes in leaf water content. The third leaf from the top was selected for each plant, which was easily identified by the order of leaves emerging from the basal stem (see Figure 3.2). Colour was measured from the third leaf because it provides a representative measure for the whole plant (see Appendix A). The colour reading taken in the first survey was used to explain presence of all herbivores, expect for *P. rapae* and *P. brassicae* where the colour reading from the second summer survey was used because these herbivores were only found in the second summer survey.

Additional leaf samples were also taken for CN and glucosinolate analysis. Samples were taken from 15 plants randomly selected at each site. For CN analysis, the fourth leaf down from the top was excised from the plant (see Figure 3.2) and placed in a paper bag to be taken back to the laboratory at the University of Sussex. Sampling of leaves for glucosinolates analysis was complicated by the fact that glucosinolates hydrolyse when plant tissues are disrupted (Halkier and Gershenzon 2006). To prevent hydrolysis from occurring, leaf samples were treated in the following way. In the lab, 5ml glass vials were washed with methanol and then filled with 1ml HPLC grade acetonitrile (ACN) (Rathburn chemicals). These vials were then labelled, weighed and sealed with parafilm. The vials were then stored at -20^{o}C until they were taken to the field sites. At each field site, the 15 pre-labelled tubes corresponding to that field site were put onto dry ice in a cryocontainer, which freezes the ACN and provides a suitable means of preserving the leaf sample. To collect a leaf sample, a 1cm metal cutter was used to punch out three tissue samples from three different leaves, chosen at random from the *B. oleracea* plant. These discs were cut into quarters and pushed into the tube with gloved hands. The vial was shaken to ensure that the sample was immersed in the ACN and then put straight back onto the dry ice. On returning from the field sites, the samples were stored at -80^{o}C and then analysed (see 'Glucosinolate extraction and analysis').

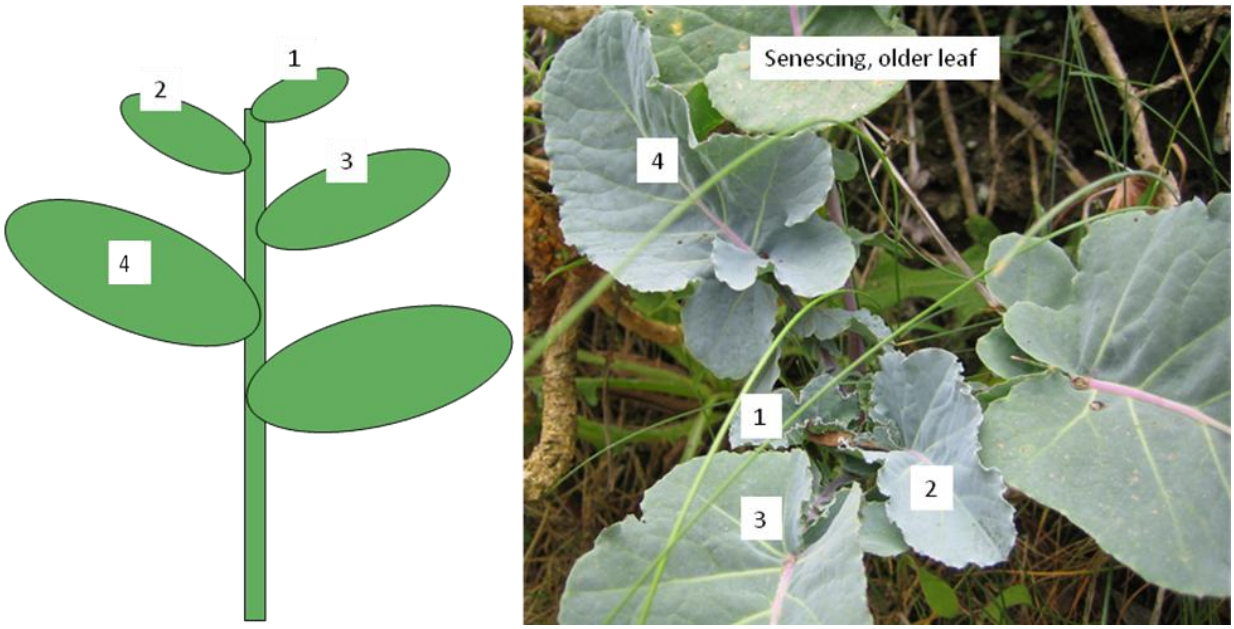

Figure 3.2. Identification of the third leaf from the top of the plant, sampled for colour measurement. The fourth leaf taken for CN analysis can also be seen.

3.2.1.2. Second summer survey

In the second survey in late summer, 312 plants identified in the first survey were successfully relocated. Leaf samples were again taken for colour measurements. The presence of herbivores on the plants was again recorded but no further morphological data were collected.

3.2.1.3. Colour measurements

Leaves sampled for colour measurements were excised from the plant with scissors and stored in a white paper bag until colour readings were taken. Colour was measured using a spectrophotometer (Ocean Optics USB-2000). The leaf was placed on a white tile and a small disc (diameter 0.5cm) was cut out with a metal cutter, taking care to avoid major leaf veins. The disc was transferred by tweezers to the holder of the spectrophotometer, taking care not to rub the leaf and remove surface waxes, which could potentially affect the colour readings. After every 10 samples a light and dark recording was taken for calibration of the spectrophotometer. Sodium barate was used as a white standard. Samples were illuminated with a UV light source (Ocean Optics UV xenon lamp). Integration time (the amount of time the spectrophotometer recorded the reflectance) was adjusted as required to prevent saturation where too much light is recorded. Colour was modelled according to the spectral sensitivities of *P. rapae* and *B. brassicae* (see below).

3.2.1.4 CN Analysis

CN analysis was carried out following the method described in Ewald et al. (2011). Leaves collected for CN analysis were dried to a constant weight for three days in a drying oven at 60^{o}C. Samples were then ground using a pulveriser. Leaves were ground to a powder by pulverising at 50 oscillations for 30 seconds. Approximately 1.5g of this homogenised powder was weighed for CN analysis. CN content was determined by flash combustion and chromatographic separation, calibrated against a standard compound ($C_{26}H_{26}N_2O_2S$) using an elemental combustion system (Costech Intruments, Milan, Italy).

3.2.1.5 Glucosinolate extraction and analysis

Extraction

The glucosinolates were extracted and converted to desulphoglucosinolates on a Sephadex column following published methods (van Dam et al. 2004). All solvents were obtained from Rathburn chemicals and were HPLC grade.

Sephadex columns were prepared in advance. The Sephadex column was made from a Pasteur pipette (5.75in Fisherbrand) with a small ball of glass wool pushed down in with a wooden stick. 1ml of DEAE Sephadex A25 (Fisher) solution was pipetted into the column and washed in with 1ml water. The columns, 50 at a time, were held in a Perspex rack which also contained a shelf to hold the tubes to collect eluted solutions (see Figure 3.3).

Figure 3.3. Sephadex columns held in Perspex rack for glucosinolate extraction. The eppendorf tubes can be seen under the columns to collect the eluted solution.

Leaf samples were ground in liquid nitrogen using a pestle and mortar placed in a pre-labelled 2ml eppendorf tube and weighed. 1ml of 70% methanol was added and vortexed for 10 seconds and the tubes were then placed in a 90^{o}C hot water bath for six minutes to boil the

methanol and denature the myrosinase enzyme. The tubes were then transferred to an ultrasonic bath for 15 minutes after which they were centrifuged for 10 minutes at 6500rpm. The supernatant was removed with a Pasteur pipette and put into the Sephadex column. A second extraction using 1ml 70% methanol with ultrasonicating and centrifuging then followed. The columns were then washed with 2ml 70% methanol, 1ml water and 2ml 5mM pH5.5 NaOAC solution. To desulphatase the glucosinolates 0.2μl of sulfastase solution (prepared from Sigma-Aldrich type H-1 aryl sulphatase of *Helix pomatia*) was then added with 0.5μl NaOAC solution to wash it onto the column. The columns were covered with aluminium foil and left to stand overnight.

The following day the desulphoglucosinolates were eluted with 2ml of water. This solution was freeze-dried overnight. The residue was dissolved in 1ml water by 30 seconds of vortexing followed by five minutes of ultrasonicating. The solution was filtered with a 0.2μm Nylon syringe filter (13mm diameter, Acrodisc from Sigma-Aldrich) and put into a 2ml amber HPLC vial. This was stored at -20^{o}C until HPLC analysis.

HPLC

Samples were analysed on an Agilent 1100 series machine. A C18 column (source: Phenomenex, dimensions: length 150mm, ID 4.6mm, 3μl grain size) was used to separate glucosinolates. Glucosinolates were detected with a UV detector at 229nm with a flow rate of 0.75ml/min. The mobile phases were acetonitrile (ACN) and water with the following programme:

Table 3.1. Programme for glucosinolate analysis with HPLC. All solvents were HPLC grade.

Time (min)	% of ACN	% water
0	2	98
30	35	65
35	2	98
42	2	98

Peaks were identified by their retention times (see Table 3.2). Glucosinolate identity was confirmed using mass spectrometry (see below).

Standard

The external standard, sinigrin (sinigrin hydrate from Sigma Aldrich) was prepared as above. The calibration curve (see Figure 3.4) was obtained after HPLC analysis, which enabled quantification of glucosinolates. The peak area of each glucosinolate was converted into concentration by dividing by 3.34 (obtained from the calibration curve; see Figure 3.4).

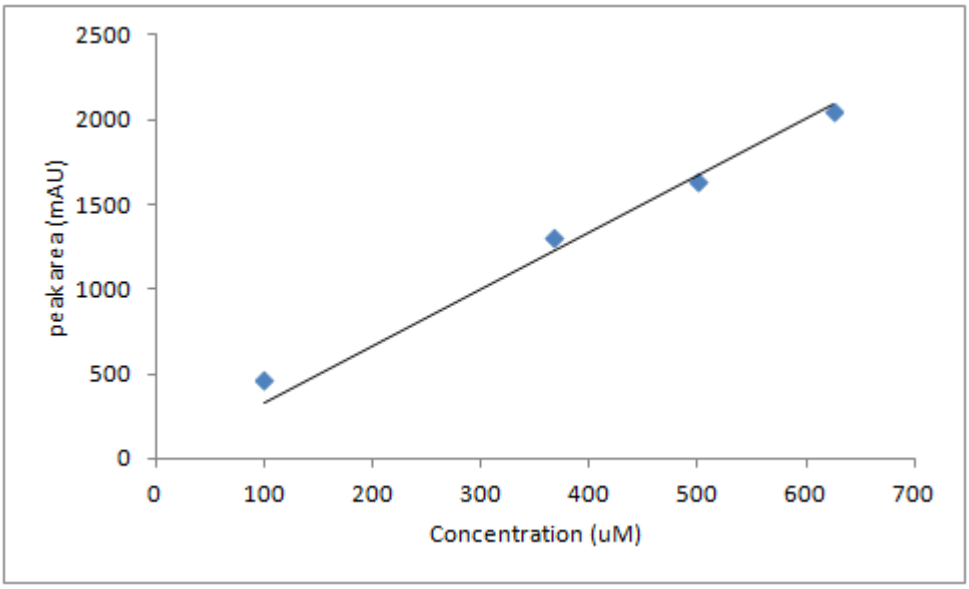

Figure 3.4. Calibration curve using sinigrin showing concentration of glucosinolate to corresponding peak area. Y=3.34x, r^2=0.981.

Mass spectrometry

Glucosinolates were identified using mass spectrometry with UPLC (ultra high pressure liquid-chromatography). The machine used was a Waters Ultima Q-ToF, with a capillary voltage of 2.7kV, a cone voltage of 35V, desolutation temperature of 300^{o}C and a gas flow rate of 400L/hr. All glucosinolates contain a sulphate moiety but during the ionisation process in LC-MS the long weak bond between the N-O breaks, meaning that the molecular mass of the glucosinolate was identified minus the sulphate group (see Table 3.2). The glucosinolates could be identified in positive and negative modes (where an electron is added or removed to make the molecule positively or negatively charged respectively) but negative mode was used because the sensitivity was ten times higher. Molecule identity from molecular mass was confirmed with the likely elemental composition function found in the computer programme MassLynx version 4.1. PPM values (the difference between theoretical and actual mass) were taken from a peak section where saturation did not occur because peak saturation gives a less accurate mass (see Table 3.2). PPM values lower than five represent a high level of accuracy (see MassLynx instructions). Four samples were compared to ensure retention times were consistent. As with HPLC, sinigrin was used as an external standard.

Table 3.2. Identification of the eight glucosinolates. The retention times are shown as used to identify the glucosinolates during HPLC. The theoretical mass and associated PPM value can also be seen.

Compound	Mass ($-SO_3$)	Retention time LC-MS	Retention time HPLC	Formula ($-SO_3$)	PPM
Glucoiberin	342.068	4.18	5.0	$C_{11}H_{20}NO_7S_2$	1.8
Progoitrin	308.081	4.91	6.2	$C_{11}H_{18}NO_7S$	1.6
Glucoraphanin	356.083	5.12	6.7	$C_{12}H_{22}NO_7S_2$	1.4
Sinigrin	278.069	5.92	7.2	$C_{10}H_{16}NO_6S$	3.2
Gluconapin	292.085	9.13	11.1	$C_{11}H_{18}NO_6S$	0.3
Glucobrassicin	367.096	15.96	13.4	$C_{16}H_{22}N_2O_7S$	0.8
4-Methoxyglucobrassicin	397.106	17.96	18.3	$C_{17}H_{21}N_2O_7S$	2.3
Neoglucobrassicin	397.106	22.09	24.6	$C_{17}H_{21}N_2O_7S$	4.5

3.2.1.6. Modelling herbivore spectral sensitivity

Pieris rapae

The butterfly colour output was modelled using quantum catch, which is the standard measure used when modelling insect vision (e.g. Kelber 1999). Quantum catch is the number of photons that cause isomerisation of photopigment molecules within a receptor per unit time, and so provides information about photoreceptor output. The model shows the extent to which each receptor is stimulated by the reflectance of a surface by an illuminant and is therefore specific for receptor type, reflectance spectrum and light source. The reflectance spectrum takes into account both wavelength and intensity. The model and the software to run the model were developed by Lucas Wilkins (University of Sussex) and is based on the simple model of Stavenga and Arikawa (2011).

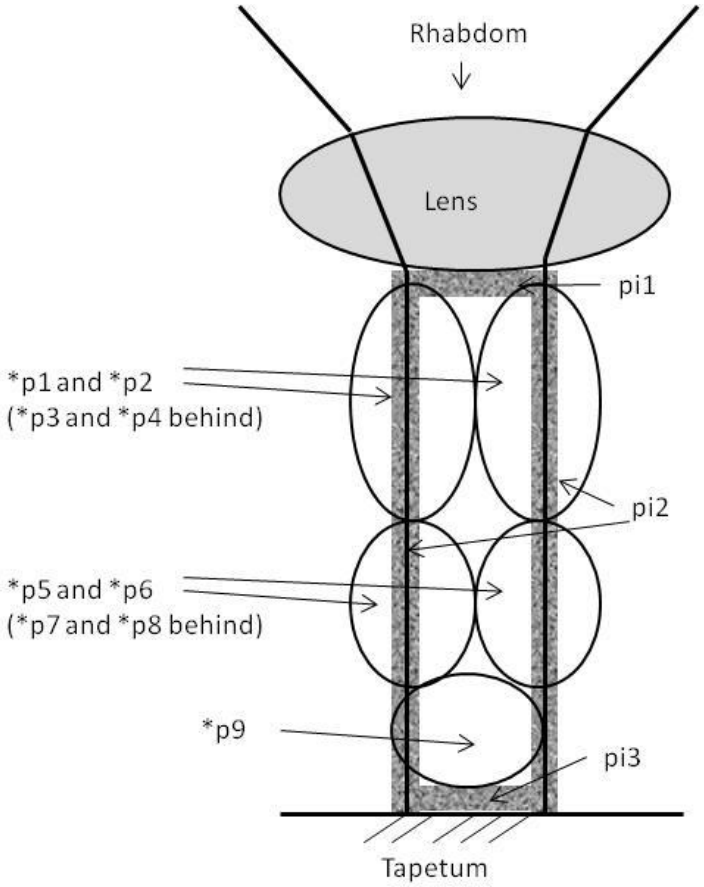

Figure 3.5. A simplified diagram of an ommatidium. Numbers pi1, pi2 and pi3 represent pigments and the starred areas represent photoreceptors *p1-*p9.

As a first step in explaining how the quantum catch model works, it is necessary to give a brief account of a butterfly ommatidium. The main structure of the ommatidium is the rhabdom, which is a fused tube that acts as a waveguide for the photons. The lens (seen at the top of the rhabdom in Figure 3.5) channels the light into the rhabdom. The size of the rhabdom affects the resonance of the wavelengths and so this parameter was included in the model. Light passes down the rhabdom and through the pigments and photoreceptors. The light hits the tapetum at the back of the eye, which acts as a mirror and reflects the light back up the rhabdom and again through the photoreceptors. There are therefore three main parts to the path of the light to be modelled:

1) Light absorbed by pigments
2) Amount of light reflected from tapetum
3) The order in which the light travels through the pigments and tapetum.

1) Pigments

There are three different absorbing pigments and nine photoreceptor pigments that filter the light wave. The only difference between the photoreceptor pigments and the absorbing pigments that is of concern of the model is that the photoreceptor pigments cause a nerve stimulation. As the photoreceptor pigments are stacked, it is necessary to model their light absorption because this will affect the light reaching the receptor cells underneath. Specifically, it is important to know what wavelengths of light and how much of light are absorbed by each pigment so that light available after it passes through the pigment can be determined. The three absorbing pigments are found (a) under the lens, (b) surrounding the rhabdom and (c) above the tapetum (pi1, pi2 and pi3 respectively on Figure 3.5). Figure 3.6 illustrates how absorbing pigments (in this case pi2) can filter the light as it passes down the rhabdom.

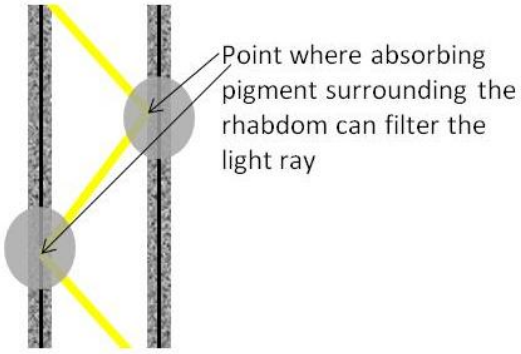

Figure 3.6. Light (yellow line) travelling down the rhabdom. Grey circled areas show where the light ray hits the pigment wall and is therefore filtered.

Beer's law describes the transmission of light through a uniformly absorbing substance:

$$\frac{I(\lambda)}{I_o(\lambda)} = e^{-ka(\lambda)}$$

$I(\lambda)$ is the amount of light that goes into the substance and $I_o(\lambda)$ is the light that comes out, and so by dividing out by in, the amount of light absorbed is calculated. K is the optical density of the substance, which incorporates information about the pathlength (how long the pigment is) and the concentration of the pigment (how dense it is). a is the parameter for what spectra are absorbed by the pigment. K and a for each pigment are parameters required for the model.

2) Tapetum

An estimation for a (what spectra are absorbed in Beer's law) is required. This tells us what spectra are reflected and thus are available for the photoreceptor cells as the light travels back up the rhabdom.

3) Component order

As the pigments are stacked together, it is necessary to consider the order of the pigments because $I_o\ (\lambda)$ of a given pigment is equivalent to $I\ (\lambda)$ for the pigment beneath it. Figure 3.7a presents the rhabdom from above so that the stacking of the photoreceptor cells can be seen, and Figure 3.7b shows this from the side. An additional complication arises when considering the fact that light travels down the rhabdom and is then reflected back up the tapetum. Returning from the tapetum, the light waves thus encounter the pigments in reverse order. In addition, overall stimulation to each receptor must be summed for the light travelling both up and down the rhabdom to give total quantum catch.

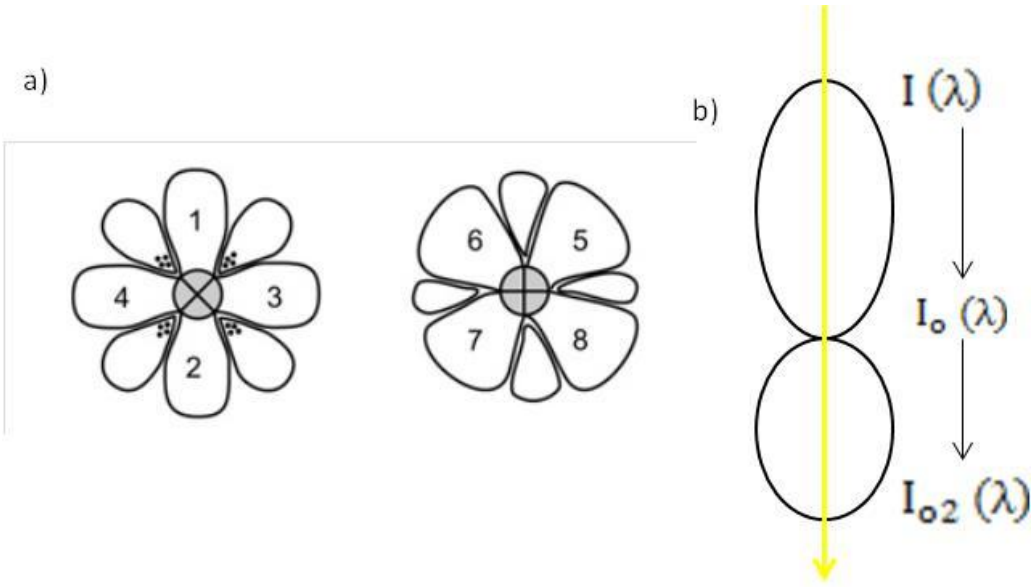

Figure 3.7. Light filtered by photoreceptors due to stacking. (a) photoreceptors as seen from above, showing how they cover and therefore filter each other. From Stavenga and Arikiawa (2006). (b) light ray passing through photoreceptors from the side. This shows how $I\ (\lambda)$ (light in) is absorbed (according to Beer's law) by the photoreceptor and becomes $I_o\ (\lambda)$. This then enters the photoreceptor below, again is filtered, and becomes $I_{o2}\ (\lambda)$.

The parameters discussed (absorbing pigment sensitivity, photoreceptor sensitivities, order of the pigments, and tapetum reflectance) were obtained or calculated from recently published data on *Pieris rapae* eye physiology (Stavenga and Arikawa 2011).

Brevicoryne brassicae

As with P*. rapae*, a model for *B. brassicae* spectral sensitivities was constructed based on quantum catch. This model is simpler than that for *P. rapae*, firstly because aphid eyes are much simpler than butterfly eyes and have fewer photoreceptors (Doring and Chittka 2007) and secondly because details of the eye physiology, including the number and location of visual pigments, have not yet been described for *B. brassicae*. Consequently, all that can be modelled for *B. brassicae* is a hypothetical filter pigment found at 300nm and the sensitivities of the receptors (530nm, 460nm and 325nm). This was modelled from data presented in Kirchner et al. (2005). The model and the software to run the model were developed by Lucas Wilkins (University of Sussex).

Ratios

Colour, it can be reasonably hypothesised, is interpreted by the insect's brain through comparisons of receptor excitations (Vorobyev et al. 2001). Therefore, ratios between receptor inputs need to be calculated as this is how the insect processes colour. For example, the red to green quantum catch ratio is the ratio of red receptor excitation compared to the green receptor excitation and provides a measure of redness from the insect's perspective.

3.2.2. Statistical analysis

All statistical analysis was carried out in R (2.10.0). P values are presented to two significant figures (or less than 0.001), and test statistics to three significant figures.

1) What features of wild *B. oleracea* affect colour?

Five measurements for colour were calculated from the quantum catch receptor outputs from the models. For *B. brassicae*, these were the blue to green ratio (blue receptor quantum catch divided by green receptor quantum catch) and brightness (green receptor output (Lehrer 1994)). For *P. rapae*, these were the dark red to green ratio (dark red receptor quantum catch divided by green receptor quantum catch), the pale red to green ratio (the pale red receptor quantum catch divided by the green receptor quantum catch) and the blue to green ratio (the blue receptor quantum catch divided by the green receptor quantum catch). As aphids do not have a red photoreceptor the red to green ratios could not be calculated for these insects.

The blue to green ratio from aphid vision and butterfly vision was very highly correlated ($r=0.997$, $df=546$, $p<0.001$) so only one measurement will be used for both

interpretations when looking at blue to green ratios of plant colouration and plant parameters. Correlations between plant size and colour were calculated with a Pearson's correlation test or a Spearman's rank if the data could not be normalised through square root transformation. Density of plants and the number of flowers could not be normalised and so correlations between these variables and colour were calculated using Spearman's rank. Differences in plant colouration between sites were explored using an ANOVA. Significant differences were further investigated using a Tukey HSD (Honest Significant Differences) post hoc test, which adjusts the p-value depending on how many multiple comparisons are carried out.

The relationship with total glucosinolates and site was analysed using an ANOVA following normalisation with a log transformation. Significant differences were further investigated using a Tukey HSD (Honest Significant Differences) post hoc test. Differences in the CN ratio (calculated as the carbon content divided by the nitrogen content) between sites were measured with an ANOVA followed by a Tukey HSD. Correlations between CN ratio and colouration were analysed using a Pearson's correlation test following normalisation with a log transformation. Correlations between colouration and glucosinolate concentration were carried out with a Pearson's correlation test or a Spearman's rank test if normalisation through log transformation was not possible. P-value corrections were required to minimise type I errors as a consequence of multiple testing and were carried out using the Benjamini-Hochberg method (henceforth B-H) (Benjamini and Hochberg 1995). Though the Bonferroni method is perhaps more well-known, its application has been criticised by a number of authors on account of it being highly conservative (Benjamini et al. 2001, Nakagawa 2004). The B-H is employed here as it is a widely-used method in all areas of biology (over 10,000 citations on Web of Science 10/9/2012) and is much less conservative correction (Benjamini and Hochberg 1995, Benjamini et al. 2001).

2) How do herbivores respond to this information about hosts?

The importance of plant colouration, CN ratio and concentration of eight glucosinolates (glucoiberin, progoitrin, glucoraphanin, sinigrin, gluconapin, glucobrassicin, neoglucobrassicin and 4-methoxyglucobrassicin) on herbivory was investigated using GLMs (generalised linear models) with either a binomial error structure or a quasibinomial error structure where there was evidence of overdispersion in the data. The number of cabbages sampled for colouration, CN ratio and glucosinolate concentration varied (n=550 for colouration, n=179 for CN ratio and n=129 for glucosinolate concentration). Separate GLMs were therefore performed to test for effects of these variables on herbivory to gain the

maximum amount of power. In each model, herbivore presence or absence was the response variable and either (a) colouration, (b) CN ratio or (c) all eight glucosinolate concentrations fitted as explanatory variables together with basal stem diameter (a measure of plant size), number of flowers and site. Model simplification proceeded by backwards deletion of non-significant terms until further removals lead to significant ($p<0.05$) increases in deviance assessed using Chi squared values (or F values where quaisbinomial GLMs were used). Following standard procedures, significance levels are reported on the addition of non-significant terms and removal of significant terms from the minimum adequate model (Crawley 2009). The minimum adequate model describes the model which best fits the data, produces the least unexplained variation (the minimum residual deviance) and where all parameters in the model are significant (Crawley 2009).

All significant results are presented for the model looking at colouration. For simplicity only CN results are presented from the results of the CN model, and only significant effects of glucosinolates in the glucosinolate model. Full two-way interaction models were carried out for the colouration and CN GLMs. However, when modelling the effect of the eight glucosinolates on herbivory, only the interaction between glucosinolate and site was included. Results for all eight glucosinolates are presented for *P. rapae* and *B. brassicae*; for simplicity, only significant differences are presented for the other herbivores. Results for all colour, CN and glucosinolate variables from the glucosinolate model (n=129, all data present) for *P. rapae* and *B. brassicae* can be found in Appendix B to see how the variables influence each other. For simplicity, this is not presented in the main results section.

In the GLMs, herbivore presence or absence was fitted as the response variable. The stem weevil and seed weevil were combined ("weevil herbivory") as they showed the same relationship with colouration when considered separately. The striped flea beetle and the stem flea beetle were also combined ("flea beetle herbivory"). In the case of the Lepidoptera, almost all herbivory was detected in the late summer survey. Colouration data collected in late summer was therefore used in the analysis of colour on Leipidoptera herbivory (note that in all other cases, plant measurements taken in the early summer survey were used). As vision is not thought to be important in snail host choice (Land and Nilsson 2002), the effect of plant colouration on mollusc herbivory was not explored. In the analyses of colouration and herbivory by weevils, flea beetles, whitefly and aphids, colouration was calculated from the *B. brassicae* spectral sensitivity model. These herbivores have not been tested for spectral sensitivity but possession of a blue, green and UV receptor is the most common pattern across insects and so this estimation is appropriate (Briscoe and Chittka 2001, Doring and Skorupski 2007). In the case of *P. rapae* and *P. brassicae*, colouration was calculated using *P. rapae*

spectral sensitivities. The spectral sensitivity of *P. brassicae* has not been explored and so these results must be treated with caution. As the butterflies are very closely related, however, it is likely that they share similar spectral sensitivity.

Correlations between the measures of colouration were calculated with a Pearson's correlation test or a Spearman's rank if the data could not be normalised through square root or log transformations.

3.3. Results

3.3.1. What features of wild *B. oleracea* affect colouration?

3.3.1.1. Colour and site

The blue to green ratio did not vary between plants at the different sites. In contrast, the pale red and dark red to green ratios did vary between sites, with the reddest plants found in Kent and the least red plants in Devon and Cornwall (Figure 3.8a). Plant brightness was also found to vary significantly between sites, with the brightest plants found in Dorset and Devon and the dullest plants found in Cornwall and Kent (Figure 3.8b) (see Table 3.3 for test statistics and p-values).

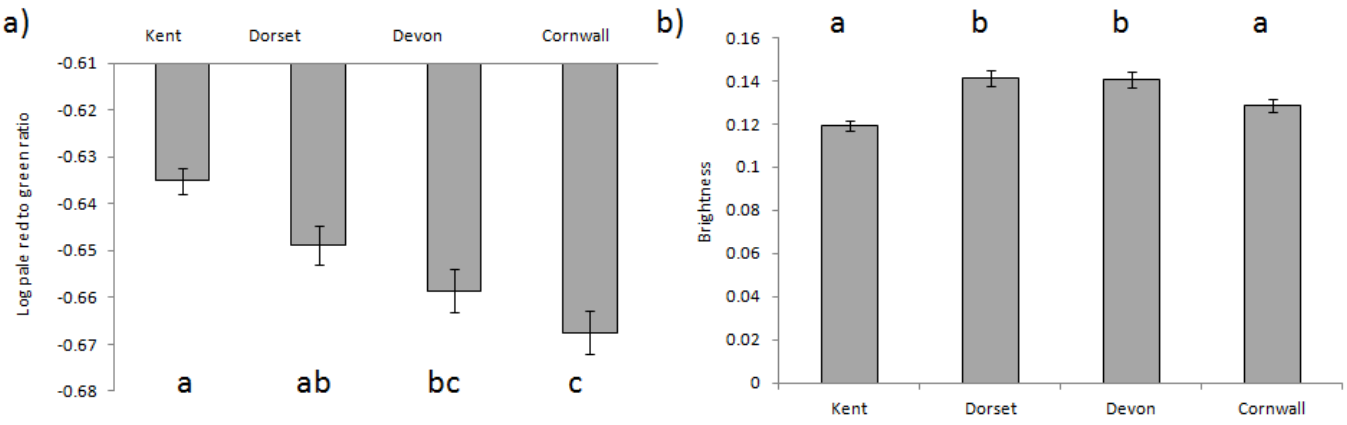

Figure 3.8. Colouration versus plant site. Letters denote significant differences from Tukey HSD tests (pale red: Devon-Cornwall p=0.44, Dorset-Cornwall p=0.0052, Kent-Cornwall p<0.001, Dorset-Devon p=0.33, Kent-Devon p<0.001, Kent-Dorset p=0.058; brightness: Devon-Cornwall p=0.047, Dorset-Cornwall p=0.019, Kent-Cornwall p=0.13, Dorset-Devon p=1.0, Kent-Devon p<0.001, Kent-Dorset p<0.001). Error bars show SEM. Note the pale red to green is on a log scale as with the ANOVA test, therefore Kent is the reddest location and Cornwall the least red.

3.3.1.2. Colour and plant size

There was no significant correlation between plant size and any of the colour measures (see Table 3.3 for test statistics and p-values).

3.3.1.3. Colour and plant density

Of the four types of colour, only the pale red to green ratio varied significantly with density (see Table 3.3). Focal plants were redder when the density of surrounding plants was lower (Figure 3.9).

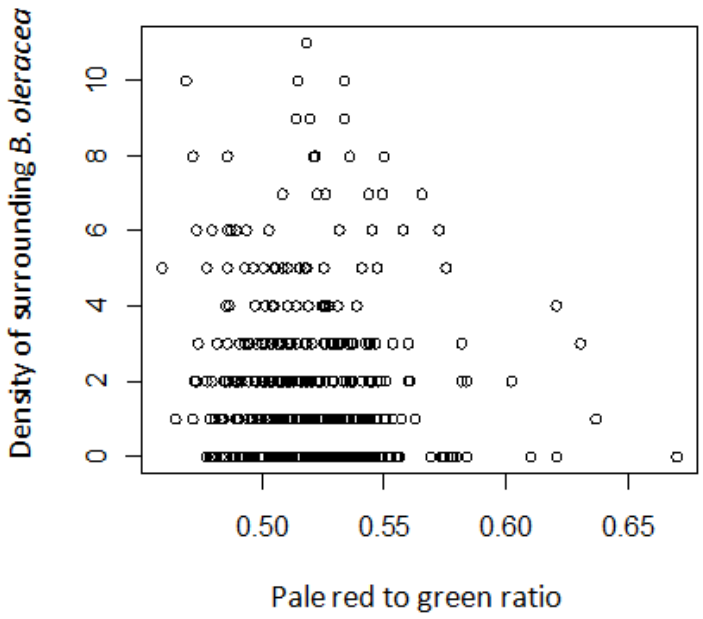

Figure 3.9. Correlation between density and pale red to green ratio (r_s=-0.104, p=0.029).

3.3.1.4. Colour and number of flowers

As the number of flowers present on a plant increased, the pale red to green ratio and dark red to green ratio also increased (see Figure 3.10a), but as the number of flowers decreased the blue to green ratio increased. Independently, the number of flowers was also negatively related to the brightness of the leaves (see Figure 3.10b) (see Table 3.3 for all test statistics and p values). Thus, plants with greater numbers of flowers had more red colouration and less blue colouration and were also duller than those with fewer flowers.

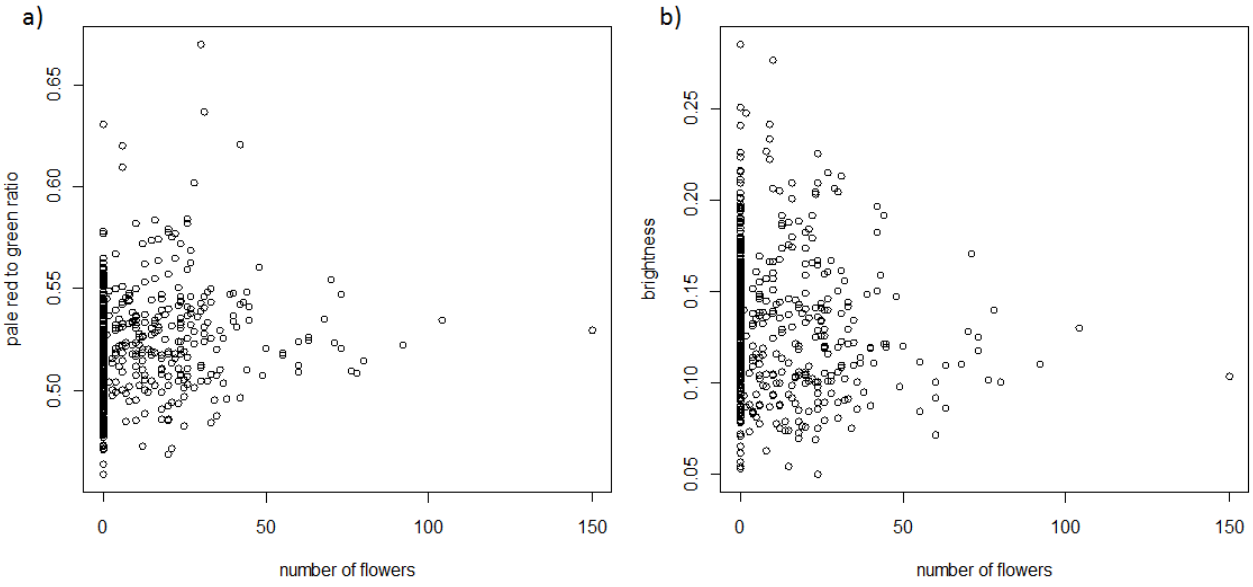

Figure 3.10. Correlations between number of flowers and a) pale red to green ratio (r_s=0.248, p<0.001) and b) plant brightness (r_s=-0.143, p<0.001).

Table 3.3. Relationship between colour parameter and size, density, number of flowers and site. df=546 in all cases. r = pearson's correlation coeffient, r_s=spearman's rank correlation rho, F = F test from an ANOVA, KW-X^2 = Kruskal Wallis chi-squared which is a non-parametric version of ANOVA.

	Size	Density	Flowers	Site
Brightness	r=-0.0819, p=0.055	r_s=0.0501, p=0.29	r_s=-0.143, p<0.001	F=12.2, p<0.001
Blue to green ratio	r=-0.0689, p=-0.11	r_s=0.0774, p=0.10	r_s=-0.169, p<0.001	F=1.16, p=0.32
Pale red to green ratio	r=0.0679, p=0.11	r_s=-0.104, p=0.029	r_s=0.248, p<0.001	F=12.2, p<0.001
Dark red to green ratio	r_s=-0.0481, p=0.27	r_s=-0.0669, p=0.16	r_s=0.153, p<0.001	KW-X^2=22.4, p<0.001

3.3.1.5. Colouration and CN

Colouration was strongly correlated with the CN ratio in *B. oleracea*. The blue to green ratio decreased as the CN ratio decreased (r=0.328, df=176, p<0.001; Fig 3.11a), whereas the pale red to green ratio increased with the CN ratio (r=-0.390, df=176, p<0.001; Fig 3.11b). These findings indicate that bluer plants tend to have a lower CN ratio, while redder plants have higher CN ratio. The dark red to green ratio did not correlate with the CN ratio (r=0.0247, df=176, p=0.74). The amount of carbon within plants was also positively correlated with leaf brightness (r=0.347, df=176, p<0.001).

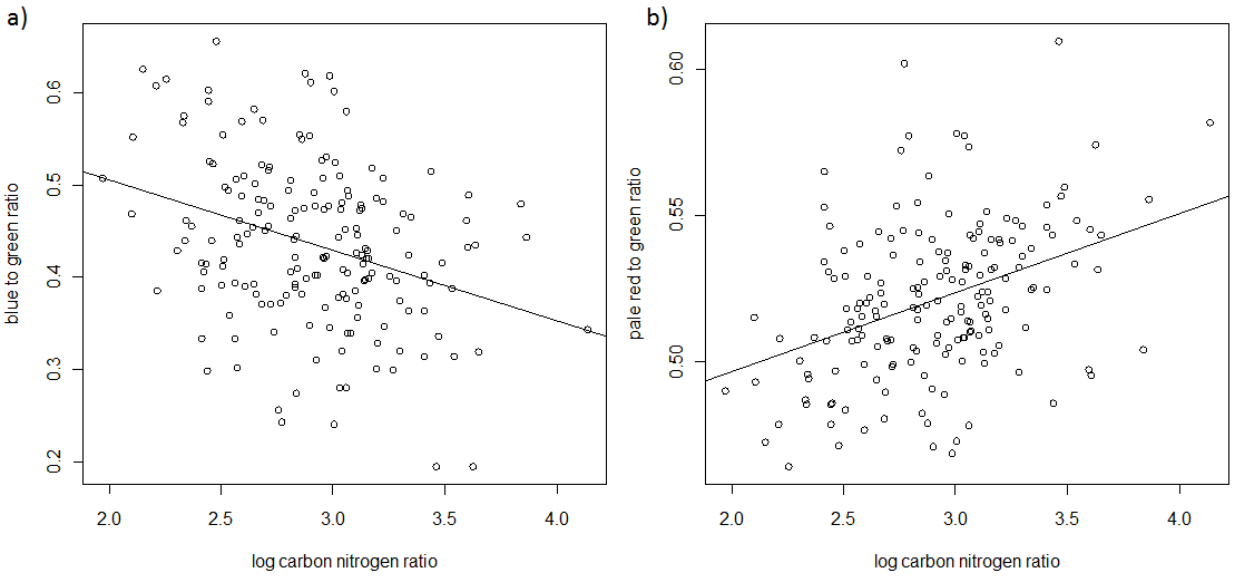

Figure 3.11. Correlations between CN ratio and colouration. a) blue to green ratio and CN ratio, b) pale red to green ratio and CN ratio. The line is a least-squares regression line produced from a linear model where colouration was fitted as the response variable and CN ratio as the predictor.

3.3.1.6. Glucosinolates and CN

A total of eight different glucosinolates were identified from the samples of *B. oleracea* taken in the field. Of the five aliphatic glucosinolates (glucoiberin, progoitrin, glucoraphanin, sinigrin, gluconapin) identified, gluconapin and progoitrin were found at the highest concentrations. Three indole glucosinolates (glucobrassicin, neoglucobrassicin and 4-methoxyglucobrassicin) were identified, of which 4-methyoxy glucobrassicin was found at the highest concentrations in the plants.

None of the glucosinolates were significantly correlated with the CN ratio and levels of glucosinolates explained only 7-24% of the variation in CN ratio among plants. The largest effect was seen for glucobrassicin (r_s= 0.236, n=126, p=0.0071, NS with B-H correction).

3.3.1.7. CN ratio and site

Plant CN ratio did not differ between sites ($F_{1,175}$=2.36, p=0.073), suggesting that plants are able to obtain enough nitrogen from the soil regardless of the plants location. In addition, there was no correlation between the CN ratio the either the number of flowers (r_s=0.0504, df=177, p=0.50) or the size of the plant (r=-0.0504, df=177, p=0.50).

3.3.1.8. Glucosinolates and site

There was significant variation in total glucosinolate levels between sites (F=21.8, df=125, p<0.001; Fig 3.12), which was attributable to differences in levels of indole glucosinolates between sites (F=28.1, df=125, p<0.001). Total levels of aliphatic glucosinolates did not differ between sites (F=0.935, df=125, p=0.43).

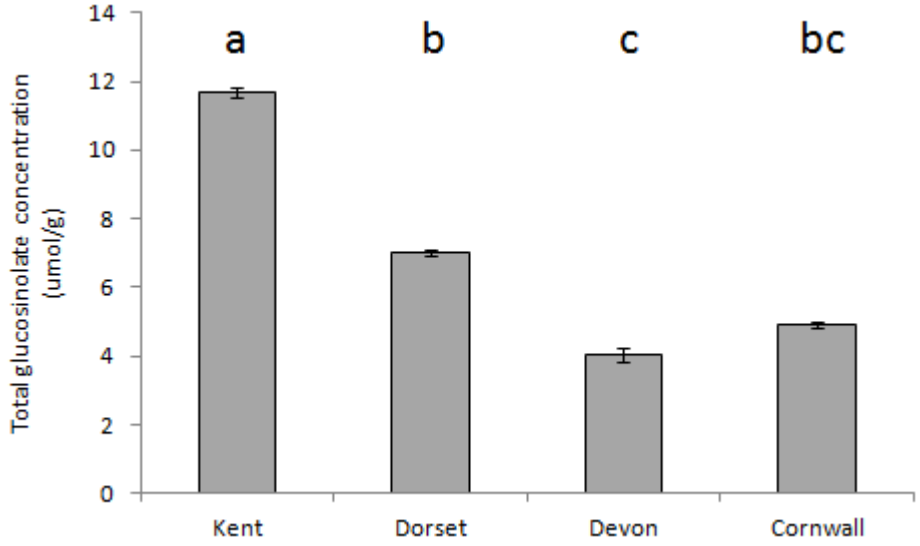

Figure 3.12. Mean total glucosinolate concentration at each of the four locations along the south coast. Letters denote significant differences calculated by a Tukey HSD following ANOVA (Devon-Cornwall p=0.25, Dorset-Cornwall p=0.095, Kent-Cornwall p<0.001, Dorset-Devon p<0.001, Kent-Devon p<0.001, Kent-Dorset p<0.001). Error bars represent SEM.

3.3.1.9. Colouration and glucosinolate concentration

Plant brightness was positively correlated with concentrations of several of the glucosinolates measured (see Table 3.4). This correlation was significant in the case of sinigrin and neoglucobrassicin, and was close to significance for glucoiberin and glucoraphanin. The total concentration of indole glucosinolates was also positively correlated with plant brightness. However, when applying a correction for multiple comparisons, all correlations between brightness and glucosinolate levels were non-significant.

Table 3.4. Correlation coefficients, associated p-values and significance corrections with B-H for the correlations between glucosinolate concentrations and plant brightness. Df=127 in all cases. Spearman's rank correlation coefficient is presented for all cases except 4-MeOH and the three total measures, where the Pearson's correlation coefficient is given. 4MeOH= 4-methyoxy glucobrassicin, neo= neoglucobrassicin.

Glucosinolate	Correlation	P value	Significant with B-H?
Glucoiberin	0.154	0.081	NS
Progoitrin	-0.0600	0.50	NS
Glucoraphanin	0.161	0.069	NS
Sinigrin	0.174	0.049	NS
Gluconapin	-0.0975	0.27	NS
Glucobrassicin	-0.00522	0.95	NS
4MeOH	0.0631	0.48	NS
Neo	0.223	0.011	NS
Total aliphatics	0.0482	0.590	NS
Total indoles	0.192	0.030	NS
Total	0.169	0.056	NS

In contrast to the results obtained for plant brightness, the blue to green ratio was not significantly correlated with any of the glucosinolates (see Table 3.5).

Table 3.5. Correlation coefficients, associated p-values and significance corrections with B-H for the correlations between glucosinolate concentration and blue to green ratio. Df= 127 in all cases. Spearman's rank correlation coefficient is presented for all cases except 4-MeOH and the three total measures, where the Pearson's correlation coefficient is given. 4MeOH= 4-methyoxy glucobrassicin, neo= neoglucobrassicin.

Glucosinolate	Correlation	P value	Significant with B-H?
Glucoiberin	-0.0791	0.37	NS
Progoitrin	0.0655	0.46	NS
Glucoraphanin	-0.0586	0.51	NS
Sinigrin	-0.121	0.17	NS
Gluconapin	0.0657	0.46	NS
Glucobrassicin	-0.129	0.15	NS
4MeOH	0.0878	0.32	NS
Neo	-0.0169	0.85	NS
Total aliphatics	-0.0978	0.27	NS
Total indoles	-0.0306	0.73	NS
Total	-0.0116	0.90	NS

There was also no significant correlation between glucosinolate levels and the pale red to green ratio (see Table 3.6). As with the blue to green ratio, this suggests that the pale red to green ratio provides little information about glucosinolate concentration. It is, however, interesting to note that, while non-significant, correlations between glucosinolates and the blue to green ratio tend to be negative, whereas those between glucosinolates and the pale red to green ratio tend to be positive.

Table 6. Correlation coefficients, associated p-values and significance corrections with B-H for the correlations between glucosinolate concentration and the pale red to green ratio. Df= 127 in all cases. Spearman's rank correlation coefficient is presented for all cases except 4-MeOH and the three total measures, where the Pearson's correlation coefficient is given. 4MeOH= 4-methyoxy glucobrassicin, neo= neoglucobrassicin.

Glucosinolate	Correlation	P value	Significant with B-H?
Glucoiberin	0.161	0.069	NS
Progoitrin	-0.0747	0.40	NS
Glucoraphanin	0.179	0.042	NS
Sinigrin	0.133	0.13	NS
Gluconapin	-0.172	0.051	NS
Glucobrassicin	0.226	0.010	NS
4MeOH	-0.00397	0.96	NS
Neo	0.190	0.031	NS
Total aliphatics	0.106	0.23	NS
Total indoles	0.200	0.024	NS
Total	0.171	0.052	NS

In contrast to the result obtained for the pale red to green ratio, there were significant positive correlations between the dark red to green ratio and concentrations of two glucosinolates, glucoraphanin and neoglucobrassicin. Total glucosinolate concentration and total indole concentration were also positively correlated with the dark red to green ratio (see Table 3.7

and Figure 3.13). Interestingly, the dark red to green ratio also varied significantly with a third glucosinolate, gluconapin, though here the correlation between redness and glucosinolate levels was negative.

Table 3.7. Correlation coefficients, associated p-values and significance corrections with B-H for the correlations between glucosinolate concentration and the dark red to green ratio. Df= 127 in all cases. Spearman's rank correlation coefficient is presented for all cases except 4-MeOH and the three total measures, where the Pearson's correlation coefficient is given. 4MeOH= 4-methyoxy glucobrassicin, neo= neoglucobrassicin.

Glucosinolate	Correlation	P value	Significant with B-H?
Glucoiberin	0.206	0.23	NS
Progoitrin	-0.0119	0.89	NS
Glucoraphanin	0.204	0.020	*
Sinigrin	<0.001	1.0	NS
Gluconapin	-0.230	0.0089	**
Glucobrassicin	0.130	0.14	NS
4MeOH	0.118	0.18	NS
Neo	0.297	<0.001	**
Total aliphatics	0.0390	0.66	NS
Total indoles	0.249	0.0046	**
Total	0.266	0.0023	**

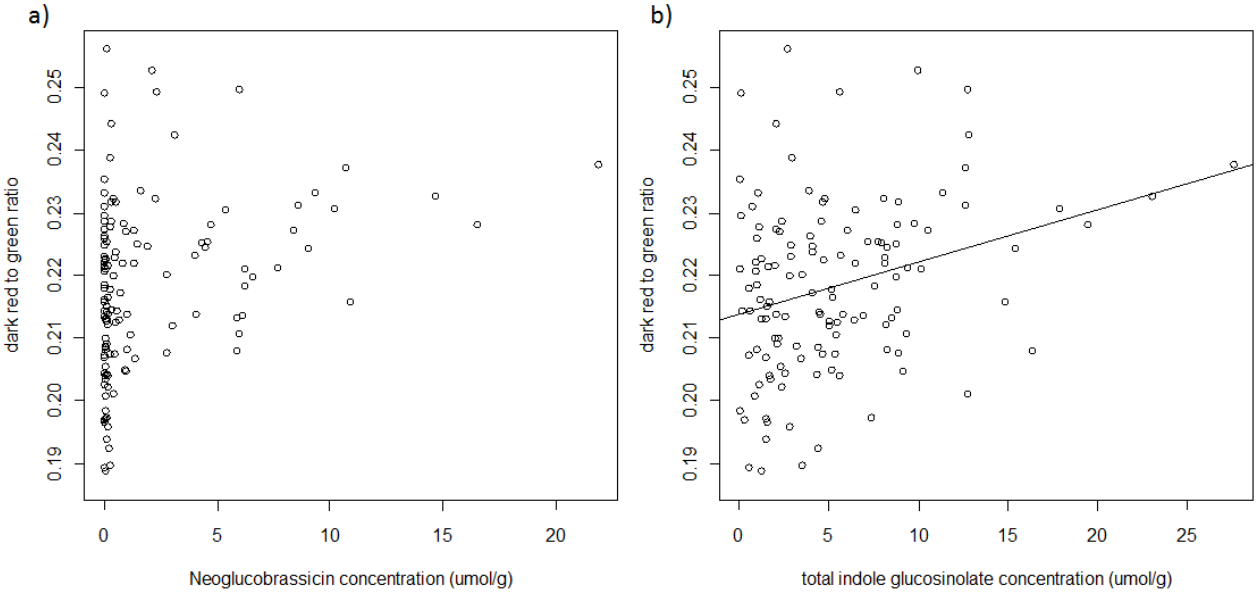

Figure 3.13. Correlations between the dark red to green ratio and a) neoglucobrassicin concentration and b) total glucosinolate concentration. Line is a least squares regression line (y=0.00118x + 0.216), produced from a linear model in which glucosinolate concentration was fitted as the predictor and colouration as the response.

3.3.2. How do herbivores respond to this information about hosts?

3.3.2.1. Snail herbivory

The effect of plant colouration on snail herbivory was not investigated, as snails probably do not use vision in host choice (Land and Nilsson 2002). Within

the wild *B. oleracea* populations studied, snails were significantly more likely to be found on plants with higher CN ratios ($F_{1,175}$=5.09, p=0.025; Fig 3.14a). In addition, snails were more likely to occur on larger plants ($F_{1,175}$=10.6, p=0.0013). Finally, snails were more common within sites in the east of the country than in the west ($F_{1,175}$=21.5, p<0.001; Fig 3.14b).

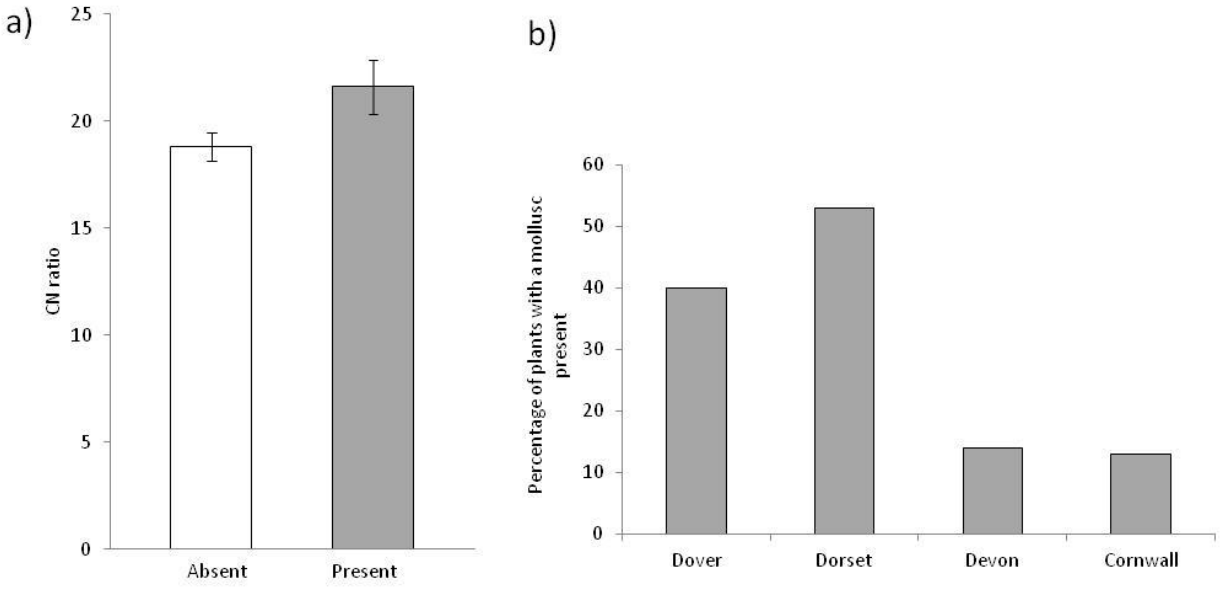

Figure 3.14. Factors affecting snail herbivory. a) mean CN ratio when snails were absent (white bars) and present (grey bars). Error bars show SEM. b) percentage of plants with a snail present at the four locations along the south coast.

Of the eight glucosinolates, only two aliphatic glucosinolates significantly predicted snail presence (see Figure 3.15). Concentrations of glucoiberin were significantly higher in plants occupied by snails, though this was only the case for plants at certain sites (glucoiberin x site interaction, $F_{1,121}$=5.05, p=0.026). In contrast, levels of progoitrin were significantly lower in plants occupied by snails (progoitrin x site interaction, $F_{1,121}$=6.74, p=0.011), though again this was only the case for plants at certain sites.

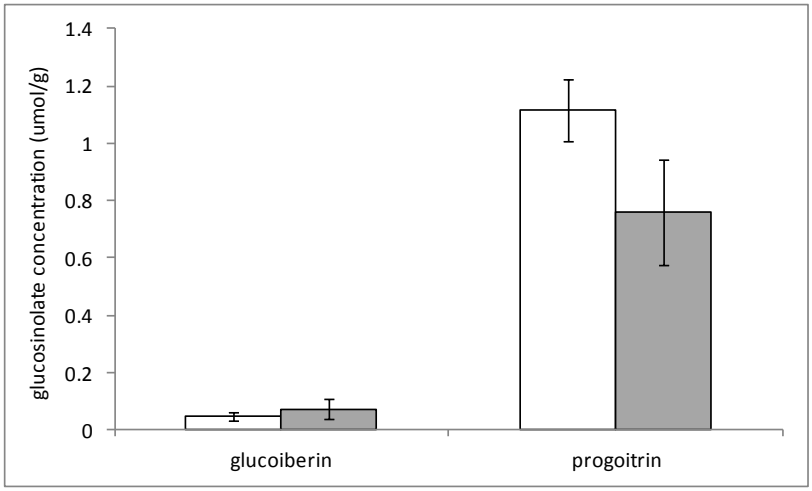

Figure 3.15. Mean glucosinolate concentration when snail was absent (white bars) and present (grey bars). Error bars show SEM.

3.3.2.2. Whitefly herbivory

The presence of whiteflies (*Aleyrodes proletella)* was significantly predicted by the blue to green ratio, with more whitefly present on bluer plants ($F_{1,529}$=8.07, p=0.0047; Fig 3.16). Plant brightness, however, did not significantly affect the presence of whitefly ($F_{1,529}$= 3.77, p=0.053). Whiteflies were more likely to be found on larger plants in the west of the country than in the east (size x site interaction, $F_{1,529}$=4.20, p=0.041). Whiteflies were also more likely to be found on larger plants without flowers than on smaller plants with flowers (size x number of flowers interaction, $F_{1,529}$=5.64, p=0.018).

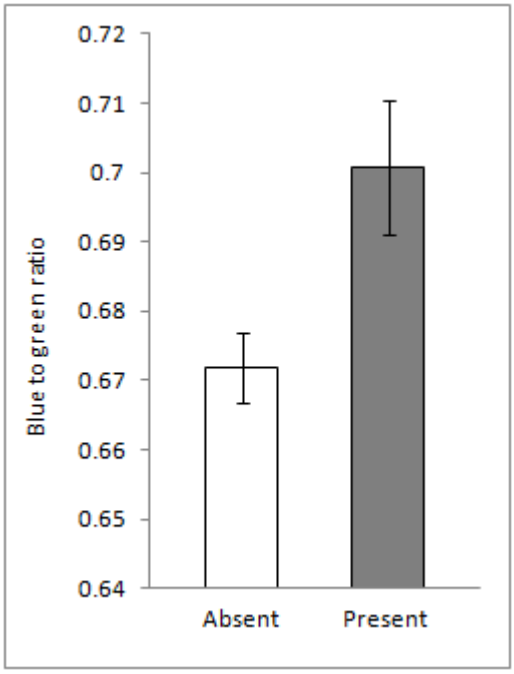

Figure 3.16. Mean blue to green ratio when *A. proletella* was absent (white bar) and present (grey bar). Error bars show SEM.

The CN ratio was important to whitefly herbivory; whiteflies were more likely to be found on plants with a lower CN ratio ($F_{1,176}$=8.40, p=0.0042; Fig 3.17a).

Of the aliphatic glucosinolates, only gluconapin significantly varied with whitefly herbivory, with increased concentrations in plants occupied by whitefly ($F1,_{116}$=5.29, p=0.023). In contrast, all three indole glucosinolates significantly predicted whitefly presence (glucobrassicin: $F_{1,128}$=8.81, p=0.0036; 4-methyglucobrassicin x site interaction: $F_{1,128}$=5.63, p=0.019; neoglucobrassicin: $F_{1,128}$=8.38, p=0.0045; Fig 3.17b). Interestingly, glucobrassicin and neoglucobrassicin differed in their effect from 4-methyoxyglucobrassicin. While glucobrassicin and neogluobrassicin were found in higher levels in plants without whitefly, the opposite pattern was seen for 4-methyoxyglucobrassicin.

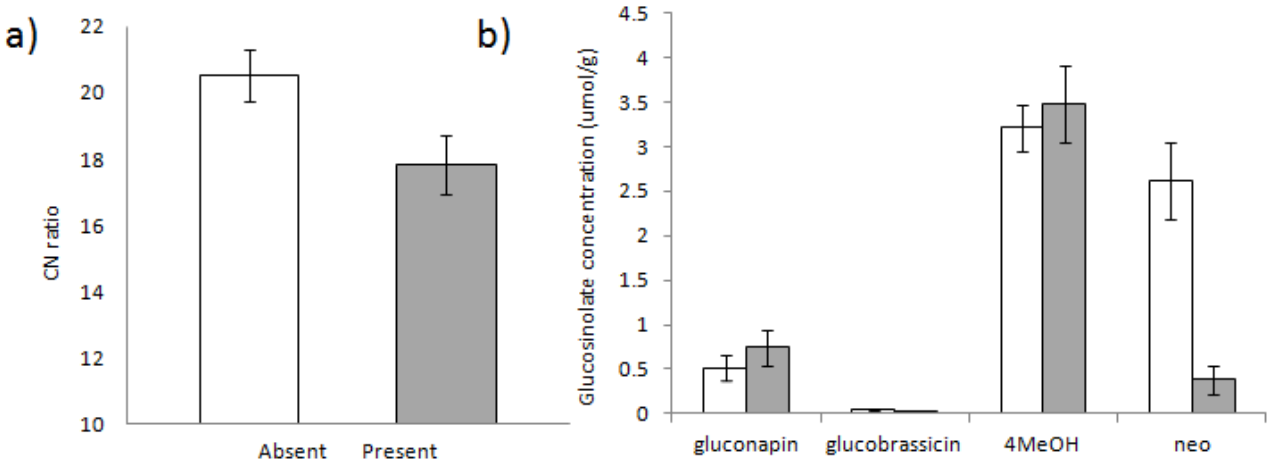

Figure 3.17. Plant chemistry and whitefly herbivory. a) mean CN ratio when whitefly was absent (white bars) and present (grey bars). Error bars show SEM. b) mean glucosinolate concentration when whitefly was absent (white bars) and present (grey bars). Error bars show SEM. 4MeOH= 4-methyoxy glucobrassicin, neo= neoglucobrassicin.

3.3.2.3. Weevil herbivory

Neither plant brightness nor the blue to green ratio significantly predicted the presence of weevils (*Ceutorhynchus quadridens* and *C. assimilis*) on *B. oleracea* (brightness: $X^2_{1,531}$=-0.613, p=0.43, blue to green ratio: $X^2_{1,531}$=-3.27, p=0.071). Weevils, however, were significantly more likely to be found on larger plants than smaller plants, though only where larger plants also had more flowers (size x number of flowers: $X^2_{1,531}$=-13.5, p<0.001), which may be expected as the seed weevil (*C. assimilis*) lays its eggs in seed pods. Across all sites, weevils were most likely to be found in Dorset ($X^2_{1,531}$=5.17, p=0.023).

Surprisingly, the CN ratio did not predict weevil presence ($X^2_{1,172}$ =-0.266, p=0.61). Of the glucosinolates, only 4-methyglucobrassicin varied significantly with weevil herbivory and was found at higher levels where weevils were present ($F_{1,121}$=9.86, p=0.0017; Fig 3.18).

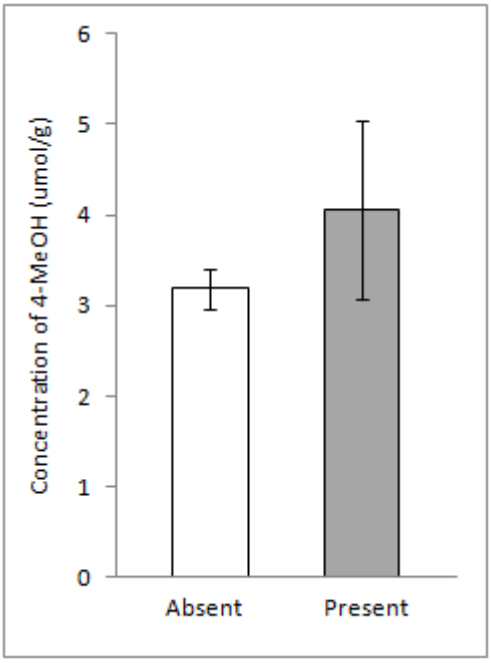

Figure 3.18. Mean concentration of 4-methyoxyglucobrassicin (4MeOH) when weevils were absent (white bars) and present (grey bars). Error bars show SEM.

3.3.2.4. Flea beetle herbivory

Flea beetles were found to respond differently to the weevils. The presence of flea beetles (*Psylliodes chrysocephala* and *P. undulata*) was significantly predicted by the blue green ratio, with more beetles occurring on bluer plants ($F_{1,531}$=4.75, p=0.030; Fig 3.19a). Flea beetles were also more likely to be found on brighter plants, though only if these had many flowers (brightness x number of flowers interaction, $F_{1,531}$=5.53, p=0.019; Fig 3.19b). Site also significantly predicted flea beetle presence, with greater numbers of flea beetles found in Dorset ($F_{1,531}$=4.77, p=0.029).

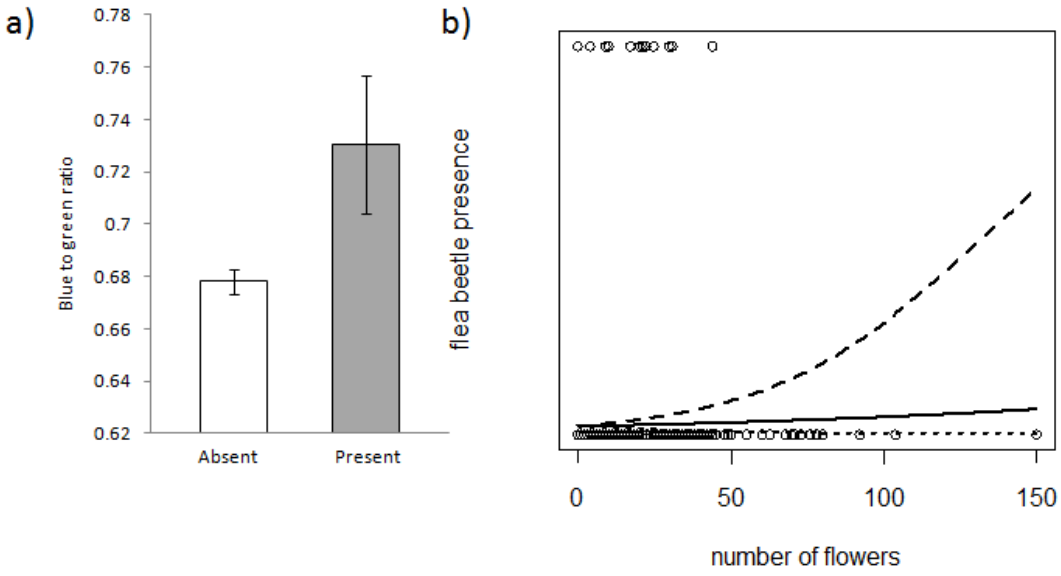

Figure 3.19. Factors predicting flea beetle herbivory. a) Mean blue to green ratio when beetles were absent (white bars) and present (grey bars). Error bars show SEM. b) interaction with number of flowers and plant brightness to predict beetle presence as predicted from the GLM. Solid black line shows relationship with number of flowers and flea beetle presence at mean level of plant brightness, dotted line at the lower interquartile range level of brightness and the dashed line at the upper interquartile range of brightness.

The CN ratio did not significantly predict the presence of flea beetles (GLM, $X^2_{1,169}$=0.0726, p=0.79). Unfortunately, too few flea beetles were found on plants sample for glucosinolate analysis to test for an effect of glucosinolates on flea beetle herbivory.

3.3.2.5. Pieris brassicae herbivory

Significant effects of colouration on *P. brassicae* presence occurred through interactions with other predictors. Thus, the blue to green ratio influenced *P. brassicae* presence via an interaction with site (site x blue to green ratio interaction, $X^2_{1,288}$=5.84, p=0.016; Fig 3.20a) such that *P. brassicae* was more likely to be found on less blue plants moving west along the coast until the Cornwall sites where the butterfly was more likely to be found on bluer plants. The pale red to green ratio also affected *P. brassicae* presence, this time through an interaction with the number of flowers (number of flowers x pale red to green ratio interaction, $X^2_{1,288}$=3.88, p=0.049; Fig 3.20b), such that larvae were more likely to occur on less red plants with many flowers . Finally, the dark red to green ratio also affected *P. brassicae* presence, through an interaction with plant size (dark red to green ratio x size interaction, $X^2_{1,288}$=4.44, p=0.035; Fig3.21c), such that larvae were more likely to be present on large plants with less red colouration.

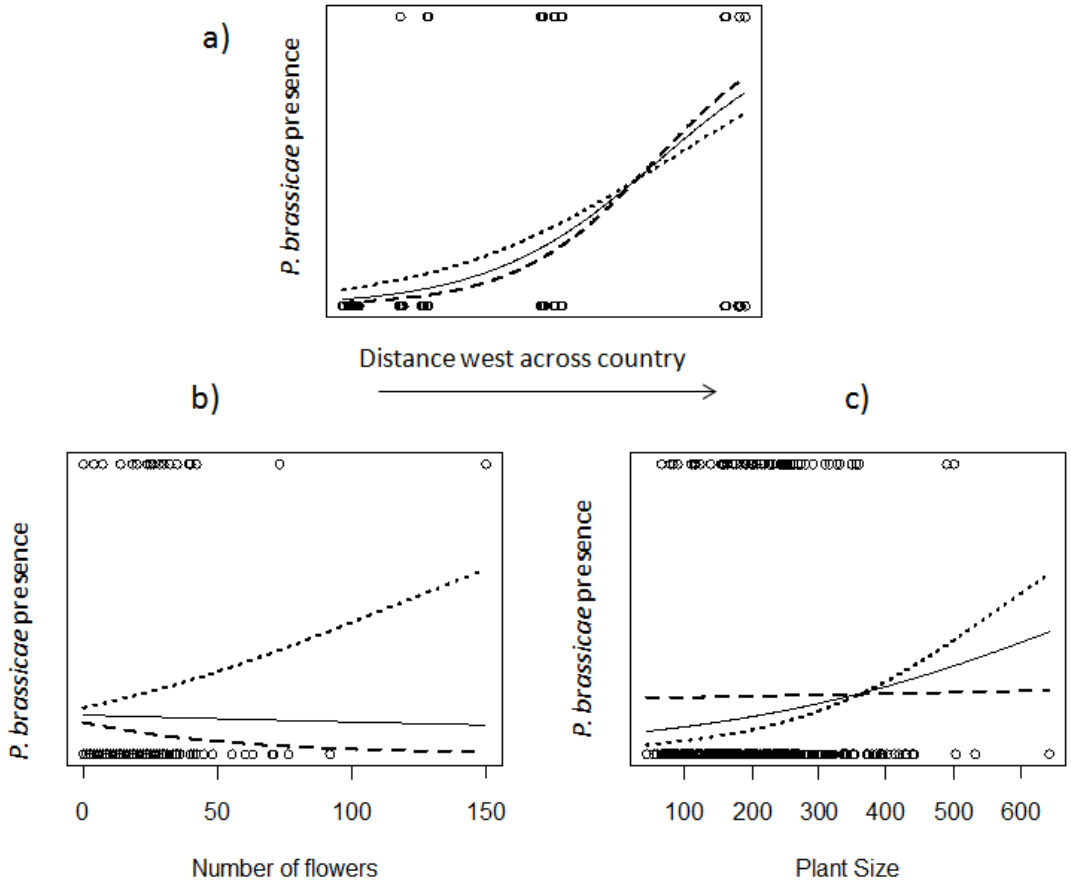

Figure 3.20. Interactions with colouration to predict *P. brassicae* presence. a) interaction with location and blue to green ratio to predict *P. brassicae* presence as predicted from the GLM. Solid black line shows relationship with a mean level of blue to green ratio and *P. brassicae* presence, dotted line at the lower interquartile range level of the blue to green ratio and the dashed line at the upper interquartile range of blue to green ratio. b) interaction with number of flowers and pale red to green ratio to predict *P. brassicae* presence as predicted from the GLM. Solid black line shows relationship with a mean level of pale red to green ratio and *P. brassicae* presence, dotted line at the lower interquartile range level of the pale red to green ratio and the dashed line at the upper interquartile range of pale red to green ratio. c) interaction with plant size and dark red to green ratio to predict *P. brassicae* presence as predicted from the GLM. Solid black line shows relationship with a mean level of dark red to green ratio and *P. brassicae* presence, dotted line at the lower interquartile range level of the dark red to green ratio and the dashed line at the upper interquartile range of dark red to green ratio.

Pieris brassicae larvae were more likely to occur on plants with a lower CN ratio ($F_{1,111}$= 7.96, p=0.0057; Fig 3.21). Surprisingly, and in contrast with the results for *P. rapae*, there was no significant effect of any glucosinolate on *P. brassicae* presence.

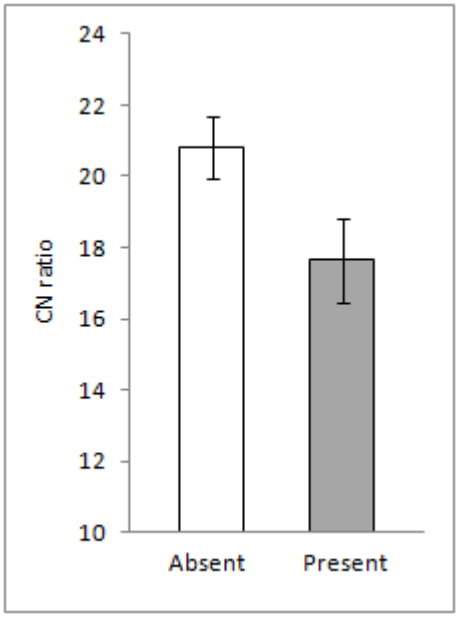

Figure 3.21. Mean CN ratio when *P. brassicae* was absent (white bars) and present (grey bars). Error bars show SEM.

3.3.2.6. Pieris rapae herbivory

Figure 3.22 displays the variation present in the blue to green ratio and the red to green ratios based on the spectral sensitivities of *P. rapae*. Variation in the blue to green ratio is much greater than in either the pale red to green or dark red to green ratios (pale red: $F_{1,547}$=9.97, p<0.001, dark red: $F_{1,547}$=24.5, p<0.001; Fig 3.22a). A strong negative correlation was found between the pale red to green and blue to green ratios (r_s=-0.779, p<0.001), while a significant positive correlation was found between the dark red to green and blue to green ratios (r_s= 0.246, p<0.001) (Figure 3.22b). The fact that both the red to green ratios are correlated with the blue to green ratio suggests that both aspects of plant colouration are likely to reflect similar information about aspects of a plant's phenotype, though the weaker correlation between the dark red to green and blue to green ratios indicates that the dark red to green may potentially provide separate information not captured by the blue to green ratio. The two red ratios were also correlated (r_s=0.392, p<0.001; Fig 3.22c), which again might suggest that these reflect similar information.

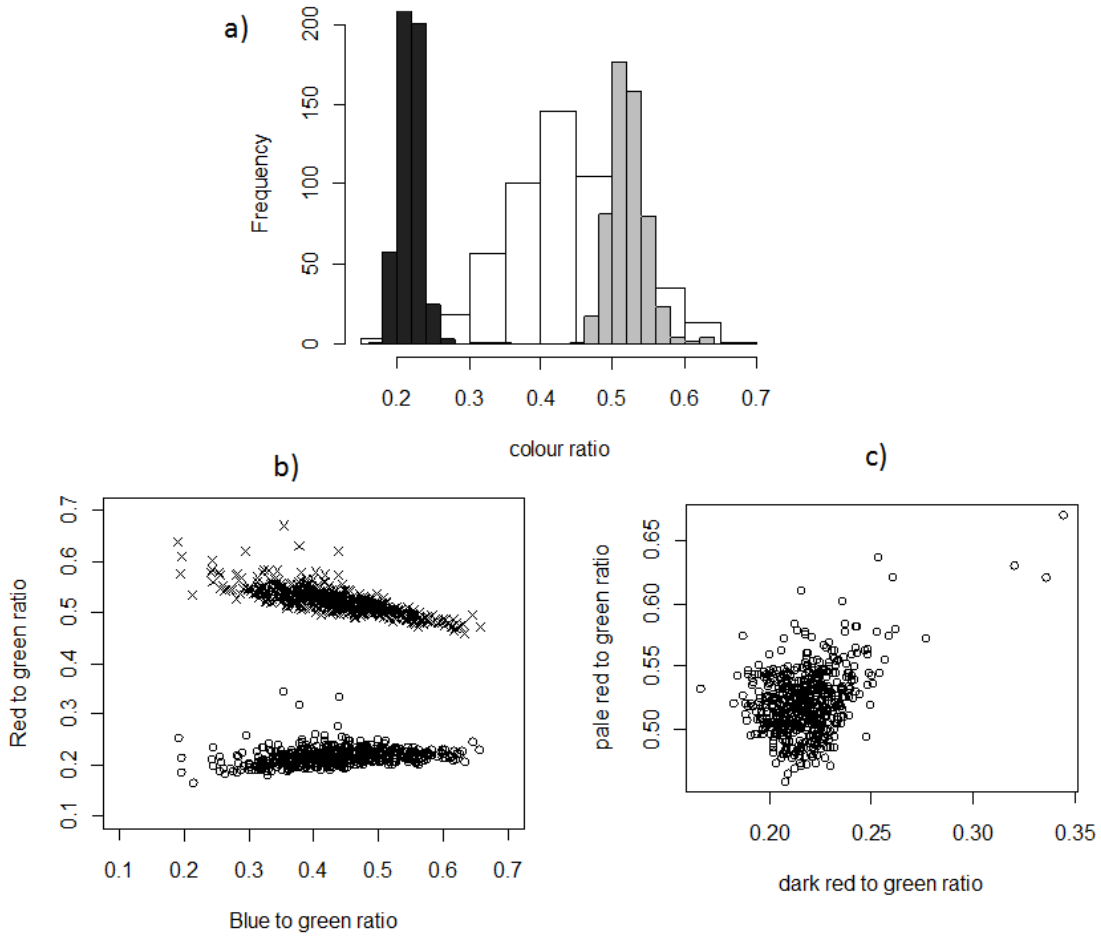

Figure 3.22. Plant colouration based on spectral sensitivities of *P. rapae*. Colouration from the perspective of *P. rapae*. a) Histogram of the three colour ratios. White bars= blue to green ratio, dark grey bars= dark red to green ratio, pale grey bars= pale red to green ratio. b) Correlation of the red to green ratios and the blue to green ratio. Open circle points= dark red to green ratio, crossed points= pale red to green ratio (Blue to green and pale red to green r_s=-0.779, p<0.001, blue to green and dark red to green r_s= 0.246, p<0.001). c) correlation between the red ratios (r_s=0.392, p<0.001).

Pieris rapae larvae were more likely to occur on plants with a high blue to green ratio (X^2_1= -5.47, p=0.019; Fig 3.23a) and a low dark red to green ratio (X^2_1=-9.25, p=0.0024; Fig 3.23b). Lower pale red to green ratios were also significantly associated with *P. rapae*, though only in more western sites (pale red to green ratio x site: X^2_1=-5.07, p=0.024; Fig 3.23c). There was also a significant interaction between plant size and site such that larger plant in more western sites were more likely to host *P. rapae* larvae (size x site interaction, X^2_1= 8.10, p=0.0044).

The relationship with colouration is similar to *P. brassicae* in that long wavelengths appear to predict the presence of larvae; however, this relationship appears stronger than that seen for *P. brassicae* where significant effects of colouration occurred only through interactions with various other predictors.

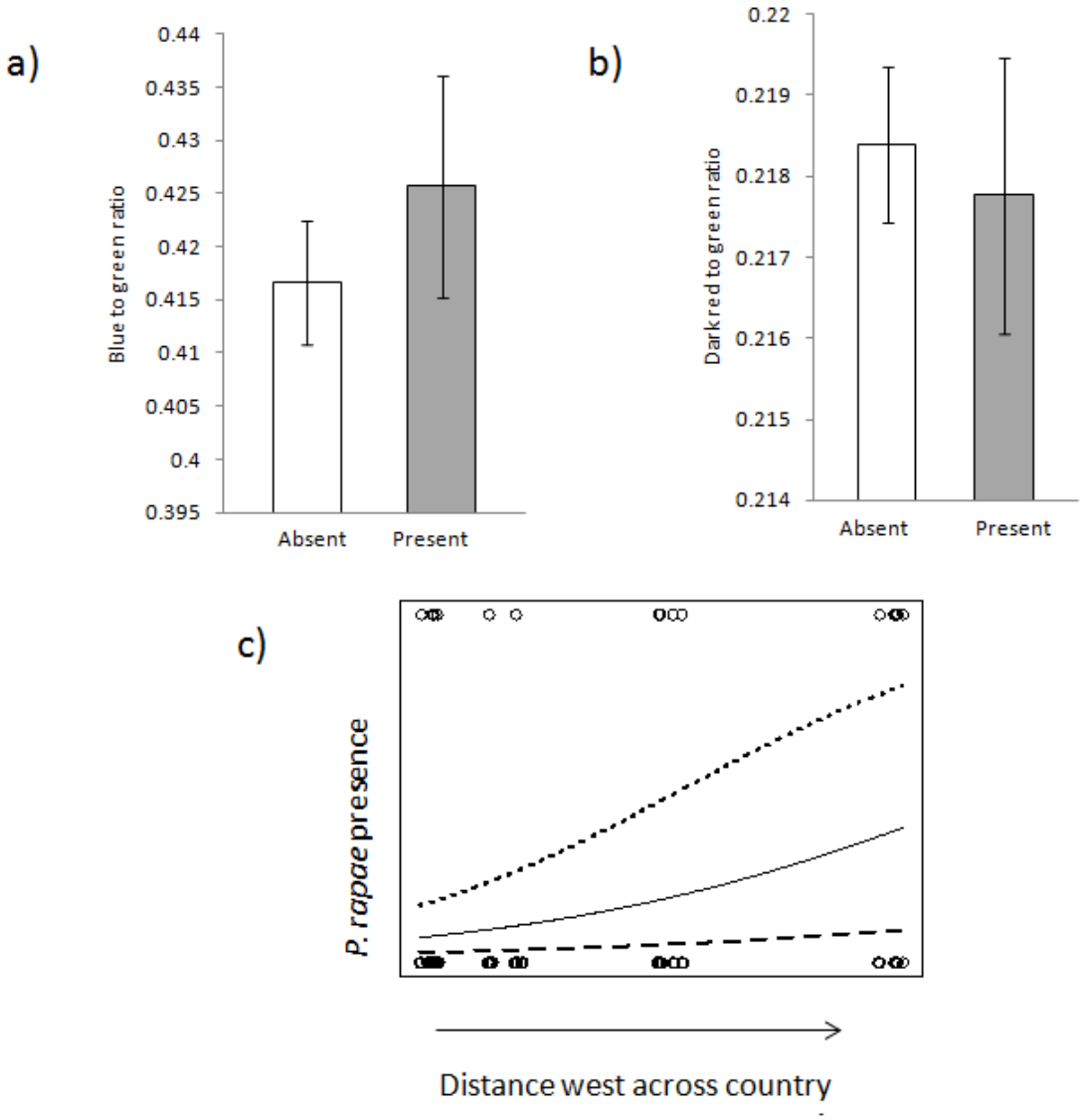

Figure 3.23. Factors affecting *P. rapae* herbivory. a) relationship between blue to green ratio and *P. rapae* herbivory. White bar= mean ratio when *P. rapae* is absent from the plant, grey bar= mean ratio when *P. rapae* is present. Error bars show SEM. b) relationship between dark to green ratio and *P. rapae* herbivory. White bar= mean ratio when *P. rapae* is absent from the plant, grey bar= mean ratio when *P. rapae* is present. Error bars show SEM. c) interaction with location and pale red to green ratio to predict *P. rapae* presence as predicted from the GLM. Solid black line shows relationship with location and *P. rapae* at mean level of pale red to green ratio, dotted line at the lower interquartile range level of redness and the dashed line at the upper interquartile range of redness.

The presence of *P. rapae* larvae was significantly predicted by an interaction between CN ratio and plant size, such that larvae were more likely to be present on large plants with low CN ratios (CN ratio x size interaction, $X^2_{1,105}$= 9.07, p=0.0026). Independently, CN ratio also varied with *P. rapae* presence through an interaction with the dark red to green ratio (CN ratio x dark red to green ratio interaction, $X^2_{1,105}$= 4.44, p=0.035), such that *P. rapae* larvae were more likely to occur on plants with lower red to green ratios and low CN ratios (see Figure 3.24a).

It was not possible to discern clear patterns between glucosinolate concentration and *Pieris rapae* herbivory (see Figure 3.24b). Gluconapin was found at lower concentrations where *P. rapae* occurred, though this was only the case for certain sites (gluconapin x site: X^2_1=6.38, p=0.012). A second glucosinolate, glucoraphanin, was also found at lower concentrations where *P. rapae* was present (X^2_1=4.50, p=0.034); however, a third glucosinolate, progoitrin, occurred at higher concentrations where *P. rapae* was present (X^2_1=3.85, p=0.050). Effects of colouration, CN and all glucosinolates of this full model can be found in Table B.2 in Appendix B.

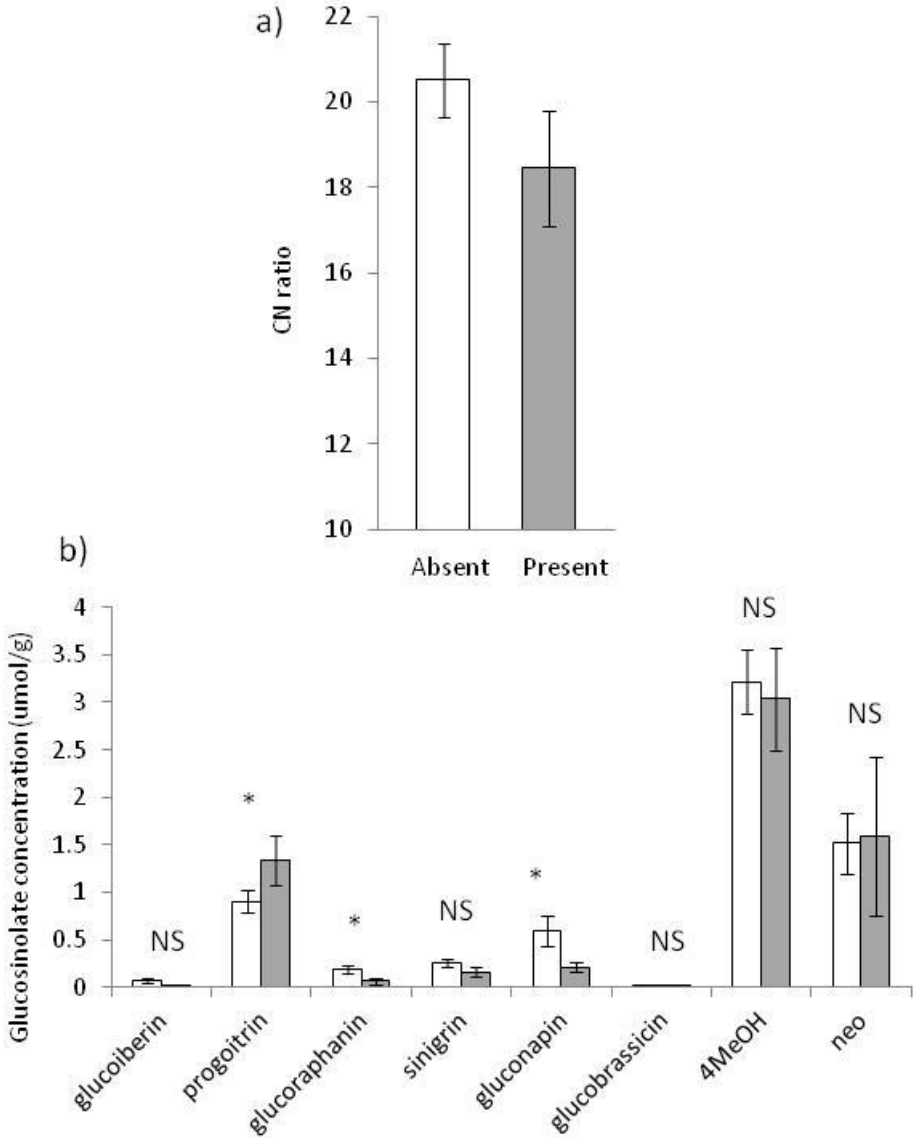

Figure 3.24. Plant chemistry and *P. rapae* herbivory. a) Mean CN ratio when *P. rapae* is absent (white bar) and present (grey bar) on the host plant. Error bars show SEM. b) Mean glucosinolate concentrations when *P. rapae* is absent (white bar) and present (grey bar) on the host plant. NS= not significant, *p<0.05. 4MeOH= 4-methyoxy glucobrassicin, neo= neoglucobrassicin.

3.3.2.7. Brevicoryne brassicae herbivory

Figure 3.25 displays frequency plots for measures of brightness and blue to green ratio based on the spectral sensitivities of *B. brassicae*. Variation among plants in the blue to green ratio was significantly greater than for brightness ($F_{1,547}$=0.123, p<0.001). The correlation between brightness and the blue to green ratio, though statistically significant, is not strong, with only 12% of the variation in brightness explained by variation in the blue: green ratio (r=0.116, df=546, p=0.0067). This indicates that brightness and the blue to green ratio may potentially reflect different aspects of the plant's phenotypic quality, and that both traits may provide important information to *B. brassicae*.

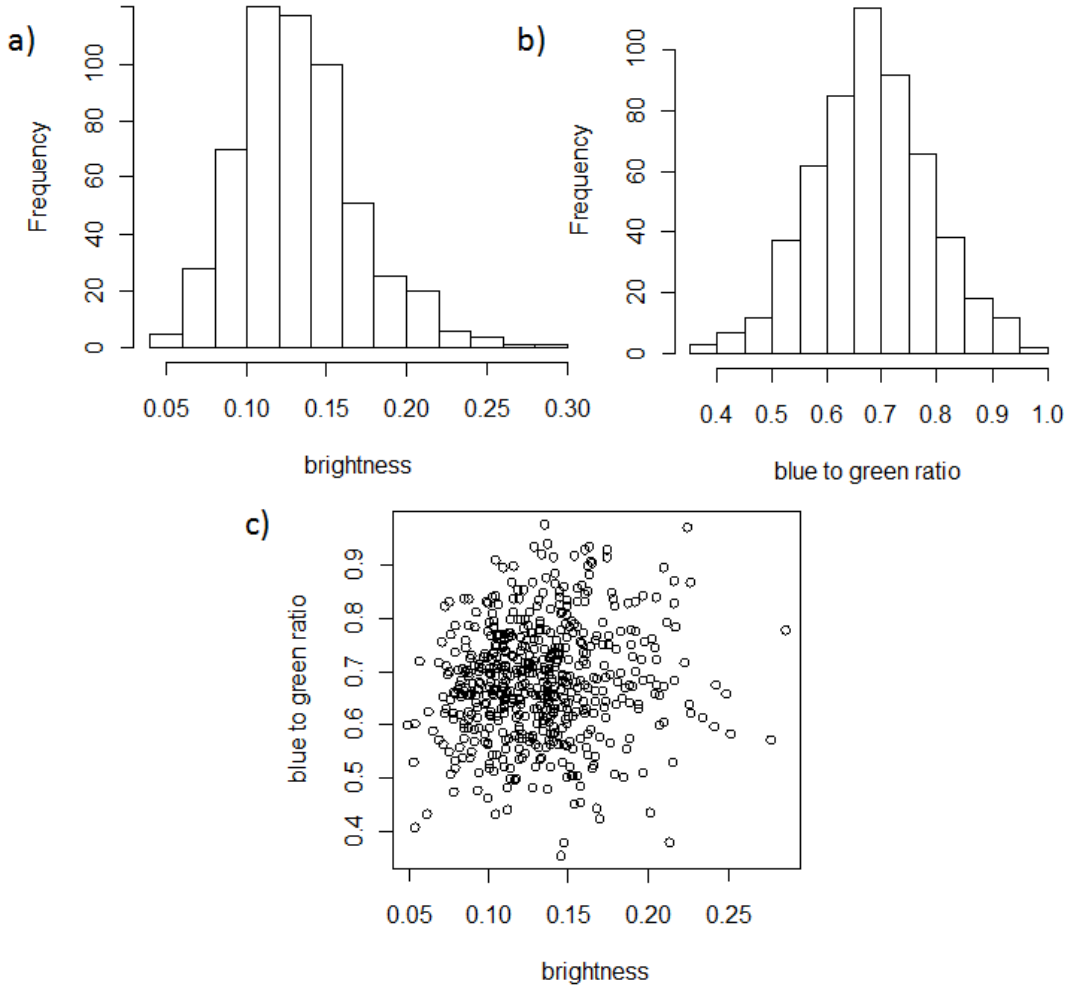

Figure 3.25. Plant colouration based on spectral sensitivities of *B. brassicae.* a) histogram of plant brightness to show spread of the data, b) histogram of the blue to green ratio to show spread of the data, c) correlation between brightness and blue to green ratio r=0.116, df=546, p=0.0067).

The presence of *B. brassicae* on cabbages was significantly predicted by an interaction between the blue to green ratio and the number of flowers ($X^2_{1,529}$=5.02, p=0.025; Fig 3.26), such that plants with a greater number of flowers and a lower blue to green ratio were more likely to host *B. brassicae*. Aphid presence was not predicted by plant brightness ($X^2_{1,529}$=0.0350, p=0.85). Aphids were more likely to be found on plants in the west of the country if they had more flowers (number of flowers x site interaction, $X^2_{1,529}$=4.01, p=0.045). In addition, larger plants were more likely to be host to aphids ($X^2_{1,529}$=12.3, p<0.001).

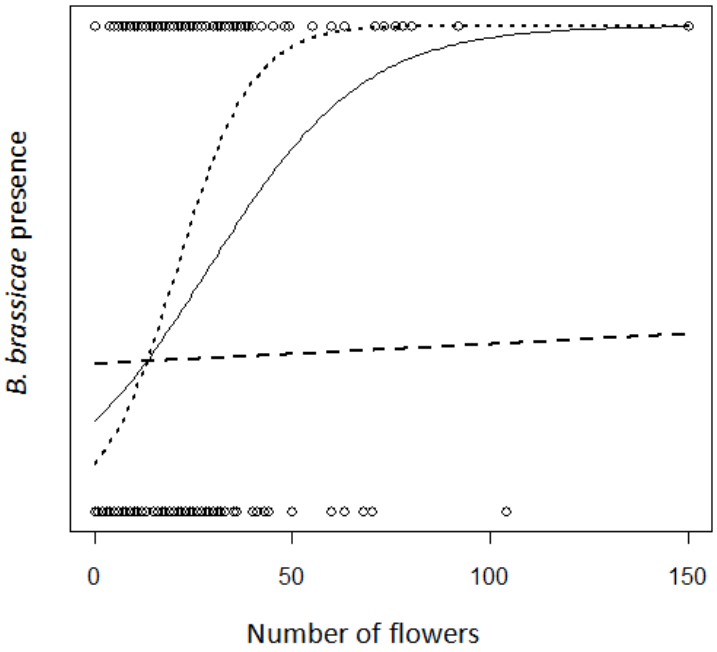

Figure 3.26. The interaction between the blue to green ratio and the number of flowers to predict aphid presence. The solid black line shows the relationship between the number of flowers and aphid presence when a mean level of colouration is considered. The dotted line represents the lower quartile value of the blue to green ratio, therefore this is the relationship between aphid presence and number of flowers when the blueness is low. The dashed line represents the upper quartile value of the blue to green ratio, and thus shows the relationship between aphid presence and the number of flowers when the blueness is high.

The CN ratio was an important predictor of aphid presence – as expected, aphids were more likely to be found where the ratio of carbon was lower (see Figure 3.27), though this effect was dependent upon the site at which the plant was growing, with greater numbers of aphids in western sites (site x CN ratio interaction, $X^2_{1,169}$=10.2, p=0.0019).

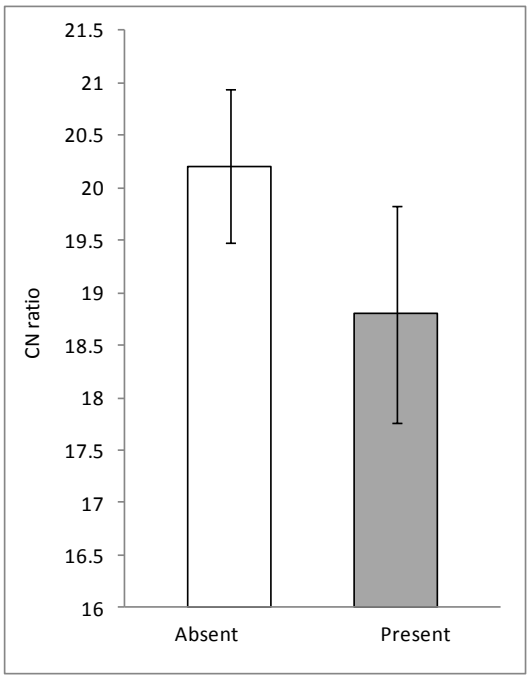

Figure 3.27. Mean CN ratio of *B. oleracea* when *B. brassicae* is absent and present on the plants. Error bars show SEM.

Only two of the eight glucosinolates significantly predicted aphid presence, glucobrassicin (F_{124}=5.93, p=0.016) and gluconapin ($F_{1,124}$=8.86, p=0.0035) (see Figure 3.28). The concentration of glucobrassicin was more than twice as high in plants where aphids were absent (mean when absent=0.044, n=87, sd=0.14, mean when present=0.013, n=42, sd=0.03). The opposite relationship was found for gluconapin, which was twice as high in plants with aphids present (mean = 0.90, n = 42, sd=1.73) than where aphids were absent (mean=0.42, n=87, sd=1.02). Effects of colouration, CN and all glucosinolates of this full model can be found in Table B.1 in Appendix B.

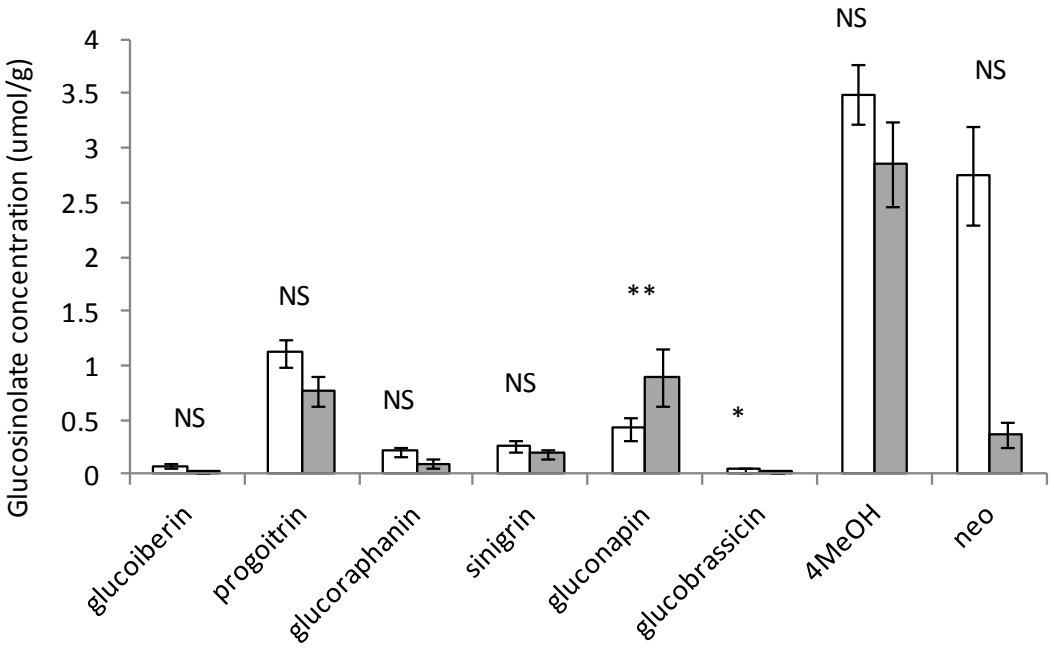

Figure 3.28. Mean glucosinolate concentrations when aphids were absent (white bars) and present (grey bars). Error bars show SEM. Significance values from GLM. NS=not significant, *p<0.05, **p<0.01. 4MeOH=4-methyoxy glucobrassicin, neo=neoglucobrassicin.

3.3.3. Summary

A summary of the effects of all plant factors on the herbivores can be found overleaf in Table 3.8. Generally, herbivores were more likely to be found on plants in the western sites. Larger plants with flowers and low CN ratio were also more likely to host herbivores. Herbivores were more likely to be found on plants with a high blue to green ratio, except for *B. brassicae* where the opposite was true. Plant brightness only affected a few herbivores and here brighter plants were preferred. The butterflies were more common on less red plants (considering both the pale red and dark red to green ratios). There was no clear patter with glucosinolates, although progoitrin, gluconapin and the indole glucosinolates most commonly influenced herbivore presence.

Table 3.8. Summary of the influences of the plant factors site, size, number of flowers, CN ratio, colour and glucosinolate concentrations on herbivore presence.
*The starred effects occur through interactions (see in text).

	Preference for plant factors:					
Herbivore type	**Site**	**Size**	**Number of flowers**	**CN ratio**	**Colour**	**Glucosinolates**
Snails	Eastern sites	Larger	No effect	High CN	N/A	High glucoiberin* Low progoitrin*
Whitefly	Western sites*	Larger*	Less flowers*	Low CN	Bluer Brightness no effect	High gluconapin Low glucobrassicin Low neo High 4-MeOH*
Weevil	Most in Dorset	Larger*	More flowers*	No effect	Blue and brightness no effect	High 4-MeOH
Flea beetle	Most in Dorset	No effect	More flowers*	No effect	Bluer Brighter*	N/A
P. brassicae	Western sites*	Larger*	More flowers*	Low CN	Less blue* Less dark red* Less pale red*	No effect
P. rapae	Western sites*	Larger*	No effect	Low CN*	Bluer Less dark red Less pale red*	Low gluconapin* Low glucoraphanin High progoitrin
B. brassicae	Western sites*	Larger	More flowers*	Low CN*	Less blue* Brightness no effect	Low glucobrassicin High gluconapin
Overall pattern?	**Western sites**	**Larger**	**More flowers**	**Low CN**	**Bluer, less red, brighter**	**No clear pattern**

3.4. Discussion

This study presents the first field survey of intraspecific plant colouration variation analysed with insect spectral sensitivity, and its association with plant defence and herbivory. Colouration was correlated with glucosinolate levels; in particular plant brightness and the dark red to green ratio, and so this may have implications for both aphid and butterfly herbivory. Plant colour and glucosinolate concentrations were found to be important in predicting the presence and absence of *B. oleracea* herbivores. These relationships suggest colour has the possibility to act as a signal of chemical defence, providing support for the ACH.

3.4.1. What features of wild *B. oleracea* affect colour?

3.4.1.1. What is the evidence that colour reflects aspects of host quality?

Colour was not correlated with plant size, which suggests that plant growth is not traded-off with colour expression. The number of flowers was positively correlated with red colouration. If colouration is an indicator of plant quality, this suggests that both the number of flowers (investment in reproduction) and the intensity of red colouration could function as potential indicators of quality. Pale redness was negatively correlated with the density of surrounding plants. High density may reflect a poorer quality individual as competition levels could be high, and this could be indicated by the increasing redness as competition decreases. Redness is thought by some authors to be an indicator of stress (Costa-Arbulu et al. 2001), but these results suggest that stress due to high density and therefore high levels of competition is unlikely to increase redness, as the reverse relationship would be expected if this was the case.

Differences in plant colouration were found between sites, which may be the result of genetic differentiation of populations. *Brassica oleracea* seeds are thought to be dispersed all along the south coast (Wichmann et al. 2009), although it is possible that populations in remote areas become genetically isolated. Differences in colouration may also be due to environmental factors differing between sites (Schaberg et al. 2003, Issarakraisila et al. 2007). The influence of genetic versus environmental factors on colouration will be explored in greater detail in Chapter Four, where these effects will be separated using a common garden design.

3.4.1.2. Could colouration be constrained by nitrogen?

A robust correlation of a medium effect size (Cohen 1988) between colouration and CN ratio was found. Given the large number of factors likely to affect CN and colouration in the field,

the size of this effect suggests that the covariation of colour and CN is likely to be a real phenomenon and potentially of biological significance. A relationship between colouration and nitrogen content has been documented elsewhere but from a physiological perspective rather than exploring the relationship between colouration and herbivory. For example, It was found that sugar maple trees (*Acer saccharum*) with a lower foliar nitrogen content turned redder at autumn time, due to an increase in anthocyanin levels (Schaberg et al. 2003). These anthocyanins are thought to increase nutrient reabsorbtion, which would be particularly important if the tree had low levels of nitrogen to begin with (Schaberg et al. 2003). Another study found that dying Norway maple (*Acer platanoides*) trees were redder and had lower levels of nitrogen, again pointing to a role of nutrient reabsorbtion by anthocyanins (Sinkkonen 2008).

It is conceivable that nitrogen content may constrain expression of colours. Though the chemicals underlying variation in colour in wild *B. oleracea* have yet to be identified, it is likely that some of this variation, especially in the red regions of the spectrum, is caused by anthocyanins (Lo Scalzo et al. 2008). The production of anthocyanins is thought to be costly to the plant (Winkel-Shirley 2001); anthocyanin-based colouration and glucosinolate content may therefore be linked through allocation costs, whereby nutrients used in defence are consequently unavailable for use in colour production (Herms and Mattson 1992, Strauss et al. 2002). Whatever the chemical basis of the link between colouration and CN ratio, colouration is at least a reliable indicator of CN ratio. Unlike communication about chemical defence, however, there is no obvious benefit to the plant of communicating CN content. This means it could be a cue for herbivores but not a signal (see Chapter One).The focus in this thesis is on relationship between colouration and plant defence (i.e. glucosinolates), which pertains to the ACH (Hamilton and Brown 2001).

3.4.1.3 Could glucosinolate concentration be constrained by nitrogen?

CN content of the plant did not correlate with glucosinolate levels. This is a surprising finding because allocation theory (Herms and Mattson 1992) suggests that if a plant has high levels of nitrogen, more nutrients will be available to invest in nitrogen-based chemical defence (Bryant et al. 1983). Previous work on *Eucalyptus cladocalyx* has shown that levels of chemical defence increase when nitrogen fertilisers are added (Simon et al. 2010). A smiliar effect has been found for glucosinolates, although this varies depending on the type of fertiliser used (Staley et al. 2010) and the type of glucosinolate analysed (Aires et al. 2006). The absence of a relationship between glucosinolates and CN ratio in the present study is based solely on

correlations, and it is unknown how the glucosinolate content of wild *B. oleracea* in the field would change with experimental manipulations of CN ratio.

The finding that glucosinolate concentration is not correlated with foliar CN ratio in the field may suggest that in some sense investment in glucosinolate-based defence might be ring-fenced i.e. wild *B. oleracea* plants must allocate a certain amount of nitrogen to defence regardless of the overall levels of nitrogen. This would imply that achieving at least a minimal level of defence is vital, and indeed glucosinolates provide essential resistance against herbivores (Hopkins et al. 2009). In a laboratory experiment with only one herbivore and with levels of aliphatic glucosinolates reduced through gene manipulation, herbivore damage was found to be negatively correlated with levels of glucosinolate (Beekwilder et al. 2008). This shows how important glucosinolates are and this effect is expected to be larger in the field with more herbivores present.

The production of glucosinolates may well be constrained by other micronutrients, in particular sulphur, which all glucosinolates contain (Halkier and Gershenzon 2006). This cannot be addressed with this data set, but previous research has shown that addition of sulphur increases glucosinolate concentration (Kestwal et al. 2011) and that that glucosinolate biosynthesis is repressed with sulphur deficiency (Hirai et al. 2005). Therefore it would be unwise to conclude from CN ratio alone either that there are no constraints on glucosinolate expression or that there are no differential costs associated with their production.

3.4.1.4 Could colouration inform insect herbivores about glucosinolate concentration?

There was evidence for some correlations between plant brightness and glucosinolate concentration. The statistical significance of these correlations depends on the application of controversial corrections (Nakagawa 2004). The correlations between six glucosinolates and brightness translated into small effect sizes. For example, 22% of the variation in levels of neoglucobrassicin was explained by plant brightness. This suggests that information about these chemicals could be provided by brightness. There was little to suggest that the blue to green ratio predicts glucosinolates in the field, however.

The strongest relationship with colouration and glucosinolate concentration was with the dark red to green ratio. This correlation was found to be positive with most glucosinolates: as red colouration increases in intensity, glucosinolate concentration also increases. This is as predicted by the ACH, that colouration, and more specifically, redness intensity, can provide information about chemical defence. It should be stressed that the use of the red to green ratio in host choice is of potential importance only for butterflies. Aphids do not have a red receptor, meaning that red to green ratios cannot meaningfully be derived for these insects.

Instead, differences in redness must be perceived through differences in the blue to green ratio (Doring and Chittka 2007). An object that is 'red' peaks in the red region of the spectrum. *Pieris rapae* can more reliably perceive this than *B. brassicae* as it has a red receptor most sensitive in this region and comparisons between the red and green receptor (red to green ratios) will detect this (Qiu and Arikawa 2003) (see Chapter Six). *Brevicoryne brassicae* will be able to 'see' this red object through stimulation of the blue receptor, and the blue to green ratio will be different for a red object compared to a green object (Doring et al. 2009). Together, these results indicate that it would be profitable in the first instance to explore the use of the dark red to green ratio as a guide to glucosinolate levels, but that it will also be important to consider the use of plant brightness as a guide to glucosinolate content, in particular for those herbivores lacking red photoreceptors (see Chapters Five and Six).

Previous studies have found a link with colouration and chemical defence, agreeing with the data presented here. For example, Cooney et al. (2012) found that the redness of the leaf margins of the New Zealand pepper tree (*Pseudowintera colorata*) increased as the levels of chemical defence increased, suggesting that the intensity of redness could provide a cue of chemical defence. Although leaf colouration and glucosinolate content have never before been linked in brassicas, flower colour and glucosinolates have (Irwin et al. 2003). Plants with more colourful flowers were found to produce higher levels of indole glucosinolates, and these plants were less preferred by leaf-eating herbivores (Irwin et al. 2003). This provides further evidence that plant colouration could indicate concentrations of glucosinolates. A mechanistic link between *B. oleracea* dark red colouration and glucosinolate content has yet to be identified. If, however, the dark red colouration is produced on the phenylpropanoid pathway (which is likely if it is caused by anthocyanins, see Chapter One for details on the production of anthocyanins), then this colouration could potentially be linked to glucosinolate production through a shared enzyme involved in both biosynthetic pathways (Hemm et al. 2003).

Addressing question one in the first part of this study suggests a link with plant defence chemistry and the intensity of colouration, as required by the ACH. I will now move to consider question two to see if these factors are important to the herbivores of *B. oleracea*.

3.4.2. How do herbivores respond to this information?

In order to determine whether *B. oleracea* colouration could potentially provide important information to herbivores, leaf colouration was modelled using *B. brassicae* and *P. rapae* spectral sensitivities. A survey of host colouration modelled to herbivore spectral sensitivity

has never before been carried out, to my knowledge, and so is a significant contribution to the field. In the case of *B. brassicae*, variation in the blue to green ratio among hosts was higher than variation in leaf brightness, indicating that the blue to green ratio could potentially provide more wide-ranging information about plant defence than brightness. The correlation between both colour measures was very weak, however, which indicates that brightness and the blue to green ratio could both potentially provide information about separate aspects of plant defence.

In the case of *P. rapae*, the variation in the blue to green ratio was considerably greater than that in the red ratios. The reason for the evolution of the red receptors is unknown and is of great interest (Stavenga and Arikawa 2006). The red receptors are suggested to have evolved to help in the selection of oviposition sites because these ratios could provide further discrimination between plants than the blue to green ratio (Kelber 1999). The greater amount of variation in the blue to green ratio than in either of the red ratios, however, does not provide support for this idea. Furthermore, significant correlations were found between the blue to green ratio and the red to green ratios, which implies that similar information is likely to be conveyed through both blue to green and red to green ratios. Nonetheless, while the red to green and blue to green ratios are correlated, they are not perfectly so, and thus it is possible that they may provide somewhat different information to herbivores and thus be used differently (Kolb and Scherer 1982). This may be particularly the case for the dark red to green ratio, which was more weakly correlated with the blue to green ratio than was the pale red to green ratio. The use of the blue to green ratios and red ratios by *P. rapae* will be further explored in Chapter Six.

3.4.2.1. How likely it is that colouration is used by herbivores in host choice?

Blue colouration

Bluer colouration was found to have a positive relationship with all herbivores, with the exception of *B. brassicae,* which had a negative relationship. This suggests that herbivores may use this cue to choose among host plants. This is the first study to investigate plant blueness and herbivory in the field. An observational study in the field looked at the susceptibility of green versus purple tree cultivars to the Japanese beetle (*Popillia japonica),* finding that purple foliage suffered greater defoliation (Rowe et al. 2002). Unlike wild *B. oleracea*, however, these trees have undergone artificial selection and so these results may not reflect preferences for wild plants. In an experimental situation, it was found that moth larvae preferred to orientate towards green rather than blue coloured substrates (Harris et al. 1995).

The meta-analysis in Chapter Two suggests that the response to non-green colouration should be negative rather than positive. It is possible, however, that a positive blue preference may be specific to brassica herbivores. A simple explanation for this could be that glaucous colouration is typical of *B. oleracea* (Mitchell and Richards 1979) and so blueness may help to identify a brassica as a suitable host among a range of species, with bluer plants being more easily identified and therefore they are more attractive as they stand out from the usually greener background.

Unlike the other herbivores, *B. brassicae* herbivory had a negative relationship with plant blueness. The reason for this is unknown. *Brevicoryne brassicae* either responds differently to colouration or a factor correlating with blue colouration has opposite effects on *B. brassicae* than the other herbivores. The meta-analysis in Chapter Two suggested that all herbivore orders (with the exception of the Coleoptera) should respond in a similar way to colouration, which contradicts the result for *B. brassicae* obtained in this study. Despite possessing similar visual mechanisms, different herbivore species appear therefore to react differently to colouration. It is possible that feeding guilds e.g. phloem feeders versus leaf chewers, may have evolved different responses to host plants (Inbar et al. 2001), and that these differences are more significant than differences between insect orders. Even within the aphids as a group, different responses to colouration have been found (Kennedy et al. 1961), consistent with the results presented here, although the reason behind this is difficult to explain. Unlike other specialist brassica herbivores that only detoxify glucosinolates, *B. brassicae* sequesters these chemicals to use for its own defence (Kazana et al. 2007). Such differences in behaviour may result in different responses to plant cues between herbivores, especially in response to glucosinolate concentrations (Cole 1997, Newton et al. 2009b).

Brightness

Plant brightness was positively associated with flea beetle and *A. protella* herbivory but not with herbivory by *B. brassicae*. The finding that *B. brassicae* did not respond to plant brightness is surprising because achromatic cues (brightness) have been shown to be important in host selection by these insects (Kennedy et al. 1961). For example, beet leaves were alighted upon more frequently than cabbage leaves by *B. brassicae,* even though they are an unsuitable host, which the authors attribute to the greater achromatic reflectance of beet leaves (Kennedy et al. 1961).

Red colouration

Both *P. rapae* and *P. brassicae* responded negatively to red colouration. Previous field studies have found that red colouration is less preferred than green colouration, supporting the finding here. For example, it was found that red young leaves of *Quercus coccifera* suffered less herbivory than green leaves (Karageorgou and Manetas 2006). A study looking specifically at Lepidoptera herbivory found that the swallowtail butterfly (*Papilio glaucus*) avoided redder young leaves in preference for older, greener leaves on the same branch (Mercader et al. 2007). Controlled experimental work has also found a preference for less red oviposition substances. A study using coloured card for oviposition found that red was avoided by *P. rapae* in preference for any other colour (Traynier 1979). Potential explanations for this avoidance are discussed below when considering the link between defensive chemistry and colouration.

The finding that colouration predicts herbivory provides support for the ACH because it suggests that herbivores may be using colouration to chose a host, one of the key predictions of this hypothesis. The ACH, however, proposes that intense colouration should be avoided. However, in the case of blue colouration and brightness, herbivores were more likely to be found on the most intensely coloured blue plants and the brightest plants. Further exploration of this finding is required, as there may be confounding influences of cause and effect; for example does brightness increase in response to herbivory, or does herbivory increase in response to brightness? The lepidopteran herbivores, on the other hand, appear to respond negatively to intense redness, which is compatible with the ACH (i.e. that red colouration could signal levels of chemical defences to herbivores).

3.4.2.2. Is nitrogen important to herbivores?

It was predicted that CN ratio would be important to herbivory, as nitrogen is considered to be the ultimate limiting nutrient for most insects (Mattson 1980, White 1984). This was found to be the case for all herbivores except the flea beetles, the weevils and snails. This finding supports previous studies suggesting that herbivores choose host plants with a higher nitrogen content (Mattson 1980) as it leads to an increase in herbivore fitness, particularly in growth rate and reproduction rate (Slansky and Feeny 1977, Kerslake et al. 1998, Khan and Port 2008). Aphids, for example, are thought to select and accept plants with the lowest CN ratio by inserting their stylets to sample plant sap before starting ingestion (Powell et al. 2006).

One possible reason why the CN ratio did not influence coleopteran herbivory (Blake et al. 2011) is because these herbivores are very mobile (pers. obs.). *Pieris rapae* and *P. brassicae* early-instar larvae generally stay on one plant, snails are very slow moving and *B. brassicae* colonies do not disperse in the summer until overcrowding occurs (Dixon 1973); all

these herbivores therefore feed on a single host plant for a long period of time. The coleopteran herbivores can readily disperse onto different hosts, however, meaning that the fitness costs of selecting a host low in nitrogen may be lower compared with other herbivores (Kirk 1992). A second possible explanation concerns the utilisation of seeds as oviposition sites by the weevils. Seeds are among the plant tissues most rich in nitrogen (Mattson 1980) and so these herbivores may not be as selective in choosing a host rich in nitrogen because the seeds have a plentiful supply.

3.4.2.3. Interactions with glucosinolates and herbivores

In this study, a novel method for collecting samples for chemical analysis in the field was used and can be recommended for future work. As a box of dry ice can last for up to three days, a field biologist could be away on field work for a prolonged period of time; substantially longer than if using liquid nitrogen to flash freeze samples (Gols et al. 2008a). Dry ice is also easier and safer to transport.

That certain aspects of colouration were found to correlate with glucosinolate concentration indicates that colouration could function as a cue (or even a signal) of glucosinolate levels. For this information to be useful, however, glucosinolate levels must be important to the herbivore. Glucosinolates significantly predicted the presence of most herbivores; however, a common pattern across all herbivores could not be discerned. *Brevicoryne brassicae* was more likely to be found on plants with low levels of glucobrassicin and high levels of gluconapin. The negative correlation between levels of glucobrassicin, an indole glucosinolate, and *B. brassicae* herbivory agrees with previous findings (Cole 1997). Indole glucosinolates differ from aliphatic glucosinolates in that hydrolysis of the latter depends upon the presence of the enzyme myrosinase, which is brought into contact with the glucosinolate when herbivores damage host tissues (Halkier and Gershenzon 2006). In the case of herbivory by aphids, which are phloem feeders and avoid causing extensive tissue damage, it is likely that indole glucosinolates have a more pronounced effect on herbivory than aliphatic glucosinolates (Hopkins et al. 2009). Experimental work has shown that *B. brassicae* performance is negatively impacted by high levels of indole glucosinolates (Cole 1997). A previous study looking at *B. brassicae* herbivory in the field found that *B. brassicae* was less likely to be found on plants that did not produce sinigrin (Newton et al. 2009b). Newton et al. (2009b), however, only examined the presence or absence of sinigrin production, which is unaffected by chemical induction. In the present study, I looked at overall concentrations in the plant, which are expected to vary with chemical induction. It is possible that *B. brassicae*

prefers plants with low levels of sinigrin but that upon colonisation this chemical is induced, and so the concentration increases, concealing the initial association with low levels of sinigrin.

It was found that *P. rapae* preferred low levels of gluconapin and glucoraphanin. Previous field studies have found no effect of glucosinolates on *P. rapae* herbivory (Moyes et al. 2000, Newton et al. 2009b). This is perhaps surprising, as experimental work has shown that plants with high levels of glucosinolates experienced fewer oviposition events (Bruinsma et al. 2007), particularly in the case of indole glucosinolates (de Vos et al. 2008). The toxicity of aliphatic glucosinolates to *P. rapae* is debated as *P. rapae* is able to detoxify these glucosinolates (Wittstock et al. 2004). Recent work, however, has reported decreased survival of *P. rapae* on a transformed line of *Arabidopsis thaliana* expressing high levels of aliphatic glucosinolates (Kos et al. 2012), which may explain the preference of *P. rapae* for plants with low levels of aliphatic glucosinolates in this field survey.

Previous field studies have found that *Ceutorhynchus assimilis* (seed weevil) is more common on plants with high levels of gluconapin, which is thought to be because this chemical assists the weevil in locating host *B. oleracea* plants (Moyes and Raybould 2001). The reason for the absence of a relationship between weevil herbivory and gluconapin levels in this study is therefore unclear. The presence of both *C. assimilis* and *A. Protella,* however, was found to be positively associated with high levels of 4-methyoxyglucobrassicin. Indole glucosinolates are often induced to high levels when hosts suffer damage by herbivores (Poelman et al. 2008b) and so this could explain the positive association between these herbivores and 4-methyoxyglucobrassicin. It is unlikely that the herbivores prefer high levels of 4-methyoxyglucobrassicin, as indole glucosinolates have been shown to be toxic to specialist herbivores (Agerbirk et al. 2006). Support for this comes from the negative correlation between *A.protella* presence and the other two indole glucosinolates, glucobrassicin and neoglucobrassicin.

On the whole, generalist herbivores are thought to be more strongly affected by aliphatic glucosinolates than specialist herbivores (Li et al. 2000, Hopkins et al. 2009). In a study by Mithen et al. (1995b), data were obtained which led the authors to suggest that contrasting herbivore pressures in their two different sites caused divergent evolution of glucosinolate types. In a sheltered site where herbivory by generalists (including snails) is high, this pressure selects for progoitrin and other aliphatic glucosinolates which are effective against generalist feeders (Mithen et al. 1995b). This is supported by the finding in this study that snails were more likely to be found on plants with low levels of progoitrin. Other previous research found no association between snail herbivory and progoitrin in the field (Moyes et al. 2000, Newton et al. 2009b). In the case of Newton et al. (2009b), this may because the auothrs

looked only at presence or absence of progoitrin, which may not be subtle enough to capture preferences of snails.

Although closely related to *P. rapae*, there was no evidence for a similar preference for low levels of aliphatic glucosinolates by *P. brassicae*. Despite the fact that *P. brassicae* is gregarious and aposematically coloured, traits typical of many insects that sequester toxic plant chemicals, *P. brassicae* has not been found to sequester glucosinolates (Muller et al. 2003). Indeed, the absence of a significant association between *P. brassicae* and glucosinolate levels, though unexpected, agrees with data from other field surveys (Moyes et al. 2000).

A significant problem associated with measuring glucosinolate concentrations in the field is that of chemical induction. It is impossible to separate the scenarios that either *P. rapae* prefers plants with high levels of progoitrin, or that upon damage by *P. rapae* progoitrin is induced to higher levels. Moreover, there is evidence to suggest that herbivore damage early on in the season could affect glucosinolate concentrations a long period of time later (Poelman et al. 2008a). To overcome this problem, previous studies have looked at the presence or absence of these glucosinolates (Newton et al. 2009b), though this is clearly a somewhat crude measure of chemical defence. While it is not possible to distinguish unequivocally between herbivore preference for, and host induction of, glucosinolates, it is nonetheless likely that the association between herbivory and reduced glucosinolate concentrations reflects a real preference among herbivores for low glucosinolate levels. The alternative explanation is that the plant reduces levels of chemical defence upon herbivore attack. This could occur if a plant reabsorbed a particular glucosinolate, thus reducing the concentration, in order to divert production to a different kind of glucosinolate. Glucosinolate induction has been shown to be specific to different types of herbivores, but importantly no decrease in glucosinolate concentration has been observed. For instance, when *P. rapae* was introduced to *B. oleracea* under controlled conditions, levels of every glucosinolate were found to increase (Poelman et al. 2008b). This suggests that a strategic reduction in glucosinolate levels following herbivore attack is unlikely, and that the association between low glucosinolate levels and herbivory presence reflects a preference by the herbivore (see Chapters Five and Six).

A second limitation of the field survey presented here is that it is not possible to distinguish between the effects of glucosinolate levels on herbivore preference and performance. For example, does the negative correlation between *P. rapae* herbivory and gluconapin levels reflect an active avoidance of hosts with high gluconapin levels (or an associated colour cue) by the ovipositing adult or decreased survival of larvae growing on plants with high gluconapin levels? This problem is impossible to overcome in the field and

thus requires experiments exploring the relationships between levels of different glucosinolates and herbivore preference and performance (see Chapters Five and Six).

3.4.3. Summary

Despite the complex nature of plant-herbivore interactions, evidence for relationships between plant colouration and insect herbivory were found in a large-scale study of wild *B. oleracea* and its herbivores. Importantly, this result suggests that leaf colouration may play a role in host choice. In general, bluer plants and less red plants were preferred across a range of herbivore groups. This is the first survey of plant colouration in natural conditions that used colour from the herbivore's perspective and so provides the most accurate assessment to date of the relationship between herbivory and host colouration in the wild from the perspective of the herbivore. Plant colouration was also correlated with glucosinolate content, which raises the intriguing possibility that colouration could be used by herbivores to gain information about the chemical defence levels within the plant. Glucosinolates had an impact on insect herbivory, including specialist herbivores, showing that it is important to the herbivores to attend to information about chemical defence.

The following chapter (Chapter Four) attempts to disentangle the effects of fixed genetic and environmental influences on colouration to determine whether selection can act on wild *B. oleracea* colouration (a necessary condition for the evolution of signalling under the ACH). Chapters Five and Six will attempt to separate the causes of herbivore preference and performance and will also look for chemical differences between differently-coloured plants in the absence of the confounding influence of chemical induction. Chapters Five and Six will focus on the herbivores *B. brassicae* and *P. rapae*, both of which were found to vary in abundance with host colouration. In its initial formulation by Hamilton and Brown (2001), the ACH focused on aphids so it is important that an experiment in this thesis explores aphid host choice in relation to colour. Aphid responses to host colouration will then be compared to those of *P. rapae*, an insect with a more sophisticated colour vision system. As *P. rapae* has six peak spectral sensitivities compared with the three seen in aphids, it is expected this insect will be better able to discriminate between plants differing in colour.

Chapter Four. Does genotypic variation in *B. oleracea* colouration explain herbivore presence?

4.1. Introduction

The idea that plants signal their investment in chemical defence through visual and olfactory cues to their herbivores in order to deter colonisation is an area of much debate (Hamilton and Brown 2001, Wilkinson et al. 2002, Archetti et al. 2009b). The use of cues during host selection by insect herbivores is well-studied, particularly in the case of olfactory cues (Visser 1986). For example, the release of plant volatiles can deter herbivores from colonising a host (Himanen et al. 2010). In contrast, the use of visual cues by insect herbivores is less well understood (Reeves 2011). Interest in this topic was ignited by the autumn colouration hypothesis (ACH) (Hamilton and Brown 2001). This controversial idea posits that autumn colouration signals a tree's commitment to chemical defence, and autumn-colonising aphids will therefore avoid the most intensely-coloured trees as these will be most defended (Hamilton and Brown 2001) (for a review of the ACH see Chapter One). Despite this interest, the possibility that plants signal to herbivores through visual means remains largely unexplored: while there is some empirical evidence in support of this idea (Archetti and Leather 2005, Rolshausen and Schaefer 2007), the data necessary to test the assumptions of the ACH are still lacking (see Chapter One).

In the previous chapter, an examination of plant-herbivore interactions in the *B. oleracea* system revealed correlations between plant colour, defensive chemistry and herbivory that supported several of the assumptions of the ACH. The blue to green ratio was found to predict aphid presence and the red to green ratios were found to predict butterfly presence. All herbivores were found to respond to glucosinolate levels within the plant. Most interestingly plant brightness and the dark red to green ratio were found to correlate with glucosinolate levels, thus providing support for the idea that plant colouration could act as signal of chemical defence. Importantly, these effects were detectable despite sampling of plants and herbivores in the field, where there are likely to be many complex interactions between colour, chemistry and herbivory, as well as many confounding effects of other factors. However, in order to explore the feasibility of the ACH in more detail, manipulation of these factors is required to determine what is driving these correlations in the field.

Arguably the most basic assumption of the ACH is that there is variation in plant colouration, which should have a fixed genetic component. Such variation is required for selection to act upon plant colouration and for signalling to evolve. A fixed genetic component to both colouration and chemical defence is also required to maintain the reliability of a colour trait signalling levels of chemical defence: if the genetic influence on plant colouration varied completely with environmental conditions (i.e. exhibited total environmental plasticity), it

would likely fluctuate too erratically for a constant correlation with chemical defence to be maintained.

Due to our extensive knowledge of the brassica *Arabidopsis*, much is known about glucosinolate control and metabolism (Wittstock and Halkier 2002). Aliphatic glucosinolates are under strong genetic control by five loci. The GSL-elong loci control elongation of the molecule by adding one to nine methylene groups. The other four loci control modifications to the side-chain: GSL-pro adds a propyl group, GSL-oh causes hydroxylation of the side chain, GSL-oxid oxides the side chain and GSL-alk converts to alkenyl glucosinolate by saturation (Giamoustaris and Mithen 1996). The combinations of null and functional alleles can explain variation of aliphatic glucosinolates (Mithen et al. 1995a, Mithen 2001). If a molecule is not elongated, glucoiberin and sinigrin are produced whereas if GSL-elong is active the longer molecules of gluconapin and progoitirin are produced (Giamoustaris and Mithen 1996)(see Figure 4.1). A study of the profiles of wild *B.oleracea* across three counties in the UK found that all plants had functional GSL-alk and GSL-oxid loci, and that most variation was caused by null alleles at GSL-pro and GSL-oh (Newton et al. 2009b). Non-functional GSL-pro loci were found to result in the absence of sinigrin and its precursor glucoiberin, while non-functional GSL-oh loci resulted in the absence of progoitrin. In the field, most plants were found to produce both progoitrin and sinigrin present, but around 10% produced no sinigrin and around 10% produced no progoitrin (Newton et al. 2009b). A schematic diagram of the loci and the production of the glucosinolate can be seen in Figure 4.1. From the figure, it can be seen that alleles active at different loci can result in different concentrations of the glucosinolates between plants.

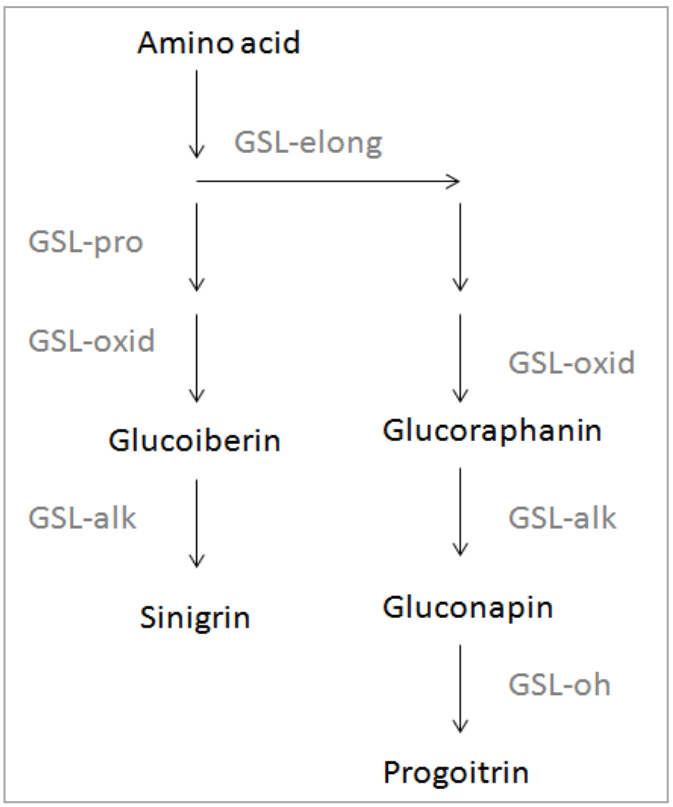

Figure 4.1. Diagram of aliphatic genetics (adapted from Giamoustaris and Mithen 1996). Names of loci regulating biosynthesis of glucosinolates are shown in grey.

A study investigating mutations that block aliphatic glucosinolate biosynthesis found that blockage of this pathway resulted in a compensatory increase in indole glucosinolates (Hemm et al. 2003). This suggests that both classes of glucosinolates are interconnected in a negative feedback system. As with aliphatic glucosinolates, much is known about the genes involved in indole glucosinolate biosynthesis (Grubb and Abel 2006). Total indole concentration is controlled by six loci, and there are five additional loci that are specific to the subsets of indole glucosinolates (Kliebenstein et al. 2001). An example is the *FD167L* allele which controls the ratio of production of gucobrassicin and 4-methyoxyglucobrassicin, thereby affecting overall indole concentrations (Kliebenstein et al. 2001). The heritability of indole glucosinolates is slightly lower than that of aliphatic glucosinolates (67% heritability compared to 81%) (Kliebenstein et al. 2001), suggesting that indole glucosinolate expression is more sensitive to seasonal and environmental factors (Gols et al. 2007).

In contrast to the genetics of glucosinolates, there is very little known about the genetic variation in plant colouration (Sinkkonen et al. 2012). Leaf colour of domesticated brassicas has been found to be heritable (Luo et al. 2011). Some of the enzymes involved in the pathways of production of carotenoids and anthocyanins have been identified (Hirschberg 2001, Winkel-Shirley 2001); if these pigments are important in leaf colouration in wild *B.*

oleracea, we can therefore gain insights into the genetic control of colouration by studying these pathways (Walker et al. 1999). A wide variety of environmental factors may potentially influence colouration, for example, plant stress can increase leaf redness (Costa-Arbulu et al. 2001), while high nitrogen availability can decrease leaf redness (Schaberg et al. 2003) and waterlogging can make leaves darker (Issarakraisila et al. 2007). Therefore, it is important to determine the extent of colouration variation that is the result of fixed genetic effects.

Four different aspects of colouration will be investigated here, modelled from the perspective of *B. oleracea* herbivores (see Chapter Three). For aphid vision, these are brightness, which has been shown to be important in herbivore host choice (Kennedy et al. 1961), and the blue to green photoreceptor quantum catch ratio, which is predicted from modelling to be important in host choice (Doring and Chittka 2007). Since aphids do not have a red receptor, red to green ratios are not of relevance to these herbivores. For butterfly vision, the ratios of interest are the blue to green photoreceptor ratio and in addition the pale red to green ratio and the dark red to green ratio. The study of butterfly red receptors is of particular interest because it is currently unclear why some butterfly species have evolved two red receptors, when many insects have none (Kelber 1999, Briscoe and Chittka 2001). One hypothesis is that red receptors have evolved for fine-tuned discrimination between plants for oviposition (Stavenga and Arikawa 2006), but further evidence for this is required.

To investigate the extent to which variation in plant colouration is under fixed genetic control, this study uses a common garden experiment. Common garden experiments are a frequently deployed tool to investigate ecological mechanisms (Johnson and Agrawal 2005). Phenotypes are a product of fixed genotypes, maternal effects and the effects of the environment on gene expression. Common garden experiments allow the separation of fixed genetic effects and plastic genetic responses to environmental influences. Because all study species are placed in a common environment, the environmental conditions are consistent across genotypes (Ferguson and Talent 1993). Therefore, if differences in colouration among plants are maintained, this suggests that this variation has a fixed genetic component. Maternal effects describe the influence on offspring phenotype of the maternal environment and phenotype (Roach and Wulff 1987). It is possible maternal effects may influence plant colouration, for example more nutrients deposited in seeds could lead to more colourful plants. In practise, it is difficult to separate maternal effects from fixed genetic effects.

This chapter aims to investigate two of the basic assumptions of the ACH. First, is there fixed genotypic variation in colour of *B. oleracea*? Second, does this genetic variation explain herbivore presence? Genetic variation in plant colouration has been shown to covary with herbivory (Sinkkonen et al. 2012), so it is expected that this will also be the case in this system.

The influence of genetic variation of colouration on herbivore presence will not be confounded by varying herbivory abundance found at different sites as in the field because the common garden will provide consistent herbivore exposure across genotypes.

4.2. Methods

4.2.1. The common garden

The common garden experiment was established at the University of Exeter in Falmouth by Dr D. Hodgson. Seeds were collected by Erika Newton (University of Exeter) in 2008 from wild *B. oleracea* populations in Cornwall (50°10'N, 5°42'W; 50°12'N, 5°42'W; 50°10'N, 5°41'W), Devon (50°50'N, 3°51'W; 50°46'N, 3°49'W; 50°34'N, 3°56'W) and Dorset (50°64'N, 1°92'W; 50°62'N, 2°27'W; 50°69'N, 2°05'W; 50°59'N, 2°03'W; 50°58'N, 2°04'W; 50°60'N, 2°13'W). To investigate genotypic variation, ten seeds from the same mother plant were sown. The seeds were initially planted individually into multicell seed trays and then transferred to six-inch pots after three weeks. Plants were watered as required. Plants were labelled with a unique number and a number corresponding to the mother plant.

Three herbivore surveys were undertaken between June-August 2010. Plants were thoroughly searched for herbivores by Nicole Goodey (University of Exeter) and identified according to Kirk (1992). The presence of the herbivores was pooled over the three surveys. The herbivores identified from the surveys were *Pieris rapae* (small cabbage white butterfly), *Pieris brassicae* (large cabbage white butterfly), *Brevicoryne brassicae* (cabbage aphid), *Myzus persicae* (peach-potato aphid), and *Psylliodes chrysocephala* (cabbage stem flea beetle).

Colour readings were taken from the third leaf down from the top of the plant using a spectrophotometer and modelled to aphid (Kirchner et al. 2005) and *P. rapae* (Stavenga and Arikawa 2011) spectral sensitivities. For the aphid species, quantum catches were obtained for the blue, green and UV photoreceptors. Using these values, the blue to green ratio and brightness (i.e. green photoreceptor excitation (Lehrer 1994)) were then calculated for each plant. As in Chapter Three, beetle vision was modelled using aphid spectral sensitivities. Quantum catches for UV, violet, blue, green, pale red and dark red photoreceptors were obtained from the butterfly model. Using these values, the blue to green, pale red to green and dark red to green ratio were then calculated for each plant. *P. brassicae* vision was modelled according to the *P. rapae* spectral sensitivity model as in Chapter Three.

4.2.2. Statistical analyses

To maximise the sample sizes available for each genotype, only mother groups with five or more progeny that had successfully grown to mature plants were included in the analysis. Differences in colouration between plant genotypes (i.e. between different mother plant groups) were analysed using a Kruskal-Wallis test as the data were not normally distributed. In order to determine the relationship between genetic variation in host colouration and

herbivory, the mean probability of herbivore presence was calculated for each herbivore in each mother group. This was then fitted as the response variable in linear models, with leaf colouration (calculated as the median for each mother group) fitted as the explanatory variable. Model simplification proceeded by backwards deletion of non-significant terms until further removals led to significant ($p<0.05$) increases in deviance, assessed using F values. Significance levels are reported on the addition of non-significant terms and removal of significant terms from the minimum adequate model. Butterflies were scored as present if either eggs or larvae (or both) were present on the host plant.

4.3. Results

4.3.1. Genetic variation in colouration

All four aspects of plant colouration measured in the study were found to differ among *B. oleracea* mother groups: blue to green ratio (KW-X^2=136, df=91, p=0.0016; Fig 4.2), brightness (KW-X^2=117, df=91, p=0.033; Fig 4.3), the pale red to green ratio (KW-X^2=134, df=91, p=0.0022; Fig 4.4) and dark red to green ratio (KW-X^2=147, df=91, p<0.001; Fig 4.5). Given that variation in these aspects of host colouration is maintained in a common garden, it is highly likely that at least some of this variation arises from fixed genetic effects, rather than having a purely environmental origin.

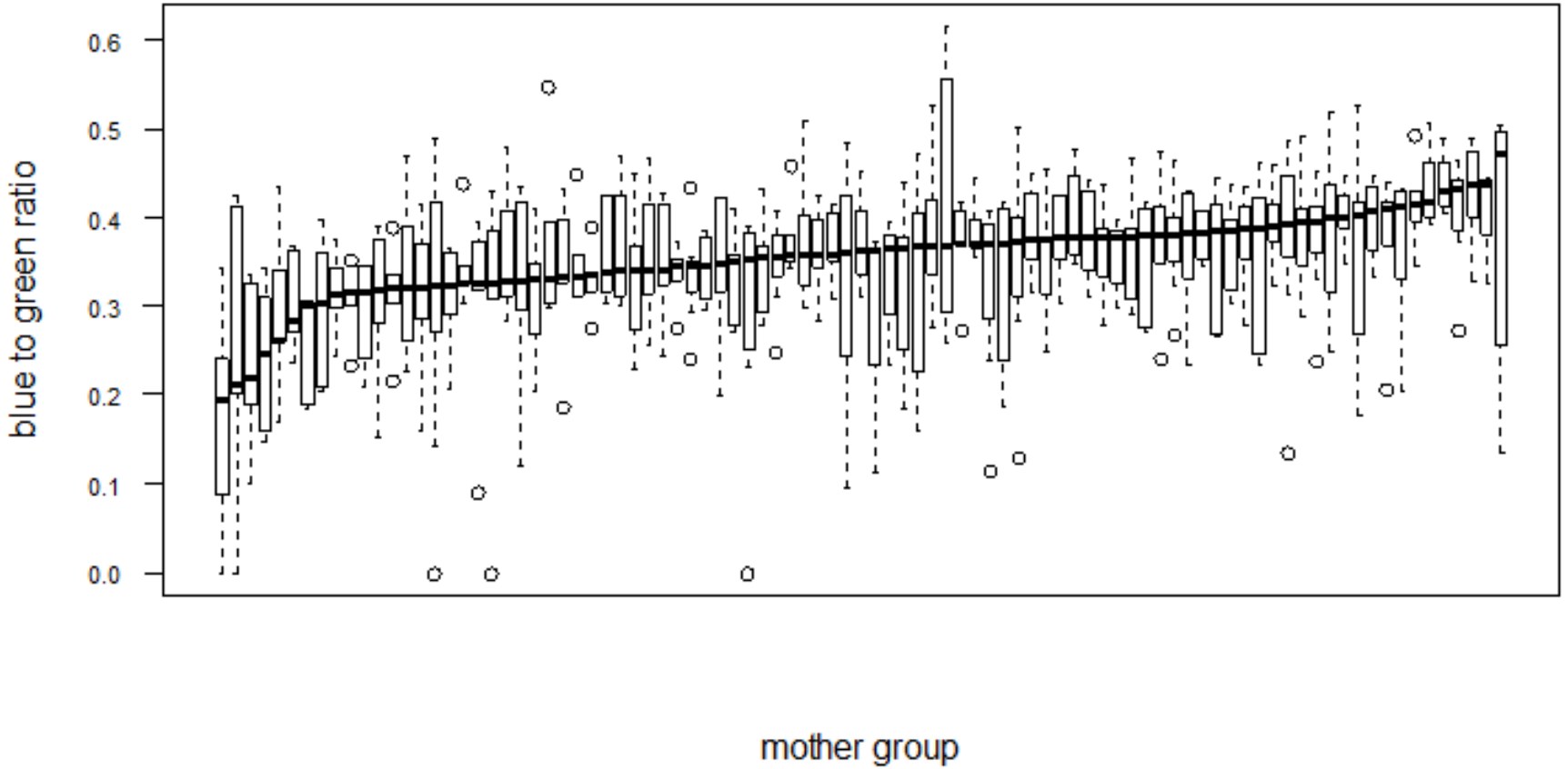

Figure 4.2. Variation in the blue to green ratio between mother groups. Bars are ranked from lowest blue to green ratio median to the highest. Data are shown for 566 offspring from 92 mothers each with a minimumof 5 offspring. Each box represents a single mother group i.e. all offspring from one mother. Box shows interquartile range with the central, heavy line representing the median. The tails from the bar represent approximately 2 standard deviations around the interquartile range, and dots represent outliers from this.

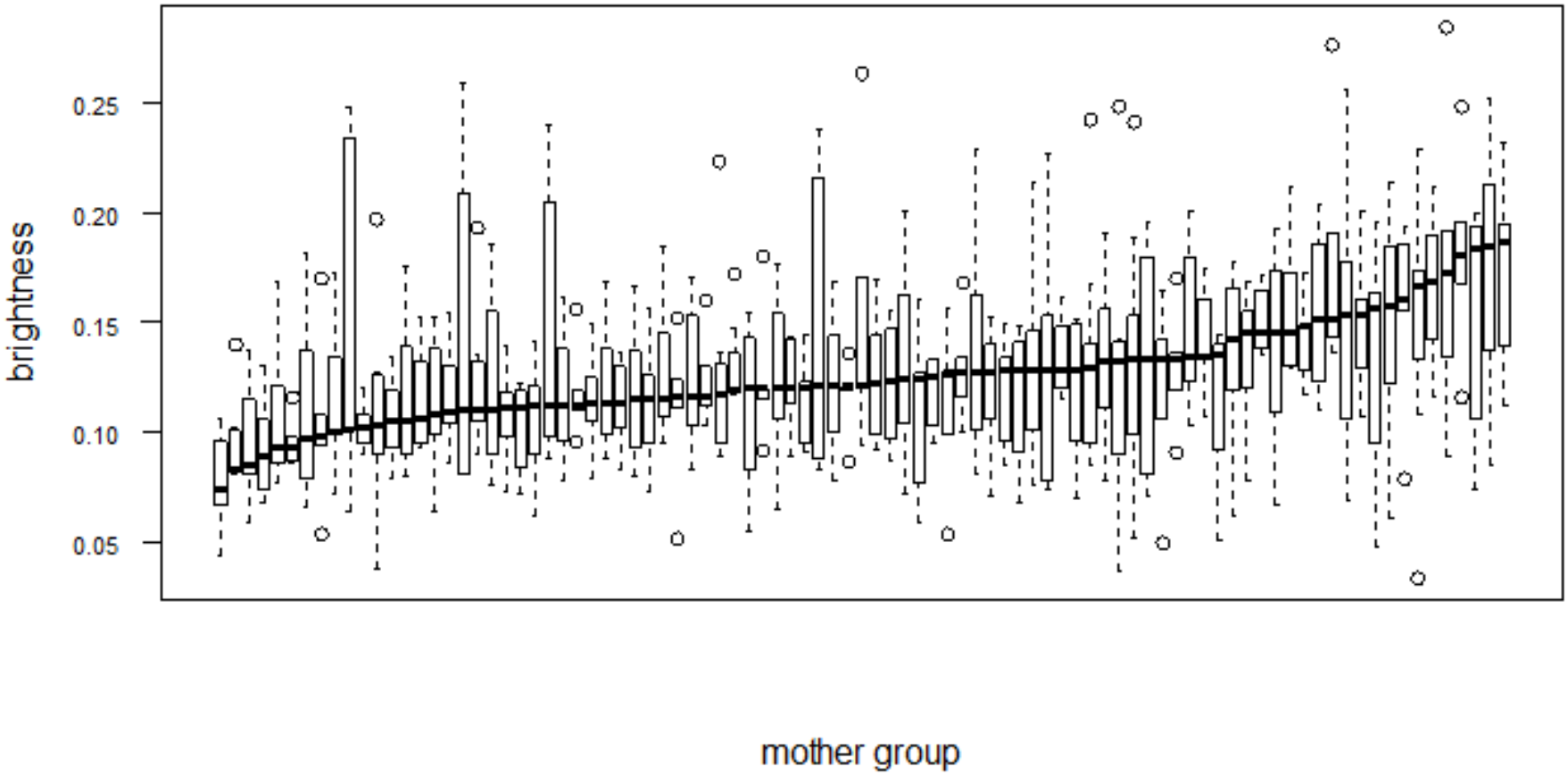

Figure 4.3. Variation in brightness between mother groups. Bars are ranked from dullest plant median to the highest. Data are shown for 566 offspring from 92 mothers each with a minimumof 5 offspring. Each box represents a single mother group i.e. all offspring from one mother. Box shows interquartile range with the central, heavy line representing the median. The tails from the bar represent approximately 2 standard deviations around the interquartile range, and dots represent outliers from this.

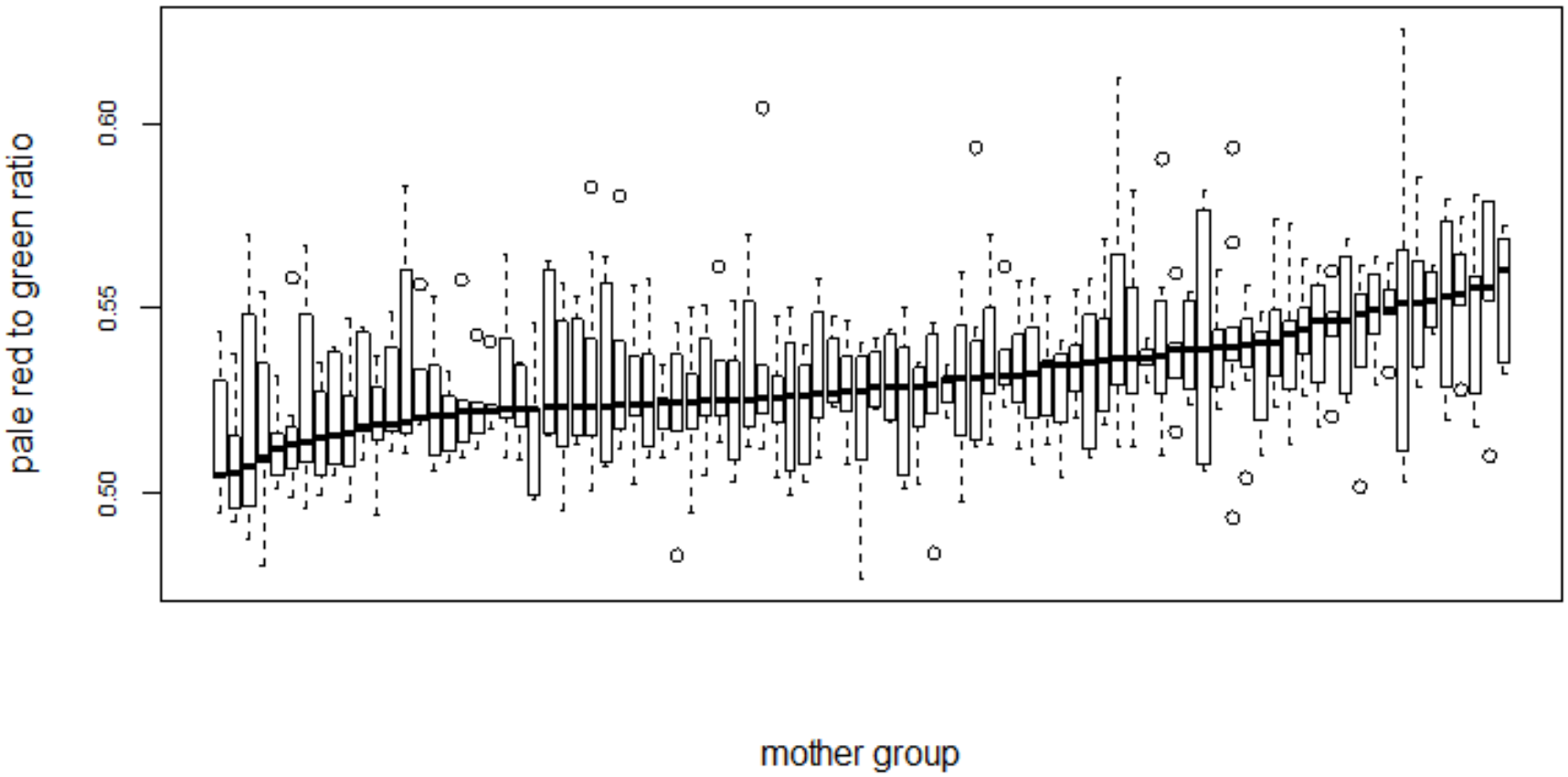

Figure 4.4. Variation in the pale red to green ratio between mother groups. Bars are ranked from lowest pale red to green ratio median to the highest. Data are shown for 566 offspring from 92 mothers each with a minimumof 5 offspring. Each box represents a single mother group i.e. all offspring from one mother. Box shows interquartile range with the central, heavy line representing the median. The tails from the bar represent approximately 2 standard deviations around the interquartile range, and dots represent outliers from this.

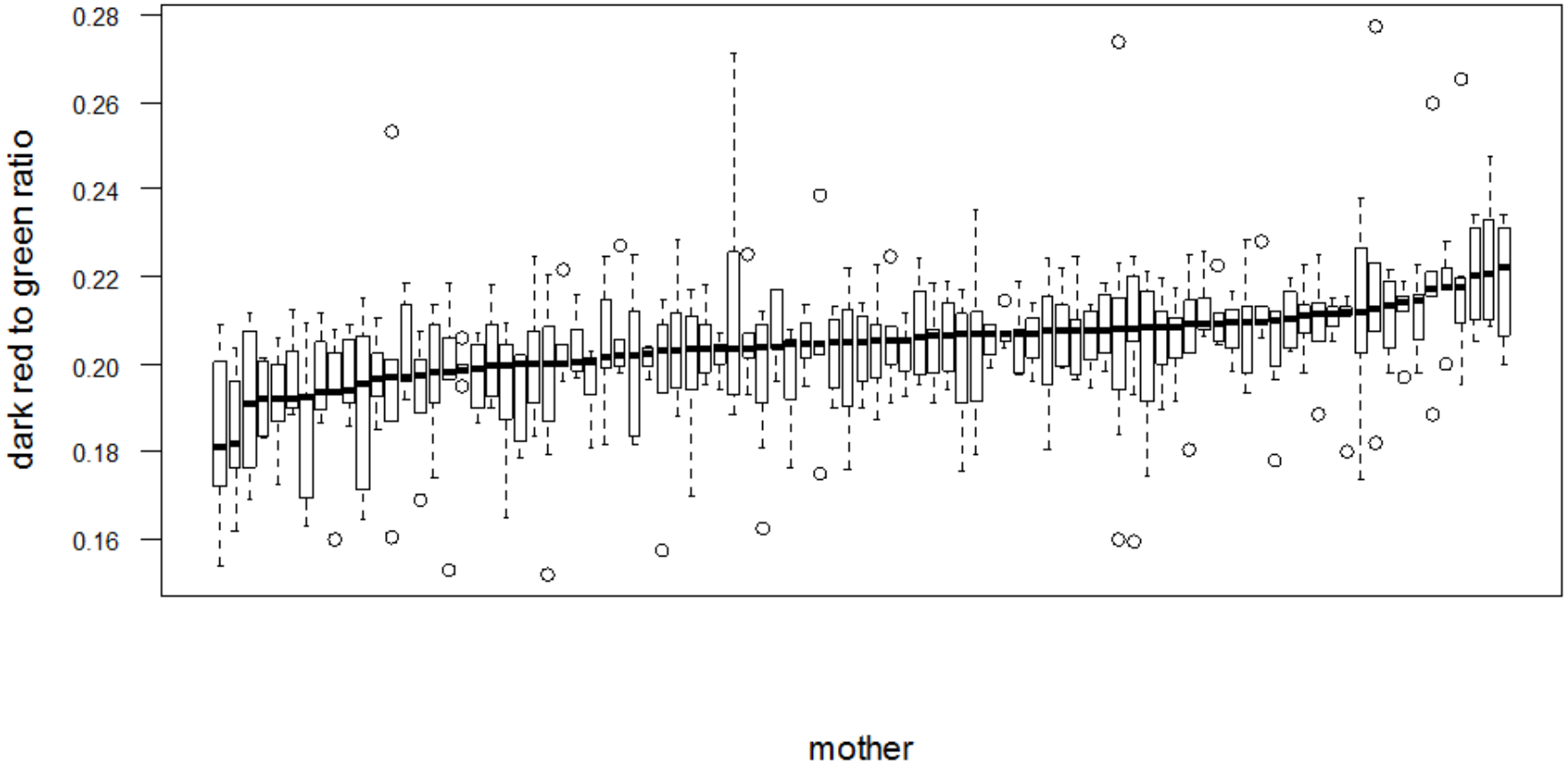

Figure 4.5. Variation in the dark red to green ratio between mother groups. Bars are ranked from lowest dark red to green ratio median to the highest. Data are shown for 566 offspring from 92 mothers each with a minimumof 5 offspring. Each box represents a single mother group i.e. all offspring from one mother. Box shows interquartile range with the central, heavy line representing the median. The tails from the bar represent approximately 2 standard deviations around the interquartile range, and dots represent outliers from this.

4.3.2. Genetic variation in colouration and herbivore presence

None of the colour measurements recorded predicted the presence of *P. rapae* eggs and larvae (pale red to green ratio $F_{1,90}$<0.001, p=0.98; blue to green ratio $F_{1,90}$=0.196, p=0.66; dark red to green ratio $F_{1,90}$=0.00830, p=0.93).

This was also the case for *P. brassicae* (pale red to green ratio $F_{1,90}$=0.0592, p=0.81; blue to green ratio $F_{1,90}$=0.0172, p=0.90; dark red to green ratio $F_{1,90}$=2.66, p=0.11). When the data for *P. rapae* and *P. brassicae* were combined, the effect of the blue to green ratio on the probability of egg presence was closer to statistical significance ($F_{1,90}$=2.92, p=0.091), with the probability of butterfly presence increasing with increasing plant blueness. These results provide little evidence that genetic variation in red colouration is related to butterfly herbivory but suggest that blue colouration could possibly be important.

Neither the blue to green ratio nor brightness significantly predicted B*. brassicae* presence (blue to green: $F_{1,90}$= 0.984, p=0.32; brightness: $F_{1,90}$=0.247, p=0.62; see Figure 4.6a). *Myzus persicae*, in contrast, was significantly more likely to be found on brighter plants ($F_{1,90}$=6.08, p=0.016; Fig 4.6b) though not on bluer plants ($F_{1,89}$=0.429, p=0.51). Therefore brightness seems to be the most important aspect of the genetic variation in plant colouration determining *M. persicae* herbivory. Plant brightness also positively predicted the presence of *P. chrysocephala* ($F_{1,89}$=4.40, p=0.038; Fig 4.6c), though as before the blue to green ratio did not ($F_{1,90}$=0.258, p=0.61).

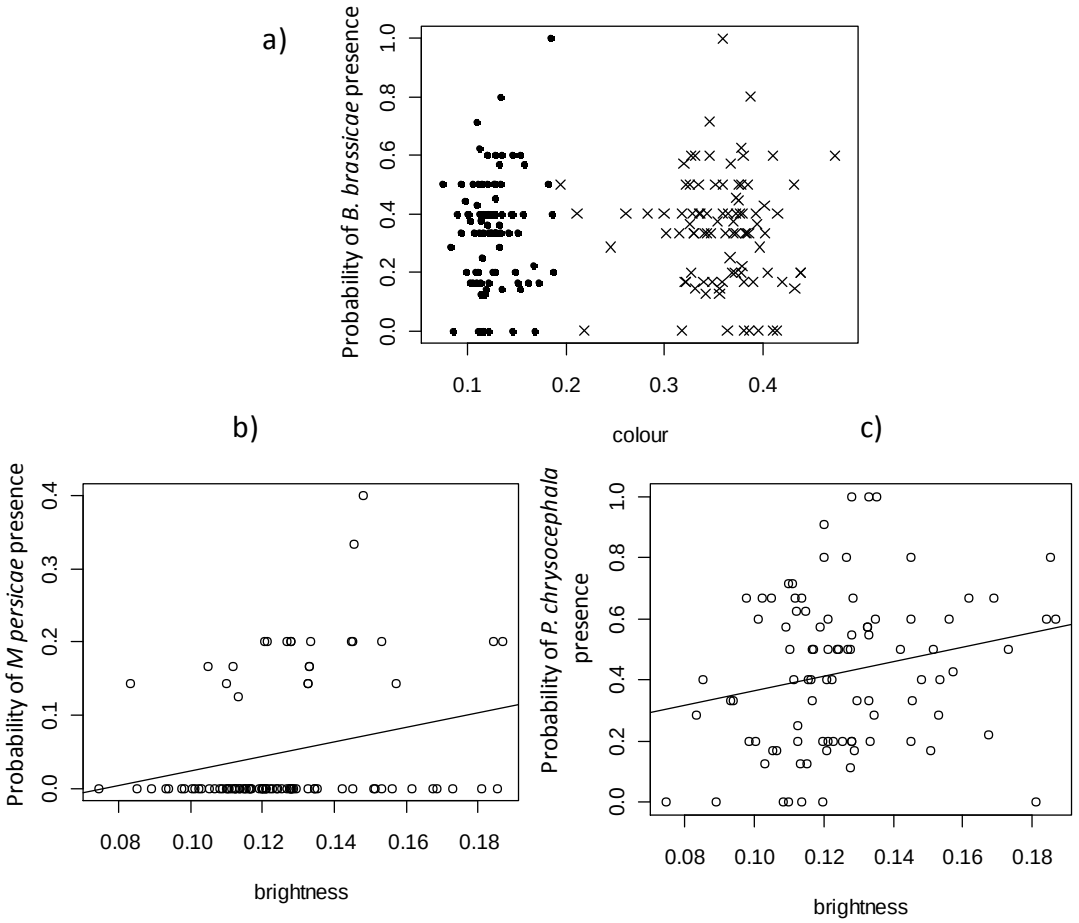

Figure 4.6. The relationship between plant colouration and the presence of *B. brassicae, M. persicae* and *P. chrysocephala*. Median colouration and mean probabilities of herbivore presence were calculated for all offspring of each mother plant. a) brightness (filled circles) and blue to green ratio (crosses) versus *B. brassicae* presence. b) brightness versus *M. persicae* presence c) brightness versus *P. chrysocephala* presence. Predicted least squares regression lines are shown.

4.4. Discussion

This chapter aimed to test the most basic assumptions of the ACH - that there is genetic variation in plant colouration and the presence of herbivores can be predicted by this colouration. Using a common garden approach, evidence for fixed genetic variation in *B. oleracea* leaf colour was found. Some aspects of this plant colouration predicted herbivore presence, notably positive effects of brightness on M*. persicae* numbers and of blueness on numbers of butterflies.

4.4.1. Genetic variation in leaf colour

Differences in leaf colouration between mother groups persisted following transplantation to a common garden, indicating a fixed genetic component to the variation in leaf colouration in *B. oleracea*. While this result does not exclude the possibility that environmental factors also influence plant colour, it nevertheless demonstrates a significant genetic component to variation in leaf colouration upon which selection may act during coevolution between *B. oleracea* and its herbivores.

Previous studies have also demonstrated genetic variation in leaf colour. For example, the leaf colour of Copper Beech *(Fagus sylvatica)* is thought to be caused by a single dominant gene (Heinze and Geburek 1995). When the Cabbage Tree (*Cordyline australis*) was taken from a range of sites across New Zealand and grown in a common garden, it was found that the variation in the purpleness of the leaf base was maintained, suggesting a fixed genetic component to colour variation (Harris and Beever 2002). Cornelius et al. (1995) found that the purpleness of young *Eucalyptus degluptu* saplings had a high level of additive genetic variation – the heritability of leaf colour was estimated to be around 77%. This means that most of the variation in colour was found between mother groups, as found in the present study (Cornelius et al. 1995).

Little is known about the mechanisms underlying genetic variation in colouration. Some of the enzymes involved in the biosynthesis of carotenoids and anthocyanins have been identified. Anthocyanins are produced in the phenylpropanoid pathway (see Chapter One) which involves many enzymatic steps (Winkel-Shirley 2001). Carotenoids are synthesized in the DOXP pathway (see Chapter One), which again requires many enzymes to produce these large molecules (Hirschberg 2001). Some of the genes controlling the production of these enzymes have been identified in Arabidopsis by creating knock-out mutants differing in appearance from wild-type plants (Hemm et al. 2003). For example, it had been found that the TTG1 locus regulates the biochemical pathways in the production of anthocyanin production (Walker et al.

1999). The potential for genetic control of leaf pigments is apparent, although the precise quantitative and qualitative control of leaf pigments in wild *B. oleracea* is unknown. It is uncertain what pigments contribute to the colour variation *B. oleracea* explored here (red to green ratios, blue to green ratio and brightness) but it is possible that varying levels of anthocyanins and carotenoids are important.

4.4.2. The influence on herbivory of genetic variation in colour

To my knowledge, only one previous study has sought to test for genetic variation in leaf colour and the influence of this variation on herbivory (Sinkkonen et al. 2012). This study found that there was significant genetic variation in the yellowness of autumn leaf colours in *Betula pendula* (Silver birch), and that this predicted the abundance of egg-laying female aphids (*Euceraphis betulae*) (Sinkkonen et al. 2012). Previous studies looking at herbivory and plant colouration in common garden designs have found varying results of the effect of colouration on herbivory (e.g. Campbell 1991, Holopainen et al. 2009). In this chapter, no relationship was found between red to green ratios and the presence of *P. rapae* and *P. brassicae*. This finding is similar to that of a previous study by Hughes et al. (2010), who found no association with herbivory and the redness of the leaf margins of *Veronica* (speedwell) when five species of *Veronica* was grown in a common garden (Hughes et al. 2010). These findings are somewhat surprising because red colouration has generally been suggested to be avoided by herbivores (Hamilton and Brown 2001), and there is evidence to support this in studies of autumn colouration (Archetti and Leather 2005; Archetti 2009) and in other situations (Karageorgou and Manetas 2006, Wong and Srivastava 2010). It is unclear why redness was not avoided in this study, especially as redness did predict *P. rapae* and *P. brassicae* presence in the field study in Chapter Three. It is possible that the variation in the red to green ratio in *B. oleracea* in the common garden is reduced compared with variation among plants in the field and may not be sufficient to allow discrimination between plants. Colour variation in the common garden was significantly lower than that in the field (e.g. dark red to green ratio, $F=2.471$, $df= 547$, $p<2.2x10^{-16}$); however, despite the high statistical significance, the size of this difference was very small ($r = 0.04$; traditionally, effect sizes of 0.1 are considered to be small (Cohen 1988)). It thus seems unlikely that the difference in variation in colouration could account for the differences in the correlation between redness and butterfly presence in the common garden and in the field.

Instead, it may be that plant colouration evokes a response only in some environments. Insect responses to host plants are often found to vary between environments (Johnson and Agrawal 2005, Rolshausen and Schaefer 2007, Newton et al. 2009b). As wild *B.*

oleracea is not naturally found in the common garden area (the nearest natural population is 20 miles away), the herbivores in the common garden may not respond to colouration of wild *B. oleracea* in the same way as herbivores which frequently encounter it (Chew 1977). This is an important problem with such experiments; although common gardens are successful in removing sources of environmental variation across genotypes, in this study the experiment provides only a snap shot of gene expression and herbivore response to colouration in the one environment in which they are grown. This problem can only be addressed by establishing more than one common garden (e.g. Maddox and Root 1990).

In this study, genetic variation in leaf brightness (green receptor excitation (Lehrer 1994)) was found to predict the probability of *M. persicae* presence. Leaf brightness is known to be important in host choice by other herbivores. For example, the Marsh Fritillary butterfly (*Euphydryas aurinia*) was found to select the greenest, and therefore most chlorophyll rich, honeysuckle (*Lonicera implexa*) leaves on which to oviposit (Stefanescu et al. 2006). The authors suggested that this preference occurs because chlorophyll content correlates positively with nitrogen content and therefore the butterflies are choosing the most nutritious leaves (Stefanescu et al. 2006). This may also be the reason why *M. persicae* was more likely to be found on brighter leaves. Higher levels of chlorophyll may result in higher levels of photosynthesis, which in turn would be expected to result in increased sugar and nitrogen levels. Brighter plants may therefore represent better hosts for herbivores such as *M. persicae* (Penuelas and Filella 1998).

Although the results of this study point to a fixed genetic component to the variation in plant colour, in practice it is impossible to separate fixed genetic effects from possible maternal effects, such as nutrients or pigments placed in the seeds that influence the development of the resulting plant (Roach and Wulff 1987). To my knowledge, the transgenerational effect of leaf colour has not been documented. Other leaf characteristics, however, such as size and shape can be influenced by maternal effects (Helenurm and Schaal 1996). The transmission of herbivore resistance via maternal effects has also been documented (e.g. Agrawal 2001), with levels of plant defences deposited in seeds varying with the maternal environment (Lammerink et al. 1984). It is difficult to see how maternal effects could have large influences on plant colouration in this study: maternal effects are normally most influential during the first few months of a seedling's growth (Helenurm and Schaal 1996), whereas measurements of colouration in this study were taken from two-year old plants. Over this period, any pigments deposited in the seed would likely have been turned over, with nutrients from the soil expected to play a more important role than nutrients gained

from the seed. For these reasons, it is likely that most colour variation seen in this experiment is due to fixed genetic rather than maternal effects.

4.4.3. Consequences of genetic variation for signalling

This study provides evidence for genetic variation in leaf colouration and several of the potential genetic mechanisms responsible for this variation have been discussed. An important question to address now is what fixed genetic variation in both leaf colouration and chemical defence means for the ACH, and in particular for the idea that plant colouration has evolved as a signal to herbivores of chemical defence levels. The fixed genetic component to both glucosinolates and leaf colouration provides a potential substrate on which selection could act in the evolution of a colour-based signal of chemical defence (Bradbury and Vehrencamp 1998). Furthermore, the fixed genetic control of colour means that colouration is likely to be sufficiently robust against environmental perturbations that a consistent correlation with glucosinolates could be maintained.

In the following chapters, I will move to test further assumptions underyling the ACH. First, I will test whether responses to visual stimuli are important in host selection by herbivores. Significant relationships between colouration and herbivory have been identified in Chapters Three and Four. It remains unclear, however, whether the effect of colouration on herbivory is due to an effect of colour on host selection and/or on host performance. In the following chapters, a combination of preference and performance experiments that separate these two potential sources of variation in herbivory were carried out. Chemical differences between plants varying in colouration will be explored in greater detail under controlled conditions, which will remove the confounding effect of chemical induction. These experiments were conducted on three herbivores: *B. brassicae, M. persicae* (Chapter Five), and *P. rapae* (Chapter Six). Since fixed genetic variation in plant brightness has been shown to be important to *M. persicae* in this chapter and correlations with brightness and glucosinolates were found in Chapter Three, I tested aphid preference and performance in relation to plant brightness in Chapter Five. In Chapter Three, significant effects of red colouration on herbivory by butterflies were reported, while in this chapter there is some evidence that blue colouration may also be important. In Chapter Six, I again use a combination of preference and performance experiments to test the importance of host colouration to host selection and offspring success in *P. rapae*.

Chapter Five. Host choice by aphids: can aphids use achromatic cues as a guide to host chemical defence?

5.1. Introduction

Aphids are serious agricultural pests, which damage and destroy many crops (see Figure 5.1) by extracting nutrients from the phloem and transmitting disease (Dixon 1987). The study of aphids thus has important economic implications, in particular the study of host-finding behaviour, which may shed light on ways to prevent colonisation of these pests.

Figure 5.1. Damage of *Brassica oleracea* caused by *Brevicoryne brassicae*. The leaf curling and discolouring can be seen and leaves are almost entirely covered by the aphids.

Aphids use plant cues to detect and choose their host (Powell et al. 2006). This includes a combination of visual (Doring and Chittka 2007) and olfactory cues (Visser 1986). Plants could therefore potentially signal information to aphids about their physiological state in order to bias host choice. The autumn colouration hypothesis (ACH) (Hamilton and Brown 2001) suggests that plants could signal their defence levels to herbivores through visual channels thus deterring colonisation. The ACH focused on aphids because they are a well documented pest and have a long evolutionary association with trees. Aphids are discriminating and can perceive host colouration (Doring and Chittka 2007), and the autumn colouration of trees coincides with their peak migration of many host-alternating aphids (Archetti and Leather 2005). With respect to the ACH, specialist aphids are of particular interest because they tend to be the most damaging (Coley and Barone 1996). For example, the grain aphid (*Sitobion avenae*) is a specialist of grasses and cereals and can cause major damage of wheat, significantly reducing yields (Dixon 1987).

The ACH posits that the intensity of leaf colouration functions to communicate levels of chemical defence to potential herbivores, which are expected to avoid the most intensely-coloured (and thus heavily-defended) trees. In Chapter One, two potential sources of variation in colour intensity were discussed: achromatic cues (i.e. brightness, perceived as total

photoreceptor stimulation) and chromatic cues (perceived by ratios of photoreceptor excitation). Overall green receptor stimulation is often used to detect achromatic signals (Giurfa et al. 1997). Plants differ most in the green region of the spectrum (Lythgoe and Partridge 1989) and brightness has significant genetic variation (Chapter Four) and so it is plausible that hosts may select among potential hosts using brightness as a cue. Alternatively, the herbivore may compare the level of stimulation of the green receptor with that of a second receptor, e.g. the blue receptor. The resulting blue to green ratio would constitute a chromatic response, which represents the spectral composition of the wavelength. Both achromatic and chromatic cues were investigated in Chapter Three and it was found that brightness had correlations with glucosinolate concentrations. It is important that this relationship is futher investigated, and here levels of plant brightness will be manipulated in controlled conditions to find associations with glucosinolates and aphid responses to this.

It is interesting to begin an investigation of the ACH with aphid responses as the original hypothesis was formulated with these insects in mind. Although aphids do not have as many spectral sensitivity types as butterflies (Qiu and Arikawa 2003), they nonetheless possess colour vision and also the ability to respond to achromatic cues (Doring and Chittka 2007). Chapter Four provided evidence that *Myzus persicae* has an association with brighter coloured plants and so achromatic cues are likely to be important to this herbivore. Much visually-guided behaviour in bees and other insects is based on achromatic mechanisms driven by the green cone (for a review see Lehrer 1994), and so it is possible that achromatic cues could serve a similar role in aphid behaviour. In butterfly host choice, it has been found that achromatic cues are essential in host landing behaviour (Koshitaka et al. 2011). Some evidence for the green receptor controlling host preference of aphids is provided by Kennedy et al. (1961): beet leaves that stimulated the green receptors more strongly than cabbage leaves were preferred by aphids, which is consistent with the hypothesis that the green receptor controls preference (Kennedy et al. 1961). Further evidence for the use of achromatic cues in this study was provided by aphid responses in a flight chamber where the insects alighted six times as often on white areas compared to black areas (Kennedy et al. 1961). The available evidence therefore suggests that investigation of achromatic cues would be worthwhile.

Basic responses to host chromatic cues are also investigated in this chapter. Aphid preferences for different colours are frequently reported in the literature. For example, it was found that *Phorodon humuli* aphids preferred to land on yellow rather than green coloured hops (Campbell 1991). In the laboratory, *Neuquenaphis staryi* aphids preferred to stay in the region of Petri dish illuminated with green rather than red light (Ramirez et al. 2008). Based on models of receptor excitation by Doring and Chittka (2007), it is predicted that increased

stimulation of the green receptor relative to the other receptors will result in a positive input, while increased stimulation of the blue receptor will produce a negative input, thereby resulting in a preference for greener colouration (Doring and Chittka 2007). Evidence from the field in Chapter Three, however, suggested the opposite pattern, as *B. brassicae* was more likely to be found on bluer plants. It will be necessary to interpret preference for human colours in terms of blue and green aphid receptor excitation.

Olfactory cues are sometimes found to be the primary cue used in host location (McMenemy et al. 2012), but in other contexts visual cues can play a more important role (Gish and Inbar 2006). To date, however, there have been no choice tests with aphids using whole plants where olfactory cues have been eliminated. This is an important requirement when looking at cue use. For example, Yue and Liu (2000) found that the turnip aphid (*Lipaphis erysimi*) preferred green varieties to red varieties of cultivated *Brassica oleracea*. Unfortunately, while Yue and Liu (2000) took care to test aphid responses to natural substrates, their design did not separate visual and olfactory cues, making it impossible to determine whether choices were based on the colour of the plant or a correlated odour.

Preference-performance experiments are commonly used to reveal the fitness consequences of herbivore preferences – do herbivores make the right host choice in terms of fitness payoffs (Valladares and Lawton 1991)? It is expected that aphid host preferences will match their performance, because the fitness benefits accrued from selecting the most suitable hosts create a selection pressure for the ability to detect these hosts (Thompson 1988, Gripenberg et al. 2010). This matching of preference and performance is commonly found (Leather 1986, Minkenberg 1990, Johnson et al. 2003), although there may be some instances where the herbivore does not choose the host which maximises its fitness (Broekgaarden et al. 2012, McMenemy et al. 2012). A preference-performance experiment is important to do here, because it is impossible to separate these effects in the field and so it is unclear whether there is a preference for certain colours in the field or that the herbivores perform poorly on other colours (see Chapter Three).

In this study, I explore the potential for host selection based on colour cues using *Brassicae oleracea* and two of its aphid herbivores, *Brevicoryne brassicae* and *Myzus persicae* (see Chapter One for an introduction to these species). *Brevicoryne brassicae* is a specialist aphid of brassicas and has an effective glucosinolate detoxification and sequestration system, enabling it to reach high densities on host plants (Kazana et al. 2007). *Myzus persicae* is a generalist aphid which utilises a large range of hosts including brassicas. Using both these aphids thus permits a comparison of host preference and performance between a specialist herbivore and a generalist herbivore. It is predicted that *B. brassicae* is more likely to be

discriminating of hosts because its reliance on one particular host means that any changes in the ability of the host to defend itself are likely to have larger fitness consequences for *B. brassicae* than for *M. persicae*. Chapter Four, however, suggested that *M. persicae*, not *B. brassicae*, had an association with plant brightness and so it is possible *M. persicae* is more selective with plants differing in brightness. It is also predicted that glucosinolates, in particular indole glucosinolates, will have a greater impact on the performance of *M. persicae* than *B. brassicae* (Cole 1997, Mewis et al. 2005), because *B. brassicae* , unlike *M. persicae,* has a detoxification system specific to glucosinolates (Kazana et al. 2007).

The impacts of visual cue use by *B. brassicae* and *M. persicae* will be investigated to test the ideas behind the ACH. *Brassica oleracea* plants were grown to produce natural variation in colouration to enable four questions to be explored. Firstly, do *B. brassicae* and *M. persicae* have a preference for plants differing in achromatic cue? If these aphids select hosts based on achromatic cues it would suggest that selection has favoured the ability to discriminate hosts varying in achromaticity. The experimental design used in this study presents a novel technique for measuring aphid visual preference. The design allows complete control over visual and olfactory cues, agrees with natural aphid movement behaviour, and removes any possible sources of bias. It is the first experiment of its kind to use a natural stimulus and eliminate olfactory cues. Secondly, do *B. brassicae* and *M. persicae* perform differently on plants varying in achromatic cue? Fitness differences (here estimated using differences in reproduction and survival) between these plant types will suggest a need for selectivity. Thirdly, do glucosinolate defence levels differ between bright and dull plants? If plants signal levels of chemical defence through colouration, a correlation between these variables is expected. Finally, can *B. brassicae* use chromatic cues in host choice? A basic investigation into the response to chromatic cues will take place to see if *B. brassicae* uses this type of colouration in host choice.

5.2 Methods

5.2.1. Plants

Three hundred *B. oleracea* plants were grown from seeds collected in the field in September 2010 from three sites in Dorset: Old Harry (50°64'N, 1°92'W) Winspit (50°59'N, 2°03'W) and Kimmeridge (50°60'N, 2°13W). The plants were grown in six-inch pots in John Innes potting compost No. 2 in a greenhouse at 15°C in a 16:8 light regime. Plants were watered as required and Hoaglands solution was applied weekly for the first month. After three months, plants had matured as the leaves had their waxy bloom (Bleasdale 1982), (Growth Stage Five since the inner leaves do not form a heart (Andaloro 1983), see Figure 5.2) and colour readings were taken.

5.2.2. Aphids

The specialist aphid, *B. brassicae* was cultured from populations which infested non-experimental greenhouse plants naturally. The generalist *M. persicae* was obtained from a culture maintained at Rothamsted research (provided by C. Woodcock, BCH division, Rothamsted research). Both aphids were reared on Chinese cabbage plants so that they were naive to wild *B. oleracea*. Fresh plants were added when required. Plants and aphids were contained in a Perspex box (60x50x70cm) with a fan in to provide fresh air flow. The boxes were kept in the greenhouse at 20°C in a 16:8 light regime. Apterous aphids were used in the trials and were starved for two hours prior to testing so they were motivated to find a host.

5.2.3. Colour measurements

Colour was measured using a spectrophotometer and the reflectance spectra modelled to aphid quantum catch (Kirchner et al. 2005) to give an output of each of the three aphid photoreceptors: UV, green and blue (see Chapter Three for details). Colour was measured using the third leaf from the top of the plant. The 30 plants with the highest green receptor quantum catch were considered the "brightest" plants, and the 30 plants with the lowest amount of green receptor quantum catch were considered the "dullest." Chromatic readings were taken from the leaf prior to testing for response to chromatic cues, so that that the colour from the aphid's perspective could be analysed.

5.2.4. Achromatic preference trials

Preference trials consisted of a choice between a bright and a dull plant (see Figure 5.2). A Y-shaped choice stick was used to determine the preference and a choice was considered to

have been made when the aphid walked 1cm onto the right or left arm. For each trial a new stick was used so that the aphid could not follow any trail left by the previous aphid. The stick was made from white cardboard and cut out with a laser so that there was no bias in angle at the Y fork. For experimental set-up see Figure 5.2.

Three types of cue from the host plant were used: visual (plant in full sight but no plant volatiles), olfactory (plant concealed but volatiles available), and visual and olfactory (plant in full sight and volatiles available). Each plant pair (bright versus dull) was tested using the three types of cue with both types of aphid (e.g. plant pair 1: visual for *B. brassicae*, olfactory for *B. brassicae*, both for *B. brassicae*, visual for *M. persicae*, olfactory for *M. persicae*, both for *M. persicae*), so that there were six trials per plant pair, resulting in 180 trials overall. Null tests were carried out before experimentation to ensure there was no side bias and to ensure that the aphids could discriminate between plant and no stimulus meaning that the experimental design was effective in giving access to the plant cues.

Plants were placed in a plastic box (56cmx40cmx30cm) with cling film in place of the lid. Cling film absorbs only about 25% of UV light (see Appendix B) and so is within acceptable variation of solar radiation (Endler 1993). This is a practical alternative to UV transparent quartz glass, which is costly. An aquarium pump was placed inside the box to extract plant volatiles, which could then be blown through a tube onto the Y stick to provide olfactory cues. The tubes were clamped in place, and located so not to obscure the view of the plant.

The plant was placed in the box for five minutes prior to testing so that the volatiles emitted from the plant could fill the experimental box. In tests omitting olfactory cues, the pump was removed from the box and placed outside where it still supplied a constant flow of air. The box was covered with an opaque sheet in tests omitting visual cues. The arena was illuminated with a UV light (Arcadia compact bird lamp, 20W, FBC20X) which was placed directly above the Y-choice stick. The overhead illumination encouraged the aphid to walk along the stick as did the airflow, since aphids walk up-wind and towards light. It is important that UV light is present as UV-negative environments result in extended task completion time (Dyer 2006). The aphids were kept in a Petri dish on damp filter paper when not in use and were introduced onto the choice stick with a paintbrush. Aphids that climbed off the stick were ignored and replaced (only about 10%). If after five minutes an aphid had not made a choice, the aphid was removed and replaced (a single occurrence). Only one aphid was used per trial.

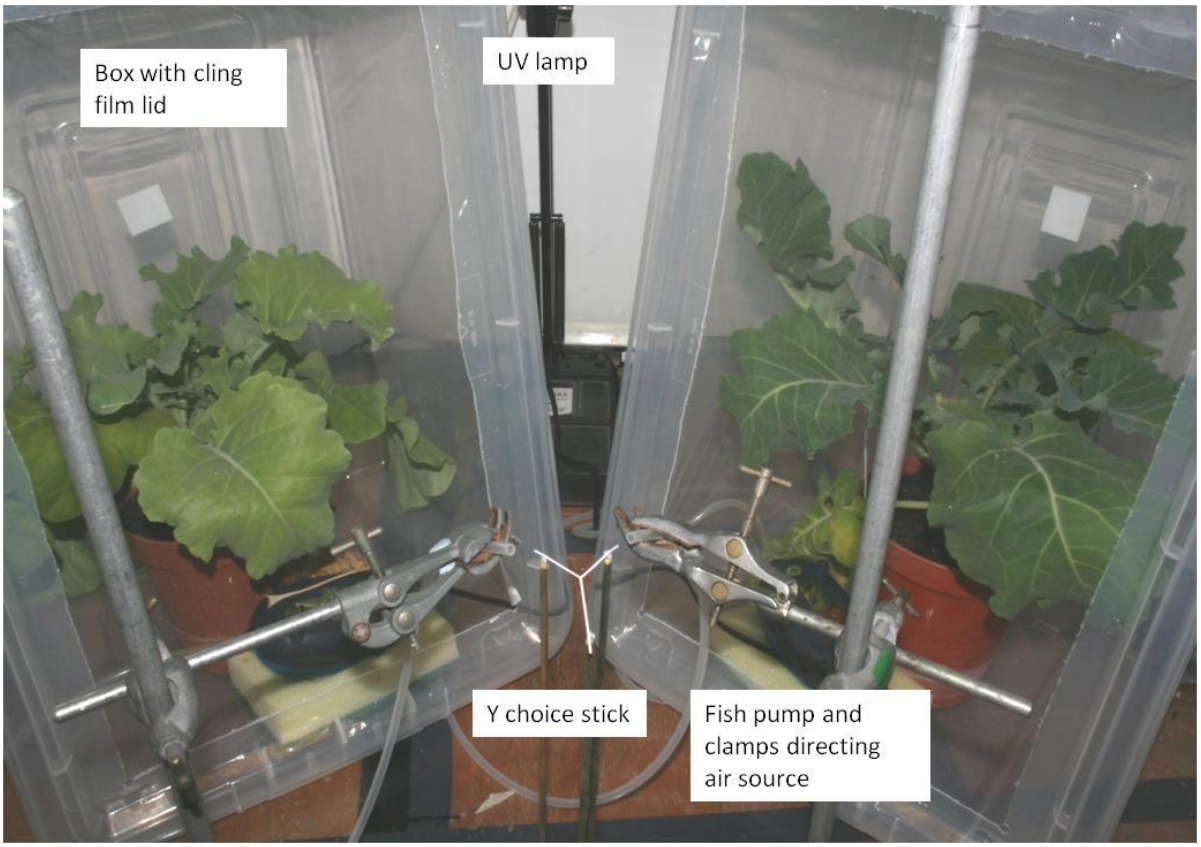

Figure 5.2. A photograph of the experimental set-up. The bright plant is on the left and dull on the right. Plant is in sight and pump inside the experimental box to extract volatiles so this demonstrates a set-up involving both visual and olfactory cues.

5.2.5. Performance trial

After the preference trial the same 60 plants (30 pairs, one bright plant and one dull plant) were inoculated with 10 aphids. Fifteen pairs of plants were inoculated with *B. brassicae* and 15 with *M. persicae*. Aphids were contained on the plants within a muslin bag. Plants were randomised with respect to greenhouse position and kept in the greenhouse as before. The plants with *M. persicae* were re-inoculated with 20 aphids after one week to ensure they had been colonised. After five weeks, the aphids were removed, placed in alcohol and counted. The aphids were separated into alate and apterate morphs, and those that had died prior to removal.

5.2.6. Glucosinolate differences between bright and dull plants

The fourth leaf from the top was excised with scissors and snap frozen in liquid nitrogen. The leaves were stored in a -80^{o}C freezer until analysis. Glucosinolates were extracted using methanol and converted to desuplhoglucosinolates with sulfatase on a sephadex column. Desulphoglucosinolates were separated on a C-18 reverse phase column on HPLC and detected at 229nm. Sinigrin was used an external standard. See Chapter Three for a detailed method of chemical analysis.

5.2.7. Chromatic preference trials

The different responses to red, yellow, green and blue leaves were investigated (see Figure 5.3a) to see if chromatic cues could be used by *B. brassicae*. A whole plant could not be used because wild *B. oleracea* does not turn completely yellow or red. The preference trial was therefore carried out using single leaves only, meaning that it was not possible to follow up this test with a performance trial. The four coloured leaves were combined in all pairing options and 30 individual *B. brassicae* were tested for the preference of colour in each pair. Again, each aphid was only used once. The trial was carried out over three days and on each day a new set of four leaves was gathered. A Y-choice stick was used as before and the leaves were covered in cling film so that no olfactory cues were present. The leaves were held into place with clamps (see Figure 5.3b)

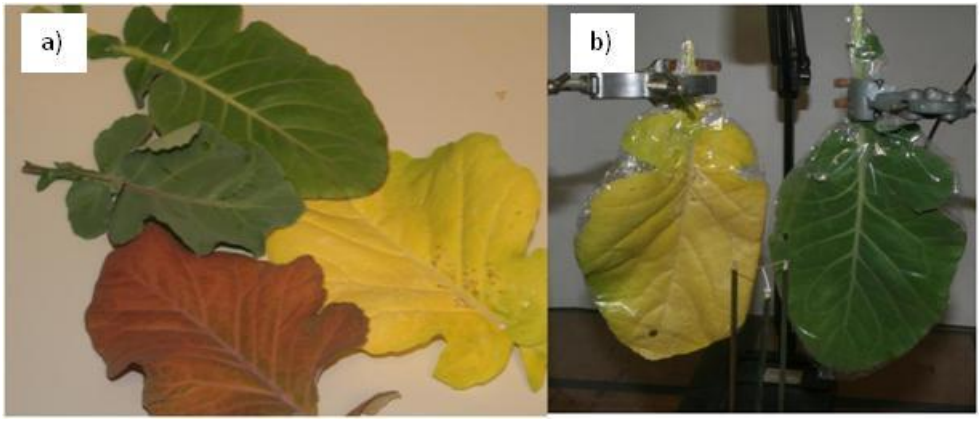

Figure 5.3. Equipment for *B. brassicae* chromatic choice experiment. a) The four leaf colours used in the experiment. b) Equipment set up for experiment; leaves wrapped in cling film and the Y choice stick can be seen.

5.2.8. Statistics

Data were analysed using R (version 2.10.00). Side bias checks (no plants present to check for a tendency to walk to one side) and null checks (plant vs. no plant to check the equipment allowed detection of plants) were analysed with a binomial test.

5.2.8.1. Achromatic cues

Aphid responses in preference trials were analysed with a generalised mixed effects model: lmer (library lme4) with choice (bright or dull) as the response factor, aphid (*B. brassicae* or *M. persicae*), cue (visual, olfactory, or visual and olfactory) and side (whether the aphid went left or right) as fixed effects and cabbage pairing as a random effect. Model simplification proceeded by backwards deletion of non-significant terms until further removals lead to significant ($p<0.05$) increases in deviance assessed using Chi squared values. Significance levels

are reported on the addition of non-significant terms and removal of significant terms from the minimum adequate model. Performance tests were analysed with ANOVA with number of aphids (log-transformed) as the response variable and cabbage (bright or dull) as the explanatory variable. Proportions of total aphids that were alate, apterate or dead as predicted by plant brightness were analysed with a generalised linear model with quasibinomial errors to correct for overdispersion.

Chemical concentrations were compared for bright and dull plants using a t-test or a Mann Whitney U-test if assumptions of normality could not be met through transformation. Benjamini-Hochberg corrections for multiple comparisons were applied (Benjamini and Hochberg 1995, Benjamini et al. 2001).

5.2.8.2. Chromatic cues

Preference for the choice of the four colours (in sets of pairs) in the chromatic choice experiment was analysed with binomial tests. Chi-squared tests confirmed that there were no significant differences between the results found over the three days for colour preferences so it was acceptable to combine the days to get an overall p-value from a binomial test per colour combination (in total 6 p-values). To investigate if there was an overall significant effect of colour the p-values of the binomial tests were combined using an unweighted z-transformation (Whitlock 2005). This was carried out on Mathematica version 7. This procedure converts the p-values into standard normal deviates which are then summed and the result divided by the number of tests combined. This results in another standard normal deviate which can be used to infer the probability for the combined tests. The colour choices were ranked to find an overall pattern of preference.

5.3. Results

There was no evidence for side bias and aphids could discriminate between plant and non-plant (an empty container of one side of the choice apparatus) with both olfactory and visual cues in the experimental set-up. Furthermore, the visual cue was not simply a silhouette as the aphids could discriminate between a plant and a piece of cardboard when olfactory cues were removed (see Table 5.1).

Table 5.1. Results for side bias and null experiments.

Species	Condition	Total n	n to plant	p-value of choice
B. brassicae	Nothing vs nothing	30	16	0.86
B. brassicae	Plant vs nothing (visual)	32	23	0.020
B. brassicae	Plant vs nothing (olfactory)	31	22	0.029
B. brassicae	Plant vs cardboard	30	25	<0.001
M. persicae	Nothing vs nothing	30	15	1.0
M. persicae	Plant vs nothing (visual)	34	26	0.0029
M. persicae	Plant vs nothing (olfactory)	32	22	0.050
M. persicae	Plant vs cardboard	30	21	0.043

5.3.1. Achromatic preference

There was no significant difference between the proportion of aphids choosing bright or dull plants when considering the type of potential cue (X^2_2=0.138, p=0.93, Fig. 5.4a), the aphid species (X^2_1=1.48, p=0.22, Fig 5.4b) or the interaction between cue type and species (X^2_5= 3.91, p=0.56). This provides strong evidence that aphid preference for hosts is not based upon any differences in visual or olfactory stimuli between bright and dull plants.

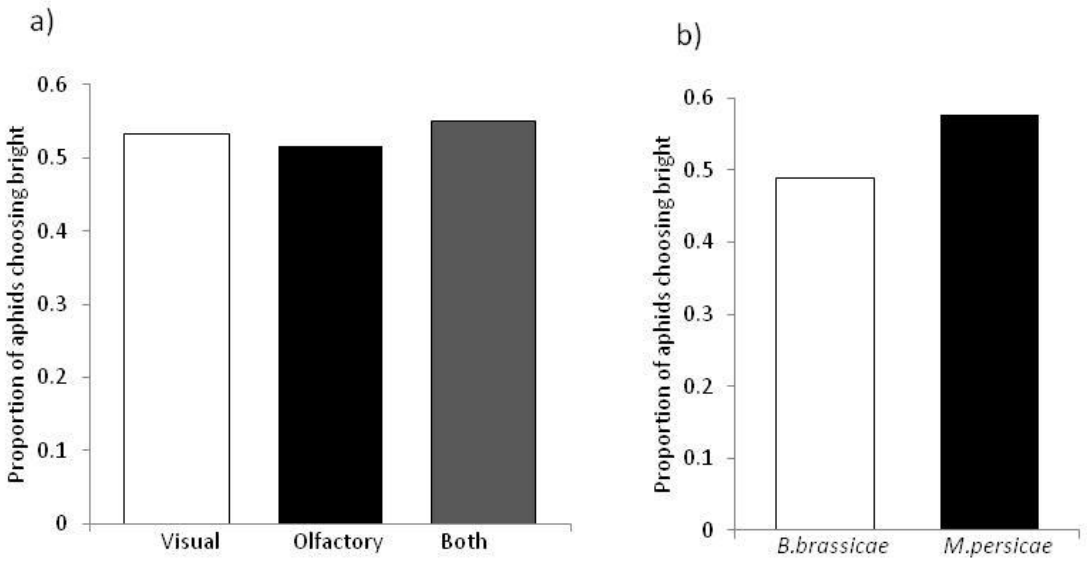

Figure 5.4. Proportion of aphids choosing bright cabbages. a) the proportion with each cue type, and b) the choice difference between each species of aphid.

5.3.2. Performance

The mean number of *B. brassicae* per plant (+/- 1SEM) was 1804 +/- 261. There were far fewer *M. persicae* with a mean of 281+/-40, confiriming that *B. brassicae* is better able to exploit brassica hosts.

There was no significant difference in the total numbers of *B. brassicae* between bright and dull cabbages ($F_{1,28}$=0.529, p=0.47). This result indicates that there are no differences in aphid reproduction and survival between the two plants types. There was also no significant difference when comparing numbers of alate ($F_{1,28}$=1.17, p=0.29), apterate ($F_{1,28}$=0.415, p=0.52) and dead aphids ($F_{1,28}$=0.689, p=0.41). When these classes were analysed as a proportion of the total aphid numbers, there was no significant difference between the proportion of alate *B. brassicae* on bright or dull plants (GLM, $F_{1,28}$=0.474, p=0.50) nor the proportion of dead aphids (GLM, $F_{1,28}$=0.931, p=0.34). This suggests bright and dull plants are equally suitable hosts for *B. brassicae* as neither aphid mortality nor the numbers of dispersing morphs produced varied between them.

In contrast, however, there were significantly greater numbers of *M. persicae* on dull cabbages than on bright cabbages ($F_{1,28}$=5.30, p=0.029, Fig. 5.5a). This difference is to be attributed to the number of apterate morphs ($F_{1,28}$=5.41, p=0.028), rather than the number of dead aphids ($F_{1,28}$=0.131, p= 0.72) or alate morphs ($F_{1,28}$=1.51, p=0.23), indicating that reproduction is lower on bright plants. When examined as a proportion of the total number of aphids, the proportion of dead *M. persicae* was higher on bright plants (GLM, $F_{1,28}$=11.4, p=0.0022, Fig. 5.5b), indicating that survival is also reduced on bright plants. There was no significant difference in the proportion of flying morphs (GLM, $F_{1,28}$=0.270, p=0.61), and so even though survival was lower on bright plants this did not result in an increase in the production of dispersing morphs that are able to seek alternative hosts.

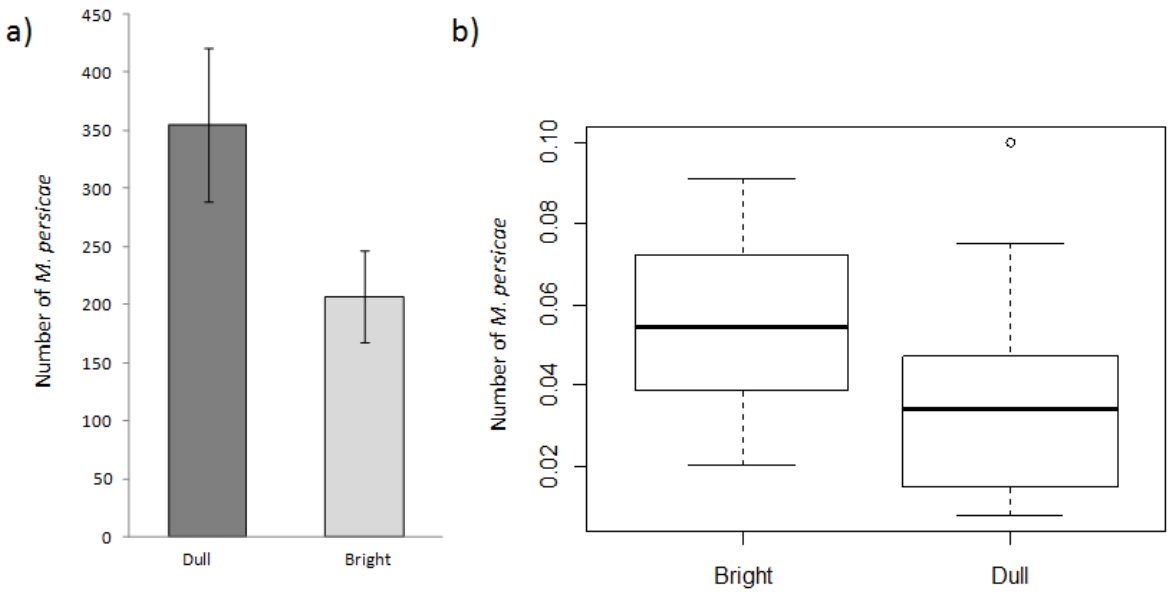

Figure 5.5. Results of *M. persicae* performance experiments. a) Mean total number of *M. persicae* on dull and bright plants. Error bars show SEM. b) Proportion of dead *M.persicae* out of the total number found on both bright and dull cabbages. Box shows interquartile range with the central, heavy line representing the median. The tails from the bar represent approximately 2 standard deviations around the interquartile range.

5.3.3. Chemical differences in bright and dull plants

A total of eight different glucosinolates were identified. Of the five aliphatic glucosinolates (glucoiberin, progoitrin, glucoraphanin, sinigrin, gluconapin) identified, gluconapin was at the highest concentrations. Three indole glucosinolates (glucobrassicin, neoglucobrassicin and 4-methoxyglucobrassicin) were identified, of which 4-methyoxy glucobrassicin was present in the highest concentrations.

There was no significant difference in the total glucosinolate concentration between bright and dull plants (W=714, df=78, p=0.76). When individual glucosinolates were analysed, there was no significant difference between bright and dull plants in concentrations of glucoiberin (W=778, df=78, p=0.23), progoitrin (W=604, df=78, p=0.37), glucoraphanin (W=696, df=78, p=0.90), gluconapin (W=718, df=78, p=0.72), neoglucobrassicin (W=639, df=78, p=0.63) or 4-methoxyglucobrassicin (t=1.20, df=78, p=0.24) (see Figure 5.6b). Brightly coloured plants, however, had significantly higher levels of sinigrin (W=918, df=78, p=0.012, Fig. 5.6a) and glucobrassicin (t=2.56, df=78, p=0.013). These were still significant when Benjamini-Hochberg corrections were applied. This indicates that brightness and dullness could be used as a cue for concentrations of these chemicals.

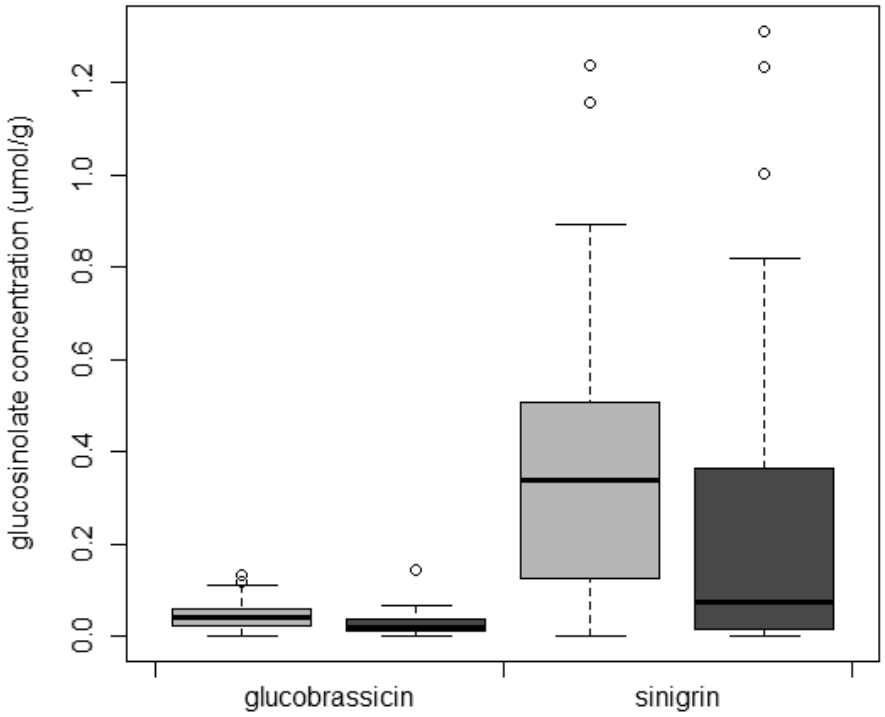

Figure 5.6. Glucobrassicin and sinigrin concentrations in bright (pale grey box) and dull (dark grey box) plants. Both significant with Benjamini-Hochberg corrections (see in text for test statistics). Box shows interquartile range with the central, heavy line representing the median. The tails from the bar represent approximately 2 standard deviations around the interquartile range, and dots represent outliers from this.

5.3.4. Chromatic preference

There was a highly significant overall effect of colour on leaf choice (Combined p-value using Z-transformation, $p<0.001$) (see Table 5.2), indicating that chromatic cues play a role in host choice.

Table 5.2. Preference of colour from each pairing in the chromatic preference trial, with associated proportion of aphids choosing the colour and the p-value.

Pairing	Preference	Proportion	P-value
Red vs yellow	Red	60/94	0.0095
Red vs green	Red	57/94	0.049
Red vs blue	Red	54/93	0.15
Green vs blue	Blue	50/91	0.40
Yellow vs blue	Blue	56/91	0.035
Green vs yellow	Green	57/93	0.038

The colour preference ranking of red>green=blue>yellow was produced from the results in Table 5.2 (see Figure 5.7). Red was the most preferred colour and yellow the least preferred. Blue and green were equally preferred.

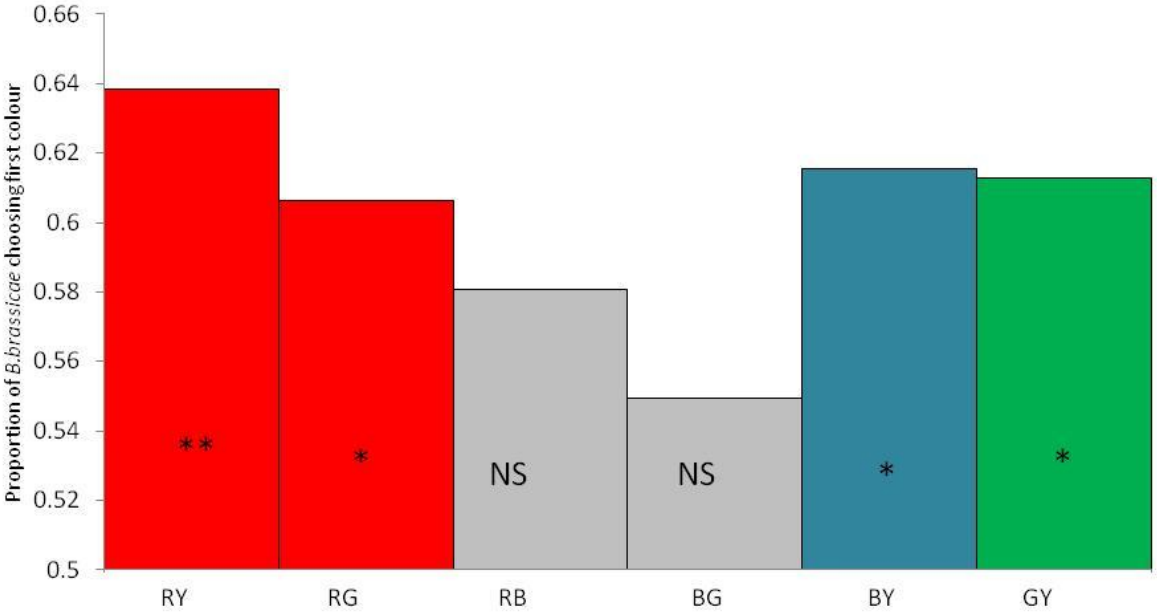

Figure 5.7. *Brevicoryne brassicae* preference of coloured leaves. The X-axis shows the pairings RY (red vs yellow), RG (red vs green), RB (red vs blue), BG (blue vs green), BY (blue vs yellow), GY (green vs yellow). The starting letter of the pairing is the preferred colour, and the stars on the bars denotes the significance (**<0.01, *<0.05, NS not significant) (also see Table 5.2).

5.4 Discussion

This chapter aimed to test whether the ACH could be applied to achromatic cue use in *B. oleracea* by two of its aphid herbivores, *M. persicae* and *B. brassicae*. In order to support the ACH, a number of requirements must be met. First, there must be variation in colouration among hosts, which herbivores must then use to choose among hosts. Second, host colouration must be a reliable signal of defence levels. Third, herbivore fitness must vary as a function of host defence levels, such that attending to cues of defence levels is beneficial. The extent to which these requirements were supported in this experiment will now be discussed.

5.4.1. Is there a host preference of aphids when offered a choice of bright or dull plants?

Green sensitive receptors are often the most abundant type in insect eyes (Osorio and Vorobyev 2005) and the literature has many examples of choices involving achromatic cues. For example, flowers in sunny locations, which are therefore brighter, receive more visits by pollinators than flowers in the shade (Kilkenny and Galloway 2008). This is because intensity contrasts are important in flower detection (Lehrer and Bischof 1995, Giurfa et al. 1997). Brightness is important in host choice in marsh fritillary butterflies (*Euphydryas aurinia)*, with the brightest leaves preferred by ovipositing females (Stefanescu et al. 2006). Aphid preference for yellow sticky traps is thought to occur because they provide a strong achromatic cue (Prokopy and Owens 1983) and preferences for yellow based on this achromatic stimulation have been reported. For example, *M. persicae* and *B. brassicae* were found to alight more frequently on a pure yellow colour than on a yellow stimulus mixed with green (Kring 1967). This evidence suggests that the null response to achromatic cues found in this study did not occur because the aphids are unable to use this mechanism; indeed, insensitivity to achromatic colouration in this context may well be adaptive (see below).

It is often found that achromatic cues are not the most important in host choice. For example, the parasitic wasp *Venturia canescens* is unable to associate a reward with an achromatic cue, but shows a strong learning ability when trained to a chromatic cue (Desouhant et al. 2010). For aphids too, there is evidence to suggest that achromatic cues do not solely guide host choice behaviour. Moericke (1950) found that there was no attraction of aphids to white targets, even though they stimulate the green receptor more than any other colour. Chromatic cues as well as achromatic cues are suggested to drive host choice behaviour in aphids (Doring and Chittka 2007), but achromatic cues are thought to have a greater role in distinguishing a plant from the sky rather than distinguishing between plants (Kennedy et al. 1961).

It may be that achromatic cues are often not used to distinguish between plants because they are less reliable that chromatic cues (Osorio and Vorobyev 2005). Chromatic cues will be constant under different lighting conditions (e.g. the red to green ratio will be the same whether it is cloudy or sunny) whereas achromatic cues will be strongly affected by lighting conditions (Lythgoe 1979). For example, it has been suggested that fruit detection by frugivorous birds does not involve achromatic cues because they are not reliably detected with variable light conditions in forest canopy (Cazetta et al. 2009). A bright leaf in the shade will have the same achromatic signal as a dull leaf in the sunshine and so the plant may not be able to reliably signal to herbivores through this channel. Therefore, it may be adaptive for the aphids not to use achromatic cues as information cannot be reliably conveyed as the cues vary too much with lighting conditions.

5.4.2. Is the preference reflected in aphid performance?

To my knowledge, no study of plant host choice has to date undertaken both a preference experiment testing responses to purely visual stimuli *and* a performance trial. Therefore, the potential fitness payoffs to choosing hosts based on visual cues are unknown. Across the literature as a whole there is generally a matching of preference and performance (Gripenberg et al. 2010), but this did not apply here. *Myzus persicae* had no preference for bright or dull plants, yet the aphid performed better on dull plants where aphid numbers were 41% higher. It might be expected that the increased reproduction and survival on dull plants would have selected for a preference. As discussed above, this lack of preference may be due to the unreliable nature of achromatic cues; however, there may be other reasons why aphids showed no preference. *Myzus persicae* may encounter wild *B. oleracea* relatively infrequently in the field, and so the selection pressures to detect and respond to cues of this host may be relatively weak (Chew 1977). This can be supported by data from Chapters Two and Three, where *M. persicae* was only found rarely on *B. oleracea* in the field and in the common garden experiment.

An alternative explanation is that the preference experiment did not allow *M. persicae* sufficient time to make the optimal decision. In order to separate visual cues from gustatory cues, contact and feeding from the plant was prevented, and a choice was recorded when the aphid walked towards the plant. Spontaneous choices have been recorded in previous studies looking at aphid preference of plants (e.g. McMenemy et al. 2012). Other studies, however, have explored preferences over longer time periods by allowing aphids to move freely between stimulus plants within a cage (e.g. Johnson et al. 2003; Ewald et al. 2011). Unfortunately, using such experimental designs, it is not possible to disentangle the initial

responses to visual or olfactory cues from responses to gustatory cues occurring when aphids sample host tissues. For example, 77% of aphids (*Sitobion avenae)* were found to prefer host plants (*Holcus lanatus*) parasitised by the hemiparasite *Rhinanthus minor* over unparasitised hosts, and aphids were also found to perform better on parasitised plants than unparasitised plants (Ewald et al. 2011). In that study, aphids were allowed 24 hours to choose among hosts, in which time phloem fluid could have been tested and rejected for nutritional content (Powell et al. 2006). If in general gustatory cues are required for aphids to select hosts, colouration may consequently be of little importance as a plant will only be accepted once phloem has been tested.

5.4.3. Are there differences in glucosinolate concentrations between bright and dull plants?

Chemical differences were found between bright and dull plants. Moreover, the plants with higher chemical defence were also the brightest, thus matching the prediction of the ACH that the intensity of colouration should be positively correlated with chemical defense levels (Hamilton and Brown 2001). The absence of a behavioural preference is, however, inconsistent with the ACH because even though there are chemical differences between bright and dull plants the aphids choose not to, or cannot, discriminate between them. *Brevicoryne brassicae* showed no differences in performance between bright and dull plants, suggesting that the extent of the difference in glucosinolates levels between these plants is not sufficient to influence reproductive rate. A signal could not evolve in this situation as it would not pay *B. brassicae* to attend to information about levels of these glucosinolates. The relationship between visual stimuli and chemical defence is too weak, and due to the temporal instability of achromatic cues, too unreliable.

Chemical differences have been found in previous studies seeking a correlation between visual stimuli and chemical defence, although none have specifically compared plants differing in brightness. In this study the relationship was quite weak, with only two out of the eight glucosinolates differing significantly between bright and dull plants and sinigrin had the larger difference (effect size sinigrin r=0.39, glucobrassicin r=0.29, medium effect sizes as by Cohen (1988)). This appears to be a common finding: there are some studies that find a strong correlation between colouration and plant defence chemistry (e.g. Cooney et al. 2012), but some find a weaker or null relationship (e.g. Lempa et al. 2001, Hughes et al. 2010). Correlations have been reported between defensive chemistry, especially tannins, and colouration in the mountain birch tree, although chemical defence also varied throughout the season (Lempa et al. 2001). Phenolics were found to correlate with colouration in the Kermes oak (*Quercus coccifera*), indicating that redness could potentially signal chemical defence in

this species (Karageorgou and Manetas 2006). The opposite, however, was found in a *Veronica* species where red leaf margins did not correlate with phenolics, although it is currently unclear how important phenolics are in defence against herbivores in *Veronica* (Hughes et al. 2010). As the phenolics are such a large group of chemicals, the difference in result of these two studies may be explained by the presence of other phenolics that do not provide a defensive function.

The difference in brightness between plants is probably caused by differing chlorophyll concentrations. Chlorophyll content is an important predictor of nitrogen availability and leaf reflectance in the green region can determine nutrient status of the plant (Penuelas and Filella 1998). Therefore a brighter plant with more chlorophyll may have more nutrients available to make costly chemical defences (Stamp 2003), thus providing a link between a visual cue and chemical defence. In this study, brighter plants were found to have slightly higher chemical defence. When organically fertilised, *B. oleracea* has been found to increase in glucosinolate levels (Hsu et al. 2009, Staley et al. 2010, Stafford et al. 2012); this provides further evidence that when more nutrients are available chemical defence may be higher. Though there may be a link with chemical defence and achromatic stimuli, as required for a signal to evolve, as noted above such a signal is unlikely to evolve as achromatic cues are too variable with lighting conditions to transmit reliable information to a receiver.

Aphids are phloem feeders; measurements of glucosinolate concentrations in the leaves therefore provide only an estimation of what the aphids are encountering. Previous studies looking at the impact of defence chemicals on aphid performance in *B. oleracea* have tended to measure levels of glucosinolates in the leaves (e.g. Kim and Jander 2007) because of the difficultly in extracting chemicals from the phloem and obtaining accurate concentrations of these chemicals (Chen et al. 2001). This is important to consider as aphids may encounter lower concentrations of chemical defence by feeding on phloem rather than leaf tissue (Martin et al. 1994). It is has been shown, however, that defence chemicals in the leaves often correlate with the phloem (Bentz et al. 1995, Merritt 1996), so measuring concentrations of glucosinolates in leaf tissue probably provides a relatively good estimate of glucosinolate concentrations in the phloem.

It was predicted that specialists would be less influenced by the toxicity of chemical defence than generalists (Ali and Agrawal 2012). Indeed, there is convincing evidence from the literature that specialists cope better with defensive chemicals on a normal host than generalists, leading to a higher feeding success (Cornell and Hawkins 2003, Staley et al. 2010). The findings in this chapter support this: populations of the generalist *M. persicae* were five times smaller than those of the specialist *B. brassicae*, and unlike *B. brassicae*, *M. persicae* exhibited reduced performance on plants with higher levels of chemical defence.

It was predicted that indole glucosinolates would have a stronger effect on the aphids than aliphatic glucosinolates. This is because indole glucosinolates hydrolyse without the myrosinase enzyme (see Chapter One). Aliphatic glucosinolates, however, hydrolyse only when in contact with myrosinase, which occurs upon tissue disruption, and as the aphids are phloem feeders they avoid such disruption (Kim and Jander 2007). The only indole glucosinolate that differed between bright and dull plants was glucobrassicin. This difference, surprisingly, did not affect *B. brassicae* performance. Previous studies have found a negative impact of indole glucosinolates on *B. brassicae* performance (Cole 1997, Mewis et al. 2005, Kim and Jander 2007). As glucobrassicin was only slightly higher in bright plants and as no other indole glucosinolates differed, these small differences may explain this null finding. Sinigrin concentrations were four times higher in bright plants and glucobrassicin was a third higher in bright plants and so it is presumed that singirin had the strongest effect on the low numbers of *M. persicae* found on bright plants. This is in agreement with other studies which have a found a negative correlation with *M. persicae* and aliphatic glucosinolates (Mewis et al. 2005, Staley et al. 2010). Sinigrin had a negative impact on *B. brassicae* colony size in wild *B. oleracea* (Newton et al. 2009a), therefore it is surprising that sinigrin did not impact *B. brassicae* in this study.

There is very little work on the different use of visual host cues by specialist and generalist aphids. In this study it was found that both the specialist and the generalist aphids had no preference between plants differing in achromatic cues. The majority of studies investigating aphid host choice using vision on natural plants, study only specialist aphids and so it is difficult to make a comparison of preference between these herbivore types. For example it was found that the specialist turnip aphid preferred green cabbages over red plants (Yue and Liu 2000), and a specialist hop aphid preferred yellow hops to green hops (Campbell 1991), but would generalist aphids have made the same decisions? Green water traps placed on differently coloured backgrounds captured both *B. brassicae* and *M. persicae*, enabling a comparison to be made with an artificial substrate (Doring et al. 2004). It was found that there was a general pattern of a negative correlation with aphid number and the intensity of UV reflectance of the background sheet for both *B. brassicae* and *M. persicae* and so it is likely that specialist and generalist aphids have similar overall rules.

5.4.4. If achromatic cues are not used, do aphids use any colour visual cues?

There is plentiful evidence to suggest that aphids use chromatic cues in host choice (e.g. Doring et al. 2004, Ramirez et al. 2008). The ranked colour preferences of R>G=B>Y found in this experiment were surprising, as usually an aversion to red and a preference for yellow is

found in aphid host preference. For example, it was found that more *Euceraphis betulae* (silver birch aphid) were found on yellow leaves of the silver birch tree compared with green leaves (Holopainen et al. 2009) and in a separate study fewer *Dysaphis plantaginea* (rosy apple aphid) were found on red apple tree leaves compared to green and yellow leaves (Archetti 2009).

Although an aversion to red is expected, there may be different responses between studies depending on the wavelength composition of the red colour. Discrepancies could arise because what looks consistently red and yellow to human vision is actually different by aphid perception from one pair of leaves to another and so comparisons between studies are not the same 'red' and 'yellow'. The attractiveness of a red versus a green leaf in two types of tree are described by Doring and Chittka (2007) where a surprising preference for red was found. It is suggested that the red leaf of *Euonymus europaeus* is likely to be preferred by aphids to the green leaf as it excites the green receptor (which has a positive input) to a higher level, so what is 'red' to humans is actually 'greener' to aphids (Doring and Chittka 2007). This shows the importance of examining and comparing plant colouration in terms of aphid perception.

Therefore, it is important to discuss the red, green, blue and yellow leaves used in this experiment from the aphid's perspective. Figure 5.8 shows the spectral reflectance and aphid spectral sensitivities of these coloured leaves, and the calculated blue to green ratios of the receptor quantum catches are shown underneath. It is likely that the green to blue ratio has a role in host selection, with blue having a negative input and green a positive one (Hardie 1989, Doring and Chittka 2007). The blue to green ratio appears to explain some of the choices in the experiment but in the opposite direction expected by theory (Doring and Chittka 2007). The yellow leaf (the least preferred colour) has the lowest blue to green ratio and blue and red leaves (the most preferred colours) had the highest blue to green ratios. This can be visualised in the spectral reflectance images; the yellow leaf begins to peak in the green region, whereas the red leaf peaks earlier. It thus appears that *B. brassicae* prefers leaves with a high level of blue receptor stimulation in comparison with the green receptor (bluer leaves from the perspective of *B. brassicae*). This is also supported by data in Chapter Three where it was found that *B .brassicae* was more likely to be found on plants with a higher blue to green ratio.

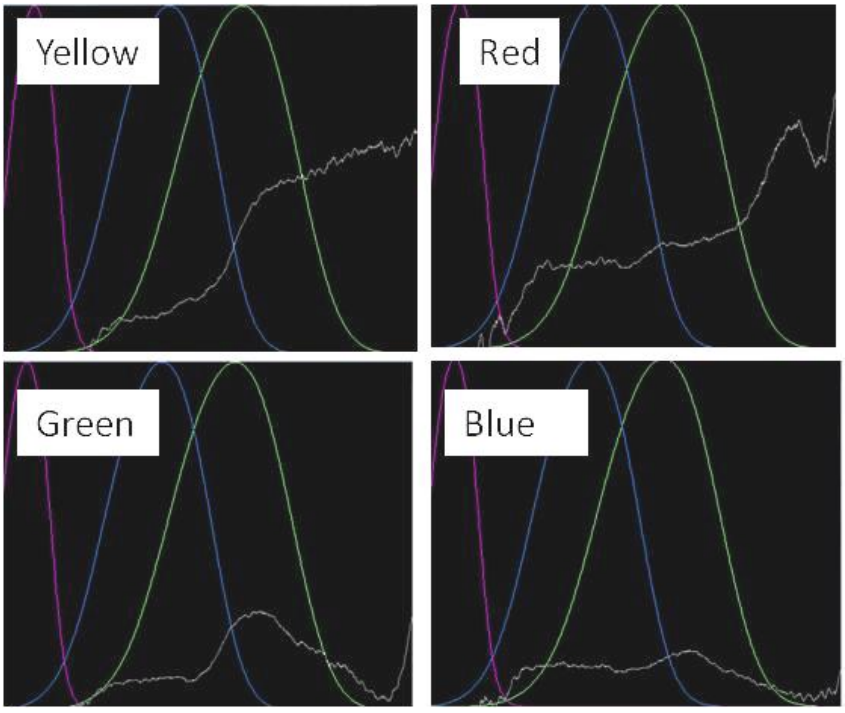

Figure 5.8. Spectral reflectance curves for yellow, red, green and blue leaves using in the chromatic preference experiment. The white line shows the reflectance pattern and the purple, blue and green peaks show the spectral sensitivities of the aphids. Mean blue to green ratios: yellow 0.45, green 0.53, red 0.76, blue 0.84.

It is unknown why *B. brassicae* in this study may have the opposite preference for leaves than expected by theory i.e. a positive rather than negative reaction to blue receptor stimulation (Doring and Chittka 2007). A weaker preference of *B. brassicae* to some colours in comparison with other aphids such as *A. fabae* and *M. persicae* has been found in a previous study (Kennedy et al. 1961), suggesting that it is expected that *B. brassicae* may respond differently to colours. One hypothesis may be that the host plant, *B. oleracea,* is unusual because it is glaucous in colour, meaning it is often bluer than its surrounding plants. Therefore it might be advantageous for *B. brassicae* to respond positively to blueness as it will help distinguish its host from other vegetation.

So why might it be adaptive for *B. brassicae* to prefer red leaves over yellow leaves? A wild *B. oleracea* plant that is completely red or yellow is not found in the field, therefore it is likely that these preferences represent within plant choices. Once a colony is established, they tend to stay on the same leaf (pers. obs.) and so within plant choices are probably very important as well as between plant choices, as there are within plant nutrient and chemical differences (Bentz et al. 1995, Van Dam et al. 1996). Figure 5.9 shows a possible explanation for within plant preferences. A lack of preference for yellow may occur because most dying leaves of *B. oleracea* turn yellow (Figure 5.9a). A dying leaf is a short lived host and would not be able to provide nutrients for a long period. Therefore yellow leaves are best avoided in

favour of any other colour because an alternative would be more permanent. Figure 5.9b shows a *B. oleracea* plant that had undergone drought stress treatment. Many of the leaves had turned red so it is possible that redness could indicate a stressed leaf. A stressed leaf could be a more preferable host because this state involves mobilisation of nutrients and especially more free amino acids (White 1969, 1984, Neuvonen and Lindgren 1987).

Figure 5.9. Within plant colour variation of *B. oleracea*. (a) a yellow dying leaf can be seen, (b) red leaves can be seen. This plant had not been watered for two weeks and so is likely to be suffering from drought stress.

5.4.5. Conclusions

This study provides limited support for the ACH. The first requirement, that plants differ in achromatic cue, was met, but I provide strong evidence that aphids do not discriminate between hosts based on this type of colouration. Only *M. persicae* performance differed between bright and dull plants and so there is unlikely to be selection for *B. brassicae* to respond to achromatic colouration. There were glucosinolate differences between bright and dull plants, notably sinigrin was higher on bright plants, and therefore brightness may act as a signal of these defence levels in agreement with the ACH. It has been discussed, however, that achromatic cues are unlikely to be a reliable informant and therefore it is highly unlikely they have evolved as a signal of chemical defence.

When testing if *B. brassicae* is able to use any visual cues in host choice, a surprising lack of preference for yellow leaves was found and a preference for red leaves. The data can be explained when looking at the colours from the aphid's perspective and this calls for authors to take this into account when planning future experiments. As chromatic rather than achromatic cues appear to be more important in host choice, this aspect of colouration will be explored in detail in the following chapter. The herbivore *Pieris rapae* will be used. This herbivore has six peak spectral sensitivities, rather than only three as in the aphids (Kirchner et al. 2005, Stavenga and Arikawa 2011). This means it has the potential to be more

discriminating between colours than the aphids and therefore may use colour cues to choose between hosts unlike the data presented there. In comparison to aphid vision, *P. rapae* has red receptors which have been suggested for subtle discrimination in oviposition (Kelber 1999) and associations with the red to green ratio and *P. rapae* have been found in Chapter Three suggesting it may be used in host choice. These reasons mean that *P. rapae* is a good candidate to compare colour use in host choice to the aphids in the final experimental chapter.

Chapter Six. Oviposition by *Pieris rapae*: cabbages, chemistry and colour

6.1. Introduction

Oviposition is a very important process for *Pieris rapae* (Renwick and Chew 1994). *Pieris rapae* larvae, especially early instar larvae, are relatively immobile and so adult oviposition on a suitable host plant is fundamental to survival and growth (Thompson and Pellmyr 1991).

The role of olfactory cues in the selection of a host plant for oviposition is well documented, although studies usually focus on discrimination between host and non-host species, rather than choices between suitable hosts (Nottingham et al. 1991). The use of glucosinolates in host acceptance for *P. rapae* is also well documented, with one of these compounds, glucobrassicin, having a notable stimulatory effect (Renwick et al. 1992). Visual cues are also widely used in host selection for oviposition (e.g. Zheng et al. 2010), and are surprisingly well studied, given the neglect of visual cue use by herbivores in other contexts (Reeves 2011). Extensive previous work has investigated the role of learning in oviposition by *P. rapae* using coloured paper. This work has demonstrated preferences for certain colours (yellow, green and blue) over others (white, red, violet and black) and has shown that a learned association with colour can overcome chemical stimuli (Traynier 1984, 1986, Traynier and Truscott 1991). It is unknown, however, whether such colour choice and learning occurs in the field. Snell-Rood and Papaj (2009) have shown host choice using colour can occur: when offered plants in a greenhouse study, *P. rapae* could become as effective at finding a novel host (a red cabbage) as the innately preferred green cabbage within only a few hours of host searching (Snell-Rood and Papaj 2009), thereby demonstrating an ability to use and learn natural intraspecific colour cues.

According to the autumn colouration hypothesis (ACH) (Hamilton and Brown 2001), plants with the most intense colouration should be avoided by herbivores as colouration is a honest signal of the plant's commitment to defence; more intense colouration signals greater defence investment. In this study, the use of dark red colouration (measured as dark red to green photoreceptor quantum catch ratio) and blue colouration (measured as blue to green photoreceptor quantum catch ratio) will be investigated. To test this idea, redder plants (those with a high dark red to green quantum catch ratio, henceforth "high red plants") will be compared to less red plants (lower dark red to green quantum catch ratio, "low red plants"), and bluer plants (high blue to green quantum catch ratio, "high blue plants") will be compared to less blue plants (lower blue to green quantum catch ratio, "low blue plants"). It is predicted from the ACH that plants with the most intense colours (the reddest and the bluest) will be avoided as they will have the highest levels of chemical defence. Chapter Two provided evidence that colours that differ from green are avoided by insect herbivores and this was

supported from data in the field (Chapter Three) where it was found that *P. rapae* was less likely to be found on plants with a high red to green ratio. In experimental conditions, however, it was found that *B. brassicae* was unable to discriminate between plants despite relationships with plant colouration in the field (Chapter Five) and so it remains to be seen if this is also the case for *P. rapae*.

The red to green ratio is of particular interest when looking at oviposition as Lepidoptera, unlike many other insects, possess a red photoreceptor (Briscoe and Chittka 2001), which is thought to have evolved for subtle discrimination between potential oviposition sites (Kelber 1999, Qiu and Arikawa 2003). There is evidence to support this prediction: preference trials by Kelber (1999) using coloured card found the colours avoided had either high blue or high red quantum catches. In this study, the effect of colour preference of *P. rapae* will be investigated further by exploring the consequences of host selection on larval performance. In the field (Chapter Three) it is impossible to separate the effects of plant colour preference and performance: were *P. rapae* less likely to be found on less red plants because the herbivore has a preference for less red plants or because larvae survival is reduced on redder plants? These factors will be separated here in controlled experiments.

It is predicted that female preference will match larval performance, as host preferences that generate the highest fitness of offspring will be selected for (the 'mother knows best' hypothesis) (Valladares and Lawton 1991, Gripenberg et al. 2010). In this study, larval growth rate, pupal weight and time to pupation will be used to estimate offspring performance. Female pupal weight is strongly correlated with fecundity, and is thus a good indicator of potential fitness (Honek 1993; but see Leather 1988). Developmental time is also an important life-history trait: the longer spent as a pre-adult, the less likely the larva is to reach adulthood due to increased mortality factors (Nylin and Gotthard 1998). As a further test of the ACH, the glucosinolate content of the plants will be analysed to determine whether there is a correlation between glucosinolate defence levels and colouration. Such a correlation would imply that colour has the potential to be a cue, or even a signal, conveying information about levels of chemical defences to ovipositing females. The dark red to green ratio was found to have correlations with glucosinolate defence in Chapter Three and so it important that this relationship is further investigated here in more controlled conditions to see if this can explain *P. rapae* preference.

This investigation will be the first to study oviposition preferences using natural plants where (a) visual and olfactory cues are separated; (b) visual cues are modelled using the spectral sensitivities of the herbivore and (c) preferences are followed up by a performance experiment. It is important to use natural plants, for while the use of artificial substrates

makes it easier to separate olfactory and visual stimuli, such substrates provide stimuli at levels not naturally encountered and so may not produce a realistic outcome. It will, however, still be necessary to separate visual and olfactory stimuli in order to determine the type of stimulus on which oviposition choices are based. This issue is frequently neglected in oviposition studies (Stefanescu et al. 2006, Mercader et al. 2007). This study aims to investigate the use of chromatic cues of *Brassica oleracea* by *Pieris rapae* for oviposition by exploring four questions:

1) Are dark red to green ratios used to select betwen hosts?

   Does *P. rapae* use this kind of chromatic cue to choose between potential wild *B. oleracea* hosts? Here, the dark red to green ratio will be used, as this was the red receptor that potentially provides most information about glucosinolate content (see Chapter Three). In the field, a negative association with red colouration and *P .rapae* presence was found, so it is expected the butterfly will prefer to oviposit on plants with the lowest red to green ratios.

2) Are blue to green ratios used to select between hosts?

   Does *P. rapae* use this kind of chromatic cue to choose between potential wild *B. oleracea* hosts? In the field, it was found there were positive associations with plant blueness although this was the opposite of what was expected, as Chapter Two suggested all colours that differed from green would be avoided. It will be interesting to see the effect of the blue to green ratio in controlled conditions.

3) Are there fitness consequences for chromatic cue use?

   Does the performance of offspring vary with plant colouration, and does offspring performance match adult oviposition preferences?

4) Are there glucosinolate differences between *B. oleracea* that differ in chromatic stimuli?

   If levels of glucosinolates varied with plant colouration, colouration could then provide information about chemical defence. To test this idea, glucosinolate concentrations in plants differing in the dark red to green ratio and the blue to green will be compared. In addition, differences in levels of glucosinolate induction (levels measured after larval performance) will be explored to determine whether induced levels of glucosinolates vary with plant colouration.

6.2. Methods

6.2.1. Plants

Three hundred wild *B. oleracea* plants were grown in 6-inch, 1.8L pots in John Innes potting compost no. 2. Pots were spaced in a greenhouse at 15^{o}C in a 16:8 light regime. Plants were grown from a mixture of seeds collected in September 2010 from three sites in Dorset: Old Harry (50^{o}64'N, 1^{o}92'W) Winspit (50^{o}59'N, 2^{o}03'W) and Kimmeridge (50^{o}60'N, 2^{o}13W). The mixture ensured that there was a mixture of genotypes. Plants were watered as required and full strength Hoaglands solution was applied weekly for the first month to provide the developing plants with sufficient nutrients.

6.2.2. Butterfly rearing

The *P. rapae* culture originated from 10 wild-caught individuals (sex unknown). The butterflies were placed in a Perspex cage (50x60x70cm) with a *B. oleracea* plant and oviposition was allowed to take place freely over one week. *Buddleia* flower heads were provided for the adults as a nectar source. Eggs were housed in the same cage, at 18^{o}C and in a 16:8 light regime. Caterpillars were reared on commercial, cropped, savoy cabbage which was kept moist by spraying with water, and the larvae were allowed to pupate on the cage walls. The emerging adults were fed *ad libitum* on sugar solution. Adults had no contact with leaves of any kind, as previous host experience has been shown to be very influential when choosing oviposition sites (Traynier 1984). The adults were allowed to fly freely in the greenhouse (380x310x240cm) to ensure they had enough room for normal courtship behaviour. Forty-nine females were used in the preference test.

6.2.3. Colour measurements

Colour readings were taken three months after germination when plants were mature, as judged by the waxy bloom on their leaves (Bleasdale 1982) (Growth Stage Five since the inner leaves do not form a heart (Andaloro 1983); see Figure 6.1). Colour was measured using a spectrophotometer and the reflectance spectra modelled to *P. rapae* vision (Stavenga and Arikawa 2011) to give an output of intensity of stimulation to each of the six photoreceptors of this butterfly (see Chapter three for details of colour modelling). All plants were measured using the 3rd leaf down from the highest leaf on the plant. Dark red to green quantum catch ratios (dark red receptor excitation divided by green receptor excitation) and green to blue quantum catch ratios (blue receptor excitation divided by green receptor excitation) were calculated. Of the 300 plants grown in the greenhouse, the 30 plants with the highest values of

each of these ratios and the 30 plants with the lowest values were selected for use in the preference and performance trials, such that the reddest plants were tested against the least red, and the bluest against the least blue. Thus, a total of 60 red-group plants (30 low red and 30 high red) and 60 blue-group plants (30 low blue and 30 high blue) were used. In order to maintain consistent differences in ratio values between pairs of plants from the low and high groups in the preference test, plants in each group were ranked according to their ratio values and the plants were then paired by rank (e.g. R1 high red + R1 low red, R2 high red + R2 low red etc.).

6.2.4. Preference test

Preference tests were carried out 21-23rd August 2011. Butterflies were given the choice of two *B. oleracea* plants differing in colour (see above). Plant size was recorded. The plants were placed in the greenhouse with the free flying butterflies. Using free flying butterflies ensured that behaviour was as natural as possible – much more so than within an enclosed choice chamber (R. Foster, *pers.obs*). The greenhouse door was removed and replaced with mesh to ensure the full spectrum of sunlight was present (including UV, which is absorbed by glass). An absence of UV light could potentially have interfered with natural behaviour (Bennett et al. 1994, Church et al. 1998) and studies not considering this have been criticised (Cuthill and Bennett 1993). No artificial illumination was used. The plants were placed at the back of the greenhouse and a fan blowing air over the plants from the front of the greenhouse through a vent behind the plants was used to remove olfactory cues. The plants were initially covered by a sheet, which was then removed at the start of the experiment so that both plants were presented to the butterflies simultaneously (see Figure 6.1). The side of the greenhouse on which the high red/blue plant (left or right) was placed was randomised. A choice was recorded when a butterfly landed on one of the plants. Butterflies had been deprived of contact with host plants until this point (see above) and in the majority of cases butterflies oviposited rapidly after landing on a plant. Butterfly choices were recorded according to their landing behaviour rather than oviposition itself, as the decision to oviposit is likely to be reinforced by contact with the leaf surface, which would involve the use of mechanical and/or olfactory cues in addition to any visual cues. To prevent re-sampling of the same individuals, butterflies were captured after landing on the plants and placed in a cage. This was important as it ensured that all choices were independent and the result of innate preferences, rather than preferences learnt during repeated exposure to hosts. A new pair of plants was used in each trial. Following choice by one butterfly, the plant pair was removed and the next pair placed in position. Thirty butterflies were tested with the 30 red plant pairs. At the end of the

experiment, captured butterflies were released back into the free-flying population. The full free-flying population was then present for the blue plant pairs, included those 30 individuals that chose between the red plant pairs. As with the red pairs, 30 butterflies were tested with the 30 blue plant pairs.

Figure 6.1. Experimental set up for the oviposition preference trial. The photograph shows a plant with low blue to green ratio (right) next to a plant with high blue to green ratio (left). A fan behind blew air over the plants to remove olfactory cues.

6.2.5. Larval performance test

The test of larval performance was carried out from 6th September to 11th October 2011. Two second instar caterpillars were placed on the third leaf down of each of the 60 plants used in the preference experiment and contained on the plants with an insulating garden fleece covering (see Figure 6.2). Caterpillars were weighed after 9, 15, 20 and 24 days to measure growth. The percentage of leaves eaten (estimated by eye) was also recorded. If after 9 days a caterpillar had died it was replaced. This was to ensure that damage levels were even across the plants – chemical induction may have been lower if only one caterpillar was feeding. Because replacement caterpillars were added to the plant on different dates to the original larvae, these individuals were not included in the growth rate calculations. Plants were checked daily for pupae. Any pupae found were weighed and then placed in sample pots. Emerging adult butterflies were sexed by examining wing spots (see Chapter One). Relative growth rates (RGR) were measured at 15 days (before any larvae pupated) and were calculated as mg/mg/ day ([weight at 15 days- weight at 0 days /weight at day 0]/15 days) as in previous studies (e.g. Berdegue et al. 1998).

Figure 6.2. *Brassica oleracea* plants contained in fleece covering for the performance trial.

6.2.6. Chemical analysis

The 4th leaf down from the highest leaf on every plant was excised and snap-frozen in liquid nitrogen before the performance test and stored at -80°C for HPLC analysis. After the performance experiment, 10 leaves from individual plants of each colour group were also snap-frozen to investigate if there were any differences in glucosinolate levels after larval feeding i.e. if there are any differences in glucosinolate induction levels. The glucosinolates were extracted from the frozen leaves using methanol and converted to desuplhoglucosinolates with sulfatase on a sephadex column. Desulphoglucosinolates were separated on a C-18 reverse phase column on HPLC and detected at 229nm. Sinigrin was used an external standard. Details of the procedure for chemical analysis are given in Chapter Three.

6.2.7. Statistical analysis

Data were analysed using R (version 2.10.00) (http://www.r-project.org/). Butterfly preferences were analysed with a binomial test (counts of preferences compared between the colour groups). Plant size differences between selected and non-selected plants were compared with a paired t-test. Larval performance was analysed with linear models with colour as the explanatory variable and days to pupation, weight at pupation, leaf area eaten or relative growth rate as the response variable. Normalisation was carried out by log transformation if necessary. Normality tests were performed using the Anderson-Darling test. Glucosinolate concentrations (at constitutive levels – prior to the performance experiment, and at induced levels – after the performance experiment) in high blue plants were compared

to those in low blue plants, and those in high red plants were compared to those in low red plants using a t-test, or else a Mann Whitney U-test if normalisation of the data through the reciprocal transformation (1/x) was not possible. Benjamini-Hochberg (B-H) corrections were carried out as multiple comparisons were made (Benjamini and Hochberg 1995, Benjamini et al. 2001). To determine whether glucosinolate levels within plants had increased after larval feeding, levels of constitutive and induced glucosinolates were compared with a paired Wilcoxon test. Means are presented +/- SEM.

6.3. Results

6.3.1. Oviposition preference

A butterfly made a choice between every plant pair. The length of time it took for a choice to occur was between two seconds and 45 minutes (median=5 minutes). There was no significant difference in the proportion of butterflies choosing high red and low red plants (n=30, p=0.20, probability=0.63; Fig. 6.3a), or in the proportion choosing high blue and low blue plants (n=30, p=0.86, probability=0.53; Fig. 6.3b). This suggests that butterflies do not choose plants based on their red to green or blue to green ratios. Plant size had no influence on butterfly preference, either for blue plants (t=0.0280, df = 55, p=0.98) or for red plants (t=-1.27, df = 29, p=0.21).

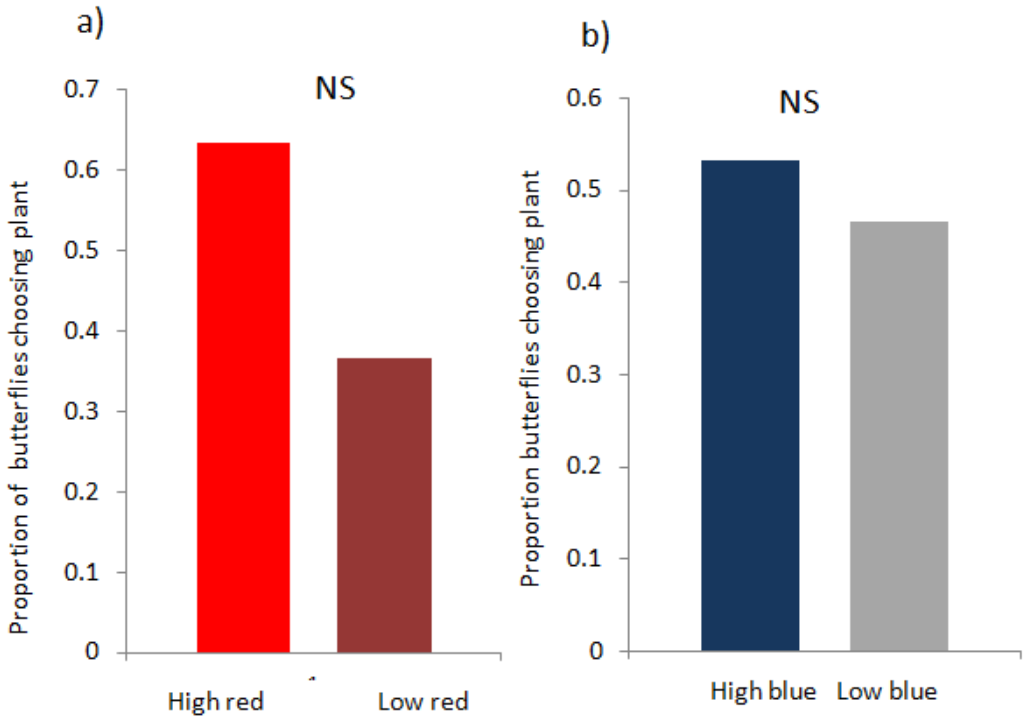

Figure 6.3. Proportion of butterflies choosing plants either with (a) high or low red ratio and (b) high or low blue ratio.

6.3.2. Larval performance

6.3.2.1. Relative growth rate

Pieris rapae larvae gained an average of 11+/-0.55mg/mg/day. There was no significant difference in RGR between high red and low red plants ($F_{1,56}$=0.326, p=0.57; Fig. 6.4a). Caterpillars, however, had a higher RGR on high blue plants than low blue plants ($F_{1,54}$= 5.48, p=0.023; Fig 6.4b).

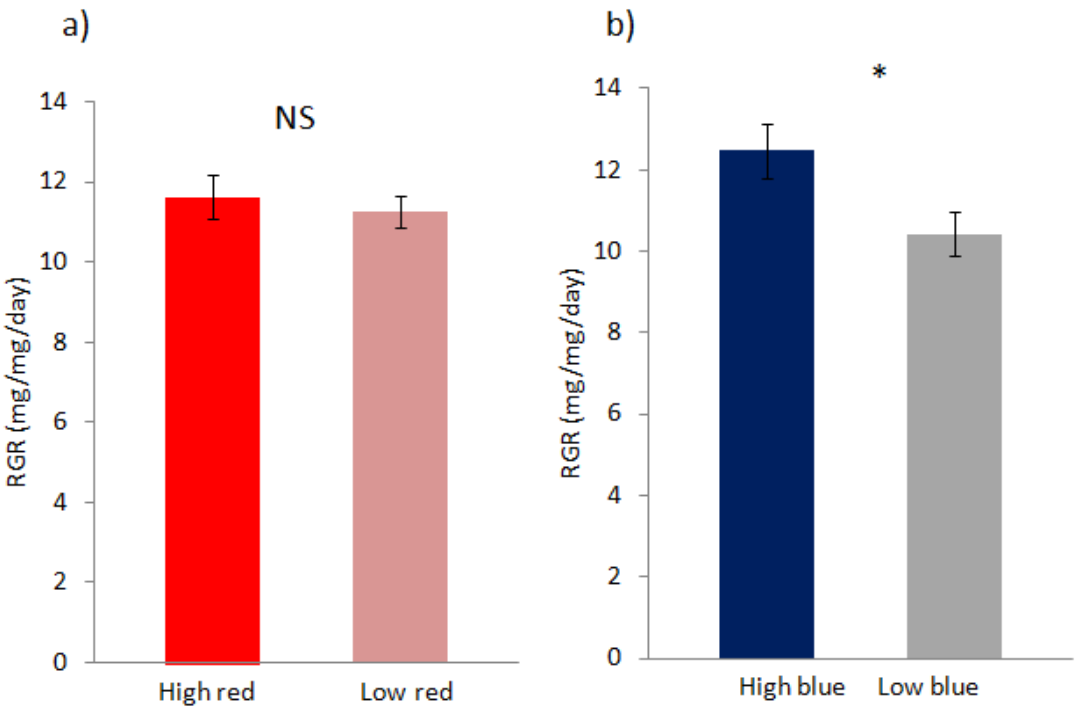

Figure 6.4. Mean relative growth rate (mg/mg/day) of caterpillars on the *B. oleracea* plants. Error bars show SEM. (a) RGR on high red vs. low red plants and (b) RGR on high blue vs. low blue plants.

6.3.2.2. Leaf area eaten

All plants had a large quantity of leaves remaining (30-95%), indicating that caterpillars were not restricted by the amount of food available. Caterpillars consumed the same leaf area from high red plants as from low red plants at all time intervals: 9 days ($F_{1,61}$= 0.433, p=0.51), 15 days ($F_{1,61}$= 0.745, p=0.39), 20 days ($F_{1,61}$= 1.67, p=0.20) and 24 days ($F_{1,61}$=3.59, p=0.063) (see Figure 6.5a). Similarly, mean leaf area eaten did not differ significantly between high and low blue plants at any time points: 9 days ($F_{1,58}$= 0.191, p=0.66), 15 days ($F_{1,58}$= 0.887, p=0.35), 20 days ($F_{1,58}$= 0.831, p= 0.37) and 24 days ($F_{1,58}$= 2.92, p=0.093) (see Figure 6.5b).

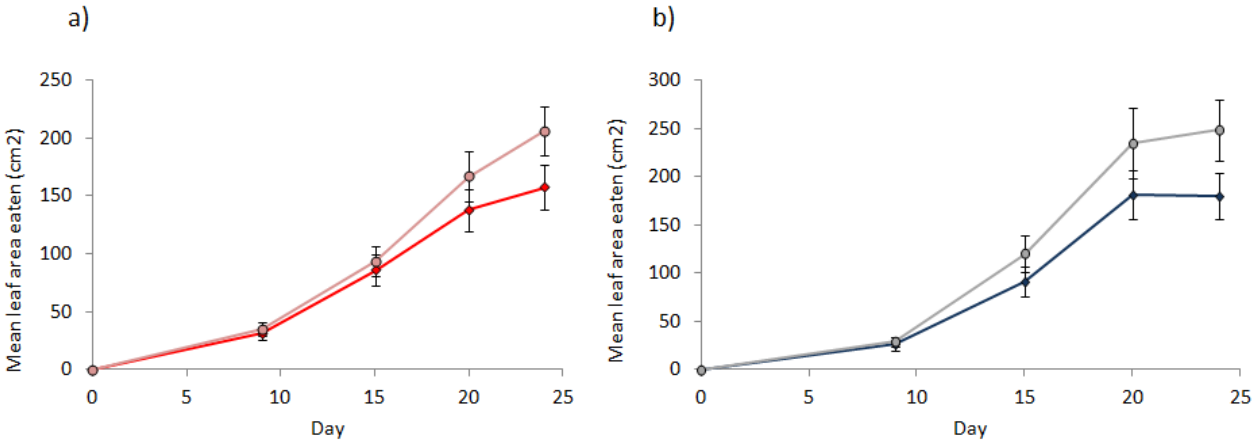

Figure 6.5. Leaf area consumed of *B. oleracea* plants with a (a) with a high dark red to green ratio and a low dark red to green and (b) high blue to green ratio compared with a low blue to green ratio. Error bars show SEM.

6.3.2.3. Time to pupation

Overall, caterpillars took a mean time of 22.5 +/- 0.34 days to pupate. There was, however, no difference in the mean time the caterpillars took to pupate when feeding on high red compared with low red plants ($F_{1,57}$=0.431, p=0.51), nor on high blue compared with low blue plants ($F_{1,56}$=0.960, p=0.33).

6.3.2.4. Weight at pupation

Pupae were significantly heavier on high blue plants compared with low blue plants ($F_{1,56}$=10.1, p=0.0025; Fig. 6.6a) and this difference was more pronounced when considering just the females ($F_{1,38}$=10.6, p= 0.0024; Fig. 6.6b).Pupae reared on high red plants, however, were not significantly heaver than pupae reared on low red plants ($F_{1,57}$=1.53, p=0.22; Fig. 6.6c) nor were any differences in pupal weight observed when considering only females ($F_{1,30}$=0.0327, p=0.86; Fig 6.6d). As female pupal weight generally predicts fecundity (Honek 1993), these results indicate that *P. rapae* developing on more intensely blue plants are likely to be more fecund that those developing on plants with a lower blue to green ratio. The pupae on high blue plants were 13% heavier than those on low blue plants, which translates into a large effect size of 0.49 (Cohen 1988) (small effect size=0.1, medium=0.3, large=0.5; also see Chapter Two).

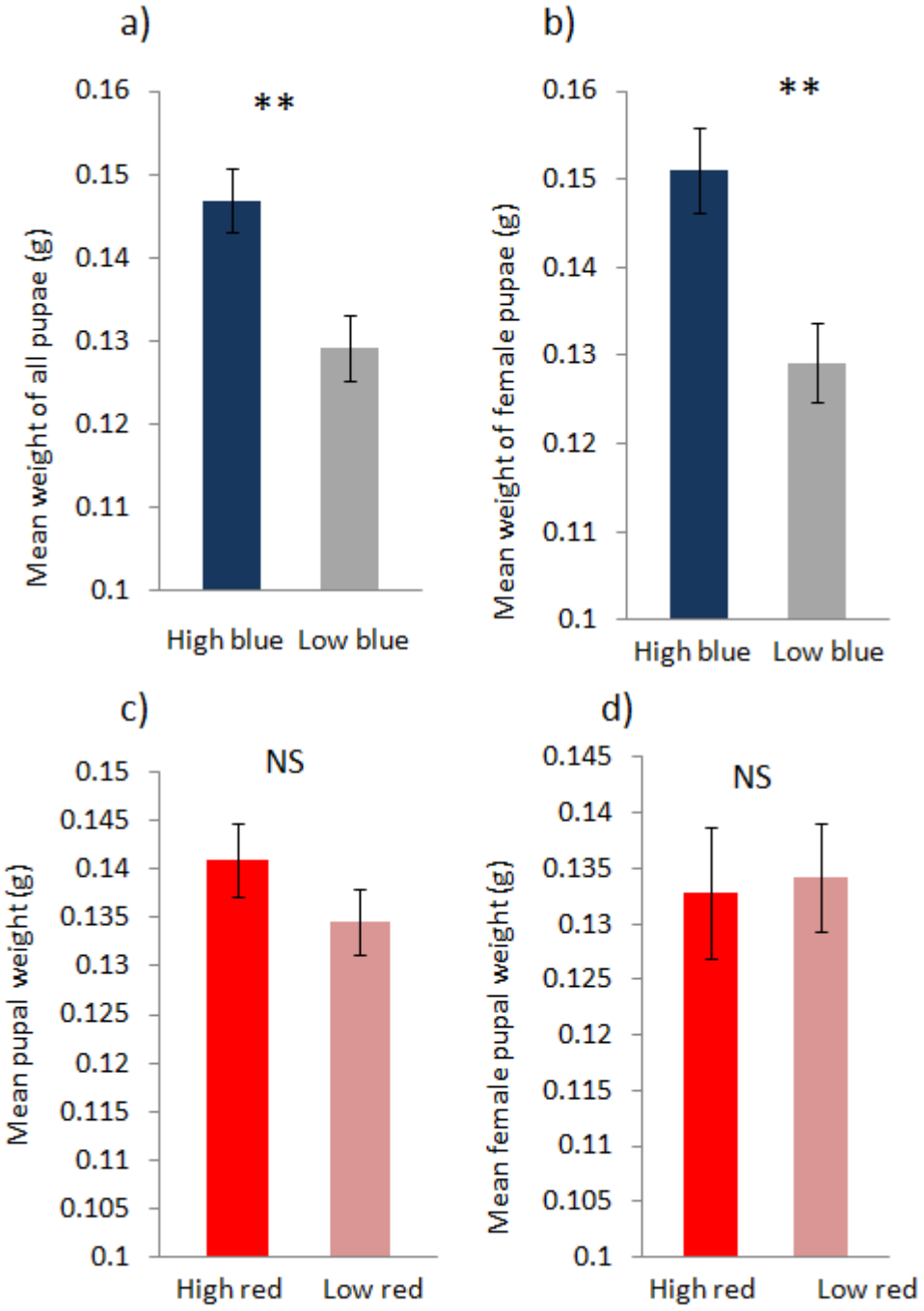

Figure 6.6. Mean weight at pupation of caterpillars reared on high vs. low blue plants (a) all pupae, (b) only female pupae and on high vs. low red plants (c) all pupae, (d) only female pupae. Error bars show SEM. NS= not significant, **<0.01.

6.3.3. Chemical analysis of glucosinolates

A total of eight different glucosinolates were identified. Of the five aliphatic glucosinolates (glucoiberin, progoitrin, glucoraphanin, sinigrin, gluconapin) identified, gluconapin was found at the highest concentrations. Three indole glucosinolates (glucobrassicin, neoglucobrassicin and 4-methoxyglucobrassicin) were identified, of which 4-methyoxyglucobrassicin was present at the highest concentrations.

6.3.3.1. Constitutive glucosinolates (prior to performance experiment)

There was no significant difference in the concentration of seven glucosinolates (glucoiberin, progoitrin, sinigrin, glucoraphainin, glucobrassicin, 4-methyoxyglucobrassicin and neoglucobrassicin) between high blue plants and low blue plants (see Figure 6.7b and Table 6.1). There was no difference in the levels of indole glucosinolates between low blue vs. high blue plants, suggesting that the blue to green ratio is unlikely to convey any information about these chemicals. The concentration of total aliphatic glucosinolates was seven times higher in low blue plants (see Figure 6.8a). More specifically, the concentration of the aliphatic gluconapin was significantly higher in low blue plants.

There was no significant difference in concentrations of any of the individual aliphatic or indole glucosinolates between high red and low red plants (see Figure 6.7d and Table 6.1). There was also no difference when these individual glucosinolates were combined into total aliphatic or total indole glucosinolates (see Figure 6.7c).

Table 6.1. Glucosinolate differences in high vs. low blue plants and high vs. low red plants. df=57 in all cases. W= Mann-Whitney test statistic, t=student's t-test statistic. NS= Not significant using B-H corrections. 4MeOH= 4-methyoxyglucobrassicin, neo=neoglucobrassicin.

Glucosinolate	Blue	Test stat. and p value	Red	Test stat. and p value
Glucoiberin	NS	W=412, p=0.70	NS	W=454, p=0.67
Progoitrin	NS	W=397, p=0.54	NS	W=437, p=0.99
Glucoraphain	NS	W=296, p=0.022	NS	W=394, p=0.47
Gluconapin	Higher in low blue	W=158, p<0.001	NS	t=1.65, p=0.11
Sinigrin	NS	W=356, p=0.23	NS	W=399, p=0.59
Glucobrassicin	NS	W=342, p=0.16	NS	t=2.95, p=0.0052
4MeOH	NS	t=0.938, p=0.35	NS	t=1.79 p=0.08
Neo	NS	W=577, p=0.031	NS	W=532, p=0.14
Total aliphatic	Higher in low blue	W=159, p<0.001	NS	t=2.10, p=0.040
Total indole	NS	t=0.511, p=0.61	NS	t=1.51, p=0.14
Total	Higher in low blue	t=3.61, p=0.0072	NS	t=2.23, p=0.030

From these results, it appears that the blue to green ratio conveys more information about *B. oleracea* chemical defence than the red to green ratio. Aliphatic glucosinolates tended to be different between high and low plants in both red and blue groups. In the case of blue plants, however, the difference between high and low plants was seven-fold, while for red plants this difference was only two-fold and was non-significant following correction for multiple comparisons. In addition, when analysing individual glucosinolates, there were significant differences gluconapin between high and low blue plants, but no difference in red plants. Gluconapin was 25 times higher in plants with low blue to green ratio compared with a high blue to green ratio.

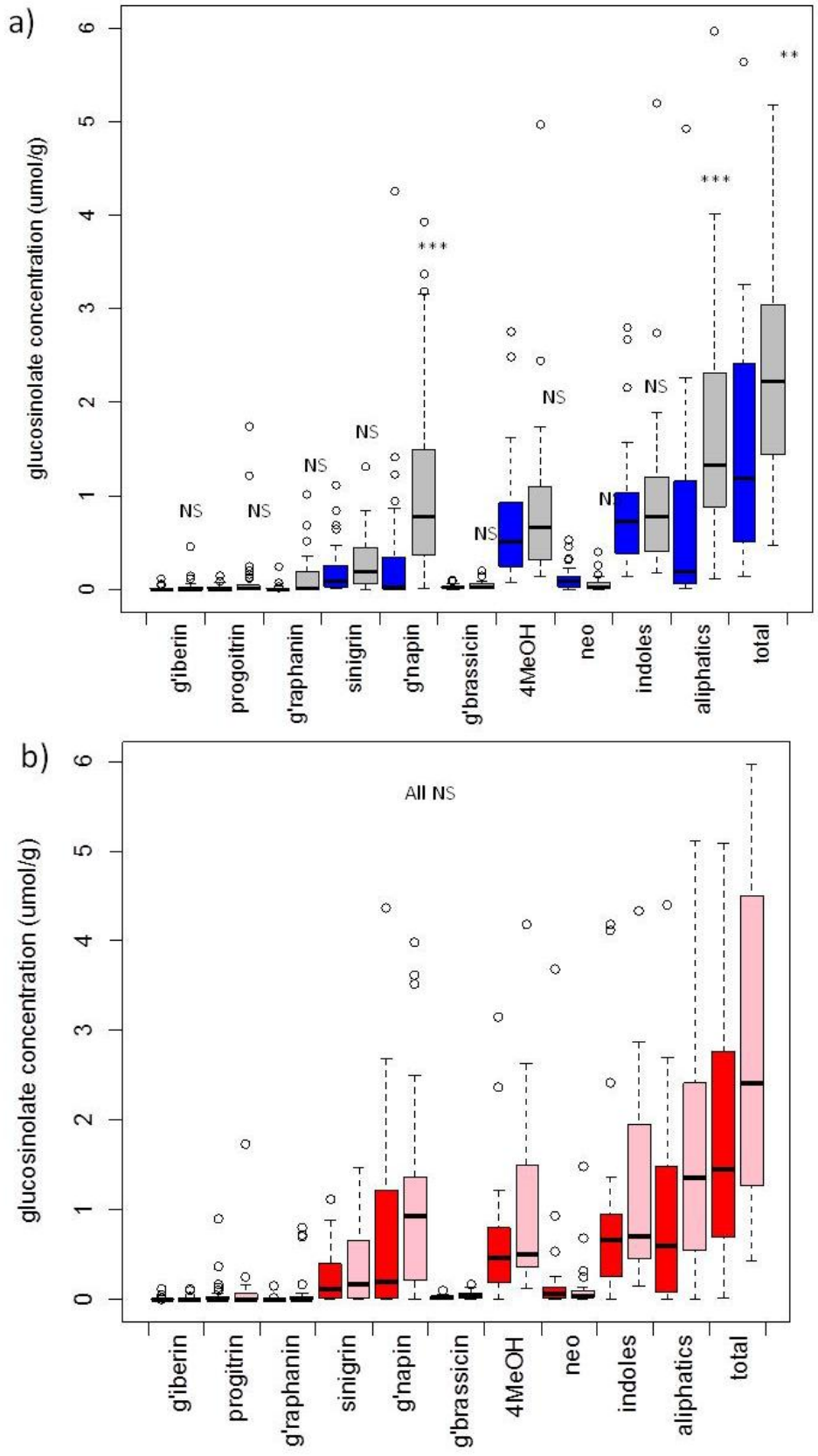

Figure 6.8. Consitiutive glucosinolate concentrations in blue plants (a) and red plants (b). Bright blue bars represent plants with high blue to green ratio and grey bars plants with low blue to green ratios. Bright red bars represent plants with high red to green ratios and pale red bars low red to green ratios. G'iberin= glucoiberin, g'raphanin=glucoraphanin, g'brassicin=glucobrassicin, indoles= total indoles, aliphatics=total aliphatics, 4MeOH= 4-methyoxyglucobrassicin, neo=neoglucobrassicin. NS=not significant, **<0.01, ***<0.001. Box shows interquartile range with the central, heavy line representing the median. The tails from the bar represent approximately 2 standard deviations around the interquartile range, and dots represent outliers from this.

6.3.3.2. Induced glucosinolates

All glucosinolates were found to be induced to higher levels than found at constitutive levels, as reported elsewhere (van Dam et al. 2004). Indole glucosinolates, however, were generally induced to higher levels in comparison to the constitutive levels (aliphatic: V=646, df=156, p<0.001, indole: V=697, df=156, p<0.001; Fig 6.9) than were aliphatic glucosinolates, which again agrees with previous work (Textor and Gershenzon 2006).

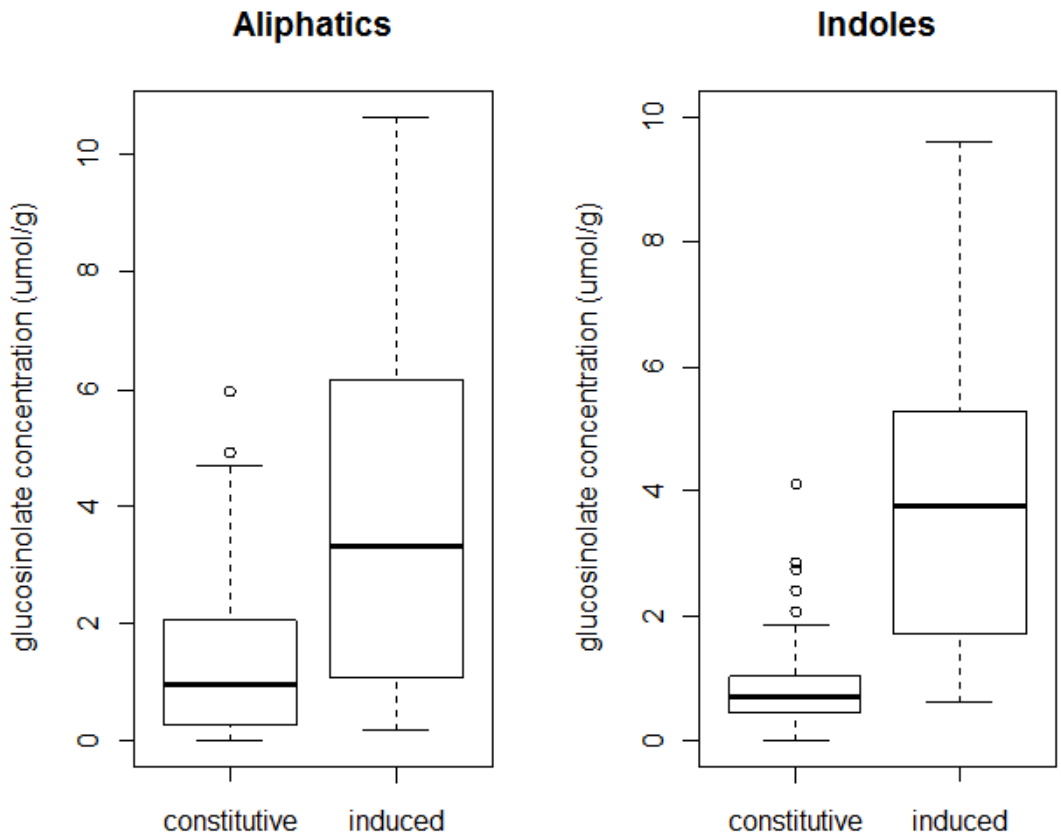

Figure 6.9. Concentration of aliphatic glucosinolates (left) and indole glucosinolates (right) found prior to herbivore feeding (at constitutive levels) and post herbivore feeding (at induced levels). Box shows interquartile range with the central, heavy line representing the median. The tails from the bar represent approximately 2 standard deviations around the interquartile range, and dots represent outliers from this.

There were no significant differences in induced glucosinolate concentrations between low red and high red plants. Neither were there any differences in induced glucosinolate concentrations between high blue and low blue (see Table 6.2). This suggests that glucosinolates are induced to similar levels regardless of plant colour.

Table 6.2. Induced glucosinolate differences in high blue compared to low blue plants and high red compared to low red plants. df=18 in all cases. W= Mann-Whitney test statistic, t= student's t-test statistic. NS= Not significant using B-H corrections. 4MeOH=4-methyoxyglucobrassicin, neo=neoglucobrassicin.

Glucosinolate	Blue	Test stat. and p value	Red	Test stat. and p value
Glucoiberin	NS	t=0.821, p=0.43	NS	W=46.0, p=0.73
Progoitrin	NS	t=1.14, p=0.27	NS	t=0.322, p=0.75
Glucoraphain	NS	t=-2.24, p=0.051	NS	t=-0.173 p=0.86
Gluconapin	NS	t=-1.51, p=0.15	NS	t=0.486, p=0.63
Sinigrin	NS	t=0.00720, p=0.99	NS	t=0.766, p=0.45
Glucobrassicin	NS	t=-0.435, p=0.67	NS	t=0.0310, p=0.98
4MeOH	NS	t=0.174, p=0.86	NS	t=0.731, p=0.72
Neo	NS	t=2.31, p=0.036	NS	t=-0.252, p=0.80
Total aliphatic	NS	t=-1.95, p=0.036	NS	t=0.504, p=0.62
Total indole	NS	t=1.53, p=0.15	NS	t=0.367, p=0.73
Total	NS	t=-0.317, p=0.76	NS	W=36.0, p=0.31

6.4. Discussion

These results are the first from a lepidopteran oviposition choice experiment based on natural colour cues (with olfactory cues eliminated), combined with a test of larval performance on the preferred and non-preferred host. It was found that chromatic colouration (high red vs low red plants and high blue vs low blue plants) did not influence oviposition choices. Levels of glucosinolates, however, differed between high blue and low blue plants, with higher levels of aliphatic glucosinolates being found on less blue plants, suggesting that chromatic cues could convey information about defence levels in accordance with the ACH. In addition, larvae had a higher fitness on high blue plants and this could be due to lower levels of aliphatic glucosinolates.

6.4.1. Does *P. rapae* use blue to green ratios or red to green ratios of colour to select a host?

The finding the butterflies did not use the red to green ratio in their host selection is very surprising. Previous work has suggested that red colouration is the chromatic cue most important in deterring herbivory. The ACH suggests that strong red colouration at autumn time should be avoided by colonising aphids (Hamilton and Brown 2001) and many studies have found that there are fewer herbivores on redder autumn trees in support of this idea (Hagen et al. 2003, Archetti and Leather 2005, Archetti 2009). Red colouration of young, immature leaves, especially in the tropics, is also suggested to deter herbivores (Kursar and Coley 1992), and there has been some experimental evidence to support this (Karageorgou and Manetas 2006; see also Chapter Two).

More specifically, it has been shown that the red to green ratio is commonly used in host selection by ovipositing butterflies, again with red colouration generally avoided. Research by Traynier (1986) has demonstrated that red paper discs are not readily accepted for oviposition by *P. rapae*. Furthermore, when *P. rapae* is given the choice of red, orange yellow, green, blue and violet cards following contact with a cabbage leaf, females do not oviposit on the red card (Traynier 1979). There are few host plants in the field that will produce as high a photoreceptor quantum catch for the red receptor as coloured cards. An aversion for redder plants, however, can still be found in the field. For example, Mercader et al. (2007) found that the swallowtail butterfly (*Papilio glaucus*) avoided redder young leaves in preference for older, greener leaves on the same branch. The red to green ratio can also be used for more subtle discrimination between different greens as the right-hand tail of a green spectrum overlaps with the red region of the spectrum (Kelber 1999).

It is possible that an effect of the red to green ratio on host selection was not found in this study because the colour differences between wild *B. oleracea* were not large enough to allow discrimination. Oviposition trials finding a preference using the red green ratio previously have used artificial substrates which create much larger differences (Traynier 1979). The colour variation available in this study was the largest that could be obtained through greenhouse-grown plants. Given this result, it seems unlikely that the red to green ratio could be used as a cue for chemical defence of wild *B. oleracea* as the variation in defence could not be adequately matched in variation in detectable colour. This seems the most likely explanation for the finding of this study as previous works suggests that butterflies are motivated to select hosts based on red to green ratio, but in this experimental situation they may simply be unable to use it.

Although seemingly unsuitable for discriminating between wild *B. oleracea* hosts, the red to green ratio is clearly used by *P. rapae* (Traynier 1979), and may instead be used to discriminate between hosts and non-hosts which may differ more dramatically in colour (Doring and Skorupski 2007), or between alternative hosts that have larger chromatic differences such as commercial red and green cabbage (Snell-Rood and Papaj 2009).

Brassica oleracea is glaucous, meaning that the plants are often bluish. Lythgoe and Partridge (1989) have shown that optimal discrimination between leaves can occur for insects with one visual pigment sensitive in the blue and one in the green as this is where much colour variation occurs. The blue to green receptor is an obvious choice for discrimination and so it is surprising that *P. rapae* was found to have no preference for plants differing in blueness. It was expected, in agreement with the ACH, that the bluest plants would be avoided (also see Chapter Two). Modelling by Kelber (1999) suggested that the blue receptor would have a negative neural response and the green receptor a positive one in response to coloured substrates for oviposition choices. An oviposition experiment of the common grass yellow butterfly (*Eurema hecabe*) using model plants made from coloured paper “leaves” mounted on a “stem” (Hirota and Kato 2001) showed that the oviposition rate was 2.2 times higher on green compared to blue models. No studies to my knowledge explore blueness using natural plants and so it is unknown whether these model predictions or experiments using artificial substrates are verified in field conditions. The results from this study suggest they are not, and that the blue to green ratio is not used in host choice, at least not with intraspecific choices of wild *B. oleracea* for *P. rapae*.

As with the red receptor, the lack of preference for plants differing in the blue to green ratio could be explained if the variation between plants is too small to be detected. Larvae developing on bluer plants had a higher level of fitness, indicating a selection pressure for

discrimination. This experiment suggests that performance rather than preference effects are more likely to explain the positive association with *P. rapae* and bluer plants in the field in Chapter Three. It is surprising, however, that *P. rapae* did not show a preference for bluer plants given the performance impact. The mismatching between preference and performance will be discussed below.

6.4.2. Are there fitness consequences of chromatic cue use of *B. oleracea*?

Pieris rapae larvae did not perform differently on high red compared to low red plants and this was matched by the absence of a preference of ovipositing adults. Glucosinolate levels did not vary with the red to green ratio. This suggests that even though the red to green ratio is thought to be important for host choice, it has no function in this context. Therefore, this aspect of the experiment does not provide support for the ACH: the red to green ratio is not attended to and in any case provides no useful information about chemical defence.

This contrasts with the data with the blue plants. The relative growth rate of *P. rapae* larvae was 20% higher on bluer plants than less blue plants. The difference in pupal weight between plants varying in the blue to green ratio was also large (effect size r=0.49). Pupae on bluer plants were 13% larger, which, according to Honek (1993), translates into a proportional fecundity increase. The ACH requires that plants vary in colour intensity and that this variation correlates with defensive chemistry, two conditions that are supported here (see below). Moreover, according to the ACH and signalling theory, a herbivore should attend to the colour signal if there are fitness benefits and, again, a clear advantage to choosing bluer plants has been found in this study. Although *P. rapae* larvae were found to perform better on bluer plants, this was not, however, matched by an adult oviposition preference for this plant type.

Throughout the literature as a whole, adult oviposition preferences generally match larval performance (Gripenberg et al. 2010) and so it appears that mother really does know best (Valladares and Lawton 1991). There are many instances, however, as with this study, when no such correspondence is found (Courtney and Kibota 1990). For example, Rausher (1979) found that larvae of three Pierid butterflies had a higher survival rate on hosts in shady locations but oviposition occurred mostly on plants in the sunshine. Within-plant species variation is of more relevance for this study, and mismatches between preference and performance have also been reported in this context. For example, differences in Madrone tree (*Arbutus xalapensis*) quality did not correspond with offspring survival of *Eucheria socialis* (Underwood 1994). This finding could be explained by the suggestion that preference and performance coupling would be tighter between species than within plant species because larvae may not even be able to consume the wrong host, but a recent meta-analysis does not

support this idea (Gripenberg et al. 2010). Thus, intraspecific decisions appear as important as interspecific ones. So what could explain such contradictions in preference and performance?

I found that the performance benefit on bluer plants was not matched with adult preference. There are at least three possible explanations for this: (1) female choice is non-adaptive; (2) female choice is adaptive and there is a delayed negative fitness effect on bluer plants which was not recorded, or (3) female choice is adaptive but there are opposing selecting pressures (i.e. other factors affect the decision).

Though larval fitness differed markedly between bluer and less blue plants, this might not translate into a big enough selection pressure in the wild for oviposition preference to evolve. Given that there is genetic variation in the population for host preference, selection must be strong enough to fix the gene for a bluer plant preference in the population, despite the effects of genetic drift and mutations. A 13% increase in pupal size may not be a large enough effect to meet these requirements. Body size is considered to be a strong predictor of fecundity (Honek 1993), but there may be exceptions to this rule which mean that a large body size does not result in high fitness. A body that is larger will contain more eggs, but insects in the field will very rarely complete their full reproductive potential (Leather 1988). For example, due to the combined effects of delayed mating and adult mortality, the pine beauty moth (*Panolis flammea*) is predicted to lay 47% fewer eggs than it can potentially produce (Leather et al. 1985). In addition, the preference-performance link may be weak because wild *B. oleracea* is an uncommon host in comparison with cultivated *B. oleracea* (Chew 1977), and therefore the selection pressures generated by wild cabbage may be weaker. To test this, differences in preference from butterflies captured where wild *B. oleracea* is abundant would need to be investigated.

It has been suggested that the preference-performance link will be strongest for insects that lay their eggs in batches (Gripenberg et al. 2010). This is because these insects are literally putting all their eggs in one basket, and a mistake in oviposition will result in a serious reproductive loss. *Pieris rapae*, however, lays its eggs singly and so may be opting for a risk-spreading strategy (Hopper 1999). Indeed, *P. rapae* may be spreading the risk in the face of possible delayed negative fitness effects for offspring grown on the bluer plants (Nylin and Gotthard 1998). Though the increase in offspring size associated with development on bluer plants is expected to lead to an increase in fecundity, there may be counteracting selection against large size. For example, smaller individuals may survive better if food is limited (Dingle 1992). In addition, larger individuals may be more susceptible to predation as they are more apparent and offer a better food resource (Nylin and Gotthard 1998). Aside from these costs of large body size, there may be delayed negative effects associated with development on bluer

plants which are not recorded in this study. Therefore, though the butterfly may have a preference for ovipositing on bright blue plants, it may be attempting to manage the risks associated with oviposition on bright blue plants by laying some more eggs on other plants, thereby masking the preference.

In the field there may be opposing selection pressures against bluer plant preference. One of these could be mortality caused by predation (Thompson 1988). Figure 6.10 illustrates how plant colour could influence this. In Figure 6.10a the larva is on a plant with a high blue to green ratio and is highly conspicuous compared with the larva in Figure 6.10b, which more closely mimics the leaf with a low blue to green ratio. It is therefore predicted that vertebrate predators would find *P. rapae* prey more easily on a plant with a high blue to green ratio so by choosing the less blue plant *P. rapae* is more likely to achieve enemy free space (Thompson 1988). As this contrasts the predicted preference of bluer plants due to pupal size, it may oppose the selection pressure for bluer plants, thus giving an overall null preference. Bluer plants offer increased size and fecundity but may also increase the risk of predation.

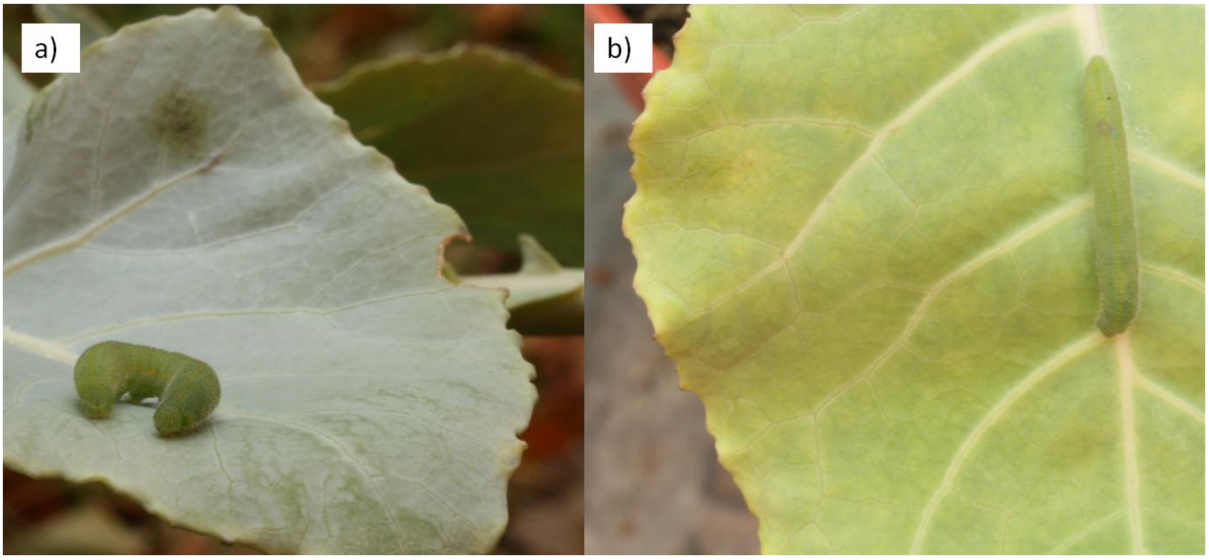

Figure 6.10. The conspicuousness of *P. rapae* larvae on a wild *B. oleracea* plant with a a) high blue to green ratio and a b) low blue to green ratio.

6.4.3. Are there glucosinolate differences between plants that differ in chromatic cues?

As there are differences in glucosinolate concentrations between plants varying in blueness, this colour could provide a potential cue, or even a signal, of levels of defensive compounds in hosts in accordance with the ACH. It has been suggested by Cooney et al. (2012) that redness of margins of the New Zealand pepper tree (*Pseudowintera colorata*) could be a signal to potential herbivores of polygodial content, the primary defense compound in leaves. Green margined leaves suffered greater herbivory than red margined leaves (Cooney et al. 2012).

However, it is premature of the authors to call this colouration a signal without an insect performance experiment to demonstrate that the larvae are affected by the defence chemical.

Other studies have demonstrated a link between leaf colour, levels of defence compounds and herbivore performance (Malone et al. 2009) suggesting colour can be a reliable signal of defence levels. Tobacco plants (*Nicotiana tabacum*) were genetically engineered to express a transcription factor that produced red leaves due to elevated levels of anthocyanins (Malone et al. 2009). Consumption of these red leaved plants caused a delay in pupation time for *Spodoptera litura* compared with green leaved plants. It was also found that these red leaves had increased levels of defensive chemicals such as caffeic acid. Together, these results suggest that the leaf redness could provide a cue about levels of defensive chemicals (Malone et al. 2009). Similarly, a study by Irwin et al. (2003) has provided evidence for a link between colouration and glucosinolates. Plants of wild radish (*Raphanus sativus*) that produced flowers lacking anthocyanins produced lower levels of glucosinolates and were preferred by herbivores, even though feeding did not occur on the flowers (Irwin et al. 2003). It is suggested that there is a pleiotropic effect of genes influencing both defensive and pigmented chemicals (Irwin et al. 2003). Further evidence is provided by Hemm et al. (2003) who produced *Arabidopsis* mutants with low levels of anthocyanins. These mutants also had lower levels of aliphatic glucosinolates, leading the authors to propose a metabolic link due to an aldoxime-oxidising enzyme between glucosinolate biosynthesis and anthocyanins, thus providing a biochemical link between colouration and glucosinolates (Hemm et al. 2003).

Glucosinolate concentrations could be used as a possible explanation for differences in *P. rapae* fitness between plants with a high blue to green and a low blue to green ratio. Aliphatic glucosinolates (and gluconapin in particular), which were found at lower concentration on bluer plants, may have had a negative impact on larval growth. *P. rapae* detoxifies glucosinolates using a nitrile-specifier protein, which diverts the hydrolysis of glucosinolates from the high toxic isothiocyanates into nitriles (Wittstock et al. 2004). The production of this protein is costly and thus will presumably divert resources from other processes essential for growth (Wittstock et al. 2004). Agrawal and Kurashige (2003) provide evidence that *P. rapae* is affected by glucosinolates. *Pieris rapae* larvae were fed a diet of synthetic allyl isothiocynate (the breakdown product from sinigrin) and it was found that the dose negatively affected larval growth rate (Agrawal and Kurashige 2003). Studies using natural glucosinolates have agreed with this effect. Plants with induced levels of chemical defence are often less prefered by ovipositing females. For example, it was found that fewer eggs were laid by *P. rapae* on induced *B. oleracea,* and the time to pupation was longer on these plants (Bruinsma et al. 2007).

Other studies, however, have found no effect of glucosinolates on *P. rapae* performance, and have suggested that this is a consequence of the butterfly's high level of specialisation on brassicas. Often these studies only look at a single glucosinolate whereas it is important to look at the whole profile of glucosinolates. For example, Blau et al. (1978) found that larval growth rate was unaffected by sinigrin but did not examine any other glucosinolates. Slansky and Feeny (1977) showed that larval growth was unaffected by glucosinolates by feeding *P. rapae* different lines of brassica which varied in glucosinolate content. The authors, however, did not measure glucosinolate concentration directly. There is great diversity in the effects of glucosinolates on *P. rapae* depending on factors such as plant type and experimental protocol, which makes identifying particular patterns of herbivore responses to host defences problematic.

6.4.5. Summary

It was found that the dark red to green photoreceptor quantum catch ratio and the blue to green photoreceptor quantum catch ratio of *B. oleracea* spectral reflectance were not used by *P. rapae* in oviposition choices. Despite patterns with the dark red to green ratio and *P. rapae* presence in the field, this experiment suggests that the red to green ratio may be unimportant in host selection and does not function as a host cue because there were no significant performance impacts of larval feeding on plants differing in dark red to green ratio. The blue to green ratio, however, could provide important information about the host because it is related to both larval performance and glucosinolate levels, so it is unclear why the females do not discriminate between hosts based on this ratio. This study is an important advance on previous work looking at visual cues in oviposition because it used natural stimuli with olfactory cues eliminated, and plants which differed according to butterfly vision not human. This study also demonstrates the importance of carrying out performance experiments as it is important to see if any chemical differences actually impact upon herbivore fitness.

Chapter Seven. Discussion.

7.1.Introduction

Previous research has shown that insect herbivores use colour in host choice (Traynier 1986, Karageorgou and Manetas 2006). Hamilton and Brown's (2001) autumn colouration hypothesis (ACH) suggested that plant colouration might function as a signal to indicate plant defence. Empirical tests of the ACH face a number of challenges, and to date convincing evidence is lacking. These challenges include: modelling colouration from the perspective of the herbivore; determining the relationship between colour and chemical defence, testing herbivore responses to colour while simultaneously eliminating other sensory cues (e.g. olfactory cues), and performing tests of both herbivore preference and performance. The aim of this thesis was to examine whether insect herbivores could use colour cues to select a host, and to investigate whether colouration could signal levels of chemical defence within the plant, while meeting the above requirements. The key findings of this thesis were:

- Throughout the literature as a whole, herbivores tend to avoid host plants that differ from green colouration.
- In the field, insect herbivory of *B. oleracea* varies with the plant's colouration and generally follows the pattern suggested in the literature. In turn, glucosinolate concentration correlated with the intensity of plant colouration.
- In a common garden, *B. oleracea* colouration was found to have a significant fixed genetic component and this predicted the presence of some insect herbivores. This suggests selection arising from herbivory pressures could act on plant colouration.
- Some glucosinolates differed between bright and dull plants in a greenhouse study. Neither the cabbage aphid, *B. brassicae*, nor the peach-potato aphid, *M. persicae,* however, chose host plants based on brightness. It is therefore unlikely that brightness functions as a signal of chemical defence.
- *Pieris rapae* did not use chromatic information to choose between *B. oleracea* hosts in an experimental situation. Chemical differences between hosts differing in chromatic colouration translated into performance effects of *P. rapae,* and so it is unclear why the butterfly did not discriminate between differently coloured plants.

These key findings were discussed in detail in the relevant chapters. This chapter considers the overall support for the ACH and raises some broader questions relating to the use of colour in host choice. The contribution of this thesis to our understanding of the use of colour cues by

insects, and the possibility that plants visually signal to avoid herbivory will be discussed. Finally, this chapter presents ideas for future work on this topic.

7.2. Could intraspecific colour variation be a signal of glucosinolate levels to insect herbivores?

For the ACH to hold, it is necessary to demonstrate three things. First, there must be a relationship between plant colouration and herbivory. Second, variation in colouration must predict herbivore performance (i.e. fitness). Third, colouration must predict levels of chemical defence. In the *B. oleracea* system, each of these requirements is met: plant colouration predicts herbivory; colouration also predicts offspring growth and survival; and finally aspects of colouration predict levels of defensive glucosinolate compounds. Given these findings, the ACH would thus predict that herbivores should use colouration when choosing hosts upon which to feed or oviposit. However, behavioural tests on three of the herbivores of *B. oleracea* did not bear out this prediction. Thus the main premise of the ACH – that leaf colour evolved as signals for herbivores – remains to be substantiated. Reasons for this finding will now be discussed.

The meta-analysis, field work and common garden experiment suggested a relationship between herbivory and plant colouration; generally bluer and brighter plants are preferred but redder plants are avoided. In addition, under controlled conditions, *M. persicae* performed better on dull plants and *P. rapae* performed better on plants with a high blue to green ratio. The fitness consequences associated with host selection, in terms of offspring growth and survival should favour host discrimination (Valladares and Lawton 1991), but in preference experiments, *P. rapae, B. brassicae* and *M. persicae* did not discriminate between plants by colour. This negative result is hard to explain. One possibility is that colouration in the field and common garden was more variable than in the greenhouse plants used for behavioural experiments. In the field, many factors such as stress and nutrient levels may additionally influence the variation in plant colouration (Costa-Arbulu et al. 2001, Sinkkonen 2008), whereas in a controlled greenhouse setting these sources of varation are minimised. Thus, it is possible the colour differences among greenhouse plants were not detectable or behaviourally relevant. Overall, in the *B. oleracea* system, insect herbivores do not respond to variation in host colouration, as predicted by the ACH.

A limited set of three colours were tested for their possible use in host choice: brightness for *B. brassicae* and the blue to green ratio and dark red to green ratio for *P. rapae*. The use of intraspecific colour variation in *B. oleracea* by these herbivores cannot be ruled out

until all possible aspects of colouration are investigated. The results of the meta-analysis in Chapter Two indicate that many different types of colouration may be linked with herbivory. Though care was taken to make sure UV light was available in the herbivore preference tests so that normal host-seeking behaviour was not adversely affected (Bennett et al. 1994), responses to UV were not tested experimentally in this thesis. Previous research has indicated that, while aphids may use UV to discriminate between the skyline and vegetation, only long wavelengths are used in host choice (Dixon 1973). The use of UV by lepidopterans has focused on mate discrimination (Stavenga et al. 2004, Arikawa et al. 2005, Giraldo and Stavenga 2007, Bybee et al. 2012) and so it is thought that this type of receptor evolved for this purpose rather than for the selection of oviposition sites. For example, male *P. rapae crucivora* wings have beads which absorb UV light and produce strong colour contrasts that are thought to be important in sexual selection (Stavenga et al. 2004). The possibility that UV is also used in host selection, however, cannot be excluded until experimentally tested.

The correlations between colouration and glucosinolates found in this thesis are shown in Table 7.1:

Table 7.1. Relationships between colouration and glucosinolates throughout the thesis.

	Colouration	Correlating glucosinolates
Chapter 3	Brightness	Total indole glucosinolates
	Redness	Gluconapin, glucoraphanin, neoglucobrassicin
	Blueness	None
Chapter 5	Brightness	Sinigrin, glucobrassicin
Chapter 6	Redness	None
	Blueness	Glucobrassicin, total aliphatic

As shown in Table 7.1, the relationship between colouration and glucosinolate concentration was variable across the different studies included in this thesis. Importantly, there does not appear to be a strong or consistent relationship between colouration and levels of the eight glucosinolates measured, suggesting that colour is perhaps unlikely to provide reliable information about chemical defences to insect herbivores. Many factors could have influenced the varying pattern of glucosinolates and colour in the field and in the greenhouse. It is possible that the effect of chemical induction influenced the relationship with colouration and glucosinolate concentration in the field survey in Chapter Three. Herbivore feeding may have induced levels of glucosinolates in some plants (Agrawal 1999) meaning that correlations with colour were confounded. Herbivory in previous seasons may have also affected levels of glucosinolates (Poelman et al. 2008a). Plants in the field varied in age, but in the greenhouse studies they were all three months old, and previous research has indicated that levels of

glucosinolates vary with the age of the plant (Wentzell and Kliebenstein 2008). Seasonal effects may also have influenced levels of glucosinolates since the levels in the field were measured in June, and levels in the greenhouse were measured in May and August the relationships (Gols et al. 2007). Despite these confounding effects, it is still expected that correlations with colour intensity and defensive chemicals would be consistent if colour was a signal of chemical defence, as predicted by the ACH.

A link with chemistry and colouration may be more likely in a system where underlying correlations are stronger and/or more consistent. Within *B. oleracea*, there are many aspects of colouration that could potentially function as cues or signals to herbivores, as well as a large number of different glucosinolates whose levels could potentially be communicated to herbivores. The complexity of the defence system may confound a simple association between defence and colour. A consistent link between defence levels and colouration was found in *Pseudowintera colorata* (the New Zealand pepper tree) by Cooney et al. (2012). A single chemical, polygodial, was found to vary with a particular colour phenotype, namely the redness of leaf margins. Such situations are probably unusual, given that plant defences normally comprise a wide diversity of defensive chemicals (Berenbaum and Zangerl 1998); for example, there are over 360 types of pyrrolizidine alkaloid in the Senecioneae (Hartmann and Dierich 1998). If the ACH is only likely to operate in simple systems consisting of a single main defensive chemical and a single colour phenotype, it is unlikely that this signalling hypothesis applies to many plant-herbivore systems.

Recent evidence suggests that the chemodiversity of plants has arisen in part because of the tolerance of secondary metabolite synthesizing enzymes to any mutations that do not affect essential, primary metabolism, which has allowed mutations to accumulate (Weng et al. 2012). This process is thought to have generated a great diversity of these enzymes, and therefore also a great diversity of defensive products. This follows from an early hypothesis of Jones and Firn (1991) arguing that plants contain a high diversity of secondary plant metabolite, but most are inactive. A high diversity of plant defence is advantageous because the chance of possessing active compounds is higher (Jones and Firn 1991). The plant is in effect bet-hedging and providing an array of metabolites with which to overcome herbivore detoxification (Hartley and Jones 1986). In light of this research on diversity of defence, it is unsurprising that a consistent link with *B. oleracea* glucosinolate defence and colour was not found: there may be many unknown chemicals that affect herbivory (Poelman et al. 2008b) and the large diversity may elude links with plant colouration.

In the study by Cooney et al. (2012) on New Zealand pepper trees (see above), the plants varied in the intensity of redness of leaf margins. These leaf margins arguably provide a more discernible contrast than the graded differences in colour ratios found in *B. oleracea* (see Figure 7.1). In general, the evolution of plant-herbivore communication systems based on leaf colouration may be restricted to those host species with high-contrast colour patterns. Other studies exploring the relationship between intraspecific host colouration and herbivory have found support for this idea. For example, Wong and Srivastava (2010) found that larger red spots on green leaves of *Columnea consanguinea* were associated with reduced herbivory, while variegated leaves of *Caladium steudnerifolium* have been shown to suffer lower levels of herbivore damage compared to non-variegated leaves (Soltau et al. 2009). In such cases, the colour patterns are characterised by high contrast elements, rather than the more subtly graded differences, as seen in wild *B. oleracea*. Efforts to extend the ACH to examples of non-transient host colouration may therefore succeed only in the relatively small number of cases where the contrast between leaf colours is high. For those species with more subtle gradations in leaf colouration, strategic signalling of chemical defence to herbivores may be unlikely to account for the variation observed in leaf colouration within host populations.

Figure 7.1. Colour contrasts in *P. colorata* compared with *B. oleracea*. From top left, going clockwise, a red margined *P. colorata*, a small red margined *P. colorata*, a low blue to green ratio *B. oleracea*, and a high blue to green ratio *B. oleracea*. *P. colorata* pictures taken from Cooney et al. (2012).

7.3. Could plant colouration be used in any aspect of host choice?

The data presented in this thesis do not support the idea that plant colouration is used as a signal of chemical defence, as suggested by the ACH. It is highly likely, however, that plant colouration is used in other aspects of host choice. For instance, it is possible that the use of plant colouration is more important in aiding herbivores to make within-plant feeding choices. In *B. oleracea*, when leaves are senescing, chlorophyll is reabsorbed and the leaves turn yellow (or red if conditions are more stressful) (see Figure 7.2).

Figure 7.2. Within-plant colour variation in *B.oleracea*. A red, stressed leaf (left) and a yellow, senescing leaf (right).

The ACH argues that variation in colour between plants provides information about levels of defensive commitment. In examining variation in colouration between plants, within-plant variation in colouration was minimised, for example, by removing senescing leaves. However, within-plant choices are known to be important for insect herbivores (Bentz et al. 1995). New leaves are often more nitrogen rich because of their high growth rate (Mattson 1980). Chemical differences within plants can also vary significantly and these can have impacts on herbivory (Lambdon et al. 2003). New leaves are often found to be most highly defended, while older leaves are generally less well-defended , in agreement with the optimal defence hypothesis (see Chapter One for a discussion of this) (McCall and Fordyce 2010). This can have consequences for herbivores, and it has been found that older leaves in the field suffer more damage (Van Dam et al. 1995). Consequently, colouration differences within plants could potentially indicate factors important to herbivore fitness and therefore may act as a cue for herbivores. In this thesis, *B. brassicae* was found to have a preference for red leaves and an aversion to yellow leaves, suggesting that variation for this colour is sufficient for within-host preferences to occur (see Chapter Five). In this context, however, colouration is

likely to function as a cue rather than a signal (as in the ACH), as there is no clear benefit to the plant for communicating the suitability of different leaves to herbivores. A benefit to both the receiver and signaller is required for signal evolution (Maynard Smith and Harper 2003).

Plant colouration is variable between species, and so interspecific host choice may involve the use of visual cues. Colour differences between hosts and non-hosts of the Colorado potato beetle have been compared and found to overlap to a large extent, leading researchers to conclude that discriminaton of host versus non-host based on colour is unlikely (Doring and Skorupski 2007). Gish and Inbar (2006) found that *Macroisponiella artemisiae* aphids are capable of visually discriminating between host and non-host plants, and use this ability to return to their host when they drop from plants to escape predators. These examples suggest plant colouration may have a role in discriminating between species, likely in combination with other sensory inputs such as olfaction and mechanoreception (Thompson and Pellmyr 1991, Powell et al. 2006), and that the relative importance of these cues will differ for different species (Vaishampayan et al. 1975, Patt and Setamou 2007, Kuhnle and Muller 2011).

7.4. What could explain differences in aphid and butterfly preference and performance?

Both *B. brassicae* and *P. rapae* host choices and performance were investigated in this thesis. Despite the original focus of the ACH on aphids, it is expected that *P. rapae* would be the best candidate for testing host choice using plant colouration because this herbivore has more peak spectral sensitivities and therefore has the potential for finer discrimination between plants. Differences in natural history and physiology (see Table 7.2) also indicate *P. rapae* may be a better candidate to study host choice using visual cues. Most significantly, *P. rapae* has the ability and opportunity to visit many hosts because it is a strong flier. Both herbivores are attacked by predators and parasitoids, which indicates that host choice may take into account factors other than host nutrition, for example enemy free space (Loader and Damman 1991), which may explain in some instances why performance did not match herbivore preference.

Table 7.2. Differences in physiology and natural history of *P. rapae* and *B. brassicae* relevant to host choice.

	P. rapae	*B. brassicae*	Consequence
Mobility as an adult	Strong flier.	Relies mainly on the wind.	*P. rapae* may have more opportunity to visit more hosts.
Mobility as larvae/apterate	Limited, especially as very young larvae.	Limited, can walk onto nearby plants.	Choice of host by mother is important for both herbivores.
Time and number of dispersal	Adults found spring-early autumn. 2-3 generations a year.	Spring. One main migration only. Some migration in response to overcrowding etc.	More dispersal events for *P. rapae* – more opportunity for natural selection to work on host choice.
Density	Mostly low.	Can reach very high densities.	Relocation may be necessary for *B. brassicae* if overcrowding occurs; enough food for *P. rapae* larvae.
Damage to plant	Consume large areas of leaf.	Extract nutrients, cause leaf curling, damage stems.	More chemical induction may occur in response to *P. rapae* feeding; fitness costs to plant of herbivore damage unknown.
Glucosinolate detoxification	Detoxification – diversion of isothiocyanates.	Sequesters.	Different glucosinolates will be effective to each herbivore.
Natural enemies	Parasitoid wasps including *Cotesia rubecula.* Vertebrate predators including birds, rodents etc.	Parasitoid wasps including *Diaeretiella rapae*. Invertebrate predators including hoverfly larvae, lacewing larvae, ladybirds.	Enemy escape mechanisms required for both herbivores e.g. the trade-off between enemy-free host but poor quality host. Both have defence mechanisms, *P. rapae* is camouflaged and *B. brassicae* sequesters glucosinolates.

7.5. The problems with the ACH

Considerable confusion and uncertainty surrounds the study of plant colouration in host selection by herbivores and this can in no small part be traced back to the original formulation of the ACH, as presented by Hamilton and Brown (2001). Several criticisms may justly be levelled at Hamilton and Brown's (2001) paper.

First, vagueness surrounds the precise aspect of leaf colouration that is argued to convey information on defensive commitment to herbivores, which has led to subsequent studies testing the hypothesis using a range of different measures of colour (e.g. human red to green ratio, Ramirez, Lavandero et al. 2008; percentage of coloured leaves, Hagen, Folstad et al. 2003; pale, medium or strong redness, Roshausen and Schaefer 2007).

Second, though Hamilton and Brown specifically invoke the ACH as an explanation for intraspecific variation in colouration within plant species, the authors nevertheless present evidence for visual signals of defensive commitment to herbivores based on interspecific differences in colouration and herbivory, a decision that has led to considerable confusion in subsequent studies seeking to test the ACH (e.g. Numata et al. 2004).

Third, the focus of Hamilton and Brown's original paper on the role of aphids in particular as the main herbivore group selecting for and responding to host colour signals is problematic since aphid vision is not optimised for leaf discrimination (Osorio and Vorobyev 2005). A red receptor is required for subtle discrimination between leaves (or trees) (Kelber 1999) and has evolved in herbivorous species that require such discrimination to locate food sources and oviposition sites (Stavenga and Arikawa 2006). For example, adult sawflies (Symphyta) are highly unusual among the Hymenoptera both for feeding on leaf material and for possessing a red receptor, which is argued to assist in discriminating among leaves (Peitsch et al. 1992). Red reflecting pigments are the most variable colour-selective reflectors in leaves, so it follows that one cone type should be optimally sensitive in this region for high discrimination (Lythgoe 1979). If searching for herbivores that are most likely to discriminate between leaves, the focus should be on herbivores with a red receptor as these are most likely to be able to discriminate between leaf colours.

Fourth, a problem becoming increasingly apparent is that that the timing of aphid arrival at their hosts in autumn does not always coincide with the expression of autumn colouration (Sinkkonen et al. 2012, but see Archetti and Leather 2005). Indeed, as Dixon (1973) notes, phloem sap has high nitrogen content when "leaves are approaching senescence" which suggests that nitrogen levels are likely to be highest before peak leaf colours change, meaning that aphids attracted to the nitrogen-rich leaves will arrive too early to be guided by autumn colours when selecting their host. Bad weather in autumn may result in leaves being blown and washed from trees before aphids migrate. For a costly signal of plant defence to have evolved, aphid arrival must coincide with peak autumn colouration consistently over years. A simple observational experiment that looks to see if aphid migration to over-wintering tree hosts coincides with peak autumn colouration over a number of years, would help to resolve this point of contention. The Rothamsted Insect Survey monitors abundance of aphids and these estimates could be correlated with measures of peak autumn colouration. Both sycamore and beech trees can undergo dramatic colour change in autumn and so the sycamore aphid (*Drepanosiphum platanoidis*) and the beech woolly aphid (*Phyllaphis fagi*) which both migrate in autumn time may be a good study species for this.

While Hamilton and Brown provided a conceptual framework in which to understand autumn colouration as a strategic signal of defensive commitment, neither their study nor the earlier study by Archetti (2000) considered explicitly how colouration and chemical defence could come to be linked (Hemm et al. 2003) or what types of chemical defence are likely to be important (Poelman et al. 2008b). A further assumption of the ACH is that the production of colouration carries a cost that acts to ensure the honesty of the signal (see Chapter One) yet such costs remain to be demonstrated. In spite of the shortcomings of the original paper, however, the autumn colouration hypothesis of Hamilton and Brown is both fascinating and important for seeking to explain a remarkable natural phenomenon – that of autumn colouration – as an adaptive process in which strategic quality signals, once considered the sole preserve of animals, have evolved in plants to dissuade herbivorous insects from attack. In that sense, at least, the ACH has succeeded in changing dramatically the way in which scientists view leaves in autumn time.

7.6. Future work

Further work investigating the link between colouration and plant defence in *B. oleracea* or another system would greatly benefit from a metabolomic approach (Jansen et al. 2009, Allwood and Goodacre 2010). Metabolomic analysis has been described as the chemical link between ecology and genetics (Macel et al. 2010) as it involves the analysis of the metabolome of an organism produced at a particular moment in time, thus linking phenotype with biotic and abiotic influences. Chemical ecologists can use this technique to look at the mechanisms at the foundations of plant-herbivore interactions (Jansen et al. 2009). A metabolomic approach has significant advantages over analysis of specific compounds because it can simultaneously detect a whole variety of biochemical changes within a plant and therefore can allow key mechanisms to be identified, even if it involves complex chemical pathways (Hartley et al. 2012).

It is also important to demonstrate fitness effects of glucosinolates on the herbivores (Agrawal and Kurashige 2003). Ideally, the glucosinolates that differed between blue cabbages in chapter six would have been fed to *P. rapae* to determine the biological relevance of these chemicals. It is still unclear which glucosinolates (if any) have performance impacts on herbivores feeding on brassicas, as the evidence from the literature is very mixed (for a review see Hopkins et al. 2009). The biological relevance of defensive chemicals is a surprisingly difficult thing to demonstrate, because if purified chemicals are fed to herbivores this may not represent what is encountered when feeding from a plant, e.g. there may be synergistic effects

of defensive chemicals. For example, Agrawal and Kurashige (2003) fed *P. rapae* isothiocyanates and concluded that survival was affected. However, when feeding from a leaf, isothiocyanates would not be encountered because the detoxification system of *P. rapae* diverts the production of isothiocyanates to less toxic nitriles (Wittstock et al. 2004) and so the study by Agrawal and Kurashige (2003) may have limited validity in natural systems. Despite these problems, it is nonetheless important to demonstrate the effects of glucosinolates on herbivore fitness where such defences are argued to exert selection pressures on herbivores.

Further work on the use of colour cues to investigate what insects are able to discriminate would be very useful. Modelling with the colour space of the herbivores could shed light on the abilities of herbivores to discriminate among different colours (Doring and Skorupski 2007). More detailed physiological work on aphid eyes would enable more sophisticated visual models to be constructed, similar to those available for *P. rapae* (Stavenga and Arikawa 2011). The spectral sensitivities of more aphid species need to be determined, as currently this is only known for *M. persicae* (Kirchner et al. 2005). Further work involving behavioural tests could explore choices either between plants of different species, within plant choices or choices with stressed plants to see if discrimination of host plants using visual cues in any context is possible.

It would be interesting to include *P. brassicae* (the large cabbage white) in behavioural experiments to compare choices with *P. rapae*. *Pieris brassicae* oviposits in clusters and therefore poor oviposition choices would have stronger evolutionary consequences, perhaps leading this herbivore to be more discriminatory (Davies and Gilbert 1985).

7.7. Conclusions

This thesis demonstrates that intraspecific host plant choices by insect herbivores are unlikely to involve the use of colour cues or signals, at least in a system like *B. oleracea* where intraspecific colour differences are relatively small. Most previous work investigating the use of colour in host choice has used human perception of colour (Hagen et al. 2003, Wong and Srivastava 2010), whereas the work presented here shows how important it is to interpret colour from the insect's perspective. The data presented here demonstrate that *B. oleracea* leaf colouration provides information about glucosinolate chemical defence levels, although this link maybe somewhat unreliable as glucosinolates do not consistently correlate with the same aspects of colouration. Indeed, plant-herbivore systems where this link may be strong are likely to be uncommon as it is likely to require a very simple system where there is one aspect of variable plant colouration and one main defensive chemical. Overall, the results of

this thesis provide evidence against the autumn colouration hypothesis. Rather, it is likely that colour cues are used in host choices in other ways than that suggested by Hamilton and Brown, for example to select suitable sites within a plant or to select an appropriate host among a number of different plant species.

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Appendix A. Does a single colour reading represent the plant as a whole?

A.1. Introduction

When carrying out surveys of plant colouration in the field, it would be less time-consuming and less destructive to remove a single leaf from the plant for colour analysis. However, a more representative measurement could be obtained by sampling from many leaves and calculating an average colour reading.

This study aims to see if taking one measurement of colour is representative of the whole and to see if most variation is found within or between plants.

A.2. Methods

Eighteen wild *Brassica oleracea* plants were randomly sampled from Margaret-at-Cliffe 2 (see Chapter Three for location) in Dover on the 1st February 2011. Seven leaves were collected for colour analysis: two new leaves, the focal leaf (the 3rd leaf from the top), two mature leaves and two old leaves. Figure A.1 shows how these leaves were identified. The leaves were placed in labelled white paper bags, placed in a cool box on ice, and transported to the University of Sussex for colour analysis.

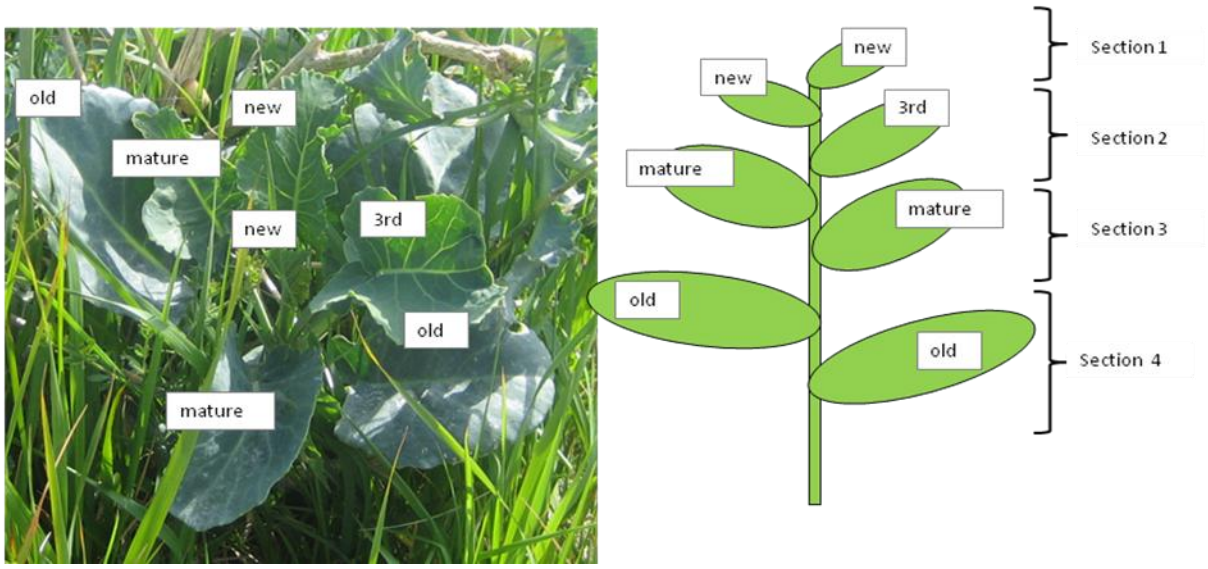

Figure A.1. The seven leaves collected for colour analysis and how these correspond to the plant sections described.

Colour was analysed and blue to green ratio and dark red to green ratios were calculated following methods described in Chapter Three. Statistical analysis was carried out in R (version 2.10.0). Colour values taken from the 3rd leaf and the plant average was compared with a paired t-test for the blue to green ratio, and a Wilcoxon paired ranks test for the red to green ratios, as these did not follow a normal distribution.

Colour variation between and within plants was analysed. Within-plant variation in colouration was explored by analysing differences in colouration between four leaf types (new leaf, the focal leaf, a mature leaf and an old leaf) (see Figure A.1) with two leaves used for each leaf type. Within-leaf colouration was also analysed by comparing three colour readings taken from the focal leaf. Colour was modelled with a linear mixed effects model (lmer, library lme4) with colour as the response variable with plant and leaf type fitted as nested, random effects. Leaf type was also fitted as a fixed effect to improve the fit of the model. Colour readings within a leaf were analysed with a concordance correlation coefficient (Zar 1999).

A.3. Results

The readings taken from the focal leaf did not differ significantly from the plant averages for either the red to green ratio or the blue to green ratio (red: V=539, df=53, p=0.08, blue: t=-1.19, df=53, p=0.24). Multiple measurements from the same leaf are very highly correlated (red: r_c=0.999; blue: r_c=0.999). These results indicate that a single colour measurement taken from the focal (3rd) leaf is representative of the plant as a whole.

There was significant variation in colouration both within and between plants. The most variation in the dark red to green ratio was found between plants (49% variation, $X^2_{1,8}$=29.1, p<0.001), but there was also significant variation in colouration between (16% variation, $X^2_{1,6}$=5.60, p=0.018), and within (23% variation, $X^2_{1,8}$=11.2, p<0.001) leaf types. Similar results were obtained for the blue to green ratio, with most variation found between plants (40% variation, $X^2_{1,7}$=35.8, p<0.001). There was also significant variation in colouration within (22% variation, $X^2_{1,7}$= 7.60, p=0.0059), though not between (10% variation, $X^2_{1,8=}$0.976, p=0.32) leaf types.

A.4. Discussion

The analysis presented here demonstrates that colour measurements based on a single reading from the 3rd leaf of a *B. oleracea* plant is representative of plant colouration as a whole, despite significant variation between and within leaves. Between-plant variation was greater than within-plant variation. Within-plant variation in colouration was nonetheless significant, and some of this variation is likely to be due to sampling a combination of old and new leaves. Compared with mature leaves, new leaves are often more brightly coloured as they have yet to establish their waxy bloom, while old leaves undergoing senescence tend to become duller and redder. The greater contribution of mature leaves to total plant volume

means that the variation in colouration between new and old leaves is greater than that found across the whole plant, however.

A.5. References

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Appendix B. The relationship with *Brassica oleracea* colouration, glucosinolates and invertebrate herbivory in the field: results of CN ratio, colouration and glucosinolates from the full GLM.

B.1. Introduction

This appendix presents an extension to the main results section in Chapter Three. In Chapter Three, separate analyses (GLMs) were carried out for each herbivore species to explore the relationship between herbivore presence and colour, CN and glucosinolates. As different numbers of plants were sampled for colour, CN and glucosinolate analysis, several analyses were performed for each herbivore to explore the effects of (a) colour (n=550), (b) colour and CN (n=179) and (c) colour, CN and glucosinolates (n=129) on herbivory. These models thus use the maximum statistical power available for each focal variable (see Chapter Three). In Chapter Three, I present the results of model (a) for colour, the results of model (b) for CN and the results of model (c) for glucosinolates. Here, I present the results for all explanatory variables (i.e. colour, CN and glucosinolates) from the full model (model c) for *Brevicoryne brassicae* and *Pieris rapae*[1] in order to determine whether and how these three variables influence one another.

B.2. Methods

For full details of the GLM analysing colour, CN and glucosinolates see Chapter Three.

B.3. Results

B.3.1. *Pieris rapae*

Results for the effects of colouration, CN and glucosinolates on *B. brassicae* presence are shown in Table B.1.

[1] In the case of *P. rapae,* the number of plants used in the analyses was reduced (see footnote in Chapter Three). Thus, for the full model containing colouration, CN and glucosinolates, n=82.

Table B.1. F values and p-values for all main variables included in GLM predicting *P.rapae* presence. df=74 in all cases

	X^2 value	p-value
Blue to green ratio	0.0157	0.90
Pale red to green ratio	0.426	0.51
Dark red to green ratio	0.246	0.62
CN ratio	-3.06	0.08
Glucoiberin	-0.0695	0.79
Progoitrin	3.85	0.05
Glucoraphanin	4.50	0.034*
Sinigrin	-0.640	0.42
Gluconapinxsite	6.38	0.012*
Glucobrassicin	-0.0282	0.87
4-methyoxyglucobrassicin	-0.637	0.42
Neo-glucobrassicin	-1.03	0.31

B.3.2. *Brevicoryne brassicae*

Results for the effects of colouration, CN and glucosinolates on *B. brassicae* presence are shown in Table B.2.

Table B.2. Fvalues and p-values for all main variables included in GLM predicting *B.brassicae* presence. df=122 in all cases.

	F value	p-value
Brightness	0.0447	0.89
Blue to green ratio	0.0404	0.84
CN ratio	4.25	0.041*
Glucoiberin	0.0269	0.87
Progoitrin	2.55	0.11
Glucoraphanin	0.293	0.59
Sinigrin	0.0128	0.91
Gluconapin	8.86	0.0035**
Glucobrassicin	5.93	0.016*
4-methyoxyglucobrassicin	1.08	0.30
Neo-glucobrassicin	3.74	0.056

B.4. Discussion

B.4.1. *Pieris rapae*

Colouration did not significantly predict the presence of *P. rapae* in the presence of glucosinolates in the full model. However, this does not rule out a role for colouration in determining herbivory by *P. rapae*. In the analysis presented in Chapter Three containing only colour in the absence of glucosinolates, the dark red to green ratio significantly predicted *P. rapae* presence. The data presented in Chapter Three also showed (a) that the dark red to green ratio was positively correlated with glucoraphanin, neoglucobrassicin and total glucosinolates and total indole glucosinolates and (b) that concentrations of glucoraphanin were higher in plants where *P. rapae* was absent. These data are internally consistent and compatible with the idea that butterflies use the dark red to green ratio as a guide to

glucoraphanin levels, and avoid high dark red to green ratios associated with high levels of glucoraphanin. The fact that the dark red to green ratio did not significantly predict *P. rapae* presence in the full model containing colour, CN and glucosinolates is likely to be the result of the positive correlation between this ratio and glucoraphanin concentration. Colinearity between explanatory variables can make it difficult to assess the relative importance of each variable in explaining variance in the dependent variable (Blalock 1963), with only the most influential factor often appearing to have a significant effect on the response variable. Thus, the fact that glucoraphanin alone is significant in this model does not exclude the fact that the red to green ratio may also have an effect on *P. rapae* presence, albeit a potentially weaker effect than that of glucoraphanin.

Intuitively, it might be expected that if colour provided a cue for plant chemistry, a stronger relationship would be observed between colour and herbivory than chemistry and herbivory. In the field, however, while colouration may influence *P. rapae* host preferences, levels of glucosinolates have the potential to affect both host preferences and offspring fitness, both of which in combination explain patterns of *P. rapae* presence on hosts. For this reason, it is possible that underlying levels of glucosinolates have a stronger effect on herbivory than does leaf colouration in the field.

B.4.2. *Brevicoryne brassicae*

It is likely that colouration is less important in host selection for *B. brassicae* than for *P. rapae*. Since only weak correlations exist between the blue to green ratio and levels of glucosinolates, it is unlikely that the effects of glucosinolates are masking an influence of colouration, as has been suggested for *P. rapae* (see above). Although leaf brightness is significantly correlated with levels of certain glucosinolates (see Chapter Three, Table 3.4), these same glucosinolates do not predict herbivory by *B. brassicae*, providing further evidence that glucosinolates are not masking an effect of brightness on herbivory in this analysis.

B.5. References

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Appendix C. How much ultraviolet light does cling film absorb?

C.1. Introduction

In Chapter Five, aphid preferences for *Brassica oleracea* leaves varying in colour were tested. It was necessary to eliminate olfactory cues, and so the plants were placed in plastic boxes (see Chapter Five, Figure 5.2.). Given that aphids may potentially use UV reflectance as a cue during host selection (Archetti et al. 2009) it was important that UV light was not excluded from the set-up as it may disrupt natural behaviour (Bennet et al. 1994). Clingfilm was therefore placed over the top of the box containing the plant. Here, I test the suitability of clingfilm for this purpose, and in particular how much UV light it absorbs.

C.2. Methods

The reflectance of three leaf samples of greenhouse grown, wild *B. oleracea* (for details of plant care see Chapter Four) were measured with a spectrophotometer and this was modelled to aphid spectral sensitivity according to methods outlined in Chapter Three. Reflectance was measured for each leaf with and without clingfilm covering the leaf. Mean UV quantum catch with and without cling film was compared with a t-test using R (version 2.10.0).

C.3. Results

UV reflectance was significantly lower when cling film covered the leaf ($t=7.14$, $df=17$, $p<0.001$; Fig C.1). The mean UV reflectance when cling film was present was 0.79 vs. 0.95 without cling film.

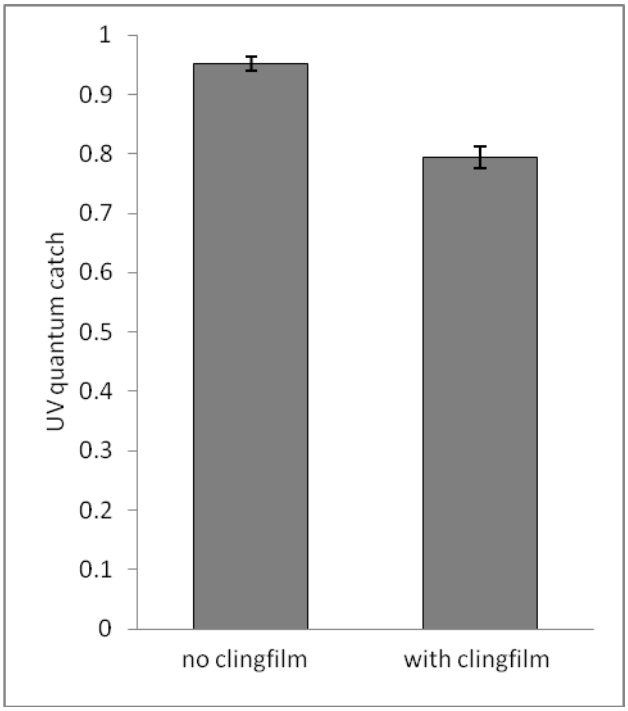

Figure C.1. Mean UV reflectance of leaves with and without clingfilm. Error bars show SEM.

C.4. Discussion

Although cling film did absorb some UV light, the reduction in UV was only 17% compared to leaves without clingfilm, which is well within the variation of solar radiation experienced in natural habitats (Endler 1993): the amount of sunlight available can decrease by 25-90% just by cloud cover and shading. The use of cling film should therefore not prevent of the use of UV cues by aphids in the preference tests.

C.5. References

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