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The Role of Phenotypic Variation on a Tritrophic Interaction.

by

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"Nature can put on a thrilling show. The stage is vast, the lighting is dramatic, the extras are innumerable, and the budget for special effects is absolutely unlimited."

Yann Martel

Life of Pi

Winner of The Man Booker Prize 2002.

THE UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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Plant defence highlights the need to approach plant-insect interactions from a tritrophic perspective. Glucosinolates are a group of defensive chemicals produced extensively by members of the Brassicaceous family. The constitutive production of glucosinolates directly deters generalist herbivores. However, specialist herbivores are attracted to these chemicals and utilise them to locate suitable host plants. Upon herbivore attack, glucosinolates are hydrolysed by the enzyme myrosinase, which causes the release of a specific blend of volatile products. These herbivore-induced volatiles are thought to form an indirect method of plant defence via the action of natural enemies.

A wild Brassica system was identified to investigate the differences in glucosinolate phenotype on a specialist herbivore and its parasitoid. HPLC analysis revealed that *Brassica oleracea* subsp. *oleracea* (wild cabbage) at Kimmeridge and Durdle Door produce quantitative and qualitative differences in aliphatic glucosinolates. Air entrainment experiments revealed that 3-butenylisothiocyanate was released by wild cabbages from Durdle Door, however when Durdle Door wild cabbages were grown in soil collected from Kimmeridge, decreased quantities were released. Laboratory and field investigations concurred to reveal that *Brevicoryne brassicae* and *Diaeretiella rapae* representing the second and third trophic level respectively were unable to differentiate between the glucosinolate differences produced by *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door. However, "host-line" variation occurred in *Brevicoryne brassicae*, whilst behavioural differences between laboratory-reared and field-collected *Diaeretiella rapae* were observed. The results of all of the investigations are discussed in relation to the evolution of plant chemical defence and factors responsible for the glucosinolate polymorphism observed in these wild Brassica populations.

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Chapter One

General Introduction

Chapter One

General Introduction

1.1. Plant Defence

All terrestrial communities based on living plants are composed of at least three interacting trophic levels (Price *et al.*, 1980): plants in the first trophic level, herbivores in the second, and natural enemies of the herbivore in the third. Many organisms have evolved within a multitrophic context and are influenced by organisms from other trophic levels (Poppy, 1997). Therefore, plant-insect interactions can only be fully understood when investigations are approached holistically.

Plant defence against herbivory illustrates the need to approach plant-insect interactions from a multitrophic perspective. Plants display many direct and indirect physical and chemical defence strategies in the battle against herbivory.

The presence of physical structures, particular life strategies and the production of chemicals can directly defend host plants against herbivores. Leaf domatia (Åhman, 1990) and tissue toughness (Heil & Bostock, 2002) can act to physically hinder the attachment of herbivores to their hosts. Life strategies including early or rapid growth, development and dispersion or simply by the "choice" of habitat can all help to minimise herbivore damage (Strong *et al.,* 1984). Plant chemicals can either be constitutively produced, for example, the constitutive production of high levels of toxins or low levels of nutrients (Price *et al.*, 1980) or induced by herbivore damage as in the activation of jasmonate and hydrogen peroxide (Gatehouse, 2002).

Indirect plant defence commonly involves the induction of plant volatiles (Karban & Myers, 1989; Karban & Baldwin, 1997; Dicke, 1999; 2000), which are thought to affect the natural enemies of herbivores (Thaler, 1999). Upon herbivore attack, damaged plants emit both quantitatively and qualitatively different volatile blends compared to undamaged plants (Dicke & van Loon, 2000). It is thought that natural enemies utilise these altered volatile profiles at distances to locate suitable habitats for their prey and once in that habitat, exploit more specific host-derived cues (Vet & Dicke, 1992).

The subject of plant defence continues to invoke speculation among population ecologists. Since Hairston *et al.*, published the "world is green" hypothesis in 1960, debate continues to rage over whether top-down forces (role of natural enemies), bottom-up forces (plant allelochemicals) or both, influence the dynamics of insects in the second trophic level (Hunter *et al.*, 1997). Harvey *et al.*, (2003) believe that bottom-up forces predominantly control herbivores. It is this premise that has inspired this study to investigate the role of defensive chemicals in plant-insect interactions. This thesis approaches population dynamics from a bottom-up perspective and investigates the role of a specific secondary metabolite thought to be involved in plant defence within a tritrophic context, under both controlled laboratory conditions and in a natural ecosystem. This chapter introduces the role of plant defensive chemicals involved in plant-insect interactions paying particular attention to the role of chemicals produced by members of the Brassicaceous family.

1.2. Chemical Ecology

The role of chemicals in the interactions of living organisms has received increasing recognition over the past 20 years. Much attention has focused upon a specific group of chemicals called glucosinolates that are produced extensively by members of the Brassicaceous family (Chew, 1988; Clossais-Besnard & Larher, 1991; Daxenbichler *et al.*, 1991; Aguinagalde *et al.*, 1992; Mithen, 1992; Bartlet *et al.*, 1996; 1997; Bones & Rossiter, 1996; Moyes *et al.*, 2000; Fahey *et al.*, 2001; Branca *et al.*, 2002). Dicke (2000) states that if plants are damaged, chemicals can be produced both locally and sometimes systemically, which can affect insects in the second trophic level in two ways. Studies of Brassica plants reveal that these chemical changes can either deter attack by generalist herbivores (Chew, 1988; Glen *et al.*, 1990; Louda & Mole, 1991; Mithen, 1992; Giamoustaris & Mithen, 1995; Shields & Mitchell, 1995), or attract specialist herbivores that have evolved adaptations to overcome these constitutive and induced chemical profiles (Rodman & Chew, 1980; van Emden, 1990; Landolt, 1993; Giamoustaris & Mithen, 1995; Mithen *et al.*, 1995b; Bartlet *et al.*, 1996; 1997).

Increasing evidence shows that Brassicas are able to indirectly defend themselves against specialist herbivores by attracting the natural enemies in the third trophic level (Vet & Dicke, 1992; Blaakmeer *et al.*, 1994; Geervliet *et al.*, 1994; Vaughn *et al.*, 1996; Dicke & van Loon, 2000). Research suggests that specialist natural enemies (predators,

parasitoids and pathogens) have adapted to respond to the specific chemical profiles emitted by herbivore-damaged plants (Dicke & van Loon, 2000). These so-called herbivore-induced synomones are highly specific to the action of herbivores, making them a very reliable signal for natural enemies. Therefore, it appears that a mutualistic relationship occurs between organisms in the first and third trophic levels. This is supported when Turlings *et al.,* (1990) revealed that *Cotesia marginiventris* are attracted to volatile terpenoids emitted from corn seedlings after the application of *Spodoptera* saliva.

Few studies have investigated this indirect defence mechanism against herbivory in the field, particularly in natural ecosystems (Dempster, 1975; Louda & Mole, 1991; Myers, 1981; Price & Clancy, 1986; Price, 1988; van der Meijden *et al.*, 1998). van der Meijden and Klinkhamer, (2000) highlighted this gap in knowledge of plant-insect interactions in the field by stating, "field studies in natural ecosystems will be highly valuable to demonstrate the importance of herbivore-induced plant volatiles." This study aims to address this lack of research by investigating chemical plant defence in a natural ecosystem. This will be related to controlled laboratory investigations to discover if they represent a realistic reflection of plant-insect interactions in the field.

1.3.First Trophic Level

1.3.1. Brassicaceous Plants

The Brassicaceous family encompasses a diverse range of genera including the genus Brassica. This genus contains commonly consumed species and thus economically important plants such as cabbage, broccoli and Brussels sprout. Brassicas also provide animal fodder and are a source for margarine and cooking oils.

Brassica oleracea subsp. *oleracea*, most commonly known as the wild cabbage, is the Brassica species under investigation. *Brassica oleracea* subsp. *oleracea* was first recorded growing wild in 1551 by Turner in Dover and areas of East Kent, England, UK (Mitchell, 1976). In fact, it is thought that *Brassica oleracea* subsp. *oleracea* is the progenitor of many crop plants (Raybould *et al.*, 1999b).

The present day record was complete by the beginning of the 19th Century (Mitchell, 1976) and reveals that wild cabbage populations are found on the coasts of Dorset,

Cornwall, Kent, Gwynedd, Glamorgan (Snogerup *et al.*, 1990) and on the Isle of Wight (Mitchell, 1976). In the UK, populations of *Brassica oleracea* subsp. *oleracea* occur mainly on calcareous cliffs on the coasts in the South and South-West of England and Wales (Raybould *et al.,* 1999a). However, there are reports that *Brassica oleracea* subsp. *oleracea* occurs as far north as the East coast of Scotland (Mitchell & Richards, 1979).

Brassica oleracea subsp. *oleracea* populations along the Dorset coastline in the UK are the focus for this study. This choice was made for several reasons. As the focus of this thesis is plant chemistry, a wild Brassica was chosen instead of a cultivated variety as it is documented that wild plants produce higher quantities of secondary compounds, including glucosinolates (Mithen *et al.*, 1987). Secondly, the wild cabbage system is an ecologically interesting model which has been well characterised, both in terms of its history and ecology and importantly is genetically relevant to this investigation (Mitchell, 1976; Mitchell & Richards, 1979; Mithen *et al.*, 1995b; Raybould *et al.*, 1999a; 1999b). These factors, in addition to the practical location of these populations, make the wild cabbage system a very powerful model within which to further expand our knowledge on the role of plant genetics in a tritrophic context.

Brassica oleracea subsp. *oleracea* is a woody, evergreen perennial plant, which can survive for up to 20 years (Figure 1.1.). Growth continues throughout the year, being most rapid between March and August. Flowering is generally over by mid-June with seed dispersal in August and September (Mitchell & Richards, 1979). Wild cabbage plants were initially thought to flower after 4 - 5 years and in each subsequent year, however it has been observed that plants growing along the Dorset coastline do not necessarily flower every year (Moyes, 1997). *Brassica oleracea* subsp. *oleracea* is an obligate outbreeder, generally pollinated by bees, although butterflies and flies are also attracted to the flowers. Plants are usually single-stemmed, but can become multi-stemmed if damaged by herbivores early on during its life cycle (Mitchell & Richards, 1979). However, Moyes (1997) observed that mature plants are also able to produce new branches. There are generally fewer than 10 flowering stems per plant, but 20 racemes have been noted on exception. They can reach one metre in height and commonly produce between 10-100 yellow flowers. The fruit of *Brassica oleracea* subsp. *oleracea* is a two-celled siliqua containing 8-16 black/brown seeds. The leaves of non-flowering stems are broad and rounded in shape (Mitchell & Richards, 1979).

Figure 1.1. *Brassica oleracea* subsp. *oleracea*

The overall size of the plant is thought to be influenced by the soil type in which it is growing. Mitchell and Richards (1979) found that plants growing in shale were generally larger than those growing on calcareous soils. This observation was supported by Moyes (1997) who noted that *Brassica oleracea* subsp. *oleracea* growing in shale at Kimmeridge along the Dorset coastline had a larger overall leaf area than individuals growing in limestone soils in nearby areas. The effect of soil type will be explored in greater depth in Chapter Two and Chapter Five.

1.3.2. Dorset Flora

Along the Dorset coastline in the UK, *Brassica oleracea* subsp. *oleracea* is found growing along the cliff tops in large discrete populations. It has been documented that wild cabbage populations have been growing in Dorset in excess of 100 years. The first record was of a population at Portland in 1813 (Mansel-Pleydell, 1874). Populations at Kimmeridge, St. Aldhelm's Head, Winspit, Lulworth Cove and Durdle Door were recorded in 1874. It is not fully understood whether the populations of wild cabbage are a result of partial or complete domestic escapes (Aguinagalde *et al.*, 1992; Mitchell & Wass, 1996) or are the progenitor of many cultivated varieties (Raybould *et al.*, 1999b).

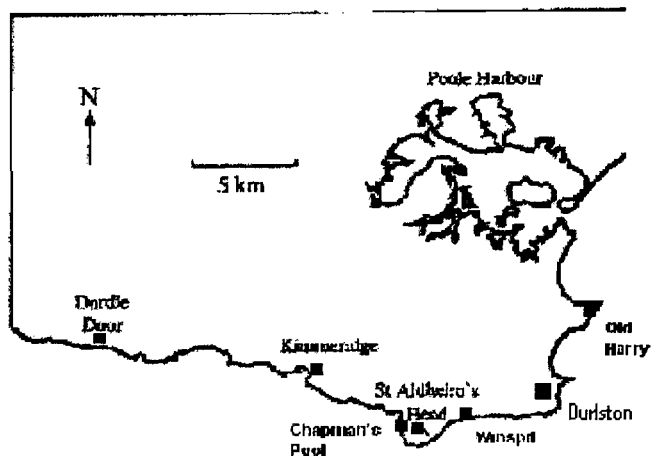


Figure 1.2. Map of the Dorset Coastline.
(Modified from Raybould *et al.*, 1999a)

There are six main sites that span the geographical range of *Brassica oleracea* subsp. *oleracea* in the Dorset region, thus explaining the reason for their initial identification (Raybould *et al.*, 1999a) (Figure 1.2.).

Old Harry population of *Brassica oleracea* subsp. *oleracea* has approximately 4000 plants, of which the majority are found growing on the face and edge of calcareous cliffs. The next population is at Winspit. This is a very large population, often thought to host as many as several tens of thousands of plants. Like the plants growing at Old Harry, wild cabbages at Winspit grow on the edges of calcareous cliffs. The next site along the coast following a westerly direction is St. Aldhelm's Head. A large population of wild cabbages grows mainly in grassland at this site. The population at Chapmans Pool is roughly the same size as that at Old Harry. It is thought to host approximately 5000 plants, which grow on cliff faces and extend into the grassland onto the cliff tops. Kimmeridge boasts approximately 4000-5000 wild cabbage plants. However, unlike the other populations previously mentioned which all grow on chalk substrate, the plants at Kimmeridge grow in clay-based soils. The final site is Durdle Door, which is the second largest site along the Dorset coastline. These plants are mostly found growing in limestone soils, although in some regions, a layer of clay is found on top of the chalk (Raybould *et al.*, 1999a).

Several reports have indicated that southern populations of *Brassica oleracea* subsp. *oleracea* in the UK are morphologically more variable and have higher levels of

glucosinolates than populations found elsewhere in the British Isles (Greenhalgh & Mitchell, 1976; Mithen *et al.*, 1995b). As the glucosinolate phenotype of wild populations of *Brassica oleracea* is fundamental to this study, the putative higher levels of this secondary metabolite in southern populations make the sites along the Dorset coastline ideal.

Despite the presence of six discrete *Brassica oleracea* subsp. *oleracea* populations along the Dorset coastline, this study is a comparative study, therefore only two sites are focused on. The two sites of interest are the population at Durdle Door and the population at Kimmeridge (Figure 1.3. and 1.4.).

Figure 1.3. Durdle Door Field Site.

Figure 1.4. Kimmeridge Field Site.

Durdle Door wild cabbage plants are thought to produce the highest quantities of total glucosinolates and aliphatic glucosinolates whilst Kimmeridge plants produce the lowest levels (Moyes, 1997). Based on this finding, Kimmeridge and Durdle Door were deliberately chosen as the wild cabbage plants are putatively at opposite ends of the glucosinolate spectrum. Interestingly, *Brassica oleracea* subsp. *oleracea* at these two field sites are also known to differ in a compound (3-butenylglucosinolate) (Moyes, 1997) whose volatile breakdown product (3-butenylisothiocyanate) is suggested to be important in plant-insect interactions (Bradbourne & Mithen, 2000; Blande, 2004). Thus a natural ecosystem was identified to investigate the bottom-up effects of these extreme levels of glucosinolates and the impact of an individual glucosinolate on the dynamics of insects in the second and third trophic levels.

1.3.3. Glucosinolates

1.3.3.1. Glucosinolate Biosynthesis

Glucosinolates (β-thioglucoside-*N*-hydroxysulfates) are plant secondary metabolites present in 16 families of dicotyledous angiosperms (Fahey *et al.*, 2001), but they are most commonly associated with members of the Brassicaceous Family (Daxenbichler *et al.,* 1991) including *Brassica oleracea* subsp. *oleracea.* It is known that glucosinolates have fungicidal, bacteriocidal, nematocidal and allelopathic properties (Fahey *et al.*, 2001). Studies have also shown that certain glucosinolate-breakdown products can have serious adverse effects on livestock causing thyroid, liver and kidney problems (Raybould & Moyes, 2001). However, glucosinolates and some of their degradation products are thought to have beneficial effects for humans due to their role in protecting against certain cancers by stimulating the production of enzymes that metabolise xenobiotic chemicals (Michaud *et al.*, 1999; Cohen *et al.*, 2000; Mithen, 2001).

It is well known that quantitative and qualitative glucosinolate differences are produced by different *Brassica* species (See reviews by Daxenbichler *et al.,* 1991 and Fahey *et al.*, 2001). Mithen *et al.*, (1995b) worked on wild populations of *Brassica oleracea* along the Dorset coastline and found that three aliphatic glucosinolates predominated; 2-propenylglucosinolate, 3-butenylglucosinolate and 2-hydroxy-3-butenylglucosinolate. Relatively high levels of 3-indolylmethylglucosinolate and 1-methoxy-3-indolylmethylglucosinolate were also produced. Work on *Brassica napus* revealed that the majority of glucosinolates produced were butenyl- and pentenyl- glucosinolates with only marginal levels of methylsulphinylpentylglucosinolates detected (Magrath *et al.*, 1994).

It has since been discovered that glucosinolate profiles not only differ between species, but also within species. Moyes *et al.,* (2000) revealed that different concentrations of five individual glucosinolates were produced among wild *Brassica oleracea* populations along the Dorset coastline. This revelation is highly important to this study as it highlights the need to confirm the profile of glucosinolates, both in terms of the profile of individual glucosinolates and the total quantity of glucosinolates produced by individuals of the same species.

The quantity and quality of glucosinolates produced by *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door is determined in Chapter Two. This is critical to this investigation as it will reveal the exact glucosinolate profiles produced by wild cabbage plants at these two sites and importantly, will reveal whether the quantity of glucosinolates produced by plants at Kimmeridge and Durdle Door is the same as that discovered eight years ago by Moyes (1997).

The characteristic profile of glucosinolates produced by each Brassica species is thought to be controlled by a range of genetic and environmental factors.

The glucosinolate molecule consists of a basic common structure of a glycone moiety and a variable aglycone side chain that is derived from amino acids (Mithen *et al.*, 1995a) (Figure 1.5.).

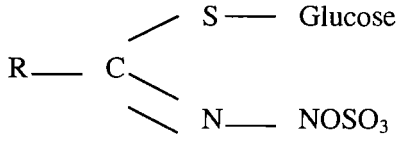


Figure 1.5.
Glucosinolate Molecule.
R = amino acid side chain

The variable aglycone side chain can be divided into three subgroups. Aliphatic glucosinolates (aliphatic side chain) are formed from methionine, glucosinolates with an indole side chain are formed from tryptophan and aromatic glucosinolates are formed from phenylalanine (Mithen *et al.*, 1995a). Aliphatic glucosinolates are the most abundant glucosinolates in *Brassica* tissue and hence are the most widely studied (Giamoustaris & Mithen, 1996).

It is generally accepted that there are three stages to aliphatic glucosinolate biosynthesis:

- Side chain elongation
- Glycone biosynthesis
- Side chain modification

Biochemical studies using *Brassica* plants are progressing, but work on *Arabidopsis thaliana* has initially helped to identify any genes that are common in glucosinolate biosynthesis.

The first step involves converting the amino acid into an oxime and is initiated by the elongation of the side chain. The basic glucosinolate structure is formed by the repeated addition of methyl carbon from acetate to form methionine molecules. This enters methionine into the aliphatic glucosinolate biosynthetic pathway. This process can be repeated to form an elongated compound, homomethionine, which forms the precursor of butyl-glucosinolates. Homomethionine can be further elongated to form 2-amino-6-methylthiohexanoic acid (Kroymann *et al.*, 2001).

Once the amino acid side chain has been elongated, it needs to be converted into an oxime. Biochemical studies on *Arabidopsis thaliana* indicates that this step is catalysed by a cytochrome P450 mono-oxygenase in the CYP79 family (Mithen, 2001). It is believed that a comparison can be made between the biosynthesis of cyanogenic glycosides and glucosinolates as both involve oximes as intermediates. The cytochrome P450 CYP79A1 catalyses the conversion of tyrosine to p-hydroxyphenylacetaldoxime during the biosynthesis of the cyanogenic glycoside dhurrin in *Sorghum bicolor* (Mithen, 2001). Bak *et al.,* (1999) demonstrated that when cytochrome P450 CYP79A1 is expressed in *Arabidopsis thaliana*, tyrosine-derived glucosinolates (benzyl glucosinolates) were produced in unusually large amounts. It was suggested that the enzymes involved have low substrate specificity based on the evidence that p-hydroxyphenylacetaldoxime is efficiently channelled into the glucosinolate biosynthetic pathway.

Glucosinolates from chain-elongated methionine are thought to be converted to the corresponding oximes by a minimum of two flavin-containing mono-oxygenases (Mithen, 2001). The first has substrate specificity for homophenylalanine and the second is specific for di- and tri-homomethionine, which are the precursors for many aliphatic glucosinolates such as 3-butenylglucosinolate (Bennett *et al.*, 1993; Dawson *et al.*, 1993). Oximes are then thought to converted to thiohydroximates (Mithen, 2001), however, the steps involved in this process are unknown at this stage.

The second step of glucosinolate biosynthesis is glycone biosynthesis. Thiohydroximate is converted to desulphoglucosinolate by UDPG:thiohydroximate glucosyltransferase (Matsuo & Underhill, 1969; Reed *et al.*, 1993; GrootWassink *et al.*, 1994; Guo & Poulton, 1994). This is then sulphated by a second enzyme, desulphoglucosinolate sulphotransferase (Jain *et al.*, 1990). The two enzymes involved

in glycone biosynthesis have been investigated in *Arabidopsis* and a number of *Brassica* species (Jain *et al.*, 1990; Guo & Poulton, 1994), however, it is yet to be determined as to whether the enzymes exist as a complex since contrasting results have been revealed according to the species worked on.

The final step in glucosinolate biosynthesis is side chain modification. It is believed that a small number of genes control side chain biosynthesis and modification. Magrath *et al.,* (1994) found that the length of the side chain is regulated by product(s) from the *Gsl-elong* genes. One outcome of this is the elongation and subsequent conversion of 2-propenylglucosinolate into 3-butenylglucosinolate. Alleles at three other loci thought to be involved in side chain modification have been determined (Parkin *et al.*, 1994; Mithen *et al.,* 1995a; Giamoustaris & Mithen, 1996). *Gsl-oxid* results in the oxidation of the methylthio- side chains to methylsulphinyl- side chains. *Gsl-alk* desaturates alkyl side chains to alkenyl- side chains, which are then hydroxylated by *Gsl-oh*. Figure 1.6. indicates the steps of glucosinolate biosynthesis.

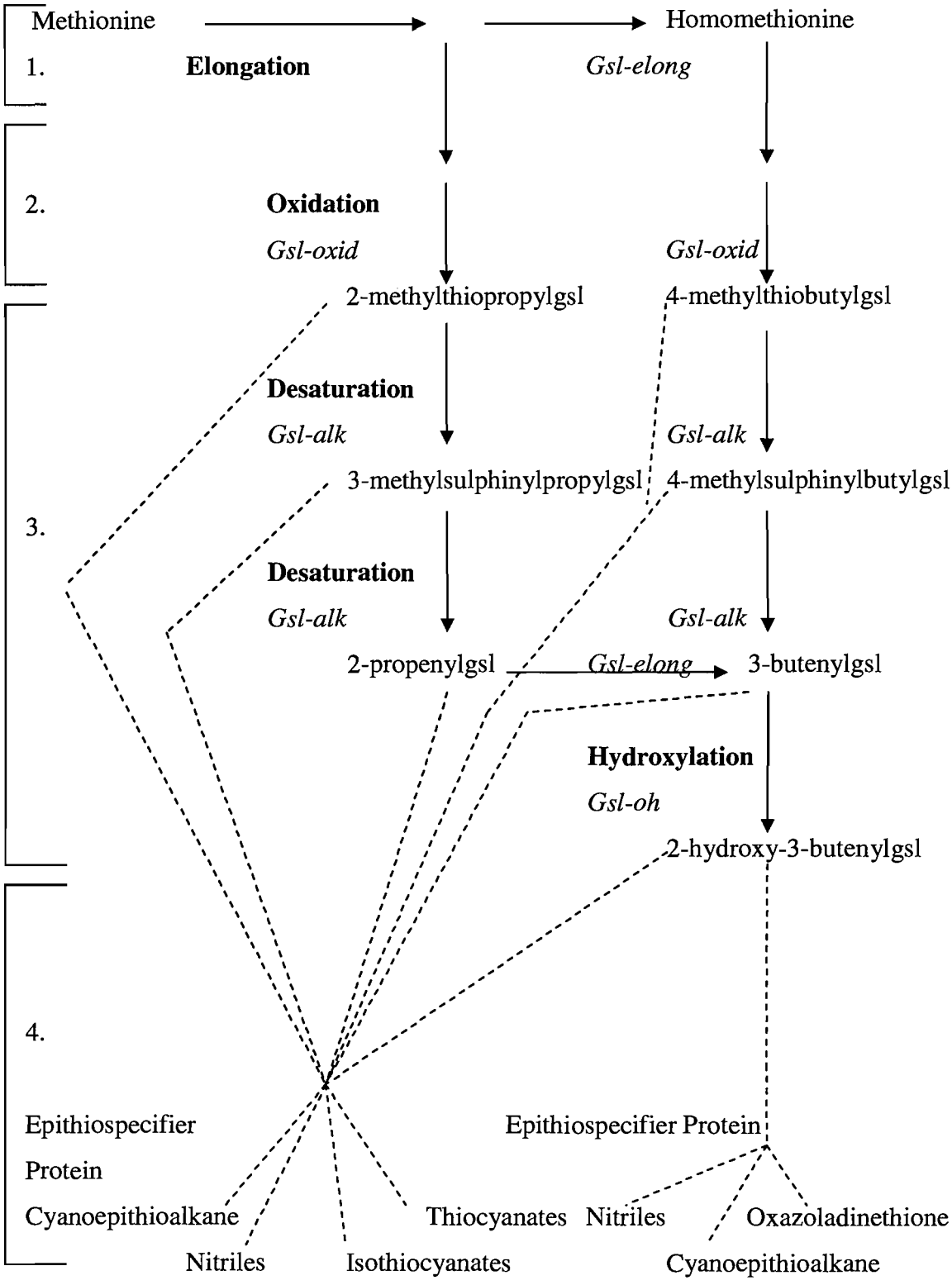


Figure 1.6. Glucosinolate Biosynthesis and Hydrolysis (adapted from Mithen & Campos, 1996; Moyes, 1997, Raybould & Moyes, 2001). 1=Side chain elongation. 2=Development of glycone moiety. 3=Side chain modification. 4=Glucosinolate hydrolysis products. Gsl=Glucosinolates. Solid arrows indicate glucosinolate biosynthesis, dashed lines indicate glucosinolate hydrolysis.

Whilst research indicates that the profile of glucosinolates is under strong genetic control, environmental factors are thought to influence the total level of glucosinolates produced by Brassica plants (Mithen, 2001). This reaffirms the necessity to confirm the quantity and quality of glucosinolates produced by *Brassica oleracea* subsp. *oleracea* at Kimmeridge and Durdle Door.

Soil nutrient availability may affect the chemicals produced by the plant. Siemens and Mitchell-Olds (1996) stated that plants growing in poorer nutrient conditions produce more carbon-based defensive compounds relative to lower levels of nitrogen-based compounds. This can be applied to glucosinolates, as nitrogen is one of the main components of this compound. Sulphur is another major constituent of glucosinolates and so in relation to this, plants growing in sulphur deficient soil may have different profiles compared to plants growing in optimum soil sulphur conditions. Specific to this study, Moyes (1997) revealed that the soil type at Durdle Door is limestone, but at Kimmeridge the soil is primarily shale. The differences in soil type between the two field sites could affect the glucosinolate profile of *Brassica oleracea* subsp. *oleracea.* Indeed, a tentative relationship exists between the soil type at Kimmeridge and St. Aldhelm's Head and the concentration of indole glucosinolates produced by wild cabbage seedlings (Moyes, 1997). This preliminary study provides supportive evidence for the effect of environmental conditions on the profile of glucosinolates produced by Brassica plants (Mithen, 2001). The effect of soil type will be addressed during Chapter Two.

Inglis *et al.*, (1992) discovered a significant difference in the concentrations of S-methylcysteine sulphoxide and glucosinolates of low (0) and double-low (00) varieties of oilseed rape plants grown in the same field. This led to the suggestion that climatic factors, geographical location, soil type and cultivation may have greater effects on plant secondary metabolite chemistry than the plant species itself.

Another factor thought to affect the profile of glucosinolates is the treatment of chemical elicitors. Kiddle *et al.*, (1994) noted a systemic increase in glucosinolate content in oilseed rape plants when treated with salicylic acid. Bartlet *et al.*, (1999) applied jasmonic acid to the cotyledons of oilseed rape and noted a distinct increase in the total levels of glucosinolates. It is interesting to note that the glucosinolate content produced concurs with that produced upon herbivore damage.

Research detailing the effect of environmental and chemical factors on glucosinolates reveals a trend. It appears that indole glucosinolates are most affected by external factors. This is in contrast to aliphatic glucosinolates which are genetically regulated and seem to be relatively unaffected by external factors. For this reason, research tends to focus on aliphatic glucosinolates (Mithen *et al.*, 1995a; Moyes, 1997). Therefore, due to the high heritability observed in aliphatic glucosinolates, this group may act as genetic agents that drive tritrophic interactions. For this reason, the variation in aliphatic glucosinolates will be concentrated on during this study.

1.3.3.2. Glucosinolate Hydrolysis

Although glucosinolates form only one aspect of the battery of chemical defences by Brassica plants, this group of secondary metabolites are the focus of this study. This is due to the growing implication of their role in plant defence (Landolt, 1993; Mithen *et al.*, 1995b; Bartlet *et al.*, 1996; 1997; Bradbourne & Mithen, 2000; Fahey *et al.*, 2001; Mithen, 2001). Glucosinolates are hydrolysed to form a variety of volatile compounds which then appears to form the second component of the plants' defence against herbivory.

Glucosinolates are thought to be located in the vacuoles of plant cells and only come into contact with myrosinases, the enzymes responsible for their hydrolysis, upon tissue damage (Moyes, 1997). Glucosinolate hydrolysis has been well documented (Mithen *et al.,* 1995a; Giamoustaris & Mithen, 1996; Moyes, 1997; Fahey *et al.*, 2001; Mithen, 2001). Myrosinase hydrolyses the β-thioglucosyl bond (thioester bond) of the molecule, which releases glucose and sulphate. The removal of the glucose molecule from the structure renders the intermediate unstable and immediately undergoes a Lossen rearrangement (Fahey *et al.*, 2001). This generates a number of glucosinolate-breakdown products, including isothiocyanates, nitriles and thiocyanates (Fahey *et al.*, 2001).

The breakdown products released depends upon the glucosinolates produced by the plant, which as previously described is under a high degree of genetic control. However, the proportion of breakdown products released depends upon the reaction conditions the hydrolysis occurs in (Cole, 1976). In neutral conditions, the main breakdown products are isothiocyanates, whilst under acidic conditions, nitriles are

released (Gil & MacLeod, 1980). Other reaction conditions are thought to influence the breakdown products released, such as the presence of Fe^{2+} ions, ascorbic acid and epithiospecifier proteins (Mithen, 2001). Figure 1.6. indicates some of the products released by glucosinolate hydrolysis.

1.3.3.3. The Effect of Glucosinolates on Plant-Insect Interactions

Due to the nature of how glucosinolates are induced and their breakdown products released, glucosinolates are thought to have a number of roles in mediating plant-insect interactions. Glucosinolates can either deter generalist herbivores by decreasing the palatability of the leaf tissue (Chew, 1988; Glen *et al.*, 1990; Louda & Mole, 1991; Mithen, 1992; Giamoustaris & Mithen, 1995) or attract and stimulate oviposition by specialist herbivores (Landolt, 1993, Mithen *et al.*, 1995b).

Brassica plants probably evolved to produce glucosinolates and their breakdown products as defensive chemicals against general herbivory (Lamb, 1989). This is supported by numerous studies. Weber *et al.*, (1986) demonstrated that *Sinapis alba* plants containing high levels of sinalbin were less susceptible to damage by pests than *Brassica napus* plants with low glucosinolate levels. However, the major flaw of this study is that two different plant species were compared so the differences observed could be due to a number of physical and chemical factors. This is addressed in Chapter Two when various physical traits are determined in addition to the glucosinolate phenotype of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door.

Shields and Mitchell (1995) found that sinigrin, a simple glucosinolate, deterred feeding by *Trichoplusia ni* (female cabbage looper moth) under certain conditions. Interestingly, these insects possess sinigrin-sensitive cells whose activity correlates with feeding behaviour. This suggests an active response to these plant-produced chemicals. Molluscs and pigeons are also deterred by glucosinolates, proving that insects are not the only organisms affected by glucosinolates (Giamoustaris & Mithen, 1995).

There is an extensive literature highlighting the role of glucosinolates as attractants for specialist herbivores. Differences in glucosinolate profiles among *Brassica* plants have been linked to host preferences of specialist herbivores (Rodman & Chew, 1980). It is thought that it is the glucosinolate-breakdown products, in particular the highly volatile

isothiocyanates, which attract herbivores (Blaakmeer *et al.*, 1994). Theory suggests that induced plant volatiles such as isothiocyanates, make the plant more apparent among surrounding undamaged plants not emitting the same blends, thus resulting in the damaged plant attracting more herbivorous insects (Dicke, 2000).

One of the many examples on specialist insects is work by Bartlet *et al.*, (1996). The cabbage flea beetle, *Psylliodes chrysocephala* was observed to be stimulated to feed by a glucosinolate enriched agar medium. Giamoustaris and Mithen (1995) demonstrated that flea beetle damage increased with a nine-fold increase in glucosinolate concentration across 28 *Brassica napus* lines tested. A further example is a study by Landolt (1993). The female cabbage looper moth, *Trichoplusia ni* is attracted to volatiles emitted from larvae-infested plants. It was deduced that female moths use olfactory cues from damaged plants to locate suitable plants on which to oviposit. However, this study did not determine whether the insects were attracted to the changes in plant volatiles resulting from larval feeding, mechanical damage, larval frass or all of the above. The next logical step would have been to determine what chemical the insect was attracted to. Finally, relating to this study, the specialist cabbage aphid, *Brevicoryne brassicae* is stimulated to feed by glucosinolates present in *Brassica* plants in the laboratory (van Emden, 1990).

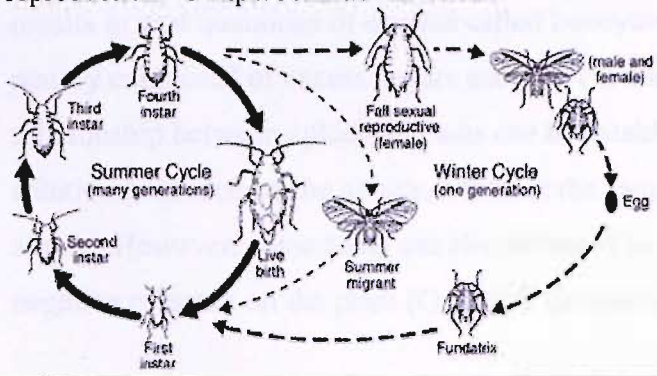
To summarise, *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door are thought to differ in terms of their glucosinolate phenotype. This study aims to reaffirm the quantity and quality of glucosinolates produced by wild cabbages both between and within sites. The impact of glucosinolates on tritrophic interactions is investigated in both laboratory and field conditions.

1.4. Second Trophic Level

A specialist aphid species, *Brevicoryne brassicae*, is the herbivore representing the second trophic level.

Aphids are classified under the Sub-Order *Homoptera* in the Order *Hemiptera*. *Homoptera* is further divided into two divisions, the *Auchenorrhyncha* and the *Sternorrhyncha*, with the latter division hosting the aphids.

Figure 1.7. Aphid Life Cycle. Summer cycle depicts asexual reproduction, winter cycle depicts sexual reproduction. ©http://bohart.ucdavis.edu



Aphids display a variety of life cycles. A common strategy involves the alternation of the production of both asexual and sexual morphs at different times of the year, typically during summer and winter respectively. This holocyclic life cycle is observed in *Brevicoryne brassicae* (Figure 1.7.). Eggs laid during the previous autumn hatch in the spring and a fundatrix emerges. This aphid is distinguishable from other generations by its appearance. The fundatrix helps found the colony by producing the "first" generation of apterous (wingless) female aphids. During spring and summer, apterous females reproduce parthenogenetically, meaning that live nymphs who are clones of the mother are produced. This allows a colony to rapidly exploit a suitable host and habitat without the need to find a mate. This important aspect in the life cycle of an aphid enhances its pest status. Colony overcrowding and/or a decline in host plant quality leads to the development of winged females (alates), which enables the colony to spread to surrounding suitable host plants. Autumn brings shorter days, decreased temperatures and often decreased plant quality, which coincides with the appearance of a sexual generation of males and females. These mate and produce eggs, which are the overwintering stage of this insect and the cycle repeats. However, during mild winters virginoparae survive leading to early infestations (Gullen & Cranston, 1994).

Aphids are generally oval-shaped, reaching a maximum of 2.5mm in length. All aphid species possess a pair of structures on their fifth abdominal segment called siphunculi. It is through these structures that aphids are able to communicate chemically with other individuals. The aphid sex pheromone (nepetalactone) and the alarm pheromone (α-farnesene) are released from these organs (Gullen & Cranston, 1994).

Aphids are phloem-feeders so their mouthparts are specifically adapted to pierce leaf tissue and penetrate phloem vessels. The proboscis contains the stylet that is inserted into the leaf. Aphids secrete a liquid from the stylet tip, which hardens as the stylet penetrates the leaf. This functions to protect the delicate stylets from damage. Once the

phloem vessel has been reached, pressure forces the phloem up into the stylet channel. This method of feeding, results in vast quantities of phloem being ingested, which also results in vast quantities of excreta called honeydew being produced. Honeydew is mainly composed of excess sugars and it is via this substance that a symbiotic relationship between aphids and ants can be established. The ants feed on the sugary solution produced by the aphids, whilst at the same time help to protect the aphids from attack. However, some fungi are also attracted to honeydew thus adding an increased negative pressure on the plant (Gullen & Cranston, 1994).

An interesting aspect to aphid morphology is the presence of endocellular symbionts present in bacteriocytes in the gut of aphids. *Buchnera aphidicola*, a relative of *Escherichia coli*, is transferred from mother to embryo during early development and forms a vital relationship for both organisms. Aphids benefit as *Buchnera* spp. provide vital amino acids, sterols and vitamins, whilst *Buchnera* spp. benefit by being "housed" inside aphids as they lack vital organelles (Gullen & Cranston, 1994).

All *Homopterans* are herbivores, thus explaining the presence of so many serious pests within this Sub-Order. As mentioned, aphids are highly adapted to their pest role by means of their parthenogenetic reproduction, which results in very short life cycles producing numerous generations during a season. Aphids are phloem-feeders whose feeding directly robs the plant of significant quantities of water and minerals causing additional stress for the plant. Aphids can also cause huge problems by acting as vectors for some viruses. The detrimental impact aphids have on plants is one of the reasons for choosing an aphid species to represent the second trophic level during this study. The greater understanding of aphids and their interactions with host plants could lead to the development of alternative methods to control aphids. A second important reason for choosing aphids, particularly *Brevicoryne brassicae*, is because they are commonly observed feeding on wild populations of *Brassica oleracea* at Kimmeridge and Durdle Door. Finally, much data are available investigating the interactions between aphids and their hosts, with much attention of Brassica systems (Rodman & Chew, 1980; van Emden, 1990; Ronquist & Åhman, 1990; Cole, 1996; 1997).

1.4.1. *Brevicoryne brassicae*

The aphid under investigation is *Brevicoryne brassicae*, more commonly known as the cabbage aphid. This species is host-specific, feeding almost exclusively on *Brassica* plants (Cole, 1996), causing significant losses in temperate regions all over the world (Ronquist & Åhman, 1990).

Brevicoryne brassicae can reach approximately 2.5mm in length when fully mature. It passes through four instar stages taking between 7-14 days before reaching maturity, however this figure invariably depends on the host plant of the aphid and other conditions such as temperature (DeLoach, 1974). Interestingly, if the winter months are particularly cold the number of *Brevicoryne brassicae* found during the summer is greatly reduced (Moyes, 1997).

Like all aphid species, certain generations of *Brevicoryne brassicae* are regularly wingless (apterous), however winged individuals (alates) are produced, often as a result of colony overcrowding. Apterous aphids are grey-green in colour with a mealy covering (Figure 1.8.). Eight dark spots are visible on the abdomen, which gradually increase in size towards the posterior end. Alates are smaller in size, pale green in colour with no mealy covering (Figure 1.9.).

Figure 1.8. Apterous *Brevicoryne brassicae* © http://www.extento.hawaii.edu.

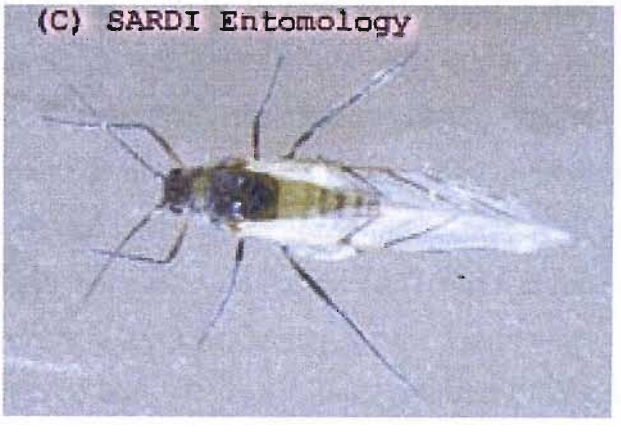


Figure 1.9. Alate *Brevicoryne brassicae* © *SARDI Entomology*

As expected for phloem-feeders, aphids are exposed to plant secondary metabolites, including glucosinolates, translocated in the phloem. It is suggested that these plant-

specific chemicals act as host selection cues for host-specific aphids (Cole, 1997). This reaffirms the choice of *Brevicoryne brassicae* as the experimental herbivore. Differences in glucosinolate profile among *Brassicas* have been linked to host preferences by specialist herbivores (Rodman & Chew, 1980). There have been numerous studies investigating how various aphid species, including specialist aphids respond to these compounds and whether they influence their performance in the laboratory (examples include van Emden, 1990; Ronquist & Åhman, 1990; Cole, 1996; 1997). However, a notable downfall is how little work has been done observing the response of these insects in the field and indeed linking these results to those obtained in the laboratory. One of my main aims is to observe the response and monitor the performance of *Brevicoryne brassicae* on *Brassica oleracea* subsp. *oleracea* both in the laboratory and at the two field sites in Dorset. I will then proceed to determine whether a relationship exists to link the activity of *Brevicoryne brassicae* to glucosinolates produced by wild populations of *Brassica oleracea*. This is discussed in greater detail during section 1.6 of this chapter.

1.5. Third Trophic Level

Interest is growing on the role of plant-produced chemicals and their influence on insects in the third trophic level. It is generally accepted that natural enemies use chemical information from both trophic levels, however debate continues as to how this information is utilised. One explanation is the "reliability-detectability hypothesis" proposed by Vet and Dicke (1992). It states that the value of chemical information depends on two factors. Firstly, the reliability of the stimulus in that it should indicate specific information about the herbivore, and secondly, the level of detectability of the stimulus. Vet and Dicke (1992) proposed that plant volatiles play an important role in mediating searching behaviour, particularly at distances among natural enemies of herbivores. Plant volatiles are thought to be utilised by parasitoids at longer distances to locate suitable habitats where herbivores may be present and then use herbivore-derived chemicals once in the habitat to locate their prey (Dicke, 2000).

This is supported by Geervliet *et al.*, (1994). *Cotesia glomerata,* a generalist parasitoid of *Pieris* caterpillars appears to utilise plant-derived chemicals to assess the suitability and profitability of patches of plants.

Herbivore-damaged plants emit both quantitatively and qualitatively different volatile blends compared to intact plants (Dicke & van Loon, 2000). Recent studies have shown that parasitoids are attracted to specific volatile profiles from herbivore-damaged plants. Bradbourne and Mithen (2000) investigated the differential attraction of *Diaeretiella rapae* to two near-isogenic lines of *Brassica oleracea* differing only in the structure of an isothiocyanate produced under semi-field conditions. Results obtained were confirmed when Blande (2004) showed an attraction of *Diaeretiella rapae* to synthetic 3-butenylisothiocyanate in the laboratory. Interestingly, Vaughn *et al.*, (1996) found that both male and female *Diaeretiella rapae* respond to a component of green-leaf volatiles, (Z)-3-hexanol-1-ol, but only females respond to isothiocyanates.

An important factor of parasitoid biology is their putative ability to learn to respond to chemical stimuli after an experience of their plant-host complex (PHC). This behavioural plasticity has been documented in several papers (review by Turlings *et al.*, 1993; Vet *et al.*, 1995). This learning ability could be important in tritrophic interactions as it suggests that in addition to their apparent innate response to chemical stimuli, parasitoids can also adapt to changes in their environment. This phenomenon could have important implications on the population dynamics of both the parasitoid and its herbivore.

This form of indirect chemical plant defence linking plants and natural enemies is an interesting concept and highlights a number of factors. These include the need to study plant-insect interactions from a multitrophic perspective (Price *et al.*, 1980), the plasticity of learning in natural enemies, particularly parasitoids (Vet, 1999) and the effect intraspecific genetic variation in plants has on plant-insect interactions (Raybould & Moyes, 2001).

1.5.1. Life Strategies of Parasitoids

The majority of parasitoids belong to the Order *Hymenoptera* of which *Symphyta* and *Apocrita* are two Sub-Orders. *Apocrita* is further divided into *Parasitica* and *Aculeata.* The sub-division *Parasitica* comprises mostly parasitoids and it is in this division that the *Ichneumonoida* are found, which in turn contains the *Braconidae* to which *Diaeretiella rapae*, our parasitoid belongs. Members of the *Braconidae* are

distinguishable from *Ichneumonoidae* by the lack of a cross-vein, 2m-cu, in the forewing (Chinery, 1993).

Parasitoids follow a holometabolous life cycle, consisting of an egg, larva, pupa and adult stage (Figure 1.10.). Parasitoids can be divided into two classes based on the feeding habit of the larval stage. Endoparasitoids lay their eggs inside their host whilst ectoparasitoids live externally, but have their mouthparts buried in the body of their host (Godfray, 1994).

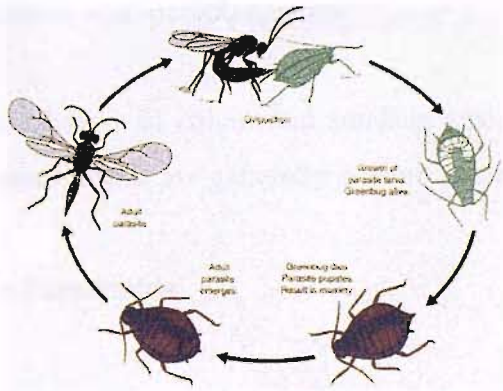


Figure 1.10. Life Cycle of a Parasitoid.
© http://www.entomology.wisc.edu

1.5.2. *Diaeretiella rapae*

Diaeretiella rapae (M^{c}Intosh) (Hymenoptera: Braconidae) is the parasitoid representing the third trophic level. *Diaeretiella rapae* is an endoparasitoid primarily of, but not exclusively, *Brassica*-feeding aphids including *Brevicoryne brassicae*. Once a suitable aphid host has been found, the adult female oviposits inside the body cavity using her adapted ovipositor (Figure 1.11.). The larvae then emerge from the eggs and proceed to feed internally, gradually killing the aphid. This results in the outer skin of the aphid being modified to form a tough, protective brown-yellow shell. These "mummies" usually appear after 8-10 days depending on the species and remain on the leaves of a plant until the larvae have pupated (Figure 1.12.). The adult wasp emerges from the mummified cases shortly afterwards, usually after 9-12 days, which is species specific and dependent on various conditions including temperature. The generation time for aphid parasitoids varies between 10-14 days (G. Poppy. pers. comm.).

Figure 1.11. *Diaeretiella rapae* ovipositing inside an aphid.
©http://ccvipmp.ucdavis.edu/insects/index.html

Figure 1.12. Mummified aphids.
© www.vegedge.umn.edu

Braconid wasps are black-brown in colour and small in size, ranging from 2-3mm to 15mm in length. *Diaeretiella rapae* are generally 2-3mm in size (Godfray, 1994).

1.5.3. Host Selection by Parasitoids

Many insect parasitoids appear to locate their hosts using a series of physical and chemical cues (Vinson, 1976). These cues enable the female to search more restricted habitats thus maximising the probability of locating suitable hosts.

The process of host selection resulting in successful parasitism by parasitoids has been grouped under five headings (Vinson, 1976):

- Host-habitat location
- Host location
- Host acceptance
- Host suitability
- Host regulation

Numerous studies indicate that chemicals play a major role for parasitoids during host-habitat location (Price *et al.*, 1980; Vet & Dicke, 1992; Du *et al.*, 1996; 1997; Thaler, 1999; Vet, 1999; Dicke, 1999; 2000; Agrawal, 2000; Dicke & van Loon, 2000). It is increasingly accepted that *Hymenopterous* parasitoids use long-range chemical cues such as plant volatiles to locate a suitable host habitat. Storeck *et al.*, (2000) demonstrated that female *Aphidius colemani* use plant chemical cues to locate the habitat of their host. This directed response is more common in mono- and oligophagous parasitoids than polyphagous parasitoids. Polyphagous parasitoids have a

wider host range, therefore detecting specific chemicals from a number of plants would be counter-productive (Powell & Wright, 1992). This is promising for *Diaeretiella rapae* as it is generally accepted that it is a specialist parasitoid with a relatively narrow host range, therefore, theoretically this species should show a direct response to a small number of host and plant volatiles. This explains why this insect was chosen to represent the third trophic level.

Once a suitable host-habitat has been selected, the female parasitoid needs to locate its host. In addition to visual cues, it is suggested that plant chemicals are involved in host location (Vinson, 1976). This could be explained by the notion that plants are more apparent in space and time in terms of the quantities of chemicals produced due to their larger biomass. In addition to plant odours, numerous studies indicate that host odours such as frass, sex pheromones and alarm pheromones are important during host location (Vinson, 1976; Vet & Dicke, 1992). Host odours are undoubtedly more reliable indicators of host presence than plant odours. These host-derived odours are referred to as short-range cues that the parasitoid utilises once in a suitable habitat to locate its host (Vet & Dicke, 1992).

Specific volatile blends released by plants when they are damaged by herbivores (host-induced synomones), benefit both the plant and the parasitoid. The plant benefits by attracting the parasitoid, which acts to reduce the number of herbivores feeding on the plant, whilst the parasitoid benefits by locating suitable hosts in which to oviposit. A review by Powell *et al.*, (1998) highlights that when *Vicia faba* is damaged by *Aphis pisum*, chemicals are released which attract the parasitoid, *Aphidius ervi.*

The decision as to whether to oviposit inside the host is the next stage of host selection and is termed host acceptance (Vinson, 1976). This stage mainly concerns visual and olfactory cues emanating from the host, therefore plant chemical cues have a less significant role and will not be discussed in further depth.

Host suitability and host regulation are the final steps for successful parasitism by parasitoids (Vinson, 1976). Host suitability is confirmed when the parasitoid decides that an insect is suitable to be utilised as prey. Nutritional and physical characteristics of the host are important during this stage (Vinson, 1976). Host regulation concerns the physiological changes occurring to the host induced by the parasitoid which cause it to

behave differently (Vinson, 1976). These two final stages do not generally involve chemical plant cues and so will not be discussed.

Parasitoids are becoming more important in the biological control of pests as the realisation of the impact insecticides are having on the environment dawns. Studying these specialist insects in a tritrophic context will increase our knowledge on its biology and ecology, which will hopefully enable us to exploit its life cycle for maximum effectiveness in controlling aphids. The end result will ultimately be to utilise parasitic wasps in integrated pest management programmes.

1.6. Thesis Aims and Hypotheses

This study combines behavioural and performance bioassays with chemical analyses in controlled laboratory conditions with performance investigations in a natural ecosystem. Experiments were designed to investigate how the glucosinolate phenotype of *Brassica oleracea* subsp. *oleracea* influences a specialist aphid species, *Brevicoryne brassicae* and its parasitoid, *Diaeretiella rapae*. The aim is to take a bottom-up approach to this work, investigating whether the glucosinolate genotype acts as a selection pressure on the insects within a tritrophic context both in the laboratory and in the field. In brief, do plant genetics drive tritrophic interactions?

Chapter Two investigates the variation of physical and chemical traits in the experimental plant. A variety of morphological traits including leaf waxiness and trichome density are investigated in *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door. Glucosinolate analysis was carried out to determine the quantitative and qualitative differences between wild cabbage plants from Kimmeridge and Durdle Door. Volatile entrainments were also conducted, paying attention to the release of 3-butenylisothiocyanate in order to understand the role of this volatile on insects in the third trophic level.

Issue One: Hypotheses:

- *Brassica oleracea* subsp. *oleracea*, both within and between field sites (Kimmeridge and Durdle Door), will differ both quantitatively and qualitatively in terms of its glucosinolate content and volatile breakdown products.
- *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door will not differ extensively in terms of its physical defensive features.

Issue Two: Hypothesis:

- A trade-off exists regarding the investment *Brassica oleracea* subsp. *oleracea* makes between chemical and physical defences.

Chapter Three investigates the selection and performance of *Brevicoryne brassicae* collected from both field sites to *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door under controlled laboratory conditions.

Hypotheses:

- *Brevicoryne brassicae* will be able to differentiate between Brassicas and select the host producing the highest total quantity of aliphatic glucosinolates.
- The highest intrinsic rate of natural increase of *Brevicoryne brassicae* will occur on *Brassica oleracea* subsp. *oleracea* producing the highest quantity of aliphatic glucosinolates.
- "Host-line" variation in *Brevicoryne brassicae* will be observed.

Chapter Four investigates the behavioural response of *Diaeretiella rapae* collected from both field sites to odours emitted from the experimental plant and their acceptance of *Brevicoryne brassicae* under controlled laboratory conditions.

Hypotheses:

- *Diaeretiella rapae* will be able to differentiate between plant-host complexes (PHC) from Kimmeridge and Durdle Door. *Diaeretiella rapae* will preferentially select odours emitted from the "typical" plant-host complex (PHC) from Durdle Door.
- When synthetic 3-butenylisothiocyanate is added to the "typical" plant-host complex (PHC) from Kimmeridge, the response of *Diaeretiella rapae* will be equal to that observed towards the "typical" plant-host complex (PHC) from Durdle Door.
- Regardless of parasitoid origin, *Diaeretiella rapae* will differentially attack the plant-host complexes (PHC) from Kimmeridge, Durdle Door and the control, displaying greater attack rates on the plant-host complex (PHC) from Durdle Door.

Chapter Five aims to relate the quantity and quality of glucosinolates produced by *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door to the

performance of *Brevicoryne brassicae* and the activity of *Diaeretiella rapae* observed in the laboratory to observations in a natural ecosystem.

Hypothesis:

- *Brassica oleracea* subsp. *oleracea* at Durdle Door will host the greatest activity of *Brevicoryne brassicae* and *Diaeretiella rapae*, both in terms of the proportion of plants infested and the severity of infestation, compared to *Brassica oleracea* subsp. *oleracea* at Kimmeridge.

Chapter Two

First Trophic Level

Physical and Chemical Defence Characteristics of *Brassica oleracea* subsp. *oleracea*.

Chapter Two

Chemical and Physical Defence Characteristics of *Brassica oleracea* subsp. *oleracea*.

2.1. Introduction

Moyes (1997) discovered that *Brassica oleracea* subsp. *oleracea* at Kimmeridge and Durdle Door are extreme producers of glucosinolates (Chapter One). The primary aim of this chapter is to determine the glucosinolate phenotype of wild cabbages at these two sites, to ascertain whether these profiles remain.

It is thought to be rare for a plant to simultaneously invest in physical and chemical defences in its resistance against herbivory (Rosenthal & Kotanen, 1994). This suggests that a trade-off occurs between the chemical and physical defensive weapons of choice. Therefore, the second aim of this chapter is to investigate certain physical features of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and to address whether a trade-off between physical and chemical defences occurs in this species.

2.1.1. Chemical Defence Characteristics

- **Constitutive Chemical Defence**

Glucosinolates are secondary plant metabolites, found in 16 families of dicotyledons (Fahey *et al.*, 2001), however, they are extensively detected in members of the Brassicaceous family (Daxenbichler *et al.*, 1991; Brudenell *et al.*, 1999). A unique profile of glucosinolates is produced by each Brassica species (Branca *et al.*, 2002), which is under a high degree of genetic (Jain *et al.*, 1990; Reed *et al.*, 1993; Magrath *et al.*, 1994; Bak *et al.*, 1999; Mithen, 2001) and environmental regulation (Wolfsen, 1982; Inglis *et al.*, 1992). Glucosinolates are constitutively produced, which directly deters generalist herbivores (Chew, 1988; Glen *et al.*, 1990; Louda & Mole, 1991; Giamoustaris & Mithen, 1995; Shields & Mitchell, 1995). However, studies also reveal that an increase in glucosinolate content occurs in response to plant damage, which forms an additional aspect of the plants' direct chemical defence armour against herbivory. Types of plant damage causing this increase in glucosinolate content include

herbivore damage (Bartlet *et al.*, 1996; 1999), pathogen infection (Butcher *et al.*, 1974) and abiotic factors such as the application of jasmonic acid (Thaler, 1999) and salicylic acid (Kiddle *et al.*, 1994).

Studies demonstrate that the glucosinolate profile varies between Brassica species (See reviews by Daxenbichler *et al.*, 1991; Fahey *et al*, 2001). However, it is revealed that the glucosinolate profile also varies within Brassica species (Moyes, 1997; Raybould & Moyes, 2001). This high degree of glucosinolate variation in Brassicas could differentially affect insects in the second and third trophic level, to the extent whereby plant chemistry could act as a bottom–up selecting agent that could drive tritrophic interactions. For this reason the quantity and quality of glucosinolates produced by populations of wild cabbages from Kimmeridge and Durdle Door will be determined.

Several studies have investigated the glucosinolate content of Brassicas (Magrath *et al.*, 1994; Mithen, 1992; 2001; Moyes *et al.*, 2000). The most widely used method to determine the glucosinolate content is by High Performance Liquid Chromatography (HPLC) (for example Magrath *et al.*, 1994; Moyes *et al.*, 2000). Therefore, HPLC will be used to determine the desulphoglucosinolate content of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door.

- **Inducible Chemical Defence**

As stated, numerous studies demonstrate that glucosinolates within plant tissue deter feeding by generalist herbivores (Chew, 1988; Glen *et al.*, 1990; Louda & Mole, 1991; Mithen, 1992; Bartlet *et al.*, 1996; 1997). However, specialist herbivores have adapted to recognise the glucosinolate profiles produced by specific Brassica plants (Rodman & Chew, 1980; van Emden, 1990; Landolt, 1993, Mithen *et al.*, 1995b). In response, it appears that plants have evolved a further level of chemical defence to counteract this.

Herbivore damage causes the hydrolysis of glucosinolates to volatile and non-volatile breakdown products including isothiocyanates, nitriles and thiocyanates (Cole, 1976) (Chapter One). This inducible level of chemical defence is thought to indirectly defend the plant against specialist herbivores by attracting natural enemies in the third trophic level (Vet & Dicke, 1992; Thaler, 1999; Dicke, 1999; 2000; Dicke & van Loon, 2000). Therefore, it seems as if a mutualistic relationship has occurred between organisms in

the first and third trophic levels. Evidence is provided by work on aphid parasitoids, which reveals that they are able to utilise these volatile products (Vaughn *et al.*, 1996). *Diaeretiella rapae* exhibit a positive directed response to a specific isothiocyanate (3-butenylisothiocyanate) produced from Brassica plants (Bradbourne & Mithen, 2000). The role of synthetic 3-butenylisothiocyanate during foraging by *Diaeretiella rapae* was confirmed by Blande (2004).

Despite the putative importance of indirect plant chemical defence, research of headspace volatiles produced by Brassicas is limited (Cole, 1976; Tollsten & Bergström, 1988). Previous research has concentrated on detecting volatiles emitted from specific plant parts (Tollsten & Bergström, 1988) or from mechanically damaged plants (Cole, 1976; Tollsten & Bergström, 1988). This could provide misleading insights as damaging the plant causes changes in the volatile composition of the sample (Blight, 1990). Despite this, work on headspace volatiles emitted from clean, undamaged plants remains extremely under-researched. Therefore, the volatiles present in the immediate vicinity of whole wild cabbage plants, the headspace volatiles, are investigated in this chapter. Volatiles emitted from both clean, undamaged and aphid-infested wild cabbage plants will be collected using a system called air entrainment and analysed using Gas Chromatography. The presence of 3-butenylisothiocyanate released from *Brassica oleracea* subsp. *oleracea* will be concentrated on because of its putative importance during parasitoid foraging (Bradbourne & Mithen, 2000; Blande, 2004). The aim is to reveal any differences in the release and/or concentration of this compound from wild cabbage populations at Kimmeridge and Durdle Door and whether aphid infestation has any effect.

Wolfsen (1982) and Inglis *et al.*, (1992) revealed that soil conditions could affect the quantity and quality of glucosinolates produced by Brassica plants. Therefore, it is possible that the profile of headspace volatiles emitted from Brassicas is affected by the soil conditions the plants are growing in. This is the first known study to address this. Headspace volatiles will be collected from *Brassica oleracea* subsp. *oleracea* grown in soil collected from both Kimmeridge and Durdle Door. Any differences in terms of the release and/or concentration of 3-butenylisothiocyanate will be focused upon.

2.1.2. Physical Defence Characteristics

Morphological features including thorns, spines, domatia (Agrawal *et al.*, 2000), tissue toughness (Heil & Bostock, 2002) and waxblooms (Eigenbrode & Kabalo, 1999) can form part of a plants' physical defensive armour against herbivore attack. Physical plant defence is under-researched, with only a few known studies published (Åhman, 1990; Shepherd *et al.*, 1995; Lambdon *et al.*, 1998; Maurico, 1998; Eigenbrode & Kabalo, 1999; Agrawal *et al.*, 2000; Traw & Bergelsom, 2003).

Physical features can directly defend plants against herbivore attack. Surface wax can either hinder the attachment of insects (Åhman, 1990) or decrease feeding by specialist insects (Lambdon *et al.*, 1998). Physical traits can also indirectly defend plants against herbivory by providing protective environments (Agrawal *et al.*, 2000) or by aiding the attachment (Eigenbrode & Kabalo, 1999) of insects in the third trophic level. Trichomes (Traw & Bergelson, 2003) and spines (Cooper & Ginnett, 1998) can also be induced in response to herbivory to protect against further damage. In addition, changes in leaf size and branch density can occur in response to damage (Grubb, 1992).

Despite the putative important role physical defences play in the battle against herbivory, research into Brassicas appears to concentrate on the chemical aspect of plant defence (Chew, 1988; Glen *et al.*, 1990; Louda & Mole, 1991; Mithen, 1992; Vet & Dicke, 1992; Bartlet *et al.*, 1996; 1997; Thaler, 1999; Dicke, 1999; 2000; Dicke & van Loon, 2000). This suggests that chemical defence is considered to be the most important aspect of a plant's defensive shield. However, few studies have investigated aspects of both physical and chemical defence (Maurico, 1998; Steward & Keeler, 1988; Traw & Bergelson, 2003). This chapter aims to address this lack of knowledge by investigating both forms of plant defence in a single Brassica species. The first section of this chapter investigates a specific aspect of constitutive and induced chemical defence, whilst the latter section investigates certain morphological features of the wild cabbage.

Ultimately, this chapter concentrates on two important issues. The first issue aims to investigate the chemical and physical defensive strategies observed in *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door. The second issue attempts to

determine whether a trade-off between these two defensive strategies occurs in this species.

Issue One: Hypotheses:

- *Brassica oleracea* subsp. *oleracea*, both within and between field sites (Kimmeridge and Durdle Door), will differ both quantitatively and qualitatively in terms of its glucosinolate content and volatile breakdown products.
- *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door will not differ extensively in terms of its physical defensive features.

Issue Two: Hypothesis:

- A trade-off exists regarding the investment *Brassica oleracea* subsp. *oleracea* makes between chemical and physical defences.

2.2. Materials and Methods

2.2.1. Chemical Defence Characteristics

Investigating the Glucosinolate Profile of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door.

- Seed Collection – Natural Population

Seeds were collected from each labelled flowering plant along the transects at both field sites in August 2002. A description of the transects at Kimmeridge and Durdle Door is stated in Chapter Five.

- Seedling Growth

A sample of ten seeds, collected from each labelled flowering plant at Kimmeridge and Durdle Door were sown in individual plastic pots (7x7x8cm, Sopodex, UK) in Levingtons® compost. The plants were sown and grown at the University of Southampton glasshouses (20°C ± 3°C, L12:D12) and watered as required.

- Seedling Collection

The method described follows that stated by Moyes (1997). Five seedlings from each of the ten seeds sown collected from each flowering plant at Kimmeridge and Durdle

Door were randomly selected for glucosinolate analysis. Leaves of the five seedlings chosen were harvested and pooled together when the plants were at the 3-4 leaf stage. The pooled leaves from each plant were kept separate, wrapped in aluminium foil and immediately frozen at -80°C. The leaf samples were then transferred to individually labelled flower sleeves (Avoncrop, Bracknell, UK) and freeze-dried using a freeze drier for 2–3 days. The freeze-dried plant samples were finely milled and stored in individually labelled air-tight containers (Bibby Sterilin LtD, Staffordshire, UK) until glucosinolate analysis could be carried out. The glucosinolate content of seedlings was determined as evidence suggests that young *Brassica napus* leaves have higher amounts of glucosinolates than mature leaves (Lambdon *et al.*, 2003). In addition, Moyes (1997) revealed that glasshouse-grown wild *Brassica oleracea* seedlings contained the same glucosinolates found in wild populations, albeit in smaller concentrations.

- Glucosinolate Analysis

Glucosinolate analysis was carried out in collaboration with Julietta Marquez at Nottingham University. The process is described by Magrath *et al.,* (1994).

Preparation of the A25 Sephadex Columns

A reservoir, frit and a needle were assembled to make the columns and were suspended vertically. 0.5ml of distilled water was added to the column. This wets the frit and ensures the needle on the column is "dripping." 13g of A25 Sephadex (Amersham Biosciences, Sweden) was added to 100ml of sodium acetate and left to expand. The resin was then filtered and the filtrate removed from the filter paper and suspended in fresh sodium acetate. The resin was filtered a second time, washed with distilled water, resuspended in excess sodium acetate and stored at 4°C. 0.5ml of the suspended resin (prepared A25 Sephadex) was added the column and left to settle. The column was then washed with 0.5ml of distilled water.

Sulphatase Purification

300mg of sulphatase (Sigma Chemicals, Poole, Dorset, UK) was added to 12ml of cold distilled water and stirred until dissolved. 12ml of cold ethanol was added and centrifuged at 3000rpm for 6 minutes. The precipitate was discarded and 1.5 volumes of cold ethanol was added to the supernatant and stirred. This was centrifuged cold at 3000rpm for 6 minutes. The supernatant was discarded and the precipitate was dissolved in 8ml of distilled water. 2ml batches were run

through 4 A25 Sephadex columns and then repeated to pass through 4 C25 Sephadex columns.

Glucosinolate Leaf Extraction

0.3g - 0.4g of milled, freeze-dried leaf tissue was added to 10ml of 70% methanol at 70°C. 100µl of the internal standard (glucotropaeolin) was added and the samples vortexed. The samples were incubated at 70°C for 20-30 minutes. After cooling the samples were centrifuged at 3000rpm for 5 minutes. 3ml of the supernatant was dripped through an A25 Sephadex column. This was washed twice with 0.5ml of distilled water and then washed twice with 0.5ml of 0.02M sodium acetate. Collecting vials were placed underneath the columns whilst 75µl of prepared sulphatase was added to the surface of the Sephadex and left overnight at room temperature. The resulting desulphoglucosinolates collected in the vials were eluted with a total of 1.25ml of distilled water. The samples were stored at –20°C until they could be separated and quantified using High Performance Liquid Chromatography (HPLC).

High Performance Liquid Chromatography (HPLC)

Glucosinolate content was quantitatively and qualitatively analysed in collaboration with Julietta Marquez at Nottingham University (School of Biosciences) using an Agilent (UK) HPLC-MS machine.

Glucosinolate Identification

The separated peaks from HPLC analysis were identified by mass spectrometry by Julietta Marquez at Nottingham University (School of Biosciences).

- Statistical Analysis

All data was analysed using Minitab, Version 13. All quantitative and qualitative differences in glucosinolates were analysed using a 2-sample t-test.

Investigating the Headspace Volatiles of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door.

- Plants

As stated, soil conditions are thought to affect the quantity and quality of glucosinolates produced by Brassicas (Wolfsen, 1982; Inglis *et al.*, 1992). Therefore, it is likely that soil conditions affect the quantity and quality of glucosinolate-breakdown volatiles released. This is the first known study to investigate whether the soil conditions affect

the headspace volatiles released by *Brassica oleracea* subsp. *oleracea*. Ten seeds of wild *Brassica oleracea* from Kimmeridge and Durdle Door, both "typical" and "atypical" of their field site (See Results for explanation of "typical" and "atypical" plants) were sown in soil collected from Kimmeridge. This was repeated on a separate occasion, when the seeds were sown in soil collected from Durdle Door. Plants were grown in the glasshouses at the University of Southampton (20°C ± 3°C, L12:D12) and watered when required. Plants were used in experiments when at the 4-5 leaf stage.

Four plants from each of the four types investigated were selected from the ten seeds initially sown. Only undamaged plants, free from chlorosis, mechanical damage or herbivore attack were selected and used for volatile collection. The plants were moved to a controlled temperature room (20°C ± 3°C, L16:D8) 48 hours prior to volatile collection where they remained for the duration of the experiment. The headspace volatiles of four clean, undamaged plants of each type (Kimmeridge "typical," "atypical," Durdle Door "typical" and "atypical") were collected each day. After collection, two of the four plants of each type were infested with a minimum of 150 *Brevicoryne brassicae*. All four plants were enclosed in flower sleeves (30x50x10cm, 40 micron punched, clear plastic. Avoncrop, Bracknell, UK) and secured at the top and bottom with masking tape and Sellotape™ respectively. Volatile collection for the four plants of each type was repeated exactly four days after infestation, when the aphids from the two infested plants were removed using a fine paintbrush. Therefore, volatiles were collected from the two clean, undamaged plants and the two previously infested plants. Plants were watered when required.

- Insects

Brevicoryne brassicae, reared on Brussels sprout var. Montgomery (Moles Seeds, UK), were used to infest the experimental plants. Insects were reared in individually heated cages (20°C ± 3°C, L16:D8) and watered when required. Aphids were transferred to experimental plants using a fine paintbrush.

- Air Entrainment Preparation

Thermal desorption tubes used for volatile collection were filled with 50mg of Tenax material (Tenax TA 60/80 Supelco, Bellefonte, PA, USA). The tubes were attached to Teflon tubes connected to a nitrogen supply (0.2ml/min) and heated in a heating block overnight (225°C). A charcoal filter was activated by attaching it to Teflon tubing via a

Swagelock fitting (Swagelock, UK) connected to nitrogen supply (0.2ml/min) and heated overnight. All entrainment glassware was washed in warm water and detergent (Decon Laboratory LtD, East Essex, UK), rinsed with distilled water, rinsed with acetone, followed with a final rinse of distilled water before being baked overnight at a temperature of 220°C.

- Volatile Collection - Air Entrainment

Headspace volatiles from each of the experimental plants were collected using a system called air entrainment. The aerial parts of each plant were enclosed inside a glass vessel (87mm inner diameter, 100mm outer diameter, length 230mm, volume 1.55L). A second glass vessel encapsulated the stem of the plant, thus acting as a lid for the glass chamber. The two glass vessels were attached together by the use of bulldog clips. The procedure described follows the method stated by Agelopoulos and Pickett (1998) and Agelopoulos *et al.*, (1999). Clean, filtered air was pushed though the base of the vessel via Teflon tubing connected via a Swagelock fitting at a rate of 1.2 litres per minute. Purified air was then pulled over the plant contained in the glass chamber and the volatiles within the chamber were collected on the Tenax tube situated at the top of the vessel connected to Teflon tubing via a Swagelock fitting at a rate of 1 litre per minute. Two entrainments were performed simultaneously from the same airflow. Therefore, 1.2 litres of filtered air was pushed into both chambers resulting in a flow of 600 litres of airflow per minute pushed in, with 500 litres of air per minute flowing out of the two chambers (Figure 2.1.).

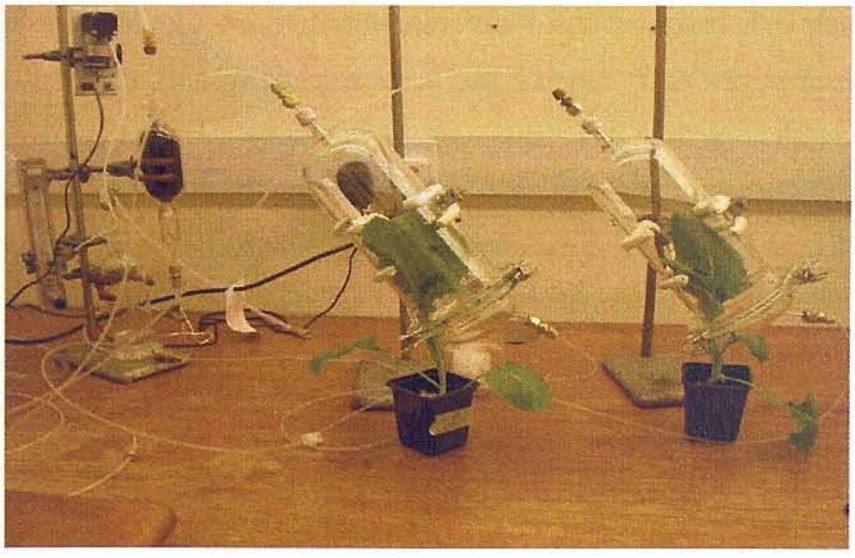


Figure 2.1. Air Entrainment Apparatus.
Photograph by T. Stirrup, 2004.

Headspace volatiles were collected for two hours for each plant, after which the airflow was stopped and the Tenax tubes removed. The volatiles collected on to the Tenax tubes were analysed by Gas Chromatography.

- Gas Chromatography

The headspace volatiles collected were analysed using a Hewlett Packard 6890N Gas Chromatograph, fitted with an OPTIC 2 programmable injector, a capillary column (50m x 0.32mm inner diameter) and an FID (Flame Ionisation Detector). The injector temperature (30°C) and pressure (18psi) was equilibrated for 1 minute. The injector temperature was then increased to 220°C at 16°C per second for 6 minutes. The carrier gas was helium (18psi). The oven temperature was set to 30°C for 2 minutes and then programmed to increase at a rate of 5°C per minute until it reached 150°C. The temperature was further programmed to increase by 10°C per minute to 250°C and maintained for 6 minutes. The headspace volatile samples were introduced to the column by thermal desorption. The data were analysed using ChemStation Plus.

The principle aim was to identify and detect the presence of 3-butenylisothiocyanate in the headspace samples of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door. 1µl of pure synthetic 3-butenylisothiocyanate (Rothamsted Research, Harpenden, Herts, UK), concentration 50ng/µl, was injected into the column. This was repeated four times.

Each headspace sample was then analysed for the presence/absence of 3-butenylisothiocyanate. An external calibration curve was constructed to quantify the amount of 3-butenylisothiocyanate present in each sample. This was performed by Dr. Alex Stewart-Jones at the University of Southampton. The calibration curve was constructed by adding 12.5ng, 25ng, 50ng and 100ng of synthetic 3-butenylisothiocyanate (Rothamsted Research, Harpenden, Herts, UK) onto Tenax filled thermal desorption tubes. The samples were then introduced to the GC by thermal desorption as previously stated. A calibration curve was then plotted and a line equation generated. The peak areas of 3-butenylisothiocyanate present in the headspace samples present were converted to ng by means of the line equation. The amount of 3-butenylisothiocyanate was adjusted to account for the percentage of sample lost due to the differing inlet/outlet air-flow over the plant and the sample collection time. This gives the amount of compound present per plant per hour (ng/plant/hour).

2.2.2. Physical Defence Characteristics

Investigating Common Morphological Features of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door.

- Plants

60 wild cabbage seeds collected from Kimmeridge were sown in Levingtons® compost in 7x7x8cm pots (Sopodex, UK) at the University of Southampton glasshouses (20°C ± 3°C, L12:D12). 30 of these seeds were collected from a plant revealed to be low in total glucosinolate content and thus declared "typical" of their field site. The remaining 30 seeds were "atypical" of the field site as they were collected from a plant known to have a high total glucosinolate content (See 2.3. Results). 60 wild cabbage seeds from Durdle Door were sown in the same way. 30 seeds "typical" of Durdle Door, collected from a high glucosinolate plant and 30 seeds "atypical" of Durdle Door, collected from a low glucosinolate plant. 60 Brussels sprout var. Montgomery (Moles Seeds, UK) seeds were sown to act as the control. Leaves one - six for each plant type were measured for leaf length, leaf area, leaf width, trichome density and amount of waxiness. Ten replicates per leaf were completed.

- Leaf Size

The morphological features of each plant type were determined when leaves reached a certain length. Leaves one and two were selected when they measured 40 - 70cm in length, leaves three and four were selected when they measured 70 - 90cm in length and leaves five and six were selected when they measured 90+ cm in length.

The length, width and area of each leaf were measured using an AM200 Portable Leaf Area Meter (ADC Bioscientific, Hoddesdon, Herts, UK).

- Trichome Density

Whole leaves were used to determine the total number of trichomes present on the surface of wild *Brassica oleracea* leaves from Kimmeridge and Durdle Door and Brussels sprout var. Montgomery (Moles Seeds, UK). The upper and lower leaf surfaces were observed under a light microscope, magnification x250 and the number of trichomes present per leaf was counted.

- Leaf Waxiness

The amount of wax present on the experimental leaves was determined following a modified method proposed by Eigenbrode *et al.,* (1991). Aluminium foil was cut to fit 9cm diameter glass Petri dishes. The Petri dishes containing the foil pieces were weighed using an Explorer OHAUS balance to give the initial weights. 20ml of Dichloromethane (Fisher Chemicals) was added to each Petri dish. One leaf per Petri dish was completely immersed in the solvent for 20 seconds, ensuring that both surfaces of the leaf were coated. The petiole of the leaf was not immersed to prevent the inner contents of the leaf coming into contact with the solvent. The Petri dishes were left undisturbed in a fume cupboard for 24 hours. This allowed time for the solvent to completely evaporate leaving the wax residue. The Petri dishes were then reweighed to give the final weights. The amount of wax was determined by subtracting the initial weight from the final weight ($\mu g/cm^2$).

- Statistical Analysis

Leaf morphological data were analysed using Principal Component Analysis, to collectively analyse all of the morphological features of each leaf. Non-normal data were transformed by Box-Cox transformation. (Minitab, Version 13).

2.3. Results

2.3.1. Chemical Defence Characteristics

Investigating the Glucosinolate Profile of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door.

Quantitative and qualitative differences in glucosinolate profile were revealed between populations from Kimmeridge and Durdle Door. The mean total glucosinolate content was greatest in seedlings from Durdle Door, whilst wild populations of *Brassica oleracea* from Kimmeridge produced the lowest total glucosinolate content (Figure 2.2.). Despite this, the difference in glucosinolate content between the two field sites was not shown to be statistically significant ($t = -0.64$, $p = 0.526$, $DF = 23$).

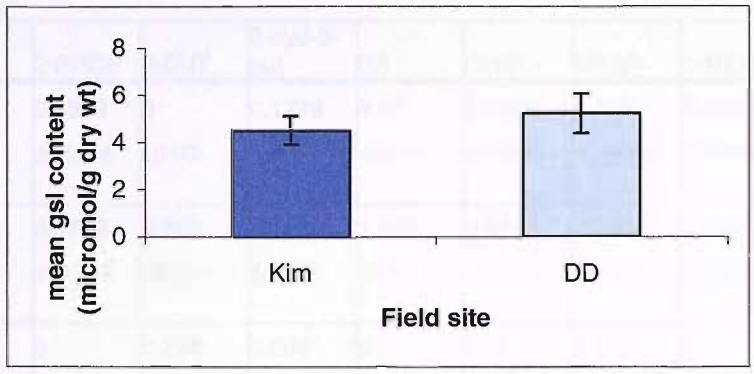


Figure 2.2. The mean total glucosinolates produced by *Brassica oleracea* subsp. *oleracea* collected from seed from Kimmeridge (Kim) and Durdle Door (DD). Quantities are expressed in µm/g dry wt (±SE). (t-test = -0.64, p = 0.526, DF = 23).

HPLC-MS analysis revealed that seven principle glucosinolates belonging to two of the three major classes of glucosinolates are produced in wild cabbages at Kimmeridge and Durdle Door. The aliphatic glucosinolates present were 2-propenylglucosinolate, 3-butenylglucosinolate and 2-hydroxy-3-butenylglucosinolate, whilst the indole glucosinolates present were hydroxyindolylmethylglucosinolate, indolylmethylglucosinolate, 1-methoxyindolylglucosinolate and 4-methoxyindolylglucosinolate (Table 2.1.). This is consistent with previous work (Mithen *et al.*, 1995b; Moyes *et al.*, 2000).

Sample	2-PROP	3-BUT	2-hyd-3-but	BR	OHBR	MEBR	1-MEBR	Total
Kimmeridge	0.6519	0	0.1779	3.07	0.0382	0.484	0.1083	4.531
B.oleracea	±0.0936	±0.00	±0.0640	±0.414	±0.0138	±0.0503	±0.0286	±0.588
N = 18								
Durdle Door	0.0759	0.693	2.221	1.937	0.011	0.2803	0.00743	5.225
B.oleracea	±0.0759	±0.131	±0.370	±0.344	±0.0110	±0.0590	±0.00743	±0.834
N = 7								
Brussels Sprout var. Montgomery N = 1	0	0.238	0.203	0	0.08	0.11	0	0.631

Table 2.1. The mean quantities of the individual glucosinolates present in *Brassica oleracea* subsp. *oleracea* collected from seed from Kimmeridge and Durdle Door. Brussels sprout var. Montgomery acts as the control. Quantities are expressed in µm/g dry wt (±SE). Aliphatic glucosinolates are shown in black, indole glucosinolates, total glucosinolates.

2-PROP = 2-propenylglucosinolate
3-BUT = 3-butenylglucosinolate
2-hyd-3-but = 2-hydroxy-3-butenylglucosinolate
BR = Indolylmethylglucosinolate
OHBR = Hydroxyindolylmethylglucosinolate
MEBR = 4-methoxyindolylmethylglucosinolate
1-MEBR = 1-methoxyindolylmethylglucosinolate

Statistical analysis reveals that there are quantitative differences in the amount of aliphatic and indole glucosinolates produced by *Brassica oleracea* subsp. *oleracea* both between and within field sites (Table 2.2. and Figure 2.3.). Wild cabbages from Kimmeridge differ significantly in the quantity of aliphatic and indole glucosinolates (t-test = -5.78, $p < 0.001$, DF = 34), however, this is not significant in plants from Durdle Door (t-test = 1.23, $p = 0.243$, DF = 12) (Figure 2.3.).

Sample	Mean Total Aliphatic Gsl	Mean Total Indole Gsl
Kimmeridge *B.oleracea* N = 18	0.83 ±0.141	3.701 ±0.476
Durdle Door *B.oleracea* N = 7	2.989 ±0.467	2.236 ±0.399
Brussels Sprout var. Montgomery N = 1	0.441	0.19

Table 2.2. Mean quantities of aliphatic and indole glucosinolates (gsl) produced by *Brassica oleracea* subsp. *oleracea* collected from seed from Kimmeridge and Durdle Door. Brussels sprout var. Montgomery acts as the control. Quantities are expressed in µm/g dry wt (±SE).

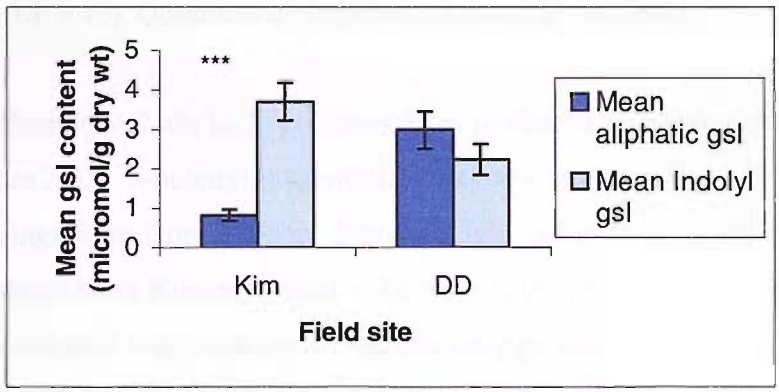


Figure 2.3. Quantitative differences in the production of aliphatic and indole glucosinolates produced by *Brassica oleracea* subsp. *oleracea* collected from seed from Kimmeridge and Durdle Door. (Kimmeridge; t-test = -5.78, p <0.001, DF = 34. Durdle Door; t-test = 1.23, p = 0.243, DF = 12). Quantities are expressed in µm/g dry wt (±SE).

Analysis reveals that wild *Brassica oleracea* seedlings from Kimmeridge and Durdle Door differ significantly in the amount of aliphatic glucosinolates produced (Figure 2.4.). Seedlings collected from Durdle Door produce significantly greater quantities of aliphatic glucosinolates compared to those collected from Kimmeridge (t = -5.96, p<0.001, DF = 23).

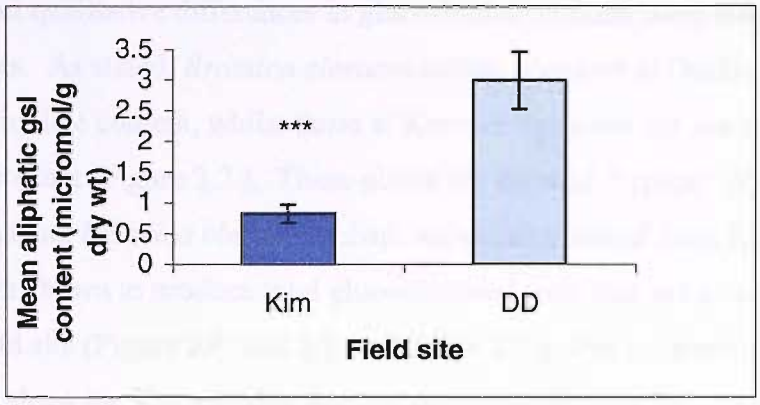


Figure 2.4. Mean quantities of aliphatic glucosinolates produced by *Brassica oleracea* subsp. *oleracea* from Kimmeridge (Kim) and Durdle Door (DD). ($t = -5.96$, $p<0.001$, $DF = 23$). Quantities are expressed in µm/g dry wt (±SE).

Qualitative differences of aliphatic glucosinolates produced between populations were revealed (Figure 2.5.). 3-butenylglucosinolate was only produced by wild *Brassica oleracea* seedlings from Durdle Door. 2-propenylglucosinolate was principally produced by plants from Kimmeridge ($t = 3.63$, $p < 0.001$, $DF = 23$), whilst 2-hydroxy-3-butenylglucosinolate was produced in significantly greater quantities by plants at Durdle Door ($t = -8.31$, $p < 0.001$, $DF = 23$). (See Appendix 1 for compound structures).

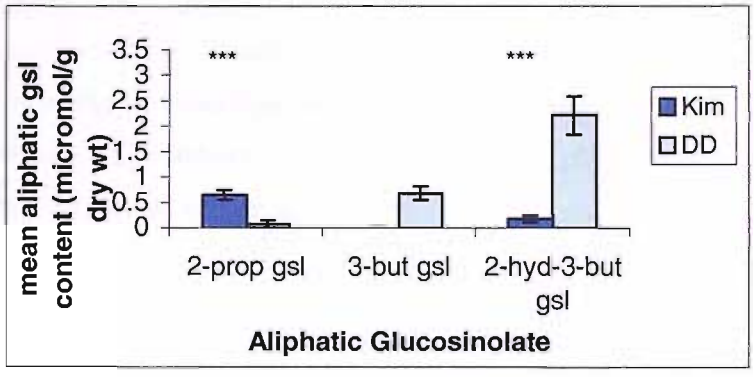


Figure 2.5. Qualitative differences in individual aliphatic glucosinolates produced by wild cabbage plants from Kimmeridge (Kim) and Durdle Door (DD). (2-propenylglucosinolate; $t = 3.63$, $p < 0.001$, $DF = 23$; 2-hydroxy-3-butenylglucosinolate; $t = -8.31$, $p < 0.001$, $DF = 23$). Quantities are expressed in µm/g dry wt (±SE).

2-prop gsl = 2-propenylglucosinolate

3-but gsl = 3-butenylglucosinolate

2-hyd-3-but gsl = 2-hydroxy-3-butenylglucosinolate

Quantitative and qualitative differences in glucosinolate content were also revealed within field sites. As stated, *Brassica oleracea* subsp. *oleracea* at Durdle Door have the greatest glucosinolate content, whilst those at Kimmeridge have the lowest glucosinolate content (Figure 2.2.). These plants are deemed "typical" of their field site. However, individual *Brassica oleracea* subsp. *oleracea* collected from Kimmeridge and Durdle Door are shown to produce total glucosinolate levels that are more characteristic of the other field site (Figure 2.6. and 2.7. and Table 2.3.). For example specific wild cabbage plants along the Kimmeridge transect produce high total levels of glucosinolates more commonly produced by plants at Durdle Door. These plants are termed "atypical."

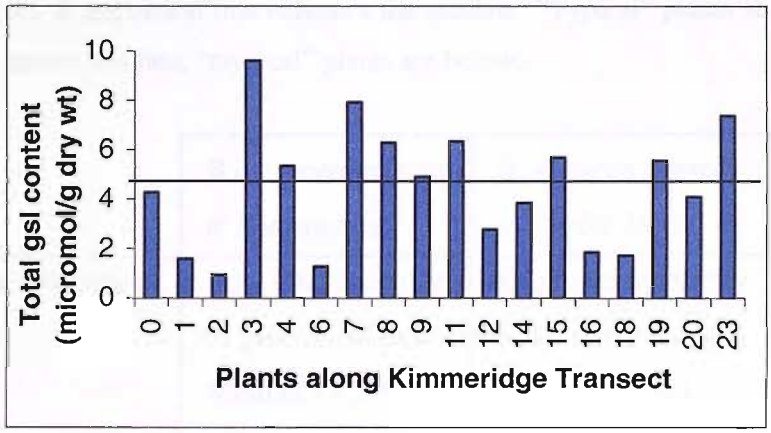


Figure 2.6. Quantitative differences in total glucosinolate content within *Brassica oleracea* subsp. *oleracea* from Kimmeridge. Quantities are expressed in µm/g dry wt. A horizontal line indicates the median. "Typical" plants are below this line, "atypical" plants are above.

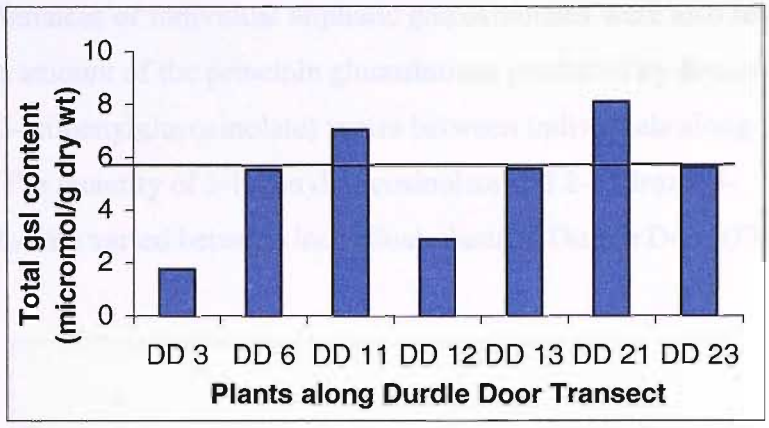


Figure 2.7. Quantitative differences in total glucosinolate content within *Brassica oleracea* subsp. *oleracea* along the transect at Durdle Door. Quantities are expressed in µm/g dry wt. A horizontal line indicates the median. "Typical" plants are above this line, "atypical" plants are below.

	B.oleracea present at Kimmeridge	*B.oleracea* present at Durdle Door
"Typical" Characteristics	Low total quantity of glucosinolates Median <4.593	High total quanitity of glucosinolates Median >5.563
"Atypical" Characteristics	High total quantity of glucosinolates Median>4.593	Low total quantity of glucosinolates Median<5.563

Table 2.3. Defining the characteristics of "typical" and "atypical" *Brassica oleracea* individuals along the transect at Kimmeridge and Durdle Door. The median value is stated, quantities are stated in µm/g dry wt.

Qualitative differences of individual aliphatic glucosinolates were also revealed within field sites. The amount of the principle glucosinolate produced by *Brassica oleracea* at Kimmeridge (2-propenylglucosinolate) varies between individuals along the transect (Figure 2.8.). The quantity of 3-butenylglucosinolate and 2-hydroxy-3-butenylglucosinolate varied between individual plants at Durdle Door (Figure 2.9.).

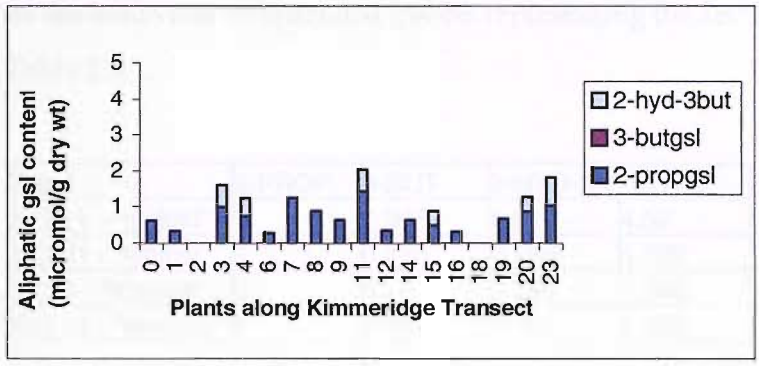


Figure 2.8. Qualitative differences in aliphatic glucosinolate content in *Brassica oleracea* from Kimmeridge. Quantities are expressed in µm/g dry wt.

2-hyd-3-but = 2-hydroxy-3-butenylglucosinolate

3-butgsl = 3-butenylglucosinolate

2-propgsl = 2-propenylglucosinolate

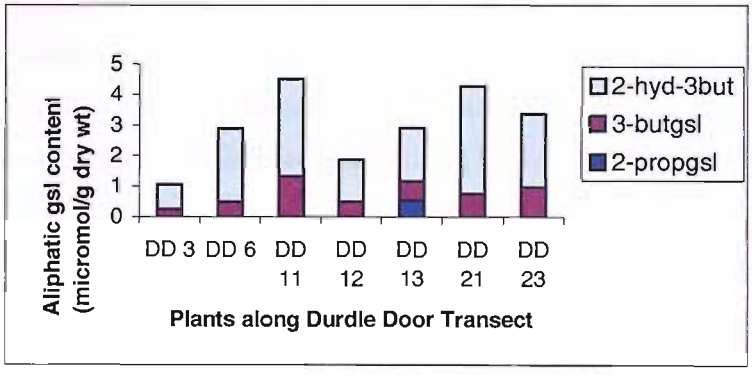


Figure 2.9. Qualitative differences in aliphatic glucosinolate content in *Brassica oleracea* within Durdle Door. Quantities are expressed in µm/g dry wt.

2-hyd-3-but = 2-hydroxy-3-butenylglucosinolate

3-butgsl = 3-butenylglucosinolate

2-propgsl = 2-propenylglucosinolate

As stated, HPLC analysis enabled the glucosinolate profile of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door to be determined. As revealed, huge

variations in glucosinolate profile occur between individuals both within and between field sites. This allowed the selection and use of specific wild *Brassica oleracea* individuals from Kimmeridge and Durdle Door, which are known to be extreme producers of glucosinolates in this model system, to be used in future experiments. It was decided that *Brassica oleracea* subsp. *oleracea* both "typical" and "atypical" of their field site will be used in laboratory experiments to investigate the effect of glucosinolates on the behaviour of specialist insects representing the second and third trophic levels (Table 2.4.).

Plant	2-PROP	3-BUT	2-HYD-3B	TOTAL
DD 11 - "typical"	0	1.306	3.214	4.52
DD 21 - "typical"	0	0.736	3.556	4.292
DD 3 - "atypical"	0	0.26	0.799	1.059
DD 12 - "atypical"	0	0.489	1.397	1.886
Kim 1 - "typical"	0.341	0	0	0.341
Kim 7 - "atypical"	1.252	0	0	1.252
Kim 11 - "atypical"	1.445	0	0.593	2.038
BS	0	0.238	0.203	0.441

Table 2.4. Individual wild cabbage plants to be used during laboratory experiments as determined by the qualitative differences in aliphatic glucosinolate content (µm/g dry wt). Durdle Door *B. oleracea*, Kimmeridge *B. oleracea* and the control plant (Brussels sprout).

2-PROP = 2-propenylglucosinolate

3-BUT = 3-butenylglucosinolate

2-HYD-3B = 2-hydroxy-3-butenylglucosinolate

Analysis reveals that *Brassica oleracea* var. *gemmifera* var. Montgomery (Brussels sprout), the control plant, produced low total levels of glucosinolates. It produced quantities and qualities of glucosinolates similar to "typical" *Brassica oleracea* from Kimmeridge (Table 2.4.).

Investigating the Headspace Volatiles of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door.

The peaks of 3-butenylisothiocyanate present in all sample chromatographs were quantified by external calibration. The amounts present were classified according to three categories:

- Trace Amounts = <3ng
- Minimum Amounts = 3ng-10ng
- Maximum Amounts = >10ng

The mean amount of 3-butenylisothiocyanate for each category for each *Brassica oleracea* subsp. *oleracea* from each field site/soil combination was calculated. The number of plants where 3-butenylisothiocyanate was not detected is also stated.

Results reveal that significant quantities of 3-butenylisothiocyanate are present in the headspace of *Brassica oleracea* subsp. *oleracea* both "typical" and "atypical" in glucosinolate profile from Durdle Door when grown in soil collected from Durdle Door. However, when *Brassica oleracea* subsp. *oleracea* from Durdle Door were grown in soil collected from Kimmeridge, decreased amounts of 3-butenylisothiocyanate, if any at all, were detected in the headspace (Table 2.5.) (Figure 2.10).

Plant/Soil	Trace Amts	Minimum Amts	Maximum Amts	Number of plants 3-butiso not detected
DD/DD Soil	2.655 n=4	4.5 n=3	41.38 n=4	5
DD/Kim Soil	1.65 n=7	6.16 n=3	13.92 n=2	4

Table 2.5. The mean amount (amt) of 3-butenylisothiocyanate present in the headspace of *Brassica oleracea* subsp. *oleracea* from Durdle Door when grown in soil collected from Durdle Door and Kimmeridge. Quantities are expressed in ng/hour. The number of plants where 3-butenylisothiocyanate (3-butiso) was not detected is stated.
DD/DD Soil = Durdle Door *Brassica oleracea* subsp. *oleracea* grown in Durdle Door soil.
DD/Kim Soil = Durdle Door *Brassica oleracea* subsp. *oleracea* grown in Kimmeridge soil.

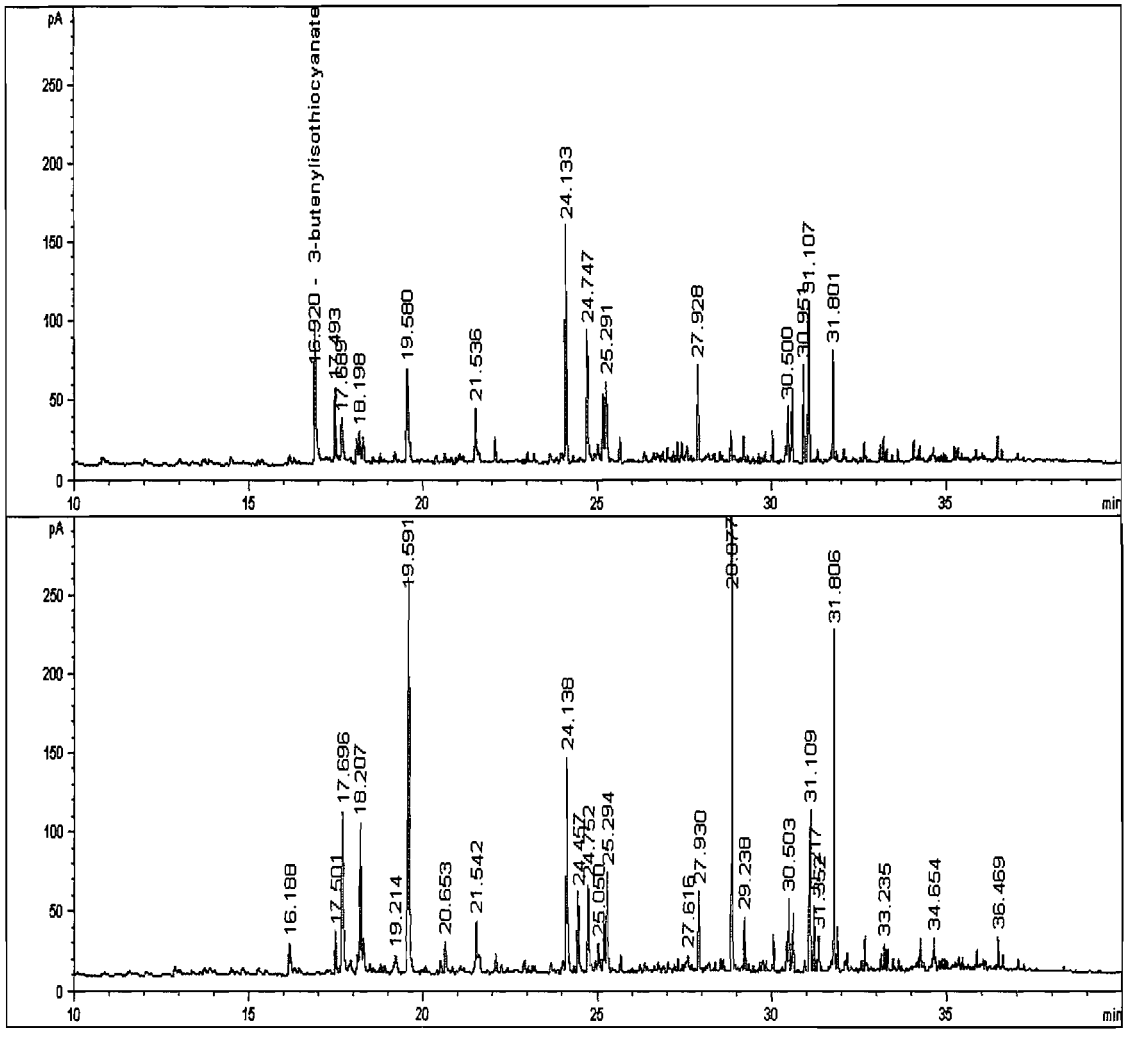


Figure 2.10. GC Chromatograms showing the presence of 3-butenylisothiocyanate in the headspace of *Brassica oleracea* subsp. *oleracea* from Durdle Door. The top profile is from a Durdle Door wild cabbage grown in Durdle Door soil, the bottom chromatogram is a Durdle Door wild cabbage grown in soil collected from Kimmeridge.

Regardless of soil type, 3-butenylisothiocyanate was either not detected or detected in small amounts in the headspace of "typical" and "atypical" *Brassica oleracea* subsp. *oleracea* from Kimmeridge (Table 2.6.) (Figure 2.11.).

Plant/Soil	Trace Amt	Minimum Amt	Maximum Amt	Number of plants 3-butiso not detected
Kim/DD Soil	1.74 n=2	4.47 n=2	0 0	12
Kim/Kim Soil	2.01 n=1	3.42 n=2	0 0	13

Table 2.6. The mean amount (amt) of 3-butenylisothiocyanate present in the headspace of *Brassica oleracea* subsp. *oleracea* from Kimmeridge when grown in soil collected from Durdle Door and Kimmeridge. Quantities are expressed in ng/hour. The number of plants where 3-butenylisothiocyanate (3-butiso) was not detected is stated.

Kim/DD Soil = Kimmeridge *Brassica oleracea* subsp. *oleracea* grown in Durdle Door soil.

Kim/Kim Soil = Kimmeridge *Brassica oleracea* subsp. *oleracea* grown in Kimmeridge soil.

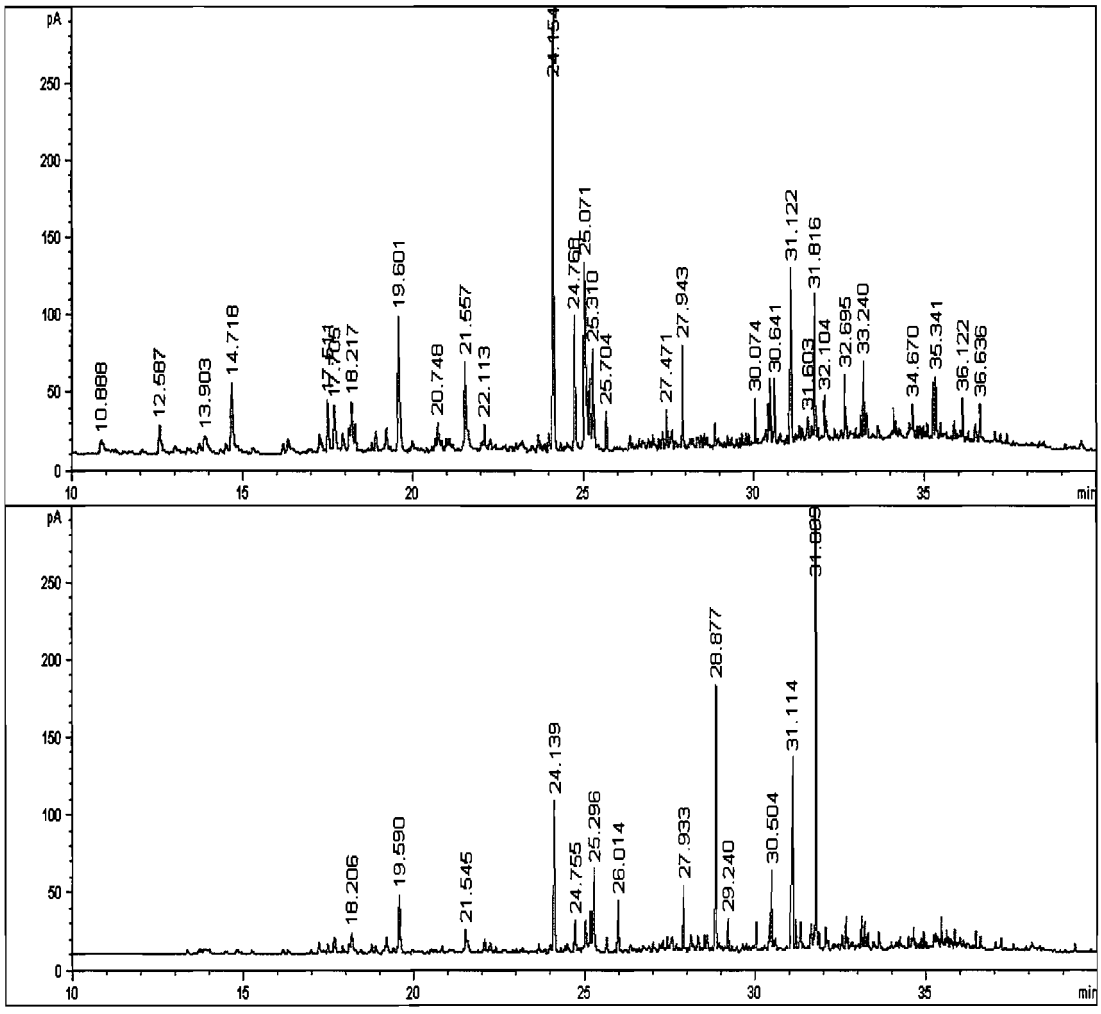


Figure 2.11. GC Chromatograms highlighting the absence of 3-butenylisothiocyanate in the headspace of *Brassica oleracea* subsp. *oleracea* from Kimmeridge. The top profile is from a Kimmeridge wild cabbage grown in Durdle Door soil, the bottom chromatogram is a Kimmeridge wild cabbage grown in soil collected from Kimmeridge.

2.3.2. Physical Defence Characteristics

Investigating Common Morphological Features of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door.

Leaf morphological analysis was conducted on clean *Brassica oleracea* subsp. *oleracea* plants collected from seed from Kimmeridge and Durdle Door, both "typical" and "atypical" of site in terms of their glucosinolate profile. As stated, leaves one - six of all plants were measured in terms of their size, shape, trichome density and degree of waxiness. Principal Component Analysis (Minitab, Version 13) was used to analyse the data to determine the degree of physical variability of the plants investigated.

The first and second principal factors (PC1 and PC2 respectively) explain the highest amount of variation of the total data for each leaf. The other factors were ignored, due to having eigenvalues of <1. The eigenvalues reveal that PC1 (factor 1) indicates leaf size, whilst PC2 (factor 2) reflects the morphological structures of the leaves.

The principal factors for each leaf are presented graphically (Figure 2.12.). The data does not display any distinct groups for any morphological features in any of the experimental plants for leaves one - three. However, leaves four, five and six of *Brassica oleracea* subsp. *oleracea* from Kimmeridge, "atypical" in its glucosinolate content, appear to be distinct from the remaining experimental plants in terms of PC1. This indicates a difference in size compared to the other plants investigated.

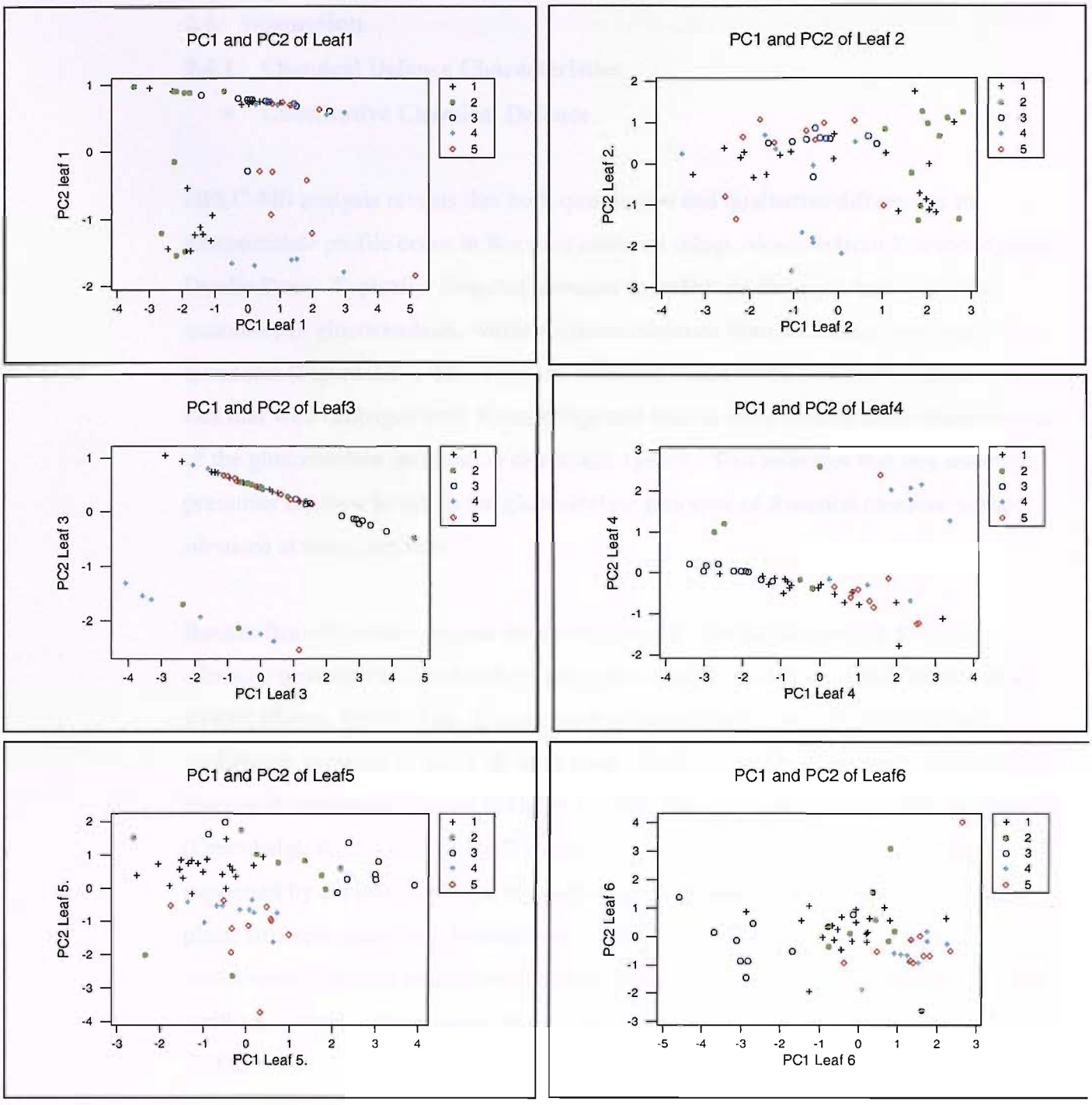


Figure 2.12. Principal Component Factors (PC1 and PC2) for the morphological features investigated for leaves 1 – 6 for all experimental plants.

- ● Kimmeridge *Brassica oleracea* subsp. *oleracea* "Typical"
- ◆ Durdle Door *Brassica oleracea* subsp. *oleracea* "Typical"
- ○ Kimmeridge *Brassica oleracea* subsp. *oleracea* "Atypical"
- ◊ Durdle Door *Brassica oleracea* subsp. *oleracea* "Atypical"
- \+ Brussels sprout var. Montgomery.

2.4. Discussion

2.4.1. Chemical Defence Characteristics

- **Constitutive Chemical Defence**

HPLC-MS analysis reveals that both quantitative and qualitative differences in glucosinolate profile occur in *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door. Typically, *Brassica oleracea* from Durdle Door produce high total quantities of glucosinolates, whilst *Brassica oleracea* from Kimmeridge produce low quantities (Figure 2.2.). This supports results revealed by Moyes (1997), confirming the fact that wild cabbages from Kimmeridge and Durdle Door remain at the extreme ends of the glucosinolate spectrum in this model system. This indicates that any selection pressures are slow to act on the glucosinolate genotype of *Brassica oleracea* subsp. *oleracea* at these two sites.

Results from this study support the revelation that wild populations of *Brassica oleracea* produce variable levels of glucosinolates (Mithen *et al.*, 1987; Mithen *et al.*, 1995b; Moyes, 1997). This glucosinolate polymorphism is also observed in wild cauliflower growing in Sicily (Branca *et al.*, 2002). In addition, previous studies show that the glucosinolate content is higher in wild Brassicas compared to cultivated species (Greenhalgh & Mitchell, 1976; Dawson *et al.*, 1993; Branca *et al.*, 2002). This is supported by the low quantities of glucosinolates produced by the cultivated control plant, Brussels sprout var. Montgomery (Table 2.1.). The low levels of chemical compounds produced by cultivated plants could be explained by the fact that increased yield and growth rate are selected for rather than defence traits (Massei & Hartley, 2000).

Interestingly, results reveal that specific wild cabbage plants at Kimmeridge and Durdle Door produce glucosinolate quantities more characteristic of the other field site. For example, as stated, *Brassica oleracea* subsp. *oleracea* from Kimmeridge typically produce low total levels of glucosinolates. These are termed "typical." However, certain individuals at Kimmeridge produce high quantities of glucosinolates, more commonly observed in *Brassica oleracea* subsp. *oleracea* from Durdle Door (Figure 2.6.). These plants are deemed "atypical" of their field site. This is also evident in *Brassica oleracea* from Durdle Door (Figure 2.7.). This polymorphism in glucosinolate

concentration in wild cabbage populations is consistent with Mithen *et al.*, (1995b) and Moyes (1997).

Significant genetic differences were revealed both between and within wild cabbages from Kimmeridge and Durdle Door, both in terms of the total levels of aliphatic glucosinolates and the ratio of individual aliphatic glucosinolates produced. *Brassica oleracea* subsp. *oleracea* from Durdle Door produce 3.5 times the quantity of aliphatic glucosinolates observed at Kimmeridge (Figure 2.4.). Unlike indole glucosinolates, whose profile is dependent upon seasonal and environmental variations (Mithen *et al.*, 1995b), aliphatic glucosinolates are under strong genetic control and are hence believed to be highly heritable (Raybould & Moyes, 2001). Due to their genetic regulation, the potential for host choice by insects exists, highlighting a possible reason why research concentrates on the role of aliphatic glucosinolates in plant-herbivore interactions (Rucker & Robbelen, 1994; Mithen *et al.*, 1995b; Moyes, 1997; Mithen, 2001; Raybould & Moyes, 2001). The fact that *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door produce significantly different quantities of aliphatic glucosinolates, provides a model wild system within which to investigate the effect of glucosinolate differences within a tritrophic context (Chapters Three, Four and Five).

The ratio of individual aliphatic glucosinolates also varies between wild cabbages from Kimmeridge and Durdle Door. Results show that 2-propenylglucosinolate is principally produced by *Brassica oleracea* subsp. *oleracea* from Kimmeridge, whilst 3-butenylglucosinolate is only produced by plants from Durdle Door (Figure 2.5.). This discovery is very interesting as several studies have indicated the role of 3-butenylglucosinolate and its volatile, 3-butenylisothiocyanate in Brassica plant-insect interactions (Bradbourne & Mithen, 2000; Blande, 2004). The presence/absence of 3-butenylglucosinolate in wild cabbages from Durdle Door and Kimmeridge respectively, enables the putative utilisation of this compound and its breakdown volatile by foraging parasitoids to be investigated both in the laboratory and in a natural ecosystem (Chapters Four and Five).

There are several possible explanations for the polymorphism in aliphatic glucosinolate profile between wild populations of *Brassica oleracea* (Mithen *et al.*, 1995b; Moyes, 1997). Firstly, Mithen *et al.*, (1995b) proposed that a combination of founder effects and genetic drift could account for the genetic variation observed. It is possible that

gene flow can occur between spatially distinct populations due to foraging by bees (Warman, 1994). However, minimal differences between populations at isozyme loci was observed and was hence rejected by Mithen *et al.*, (1995b). However, Moyes (1997) stated that the "exact level of gene flow as indicated by the isozyme data is unclear," and suggested that founder effects and genetic drift should not be ruled out completely.

Selection is the most likely possibility to account for the variation in glucosinolate genotype between *Brassica oleracea* subsp. *oleracea* populations (Mithen *et al.*, 1995b; Moyes, 1997). Selection can occur as a result of many factors including differences in the physical habitats of the populations and differences in the composition of the surrounding vegetation.

The physical differences in habitat between Kimmeridge and Durdle Door are dramatic. *Brassica oleracea* subsp. *oleracea* are present on cliff faces and cliff tops at Kimmeridge. This is an extremely unstable environment due to the fact that the plants are exposed to sea spray and high winds. Plants are also highly vulnerable to subsidence due to their position on the cliffs. This hostile environment inevitably decreases the diversity of generalists able to feed on the wild cabbage plants. Mithen *et al.*, (1995b) stated that plants might not invest in chemical defences against feeding by generalist herbivores as it is relatively less important compared to the pressures imposed by the environment. The low quantity of glucosinolates produced by wild cabbages at Kimmeridge appears to support this view. The habitat at Durdle Door is equally unstable, but is under different environmental pressures. Wild *Brassica oleracea* populations are commonly found growing at the base of cliffs on the coast. This results in higher exposure to sea spray and at times, the sea. This would affect the species of plants able to survive in such conditions, generally hosting a high proportion of halophytic vegetation, which would inevitably influence the herbivores present in that habitat. An additional factor that could influence the genotype of *Brassica oleracea* subsp. *oleracea* is the presence of tourists at Durdle Door. It is a popular attraction in the UK and inevitably, plants are damaged through trampling, thus contributing to the unstable nature of the environment. The different habitats observed at Kimmeridge and Durdle Door could result in different environmental pressures, which could act as bottom-up selecting forces on the glucosinolate genotype of wild cabbage populations and explain the polymorphism observed.

In addition, the composition of the surrounding vegetation at both field sites could be an important factor in influencing the genetic variation of glucosinolates observed (Mithen *et al.*, 1995b; Moyes, 1997). The presence and diversity of plant species could be a result of the physical environment, which would inevitably influence the species of generalist and specialist insects present within that habitat. Theoretically, the ratio of generalist to specialist herbivores could differ between Kimmeridge and Durdle Door and thus impose top-down selection pressures on the genotype of *Brassica oleracea* subsp. *oleracea*. The difference in habitat and vegetation diversity at Kimmeridge and Durdle Door is addressed in Chapter Five.

As stated, "typical" and "atypical" wild cabbages are present at both field sites. This reveals polymorphism in glucosinolate phenotype also occurs within populations of wild cabbages at Kimmeridge and Durdle Door. This concurs with previous work (Mithen *et al.*, 1995b; Moyes, 1997). Possible explanations for this intra-site variation in glucosinolate profile could be microsite differences and prior herbivory (Shelton, 2004). It is suggested that chemical variation within and among plants is important for decreasing the development of herbivore resistance, which is extremely likely in herbivores with short generation times (Whitham & Slobodchikoff, 1981). This could be applied to the wild cabbage model as *Brevicoryne brassicae*, which has a reproductive development time of 8-10 days (pers. obs), is a common herbivore of wild cabbages at Kimmeridge and Durdle Door. The interaction between *Brassica oleracea* subsp. *oleracea* and *Brevicoryne brassicae* is explored in Chapters Three and Five.

This thesis concentrates on the role of glucosinolates in plant-insect interactions. Therefore, this chapter serves as an important prelude to all subsequent chapters. It was imperative to determine the genetic variation in glucosinolate profile produced by *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door before behavioural studies could commence. Knowledge of the glucosinolate phenotype of the wild cabbage will enable possible links between glucosinolates and the behaviour of *Brevicoryne brassicae* and *Diaeretiella rapae* to be made. This is investigated in Chapters Three and Four.

It is generally accepted that generalist herbivores preferentially attack plants with lower concentrations of glucosinolates, whilst specialist herbivores are attracted to plants producing higher glucosinolate concentrations (Raybould & Moyes, 2001). However,

very few studies have determined whether insects in the second and third trophic levels are responding to total glucosinolate quantity or the presence of certain individual glucosinolates (Cole, 1976; Moyes *et al.*, 2000). This study aims to determine whether the overall content of aliphatic glucosinolates or differences in the profile of aliphatic glucosinolates drive tritrophic interactions (Chapters Three, Four and Five).

- **Inducible Chemical Defence**

The aim was not to determine the differences in headspace composition of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door, but to concentrate on the release of one individual volatile, 3-butenylisothiocyanate. As expected results reveal that 3-butenylisothiocyanate is significantly released from *Brassica oleracea* subsp. *oleracea* from Durdle Door, but not from wild cabbages from Kimmeridge when the plants are grown in soil collected from Durdle Door. However, when *Brassica oleracea* subsp. *oleracea* from Durdle Door are grown in soil collected from Kimmeridge, decreased amounts or trace amounts of this compound are detected (Table 2.5.). This reveals two things: 3-butenylisothiocyanate appears to only be significantly released from wild cabbages from Durdle Door compared to those from Kimmeridge. Secondly, the soil the plant is growing in appears to affect the quantity of 3-butenylisothiocyanate released.

The apparent presence/absence of 3-butenylisothiocyanate from populations of wild cabbage at Durdle Door and Kimmeridge respectively concurs with results obtained from analysing the glucosinolate content of these populations. 3-butenylglucosinolate was only detected in plants from Durdle Door. As stated, 3-butenylglucosinolate is hydrolysed by a myrosinase enzyme upon herbivore attack to produce 3-butenylisothiocyanate (Chapter One). This explains the presence of 3-butenylisothiocyanate from wild cabbages from Durdle Door in contrast to those from Kimmeridge. This is a very interesting revelation as Bradbourne and Mithen (2000) and Blande (2004) highlighted the importance of 3-butenylisothiocyanate during foraging by *Diaeretiella rapae*. Therefore, this work reveals a natural wild system within which to investigate the role of this compound on *Diaeretiella rapae*. This is investigated in both controlled laboratory experiments (Chapter Four) and in a natural ecosystem (Chapter Five). It should be noted that the fact that 3-butenylisothiocyanate is detected at all in "non-damaged" plants could be due to the minimal unavoidable

damage caused to the plants whilst conducting the air entrainment procedure. Care was taken to minimise any damage caused to the plants.

The second important discovery is the apparent soil effect on the release of 3-butenylisothiocyanate, which could affect the interactions of insects in the higher trophic levels. This study supports the fact that soil conditions can affect the chemical profile of plants (Wolfsen, 1982; Inglis *et al.*, 1992). Cole (1976) stated that the dominant glucosinolate-breakdown products released depends upon the conditions the hydrolysis reaction occurs in. For example, in a neutral pH, a high proportion of isothiocyanates is produced, whilst in an acidic pH, nitriles are the dominant volatile compounds. Therefore, relating to this, the results obtained could suggest that the soil at Durdle Door has a higher pH compared to the soil at Kimmeridge. This is supported by Mitchell and Richards (1979) who revealed that Durdle Door has a calcareous substrate, whilst at Kimmeridge the soil is predominantly shale. It would be interesting to determine if the pH of the soil affects the glucosinolate and subsequently the isothiocyanate content of *Brassica oleracea* subsp. *oleracea.* The pH of the soil collected from Durdle Door could be increased to determine whether the quantity of 3-butenylisothiocyanate increases. Secondly, as glucosinolates are nitrogen-rich compounds (Chapter One), it would be interesting to determine if the amount of available nitrogen present in the soil also affects the volatile composition released by the wild cabbage. Nitrogen could be added to soil collected from Durdle Door, headspace volatiles of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door could then be collected to determine the effect on the profile of 3-butenylisothiocyanate.

It should be noted that previously infesting the plants with *Brevicoryne brassicae* did not appear to affect the release or concentration of 3-butenylisothiocyanate. This can be explained by the fact that aphids are phloem-feeders, which is a relatively less invasive form of feeding. Therefore, it is possible that whilst relatively low numbers of aphids (>150) may sufficiently damage the plant necessary for glucosinolate hydrolysis to occur, the amount of 3-butenylisothiocyanate released may be very small and therefore any differences may be difficult to confidently determine.

2.4.2. Physical Defence Characteristics

Principal Component Analysis reveals no significant differences in the morphology of leaves one to three of the experimental plants. At this early stage of plant development, *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and the control plant, *Brassica oleracea* var. *gemmifera* are similar in size and shape and are predominantly glabrous. The only difference observed is a slight variation in the size of leaves four, five and six of *Brassica oleracea* subsp. *oleracea* from Kimmeridge, "atypical" in glucosinolate profile, in comparison to the remaining plant types. This confirms that "atypical" Kimmeridge cabbage plants grow slightly faster (pers. obs.). This is interesting as this plant type produces high levels of total glucosinolates, thus appearing to invest equal resources into both chemical and physical defences. This seems to support the "apparency theory" proposed by Feeny (1976). This states that plants that are spatially or temporally more "obvious" should invest more in defence against herbivory. However, the fact that the remaining Brassicas investigated have similar sized leaves means that this theory cannot be confidently applied to this work.

As stated, results reveal that *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door have highly glabrous leaves, supporting previous research (Mitchell & Richards, 1979; Moyes, 1997). Interestingly, Åhman (1990) discovered that high levels of leaf wax hindered the movement of *Lipaphis erysimi*, but did not affect *Brevicoryne brassicae*. This difference could be explained by the fact that *Lipaphis erysimi* is a specialist of *Brassica rapa* (turnip) and is likely to be adapted to its high trichome density and less glabrous morphology. In contrast, *Brevicoryne brassicae* is a specialist of *Brassica oleracea* and subsequently is likely to be adapted to its highly glabrous leaves. *Lipaphis erysimi* is commonly chosen to investigate interactions with Brassicas. However, *Brevicoryne brassicae* was chosen as the specialist aphid to represent the second trophic level during this study, as this species is adapted to feed on wild cabbages and importantly is commonly observed feeding at Kimmeridge and Durdle Door. Therefore, the behaviour of *Brevicoryne brassicae* observed in this study is likely to be due to the differences between the host plants, rather than a consequence of its adaptation to a less suitable host species.

The overall similarities in plant morphology suggest that *Brassica oleracea* subsp. *oleracea* and *Brassica oleracea* var. *gemmifera* do not heavily invest resources into

displaying physical defences in the resistance against herbivory, either by acting to deter herbivores or attracting natural enemies. In addition, the overall similarities in physical defences between *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and the control plant suggest that any differences observed in insect behaviour are unlikely to be a result of surface morphological traits.

The similarities in physical features, but high variation in terms of the profile of glucosinolates and subsequent breakdown products produced by *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door, implies that a trade-off between chemical and physical defence strategies does occur in this species. This putative trade-off of resistance characters supports previous studies (Rehr *et al.*, 1973; Massei & Hartley, 2000). The allocation of resources for chemical defences in preference to physical defences appears to support the "growth differentiation theory" (Herms & Mattson, 1992) and the "resource availability theory" (Coley *et al.*, 1985). Both state that slow growing plants usually have higher levels of chemical compounds compared to faster growing plants (Massei & Hartley, 2000). Indeed *Brassica oleracea* subsp. *oleracea* is a relatively slow growing perennial plant, which can survive for up to twenty years (Mitchell & Richards, 1979), thus explaining the high levels of glucosinolates produced. This is investigated in Chapter Five.

The trade-off observed in wild cabbage seedlings could have evolved as a result of high levels of damage caused by feeding of generalist herbivores in the early part of the plants' life cycle (Moyes, 1997). Molluscs commonly feed on Brassica seedlings, therefore, it is suggested that greater resources are invested in chemical defence, principally in glucosinolate content, to deter generalists during this early stage of development. An expansion on this work would be to repeat the investigation into chemical and physical defences on mature *Brassica oleracea* subsp. *oleracea* at Kimmeridge and Durdle Door to determine if this trade-off remains in mature plants in natural populations. In addition, it would be interesting to repeat the experiment using mature wild cabbage plants at different times of the year to see if the balance in this trade-off changes depending on the time of year, as observed in wild olives (Massei & Hartley, 2000).

Summary

- *Brassica oleracea* subsp. *oleracea* from Durdle Door produce high levels of aliphatic glucosinolates compared to those from Kimmeridge. 3-butenylglucosinolate is only produced from *Brassica oleracea* subsp. *oleracea* from Durdle Door.
- 3-butenylisothiocyanate is released in significantly greater quantities from *Brassica oleracea* subsp. *oleracea* from Durdle Door compared to those from Kimmeridge. However, when wild cabbages from Durdle Door are grown in soil collected from Kimmeridge, decreased amounts of this compound are detected.
- *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door do not differ in terms of their physical defences.
- A trade-off between chemical and physical defences is suggested to occur in *Brassica oleracea* subsp. *oleracea.*
- Any differences observed in the behaviour of *Brevicoryne brassicae* and *Diaeretiella rapae* during Chapters Three and Four, are likely to result from the chemical differences between the plants, due to the lack of physical differences revealed.

Chapter Three

Second Trophic Level

Host Selection and Performance of *Brevicoryne brassicae.*

Chapter Three

Host Selection and Performance of *Brevicoryne brassicae*.

3.1. Introduction

Brevicoryne brassicae (L.) (Homoptera: Aphididae) is a specialist aphid of Brassica plants, including *Brassica oleracea* subsp. *oleracea* (Ellis & Singh, 1993). It is an important pest in temperate regions of the world (Ronquist & Åhman, 1990), causing both direct and indirect damage to plants. High densities of *Brevicoryne brassicae* can directly cause the curling of leaves, wilting, stunted growth and even the death of seedlings (Yue & Liu, 2000). Indirect damage occurs because *Brevicoryne brassicae* can act as a vector, transmitting approximately twenty viruses (Ellis & Singh, 1993), including the cauliflower mosaic virus and turnip mosaic virus (Raybould *et al.*, 1999a). Currently, aphid control is achieved by the application of insecticides (Cole, 1997) with 95% of horticultural Brassica crops being treated with insecticides in the UK (Singh *et al.*, 1994). Environmental concerns and the increased probability of resistance occurring in response to heavy insecticide use, has driven the need for alternative aphid control methods to be investigated, such as the production of resistant cultivars (Ellis & Singh, 1993; Singh *et al.*, 1994; Cole, 1994; 1997; Ellis *et al.*, 2000).

Physical plant traits including leaf waxiness (Åhman, 1990) and trichome density (Agrawal *et al.*, 2000) and chemical plant traits such as nutritional factors including amino acid concentration (van Emden, 1990) and biochemical factors such as glucosinolate content (Cole, 1996; 1997) are thought to influence aphid behaviour and performance.

Brassica oleracea subsp. *oleracea* from Kimmeridge and Durdle Door and the control plant *Brassica oleracea* var. *gemmifera* var. Montgomery display minimal physical differences, but significant chemical differences in terms of the quantity and quality of glucosinolates produced (Chapter Two). Cole (1997) suggested that glucosinolates act as major determinants on the behaviour of *Brevicoryne brassicae.* This chapter aims to investigate whether the glucosinolate profile of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door affects the behaviour of *Brevicoryne brassicae* in terms

of host selection and performance. Few studies have addressed this issue in a wild Brassica species (Ellis & Singh, 1993; Singh *et al.*, 1994; Cole, 1996; 1997; Ellis *et al.*, 2000). Continued research into the glucosinolate profile of Brassicas and how aphids are influenced by these defensive compounds is important to increase our understanding of the interaction between these two trophic levels. Ultimately, greater understanding could lead to alternative methods of aphid control other than insecticides.

3.1.1. Host Selection

Selecting the most suitable host plant is an important decision for an aphid as it can ultimately determine the overall performance of a population (Nikolakakis *et al.*, 2003). Nikolakakis *et al.*, (2003) investigated host selection in the generalist aphid species, *Myzus persicae*, whilst Yue and Liu (2000) worked on a specialist aphid, *Lipaphis erysimi*. Both studies revealed that aphids can differentiate between hosts, suggesting that physical and/or chemical plant features can be recognised and exploited. However, no effort was made to determine the host features that influence selection.

Cole (1994) suggested that aphids might detect chemical cues either translocated in the phloem or present in the plant cuticle upon probing. Thus, it is likely that host-specific chemicals such as glucosinolates act as host selection cues for specialist aphids (Cole, 1997), including *Brevicoryne brassicae*. However, an interesting aspect of aphid behaviour was discovered by Nikolakakis *et al.*, (2003). There was evidence of interclonal variation in the preference and ultimate performance of *Myzus persicae*. Therefore, it is possible that interclonal variation also exists in a specialist aphid. In fact the differences may be enhanced as specialist aphids may utilise host-specific chemicals to select their desired hosts to a greater degree than generalist aphids.

This study is the first to investigate initial host selection by *Brevicoryne brassicae* in a single host species. The first aim is to determine if host selection differs for the three "host-lines" (group of aphids collected from a common plant and reared separately in the laboratory) of *Brevicoryne brassicae*. This will reveal whether "host-line" variation exists, implicating the existence of interclonal variation in *Brevicoryne brassicae,* which could have strong implications for aphid control. The second aim is to determine if *Brevicoryne brassicae* differentially selects between *Brassica oleracea* subsp. *oleracea*

from Kimmeridge and Durdle Door and the control plant and whether any differences observed relate to the glucosinolate profile produced by the hosts.

3.1.2. Aphid Performance

Once a suitable host has been selected, the next step is to determine how *Brevicoryne brassicae* performs on its host.

There are two common methods for measuring aphid performance. Both are indicative of aphid fitness and both commonly involve the use of clip cages. The first method involves determining the intrinsic rate of natural increase of a population (r_m). It was first proposed by Birch (1948), but later simplified by Wyatt and White (1977). This method involves measuring the development time and the fecundity of an aphid and calculating the population rate of increase using a constant determined by Wyatt and White (1977). The second method, proposed by van Emden (1969) determines the mean relative growth rate (MRGR) of an aphid. It involves measuring the individual weight of the aphid at the start and end of the experiment and subtracting the natural log of the initial weight from the natural log of the final weight.

Both methods of aphid performance have been extensively researched (van Emden, 1969; Wyatt & White, 1977; Leather & Dixon, 1984; Ronquist & Åhman, 1990; Amjad & Peters, 1992; Wojciechowicx-Zytko & van Emden, 1995; Cole, 1997; Guldemond *et al.*, 1998; Kift *et al.*, 1999; Edwards, 2001; Petersen & Sandström, 2001; Nikolakakis *et al.*, 2003). Due to the general acceptance of using clip cages to assess aphid performance, preliminary experiments were conducted for *Brevicoryne brassicae* on *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door (Appendix 2). However, numerous problems were revealed resulting from using clip cages. Firstly, aphids are placed on parts of the host plant, which may not be the most desirable for aphid growth. For example, *Brevicoryne brassicae* aggregate on the actively growing parts of the plant (pers. obs.), thus clipping the aphids to the underside of older leaves could result in an under-estimation of aphid performance. Secondly, the use of clip cages commonly resulted in the appearance of chlorotic lesions around the cage (pers. obs.), which inevitably caused increased stress for the plant and decreased the quality of the host, possibly leading to an under-estimate of aphid performance. Increased stress on the plant may have implications on the production of secondary defensive

compounds such as glucosinolates, which could result in an induction of sinks leading to either an over-estimate or under-estimate of aphid performance. However, the main problem associated with clip cages relates to the ecology of *Brevicoryne brassicae*. This species prefers to develop and reproduce within a colony (pers. obs.), thus confining single aphids to an undesirable part of a plant leads to a longer developmental period and inevitably results in an under-estimate of the performance of *Brevicoryne brassicae*.

A method was adapted in order to work with *Brevicoryne brassicae*. It involves infesting whole plants with a specific number of *Brevicoryne brassicae* and counting the number of aphids at given time periods (Guldemond *et al.*, 1998; Kift *et al.*, 1999). The intrinsic rate of natural increase (r_m) is then estimated using the exponential aphid population growth for each individual plant (Southwood, 1978).

The overall aim is to determine if the three "host-lines" of *Brevicoryne brassicae* display differing intrinsic rates of natural increase on *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and whether performance is related to the glucosinolate profile of the host plants.

Hypotheses:

- *Brevicoryne brassicae* will be able to differentiate between Brassicas and select the host producing the highest total quantity of aliphatic glucosinolates.
- The highest intrinsic rate of natural increase of *Brevicoryne brassicae* will occur on *Brassica oleracea* subsp. *oleracea* producing the highest quantity of aliphatic glucosinolates.
- "Host-line" variation in *Brevicoryne brassicae* will be observed.

3.2. Materials and Methods

- Plants

Brassica oleracea subsp. *oleracea* seeds were collected from plants from Kimmeridge and Durdle Door, which were both "typical" and "atypical" in terms of their total glucosinolate content (Chapter Two). Seeds were sown in Levingtons® compost in 7x7x8cm pots (Sopodex, UK) at the University of Southampton glasshouses (20°C ± 3°C, L12:D12) and watered when required. *Brassica oleracea* var. *gemmifera* var. Montgomery (untreated F_1 hybrid seed, Moles Seeds, Essex, UK) seeds were sown to act as the control plant. Clean, undamaged plants at the 4–5 leaf stage were selected 48 hours prior to conducting experiments and transported to a temperature controlled room (22°C ± 3°C, L16:D8) where they remained for the duration of the experiments.

- Insects

Cultures of *Brevicoryne brassicae* were established with aphids collected from *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door in 2002. Aphids were reared on *Brassica oleracea* subsp. *oleracea* grown from seed collected from their original field site to enable separate "host-lines" of *Brevicoryne brassicae* to be established. The control culture was started from stocks of *Brevicoryne brassicae* reared on Brussels sprout var. Montgomery (*Brassica oleracea* var. *gemmifera*) from HRI Wellesbourne, Warwick, UK. All *Brevicoryne brassicae* cultures were reared in individual cages (22°C ± 3°C, L16:D8) to ensure that the separate "host-lines" remained.

- **Experiment One: Host Selection**

Petri dishes (9cm diameter, Bibby Sterilin LtD, Staffordshire, UK) were lined with two pieces of filter paper (9cm diameter, Millipore, UK) and moistened with 2ml of distilled water. Two 4cm diameter leaf discs were added to the Petri dishes, leaving a 2cm space between them. Five 2^{nd}–3^{rd} instar apterous aphids were added to the centre of the experimental arena between the two leaf discs (Figure 3.1.). The Petri dishes were laid out under a laminar light source in a controlled environment room (22°C ± 3°C, L16:D8). Apterous morphs were investigated as they have the ability to recognise potential hosts (Hodgson, 1991; Nikolakakis *et al.*, 2003).

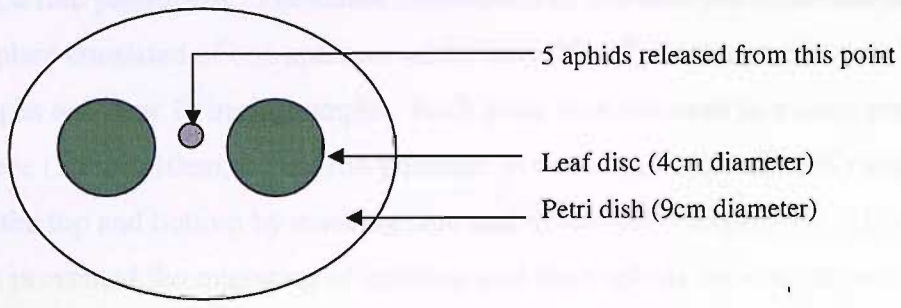


Figure 3.1. Host Selection Arena. Leaf disc position and aphid release site are indicated.

Aphid position was noted 30 minutes, 1 hour, 2 hours and 24 hours after the start of the experiment. The leaf disc selected by each individual aphid was deemed the most desirable host choice at each time period investigated.

Six plant choices were investigated:

- Kimmeridge *B.oleracea* "typical"–Durdle Door *B.oleracea* "typical"
- Kimmeridge *B.oleracea* "atypical"–Durdle Door *B.oleracea* "atypical"
- Kimmeridge *B.oleracea* "typical"–Control (*B.oleracea* var. *gemmifera)*
- Kimmeridge *B.oleracea* "atypical"–Control (*B.oleracea* var. *gemmifera)*
- Durdle Door *B.oleracea* "typical"–Control (*B.oleracea* var. *gemmifera)*
- Durdle Door *B.oleracea* "atypical"-Control (*B.oleracea* var. *gemmifera)*

Host selection was investigated for each *Brevicoryne brassicae* "host-line" (Kimmeridge *B.brassicae*, Durdle Door *B.brassicae* and control *B.brassicae*) for each of the plant combinations stated. Each plant combination–aphid "host-line" combination was replicated twenty times on two separate occasions. The position of the leaf discs was alternated every ten replicates to avoid positional bias.

- Statistical Analysis

The results obtained for each plant combination–aphid "host-line" investigated were analysed using χ^2 2x3 contingency tests (Minitab, Version 13).

- **Experiment Two: Aphid Performance – Method One**

This method was modified from Guldemond *et al.*, (1998) and Kift *et al.*, (1999). Ten *Brevicoryne brassicae* of mixed instars were transferred from stock cultures to each

plant using a fine paintbrush. The stable distribution of *Brevicoryne brassicae* released onto each plant consisted of one apterous adult, three 3rd–4th instar nymphs, two 2nd instar nymphs and four 1st instar nymphs. Each plant was enclosed in a clear perforated flower sleeve (30x50x10cm, 40 micron-punched, Avoncrop, Bracknell, UK) and secured at the top and bottom by masking tape and Sellotape™ respectively (Figure 3.2.). This prevented the migration of apterous and alate aphids between experimental plants. The plants were housed in water-filled plant trays; five plants per tray to further prevent aphid migration.

Figure 3.2. Aphid Performance using whole plants.

The total number of aphids on each plant was counted 3 days, 5 days, 7 days, 9 days and 10 days after the start of the experiment. All aphid performance experiments were terminated after 10 days, as this is the development time for *Brevicoryne brassicae* to reach reproductive maturity under controlled laboratory conditions (22°C ± 3°C, L16:D8) (pers. obs.). Five replicates were completed at one time for each plant type-aphid "host-line" combination, until a minimum replication size of twenty was collected. This was carried out for each of the three *Brevicoryne brassicae* lines on each of the five plant types under investigation:

Brassica	Abbreviation	Aphid "Host-line"
Control plant (Brussels sprout var. Montgomery)	BS	
Kimmeridge *Brassica oleracea* "typical"	Kim Typ	Control *B.brassicae*
Kimmeridge *Brassica oleracea* "atypical"	Kim Atyp	(BSA)
Durdle Door *Brassica oleracea* "typical"	DD Typ	
Durdle Door *Brassica oleracea* "atypical"	DD Atyp	
Control plant (Brussels sprout var. Montgomery)	BS	
Kimmeridge *Brassica oleracea* "typical"	Kim Typ	Kimmeridge *B.brassicae*
Kimmeridge *Brassica oleracea* "atypical"	Kim Atyp	(KimA)
Durdle Door *Brassica oleracea* "typical"	DD Typ	
Durdle Door *Brassica oleracea* "atypical"	DD Atyp	
Control plant (Brussels sprout var. Montgomery)	BS	
Kimmeridge *Brassica oleracea* "typical"	Kim Typ	Durdle Door *B.brassicae*
Kimmeridge *Brassica oleracea* "atypical"	Kim Atyp	(DDA)
Durdle Door *Brassica oleracea* "typical"	DD Typ	
Durdle Door *Brassica oleracea* "atypical"	DD Atyp	

- Statistical Analysis

Southwood (1978) demonstrated that if an aphid population increases exponentially, then the resulting straight line of the slope would be an estimate of the intrinsic rate of natural increase (r_m) for that population. The natural log +1 (ln+1) of the total number of aphids per plant was plotted against days after inoculation and the r_m value calculated.

The natural log+1 (ln+1) transformed aphid numbers for day 10 only and the r_m values for each plant type-aphid "host-line" combination were analysed using ANOVA General Linear Model (Minitab, Version 13). Plant type, aphid "host-line" and plant type*aphid "host-line" acted as model factors.

One-way ANOVA was also performed on all of the ln+1 transformed aphid totals for day 10 only and r_m data, comparing each aphid "host-line" and plant type separately. Fisher's Pairwise Comparison analysed any significant results ($p<0.05$).

- **Experiment Three: Aphid Population – Method Two**

Method two was conducted in the exact same way as method one. The only difference between the two methods is that the total number of aphids on each plant was noted on the tenth and final day only.

- Statistical Analysis

The natural log+1 (ln+1) of aphid numbers for day zero and day ten were plotted. A linear regression was plotted through the two points to give an estimate for the intrinsic rate of natural increase (r_m) for each aphid population per plant. Results obtained from method two were analysed as for method one. In addition, the aphid performance and rate of increase results obtained from both methods were analysed using a two-sample t-test to compare the success of the two methods.

3.3. Results

3.3.1. Host Selection

χ^2 analysis revealed that only 5% of results were significant ($p<0.05$). During replicate one, *Brevicoryne brassicae* was able to distinguish between wild *Brassica oleracea* from Durdle Door that was "atypical" in its glucosinolate content and the control plant, 30 minutes after the start of the experiment ($\chi^2 = 6.060$, $p = 0.048$, $DF = 2$). Replicate two revealed that *Brevicoryne brassicae* significantly preferred wild *Brassica oleracea* from Durdle Door, "typical" in terms of its glucosinolate content compared to the control plant, 30 minutes into the start of the experiment ($\chi^2 = 8.803$, $p = 0.016$, $DF = 2$). The significant results obtained from both replicates do not correlate and the fact that only 5% of results are significant suggests that they are viewed cautiously.

However, 95% of results indicate that *Brevicoryne brassicae,* regardless of "host-line," is unable to significantly distinguish between *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and the control plant, *Brassica oleracea* var. *gemmifera* var. Montgomery (Table 3.1.). This is accepted with confidence.

Plant combination	30 minutes		1 hour		2 hours		24 hours	
	χ^2	p	χ^2	p	χ^2	p	χ^2	p
Kim Typ-DD Typ	5.000	0.082	4.104	0.128	4.504	0.105	2.940	0.230
	1.457	0.483	1.513	0.469	1.468	0.480	1.666	0.465
Kim Atyp-DD Atyp	1.679	0.432	1.350	0.509	1.178	0.555	0.136	0.934
	0.637	0.727	0.504	0.777	1.312	0.519	0.027	0.986
DD Typ – BS	0.481	0.786	1.060	0.589	3.591	0.166	2.798	0.247
	8.803	0.016*	5.308	0.070	4.781	0.092	0.883	0.643
Kim Typ – BS	5.963	0.051	3.109	0.211	6.041	0.049	2.602	0.272
	1.029	0.598	1.589	0.452	1.419	0.492	1.757	0.415
DD Atyp – BS	6.060	0.048*	5.223	0.073	1.765	0.414	4.519	0.104
	3.036	0.219	2.705	0.259	2.326	0.313	4.969	0.083
Kim Atyp – BS	0.360	3.333	0.189	1.530	0.465	0.816	0.665	2.041
	0.028	0.986	0.176	0.916	0.026	0.987	1.227	0.542

Table 3.1. Host selection by *Brevicoryne brassicae* between *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and *Brassica oleracea* var. *gemmifera* var. Montgomery. The χ^2 and p values for each of the investigated plant choice combination are stated. Significant differences are highlighted ($p<0.05$*). N = 20, DF = 2 for each replicate. Replicate one is highlighted in black; replicate two is highlighted in blue.

Results suggest that *Brevicoryne brassicae* quickly locates a host (30 minutes, 1 hour and 2 hours) and if the host is deemed suitable, remains on the host initially selected (24 hours).

3.3.2. Aphid Performance

- **Method One**
- Aphid Population

General Linear Model analysis of the ln+1 transformed total number of the three "host-lines" of *Brevicoryne brassicae* on the five plant types investigated reveal that plant type and aphid "host-line" do not significantly affect the total number of *Brevicoryne brassicae*. However, plant type*aphid "host-line" does have a significant effect on aphid performance. This indicates that the total number of aphids present on the investigated plant types is influenced to a certain degree by an interaction of these two factors (Table 3.2.).

Source	DF	Seq. SS	Adj. SS	Adj. MS	F	p
Plant Type	4	1.5175	1.2816	0.3204	1.42	0.228
Aphid "Host-line"	2	1.6434	0.3129	0.1564	0.69	0.501
Plant*Aphid	8	4.5008	4.5008	0.5626	2.49	0.012*
Error	319	72.1303	72.1303	0.2261		
Total	333	79.7920				

Table 3.2. General linear model on ln+1 transformed total number of aphids. Plant type and aphid "host-line" and plant type*aphid "host-line" act as model factors. Significant differences at $p<0.05$ are indicated (*).

- Aphid Population – Plant Type

One-way analysis of variance of the ln+1 transformed total aphid numbers was conducted for each plant type individually. "Host-line" differences in the performance of *Brevicoryne brassicae* on the control plant were revealed ($F_{2,119} = 8.82$, $p<0.001$). However, the performance of the three lines of *Brevicoryne brassicae* do not significantly differ on "typical" *Brassica oleracea* from Kimmeridge ($F_{2,46} = 1.96$, $p = 0.153$), "typical" *Brassica oleracea* from Durdle Door ($F_{2,39} = 0.30$, $p = 0.790$), "atypical" *Brassica oleracea* from Kimmeridge ($F_{2,78} = 2.57$, $p = 0.083$) and "atypical" Durdle Door ($F_{2,41} = 0.11$, $p = 0.892$) (Figure 3.3.).

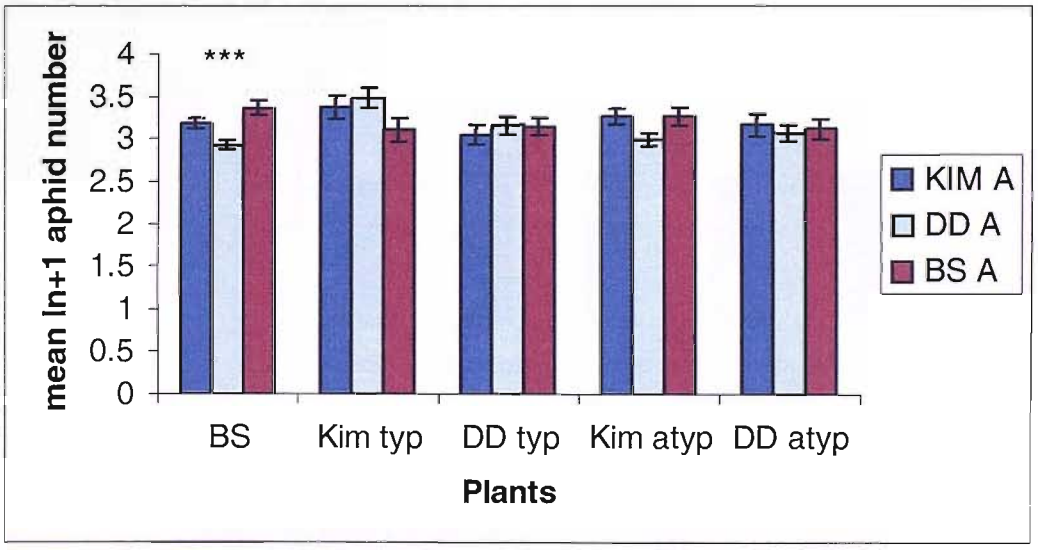


Figure 3.3. One-way analysis of variance carried out on the performance of the three *Brevicoryne brassicae* "host-lines" on the five plant types. Significant differences ($p<0.001$***) and standard error values (±SE) are indicated.

- Aphid Population – Aphid "Host-Line"

One-way analysis of variance was carried out on each aphid "host-line" separately. Results indicate that *Brevicoryne brassicae* originated from Kimmeridge do not differ significantly in terms of their performance on the five plant types (Figure 3.4.)

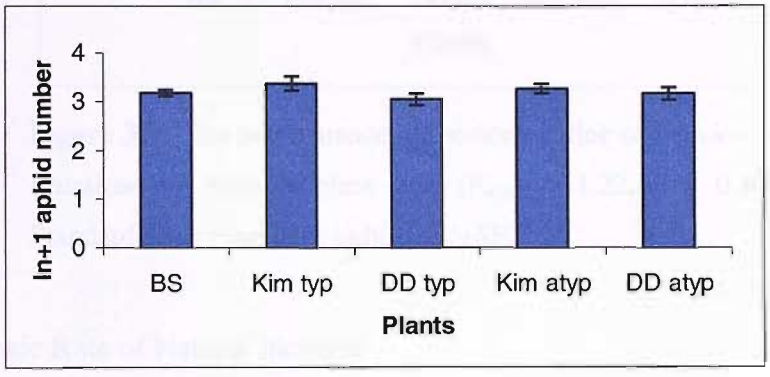


Figure 3.4. Performance of *Brevicoryne brassicae* from Kimmeridge on each plant type ($F_{4,120}$ = 0.95, p = 0.436). Standard error values are indicated (±SE).

The performance of *Brevicoryne brassicae* originated from Durdle Door differs significantly between *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and the control plant ($p<0.001$). Analysis reveals that the highest number of aphids is present on *Brassica oleracea* from Kimmeridge, "typical" in its total glucosinolate content ($F_{4,96} = 6.66$, $p<0.001$) (Figure 3.5.).

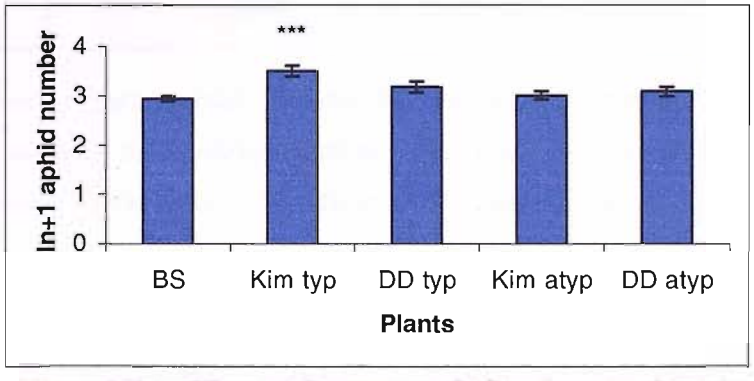


Figure 3.5. The performance of *Brevicoryne brassicae* originated from Durdle Door on the five plant types ($F_{4,96} = 6.66$, $p<0.001$***). Standard error values are indicated (±SE).

Brevicoryne brassicae reared on the control plant, *Brassica oleracea* var. *gemmifera* var. Montgomery (Brussels sprout), do not differ significantly in terms of their performance on the investigated plants types ($F_{4,103} = 1.22$, p = 0.306) (Figure 3.6.).

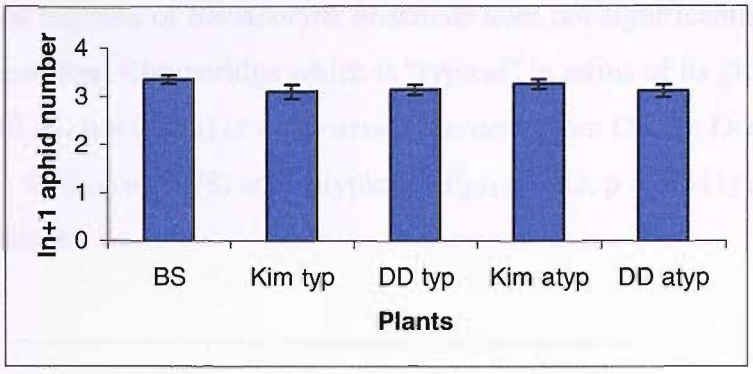


Figure 3.6. The performance of the control line of *Brevicoryne brassicae* on each of plant type ($F_{4,103}$ = 1.22, p = 0.306). Standard error values are indicated (±SE).

- Intrinsic Rate of Natural Increase

The r_m values indicating the intrinsic rate of natural increase of *Brevicoryne brassicae* obtained for each plant type-aphid "host-line" combination were analysed using a General Linear Model. Results reveal that only plant type*aphid "host-line" significantly affects the intrinsic rate of natural increase of *Brevicoryne brassicae* (Table 3.3.).

Source	DF	Seq. SS	Adj. SS	Adj. MS	F	p
Plant Type	4	0.08549	0.07601	0.01900	1.86	0.118
Aphid "host-line"	2	0.12483	0.05925	0.02963	2.90	0.057
Plant*Aphid	8	0.19240	0.19240	0.02405	2.35	0.018*
Error	319	3.26178	3.26178	0.01023		
Total	333	3.66450				

Table 3.3. General linear model analysis on the intrinsic rate of natural increase of *Brevicoryne brassicae* on the investigated plant types. Plant type, aphid "host-line" and plant type*aphid "host-line" act as factors. Significant differences are indicated (p<0.05*).

- Intrinsic Rate of Natural Increase – Plant Type

The intrinsic rate of natural increase (r_m) values were analysed by one-way analysis of variance for each plant type separately. The rates of increase of the three "host-lines" of *Brevicoryne brassicae* differ significantly on the control plant ($F_{2,119}$ = 7.69, p<0.001***) and on *Brassica oleracea* from Kimmeridge that is "atypical" in terms of its glucosinolate content ($F_{2,78}$ = 4.29, p = 0.017*). For both plants, *Brevicoryne brassicae* originally from Durdle Door displays the lowest rate of increase compared to the *Brevicoryne brassicae* "host-lines" from Kimmeridge and the control plant. (Figure

3.7.). The rate of increase of *Brevicoryne brassicae* does not significantly differ on *Brassica oleracea* from Kimmeridge which is "typical" in terms of its glucosinolate content ($F_{2,44} = 2.62$, $p = 0.084$) or on *Brassica oleracea* from Durdle Door both "typical" ($F_{2,37} - 0.02$, $p = 0.978$) and "atypical" ($F_{2,41} = 0.62$, $p = 0.541$) in its glucosinolate content.

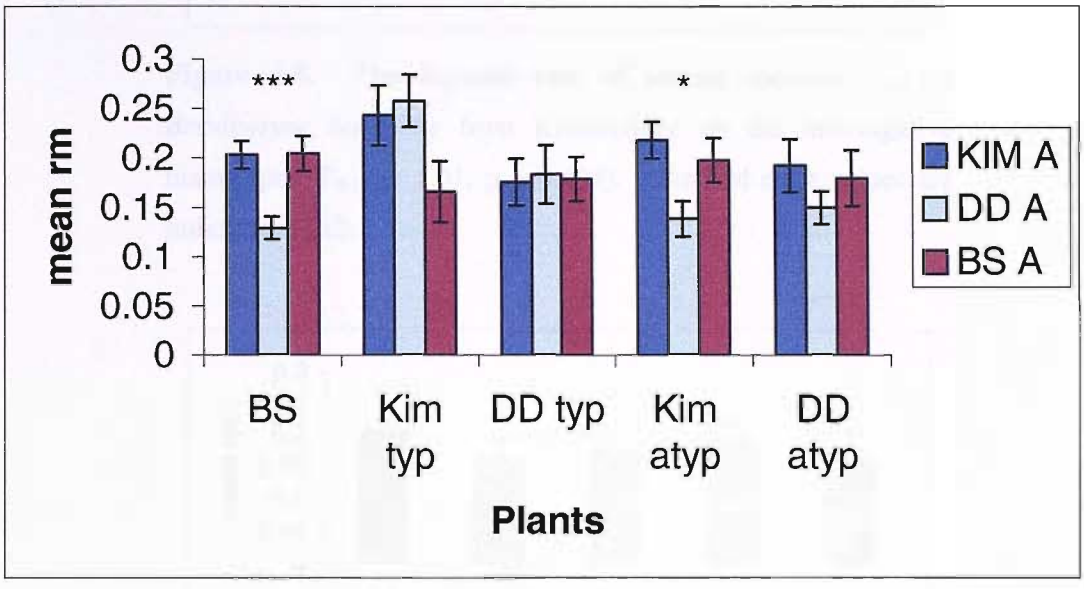


Figure 3.7. One-way analysis of variance carried out on the intrinsic rate of natural increase (r_m) of the three *Brevicoryne brassicae* "host-lines" on the five plant types. Significant differences ($p<0.05$*, $p<0.001$***) and standard error values (±SE) are indicated.

- Intrinsic Rate of Natural Increase – Aphid "Host-Line"

One-way analysis of variance revealed that aphids from Kimmeridge ($F_{4,120} = 1.01$, $p = 0.404$) (Figure 3.8.) and the control line ($F_{4,103} = 0.44$, $p = 0.777$) (Figure 3.9.) do not display different rates of increase on the five plant types ($p<0.05$).

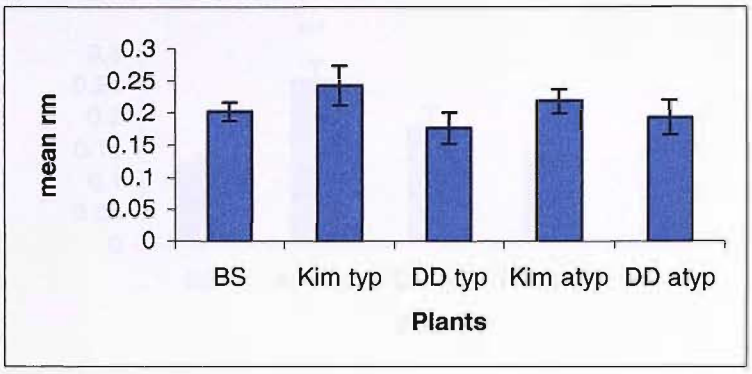


Figure 3.8. The intrinsic rate of natural increase (r_m) of *Brevicoryne brassicae* from Kimmeridge on the investigative plant types ($F_{4,120} = 1.01$, $p = 0.404$). Standard error values are indicated (±SE).

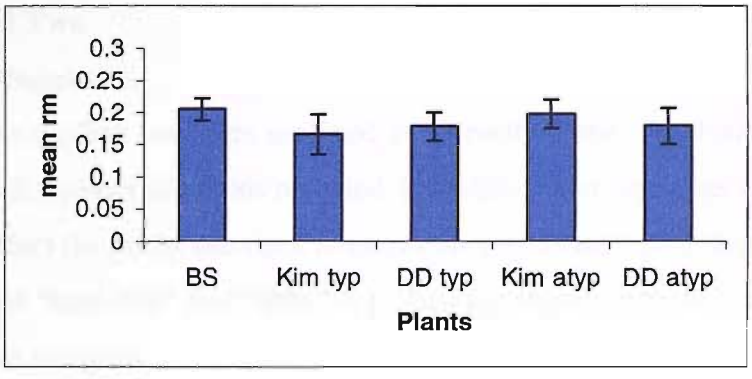


Figure 3.9. The intrinsic rate of natural increase (r_m) of the control line of *Brevicoryne brassicae* on *Brassica oleracea* from Kimmeridge "typical" and "atypical," *Brassica oleracea* from Durdle Door "typical" and "atypical" and the control plant, Brussels sprout ($F_{4,103} = 0.44$, $p = 0.777$). Standard error values are indicated (±SE).

Brevicoryne brassicae originated from Durdle Door display significantly different rates of natural increase (r_m) on the experimental plants. The highest rate of increase occurs on "typical" *Brassica oleracea* from Kimmeridge ($F_{4,96} = 7.43$, $p<0.001$) (Figure 3.10.).

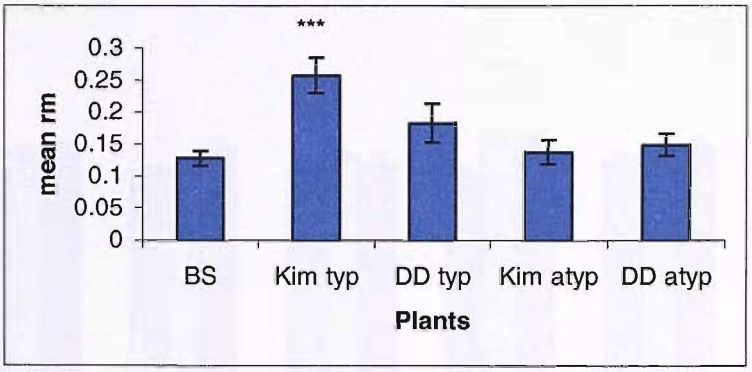


Figure 3.10. The intrinsic rate of natural increase (r_m) of *Brevicoryne brassicae* originated from Durdle Door on each of the investigative plant types. ($F_{4,96} = 7.43$, $p<0.001$***). Standard error values are indicated (±SE).

- **Method Two**
- Aphid Population

The results from method two were analysed as for method one. Analysis of ln+1 transformed total number of aphids revealed that whilst plant type does not significantly affect the performance of *Brevicoryne brassicae*, aphid "host-line" and plant type*aphid "host-line" do (Table 3.4.). This highlights an inconsistency between the two methods.

Source	DF	Seq. SS	Adj. SS	Adj. MS	F	p
Plant Type	4	2.4365	2.3625	0.5906	2.08	0.082
Aphid "Host-line"	2	3.2502	3.3077	1.6539	5.83	0.003**
Plant*Aphid	8	4.5949	4.5949	0.5744	2.03	0.042*
Error	421	119.3727	119.3727	0.2835		
Total	435	129.6543				

Table 3.4. Analysis of ln+1 transformed total number of aphids by general linear model. Plant type, aphid "host-line" and plant type*aphid "host-line" act as model factors. Significant results are indicated. ($p<0.05$*, $p<0.01$**).

- Aphid Population – Plant Type

Results obtained from method one reveal that the performance of each "host-line" of *Brevicoryne brassicae* differs significantly on the control plant only. The results revealed for method two do not support this, as the only significant difference in performance occurs on *Brassica oleracea* from Kimmeridge, which is "atypical" in its glucosinolate profile ($F_{2,83} = 8.83$, $p<0.001$) (Figure 3.11.).

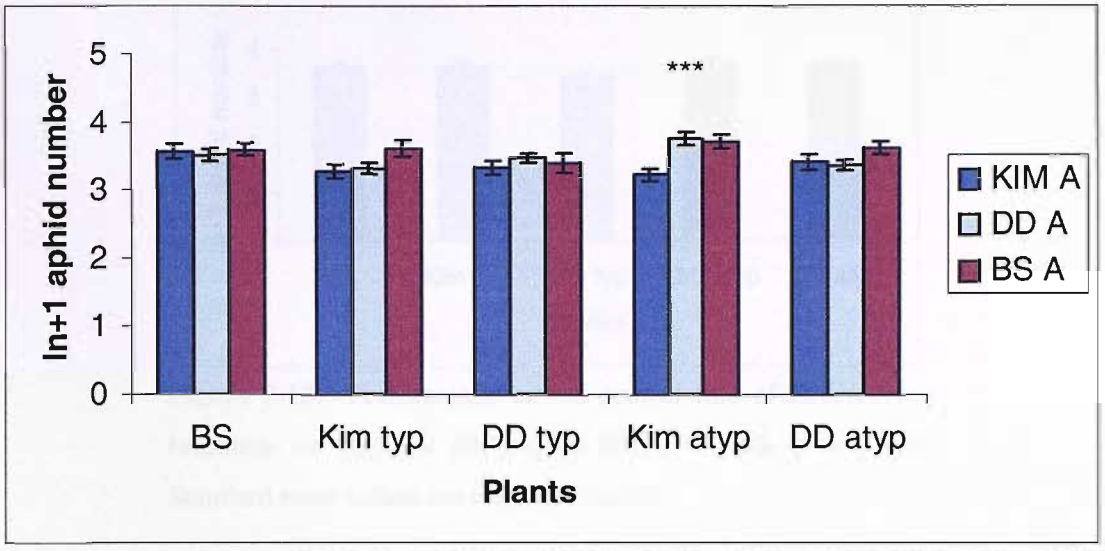


Figure 3.11. Performance of the three "host-lines" of *Brevicoryne brassicae* on each of the five plant types. Significant differences ($p<0.001$***) and standard error values (±SE) are highlighted.

- Aphid Population – Aphid "Host-Line"

When each aphid "host-line" was analysed individually, differences in *Brevicoryne brassicae* performance were clearly revealed. *Brevicoryne brassicae* from Kimmeridge ($F_{4,138} = 1.99$, $p = 0.099$) (Figure 3.12.) and the control line ($F_{4,139} = 0.93$, $p = 0.449$) (Figure 3.13.) do not differ in terms of their performance on either "typical" and "atypical" *Brassica oleracea* from Kimmeridge and Durdle Door or on the control plant ($p<0.05$).

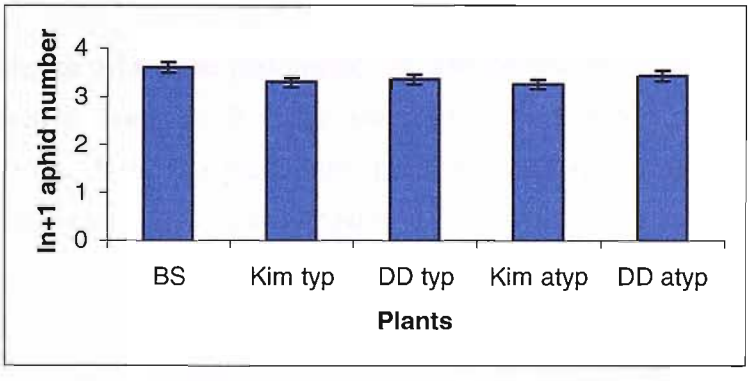


Figure 3.12. Performance of *Brevicoryne brassicae* from Kimmeridge on each plant type ($F_{4,138} = 1.99$, $p = 0.099$). Standard error values are indicated (±SE).

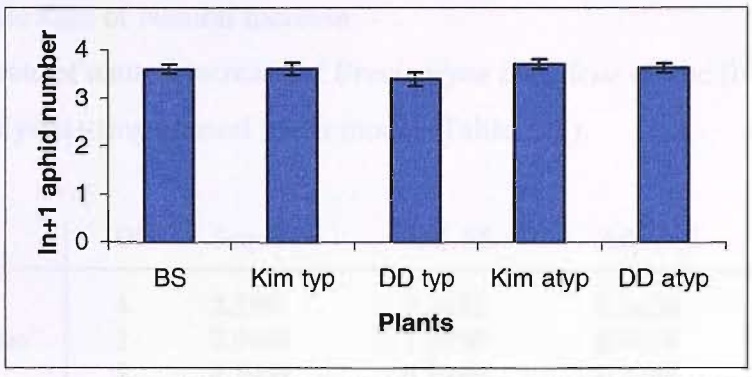


Figure 3.13. Performance of the control line of *Brevicoryne brassicae* on the five plant types ($F_{4,139}$ = 0.93, p = 0.449). Standard error values are indicated (±SE).

Significant differences were revealed in the performance of *Brevicoryne brassicae* from Durdle Door on the five plant types investigated. The highest performance occurs on *Brassica oleracea* from Kimmeridge, which is "atypical" in its glucosinolate profile ($F_{4,144}$ = 3.41, p = 0.011) (Figure 3.14.).

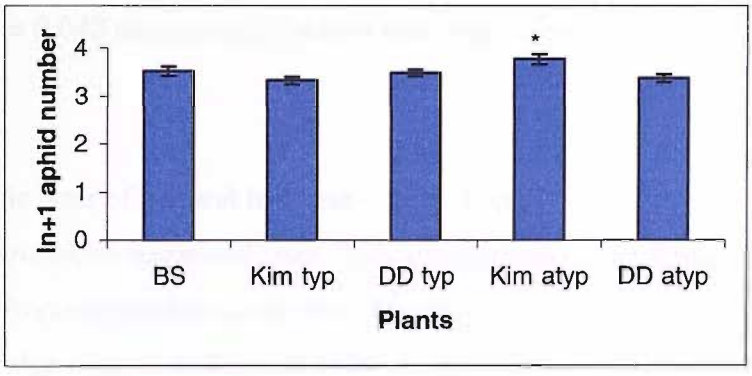


Figure 3.14. The performance of *Brevicoryne brassicae* from Durdle Door on *Brassica oleracea* from Kimmeridge and Durdle Door and the control plant, Brussels sprout. ($F_{4,144}$ = 3.41, p = 0.011*). Standard error values are indicated (±SE).

Results obtained from method two agree with results from method one, in that the only significant difference in the performance of *Brevicoryne brassicae* occurs in the "host-line" from Durdle Door. However, differences in the performance of this "host-line" do not occur on the same plant. Method one reveals that the performance of Durdle Door *Brevicoryne brassicae* differs significantly on *Brassica oleracea* from Kimmeridge, "typical" in its glucosinolate phenotype, whilst method two reveals that the performance differs on "atypical" *Brassica oleracea* from Kimmeridge.

- Intrinsic Rate of Natural Increase

The intrinsic rate of natural increase of *Brevicoryne brassicae* on the five plant types was analysed using general linear model (Table 3.5.).

Source	DF	Seq. SS	Adj. SS	Adj. MS	F	p
Plant Type	4	2.3501	2.2433	0.5608	1.94	0.104
Aphid "Host-line"	2	2.0999	1.8876	0.9438	3.26	0.039*
Plant*Aphid	8	4.5949	4.5949	0.5744	2.03	0.042*
Error	421	119.3727	119.3727	0.2835		
Total	435	129.6543				

Table 3.5. Analysis of the intrinsic rate of natural increase (r_m) of *Brevicoryne brassicae* on the investigative plant types. Plant type, aphid "host-line" and plant type*aphid "host-line" acted as model factors in the general linear model. Significant differences are highlighted ($p<0.05$*).

Results reveal that aphid "host-line" and plant type*aphid "host-line" affects the rate of increase of *Brevicoryne brassicae* to some degree ($F_{2,421} = 3.26$, $p = 0.039$ and $F_{8,421} = 2.03$, $p = 0.042$ respectively), whilst plant type does not ($F_{4,421} = 1.94$, $p = 0.104$).

- Intrinsic Rate of Natural Increase – Plant Type

Brevicoryne brassicae originated from Kimmeridge have a significantly lower rate of increase than *Brevicoryne brassicae* from Durdle Door on "atypical" *Brassica oleracea* from Kimmeridge ($F_{2,83} = 3.30$, $p = 0.042$). Regardless of "host-line," the rate of increase of *Brevicoryne brassicae* does not differ between the control plant, ($F_{2,115} = 0.62$, $p = 0.540$), *Brassica oleracea* from Kimmeridge, "typical" in terms of its glucosinolate profile ($F_{2,78} = 3.30$, $p = 0.188$), *Brassica oleracea* from Durdle Door "typical" ($F_{2,67} = 0.18$, $p = 0.859$) and "atypical" ($F_{2,77} = 2.05$, $p = 0.135$) in its glucosinolate profile (Figure 3.15.).

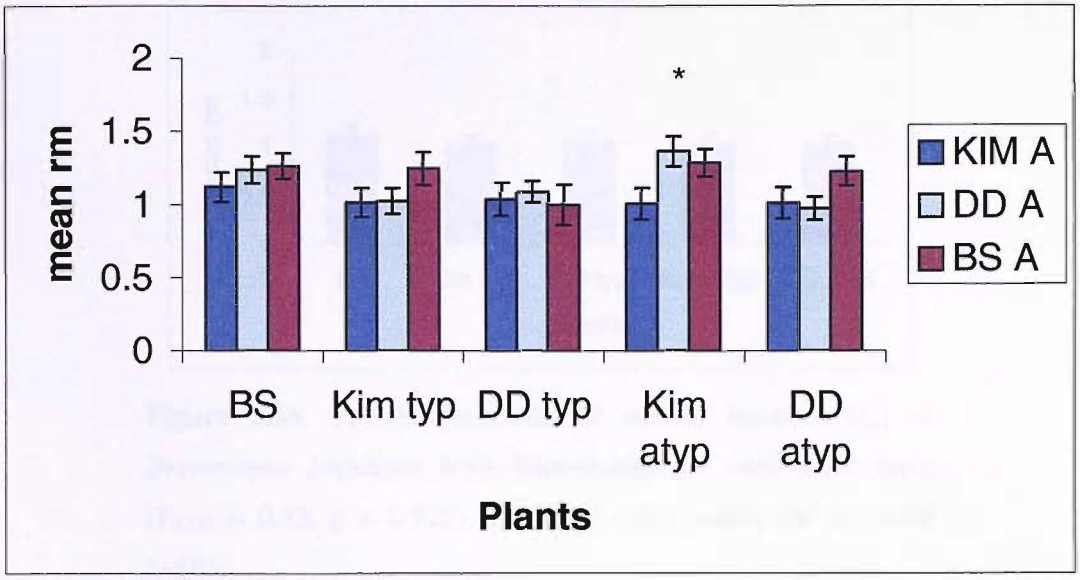


Figure 3.15. The intrinsic rate of natural increase (r_m) of the three "host-lines" of *Brevicoryne brassicae* on each of the five plant types. Significant differences ($p<0.05$*) and standard error values (±SE) are highlighted.

Analysis of the r_m values obtained during method one reveal that the rate of increase of each *Brevicoryne brassicae* "host-line" differs on the control plant and on *Brassica oleracea* from Kimmeridge "atypical" in its glucosinolate content. The only significant difference revealed during method two occurs on "atypical" *Brassica oleracea* from Kimmeridge (Figure 3.15.), again highlighting the lack of consistency between the two methods.

- Intrinsic Rate of Natural Increase – Aphid "Host-Line"

Differences in rates of increase are clearly revealed when each "host-line" is analysed separately. *Brevicoryne brassicae* from Kimmeridge ($F_{4,137} = 0.23$, $p = 0.922$) and *Brevicoryne brassicae* reared on Brussels sprout ($F_{4,139} = 0.96$, $p = 0.431$) do not display any significant differences in terms of its intrinsic rate of increase between *Brassica oleracea* from Kimmeridge and Durdle Door both "typical" and "atypical" and on the control plant (Figure 3.16 and 3.17. respectively).

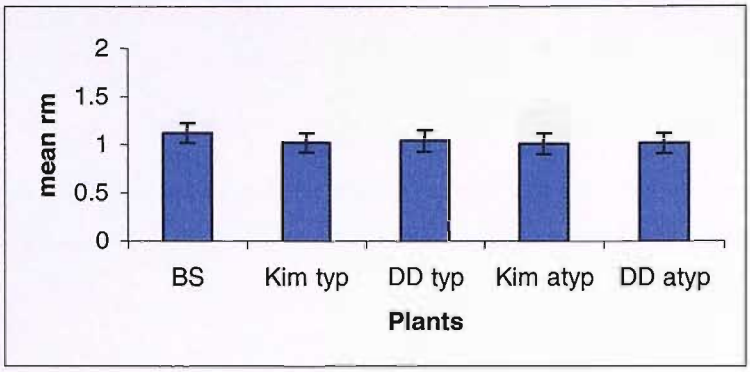


Figure 3.16. The intrinsic rate of natural increase (r_m) of *Brevicoryne brassicae* from Kimmeridge on each plant type. ($F_{4,137}$ = 0.23, p = 0.922). Standard error values are indicated (±SE).

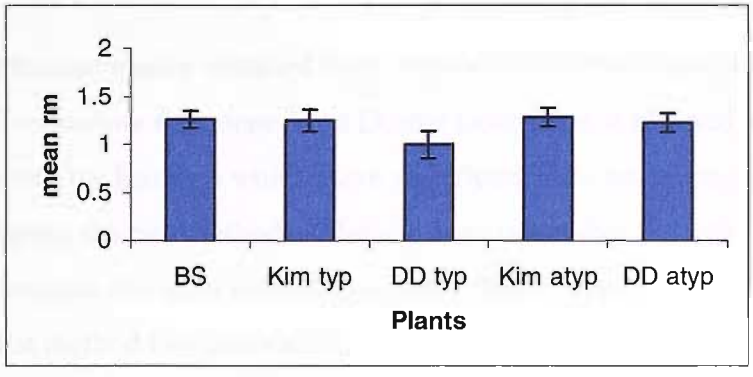


Figure 3.17. The intrinsic rate of natural increase (r_m) of the control *Brevicoryne brassicae* "host-line" on the five plant types. ($F_{4,139}$ = 0.96, p = 0.431). Standard error values are indicated (±SE).

Brevicoryne brassicae originated from Durdle Door displays significant differences in terms of the rate of increase between the investigated plants. The highest rate of increase is revealed on *Brassica oleracea* from Kimmeridge that is "atypical" in its glucosinolate content, in comparison to *Brassica oleracea* from Kimmeridge "typical" and *Brassica oleracea* from Durdle Door both "typical" and "atypical." The rate of increase of *Brevicoryne brassicae* is also significantly higher on Brussels sprout compared to *Brassica oleracea* from Durdle Door that is "atypical" in glucosinolate content ($F_{4,144}$ = 3.03, p = 0.020) (Figure 3.18.).

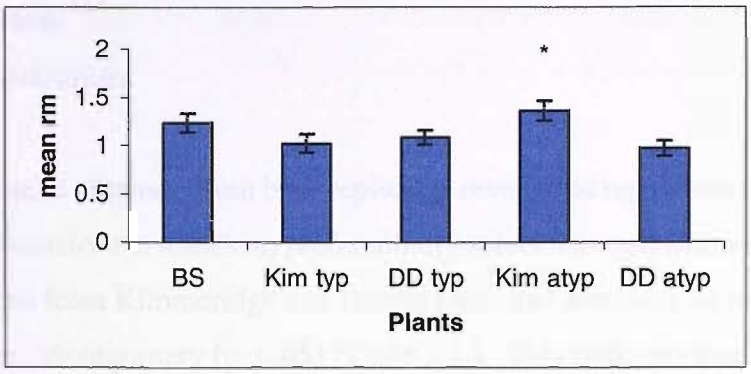


Figure 3.18. The intrinsic rate of natural increase (r_m) of *Brevicoryne brassicae* originated from Durdle Door on the five plant types ($F_{4,144} = 3.03$, $p = 0.020$). Significant differences are highlighted ($p<0.05$*). Standard error values are indicated (±SE).

The rate of increase results obtained from method two support results from method one in that *Brevicoryne brassicae* from Durdle Door is most affected by the host plant. However, the hosts on which these significant differences occur does not correlate between the two methods. Method one reveals that the rate of increase is highest on *Brassica oleracea* from Kimmeridge that is "typical" in glucosinolate content, whilst method two reveals that most variation occurs on "atypical" *Brassica oleracea* subsp. *oleracea* from Kimmeridge.

- Aphid Performance – Comparing Methods

The results obtained from both methods were analysed to determine the most effective method for assessing the population dynamics of *Brevicoryne brassicae*. Analysis reveals that method two is more effective in assessing the performance and the rate of increase of *Brevicoryne brassicae* on *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and on *Brassica oleracea* var. *gemmifera* var. Montgomery than method one (Appendix 3, Table 3.1. and 3.2.). Consistently greater aphid numbers and intrinsic rates of natural increase were observed using method two. The level of disturbance was the only difference between the two methods, therefore the apparent "preference" of method two, indicative of the higher performance values obtained, corresponds with the biology and behaviour of *Brevicoryne brassicae*. As stated, this species prefers to reproduce in a colony and with minimal disturbance (pers.obs.).

3.4. Discussion

3.4.1. Host Selection

95% of the results obtained from both replicates reveal that regardless of "host-line," *Brevicoryne brassicae* is unable to preferentially select between *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and *Brassica oleracea* var. *gemmifera* var. Montgomery ($p<0.05$) (Table 3.1.). This indicates that the glucosinolate differences produced by the hosts do not influence initial host selection or the short-term retention of *Brevicoryne brassicae* revealing that overall host selection is unaffected.

However, 5% of results do indicate significance. The host preferred during these two cases (*Brassica oleracea* from Durdle Door "atypical" and "typical" in glucosinolate profile respectively), occurs only 30 minutes after the start of the experiment. Therefore, significance could simply be explained by the fact that not all of the aphids placed within the experimental arena would have made a selection within that time, which could have easily distorted the results.

This study is the first to investigate host selection by *Brevicoryne brassicae* and the second known study to investigate host selection by a specialist Brassica aphid. However, no consistent findings between studies were obtained. Yue and Liu (2000) revealed that *Lipaphis erysimi* differentially selects between red and green varieties of *Brassica oleracea* var. *capitata*, preferring the green variety. Host differentiation could obviously have been a consequence of the differences in colour between the hosts, as it is accepted that physical differences play a key role during host selection by alate aphids (Yue & Liu, 2000). However, unlike this study, no attempt was made to ascertain whether the hosts exhibit other physical and chemical defensive traits, besides colour, which could have influenced the preferences observed by *Lipaphis erysimi.*

Two explanations for the lack of preference exhibited by *Brevicoryne brassicae* during this investigation are offered. Previously, host selection experiments have investigated the response of both alate aphids (Yue & Liu, 2000) and apterous aphids (Nikolakakis *et al.,* 2003), as debate exists as to which morph is fundamental during host selection. Hughes (1963) suggested that alates do not display specific attraction to host plants as they are often carried on the wind over long distances. This is opposed by Powell and

Hardie (2001) who revealed that alates are able to respond to volatile cues released by host plants. Nikolakakis *et al.,* (2003) suggested that both alate and apterous morphs are fundamental during the initial stages of host location and for establishing a population. Apterous *Brevicoryne brassicae* were investigated during this study based on research by Hodgson (1991) who stated that apterous morphs are important dispersal agents, allowing for a greater utilisation of the environment than by alate dispersal alone. Despite this evidence, it is possible that the lack of preference exhibited by *Brevicoryne brassicae* could be a result of the aphid morph investigated. It is possible that alates are adapted to recognise specific physical and/or chemical plant traits of suitable hosts which apterous aphids are less well adapted to detect and exploit. Due to the conflict that exits over which morph is important during host selection, it would be beneficial to repeat this experiment using alate *Brevicoryne brassicae* in controlled laboratory conditions to determine if alate females are important in host selection and whether this is linked to glucosinolates.

The "optimal host range hypothesis" proposed by Dixon (1998) provides the second explanation. According to theory, when an aphid lands on a host, its decision to stay or to locate a superior host depends on:

- The probability of locating a superior host
- The time the hosts remain suitable
- The relative rates of increase of the aphids on the hosts

Despite the fact that dispersal is hazardous for aphids, the benefits of locating a superior host outweigh the risks involved (Nikolakakis *et al.*, 2003). The lack of differential host selection, both in terms of initial selection and short-term retention, suggests that *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and Brussels sprout are suitable hosts for *Brevicoryne brassicae,* regardless of glucosinolate differences. This supports work by Ellis and Singh (1993) who revealed that these two Brassicas are true hosts of *Brevicoryne brassicae.* In addition, a positive control had previously been conducted in the laboratory, which confirmed the suitability of Brassicas as hosts for *Brevicoryne brassicae* (G. Poppy, pers. comm.).

3.4.2. Aphid Performance

Two methods were used to assess the performance and the intrinsic rate of natural increase (r_m) of *Brevicoryne brassicae* on *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and Brussels sprout var. Montgomery. The population size and rate of increase of the three *Brevicoryne brassicae* "host-lines" were consistently greater during method two (Appendix 3, Table 3.1. and 3.2.). This suggests that method two provides a clearer, more realistic estimation of the performance of *Brevicoryne brassicae*.

The differences observed between the two methods can be explained by the experimental procedure. Method one is considerably more labour intensive than method two. The number of aphids present on each plant was counted at three days, five days, seven days, nine days and ten days after the start of the experiment. Despite efforts to minimise disruption to the aphid colonies, this continued disturbance could have caused an increase in aphid mortality. As stated, *Brevicoryne brassicae* prefers to feed and reproduce within a colony (pers. obs.), thus an increase in the proportion of deaths, inevitably decreases the size of the population and consequently could increase the development time of the surviving aphids. Due to the lack of disruption to the aphid colonies, the results obtained from method two are accepted with greater confidence. This raises an unexpected question. Do trade-offs occur when designing bioassays? For example, bioassays which yield a high quantity of data, at the expense of causing high levels of disturbance and possibly presenting inaccurate insights versus obtaining a lower quantity of data with minimal disturbance, but presents a higher quality of data and more realistic insights.

Brevicoryne brassicae survives and reproduces on each plant type investigated, supporting the fact that *Brassica oleracea* subsp. *oleracea* and *Brassica oleracea* var. *gemmifera* are suitable hosts (Ellis & Singh, 1993). Method one reveals that the mean transformed population size varied from 2.9277 on the control plant to 3.480 on *Brassica oleracea* from Kimmeridge, "typical" in its glucosinolate phenotype. The highest and lowest intrinsic rate of natural increase (0.2582 and 0.1288 respectively) occurred on the same plant types. This suggests that whilst the presence of glucosinolates may influence the behaviour of *Brevicoryne brassicae* (Cole, 1997), it appears that the differences in glucosinolate profile produced by the plant types

investigated during this study are either not great enough to warrant behavioural differences or that glucosinolates may simply need to be present above a threshold concentration for the host to be deemed suitable for *Brevicoryne brassicae*.

The mean intrinsic rate of increase for the control line of *Brevicoryne brassicae* on the control plant during method one is 0.2049 (n=44). Kift *et al.*, (1999) obtained an r_m value of 0.2239 (n=5) using a similar method. This is the only other known study to assess the rate of increase of *Brevicoryne brassicae* using a stable age distribution on whole plants. However, the differences in replicate size should be noted. Despite this, the relatively high rate of increase obtained from both studies indicates that a stable population of aphids on whole plants provides a more realistic representation of the dynamics of *Brevicoryne brassicae* rather than confining single aphids in clip cages. This is supported by the low r_m value of 0.1616 obtained using clip cages (Kift *et al.*, 1999).

The results obtained from method two were consistently higher than those obtained during method one. Therefore, as stated, it is proposed that method two provides a more accurate estimation of the population dynamics of *Brevicoryne brassicae* than method one and by using clip cages. Interestingly, the highest and lowest population size (3.7098 and 3.2353 respectively) of *Brevicoryne brassicae* occurred on the same plant type, *Brassica oleracea* from Kimmeridge, "atypical" in its glucosinolate phenotype during method two. Again, this indicates that the glucosinolate differences produced by wild cabbages from Kimmeridge and Durdle Door may not be great enough to affect the behaviour of *Brevicoryne brassicae*. The intrinsic rate of increase of *Brevicoryne brassicae* varied from 0.9702 on *Brassica oleracea* from Durdle Door "atypical" in its glucosinolate profile to 1.2828 on *Brassica oleracea* from Kimmeridge "atypical" in profile. Interestingly this reveals that the population size and the intrinsic rate of natural increase of an aphid population do not always correlate. This raises the possibility that the rate of increase could be a measure of how well an aphid population recovers after being transferred to a new host in the laboratory.

A number of reasons may explain the lack of a link between the behaviour of *Brevicoryne brassicae* and glucosinolates despite evidence to the contrary.

- Cole (1997) investigated the quality of glucosinolates produced by wild and cultivated Brassica species and found a relationship between the rate of increase

of *Brevicoryne brassicae* and four individual glucosinolates. Cole (1997) concluded that Brassicas producing low concentrations of 2-propenylglucosinolate and 2-hydroxy-3-butenylglucosinolate, but high concentrations of 3-butenylglucosinolate and 4-pentenylglucosinolate should be more resistant to Brassica aphids. The individual glucosinolate profiles of plants from Kimmeridge and Durdle Door were determined in Chapter Two. According to Cole (1997), *Brassica oleracea* subsp. *oleracea* from Durdle Door should be more resistant to *Brevicoryne brassicae* and thus support smaller population sizes and lower rates of natural increase. Results do not support this. It seems that the differences in the quantity and quality of glucosinolates may not be as important as initially hypothesised or previously suggested (Rodman & Chew, 1980; van Emden, 1990; Cole, 1996; 1997). This is because all of the Brassicas investigated are susceptible to *Brevicoryne brassicae,* regardless of glucosinolate profile. Results suggest that the presence of glucosinolates at a threshold concentration may be sufficient for the host to be deemed suitable.

- The activity of myrosinase was not analysed and thus could vary between the host plants. Plant myrosinase is highly variable, in terms of its distribution (MacGibbon & Allison, 1970) and its activity (Bones, 1990). Therefore, the release rates of glucosinolates could differ between *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and consequently could affect the concentration of glucosinolates produced. Therefore, it is possible that the glucosinolate content of the hosts from the two sites may not be as extreme as originally determined.
- *Brassica oleracea* subsp. *oleracea* is an obligate outbreeder (Mitchell, 1976), and thus inevitably has variable paternal genotypes. It is likely that seeds used during laboratory experiments would not always display the same glucosinolate phenotype as its sibling. As expected, current research suggests that genetic variation does exist in seeds collected from the same mother (T. Stirrup, pers. comm.). The only way to completely determine the glucosinolate profile of each plant used in the laboratory is to analyse the profile by HPLC (Chapter Two). However, a minimum of 300 plants was used for each method of the aphid performance experiments, therefore time and resources made this an unrealistic achievement during this study.
- Chemical factors, other than glucosinolates may influence aphid performance. Previous studies have revealed a relationship between aphid population growth

and the concentration of amino acids (Jansson & Smilowitz, 1986; Mentink, 1986; van Emden, 1990; Zou *et al.*, 1992; Petersen & Sandström, 2001). It is suggested that resistant and susceptible hosts differ in amino acid concentration (Mentink, 1986) and profile (Febvay *et al.*, 1992; Kazemi & van Emden, 1992). van Emden (1990) proposed that amino acid concentration influences feeding by generalist aphids, whilst specific chemicals such as glucosinolates influence specialist aphids. However, Cole (1997) revealed a relationship between the rate of increase of *Brevicoryne brassicae* and the concentration of tyrosine, alanine, leucine and glutamic acid. This supported work by Zou *et al.*, (1992) when it was discovered that low concentrations of tyrosine and glutamic acid improved the performance of *Brevicoryne brassicae*, due to an increase in alate females. It would be interesting to investigate the differences in nutritional factors, such as the availability of free amino acids produced by *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and whether the behaviour of *Brevicoryne brassicae* is related. The differences in nutritional composition of the wild cabbage were outside the realm of this investigation as the difference in a specific biochemical factor is the primary focus of this study.

- Interestingly, results from methods one and two correlate to reveal that "host-line" variation exists for *Brevicoryne brassicae,* suggesting that the aphid species itself could be fundamental in influencing aphid behaviour.

"Host-line" variation exists in *Brevicoryne brassicae. Brevicoryne brassicae* from Kimmeridge and the control line do not differ in terms of its population size and rate of increase on *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and the control plant, Brussels sprout var. Montgomery ($p<0.05$). However, *Brevicoryne brassicae* from Durdle Door is significantly affected by its host. *Brevicoryne brassicae* from Durdle Door reproduce at a faster rate and display the greatest population size on *Brassica oleracea* from Kimmeridge which is "typical" in glucosinolate profile in method one and "atypical" in its glucosinolate content in method two.

This is the first known study to reveal that "host-line" variation occurs in *Brevicoryne brassicae.* Evidence of "host-line" variation strongly suggests that interclonal variation exists in *Brevicoryne brassicae.* Interclonal variation, in that morphological and genetic differences are observed in aphid clones has previously been reported in *Myzus persicae* (Nikolakakis *et al.,* 2003). Therefore, the next step would be to adopt a genetic

approach to determine the differences between the three "host-lines" to confirm that interclonal variation exists in *Brevicoryne brassicae*.

Possible explanations for the variation observed within *Brevicoryne brassicae* lines are proposed. Maternal effects could influence the performance of aphids and induce variation within a species. De Barro *et al.*, (1995) revealed that the performance of *Sitobion avenae* improved if its mother fed on the host. However, results do not support this as *Brevicoryne brassicae* originated from Durdle Door performed better on *Brassica oleracea* subsp. *oleracea* from Kimmeridge than Durdle Door.

A study by Cole (1994) raised an interesting question, which may indicate why "host-line" variation and interclonal variation exists in aphids. Cole (1994) monitored the feeding preference of *Brevicoryne brassicae* electronically, to investigate the resistance mechanisms of a variety of Brassicas. Initially all Brassicas were suitable hosts, but feeding activity differences were revealed once the phloem element was penetrated (Cole, 1994). It has been suggested that true host selection occurs in the pharyngeal cavity of the aphid when chemicals translocated in the phloem are sampled (Tjallingii, 1985; Wensler & Filshie, 1969). Cole (1994) proposed that the differences in aphid behaviour is either a result of a chemical deterrent, a lack of stimulant or the production of a mechanical barrier produced by the host in response to aphid feeding, which is thought to be species specific with respect to the aphid. It is possible that whilst the three "host-lines" of *Brevicoryne brassicae* are able to successfully feed and reproduce on *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and Brussels sprout var. Montgomery, each "host-line" may respond differently to the same host type. To elaborate, the "host-line" originally from Durdle Door may respond differently to either the chemical profile of the host or the aphid may invoke differential plant defences, which the other two aphid "host-lines" do not.

The myrosinase-glucosinolate system may explain why the performance of *Brevicoryne brassicae* originated from Durdle Door is most affected. Myrosinases are produced by specialist aphids including *Lipaphis erysimi* (Blande, 2004) and *Brevicoryne brassicae* (MacGibbon & Beuzenberg, 1978; Bridges *et al.*, 2002), to sequester glucosinolates produced by host plants. Work on aphid myrosinases is progressing, but plant myrosinase activity is well documented (James & Rossiter, 1991; Falk *et al.*, 1992; Bones & Rossiter, 1996; Mithen, 2001). It is accepted that myrosinases are produced

by Brassicas to hydrolyse glucosinolates to aglucones (Cole, 1977) in response to herbivore damage. The distribution and activity of plant myrosinase varies with age, between organs and species (Bones & Rossiter, 1996). It is also revealed that multiple forms of myrosinase exist (James & Rossiter, 1991). All of which can inevitably affect glucosinolate hydrolysis. Therefore, it is possible that the same variation in myrosinase production, activity and distribution occurs in aphid myrosinases. It is proposed that "host-line" variation occurs because *Brevicoryne brassicae* have evolved myrosinases, specifically adapted to hydrolyse and sequester the specific profile of glucosinolates produced by their hosts. Thus, *Brevicoryne brassicae* from Durdle Door could be adapted to the specific quantity and to a lesser extent, quality of glucosinolates produced by their hosts. This theory is further supported by the fact that *Brevicoryne brassicae* from Kimmeridge and the control line show similar performances on the investigated plant types. It is possible that these two "host-lines" may be adapted to host plants producing low quantities of glucosinolates and thus characteristically produce specific myrosinase isoenzymes to degrade these low levels. It would be extremely interesting to investigate the types of myrosinase isoenzymes produced by *Brevicoryne brassicae* to determine whether these isoenzymes differ between the aphid "host-lines." This could in turn affect the chemistry of the aphids, which could have important implications on the action of insects in higher trophic levels. The revelation that "host-line" variation exists in *Brevicoryne brassicae* could have huge implications for aphid control, as it suggests that the aphid species itself may be as important as the host plant in explaining aphid behaviour.

In conclusion, this study is not an investigation of the presence/absence of glucosinolates, rather it is a comparative study investigating the effect of glucosinolate extremes within a wild tritrophic interaction. The results obtained suggest that the behaviour of *Brevicoryne brassicae*, in terms of host selection and performance, is not influenced by the quantitative and qualitative differences in glucosinolate phenotype of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door. It is possible that glucosinolates may only be required at a threshold concentration for the host to be deemed suitable for *Brevicoryne brassicae.* Interestingly, "host line" variation exists in *Brevicoryne brassicae*, which may account for the differential behaviour observed to a greater degree than the effect of the host itself. "Host-line" variation strongly implies that interclonal variation exists, however a genetic approach is required to confirm that interclonal variation exists in this aphid species.

Summary

- Apterous *Brevicoryne brassicae* are unable to preferentially select between *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and the control plant, Brussels sprout var. Montgomery.
- The quantitative and qualitative differences in glucosinolates produced by *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door do not influence the selection behaviour or the performance of *Brevicoryne brassicae.*
- "Host-line" variation, in terms of aphid performance, exists in *Brevicoryne brassicae.*

Chapter Four

Third Trophic Level

How does the Glucosinolate Phenotype affect *Diaeretiella rapae*?

Chapter Four

How does the Glucosinolate Phenotype affect *Diaeretiella rapae?*

4.1. Introduction

Brassica oleracea subsp. *oleracea* at Durdle Door produce the highest quantity of total aliphatic glucosinolates and total glucosinolates compared with those growing at Kimmeridge (Chapter Two). The experiments described in Chapter Three investigated the preference and performance of *Brevicoryne brassicae* to *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door. This chapter aims to investigate whether there is a relationship between glucosinolate phenotype and parasitoid behaviour.

After emergence, adult female parasitoids need to locate and recognise their hosts in order to reproduce (Powell & Wright, 1992). It is generally accepted that many parasitoids locate their hosts via a series of specific physical and chemical cues emitted from their host, the plant or a combination of both (plant-host complexes: PHC) (Vinson, 1976). These cues are important during foraging as they initiate a directed response by the female parasitoid, thus restricting the habitats searched and the host species located (Vinson, 1976).

Herbivore-damaged plants emit both quantitatively and qualitatively different volatile blends compared to undamaged plants (Vet & Dicke, 1992; Dicke, 2000). It is thought that natural enemies in the third trophic level utilise these altered chemical blends at distances to locate suitable habitats where their prey are likely to be present. Once in that habitat, more specific host-derived cues, including the aphid sex pheromone (Glinwood *et al.*, 1999) and honeydew (Shaltiel & Ayal, 1998) are used to locate their hosts (Vet & Dicke, 1992).

Diaeretiella rapae (M^{c}Intosh) (Hymenoptera: Braconidae) is often regarded to be specialised on Brassica-feeding aphids (Vaughn *et al.*, 1996). However, it should be noted that despite a "preference" for Brassica aphids, *Diaeretiella rapae* is able to parasitise more than 60 aphid species (Pike *et al.*, 1999), including the Russian wheat aphid (Vaughn *et al.*, 1996). Despite the putative wide host range of *Diaeretiella rapae*,

it is an important biological control agent of the Russian wheat aphid in the USA (Vaughn *et al.*, 1996).

There is considerable evidence for the enhanced attractiveness of aphid parasitoids to plant-host complexes compared to undamaged plants (Du *et al.*, 1996; 1997; Guerrieri *et al.*, 1993; 1997; Havill & Raffa, 2000; Storeck *et al.*, 2000). With respect to *Diaeretiella rapae*, Vaughn *et al.*, (1996) revealed that both male and female parasitoids respond to a green-leaf volatile ((Z)-3-hexan-1-ol), however only females elicited a physiological and behavioural response to induced volatiles emitted from Brassica plants. This suggests that females utilise these specific plant volatiles to locate specific prey, supporting the indirect defensive theory stated by Vet and Dicke (1992).

Plant volatiles are likely to be utilised during "steps" one and two (host-habitat location and host location respectively) of parasitoid foraging (Vinson, 1976). Y-tube olfactometer experiments were designed to investigate the role of plant volatiles during these "steps" of parasitoid foraging behaviour. Relevant to this study, the importance of Brassica volatiles in the foraging behaviour of *Diaeretiella rapae* was confirmed by Bradbourne and Mithen (2000). Near-isogenic lines of *Brassica oleracea* were produced which differed only in the amount of a particular volatile produced. Laboratory-based olfactometer studies and semi-field experiments revealed that plants emitting 3-butenylisothiocyanate had increased attractiveness to *Diaeretiella rapae*. The importance of synthetic 3-butenylisothiocyanate was confirmed in the laboratory by Blande (2004). This is important to this study as wild *Brassica oleracea* from Durdle Door produce 3-butenylisothiocyanate in contrast to those at Kimmeridge that do not (Chapter Two). This provides a natural system in which to test the putative importance of Brassica volatiles during foraging by *Diaeretiella rapae*. Synthetic 3-butenylisothiocyanate will be added to the system to confirm its attractiveness.

It is documented that physical and/or chemical cues from aphids influence parasitoid behaviour. Colour and movement (Michaud & Mackauer, 1994), instar stage (Lin & Ives, 2003) and colony distribution (Lopez *et al.*, 1990) are thought to influence the host acceptance, host suitability and host regulation "steps" of successful parasitoid foraging. However, during this study attention is restricted to investigating the effect of chemistry, particularly plant chemistry on *Diaeretiella rapae* foraging. Therefore, in the second section of this chapter, a series of attack rate bioassays have been designed to

investigate these chemical effects on "step" three of the foraging behaviour of *Diaeretiella rapae* (Vinson, 1976).

Specialist Brassica aphids are thought to sequester glucosinolates (MacGibbon & Beuzenberg, 1978; Bridges *et al.*, 2002; Blande, 2004), resulting in the storage of relatively high levels of glucosinolates within the tissue of the aphid. It was suggested that the three "host-lines" of *Brevicoryne brassicae* differ in terms of their myrosinase activity, which in turn could result in differing quantities and qualities of glucosinolates stored and subsequent volatiles produced. It was proposed that *Brevicoryne brassicae* originally from Durdle Door accumulate the highest levels of glucosinolates and release the highest levels of isothiocyanates. It is hypothesised that *Diaeretiella rapae* will be adapted to respond to the higher chemical levels produced and in turn utilise them during foraging. It is expected that *Diaeretiella rapae* will exhibit greater attack rate on the plant-host complex (PHC) highest in their glucosinolate/isothiocyanate content.

The effect of conditioning and learning on the response of adult female parasitoids to odours is well researched (van Emden *et al.,* 1996; Du *et al.,* 1997; 1998; Storeck *et al.,* 2000; Blande *et al.,* 2004). Conditioning is defined as behaviour that influences events and/or experiences during larval and pupal development, but not responses that are learnt as adults (Poppy *et al.*, 1997). Emergence conditioning also occurs during emergence from mummy cases (van Emden *et al.,* 1996; Storeck *et al.,* 2000; Blande, 2004). Despite aphid parasitoids having being exposed to many conditioning cues during the early stages of their life cycle, it is the effect of post-emergence experience on the response of *Diaeretiella rapae* that is investigated in this chapter. Many studies have documented the importance of learning experiences post-emergence in influencing the response of aphid parasitoids to odours (Du *et al.*, 1996; 1997; 1998; Storeck *et al.*, 2000; Blande *et al.*, 2004). Despite the fact that parasitoids are never completely naïve due to the effects of conditioning, "naïve" parasitoids are accepted as having no prior exposure to odours from plant-host complexes (PHC) post-emergence. In contrast, experienced parasitoids are allowed plant-host complex (PHC) contact post-emergence to the point of making ovipositional attacks, several hours prior to use in experiments. The effect of this learning experience has enabled parasitoids to differentiate between infested and un-infested plants (Du *et al.*, 1997; 1998) or exhibit enhanced responses (Du *et al.*, 1997; Blande *et al.*, 2004). Both naïve and experienced adult female parasitoids will be investigated during attack rate bioassays. This will determine

whether *Diaeretiella rapae* have an innate ability to respond to Brassica volatiles or whether their behaviour is plastic and can thus be influenced by a learning experience (Poppy *et al.*, 1997).

The effect of experience has focused on laboratory-reared parasitoids (Storeck *et al.*, 2000; Blande, 2004), therefore this is under-researched in field-collected individuals (Vaughn *et al.*, 1996). This thesis aims to investigate whether exposing field-collected insects to plant-host complexes (PHC) prior to experimentation initiates the same response that is observed in laboratory-reared insects.

Hypotheses:

- *Diaeretiella rapae* will be able to differentiate between plant-host complexes (PHC) from Kimmeridge and Durdle Door. *Diaeretiella rapae* will preferentially select odours emitted from the "typical" plant-host complex (PHC) from Durdle Door.
- When synthetic 3-butenylisothiocyanate is added to the "typical" plant-host complex (PHC) from Kimmeridge, the response of *Diaeretiella rapae* will be equal to that observed towards the "typical" plant-host complex (PHC) from Durdle Door.
- Regardless of parasitoid origin, *Diaeretiella rapae* will differentially attack the plant-host complexes (PHC) from Kimmeridge, Durdle Door and the control, displaying greater attack rates on the plant-host complex (PHC) from Durdle Door.

4.2. Materials and Methods

- Plants

Brassica oleracea subsp. *oleracea* from Kimmeridge and Durdle Door, "typical" in glucosinolate genotype were investigated during Y-tube olfactometer bioassays. *Brassica oleracea* from Kimmeridge and Durdle Door both "typical" and "atypical" in glucosinolate profile were used during attack rate bioassays (Chapter Two). All plants were used at the 4–5 leaf stage. Plants were sown and grown in 7x7x8cm pots (Sopodex, UK) in Levingtons® compost at the University of Southampton glasshouses ($20^{o}C \pm 3^{o}C$, L12:D12) and watered when required.

- Aphids

Cultures of *Brevicoryne brassicae* were established using aphids collected from *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door in 2002. Stocks were replenished in 2003 and 2004. Aphids were reared on *Brassica oleracea* grown from seed collected from their "native" field site to enable separate "host-lines" of *Brevicoryne brassicae* to be established. The control aphid culture was started from stocks of *Brevicoryne brassicae* reared on Brussels sprout var. Montgomery (*Brassica oleracea* var. *gemmifera*) from HRI Wellesbourne (Warwick, UK). All aphid cultures were reared in individual cages (22°C ± 3°C, L16:D8) to ensure that the "host-lines" of *Brevicoryne brassicae* remained distinct.

- Parasitoid Rearing

Mummified *Brevicoryne brassicae* were collected from *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door when required for experiments. A control culture (laboratory-reared) of *Diaeretiella rapae* was established from stocks collected from Rothamsted Research, which were reared on *Myzus persicae* on Chinese cabbage. The original stocks were started in 1990 and supplemented in 1995 with 30 females and 20 males collected from oilseed rape (Blande, 2004). The control culture was reared on *Myzus persicae* on *Brassica napus* var. Apex in an individual cage in the insectary at the University of Southampton (22°C ± 3°C, L16:D8).

- Harvesting Parasitoids for Experiments

Mummies were transferred to plastic containers that were completely darkened by black tape. A 3cm diameter hole was cut out of the lid of the container where upon a jar was placed upside down. Adult *Diaeretiella rapae* emerged from the dark container into the jar by means of phototaxis. Every 24 hours, the jar was removed and a clean jar added for further parasitoid emergence. Cotton wool soaked in 25% honey solution was added to the removed jar containing the parasitoids to act as a food source. This was repeated until all of the parasitoids had emerged. Female *Diaeretiella rapae* were not used in experiments until they were two days old. This ensured that the females had an opportunity to mate.

Both naïve and experienced females were investigated. Naïve females were not exposed to plants or aphids prior to experimentation. Experienced females were exposed to their "native" plant-host complex (PHC). This involved releasing the

parasitoids into a cage containing their "native" host plant heavily infested with their "native" host aphids for 10 minutes. During this time the parasitoids were observed making full contact ovipositional attacks. The insects were then removed using an electric pooter and transferred to individual glass vials containing paper soaked in 25% honey solution. The parasitoids were moved to a controlled bioassay room (22°C ± 3°C, 45-60% RH) 2–3 hours before being used in bioassays as this has been shown to increase performance in bioassays (G. Poppy. pers.comm.).

4.2.1. Attack Rate Bioassays

- Attack Rate Arenas

The attack rate arena consisted of a 9cm diameter Petri dish (Bibby Sterilin LtD, Staffordshire, UK), with a 7cm diameter leaf disc embedded in agar (Sigma, Poole, UK). Twenty 2nd and 3rd instar aphids were transferred to the corresponding leaf disc using a fine paintbrush. Individual female *Diaeretiella rapae* were added to each attack rate arena (Figure 4.1.). The time of the first full contact ovipositional attack and the total number of attacks made in 10 minutes were recorded. 20 replicates were completed for each plant-host complex (PHC) investigated.

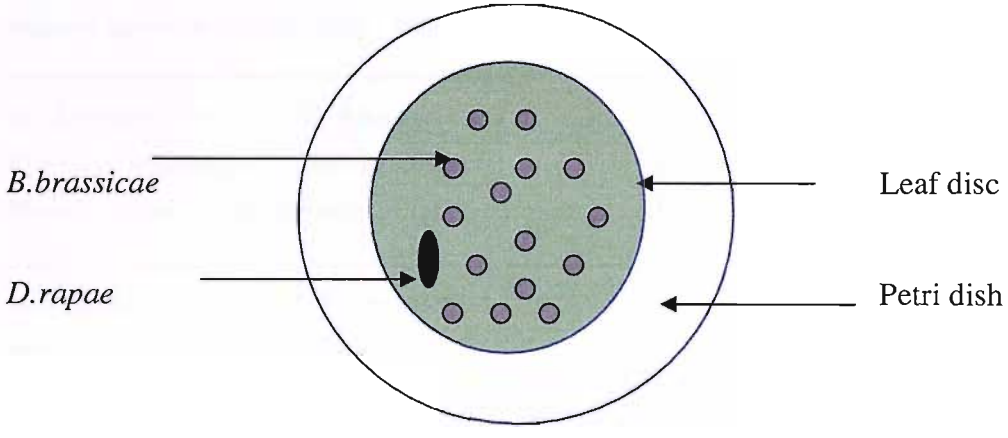


Figure 4.1. Attack Rate Bioassay Arena.

All attack rate bioassays were conducted under a laminar light source in a controlled environment room (22°C ± 3°C, 45-60% RH) in a blackened arena to minimise any external visual stimuli.

- Plant–Host Complexes (PHC).

The plant–host complexes (PHC) investigated during attack rate bioassays are listed (Figure 4.2.).

Plant-Host Complex (PHC)	*Diaeretiella rapae*
DD *B.oleracea* "typical" –DD *B.brassicae* Kim *B.oleracea* "typical" –Kim *B.brassicae* Brussels sprout var. Montgomery – Control *B.brassicae*	Naïve DD "native" *D.rapae*
DD *B.oleracea* "typical" –DD *B.brassicae* Kim *B.oleracea* "typical" –Kim *B.brassicae* Brussels sprout var. Montgomery – Control *B.brassicae*	Naïve Kim "native" *D.rapae*
DD *B.oleracea* "typical" –DD *B.brassicae* Kim *B.oleracea* "typical" –Kim *B.brassicae* Brussels sprout var. Montgomery – Control *B.brassicae*	Naïve Control *D.rapae*
DD *B.oleracea* "Atypical" –DD *B.brassicae* Kim *B.oleracea* "Atypical" –Kim *B.brassicae* Brussels sprout var. Montgomery – Control *B.brassicae*	Naïve DD "native" *D.rapae*
DD *B.oleracea* "Atypical" –DD *B.brassicae* Kim *B.oleracea* "Atypical" –Kim *B.brassicae* Brussels sprout var. Montgomery – Control *B.brassicae*	Naïve Kim "native" *D.rapae*
DD *B.oleracea* "typical" –DD *B.brassicae* Kim *B.oleracea* "typical" –Kim *B.brassicae* Brussels sprout var. Montgomery – Control *B.brassicae*	Experienced DD "native" *D.rapae*
DD *B.oleracea* "typical" –DD *B.brassicae* Kim *B.oleracea* "typical" –Kim *B.brassicae* Brussels sprout var. Montgomery – Control *B.brassicae*	Experienced Control *D.rapae*
DD *B.oleracea* "Atypical" –DD *B.brassicae* Kim *B.oleracea* "Atypical" –Kim *B.brassicae* Brussels sprout var. Montgomery – Control *B.brassicae*	Experienced DD "native" *D.rapae*

Figure 4.2. Plant–Host Combinations investigated during Attack Rate Bioassays. DD = Durdle Door. Kim = Kimmeridge. Typical glucosinolate PHC. Atypical glucosinolate PHC. Naïve DD "native" *D.rapae*. Experienced DD "native" *D.rapae*. Naïve Kim "native" *D.rapae*. Naïve Control *D.rapae*. Experienced Control *D.rapae*.

- Statistical Analysis

All analyses were conducted using Minitab, Version 13. One-way analysis of variance was used to analyse variations in attack rate and initial time of attack by each "plant-host-line" of *Diaeretiella rapae* (Kimmeridge *D.rapae*, Durdle Door *D.rapae*, Laboratory-reared *D.rapae*) on each "typical" and "atypical" plant-host complex. Bioassays not resulting in attacks were omitted from the data set before analysis was conducted. Fisher's Pairwise Comparison was used to analyse any significant results ($p<0.05$). The attack activity of the three lines of naïve *Diaeretiella rapae* (field-collected and laboratory-reared) on each plant-host complex were compared and analysed using a 2-sample t-test. The attack activity of naïve and experienced field-collected and laboratory-reared *Diaeretiella rapae* were also compared and analysed using a 2-sample t-test. The attack activity of naïve field-collected and laboratory-reared *Diaeretiella rapae* exposed to "typical" plant-host complexes were analysed using one-way analysis of variance. Fisher's Pairwise Comparison further analysed any significant results ($p<0.05$).

4.2.2. Y-tube Olfactometer Bioassays

- Plants

Undamaged, clean *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door, "typical" in glucosinolate profile, at the 4–5 leaf stage, were transferred from the glasshouse into a controlled environment room (20°C ± 3°C, L16:D8).

- Insects

Plants were infested with an excess of 250 *Brevicoryne brassicae* of mixed instars from the control culture (reared on Brussels sprout var. Montgomery), five days prior to conducting experiments. Naïve, female, laboratory-reared *Diaeretiella rapae,* cultured on *Myzus persicae* and *Brassica napus* var. Apex were used during Y-tube olfactometer bioassays. Parasitoids were harvested as previously stated.

- Y-tube Olfactometer Apparatus

A Y-tube olfactometer was used to compare the attractiveness of "typical" *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door to *Diaeretiella rapae*. Air was pumped through the system via Teflon tubing (diameter 1mm) into an activated charcoal filter, which removes any impurities present. Purified air is then pumped

through further Teflon tubing to a flow meter set at 800ml/min and then divided equally into two using a T-junction fitting (Swagelock, Ohio, USA). This results in an equal flow of air into the two glass vessels hosting the odour source. Air is then pushed out of the glass vessels at a rate of 400ml/min, accurately achieved using a flow meter via more Teflon tubing. Teflon tubing leads the air from the flow meters to the ends of the Y-tube connected via Swagelock fittings (Swagelock, Ohio, USA) (Figure 4.3. and 4.4.).

Figure 4.3. Y-tube Olfactometer Apparatus.

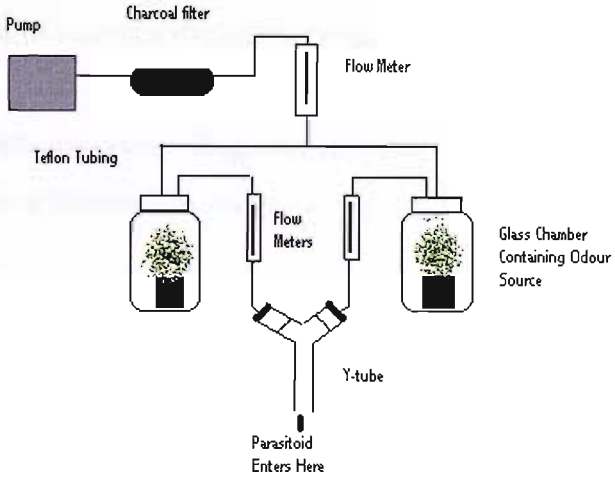


Figure 4.4. Diagrammatical Representation of the Y-tube Olfactometer Apparatus.

Female *Diaeretiella rapae* were released at the opening of the Y-tube olfactometer via glass vials. As soon as the insect entered the apparatus, its activity was monitored for five minutes. The arm the parasitoid selected was noted and the time it takes to do so was recorded (first selection). A positive selection for a specific odour was deemed if the parasitoid stayed in the upper section of the selected arm for 15 seconds (final selection) (Figure 4.5.).

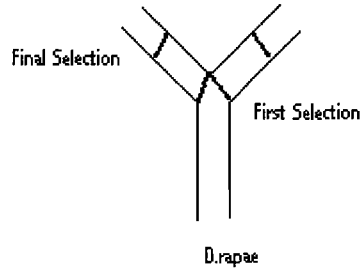


Figure 4.5. Diagrammatical representation of the selection points on the Y-tube.

All glassware was washed with hot water and Decon solution (Decon Laboratories Ltd, East Sussex, UK), followed by a rinse in distilled water, a rinse with acetone and a final rinse with distilled water. The glassware was then baked overnight at 225°C to remove any remaining impurities. The charcoal filter was reactivated by baking it overnight at 180°C with a flow of nitrogen passing through.

- **Y-tube Olfactometer – Experiment One**

  The response of *Diaeretiella rapae* to odours from PHC from Kimmeridge and Durdle Door.

Infested *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door both "typical" in glucosinolate phenotype were bioassayed opposite each other. The response of naïve laboratory-reared *Diaeretiella rapae* was investigated.

- **Y-tube Olfactometer – Experiment Two**

  The effect of synthetic 3-butenylisothiocyanate on the response of *Diaeretiella rapae* to odours from PHC from Kimmeridge and Durdle Door.

Infested *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door both "typical" in glucosinolate phenotype were compared, however synthetic 3-

butenylisothiocyanate was added to the vessel containing the complex from Kimmeridge. 3-butenylisothiocyanate was synthesised at Rothamsted Research. The volatile was made to a concentration of 50ng/µl in hexane and applied to Whatman number 1 filter paper in 50µl volumes using a syringe. A total of 1ml was added to the filter paper, which was necessary to achieve the same concentration of 3-butenylisothiocyanate stated by Blande (2004). The filter paper was attached to a glass pipette, which had previously been baked at 225°C overnight and inserted into the top layer of the soil. This was assayed against the "typical" plant-host complex from Durdle Door and the control chemical. The control chemical consisted of the same volume of hexane applied to the filter paper and inserted into the soil as before.

- Statistical Analysis

Parasitoids not making an odour selection were removed from the data set prior to analysis by Chi-squared (χ^2). The data were adjusted using Yates' Correction to account for data containing just two categories. The modified formula is shown:

$$\chi^2 = \frac{(O-E - 0.5)^2}{E}$$

O = the observed proportion of the total number of selections towards a specific odour source.
E = the expected number of selections towards an odour source if there is an equal chance of the choice occurring.

The time taken to make an initial and final selection was analysed using a 2-sample t-test.

4.2.3. Hind Tibia Measurements of *Diaeretiella rapae*

The length of the hind tibia of random samples of male and female *Diaeretiella rapae*, collected from Kimmeridge and Durdle Door and taken from the laboratory culture were measured. Measurements were recorded using a microscope with x45 magnification. The microscope was calibrated using a graticule. Each graduation represented 0.025mm.

- Statistical Analysis

The lengths of the hind tibia of *Diaeretiella rapae* were compared using one-way analysis of variance. Fisher's Pairwise Comparison was used to further analyse any significant results ($p<0.05$).

4.3. Results

4.3.1. Attack Rate Bioassays

- The Effect of Glucosinolate Phenotype on the Host Acceptance Behaviour of *Diaeretiella rapae.*

Results reveal that naïve *Diaeretiella rapae* collected from Kimmeridge differentially attacked "typical" plant-host complexes. A significantly higher number of attacks were observed on the control plant-host complex compared to "typical" plant-host complexes from Kimmeridge and Durdle Door ($F_{2,59}=3.31$, $p = 0.044$). Regardless of parasitoid origin, no further significant differences, in terms of attack rate were revealed for naïve *Diaeretiella rapae* ($p<0.05$) (Table 4.1.).

Experienced *Diaeretiella rapae* collected from Durdle Door, displayed a significantly higher attack rate on the "typical" plant-host complex from Durdle Door, compared to the "typical" Kimmeridge plant-host complex and the control plant-host complex ($F_{2,54}=6.84$, $p = 0.002$). However, no further significant differences in attack rates of experienced *Diaeretiella rapae* were observed ($p<0.05$). (Table 4.1.).

Parasitoid Origin	PHC Typ/Atyp Gsl	Total Attacks on individual plants: DD PHC	KIM PHC	BS PHC
Naïve DD *D.rapae*	Typ PHC	16.85±2.90	15.37±2.06	14.30±1.42
		$F_{2,58}$ =0.34, p=0.714		
Naïve Kim *D.rapae*	Typ PHC	11.95±1.49	14.70±1.54	19.40±2.85
		$F_{2,59}$ =3.31, p=0.044*		
Naïve LR *D.rapae*	Typ PHC	9.50±1.58	7.74±0.99	10.41±1.13
		$F_{2,57}$ =1.01, p=0.369		
Naïve DD *D.rapae*	Atyp PHC	17.20±2.25	17.50±2.99	12.60±2.62
		$F_{2,59}$=1.64, p=0.203		
Naïve Kim *D.rapae*	Atyp PHC	13.40±1.93	14.10±1.47	9.90±1.28
		$F_{2,59}$=2.02, p=0.142		
Exp DD *D.rapae*	Typ PHC	16.56±1.69	10.68±1.55	9.83±0.69
		$F_{2,54}$=6.84, p=0.002**		
Exp LR *D.rapae*	Typ PHC	16.11±2.59	16.10±2.64	19.88±3.25
		$F_{2,55}$=0.57, p=0.567		
Exp DD *D.rapae*	Atyp PHC	17.00±1.87	16.15±2.52	10.75±1.79
		$F_{2,59}$ =2.64, p=0.080		

Table 4.1. The attack rate of naïve and experienced field-collected and laboratory-reared *Diaeretiella rapae* on plant-host complexes (PHC) "typical" and "atypical" in their glucosinolate content. The mean number of attacks made on each PHC is stated, ±SE. Results of the one-way ANOVA are stated for each PHC-parasitoid combination. Significant differences are displayed in blue ($p<0.05$*, $p<0.01$**).

DD PHC = plant-host complex from Durdle Door.

Kim PHC = plant-host complex from Kimmeridge.

BS PHC = Control plant-host complex.

DD = *Diaeretiella rapae* collected from Durdle Door.

Kim = *Diaeretiella rapae* collected from Kimmeridge

LR = Laboratory-reared *Diaeretiella rapae*.

Exp = Experienced *Diaeretiella rapae*.

Regardless of parasitoid origin, the time taken to make the first full contact ovipositional attack by naïve and experienced females did not differ significantly for any plant-host complex differing in glucosinolate content ($p<0.05$) (Table 4.2.).

Parasitoid Origin	PHC Typ/Atyp Gsl	Time of first attack on individual plants: DD	KIM	BS
Naïve DD *D.rapae*	Typ PHC	126.1±25.1	213.8±28.1	185.3±30.1
		$F_{2,58}$ =2.58, p=0.085		
Naïve Kim *D.rapae*	Typ PHC	148.3±33.0	223.9±43.9	173.2±46.4
		$F_{2,59}$ =0.21, p=0.811		
Naïve LR *D.rapae*	Typ PHC	249.7±40.5	206.1±33.9	222.8±29.1
		$F_{2,57}$ =0.40, p=0.674		
Naïve DD *D.rapae*	Atyp PHC	126.1±24.2	135.5±33.7	206.2±30.5
		$F_{2,59}$=1.63, p=0.205		
Naïve Kim *D.rapae*	Atyp PHC	119.6±40.1	149.6±32.0	175.4±29.6
		$F_{2,59}$=0.47, p=0.627		
Exp DD *D.rapae*	Typ PHC	127.4±31.6	171.4±38.2	218.4±29.4
		$F_{2,54}$=2.39, p=0.102		
Exp LR *D.rapae*	Typ PHC	77.9±28.9	82.3±27.0	101.9±30.7
		$F_{2,55}$=0.57, p=0.569		
Exp DD *D.rapae*	Atyp PHC	141.4±26.6	193.1±36.7	199.7±36.1
		$F_{2,59}$ =0.91, p=0.407		

Table 4.2. The initial attack made by naïve and experienced field-collected and laboratory-reared *Diaeretiella rapae* on plant-host complexes (PHC) "typical" and "atypical" in their glucosinolate content. The time of the initial attack made on each PHC is stated, ±SE (seconds). Results of the one-way ANOVA are stated for each PHC-parasitoid combination ($p<0.05$).

DD PHC = plant-host complex from Durdle Door.

Kim PHC = plant-host complex from Kimmeridge.

BS PHC = Control plant-host complex.

DD = *Diaeretiella rapae* collected from Durdle Door.

Kim = *Diaeretiella rapae* collected from Kimmeridge

LR = Laboratory-reared *Diaeretiella rapae*.

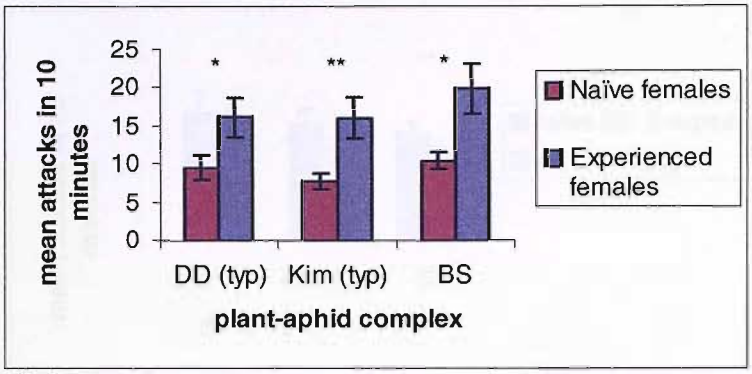
Exp = Experienced *Diaeretiella rapae*.

Despite the apparent lack of a relationship between glucosinolate phenotype and the host acceptance behaviour of *Diaeretiella rapae*, the origin of the parasitoids (field-collected and laboratory-reared insects) significantly influenced the attack behaviour observed.

- The Effect of Parasitoid Origin on the Host Acceptance Behaviour of *Diaeretiella rapae*.

Unexpected results concerning the effect of parasitoid origin and learning ability were revealed. As expected based on previous aphid parasitoid studies (Du *et al.,* 1997; 1998; Blande, 2004), experienced laboratory-reared *Diaeretiella rapae* showed significantly higher attack rates compared to naïve laboratory-reared insects on the "typical" PHC from Durdle Door ($t = -2.18$, $p = 0.038$ $DF = 30$), "typical" Kimmeridge PHC ($t = -2.97$, $p = 0.007$, $DF = 24$) and the control complex ($t = -2.75$, $p = 0.013$, $DF = 19$) (Figure 4.6.). In addition, experienced laboratory-reared females made an initial attack significantly faster on the "typical" Durdle Door PHC ($t = 3.45$, $p = 0.001$, $DF = 36$), "typical" Kimmeridge PHC ($t = 2.85$, $p = 0.007$, $DF = 34$) and the control complex ($t = 2.86$, $p = 0.008$, $DF = 31$) compared to naïve laboratory-reared insects (Figure 4.6.).

(a)



(b)

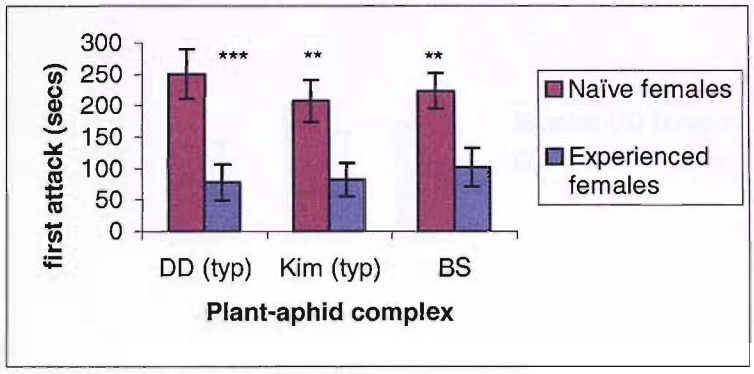


Figure 4.6. Comparing the attack rate (a) and time taken for an initial full contact ovipositional attack (b) by naïve and experienced laboratory-reared *Diaeretiella rapae* in arenas hosting "typical" glucosinolate PHC. BS acts the control. ($p<0.05$*, $p<0.01$**, $p<0.001$***). Standard error values are indicated (±SE).

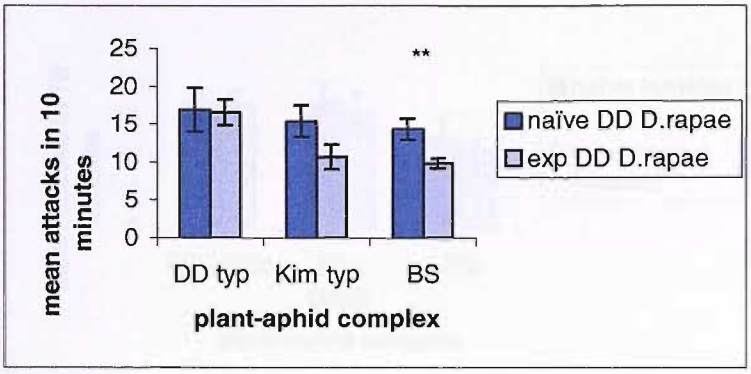
DD (typ) = "typical" Durdle Door *Brassica oleracea*

Kim (typ) = "typical" Kimmeridge *Brassica oleracea*

BS = Brussels sprout (Control)

However, this trend was not observed for naïve and experienced *Diaeretiella rapae* collected from Durdle Door. Experienced females did not display differential host acceptance behaviour on "typical" PHC, both in terms of initial attack and total attacks, compared to naïve females ($p<0.05$). The only significant difference was observed for the total number of attacks on the control PHC ($t = 2.83$, $p = 0.009$, $DF = 27$) (Figure 4.7.).

(a)



(b)

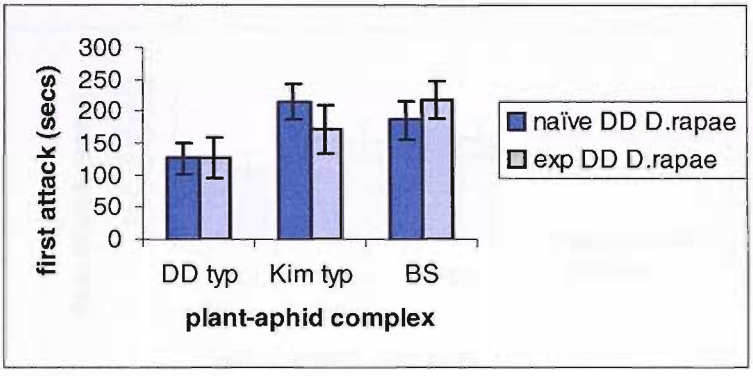


Figure 4.7. Comparing the attack rate (a) and time taken for an initial full contact ovipositional attack (b) by naïve and experienced *Diaeretiella rapae* from Durdle Door in arenas hosting "typical" glucosinolate PHC. BS acts the control. ($p<0.01$**). Standard error values are indicated (±SE).

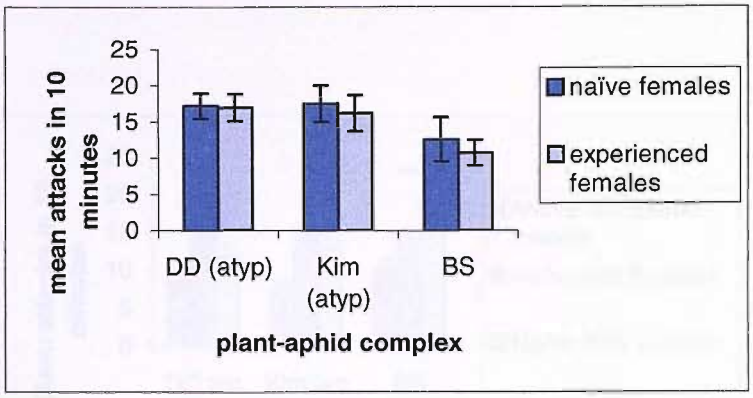
DD typ = "typical" Durdle Door *Brassica oleracea*

Kim typ = "typical" Kimmeridge *Brassica oleracea*

BS = Brussels sprout (Control)

The same trend was revealed when naïve and experienced *Diaeretiella rapae* collected from Durdle Door did not display significantly greater attack rates or initial ovipositional attacks on "atypical" PHC ($p<0.05$) (Figure 4.8.)

(a)



(b)

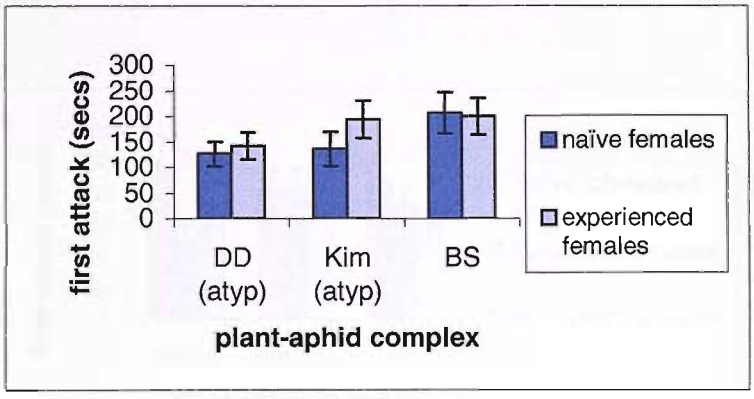


Figure 4.8. Comparing the attack rate (a) and time taken for an initial full contact ovipositional attack (b) by naïve and experienced *Diaeretiella rapae* from Durdle Door in arenas hosting "atypical" glucosinolate PHC. BS acts the control. Standard error values are indicated (±SE).

DD (atyp) = "atypical" Durdle Door *Brassica oleracea*

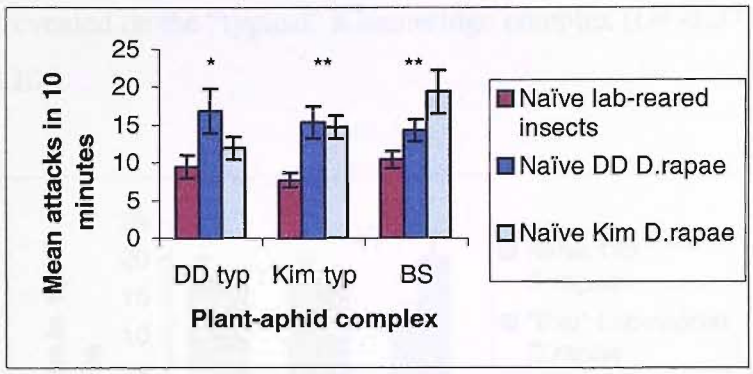
Kim (atyp) = "atypical" Kimmeridge *Brassica oleracea*

BS = Brussels sprout (Control)

Interestingly, significant differences in attack rates were revealed between naïve *Diaeretiella rapae* collected from Durdle Door and Kimmeridge and laboratory-reared insects. Naïve females reared in the laboratory displayed significantly lower attack rates on the "typical" PHC from Durdle Door ($F_{2,61} = 3.30$, $p = 0.044$), "typical" PHC from Kimmeridge ($F_{2,57} = 7.00$, $p = 0.002$) and the control complex ($F_{2,56} = 4.78$, $p = 0.012$) compared to naïve *Diaeretiella rapae* collected from Kimmeridge and Durdle Door. The time taken to make the initial full contact ovipositional attack also differed between the three parasitoid lines. Naïve laboratory-reared females took significantly longer to make the first attack on the "typical" PHC characteristic of Durdle Door ($F_{2,61}$

= 3.86, p = 0.027). No further significant differences were revealed ($p<0.05$) (Figure 4.9.).

(a)



(b)

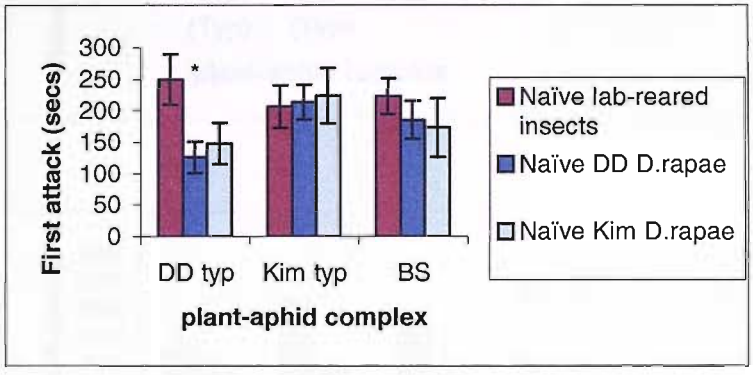


Figure 4.9. Comparing the attack rate (a) and time taken for an initial full contact ovipositional attack (b) by naïve *Diaeretiella rapae* from Kimmeridge and Durdle Door and laboratory-reared females in arenas hosting "typical" glucosinolate PHC. BS acts as the control. ($p<0.05$*, $p<0.01$**). Standard error values are indicated (±SE).

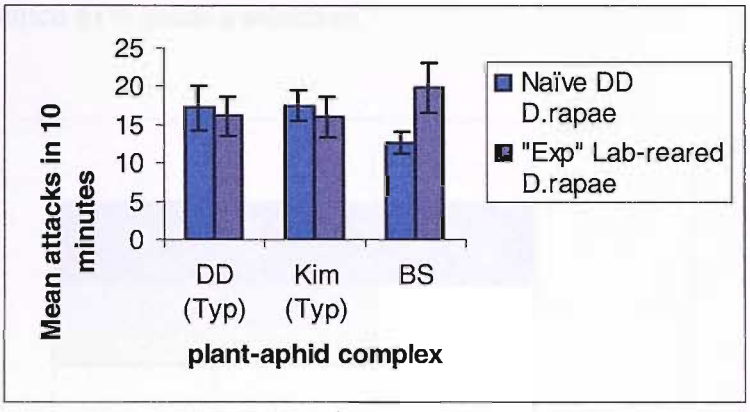
DD typ = "typical" Durdle Door *Brassica oleracea*
Kim typ = "typical" Kimmeridge *Brassica oleracea*
BS = Brussels sprout (Control)

It should be noted that naïve field-collected females did not display significantly different attack behaviour (attack rate or time for initial attack) between "typical" and "atypical" plant-host complexes ($p<0.05$) (Figure 4.9.).

Interestingly, experienced laboratory-reared females did not significantly attack "typical" Durdle Door PHC (t = -0.19, p = 0.849, DF = 36), "typical" Kimmeridge PHC (t = 0.22, p = 0.828, DF = 35) or the control complex (t = 1.58, p = 0.129, DF = 22), to a greater extent than naïve insects collected from Durdle Door. The time taken to make

an initial ovipositional attack did not differ significantly between naïve field-collected and experienced laboratory-reared insects on "typical" Durdle Door PHC ($t = -1.26$, $p = 0.215$, $DF = 35$) or the control complex ($t = -1.94$, $p = 0.061$, $DF = 34$), however a difference was revealed on the "typical" Kimmeridge complex ($t = -3.37$, $p = 0.002$, $DF = 36$) (Figure 4.10.).

(a)



(b)

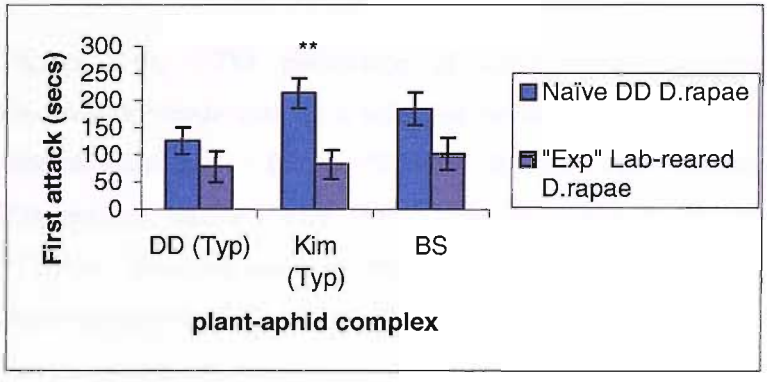


Figure 4.10. Comparing the attack rate (a) and time taken for an initial full contact ovipositional attack (b) by naïve *Diaeretiella rapae* from Durdle Door and experienced *Diaeretiella rapae* reared in the laboratory in arenas hosting "typical" glucosinolate PHC. BS acts the control. ($p<0.01$**). Standard error values are indicated (±SE).

DD (Typ) = "typical" Durdle Door *Brassica oleracea*

Kim (Typ) = "typical" Kimmeridge *Brassica oleracea*

BS = Brussels sprout (Control)

4.3.2. Y-tube Olfactometer Bioassays

Naïve laboratory-reared *Diaeretiella rapae* (*Myzus persicae–Brassica napus* var. Apex) did not preferentially select between odours emitted from *Brevicoryne brassicae* infested "typical" wild cabbages from Kimmeridge or Durdle Door ($\chi^2 = 0.85$, DF = 1, $p<0.05$) (Figure 4.11.). However, it should be noted that only 33 replicates were completed, of which 61% made a selection.

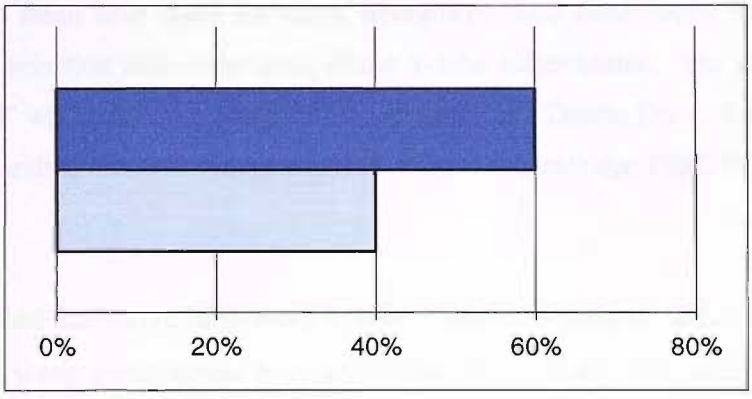


Figure 4.11. The percentage of naïve laboratory-reared *Diaeretiella rapae* making a selection between odours in a Y-tube olfactometer. □ = "Typical" *Brassica oleracea* from Kimmeridge infested with *Brevicoryne brassicae*. ■ = "Typical" *Brassica oleracea* from Durdle Door infested with *Brevicoryne brassicae*. 33 replicates were completed, 61% of insects made a selection.

The time taken by *Diaeretiella rapae* to make an initial and final selection between odour sources was not significant at either time period ($p<0.05$) (Table 4.3.).

Odours	Time taken for initial Selection (seconds ± SE)		Time taken for final Selection (seconds ± SE)	
KIM DD	KIM	DD	KIM	DD
	114.9 (±34)	52.3 (±9.5)	163 (±38)	88.8 (±20)
t-value	1.76		1.72	
p value	0.117		0.116	
DF	8		10	

Table 4.3. The mean time taken for naïve laboratory-reared *Diaeretiella rapae* to make an initial and final selection into either arms of the Y-tube olfactometer. The odour sources are infested "typical" wild cabbage plants from Kimmeridge and Durdle Door. Times are stated in seconds, with standard errors in brackets (±SE). Kim = Kimmeridge PHC, DD = Durdle Door PHC.

Analyses revealed that naïve laboratory-reared *Diaeretiella rapae* did not significantly differentiate between *Brevicoryne brassicae* infested "typical" wild cabbage from Kimmeridge with synthetic 3-butenylisothiocyanate added and *Brevicoryne brassicae* infested "typical" wild cabbage from Durdle Door with the same amount of solvent added ($p<0.05$) (Figure 4.12.). Only 39 replicates were completed with a 51% success rate of parasitoids making a selection.

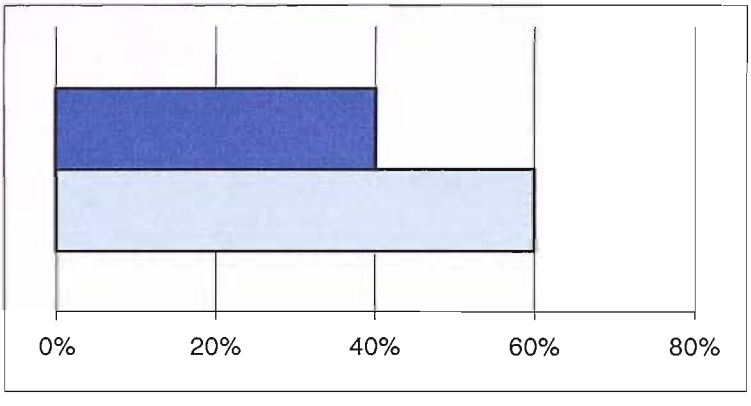


Figure 4.12. The percentage of naïve laboratory-reared *Diaeretiella rapae* making a selection between odours in a Y-tube olfactometer. □ = "Typical" *Brassica oleracea* from Kimmeridge infested with *Brevicoryne brassicae* + synthetic 3-butenylisothiocyanate. ■ = "Typical" *Brassica oleracea* from Durdle Door infested with *Brevicoryne brassicae* + solvent (hexane). 39 replicates were completed, 51% of insects made a selection.

The time taken by *Diaeretiella rapae* to make an initial and final selection between odour sources was not significant at either time period ($p<0.05$) (Table 4.4.).

Odours	Time taken for initial Selection (seconds ± SE)		Time taken for final Selection (seconds ± SE)	
KIM DD	KIM	DD	KIM	DD
	83.7 (±22)	124.0 (±33)	122.6 (±21)	186.9 (±28)
t-value	-1.02		-1.85	
p value	0.326		0.086	
DF	12		14	

Table 4.4. The mean time taken for naïve laboratory-reared *Diaeretiella rapae* to make an initial and final selection into either arms of the Y-tube olfactometer. "Typical" *Brassica oleracea* from Kimmeridge infested with *Brevicoryne brassicae* + synthetic 3-butenylisothiocyanate and "typical" *Brassica oleracea* from Durdle Door infested with *Brevicoryne brassicae* + solvent (hexane) are the two odour sources. Times are stated in seconds, with standard errors in brackets. Kim = Kimmeridge PHC, DD = Durdle Door PHC.

However, despite the lack of significant results, adding 3-butenylisothiocyanate reversed the selection of odours observed by *Diaeretiella rapae*. This provides promise for the possibility that *Diaeretiella rapae* utilises this compound during foraging.

4.3.3. Hind Tibia Measurements

Significant size differences were revealed between field-collected and laboratory-reared insects (Table 4.5.).

Parasitoid	Mean hind tibia length of *Diaeretiella rapae* (mm±SE)	
	Female	Male
Kim *D.rapae*	0.6570 ±0.0266	0.6117 ±0.0160
DD *D.rapae*	0.6850 ±0.0205	0.6350 ±0.0199
LR *D.rapae*	0.5050 ±0.0147***	0.4585 ±0.0142***

Table 4.5. The mean hind tibia lengths of female and male *Diaeretiella rapae* collected from Kimmeridge and Durdle Door and laboratory-reared insects (mm±SE). N = 15. Significant differences are indicated by *** ($p<0.001$).
Kim *D.rapae* = *Diaeretiella rapae* collected from Kimmeridge.
DD *D.rapae* = *Diaeretiella rapae* collected from Durdle Door.
LR *D.rapae* = Laboratory-reared *Diaeretiella rapae*.

The hind tibia of female *Diaeretiella rapae* collected from Kimmeridge and Durdle Door were significantly longer than insects reared in the laboratory ($F_{2,44} = 20.90$, $p<0.001$) (Figure 4.13.).

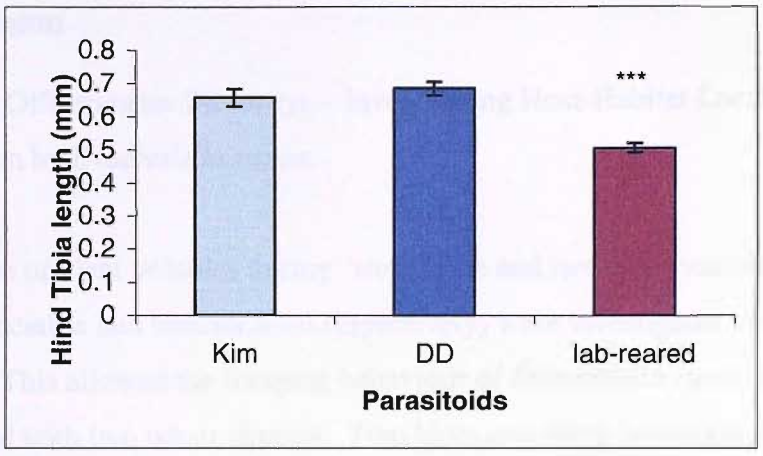


Figure 4.13. The mean hind tibia lengths of female *Diaeretiella rapae* collected from Kimmeridge (Kim), Durdle Door (DD) and laboratory-reared insects. N = 15 for each origin of parasitoids. ($F_{2,44}$ = 20.90, p<0.001***). Standard error values are indicated (±SE).

The same significant size differences were revealed for male *Diaeretiella rapae*. The hind tibia of males from Kimmeridge and Durdle Door were significantly longer than the hind tibia of laboratory-reared males ($F_{2, 44}$ = 32.24, p<0.001) (Figure 4.14.).

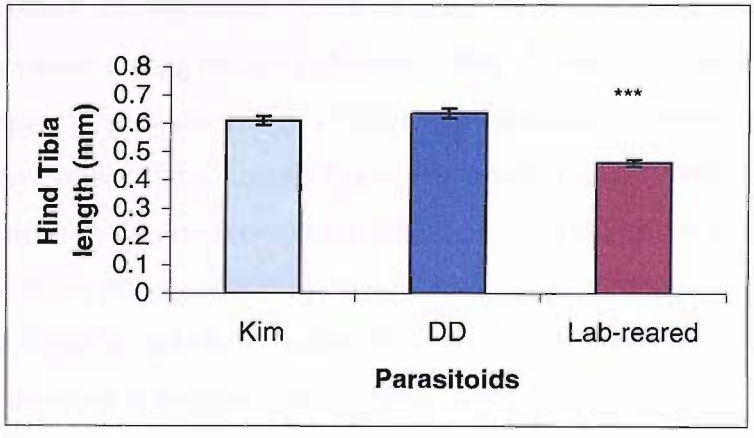


Figure 4.14. The mean hind tibia lengths of male *Diaeretiella rapae* collected from Kimmeridge (Kim), Durdle Door (DD) and laboratory-reared insects. N = 15 for each origin of parasitoids. ($F_{2,44}$ = 32.24, p<0.001***). Standard error values are indicated (±SE).

4.4. Discussion

- Y-tube Olfactometer Bioassays – Investigating Host-Habitat Location and Host Location by *Diaeretiella rapae*.

The importance of plant volatiles during "steps" one and two of parasitoid foraging (host-habitat location and host location respectively) were investigated using a Y-tube olfactometer. This allowed the foraging behaviour of *Diaeretiella rapae* to be observed when presented with two odour choices. Two bioassays were investigated. The first bioassay observed the behaviour of *Diaeretiella rapae* when presented with "typical" plant-host complexes (PHC) from Kimmeridge and Durdle Door. It was hypothesised that *Diaeretiella rapae* would preferentially select the Durdle Door plant-host complex (PHC) as it produces greater quantities of 3-butenylisothiocyanate (Chapter Two), the volatile thought to be attractive to *Diaeretiella rapae* during foraging (Bradbourne & Mithen, 2000; Blande, 2004). However, *Diaeretiella rapae* did not preferentially select between the two odour sources from Kimmeridge and Durdle Door, either in terms of initial choice or final odour selection (Figure 4.11. and Table 4.3.).

The second bioassay investigated the putative attractiveness of synthetic 3-butenylisothiocyanate during foraging (Blande 2004). It was hypothesised that the plant-host complex from Kimmeridge + 3-butenylisothiocyanate would be as attractive as the plant-host complex from Durdle Door. However, results revealed no significant differentiation between the two complexes (Figure 4.12. and Table 4.4.). Despite the lack of significant support for the utilisation of 3-butenylisothiocyanate during foraging by *Diaeretiella rapae* in this study, adding this compound completely reversed the original trend observed in bioassay one. 60% of insects preferred the plant-host complex from Durdle Door compared to the Kimmeridge plant-host complex in bioassay one, whilst during bioassay two, 60% of insects preferred the Kimmeridge complex containing the synthetic 3-butenylisothiocyanate. Therefore, it is proposed that the results obtained are preliminary results as they provide promise for the role of isothiocyanates during foraging by *Diaeretiella rapae* (Bradbourne & Mithen, 2000; Blande, 2004).

The lack of significance observed during the Y-tube olfactometer bioassays could be a result of the small replication size. Due to time constraints, only 20 replicates per

bioassay were completed. Therefore, it would be beneficial to repeat this experiment when more replicates can be completed.

Despite this, it is possible that the insignificant results obtained reflect the fact that the differences in glucosinolate-breakdown volatiles are not great enough to initiate differences in foraging behaviour by *Diaeretiella rapae*. This could be because the two complexes investigated during both bioassays may not greatly differ in terms of the quantities of isothiocyanates produced. This could result from the fact that the plants used during laboratory experiments were collected from individual plants along the transects deemed "typical" in terms of their glucosinolate profile. However, the plants used in laboratory experiments may not produce the same profile of glucosinolates and volatile isothiocyanates as their siblings and thus may not be the extreme producers of glucosinolates initially thought (Chapter Three).

It should be noted that during this study, the same aphid "host-line" (*Brevicoryne brassicae* reared on Brussels sprout var. Montgomery) was used to infest *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door. This was to ensure that the same volatiles would be induced in both complexes. This was a concern as Du *et al.*, (1997), (1998) and Guerrieri *et al.,* (1999) revealed that plants release different volatiles in response to infestation by different aphid species, suggested to be a result of the differences in aphid saliva between the species. I have found "host-line" variation in *Brevicoryne brassicae* (Chapter Three). Therefore, using the same control aphid "host-line" to infest the experimental plants decreased the possibility of different volatiles being induced in response to the three aphid lines. Despite these precautions, a further explanation for the lack of differential selection exhibited by *Diaeretiella rapae* could be a result of the number of aphids used to infest the plants. Aphids are phloem-feeders and thus insert their stylets into the phloem vessel of the host. This method of feeding is relatively unobtrusive and is unlikely to cause high levels of damage unless large aphid populations are feeding on the host. Powell *et al.,* (1998) revealed that broad bean plants infested with 40 *Acrythosiphum pisum*, 72 hours previously was sufficient to elicit a response from *Aphidius ervi*. However, it is possible that Brassicas "need" to be infested with larger numbers of aphids in excess of 250 for a longer period of time in order to cause sufficient damage necessary to induce the isothiocyanate defences thought to be utilised by aphid parasitoids. Future experiments could

investigate the number of aphids needed and for how long the infestation should occur to initiate the induction of isothiocyanates by *Brassica oleracea* subsp. o*leracea.*

An obvious third explanation could be the fact that females utilise plant volatiles other than isothiocyanates during foraging, which supports the idea by Vaughn *et al.*, (1996). Despite being referred to as a specialist parasitoid of Brassica-feeding aphids, *Diaeretiella rapae* successfully parasitises the Russian wheat aphid feeding on cereal crops. As cereal crops do not emit isothiocyanates, this could indicate that these volatiles are not as important during foraging as previously suggested (Bradbourne & Mithen, 2000; Blande, 2004). Vaughn *et al.*, (1996) proposed that green-leaf volatiles such as (Z)-3-hexan-1-ol could be important during the initial stages of parasitoid foraging. Future work could determine the differences in composition of green-leaf volatiles between *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door. This would reveal any differences, besides isothiocyanates, which could influence parasitoid behaviour.

Finally, the use of laboratory-reared insects could explain the lack of significant selection of odour sources by *Diaeretiella rapae.* The extensive, continuous rearing of insects in laboratories could affect the behaviour, physiology and genetics of individuals. This would undoubtedly affect their performance in controlled experiments leading to unrealistic insights and conclusions about their behaviour in the field. This is explained in greater depth in the last section of this chapter.

- Attack Rate Bioassays – Investigating Host Acceptance by *Diaeretiella rapae.*

The majority of this chapter focused on investigating "step" three (host acceptance) of parasitoid foraging behaviour (Vinson, 1976), during which aphid chemistry in addition to plant chemicals are thought to play an important role. For this reason, a series of attack rate bioassays were designed to investigate the role of plant-host chemistry on the attack behaviour of *Diaeretiella rapae.*

Chapter Three revealed "host-line" variation in *Brevicoryne brassicae*, highlighting that the three aphid lines differ in terms of their performance and intrinsic rate of natural increase on their host plants. It was suggested that the "host-line" originally from Durdle Door is most affected by its host chemistry, being adapted to high levels of

glucosinolates and may thus sequester glucosinolates to higher levels. It was hypothesised that the foraging behaviour of *Diaeretiella rapae* would be influenced by aphid chemistry and would preferentially attack the plant-host complex highest in its glucosinolate/isothiocyanate content.

The only significant results were revealed for naïve *Diaeretiella rapae* from Kimmeridge and experienced *Diaeretiella rapae* from Durdle Door when exposed to "typical" plant-host complexes. Naïve Kimmeridge *Diaeretiella rapae* displayed a significantly higher number of attacks on the control complex, whilst experienced Durdle Door *Diaeretiella rapae* preferentially attacked the Durdle Door plant-host complex (Table 4.1.). No further significant results were found to suggest that *Diaeretiella rapae* differentially attacks between plant-host complexes from Kimmeridge, Durdle Door or the control, either in terms of initial attack made or total attacks (Table 4.1. and 4.2.). This suggests that the differences in glucosinolate-breakdown volatiles emitted by the plant-host complexes investigated are not great enough to elicit differences in the foraging behaviour of *Diaeretiella rapae*. The importance of glucosinolates and their breakdown volatiles is accepted (Vaughn *et al.*, 1996; Bradbourne & Mithen, 2000; Blande, 2004), however, this study investigated the next step in the impact of glucosinolates in a tritrophic context by investigating the differences in genotype and subsequent phenotype, using the wild cabbage as the model system. Several explanations are offered for the lack of significant results produced by this study.

As stated, seeds taken from characteristic individuals at the two field sites were used during laboratory experiments. However, it is possible that the glucosinolate profiles of plants used may not be at the extreme ends of the glucosinolate spectrum and may be similar in content (Chapter Two), which would inevitably affect the volatile profile emitted by the plants. Current work reveals that genetic variability exists in seeds collected from a common mother (T.Stirrup. pers.comm.). *Diaeretiella rapae* may be unable to detect these subtle chemical differences in plant-host complex chemistry, therefore explaining the lack of differentiation.

A second explanation depends on aphid chemistry. It is known that specialist aphids such as *Lipaphis erysimi* (Blande, 2004) and *Brevicoryne brassicae* (MacGibbon & Beuzenberg, 1978; Bridges *et al.*, 2002) store differential levels of glucosinolates.

However, it remains to be determined whether this occurs in individuals of the same species. Plants of the same Brassica species produce different glucosinolate profiles (Chapter Two). Therefore, it is likely that aphids of the same species differ in the amount of glucosinolates sequestered as a result of the glucosinolate content of its host (Chapter Three). However, results from this investigation do not support this as *Diaeretiella rapae* failed to display differential attack activity against *Brassica oleracea-Brevicoryne brassicae* complexes from Kimmeridge and Durdle Door. It is possible that specialist aphids, including *Brevicoryne brassicae,* store higher levels of glucosinolates, however a threshold concentration may be sufficient to "declare" the aphid species suitable for oviposition by *Diaeretiella rapae.* It would be interesting to determine the glucosinolate content of the three "host-lines" of *Brevicoryne brassicae* to determine whether differences occur. Following on from this, the attack rate bioassays could be repeated using aphids that are reared on diets with different glucosinolate contents.

A third explanation could depend on the amount of priming cues received from the mummy case upon emergence. Parasitoids receive priming cues present on mummy cases, the intensity of which depends on the chemical background the parasitoid develops in (van Emden *et al.*, 1996; Storeck *et al.*, 2000; Blande, 2004). Therefore, aphids adapted to hosts with high glucosinolate concentrations may influence the quantity of glucosinolates sequestered, thus affecting the intensity of the priming cue received and ultimately influencing the host accepted by the parasitoid. However, this study investigated the attack rate of *Diaeretiella rapae* between plants and hosts of the same species. Therefore, it is possible that the three aphid "host-lines" do not differentially store glucosinolates or that a threshold level of stored glucosinolates is all that is required to result in similarly intense priming cues present on the mummy cases which are available to the parasitoid.

Finally the lack of differences in attack activity could be due to the relatively wide host range of *Diaeretiella rapae.* As stated, this species is able to successfully parasitise cereal aphids (Vaughn *et al.*, 1996) in addition to Brassica specialists. It is possible that other semiochemicals such as green-leaf volatiles and amino acid concentration may be more important than isothiocyanates which are specific to only one host family.

- Differential Foraging Behaviour of Wild and Laboratory-reared *Diaeretiella rapae*.

Unexpected differences in the attack behaviour of field-collected and laboratory-reared insects were revealed. The effect of experience on the behaviour of aphid parasitoids is well documented (Powell & Wright, 1992; Wickremansinghe & van Emden, 1992; Du *et al.,* 1997; Poppy *et al.,* 1997; Powell *et al.*, 1998; Olsen *et al.*, 2003; Blande, 2004). Previous experiments revealed that exposing parasitoids to its plant-host complex prior to experimentation enhances its behaviour (Vaughn *et al.*, 1996; Du *et al.*, 1997; 1998; Blande, 2004). Theory suggests that parasitoids possess an ability to learn olfactory cues (Poppy *et al.*, 1997; Powell *et al.*, 1998) associated with suitable prey, which allows for more efficient foraging (Du *et al.*, 1997).

As expected, experienced laboratory-reared *Diaeretiella rapae* displayed enhanced attack activity compared to naïve laboratory-reared insects (Figure 4.6.). However, when the attack behaviour of naïve and experienced field-collected *Diaeretiella rapae* were compared, no such enhanced response occurred (Figure 4.7. and 4.8.). This supports Vaughn *et al.*, (1996) who observed that naïve and experienced field-collected *Diaeretiella rapae* showed similar responses to cereal aphids. This suggests that the ability for parasitoids to learn is more apparent in laboratory-reared insects than wild insects.

An obvious explanation for this discovery is the continuous rearing of insects in the laboratory. As stated, the laboratory culture of *Diaeretiella rapae* was started from stocks from Rothamsted Research, which had been continuously cultured since 1990. The culture was only supplemented with 30 females and 20 males in 1995 (Blande, 2004). This continuous rearing of *Diaeretiella rapae* whereby unlimited suitable resources are provided, could inevitably affect the insects' ability to respond to olfactory and/or physical cues emanating from the host, the plant or a combination of both. The fitness of laboratory-reared insects has previously been questioned. Wild strains of *Musca domestica* (Bryant & Cowles, 2000) and *Plutella xylostella* (Mohan & Gujar, 2003) are more resistant to insecticides than laboratory strains. This is the first known study to reveal a differential behavioural response between wild and laboratory-reared *Diaeretiella rapae*.

This extensive laboratory rearing could have significant effects on the fitness of the insect. One such physical effect is evident. The mean hind tibia length of laboratory-reared *Diaeretiella rapae* is 0.5050mm for females and 0.4585mm for males, which is concurrent with Blande (2004). The hind tibia lengths of insects collected from Kimmeridge and Durdle Door do not differ, however a significant difference is observed between field-collected and laboratory-reared insects. Both female and males collected from Kimmeridge and Durdle Door are significantly bigger than those reared in the laboratory ($p<0.001$) (Figure 4.13. and 4.14. respectively). This suggests that bigger insects (wild insects) are fitter than smaller individuals (laboratory-reared insects). The exposure of a physical difference between laboratory-reared and field-collected insects leads to an inevitable question. "How else does this continuous rearing affect the behaviour, physiology and genetics of *Diaeretiella rapae*?"

Evidence for an effect on their behaviour is revealed when the attack behaviour of naïve field-collected and naïve laboratory-reared insects was compared. Naïve field-collected individuals display significantly greater attack rates and attack in significantly shorter periods of time compared to naïve laboratory-reared insects (Figure 4.9.). It is proposed that field-collected *Diaeretiella rapae* are fitter and in turn better adapted to respond to differences in their hosts and their host-habitat. It is suggested that extensive laboratory rearing decreases the ability of insects to respond to chemical and/or physical cues, possibly resulting from the continuous supply of suitable hosts, which decreases the need for efficient foraging.

Interestingly, the attack rate behaviour of experienced laboratory-reared *Diaeretiella rapae* does not significantly differ to that observed by naïve field-collected insects (Figure 4.10.). This suggests laboratory-reared insects are made more fit through learning as supported by their enhanced response after being allowed prior experience. It is suggested that giving laboratory-reared *Diaeretiella rapae* an experience prior to experimentation in controlled laboratory experiments is the only way to observe parasitoid behaviour, which is realistic of their behaviour in natural field conditions. All studies investigating the learning ability of parasitoids were conducted with laboratory-reared insects. Therefore, it is possible that the learning ability of parasitoids (Poppy *et al.*, 1997; Du *et al.*, 1997; Storeck *et al.*, 2000, Blande, 2004) is a laboratory phenomenon.

Summary

- *Diaeretiella rapae* does not exhibit differential foraging behaviour in response to isothiocyanate differences produced from its host, its host-habitat or a combination of the two (PHC).
- Allowing field-collected *Diaeretiella rapae* from Kimmeridge and Durdle Door prior experience of its plant-host complex (PHC) does not enhance its attack behaviour.
- It is proposed that laboratory-reared *Diaeretiella rapae* are less well adapted to respond to chemical and/or physical cues compared to field-collected insects. Only when laboratory-reared insects are given prior exposure does their attack behaviour resemble the behaviour observed by field-collected insects.

Chapter Five

Tritrophic Interactions in a Natural Ecosystem.

Chapter Five

Tritrophic Interactions in a Natural Ecosystem

5.1. Introduction

The role of plant secondary metabolites, including glucosinolates, in defence against herbivory is well documented (review by Bennett & Wallsgrove, 1994). However, relevant to this study, research that highlights the role of glucosinolates in plant-insect interactions is from controlled laboratory experiments (van Emden, 1990; Vet & Dicke, 1992; Bartlet *et al.,* 1996; 1997; 1999; Cole, 1996; 1997; Blande, 2004). This means that very few studies have investigated the impact of plant chemicals including glucosinolates, in natural field conditions. Research conducted in natural ecosystems is vital to determine whether the role of plant chemicals is as important in the field as suggested by laboratory experiments. This view is supported by van der Meijden and Klinkhamer (2000) who stated; "field studies in natural ecosystems will be highly valuable to demonstrate the importance of herbivore-induced plant volatiles." Furthermore, significant interactions observed in controlled experiments may not be ecologically significant (Gurevitch & Collins, 1994).

Six known studies have highlighted the influence of glucosinolates on insects in the field. Two studies were conducted under semi-field conditions (Giamoustaris & Mithen, 1995; Bradbourne & Mithen, 2000), whilst the remaining research investigated their role in natural ecosystems (Root, 1973; Louda & Rodman, 1983; Mithen *et al.*, 1995b; Moyes *et al.*, 2000).

Root (1973) investigated the diversity of arthropods associated with wild populations of *Brassica oleracea* in Ithaca, New York. Results showed that herbivore density varied greatly both in time and space. However, no attempts were made to determine the glucosinolate composition of the plants or to link the variation in herbivore diversity and density to glucosinolates.

Louda and Rodman (1983) investigated the glucosinolate concentration produced by a natural population of *Cardamine cordifolia*, but found no significant link with herbivory. This result could be explained by the fact that only a small number of plants

were investigated. In addition, the profile of individual glucosinolates was not determined, possibly due to the methods available at that time.

Mithen *et al.,* (1995b) investigated the quantity and quality of glucosinolates produced in four distinct populations of *Brassica oleracea* subsp. *oleracea* along the Dorset coastline. Genetic differences controlling aliphatic glucosinolates were found between these populations. It was proposed that these differences were a result of selection pressures imposed by the variations in herbivores feeding at each site. However, it should be noted that no measure of insect activity was made. This was later addressed by Moyes *et al.*, (2000). A detailed top-down study was conducted to ascertain the diversity and density of insects present at the sites and whether feeding preference is related to the quantity and quality of glucosinolates produced by their hosts. However, it was revealed that selection pressures imposed by herbivores were unlikely to account for the glucosinolate profiles observed in the wild cabbage.

This thesis continues the research by Mithen *et al.*, (1995b) and Moyes *et al.,* (2000), but approaches it from the opposite perspective, the bottom-up perspective. As stated, it is proposed that the extreme glucosinolate profiles produced by *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door influence the presence and/or behaviour of insects in the second and third trophic levels (Chapter One). Experiments detailed so far have investigated this in controlled laboratory experiments (Chapters Two, Three and Four). The aim of this chapter is to investigate this interaction in a natural ecosystem. This attempts to link the behaviours of *Brevicoryne brassicae* and *Diaeretiella rapae* to glucosinolates observed in controlled laboratory experiments to those observed in a natural ecosystem, thus determining whether the results from laboratory experiments are a realistic reflection of interactions in the field.

Hypothesis:

- *Brassica oleracea* subsp. *oleracea* at Durdle Door will host the greatest activity of *Brevicoryne brassicae* and *Diaeretiella rapae*, both in terms of the proportion of plants infested and the severity of infestation, compared to *Brassica oleracea* subsp. *oleracea* at Kimmeridge.

5.2. Materials and Methods

- Transect Set-Up

A 25 metre transect was set up at Kimmeridge and Durdle Door in August 2002. The transect at Kimmeridge was situated on the cliff top, approximately 3 metres from the edge (Figure 5.1.). The transect at Durdle Door was situated at the base of a cliff, adjacent to a pebble beach (Figure 5.2.).

Figure 5.1. Kimmeridge Transect

Figure 5.2. Durdle Door Transect

Transects were marked out using canes and tape measures. At one metre intervals, starting from zero metres, a wild cabbage plant, perpendicular to the tape measure, was labelled. This resulted in 25 labelled plants at Kimmeridge and Durdle Door. The position of each plant was noted.

In 2004, a high proportion of mortalities was sustained at Kimmeridge due to coastal erosion and consequently due to the widening of the path which ran the length of the

transect (Figure 5.3.). Replacement plants were marked wherever possible, however this inevitably affected the amount of data that could be collected in the second field season.

Figure 5.3. Kimmeridge Transect in 2004

- Vegetation Survey

A transect was chosen to enable the habitat variability of the two field sites to be assessed. The diversity of vegetation was surveyed in April 2004. Using canes and tape measures, all of the species growing along the length of the transects, within a width of 1 metre, were identified.

- Climate

Weather data was provided by Durlston Country Park, Swanage, Dorset UK (Figure 1.2.). The minimum and maximum air temperatures and total rainfall were recorded daily. The mean of each climatic factor for each month was calculated from January 2002 to September 2004.

- Morphological Measurements

Both transects were visited every three weeks from March to September of 2003 and 2004. The height, width, number of racemes (elongated spray of flowers) and number of pedicels (flower stalk) of each labelled wild cabbage plant were measured. The time of flowering and pod development were also noted. The size of each plant was calculated from the height and width measurements.

The morphological data collected in April 2003 was analysed to reveal differences between the populations of wild cabbage at Kimmeridge and Durdle Door. The

differences in height, width, number of racemes and number of pedicels in individuals between the two sites were analysed using a 2-sample t-test.

- Insect Survey

(a) Foliage

The density of *Brevicoryne brassicae* infestations rendered it impossible to count the exact numbers present on each plant (Figure 5.4.).

Figure 5.4. *Brevicoryne brassicae* infestation.

To overcome this, a 5x5cm^2 grid was constructed and the aphids present within a 1cm^2 area were brushed from a leaf of an unmarked wild cabbage plant, using a fine paintbrush. The sample was stored in 100% ethanol in a labelled container to enable the aphids present to be counted in the laboratory at the University of Southampton. Aphid infestations were categorised according to density: dense, moderate, sparse. This process was repeated five times for each category of infestation. A mean number of *Brevicoryne brassicae* was then calculated for each category (Appendix 4).

The 5x5cm^2 grid was then placed over the aphid infestations present on the leaves of labelled *Brassica oleracea* subsp. *oleracea* plants at Kimmeridge and Durdle Door. The size of the colony was determined by counting the number of centimetres covered. The number of centimetres covered by the infestations was multiplied by the mean number of *Brevicoryne brassicae* determined for each density of infestation. This enabled a numerical estimation of *Brevicoryne brassicae* infesting the foliage of wild cabbages to be obtained.

The activity of *Diaeretiella rapae* was assessed at the same time by counting the number of mummified aphids present. This is an indirect measure of foraging activity, but relates to the number of parasitoids present and the attack activity of parasitoids in that area (G. Poppy. pers.comm.).

(b) Racemes

Infested racemes were again classified according to degree of infestation: dense, moderate and sparse. The racemes were further divided into size categories, both in terms of the diameter of the stem and the size of the flowers (Appendix 4). This yielded a number of categories:

Big stem-big buds
Big stem-medium buds
Big stem-small buds
Medium stem-big buds
Medium stem-medium buds
Medium stem-small buds
Small stem-big buds
Small stem-medium buds
Small stem-small buds

Five samples of each differently sized raceme and differing density were collected at the base of the buds and stored in 100% ethanol in individually labelled containers until they could be counted in the laboratory at the University of Southampton. This enabled the number of *Brevicoryne brassicae* present to be determined. The mean number of aphids present for each raceme size-aphid density category was then calculated (Appendix 4).

For dense colonies, the infestation often extended the length of the raceme (pers. obs.). 1cm sections of big, medium and small stems were removed and stored in 100% ethanol. The number of *Brevicoryne brassicae* present on the differently sized stems was then counted at the University of Southampton. The length of the colony was then measured in severe infestations to give an estimate of the aphids present.

The number of mummified aphids present on the racemes was counted at the same time as *Brevicoryne brassicae*. This enabled the activity of *Diaeretiella rapae* to be assessed.

- Statistical Analysis

Brevicoryne brassicae

The percentage of plants infested with *Brevicoryne brassicae* was determined for 2003 and 2004. The number of aphids present per plant was calculated by adding the total number of aphids present on the foliage and reproductive parts of the plants. Data collected from 2003 only was included in the analysis due to the low number of aphids present at Kimmeridge during 2004 resulting from the high percentage of wild cabbage mortality sustained. The number of aphids present (ln transformed) per transect at each time period was analysed separately using a 2-sample t-test. Un-infested plants were omitted from the analysis, as the aim of the investigation was to determine differences in infestation densities between the two sites.

Diaeretiella rapae

The percentage of plants hosting parasitised *Brevicoryne brassicae* colonies was determined for 2003 and 2004. The number of mummified aphids at both sites in 2003 was calculated and the ln+1 total number for each site for each time period was analysed using a 2-sample t-test.

Glucosinolates and Insect Activity

Data collected from both field sites in 2003 were pooled together to assess a relationship between insect presence and glucosinolate concentration. The total concentration of each individual glucosinolate, total aliphatic glucosinolates, total indole glucosinolates and total glucosinolates produced by aphid infested and un-infested and *Diaeretiella rapae* colonised and un-colonised wild cabbage plants was analysed using a 2-sample t-test. A further 2-sample t-test was conducted on high and low *Brevicoryne brassicae* infestations. Plants hosting infestations less than 1000 aphids were deemed low. Chi-squared tests were conducted for the number of plants with/without aphid infestations, high and low aphid infestations and with/without mummified aphids containing each individual aliphatic glucosinolate.

5.3. Results

5.3.1. Vegetation Survey

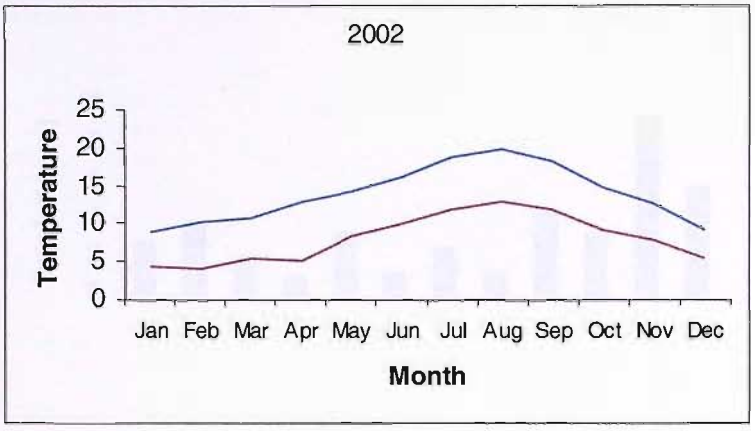
The plant species found at both sites are listed in Appendix 5. Kimmeridge has a greater species diversity compared to Durdle Door. Species were classified into seven habitat categories in accordance to Moyes (1997) (Table 5.1), however, the acid soil habitat was replaced by the grassy habitat due to the high proportion of species present at both sites that are typical of grassy habitats.

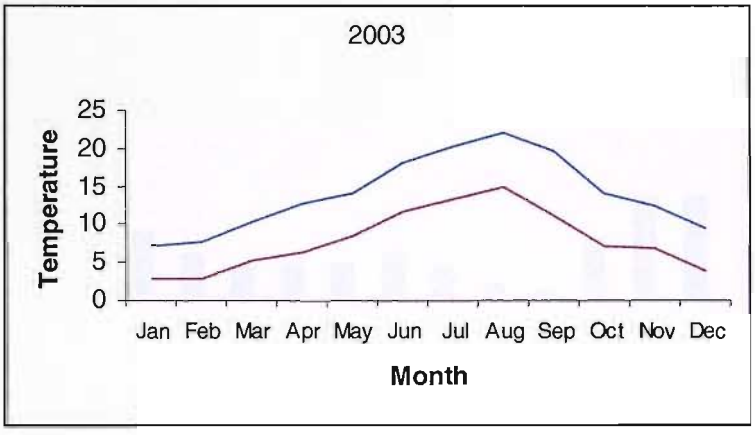
Habitat	Kimmeridge	Durdle Door
Cliffs/Coast/Dunes	19	21
Waste/Disturbed	40	26
Grassy	29	33
Alkaline Soil	3	4
Wet/Damp	3	8
Dry	6	8
Species Number	31	24

Table 5.1. The percentage of plant species found at Kimmeridge and Durdle Door classified by habitat. The total number of species is indicated.

5.3.2. Climate

Air temperatures varied throughout the duration of this study (Figure 5.5.). The temperatures most relevant to this work are the temperatures from March to September when fieldwork was conducted. 2003 was hotter than 2002, which was slightly hotter than 2004. The winter of 2003/2004 was colder than 2002/2003. 2002 was wetter than 2003 and 2004. The heaviest rainfall was recorded for the winter of 2002/2003 (Figure 5.6.).





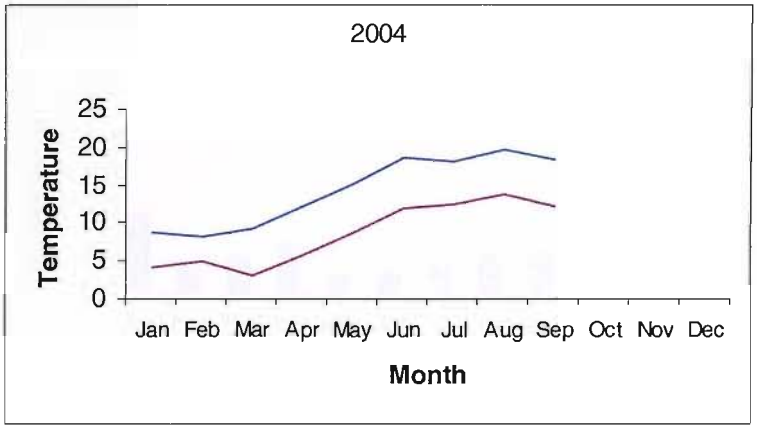


Figure 5.5. Mean monthly temperature recordings for the duration of this study (°C). Maximum temperatures, Minimum temperatures.

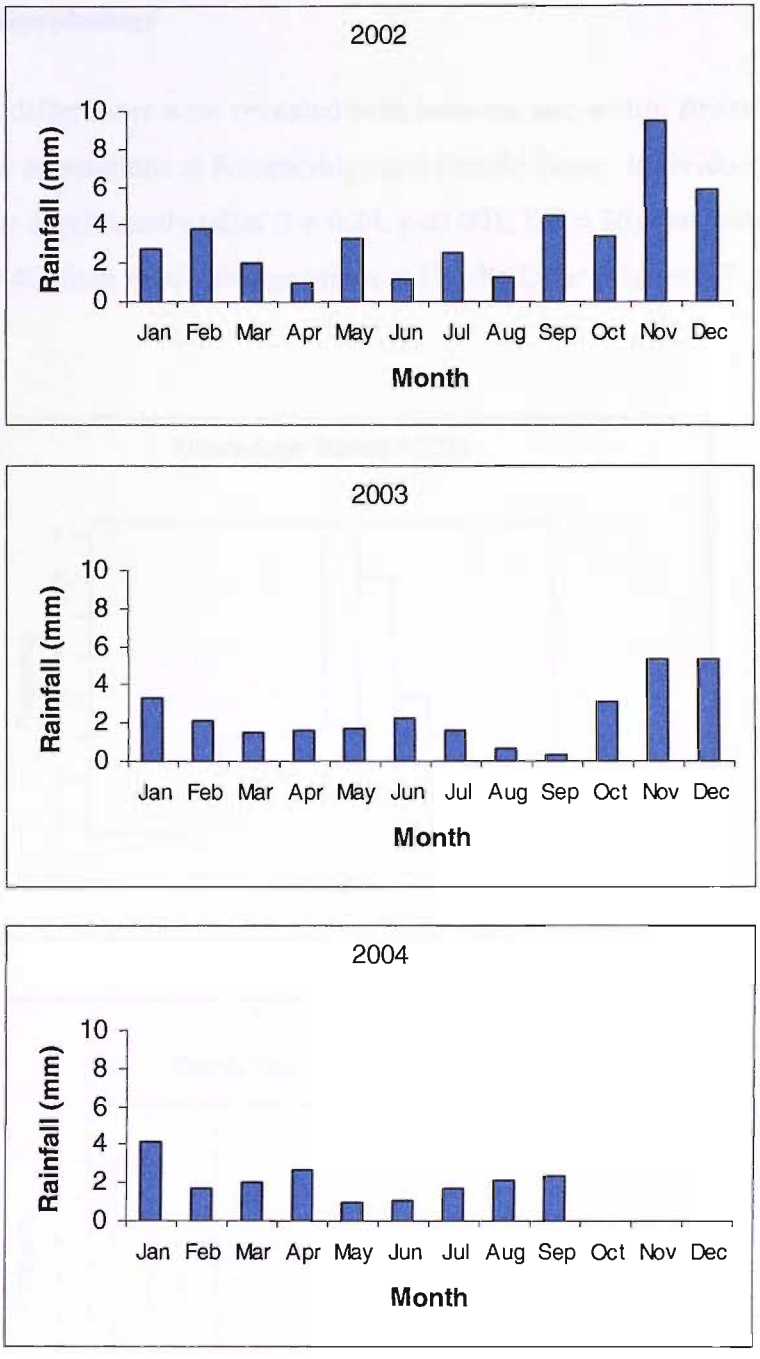


Figure 5.6. Mean monthly rainfall for the duration of this study (mm).

It should be noted that climate data was obtained from Durlston Country Park, Swanage, 12km from Kimmeridge and 22.5km from Durdle Door (Figure 1.2.). Therefore, it is possible that microclimate differences occur between the sites. Future experiments could determine any microclimate differences using sensors.

5.3.3. Plant Morphology

Morphological differences were revealed both between and within *Brassica oleracea* subsp. *oleracea* populations at Kimmeridge and Durdle Door. Individual plants at Kimmeridge are significantly taller ($t = 6.24$, $p<0.001$, $DF = 36$) and wider ($t = 4.28$, $p<0.001$, $DF = 40$) than wild cabbage plants at Durdle Door (Figure 5.7. and 5.8. respectively).

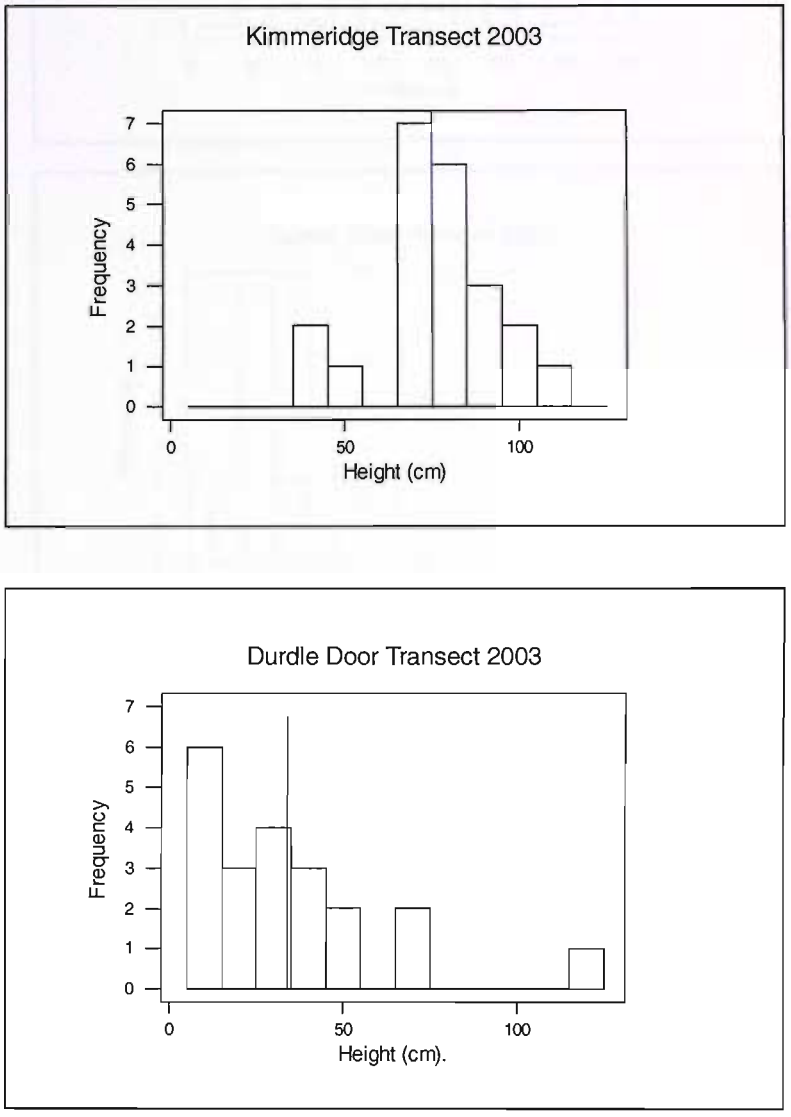


Figure 5.7. Height of the labelled wild cabbage plants along the transects at Kimmeridge and Durdle Door in April 2003. A blue vertical line indicates the mean.

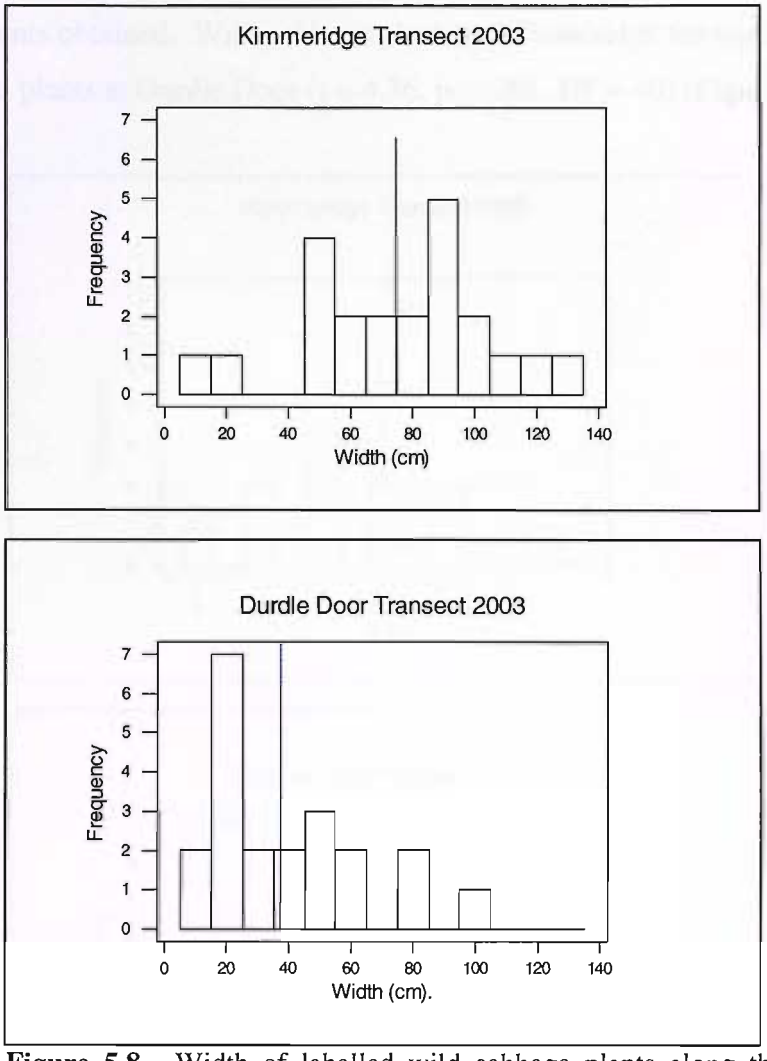


Figure 5.8. Width of labelled wild cabbage plants along the transects at Kimmeridge and Durdle Door in April 2003. A blue vertical line indicates the mean.

The size of each individual plant was calculated from the height and width measurements obtained. Wild cabbage plants at Kimmeridge are significantly bigger than plants at Durdle Door ($t = 4.76$, $p<0.001$, $DF = 40$) (Figure 5.9.).

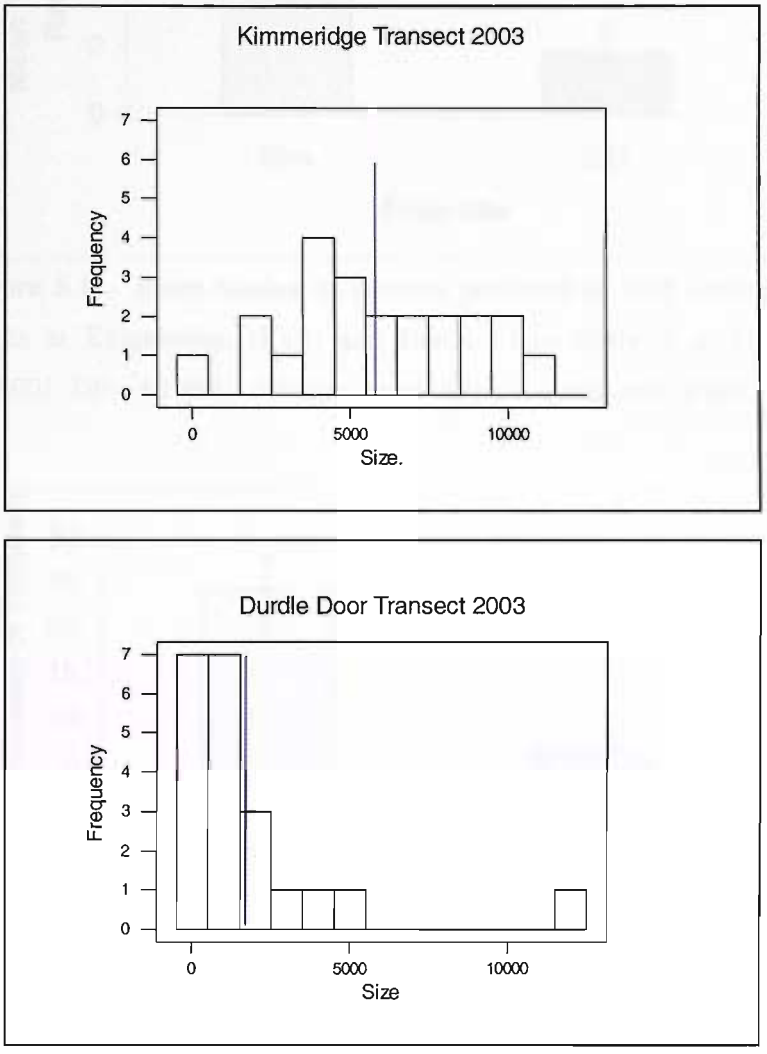


Figure 5.9. Size of labelled wild cabbage plants along the transects at Kimmeridge and Durdle Door in April 2003. A blue vertical line indicates mean size.

The number of flowering plants varied between Kimmeridge and Durdle Door. There are a higher proportion of reproductive plants at Kimmeridge. 100% of the surviving plants at Kimmeridge were flowering and able to reproduce at the time surveyed compared to only 38% of plants at Durdle Door. *Brassica oleracea* subsp. *oleracea* at Kimmeridge produced a significantly higher number of racemes ($t = 3.92$, $p<0.001$, $DF = 40$) and pedicels ($t = 3.36$, $p<0.002$, $DF = 40$) compared to those at Durdle Door (Figure 5.10. and 5.11. respectively).

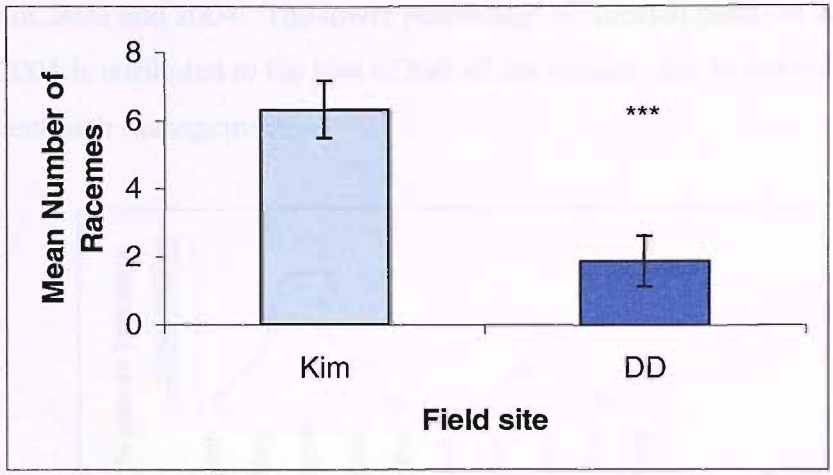


Figure 5.10. Mean number of racemes produced by wild cabbage plants at Kimmeridge (Kim) and Durdle Door (DD) (t = 3.92, p<0.001, DF = 40 ***). Standard error values are indicated (±SE).

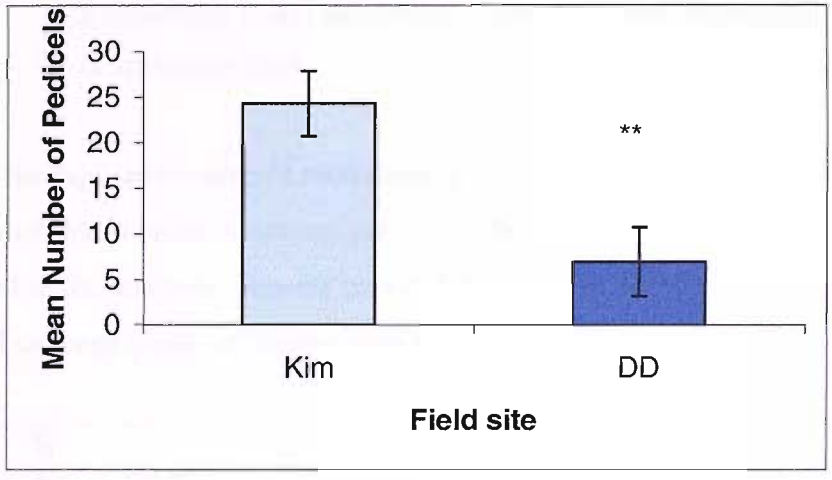


Figure 5.11. Mean number of pedicels produced by wild cabbage plants at Kimmeridge (Kim) and Durdle Door (DD) (t = 3.36, p<0.002, DF = 40 **). Standard error values are indicated (±SE).

No differences were observed for the time of flowering at both Kimmeridge and Durdle Door. For both sites, flowering occurred in April 2003 and in March 2004.

5.3.4. Insect Survey

- *Brevicoryne brassicae*

Brevicoryne brassicae infestations were found on both the foliage and the racemes of *Brassica oleracea* subsp. *oleracea* at Kimmeridge and Durdle Door. The data was combined to give the total number of aphids present per plant. Figure 5.12. highlights the percentage of plants infested at Kimmeridge and Durdle Door during the summer

seasons of 2003 and 2004. The lower percentage of infested plants at Kimmeridge during 2004 is attributed to the loss of half of the transect due to coastal erosion and subsequent path management.

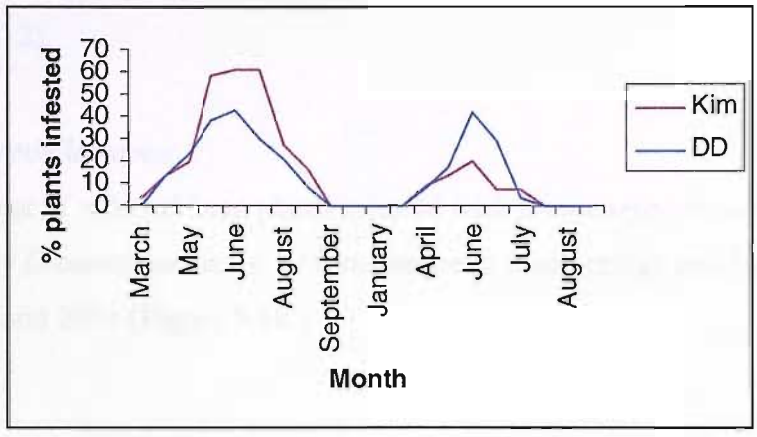


Figure 5.12. The percentage of infested cabbage plants at Kimmeridge (Kim) and Durdle Door (DD) from March 2003 to September 2004.

Due to the high percentage of mortalities at Kimmeridge in 2004, the differences in the number of *Brevicoryne brassicae* present at the two field sites during 2003 only were included in the analysis. Results reveal differences in the severity of infestations found on wild cabbage plants at the two sites (Figure 5.13.)

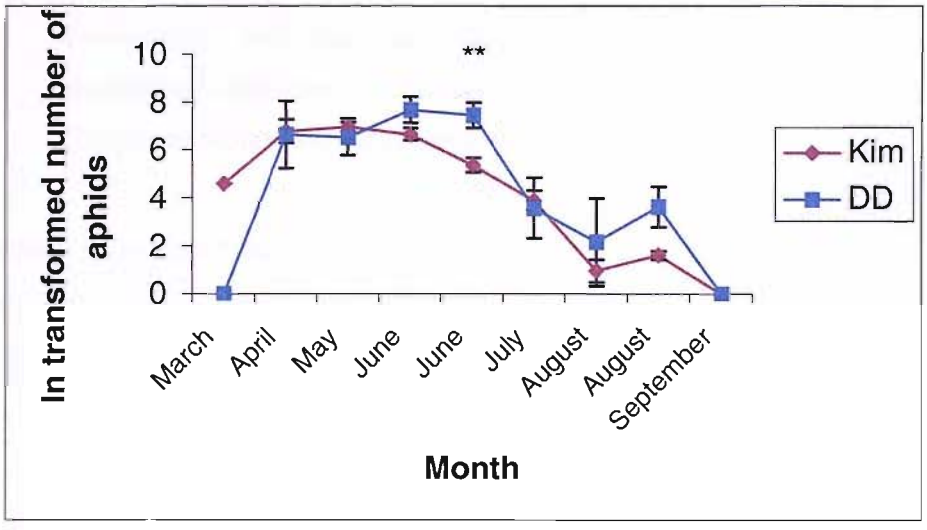


Figure 5.13. ln transformed number of *Brevicoryne brassicae* on *Brassica oleracea* subsp. *oleracea* at Kimmeridge (Kim) and Durdle Door (DD), from March to September 2003. Significant differences are indicated by ** ($p<0.01$). Standard error values are highlighted (±SE). NB. No statistical test could be performed for the number of aphids surveyed in March.

The number of aphids present at both sites at each time period were analysed separately (ln transformed, t-test). The only significant difference in the severity of aphid infestations between the sites was revealed for time period five (June II). A higher number of *Brevicoryne brassicae* was present at Durdle Door ($t = -3.36$, $p = 0.006$, $DF = 12$).

- *Diaeretiella rapae*

The percentage of wild cabbage plants infested with *Brevicoryne brassicae* that were parasitised by *Diaeretiella rapae*, differed between Kimmeridge and Durdle Door during 2003 and 2004 (Figure 5.14.).

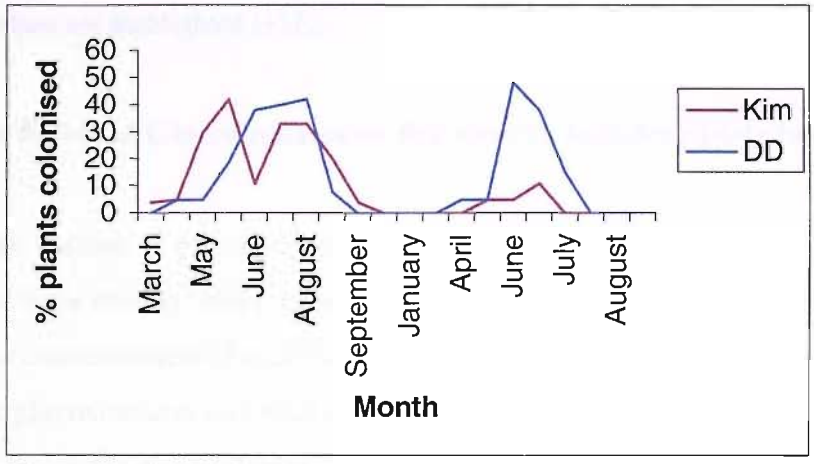


Figure 5.14. The percentage of wild cabbage plants infested with *Brevicoryne brassicae* parasitised by *Diaeretiella rapae* (1 mummified aphid present) at Kimmeridge (Kim) and Durdle Door (DD) from March 2003 to September 2004.

The number of mummified aphids present at Kimmeridge and Durdle Door was analysed for each time period separately (ln+1 transformed, t-test). Results reveal significant differences at two time periods. There was a higher number of mummies present at Durdle Door at time six (July) ($t = -3.07$, $p = 0.013$, $DF = 9$) and time period eight (August II) ($t = -3.32$, $p = 0.045$, $DF = 3$) compared to Kimmeridge (Figure 5.15.).

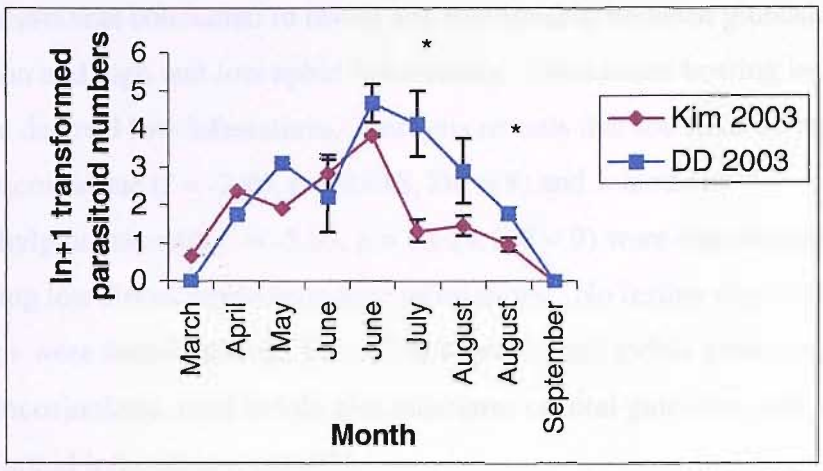


Figure 5.15. In+1 transformed number of mummified aphids present at Kimmeridge (Kim) and Durdle Door (DD) from March to September 2003. Significant differences are indicated * ($p<0.05$). Standard error values are highlighted (±SE).

5.3.5. The Effect of Glucosinolates on *Brevicoryne brassicae* Distribution

Only a small number of plants supported aphid infestations, therefore the following results need to be treated with caution. Analysis was performed to reveal links between the concentration of individual glucosinolates, total aliphatic glucosinolates, total indole glucosinolates and total glucosinolates with the presence of *Brevicoryne brassicae*. However no significant differences were found between the concentration of 2-propenylglucosinolate ($t = 0.88$, $p = 0.445$, $DF = 3$), 3-butenylglucosinolate ($t = -2.06$, $p = 0.175$, $DF = 2$), 2-hydroxy-3-butenylglucosinolate ($t = -0.27$, $p = 0.791$, $DF = 14$), total aliphatic glucosinolates ($t = -0.27$, 0.791, $DF = 14$), hydroxyindolylmethylglucosinolate ($t = -0.07$, $p = 0.950$, $DF = 7$), indolylmethylglucosinolate ($t = 0.13$, $p = 0.899$, $DF = 6$), 4-methoxyindolylglucosinolate ($t = -0.40$, $p = 0.704$, $DF = 6$), total indole glucosinolates ($t = -0.01$, $p = 0.995$, $DF = 6$) or total glucosinolates ($t = -0.34$, $p = 0.737$, $DF = 13$) between infested and un-infested wild cabbage plants.

When the presence of each individual aliphatic glucosinolate was analysed in relation to the presence or absence of aphids, it was found that the presence of 2-propenylglucosinolate ($\chi^2 = 0.004$, $p = 0.949$, $DF = 1$), 3-butenylglucosinolate ($\chi^2 = 0.382$, $p = 0.536$, $DF = 1$) and 2-hydroxy-3-butenylglucosinolate ($\chi^2 = 1.036$, $p = 0.309$, $DF = 1$) were not linked to aphid infestation.

Further analysis was conducted to reveal any relationship between glucosinolate concentration and high and low aphid infestations. Infestations hosting less than 1000 aphids were deemed low infestations. Analysis reveals that the concentration of 2-propenylglucosinolate ($t = -2.96$, $p = 0.018$, $DF = 8$) and 1-methoxy-3-indolylmethylglucosinolate ($t = -3.03$, $p = 0.014$, $DF = 9$) were significantly higher in plants hosting low *Brevicoryne brassicae* infestations. No further significant relationships were found between individual aliphatic and indole glucosinolates, total aliphatic glucosinolates, total indole glucosinolates or total glucosinolates and the severity of aphid infestations ($p<0.05$).

Chi-squared analysis revealed that the presence of 3-butenylglucosinolate ($\chi^2 = 1.029$, $p = 0.310$, $DF = 1$) and 2-hydroxy-3-butenylglucosinolate ($\chi^2 = 0.171$, $p = 0.679$, $DF = 1$) were not linked to the presence of high and low aphid infestations. However, analysis reveals a significant relationship between the presence of 2-propenylglucosinolate and high and low aphid infestations ($\chi^2 = 5.182$, $p = 0.023$, $DF = 1$).

5.3.6. The Effect of Glucosinolates on *Diaeretiella rapae* Activity

Only a small number of plants supported parasitised aphid colonies, thus the following results need to be viewed cautiously. Analysis was conducted to investigate a relationship between *Diaeretiella rapae* activity and glucosinolate concentration. Results reveal that no significant differences occurred in plants hosting parasitised and un-parasitised *Brevicoryne brassicae* colonies and the concentration of 2-propenylglucosinolate ($t = 0.25$, $p = 0.812$, $DF = 6$), 3-butenylglucosinolate ($t = -0.46$, $p = 0.633$, $DF = 14$), 2-hydroxy-3-butenylglucosinolate ($t = -0.27$, $p = 0.794$, $DF = 14$), total aliphatic glucosinolates ($t = -0.26$, 0.795, $DF = 14$), hydroxyindolylmethylglucosinolate ($t = -0.07$, $p = 0.950$, $DF = 7$), indolylmethylglucosinolate ($t = -0.08$, $p = 0.941$, $DF = 7$), 4-methoxyindolylglucosinolate ($t = -0.12$, $p = 0.910$, $DF = 6$), 1-methoxy-3-indolylmethylglucosinolate ($t = -1.45$, $p = 0.172$, $DF = 12$), total indole glucosinolates ($t = -0.13$, $p = 0.902$, $DF = 6$) or total glucosinolates ($t = -0.43$, $p = 0.674$, $DF = 13$).

When the presence of each individual aliphatic glucosinolate was analysed in relation to the presence or absence of mummified aphids, it was found that the presence of 2-

propenylglucosinolate ($\chi^2 = 0.004$, p = 0.949, DF = 1), 3-butenylglucosinolate ($\chi^2 = 0.382$, p = 0.536, DF = 1) and 2-hydroxy-3-butenylglucosinolate ($\chi^2 = 1.036$, p = 0.309, DF = 1) were not linked to *Diaeretiella rapae* activity.

5.4. Discussion

5.4.1. The Habitat and Morphology of *Brassica oleracea* subsp. *oleracea*

Results indicate that the habitat and morphology of *Brassica oleracea* subsp. *oleracea* differs between Kimmeridge and Durdle Door. A survey of the vegetation at the two sites reveals that Kimmeridge hosts the greatest diversity (Appendix 5). Species found were often in agreement with Moyes (1997), however a small number of species were present which have not been documented by Moyes (1997). Species were classified according to habitat. The trends found support those previously documented by Mitchell and Richards (1979) and Moyes (1997). As expected, there were a relatively large percentage of plants characteristically found in coastal habitats. Despite a relatively lower percentage of species typical of damp/wet habitats at Kimmeridge and Durdle Door, their presence indicates the conditions within which the wild cabbage grows. A higher percentage is found at Durdle Door, highlighting the amount of water flowing over the transect. This was expected due to the location of the transect at Durdle Door. Interestingly, there were a high percentage of plants at both sites typical of waste and/or disturbed habitats. This supports Moyes (1997) in that it indicates the unstable environment the wild cabbage inhabits. The high level of plant mortality observed, particularly at Kimmeridge highlights the degree of instability of the environments. As described, the transect at Kimmeridge is situated on the cliff top, however in 2004 approximately half of the transect was lost due to coastal erosion and subsequently due to path management. Prior to this event, it was common to lose individuals resulting from wind damage and by the action of humans (pers. obs.). Both Kimmeridge and Durdle Door are popular tourist sites and inevitably plants were damaged or lost completely due to trampling.

The soil type of both sites could have important implications, both in terms of influencing the diversity of the vegetation and the morphology of the wild cabbage. As stated, Kimmeridge has a shale soil type, whilst Durdle Door is on a calcareous substrate (Mitchell and Richards, 1979; Moyes, 1997; Raybould *et al.*, 1999a) (Chapter One). It is documented that plants growing in shale substrates are larger than plants

growing in limestone-based soils (Mitchell & Richards, 1979). Results investigating the morphology of the mature wild cabbage support this. It was found that the Kimmeridge site hosts larger individuals compared to Durdle Door. In addition, a higher proportion of *Brassica oleracea* subsp. *oleracea* individuals produced racemes at Kimmeridge. Mitchell and Richards (1979) stated that the wild cabbage is able to flower after four to five years. Therefore, the morphological variability between the two sites, both in terms of plant size and the percentage of flowering plants, could be explained by the fact that the individuals at Durdle Door may be younger than those at Kimmeridge. The variation in wild cabbage development between the two sites could be a result of their spatial apparency. Prior to the coastal erosion that occurred at Kimmeridge in 2004, it was noted that a higher degree of plant deaths occurred at Durdle Door (pers. obs.). This was attributed to the fact that the overall lower proportion of wild cabbage plants present and indeed due to the lower diversity of surrounding vegetation at this site results in individuals being more visually and chemically apparent (Feeny, 1976). This is compounded by the fact that Durdle Door is more popular tourist attraction compared to Kimmeridge, and thus a higher percentage of plants were damaged, often to a greater extent, as a result of human disturbance.

A further explanation for the presence of smaller wild cabbages at Durdle Door compared to Kimmeridge relates to a trade-off between growth and defence. It is thought that slow-growing plants usually produce higher levels of secondary compounds than faster growing plants (Massei & Hartley, 2000). Results of this study support this as *Brassica oleracea* subsp. *oleracea* from Durdle Door produce higher levels of total and aliphatic glucosinolates compared to those from Kimmeridge (Chapter Two). It is likely that due to the lower vegetation diversity and the greater apparency of wild cabbages at Durdle Door, greater resources appear to have been invested in chemical defence rather than growth which appears to be the opposite to what has seemed to occur at Kimmeridge.

The high degree of variability in terms of habitat, vegetation and morphology of *Brassica oleracea* subsp. *oleracea* between Kimmeridge and Durdle Door could inevitably affect the presence and the activity of insects to a greater degree than the glucosinolate genotype and subsequent phenotype observed in this species.

5.4.2. Brevicoryne brassicae

Results support Root (1973) and Moyes (1997) in that *Brevicoryne brassicae* infestations differ both between and within field sites and also from year to year. A possible explanation for the severity of infestations could be due to the ecology of *Brevicoryne brassicae*. The development of the cabbage aphid is influenced by temperature (Chapter One). The weather data reveals that the winter of 2002/2003 was extremely mild which could have resulted in earlier infestations in spring 2003. This is supported by the appearance of *Brevicoryne brassicae* at Kimmeridge in March 2003. In addition the lower number of *Brevicoryne brassicae* observed in 2004 could be a reflection of the cooler temperatures. This would inevitably increase the time needed for the aphid to reach reproductive maturity, thus resulting in fewer generations over the season and inevitably fewer aphid infestations and/or decreased aphid numbers.

A second reason accounting for the variation in aphid infestations from year to year relates to the high percentage of wild *Brassica oleracea* mortalities observed, particularly at Kimmeridge in 2004. However, replacement plants were marked wherever possible as soon as a plant died.

A final explanation could be due to the secondary chemistry of the wild cabbage. It is well documented that the glucosinolate phenotype varies within *Brassica oleracea* subsp. *oleracea* (Mithen, *et al.*, 1995b; Moyes, 1997; Moyes *et al.*,2000; Chapter Two). The glucosinolate content is of primary importance to this study and so efforts were made to link the presence and concentration of individual and total glucosinolates to the presence of *Brevicoryne brassicae*. This is explained in a latter section of this chapter.

It was noted that flowering wild cabbages were more highly infested than non-flowering plants (pers. obs.). The time of flowering (April-June) coincided with a peak in the number of plants infested and the severity of infestations. This observation agrees with Moyes (1997). She linked this observation with work by van Emden (1990) who documented that plants have increased amino acids present in the phloem at this time. Future work should determine if the quality of the phloem in mature wild cabbages increases during flowering and whether the coincidental increase in cabbage aphid infestations is linked. A further explanation could be due to the fact that reproductive tissues are often associated with higher levels of secondary plant compounds (van Dam

et al., 1996; Zangerl & Rutledge, 1996). Therefore, it is possible that the racemes of wild cabbages produce higher constitutive levels of glucosinolates, which are utilised by alate aphids during host location. It would be interesting to analyse the glucosinolate content of racemes and foliage to determine whether the concentrations differ.

Despite the fact that the number of wild cabbage plants infested with *Brevicoryne brassicae* differs between the sites, the severity of infestations was similar (Figure 5.12. and 5.13.). This agrees with the results obtained from performance experiments conducted under controlled laboratory conditions (Chapter Three). This is interesting as the genotype of wild cabbages in the field would have expressed optimum phenotypes as far as the glucosinolate content is concerned. Therefore, the possibility that the plants investigated in the laboratory may not have reached their full glucosinolate phenotypic potential is unlikely to be a problem, in terms of the extrapolation of laboratory results to observations in the field. This is the first known study to link the behaviour of *Brevicoryne brassicae* in laboratory experiments to the performance in a natural ecosystem. A significant result was observed for one time period only. A significantly higher number of aphids were present at Durdle Door in comparison to Kimmeridge at time five in June 2003 (Figure 5.13.). A possible explanation could be due to the aphid "host-line" itself. As revealed, the "host-line" originally from Durdle Door showed greater variability in performance on the investigative plants than the control line and the line from Kimmeridge (Chapter Three). Therefore, the difference in the number of aphids between the sites at this time could be a result of the aphid species itself and not the host plant. A further explanation could be due to the impact of predators and parasitoids at this time. This study concentrated on the influence of *Diaeretiella rapae* on *Brevicoryne brassicae.* (This is discussed in a latter section of this chapter). However, it would be interesting to note the influence of specialist predators such as certain species of syrphids, on the population dynamics of *Brevicoryne brassicae* in the field.

5.4.3. The Effect of Glucosinolates on *Brevicoryne brassicae*

There are considerable data to support the fact that specialist Brassica herbivores are able to detect and respond to both the total content of glucosinolates and to individual glucosinolates under controlled laboratory conditions. Examples include *Psylliodes chrysocephala* which exhibits increased feeding to plants producing high levels of glucosinolates (Bartlet *et al.*, 1999). *Pieris rapae* (Traynier & Truscott, 1991; Renwick *et al.*, 1992) and *Pieris brassicae* respond to 3-indolylmethylglucosinolate (van Loon *et al.*, 1992). In semi-field trials, *Psylliodes chrysocephala* is attracted to higher levels of total glucosinolates and to 3-butenylglucosinolate (Giamoustaris & Mithen, 1995). In a natural ecosystem, *Selania leplastriana* responds to increased levels of 2-hydroxy-3-butenylglucosinolate and 3-indolylmethylglucosinolate produced by *Brassica oleracea* subsp. *oleracea* (Moyes *et al.*, 2000). High levels of 3-butenylglucosinolate produced by *Brassica oleracea* subsp. *oleracea* initiated high oviposition rates of *Ceutorhynchus assimilis* (Raybould & Moyes, 2001).

Analysis was conducted to link the presence and performance of *Brevicoryne brassicae* to the glucosinolate phenotype observed in *Brassica oleracea* subsp. *oleracea* at Kimmeridge and Durdle Door. Results reveal no significant differences in the concentration, either in terms of the total quantity or individual quality of glucosinolates produced by infested and un-infested *Brassica oleracea* individuals. Tests of association support this as no relationship was found between the number of plants producing individual aliphatic glucosinolates and aphid infestation. As the wild cabbages at Kimmeridge and Durdle Door both produce glucosinolates, it was never the intention of this thesis to investigate the presence/absence of glucosinolates in plant-herbivore interactions, as this is already well documented (Bartlet *et al.*, 1994; 1996; 1999; Giamoustaris & Mithen, 1995; Mithen *et al.*, 1995b; Cole, 1997). The primary objective was to ascertain the impact of plants producing extreme levels of glucosinolates on insects in a tritrophic interaction. The lack of a link between the quantity and quality of glucosinolates and the presence/absence of *Brevicoryne brassicae* indicates that these differences produced by wild cabbages at Kimmeridge and Durdle Door may not be great enough to influence host selection by *Brevicoryne brassicae*. This result supports those obtained from laboratory performance experiments (Chapter Three). However, when analysis between glucosinolate concentration and high and low aphid infestations was conducted, a tentative link was

revealed between a high concentration of 2-propenylglucosinolate and 1-methoxy-3-indolylmethylglucosinolate and low infestations of *Brevicoryne brassicae*. This was supported by the results of the association test of wild *Brassica oleracea* producing 2-propenylglucosinolate and the severity of aphid infestations. Results indicate that a high concentration of 2-propenylglucosinolate decreases the performance of *Brevicoryne brassicae*. However, when the total concentration of aliphatic glucosinolates and the presence of *Brevicoryne brassicae* infestations were considered, this relationship was lost. This indicates that despite the variations in terms of the quantity and quality of glucosinolates produced by *Brassica oleracea* subsp. *oleracea* at Kimmeridge and Durdle Door, the differences in individual glucosinolates may be counter-balanced by the total concentration of aliphatic, indole and/or total glucosinolates produced.

The only other known study to link the behaviour of *Brevicoryne brassicae* and glucosinolate content is a laboratory experiment conducted by Cole (1997). It was revealed that Brassicas displaying increased resistance to *Brevicoryne brassicae* produce low concentrations of 2-propenylglucosinolate and 2-hydroxy-3-butenylglucosinolate and high concentrations of 3-butenylglucosinolate and 4-pentenylglucosinolates. Results obtained from this study contradict this work as based on results obtained from HPLC analysis (Chapter Two), *Brassica oleracea* from Durdle Door should be more resistant to *Brevicoryne brassicae*. However, results from both laboratory experiments and field surveys reveal that wild *Brassica oleracea* at Kimmeridge and Durdle Door display similar levels of aphid susceptibility.

The correlation between the performance of *Brevicoryne brassicae* in the laboratory and in the natural environment suggests that laboratory experiments represent a realistic reflection of the behaviour of *Brevicoryne brassicae* in the field.

The possible reasons for the differences in the activity of *Brevicoryne brassicae*, other than as a result of the glucosinolate genotype and phenotype of *Brassica oleracea* subsp. *oleracea* at Kimmeridge and Durdle Door have previously been stated and thus will not be discussed further (Chapter Three).

5.4.4. Diaeretiella rapae

The number of plants hosting parasitised aphid infestations varies between years, which supports Moyes (1997) (Figure 5.14.). The earlier appearance of mummies at Kimmeridge could be due to the earlier appearance of *Brevicoryne brassicae* at this site. Analysis reveals that the number of mummified aphids is significantly greater at Durdle Door than Kimmeridge on two occasions in 2003 (Figure 5.15.). This indicates that the number of *Diaeretiella rapae* adults and/or the activity of *Diaeretiella rapae* are higher at Durdle Door. The significant increase in mummified aphids at time six at Durdle Door closely follows the high number of *Brevicoryne brassicae* at time five. This suggests that *Diaeretiella rapae* is able to respond to either visual cues from its host and/or host plant or to olfactory stimuli emitted from its host or from the increased production of secondary volatiles from the plant resulting from herbivore damage. This supports previous studies of aphid parasitoids in the laboratory (Du *et al.*, 1996: 1997: Blande, 2004) and investigations conducted in Chapter Four of this study. This observation is typical of how parasitoids locate aphid hosts (G. Poppy. pers.comm.). Importantly, it appears that the trends observed in Chapter Four are manifesting themselves in the field.

Visual cues emanating from the host plant could influence the activity of *Diaeretiella rapae*. Chew and Courtney (1991) revealed that *Pieris* spp. infest hosts that are spatially more apparent. It was found that apparent plants host greater numbers and more specialised herbivores, supporting the "plant apparency theory" proposed by Feeny (1976). This could be applied to the wild cabbage system and natural enemies, as plants at Durdle Door are larger than the surrounding vegetation and are hence more visually and chemically apparent. Future work could investigate the impact of plant apparency on the activity of *Diaeretiella rapae*. Mature infested wild cabbages could be grown in differing in amounts of vegetation and the number of mummified aphids could be determined.

5.4.5. The Effect of Glucosinolates on *Diaeretiella rapae*

A second explanation for the variability in the activity of *Diaeretiella rapae* between Kimmeridge and Durdle Door relates to the glucosinolates and their volatile breakdown products produced by the wild cabbage. Analysis was conducted to identify

relationships between the activity of parasitoids and glucosinolate concentration. However, analysis revealed no significant links. There was no difference in the concentration of individual and total glucosinolates produced by plants hosting parasitised and un-parasitised aphid infestations. No association was revealed between the number of plants producing individual aliphatic glucosinolates and *Diaeretiella rapae* activity. This contradicts previous work, when an increased attraction by *Diaeretiella rapae* was shown towards plants producing 2-propenylisothiocyanate (Read *et al.*, 1970; Moyes, 1997) and/or 3-butenylisothiocyanate (Bradbourne & Mithen, 2000). However, it should be noted that the number of plants was relatively low and so the statistical power of the tests may be low. However, this work confidently disagrees with Moyes (1997) who stated that all plants hosting parasitised aphid infestations produced 3-butenylglucosinolate. 3-butenylglucosinolate was not produced in any of the wild cabbage individuals at Kimmeridge (Chapter Two), however mummified aphids were present at this site. The lack of relationship between *Diaeretiella rapae* activity and glucosinolate phenotype indicates that the differences in glucosinolates produced by *Brassica oleracea* subsp. *oleracea* at Kimmeridge and Durdle Door are not great enough for *Diaeretiella rapae* to differentiate between and utilise during host-habitat and host location in a natural ecosystem. This supports results obtained from attack rate bioassays investigated in Chapter Four. Possible reasons explaining the apparent lack of glucosinolate utilisation by *Diaeretiella rapae* and the possible role of other secondary metabolites have previously been stated and so will not be discussed further (Chapter Four).

Additional reasons for the lack of a relationship which have not been offered previously includes the difference in quality of *Brevicoryne brassicae* and the genetic variability thought to exist in wild populations of parasitoids.

It was revealed that *Brevicoryne brassicae* originally from *Brassica oleracea* from Kimmeridge and Durdle Door exhibit differences in performance on their host plants (Chapter Three). This was attributed to the fact that the two aphid "host-lines" are exposed and may thus be adapted to the differences in glucosinolates produced by their hosts. This could influence the type of myrosinases present in the aphid needed to sequester glucosinolates and in turn affect the concentration and quality of glucosinolates stored within the aphid. This would influence the quality of the aphid and hence may affect its interaction with its natural enemies, including *Diaeretiella*

rapae. It would be interesting to take samples of mummified aphids from Kimmeridge and Durdle Door and determine whether the percentage of adults emerging and the sex ratio of males to females differ. Either of these could affect the number and activity of *Diaeretiella rapae* present at both sites.

Secondly, Powell and Wright (1992) stated that due to the genetic variability that exists in wild populations of parasitoids (Unruh *et al.*, 1983; Nemec & Stary, 1986 from Powell & Wright, 1992), specialist and generalist individuals exist in wild populations. This was stated for oligophagous species, however since it was documented that *Diaeretiella rapae* is able to parasitise both Brassica and cereal aphids (Vaughn *et al.*, 1996), it is possible that specialist and generalist individuals occur in this species. Future work could investigate the genetic differences in *Diaeretiella rapae* collected from Kimmeridge and Durdle Door to determine whether they are genetically different. This would link the observations of the behavioural experiments investigated in Chapter Four.

Summary

- Kimmeridge and Durdle Door differ in terms of the vegetation diversity, habitat and morphology of mature *Brassica oleracea* subsp. *oleracea*.
- A tentative relationship between the concentration of 2-propenylglucosinolate and *Brevicoryne brassicae* was revealed.
- No relationship was found between glucosinolate phenotype of *Brassica oleracea* subsp. *oleracea* and the activity of *Diaeretiella rapae*.
- Field investigations support the laboratory findings in that the differences in glucosinolates produced by wild cabbages at Kimmeridge and Durdle Door may not be great enough to influence the behaviour of *Brevicoryne brassicae* and *Diaeretiella rapae*.

Chapter Six

General Discussion

Chapter Six

General Discussion

6.1. Discussion

A detailed investigation into the glucosinolate phenotype of *Brassica oleracea* subsp. *oleracea* has been conducted in this thesis. It was accepted that insects are attracted to glucosinolates (examples include Traynier & Truscott, 1991; Renwick *et al.*, 1992; Cole, 1994; 1997; Bartlet *et al.*, 1996; Vaughn *et al.*, 1996), however this work is one of only a few known comparative studies which have investigated the effect of glucosinolate differences produced by a Brassica species in a tritrophic context (Moyes, 1997; Bradbourne & Mithen, 2000).

As predicted from previous data collected by Moyes (1997), *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door produce quantitative and qualitative differences in total aliphatic glucosinolates and total glucosinolates (Chapter Two). An important discovery was the presence/absence of 3-butenylisothiocyanate released from Durdle Door and Kimmeridge respectively (Chapter Two), which is a putative attractive volatile for foraging *Diaeretiella rapae* (Bradbourne & Mithen, 2000; Blande, 2004). To put this study into an ecological context, it was necessary to extend work conducted in the laboratory to a natural ecosystem. This explains why a wild Brassica model was chosen to investigate the effect of quantitative and qualitative glucosinolate differences on the interactions of insects in a tritrophic context.

Despite this, results from laboratory and field investigations concur to reveal that the performance of *Brevicoryne brassicae* (Chapter Three and Chapter Five) and the foraging behaviour of *Diaeretiella rapae* (Chapter Four and Chapter Five) are not affected by the differences in glucosinolate phenotype of wild populations of *Brassica oleracea* at Kimmeridge and Durdle Door. The lack of a relationship observed in this particular tritrophic interaction suggests that the differences in glucosinolate phenotype are either not great enough or important enough to warrant behavioural differences. Compounds other than glucosinolates may be more important in the population dynamics of these insects.

The lack of a relationship between glucosinolate differences and these specialist insects raises two important questions:

- Why do these polymorphisms in glucosinolate phenotype observed in discrete wild cabbage populations exist?
- What selecting agent(s) enforces this polymorphism?

6.2. First Trophic Level

Moyes (1997) conducted a detailed study of the herbivory of the wild cabbage. She concluded that herbivores do not act as top-down selecting agents on the glucosinolate genotype in populations along the Dorset coastline. This raises the possibility that bottom-up agents impose the selecting force.

Several hypotheses have been presented to explain the evolution of plant chemical defence:

- Classical plant defence suggests that plants and herbivores are involved in an "evolutionary arms race" (Ehrlich & Raven, 1965). Plants evolve defences to fight against herbivore attack, which then act as selection pressures on herbivores to evolve adaptations to overcome these defences. It is this continued co-evolution of attacks and counter-attacks by plants and herbivores that can explain the defences observed.
- The "plant apparency theory" (Feeny, 1976; Rhoades & Cates, 1976) depends on the ease at which plants can be found by herbivores. Plants are either deemed "apparent" or "non-apparent" in space and time, which are chemically defended to differing degrees. Theory suggests that "apparent" plants (easily found by herbivores) are "quantitatively" defended, meaning that costly secondary chemicals, which make up a large proportion of the plants' dry weight, are produced to defend against herbivores. "Unapparent" plants (hard for herbivores to find) are "qualitatively" defended, producing chemicals that are effective at low concentrations. "Qualitative" chemicals do not contribute heavily to the overall dry weight of the plant and are therefore deemed to have a lower cost to the plant. Feeny (1976) classically compared a long-lived oak tree and an annual mustard species to provide evidence for the "plant apparency theory," however many argue that the differences observed could be a result of the oversimplification of investigating different model species and the differing

life strategies of these plants (Futuyma, 1976; Coley, 1983). For the reason that only one plant species is investigated and that only one chemical defensive chemical is focused upon, the "plant apparency theory" was not applied to this study.

- The "optimal defence theory" proposed by McKey (1974), relates to the "plant apparency theory." It proposes that plant parts are not of equal value, therefore an optimal level of chemical defence is commonly only found in critical tissue only such as reproductive tissue. This suggests that herbivory is the primary selecting agent accounting for the intra-chemical variation observed in plants. Several studies disprove the "optimal defence theory" (examples include Moyes, 1997; Strauss *et al.*, 2004), arguing the need to take into account the life strategy of the plant species under investigation rather than making a generalisation. Glucosinolates are produced in all organs of certain Brassica species, including *Brassica oleracea* (Sang *et al.,* 1984), whilst Moyes (1997) found high concentrations of glucosinolates in mature vegetative tissues, which raises questions about the application of this theory to this system. However, this study cannot support or reject the "optimal defence theory" as the glucosinolate profile of various organs of the wild cabbage was not determined. A comparison between the glucosinolate profile of reproductive and vegetative tissues could be an area for future work.
- The "resource availability hypothesis" (Coley *et al.,* 1985), the "growth differentiation hypothesis" (Herms & Mattson, 1992) and the "carbon-nutrient balance hypothesis" (Bryant *et al.*, 1983) are three theories that consider the resources available to the plant.

  The "carbon-nutrient balance hypothesis" (Bryant *et al.*, 1983) states that plants in resource-rich environments have more nutrients available to produce secondary metabolites. For example, when light intensity is high, but nutrients are limited, carbon accumulates in the tissues and leads to an increased production of carbon-based defences. However, in light-limiting, high nutrient environments, all carbohydrates are allocated to primary processes, decreasing the carbon:nutrient ratio leading to an increased production of nitrogen-based defence chemicals. The nutrient content of *Brassica oleracea* subsp. *oleracea* was not investigated during this study, therefore the carbon-nutrient balance was not applied to explain the evolution of glucosinolate polymorphism in the wild cabbage system. In addition, the application of this theory may be unwise as it

has largely been discredited as it is based on several assumptions now known to be incorrect (See review by Hamilton *et al.*, 2001).

- The "resource availability hypothesis" (Coley *et al.*, 1985) and the "growth differentiation hypothesis" (Herms & Mattson, 1992) make similar predictions. Therefore, the application of these theories to the wild cabbage system is discussed simultaneously. The "resource availability hypothesis" suggests that the resources available limit the defence capabilities of plants. The "growth differentiation hypothesis" predicts that environmental factors affect the trade-off between growth and photosynthesis, which in turn affects the resources available for secondary metabolism. Both theories suggest that bottom-up forces may act as the selecting agent(s). In addition, both predict that plants in low-resource environments will display slow growth rates, but will heavily invest in chemical defence. It is accepted that *Brassica oleracea* subsp. *oleracea* is a slow growing perennial plant (Mitchell & Richards, 1979), but this study reveals that the size of mature wild cabbages differs between populations (Chapter Five). *Brassica oleracea* subsp. *oleracea* are smaller at Durdle Door compared to Kimmeridge, which in turn can imply that Durdle Door wild cabbages exhibit slower growth rates. According to both theories, slower-growing plants have more resources allocated to chemical defence. This study supports this as the smaller wild cabbages at Durdle Door produce the highest quantities of glucosinolates when compared to Kimmeridge plants. Therefore, the "resource availability hypothesis" and the "growth differentiation hypothesis" may apply to this system. However, despite promising support for the application of these theories, the wild cabbage system needs more research to confidently accept their application.

It should be noted that glucosinolates are nitrogen, not carbon-based defences, which are commonly associated with resource-rich habitats (Mitchell & Richards, 1979; Herms & Mattson, 1992; Moyes, 1997). Whilst it is accepted that Kimmeridge and Durdle Door are both resource-rich environments, it is possible that rich environments differ in terms of the resources available. It is suggested that a lower concentration of nutrients is available or nutrients are taken up in smaller quantities by *Brassica oleracea* subsp. *oleracea* at Durdle Door relative to Kimmeridge. This could explain the presence of smaller plants at Durdle Door, which produce the highest concentrations of glucosinolates necessary for defence against herbivory. It is proposed that the available

nutrients in the soil act as the bottom-up selecting force on the glucosinolate genotype of the wild cabbage, which accounts for the phenotypic variation observed between the two populations (Figure 6.1.). This is supported by results in Chapter Two. 3-butenylglucosinolate is only produced by wild cabbages at Durdle Door. This could suggest that the allele controlling the elongation of 2-propenylglucosinolate to 3-butenylglucosinolate, *Gsl-elong* (Magrath *et al.*, 1994) is only expressed by plants at Durdle Door (Figure 1.6.). This particular gene could be very important in Brassica-insect interactions, particularly upon natural enemies, as it is known that 3-butenylglucosinolate is hydrolysed to 3-butenylisothiocyanate upon herbivore damage. This volatile is suggested to be important during foraging by *Diaeretiella rapae* (Bradbourne & Mithen, 2000; Blande, 2004). Interestingly this study reveals that 3-butenylisothiocyanate is only produced in significant quantities by wild cabbages at Durdle Door. However, when Durdle Door plants are grown in soil collected from Kimmeridge, 3-butenylisothiocyanate was either not released or released in decreased amounts. This suggests that the soil and the nutrients available greatly affect the chemical defences, particularly induced defences, observed in the wild cabbage. This theory is supported by Mithen *et al.*, (1995b) who documented that the *Gsl-elong* locus is polymorphic between and within wild *Brassica oleracea* populations along the Dorset coastline. Future work could investigate the soil nutrients at Kimmeridge and Durdle Door to determine any differences. Secondly the alleles controlling the glucosinolates observed in wild cabbage populations at Kimmeridge and Durdle Door could be investigated to reveal whether *Gsl-elong* is significantly present at Durdle Door relative to Kimmeridge.

6.3. Second and Third Trophic Levels

However, despite the differences in glucosinolate phenotype between the two populations, no relationship between glucosinolates and the behaviour of *Brevicoryne brassicae* and *Diaeretiella rapae* was revealed in this study.

Brevicoryne brassicae was unable to differentiate between *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door (Chapter Three and Chapter Five) both in controlled laboratory experiments and in a natural ecosystem. However, "host-line" variation exists in *Brevicoryne brassicae* (Chapter Three). Interestingly, the "host-line" from Durdle Door is most affected by its host, in terms of its reproduction. Chemicals

are thought to be vertically transferred through trophic systems (Harvey *et al.*, 2003). The evidence from this study suggests that the effect of the differences in glucosinolate profile produced by *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door "stops" at the second trophic level (Figure 6.1.). This is supported by the lack of differential foraging behaviour exhibited by *Diaeretiella rapae* towards plant-host complexes from the two sites (Chapter Four and Chapter Five). It is proposed that *Brevicoryne brassicae* adapted to high glucosinolate hosts (for example Durdle Door *Brevicoryne brassicae*) are able to eliminate glucosinolates to greater levels in order to achieve concentrations more indicative of aphids infesting low glucosinolate hosts. I propose that *Brevicoryne brassicae* sequester glucosinolates to a threshold concentration. Aphids that are adapted to high glucosinolate hosts eliminate the excess levels of toxic glucosinolates in similar ways to generalist aphids such as *Myzus persicae*. The effect of this could be that regardless of host plant chemistry, *Brevicoryne brassicae* store glucosinolates to similar levels. It is suggested that the variation in glucosinolate quantity produced by wild cabbages at Kimmeridge and Durdle Door are "equalised" by *Brevicoryne brassicae*. Essentially, the transfer of chemicals through the tritrophic system is altered by this aphid species. The overall effect of this is to "evade" or minimise location by its natural enemy, *Diaeretiella rapae*. This would explain the lack of behavioural differentiation exhibited by *Diaeretiella rapae* during this study (Chapter Four and Chapter Five) and data collected by Blande (2004). However, in an effort to overcome this proposed adaptation by *Brevicoryne brassicae*, the induced release of 3-butenylisothiocyanate by the slower-growing plants at Durdle Door could be an evolutionary adaptation to attract greater numbers of parasitoids such as *Diaeretiella rapae* as an indirect defence strategy against aphid infestations (Figure 6.1.).

Despite the fact that no significant relationships were revealed between the glucosinolate phenotype and the insects investigated in this study, the possibility that these different chemical profiles are important in the tritrophic interaction of other Brassica specialists, for example *Pieris rapae* and *Cotesia rubecula*, has not yet been investigated and thus continues to be an area of great interest.

This study has not revealed a relationship between the behaviour of *Brevicoryne brassicae* and *Diaeretiella rapae* and the glucosinolate phenotype of *Brassica oleracea* subsp. *oleracea* at Kimmeridge and Durdle Door. However, important discoveries have

been made which raise the possibility that the soil and the available nutrients may act as the selecting agent on the polymorphic glucosinolate genotype observed in the wild cabbage. Future work should exploit this wild natural system to investigate the impact of bottom-up forces, particularly soil nutrient availability on the fitness of the populations of wild cabbage at Kimmeridge and Durdle Door.

Despite this, an unexpected major finding of this study questions the learning ability of *Hymenopterous* parasitoids (Chapter Four). Previous evidence supporting parasitoid learning is based on controlled laboratory experiments using continuously reared insects (Poppy *et al.*, 1997; Du *et al.*, 1997; Storeck *et al.*, 2000; Blande, 2004). It is known that allowing parasitoids prior-exposure to plants, hosts or a combination of both can enhance their response, leading to more efficient foraging (Du et al., 1997; Blande, 2004; Chapter Four). However, this study revealed that the ability of *Diaeretiella rapae* to learn was not observed in field-collected insects, suggesting that learning may be a laboratory phenomenon. It is suggested that wild *Diaeretiella rapae* are well adapted, possessing an innate response to the visual and chemical cues emanating from their hosts, plants or both. I propose that the continued evolution and selection of genes, which allows for the efficient foraging by these insects does not occur in continuously reared cultures. This is likely to be a result of the continued supply of suitable hosts that decreases the need for efficient foraging. This is the first known study to question the fitness and the learning ability of laboratory-reared parasitoids. It is suggested that in order to achieve realistic insights into the behaviour of parasitoids in the field, wild insects should be utilised in laboratory experiments.

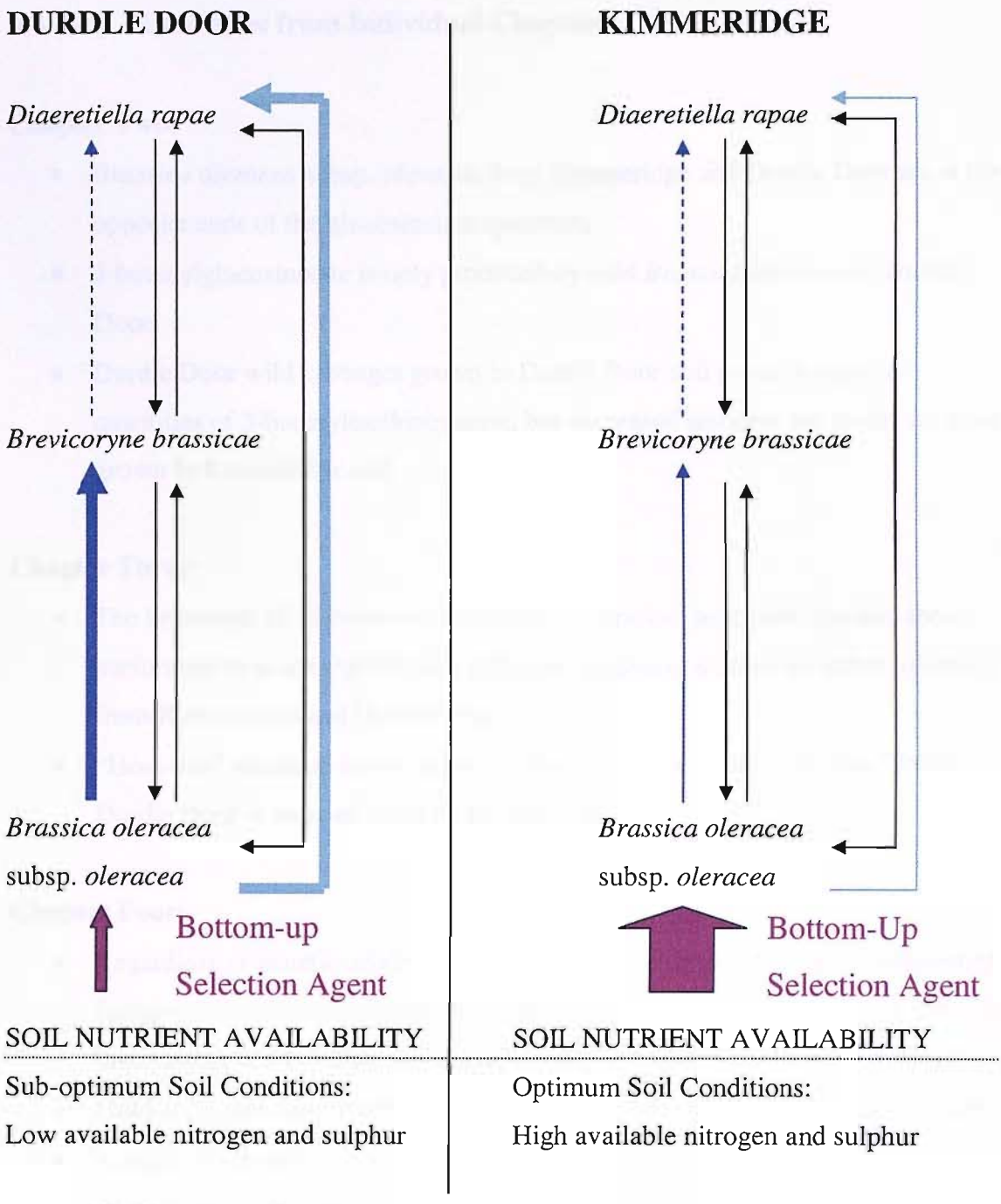


Figure 6.1. Diagram to show the possible effect of soil nutrient availability on the constitutive and induced glucosinolate chemical defences by *Brassica oleracea* subsp. *oleracea* at Durdle Door and Kimmeridge.

- Trophic interactions.
- Greater profile of constitutive defences produced (glucosinolates).
- Decreased profile of constitutive defences produced (glucosinolates).
- *Brevicoryne brassicae* sequesters constitutive defences to similar threshold concentrations.
- Greater profile of induced defences produced (glucosinolate volatiles)
- Decreased profile of induced defences produced (glucosinolate volatiles)

6.4. Key Discoveries from Individual Chapters

Chapter Two:

- *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door are at the opposite ends of the glucosinolate spectrum.
- 3-butenylglucosinolate is only produced by wild *Brassica oleracea* at Durdle Door.
- Durdle Door wild cabbages grown in Durdle Door soil produce significant quantities of 3-butenylisothiocyanate, but decreased amounts are produced when grown in Kimmeridge soil.

Chapter Three:

- The behaviour of *Brevicoryne brassicae* (in terms of host selection and aphid performance) is not significantly different on *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door.
- "Host-line" variation exists in *Brevicoryne brassicae*. The "host-line" from Durdle Door is most affected by its host plant.

Chapter Four:

- Regardless of genetic origin, *Diaeretiella rapae* is unable to display differential foraging behaviour in response to volatile differences produced from its host, its host-habitat or a combination of the two.
- Field-collected *Diaeretiella rapae* are larger than laboratory-reared individuals.
- A major discovery is that allowing field-collected *Diaeretiella rapae* prior experience of its plant-host complex does not enhance its attack rate activity.
- Importantly, a learning experience enhances the attack activity of laboratory-reared *Diaeretiella rapae* to similar levels observed by field-collected insects.

Chapter Five:

- Field investigations concur with laboratory experiments to reveal that *Brevicoryne brassicae* and *Diaeretiella rapae* are unable to detect differences in glucosinolate phenotype of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door.
- Kimmeridge and Durdle Door differ in terms of vegetation diversity and habitat.

6.5. Future Work

First Trophic Level: *Brassica oleracea* subsp. *oleracea*

- Analyse the glucosinolate content of racemes and foliage of mature wild cabbages to determine any differences.
- Investigate the myrosinase activity of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door to determine differences in the hydrolysis of glucosinolates.
- Investigate whether the quality of the phloem in mature wild cabbages increases during flowering to determine whether the coincidental increase in aphid infestation is linked.
- Investigate any other chemical differences of *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door, such as nutritional composition and green-leaf volatiles.
- Investigate the number of *Brevicoryne brassicae* required and for how long the infestation should occur to cause sufficient damage necessary to induce isothiocyanate defences from *Brassica oleracea* subsp. *oleracea.*
- Determine the soil nutrient availability at Kimmeridge and Durdle Door. How does this affect the fitness of *Brassica oleracea* subsp. *oleracea*?

Second Trophic Level: *Brevicoryne brassicae*

- Repeat host selection experiment using alate *Brevicoryne brassicae* to determine which morph is fundamental during host selection.
- Determine the glucosinolate content of the three "host-lines" of *Brevicoryne brassicae.*
- Investigate the distribution and activity of myrosinase isoenzymes produced by *Brevicoryne brassicae* to determine any differences.
- Determine the basis of "host-line" variation in *Brevicoryne brassicae* by adopting a genetic approach.
- Investigate the influence of other specialist natural enemies of *Brevicoryne brassicae.*

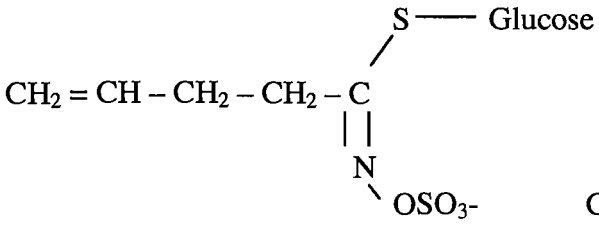
Third Trophic Level: ***Diaeretiella rapae***

- More replicates are needed to determine the effect of adding synthetic 3-butenylisothiocyanate to plant-host complexes from Kimmeridge assayed against plant-host complexes from Durdle Door in a Y-tube olfactometer.
- Repeat attack rate bioassays using *Brevicoryne brassicae* reared on diets with different glucosinolate contents.
- Take samples of mummified aphids from Kimmeridge and Durdle Door and determine whether the percentage of adults emerging and the sex ratio of males to females differ between sites.

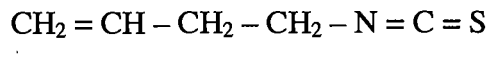
Appendices

Appendix 1

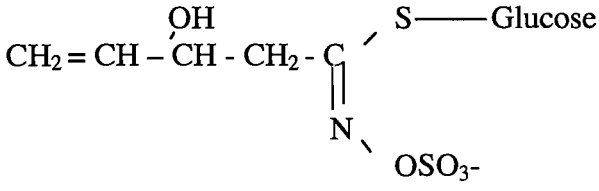
Glucosinolate Structures



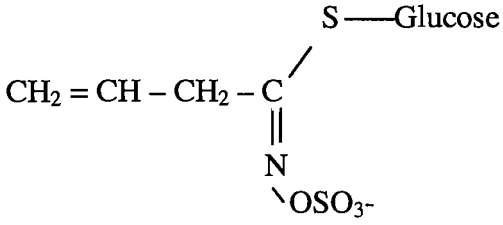
3-butenylglucosinolate



3-butenylisothiocyanate



2-hydroxy-3-butenylglucosinolate



2-propenylglucosinolate

2-propenylisothiocyanate

Appendix 2

Rate of Intrinsic Increase of *Brevicoryne brassicae* using clip cages (Wyatt & White, 1977).

$$Rm = 0.74 \frac{(\ln F_D)}{D}$$

F_D = number of nymphs produced over a period of time equal to that of the pre-reproductive period (D)

0.74 = Constant.

Preliminary experiments using control line of *Brevicoryne brassicae* on *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door. 8 replicates for each plant type were set up, however only 5 replicates could be completed due to the many problems associated with clip cage use.

Plant	F_D	D	Rm
Kimmeridge 1	10	10	0.170
Kimmeridge 2	6	10	0.133
Kimmeridge 3	15	10	0.200
Durdle Door 1	9	10	0.163
Durdle Door 2	7	10	0.144

Appendix 3

Plant-Aphid Combination	ln+1 number of aphids		t-test value	DF	p
	Method 1	Method 2			
BS-KIMA	3.177 ±0.0636	3.582 ±0.104	-3.33	57	0.002**
BS-DDA	2.928 ±0.0554	5.517 ±0.0916	-5.51	64	<0.001***
BS-BSA	3.357 ±0.0838	3.602 ±0.0884	-2.02	83	0.047*
Kim Typ-KIMA	3.369 ±0.149	3.286 ±0.100	0.46	28	0.651
Kim Typ-DDA	3.48 ±0.117	3.325 ±0.0774	1.10	30	0.278
Kim Typ-BSA	3.095 ±0.146	3.609 ±0.119	-2.73	27	0.011*
DD Typ-KIMA	3.042 ±0.124	3.336 ±0.103	-1.83	29	0.078
DD Typ-DDA	3.156 ±0.108	3.475 ±0.0713	-2.46	22	0.022*
DD Typ-BSA	3.138 ±0.103	3.397 ±0.137	-1.51	30	0.143
Kim Atyp-KIMA	3.26 ±0.0931	3.336 ±0.0884	0.19	59	0.848
Kim Atyp-DDA	2.987 ±0.0814	3.76 ±0.103	-5.87	50	<0.001***
Kim Atyp-BSA	3.257 ±0.104	3.71 ±0.0976	-3.17	48	0.003**
DD Atyp-KIMA	3.156 ±0.137	3.409 ±0.106	-1.46	34	0.153
DD Atyp-DDA	3.068 ±0.0944	3.368 ±0.0805	-2.42	24	0.023*
DD Atyp-BSA	3.107 ±0.122	3.622 ±0.0983	-3.28	30	0.003*

Table 3.1. Comparing the performance of the three host-lines of *Brevicoryne brassicae* on *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and the control plant. T values, DF, and p values are stated. Significant differences are highlighted ($p<0.05$*, $p<0.01$**, $p<0.001$***). Standard error values are highlighted (±SE). Method one is stated in black, method two is stated in blue.

Plant-Aphid Combination	r_m value Method 1	Method 2	t-test value	DF	p
BS-KIMA	0.2033 ±0.137	1.119 ±0.101	-8.97	35	<0.001***
BS-DDA	0.1288 ±0.116	1.235 ±0.0925	-11.87	41	<0.001***
BS-BSA	0.205 ±0.0178	1.262 ±0.0879	-11.97	44	<0.001***
Kim Typ-KIMA	0.243 ±0.0302	1.017 ±0.0989	-7.48	32	<0.001***
Kim Typ-DDA	0.258 ±0.0267	1.023 ±0.0882	-8.30	28	<0.001***
Kim Typ-BSA	0.166 ±0.0311	1.248 ±0.112	-9.32	29	<0.001***
DD Typ-KIMA	0.1761 ±0.0238	1.038 ±0.111	-7.56	25	<0.001***
DD Typ-DDA	0.184 ±0.0298	1.088 ±0.0738	-11.36	32	<0.001***
DD Typ-BSA	0.1785 ±0.0222	0.999 ±0.137	-5.90	19	<0.001***
Kim Atyp-KIMA	0.218 ±0.0188	1.007 ±0.107	-7.27	28	<0.001***
Kim Atyp-DDA	0.1382 ±0.0182	1.362 ±0.103	-11.66	29	<0.001***
Kim Atyp-BSA	0.198 ±0.0226	1.283 ±0.0956	-11.05	31	<0.001***
DD Atyp-KIMA	0.193 ±0.0268	1.011 ±0.106	-7.50	28	<0.001***
DD Atyp-DDA	0.1494 ±0.0170	0.97 ±0.0805	-9.98	29	<0.001***
DD Atyp-BSA	0.179 ±0.0283	1.224 ±0.0983	-10.21	28	<0.001***

Table 3.2. Comparing the intrinsic rate of natural increase (r_m) of the three host-lines of *Brevicoryne brassicae* on *Brassica oleracea* subsp. *oleracea* from Kimmeridge and Durdle Door and the control plant. t values, DF, and p values are stated. Significant differences are highlighted (p<0.001***). Standard error values are highlighted (±SE). Method one is stated in black, method two is stated in blue.

Appendix 4

Brevicoryne brassicae Infestations – Estimation of Population Densities

- Foliage

Number of aphids in a $1cm^2$ area in each of the populated densities.

	Aphid Density		
Replicate	Dense	Moderate	Sparse
1	192	127	59
2	186	174	103
3	132	191	103
4	197	136	60
5	222	146	75

Mean number of aphids in a $1cm^2$ area in each of the populated densities.

	Aphid Density		
Replicate	Dense	Moderate	Sparse
Mean	186	155	80

- Racemes

Determination of the size categories.

Raceme Size

	Size (mm)
Big Buds	>4
Medium Buds	3
Small Buds	<2
Big Stem	>4
Medium Stem	3
Small Stem	<2

Number of aphids in each of the aphid density-raceme size categories

Big Stem, Big Buds	Dense	Moderate	Sparse
1	624	194	189
2	584	328	225
3	526	273	258
4	503	248	168
5	578	265	123
mean	563	262	193

Big Stem, Medium Buds	Dense	Moderate	Sparse
1	496	252	167
2	543	228	185
3	568	239	159
4	575	263	187
5	603	256	143
mean	557	248	168

Big Stem, Small Buds	Dense	Moderate	Sparse
1	483	224	156
2	372	258	203
3	494	257	178
4	537	206	167
5	548	237	150
mean	487	236	171

Medium Stem, Big Buds	Dense	Moderate	Sparse
1	485	343	136
2	594	304	192
3	506	286	211
4	524	367	183
5	547	352	226
mean	531	330	190

Medium Stem, Medium Buds	Dense	Moderate	Sparse
1	524	293	199
2	546	315	176
3	528	283	173
4	567	347	194
5	598	354	167
mean	553	318	182

Medium Stem, Small Buds	Dense	Moderate	Sparse
1	494	272	101
2	523	263	123
3	527	233	98
4	536	246	142
5	517	245	138
mean	519	252	120

Small Stem, Big Buds	Dense	Moderate	Sparse
1	497	411	323
2	504	457	307
3	483	398	403
4	472	424	394
5	486	427	352
mean	488	423	356

Small Stem, Medium Buds	Dense	Moderate	Sparse
1	473	342	222
2	526	327	254
3	573	384	279
4	485	337	192
5	494	294	226
mean	510	337	235

Small Stem, Small Buds	Dense	Moderate	Sparse
1	488	234	161
2	523	301	110
3	463	262	149
4	483	341	69
5	593	210	130
mean	510	270	124

Mean number of aphids in each of the aphid density-raceme size categories.

	Mean Aphid Density		
	Dense	Moderate	Sparse
Big Stem, Big Buds	563	262	193
Big Stem, Medium Buds	557	248	168
Big Stem, Small Buds	487	236	171
Medium Stem, Big Buds	531	330	190
Medium Stem, Medium Buds	553	318	182
Medium Stem, Small Buds	519	252	120
Small Stem, Big Buds	356	423	356
Small Stem, Medium Buds	510	337	235
Small Stem, Small Buds	510	270	124

Appendix 5

Vegetation Survey of Kimmeridge and Durdle Door

Plant species growing the length of the 25m transect at Kimmeridge and Durdle Door. (*X* indicates the presence of a species). Habitat is classified as Moyes (1997). Additional references used for habitat classification include Phillips (1987) and Chinery (1998).

Species	Kimmeridge	Durdle Door	Habitat
Arrhenatherum elatius	X		waste, hedge
Atriplex hastata	X	X	seashore, disturbed
Avena fatua	X		arable, waste
Bellis perennis		X	short turf
Brassica nigra	X		waste, cliff, damp
Brassica oleracea	X	X	cliff
Campanula rotundifolia	X		dry, grassy, heath
Carpobrotus edulis		X	cliff, coasts
Carex distans	X		coasts
Centaurium erythraea		X	grassy, dune
Cirsium vulgare	X		bare, waste
Dactylis glomerata	X	X	meadow
Daucus carota	X	X	grassy, sea
Galium aparine	X	X	disturbed, shingle
Holcus lanatus	X	X	widespread
Lamium album	X		waste
Lamium purpureum	X		cultivated
Lolium perenne	X	X	pasture, waste
Lotus corniculatus	X	X	grassy
Malva sylvestris	X		waste
Medicago lupulina	X	X	bare, grassy
Pastinaca sativa	X		bare, grassy
Pimpinella saxifraga	X	X	dry, chalk soils, waste
Plantago coronopus		X	coast, sandy
Plantago lanceolata	X	X	grassy, waste
Plantago major		X	waste, paths

Poa pratensis		X	widespread
Polygonum aviculare	X		bare, seashore
Potentilla anserina		X	damp, grassy
Rumex acetosa	X	X	grassy, woods
Rumex acetosella	X	X	dry, bare
Rumex crispus	X		bare, disturbed
Senecio jacobaea		X	dry, grassy
Sonchus asper	X		bare, waste
Taraxacum officinale	X	X	grassy, waste
Thymus drucei	X	X	heaths, banks, dry, grassy
Trifolium dubium	X		dry, grassy
Trifolium pratense	X		grassy
Trifolium repens		X	grassy
Urtica dioica	X		woods, waste

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